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Source: Journal of Wildlife Diseases, 39(1) : 105-113

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-39.1.105

Journal of Wildlife Diseases, 39(1), 2003, pp. 105–113

SPONTANEOUS GALLSTONE FORMATION IN DEER MICE: INTERACTION OF CHOLESTEROL, BILE ACIDS, AND DIETARY FIBER

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ABSTRACT: A study of the physiologic and ecologic factors involved in a spontaneous seasonal gallstone cycle of deer mice (*Peromyscus maniculatus gambelii*) was conducted at the Tulelake National Wildlife Refuge (California, USA) from March 1991 to June 1992. The specific hypothesis examined was whether or not seasonal increases in dietary fiber intake provides the necessary conditions for a solubility defect, or supersaturation mechanism, resulting in precipitation of cholesterol gallstones. Results indicated that in addition to the seasonal gallstone prevalence cycle, these deer mice exhibit significant seasonal cycling in serum cholesterol, serum bile acids, fecal bile acids, and diet composition. These physiologic and dietary cycles were phase-advanced 3 mo over the gallstone prevalence cycle, indicating an approximate 3 mo time period for gallstone formation under field conditions. Further, seasonal dietary fiber (plant material and seeds) was positively correlated with both serum cholesterol and the fecal bile acids. This suggests that in wild deer mice, variations in dietary fiber may significantly affect the resorption of bile acids, thereby providing a potential physiologic and nutritional mechanism for spontaneous cholesterol gallstone formation.

Key words: Bile acids, cholelithiasis, cholesterol, deer mouse, fiber, gallstones, *Peromyscus maniculatus.*

INTRODUCTION

Under natural conditions, spontaneous gallstone formation (cholelithiasis) appears to be relatively rare in wild mammals (Anver et al., 1972; Gurell and Denbesten, 1979; Pissinatti et al., 1992). However, spontaneous cholesterol gallstone formation has been reported in two wild rodent species. Pence et al. (1978) reported 71% prevalence of gallstones in cotton rats (*Sigmodon hispidus*) collected from xeric sites in Lubbock County (Texas, USA), and Schwab and Theis (1989) reported a repeatable annual cycle of gallstone prevalence in a deer mouse (*Peromyscus maniculatus gambelii*) population collected from xeric sites at the Tulelake National Wildlife Refuge (Siskiyou County, California, USA). In both studies, there was no difference in gallstone prevalence between ages or genders and the gallstones were primarily cholesterol in composition. The association of gallstone prevalence with season, xeric habitats, forage quality, and/or population abundance in both rodent species led these authors to recommend that further studies of spontaneous gallstone formation in wild rodents include both dietary composition analysis and laboratory dietary trials.

Animals with a gallbladder have the potential to form gallstones from precipitated cholesterol, bile pigments, or calcium salts. The biochemical abnormalities that can result in gallstone formation have been classified into the three broad categories of: 1) a solubility defect, or supersaturation of bile with cholesterol; 2) a kinetic factor defect, or formation of a crystal nucleus; and 3) a residence time defect, or hypomotility of the gallbladder (Hay and Carey, 1990; Busch and Matern, 1991; Konikoff, 1993).

Many sources of dietary fiber are known to adsorb bile acids, thus increasing the amount of bile acids excreted in the feces and reducing the bile acid pool. Lignin contributes to the bile acid absorption characteristics of dietary fiber (Story and Kritchevsky, 1978; Story, 1981; Gallaher and Schneeman, 1986). Lignin content in-

creases in both woody and herbaceous plants throughout the growing season (Harkin, 1973; Eastman, 1983). This seasonal change in the dietary fiber content of the forage could affect the bile acid synthesis from cholesterol and recycling via the adsorption and excretory mechanisms. Deer mice are opportunistic omnivores and adaptable to seasonal variations in food availability. In xeric habitats, arthropods are the primary prey over all seasons, with seeds and green plant material also utilized extensively on a seasonal basis (Flake, 1973; Harris, 1986; Koehler and Anderson, 1991). Seasonal variation in the dietary fiber component of deer mouse diets would provide a potential mechanism for cholesterol supersaturation of bile and formation of cholesterol gallstones.

