REVERSIBLE INSULIN SENSITIVITY IN GRIZZLY BEARS (*URSUS ARCTOS HORRIBILIS*): THE ROLES OF CELL AUTONOMOUS AND EXOGENOUS FACTORS IN SEASONAL GLUCOSE METABOLISM

By

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Abstract

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Grizzly bears (*Ursus arctos horribilis*) have evolved remarkable metabolic adaptations to extended periods of low food availability including hyperphagia and massive fat accumulation during the fall followed by months of fasting throughout hibernation. Seasonal fluctuations in body mass are accompanied by changes in glucose and lipid metabolism, yet bears do not appear to suffer from the harmful effects associated with obesity in humans, such as type 2 diabetes mellitus (T2DM). To better define the metabolic transitions that occur annually in bears, we performed intravenous insulin tolerance tests (ivITTs) in anesthetized grizzly bears and oral glucose tolerance tests (oGTTs) in unanesthetized animals during the hibernation (January/February), active (May), and hyperphagic (September/October) seasons. To further investigate this system, we derived an *in vitro* model in which stromal vascular fraction (SVF) cells were isolated from subcutaneous fat biopsies during the three seasons. Cells were differentiated into adipocytes and stimulated with insulin under standard culture conditions and following chronic exposure to seasonal bear serum. Bears exhibited seasonal transitions in

insulin sensitivity shifting from a sensitive state in the active and hyperphagic seasons to an insulin resistant state during hibernation. This change was accompanied by glucose intolerance and hyperinsulinemia in hibernating bears although euglycemia was maintained throughout the year. Adipocyte cultures mirrored the metabolic states observed *in vivo* only when exposed to season-matched bear serum. Hibernation serum suppressed insulin-mediated glucose uptake in all cells while hibernation cells exposed to active serum were highly insulin responsive. Active and hyperphagic serum also elevated expression of insulin receptor (INSR) following insulin administration in active cells with corresponding increases in glucose uptake. Protein kinase B (AKT1) expression was suppressed in hibernation cells with season-matched serum, yet elevated in active cells with hibernation serum at baseline (no insulin). Thus, both serum factors and cell autonomous mechanisms play a role in seasonal insulin sensitivity. Results indicate the importance of serum proteins in regulating glucose metabolism and greatly expand our understanding of bear hibernation physiology. Furthermore, identification of mechanisms responsible for metabolic changes may provide insight into human metabolic disorders.

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INTRODUCTION

Hibernation has evolved as an extraordinary adaptation to long periods of food scarcity. Grizzly bears (*Ursus arctos horribilis*) hibernate for 5 to 7 months during which they do not eat, drink, or urinate (Folk et al. 1972; Hellgren 1998; Nelson 1973). Bears represent one end of the hibernation spectrum as shallow hibernators, in that they are responsive to external stimuli and maintain a relatively high body temperature (33°C) compared to hibernating rodents (-1.9-5°C) (Barnes 1989; Folk et al. 1972; Hissa et al. 1994). Nevertheless, bears undergo impressive physiological transformations including a 75% reduction in metabolic rate throughout hibernation and decreased cardiac output (Folk et al. 1972; Nelson and Robbins 2010; Tøien et al. 2011). Thus, grizzly bears represent an extreme model of metabolic adaptation, experiencing dramatic seasonal changes in body mass of over 50% in some cases within a single year (Kingsley et al. 1983). The bear's annual cycle consists of at least three distinct phases: 1) lean mass gain during the summer active season, 2) fat accumulation during the fall hyperphagic period, and 3) significant weight loss during hibernation (Hilderbrand et al. 1999; Nelson et al. 1983). Eighty-one percent of the fall mass gain is due to increased fat content facilitated by an almost insatiable appetite (Hilderbrand et al. 1999) during which bears may eat 20,000 kcal or more (Nelson et al. 1983) and gain up to 4 kg per day (Erlenbach et al. 2014). Similarly, hibernation weight loss occurs only in adipose tissue (Nelson et al. 1975; Nelson et al. 1983).

Fat accumulation is therefore essential for survival during winter when food resources are scarce, and lipolysis serves as the sole source of energy during hibernation (Carey et al. 2003; Nelson et al. 1973). It is also important to ensure propagation of the species, as females with higher body fat content produce more cubs, which are typically larger and more likely to survive (Atkinson and Ramsay 1995; Belant et al. 2006; Robbins et al. 2012). This fitness advantage has

created selection for morbidly obese bears by human standards (Atkinson and Ramsay 1995; Dahle et al. 2006; Derocher and Stirling 1996; Robbins et al. 2012). Surprisingly, these obese bears appear healthy without any detectable circulatory, respiratory, or metabolic pathologies (e.g. hyperglycemia and T2DM) seen in obese humans and other animals (Kamine et al. 2012a; McCain et al. 2013; Nelson 1973; Nelson et al. 2003; Palumbo et al. 1983; Stenvinkel et al. 2012). Thus, investigating these seasonally programmed mechanisms of fat deposition and usage could yield important insights into the physiology of how resource allocation patterns have been optimized for life histories that include a hibernation phase.

The utilization of fat and sparing of glucose by bears during hibernation may have led to selection for specific adaptations within the insulin-stimulated glucose uptake pathway. In healthy individuals, insulin facilitates the uptake of glucose into cells by inducing a conformational change in INSR and activating the glucose transporter, GLUT4, through the actions of phosphatidylinositol 3-kinase (PI3K) and AKT (Hers et al. 2011; Lewis et al. 2002; Pessin et al. 1999; Wilcox, 2005). Insulin thereby plays a significant role in maintaining normal blood glucose levels. It also regulates carbohydrate, lipid, and protein metabolism and promotes cell division and growth (Wilcox, 2005). The primary target tissues of insulin are skeletal muscle, liver, and adipose tissue. Here, insulin inhibits lipolysis and glucose secretion by prohibiting the processes of glycogenolysis and gluconeogenesis, and it stimulates glycogen synthesis and lipogenesis, among other actions (Hers et al. 2011; Logie et al. 2007). Insulin is the major regulator of adipose fat content due to 1) inhibition of hormone sensitive lipase causing reduced hydrolysis of intracellular triglycerides, and 2) stimulation of lipoprotein lipase, which mediates the hydrolysis of lipoprotein triglycerides to free fatty acids (FFAs) for intracellular storage (Hajer et al. 2008; Lewis et al. 2002). Loss of insulin sensitivity and the resulting

derangement of glucose homeostasis are primary factors in the etiology of T2DM (Reaven 2004; Wilcox 2005). This results in hyperglycemia, hyperinsulinemia, and the eventual death of pancreatic beta cells if left untreated, along with a host of other pathologies including hypertension and coronary artery disease (Malnick and Knobler 2006; Pi-Sunyer F.X. 2002; Reaven 2004).

Evidence suggests that insulin resistance in humans is caused by problems in insulin signaling via post-receptor mechanisms (Wheatcroft et al. 2003; Wilcox 2005). Receptor deficits also may play a role in the development of T2DM because INSR expression and tyrosine kinase activity are decreased in the adipocytes of obese humans (Olefsky 1976). However, restoration of receptor kinase activity and expression does not fully restore insulin sensitivity, implicating other factors in the development of insulin resistance (Freidenberg et al.1988; Virkamäki et al. 1999). For example, polymorphisms in insulin receptor substrate-1 (IRS-1) and in the p58 regulatory subunit of PI3K have also been associated with insulin resistance in humans (Virkamäki et al. 1999). In obese rats and humans, insulin is less effective at phosphorylating INSR and PI3K, and expression of these proteins is low in obese subjects compared to lean ones (Goodyear et al. 1995; Kim et al. 2000). Dysfunction of AKT has also been implicated in creating a diabetic state (Hers et al. 2011). Hyperactivation of AKT signaling in adipose tissue caused by mutations in phosphatase and tensin homolog (PTEN) results in an obese yet insulin sensitive phenotype (Pal et al. 2012). Mice with AKT2 mutations have impaired glucose uptake in response to insulin, and a rare R274H mutation in AKT2 in humans results in T2DM and severe hyperinsulinemia (Cho et al. 2001; George et al. 2004; Hers et al. 2011). However, Kim et al. (2000) found PI3K activity to be much more impaired than that of AKT isoforms 1 and 2 in obese rats. Another mechanism by which glucose uptake may be impaired in a diabetic state is

through the increased circulation of FFAs and inflammatory cytokines. Both cause serine phosphorylation and uncoupling of IRS-1 leading to decreased activation of AKT and PI3K (Hers et al. 2011; Le Marchand-Brustel et al. 2003). FFAs, through the concomitant accumulation of the palmitate derivative ceramide, may also activate phosphatases that inhibit the insulin signaling pathway by preventing insulin-stimulated phosphorylation of AKT (Chavez et al. 2003; Schmitz-Peiffer et al. 1999; Ueki et al. 2004).

Insulin resistance is not unique to humans. Along with rising obesity rates, this phenomenon has been extensively documented in companion animals such as cats and dogs (German et al. 2009; German et al. 2010; Hoenig et al. 2006). Natural and reversible insulin resistance has also been observed in yellow-bellied marmots (*Marmota flaviventris*), which exhibited hyperinsulinemia and insulin resistance while obese in the fall after several weeks of fasting but prior to the onset of hibernation. Interestingly, insulin responsiveness returned after weight loss during hibernation (Florant et al. 1985). Thus, hibernating animals could serve as excellent models for studying the factors involved in the loss and return of insulin sensitivity (Martin 2008). Unlike marmots, both wild and captive American black bears (*Ursus americanus*) and Japanese black bears (*Ursus thibetanus japonicas*) were insulin sensitive throughout the active season yet became insulin resistant during hibernation (Kamine et al. 2012a; McCain et al. 2013; Palumbo et al. 1983). This metabolic transition occurred regardless of consistent food availability in American black bears, implying endogenous regulation of seasonal changes (McCain et al. 2013). Studies in marmots suggest that seasonal changes in metabolism may be regulated by differential gene expression in white adipose tissue (Wilson et al. 1992). Adipose from hibernating mammals may therefore represent a unique tool to understand insulin regulation. In particular, the distinct hibernation physiology of bears suggests that they may

possess physiological abilities absent in non-hibernating mammals including humans. However, numerous unanswered questions remain regarding the relationship between insulin, lipid metabolism, and adipose function in grizzly bears.

The current study investigated the seasonal metabolic transitions that occur in captive grizzly bears as well as the cellular mechanisms regulating these cycles. Because anesthetics can potentially confound glucoregulatory results by causing hyperglycemia and hypoinsulinemia (Kamine et al. 2012b), we sought to examine the seasonal variation in glucose homeostasis in both anesthetized and unanesthetized grizzly bears. We also assessed changes in adipose function and insulin sensitivity of fat tissue in order to investigate the underlying processes responsible for seasonal transitions. We hypothesized that glucose homeostasis would vary seasonally in bears as well as in bear adipose tissue. Specifically, active bears would be insulin sensitive to support intracellular glucose storage and fatty acid synthesis while hibernating bears would be insensitive to insulin to prevent hypoglycemia and promote lipolysis. *In vitro* results were predicted to recapitulate responses observed during *in vivo* experiments. Furthermore, we explored the possibility that seasonal changes in insulin sensitivity are mediated by a season-specific serum factor(s) or alternatively by autonomous mechanisms within the adipocyte.

METHODS

Animals

Nine grizzly bears, ranging in age from 2 to 11 years, were used in this research. Data for ivITTs were collected between January 2013 and October 2015 from 2 adult females (age 10 to 12 years), 2 adult males (age 11 to 13 years), and 2 juvenile males (age 3 to 5 years). For oGTTs, 3 adult females (age 9 to 11 years) were studied from October 2014 to May 2015. All *in vitro* work was completed using samples from 4 bears (2 adult females, 2 adult males) collected over a 2 year period (ages 10 to 11 years). Animals were housed at the Washington State University Bear Research, Education, and Conservation Center in Pullman, WA where they were maintained according to ASM guidelines (Sikes et al. 2011) and the *Bear Care and Colony Health Standard Operating Procedures*. All procedures were approved by the Washington State University Institutional Animal Care and Use Committee (IACUC) (ASAF # 03802-017). Throughout October, food availability was reduced, and feeding was discontinued by early November. Bears were hibernated in their home dens with access to both indoor pens (3m x 3m x 2.5m) and outdoor runs (3m x 3m x 5m). Thus, bears were exposed to natural temperature and light conditions during hibernation and throughout the year.

IN VIVO EXPERIMENTS

Insulin Tolerance Test (ivITT)

We conducted ivITTs during each of the three seasonal metabolic phases: active (May), hyperphagia (October), and hibernation (January). All bears were fasting prior to ivITT procedures. In May and October bears were fasted overnight whereas hibernating bears were naturally fasting. Bears were anesthetized with a combination of dexmedetomidine (Zoetis,

Florham Park, NJ, 6 μg/kg in May and October and 3.5 μg/ kg in January) and tiletamine HCl/zolazepam HCl (Telazol; Zoetis, 2.5 mg/kg in May and October and 1.5 mg/kg in January) administered intramuscularly in the shoulder region via pneumatic dart according to protocols described in Ware et al. (2012). Serial blood samples were collected at 5 minute intervals from the jugular vein using a peripheral venous catheter (14G, 14 cm; BD Angiocath). Blood glucose was measured at baseline (time = 0) with the use of a standard commercial glucometer (Accu-Chek Active, Roche Diagnostics, Indianapolis, IN). Immediately thereafter, insulin (0.015 U/kg; Humalin- R, Eli Lilly, Indianapolis, IN) was administered intravenously, and blood glucose was assessed every 5 minutes for a total of 40 minutes post injection. Serum was collected at baseline and again at the glucose nadir and was stored at -80°C in order to verify glucometer readings. Dextrose (50%) was administered intravenously if blood glucose levels fell below 40 mg/dl. Blood glucose values collected after dextrose supplementation were not used in subsequent analyses.

