SEASONAL CHANGES IN ADIPOKINES, FAT UTILIZATION, AND REGULATION OF FOOD INTAKE IN GRIZZLY BEARS (*URSUS ARCTOS HORRIBILIS*)

By

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To the Faculty of Washington State University:

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Abstract

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Many hibernating species have evolved physiological adaptations not found in more active organisms, including the ability to amass enormous adipose deposits. In humans, large amounts of adipose tissue are associated with the onset of metabolic diseases. However, in hibernating grizzly bears (*Ursus arctos horribilis*), obese animals are not only healthy, they are more reproductively fit than their leaner counterparts. Because we hypothesized that adipokines might reflect adiposity, we determined the annual serum profiles of leptin and adiponectin relative to body fat content. Both adipokines were present at relatively low levels throughout the active season but peaked in mid-October just prior to hibernation. Neither strongly correlated with body fat content throughout the year. Since leptin is an appetite suppressant in other animals, we tested the ability of centrally administered leptin to alter food intake at the beginning (August) and end (October) of the annual hyperphagic period. Bears treated with leptin in October significantly reduced their food intake whereas in August intake was unchanged, suggesting that the brain's sensitivity to leptin varies seasonally. We also cultured adipocytes

from the active season and from hibernation under a variety of conditions to measure changes in gene expression and cellular lipolytic ability. Following treatment with bear serum collected at different times of year, leptin and its receptor responded differentially to catecholaminergic stimulation. The expression of genes closely related to lipolysis was significantly higher in hibernation cells, revealing cell autonomous changes. Taken together, the results reveal both cell-specific and systemic seasonal changes in bear adipose physiology.

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INTRODUCTION

Grizzly bears (*Ursus arctos horribilis*) exhibit extraordinary physiological adaptations which allow them not only to survive extended fasts by using stored energy but also to give birth and lactate during hibernation (Stringham 1990). Bears can gain and lose up to 40% of their body mass during the annual cycle of pre-hibernation mass gain and subsequent loss during hibernation (Kingsley et al. 1983). Importantly, fatter females produce more and larger cubs that have an increased chance of survival compared to cubs born to leaner females (Dahle et al. 2006; Derocher and Stirling 1996; Robbins et al. 2012). Females with less than 20% body fat entering hibernation generally do not produce cubs (Robbins et al. 2012). Although female mate choice somewhat depends on population density (Steyaert et al. 2012), females generally mate with larger males (Bellemain et al. 2006). Thus, the selective benefits accrued by fatter females and larger males have resulted in large appetites and almost unlimited fat accumulation in grizzly bears. By human standards, many bears would be considered morbidly obese as they prepare to enter hibernation. Nevertheless, they are completely healthy with no detectable circulatory, respiratory, or other pathologies. Despite the potential significance of this adaptation, the precise relationship between adipose tissue and the control of food intake in grizzly bears has not been studied thoroughly.

Given the need for bears to gain body fat, we hypothesized that many of the systems that other animals use to suppress appetite would be inoperative in grizzly bears during the active, feeding season. Hibernation lasts 3 – 7 months in grizzly bears, yet they do not eat or drink during this time (Hilderbrand et al. 2000; Nelson et al. 1983). Thus, a seasonal change in appetite must occur. How bears transition from hyperphagia to anorexia is unknown but could be facilitated by increases in adipose-derived appetite-suppressing hormones like leptin. By linking

amplitude of the signal to the quantity of adipose tissue, female bears could potentially determine the number of embryos to implant at the beginning of hibernation (Friebe et al. 2014).

Adipose is a metabolically active endocrine organ that secretes a wide variety of peptides, termed adipokines, which influence diverse physiological processes such as immune response, energy homeostasis, and neuroendocrine function (Galic et al. 2010; Kershaw and Flier 2004; Trayhurn and Beattie 2001). Adipokines are capable of acting in an autocrine, paracrine, or endocrine fashion, thereby serving as both local and global mediators of energy status (Ahima and Flier 2000; Frühbeck et al. 2001; Gregoire et al. 1998). Thus, seasonal variation in adipokine concentrations is likely of critical importance to mammalian hibernators. One of these adipokines, adiponectin, may prove to be a particularly valuable indicator of body fat content and, therefore, reproductive fitness. Adiponectin is produced almost exclusively by adipose tissue (Havel 2002). Although its mechanisms of action have not yet been fully elucidated, adiponectin is thought to be an endogenous insulin sensitizer that also inhibits inflammation, atherogenesis, and apoptosis (Blüher 2012; Ziemke and Mantzoros 2010). In humans, circulating adiponectin concentrations are negatively correlated with body weight (Arita et al. 1999; Bulló et al. 2005; Meier and Gressner 2004). However, in American black bears (*U. americanus*) serum adiponectin was lowest during hibernation (Hill 2013) and higher at other times of the year (McGee-Lawrence et al. 2015). Because adiponectin levels rose as bears emerged from hibernation, they did not correlate with adiposity as these bears could not yet have regained fat stores comparable to pre-hibernation levels. Therefore, serum adiponectin concentrations in hibernating mammals may be partially governed by a circannual rhythm rather than strictly by fat mass.

Another adipokine, leptin, a product of the *ob* gene (Tartaglia et al. 1995), is produced mainly by white adipose tissue (WAT), although it and its receptors are also produced in other organs (Reidy and Weber 2000; Trayhurn and Beattie 2001). Leptin is an important regulator of appetite in humans and rodents, with increased serum concentrations decreasing food intake (Keim et al. 1998; Trayhurn et al. 1999; Wang et al. 1997). One of its most potent and well-studied mechanisms of action is its inhibition of gene expression of the orexigen neuropeptide Y in the arcuate nucleus of the hypothalamus (Cowley et al. 2001; Mantzoros et al. 2011). However, leptin is more than simply a satiety signal – it is a highly pleiotropic hormone that influences multiple biological pathways. It is possible that, like adiponectin, serum leptin concentrations may be uncoupled from WAT mass (Ahima et al. 1998; Dark 2005; Kronfeld-Schor et al. 2000). In addition to its hypothalamic influence on appetite, leptin is also involved more directly in lipolysis (Frühbeck et al. 2014). Administration of leptin to mice activates sympathetic nerves that innervate WAT, increasing lipolysis, local catecholamine release, and phosphorylation of hormone sensitive lipase (Zeng et al. 2015). However, earlier studies showed that leptin administered peripherally to rodents with denervated adipose tissue depots did not diminish leptin's lipolytic effect (Rooks et al. 2005), suggesting that leptin's actions can occur independently of sympathetic innervation.

Lipolysis is induced by catecholamines acting through the 3 main β-adrenergic receptor subtypes (Arner 2005; Carpéné et al. 1998), which are differentially expressed depending on species and sex (Lafontan and Berlan 1993). It is unknown which of these receptors are expressed in grizzly bear adipose tissue. β-adrenergic receptors mediate lipolysis via endogenous catecholamines or synthetic agonists such as isoproterenol, ultimately activating lipases like adipose triglyceride lipase (ATGL). Lipases hydrolyze triacylglycerol (TAG) to liberate free

fatty acids (FFA) and glycerol (Ahmadian et al. 2010; González-Yanes and Sánchez-Margalet 2006; Lafontan et al. 1997). ATGL is the rate-limiting enzyme in TAG hydrolysis, as it removes the first FFA (Bézaire et al. 2009; Haemmerle et al. 2006). The importance of lipolysis as an energy source can be seen in fasting ATGL-deficient mice, which exhibit hypothermia within 5 hours of cold exposure at 4°C (Haemmerle et al. 2006). Thus, ATGL-induced lipolysis likely plays an important role in hibernating bears.

Another important metabolic issue for hibernating bears is the limited supply of glucose precursors. Because bears do not eat during hibernation, glycerol in lipids and amino acids in tissue proteins are two potential substrates for glucose synthesis. However, since tissue proteins are conserved during hibernation (Hellgren 1998; Lohuis et al. 2007), fatty acids and glycerol released during lipolysis likely provide both the energy for hibernation and the glucose necessary to conserve lean body mass (Ahlquist et al. 1984; Nelson et al. 1983).

