ABSTRACT

A Model of Early Vitamin A Deficiency in a Hibernator, the 13-lined Ground Squirrel

(*Ictidomys tridecemlineatus*)

By: Ryan J. Sprenger

Hibernators, such as the 13-lined ground squirrel (*Ictidomys tridecemlineatus*), undergo dramatic changes in adipose tissue mass during their circannual cycle. Retinoic acid (RA), the biologically active derivative of vitamin A, plays key roles in the development and growth of adipose tissue. We developed a model of early vitamin A deficiency in juvenile ground squirrels to determine RA's effects on the ability to adequately build adipose mass prior to the first season of hibernation. Deficient squirrels (VAD) were maintained on the diet until 8 weeks of age when severe symptoms of hypovitaminosis were observed, including stunted growth and limb weakness. Liver retinoid analysis showed that these animals had essentially no retinoid stores. Even when placed on a normal diet, the VAD squirrels never reached the retinoid stores of controls. Control retinoid stores were high compared to other rodents, but in the normal range for the species. Serum retinol binding protein (RBP) was not affected by diet, although decreased levels were found in torpid hibernators. Early deficiency was associated with decreased mass of white (WAT) and brown (BAT) adipose tissue depots, decreased expression of resistin, altered seasonal expression of the RXR-β retinoid receptor and increased expression of BAT uncoupling protein. Our results suggest that ground squirrels require high levels of retinoids for normal function and are especially susceptible to deficiency as it affects the seasonal accumulation of adipose tissue.

A Model of Early Vitamin A Deficiency in a Hibernator, the 13-lined Ground Squirrel

(*Ictidomys tridecemlineatus*)

by

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Chapter 1: Introduction

I. Hibernation

Mammalian hibernators display a suite of astounding physiological and behavioral changes during their annual cycle. Hibernation has evolved as a way to avoid unfavorable environmental conditions which result in a high metabolic need. Showing great physiological plasticity, hibernating mammals can effectively accumulate adequate energy stores and reduce metabolic need to persist through the harsh environmental conditions of winter. Hibernators characteristically, with the exception of some larger organisms such as the black bear (*Ursus americanus*) (21), allow their body temperature to mirror ambient temperature, staying only a few degrees higher. In conjunction with a reduction in body temperature, fat storing hibernators will significantly increase fat mass, in some cases doubling their body weight prior to hibernation. During pre-hibernating and hibernating states, these animals gain and lose adipose tissue, respectively, at an astounding rate, a situation that would be detrimental to non-hibernating mammals such as humans.

The hibernating season is characterized by cyclical periods of torpor and arousal. Torpor is characterized by a reduction in metabolic rate (MR) followed by a reduction in body temperature (T_B). While in a torpid state, basal metabolic rate is approximately 2-4% of euthermic rates and T_B ranges from 2-10°C in small hibernators but can drop as low as -3°C in arctic ground squirrels (*Urocitellus parryii*)(4). T_B does, however, remain above ambient temperature for the duration of the winter season. Breathing rate during

torpor is also reduced drastically to 4-5 breaths/min and some species display episodic breathing with long apneic periods broken up by periods of a few rapid breaths (40). Hibernators do not maintain torpor for the duration of a hibernating season however. Torpor is regularly disrupted by periods of intense metabolic activity called interbout arousals. Arousals can last 12-24 hours and are characterized by a rapid return to euthermic T_B and metabolic rate. Arousals are cyclic and regular during the hibernating season. As the season progresses, however, the time between arousals increases, particularly during the colder months of the winter season. During the early and late stages of hibernation, arousals are more frequent as the organism prepares for either torpor cycles or spring emergence. Some hypotheses have been proposed in an attempt to justify periodic arousals. These include clearing metabolic waste, restoring blood glucose, avoiding muscle atrophy, and restoration of immune function (52). The function of interbout arousals is still not fully understood but considering the large amount of energy stores that are used in order to arouse, it must be physiologically important for the animal.

Adequate energy storage is invaluable to hibernating mammals going into the winter. Hibernators rely heavily on these stores as most of them consume no food or water for the duration of the hibernation season. To ensure adequate energy storage, fat storing hibernators will become hyperphagic in the months prior to immergence and in some cases nearly double their body weight (12). The primary energy storage organ employed by hibernators, as well as non-hibernating mammals, is adipose tissue.

II. Adipose Tissue

White adipose tissue (WAT) is composed of cells called white adipocytes. These cells, in contrast to other cell types, have a limited peripheral cytoplasm containing the nucleus surrounding a large, central globule of lipids, mainly in the form of triglycerides (Figure 1). These triglycerides are hydrolyzed by lipase enzymes during periods of fasting and broken down into fatty acids and glycerol, which can be used in the citric acid cycle to generate ATP. Increased energy intake, such as the hyperphagia prior to hibernation, leads to an increase in WAT mass. WAT mass can be increased in two ways – hypertrophy, or increase in adipocyte size, and hyperplasia, or increase in adipocyte number. Hypertrophy occurs when excess triglycerides are stored in an existing lipid droplet. This mechanism of increased WAT mass is the most common in adult mammals as proliferation of adipocytes is greatly reduced. The hormone insulin specifically facilitates the uptake of glucose into adipocytes by initiating the movement of GLUT4 carrier proteins to the plasma membrane (54). GLUT4 is necessary to transport glucose from the bloodstream into the cytoplasm where it is converted into glycerol and then incorporated into triglycerides. Hyperplasia of adipose, or adipogenesis, can be mediated by peroxisome proliferator-activated receptor-γ (PPAR-γ), which acts as a transcription factor in conjunction with retinoid X receptors (RXR) (1). PPAR-γ mediates many physiological processes in multiple cell types, but it is considered the master regulator of adipose proliferation as well as insulin sensitivity (64). PPAR-γ is highly expressed in both WAT and brown adipose tissue (BAT) and exists in two isoforms, PPAR-γ1 and PPAR-γ2, with PPAR-γ2 being exclusive to adipose tissue (1). Fatty acids can act as

ligands for PPAR-γ, however, the specific ligands and downstream consequences of ligand-receptor binding are ambiguous (1). In non-hibernating mammals adipogenesis is reduced in adulthood, but hibernators maintain this process as adults (1), presumably due to the importance of energy storage and the need for rapid lipogenesis. WAT is organized in several depots throughout the body. Each depot is innervated and vascularized differently causing each to have unique properties. In rodents, the depots consist of two main groups, subcutaneous and visceral depots (17). Subcutaneous WAT depots serve two important roles. The first role is housing epithelial ducts which form lactating nipples in pairs (17), and the second role is triglyceride accumulation as subcutaneous adipose tissue is efficient at short- and long-term fat accumulation when compared to other depots (6). Most WAT depots are visceral WAT depots. In comparison to subcutaneous depots, visceral depots are more lipolytically active and involved in adipose expenditure. These depots are therefore responsible for the release of free fatty acids and glycerol into the bloodstream (6). The main visceral WAT depots are the intra-abdominal (iaWAT, also called epididymal or eWAT), retroperitoneal (rWAT), omental (oWAT), and mesenteric (mWAT) depots. iaWAT represents the largest depot with regard to mass and is located in the peritoneal cavity on the ventral side of the abdominal organs (17). Located laterally to iaWAT to the left and right are the two rWAT depots. Among the mesentery holding the visceral organs in place is the mWAT and the oWAT is anterior to the iaWAT, just posterior to the stomach (17). The oWAT and mWAT depots play a key role in the development and severity of cardiovascular disease as well as type 2 diabetes mellitus in humans (6).

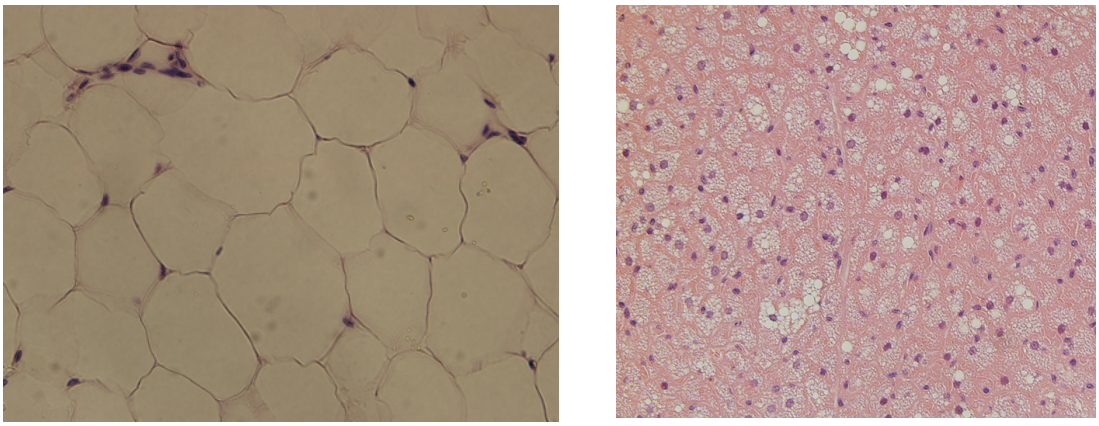

Figure 1. White adipose tissue (WAT, left) and brown adipose tissue (BAT, right). *WAT contains large lipid droplets. BAT contains small lipid droplets. Note the darker staining and smaller cell size in BAT compared to WAT cells.*

In contrast to the role of WAT in energy storage, the primary role of the other type of adipose tissue, brown adipose tissue or BAT, is the breakdown of triglycerides for heat production. BAT is a unique tissue rich with mitochondria (which give the tissue its brown appearance) and containing numerous small lipid droplets, different from the one large droplet found in WAT (Figure 1). While mitochondria are found in all animal cells, the mitochondria of BAT contain large amounts of uncoupling protein 1 (UCP1). UCP1 uncouples oxidative phosphorylation allowing H^+ ions to leak out of the intermembrane space across the inner mitochondrial membrane and bypass ATP synthase. Thus, the majority of the energy released from the metabolism of different energy sources, primarily lipids, in BAT is lost as heat (11).

Although it was once thought that WAT and BAT were influenced only by hormones secreted by distant tissues, both are now known to secrete their own regulatory molecules called adipokines. Adipokines act like hormones and help regulate several physiological functions associated with nutrient metabolism, including hunger and insulin resistance (19; 59). Adipokines act on distant organs and tissues, including the brain, pancreas, liver and immune system (19; 51; 59; 66). These adipokines include leptin, resistin, and retinol binding protein 4 (RBP4), among others. Leptin is produced by WAT and BAT, as well as stomach, and helps control satiety. Leptin receptors are found on an array of tissues including the hypothalamus, small intestine, WAT, BAT and pancreas (19). High leptin levels result in a loss of body weight (19). Leptin is also known to enhance insulin sensitivity and therefore glucose removal from circulation and is increased in obese states due to the increased production from larger WAT depots (61). Leptin resistance has been documented in pregnancy and hibernation presumably due to the physiological need for excess adipose accumulation (45). Resistin is a small protein hormone produced by WAT and BAT that acts on several tissues, most notably skeletal muscle and adipose tissue, to decrease insulin sensitivity (22; 50). Insulin insensitivity refers to any interaction with the insulin signaling pathway that leads to an interruption of the metabolic process of insulin by failure to respond to the signal (22; 50). Resistin specifically acts as an antagonist to insulin mediated glucose uptake (22; 59) as well as an inhibitor of new adipocyte formation (22; 33). Resistin levels are usually increased in obese states thus reducing the amount of glucose stored in sinks such as adipose and skeletal muscle (61). Retinol binding protein 4 (RBP4) was once thought to only serve as

a transport protein carrying retinol in the blood. It has now been shown, however, that RBP4 secreted from adipose could be involved in initiating systemic insulin resistance (66). The hormones and adipokines described can, not only alter the action of each other in adipose tissue, but also act on other pathways in exogenous tissue, providing support for the notion that adipose tissue is an important organ system with many complex physiological actions.