Glucose Tolerance Test (oGTT)

To facilitate the safe collection of blood samples in conscious animals without the use of anesthetics, three adult female bears were trained to enter a crate and extend a hind leg where blood was taken from a peripheral vein (Joyce-Zuniga et al. in press). These individuals were used to perform oGTTs during the same seasonal time periods as those used for ivITTs (see above) with the exception that hyperphagic sampling began in late September and extended into October. Hibernation samplings extended from late January into early February. All animals were fasted prior to testing as described above. Upon entering the crate bears were fed dextrose (1 g/kg) and were subsequently given a non-caloric reward (Truvia® to saturation) if needed to collect baseline blood samples. Blood was collected at baseline (0 min) and again at 15, 60, and

120 minutes post consumption. Following dextrose administration, blood samples were taken while the bear received a honey reward. Blood collection took less than 5 minutes for each time point. No more than 2 blood samples (baseline plus additional time point) were collected from each bear in a single day to avoid influencing subsequent samples with honey intake. Sampling was repeated on subsequent days until the entire 120 minute profile for each bear was obtained. During hibernation, sampling took place over the span of approximately 4 weeks with a 2 week honey/dextrose-free interval to avoid disrupting the bears' normal hibernation physiology.

Blood glucose was measured immediately after sampling as described above, and the remaining blood was placed into vacutainer tubes and centrifuged (1300 x g for 20 min at 4°C). Serum was stored at -80°C for further analysis. A porcine/canine ELISA (ALPCO, Salem, NH) was used to measure serum insulin concentrations according to instructions provided by the manufacturer. Serum glucagon was quantified using a multi-species glucagon EIA according to the manufacturer's instructions (Phoenix Pharmaceuticals, Inc., Burlingame, CA).

IN VITRO EXPERIMENTS

Cell Culture

Bears were anesthetized using the protocol described above. Biopsy sites were shaved and scrubbed with chlorhexidine and 70% ethanol. Punch biopsies (6mm; Miltex, York, PA) were used to obtain samples of subcutaneous adipose tissue from gluteal depots. The collected adipose tissue was then processed to isolate mesenchymal stem cells from the SVF. Cells were plated or cryopreserved in liquid nitrogen using protocols described in Gehring et al. (in review). We also collected jugular blood samples at the time of biopsy. Serum was stored at -80°C. For all experimental cultures, cells were seeded at 1.0×10^4 cells/well (5.2×10^3 cells/cm^2) in 24 well

culture plates. Cultures were maintained and differentiation to the adipogenic lineage induced following culture protocols and medium formulas detailed in Gehring et al. (in review). All mature adipocyte cultures were processed on day 11 post-differentiation for retrieval of mRNA for gene expression assays or cell lysates from which glucose uptake could be quantified. All experiments were performed in duplicate wells with cells obtained from the same individuals (N = 4).

Insulin Dose Response

To produce dose response curves, cells originating from the active season (May), hyperphagia (September/October), and hibernation (January) were cultured according to the protocols described above. Passage 0 to 2 cells were used because grizzly bear adipocytes cultured to low passage numbers retain lipogenic characteristics similar to those of passage 0 cells plated directly from the animal without subculturing (Gehring et al. in review). Cells were processed for the quantification of glucose uptake by colorimetric assay using a minor modification of the procedures described by the manufacturer (BioVision Inc., Milpitas, CA). Volumes for washes and incubations were scaled up by a factor of 6.67 to accommodate our increased cell number. On day 10 post-differentiation, we removed medium and washed cells once with 1x PBS. Cells were then incubated overnight at 37°C and 5% CO_2 in starve medium (89% DMEM/F-12 containing GlutaMAX^{TM} (Thermo Fisher Scientific Inc., Waltham, MA) and 1% 100X antibiotic/antimycotic (Sigma, St. Louis, MO) with final concentrations of 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B). On the following day, starve medium was removed and cells were washed twice with 1x PBS at room temperature. Cells were then incubated in KRPH (20 mM HEPES, 5 mM KH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 136 mM NaCl, and 4.7 mM KCl, pH =7.4) with 2% bovine serum albumin (BSA; Sigma)

for 40 minutes. All incubations were performed at 37°C unless otherwise specified. Next, duplicate wells were treated with insulin (0, 10, 100, 300, 500, 700, or 1000 nM) in KRPH containing 2% BSA for 20 minutes. A 10mM 2-deoxyglucose (2-DG) solution was then added and the cells were incubated for an additional 20 minutes. Following the incubation, wells were washed twice with 1x PBS and scraped to lyse the cells. The cell lysates were stored at -80°C until lyophilized. To lyophilize, 2.0 ml microcentrifuge tubes containing the samples were frozen in ice blocks at -20°C in 600 ml Fast-Freeze flasks (Labconco, Kansas City, MO). Samples were cooled to -80°C to prevent thawing and subsequently attached to a FreeZone Cascade Freeze Dryer (Labconco) for approximately 96 hours. Lyophilized samples were stored at -20°C for up to 2 weeks until analyzed by colorimetric assay. Samples were diluted 1:3, 1:5, 1:6, or 1:10 as needed to place absorbance values into the detectable range of the assay's standard curve. All samples were assayed in duplicate.

Adipocyte Differentiation

To examine effects of differentiation on the isolated SVF, undifferentiated cells from the active season and hibernation were expanded in preadipocyte medium (Gehring et al. in review) and processed as described above upon reaching confluence. Control wells were incubated in KRPH with 2% BSA while treated wells were incubated in 300 nM insulin. Cells were processed for glucose uptake as described above with a minor modification where samples were only diluted 1:1.2, 1:3, or 1:5. Replicate wells were also processed for mRNA as describe below in order to measure expression of INSR and peroxisome proliferator-activated receptor gamma (PPARG).

Real-Time Quantitative PCR

After the 20 minute insulin treatment, cells were stored in 300 µl of QIAzol (QIAGEN, Venlo, Netherlands) at -80°C. Total RNA purification, reverse transcription, and real-time quantitative PCR (RT qPCR) were performed according to protocols provided in Gehring et al. (in review). Real time qPCR was performed using RPS18 as a reference gene to normalize expression. Gene expression was assayed using canine TaqMan probes (Thermo Fisher Scientific Inc., Waltham, MA). Probe specificity was confirmed with comparisons of canine TaqMan probe sequences to the polar bear (*Ursus maritimus*) genome performed with a BLASTn search of the National Center for Biotechnology Information database (Table 1) and by the appearance of amplicons within 40 cycles.

Culture Temperature

Mature (differentiated) adipocytes derived from active and hibernation biopsies were cultured according to the previously described protocols. However, on day 8 post differentiation, cultures were transferred to a 34.7 °C incubator to mimic the body temperature of a hibernating bear (Hissa et al. 1994; Jansen unpublished observations; Tøien et al. 2011). These cells were processed for 2-DG uptake measurements with all incubations performed at 34.7 °C. Control wells were incubated in KRPH with 2% BSA and treated wells incubated in 300 nM insulin. Glucose uptake assays were performed as described using samples diluted 1:3, 1:5, 1:6, or 1:10.

Use of Bear Serum in Culture

Cells originating from the active season and hibernation were cultured according to protocols described above. All experiments were performed using low passage cells (0 or 1). At the time of differentiation, cells were divided into different serum treatment groups. Cells were placed in differentiation medium containing either 10% FBS or 10% bear serum from one of the three seasonal phases. Bear serum was filtered twice through 0.22 µm filters and once through a

0.1 μm filter prior to use in cell cultures. Each cell type (active or hibernation) was cultured with each of the four types of serum. Maintenance medium was supplemented with the assigned serum type for the remaining 8 days in culture prior to incubation in starve medium. Cells were processed for glucose uptake quantification using procedures described above with slight modifications due to the enlarged lipid droplets observed in cultures with hyperphagic and active season serum, which made them prone to peeling. This tendency was not due to cell death as evidenced by total RNA concentrations (see results). Thus, all cultures were transferred directly from maintenance medium to starve medium and were washed once with 1x PBS prior to the 40 minute incubation in KRPH containing 2% BSA. Control wells were incubated in KRPH with 2% BSA whereas treatment wells were incubated in 300 nM insulin. Each cell type, serum, and insulin dose combination was cultured in duplicate. Glucose uptake colorimetric assays were performed as described above. Samples were assayed in duplicate at one of the following dilutions: 1:3, 1:10, 1:15, or 1:20. Correction factors of 1.7 and 2.58 were applied to samples assayed at 1:15 and 1:20 dilutions respectively due to nonlinearity. Replicates were also cultured in order to measure gene expression. Cells were processed and RT q PCR run as described above. Samples were assayed for expression of INSR, AKT1, PTEN, and PPARG using canine TaqMan probes. Probe specificity was confirmed as previously described (Table 1).

Preliminary Serum Purification

To explore the potential identity of serum factors responsible for season-dependent effects on glucose uptake, active season serum from two adult bears (1 male, 1 female) was treated to remove putative active constituents. All sera were first filtered through a 0.22 μm filter. Then, serum was either incubated for 30 minutes at 65°C to denature endogenous proteins and re-filtered or charcoal-stripped in order to remove steroid and other non-protein molecules as

follows. Activated charcoal suitable for cell culture (Sigma) was added to samples at approximately 40mg/ml. The serum was vortexed and then incubated with agitation at 4°C for 3 hours. Following incubation, samples were centrifuged at 24,310 x g for 10 minutes. The supernatant was collected and then filtered sequentially to remove all charcoal using 0.45, 0.22, and 0.1 μm filters. Hibernation cells, originating in the same bears from whom serum was collected, were cultured according to the protocols outlined above. Upon reaching confluence, cells were induced to differentiate with medium containing either FBS, active season serum, heat-inactivated active season serum, or charcoal-stripped active season serum at a 10% concentration. Cells were maintained in medium with the respective serum type for an additional 8 days post-differentiation. Cells were processed for insulin stimulated 2-DG uptake as described above for serum swap cultures with samples assayed at a 1:10 or 1:20 dilution.

To corroborate results of the glucose uptake colorimetric assay, glucose concentrations of culture medium were also measured using the same glucometer used for blood glucose measurements. We measured glucose in unused maintenance medium and in the same medium 48 hours after its addition to culture wells on day 10 post-differentiation. Values were collected from hibernation cells treated with FBS, active season serum, heat-inactivated active season serum, and charcoal-stripped active season serum.

Statistical Analysis

For statistical tests on ivITT data, percent baseline values were used. Raw data from oGTTs were analyzed. For ivITTs and oGTTs, effect of season and time post insulin or dextrose administration was analyzed using two-way ANOVAs. Tukey's multiple comparison tests were run to compare blood glucose, insulin, or glucagon values at each time point between the three seasons. A one-way ANOVA and Dunnett's multiple comparisons tests were used to determine

seasonal differences in baseline insulin values. Baseline subtracted values were calculated for insulin treated adipocytes for all glucose uptake measurements from cell cultures. Effect of cell season and insulin dose were determined with a two-way ANOVA for insulin dose response curves. Glucose uptake values from undifferentiated cells, cells cultured at 34.7°C, and serum swap cultures were also analyzed using two-way ANOVAs and subsequent Sidak's multiple comparisons tests to determine effect of cell season and differentiation, temperature, or serum type. A Kruskal-Wallis test and Dunn's multiple comparisons were performed to compare medium glucose concentrations in cultures treated with different forms of active season serum. A two-way ANOVA was used to assess differences in total RNA between cultures grown under different serum conditions.

For gene expression, fold changes were calculated in two ways: 1) under insulin stimulation (300nM) with respect to expression in untreated cells (no insulin) to obtain a measure of the cells response to insulin for each gene and 2) in the absence of insulin with respect to expression in hibernation cells cultured with FBS, to obtain an estimate of the relative expression at baseline between different serum types. For undifferentiated cells and differentiated cells cultured with FBS, fold change was also calculated with respect to expression in undifferentiated cells derived from hibernation (no insulin) to determine relative expression at baseline. Two-way ANOVAs were used to analyze effect of cell season and either serum type or differentiation on response to insulin or on baseline expression. Sidak's or Tukey's multiple comparisons tests were used to examine differences between the cell season and serum treatment groups. Differences with $P \leq 0.05$ were considered statistically significant. All data are presented as mean ± SEM.

RESULTS

IN VIVO EXPERIMENTS

Insulin Tolerance Test (ivITT)

Hibernating bears exhibited a minimal response to insulin with blood glucose only falling to 95 ± 5% of baseline at 40 minutes post injection (Figure 1). During May sampling, blood glucose levels dropped to a low of 60 ± 4% at 35 minutes, and rose slightly to 63 ± 4% at 40 minutes post injection. There was a more pronounced decrease in blood glucose levels in response to insulin administration during October ivITTs. During this season, blood glucose levels fell to 47 ± 2% of baseline at 40 minutes post injection. This value likely underestimates the reduction in blood glucose because measurements were terminated for three bears after 30 minutes due to severe hypoglycemia, at which time they were administered dextrose. There was a significant effect of both time post insulin injection and season on glucose concentrations [two-way ANOVA; time post insulin $F(8,232) = 58.50$, $P < 0.0001$; season $F(2, 232) = 333.30$, $P < 0.0001$]. There was also a significant interaction between time point and season when the sample was collected [two-way ANOVA; $F(16,232) = 9.98$, $P < 0.0001$]. Significant differences existed between January and May ($P = 0.0020$) as well as January and October ($P = 0.0012$) glucose concentrations at 5 minutes post insulin administration and for all subsequent time points at the $P< 0.0001$ level. There were several differences between blood glucose levels in May and October at the 25 ($P = 0.0065$), 30 ($P = 0.0037$), and 40 ($P = 0.0059$) minute time points.

Glucose Tolerance Test (oGTT)

In unanesthetized bears following a 1g/kg oral dose of dextrose, blood glucose peaked at 15 minutes in May (117 ± 5 mg/dL) and October (104 ± 7 mg/dL) and declined throughout subsequent sampling, reaching near baseline levels at 120 minutes (Figure 2). In contrast,

glucose increased continuously over the entire 120 minute sampling period in January and remained elevated at this time point compared to the other seasons ($P < 0.0001$). Due to a delayed rise in blood glucose during hibernation, January values were significantly lower than those in October and May at the 15 minute time point ($P < 0.0001$). Both time post dextrose administration and season had a significant effect on blood glucose concentrations [two-way ANOVA; time post dextrose $F(3,53) = 27.20$, $P < 0.0001$; season $F(2, 53) = 6.19$, $P = 0.0038$]. There was also a significant interaction between these factors [two-way ANOVA; $F(6,53) = 15.61$, $P < 0.0001$]. We observed no significant difference between baseline glucose concentrations in May (66 ± 2 mg/dL), October (58 ± 1 mg/dL), and January (60 ± 4 mg/dL).

