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Habitat effects on blood adiponectin isoforms in black bears.

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Habitat Effects on Blood Adiponectin Isoforms in Black Bears

By

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Lay Summary

Adiponectin is a hormone closely associated with symptoms of obesity and diabetes. This hormone has multiple forms: a low molecular weight form (LMW) and a high molecular weight form (HMW). Levels of the HMW form directly correlate to symptoms of the aforementioned conditions. Blood samples were taken from black bears of two different habitats in eastern Kentucky. One population from Pine Mountain (PM), and the other from Big South Fork (BSF). These populations were utilized under the assumption that they would differ in potential sources of nutrition. The PM population is located in an area with greater access to highly palatable and energy rich human refuse. Therefore, this population was thought more likely to have a poor diet compared to the BSF population, which was assumed to be more reliant on natural sources of nutrition. The adiponectin in the blood samples was separated into its two different forms and the concentrations of the two forms were compared between the two habitats. The concentrations of the adiponectin forms were also compared between sexes of the bears sampled.

This study found that bears from PM had significantly higher concentrations of total adiponectin than bears from BSF. Male bears from PM had significantly higher levels of the HMW form, and lower levels of the LMW form, when compared to male bears from BSF. No other significant differences were found with respect to habitat or sex. This suggests that a significant difference does exist between the two habitats. Possible explanations for this difference may include effects of age, percent body fat, diet, and seasonality of hormones.

Summary

Adiponectin is a 244-amino acid protein hormone predominantly produced and secreted by white adipocytes. This hormone plays a variety of roles in the regulation of metabolic processes that may be involved in obesity and copathologies, such as diabetes and cardiovascular disease. Adiponectin is synthesized in white adipocytes as a 30 kilodalton (kDa) monomer. Post-translational modifications transform this monomer into higher molecular weight multimers, including hexamers (known as the low molecular weight form, LMW) and 12-18 unit oligomers (known as the high molecular weight form, HMW). Although both multimeric forms are found in circulation, it has been established that the LMW adiponectin isoform is found at very minute levels. Furthermore, changes in HMW adiponectin correlate more with changes in total serum adiponectin levels, suggesting the HMW isoform is more metabolically active in both human and animal models.

Blood serum samples were obtained from two populations of radio-collared black bears (*Ursus americanus*) in Eastern Kentucky. HMW adiponectin was separated from LMW adiponectin utilizing a centrifugation protocol. Serum and isoform levels of adiponectin were determined with a sandwich ELISA (enzyme-linked immunosorbent assay) kit (Rat) from ALPCO Diagnostics. Mean ± standard error values for serum and isoform adiponectin levels were determined for the Pine Mountain (PM) and Big South Fork (BSF) populations. These values were compared according to sex and habitat utilizing a one-tailed student t-test with $p < 0.05$ considered significant.

The findings of this study are as follows. First, black bears from the PM population had a significantly higher total serum adiponectin concentration than bears from the BSF population. After the multimeric analysis was performed, it was found that the percentage of HMW adiponectin was significantly greater in the PM population than in the BSF population. In contrast, the percentage of LMW adiponectin was significantly lower in the PM population than in the BSF population. Two significant findings concerning sex were found. The first was that males from PM had significantly higher percentages of HMW adiponectin than did males from BSF. The second was that males from PM had significantly lower percentages of LMW adiponectin than did males from BSF. No significant difference was found between any other combination of habitat and sex.

These findings suggest that black bears from different habitats with potentially different dietary regimens may have distinct metabolic mechanisms involving the levels of adiponectin in its multimeric forms. Further studies should be conducted with focus on the multimeric analysis of adiponectin in animal models that exhibit reversible obesity. This will provide potentially more information on the relationship between adiponectin, obesity, diabetes, and metabolic syndrome in humans.

INTRODUCTION

Adiponectin is a 244-amino acid protein hormone predominantly produced and secreted by white adipocytes, or white fat cells. This hormone plays a variety of roles in the regulation of metabolic processes. Among these roles, adiponectin appears to enhance insulin sensitivity, exhibits anti-atherosclerotic effects, increases β-oxidation of fatty acids in liver and muscle tissue, and reduces inflammation (Sowers, 2008). These functions of adiponectin may ultimately provide a link to its role in the development and control of type II diabetes, heart disease, and obesity. Uniquely, in comparison to other adipokines, serum adiponectin concentration is inversely related to percent body fat and insulin levels in humans and most animal models (Bullen Jr. et al., 2007). This trend is affiliated with the exacerbating effects that obesity has on the conditions of heart disease, diabetes, and metabolic syndrome.