Adipose tissue is also involved in immune modulation. Specifically, adipocytes secrete and respond to immune-related cytokines (51). Leptin has been shown to differentially regulate T-cell proliferation in periods of fasting or starving (38). Additionally, adipose tissue has been shown to release several cytokines. One such cytokine is tumor necrosis factor-α (TNF-α), which in turn recruits macrophages inducing a pro-inflammatory condition (51). Another cytokine secreted by adipose tissue is interleukin-6 (IL-6) which functions to signal T and B lymphocyte proliferation and activation (51).

III. Adipose in Hibernators

White adipose tissue (WAT) is the primary fat-storage organ for mammals. Hibernating mammals undergo periods of lipogenesis that is mainly facilitated by hypertrophy of adipose cells with some hyperplasia occurring prior to hibernation to store excess adipose tissue (12). Although basal metabolic rate is greatly reduced during torpor bouts, metabolic activity nevertheless persists which requires ATP. Large WAT depots allow hibernators to survive long periods of complete fasting (12). The composition of

triglycerides stored in adipocytes is based on the type of fatty acid consumed in the diet and in hibernators this can be important for successful torpor-arousal cycles. Polyunsaturated fatty acids (PUFA) cannot be synthesized *in vivo* and must be consumed in the diet. These PUFAs seem to be pivotal in the maintenance of "proper" torpor-arousal cycles by maintaining membrane fluidity during torpor, allowing cells of hibernators to continue to function at lower temperatures (55).

BAT is also essential for successful hibernation. Hibernating mammals rely on BAT to regulate T_B at the lower set point of torpor, and to initiate regular arousals in which euthermic T_B is restored (12). Nonshivering thermogenesis in BAT is essential to warm the hibernator's body until T^B reaches ~16 C, at which point skeletal muscle shivering can adequately function (11).

Hibernators undergo an annual cycle of weight change based on metabolic need. During the lipogenic stage in the summer, hibernators become hyperphagic, a phenomenon that decreases drastically in the fall just prior to the onset of the first torpor (39). The lipolytic stage occurs during the winter months when hibernators are heavily reliant on energy stores contained in WAT. Hormones such as insulin, and leptin facilitate this cyclical change in body weight. Hibernating mammals become insulin resistant in obese states prior to torpor cycles, but are able to reverse this resistance post-hibernation during the spring season (39). In hibernators, leptin decreases during the spring and summer seasons primarily to aid in lipogensis then falls just before hibernation to inhibit hyperphagic states and end pre-hibernation fattening (24; 49). Interestingly, hibernators display pathologies that, in humans, would be classified as

diabetes with regard to weight gain, hyperinsulinaemia, and reduced insulin sensitivity (39). However, this drastic shift in homeostasis is necessary to produce rapid lipogenic and lipolytic stages found in the annual cycle of weight change. Another signaling molecule that could potentially regulate adipose metabolism in hibernators is retinoic acid.

IV. Retinoic Acid

Retinoic acid (RA) is the biologically-active derivative of vitamin A. Vitamin A must be obtained from the diet, as it cannot be synthesized in the body (47). Vitamin A is fat soluble and found in foods such as carrots, leafy vegetables, and eggs. However, even when consumed vitamin A is not biologically accessible and must be converted into retinoic acid and other forms. In the bloodstream, retinol is found bound to RBP4, the main transport protein responsible for moving retinol to target tissues, such as the liver, for storage (66). Most vitamin A stores in mammals are found in the liver in the form of retinyl esters. All-*trans*-retinol (vitamin A) is esterified into retinyl oleate, retinyl laurate, retinyl stearate, and retinyl palmitate, among others, prior to storage (47). Lecithin:retinol acyltransferase (LRAT) is largely responsible for the esterification and storage of retinol (5) (Diagram 1). LRAT is a membrane-bound protein and facilitates the uptake and storage of retinyl esters in several tissues including liver, lung, and intestine (5). LRAT also preferentially esterfies retinol or retinal bound to cellular retinol binding proteins (CRBP) a class of proteins involved in the transport of retinol inside the cell as well as the esterification of the carried retinol (47).

The conversion of vitamin A to RA is facilitated by dehydrogenases (Diagram 2). Initially vitamin A (retinol) is converted into retinal (retinaldehyde: Rald) via aldehyde dehydrogenases (ALDHs) (35). Rald itself can serve several functions, including repression of adipogenesis via inhibition of the PPAR-γ/RXR heterodimer (67). Rald can also be converted into RA, a process facilitated by retinaldehyde dehydrogenases (RALDH)-1, -2 and-3 (14). RA acts like a hormone, circulating in the bloodstream and binding to nuclear receptors on distant cells. Once transported into the cell, RA is transported to various locations in the cell bound to cellular retinoic acid binding proteins (CRABP)(15; 47). RA can exist as all-*trans* retinoic acid, 9-*cis*-retinoic acid, or 13-*cis*-retinoic acid (47). These different forms bind to different classes of RA receptors and are responsible for all RA-associated functions.

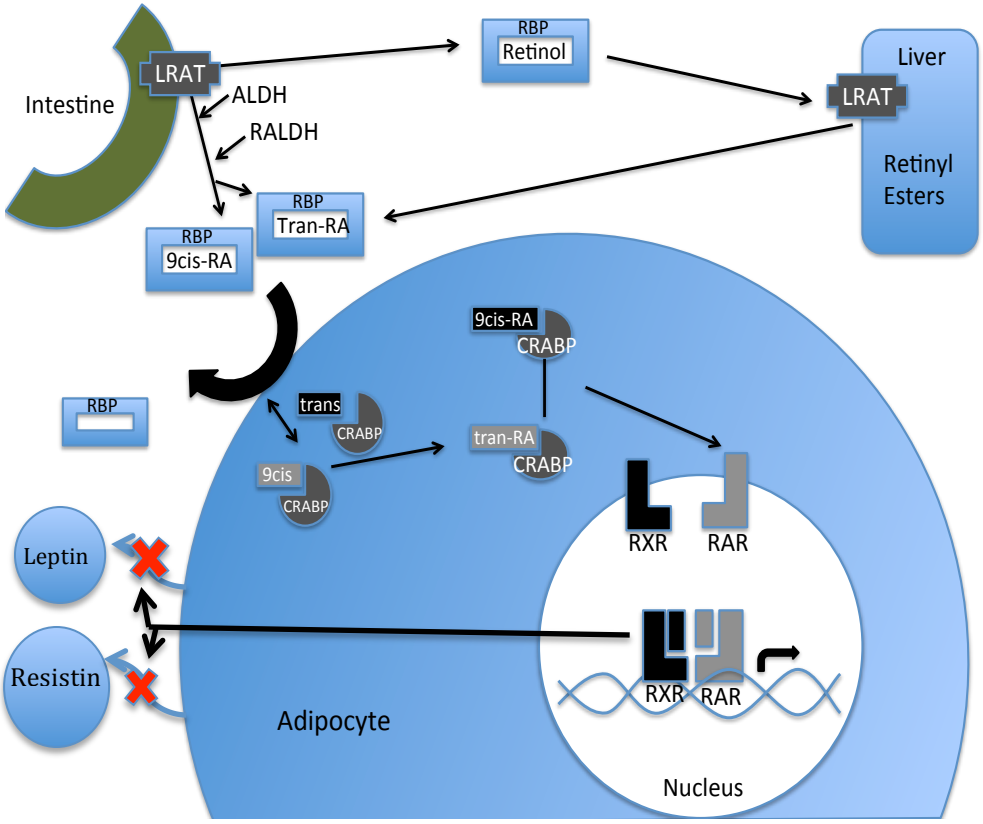

Diagram 1. A model of RA signaling in an adipocyte. *RA binding to its receptors leads to increased expression of those receptors and a decrease in the expression of leptin and resistin. LRAT: Lecithin Retinol Acytltransferase, ALDH: Alcohol Dehydrogenase, RALDH: Retinaldehyde Dehydrogenase, RBP: Retinol Binding Protein, 9cis-RA: 9cis Retinoic Acid, Tran-RA: All-trans Retinoic Acid, CRAPB: Cellular Retinoic Acid Binding Protein.*

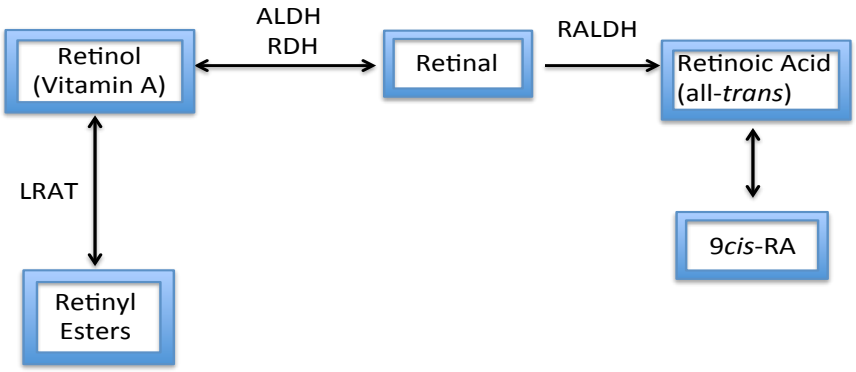

Diagram 2. A model of RA conversion *ALDH: Aldehyde Dehydrogenase, RDH: Retinol Dehydrogenase, RALDH: Retinaldehyde Dehydrogenase, LRAT: Lecithin Retinol Acytltransferase*

RA receptors are transcription factors that up- or down-regulate transcription. There are two classes of RA receptors — retinoic acid receptors (RAR) and retinoid X receptors (RXR). RAR and RXR each consist of three subtypes, α, β, and γ (56). Different forms of RA bind to different receptor types. RAR binds both all-*trans*-RA and 9-*cis*-RA whereas RXR binds only 9-*cis*-RA (56). Additionally, these receptors can form RAR-RXR or RXR-RXR dimers before regulating target genes (56). The receptors consist of a ligand-binding domain and a DNA-binding domain. When two receptors bind their respective ligands they attach to half sites on the target gene via the DNA-binding domain up-regulating transcription of the target gene (56). RXR also forms a heterodimer with PPAR-γ to promote adipogenesis and increase insulin sensitivity (1). These nuclear receptors are differentially expressed in several tissue types as well as

under different conditions. For example, vitamin A-deficiency reduces expression of RARα, β, and γ in liver, kidney, intestine and lung (65). Additionally, WAT and BAT differentially express receptor subtypes based on the signaling of the expressed receptors and the main functional processes of the tissue. RARβ and RXRγ are expressed at low levels in WAT but are abundant in BAT, and both RAR and RXR are represented by at least one isoform in each adipose tissue type (8).

Adipose tissue differentiation is directly influenced by RA signaling facilitated by RAR and RXR receptors. At high concentrations, both all-*trans*-RA and 9-*cis*-RA, accounting for RA signaling, inhibit preadipocyte differentiation (56). However, at low concentrations RA signaling acts in a positive manner and increases preadipocyte differentiation into adipocytes (56). The concentration-dependent action of RA suggests a tightly regulated feedback loop for the depletion and accumulation of adipose tissue. Additionally, RA has been shown to reduce leptin production, which can increase hunger and lead to increases in adipose mass (8). In BAT, RA signaling has two main effects. The first is upregulating UCP1 and UCP2 expression, which increases thermogenic capacity and energy expenditure (8). The second effect, like WAT, is decreased adipogensis by inhibition of new adipocyte formation (8).

RA signaling is involved in an array of processes outside of adipose tissue as well. RAR, for example, is heavily involved in multiple facets of embryonic development. During development, RAR helps control neural crest development, axial patterning, and limb and eye development (31). In adults, RARs affect the maintenance of skin and reproductive tissues (31). Immune function is strongly influenced by RA

signaling as well. RA signaling via RAR-α recruits lymphocytes to the small intestine (28; 30), another phenomenon that is seen in hibernators (37).