Serum insulin also peaked 15 minutes after dextrose consumption in October (0.36 ± 0.09 ng/ml) and May (0.26 ± 0.05 ng/ml). Similar to glucose (Figure 2a), insulin concentrations increased during hibernation reaching a high of 0.23 ± 0.10 ng/ml at 60 minutes and remained constant through 120 minutes (Figure 2b). Time post dextrose consumption had a significant effect on serum insulin [two-way ANOVA; $F(3,24) = 7.61$, $P = 0.0010$], and there was a significant interaction of time point and season on insulin concentration [two-way ANOVA; $F(6,24) = 2.75$, $P = 0.0352$]. January insulin was significantly lower than October insulin at 15 minutes ($P = 0.0337$). Differences between January insulin and May and October concentrations approached significance at the 120 minute time point (January vs. May, $P = 0.0582$; January vs. October, $P = 0.0518$). The seasonal effect on baseline insulin approached significance [one-way ANOVA; $F(2,6) = 4.76$, $P = 0.0578$]. January insulin was greater than October and May ($P = 0.0392$) values by factors of 1.65 and 2.64 respectively.

Unlike glucose and insulin, glucagon levels were unchanged following dextrose administration. Overall, glucagon concentrations were significantly higher in January compared

to May or October [two-way ANOVA; $F(2,30) = 9.53$, P = 0.0006]. May values (1.13 ± 0.02 ng/ml) were consistently lower than those in October (1.35 ± 0.02 ng/ml; P = 0.0061) and January (1.45 ± 0.003 ng/ml; P = 0.0002) (Figure 2c). The ratio of glucagon to insulin was lowest in January (10.03 ± 0.99) and elevated in May (20.09 ± 0.82) and October (15.59 ± 1.59) by factors of 2.0 and 1.55 respectively.

IN VITRO EXPERIMENTS

Insulin Dose Response

When differentiated adipocytes were treated with varying doses of insulin, there was a significant main effect of season from which the cells were derived on 2-DG uptake [two-way ANOVA; $F(2,45) = 3.23$, P = 0.0487] (Figure 3). At baseline, cells from the hyperphagic period had the highest 2-DG uptake at 211.36 ± 39.36 pmol per well. Uptake was 30.14 ± 7.21 pmol greater in hyperphagic cells than cells derived from hibernation. The most pronounced seasonal differences existed at the 1000 nM insulin dose with hibernation cells having the highest baseline subtracted 2-DG uptake at 243.34 ± 82.26 pmol. At this dose, hibernation cells expressed a significantly greater rate of uptake by 232.59 ± 8.26 pmol compared to active season cells (P = 0.0029). Uptake was 129.51 ± 9.00 pmol higher in hibernation cultures than in hyperphagic (October) cells, although this did not reach statistical significance (Tukey post-hoc analysis, P = 0.1491).

Adipocyte Differentiation

We observed a significant effect of cell type (undifferentiated vs. differentiated) on 2-DG uptake [two-way ANOVA; $F(1,28) = 7.54$, P = 0.0104] (Figure 4). Baseline-subtracted 2-DG uptake was significantly lower in undifferentiated cells during both hibernation (11.09 ± 3.99

pmol) and active season (9.01 ± 3.13 pmol) compared to differentiated cells (hibernation, 99.15 ± 63.04 pmol; active, 114.60 ± 31.18 pmol).

There were no significant differences on INSR or PPARG expression in response to insulin treatment across cell season or between undifferentiated or differentiated cultures. However, baseline (no insulin) expression of INSR [two-way ANOVA; $F(1,28) = 7.74$, $P = 0.0096$] and PPARG [two-way ANOVA; $F(1,28) = 73.87$, $P < 0.0001$] varied significantly with cell type, with differentiated cells having higher expression than undifferentiated cells (Figure 5). Expression of PPARG was 18.39 times higher in differentiated cells than in undifferentiated cells for active season cultures. There was a 13.19 fold difference in hibernation cells.

Culture Temperature

To more closely mimic the body temperature of hibernating and active bears, we measured 2-DG uptake at 37ºC and 34.7ºC (Figure 4). In active season cells, 2-DG uptake was similar between mature adipocytes cultured at 37°C (114 ± 31.18 pmol) and 34.7°C (109.82 ± 32.60 pmol). Uptake was higher in hibernation cells cultured at 37°C (99.15 ± 63.04 pmol) compared to those grown at 34.7°C (-8.33 ± 36.21 pmol). However, due to high variability between individual bears, this difference did not reach statistical significance.

Use of Bear Serum in Culture

Differentiated cells obtained during the active and hibernation seasons were treated with insulin (300nM) in the presence of bear serum obtained at different times of the year (active, hyperphagic, and hibernation) (Figure 4). The results revealed a significant effect of serum type on glucose uptake [two-way ANOVA; $F(3,55) = 9.91$, $P < 0.0001$]. There was also a significant interaction between serum type and season during which the cells were obtained [two-way ANOVA; $F(3,55) = 7.76$, $P = 0.0002$]. For cells cultured with bear serum, adipocytes grown in

hyperphagic and active season serum had higher 2-DG uptake than those cultured in FBS for cells from both seasons. Active cells with hyperphagic serum and active serum had 229.82 ± 13.12 pmol and 307.83 ± 13.36 pmol higher 2-DG uptake respectively than active cells in FBS (Table 2). Uptake in hibernation cells with hyperphagic serum was 438.57 ± 22.46 pmol greater than cells of the same season cultured in FBS. Hibernation cells in active serum had 901.62 ± 21.95 pmol higher 2-DG uptake than hibernation cells in FBS. For active and hibernation cultures, cells matched with serum from the same season had significantly different uptake between seasons ($P = 0.0464$). There were also significant seasonal differences between cells grown in serum from the opposite season ($P = 0.0005$) (Figure 4). To determine if differences in 2-DG uptake were the result of variation in live cell concentrations, total RNA was compared between seasons and serum type. There were no significant differences in amount of RNA.

Gene expression of INSR, PTEN, and AKT1 was measured in active and hibernation cell cultures grown with all serum types. For INSR, there was a significant effect of serum type on response to insulin [two-way ANOVA; $F(3,56) = 3.37$, $P = 0.0248$]. There was also a significant interaction between cell season and serum type [two-way ANOVA; $F(3,56) = 7.65$, $P = 0.0002$]. For active season cells, the INSR response to insulin was greater following active and hyperphagic serum exposure compared to FBS and hibernation serum treatment (active vs. hibernation, $P = 0.0131$; active vs. FBS, $P = 0.0278$; hyperphagic vs. hibernation, $P = 0.0022$; hyperphagic vs. FBS, $P = 0.0052$) (Figure 6a). Active cells cultured with hyperphagic serum also had significantly greater INSR response than hibernation cells with active serum or FBS at the $P < 0.05$ level. We found no differences in insulin response among hibernation cells cultured in different sera. The response of PTEN to insulin did not differ significantly between cell seasons or sera. Expression of AKT1 in response to insulin was significantly influenced by cell season

[two-way ANOVA; $F(1,56) = 4.14$, P = 0.0467]. There was also a significant interaction between cell season and serum type on the response of AKT1 to insulin [two-way ANOVA; $F(3,56) = 8.17$, P = 0.0001]. Within the active season, cells grown in hyperphagic serum had significantly greater AKT1 response compared to cells exposed to hibernation serum (P = 0.0066). Hibernation cells cultured in hibernation serum exhibited a significantly higher AKT1 response compared to active cells with hibernation serum (P = 0.0002) and FBS (P = 0.0206). No differences were found for any serum exposure within hibernating cells (Figure 6b).

Baseline expression of INSR, PTEN, and AKT1 relative to hibernation cells cultured in FBS was significantly influenced by serum type [two-way ANOVA; INSR $F(3,55) = 9.39$, P < 0.0001; PTEN $F(3,55) = 6.00$, P = 0.0013; AKT1 $F(3,56) = 5.20$, P = 0.0031] . There was also a significant effect of cell season on AKT1 expression [two-way ANOVA; $F(1,56) = 8.70$, P = 0.0046] as well as a significant interaction between cell season and serum type [two-way ANOVA; $F(3,56) = 4.41$, P = 0.0075] (Figure 7). INSR expression for active season cells cultured with bear serum was significantly lower than in FBS cultures (active vs. FBS, P = 0.0021; hyperphagic vs. FBS, P = 0.0006; hibernation vs. FBS, P = 0.0228) (Figure 7a). For PTEN, all hibernation cells cultured in bear serum exhibited significantly lower baseline expression compared to FBS (active vs. FBS, P = 0.0319; hyperphagic vs. FBS, P = 0.0209; hibernation vs. FBS, P = 0.0366) (Figure 7b). Active season cells cultured with bear serum generally expressed lower levels of PTEN compared to FBS, but these differences did not reach statistical significance. Baseline expression of AKT1 was high in active season adipocytes grown with FBS compared to active cells cultured with active and hyperphagic serum (FBS vs. active, P = 0.0248; FBS vs. hyperphagic, P = 0.0063). All hibernation cells grown in bear serum also had significantly lower AKT1 expression at baseline than active season FBS cultures (FBS vs. active,

P = 0.0328; FBS vs. hyperphagic, P = 0.0231; FBS vs. hibernation, P = 0.0008) (Figure 7c). In the absence of insulin stimulation, active cells grown in hibernation serum also had high levels of AKT1 expression compared to active season cultures with hyperphagic serum (P = 0.0381) and hibernation cells matched with hibernation serum (P = 0.0054).

Preliminary Serum Purification

Heat inactivation and charcoal stripping were used to determine if the active substances in bear serum responsible for effects on glucose uptake were protein or non-protein. When compared to hibernation cells cultured in untreated active serum, 2-DG uptake (baseline subtracted) was reduced >50% in cells treated with heat-inactivated active serum whereas charcoal stripped serum only resulted in an approximate 21% reduction. Similarly, 2-DG uptake was approximately 38% less in FBS cultures compared to cells grown in active serum, although this did not attain statistical significance.

There was a significant effect of serum type on glucose usage, estimated from glucose concentrations of culture medium after a 48 hour incubation [Kruskal-Wallis test, P = 0.0008]. Glucose usage was significantly greater in hibernation cells grown with untreated active season serum (P = 0.0005) and charcoal-stripped active serum (P = 0.0217) compared to those grown in FBS (Figure 8) by 131.50 ± 9.01 mg/dL and 108.25 ± 8.60 mg/dL respectively. Active serum cultures utilized 81.13 ± 10.09 mg/dL more glucose than cells grown in heat-inactivated serum although this difference did not reach statistical significance. Regression analysis revealed a strong positive relationship between glucose uptake and estimated glucose usage measured in culture medium for cells treated with insulin (R^2 = 0.992) and for untreated cells (R^2 = 0.983).

DISCUSSION

Both whole animal and cell culture results revealed a naturally reversible state of insulin sensitivity characterized by resistance during hibernation and enhanced insulin responsiveness during the active season. Insulin sensitivity and glucose metabolism were altered under different culture and treatment conditions. Results from cultures exposed to bear serum suggest that serum factors play a central role in the seasonal metabolic changes observed, and preliminary serum purification experiments point to the importance of serum protein(s) in regulating glucose metabolism in adipocytes. Serum also affected expression of genes directly involved in the insulin signaling pathway including INSR and AKT1.

Bears were most responsive to insulin administration during the hyperphagic and active seasons while hibernating bears displayed a muted response to insulin. These findings corroborate results from black bears, which indicate delayed glucose absorption following insulin administration during hibernation (Palumbo et al. 1983). These results were supported by our findings from the oGTTs performed in unanesthetized bears. Blood glucose and insulin peaked at 15 minutes during May and October after which glucose disappeared from the blood quickly over the 120 minute time span. In contrast, during hibernation, glucose concentrations rose continuously throughout the sampling period, and insulin increased slowly over the two hour time span. This is likely due to inefficient gut absorption of glucose since insulin peaked at 30 minutes for all seasons during intravenous GTTs in anesthetized Japanese black bears (Kamine et al. 2012a). However, a rise in glucose concentrations despite elevated serum insulin in the unanesthetized bears supports a general state of peripheral insulin resistance leading to an absence of glucose clearance from the blood.

Together, results of ivITTs and oGTTs indicate that bears exhibit insulin resistance and impaired glucose metabolism during hibernation and regain sensitivity in the spring. They remain insulin sensitive, perhaps even more so, during the hyperphagic period when glucose storage and fat accumulation are at a maximum (Hilderbrand et al. 1999; Kingsley et al. 1983; Nelson et al. 1983). The transition between fatty acid and carbohydrate metabolism has been extensively studied in ground squirrels revealing severely depressed lipogenesis during hibernation (Tashima et al. 1970). Seasonal changes in lipogenic and lipolytic enzyme expression and activity have also been observed in the white adipose tissue of hibernating rodents revealing higher levels of lipolytic enzymes during hibernation (Bauer et al. 2001; Carey et al. 2003; Martin 2008; Mostafa et al. 1993; Wang et al. 1997; Wilson et al. 1992). In bears, insulin resistance during hibernation could inhibit the anti-lipolytic effects of insulin and facilitate lipolysis. This state would also prevent insulin from clearing glucose from the blood to maintain euglycemia seen in the unanesthetized hibernating bears.