Adiponectin is synthesized in white adipocytes as a 30 kilodalton (kDa) monomer. Post-translational modifications transform this monomer into higher molecular weight multimers, or aggregates of monomers. These include hexamers (known as the low molecular weight form, LMW), which are compounds of 6 adiponectin monomers, and 12-18 unit multimers (known as the high molecular weight form, HMW). In Figure 1, Goldstein et al. (2009) illustrated the structure of adiponectin after post-translational modifications. Levels of HMW adiponectin correlate more with obesity and type II diabetes, suggesting the HMW isoform is more metabolically active in both humans and animals. Schraw et al. (2015) demonstrated that there is no interconversion between these multimeric forms in the circulation following secretion. There is a high degree of correlation between the levels of the HMW isoform and the previously discussed conditions (Lara-Castro et al., 2006). This discovery suggests that while total serum adiponectin levels are obviously important, the multimeric distribution of adiponectin should be explored.

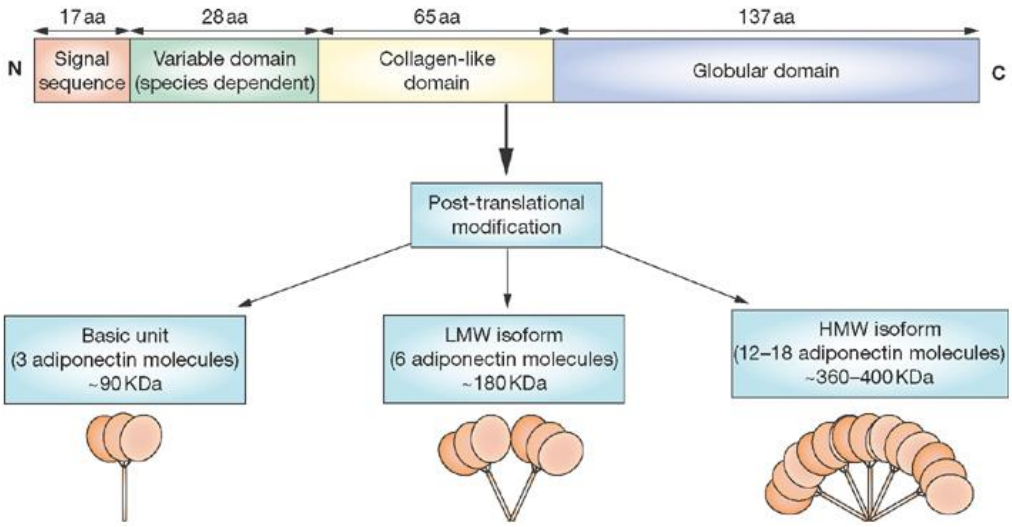

Figure 1. The structure of the various adiponectin isoforms (Goldstein et al., 2009).

Interestingly, human studies and rodent models show females to have significantly higher levels of total serum adiponectin than their male counterparts (Schraw et al., 2015). Similarly, Waki et al. (2003) demonstrated that females have significantly higher levels of the HMW isoform than males. However, no significant difference in the LMW isoform has been found between sexes. Xu et al. (2005) demonstrated the theory that testosterone selectively inhibits the secretion of HMW adiponectin, accounting for this difference between sexes.

Animal studies have proven effective to further investigate the relationship between HMW adiponectin and the onset of heart disease, diabetes, and obesity (Shafrir & Ziv, 2009). Most studies reiterate that, through diet-induced obesity, reduction in HMW adiponectin is associated with insulin resistance and diabetic symptoms. Kennedy et al. (2010) showed that populations with metabolic syndrome exhibit double the chance of having a heart attack or stroke, as well as a fivefold increase in the risk of developing diabetes. These animal models utilize mostly small rodents such as rats and mice and have brought about progress in the understanding of the effects of adiponectin.

Overhunting and loss of habitat significantly reduced the population of the once abundant black bear (*Ursus americanus*) in the eastern United States. Due to concerted efforts by wildlife management specialists, the population has risen sufficiently that some states, such as Kentucky, allow a limited hunting season. Two populations of the black bear in Kentucky are of interest in this study. The Big South Fork (BSF) population in McCreary County has limited contact with human populations due to the more isolated nature of the area. In contrast, the Pine Mountain (PM) population in Harlan, Letcher, and Pike counties is in an area of greater human habitation and tourist visitation. This affords the PM population a greater potential for access to highly palatable and energy rich sources of human food and refuse. Some of the bears in the PM population, due to their more frequent contact with humans, were noted to be nuisance animals. This difference in habitat and nutrition is a key component of my study. In a recent study on rodents, those given the option of consuming highly palatable, energy dense foods prevalent in Western society developed symptoms similar to those found in humans afflicted with obesity and metabolic syndrome (Sampey et al., 2011). These symptoms include insulin resistance, hyperglycemia, higher levels of nonesterified fatty acids, and systemic inflammation. The black bear, given a similar type of diet, may parallel these results.

