Comparative Medicine
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Vol 68, No 3
June 2018
Pages 196–203

Original Research

Developing a Model of Vitamin A Deficiency in a Hibernating Mammal, the 13-Lined Ground Squirrel (*Ictidomys tridecemlineatus*)

Ryan J Sprenger,[1] Sherry A Tanumihardjo,[2] and Courtney C Kurtz[1,*]

Retinoic acid, a bioactive metabolite of vitamin A, plays key roles in immune function and vision and adipose tissue development. Our goal was to study the effect of vitamin A deficiency in physiologic changes seen in hibernating 13-lined ground squirrels (*Ictidomys tridecemlineatus*). In this study, we first developed a model of vitamin A deficiency that was based on published mouse models; we then examined the role of RA in the circannual cycle of and adipose accumulation in this hibernating species. Gravid female ground squirrels began consuming a deficient diet during the last 2 wk of their 4-wk gestation; pups received the diet until they were 8 wk old, when severe symptoms of hypovitaminosis were observed, requiring the animals' removal from the protocol. Body size and adipose mass were significantly lower in vitamin-deficient pups than controls. To avoid these complications, we developed a second model, in which pups started on the deficient diet after weaning. The revised model produced few symptoms of deficiency, and squirrels were able to remain on the diet through spring emergence. Liver retinol analysis showed that deficient squirrels essentially had no vitamin A stores. Our data suggest that 13-lined ground squirrels maintain higher concentrations of stored retinol than other rodent species, such that their dietary needs may differ from those of traditional laboratory rodent models. Our results indicate that ground squirrels are especially susceptible to vitamin A deficiency, and ground squirrels should not be fed a deficient diet until after weaning, to avoid severe symptoms. Interestingly, vitamin A deficiency does not seem to affect this species' ability to hibernate successfully.

Abbreviations: BAT, brown adipose tissue; RA, retinoic acid; VAC, vitamin A control diet; VAD, vitamin A deficient diet; WAT, white adipose tissue

DOI: 10.30802/AALAS-CM-17-000113

Hibernating mammals have become a valuable laboratory model for a number of physiologic, behavioral, and biomedical studies. These animals have the remarkable ability to survive long periods of reduced resource availability by entering hibernation. The majority of the hibernation season is spent in torpor, a state associated with decreased body temperature and metabolic rate, fasting, and inactivity. To survive hibernation, these animals display a suite of significant physiologic, behavioral, and morphologic changes. These changes make them unique and valuable as models for various aspects of human physiology and disease, including eye function,[32,38] immunology,[2,5,6] obesity,[18,27] and the role of the microbiome.[9,13,41,42] Exploration of these physiologic systems often necessitates keeping hibernators in captivity and manipulating their physiology through dietary changes or pharmacologic treatments, but these regimens can be problematic. Hibernators often do not respond to treatments in the same way as do inbred laboratory mice and rats, and little is known about the nutritional needs and dietary components of wild hibernating mammals. Our laboratory studies the physiology of hibernating 13-lined ground squirrels (*Ictidomys tridecemlineatus*), which we want to use to examine the role of retinoic acid (RA), an active metabolite of vitamin A, in various changes that are characteristic of hibernation.

RA signaling plays an important role in several organ systems, and its effects mirror many of the changes in preparation for hibernation. RA signaling affects the growth of white adipose tissue (WAT)[4,29,31,37] and development of brown adipose tissue (BAT),[3,30,37,47] both of which are essential to successful hibernation.[7,15,19] In addition, RA promotes recruitment of T cells to the small intestine,[17,21,22,35] a phenomenon observed in hibernating mammals.[14,26] WAT is an essential energy source for hibernators that do not eat throughout the cold months, and BAT provides thermogenesis for periodic arousals that are necessary to survive hibernation.[7,25] A large gut immune-cell population may provide an effective defense against microbes at a time when the epithelial barrier is 'leaky.'[8] Given the importance of adequate adipose stores in hibernation, we sought to determine whether decreased RA signaling affected the ability of ground squirrels to accumulate adipose mass prior to hibernation and thus altered the ability of the animals to hibernate.

Studies examining the effects of RA signaling in animals suggest that inducing vitamin A deficiency is the most efficient way to halt RA signaling. Therefore, using information from a literature review of the most common vitamin A deficiency models in rats and mice,[10,22,33,44] we designed a model of vitamin A deficiency where the diet was introduced to pregnant 13-lined

Received: 21 Nov 2017. Revision requested: 08 Dec 2017. Accepted: 22 Jan 2018.
[1]Department of Biology, University of Wisconsin–Oshkosh, Oshkosh, Wisconsin, and [2]Department of Nutritional Sciences, University of Wisconsin–Madison, Wisconsin
[*]Corresponding author. Email: kurtzc@uwosh.edu

ground squirrels approximately halfway through gestation. This resulted in a rapid and severe onset of deficiency after weaning; this profound deficiency occurred much earlier than in rats and mice. In the following breeding season, we modified our protocol to begin the deficient diet after weaning, allowing us to successfully maintain the deficiency throughout hibernation (8 mo, approximately September through April) and into spring emergence. Here we describe both protocols that we used to induce vitamin A deficiency in 13-lined ground squirrels. We hope that this information will help to refine future diet-based studies in this species and improve our understanding of the dietary needs of hibernating species throughout the circannual cycle.