The natural history of cholesterol gallstone disease in both cotton rats (Pence et al., 1978) and deer mice (Schwab and Theis, 1989) at xeric sites suggests that dietary fiber intake plays a crucial role in gallstone formation. The proposed mechanism involves bile acid adsorption to dietary fiber in the intestinal lumen resulting in supersaturation of bile with cholesterol and precipitation of cholesterol. The goal of this study was to investigate the physiologic and ecologic factors involved in a spontaneous seasonal gallstone formation cycle of deer mice at the Tulelake National Wildlife Refuge. In particular, the interactions between cholesterol gallstone formation, serum cholesterol, serum and fecal bile acids, and dietary fiber under field conditions were examined. The specific hypothesis tested was whether or not seasonal increases in dietary fiber intake provide the necessary conditions for a solubility defect, or supersaturation mechanism, resulting in precipitation of cholesterol gallstones.

MATERIALS AND METHODS

The study area was a site of known gallstone occurrence in deer mice (Schwab and Theis, 1989) along the tour route dike roads of the Klamath Basin Refuge Complex in Siskiyou County (California, USA, 41°50′N, 121°30′W, elevation 1,230 m). The tour route ditch bank is 40 m wide and 7 km long, and is bounded on the long axis by Tulelake and an irrigation canal, and on both ends by contiguous habitat. The vegetation and climate of the Tulelake area can be characterized as Great Basin sagebrush scrub. However, the tour route study site was dominated by mixed annual weeds including: summer cypress (*Kochia scoparia*), tansy-mustard (*Descurainia sophia*), tumble-mustard (*Sisymbrium altissimum*), giant wildrye (*Elymus cinereus*), lamb's-quarters (*Chenopodium album*), and nettle (*Urtica holosericea*) (Schwab and Theis, 1989). This vegetation formed a relatively dense cover during spring and early summer, but was sparsely covered by senescent vegetation during fall and winter. The primary precipitation in this area occurs as winter snow; the summers are generally warm and dry. However, during the study period, the Tulelake area was affected by an extended drought, which increased in severity in the spring and summer of 1992.

The deer mice were collected using rolled-oat baited Sherman live traps (H. B. Sherman Traps, Inc., Tallahassee, Florida, USA) during the new moon phase of the lunar cycle in order to maximize trap success (Schwab and Theis, 1989). The collections were made on a seasonal basis (3 mo intervals) from March 1991 to June 1992. Traps were spaced at 8 m intervals along the edge of the tour route dike road. Deer mice were maintained on rolled-oats and potatoes (water source) during the interval between capture and sampling.

Animals were killed by cervical dislocation and were weighed, measured, and their age class and reproductive status were determined (Schwab and Theis, 1989). Blood samples were collected from the heart of freshly killed deer mice in microhematocrit capillary tubes, allowed to clot, centrifuged, and the serum was frozen. The liver and gallbladder were removed and stored in 10% formalin. The carcasses were then stored frozen for later analysis.

Stored gallbladders were later examined for gallstones under a dissecting microscope. Gallstone status was recorded as positive if one or more discrete round gallstones were visible. The chemical composition of gallstones was analyzed by infrared spectrophotometry and high performance liquid chromatography (Arawak Laboratories, Inc., Inglewood, California) as per Schwab and Theis (1989).

A 10 μl aliquot of serum from each animal was analyzed for serum cholesterol utilizing a Kodak Ektachem® DT60 Analyzer (Eastman Kodak Company, Rochester, New York, USA). Total serum bile acids (tSBA) were analyzed from pooled samples, because sufficient vol-

TABLE 1. Seasonal changes in sample size, relative abundance (percent trap success), and sex ratios for *Peromyscus maniculatus* at Tulelake National Wildlife Refuge, California.

