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Thermoneutral temperature reduces liver volume but increases fat content in a mammalian hibernator

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ABSTRACT

Hibernators survive challenging winters by entering torpor, which lowers body temperature (T_b) to ~5 °C for 12–14 days, followed by spontaneous arousals where T_b increases to ~37 °C for 10–12 h before entering another torpor bout. This T_b cycle is accompanied by significant fluctuations in metabolic rate. Little is known about the role of the liver in lipid metabolism during hibernation. In this study we measured the effect of ambient temperature on liver volume and lipid content in 13-lined ground squirrels (*Ictidomys tridecemlineatus*). We housed animals at thermoneutral (25 °C) or cold (5 °C) ambient temperatures, with the same photoperiod (12 h light:12 h dark) for an entire year. We determined volume and water-fat ratio of the liver using magnetic resonance imaging (MRI). Ambient temperature significantly affected both liver volume and fat content. From October to August squirrels housed at 25 °C had 25% smaller livers compared to the squirrels housed at 5 °C, but their average lipid content (13.3%) was 37% higher. Because the squirrels housed at 25 °C appeared to continue feeding throughout the winter but did not enter extended torpor, more carbohydrates may have been diverted to lipid stores. By contrast, animals housed at 5 °C did not appear to feed, and carbohydrates would likely be preferentially stored in the liver as glycogen to supply glucose for brain metabolism. These results suggest that the fat burden caused by hibernators preparing for winter can lead to symptoms of metabolic syndrome, but that these symptoms are reversible in the spring.

1. Introduction

Hibernators, such as the arctic ground squirrel, display seasonal increases in caloric intake that correspond with significant increases in body mass and adiposity prior to the hibernation season (Sheriff et al., 2013). The 13-lined ground squirrel (*Ictidomys tridecemlineatus*) hibernates from late autumn to early spring. They cope with the combined environmental stresses of low temperature and low food availability by cycling between states of torpor and interbout euthermia (IBE) (Russell et al., 2010). During the hibernation season, periods of torpor with reduced core body temperature (T_b) of ~5 °C last for 12–14 days, are interrupted by spontaneous arousals to IBE, which raise T_b to approximately 37 °C for 10–12 h. During IBE metabolic rate, heart rate and T_b are comparable to the summer euthermic state (Reviewed in Staples, 2014). Prior to the winter months these hibernators also experience significant increases in body mass, up to 60% of which is comprised of lipid (MacCannell et al., 2017; Sheriff et al., 2013).

The liver is a highly versatile metabolic organ but experiences significant metabolic suppression during torpor, as judged by mitochondrial function (Mathers et al., 2017). Many hibernators, including the arctic ground squirrel, fast throughout the winter. In this species liver glycogen is depleted over the course of a torpor bout and replenished during arousal, by gluconeogenesis primarily using lactate and glycerol (derived from triglycerides) as well as substrates derived from amino acids (Galster and Morrison, 1975). Although no such data exist for 13-lined ground squirrels, this species can also fast during the hibernation season, so it is reasonable to assume that liver glycogen stores are regularly depleted and replenished.

In fed mammals a high insulin/glucagon ratio stimulates the liver to take up plasma glucose, storing it as glycogen (Flatt, 1995). When liver glycogen stores are replete, glucose taken up by the liver is transformed into fatty acids for long-term storage. Although these fatty acids are

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typically delivered to adipocytes within white adipose tissue (WAT), the liver can also store triglycerides. In other rodents, dietary manipulations lead to excess fat stored within the liver which can lead to conditions such as non-alcoholic fatty liver disease (NAFLD) and metabolic syndrome, a cluster of conditions including increased blood pressure, insulin resistance, excess body fat, and abnormal blood cholesterol or triglyceride levels (Van Herck et al., 2017). Our recent research suggested that housing ground squirrels at a constant, thermoneutral ambient temperature (T_a) (25 °C) (Long et al., 2005; Vispo and Bakken, 1993) increased WAT deposits and the animals showed no indications of torpor (MacCannell et al., 2018). We hypothesized that this treatment might also lead to high liver lipid levels.