Black bears and humans are both large, omnivorous mammals. Depending on the utilization of conserved amino acids, the amino acid sequence of the adiponectin gene in black bears and humans is somewhere between 88% and 94% similar. Furthermore, black bears, as true hibernators, may serve as natural reversible obesity models (Martin, 2015). Hibernating mammals serve as unique metabolic models because of their ability to store exceedingly high amounts of fat prior to hibernation without signs of adverse health effects typically observed in obese non-hibernating species.

Using the bear as a study animal allows for the long-term tracking of changes in both total and multimeric serum adiponectin levels. In a recent study, Nelson et al. (2014) demonstrated that grizzly bears exhibit increased sensitivity to insulin in the obese, pre-hibernation state prior to a reversible insulin resistant state during winter hibernation. Despite the increasing use of hibernating animal models in research, the multimeric analysis of the different isoforms of adiponectin has not yet been conducted among them (Florant et al., 2004). The purpose of this study was to examine the multimeric distribution of serum adiponectin in two summer-active populations of black bears living in different habitats with potentially different sources of nutrition. It was hypothesized that bears from the PM population would weigh more than bears from BSF, and thus would exhibit lower levels of both total serum adiponectin and specifically the HMW isoform. It was also hypothesized that female bears from both populations would have higher levels of both total serum adiponectin and specifically the HMW isoform compared to males.

METHODS

Dr. John Cox and his research team from the University of Kentucky Department of Forestry collected blood serum samples from the two populations of radio-collared black bears (*Ursus americanus*) in Eastern Kentucky. Animals of both sexes and different ages were trapped utilizing Aldrich spring-activated foot snares, culvert traps or free-range darting. Captured bears were immobilized with Telazol (mg/kg) administered utilizing a dart rifle or a jab stick. Blood was collected by vacutainer tube in two 10 mL aliquots form the femoral vein and maintained on ice. Samples were centrifuged within one hour of collection to separate the serum, which was placed in a new vacutainer and immediately frozen at -20°C. Samples were transported from the field to a laboratory in Lexington on ice to maintain the frozen state and immediately placed in a -20°C

freezer. Frozen samples were transferred to Louisville on ice to maintain the frozen state and stored at -80°C until utilization.

HMW adiponectin was separated from LMW adiponectin utilizing a centrifugation protocol employing tubes from Pall Corporation (Ann Arbor, MI). These tubes contain filters having a molecular weight cutoff of 300kDa. Serum samples were placed into the upper chamber of the tubes, which were centrifuged at 12,000 rpm for 15 minutes, or until about 75% of the initial volume went through the filter. Because the HMW isoform of adiponectin has a molecular weight greater than the filter cut-off limit, it remained in the upper chamber while the LMW isoform was filtered into the lower chamber.

Serum adiponectin was determined with a sandwich ELISA (enzyme-linked immunosorbent assay) kit (rat) from ALPCO Diagnostics (Salem, NH). The amino acid sequence of adiponectin was compared between rats and polar bears. Including conserved amino acids, the amino acid sequences are 91% similar. Assay instructions provided by the manufacturer were followed explicitly utilizing a pre-coated microtiter plate. Aliquots, in duplicate, from each standard, control, and bear sample were loaded into the 96-well plate. The absorbance values of the standards, controls, and bear samples were determined. The absorbance values were measured at 450 nm utilizing an EL_x808 microplate reader. The absorbance values of each standard corrected for background were utilized to produce a standard curve ($r^2 = 0.988$) from which the serum adiponectin concentrations were calculated.

Black bear samples obtained in summer 2011 were organized according to the two different habitats. Of the total samples collected from PM (*N* = 23) and BSF (*N* = 14), 10 samples from PM and 9 samples from BSF were chosen to analyze due to the limited number of wells in the microtiter plate. Two samples were strikingly inconsistent with the others, so one sample from each

population was thrown out as an outlier. All samples used in this study were taken from adult black bears in an effort to minimize any effects age may have on serum and isoform levels of adiponectin, though ages still varied. Of the 9 remaining samples from PM, 5 were from male specimens and 4 from female specimens. Of the 8 remaining samples from BSF, 3 were from male specimens and 5 from female specimens. Mean ± standard error values for serum adiponectin were determined for the PM and BSF populations. These values were compared according to sex and habitat utilizing a one-tailed student t-test with $p < 0.05$ considered significant.