Materials and Methods

Animals. Trapping permits were obtained from the Wisconsin Department of Natural Resources. Gravid female 13-lined ground squirrels (*Ictidomys tridecemlineatus*) were wild-caught (age and mass unknown) from various locations around northeastern Wisconsin in mid-May after breeding occurs (late April to early May) and transferred to the animal facility at University of Wisconsin–Oshkosh. On arrival, squirrels received IACUC-mandated treatment with ivermectin (0.4 mg/kg SC; to remove endoparasites) and flea spray (to remove ectoparasites). Squirrels were housed individually, at approximately 20 °C, with unlimited access to water, and on a lighting cycle (updated weekly) that roughly corresponded to the natural photoperiod.[46] Gravid squirrels were housed in cages (0.21 m^2; 38 cm × 55 cm × 15 cm), which provided adequate space for mother and pups. Bedding comprised hardwood shavings (SaniChips, PJ Murphy, Montville, NJ), and paper nesting material (Enviro-Dri, Shepherd Specialty Papers, Watertown, TN) and PVC pipe were provided for enrichment.

After weaning at approximately 1 mo of age (approximately 1 July), pups were group-housed for an additional 2 to 3 wk in the same cages before being allocated into single housing in standard rat cages (0.12 m^2; 48 cm × 25.4 cm × 20 cm). From 4 through 5 wk of age and until entrance into hibernation, all active pups were weighed weekly. All procedures involving animals were IACUC-approved (protocol nos. 0026-000276 and 0026-000283).

Diet details. Custom diets were formulated to meet known nutrient needs of the squirrels and to ensure a vitamin A–free diet. The vitamin A–deficient (VAD) diet contained vitamin-free casein. The vitamin A control (VAC) diet contained the same nutrients and casein but had retinyl palmitate (500,000 IU/g) added to it (0.04 g retinyl palmitate per kilogram of diet), which provided squirrels 64 to 86 µg retinol daily according to a feed intake of 75 to 100 g weekly. Because we deemed this intake to be excessive, the revised chow contained 0.016 g retinyl palmitate per kilogram of diet. For other laboratory rodents, the recommended amount of vitamin A is 4000 IU/kg,[36] resulting in 13 to 17 µg retinol daily, similar to that in regimen 2. Except for retinyl palmitate, macronutrient composition was identical among diets (protein, 24.6%; carbohydrate, 47.1%; fat, 12.0%, by weight; Teklad Specialty Diets/Envigo, Madison, WI). Squirrels did not receive any food items other than the formulated diets.

Diet regimens. For regimen 1 (that is, early-onset deficiency; Figure 1 A), 6 gravid squirrels were randomly assigned at 1 wk after capture (that is, approximately halfway through their 28-d gestational period) to either a VAC or VAD diet. Once litters were born, dams remained on their assigned diets until the pups were weaned, when the dams were removed from the study. At 2 wk after weaning (after the onset of severe symptoms of deficiency), pups were put on a diet of dog food (ProHealth chunks, Iams, Lewisburg, OH) supplemented with sunflower seeds (approximately 15 g, twice weekly), dried vegetables (approximately 15 g weekly), and peanuts (one peanut, once weekly). After removal from the deficient diet, VAD pups were treated with retinyl palmitate (6 mg PO) 3 times over 1 wk.

For regimen 2 (that is, late-onset deficiency; Figure 1 B), pups from 7 litters were assigned to either the VAD or VAC diet so that some from each litter were on each diet and a relatively even distribution of male and female pups was achieved. This allocation occurred approximately 1 wk after weaning, and the pups continued receiving the assigned diet throughout the study. Food was removed during hibernation.

Initiation of hibernation. All squirrels were challenged to induce torpor between 4 September and 15 October. Regimen 1 squirrels were challenged approximately 9 wk after removal from the diets, and regimen 2 animals were challenged 9 to 15 wk after starting on the diets. Feed and water were removed, and the squirrels were placed in a 3 to 7 °C hibernaculum (that is, constant darkness) for 7 d. Squirrels that entered torpor for at least 2 consecutive days remained in the hibernaculum. Hibernating squirrels were housed in plastic containers (48 in^2; 8 in. × 6 in. × 5.5 in.; Handi-Box, Tupperware, Roanoke, VA) lined with Enviro-dri (Shepherd Specialty Papers), and Tek-Fresh (Harlan Teklad/Envigo), as previously described.[46] Squirrels that did not enter torpor within 7 d were removed to normal housing with food and water and challenged again a week later. After a third challenge, all but 2 squirrels (one VAD and one VAC, both from regimen 1 and removed from study) had entered hibernation. Hibernating squirrels were checked daily under red light (maximum, 10 min) and monitored for interbout arousals by using bedding chips sprinkled on their backs as an indicator of movement or activity.