V. Retinoic Acid Signaling is Important to Hibernators

Little is known about vitamin A, much less retinoic acid, in hibernators. Hibernating mammals, such as the 13-lined ground squirrel *(Ictidomys tridecemlineatus)* are faced with two major challenges that they must overcome in order to successfully hibernate. The first of these challenges is adequate energy storage and the second is production of heat. To accomplish these physiological processes hibernators rely on WAT and BAT. Given the intimate relationship of RA signaling and adipose accumulation and thermogenesis in BAT, the regulation of RA signaling is likely vital to a hibernating mammal. The conversion, transport and storage of retinol are likely to also be important.

The complex interaction of RA with adipose tissues and its role in regulating the adipokines these tissues produce suggests an important role for RA in the physiology of successful hibernation. The consumption and proper storage of vitamin A prior to the hibernating season may be pivotal to hibernating mammals. Understanding RA signaling in a hibernator could prove beneficial to understanding the adipose tissue as a whole and the complex pathways RA signaling can alter.

Chapter 2: Body

Introduction

Hibernation is a seasonal adaptation characterized by extreme changes in physiology. The majority of the hibernation season is spent in torpor, a state associated with reduced body temperature and metabolic rate, fasting, and inactivity. During the hibernation season, these animals also undergo periodic arousals which are characterized by a rapid increase in body temperature, metabolic rate, and mobility. Arousals generally last for 18-24 hours and occur many times throughout the hibernation season with the greatest frequency of arousal in the weeks after immergence and the weeks before emergence and the lowest frequency in the middle of the hibernation season. Hibernators require adequate energy stores to successfully survive the hibernation season and the majority of that energy is used during the periodic arousals. This energy is stored in white adipose tissue (WAT), although brown adipose tissue (BAT) can also store significant amounts of lipids that are used to power rewarming during periodic arousals (12). Therefore, adequate adipose mass prior to hibernation is essential to survival.

In order to store adequate amounts of lipids to survive the winter, hibernating mammals become hyperphagic in the late summer and early autumn. Little is known about the nutrient needs of hibernating mammals or the exact contents of a wild diet. Studies have focused on the importance of various saturated and unsaturated fatty acids and their role in the mechanisms of hibernation (25)(Reviewed in (55)), but the vitamin needs of these animals are largely unknown. One fat-soluble vitamin, vitamin A (i.e., retinol), plays an integral role in nervous system function, vision, energy storage,

thermoregulation and immunity. The biological effects of vitamin A are carried out primarily by its metabolite, retinoic acid (RA), via signaling through specific retinoid receptors. Retinol is converted into RA via several enzymatic processes conducted by alcohol dehydrogenases (ALDH) and retinaldehyde dehydrogenases (RALDH) (32; 35). RA signaling is involved in many functions of WAT and BAT, including regulation of adipogensis and lipid mobilization (8; 9; 42; 56), adipokine secretion (22; 36) and uncoupling protein expression (7; 42; 57).

Given the importance of RA signaling in the development, storage and use of adipose tissue, proper consumption and storage of retinoids could prove vital to hibernating mammals prior to the wintering season. To test this, we developed a model of early vitamin A deficiency in 13-lined ground squirrels (*Ictidomys tridecemlineatus)*. We hypothesized that squirrels which were deficient in vitamin A during the hyperphagic period would accumulate more adipose mass compared to controls. We also hypothesized that expression of retinoid receptors and adipokines would be altered in the WAT of deficient animals and that uncoupling protein 1 (UCP1) expression would be significantly lower in BAT of deficient squirrels.

Materials and Methods

Animals. Gravid female 13-lined ground squirrels were caught from various locations around northeastern Wisconsin in mid-May and transferred to the animal facility at UW Oshkosh. Squirrels were treated with ivermectin (0.4 mg/kg, s.c.) and flea

spray upon arrival. Squirrels were housed individually with unlimited access to water at ~20°C with a light cycle roughly corresponding to the natural photoperiod. All procedures involving animals were approved by the UW Oshkosh Institutional Animal Care and Use Committee (Protocol #0026-000276-04-11-14).

Vitamin A Diet Regimen. Gravid females were housed in cages (330 in^2, 15W X 22L X 6H) to provide adequate space for mother and pups. One week after capture, squirrels were assigned to either a Vitamin A Control (VAC, containing 0.04 g/kg retinyl palmitate) or Vitamin A Deficient (VAD, using "Vitamin-Free" test casein to eliminate vitamin A sources) diet. The diets had similar macronutrient composition (24.6% protein, 47.1% carbohydrate, and 12.0% fat, by weight). Once litters were born, mothers remained on their respective diets until the pups were weaned at which point they were returned to a normal diet and removed from the study. Weaned pups continued on their respective diets for 2 weeks after weaning (until ~7-8 weeks of age) before being returned to a normal diet of dog food (Iams ProHealth chunks) supplemented with sunflower seeds (2 tbsp, twice per week), dried vegetables (2 tbsp, once per week) and peanuts (one peanut, once per week). After removal from the deficient diet, VAD pups were treated with retinyl palmitate (6 ug) three times over the span of a week. From 4-5 weeks of age through entrance to hibernation, all active pups were weighed weekly.

Initiation of Hibernation. Nine weeks after removal from the VAD or VAC diet, all remaining squirrels were challenged in an attempt to induce torpor. Food and

water were removed and the squirrels were placed in a 3-7°C hibernaculum for 7 days. Squirrels that entered torpor for at least 2 consecutive days remained in the hibernaculum. Squirrels that did not enter torpor within 7 days were removed to normal housing with food and water. Squirrels were challenged again at 12 weeks, 15 weeks post removal from diet. After the last challenge, all but two squirrels (one VAD and one VAC) from the study had entered hibernation. These squirrels were removed from the study. Hibernating squirrels were checked daily under red light (< 10 min) and monitored for interbout arousals using bedding chips as an indicator of movement or activity.

Tissue Collection. Tissues taken during hibernation (HIB) were collected from individuals that had been in hibernation for 7-13 weeks and were torpid (T) for at least 2 consecutive days. Other seasons in which tissues were collected include summer (while still on the assigned diet), pre-hibernation (2-4 weeks prior to first cold room challenge), and spring (1 week post-emergence). Squirrels were euthanized by rapid decapitation and body weight, shoulder-to-hip length & Tb were measured. Serum was collected. Three WAT depots (intra-abdominal/gonadal [iaWAT], retroperitoneal [rWAT], omental [oWAT]) and the interscapular BAT depot were removed and weighed. Adiposity index was measured by dividing the iaWAT mass by total body mass. A small portion of iaWAT and BAT were preserved in RNA Later (ThermoFisher Scientific, Waltham, MA). Jejunum and liver were removed and a portion was flash frozen in liquid nitrogen. All tissues were stored at -80 °C until use.

Total Vitamin A Quantification (HPLC). Total Vitamin A concentrations were determined by high performance liquid chromatography (HPLC). One gram of liver tissue for each replicate was ground up in sodium sulfate and standardized with retinyl butyrate. Samples were then filtered with dichloromethylate, evaporated down under nitrogen and re-solubilized with 75% MeOH/25% DCE before being loaded into a Waters HPLC system with a Resolve C18 Column (90Å, 5 μm; Waters Corporation, Milford, MA) for total retinoid analysis.

ELISA. Serum samples taken from each squirrel were analyzed for rentinol binding protein 4 (RBP4) using the human/mouse/rat RBP4 competitive enzyme linked immunoassay kit (RayBiotech, Norcross, GA) following the manufacturer's recommended protocol. Briefly, microtiter plates were coated with anti-RBP4 antibody then incubated with diluted serum samples for 2.5 hours. An HRP-streptavidin enzyme was then added and color was produced using 3, 3', 5, 5'- tetramethylbenzidine (TMB) substrate. Absorbance was read using an iMark plate reader and Microplate Reader 6.1 software (BIO-RAD, Hercules, CA).

Immunoblotting. Protein lysates were separated via SDS-PAGE, transferred to a PVDF membrane and incubated with antibodies to lecithin:retinol acyltransferase (LRAT, H-83) (Santa Cruz Biotechnology, Santa Cruz, CA). Bands were detected using chemiluminescent substrate and relative intensity of bands was quantified using Quantity-

One 1-D analysis software (BIO-RAD, Hercules, CA). LRAT densitometry was normalized to β-actin (Novus Biologicals, Littleton, CO).

Quantitative RT-PCR. RNA was isolated using a total tissue RNA mini kit (IBI Scientific, Peosta, IA), following the manufacturer's suggested protocol except for the tissue lysis step. To adequately lyse adipose tissue, we used 0.5 mm RNAse/DNAse-free stainless steel beads in conjunction with a TissueLyser unit (Qiagen, Hilden, Germany). RNA was quantified with a ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). RNA (350 ng) was used to make first strand cDNA using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega, Madison, WI). Quantitative RT-PCR was run on a Step One Real-Time PCR System (Applied Biosystems/Life Technologies, Grand Island, NY) using Bullseye EvaGreen (2x) qPCR master mix (Midwest Scientific, Valley Park, MO). Samples were run in duplicate and normalized to the housekeeping gene beta-actin. Primers (Table 1), were designed from the 13-lined ground squirrel annotated genome on Ensembl.org. Annealing temperature used for all primers was 60.0°C.

Table 1. Primers

RAR-α	F--TACGGATCTGCACGAGGTAC	R--TCCTGCCGGTCTCCACAG
RXR-β	F--GAGTTTACAGCTGTGAGGGC	R--CCTCCTGTACAGCTTCCCTC
Leptin	F-ATGTTCAAGCTGTGCCCATC	R-AAGGCAGACTGGTGAGGATC
Resistin	F- CTCTCTGCGTCCTTCTCCTC	R- CCTGGAGGTGAGGGTCTTG
UCP1	F-AGTCCGGCTACAGATCCAAG	R- CCCCTGAGGTGAAGAACTCC
Actin	F- GGAAATCGTGCGTGACATCA	R- AGGATTCCATGCCCAGGAAA

Statistics. Multifactor ANOVA was used to simultaneously analyze the effects of diet and season as well as the interaction between diet and season. If seasonal comparisons were significant ($p \leq 0.05$), Tukey's pairwise comparison was used. Before performing ANOVA, a regression analysis was performed on the data and the residuals were determined to be normally distributed. If residuals were not normally distributed, the data was log transformed (mass of iaWAT, rWAT, oWAT and BAT; adiposity index; RBP4; all WAT and BAT qPCR). To determine if there was a correlation between total liver retinoids and serum RBP4, the Pearson total correlation coefficient was determined.

Results

Vitamin A *deficient diet induces rapid hypovitaminosis and stunted growth in ground squirrels.* Squirrel pups were weighed weekly starting at 4-5 weeks of age until entering hibernation. Early in this period, at approximately 8 weeks of age, deficient (VAD) squirrel weights diverged from their control counterparts as they began to gain weight at a slower rate (Figure 2). At the summer tissue collection, VAD pups were significantly smaller in length and mass (Table 2). While on the VAD diet, squirrels had shaky movements and tremors that suggested a lack of hind limb coordination. Behavior (responding to noise but not movement around the cage) indicated possible blindness and corresponded to the appearance of a milky discharge from the eyes. Vocalizations in VAD animals were unusually dry and weak compared to normal squirrel chirping sounds, an effect of deficiency on vocal cords that has been noted in other animals (41; 63). The summer tissue collection was completed at the onset of these symptoms and all remaining

pups were removed from the study diets and put on a normal diet by approximately 8-9 weeks of age. After 6-8 weeks on a normal diet, weight gain of VAD squirrels started to mirror VAC squirrels (Figure 2). It is important to note, however, that percent weight change does not reflect total body weight, as hibernating VAD squirrels did not reach similar weights compared to controls (Table 2).