	Spring 1991	Summer 1991	Fall 1991	Winter 1991	Spring 1992	Summer 1992
Number of mice	50	50	80	80	82	83
Relative abundance (%)	42	31	23	34	45	65
Sex ratio (male : female)	1:1.38	1:0.67	1:0.82	1:0.43	1:0.61	1:0.57

ume (200 μl) could not be obtained from a single animal. Serum from groups of four mice was pooled, with individuals contributing equal volumes (50 μl). Pooled samples were grouped by season, sex, and gallstone status. An Enzabile® kit (American Chemical and Scientific Corporation, Westbury, New York) was utilized for the enzymatic, colorimetric analysis of tSBA. Absorbance was read at 540 nm with a Gilford-Beckman Automatic Spectrophotometer Model 2000 (Gilford Instrument Laboratories, Inc., Oberlin, Ohio, USA).

Formed feces were collected from the large intestines and ceca of mice and dried at 60 C for 24 hr. Dried feces were pooled utilizing the same groupings of four mice as in the tSBA analysis. Bile acids were extracted from the feces utilizing a methanol extraction methodology provided by Enzabile® and modified from Setchell et al. (1983). A 200 μl aliquot of the supernatant was analyzed for total fecal bile acids (tFBA) utilizing the Enzabile® method described above.

Seasonal field collections of deer mice for stomach contents analysis were made during the new moon phase of the lunar cycle utilizing Purina rodent chow pellets (Purina Mills, Richmond, Indiana, USA) encased in wire mesh bait exclusion cages, which prevented bait ingestion. Deer mice were killed by cervical dislocation and the carcasses were immediately frozen. Direct analysis of stomach contents via standard fiber determination methods was impractical due to the small volume of individual stomach contents, inability to separate diet fractions, and partial digestion of stomach contents. Thus, indirect measurements of dietary fiber were utilized. Stomach contents were treated as in Williams (1959, 1962). Sub-samples of stomach contents were placed on two slides and cleared with glycerin. Ten randomly selected fields were examined per slide under a compound microscope (100×), for a total of 20 fields per stomach. In each field, the percent volume of each food category was estimated to the nearest 5%. Fields containing no fragments were not counted toward the 20 randomly selected fields per stomach. Diet composition was divided into four major categories (Martell and Macaulay, 1981): arthropod, plant material, seeds, and other (fur, feathers, fungi, dirt). No attempt was made to identify food items to species. The average percent volume of each food category was calculated for each season.

Gallstone prevalence data were analyzed by logistic regression (Statistical Analysis System, SAS Institute Inc., Cary, North Carolina, USA) for season, sex, and age effects. Relative abundances, age classes, sex ratios, and reproductive status were analyzed by Chi-square (Minitab, Minitab Inc., State College, Pennsylvania, USA) for seasonal variation. Body mass, serum cholesterol, tSBA, and tFBA data were analyzed by two-way analysis of variance, (ANOVA, QuatroPro, Corel Corporation, Ottawa, Ontario, Canada) for season and sex effects. In addition, correlations were run to examine the interactions among gallstones, serum cholesterol, tSBA, tFBA, and diet composition. The diet composition data (percent volume) were analyzed by MANOVA (Statistical Analysis System) for season and sex effects. Compositions, such as stomach contents, present a unique statistical problem due to the fact that the proportions sum to 100% (Aitchison, 1986). Therefore, these compositional data were transformed into natural log ratios, utilizing one proportion as the denominator, prior to MANOVA analysis as described by Aebischer et al. (1993) and Aitchison (1986).

RESULTS

The field collections and assigned seasons were: March (spring), June (summer), September (fall), and December (winter). The relative abundance of deer mice varied with season (Table 1; $\chi^2=97.480$, df=5, $P<0.001$). It was lowest in fall 1991 and highest in spring 1991 and 1992 and summer 1992. Sex ratios also varied with season ($\chi^2=11.595$, df=5, $0.025<P<0.050$) and were generally skewed in favor of males, with the exception of spring 1991.

Reproductive status of adult deer mice varied by season for both sexes (Table 2;

TABLE 2. Seasonal changes in sample size, percent reproduction, and body mass (mean±1 SE) for adult female and adult male *Peromyscus maniculatus* at Tulelake National Wildlife Refuge, California.