Tissue collection. Tissues were collected during summer, hibernation (hibernating for 7 to 13 wk and torpid for at least 2 consecutive days at the time of collection), and spring (1 wk after emergence, regimen 2 only). Squirrels were randomly assigned to tissue collection cohorts (for example, summer, hibernating), and all squirrels on each diet eventually were used for tissue collection. Squirrels were euthanized by rapid decapitation, and body mass, shoulder-to-hip length, and body temperature were measured. Three WAT depots (intraabdominal–gonadal, retroperitoneal, and omental) and the interscapular BAT depot were removed and weighed. The adiposity index was obtained by dividing the intraabdominal–gonadal WAT mass by total body mass. The liver was removed and flash-frozen in liquid nitrogen. All tissues were stored at –80 °C until analysis.

Quantification of total vitamin A. Total vitamin A (retinol plus all identifiable retinyl esters, hereafter referred to as total retinol) concentrations were determined by using HPLC. For each replicate, 1 g of liver was ground in sodium sulfate and internally standardized by using retinyl butyrate to determine extraction efficiency. Samples were extracted with dichloromethane and filtered into volumetric flasks. An aliquot was evaporated to dryness under nitrogen and resolubilized with 75% methanol: 25% dichloroethane before being loaded into a photodiode array (HPLC system with a Resolve C18 column [90 Å, 5 µm], Waters, Milford, MA) for total retinol analysis. The minimal detectable limit of this equipment is 1 pmol.

Statistics. Multifactor ANOVA was used to simultaneously analyze the effects of diet and season as well as the interaction between diet and season. When differences were significant ($P \leq 0.05$), Tukey pairwise comparison was used. Before ANOVA, a regression analysis was performed on the data, and the residuals were examined for normal distribution. When values were not normally distributed, the data were log-transformed