Invasive liver biopsies have been replaced with quantitative assessments of liver composition and volume using magnetic resonance imaging (MRI) (Lee and Park, 2014). This non-invasive technique does not use ionizing radiation, and thus can be repeated on individual subjects. ***I***terative ***D***ecomposition of water and fat with ***E***cho ***A***symmetry and ***L***east squares estimation (IDEAL) is an MRI pulse sequence that provides a sensitive and quantitative measure of lipid content (Hu et al., 2010). This pulse sequence allows identification of the proton density fat fraction (PDFF), which is the percent of lipid in the tissue (Prakash et al., 2016). A PDFF value for of 5% or below is indicative of a normally functioning (Byrne and Targher, 2015), non-diseased liver, whereas higher PDFF values indicate that the liver is storing lipid.

We used IDEAL MRI to repeatedly measure liver volume and PDFF from 13-lined ground squirrels housed at two different temperatures over an entire year. We subjected squirrels to constant cold (5 °C) or thermoneutral (25 °C) with a photoperiod of 12 h light (L):12 h dark (D). We hypothesized that animals exposed to higher environmental temperatures would have livers with a larger fat fraction and volume. We measured these values regularly over an entire year to identify potential circannual patterns.

2. Materials and methods

2.1. Experimental animals

All procedures were approved by the University of Western Ontario Animal Care Committee (protocol 2012-016) and followed Canadian Council on Animals Care guidelines. Details of ground squirrel trapping and husbandry can be found in our recent publication (MacCannell et al., 2017).

Our experimental design was the same as that recently used to assess adipose tissue dynamics (MacCannell et al., 2018). In brief, eight juvenile males from the same litter were housed at 22 °C until weaning, when they were divided randomly into two conditions, cold-housed (5 °C; n = 4) and warm-housed (thermoneutral 25 °C; n = 4). After the initial MRI of the cold-housed squirrels on 19 August 2016 the temperature was decreased 1 °C/day until the T_a reached 5 °C (6 September 2016). On 26 August 2016, immediately after the first MRI of the warm-housed squirrels, temperature was increased to 25 °C. For both groups the photoperiod remained at 12L:12D throughout the year to eliminate any interaction with ambient temperature effects. Both food (LabDiet 5P00, Iams dry dog food), and water were provided *ad libitum*, with sunflower seeds and corn provided three times a week. Animals were weighed approximately every week (± 0.01 g) during cage changes unless animals were hibernating, to minimize disturbance. Potential torpor bouts were identified using the sawdust technique (Pengelley and Fisher, 1961), in which a small amount of fine sawdust is placed on the back of a torpid squirrel and animals were observed daily for the presence of the sawdust. We used this technique because instrumenting these animals with T_b telemeters or loggers would have interfered with MRI.

2.2. MRI scanning

MRI scans for T1-weighted images and IDEAL water-fat images were conducted on each group approximately every three weeks using a 3 T (MR750, GE, Waukesha, WI, USA) 32-element cardiac receiver array (Invivo, Gainesville, FL, USA). IDEAL identifies the PDFF through the lipid to water ratio within the tissue (Equation (1)). Isoflurane anaesthesia was used when necessary to prevent movement within the scanner, particularly for warm-housed animals. Torpid animals were kept cool using temperature-controlled blankets and remained immobile throughout the scan without requiring anaesthesia. The two treatment groups were scanned on subsequent weeks. MRI scanning details can be found in our recent publications (MacCannell et al., 2017, 2018).

$$\text{Proton Density Fat Fraction} = \frac{\text{Lipid } ^1\text{H signal}}{\text{Lipid } ^1\text{H signal} + \text{Water } ^1\text{H signal}} \quad (1)$$

2.3. Image segmentation

MRI data were analyzed using the digital image viewer Osirix (Bernex, Switzerland; v.5.8.1). The MRI data were collected from the complete volume of squirrels positioned on their ventral surface. Axial images were acquired with 0.9 mm thickness and 0.55 mm by 0.55 mm in-plane resolution. The 3D liver volumes were manually outlined (segmented) on anatomical T1-weighted images and copied onto corresponding IDEAL images to quantify PDFF or segmented directly onto IDEAL MRIs (Fig. S2). The liver area and PDFF were recorded for each slice. Total liver volume was calculated by taking the sum of the areas and multiplying by image thickness of each slice (0.9 mm). Liver PDFF was calculated as the mean for each slice.