Glucose and insulin curves in bears during the active and hyperphagic seasons generally followed trends observed in lean humans while hibernation profiles resembled those seen in individuals suffering from T2DM or with impaired glucose metabolism (Eriksson et al. 1989; Mari et al. 2001; Vaag et al. 1992). However, glucose levels increased more slowly and started at a lower baseline concentration in hibernating bears than in diabetic humans. Insulin also peaked and declined more rapidly in active bears than lean humans. Basal levels of glucose did not differ significantly between seasons, but insulin was found at higher concentrations (approximately 2.5-fold) during hibernation than in the active season which is comparable to the hyperinsulinemia seen in diabetic humans (approximately 2-fold) (Mari et al. 2001; Shanik et al. 2008). This contrasts with previous research in black bears, which found the highest serum

insulin concentrations in the fall (Kamine et al 2012a; Nelson et al. 1973; Palumbo et al. 1983) and in ground squirrels where insulin reached a maximum in August just prior to hibernation (Boswell et al. 1994; Tokuyama et al. 1991). However, our insulin measurements were obtained during different times of the year than those from Japanese black bears, which did not include a mid-hibernation (January and February) sample (Kamine et al. 2012a). Varying seasonal effects of different drug combinations could also have affected serum insulin in previous studies (Kamine et al. 2012b). Indeed, a recent study, using a large sample size of captive and wild bears, also found the highest insulin concentrations during hibernation, which is consistent with an insulin resistant state (McCain et al. 2013) and our current findings.

The hyperinsulinemia observed during hibernation may play an important role in the development of the insulin resistant state (Shanik et al. 2008). For example, transgenic mice expressing extra copies of the human insulin gene displayed reduced glucose clearance during ITTs along with a prolonged elevation in postprandial blood glucose (Marban and Roth 1996). One possible mechanism for this has been revealed in human lymphocytes and obese insulin resistant mice where chronic elevated insulin levels resulted in a reduced number of insulin receptors (Gavin et al. 1974). Although our results of INSR expression do not support this, the reduction in AKT1 in hibernation cells cultured with hibernation serum (no insulin) compared to active cells suggest a possible downstream influence.

Insulin insensitivity in hibernating bears may also be driven by the high levels of glucagon. Glucagon is a counterregulatory hormone that stimulates lipolysis and hepatic glucose production while blocking insulin's metabolic actions. Increased protein degradation and amino acid reutilization in hibernating bears (Barboza et al. 1997) could drive the high levels of glucagon observed (Pipeleers et al. 1985; Rocha et al. 1972). The absolute level of glucagon as

well as the ratio of circulating glucagon to insulin are very important for maintaining normal blood glucose in healthy individuals, and both are elevated with T2DM (Bratusch-Marrain 1983; Jiang and Zhang 2003; Unger 1978). In contrast, we found that the glucagon to insulin ratio in bears was lower during hibernation compared to the active and hyperphagic seasons by factors of 2 and 1.5 respectively due in part to the increased insulin secretion in hibernating bears. In bears, high levels of circulating glucagon and insulin together may facilitate maintenance of normal blood glucose during fasting and contribute to the observed insulin resistant state. Peripheral insulin resistance combined with possible enhanced glucagon sensitivity in hibernating bears could stimulate lipolysis in subcutaneous adipose tissue leading the release of glycerol, an important substrate for gluconeogenesis. Glucagon also stimulates glycogenolysis and gluconeogenesis in the liver (Exton and Park 1968; Stevenson et al. 1987) potentially accounting for the euglycemia observed in hibernating bears. Glucagon-stimulated FFA and glucose release could provide a significant fuel source for muscle and brain tissue throughout the winter fast (Figure 9).

The metabolic transitions that occur in grizzly bears throughout the year may be modulated by neural and/or gastrointestinal mechanisms responding to food availability. Indeed, we found evidence suggesting that the insulin resistance characteristic of hibernating bears may be reversible with prolonged feeding. Repeated dextrose administration over the span of several weeks during hibernation caused blood glucose in the unanesthetized bears to decline after 60 minutes, rather than remain elevated. When feeding was discontinued and bears were fasted for approximately two weeks, the response to oral dextrose administration again resembled that observed in an insulin resistant state (i.e., blood glucose rose continuously; Rigano unpublished observations). This observation is not entirely surprising given that captive bears will continue to

eat (although with a greatly reduce appetite) during the winter (Hill 2013; McCain et al. 2013), unlike marmots which will not eat throughout winter even when presented with food (Florant et al. 2010). Male bears on Kodiak Island have also been reported to forgo denning during winter months likely due to a prolonged period of food availability during the year (Van Daele et al. 1990). These bears appeared to be in state of "walking hibernation" described by Nelson et al. (1983) in which they were active yet exhibited suppressed metabolic activity and decreased food intake. These seasonally-specific metabolic changes are further supported by observations that fasted bears in the active season do not develop the fat-based metabolism found in hibernating bears and catabolize lean mass for energy similarly to humans and nonhibernating mammals in a fasted state (Nelson et al. 1975).

Investigation into specific endogenous factors affecting seasonal states of insulin responsiveness indicate that adipocytes provide a viable *in vitro* system to examine this in bears, but only when cultured with serum from the matching season. Hibernation cells grown in FBS and treated with supraphysiological doses of insulin (≥ 500 nM) displayed the greatest response in glucose uptake compared to hyperphagic or active season cells. This exaggerated response to insulin is discordant with the insulin resistance exhibited in hibernating bears *in vivo*. Thus, the hibernation physiology of adipocytes also appears to be reversible under conditions of intense lipogenic stimulation. Temperature had no significant effect on cellular response to insulin suggesting a minimal role of decreased body temperature on inducing hibernation physiology in bear adipocytes. This is supported by the observation that metabolic suppression in black bears was also independent of temperature changes (Tøien et al. 2011). Thus, endogenous serum factors appear to be extremely important in the regulation of seasonal transitions in insulin sensitivity.

Active season serum had the most striking effect on glucose uptake *in vitro*. In hibernation derived adipocyte cultures, there was an approximate 1000-fold difference in glucose uptake in response to insulin between serum matched and serum opposite groups. Hibernation cells cultured with active season serum had the highest glucose uptake values of any cell season and serum combination. Hibernation serum caused a reduction in glucose uptake compared to active serum in cells originating from the active season, although the decrease was less pronounced. The effects of serum factors from hibernating animals have been demonstrated in ground squirrels where hibernation was induced in active animals by the serial transfusion of serum from hibernating individuals (Dawe and Spurrier 1969; Spurrier et al. 1976). Summer serum also inhibited hibernation in the fall when ground squirrels would typically begin fasting (Spurrier et al. 1976). Serum from hibernating black bears and polar bears at different times of the year had a similar effect causing the onset of hibernation in ground squirrels (Bruce et al. 1990; Rutt et al. 1987), while November polar bear serum induced hyperphagia (Bruce et al. 1990). However, other studies were not able to replicate this effect with intraspecies transfusions in ground squirrels or with European brown bear (*Ursus arctos arctos*) serum in Djungarian hamsters (*Phodopus sungorus*) and rats (Karjalainen et al. 1994; Wang et al. 1988).

Here, we have demonstrated the pronounced effect of grizzly bear serum on glucose metabolism in fat cells. Serum from the active and hyperphagic seasons caused increased glucose uptake in response to insulin in both active and hibernation cells suggesting these sera possibly contain increased levels of insulin sensitizing agents compared to serum from hibernating bears. The response to insulin was slightly reduced in charcoal-stripped serum cultures compared to untreated active serum cultures suggesting steroids such as thyroxine or glucocorticoids may be involved in the initiation of the insulin response. Elevated levels of FFAs found in hibernation

serum (Ahlquist et al. 1984; Hellgren et al. 1993; Nelson et. al. 1973) are commonly associated with insulin resistance (Delarue and Magnan 2007; Shulman 2004; Wilcox 2005). FFAs inhibit glucose uptake by preventing glucose transport or phosphorylation activity (Roden et al. 1996).

However, adipocytes cultured with heat-inactivated serum had dramatically lower glucose uptake in response to insulin than cultures grown in untreated active season serum indicating that these changes are being driven by serum protein. Bruce et al. (1990) also found that hibernation was triggered by a factor in the albumin fraction of polar bear serum. The adipocyte produced hormone leptin has been found to vary seasonally in bears with lower levels during hibernation compared to the active season and spiking prior to the onset of hibernation (Gardi et al. 2011; Gehring et al. in prep; Hissa et al. 1998; Tsubota et al. 2008). Leptin production is positively correlated with adiposity (Havel et al. 1996), and prolonged exposure to leptin decreases insulin stimulated glucose uptake and lipogenesis (Harris 2014; Muller et al. 1997). Thus, the rise in leptin in the fall may promote insulin resistance in hibernating bears. Apolipoprotein A-IV (apoA-IV) has a significant role in regulating glucose homeostasis and is downregulated during hibernation (Chow et al. 2013; Morgenstern et al. unpublished observations). ApoA-IV knockout mice exhibited impaired glucose tolerance (Wang et al. 2012) consistent with the metabolic state observed in unanesthetized hibernating grizzly bears and adipocytes when cultured with serum from hibernating bears. Recently insulin growth factor binding protein-2 (IGFBP-2) and enzymes involved in the production of reactive oxygen species (ROS) were also found to be elevated in bear serum during hibernation (Blumenthal et al. 2011; Morgenstern et al. unpublished observations). The balance between these peptides may be vital to maintaining the euglycemia observed in insulin resistant bears during hibernation since IGFBP-2 has been shown to prevent insulin resistance and glucose intolerance (Hedbacker et al.

2010; Wheatcroft et al. 2007) while ROS have been implicated in the development of insulin insensitivity (Bashan et al. 2009).

Serum treatments did not account for all of the variation in adipocyte glucose metabolism. There were seasonal differences in insulin response of glucose uptake and AKT1 expression in cells treated with the same type of bear serum. Active cells treated with hibernation serum maintained some sensitivity to insulin while hibernation cells cultured with matched seasonal serum were unresponsive to insulin treatment based on glucose uptake results. Active season serum in hibernation cells caused a prominent spike in glucose uptake compared to active cells with active serum. This indicates that cells derived from active and hibernating bears are inherently different and respond differently to the same serum and treatment conditions. Thus, glucose metabolism and insulin response are likely regulated by a combination of cell autonomous and serum factors.

Hibernation evolved multiple times across a wide variety of mammalian species while the insulin signaling pathway has been highly conserved. Thus, the regulation of this process is likely controlled by differential gene expression as opposed to novel proteins (Martin 2008; Srene et al. 1992). Measures of PPARG and INSR expression in undifferentiated compared to differentiated cells support conserved glucose uptake and lipogenic pathways in grizzly bears and point to the importance of these genes in stimulating adipocyte differentiation. However, lower expression of INSR and PTEN in cells cultured with bear serum compared to FBS suggests bears operate at much lower levels of circulating insulin and higher levels of sensitivity than other species (Lee et al. 2003; Shanik et al. 2008). The large response of INSR to insulin stimulation in active cultures grown with active and hyperphagic serum is consistent with the glucose uptake results indicating high sensitivity of these cells to insulin. However, hibernation

cells grown in active season serum did not exhibit a proportional increase in INSR following insulin stimulation even though they had the greatest change in glucose uptake. There were also no seasonal differences in baseline INSR expression. Thus, the regulation of insulin sensitivity is likely occurring via a post receptor mechanism.

AKT1 expression did vary with respect to cell season and serum although fold changes were relatively small compared to those observed in expression of other lipogenic enzymes following insulin stimulation in mouse 3T3-L1 cells (O'Brien and Granner 1991; Wang and Sul 1998; Weiner et al. 1991). The AKT1 response to insulin in active season cells was greatest in cells treated with active and hyperphagic serum. These cells also displayed the highest levels of glucose uptake indicating that AKT1 is likely an important factor involved in mediating this response to insulin in bear adipocytes. However, the greatest increase in AKT1 took place in hibernation cells treated with hibernation serum which exhibited minimal changes in glucose uptake following insulin treatment. Thus, the relationship between changes in AKT1 and the observed adipocyte insulin response is unclear.

The high response of AKT1 expression in cells which exhibit an insulin resistant phenotype conflicts with studies indicating suppressed AKT activity in humans with T2DM (Carvalho et al. 2000; Smith 2002). Decreased activation of AKT has also been documented in hibernating mammals (Eddy and Storey 2003; Hoehn et al. 2004; Lee et al. 2002). Although activation of AKT was upregulated in adipose tissue of marmots during the summer, expression of AKT did not change seasonally (Hoehn et al. 2004). Insulin has also been shown to increase efficiency of mRNA translation resulting in elevated protein levels without an increase in total mRNA (O'Brien and Granner 1991). Thus, expression levels measured in the current study may not mirror seasonal activity of AKT in grizzly bear adipocytes. Changes in AKT1 expression

may also reflect variation in other signaling pathways mediated by AKT1. At baseline (no insulin), AKT1 expression was greatest in active season cells grown with hibernation serum and FBS and lowest in hibernation cells with hibernation serum. This cell-specific discrepancy between adipocytes exposed to the same serum type may be the result of seasonal upregulation of other signaling events mediated by AKT1 such as immune response or cell proliferation (Brazil et al. 2004; Datta et al. 1997; Kandel et al. 2002; Okkenhaug and Vanhaesebroeck 2003; Weichhart and Säemann 2008). Thus, high AKT1 expression in active cells could indicate that these cells are primed for enhanced protein synthesis, survival, and proliferation.

Further work is needed to identify the serum factors involved in seasonal transitions in insulin sensitivity and determine the mechanisms by which insulin signaling is regulated in grizzly bears. Examining protein activity may be more important than gene expression in understanding these mechanisms. The downstream target GLUT4 might be a more appropriate indicator of insulin sensitivity than AKT because both protein and mRNA levels are depressed in fat cells from subjects with impaired insulin signaling (Carlson et al. 2003; Smith 2002). Differences in insulin sensitizing agents such as PPARG between bear serum cultures may also provide mechanistic insight into metabolic regulation during hibernation since activation of pyruvate dehydrogenase kinase isoenzyme 4 (PDK4) via PPARG was proposed as an important means of switching to fatty acid oxidation in hibernating ground squirrels (Buck et al. 2002).