To explore the seasonal control of appetite and metabolic fuel usage in grizzly bears, we used a combination of *in vivo* and *in vitro* studies. We characterized annual fluctuations in serum leptin and adiponectin, predicting that these would correlate with fat mass prior to hibernation if they provide a means for bears to sense body fat content. Since leptin is also an appetite suppressant, we hypothesized that serum leptin would exhibit a seasonal rhythm independent of body fat content during the active season but would both suppress appetite and reflect body fat content immediately before hibernation. Because embryo implantation occurs in early November at the beginning of hibernation, leptin, adiponectin, or other adipokines could serve as an important lipostatic determinant of the reproductive processes (Moschos et al. 2002). Furthermore, we anticipated that adipocytes obtained from hibernating bears would exhibit greater lipolytic activity in response to β-adrenergic stimulation, and serum factors could

influence the lipolytic potential of adipocytes (Bruce et al. 1984; Rutt et al. 1987; Vybiral and Jansky 1997).

METHODS AND MATERIALS

Animals and Facilities

Grizzly bears (adult males, adult females, and juvenile males) were housed at the Washington State University Bear Research, Education, and Conservation Center, Pullman, WA. All procedures were approved by the WSU Institutional Animal Care and Use Committee (protocols #3802, 4373, and 3054). Bears were maintained in accordance with the *Bear Care and Colony Health Standard Operating Procedures* (protocol #4259). Unless otherwise specified, anesthesia followed protocols described in Ware et al. (2012). Bears hibernated from November through early- to mid-March each year, depending on weather conditions. Early August through mid-October was considered the hyperphagic period. Beginning in mid-October food intake decreased, ceasing entirely by late October. Bears were fed lightly beginning in mid-March, with intake increasing to maintenance levels in early April.

Blood Sampling for Adipokine and Glycerol Determination

Over a period of 6 years, blood was collected from both anesthetized (n = 13) and trained unanesthetized (n = 4) grizzly bears. Blood was drawn from the jugular vein in anesthetized bears, whereas in trained bears blood was taken from a dorsal metatarsal vein while the bears were rewarded with dilute honey. All animals had been fasted for 14-16 hours prior to sampling, which occurred between 7:30AM and 10:00AM. Serum was separated from whole blood via centrifugation and samples from all bears were assayed for leptin. The serum obtained from the 4 trained bears was collected over the course of 8 months (April – November) and was assayed for adiponectin as well as leptin. Serum was collected weekly from these trained bears between August and early November to better characterize the fall patterns in adipokine concentrations seen in preliminary studies.

Percent body fat was determined via isotopic water dilution using a technique previously validated for black bears, polar bears, and grizzly bears (Farley and Robbins 1994). Briefly, a baseline blood sample was taken from a hind limb prior to injection of 8 ml of 99.8% deuterium oxide, which was followed by a sterile saline flush. A blood sample was taken 1 hour after deuterium oxide injection. Previous studies using anesthetized bears found that 2 hours were required for deuterium oxide equilibration (Farley and Robbins 1994), but our preliminary studies showed that 1 hour allowed complete equilibration in unanesthetized bears. A commercial lab (Metabolic Solutions, Inc., Nashua, NH) analyzed pre- and post-injection serum samples for deuterium oxide by cavity down-ring spectroscopy. Blood from anesthetized (n = 9) and trained unanesthetized (n = 4) bears was also assayed for glycerol.

Effect of Leptin on Food Intake

To determine if leptin produces season-specific effects on food intake, we injected 4 bears with recombinant human (rh) leptin centrally during feeding trials that occurred in August, when the bears have low serum leptin concentrations, and in October, when serum leptin concentration peaks during the transitional period prior to hibernation. Four males (3 and 4 years old, weighing from 120 to 220 kg) were used in these trials. Adult male bears were excluded because the subarachnoid space could not be accessed even with fluoroscopic techniques. Bears were housed individually in 3 m x 3 m x 2.5 m dens adjoining 3 m x 5 m x 5 m pens. During the trial, bears were given *ad libitum* access to Hill's Science Diet dry adult dog food for three days and their intake was recorded twice daily. All food was removed on the evening of the fourth day of the trial to allow the bears to fast 12 hours prior to anesthesia. The next day all bears were anesthetized, with half receiving injections of rh-leptin (R&D Systems, Inc., Minneapolis, MN)

into the cerebromedullary space while the other half received injections of artificial cerebrospinal fluid (aCSF).

Once anesthetized, the bears were placed in lateral recumbency with the head flexed ventrally. The skin overlying the cervical spine was shaved and surgically prepared with chlorhexidine and 70% ethanol. Anatomical landmarks such as the occipital protuberance and the wings of the atlas were palpated to identify the appropriate injection site. A beveled spinal needle (20- or 22-gauge, 8.89 cm in length) was inserted parallel to the ramus of the mandible and was advanced slowly into the atlantooccipital space until clear CSF began to flow from the hub, indicating that the subarachnoid space had been reached. A contrast study was performed on a 3-year-old male bear to confirm this. After collecting 20 ml of the bear's CSF, we injected 20 ml Omnipaque^{TM} (GE Healthcare Life Sciences, Marlborough, MA) and used fluoroscopy to visualize the flow of contrast. Omnipaque^{TM} entered the spinal cord, filled the subarachnoid cisterns of the cerebrum, and moved rostrally around the brain, ultimately reaching the base of the hypothalamus (Fig. 1). During the leptin injection feeding trials, a 35 cm extension tube was attached to the spinal needle and then 2-6 ml CSF was collected. Next, either rh-leptin or aCSF was injected, followed by a 2 ml flush of aCSF or the bear's own previously collected CSF. The amount of rh-leptin was based on brain allometry and previous studies in rodents and non-human primates examining the effect of central leptin on food intake (Koch et al. 2010; McCarthy et al. 2002; Seeley 1996). Based on these estimates and a BLASTp search of National Center for Biotechnology Information database, which yielded a predicted homology of 81% between human and polar bear leptin, we increased the amount of leptin by 4.5-fold. Thus, the resulting amounts of rh-leptin administered to the bears ranged from 2.44 to 3.42 mg. Leptin was delivered centrally because a prohibitively large volume would be required for intravenous or

intraperitoneal delivery. Following recovery, all 4 bears were again given free access to food for an additional 4 days, and their consumption was determined twice daily as before.

On one occasion the subarachnoid space could not be accessed. This occurred in bear C in October 2013. However, this individual was successfully injected 48 hours later using fluoroscopy. In this instance, food intake measurements from the first 3 days of the trial served as baseline intake and food ingested the day after the second round of anesthesia was considered experimental intake. Intake during the 48 hours between procedures was not used in calculations.

The average daily intake over the first three days of *ad libitum* feeding was designated baseline and was compared to intake from the day of treatment. Baseline food intake was also compared to intake from the 4 days following the injection, which was analyzed as a percentage of the averaged 3 days of baseline consumption. Bears were fasted for 8 hours after the injection procedure to ensure complete recovery before being given unlimited access to food. Thus, food intake for the day of treatment was limited to 16 hours, although previous studies indicated that bears given *ad libitum* access to food over 12 hours consumed the same amount of food as those given access for 24 hours (Hilderbrand et al. 1999).

Adipocyte Culture

Fat biopsies were performed on 2 female and 2 male adult bears in the active season (May), hyperphagic period (September and October), and during hibernation (January) to obtain adipose-derived mesenchymal stem cells (MSC). The isolated MSC were cultured under sterile conditions in 24-well plates using protocols described in Gehring et al. (2015, in review). Cells were differentiated into the adipogenic lineage upon reaching confluence. On day 10-11 post-differentiation, after cells had been in maintenance medium without rosiglitazone for the standard 48 hours, this medium was gently aspirated and the cells were rinsed with 37°C 1 X

Krebs-Ringer Phosphate Buffer (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). The cells were then maintained in 667 μL starve medium (1% antibiotic/antimycotic, 99% DMEM/F-12 containing GlutaMAX^{TM}) for 24 hours. Following another KRPH rinse, 445 μL KRPH or KRPH with isoproterenol (0.01, 0.1, 1, 10, or 50 μM) at 37°C was added to each well. The plate was incubated in a humidified environment at 37°C and 5% CO_2 for 1 hour, after which the medium was removed and stored at -80°C until assayed for glycerol to attain the median effective dose. This general culture protocol was used in the experiments described below with modifications where indicated.