2.4. Statistical analyses

All values are presented as mean ± standard error of the mean (S.E.M.). The effect of time, temperature or interaction between time and temperature on body mass, liver volume and PDFF was assessed using a repeated measures ANOVA and Greenhouse-Geisser Correction on SPSS (IBM Corp. Released, 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). Reliability of MRI segmentation volumes was confirmed by calculation of the interclass correlation coefficient (ICC) between values determined by one reader (XW) and second readers (ADVM and CFW); ICC values higher than 0.9 are indicative of excellent reliability (Koo and Li, 2016).

3. Results

3.1. Total body mass of animals

As previously noted (MacCannell et al., 2018) daily observation showed no indication that the warm-housed animals ever entered torpor. By contrast, cold-housed animals began entering torpor on 28 September 2016 and maintained consistent torpor-arousal cycles until 18 March 2017, with the last animal arousing on 3 April 2017. Moreover, daily observations showed that the warm-housed squirrels continued to eat throughout the winter months, whereas the cold-housed animals appeared to cease eating in the autumn.

Before temperature treatments began all animals gained body mass at an average of 2.7 g per day over six weeks, increasing from approximately 110.4 ± 1.3 g to 247 ± 7.4 (Fig. 1). After separation into temperature treatment groups, cold-housed animals started to enter torpor in late September and lost body mass steadily until March, when they aroused from hibernation and gained mass back to pre-hibernation levels. Warm-housed animals did not appear to enter torpor, and their body mass remained at high levels of 268 ± 3.2 g throughout the