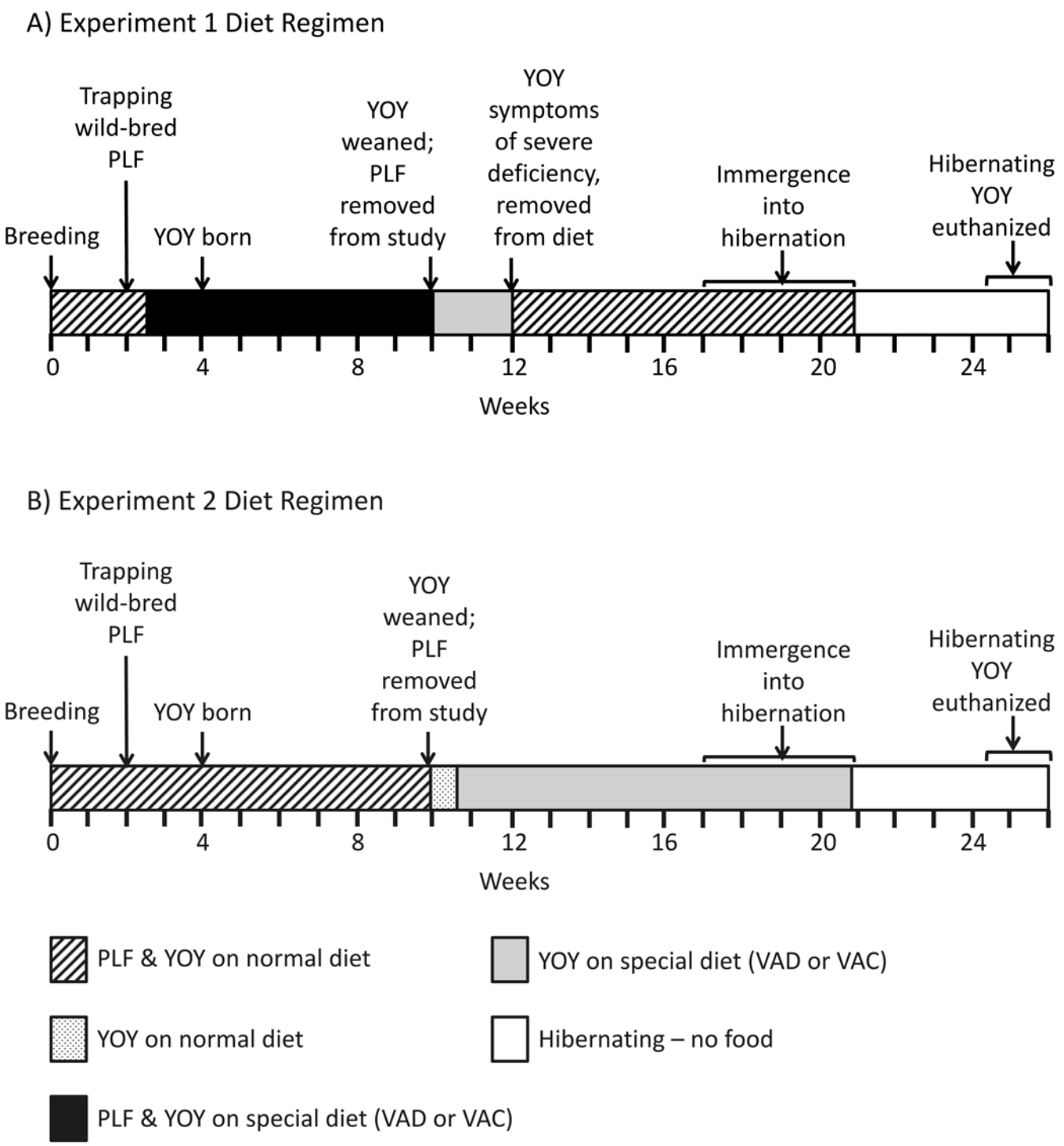

Figure 1. Timeline for diet regimens (A) 1 and (B) 2. Note that animals on regimen 2 continued receiving the vitamin-deficient diet throughout the study, whereas all squirrels on regimen 1 were removed from VAD or VAC diets by week 12 due to the onset of severe deficiency symptoms. PLF, pregnant or lactating females; YOY, young of year.

(total mass of intraabdominal–gonadal and retroperitoneal WAT depots). Analysis of body mass was performed using Student *t* tests at each time point with a Bonferroni correction. Statistical analyses were performed by using Minitab (version 16, Minitab, State College, PA).

Results

Effect of timing of vitamin A restriction on the health of juvenile 13-lined ground squirrels. For regimen 1, gravid dams began receiving the deficient diet (VAD1) or control diet (VAC1) 1 to 2 wk before litters were born. At 5 wk of age (prior to weaning), body mass of VAD1 pups diverged significantly ($P < 0.05$) from their control counterparts' and showed a slower rate of gain (Figure 2 A). In the 2 wk prior to summer tissue collection (that is, during VAD feeding), VAD1 squirrels had shaky movements and tremors that suggested a lack of hindlimb coordination. Behavior (that is, response to noise but not movement around the cage) indicated possible blindness and corresponded to the

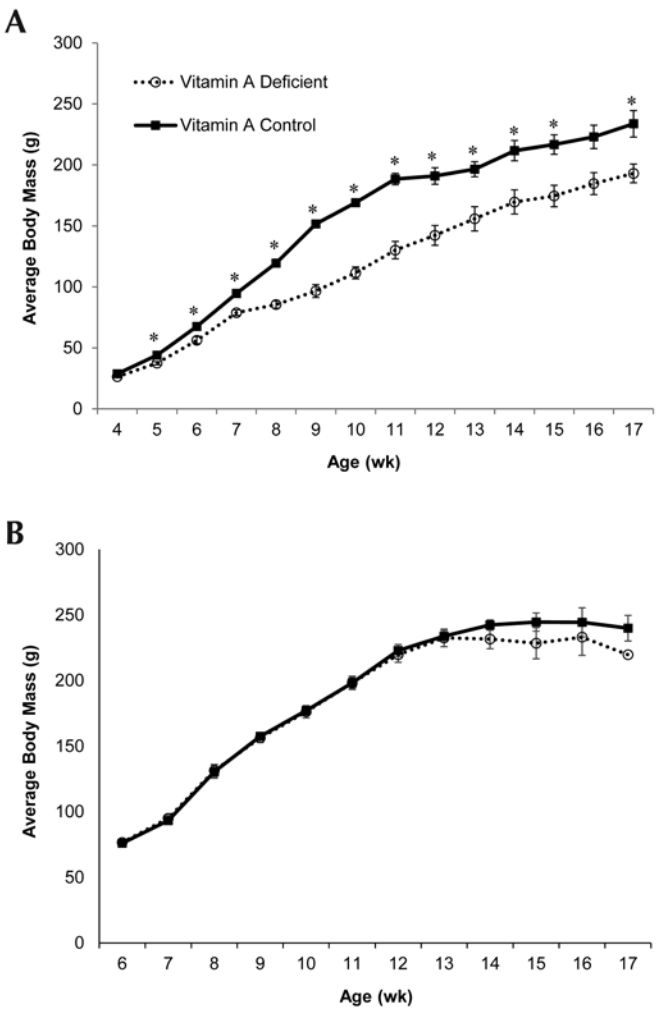

Figure 2. Body mass of juvenile ground squirrels on regimens (A) 1 and (B) 2. (A) Removal from VAD diet occurred between 7 and 8 wk of age for year 1 only. Data are given as mean ± SE. Sample sizes: (A) Prior to 8 wk of age, n = 16 to 29 per treatment; at 8 wk of age and older, n = 11 to 16 per treatment; (B) Prior to 10 wk of age, n = 17 to 24; 10 wk of age and older, n = 7 to 18. Differences in sample sizes are due to the timing of tissue collection and immergence into hibernation. *, $P < 0.05$ (t test with Bonferroni correction).

appearance of a milky discharge from the eyes (Figure 3). None of these symptoms occurred in control animals. Vocalizations in VAD1 animals were unusually hoarse and weak compared with normal squirrel chirping, an effect of deficiency noted in other animals.[28,43,44] Daily feeding logs suggested that the VAD1 pups may have consumed less chow than VAC1 pups, but this estimate was based on the food remaining in the hopper above the animals and did not account for any food on the floor of the cage (for VAD squirrels with attenuated motor function) or food cached in the nest (common in this species). In addition, gastrointestinal contents in all animals during tissue collection suggested food ingestion.