RESULTS

A summary of the characteristics of those bears used in this study can be found listed in Table 1. It can be noted that, on average, the black bears sampled from Big South Fork (BSF) weighed significantly more than those from Pine Mountain (PM) ($t = -2.43$, $p < 0.02$). Furthermore, male specimens generally weigh more than female specimens. This variance in weight may be due to the age of the specimens when samples were taken. Though all samples utilized were from adult black bears, some specimens were 7 years of age, while others were only 3 years of age.

Total Adiponectin Concentration

Figure 2 depicts a comparison between adult black bears from the two habitats with respect to the total samples available ($N = 37$) and the samples ($N = 17$) used for this study. For the total samples available, bears from PM had a significantly higher concentration of adiponectin when compared to those from BSF ($t = 3.44$, $p < 0.001$). The smaller sample population of only adults used for this study mimicked this result, with bears from PM having a significantly higher concentration of adiponectin than those from BSF ($t = 2.19$, $p < 0.03$).

Sample ID	Habitat	Sex	Weight (lbs.)	Total Adiponectin Concentration (µg/mL)
1	PM	F	135	0.7
6	PM	M	200	3.52
7	PM	M	200	1.11
10	PM	F	130	4.15
12	PM	F	110	2.31
15	PM	M	240	3.36
16	PM	F	180	0.62
18	PM	M	130	4.74
22	PM	M	230	1.89
26	BSF	F	140	1.76
28	BSF	M	200	1.78
29	BSF	F	125	0.56
30	BSF	F	200	3.44
32	BSF	M	325	0.76
33	BSF	M	330	0.52
35	BSF	F	170	0.99
36	BSF	F	200	0.05

Table 1. Characteristics of the black bears used in this study.

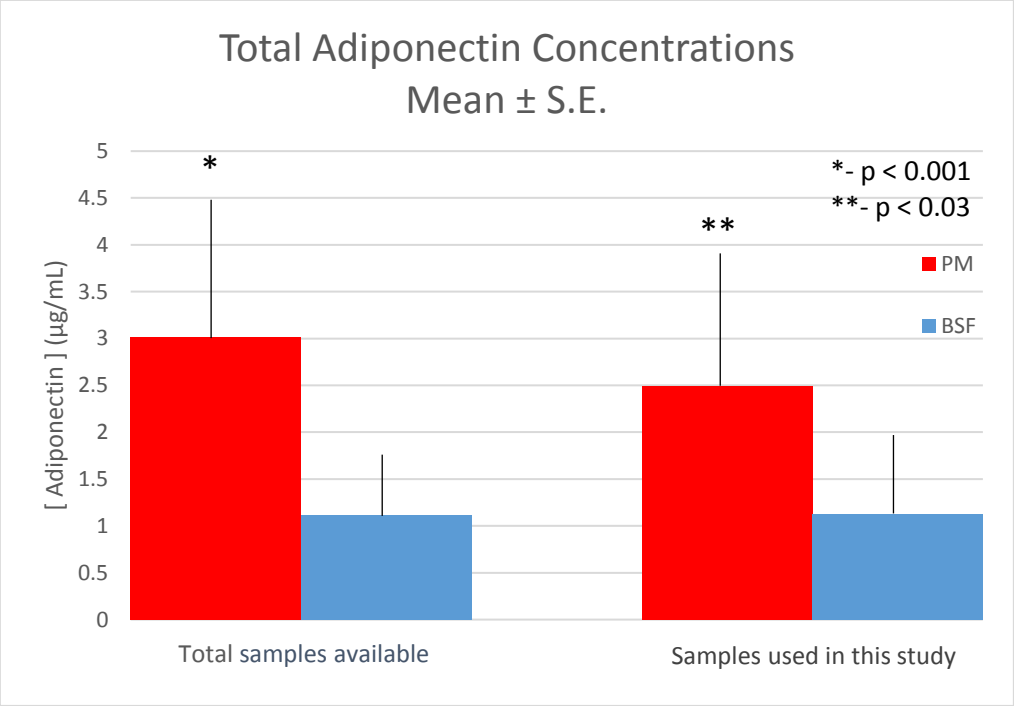

Figure 2. A comparison of total adiponectin concentrations (µg/mL) between 1) the total samples available, and 2) those samples used for the comparison of adiponectin isoforms.

Adiponectin Isoform Analysis

After correcting for the process of dilution used for the ELISA assay, the amounts of the different multimeric forms of adiponectin were calculated. The percentages of HMW and LMW adiponectin were calculated with respect to the total concentration of adiponectin. These percentages were used for the statistical tests. Though not explicitly shown in the following figures, in each population the percentage of the HMW form of adiponectin was significantly greater than that of the LMW form ($t = 44.01$, $p < 0.00001$).