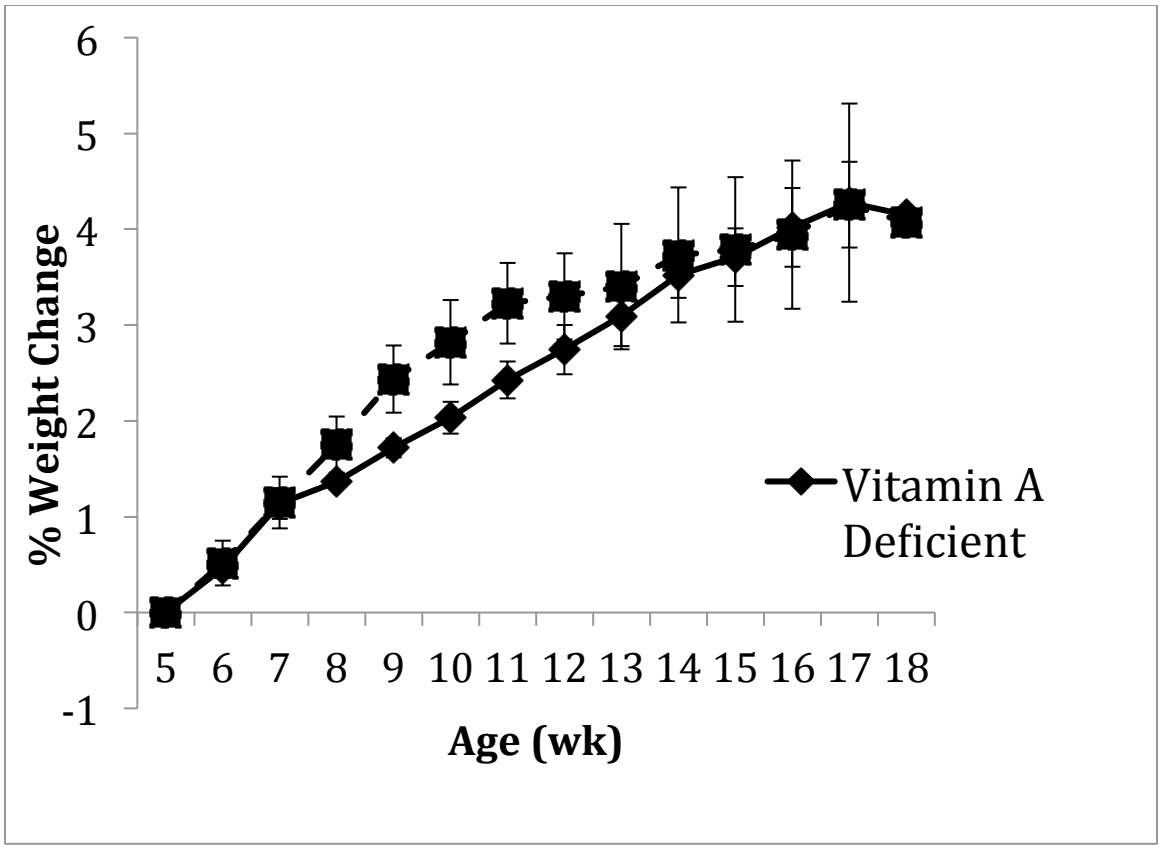

Figure 2. *Percent weight gain in ground squirrel litters based on initial weight at 5 weeks of age and continuing until squirrels entered hibernation. Because pups were co-housed until 12 wk of age, litter average was calculated. Lines represent mean ± SE of 3 litters for each treatment. $P \geq 0.05$ for each week.*

Table 2. Body Size and Temperature at Tissue Collection

VAC	Body Weight (g)	Shoulder-to-Hip (cm)	T_B (°C)
Summer (10)	118.1 ± 7.44	10.3 ± 0.23	38.6 ± 0.26
Pre-Hibernation (2)	166.2 ± 12.9	11.3 ± 0.25	38.3 ± 0.90
Hibernation (5)	173.5 ± 9.22	10.4 ± 0.40	7.8 ± 0.27
Spring (2)	138.0 ± 3.00	10.5 ± 0.50	37.5 ± 0.05
VAD			
Summer (13)	80.6 ± 3.28*	9.1 ± 0.23*	37.5 ± 0.19*
Pre-Hibernation (4)	184 ± 4.90	10.5 ± 0.00	37.5 ± 0.34
Hibernation (7)	139.7 ± 9.58*	9.3 ± 0.36	7.59 ± 0.22
Spring (6)	143.9 ± 4.38	10.3 ± 0.17	37.8 ± 0.52

*Data represent means ± SE. * indicates significant effect of diet within season. Numbers in parentheses indicate sample size.*

Vitamin A deficiency induces significant changes in adipose depot mass. In addition to overall body weight, various adipose depots in deficient squirrels were also significantly smaller in size. The depots collected and weighed during tissue collection include the intra-abdominal (iaWAT), omental (oWAT) and retroperitoneal (rWAT) white adipose tissue depots and the interscapular brown adipose depot (BAT). In addition, adiposity index was calculated for each squirrel. As expected, season had a significant impact on adipose depot mass (Table 3). Multifactor ANOVA analysis showed a significant effect of season and a significant interaction between season and diet for iaWAT, rWAT and oWAT mass as well as adiposity index (Table 3). In controls, the mass of these depots increased steadily, reaching its peak in hibernators and dropping off in spring. In early deficient squirrels, WAT mass increased from summer to pre-hibernation season, but dropped off in hibernators and again in spring. BAT mass differed significantly based on season, diet and the interaction between season and diet. In both diet groups, BAT mass increased steadily until reaching its maximum in hibernators, but mass was significantly lower in VAD compared to control. In summer VAD squirrels, decreased BAT mass was associated with lower Tb (Table 2).

Table 3. Mass of Adipose Tissue Depots

VAC	iaWAT	rWAT	oWAT	BAT	Adiposity Index
Summer (10)	2.10 ± 0.30 *a	1.25 ± 0.16 *a	0.76 ± 0.13 *a	0.48 ± 0.05 *a	1.73 ± 0.18 *a
Pre-Hibernation (2)	6.83 ± 4.76 b	3.94 ± 3.17 b	1.71 ± 0.73 b	0.92 ± 0.27 b	3.91 ± 2.56 b
Hibernation (5)	10.31 ± 0.74 b	7.03 ± 0.74 b	2.03 ± 0.44 b	1.87 ± 0.20 *c	6.07 ± 0.70 b
Spring (2)	5.89 ± 0.002 b	3.76 ± 0.25 b	1.14 ± 0.19 b	1.04 ± 0.02 b	4.27 ± 0.09 b
VAD					
Summer (13)	0.80 ± 0.09 *a	0.37 ± 0.02 *a	0.46 ± 0.48 *a	0.24 ± 0.02 *a	0.95 ± 0.08 *a
Pre-Hibernation (4)	10.3 ± 0.74 b	6.51 ± 0.73 b	2.64 ± 0.43 b	1.11 ± 0.18 b	6.15 ± 0.59 b
Hibernation (7)	7.05 ± 0.94 b	4.19 ± 0.4 b	2.02 ± 0.21 b	1.31 ± 0.11 *c	4.94 ± 0.39 b
Spring (6)	6.00 ± 0.99 b	3.35 ± 0.56 b	1.40 ± 0.28 b	0.81 ± 0.10 b	4.37 ± 0.73 b

*Data represent means ± SE. * Indicates a significant difference between diets within activity state (calculated by t-test). Letters indicate differences between seasonal groups (calculated by Tukey's post-hoc test); groups with the same letter are not significantly different.*

Total liver retinoid levels do not correlate with serum RBP4. Analysis of liver tissue from summer VAD squirrels (still on the deficient diet) showed no detectable retinoids (Figure 3A). After 7 weeks on a normal diet, total liver retinoids in pre-hibernating VAD squirrels increased significantly, but did not reach the level of controls (Figure 3A). This trend continued throughout the annual cycle as hibernating and spring VAD recovery squirrels had significantly lower retinoid stores compared to controls. This occurred despite a new diet that included an abundance of foods containing vitamin A. Because control (VAC) liver retinoid levels were high for a rodent, we assayed summer and hibernating adult squirrel livers collected during a separate study that did not manipulate diet ("Non-Study" group) and found that retinoid stores were similar to those in the older VAC (Figure 3A). Overall, the predominant retinyl ester in 13-lined ground squirrels was retinyl oleate, an 18-carbon unsaturated ester, followed by retinyl palmitate, laurate and stearate (Table 4). The relative abundance of retinol, however, was significantly lower in controls compared to non-study squirrels. Torpid hibernators on both diet regimens had a higher relative abundance of retinyl oleate compared to other seasons, a shift which occurred at the expense of retinyl palmitate and stearate (Table 4).

Serum RBP4 was examined by ELISA to determine if retinol transport was downregulated in deficient squirrels. RBP4 levels did not vary based on diet but hibernating squirrels had significantly less serum RBP4 compared to all other seasons (Figure 3B). In addition, RBP4 did not correlate with total liver retinoids suggesting expression was maintained despite the lower levels of liver retinoids in deficient squirrels (Figure 3C).

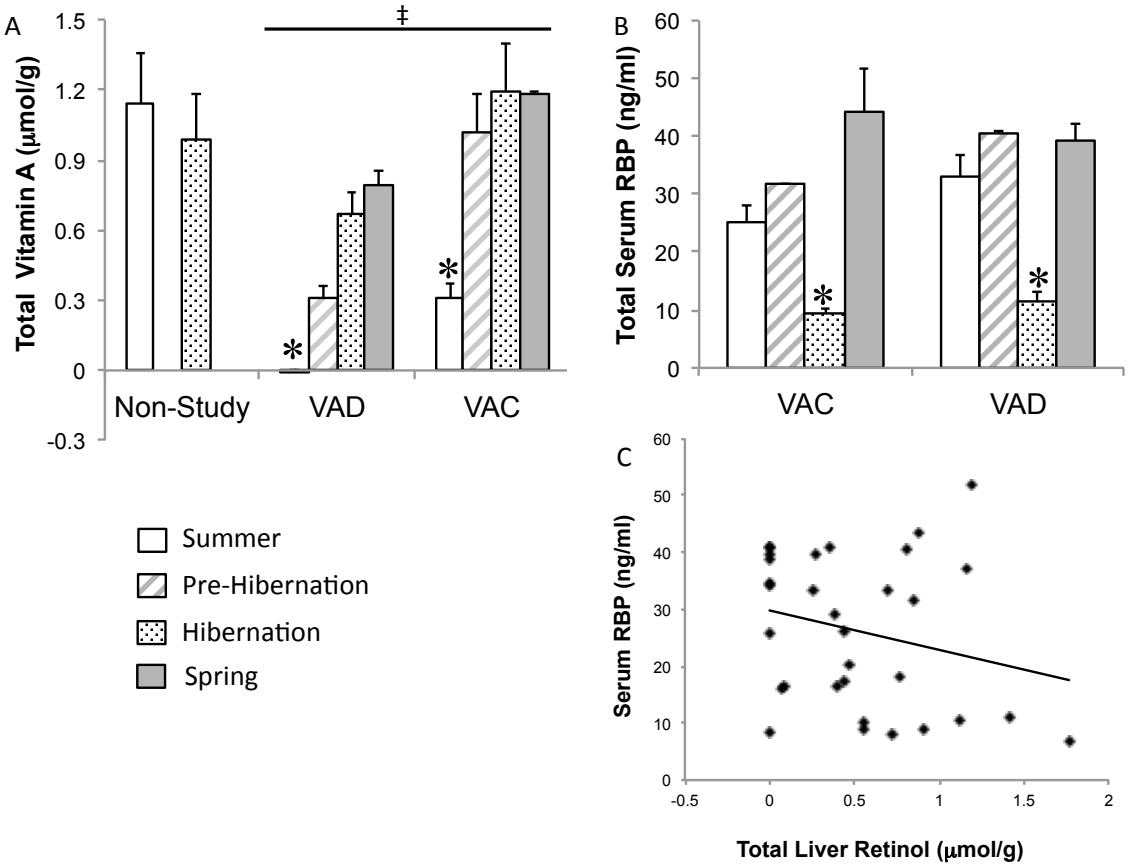

Figure 3. *Effects of early vitamin A deficiency on liver and blood retinol. (A) Total liver retinoid content. * Indicates summer different from other seasonal groups. ‡ Indicates significant effect of diet. Multi-factor ANOVA: Diet effect, $P < 0.001$; Season effect, $P < 0.001$; Diet*Season effect, $P > 0.05$. (B) Total serum RBP4. * Indicates hibernating squirrels significantly different from other groups. Multi-factor ANOVA: Diet effect, $P > 0.05$; Season effect, $P < 0.001$; Diet*Season effect, $P > 0.05$. (C) Correlation of liver retinoids to serum RBP4. Pearson correlation coefficient = -0.246, $P = 0.175$.*