	Spring 1991	Summer 1991	Fall 1991	Winter 1991	Spring 1992	Summer 1992
Adult females:						
Number[a]	25	11	30 (31)	17	24	17
Percent reproductive	0	0	37	53	21	0
Body mass (g)	20.1±0.5	17.0±0.4	23.0±0.6	21.6±0.8	21.1±0.6	17.8±0.4
Adult males:						
Number[a]	18	20	22	23	36 (43)	33 (34)
Percent reproductive	56	10	86	52	19	0
Body mass (g)	20.4±0.5	18.2±0.4	20.5±0.6	20.3±0.3	19.7±0.3	17.7±0.4

[a] When sample sizes for reproductive and body mass data differ, *n* for body mass is indicated in parentheses.

females, $\chi^2=29.816$, df=5, $P<0.001$; males, $\chi^2=60.429$, df=5, $P<0.001$). Females were reproductively active, as indicated by pregnancy or recent parturition, from fall 1991 to spring 1992, with peak reproduction in winter 1991. Males were active reproductively, as indicated by tubular epididymis, in all seasons except spring 1992. Peak male reproductive activity occurred in fall 1991. Mean adult body mass varied significantly with season (Table 2; two-way ANOVA, F=20.352, $P<0.001$) and with sex (two-way ANOVA, F=4.087, $P=0.044$). In addition, there was a significant season by sex interaction (two-way ANOVA, F=3.177, $P=0.008$). The mean female body masses were substantially higher than males during fall 1991, winter 1991, and spring 1992.

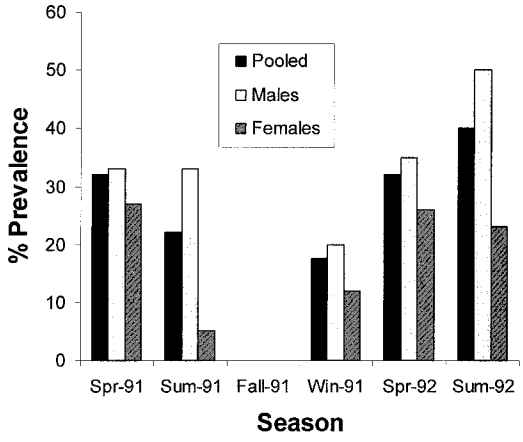

FIGURE 1. Seasonal gallstone prevalence for the Tulelake deer mouse population. Pooled population sample sizes (*n*) are spring 1991 (50), summer 1991 (50), fall 1991 (80), winter 1991 (80), spring 1992 (82), and summer 1992 (82). Male and female sample sizes (n_{male}, n_{female}) are spring 1991 (21, 29), summer 1991 (30, 20), fall 1991 (43, 37), winter 1991 (56, 24), spring 1992 (51, 31), and summer 1992 (52, 30).

Gallstone prevalence for the pooled population varied by season (Fig. 1; logistic regression, $P=0.009$) with peak prevalences in spring 1991 and 1992 and summer 1992 (32%, 32%, 40% respectively) and low prevalence in fall 1991 (0%). There was also sexual variation in gallstone prevalence (Fig. 1; logistic regression, $P=0.002$), with male deer mice exhibiting higher seasonal gallstone prevalences. There was no effect of age on gallstone prevalence. Gallstone composition was determined to be 100% cholesterol, with no nidus present.

Serum cholesterol varied by season (Table 3; two-way ANOVA, $n=418$, F=22.903, df=5, $P<0.001$), with peak values in spring 1992 and low values in summer 1991. There was no effect of gender on serum cholesterol levels. Pooled tSBA (Table 3; two-way ANOVA, F=4.71, df=5, $P=0.001$) and pooled tFBA also varied by season (Table 3; two-way ANOVA, F=3.93, df=5, $P=0.003$). There was no effect of gender on tSBA or tFBA.