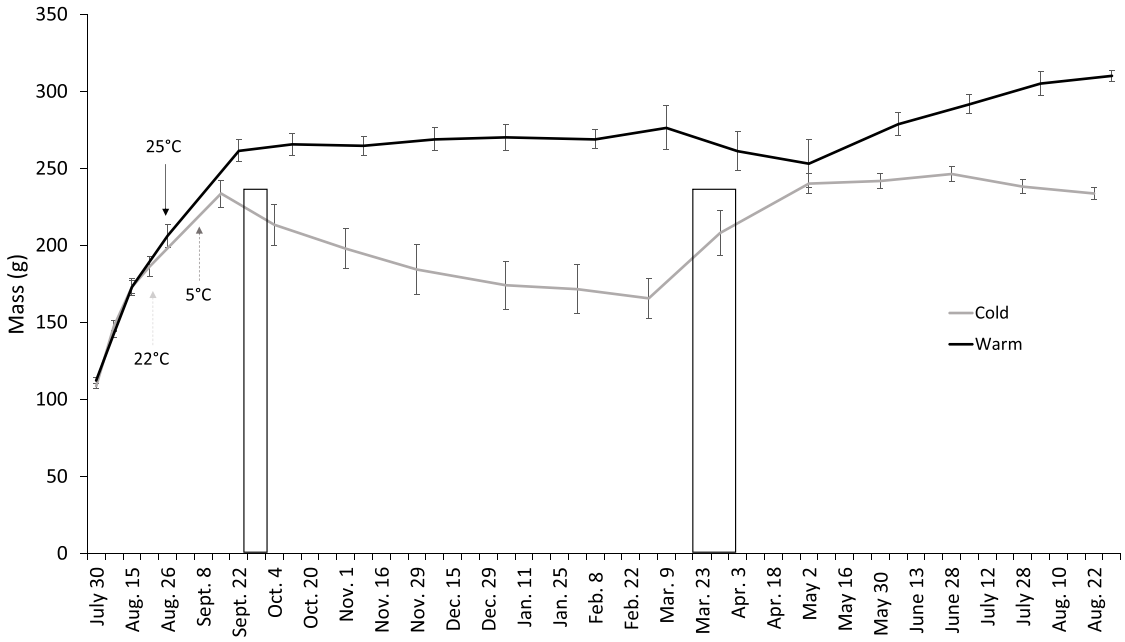

Fig. 1. Ground squirrel body mass over an entire year. The black line represents animals housed at 25 °C and the grey line represents animals housed at 5 °C, both groups had 12 h L:12 h D photoperiod. Data are presented as mean ± SEM, $n = 4$ for each group. The arrow labelled 25 °C indicates the initial MRI scan for warm animals and the day they were transferred to a T_a of 25 °C (26 August 2016). The light grey arrow labelled 22 °C indicates the initial scan for cold group, and the day T_a was dropped 1 °C/day (19 August 2016). The dark grey arrow labelled 5 °C indicates the day T_a for the cold group reached 5 °C (6 September 2016). The first box indicates range of initial torpor bouts for cold-housed animals (beginning 28 September 2016). The second box indicates the range of terminal arousal dates (18 March – 3 April 2017). There is a significant effect of time ($F_{(3.1,\ 18.8)} = 76.2$, $P < 0.001$) and T_a ($F_{(1,\ 6)} = 27.8$, $P = 0.002$), and an interaction between time and temperature ($F_{(3.1,\ 18.8)} = 15.0$, $P < 0.001$) on mass. Taken from (MacCannell et al., 2018).

winter. Further details about the fluctuating mass and adipose of these animals under these conditions can be found in (MacCannell et al., 2018).

3.2. Environmental temperature effects of liver volume

Prior to and throughout the hibernation season the absolute liver volume was 8.1 ± 0.7 ml for the cold-housed animals, and 8.0 ± 0.5 ml for the warm-housed group (Fig. 2). In the months following terminal arousal, however, the liver volume increased 23.3% in the cold-housed animals, peaking at 12.5 ± 1.0 ml in June. By contrast, these values remained stable in the warm-housed animals at 8.5 ± 0.4 ml during the same period. There was a significant effect of time on the absolute liver volume ($F_{(4.2,\ 25.2)} = 10.8$, $P < 0.001$), but no significant effect of T_a ($F_{(1,\ 6)} = 1.3$, $P = 0.300$). There was also a significant interaction between time and temperature ($F_{(4.2,\ 25.2)} = 5.2$, $P = 0.003$), suggesting that T_a influences the liver volume based on the time of year.

3.3. Percentage body mass comprised of liver

Percent body mass comprised of liver was calculated using body mass measured on the day of MRI acquisition, assuming a 1 g/cm^3 density of liver. At the beginning of the experiment in August percent body mass comprised of liver was similar in both groups, 3.5 ± 0.3% in the warm-housed animals and 3.6 ± 0.1% in the cold-housed animals. These values diverged after the animals were separated into temperature treatment conditions (Fig. 3). The cold-housed animals increased relative liver size during the hibernation season, peaking at 5.2 ± 0.4% on 31 March 2017, coinciding with terminal arousal from hibernation. There was no significant effect of time on the percent body mass ($F_{(3.3,\ 19.8)} = 2.7$, $P = 0.068$), but there was a significant effect of T_a ($F_{(1,\ 6)} = 68.4$, $P = 0.004$). The warm-housed animals maintained 3.1 ± 0.1% body mass comprised of liver, whereas the cold-housed animals maintained 4.5 ± 0.1% There was also a significant interaction between time and temperature ($F_{(3.3,\ 19.8)} = 3.3$, $P = 0.009$), resulting in more divergence in the percent body mass comprised of liver between the two groups as time progressed.

3.4. Lipid amount within livers

The liver PDFF of both the cold-housed and the warm-housed animals on the first scan was 8.1 ± 0.5% and 10.1 ± 1.8% respectively. This value in the cold-housed animals peaked in September at 14.2 ± 2.3%, after cold exposure had begun, but before torpor bouts were observed. In the cold-housed animals PDFF decreased during the hibernation period to 7.3 ± 0.6% in March. In the warm-housed animals, however, PDFF fell from its peak of 16.1 ± 1.5% to 12.0 ± 1.6% in February and stayed relatively stable thereafter. Along with the significant effect of time ($F_{(4.0,\ 24.0)} = 6.9$, $P = 0.001$), there was also a significant effect of temperature ($F_{(1,\ 6)} = 7.1$, $P = 0.037$) on PDFF.

Total liver lipid amount (ml) was calculated by multiplying the liver volume by PDFF. Warm-housed animals had smaller livers in the spring (Fig. 2) with higher lipid content (Fig. 4) throughout the year. This results in no significant differences over time or between temperature treatments of the total lipid stored within the livers of either group (Fig. S1).