Temperature Experiment

I. Chronic low temperature: Active season and hibernation cells from the same 4 bears were grown at a lower temperature to better approximate the *in vivo* condition seen in hibernating bears (Nelson et al. 1983). Thermochron® iButton® model DS1922L (Maxim Integrated, Inc., San Jose, CA) temperature loggers implanted subcutaneously in the axillae of these bears in previous years indicated an average hibernating body temperature of 34.7°C. Therefore cells were moved to a humidified incubator with 5% CO_2 at 34.7°C upon differentiation. Cells were maintained at this temperature until treatment 11 days later. Treatment with KRPH and 0.1 μM isoproterenol occurred as described above, with the 1 hour incubation again occurring at 37°C. Treatment medium and total RNA were collected and stored at -80°C.

II. Acute low temperature: We observed that the cells grown chronically at 34.7ºC had visibly smaller lipid droplets compared to cells that had been grown at 37°C for the entirety of the maturation process (not shown). Because of concerns that this may impact glycerol release, we performed a second experiment using active season and hibernation cells from the same 4 bears but transferred cells to the 34.7°C incubator 8 days after differentiation medium was added.

Thus, these cells were grown for only 3 days at the lower temperature. Treatment medium was stored at -80°C until assayed for glycerol as before.

Undifferentiated Cells

Cells from the same bears described above were grown in a humidified environment at 37°C and 5% CO_2 until they reached confluence. However, rather than being exposed to differentiation medium, the cells were immediately treated with KRPH or KRPH containing 0.1 μM isoproterenol as described above (see 'Adipocyte Culture') and medium was collected and stored at -80°C as before.

Serum Swap Experiment

We collected serum at different times of year and added it to adipocyte culture medium to investigate the possibility that a serum factor influences lipid metabolism. Active season and hibernation cells were collected from the same 4 adult bears described above and from 3 additional juvenile male bears. Blood was collected from the jugular vein during biopsies and the serum was separated via centrifugation and stored at -80°C. Each bear's own serum was used on its cells rather than pooled serum. Serum was collected during the active season (May), hyperphagic period (September and early October), and mid-hibernation (January and February). Prior to use in cell culture, thawed bear serum was filtered twice using 0.22 μm Millex-GP syringe filters (#SLGP033RS, EMD Millipore) and then once with a 0.1 μm Anotop™ filter (#6809-3112, Anopore™ Inorganic Membrane Filter & Glass Fiber Prefilter, Whatman™, GE Healthcare Life Sciences, Marlborough, MA), then treated with Primocin™ (100 μg/ml)(#ant-pm-1, Invivogen, San Diego, CA) overnight while stored at 4°C. All surfaces and instruments were sterilized with UV light and/or 70% ethanol between filtration steps.

Both active season and hibernation cells were treated with one of the three serum types or FBS upon differentiation. All medium contained 10% serum and Primocin^{TM} was added at a concentration of 100 μg/ml as before. Serum substitution and medium changes continued for 10 days following differentiation. On day 12, cells were treated with KRPH or KRPH with 0.1 μM isoproterenol as described above. Treatment medium was collected, centrifuged (10 min; 12,000 x *g*; 4°C) to remove debris, and stored at -80°C prior to use in a glycerol assay.

Total RNA was collected from cultures from the 4 adult bears and stored as previously described until assayed with TaqMan probes (Thermo Fisher Scientific, Beverly, MA) for the following genes: β2-adrenergic receptor (ADRB2), leptin (LEP), leptin receptor (LEPR), and adipose triglyceride lipase (ATGL). These genes were selected following a search of GenBank to confirm predicted polar bear homology (Table 2). Ribosomal protein S18 (RPS18) was used as a housekeeping gene to normalize expression. Gene expression was quantified as fold change.

Assays

A canine leptin ELISA (#EZCL-31K, EMD Millipore, Billerica, MA) was used to determine serum leptin concentrations. A mouse/rat adiponectin ELISA from B-Bridge International (#UM-100201, Thermo Fisher Scientific) was used to determine adiponectin concentrations in serum. Glycerol content in medium was quantified using a colorimetric glycerol assay kit from Cayman Chemical (#10010755, Ann Arbor, MI). Real-time quantitative PCR (RT-qPCR) was performed as described in Gehring et al. (2015, in review). All samples were handled following the manufacturer's recommended protocol and were run in duplicate.

Adrenergic Receptors

A search of GenBank was unable to confirm the presence of β1- or β3-adrenergic receptors in polar bears (*Ursus maritimus*), the closest relative to the grizzly bear (Welch et al.

2014). Thus, the lipolytic action of isoproterenol is likely to occur via ß2-adrenergic receptors, unless grizzly bear adipocytes express different receptors than are found in polar bears. To clarify how grizzly bears regulate lipolysis, we tested probes for β1-adrenergic receptor (ADRB1) and β3-adrenergic receptor (ADRB3) to determine whether or not these receptors are present in grizzly bear subcutaneous adipocytes.

Statistical Analysis

One-way ANOVAs were used to compare serum adipokine concentrations with baseline values while unpaired two-tailed t-tests were used to assess differences in serum glycerol. In statistical analyses of the *ad libitum* feeding trials, the first 3 days of intake were designated baseline and the 4 full days following the injection were considered experimental. Two-way ANOVAs were used to compare food intake in the feeding trials and to determine the effect of anesthesia on serum adipokine concentrations. A paired t-test was used to find the median effective dose of isoproterenol for use in cell culture and an unpaired t-test was used to compare glycerol release in active and hibernation cells.

For gene expression studies treatment groups were defined as samples derived from cells from the same season that had been cultured with the same type of serum. Fold changes were then used to examine differences in gene expression in two ways. First, to examine baseline seasonal differences among treatment groups that were not exposed to isoproterenol, fold changes were calculated relative to active season cells cultured with FBS. Second, to clarify the effect of isoproterenol stimulation on gene expression, fold change was calculated between the 0 dose and the 0.1 μM isoproterenol dose within each treatment group. Two-way ANOVAs were used to assess the effect of cell season and serum or temperature treatment by comparing gene expression of ADRB2, ATGL, LEP, and LEPR. We used Tukey's multiple comparisons tests to

determine which cell/treatment pairings had a significant effect. For LEP gene expression in isoproterenol-treated serum swap cultures, a Sidak's multiple comparisons test was performed to assess differences in fold change relating to cell season of origin. Unpaired t-tests were used to examine the relationship between cell type and serum treatment for ADRB1 expression. The α level was 0.05 and all data are presented as mean±SEM.

RESULTS

In Vivo Studies

Annual Serum Adipokine Profiles

Serum leptin concentrations varied over the annual cycle with the highest levels occurring in October ($F_{(21,156)}$ = 7.593; P < 0.0001)(Fig. 2). Average serum leptin concentrations in October (4.56±0.42 ng/ml, n = 12) were 4 times higher than those occurring during the rest of the year (1.14±0.19 ng/ml, n = 17). Leptin concentrations peaked in mid-October (5.72±0.97 ng/ml, n = 7). Anesthesia did not significantly impact leptin levels in that there was no difference between the values in anesthetized and unanesthetized bears ($F_{(1,125)}$ = 1.107; P = 0.2947). Average serum adiponectin concentrations increased from baseline levels in April and May (0.65±0.21 μg/ml, n = 4) to a high in October (5.45±0.4528 μg/ml, n = 4)($F_{(7,67)}$=12.67; P < 0.0001)(Fig. 3). Neither leptin nor adiponectin was highly correlated with body fat content (Fig. 3).

Feeding Trials

Ad libitum food intake did not differ between bears injected with leptin and the control bears in August (effect of day, $F_{(3,36)}$ = 0.6736; P = 0.5739; effect of treatment, $F_{(1,36)}$ = 1.197; P = 0.2811), while in October the leptin treatment group consumed significantly less food than the aCSF group (effect of day, $F_{(3,28)}$ = 2.272; P = 0.1020; effect of treatment, $F_{(1,28)}$ = 5.233; P = 0.0299)(Fig. 4). No significant differences occurred between average daily intake during the baseline period and that occurring on the day of the injection in all trials (effect of month, $F_{(1,9)}$ = 1.034; P = 0.3358; effect of treatment, $F_{(1,9)}$ = 0.1354; P = 0.7215; effect of individual, $F_{(9,9)}$ = 0.7578; P = 0.6569).