Figure 3 depicts a comparison between the multimeric forms of adiponectin with respect to habitat. The percentage of HMW adiponectin was significantly greater in the PM population than the BSF population ($t = 2.17$, $p < 0.03$). This trend was opposite for LMW adiponectin. The percentage of the LMW isoform was significantly less in the PM population than that of the BSF population ($t = -2.17$, $p < 0.03$).

A subsequent comparison was made between sexes. The total number of samples taken from male specimens (from both PM and BSF) were compared to the total number of female specimens, as portrayed in Figure 4. As expected, though not shown in the figure, the percentage of HMW adiponectin was significantly greater than the percentage of LWM adiponectin for males ($t = 29.69$, $p < 0.00001$) as well as females ($t = 10.34$, $p < 0.00001$). However, when HMW adiponectin was compared between males and females, no significant difference was discovered. Likewise, no significant difference was found in LMW percentage compared between males and females. Thus, no significant difference between sexes was determined in this population of black bears sampled.

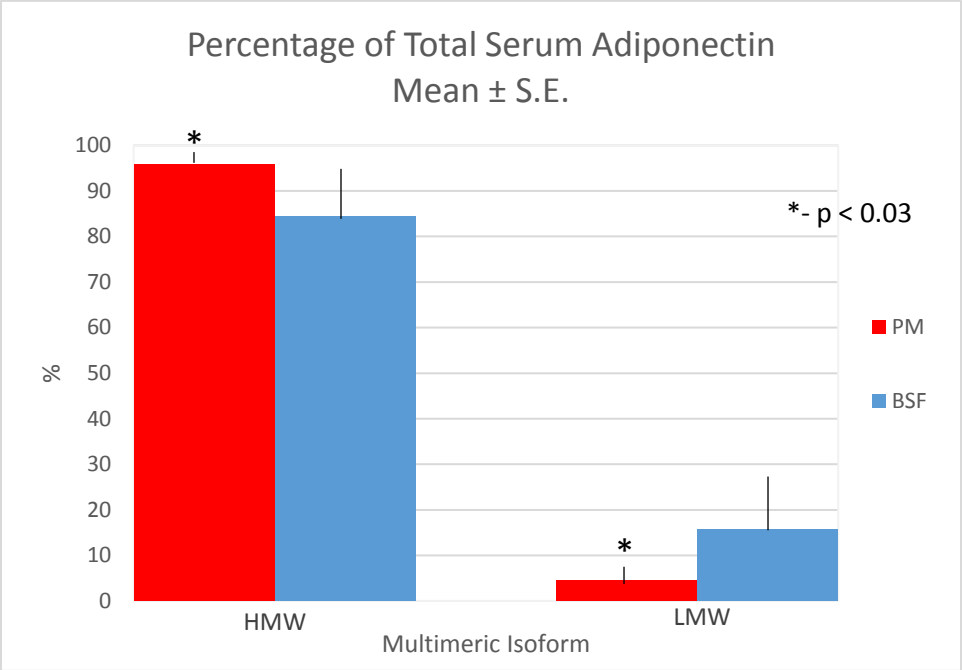

Figure 3. A comparison of the percentage of HMW and LMW adiponectin between the two different habitats with respect to the total amount of serum adiponectin.