Table 4. Retinol and Retinyl Ester Composition as Percent of Total Retinoids

VAC	%ROL	%Laurate	%Oleate	%Palmitate	%Stearate
Summer	1.8 ± 0.4* a	14.5 ± 0.4* a,b	35.3 ± 1.9 c	37.2 ± 2.0 b	11.3 ± 0.6 b
Pre-Hibernation	0.7 ± 0.2 a,b	11.5 ± 0.5 b,c	37.1 ± 4.2 b,c	39.2 ± 2.9 b	11.5 ± 0.7 b
Hibernation	0.6 ± 0.3 a,b	14.9 ± 0.9 a	54.7 ± 1.2 a	22.4 ± 0.9 a	7.4 ± 0.5 a
Spring	0.24 ± 0.01 b	8.4 ± 2.5 c	45.2 ± 2.3 b	36.7 ± 0.4 b	9.4 ± 0.6 a,b
VAD					
Summer	ND	ND	ND	ND	ND
Pre-Hibernation	0.3 ± 0.4	8.8 ± 0.2	43.1 ± 0.8 b	36.8 ± 0.8 b	11.0 ± 0.7 b
Hibernation	0.1 ± 0.1	12.3 ± 0.8	56.8 ± 1.5 a	22.8 ± 1.8 a	7.9 ± 0.5 a
Spring	0.0 ± 0.4	12.6 ± 4.6	42.6 ± 1.7 b	36.8 ± 2.9 b	8.5 ± 0.5 a
Non-Study					
Summer	17.3 ± 3.6	10.7 ± 0.7	31.1 ± 2.9	30.6 ± 3.3	10.3 ± 1.3
Hibernation	2.3 ± 1.4	14.4 ± 0.1	53.1 ± 1.7	23.4 ± 0.8	6.8 ± 0.5

Data represent means ± SE. "ND"—not determined, total retinoids were basically zero.
** Indicates different from Non-Study group of the same season. Letters indicate ANOVA of seasonal differences within diet. Groups with the same letter are not significantly different*

LRAT expression in liver and jejunum is not affected by early deficiency. In an attempt to explain the differences in retinoid storage between early deficient and control squirrels, we examined the expression of LRAT, an enzyme responsible for proper esterification of retinol for absorption and storage, in jejunum and liver. Diet and season did not affect LRAT expression in liver (Figure 4A), but if the analysis was performed without spring and pre-hibernation groups a significant difference between summer and hibernating seasons is observed. Interestingly, the expression of LRAT was higher in torpid squirrels in comparison to summer squirrels in both diet groups (Figure 4A). Jejunum LRAT expression was also not affected by diet or season (Figure 4B). However, when comparing VAC to VAD within the torpid state, VAC squirrels displayed a lower expression of LRAT in the jejunum (Figure 4B).

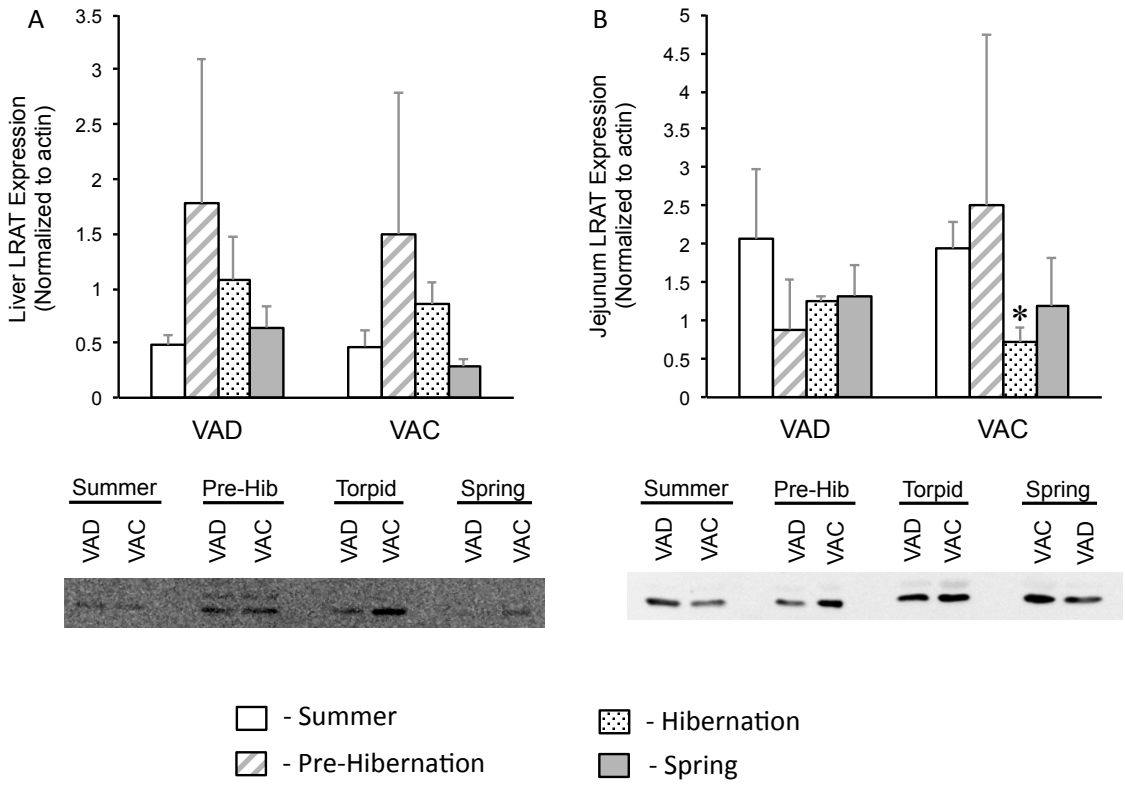

Figure 4. *LRAT protein expression in jejunum and liver. (A) LRAT expression in liver samples. Multi-factor ANOVA: Diet effect, P > 0.05; Season effect, P > 0.05; Diet*Season effect, P > 0.05. (B) LRAT expression in jejunum samples. Multi-factor ANOVA: Diet effect, P > 0.05; Season effect, P > 0.05; Diet*Season effect, P > 0.05. * indicates significant difference between VAD and VAC within torpid hibernators. Representative immunoblots are shown for jejunum and liver LRAT. All samples were normalized to β-actin.*

Early vitamin A deficiency affects expression of RXR-β and resistin in WAT. In order to determine whether RA signaling was disrupted by early deficiency, we examined the expression of two predominant RA receptors in iaWAT. Retinoic acid receptor (RAR)-α expression was not affected by diet or season (Figure 5A). Retinoid X receptor (RXR)-β expression was not affected by diet or season individually but when the interaction between season and treatment was analyzed, a significant difference was found (Figure 5B). In control squirrels, RXR-β transcripts were low in summer and increased in pre-hibernating and hibernating animals, when fat utilization is at its peak, before dropping back to low levels after spring emergence. In the deficient group, RXR-β transcript number started off high in summer, decreased in pre-hibernation season and remained low for hibernators and spring squirrels (Figure 5B). We also examined the expression of two adipokines, resistin and leptin, which have been shown to be influenced by RA signaling. The relative abundance of resistin transcripts was significantly affected by diet wherein control squirrels expressed significantly more resistin compared to deficient squirrels (Figure 5C). In addition, season had a significant effect on resistin with pre-hibernating squirrels having higher expression than summer animals for both diets. For leptin, no effect of diet was seen but, unsurprisingly, season had a significant effect (Figure 5D). Pre-hibernating squirrels had significantly higher amounts of leptin in comparison to the other seasons similar to that observed in previously published studies (19; 24; 49).

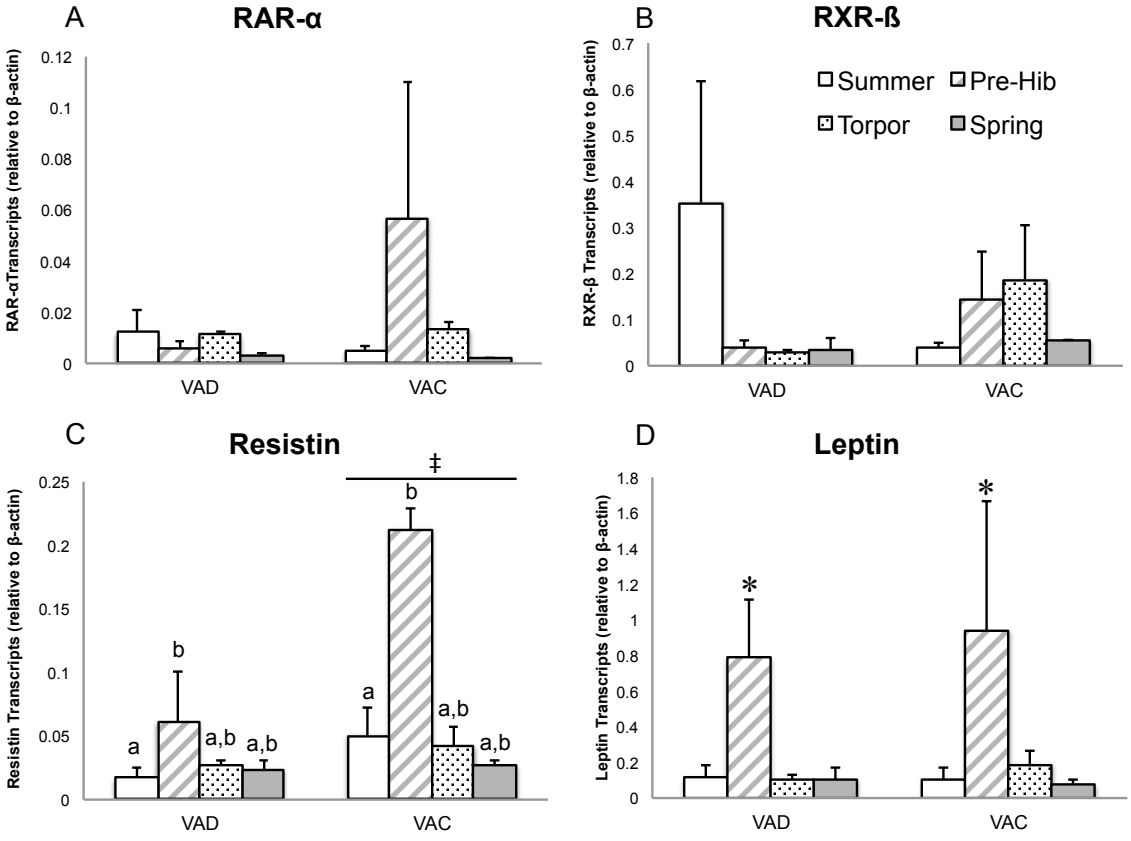

Figure 5. *Expression of retinoid receptor and adipose hormone transcripts in intrabdominal white adipose tissue (iaWAT). Expression was normalized to β-actin. (A) RAR-α, Multi-factor ANOVA: Diet effect, P > 0.05; Season effect, P = 0.079; Diet*Season effect, P > 0.05. (B) RXR-β, Multi-factor ANOVA: Diet effect, P > 0.05; Season effect, P > 0.05; Diet*Season effect, P < 0.05. (C) Resistin, Multi-factor ANOVA: Diet effect, ‡P < 0.05; Season effect, P < 0.05, groups with the same letter are not significantly different; Diet*Season effect, P > 0.05. (D) Leptin, Multi-factor ANOVA: Diet effect, P > 0.05; Season effect, P = 0.005, * indicates pre-hibernation group different from all other states; Diet*Season effect, P > 0.05.*

Early deficiency increases expression of UCP1 in BAT. UCP1 expression in BAT varied significantly by season and diet. The interaction of season with diet was also significant. Interestingly, VAD squirrels showed significantly higher levels of expression compared to control squirrels (Figure 6A). In controls, UCP1 expression was highest in summer and decreased with each subsequent season. In deficient squirrels, expression increased in the pre-hibernation group before dropping significantly in the hibernators and spring squirrels (Figure 6A). Changes in UCP1 expression and BAT mass (Table 3) induced by early vitamin A deficiency did not affect the frequency of interbout arousals (Figure 6B).