Serum Glycerol

Serum glycerol was highest during mid-hibernation and dropped to a minimum by October (Fig. 5). Serum glycerol concentrations did not differ between anesthetized and unanesthetized bears ($t_{(41,62)}$ = 1.371; P = 0.178). Outliers were identified using robust regression and outlier removal (ROUT) and were excluded from analysis.

In Vitro Studies

Median Effective Dose

Maximum glycerol release occurred when differentiated cells from all 3 seasons were exposed to 1 μM isoproterenol. Hyperphagic season cells released approximately 3 times more glycerol than active season or hibernation cells, but this difference was only statistically significant at the 0.1 μM dose (active cells $t_{(2)}$ = 6.945; P = 0.02; hibernation cells $t_{(3)}$ = 4.850; P = 0.0167). Therefore 0.1 μM isoproterenol was designated the median effective dose. Undifferentiated cells released undetectable amounts of glycerol (< 0.366 μg/ml) when induced with 0.1 μM isoproterenol.

Serum Swap Experiment

I. ***Glycerol Release*****:** Glycerol release following stimulation with isoproterenol did not differ between cells obtained during the active season and cells from hibernating bears (P = 0.125)(Fig. 6). Among active season cells, serum treatment did not influence medium glycerol content. Only the addition of isoproterenol created significant differences (effect of isoproterenol, $F_{(1,36)}$ = 43.35; P < 0.0001; effect of serum type, $F_{(3,36)}$ = 1.328; P = 0.2804). The same trends were found in hibernation cells (effect of isoproterenol, $F_{(1,44)}$ = 34.36; P < 0.0001; effect of serum type, $F_{(3,44)}$ = 0.4296; P = 0.7328).

II. Baseline Differences in Gene Expression (Fig. 7):

i. **ADRB2:** Serum treatment had a significant impact on fold change (effect of cell season, $F_{(1,52)} = 0.2353$; P = 0.6296; effect of serum treatment, $F_{(3,52)} = 12.3$; P < 0.0001). All bear sera suppressed baseline ADRB2 expression compared to FBS treatment. Active cell bear serum treatment fold changes were significantly lower than those of hibernation cells treated with FBS. Similarly, active season cells grown with FBS expressed higher levels of ADRB2 than did active season cells treated with bear serum, but this difference was only significant in cells cultured with active season serum (P = 0.029).

ii. **ATGL:** Although cells grown in FBS generally expressed more ATGL than cells incubated in bear serum, this difference was only significant in active season cells treated with active season serum, which expressed ATGL at minimal levels compared to active season cells grown in FBS. No other significant differences were found at baseline (effect of cell season, $F_{(1,54)} = 2.042$; P = 0.1588; effect of serum treatment, $F_{(3,54)} = 2.075$; P = 0.1143).

iii. **LEP:** Both serum treatment and interactions were found to be statistically significant (effect of cell season, $F_{(1,56)} = 3.786$; P = 0.0567; effect of serum treatment, $F_{(3,56)} = 4.682$; P = 0.0055; interaction, $F_{(3,56)} = 6.189$; P = 0.0010). Active season cells treated with hibernation serum had significantly increased LEP gene expression compared to any other treatment group.

iv. **LEPR:** Interaction and serum treatment both exerted significant effects on fold change (effect of cell season, $F_{(1,50)} = 3.553$; P = 0.0653; effect of serum treatment, $F_{(3,50)} = 6.490$; P = 0.0009; interaction, $F_{(3,50)} = 6.388$; P = 0.0009). All treatment groups expressed significantly smaller amounts of LEPR than active season cells treated with FBS.

III. Response to Isoproterenol (Fig. 8):

i. **ADRB2:** Statistical analysis revealed significant effects of cell season, serum type, and interaction on gene expression (effect of cell season, $F_{(1,48)} = 17.51$; P = 0.0001; effect of serum

type, $F_{(3,48)}$ = 3.918; P = 0.0140; interaction, $F_{(3,48)}$ = 3.103; P = 0.0352). No significant differences existed among serum treatments in active season cells (P = 0.9999). However, hibernation cells treated with hibernation serum (16.0252±6.7287) or FBS (15.4896±6.1581) exhibited sex-dependent responses to isoproterenol stimulation. Male bear hibernation cells treated with FBS had an average fold change of 29.5043±6.7802; in female bears, average fold change was only 1.4749±0.2416. Male bear hibernation cells treated with hibernation serum had an average fold change of 30.9856±7.8782, while female bear cells from the same treatment group averaged 1.0648±0.1129. In contrast, fold change in hibernation cells treated with active or hyperphagic season serum did not significantly differ from the responses of active season cells.

ii. ATGL: Only cell season significantly impacted gene expression following stimulation with isoproterenol (effect of cell season, $F_{(1,56)}$ = 13.90; P = 0.0005; effect of serum type, $F_{(3,56)}$ = 1.295; P = 0.2851). Hibernation cells treated with active season serum had significantly larger fold changes than did active cells treated with active serum (P = 0.047), FBS (P = 0.0081), or hibernation serum (P = 0.0283).

iii. LEP: Only cell season altered fold change among treatments (effect of cell season, $F_{(1,56)}$ = 5.684; P = 0.0205; effect of serum type, $F_{(3,56)}$ = 2.245; P = 0.0930). This difference was only significant in active season and hibernation cells treated with hibernation serum.

iv. LEPR: Interaction and serum treatment impacted gene expression (effect of cell season, $F_{(1,54)}$ = 1.528; P = 0.2218; effect of serum type, $F_{(3,54)}$ = 5.651; P = 0.0019; interaction, $F_{(3,54)}$ = 8.060; P = 0.0002). Active season cells treated with hyperphagic serum had significantly larger fold changes (4.052±0.4907) than any other treatment group. This difference was most

pronounced when compared with active cells treated with hibernation serum or FBS ($P < 0.0001$) and hibernation cells treated with hibernation or hyperphagic serum ($P = 0.0005$).

Temperature Experiments

An analysis of baseline subtracted medium glycerol measurements showed no difference in glycerol release between cells from different seasons. However, temperature treatment did influence medium glycerol (effect of cell season, $F_{(1,21)} = 1.106$; $P = 0.3048$; effect of temperature treatment, $F_{(2,21)} = 12.88$; $P = 0.0002$). Medium glycerol did not differ between groups of cells grown at 34.7°C for 3 or 11 days ($P = 0.7527$), but cells grown at the lower temperature did release less glycerol than control cells grown at 37°C (3 days, $P = 0.0003$; 11 days, $P = 0.002$). Again, gene expression did not differ between cold-cultured cells and cells grown at 37°C, nor was cell season of origin significant in cells grown at 34.7°C.

RT-qPCR for ADRB2, ATGL, LEP, and LEPR in isoproterenol-stimulated samples maintained at 34.7°C for 11 days showed no significant differences between cell seasons in cells grown at lower temperatures. Additionally, gene expression did not differ significantly between isoproterenol-stimulated cells grown at 34.7°C and 37°C.

Adrenergic Receptors

A canine probe for β3-adrenergic receptor (ADRB3) failed to amplify in mRNA samples from differentiated cells. However, a canine probe for β1-adrenergic receptor (ADRB1) did amplify in mRNA from cultured active season and hibernation adipocytes from male and female bears. RT-qPCR for ADRB1 in these samples revealed that ADRB1 is expressed at relatively low levels in bear adipocytes. We found no differences between hibernation cells treated with hibernation serum or FBS ($P = 0.2502$), active season cells treated with active season serum or FBS ($P = 0.5488$), or both cell types treated with FBS ($P = 0.9567$).

DISCUSSION

Grizzly bears are metabolically primed for lipogenesis during the active season and lipolysis during hibernation. A variety of physiological parameters were investigated to verify this both at the cellular level and in the whole animal. Bears exhibited seasonal differences in plasma adipokine concentrations that were at least partially independent of body fat content. Thus, neither leptin nor adiponectin appears useful as indicators of body fat content. However, centrally administered leptin altered ingestive behavior in pre-hibernation bears, decreasing daily food intake compared to control animals in all trials. This effect was only seen just prior to hibernation, supporting our hypothesis that bears are leptin-insensitive in August at the beginning of the fall hyperphagic period. In studies of isolated bear adipocytes, quantitative PCR showed that cell season and serum treatment can impact genes that influence adipose-derived hormones and lipolysis.