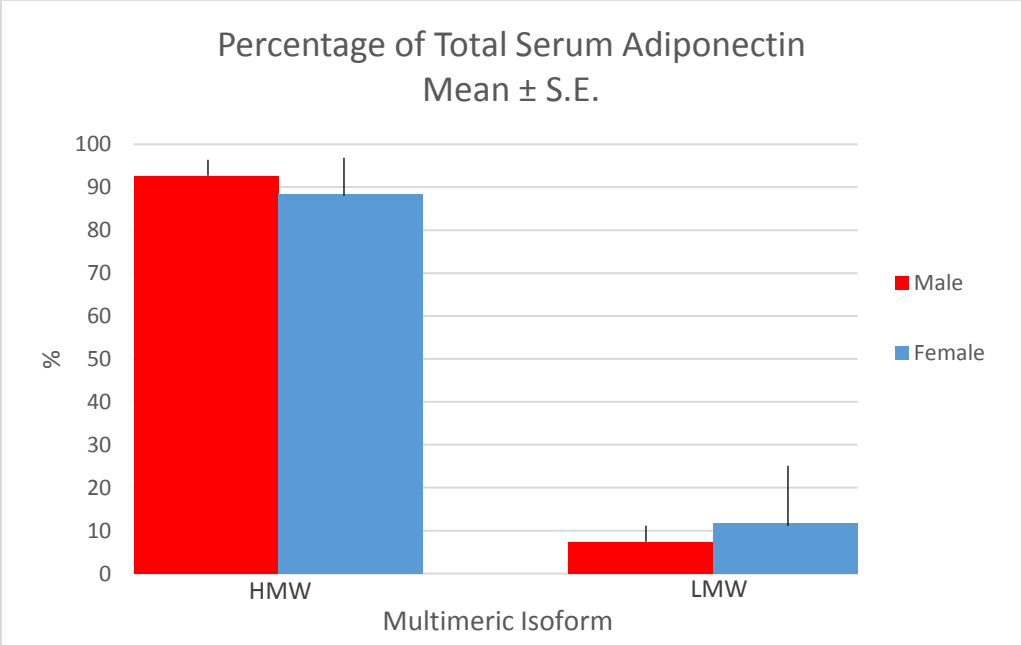

Figure 4. A comparison of the percentage of HMW and LMW adiponectin between the two sexes with respect to the total amount of serum adiponectin.

Subsequent comparisons were made within each adiponectin isoform to find any differences with respect to both habitat and sex. As Figure 5 illustrates, only one significant difference within the HMW adiponectin isoform was found between populations. The percentage of HMW adiponectin for males sampled from the PM population was found to be significantly greater than the percentage of HMW adiponectin for males of the BSF population ($t = 8.39$, $p < 0.0001$). No significant difference was found between any other combination of sex and habitat for the HMW isoform of adiponectin. Similarly, as Figure 6 illustrates, the only significant difference within the LMW adiponectin isoform was that between males of the two different populations. The percentage of LMW adiponectin for males sampled from the PM population was significantly less than the percentage of LMW adiponectin for males of the BSF population ($t = -8.38$, $p < 0.0001$). No significant difference was found between any other combination of sex and habitat for the LMW isoform of adiponectin.

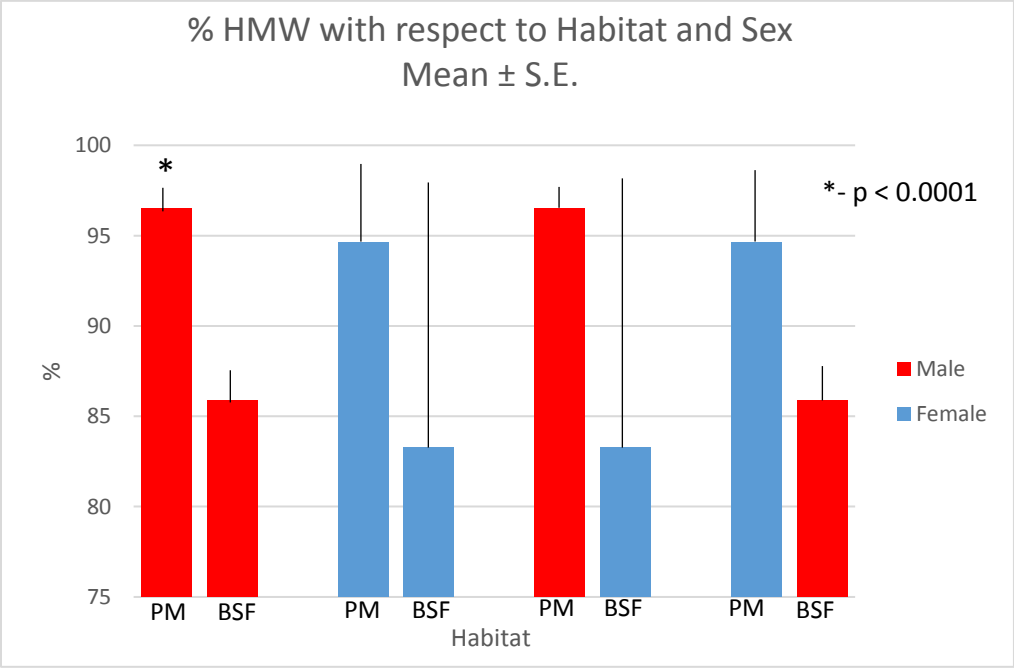

Figure 5. Comparisons of the percentage of HMW adiponectin with respect to habitat and sex.