The results presented here identify several physiological and adipocyte-specific seasonal changes in metabolism of grizzly bears. Specifically, transitions in insulin sensitivity and glucose tolerance facilitate a switch from a carbohydrate metabolism and lipogenesis to a lipolytic metabolism during hibernation. Hibernation physiology may be in part reversible with high glucose levels and lipogenic stimulation. The most important regulator of insulin sensitivity in

grizzly bears is a seasonally specific serum protein, but there are also autonomous differences between hibernation and active season derived adipocytes. Our research provides a strong foundation for understanding seasonal cycles in grizzly bear glucose metabolism and extends our understanding of bear hibernation physiology. The identification of a serum factor responsible for this regulation and the mechanisms by which it operates may lead to advances in our understanding of human metabolic disorders.

LITERATURE CITED

AHLQUIST, D.A., NELSON, R.A., STEIGER, D.L., JONES, J.D., and ELLEFSON, R.B. 1984. Glycerol metabolism in the hibernating black bear. Journal of Comparative Physiology B 155(1):5-79.

ATKINSON, S.N. and RAMSAY, M.A. 1995. The effect of prolonged fasting on the body composition and reproductive success of female polar bears (*Ursus maritimus*). Functional Ecology 9:559-567.

BARBOZA, P.S., FARLEY, S.D., and ROBBINS, C.T. 1997. Whole-body urea cycling and protein turnover during hyperphagia and dormancy in growing bears (*Ursus americanus* and *U. arctos*). Canadian Journal of Zoology 75:2129-2136.

BARNES, B.M. 1989. Freeze avoidance in a mammal: body temperatures below 0ºC in an Arctic hibernator. Science 244(4912):1593-1595.

BASHAN, N., KOVSAN, J., KACHKO, I., OVADIA, H., and RUDICH, A. 2009. Positive and negative regulation of insulin signaling by reactive oxygen and nitrogen species. Physiological Reviews 89(1):27-71.

BAUER, V.W., SQUIRE, T.L., LOWE, M.E., and ANDREWS, M.T. 2001. Expression of a chimeric retroviral-lipase mRNA confers enhanced lipolysis in a hibernating mammal. American journal of physiology. Regulatory, integrative and comparative physiology 281:R1186-R1192.

BELANT, J.L., KIELLAND, K., FOLLMANN, E.H., and ADAMS, L.G. 2006. Interspecific resource partitioning in sympatric ursids. Ecological Applications 16(6):2333-2343.

BOSWELL, T., WOODS, S.C., and KENAGY, G.J. 1994. Seasonal changes in body mass,

insulin, and glucocorticoids of free-living golden-mantled ground squirrels. General and Comparative Endocrinology 96(3):339-346.

BLUMENTHAL, S., MORGAN-BOYD, R., NELSON, R., GARSHELIS, D.L., TURYK, M.E., AND UNTERMAN, T. 2011. Seasonal regulation of the growth hormone-insulin-like growth factor-I axis in the American black bear (*Ursus americanus*). American Journal of Physiology - Endocrinology and Metabolism 301(4):E628-E636.

BRATUSCH-MARRAIN, P.R. 1983. Insulin-counteracting hormones: Their impact on glucose metabolism. Diabetologia 24(2):74-79.

BRAZIL, D.P., YANG, Z., and HEMMINGS, B.A. 2004. Advances in protein kinase B signaling: AKTion on multiple fronts. Trends in Biochemical Sciences 29(5):233-242.

BRUCE, D.S., DARLING, N.K., SEELAND, K.J., OELTGEN, P.R., NILEKANI, S.P., and AMSTRUP, S.C. 1990. Is the polar bear (*Ursus maritimus*) a Hibernator?: continued studies on opioids and hibernation. Pharmacology Biochemistry and Behavior 35(3):705-711.

BUCK, M.J., SQUIRE, T.L., and ANDREWS, M.T. 2002. Coordinate expression of the PDK4 gene: a means of regulating fuel selection in a hibernating mammal. Physiological Genomics 8:5-13.

CARLSON, C.J., KOTERSKI, S., SCIOTTI, R.J., POCCARD, G.B., and RONDINONE, C.M. 2003. Enhanced basal activation of mitogen-activated protein kinases in adipocytes from Type 2 diabetes - potential role of p38 in the downregulation of GLUT4 expression. Diabetes 52(3):634-641.

CAREY, H.V., ANDREWS, M.T., and MARTIN, A.L. 2003. Mammalian hibernation: Cellular

and molecular responses to depressed metabolism and low temperature. Physiological Reviews 83:1153-1181.

CARVALHO, E., ELIASSON, B., WESSLAU, C., and SMITH, U. 2000. Impaired phosphorylation and insulin-stimulated translocation to the plasma membrane of protein kinase B/AKT in adipocytes from Type II diabetic subjects. Diabetologia 43(9):1107-1115.

CHAVEZ, J.A., KNOTTS, T.A., WANG, L., LI, G., DOBROWSKY, R.T., FLORANT, G.L., and SUMMERS, S.A. 2003. A role for ceramide, but not diacylglycerol, in the antagonism of insulin signal transduction by saturated fatty acids. The Journal of Biological Chemistry 278:10297-10303.

CHO, H. et al. 2001. Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase AKT2 (PKBβ). Science 292(5522):1728-1731.

CHOW, B.A., DONAHUE, S.W., VAUGHAN, M.R., MCCONKEY, B. and VIJAYAN, M.M. 2013. Serum immune-related proteins are differentially expressed during hibernation in the American black bear. PLOS ONE 8(6):e66119.

DAHLE, B., ZEDROSSER, A. and SWENSON, J.E. 2006. Correlates with body size and mass in yearling brown bears (*Ursus arctos*). Journal of Zoology 269:273-283.

DATTA, R.S., DUDEK, H., TAO, X., MASTERS, S., FU, H., GOTOH, Y., and GREENBERG, M.E. 1997. AKT phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91:231-241.

DAWE, A.R. and SPURRIER, W.A. 1969. Hibernation induced in ground squirrels by blood transfusion. Science 163(3864):298-299.

DELARUE, J. and MAGNAN, C. 2007. Free fatty acids and insulin resistance. Current Opinion

in Clinical Nutrition and Metabolic Care 10:142-148.

DEROCHER, A. and STIRLING, I. 1996. Aspects of survival in juvenile polar bears. Canadian Journal of Zoology 74(7):1246-1252.

EDDY, S.F. and STOREY, K.B. Differential expression of AKT, PPARγ, and PGC-1 during hibernation in bats. Biochemistry and Cell Biology 81:269-274.

ERIKSSON, J., FRANSSILA-KALLUNKI, A., EKSTRAND, A., SALORANTA, C., WIDÉN, E., SCHALIN, C., and GROOP, L. 1989. Early metabolic defects in persons at increased risk for non-insulin-dependent diabetes mellitus. The New England Journal of Medicine 321(6):337-343.

ERLENBACH, J. A., K. D. RODE, D. RAUBENHEIMER, AND C. T. ROBBINS. 2014. Macronutrient optimization and energy maximization determine diets of brown bears. Journal of Mammalogy 95:160-168.

EXTON, J.H. and PARK, C.R. 1968. Control of gluconeogenesis in liver. The Journal of Biological Chemistry 243(16):4189-4196.

FLORANT, G.L., FENN, A.M., HEALY, J.E., WILKERSON, G.K., and HANDA, R.J. 2010. To eat or not to eat: the effect of AICAR on food intake regulation in yellow-bellied marmots (*Marmota flaviventris*). The Journal of Experimental Biology 213:2031-2037.

FLORANT, G.L., LAWRENCE, A.K., WILLIAMS, K., and BAUMAN, W.A. 1985.Seasonal changes in pancreatic B-cell function in euthermic yellow-bellied marmots. American Journal of Physiology 249:R159–R165.

FOLK JR., G.E., FOLK, M.A., and MINOR, J.J. 1972. Physiological condition of three species of bears in winter dens. Bears: Their Biology and Management 2:107-124.

FREIDENBERG, G.R., REICHART, D.R., OLEFSKY, J.M., and HENRY, R.R. 1988.

Reversibility of defective adipocyte insulin receptor kinase activity in non-insulin-dependent diabetes mellitus: Effect of weight loss. The Journal of Clinical Investigation 82:1398–1406.

GARDI, J., NELSON, O.L., ROBBINS, C.T., SZENTIRMAI, E., KAPÁS, L., and KRUEGER, J.M. 2011. Energy homeostasis regulatory peptides in hibernating grizzly bears. General and Comparative Endocrinology 172(1):181-183.

GAVIN, J.R. III, ROTH, J., NEVILLE, D.M. JR., DE MEYTS, P., and BUELL, D.N. 1974. Insulin-dependent regulation of insulin receptor concentrations: A direct demonstration in cell culture. Proceedings of the National Academy of Sciences 71(1):84-88.

GOODYEAR, L.J., GIORGINO, F., SHERMAN, L.A., CAREY, J., SMITH, R.J., and DOHM, G.L. 1995. Insulin receptor phosphorylation, insulin receptor substrate-1 phosphorylation, and phosphatidylinositol 3-kinase activity are decreased in intact skeletal muscle strips from obese subject. The Journal of Clinical Investigation 95:2195-2204.

GEORGE et al. 2004. A family with severe insulin resistance and diabetes mellitus due to a missense mutation in AKT2. Science 304(5675):1325-1328.

GERMAN, A.J., HERVERA, M., HUNTER, L., HOLDEN, S.L., MORRIS, P.J., BIOURGE, V., and TRAYHURN, P. 2009. Improvement in insulin resistance and reduction in plasma inflammatory adipokines after weight loss in obese dogs. Domestic Animal Endocrinology 37:214-226.

GERMAN, A.J., RYAN, V.H., GERMAN, A.C., WOOD, I.S., and TRAYHURN, P. 2010. Obesity, its associated disorders and the role of inflammatory adipokines in companion animals. The Veterinary Journal 185:4-9.

HAJER, G. R., VANHAEFTEN, T. W., and VISSEREN, F. L. 2008. Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. European Heart Journal 29:2959-2971.

HARRIS, R.B.S. 2014. Direct and indirect effects of leptin on adipocyte metabolism. Biochimica et Biophysica Acta 1842:414–423.

HAVEL, P. J., KASIM-KARAKAS, S., MUELLER, W., JOHNSON, P.R., GINGERICH, R.L., and STERN, J.S. 1996. Relationship of plasma leptin to plasma insulin and adiposity in normal weight and overweight women: effects of dietary fat content and sustained weight loss. The Journal of Clinical Endocrinology & Metabolism 81(12):4406-4413.

HEDBACKER, K., BIRSOY, K., WYSOCKI, R.W., ASILMAZ, E., AHIMA, R.S., FAROOGI, I.S., and FRIEDMAN, J.M. 2010. Antidiabetic effects of IGFBP2, a leptin-regulated gene. Cell Metabolism 11(1):11-22.

HELLGREN, E.C. 1998. Physiology of hibernation in bears. Ursus 10:467-477.

HELLGREN, E.C., ROGERS, L.L., and SEAL, U.S. 1993. Serum chemistry and hematology of black bears: physiological indices of habitat quality or seasonal patterns? The Journal of Mammalogy 74:304-315.

HERS, I., VINCENT, E.E., and TAVARÉ, J.M. 2011. AKT signalling in health and disease. Cellular Signalling 23:1515-1527.

HILDERBRAND, G.V., JENKINS, S.G., SCHWARTZ, C.C., HANLEY, T.A., and ROBBINS C.T. 1999. Effect of seasonal differences in dietary meat intake on changes in body mass and composition in wild and captive brown bears. Canadian Journal of Zoology 77:1623-1630.

HILL, E.M. 2013. Seasonal changes in white adipose tissue in American black bears (*Ursus americanus*). Master's Thesis, University of Tennessee, Knoxville, 93 p.

HISSA, R., HOHTOLA, E., TUOMALA-SARAMÄKI, T., LAINE, T., and KALLIO, H. 1998. Seasonal changes in fatty acids and leptin contents in the plasma of the European brown bear (*Ursus arctos arctos*). Annales Zoologici Fennici 35:215-224.

HISSA., SIEKKINEN J., HOHTOLA E., SAARELA S., HAKALA A., and PUDAS J. 1994. Seasonal patterns in the physiology of the European brown bear (*Ursus arctos arctos*) in Finland. Comparative Biochemistry and Physiology A - Comparative Physiology 109:781-791.

HOEHN, K.L., HUDACHEK, S.F., SUMMERS, S.A., and FLORANT, G.L. 2004. Seasonal, tissue-specific regulation of AKT/protein kinase B and glycogen synthase in hibernators. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 286(3):R498-R504.

HOENIG, M., THOMASETH, K., WALDRON, M., and FERGUSON, D.C. 2007. Insulin sensitivity, fat distribution, and adipocytokine response to different diets in lean and obese cats before and after weight loss. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 292:R227-R234.

JIANG, G. and ZHANG, B.B. 2003. Glucagon and regulation of glucose metabolism. American Journal of Physiology – Endocrinology and Metabolism 284(4):E671-E678.

KAMINE, A., SHIMOZURU, M., SHIBATA, H. and TSUBOTA, T. 2012a. Changes in blood glucose and insulin responses to intravenous glucose tolerance tests and blood biochemical values in adult female Japanese black bears (*Ursus thibetanus japonicus*). Japanese Journal of Veterinary Research 60(1):5-13.

KAMINE, A., SHIMOZURU, M., SHIBATA, H. and TSUBOTA, T. 2012b.Effects of

intramuscular administration of tiletamine-zolazepam with and without sedative pretreatment on plasma and serum biochemical values and glucose tolerance test results in Japanese black bears (*Ursus thibetanus japonicus*). American Journal of Veterinary Research 73(8):1282-1289.

KANDEL, E.S., SKEEN, J., MAJEWSKI, N., CRISTOFANO, A.D., PANDOLFI, P.P., FELICIANO, C.S., GARTEL, A., and HAY, N. 2002. Activation of AKT/protein kinase B overcomes a G_2/M cell cycle checkpoint induced by DNA damage. Molecular and Cellular Biology 22(22):7831-7841.

KARJALAINEN, M., HOHTOLA, E., and HISSA, R. 1994. No metabolic suppression in the djungarian hamster or rat by injections of plasma from the winter-sleeping brown bear. Journal of Thermal Biology 19(5):321-325.