In humans and rodents, serum leptin concentrations are highly correlated with body fat (Zhang et al. 2002), unlike that occurring in grizzly bears. Serum leptin concentrations were reported to be predictive of body fat in the American black bear (Spady et al. 2009). In that study, serum leptin and body fat content were assessed in January, March, June, and October. The primary difference in seasonal leptin trends in black and grizzly bears was that in grizzly bears, leptin dropped to baseline levels by January, while in black bears January leptin concentrations did not differ from the relatively high concentrations seen in October. However, only male black bears were sampled in January, and these males had access to supplemental food throughout the winter. In fact, most males were heavier in January than in October and had higher body condition indices. These factors may have altered their leptin profiles, potentially extending or delaying peak serum leptin concentrations. In our bears the rise in leptin was more

discrete, surging in October. While a relationship still existed, it is clear that the elevated leptin concentrations of the hyperphagic period account for much of the seasonal positive relationship (Fig. 3A).

Our annual serum leptin profile for the grizzly bear is strikingly similar to that of the Japanese black bear (Tsubota et al. 2008) and corroborates patterns seen in the active and hyperphagic periods in the Spady study (2009). Our research also confirms findings from less extensive studies of a variety of ursids, including the European brown bear (*Ursus arctos arctos*) (Hissa et al. 1998) and the grizzly bear (Gardi et al. 2011). This suggests that the seasonal leptin profile may be common to the 4 members of the genus *Ursus*, all of which are known to hibernate (Nelson et al. 1983; Tsubota et al. 2008).

Based on our feeding studies, it appears that bears are insensitive to leptin during hyperphagia but resensitize as hibernation approaches, implying that they are capable of reversing their leptin resistance. In humans, the leptin-resistant state is a hallmark of obesity: higher concentrations of circulating leptin in obese individuals fail to reduce body weight or suppress appetite (Galic et al. 2010; Meier and Gressner 2004; Myers et al. 2008). In our study, increased concentrations of leptin were most effective when bears were at their heaviest during their annual surge of endogenous leptin. This suggests that a threshold may need to be reached for appetite to be suppressed. Intriguingly, the fall in leptin that occurs in early hibernation does not cause bears to resume feeding. Elucidation of the mechanism bears use to alter leptin sensitivity may therefore have important ramifications for human obesity research. Unpublished results from our laboratory show that brain leptin signaling is intact during hibernation, indicating that central leptin sensitivity is independent of circulating leptin concentrations (Jansen, unpublished results).

The compensatory feeding that occurred on the day of the injection suggests that cumulative daily intake is governed by an endogenous rhythm or homeostatic response to caloric restriction. The ability to sense energy deficits would be a highly adaptive trait given the imperative for bears to gain weight prior to hibernation (Farley and Robbins 1995). The Syrian hamster (*Mesocricetus auratus*), a photoperiodic hibernator, lacks this ability and consequently can starve to death by failing to compensate for lost food intake despite having the physical capacity to do so (Silverman and Zucker 1976). Interestingly, while most animals exhibit compensatory feeding to defend a certain body weight, hyperphagic bears eat enough to not just maintain their weight, but also to continue to gain weight in preparation for hibernation despite increasing circulating leptin concentrations.

Serum adiponectin generally followed the same patterns as leptin, except adiponectin tended to increase slightly earlier and more gradually. Serum adiponectin in American black bears decreased during hibernation compared to the active season. Adiponectin is thought to inhibit bone formation, so a reduction in circulating adiponectin during hibernation could increase osteoblast activity, balancing bone formation and resorption in hibernating bears (McGee-Lawrence et al. 2015). Adiponectin also has insulin-sensitizing effects that could help bears maintain lipogenesis during the active and hyperphagic periods, when bears are storing energy as fat. Likewise, the decrease in adiponectin that occurs during hibernation might promote insulin resistance, facilitating the development of a highly lipolytic state (Kadowaki et al. 2006).

The elevated serum glycerol of hibernating grizzly bears relative to that of hyperphagic bears has not been shown in all ursids. For example, serum glycerol was significantly lower during hibernation in American black bears (Ahlquist et al. 1984). By contrast, the increased

serum glycerol seen in hibernating grizzly bears indicates that they were fully catabolizing TAG to mobilize FFAs as well as glycerol, which is consistent with a switch to a lipid-based metabolism.

Surprisingly, in our cell cultures, serum treatment and cell type did not impact glycerol release, implying that the differential glycerol release seen seasonally *in vivo* is dependent on factors not found in serum or isolated cells. In the Siberian hamster (*Phodopus sungorus*), seasonal differences in lipolytic function are controlled by photoperiod. Light-related signals are relayed to adipocytes through sympathetic nerves that originate in the spinal cord, modulating lipolysis in response to the light-dark cycle (Bartness et al. 2014). Changes in photoperiod also alter activity seasonally in grizzly bears (Ware et al. 2012). It is therefore possible that seasonal differences in glycerol release are in part governed by direct changes in sympathetic innervation or activity that were not present in isolated adipocytes.

Another factor that could influence adipocyte responsiveness is the decreased body temperatures of hibernating bears. In some mammals, cold exposure can cause physiological remodeling in fat, such as the browning of WAT (Barbatelli et al. 2010). We cultured grizzly bear subcutaneous adipocytes at lower temperatures to simulate hibernation. When stimulated with isoproterenol, adipocytes cultured at 34.7°C released less glycerol than cells cultured at 37°C. Adipocytes cultured at the lower temperature for just 3 days released the same amount of glycerol as adipocytes maintained at 34.7°C for 11 days, so it is unlikely that this decreased glycerol release is a byproduct of the smaller lipid droplets we saw in cells cultured at low temperatures for 11 days. As we saw before in serum-treated cells, cell season of origin did not impact glycerol release within each temperature treatment, which again suggests that seasonal

differences in plasma glycerol are triggered by elements that are absent from isolated subcutaneous adipocytes.

One of the genes we investigated was ADRB2, which was selected because it initiates the lipolytic cascade and can decrease leptin expression (Slieker et al. 1996). Hibernation cells treated with FBS or serum from hibernating bears expressed ADRB2 at extremely high levels following exposure to isoproterenol. The fact that these large fold changes were found in cells treated with either bear serum or FBS suggests that this response is driven by cell autonomous changes independent of specific serum factors. This increase could serve as a 'priming' effect to facilitate an enhanced lipolytic response in hibernating bears. Closer examination of the data revealed that this difference was only pronounced in male bears. Due to small sample size, further study is needed to determine whether this is a valid trend. However, adrenergic receptor expression in white adipocytes often differs greatly between species and sexes, so it is possible that male bears do express ADRB2 at much higher levels than females (Lladó et al. 2002). We also assessed ADRB1 to determine which receptor is dominant in white adipocytes. Fold changes in ADRB1 following isoproterenol stimulation were generally much smaller than those seen for ADRB2. This information taken together with the fact that ADRB1 expression did not differ by season suggests that ADRB2 is the predominant β-adrenergic receptor controlling seasonal changes in lipolytic function.

While the β-adrenergic receptors serve as the initial interface between the nervous system and the adipocyte, downstream lipases regulate the amount and type of substrate released from lipid droplets within the fat cell. We assessed ATGL because it is upregulated in fasting animals (Viscarra and Ortiz 2013) and because it liberates the first FFA from TAG molecules and thus should reflect seasonal differences in lipase activity. Prior research in murine 3T3-L1 cells

showed that exposure to isoproterenol decreased ATGL mRNA expression (Kralisch et al. 2005). However, in bear cells stimulated with isoproterenol ATGL expression increased or did not change. Because mice do not hibernate, this increase in ATGL under isoproterenol stimulation may reflect a specific adaptation found only in hibernators. Interestingly, sera from 'opposite' seasons had opposite effects on adipocytes from each season, such that gene expression from cells treated with the opposing serum type matched that of cells treated with serum from the same season. This indicates that, as we saw for ADRB2, the cells themselves are more influential than the serum treatment in altering gene expression, an observation that is consistent with hibernating bears being strongly predisposed to fat catabolism.