Comparing mean serum cholesterol to gallstone prevalence (Fig. 2a) suggests that a three mo phase-shift exists between these two seasonal cycles. This potential phase-shift relationship was examined by

TABLE 3. Seasonal serum cholesterol, pooled serum bile acids, and pooled fecal bile acids in deer mice. Sample sizes (n) for serum cholesterol are spring 1991 (46), summer 1991 (50), fall 1991 (79), winter 1991 (80), spring 1992 (80), and summer 1992 (83). Serum bile and fecal bile sample sizes ($n_{\text{serum bile}}$, $n_{\text{fecal bile}}$) are spring 1991 (12, 14), summer 1991 (11, 11), fall 1991 (17, 17), winter 1992 (18, 18), spring 1991 (18, 18), and summer 1992 (17, 17).

	Spring 1991	Summer 1991	Fall 1991	Winter 1991	Spring 1992	Summer 1992
Cholesterol (mg/dl)	143±9	94±7	149±7	173±8	209±7	180±7
Serum bile (μmol/l)	42±7	25±3	35±5	66±14	35±6	108±30
Fecal bile (μmol/g)	2.3±−0.5	0.8±0.1	1.5±0.2	2.2±0.5	3.6±0.6	2.4±0.4

shifting the mean serum cholesterol values 3 mo later (phase-advanced) and regressing them against seasonal gallstone prevalence (Fig. 2b). A significant linear relationship emerged (r^2=0.96, P=0.003), indicating that the gallstone cycle may be 3 mo phase-delayed in relation to the cholesterol cycle.

Mean tSBA was not correlated with gallstone prevalence when 3 mo phase-advanced, nor was mean tSBA correlated with mean seasonal serum cholesterol. Mean tFBA, however, was correlated with gallstone prevalence when 3 mo phase-advanced (r=0.889, P=0.024), indicating that the gallstone cycle may also be 3 mo phase-delayed in relation to the tFBA cycle. In addition, mean tFBA was positively correlated with the mean serum cholesterol (r=0.9136, P=0.011).

Deer mouse diets varied by season in percent volume (Fig. 3; MANOVA, P<0.001). The percent volume of arthropods peaked in summer 1991 and the percent volume of dietary fiber (combined plant material and seed categories) peaked in winter 1991 and spring 1992. Further, the seasonal percent volume of dietary fiber (plant material and seeds) had a significant correlation with gallstone prevalence when 3 mo phase-advanced (r=0.9583, P=0.10), indicating that the gallstone cycle may be 3 mo phase-delayed in relation to the dietary

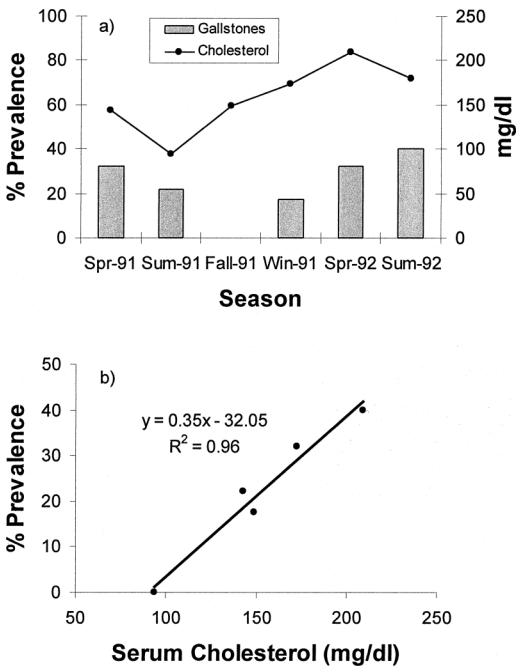

FIGURE 2. a. Seasonal serum cholesterol versus seasonal gallstone prevalence (for sample sizes, see Table 3 legend). b. Mean seasonal serum cholesterol have been phase-advanced 3 mo and regressed against seasonal gallstone prevalence.