At the summer tissue collection, VAD1 pups were significantly smaller in length and mass and had lower body temperature than controls (Table 1). The summer tissue collection was completed over 4 d and within 2 wk of the onset of deficiency symptoms, after which all remaining VAD1 and VAC1 pups were removed from the study diets and returned to a normal diet (that is, by 8 to 9 wk of age). However, despite early vitamin A deficiency, the pups that were removed from the study diets were able to hibernate successfully. Body temperature during

Figure 3. Periocular secretions in animals that received VAD diet during regimen 1. These squirrels also displayed characteristics of vision impairment, including response to sound but not movement.

hibernation did not differ significantly between VAD1 and VAC1, although this parameter was measured only once during each season and during torpor and therefore does not reflect potential differences in temperature during interbout arousals. Analysis of torpor records for these animals revealed no difference in the number of arousals (mean ± SE) during the first month of hibernation (VAD1, 7.5 ± 0.5 arousals; VAC1, 7.6 ± 0.6)

Initially we expected that regimen 1 squirrels would remain on the diet until hibernation because traditional laboratory rodents have been maintained on VAD diets for at least this long,[10,22] but the severe symptoms in our squirrel pups necessitated their removal from the diet. Therefore, to avoid these complications, we designed regimen 2 to use during the following active season. Body mass did not differ significantly between VAD2 and VAC2 pups for the duration of the study. In addition, length, mass, and body temperature were similar between VAD2 and VAC2 squirrels during the summer (Table 1). Mild hypovitaminosis symptoms (that is, dry vocalizations and mild to moderate ocular discharge) were noticed at 7 to 8 wk after pups began the VAD diet. Squirrels were challenged to induce hibernation within 1 wk of symptom onset. All regimen 2 animals hibernated successfully, and the number of arousals during the first month of hibernation did not differ significantly between groups (VAD2, 4.3 ± 0.5 arousals; VAC2, 4.3 ± 0.6 arousals).

Total liver retinol levels. Analysis of liver tissue from summer VAD squirrels on either diet regimen showed no detectable retinol (Figure 4). For VAD2 this trend continued throughout the study (Figure 4 B). However, VAD1 squirrels' liver retinol did increase after removal from the diet, although they continued to have significantly lower retinol stores compared with VAC1 (Figure 4 A). Liver mass was recorded for diet regimen 2 and no significant differences in total liver mass were found between VAC2 and VAD2 squirrels in any season (data not shown). Because VAC liver retinol levels were high compared with laboratory rodents, we assayed summer and hibernating adult squirrel livers collected during a separate study that did not manipulate diet. We found that retinol stores (summer, 1.14 ± 0.22 µmol/g [n = 7]; hibernating, 0.99 ± 0.20 µmol/g [n = 7]) were similar to those in the older (that is, hibernating) VAC squirrels (Figure 4).

Effect of vitamin A deficiency on the mass of adipose depots. As expected, season had a significant effect on adipose mass, particularly for intraabdominal–gonadal WAT, omental WAT, and BAT (Table 2). The specific differences between VAD and VAC squirrels depended on the regimen used. For regimen 1, intraabdominal–gonadal WAT mass was significantly ($P < 0.05$) lower in VAD1 than VAC1 animals during summer but did not

Table 1. Body size and temperature at tissue collection

	Mass (g)	Shoulder-to-hip length (cm)	Body temperature (°C)
Regimen 1			
Summer			
VAC1 (n = 8)	118.1 ± 7.4[b]	10.3 ± 0.2[a]	38.6 ± 0.3[a]
VAD1 (n = 8)	80.6 ± 3.3[c]	9.1 ± 0.2[b]	37.5 ± 0.2[b]
Hibernation			
VAC1 (n = 5)	173.5 ± 9.2[a]	10.4 ± 0.4[a]	7.8 ± 0.3
VAD1 (n = 7)	139.7 ± 9.6[b]	9.3 ± 0.4[a,b]	7.6 ± 0.2
Regimen 2			
Summer			
VAC2 (n = 8)	189.4 ± 4.2[a]	11.6 ± 0.3[a]	38.7 ± 0.3
VAD2 (n = 8)	185.4 ± 3.8[a]	10.8 ± 0.3[a]	39.0 ± 0.3
Hibernation			
VAC2 (n = 7)	192.2 ± 7.1[a]	10.4 ± 0.8[b]	7.5 ± 0.7
VAD2 (n = 8)	185.6 ± 5.8[a]	10.1 ± 0.2[b]	6.7 ± 0.1
Spring			
VAC2 (n = 7)	168.0 ± 5.3[b]	10.8 ± 0.3[a,b]	37.7 ± 0.4
VAD2 (n = 7)	158.2 ± 3.2[b]	10.7 ± 0.3[a,b]	36.2 ± 1.8