In summary, this study outlines seasonal changes in circulating adipokines and glycerol as well as glycerol release and gene expression *in vitro*. In cultured adipocytes, genes involved in lipolysis seem to be controlled both by serum factors and season-dependent properties of the cells themselves. We demonstrated that bears are sensitive to leptin's central effects only during certain time periods, which will provide the basis for future research into shifting adipokine sensitivity in other hibernators. Grizzly bears may therefore prove to be an invaluable model of naturally reversible leptin resistance, a condition strongly associated with obesity and metabolic dysfunction in humans. Further research is required to identify the mechanisms bears use to modulate their sensitivity to various adipokines.

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Table 1 Treatment each animal received in each trial. A = Artificial CSF; L = Leptin

	Trial Date					
	2013		2014		2015	
Bear	AUG	OCT	AUG	OCT	AUG	OCT
A	A	A				
B	A	A	L	L		
C	L	L	A	A	A	A
D	L	L	L	L	L	A

Table 2 Target genes, probe names, expected values, and predicted homology with *U. maritimus* according to a BLASTn search of the National Center for Biotechnology Information database.

Gene	TaqMan Probe ID	Interrogated RefSeq	*U. maritimus* RefSeq	E Value	Identity (%)
ADRB1*	Cf02691269_g1	NM_001008713.1	XM_011232074.1	$3\ e^{-28}$	97
ADRB2	Cf02690130_s1	NM_001003234.1	XM_008692744.1	$8\ e^{-60}$	99
ADRB3*	Cf03022965_s1	NM_001003377.1	XM_002917227	$5\ e^{-39}$	94
ATGL	Cf02660386_g1	XM_849071.1	XM_008699672.1	$2\ e^{-41}$	94
LEP	Cf02621954_m1	NM_001003070.1	XM_008705840.1	$4\ e^{-30}$	97
LEPR	Cf02622590	NM_001024634.1	XM_008690767.1	$1\ e^{-38}$	98

*The marked probes were compared to predicted sequences for the giant panda (*Ailuropoda melanoleuca*) after a search of predicted polar bear sequences failed to yield results. The giant panda is a more distant relative of the grizzly bear for which the full genome has been sequenced (Li et al. 2010), although this species does not hibernate.

FIGURES

Fig. 1 Images from a contrast study in a 3-year-old male bear. **a** The arrow indicates the needle injecting contrast into the subarachnoid space. S – skull, C1 – atlas, C2 – axis **b,c** – A false color image shows contrast (red) spreading through the subarachnoid space, with the hypothalamus indicated by (*). Arrows indicate the injection site **c** – The horizontal line on this figure represents the plane of (**b**)

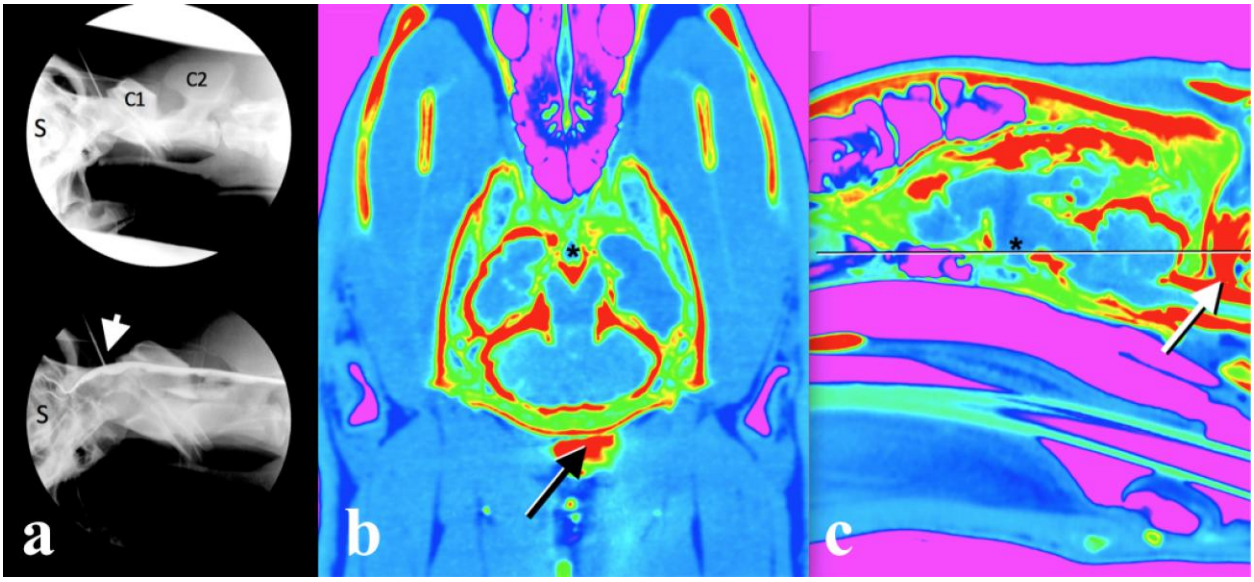

Fig. 2 Average monthly serum adipokine concentrations. All animals had been fasted for 12 hours prior to sampling. SEM is indicated at each time point. **a** – Leptin (ng/ml) in anesthetized (n = 10) and trained unanesthetized (n = 4) captive grizzly bears. (*) indicates that serum leptin concentrations differed significantly from January values ($p < 0.05$). **b** – Leptin (ng/ml) in trained unanesthetized (n = 4) captive grizzly bears **c** – Adiponectin (μg/ml) in trained unanesthetized (n = 4) captive grizzly bears

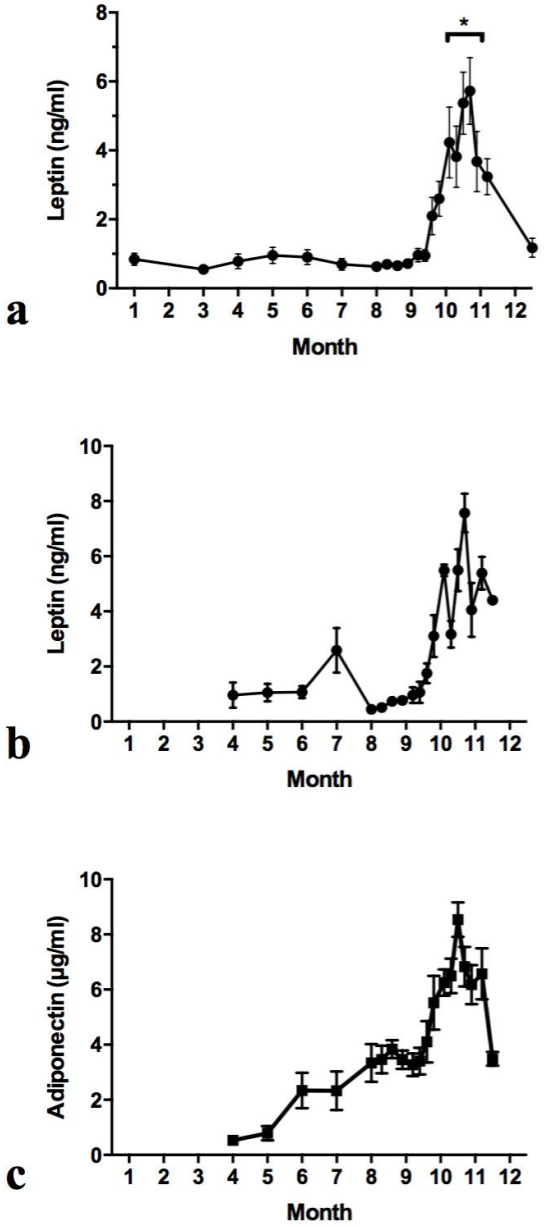

Fig. 3 Monthly serum concentrations of **a** – leptin (ng/ml) and **b** – adiponectin (µg/ml) compared to body fat percentage in 4 unanesthetized adult female bears in April through November of a single year. Square points represent samples collected in October