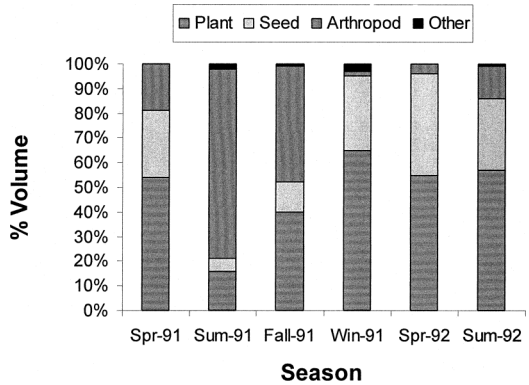

FIGURE 3. Seasonal percent volume composition of deer mouse stomach contents. Sample sizes (n) are spring 1991 (20), summer 1991 (20), fall 1991 (20), winter 1991 (20), spring 1992 (20), and summer 1992 (20).

fiber (plant material and seeds) cycle. In addition, the dietary fiber (plant material and seeds) cycle was significantly correlated with both mean seasonal serum cholesterol (r=0.8917, P=0.017), and with mean seasonal tFBA (r=0.8772, P=0.022).

DISCUSSION

The 1991/1992 seasonal gallstone cycle for the Tulelake deer mouse population was similar to the 3 yr gallstone cycle previously documented by Schwab and Theis (1989). However, the summer 1991 and 1992 field collections had unexpectedly high gallstone prevalences (22% in 1991 and 40% in 1992) as compared to the gallstone prevalences for summer 1985 to 1987 (7–9%, Schwab and Theis, 1989). The unusually high gallstone prevalences during the summer droughts of 1991 and 1992 lend support to the hypothesis that the high fiber diets present during xeric conditions contribute to cholesterol supersaturation of bile and increased gallstone formation in deer mice. In human subjects, supersaturated bile is also known to play a role in the pathophysiology of gallstone formation (Carey and Duane, 1994).

This deer mouse population also displayed significant seasonal cycling in mean serum cholesterol, tSBA, tFBA and dietary fiber (plant material and seeds) composition. These physiologic and dietary cycles were phase-advanced 3 mo over the gallstone prevalence cycle, indicating an approximate 3 mo period for gallstone formation under field conditions. In addition, high levels of dietary fiber (plant material and seeds) were significantly correlated with the serum cholesterol and fecal bile acids cycles, indicating that seasonal increases in dietary fiber intake may indeed create the necessary conditions for a solubility defect, or supersaturation mechanism (Hay and Carey, 1990; Busch and Matern, 1991; Konikoff, 1993) for cholesterol gallstone formation in wild deer mice.

The gallstones collected in this study were composed of 100% cholesterol with no nidus present. This suggests that a kinetic factor defect, or the formation of a crystal nucleus (Hay and Carey, 1990; Busch and Matern, 1991; Konikoff, 1993), was not an important mechanism in this population for gallstone formation. However, the potential gallstone formation mechanism of a residence time defect, or hypomotility of the gallbladder, may deserve further investigation. The gender differences observed in gallstone prevalences, with male deer mice exhibiting significantly higher levels than females, is not adequately explained by the supersaturation mechanism, as there was no sexual variation in cholesterol, bile acids or percent volume of dietary fiber (plant material and seeds). However, Wang et al. (1997) observed higher gallstone prevalences and enhanced cholesterol supersaturation in inbred strains of male mice on lithogenic diets. This deer mouse gender difference is in contrast to the human data where gallstone prevalences are generally higher in females due to the effect of estrogens on cholesterol saturation of bile (Diehl, 1991).

In our study, male deer mice had lower body mass than females in several seasons (fall and winter 1991 and spring 1992). It is possible males may attempt to maximize their energetic efficiency by entering into daily torpor during periods of environmental stress such as extreme cold and/or limited food supply (Nestler, 1991). In hibernating chipmunks (*Tamias amoenus*), it has been observed that increased dietary cholesterol enhances both torpor depth and length (Geiser et al., 1997). Free cholesterol forms a crystalline solid at 37 C (Small, 1988), so the decreased body temperatures characteristic of torpor may induce cholesterol precipitation. In addition, the gallbladder would empty less frequently (hypomotility) during torpor. Thus, the gallstone formation mechanism due to a residence time defect, or hypomotility, (Hay and Carey, 1990; Busch and Matern, 1991; Konikoff, 1993) deserves further investigation, and may provide an explana-

tion for the observed gender differences in deer mouse gallstone prevalences. Konikoff (1993) supports this potential role of hypomotility, stating that cholesterol gallstone formation is a multifactorial process involving cholesterol supersaturation of bile as the underlying defect, combined with either the presence of nucleating factors or gallbladder stasis. However, Wang and Carey (1996) present a conflicting model; they demonstrated that decreases in temperature to 4 C retarded cholesterol crystallization in model bile systems.