Data are presented as mean ± SE. Within each regimen and parameter, values indicated by different superscript letters are significantly different ($P < 0.05$; Tukey posthoc testing with general linear model ANOVA).

differ during hibernation. However, for both regimens, there was a trend for lower intraabdominal–gonadal WAT mass in VAD hibernators (Table 2; $0.05 < P \leq 0.1$). Mass of omental WAT increased significantly ($P < 0.05$) from summer to hibernation in VAD1, VAC1, and VAC2 squirrels but not in VAD2 squirrels (Table 2). Despite early removal from the deficient diet and at least 4 wk of fattening on a normal diet before hibernation (regimen 1), hibernating VAD1 squirrels still had less ($P < 0.05$) retroperitoneal WAT than hibernating VAC1 controls (Table 2). BAT mass increased in hibernators (both regimens) and remained high throughout the spring (regimen 2), but the increase was smaller ($P < 0.05$) in VAD1 than VAC1 squirrels.

Discussion

In this study, we found that 13-lined ground squirrels are especially sensitive to vitamin A deficiency. This study was the first that attempted to produce vitamin A deficiency in a hibernating animal, and our data confirm that vitamin A is essential for early development in these animals. Interestingly, our data suggest that this species maintains higher concentrations of stored retinol than other rodent species, suggesting the dietary needs of hibernating species may be different from traditional laboratory rodent models. Furthermore, early deficiency of vitamin A impeded the accumulation of fat prior to hibernation but not the ability to hibernate, regardless of the deficiency model.

In mammals, vitamin A (retinol) must be obtained from the diet, because it cannot be synthesized in the body.[34] Vitamin A is fat-soluble and found in foods such as carrots, leafy vegetables, and eggs. In the bloodstream, retinol is found bound to RBP4, the main transport protein responsible for moving retinol to target storage tissues, including liver, lungs, intestine, adipose, and eyes.[48] However, most vitamin A in mammals is stored in the liver in the form of retinyl esters. Lecithin–retinol acyltransferase is largely responsible for the esterification of retinol, which is required for its storage.[1] Even when consumed, vitamin A is not biologically accessible and must be converted into RA and other forms through the activity of aldehyde and retinaldehyde dehydrogenases. Intracellular and extracellular RA receptors selectively bind and mediate the activity of the various forms of RA (such as all-*trans*, 9-*cis*-, and 13-*cis*- retinoic acid).[34] The vitamin A utilization rate in ground squirrels is unknown. In Sprague–Dawley rats, the utilization rate varies greatly (19.9 to 41.0 nmol daily), depending on several factors, such as plasma retinol and nutritional status of iron.[23,24]

In regimen 1, we induced vitamin A deficiency in ground squirrels by starting gravid dams on a VAD diet approximately 1-2 wk before the pups were born. This protocol is commonly used in other rodents, including mice and rats,[10,22,33,44] and some rat studies have maintained offspring on a deficient diet for as long as 10 mo before the onset of severe deficiency.[10] Symptom onset in ground squirrels was more rapid (that is, within 2 wk), such that squirrels had to be removed from the diet and treated with retinyl palmitate to recover, thus presenting a limitation to the full assessment of developmental effects of the deficient diet in regimen 1 animals. The symptoms were characteristic of vitamin A deficiency and included hind limb weakness,[28] dry vocalizations[28,44] and periocular inflammation.[10] Regarding the species' dietary sources of vitamin A, observational studies of 13-lined ground squirrels reveal that they eat a combination of animal (for example, eggs, fledgling birds, insects) and plant matter (for example, grasses, seeds, vegetables, roots).[12] The green, leafy, and vegetable portions of their diet, as well as eggs and meat, likely provide ground squirrels with considerable amounts of vitamin A and suggest that wild squirrels may enter the hibernation season with high stores, a possibility that needs to be examined in more detail.

To avoid the rapid deterioration of health due to early dietary vitamin A deficiency, regimen 2 pups were weaned prior to being assigned to the VAD or VAC diet (at approximately10 wk of age). This modified regimen induced deficiency in all VAD2 pups, and all successfully hibernated after being fed their respective diets. VAD2 squirrels were on the diet for a maximum of 10 wk before entering hibernation and remained deficient throughout hibernation. Mild symptoms of hypovitaminosis manifested in the form of slight periocular inflammation and