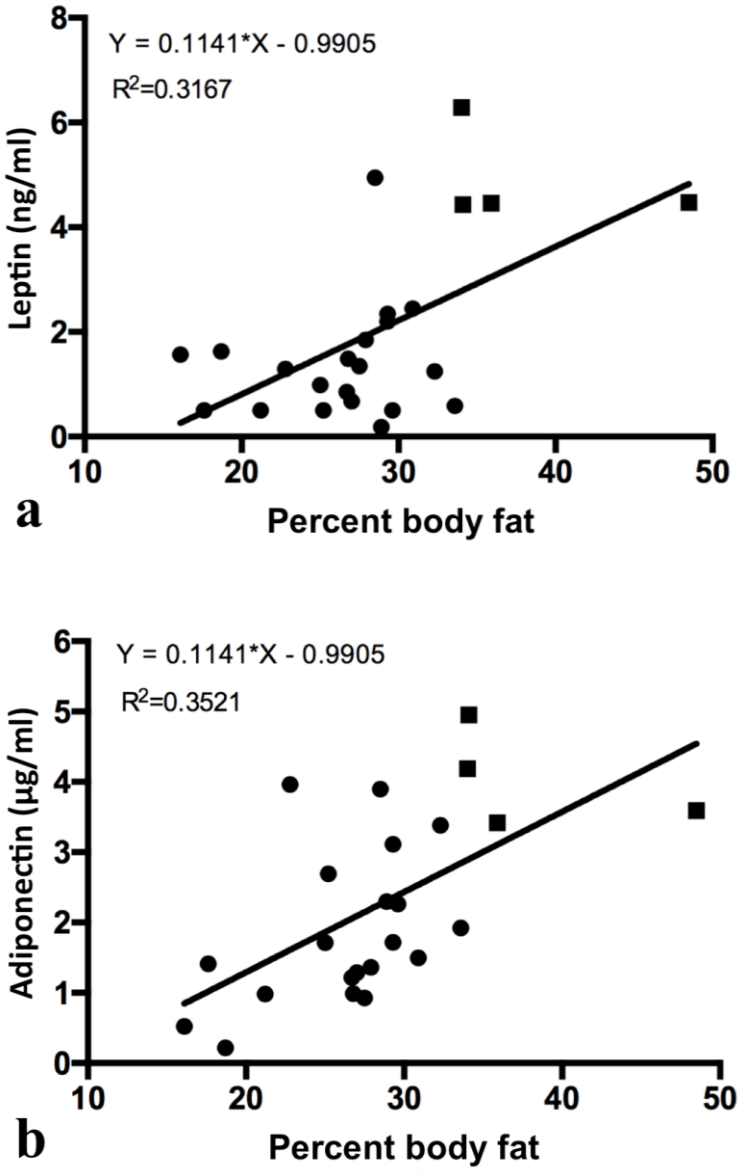

Fig. 4 Average cumulative post-injection intake in male bears in **a** – August and **b** – October presented as a percentage of average baseline intake (i.e. the first 3 days of the trial)

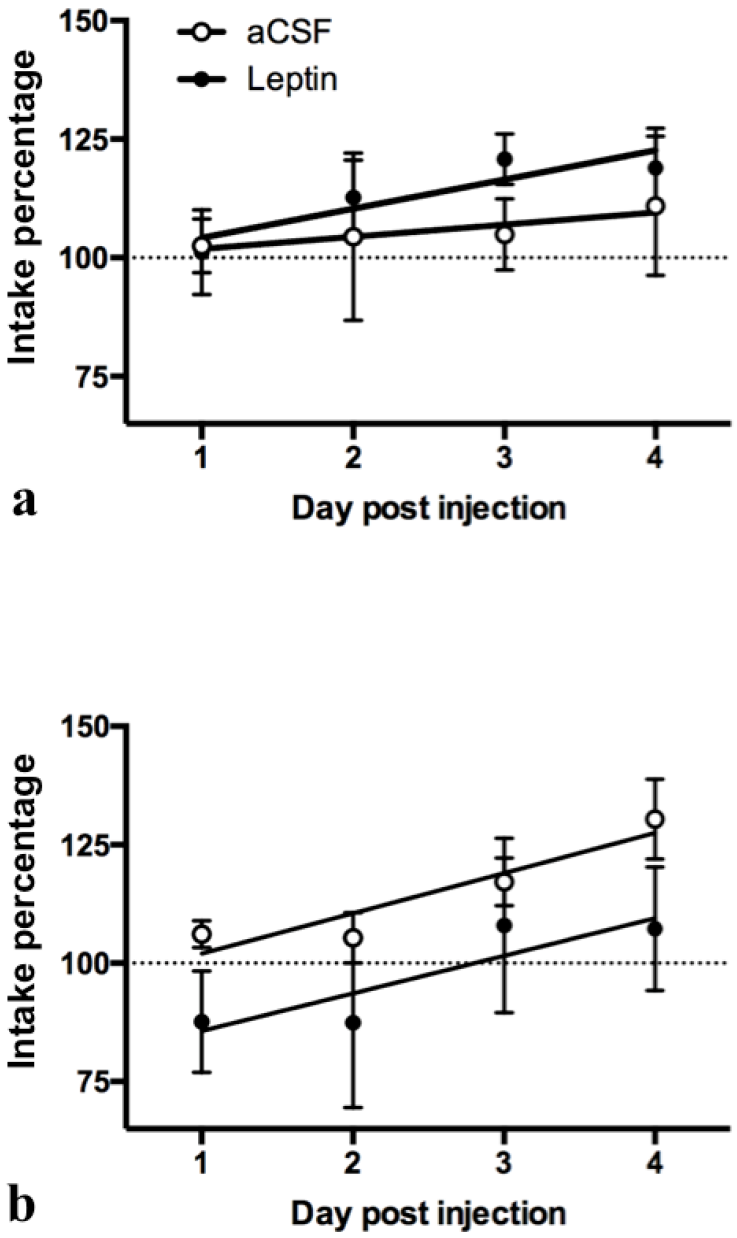

Fig. 5 Average glycerol concentrations (µg/ml) in serum collected from male (n = 7) and female (n = 6) bears that were unanesthetized (n = 4) or anesthetized (n = 9). The gray background indicates hibernation, while the white background marks the active season. The symbol ** indicates a significant difference compared to time point 10.6, when peak leptin occurred

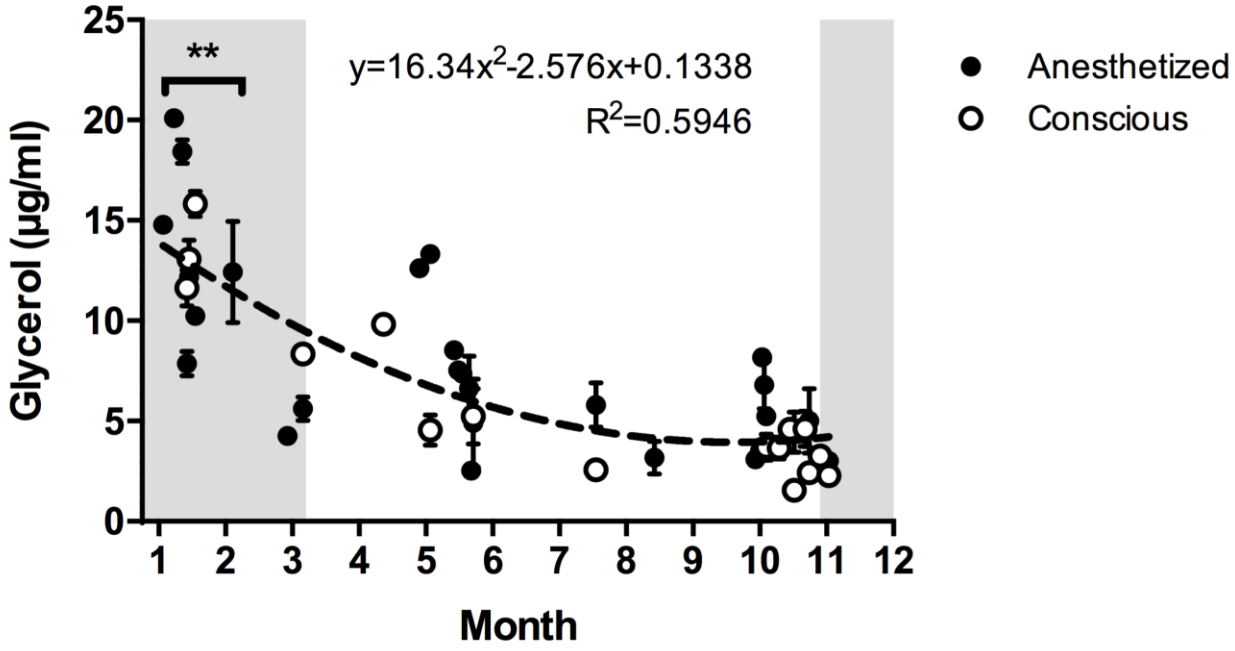

Fig. 6 Average glycerol concentrations (μg/ml) both at baseline and following a 60-minute isoproterenol treatment found in medium from **a** – active season cells and **b** – hibernation cells