4. Discussion

This study shows that T_a, especially during the hibernation season, influences both volume and lipid content of the liver in 13-lined ground squirrels. The livers of warm-housed animals had 1.8-fold higher PDFF than the cold-housed animals after acclimation to temperature treatment conditions in early October, but liver volume of the warm-housed animals was stable. In contrast, the livers of cold-housed animals had lower lipid content, but liver volumes increased after the winter months

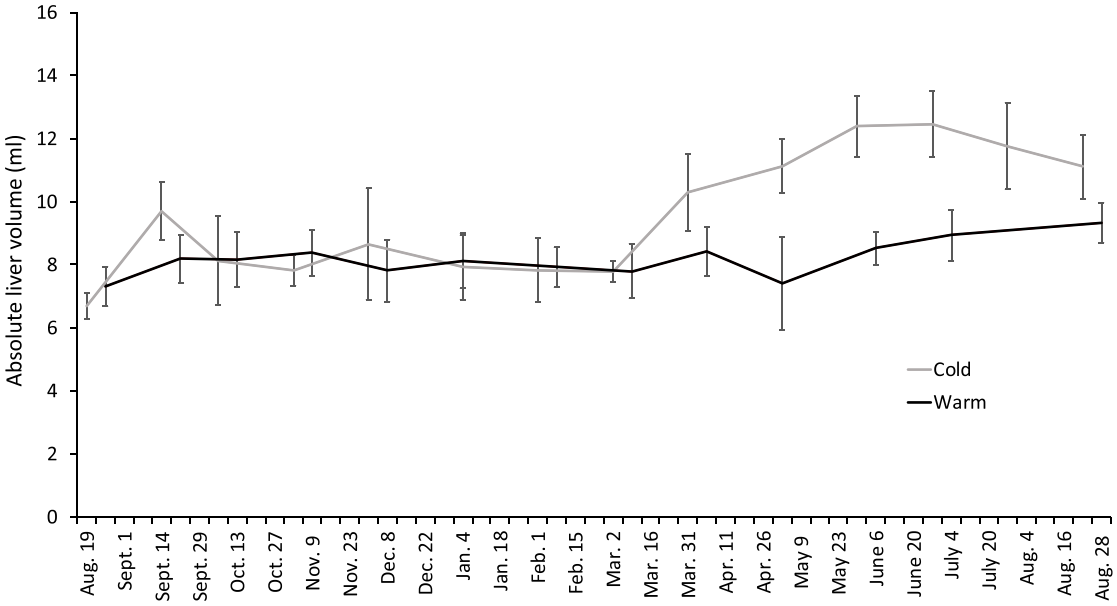

Fig. 2. Absolute liver volume of squirrels housed in cold or warm conditions. Data extracted from MR images of thirteen-lined ground squirrels. Volume was calculated as the sum of individual slice areas multiplied by slice thickness. The grey line represents animals housed at 5 °C, the black line represents those housed at 25 °C. There is a significant effect of time ($F_{(4.2,\ 25.2)} = 10.8$, $P < 0.000$), but not temperature ($F_{(1,\ 6)} = 1.3$, $P = 0.300$), and there is a significant interaction between time and temperature ($F_{(4.2,\ 25.2)} = 5.2$, $P = 0.003$) on absolute liver volume.

and following terminal arousal from hibernation.

In the 13-lined ground squirrel, initial scans of both groups showed PDFF values higher than those reported for guinea pigs, another rodent. Guinea pigs on a control diet have a liver lipid content of approximately 2–4% (Hines et al., 2012; Sinclair et al., 2018), compared to ~8–16% in the ground squirrels. After exposure to cold environmental temperatures, but before hibernation, cold-housed squirrels showed a decline in liver PDFF (Fig. 4). The increase in volume which occurred in cold-housed animals from March to August corresponds closely to when the animals arouse from hibernation. Liver glycogen stores would be replenished quickly, and excess carbohydrates would be stored as lipids. The pattern in volume closely resembles the total liver lipid (Fig. S1). By contrast, warm-housed squirrels continued to accumulate fat in the liver into October 2016. The difference in PDFF between groups was maintained throughout the winter and may reflect the fact that the warm group were observed to continue eating and did not enter extended bouts of torpor. As a result, the warm-housed animals likely maintained more stable blood glucose levels during the winter months. This prevented the squirrels from depleting liver glycogen and promoting conversion of blood glucose into fatty acids. With WAT lipid stores approaching 60% of body mass (MacCannell et al., 2018) these fatty acids were likely retained in the liver, leading to the higher PDFF.