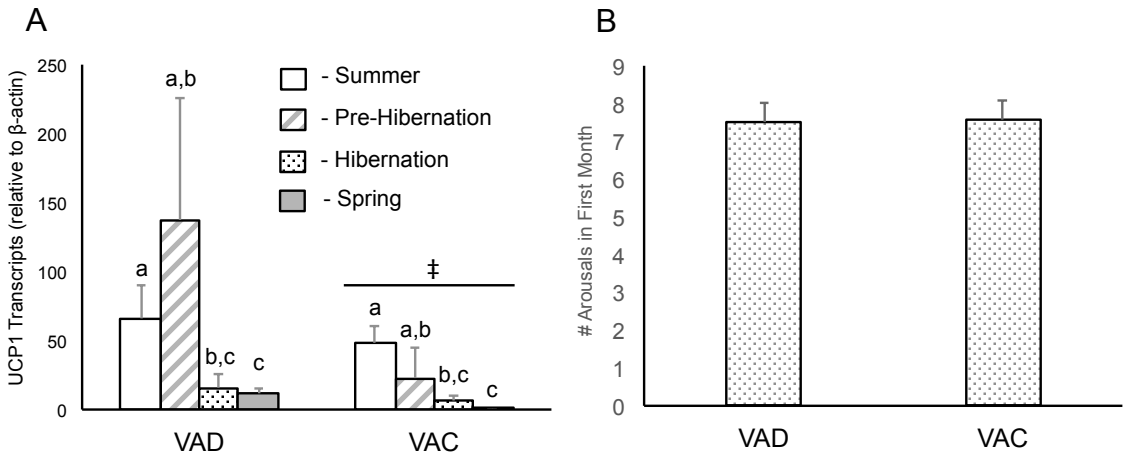

Figure 6. *(A) Expression of UCP1 transcripts in intra-scapular BAT of early deficient (VAD) and control (VAC) squirrels. Expression was normalized to β-actin. Multi-factor ANOVA: Diet effect, ‡P = 0.001; Season effect, P < 0.001, groups with the same letter are not significantly different; Diet*Season effect, P = 0.05. (B) Number of interbout arousals in the first month of hibernation in deficient and control squirrels (p > 0.05).*

Discussion

In this study, we examined the physiology of early vitamin A deficiency in a mammalian hibernator. Because of the role of RA, a vitamin A metabolite, in WAT accumulation and hypertrophy and in UCP expression in BAT and previously published studies on the effects of vitamin A deficiency in rodents (7; 43; 44; 53; 57), we hypothesized that deficient animals would gain more WAT mass and produce less UCP than controls. What we found, however, is that 13-lined ground squirrels are especially sensitive to vitamin A deficiency and that this deficiency is associated with reduced WAT accumulation and increased BAT UCP expression. This was the first study that has attempted to produce early vitamin A deficiency in a hibernating animal and our data suggest that vitamin A is essential for early development in these animals but that RA signaling may not play as important of a role in WAT and BAT as we hypothesized.

We induced deficiency in 13-lined ground squirrels by trapping gravid females and starting them on a vitamin A deficient diet approximately one week before the pups were born. This protocol is commonly used in other rodents, such as mice and rats (13; 63), and some studies with rats have kept the offspring on a deficient diet for up to 10 months before symptoms of severe deficiency appeared (13). The onset of severe deficiency was much more rapid in our ground squirrels, which developed such severe symptoms by two weeks post-weaning that they had to be removed from the diet and treated with oral retinyl palmitate in order to recover. The symptoms we observed are commonly associated with vitamin A deficiency and include hind limb weakness (41), dry vocalizations (41; 63) and periocular inflammation (13). Not surprisingly, the

squirrels also showed signs of vision loss, such as a lack of response to movement around the cage but normal response to auditory stimuli. Additionally, deficient squirrels gained weight more slowly while on their assigned diet, demonstrating the onset of deficiency very early in life. We do not fully understand why the ground squirrels developed such severe deficiency so early, but observational studies of this species' diet in the wild may provide some clues to their vitamin A requirements. Thirteen-lined ground squirrels eat a combination of animal matter (i.e., eggs, fledgling birds, insects) and plant matter (i.e., grasses, seeds, vegetables, roots) (18). The green, leafy and vegetable portions of the diet, as well as eggs and meat, can provide ground squirrels with a significant amount of vitamin A and suggest a need for high vitamin A stores going into the hibernation season.

Indeed, we found that liver retinoid stores in our control ground squirrels were high relative to other rodents (16). Additionally, when surveying liver retinoid stores in squirrels that were not associated with the current diet study, some of which were euthanized immediately after trapping from the wild, we found that these unusually high stores were normal for this species. The maintenance of high liver retinoid stores could reflect an increased need for retinoids, possibly due to a more active expenditure of stored esters for adipose accumulation and mobilization during their annual cycle. The control squirrels in this study, as well as the non-study squirrels mentioned previously, maintained levels of stored retinoids that would normally result in hypervitaminosis in other rodents, yet we observed no signs of this condition in our animals (16; 60). We examined the relative levels of retinyl esters but found little difference between control squirrels and non-study squirrels suggesting that retinoid metabolism and storage was not

affected in our control animals. The percentage of retinol was lower in control squirrels compared to non-study animals but both groups decreased prior to hibernation. When comparing the relative composition of the total retinoid pool across seasons some interesting trends were discovered. During hibernation, the percentage of retinyl oleate increases significantly at the expense of retinyl palmitate and, to a lesser extent, retinyl stearate. This is different from rats and humans where retinyl palmitate is the predominant ester (27; 62), but not surprising given that oleic acid makes up the majority of the fatty acid composition of adipose depots in ground squirrels prior to hibernation (25). Hibernators also had significantly lower levels of circulating RBP4 compared to other seasons in both the deficient and control squirrels. Although we cannot correlate the blood levels of RBP4 to changes in liver stores, it is interesting that the potential to transport vitamin A in the bloodstream is significantly diminished during torpor. We did not test interbout arousal squirrels and therefore cannot determine whether this decrease is maintained throughout the hibernation season or whether it is a consequence of torpor in some way but this would be an interesting avenue for further study. In every season, deficient animals had significantly lower liver retinoid stores than controls despite being removed from the deficient diet at about 8 weeks of age and supplemented with vitamin A-rich foods. The seasonal trends with respect to the relative percentage of the different retinyl esters held true for the VAD group as well, although actual concentrations of all esters were lower than control. This suggests a diminished capacity to absorb and/or store ingested vitamin A.

In an attempt to determine whether vitamin A absorption and/or storage was affected by the early deficiency, we examined expression of LRAT protein, which is an enzyme essential to the proper absorption of vitamin A in the small intestine (58) as well as the storage of retinoids in the liver (2; 34). Vitamin A deficiency has been shown to reduce LRAT expression in the liver (2; 68). Surprisingly, LRAT expression was largely unchanged by both diet and season in the jejunum and liver of our study animals. We did not assay the activity of LRAT so it is possible that activity was diminished in the VAD group even though expression stayed relatively constant. Another possibility is that the retinoids supplied to the VAD group after they were taken off the diet were being stored primarily in tissues other than liver, such as adipose and lung, as an artifact of deficiency (2) or a result of decreased LRAT function (34; 47). Alternatively, retinal could have been delegated for immediate use in organs of importance such as the eyes to maintain vision thereby bypassing storage in the liver (2; 8). In our model, restoration of a diet high in vitamin A was vital to reestablishing weight gain and body mass in the previously-deficient squirrels, but it took approximately 7 weeks for these squirrels to mirror the weekly weight gain seen in control animals. In fact, these recovery animals consistently displayed lower body mass until spring. Some of the mass difference can be accounted for in the WAT depots as deficient summer squirrels had significantly smaller depot masses.

The role of vitamin A in the regulation of adipocyte proliferation and thus the accumulation of adipose tissue depends on signaling via its metabolite, RA (8; 47; 56). Availability of RA is directly correlated with the availability of retinoids (47). Biological

activity of RA is mediated via retinoid receptors. RXR and RAR are the two main RA receptor types (each existing in three isotypes - α, β, and γ) and act as transcription factors (8). In our model, expression of RAR-α transcript was largely uninfluenced by early vitamin A deficiency while RXR-β expression demonstrated a significant interaction between diet and season, suggesting that normal seasonal trends in expression were disrupted by the diet. This was particularly evident in the summer group where RXR-β expression was higher in VAD squirrels, presumably as a compensatory mechanism to increase sensitivity in times of low available RA. Studies have shown that RA signaling influences adipose accumulation with low concentrations of RA promoting accumulation and high concentrations inhibiting it (8; 9; 42; 56). Additionally, it has been shown that retinoid receptor expression is influenced by a deficient diet (65). We originally hypothesized that retinoid receptor expression would change based on diet and that deficient animals would have larger adipose depot accumulations based on these studies. However, given that neither RXR-β nor RAR-α expression was affected by the diet alone, other adipose accumulation pathways may have been disrupted accounting for the mass differences seen in our early deficiency model.

The masses of the three WAT depots examined showed similar trends in response to diet and season. Previous studies of the role of vitamin A in rodents have found that deficiency increases WAT mass (53) and supplementation with RA or dietary retinoids decreases WAT mass (44; 57). In our model, iaWAT, rWAT and oWAT mass steadily increased in control squirrels from summer to pre-hibernation to torpid hibernator before dropping off again in spring. This is not unexpected given the hyperphagia of the

summer and pre-hibernating seasons and the role that these depots play in providing energy for the hibernation season. Although diet alone did not have a significant effect, the seasonal trend was different between the two diet groups. The squirrels that experienced early vitamin A deficiency had an increase in WAT mass from summer to the pre-hibernation season, but decreased prior to tissue collection during hibernation. Not surprisingly, this decrease in WAT mass continued into the spring post-emergence. The difference in these trends is interesting given that all squirrels, deficient and control, were removed from the special diet and given the same vitamin A-rich diet starting around 8 weeks of age. It is possible that the dramatic change from a vitamin A deficient to a vitamin A rich diet led to high levels of RA signaling which would act to reduce adipose accumulation (8; 9; 42; 56). Torpid hibernators were euthanized approximately 2-3 months after the initiation of their first torpor bout so it is also possible that they depleted more of their WAT stores during that time compared to the controls. Thus, these effects of the diet on seasonal changes may reflect a change in overall WAT metabolism in the early deficient squirrels.