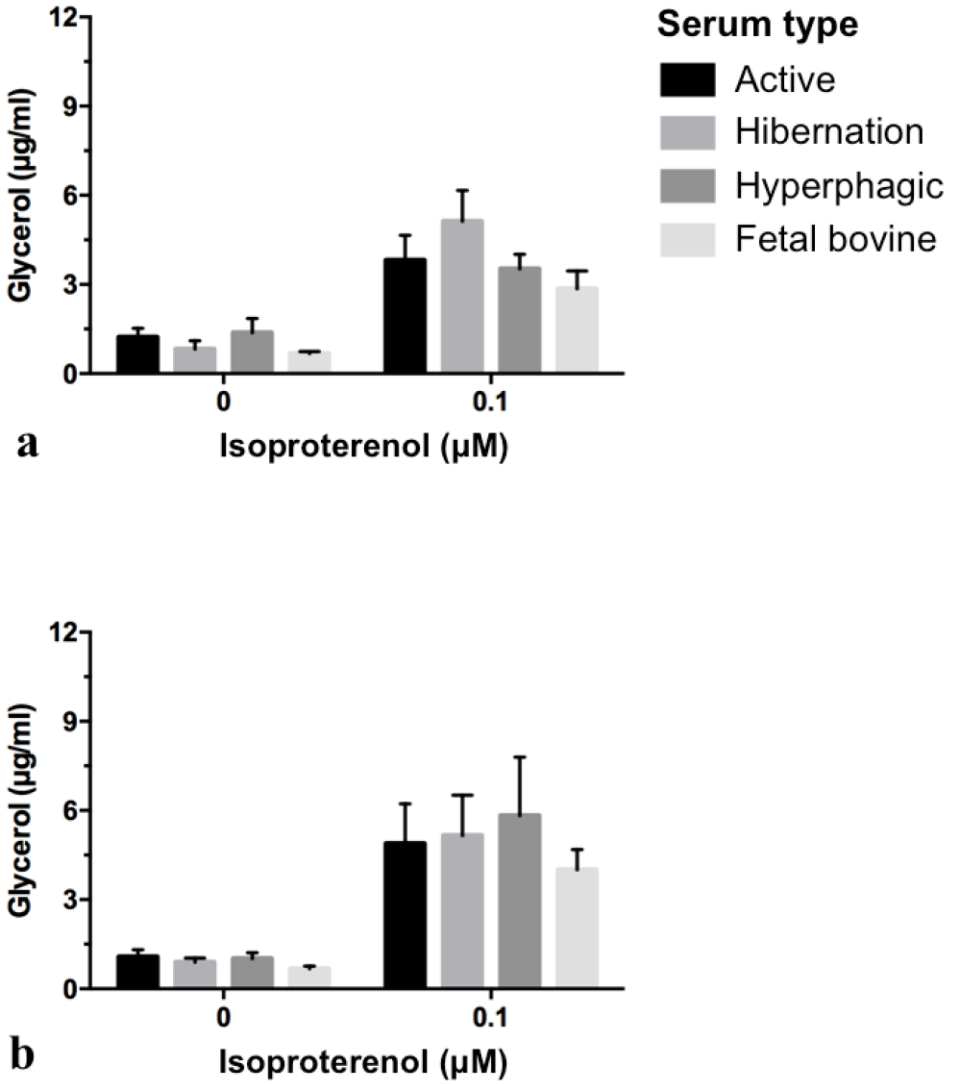

Fig. 7 Baseline differences in **a** – ADRB2, **b** – ATGL, **c** – LEP, and **d** – LEPR gene expression. Fold change was calculated by comparing all samples to active season cells grown with 10% FBS that were also not treated with isoproterenol

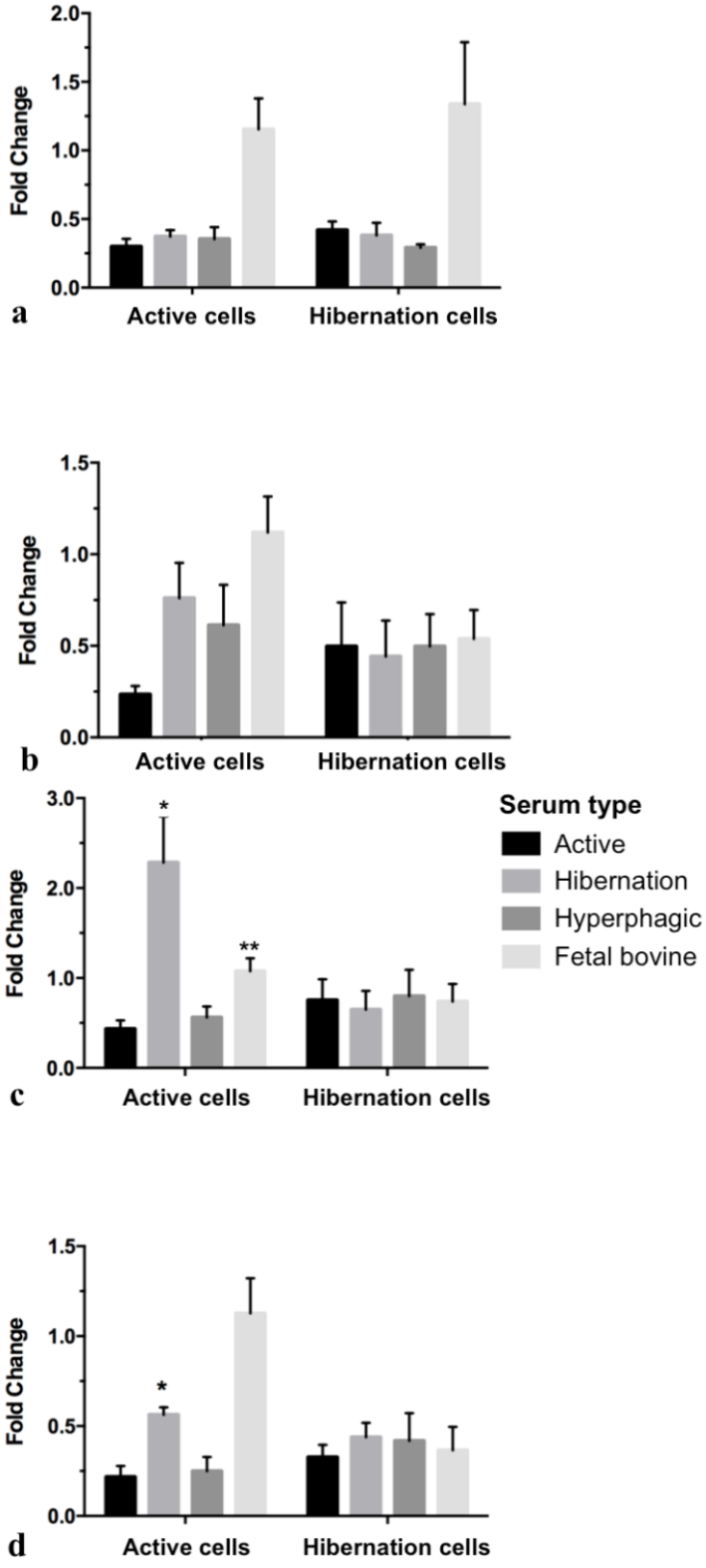
Fold Change
2.0
1.5
1.0
0.5
0.0
Active cells
Hibernation cells
a
Fold Change
1.5
1.0
0.5
0.0
Active cells
Hibernation cells
b
Fold Change
3.0
2.0
1.0
0.0
*
**
Active cells
Hibernation cells
c
Serum type
Active
Hibernation
Hyperphagic
Fetal bovine
Fold Change
1.5
1.0
0.5
0.0
*
Active cells
Hibernation cells
d

Fig. 8 Effect of isoproterenol on **a** – ADRB2, **b** – ATGL, **c** – LEP, and **d** – LEPR gene expression. Fold change was calculated by comparing samples stimulated with 0.1 μM isoproterenol with samples that were only incubated in KRPH buffer within each treatment group