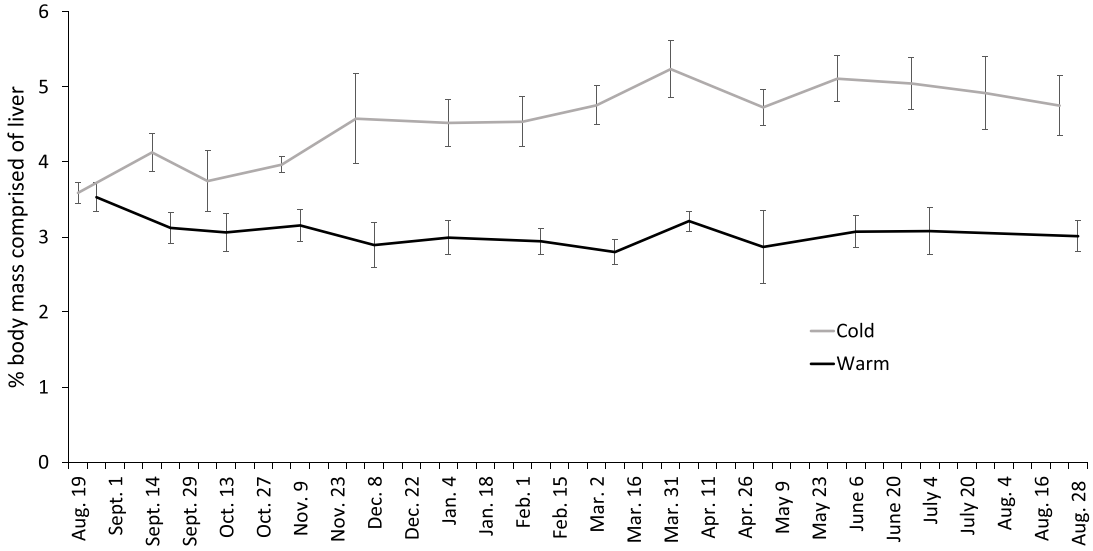

Fig. 3. Changes in percent body mass comprised of liver in ground squirrels over one year. Data extracted from MR images of thirteen-lined ground squirrels. Body mass was measured on the day of MRI and liver mass estimated from liver volume (assuming 1 cm^3 = 1 g). The grey line represents animals house at 5 °C, the black line represents those housed at 25 °C. There is no significant effect of time ($F_{(3.3,\ 19.8)} = 2.7$, $P = 0.068$), but there is a significant effect of temperature ($F_{(1,\ 6)} = 68.4$, $P = 0.004$), and interaction between time and temperature ($F_{(3.3,\ 19.8)} = 4.9$, $P = 0.009$) on percent body mass comprised of liver.