KINGSLEY, M.C.S, NAGY J.A., and RUSSELL R.H. 1983. Patterns of weight gain and loss for grizzly bears in northern Canada. Bears: Their Biology and Management 2:174-178.

KIM, Y., PERONI, O.D., FRANKE, T.F., and KAHN, B.B. 2000. Divergent regulation of AKT1 and AKT2 isoforms in insulin target tissues of obese Zucker rats. Diabetes 49:847-856.

LE MARCHAND-BRUSTEL, Y., GUAL, P., GRÉMEAUX, T., GONZALEZ, T., BARRÈS, R. and TANTI, J.F. 2003. Fatty acid-induced insulin resistance: role of insulin receptor substrate 1 serine phosphorylation in the retroregulation of insulin signalling. Biochemical Society Transactions 31:1152-1156.

LEE, J.H., CHAN, J.L., YIANNAKOURIS, N., KONTOGIANNI, M., ESTRADA, E., SEIP, R., ORLOVA, C., and MANTZOROS, C.S. 2003. Circulating resistin levels are not associated with obesity or insulin resistance in humans and are not regulated by fasting or leptin administration: Cross-sectional and interventional studies in normal, insulin-

resistant, and diabetic subjects. The Journal of Clinical Endocrinology & Metabolism 88(10):4848-4856.

LEE, M., CHOI, I., and PARK, K. 2002.Activation of stress signaling molecules in bat brain during arousal from hibernation. Journal of Neurochemistry 82(4):867-873.

LEWIS, G. F., CARPENTIER, A., ADELI, K., and GIACCA, A. 2002. Disordered fat storage and mobilization in the pathogenesis of insulin resistance and type 2 diabetes. Endocrine Reviews 23(2):201-229.

MALNICK, S.D. and KNOBLER H. 2006. The medical complications of obesity. QJM. 99(9): 565-579.

MARBAN, S.L. and ROTH, J. 1996. Transgenic hyperinsulinemia: A mouse model of insulin resistance and glucose intolerance without obesity. In: Shafrir, E. (ed) Lessons from Animal Diabetes VI. Sixth Edition. Birkhauser, Boston, Massachusetts, pp 201-224.

MARI, A., PACINI, G., MURPHY, E., LUDVIK, B., and NOLAN, J.J. 2001. A model-based method for assessing insulin sensitivity from the oral glucose tolerance test. Diabetes Care 24(3):539-548.

MARTIN, S.L. 2008. Mammalian hibernation: a naturally reversible model for insulin resistance in man? Diabetes and Vascular Disease Research 5(2):76-81.

MCCAIN, S., RAMSAY, E., and KIRK, C. 2013. The effects of hibernation and captivity on glucose metabolism and thyroid hormones in American black bear (*Ursus americanus*). Journal of Zoo and Wildlife Medicine 44(2):324–332.

MOSTAFA, N., EVERETT, D.C., CHOU, S.C., KONG, P.A., FLORANT, G.L., and

COLEMAN, R.A. 1993. Seasonal changes in critical enzymes of lipogenesis and triacylglycerol synthesis in the marmot (*Marmota flaviventris*) Journal of Comparative Physiology B 163(6):463-469.

MÜLLER, G., ERTL, J., GERL, M., and PREIBISCH, G. 1997. Leptin impairs metabolic actions of insulin in isolated rat adipocytes. The Journal of Biological Chemistry 272: 10585-10593.

NELSON, R.A. 1973. Winter sleep in the black bear: a physiologic and metabolic marvel. Mayo Clinic Proceedings 48:733-737.

NELSON, R.A., JONES, J.D., WAHNER, H.W., MCGILL, D.B., and CODE, C.F. 1975. Nitrogen metabolism in bears: urea metabolism in summer starvation and in winter sleep and role of urinary bladder in water and nitrogen conservation. Mayo Clinic Proceedings 50(3):141-146.

NELSON, R.A., WAHNER, H.W., JONES, J.D., ELLEFSON, R.D., and ZOLLMAN, P.E. 1973. Metabolism of bears before, during, and after winter sleep. American Journal of Physiology 224(2):491-496.

NELSON, O.L., MCEWEN, M., ROBBINS, C.T., FELICETTI, L., and CHRISTENSEN, W.F. 2003. Evaluation of cardiac function in active and hibernating grizzly bears. Journal of the American Veterinary Medical Association. 223(8):1170-1175.

NELSON, O.L. and ROBBINS, C.T. 2010. Cardiac function adaptations in hibernating grizzly bears (*Ursus arctos horribilis*). Journal of Comparative Physiology B - Biochemical, Systemic, and Environmental Physiology 180:465–473.

NELSON, R.A., FOLK JR., G.E., PFEIFFER, E.W., CRAIGHEAD, J.J., JONKEL, C.J., and

STEIGER, D.L. 1983. Behavior, biochemistry, and hibernation in black, grizzly, and polar bears. Bears: Their Biology and Management 5:284-290.

O'BRIEN, R. M. and GRANNER, D.K. 1991. Regulation of gene expression by insulin. Biochemical Journal 278:609-619.

OLEFSKY, J.M. 1976. Decreased insulin binding to adipocytes and circulating monocytes from obese subjects. The Journal of Clinical Investigation 57:1165-1172.

OKKENHAUG, K. and VANHAESEBROECK, B. 2003. PI3K in lymphocyte development, differentiation, and activation. Nature Reviews – Immunology 3:317-330.

PAL, A., BARBER, T.M., VAN DE BUNT, M., RUDGE, S.A., ZHANG, Q., LACHLAN, K.L., COOPER, N.S., LINDEN, H., LEVY, J.C., WAKELAM, M.J., et al. 2012. PTEN mutations as a cause of constitutive insulin sensitivity and obesity. The New England Journal of Medicine 367:1002-1011.

PALUMBO, P.J., WELLIK, D.L., BAGLEY, N.A., and NELSON, R.A. 1983. Insulin and glucagon responses in the hibernating black bear. Bears: Their Biology and Management 5:291-296.

PIPELEERS, D.G., SCHUIT, F.C., VAN SCHRAVENDIJK, C.F., and VAN DE WINKEL, M. 1985. Interplay of nutrients and hormones in regulation of glucagon release. Endocrinology 117(3):817-823.

PI-SUNYER, F.X. 2002. The obesity epidemic: pathophysiology and consequences. Obesity research 10(2):97-104.

PESSIN, J.E., THURMOND, D.C., ELMENDORF, J.S., COKER, K.J., and OKADA, S. 1999. Molecular basis of insulin-stimulated GLUT4 vesicle trafficking: Location! Location! Location! The Journal of Biological Chemistry 274(5):2593-2596.

REAVEN, G. 2004.The metabolic syndrome or the insulin resistance syndrome? Different names, different concepts, and different goals. Endocrinology and Metabolism Clinics of North America 33(2):283-303.

ROCHA, D.M., FALOONA, G.R., and UNGER, R.H. 1972. Glucagon-stimulating activity of 20 amino acids in dogs. The Journal of Clinical Investigation 51:2346-2351.

RODEN, M., PRICE, T.B., PERSEGHIN, G., PETERSEN, K.F., ROTHMAN, D.L., CLINE, G.W., and SHULMAN, G.I. 1996. Mechanism of free fatty acid-induced insulin resistance in humans. The Journal of Clinical Investigation 97: 2859-2865.

ROBBINS C.T., BEN-DAVID M., FORTIN J.K., and NELSON O.L. 2012. Maternal condition determines birth date and growth of newborn bear cubs. Journal of Mammalogy 93(2):540-546.

RUTT, K.A., BRUCE, D.S., CHIANG, P.P., OELTGEN, P.R., WELBORN, J.R., and NILEKANI, S.P. 1987. Summer hibernation in ground squirrels (*Citellus tridecemlineatus*) induced by injection of whole or fractionated plasma from hibernating black bears (*Ursus americanus*). Journal of Thermal Biology 12(2):135-138.

SCHMITZ-PEIFFER, C., CRAIG, D.L., and BIDEN, T.J. 1999. Ceramide generation is sufficient to account for the inhibition of insulin-stimulated PKB pathway in C2C12 skeletal muscle cells pretreated with palmitate. The Journal of Biological Chemistry 274(34):24202-24210.

SHANIK, M.H., XU, Y., ŠKRHA, J., DANKNER, R., ZICK, Y., and ROTH, J. 2008. Insulin resistance and hyperinsulinemia - Is hyperinsulinemia the cart or the horse? Diabetes Care 31(2):S262-S-268. .

SHULMAN, G.I. 2004. Unraveling the cellular mechanism of insulin resistance in humans- New

insights from magnetic resonance spectroscopy. Physiology 19:183-190.

SIKES, R. S., W. L. GANNON, AND THE ANIMAL CARE AND USE COMMITTEE OF THE AMERICAN SOCIETY OF MAMMALOGISTS. 2011. Guidelines of the American Society of Mammalogists for the use of wild mammals in research. Journal of Mammalogy 92:235-253.

SMITH, U. 2002. Impaired ('diabetic') insulin signaling and action occur in fat cells long before glucose intolerance - is insulin resistance initiated in the adipose tissue? International Journal of Obesity 26:897-904.

SPURRIER, W.A., FOLK, G.E. JR., and DAWE, A.R. 1976. Induction of summer hibernation in the 13-lined ground squirrel shown by comparative serum transfusions from Arctic mammals. Cryobiology 13(3):368-374.

SRENE, H.K., WANG, L.C., and MARTIN, S.L. 1992. Central role for differential gene expression in mammalian hibernation. Proceedings of the National Academy of Sciences 89(15):7119-7123

STENVINKEL, P., JANI, A.H., and JOHNSON, R.J. 2012. Hibernating bears (Ursidae): metabolic magicians of definite interest for the nephrologist. Kidney International 83:207-212.

STEVENSON, R.W., STEINER, K.E., DAVIS, M.A., HENDRICK, G.K., WILLIAMS, P.E., LACY, W.W., BROWN, L., DONAHUE, P., LACY, D.B., and CHERRINGTON, A.D. 1987. Similar dose responsiveness of hepatic glycogenolysis and gluconeogenesis to glucagon *in vivo*. Diabetes 36(3):382-389.

TASHIMA, L.S., ADELSTEIN, S.J., and LYMAN, C.P. 1970. Radioglucose utilization by

active, hibernating, and arousing ground squirrels. American Journal of Physiology 218:303-309.

TØIEN, Ø., BLAKE, J., EDGAR, D.M., GRAHN, D.A., HELLER, H.C., and BARNES, B.M. 2011. Hibernation in black bears: Independence of metabolic suppression from body temperature. Science 331:906-909.

TOKUYAMA, K., GALANTINO, H.L., GREEN, R., and FLORANT, G.L. 1991. Seasonal glucose uptake in marmots (*Marmota flaviventris*): The role of pancreatic hormones. Comparative Biochemistry and Physiology Part A: Physiology 100(4):925-930.

TSUBOTA, T., SATO, M., OKANO, T., NAKAMURA, S., ASANO, M., KOMATSU, T., SHIBATA, H., and SAITO, M. 2008. Annual changes in serum leptin concentration in the adult female Japanese black bear (*Ursus thibetanus japonicus*). Journal of Veterinary Medical Science 70(12):1399-1403.

UEKI, K., KONDO, T., and KAHN, C.R. 2004. Suppressor of cytokine signaling 1 (SOCS-1) and SOCS-3 cause insulin resistance through inhibition of tyrosine phosphorylation of insulin receptor substrate proteins by discrete mechanisms. Molecular and Cellular Biology 24(12):5434-5446.

UNGER, R.H. 1978.Role of glucagon in the pathogenesis of diabetes: the status of the controversy. Metabolism 27:1691-1709.

VAAG, A., HENRIKSEN, J.E., and BECK-NIELSON, H. 1992. Decrease insulin activation of glycogen synthase in skeletal muscles in young nonobese Caucasian first-degree relatives of patients with non-insulin-dependent diabetes mellitus. The Journal of Clinical Investigation 89(3):782-788.

VAN DAELE, L.J., BARNES, V.G., and SMITH, R.B. 1990. Denning characteristics of brown

bears on Kodiak Island, Alaska. Bears: Their Biology and Management 8:257-267.

VIRKAMÄKI, A., UEKI, K. and KAHN, R.C. 1999. Protein-protein interaction in insulin signaling and the molecular mechanisms of insulin resistance. The Journal of Clinical Investigation 103(7):931-943.

WANG, D. and SUL, H.S. 1998. Insulin stimulation of the fatty acid synthase promoter is mediated by the phosphatidylinositol 3-kinase pathway – Involvement of protein kinase B/AKT. The Journal of Biological Chemistry 273:25420-25426.

WANG, F., KOHAN, A.B., KINDEL, T.L., CORBIN, K.L., NUNEMAKER, C.S., OBICI, S., WOODS, S.C., DAVIDSON, W.S., and TSO, P. 2012. Apolipoprotein A-IV improves glucose homeostasis by enhancing insulin secretion. 109(24):9641-9646.

WANG, L.C.H., BELKE, D., JOURDAN, M.L., LEE, T.F., WESTLY, J., and NURNBERGER, F. 1988. The "hibernation induction trigger": Specificity and validity of bioassay using the 13-lined ground squirrel. Cryobiology 25(4):355-362.

WANG, P., WALTER, R.D., BHAT, G., FLORANT, G.L., and COLEMAN, R.A. 1997. Seasonal changes in enzymes of lipogenesis and triacylglycerol synthesis in the golden-mantled ground squirrel (*Spermophilus lateralis*). Comparative Biochemistry and Physiology Part B 118(2):261-267.

WARE, J. V., NELSON, O. L., ROBBINS, C. T., and JANSEN, H. T. 2012. Temporal organization of activity in the brown bear (*Ursus arctos*): roles of circadian rhythms, light, and food entrainment. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 303(9):R890-R902.

WEICHHART, T. and SÄEMANN, M.D. 2008. The PI3K/AKT/mTOR pathway in innate

immune cells: emerging therapeutic applications. Annals of the Rheumatic Diseases 67:iii70-iii74.