One other aspect of WAT metabolism that was affected by early vitamin A deficiency was adipokine expression. Resistin was significantly affected by season and diet in our model with VAD squirrels having significantly lower levels of resistin transcript in iaWAT. Given the role of resistin in promoting insulin resistance (22), it is not surprising that the highest levels of transcript expression were found in the pre-hibernating season for both diet groups, as hibernators tend to be insulin resistant during this time (23). Resistin expression is inhibited by RA via the RXR and RAR receptors so

higher levels are expected in deficient squirrels (22). However, our deficient squirrels exhibited lower levels of resistin (Figure 5C) in all seasons. We speculate this could be compensatory in attempt to increase adipose accumulation by reducing insulin resistance given that during the early deficiency VAD squirrels had diminished adipose depot size. RA signaling also reduces leptin gene expression in rats which promotes hyperphagia and obesity (36). Past studies in marmots (*Marmota flaviventris*)(24), woodchucks (*Marmota monax*)(19) and artic ground squirrels (*Urocitellus parryii*)(49) have demonstrated seasonal changes in leptin throughout the annual cycle and a probable role for leptin in increased food intake. Our results mirror the circulated levels of leptin published in other species and show an increase in leptin expression in iaWAT during the pre-hibernation period. Leptin transcript expression was unaffected by diet which is not surprising given that the high levels of leptin normally found in the pre-hibernation period suggest that RA signaling is not active or not able to suppress leptin expression during this time. It is more likely that RA signaling would work to inhibit leptin during the hibernation season, but at this point the deficiency in our model was significantly less severe as the animals had been removed from the diet.

Another adipose tissue that is subject to control by RA and important to hibernation is BAT. Of all the adipose depots measured in our study, BAT mass was most affected by early vitamin A deficiency. Although BAT mass increased after deficient squirrels were removed from the diet, mass in torpid VAD hibernators was significantly less than controls. BAT mass, like WAT mass, is affected by RA signaling (7; 42; 57). Additionally, RA signaling directly induces UCP1 gene expression in rodents

and rodent-derived cell lines raising thermogenic capacity (7; 43). Surprisingly, in our model we found higher levels of UCP1 transcript in the BAT of deficient squirrels compared to controls. This is similar to what has been shown with RA treatment of human cell lines (46). Thus, the increased UCP1 expression we observed in early deficient squirrels may be explained by signaling mechanisms other than RA that can induce transcription in BAT. Protein kinase A activation has also been shown to play a significant role in upregulating UCP1 in BAT in response to adrenergic signaling (20; 26). Interestingly, there is evidence in malignant cells that RA treatment interferes with PKA activity (48). Thus, it may be that in a hibernator's BAT, PKA signaling is the predominant force driving UCP expression in response to adrenergic signaling. The lack of RA signaling in our model could have modified the activity of the PKA pathway and promoted UCP1 expression. It is important to note, however, that the highest UCP1 levels we observed were in the pre-hibernating deficient squirrels, which were euthanized several weeks after being placed on a vitamin A rich diet. Our results could therefore reflect a compensatory mechanism to increase UCP1 expression prior to hibernation in animals, which now have restored levels of RA. The seasonal changes in UCP1 expression in our study differ slightly from published studies in hibernators, which show no difference in UCP1 mRNA between summer and hibernating arctic ground squirrels (3; 10) or increases in hibernation in 13-lined ground squirrels (29). In our model, UCP1 expression decreased between summer and hibernating seasons in both diet groups. We cannot adequately explain the differences in our UCP1 transcript expression results

compared with published studies in hibernators, but it could be due to the diet regimen or the young age of our study squirrels.

Results from our early vitamin A deficiency model in the thirteen lined ground squirrel suggest a strong dependence on vitamin A storage and RA signaling in hibernators. The rapid onset of severe deficiency and abnormally high retinoid levels in control squirrels point to the possibility that hibernating mammals require retinoid stores that would be considered hypervitamoic in other rodent species. Hibernating mammals display an annual cycle of rapid weight gain and loss associated primarily with increase and depletion of fat stores in adipose tissue. If proliferation and hypertrophy of adipocytes are essential pathways for fat storage in juvenile hibernators, having proper stores of retinoids would be essential to this process. Further studies are needed to examine the role of RA signaling in hibernation. Starting a deficient diet at weaning may allow ground squirrels to develop the deficiency more slowly and enter the hibernation season in a truly deficient condition. Additionally, a better understanding of wild ground squirrel diet may provide insight to the normal wild ingestion of vitamin A.

Chapter 3: Conclusion

Hibernators like the 13-lined ground squirrel (*Ictidomys tridecemlineatus*) rely on white adipose (WAT) and brown adipose (BAT) tissue as energy sources during their circannual torpor-arousal cycles. This makes the accumulation of adipose tissue prior to the hibernation season of vital importance for survival. Retinoic acid (RA), the biologically active form of vitamin A, plays key roles in adipose accumulation pathways by the induction of both hypertrophic and hyperplastic changes in adipose tissue (7; 43; 44; 53; 57). The direct action of RA is mediated mostly by nuclear retinoid receptors (RXR and RAR). Typically, low concentrations of RA upregulate the accumulation pathways allowing for an increase in adipose tissue and high concentrations downregulate these pathways (56). Most notably, RXR, RAR, and PPARγ heterodimer complexes rely specifically on RA signaling to facilitate this regulation. Additionally, RA signaling also serves to downregulate the expression of leptin and resistin both serving to decrease adipose mass (22; 36). Thus, high concentrations of RA would decrease both adipokines and low concentrations would increase expression. Similarly, UCP1 expression is directly influence by RA signaling as well (7; 43). RA serves to promote UCP1 expression, while an absence causes lower expression.

In our study we developed a model of early vitamin A deficiency in the 13-lined ground squirrel to determine if this would affect their ability to accumulate adequate stores of WAT and BAT prior to hibernation. Analysis of total liver retinoid content in deficient squirrels showed that their stores were basically undetectable. Interestingly,

after removal from the diet these early deficient squirrels showed consistently lower liver retinoid stores and never mirrored that of their control counterparts. This suggests that storage capabilities of the early deficient squirrels were impaired as an artifact of the deficient diet and subsequent restoration of vitamin A availability. Additionally, our control squirrels maintained liver retinoid stores of ~1.2 μmol/g which would normally be considered hypervitaminoic and toxic in other rodents (16). These levels were consistent with retinoid levels in the livers of wild-caught squirrels that were not part of the current diet study, suggesting that 13-lined ground squirrels store and rely more on vitamin A in comparison to other rodents. We speculate that this could be due to a greater reliance on RA signaling during their annual cycle of adipose accumulation and expenditure.

We examined the expression of genes related to the accumulation of adipose which included two retinoid receptors (RXR-β and RAR-α) and two adipokines (leptin and resistin) in WAT and UCP1 in BAT. Our results do not support our original hypothesis and contradict published studies in non-hibernators. These differences could possibly be explained by the early onset of severe deficiency we observed in our squirrels. Other rodents such as rats and mice maintain the same diet regimen for up to 6-9 months without severe symptoms (13; 63) while our squirrels were severely deficient by 8 weeks of age (~2 weeks after weaning). We hypothesize that the severe deficiency may have led to overcompensation in expression of retinoid receptors to increase sensitivity to RA. In addition, resistin expression is normally disrupted by RA signaling, but our deficient squirrels expressed less resistin compared to controls. Resistin is

associated with insulin resistance, which has been shown in the pre-hibernation season (23). The low resistin in early deficient squirrels suggests that they may not become as insulin resistant leading to less adipose accumulation. UCP1 expression was also higher despite the lack of RA signaling. We speculate that other pathways to upregulate UCP1, such as PKA, could have been activated in response to the diminished RA (20; 26 ;48). This may also indicate that PKA signaling is the dominant pathway mediating UCP1 expression in hibernators.

We propose that a later initiation of deficiency (not starting the squirrels on the deficient diet until weaning) in future studies will allow the squirrels to stay on the diet longer and enter the hibernation season with deficiency. This may lead to results that follow the studies in other rodents. Understanding the interaction between vitamin A and adipose could shed light on the role of RA in the annual torpor-arousal cycle of hibernators as well as the importance of diet composition prior to and after a hibernating season. Additionally, a better understanding of this interaction in a mammal that annually exhibits cycles of obesity and insulin resistance followed by reversal of these conditions could allow a better understanding of the intricate pathways involved in excessive adipose accumulation in humans.

Reference List

1. Ahmadian M, Suh JM, Hah N, Liddle C, Atkins AR, Downes M and Evans RM. PPARgamma signaling and metabolism: the good, the bad and the future. *Nat Med* 19: 557-566, 2013.

2. Amengual J, Golczak M, Palczewski K and von LJ. Lecithin:retinol acyltransferase is critical for cellular uptake of vitamin A from serum retinol-binding protein. *J Biol Chem* 287: 24216-24227, 2012.

3. Barger JL, Barnes BM and Boyer BB. Regulation of UCP1 and UCP3 in arctic ground squirrels and relation with mitochondrial proton leak. *J Appl Physiol (1985)* 101: 339-347, 2006.

4. Barnes BM. Freeze avoidance in a mammal: body temperatures below 0 degree C in an Arctic hibernator. *Science* 244: 1593-1595, 1989.

5. Batten ML, Imanishi Y, Maeda T, Tu DC, Moise AR, Bronson D, Possin D, Van Gelder RN, Baehr W and Palczewski K. Lecithin-retinol acyltransferase is essential for accumulation of all-trans-retinyl esters in the eye and in the liver. *J Biol Chem* 279: 10422-10432, 2004.

6. Bjorndal B, Burri L, Staalesen V, Skorve J and Berge RK. Different adipose depots: their role in the development of metabolic syndrome and mitochondrial response to hypolipidemic agents. *J Obes* 2011: 490650, 2011.

7. Bonet ML, Oliver J, Pico C, Felipe F, Ribot J, Cinti S and Palou A. Opposite effects of feeding a vitamin A-deficient diet and retinoic acid treatment on brown adipose tissue uncoupling protein 1 (UCP1), UCP2 and leptin expression. *J Endocrinol* 166: 511-517, 2000.

8. Bonet ML, Ribot J, Felipe F and Palou A. Vitamin A and the regulation of fat reserves. *Cell Mol Life Sci* 60: 1311-1321, 2003.

9. Bonet ML, Ribot J and Palou A. Lipid metabolism in mammalian tissues and its control by retinoic acid. *Biochim Biophys Acta* 1821: 177-189, 2012.

10. Boyer BB, Barnes BM, Lowell BB and Grujic D. Differential regulation of uncoupling protein gene homologues in multiple tissues of hibernating ground squirrels. *Am J Physiol* 275: R1232-R1238, 1998.

11. Cannon B and Nedergaard J. Brown adipose tissue: function and physiological significance. *Physiol Rev* 84: 277-359, 2004.

12. Carey HV, Andrews MT and Martin SL. Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature. *Physiol Rev* 83: 1153-1181, 2003.

13. Carter-Dawson L, Kuwabara T, O'Brien PJ and Bieri JG. Structural and biochemical changes in vitamin A--deficient rat retinas. *Invest Ophthalmol Vis Sci* 18: 437-446, 1979.

14. Chambers D, Wilson L, Maden M and Lumsden A. RALDH-independent generation of retinoic acid during vertebrate embryogenesis by CYP1B1. *Development* 134: 1369-1383, 2007.

15. Chambon P. A decade of molecular biology of retinoic acid receptors. *FASEB J* 10: 940-954, 1996.

16. Chen W and Chen G. The Roles of Vitamin A in the Regulation of Carbohydrate, Lipid, and Protein Metabolism. *J Clin Med* 3: 453-479, 2014.