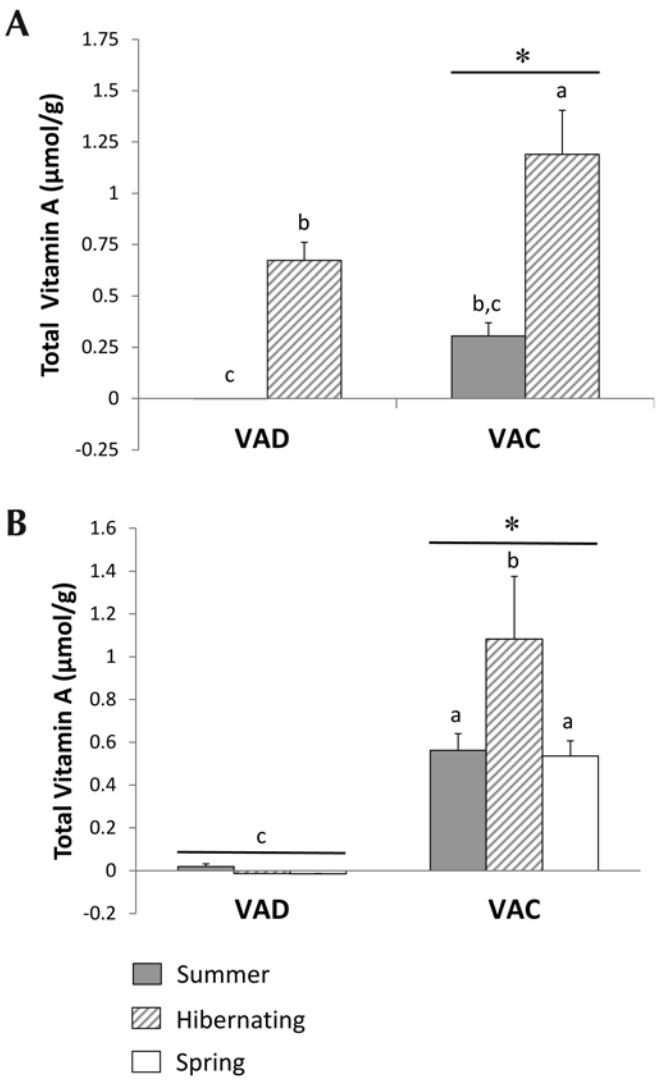

Figure 4. Hepatic total retinol concentration for squirrels on each diet regimen. (A) Regimen 1. Different letters indicate significantly different values. Multifactor ANOVA: *, diet effect ($P < 0.001$); Season effect ($P < 0.001$); diet×season effect ($P > 0.05$); n = 5–9 per group. (B) Liver total retinol concentration for diet regimen 2. Groups with the same letter are not significantly different. Multifactor ANOVA: *, diet effect ($P < 0.001$); season effect (not shown; $P = 0.054$); diet×season effect ($P < 0.05$); n = 7 or 8 per group.

weak vocalizations in a few animals shortly before hibernation, but the majority showed no signs of hypovitaminosis A. Liver retinol levels were undetectable in VAD2 animals throughout the study.

Interestingly, liver retinol stores in our VAC squirrels were high relative those in other laboratory rodents, although no signs of hypervitaminosis[11,40] were observed. Liver stores in squirrels not associated with the current study, some of which were euthanized immediately after trapping from the wild, were similarly high, suggesting that this condition is normal for this species. However, a larger survey of wild squirrels might provide more insight. The maintenance of high liver retinol may reflect an increased need for vitamin A, possibly due to increased expenditure of stored esters for adipose accumulation during their annual hibernation cycle.

Previous studies of vitamin A deficiency in laboratory rodents have found increased WAT mass, although changes in adipose mass were slight.[37] Similarly, supplementation with RA or dietary vitamin A can decrease WAT mass.[31] Others have shown obesogenic effects of vitamin A treatment.[16,39] Therefore, the role of vitamin A and its metabolites in adipose tissue growth and metabolism is incompletely understood. In our model, the mass of all WAT depots measured increased from summer to hibernation as expected, given the hyperphagia of the summer season and the role that WAT plays in providing energy for hibernation. Early deficiency (VAD1) had a significant negative effect on the mass of intraabdominal–gonadal WAT, retroperitoneal WAT, and BAT, although only the deficits of retroperitoneal WAT and BAT continued into hibernation. Of all the adipose depots measured, early deficiency had the greatest effect on BAT. Although BAT mass increased after VAD1 squirrels were removed from the diet, BAT mass was significantly less in VAD1 hibernators than VAC1 controls. RA signaling alters BAT mass[3,29,39] and thus potentially thermogenic capacity.[3,30] Decreased BAT mass in ground squirrels could affect thermogenesis during periodic arousals, although neither regimen influenced the number of arousals. Therefore, the effects on adipose mass may reflect a change in adipose metabolism in the deficient squirrels as they enter hibernation. We expected more differences in adipose tissue mass in the animals fed regimen 2, given the lack of pathology in them. Regimen 2 animals, however, showed less disparity in adipose mass between squirrels fed the VAD2 compared with VAC2 diet, with only omental WAT being significantly larger in VAC2 hibernators compared with VAD2 hibernators. This results provides support that in utero deficiency (regimen 1) causes stunted growth, which also might help explain the decreased adipose masses in those squirrels. However, VAD2 squirrels still did not show the increase in adipose mass we expected, given findings from previous studies.[31,37] Perhaps adipose hypertrophy is such an essential part of hibernation preparation that multiple pathways provide an ability to compensate for changes in one mechanism (that is, RA signaling).

Regardless of the diet regimen, liver retinol stores and adipose mass did not affect the ability of any study animals to hibernate successfully and undergo regular interbout arousals. However, arousal frequency was noticeably different between animals from regimen 1 and 2 during the first month of torpor. Ambient temperature and the time of season affect the duration of torpor bouts[45] and therefore arousal frequency. For our study, arousal frequency was assessed in the first month of hibernation for both years, so the disparity is likely due to normal variation between the 2 groups or in the room conditions or environmental disturbances from year to year.