WEINER, F.R., SMITH, P.J., WERTHEIMER, S., and RUBIN, C.S. 1991. Regulation of gene expression by insulin and tumor necrosis factor α in 3T3-L1 cells. The Journal of Biological Chemistry 266(35):23525-23528.

WHEATCROFT, S.B., KEARNEY, M.T., SHAH, A.M., EZZAT, V.A., MIELL, J.R., MODO, M., WILLIAMS, S.C.R, CAWTHORN, W.P., MEDINA-GOMEZ, G., VIDAL-PUIG, A., SETHI, J.K., and CROSSEY, P.A. 2007. IGF-binding protein-2 protects against the development of obesity and insulin resistance. Diabetes 56(2):285-294.

WHEATCROFT, S.B., WILLIAMS, I.L., SHAH, A.M., and KEARNEY, M.T. 2003. Pathophysiology implications of insulin resistance on vascular endothelial function. Diabetic Medicine 20:255-268.

WILCOX, G. 2005. Insulin and insulin resistance. The Clinical Biochemist Reviews 26:19-39.

WILSON, B.E., DEEB, S., and FLORANT, G.L. 1992. Seasonal changes in hormone-sensitive and lipoprotein lipase mRNA concentrations in marmot white adipose tissue. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 262(2):R177-R181.

Table 1. Gene names, expected values (indicating background noise), and predicted *U. maritimus* homology for canine probes used to characterize cellular response to differentiation, serum exposure, or insulin treatment. Homology and expected values were determined using a BLASTn search of the National Center for Biotechnology Information database

Gene	**TaqMan Probe ID**	**Interrogated RefSeq**	***U. maritimus* RefSeq**	**E Value**	**Identity (%)**
AKT1	Cf02694335_mH	XM_548000.2	XM_008706093.1	$6\ e^{-32}$	93
INSR	Cf02647625_m1	XM_542108.2	XM_008705466.1	$3\ e^{-49}$	96
PPARG	Cf02625640_m1	NM_001024632.2	XM_008697869.1	$6\ e^{-41}$	96
PTEN	Cf02628582_m1	NM_001003192.1	XM_008689584.1	$2\ e^{-67}$	99
RPS18	Cf02624915_g1	NM_001048082.1	XM_008693439.1	$1\ e^{-44}$	95

Table 2. 2-Deoxyglucose uptake (pmol/well ± SEM) with baseline values subtracted for active (May) and hibernation (January) cells cultured in the different serum types: FBS, hyperphagic (HYP, September/October), active, and hibernation serum. Values represent averages for 4 bears. *P < 0.05, ***P = 0.0005 vs. active cells.

	Cell Season	
Serum Type	Active	Hibernation
FBS	114.60 ± 31.18	99.15 ± 63.04
HYP	344.42 ± 109.12	537.72 ± 215.13
Serum Match	422.43 ± 123.54	-23.43 ± 88.28*
Serum Opposite	279.43 ± 108.61	1000.78 ± 283.61***

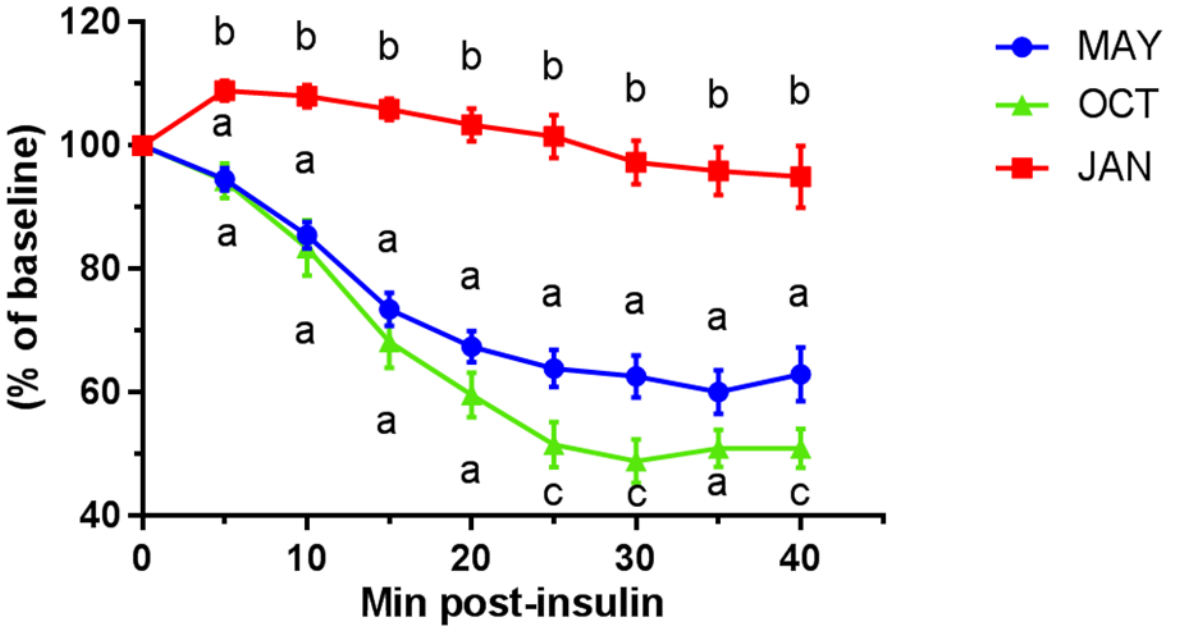

Figure 1. Blood glucose profiles given as percent of baseline after IV insulin administration (0.015 U/kg). All values represent averages from 6 bears over the 2 year sampling period. A two-way ANOVA indicated a significant effect of time post insulin and season as well as their interaction at the $P < 0.0001$ level. Glucose values that are significantly different from one another are denoted by different letters based on results of Tukey's multiple comparisons tests ($P \leq 0.05$).

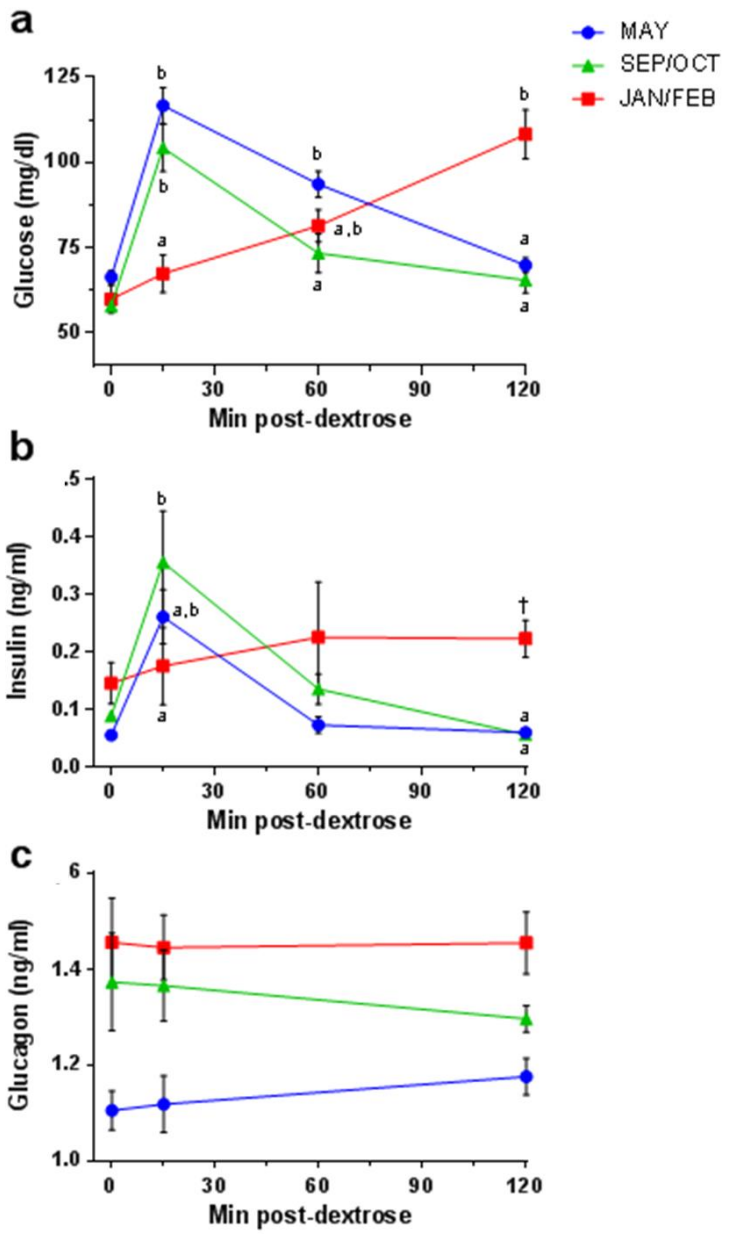

Figure 2. a – Blood glucose (mg/dL), b – serum insulin (ng/ml), and c – serum glucagon concentrations (ng/ml) for each season following oral dextrose administration (1g/kg) in 3 bears. Effect of season and time post-dextrose were assessed with two-way ANOVAs. Different letters within a time point are significantly different based on results of Tukey's multiple comparisons tests ($P \leq 0.05$). † P = 0.0582, 0.0518 vs. May and September/October.

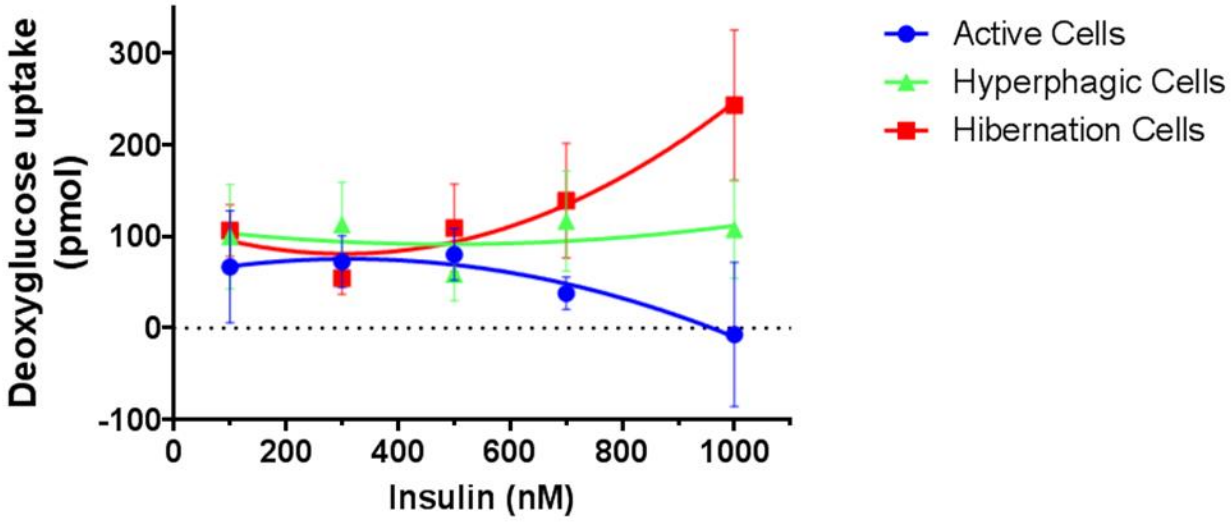

Figure 3. 2-Deoxyglucose uptake (pmol/well) with baseline (no insulin) values subtracted in response to graded insulin treatments in cells from each of the three seasonal phases: active season (May), hyperphagia (September/October), and hibernation (January). Values represent averages from 4 bears. A two-way ANOVA revealed a significant effect of season on glucose uptake ($P = 0.0487$).

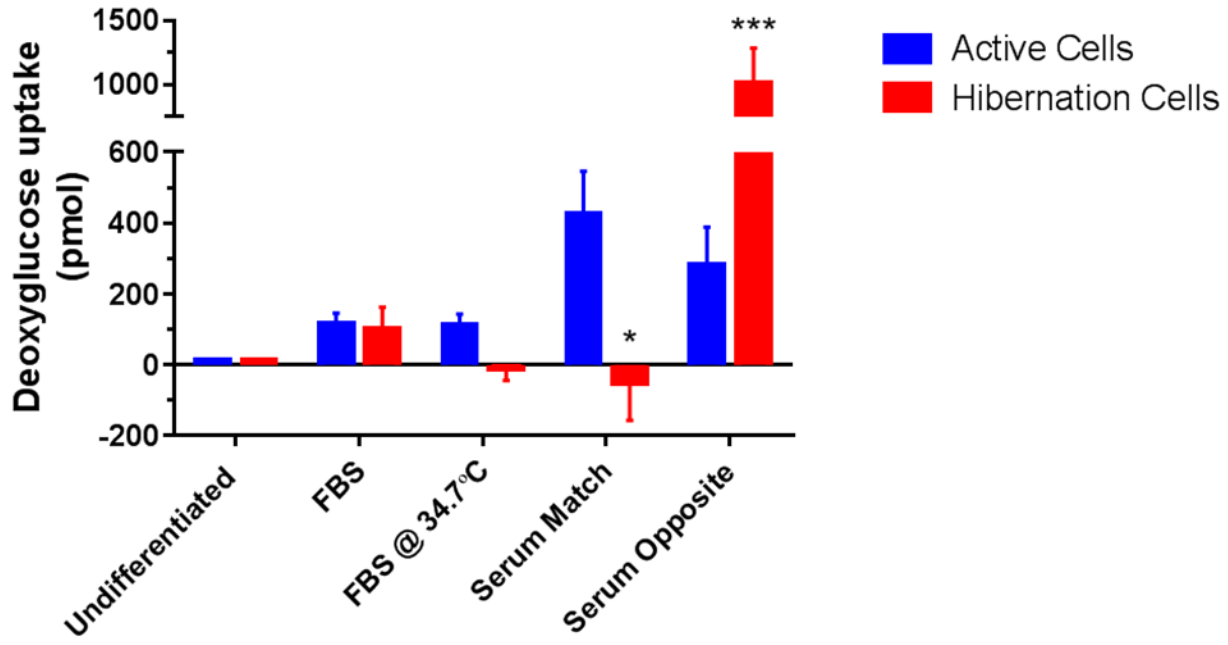

Figure 4. 2-Deoxyglucose uptake values (pmol/well) following insulin treatment (300 nM) with baseline (no insulin) values subtracted in undifferentiated and differentiated cells from the active season (May) and hibernation (January) cultured in FBS at 37°C, FBS at 34.7°C, and bear serum from the active season and hibernation. Values represent averages from 4 bears. Two-way ANOVAs and Sidak's multiple comparisons tests were run to determine differences between undifferentiated and differentiated cells, differentiated cells cultured at 37°C and 34.7°C, and differentiated cells cultured with active and hibernation bear serum. *P < 0.05, ***P = 0.0005 vs. active cells.