17. Cinti S. The adipose organ at a glance. *Dis Model Mech* 5: 588-594, 2012.

18. Cleary EC and Craven SR. Thirteen-lined ground squirrels and their control. In: Prevention and Control of Wildlife Damage, edited by Hygnstrom SE,

Timm RM and Larson GE. Lincoln, NE: University of Nebraska Cooperative Extension, 1992, p. 165-170.

19. Concannon P, Levac K, Rawson R, Tennant B and Bensadoun A. Seasonal changes in serum leptin, food intake, and body weight in photoentrained woodchucks. *Am J Physiol Regul Integr Comp Physiol* 281: R951-R959, 2001.

20. Dickson LM, Gandhi S, Layden BT, Cohen RN and Wicksteed B. Protein kinase A induces UCP1 expression in specific adipose depots to increase energy expenditure and improve metabolic health. *Am J Physiol Regul Integr Comp Physiol* ajpregu, 2016.

21. Fedorov VB, Goropashnaya AV, Toien O, Stewart NC, Gracey AY, Chang C, Qin S, Pertea G, Quackenbush J, Showe LC, Showe MK, Boyer BB and Barnes BM. Elevated expression of protein biosynthesis genes in liver and muscle of hibernating black bears (Ursus americanus). *Physiol Genomics* 37: 108-118, 2009.

22. Felipe F, Bonet ML, Ribot J and Palou A. Modulation of resistin expression by retinoic acid and vitamin A status. *Diabetes* 53: 882-889, 2004.

23. Florant GL, Lawrence AK, Williams K and Bauman WA. Seasonal changes in pancreatic B-cell function in euthermic yellow-bellied marmots. *Am J Physiol* 249: R159-R165, 1985.

24. Florant GL, Porst H, Peiffer A, Hudachek SF, Pittman C, Summers SA, Rajala MW and Scherer PE. Fat-cell mass, serum leptin and adiponectin changes during weight gain and loss in yellow-bellied marmots (Marmota flaviventris). *J Comp Physiol B* 174: 633-639, 2004.

25. Frank CL. The influence of dietary fatty acids on hibernation by golden-mantled ground squirrels (*Spermophilus lateralis*). *Phys Zool* 65: 906-920, 1992.

26. Fredriksson JM, Thonberg H, Ohlson KB, Ohba K, Cannon B and Nedergaard J. Analysis of inhibition by H89 of UCP1 gene expression and thermogenesis indicates protein kinase A mediation of beta(3)-adrenergic signalling rather than beta(3)-adrenoceptor antagonism by H89. *Biochim Biophys Acta* 1538: 206-217, 2001.

27. GOODMAN DW, HUANG HS and SHIRATORI T. Tissue distribution and metabolism of newly absorbed vitamin A in the rat. *J Lipid Res* 6: 390-396, 1965.

28. Hammerschmidt SI, Friedrichsen M, Boelter J, Lyszkiewicz M, Kremmer E, Pabst O and Forster R. Retinoic acid induces homing of protective T and B cells to the gut after subcutaneous immunization in mice. *J Clin Invest* 121: 3051-3061, 2011.

29. Hampton M, Melvin RG and Andrews MT. Transcriptomic analysis of brown adipose tissue across the physiological extremes of natural hibernation. *PLoS One* 8: e85157, 2013.

30. Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C and Song SY. Retinoic acid imprints gut-homing specificity on T cells. *Immunity* 21: 527-538, 2004.

31. Kastner P, Mark M and Chambon P. Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? *Cell* 83: 859-869, 1995.

32. Kiefer FW, Vernochet C, O'Brien P, Spoerl S, Brown JD, Nallamshetty S, Zeyda M, Stulnig TM, Cohen DE, Kahn CR and Plutzky J. Retinaldehyde dehydrogenase 1 regulates a thermogenic program in white adipose tissue. *Nat Med* 18: 918-925, 2012.

33. Kim KH, Lee K, Moon YS and Sul HS. A cysteine-rich adipose tissue-specific secretory factor inhibits adipocyte differentiation. *J Biol Chem* 276: 11252-11256, 2001.

34. Kim YK, Wassef L, Hamberger L, Piantedosi R, Palczewski K, Blaner WS and Quadro L. Retinyl ester formation by lecithin: retinol acyltransferase is a key regulator of retinoid homeostasis in mouse embryogenesis. *J Biol Chem* 283: 5611-5621, 2008.

35. Koppaka V, Thompson DC, Chen Y, Ellermann M, Nicolaou KC, Juvonen RO, Petersen D, Deitrich RA, Hurley TD and Vasiliou V. Aldehyde dehydrogenase inhibitors: a comprehensive review of the pharmacology, mechanism of action, substrate specificity, and clinical application. *Pharmacol Rev* 64: 520-539, 2012.

36. Kumar MV, Sunvold GD and Scarpace PJ. Dietary vitamin A supplementation in rats: suppression of leptin and induction of UCP1 mRNA. *J Lipid Res* 40: 824-829, 1999.

37. Kurtz CC and Carey HV. Seasonal changes in the intestinal immune system of hibernating ground squirrels. *Dev Comp Immunol* 31: 415-428, 2007.

38. Lord GM, Matarese G, Howard JK, Baker RJ, Bloom SR and Lechler RI. Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. *Nature* 394: 897-901, 1998.

39. Martin SL. Mammalian hibernation: a naturally reversible model for insulin resistance in man? *Diab Vasc Dis Res* 5: 76-81, 2008.

40. McArthur MD and Milsom WK. Changes in ventilation and respiratory sensitivity associated with hibernation in Columbian (Spermophilus columbianus) and golden-mantled (Spermophilus lateralis) ground squirrels. *Physiol Zool* 64: 940-959, 1991.

41. McCain S, Souza M, amsay E, Schumacher J, Hecht S and Thomas W. Diagnosis and surgical treatment of a Chiari I-like malformation in an African lion (Panthera leo). *J Zoo Wildl Med* 39: 421-427, 2008.

42. Mercader J, Madsen L, Felipe F, Palou A, Kristiansen K and Bonet ML. All-trans retinoic acid increases oxidative metabolism in mature adipocytes. *Cell Physiol Biochem* 20: 1061-1072, 2007.

43. Mercader J, Palou A and Bonet ML. Induction of uncoupling protein-1 in mouse embryonic fibroblast-derived adipocytes by retinoic acid. *Obesity (Silver Spring)* 18: 655-662, 2010.

44. Mercader J, Ribot J, Murano I, Felipe F, Cinti S, Bonet ML and Palou A. Remodeling of white adipose tissue after retinoic acid administration in mice. *Endocrinology* 147: 5325-5332, 2006.

45. Morrison CD. Leptin resistance and the response to positive energy balance. *Physiol Behav* 94: 660-663, 2008.

46. Murholm M, Isidor MS, Basse AL, Winther S, Sorensen C, Skovgaard-Petersen J, Nielsen MM, Hansen AS, Quistorff B and Hansen JB. Retinoic acid has different effects on UCP1 expression in mouse and human adipocytes. *BMC Cell Biol* 14: 41, 2013.

47. O'Byrne SM and Blaner WS. Retinol and retinyl esters: biochemistry and physiology. *J Lipid Res* 54: 1731-1743, 2013.

48. Ombra MN, Di SA, Abbondanza C, Migliaccio A, Avvedimento EV and Perillo B. Retinoic acid impairs estrogen signaling in breast cancer cells by interfering with activation of LSD1 via PKA. *Biochim Biophys Acta* 1829: 480-486, 2013.

49. Ormseth OA, Nicolson M, Pelleymounter MA and Boyer BB. Leptin inhibits prehibernation hyperphagia and reduces body weight in arctic ground squirrels. *Am J Physiol* 271: R1775-R1779, 1996.

50. Patel L, Buckels AC, Kinghorn IJ, Murdock PR, Holbrook JD, Plumpton C, Macphee CH and Smith SA. Resistin is expressed in human macrophages and directly regulated by PPAR gamma activators. *Biochem Biophys Res Commun* 300: 472-476, 2003.

51. Pond CM. Adipose tissue and the immune system. *Prostaglandins Leukot Essent Fatty Acids* 73: 17-30, 2005.

52. Prendergast BJ, Freeman DA, Zucker I and Nelson RJ. Periodic arousal from hibernation is necessary for initiation of immune responses in ground squirrels. *Am J Physiol Regul Integr Comp Physiol* 282: R1054-R1062, 2002.

53. Ribot J, Felipe F, Bonet ML and Palou A. Changes of adiposity in response to vitamin A status correlate with changes of PPAR gamma 2 expression. *Obes Res* 9: 500-509, 2001.

54. Rowland AF, Fazakerley DJ and James DE. Mapping insulin/GLUT4 circuitry. *Traffic* 12: 672-681, 2011.

55. Ruf T and Arnold W. Effects of polyunsaturated fatty acids on hibernation and torpor: a review and hypothesis. *Am J Physiol Regul Integr Comp Physiol* 294: R1044-R1052, 2008.

56. Safonova I, Darimont C, Amri EZ, Grimaldi P, Ailhaud G, Reichert U and Shroot B. Retinoids are positive effectors of adipose cell differentiation. *Mol Cell Endocrinol* 104: 201-211, 1994.

57. Sanchez J, Fuster A, Oliver P, Palou A and Pico C. Effects of beta-carotene supplementation on adipose tissue thermogenic capacity in ferrets (Mustela putorius furo). *Br J Nutr* 102: 1686-1694, 2009.

58. Shirakami Y, Lee SA, Clugston RD and Blaner WS. Hepatic metabolism of retinoids and disease associations. *Biochim Biophys Acta* 1821: 124-136, 2012.

59. Shojima N, Sakoda H, Ogihara T, Fujishiro M, Katagiri H, Anai M, Onishi Y, Ono H, Inukai K, Abe M, Fukushima Y, Kikuchi M, Oka Y and Asano T. Humoral regulation of resistin expression in 3T3-L1 and mouse adipose cells. *Diabetes* 51: 1737-1744, 2002.

60. Singh VN, Singh M and Venkitasubramanian TA. Early effects of feeding excess vitamin A: mechanism of fatty liver production in rats. *J Lipid Res* 10: 395-401, 1969.

61. Steppan CM and Lazar MA. Resistin and obesity-associated insulin resistance. *Trends Endocrinol Metab* 13: 18-23, 2002.

62. Tanumihardjo SA, Furr HC, Amedee-Manesme O and Olson JA. Retinyl ester (vitamin A ester) and carotenoid composition in human liver. *Int J Vitam Nutr Res* 60: 307-313, 1990.

63. Tateya T, Tateya I, Surles RL, Tanumihardjo S and Bless DM. Roles of vitamin A and macula flava in maintaining vocal folds. *Ann Otol Rhinol Laryngol* 117: 65-73, 2008.

64. Tontonoz P and Spiegelman BM. Fat and beyond: the diverse biology of PPARgamma. *Annu Rev Biochem* 77: 289-312, 2008.

65. Verma AK, Shoemaker A, Simsiman R, Denning M and Zachman RD. Expression of retinoic acid nuclear receptors and tissue transglutaminase is altered in various tissues of rats fed a vitamin A-deficient diet. *J Nutr* 122: 2144-2152, 1992.

66. Yang Q, Graham TE, Mody N, Preitner F, Peroni OD, Zabolotny JM, Kotani K, Quadro L and Kahn BB. Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. *Nature* 436: 356-362, 2005.

67. Ziouzenkova O, Orasanu G, Sharlach M, Akiyama TE, Berger JP, Viereck J, Hamilton JA, Tang G, Dolnikowski GG, Vogel S, Duester G and Plutzky J. Retinaldehyde represses adipogenesis and diet-induced obesity. *Nat Med* 13: 695-702, 2007.

68. Zolfaghari R and Ross AC. Lecithin:retinol acyltransferase expression is regulated by dietary vitamin A and exogenous retinoic acid in the lung of adult rats. *J Nutr* 132: 1160-1164, 2002.