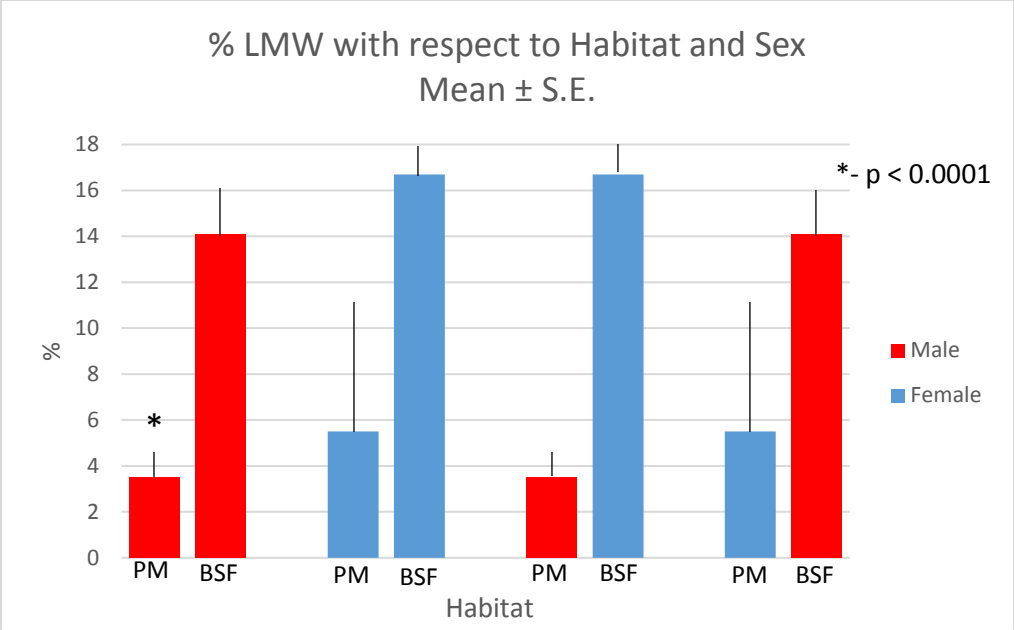

Figure 6. Comparisons of the percentage of LMW adiponectin with respect to habitat and sex. (Error bars for females from BSF extend beyond the limits of the shown graph.)

DISCUSSION

The average weight of the adult black bears in the two populations came as a surprise. Because black bears from the PM population had greater potential for access to highly palatable and energy rich sources of human food and refuse, it was hypothesized that the PM specimens would weigh more than the BSF specimens. After all, Sampey et al. (2011) found that rodents fed a high-fat cafeteria diet similar to that of humans led to a significant risk of developing obesity and metabolic syndrome. This effect was not found for the black bears sampled. Perhaps this was due to the initial assumption that nutritional resources for the two populations might differ. However, this may also be due to the differing ages of the bears sampled. Though only adult samples were utilized by this study, most of the ages of those adults were unknown.

Black bears from the PM population had a significantly higher total serum adiponectin concentration than bears from the BSF population. The values of total serum adiponectin levels obtained from the samples appear to be in a similar range in comparison to other previously tested mammals, such as rats and humans. Merl et al. (2005) demonstrated that total serum adiponectin levels in humans can range anywhere from 2 μg/mL to 11 μg/mL. Rats fed different diets show total serum adiponectin levels at a range of 1.7 μg/mL to 3.2 μg/mL (Rossi et al., 2005). The mean values of total serum adiponectin reported from the total samples available (PM: 3.02 μg/mL, BSF: 1.12 μg/mL) as well as from the samples used in this study (PM: 2.49 μg/mL, BSF: 1.13 μg/mL) appear to be within this range (see Figure 2) and argue that the utilization of a rat-based ELISA assay for analysis of adiponectin in bears is appropriate.

It was expected that, because black bears in the PM population have greater potential for access to highly palatable and energy rich sources of human food and refuse, the PM population would have decreased serum adiponectin levels when compared to bears in the BSF population. This is because, as in all previous studies, obesity has been correlated with depressed serum adiponectin concentration. As stated, it was hypothesized that the PM population specimens would weigh more, therefore demonstrating this correlation. However, the opposite was found. On average, black bears from the BSF population weighed more than bears from the PM population. Though the findings did not support the hypothesis, the inverse relationship between percent body fat and serum adiponectin concentration was supported nonetheless. Specimens from the BSF population weighed significantly more than those from the PM population, and exhibited a significantly lower concentration of serum adiponectin.

The percentage of HMW adiponectin was significantly greater in the PM population than in the BSF population. In contrast, the percentage of LMW adiponectin was significantly lower in the

PM population than in the BSF population. These findings are attributed only to differences in males between the two populations (see Figures 5 & 6). No significant difference was found between any other combination of habitat and sex. Though the percentages of respective isoform adiponectin levels appear to be more extreme in the samples used in this study, the values obtained followed the same pattern reported in previous studies. Salani et al. (2006) showed that the mean percentage of HMW adiponectin with respect to total serum adiponectin levels is roughly 60% for non-obese humans. The percentage of HMW adiponectin with respect to total serum adiponectin levels is roughly 70% for healthy rats (Amitani et al., 2013). The mean percentage of HMW adiponectin with respect to total serum adiponectin levels ranged from 80% to 95% for the samples used in this study. Although this study reports a higher percentage of HMW adiponectin than that shown in humans or rats, the higher percentage of HMW adiponectin compared to LMW adiponectin appears to be universal in mammals.