This study supports the hypothesis that seasonal increases in dietary fiber intake provide the necessary conditions for a solubility defect, or supersaturation mechanism (Hay and Carey, 1990; Busch and Matern, 1991; Konikoff, 1993) for cholesterol gallstone formation in wild deer mice. It also presents baseline data on serum cholesterol, serum bile acids, and fecal bile acids levels in a wild deer mouse population. We demonstrate how seasonal habitat cyclicity and physiologic ecology can interact to impact gallstone prevalence in a resident mammalian population.

The deer mouse provides a unique animal model system for the study of cholesterol gallstone formation because their gallstones occur spontaneously in a predictable and repeatable seasonal cycle. A companion study utilizing dietary trials to investigate the impact of fiber (lignin levels) on gallstone induction and reabsorption demonstrated gallstone reabsorption over a 3 mo time period on a fiber-free diet, and elevated serum cholesterol coupled with reduced serum bile acids on fiber-supplemented diets (5 and 10% lignin). However, gallstones were not induced under these laboratory conditions (Ginnett, 1994). Another companion study utilized high performance liquid chromatographic fractionation of serum bile acids to demonstrate: 1) chenodeoxycholic acid (CDCA) was the primary bile acid in wild deer mice; 2) sexual variation in cholic acid (CA), with females exhibiting higher CA levels in all seasons; and 3) seasonal variation in CA, CDCA, and deoxycholic acid (DCA). Increases in dietary fiber were associated with decreased CDCA and increased DCA (Ginnett, 1994).

Further study of spontaneous gallstones in deer mice should include field and laboratory trials investigating the relationship between cold stress, torpor, and gallstone formation. It would also be interesting to subfractionate the serum cholesterol and examine the role of HDL versus VLDL in gallstone formation in deer mice, because low HDL cholesterol is positively correlated with gallstone prevalence in humans (Thijs et al., 1990), and is suggested in inbred mice (Khanuja et al., 1995). In addition, laboratory studies of biliary lipid secretion rates would help to further our understanding of the proposed supersaturation mechanism (Wang et al., 1999). Recent research into gallstone formation suggests a potential role for: 1) genetic inheritance of a predisposition to gallstones in laboratory mice (Alexander and Portman, 1987; Khanuja et al., 1995; Wang et al., 1997), 2) bacteria serving as a nidus for both cholesterol and pigment gallstone formation (Vitetta et al., 2000), and 3) melatonin as an inhibitor of cholelithiasis (Reiter et al., 2001). In the future, this animal model may contribute further insights towards the mechanisms underlying spontaneous gallstone formation and the development of dietary regimens for the prevention and treatment of human gallstones.

ACKNOWLEDGMENTS

This study was made possible by the cooperation of the Tulelake National Wildlife Refuge of the Klamath Basin Refuge Complex, US Department of the Interior Fish and Wildlife Service and the Winema Hunting Lodge, Inc. This work was supported in part by a SigmaXi Grant-In-Aid of Research and J. J. Kaneko's discretionary research funds. Animal care facilities were provided by R. E. Marsh. The authors extend their appreciation to T. F. Ginnett and S. J. Taft for manuscript reviews and to M. H. Rollins, R. E. Marsh, T. F. Ginnett, K. Stewart, S. Schmidt, K. Crooks, and M. Johnson for field and/or laboratory assistance. In addition,

D.A.G. thanks the late Dr. R. G. Schwab for mentoring and field assistance.

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Received for publication 26 July 2002.