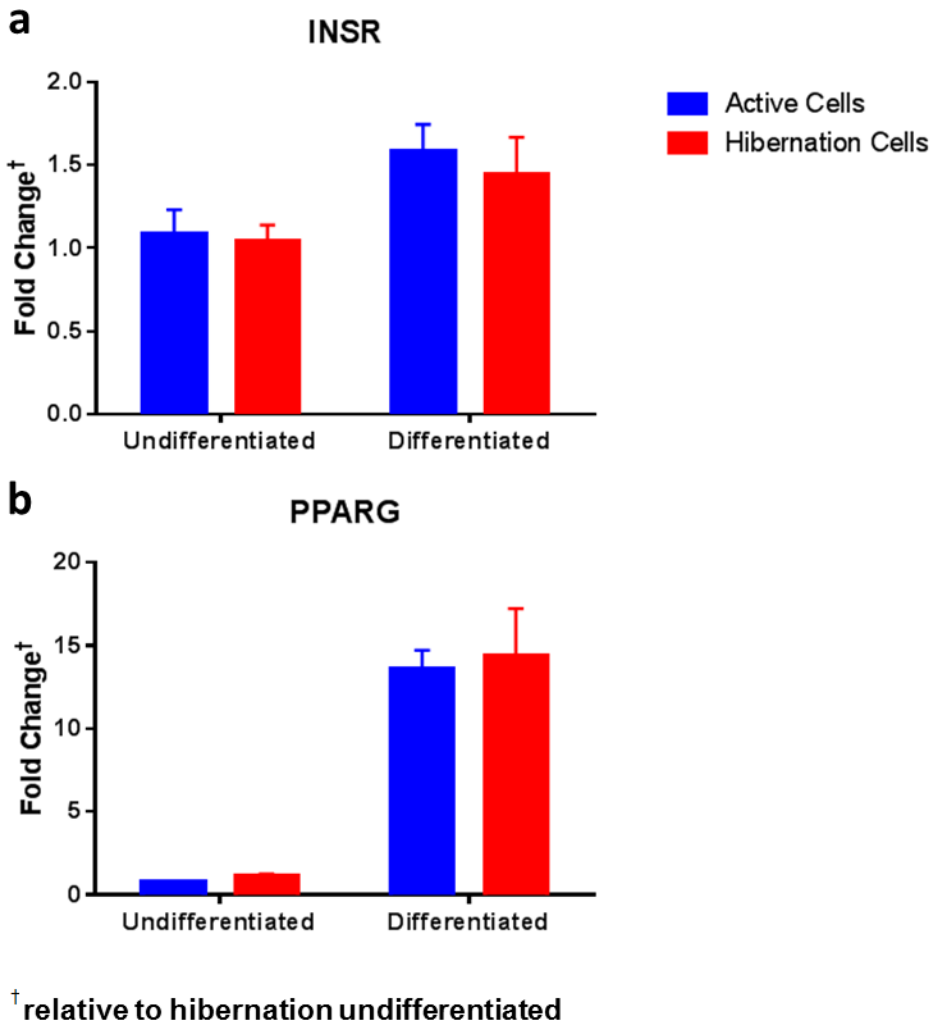

Figure 5. a – Relative expression of insulin receptor (INSR) and b – peroxisome proliferator-activated receptor gamma (PPARG) in active (May) and hibernation (January) undifferentiated and differentiated cells at baseline (no insulin). Gene expression is given as fold change calculated with respect to undifferentiated cells from hibernation and averaged for 4 bears. There was a significant difference between undifferentiated and differentiated cells for both INSR and PPARG based on results of a two-way ANOVA (INSR, $P = 0.0096$; PPARG, $P < 0.0001$).

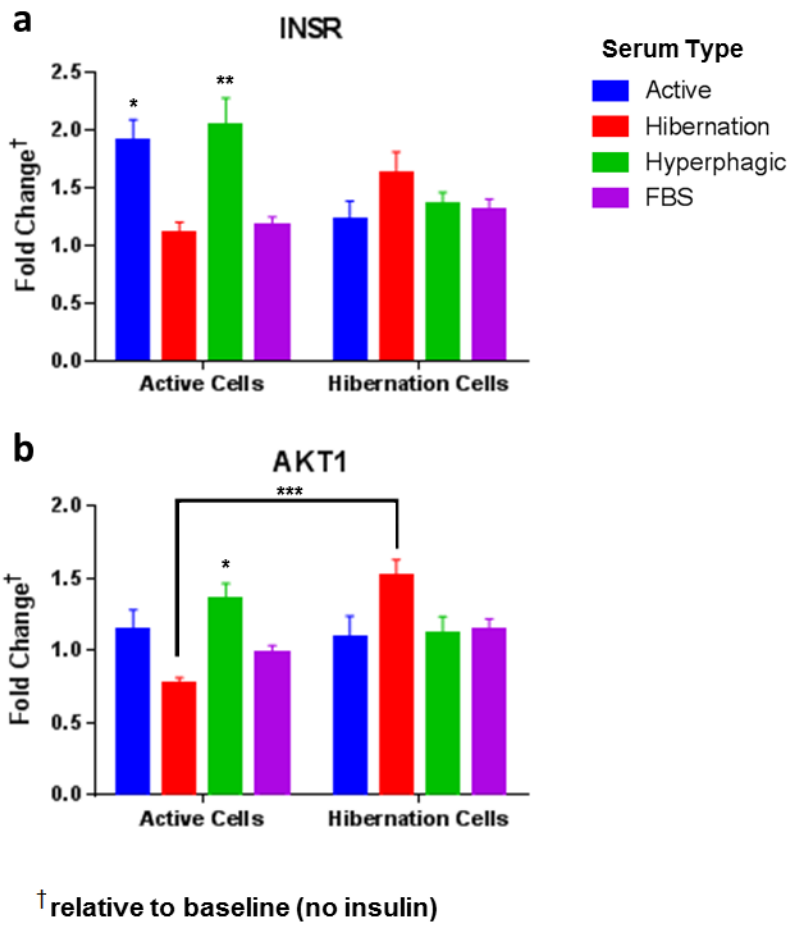

Figure 6. a – Insulin receptor (INSR) and b – protein kinase B (AKT1) expression in response to insulin stimulation (300nM) reported as fold change over baseline expression (no insulin) in cultures of matching season and serum type for both active season (May) and hibernation cells (January) cultured with all serum types: active, hibernation, hyperphagic (September/October), and FBS. Values represent averages from 4 bears. *P $\leq$ 0.01, **P < 0.005, ***P < 0.0005 vs. the hibernation serum treatment group within season unless otherwise specified.

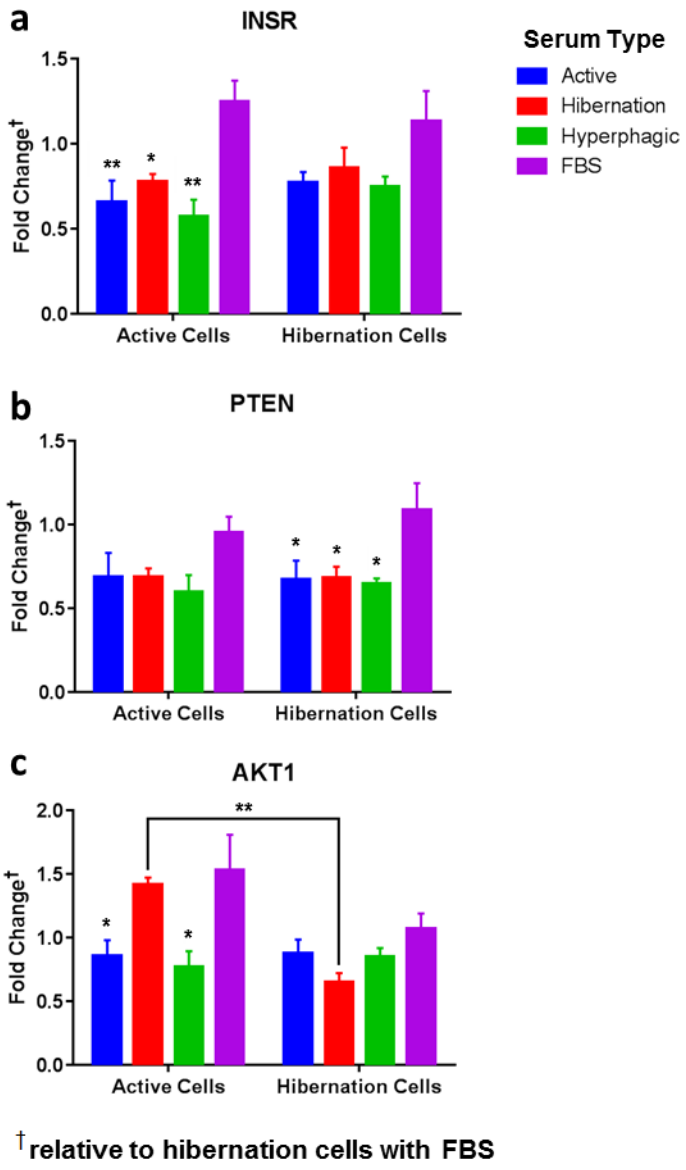

Figure 7. a – Baseline insulin receptor (INSR), b –phosphatase and tensin homolog (PTEN), and c – protein kinase B (AKT1) expression given as fold change calculated with respect to hibernation cells cultured in FBS for both active season (May) and hibernation (January) cells cultured with all serum types: active, hibernation, hyperphagic (September/October), and FBS. Values represent averages from 4 bears. *P < 0.05, **P ≤ 0.005 vs. FBS within season unless otherwise specified.

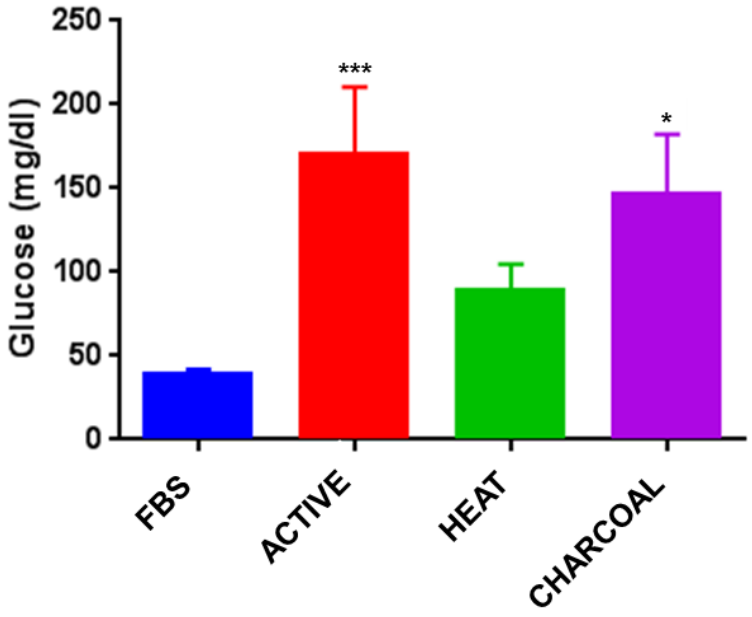

Figure 8. Glucose usage (mg/dL) measured in culture medium for hibernation cells (January) cultured in active season (May) serum (ACTIVE), heat-inactivated active serum (HEAT), charcoal-stripped active serum (CHARCOAL), and FBS. Values represent averages from 2 bears. *P < 0.05, ***P = 0.0005 vs. FBS.

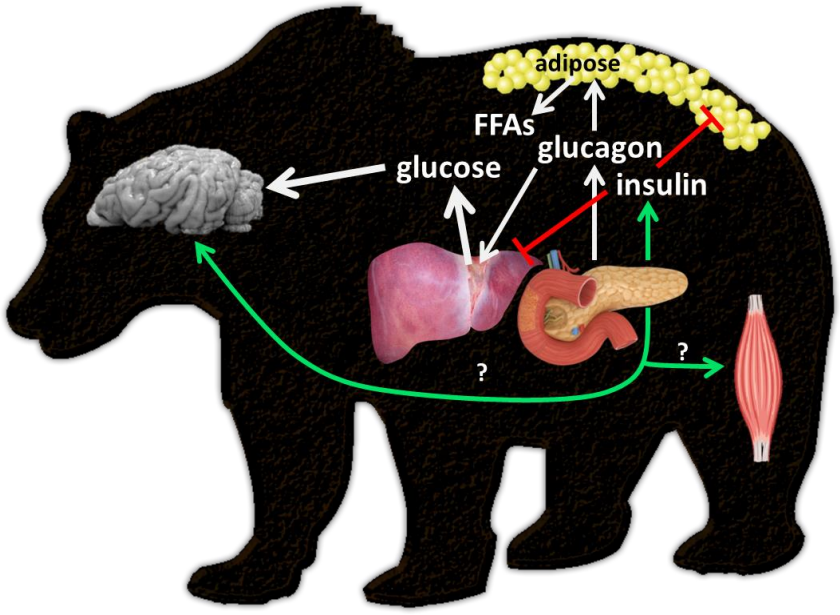

Figure 9. Proposed actions of glucagon and insulin in the hibernating grizzly bear. Heterogeneous tissue insulin sensitivity may be a mechanism for glucose sparing in hibernating animals while glucagon may promote release of glucose from the liver to maintain euglycemia through the processes of glycogenolysis and gluconeogenesis. Insulin resistance and glucagon sensitivity of peripheral adipose tissue may facilitate a lipolytic metabolism.