Females tend to show significantly greater concentrations of serum adiponectin when compared to males, specifically the HMW isoform. This is most likely because, in general, males weigh more than females. And, as stated previously, increases in percent body fat are associated with decreases in serum adiponectin concentrations. Unlike previous studies of humans and rats, the sample population used in this study failed to demonstrate any significant difference in serum adiponectin levels with respect to sex (see Figure 4). In speculation, female black bears may have some mechanism to maintain fat levels, thus depressed serum adiponectin levels, because they undergo parturition during hibernation. Hibernation prompts the switch from carbohydrate metabolism to a lipolytic state for energy consumption (Florant et al., 2004). Therefore, pregnant female black bears may need to maintain a higher fat mass to adequately nourish their young.

However, all black bears used in this study were sampled in the summer, while in a state of arousal, not hibernation.

The difference found in total serum adiponectin concentration can be attributed to differences in both isoforms between males of the different populations. Male specimens from the PM population had a significantly higher percentage of HMW adiponectin when compared to male specimens from the BSF population. In contrast, male specimens from the PM population had a significantly lower percentage of LMW adiponectin when compared to male specimens from the BSF population. No significant differences were found between females of the two populations. As indicated previously, Xu et al. (2005) showed that testosterone plays a role in the reduction of HMW adiponectin circulation. A possible explanation for these results may be that testosterone levels are decreased in male black bears during the summer and well after the breeding season, resulting in no significant difference between the sexes. This may account for a potential depression in testosterone levels and may be a factor that must be taken into account in these seasonally breeding animals compared to laboratory rodents or humans.

The results presented may have been confounded by the limitations of this study. Chiefly, a greater experimental sample size could have yielded more representative results for the two populations studied. The power of the statistical analyses conducted in this study was weakened by the low sample size. Another limitation of this study is the lack of information about or disclosure of some important variables, such as age and dietary regimen. Though this study only reported analyses from adult black bears, the ages of the black bears still varied. Age may affect a variety of factors, including accessibility to food, accumulation of total fat (and thus weight), and concentrations of hormones. Only 5 ages of the total 17 sampled black bears were known. All samples were taken in the summer of 2011, but all samples may not have been taken at the same

time of day. Some black bears may have consumed a meal before being sampled, thus altering metabolic hormone levels. And, given the inverse relationship demonstrated between serum adiponectin and insulin, this variation in diet may have confounded results. Furthermore, weight appears to have a significant relationship with the concentration of serum adiponectin and, perhaps, expression of adiponectin levels related to body mass could have revealed other interesting differences. However, the limited sample sizes used in this study were not large enough to take into account body weight.

This study sought to identify the black bear as an animal to learn more about adiponectin and its relationship to human conditions such as obesity, diabetes, and metabolic syndrome. Black bears and humans are both large, omnivorous mammals with similar amino acids sequences for the adiponectin gene as well as similar physiological temperature and cardiac output levels. Furthermore, hibernating animals, such as the black bear, may be useful to study obesity, fasting, and fat metabolism. However, a great distinction is that humans do not hibernate. Another distinction concerns the seasonality of hormones. Black bears are active during spring and summer and inactive during winter hibernation, so a flux in hormone levels like adiponectin between seasons is evident. Black bears also undergo a mating season in spring, which may affect testosterone levels in male bears. Because humans do not hibernate, they do not exhibit such seasonality differences in hormone levels. Therefore, results found in studies using the black bear as an animal model cannot be applied to human conditions with certain confidence.

This study will hopefully serve as a basis for the further study of hibernating mammals and the analysis of the different isoforms of adiponectin. Given its significant relationship to adiponectin concentration, future studies should take weight into account when determining the concentration of serum adiponectin (µg/mL/lb.). More black bear population characteristics should be examined.

For example, not only using habitat and sex, but also age, weight, and dietary regimen. Additional metabolic hormones should be examined, so they can be compared to serum adiponectin levels. These hormones might include insulin, glucagon, leptin, and ghrelin. Other possibly related hormones, such as testosterone, should be examined as well. In addition, black bears should be tested for common symptoms of obesity and metabolic syndrome. These tests might investigate systemic inflammation, insulin resistance, atherosclerosis, β-oxidation of fatty acids, and visceral/total adipose accumulation. The results of this study may point future studies to explore the relationship of obesity, diabetes, and metabolic syndrome between hibernating mammals and humans.

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