The rapid onset of symptoms associated with hypovitaminosis in squirrels fed the vitamin A-deficient diet during regimen 1 suggests that starting the deficient diet after weaning is more effective in regard to long-term maintenance of deficiency, given that animals on the deficient diet during regimen 2 showed few symptoms yet remained functionally depleted of vitamin A. It is highly likely, however, that this species cannot be maintained on this diet as long as traditional laboratory rodents, given the rapid onset of deficiency associated with regimen 1. Little is known about vitamin A deficiency in wild rodents, and the high retinol stores in VAC squirrels and rapid deficiency in VAD squirrels reported here may, at least partly, be a consequence of being a wild-bred, rather than a multigenerationally laboratory-bred, rodent.

In addition, our data provide some insight into the dietary needs of captive wild rodents that engage in seasonal hibernation. Hibernating mammals display an annual cycle of rapid mass gain and loss that are associated primarily with

Table 2. Mass (g) of adipose tissue

	White adipose tissue				
	Intraabdominal–gonadal	Retroperitoneal	Omental	Brown adipose tissue	Adiposity index
Regimen 1					
Summer					
VAC1 (n = 8)	2.1 ± 0.3[b]	1.3 ± 0.2[c]	0.8 ± 0.1[b]	0.5 ± 0.1[c]	1.7 ± 0.2[b]
VAD1 (n = 8)	0.8 ± 0.1[c]	0.4 ± 0.0[d]	0.5 ± 0.5[b]	0.2 ± 0.0[c]	1.0 ± 0.1[b]
Hibernation					
VAC1 (n = 5)	10.3 ± 0.7[a]	7.0 ± 0.7[a]	2.0 ± 0.4[a]	1.9 ± 0.2[a]	6.1 ± 0.7[a]
VAD1 (n = 7)	7.1 ± 0.9[a]	4.2 ± 0.4[b]	2.0 ± 0.2[a]	1.3 ± 0.1[b]	4.9 ± 0.4[a]
Regimen 2					
Summer					
VAC2 (n = 8)	9.0 ± 0.8	4.7 ± 0.3	2.6 ± 0.2	1.5 ± 0.1[b]	4.7 ± 0.4
VAD2 (n = 8)	9.2 ± 1.0	4.9 ± 0.3	2.3 ± 0.1	1.6 ± 0.2[b]	4.9 ± 0.5
Hibernation					
VAC2 (n = 7)	12.0 ± 0.9	5.6 ± 0.3	3.8 ± 0.3[*]	2.1 ± 0.2[a]	6.2 ± 0.3
VAD2 (n = 8)	9.9 ± 0.7	5.0 ± 0.4	2.2 ± 0.3	2.1 ± 0.1[a]	5.4 ± 0.4
Spring					
VAC2 (n = 7)	9.3 ± 1.0	5.4 ± 0.4	2.7 ± 0.2	2.0 ± 0.2[a]	5.6 ± 0.6
VAD2 (n = 7)	8.5 ± 0.6	4.5 ± 0.3	2.3 ± 0.2	1.8 ± 0.1[a]	5.3 ± 0.3

Data are presented as mean ± SE. Within each site and regimen, groups with different superscripted letters differ significantly ($P < 0.05$; Tukey posthoc test with general linear model ANOVA).
[*]This value was significantly ($P < 0.05$) different from all other omental WAT measures for regimen 2.

increases in and depletion of energy stores in adipose tissue, respectively.[7,20] If proliferation and hypertrophy of adipocytes are essential pathways for storage in juvenile hibernators, having appropriate stores of vitamin A should be important to this process. Given the high levels of liver retinol as well as the severe response to a vitamin A-deficient diet that we observed in this study, improving the quality and efficiency of captive husbandry and breeding for this species—and potentially other hibernators—may necessitate the addition of vitamin A-rich foods. Hibernating species may require different diets not only from other rodent species but also from season to season. The ability of 13-lined ground squirrels on both regimens to successfully hibernate with equivalent numbers of interbout arousals points to strong plasticity with regard to nutrient requirements for successful hibernation. Although hibernation may not be vitamin A-dependent, quality of life for hibernators in captivity might be refined through an improved understanding of diets and vitamin needs.

Acknowledgments

We thank Vishwajit Tuchscherer, Alexandra Vizcarra, Samantha Mitchell, Cassandra Duncan, John Malovrh, Jeffrey Farvour, and Laurana Rudenas for their technical expertise and husbandry assistance. We also thank Dr Dana Merriman for her expert advice on squirrel husbandry and acquisition, Dr Hannah Carey for assistance with histology and helpful comments on the manuscript, and Mike Grahn and Chris Davis for assistance with HPLC analyses. This work was funded by the UW–Oshkosh Faculty Development Program (grant nos. FDR854 and FDR890) and the UW–Oshkosh Office of Student Research and Creative Activity (to RJS).

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