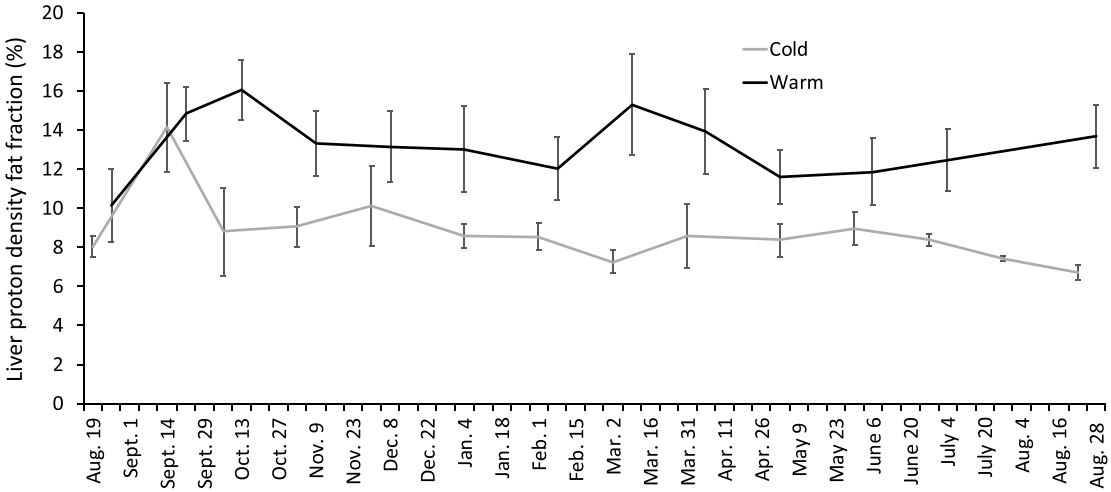

Fig. 4. Changes in liver proton density fat fraction (PDFF) in ground squirrels over one year. Data extracted from MR images of thirteen-lined ground squirrels. Proton density fat fraction was calculated as lipid signal/(lipid signal + water signal). The grey line represents animals housed at 5 °C, black line represents those housed at 25 °C. There is a significant effect of time ($F_{(4.0,\ 24.0)} = 6.9$, $P = 0.001$), temperature ($F_{(1,\ 6)} = 7.1$, $P = 0.037$), and an interaction between time and temperature ($F_{(4.0,\ 24.0)} = 3.6$, $P = 0.002$) on liver PDFF.

Regardless of differences between groups, ground squirrel liver PDFF values were up to 2-fold higher than Guinea pigs imaged using the same technique, which had liver PDFF of 2–4% on a standard diet and 5–8% on a western diet (Sinclair et al., 2018). In humans, the threshold of liver fat content for NAFLD is 5% (Byrne and Targher, 2015). Throughout the year our ground squirrels maintained liver fat 60–295% higher than this threshold. These findings suggest that, without exogenous dietary manipulation, 13-lined ground squirrels have unusually fatty livers, even when not hibernating. This is a unique finding – in mice similar significant increases in liver size and fat content require diets which combine high fat and high cholesterol (Savard et al., 2013).

Although it is unclear what happens in nature, in captivity many hibernating species cease food intake for several months and must utilize energy-dense lipid stored within WAT. Hibernating mammals increase their WAT lipid stores in anticipation of hibernation and up to ~60% of the mass of a 13-lined ground squirrel can be comprised of WAT prior to winter (MacCannell et al., 2018), similar to levels found in free-living arctic ground squirrels (Sheriff et al., 2013). Metabolomics studies in the 13-lined ground squirrel suggest that liver lipid oxidation is upregulated in the winter months (Serkova et al., 2007), and numerous studies (Reviewed in Staples, 2016) show that most metabolism during the hibernation season is fuelled by lipid oxidation. This shift towards lipid consumption and storage are similar to models of obesity in non-hibernating mammals, which may lead to metabolic syndrome (Wong et al., 2016). Indeed, arctic ground squirrels show hyperinsulinemia in the autumn, when they are hyperphagic, compared to winter, spring and summer (Buck et al., 2002). Moreover, another hibernator, the yellow-bellied marmot (*Marmota flaviventris*), exhibits both hyperinsulinemia and insulin-resistance in the autumn compared to winter, spring and summer (Florant et al., 1985). Non-hibernating models of obesity also exhibit hyperinsulinemia and insulin resistance (Corkey, 2012), but in hibernators these conditions develop only in preparation for the hibernation season and are reversible (Florant et al., 1985). The elevated fat within the liver that we observed in this study, combined with increased WAT, insulin-resistance and hyperinsulinemia support the idea that hibernators may suffer symptoms of metabolic syndrome naturally prior to hibernation, but can reverse these effects before long-term damage occurs. The increased PDFF seen in the warm-housed animals supports this idea because these animals did not appear to fast or enter torpor. As a result, liver glycogen stores were not depleted, and excess digested food could be stored as lipid. Unfortunately, the ferromagnetic nature of virtually all implantable T_b monitors are incompatible with the strong magnetic fields required for MRI, so we cannot definitively rule out the possibility of short, shallow torpor bouts in this group. In a separate study we are examining the effect of a similar T_a regime on torpor patterns in this species using implanted T_b loggers.

Our findings complement earlier observations of seasonal hyperinsulinemia and insulin-resistance in hibernators. Taken together, these results suggest that the natural, but extreme fat burden of hibernators preparing for winter can lead to symptoms of metabolic syndrome, but that these symptoms are reversible.

Conflicts of interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2019.05.015.

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