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James A. Wilson
Oklahoma State University, jameswilson@unomaha.edu

Jennifer L. Parsons
Oklahoma State University

Eric E. Jorgensen
U.S. Environmental Protection Agency

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James A. Wilson[1,*]
Jennifer L. Parsons[1,†]
Eric E. Jorgensen[2]
[1]Department of Zoology, Oklahoma State University, Stillwater, Oklahoma, 74078; [2]U.S. Environmental Protection Agency, Office of Research and Development, National Risk Management Research Laboratory, Ada, Oklahoma 74820

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ABSTRACT

Maternal nutrition during pregnancy and postnatal offspring nutrition may influence offspring traits. We investigated the effects of maternal and postweaning offspring dietary nitrogen on immune function and hematology in two species of rodent: the hispid cotton rat (*Sigmodon hispidus*), a primarily herbivorous rodent, and the fulvous harvest mouse (*Reithrodontomys fulvescens*), an omnivore. These two species responded differently to the same levels of treatment, with cotton rats primarily influenced by maternal diet and harvest mice by postweaning offspring diet. Cotton rats born to mothers on high-nitrogen diets had lower values of mean corpuscle volume and hemoglobin and greater concentrations of serum immunoglobulins. Spleen size, cell-mediated immune response, and the number of splenocytes and thymic platelets were lower in cotton rats born to mothers on low- and high-nitrogen diets. High-nitrogen offspring diet increased kidney and liver mass in cotton rats. Harvest mice had increased kidney mass on high-nitrogen maternal diets; however, changes in offspring diet after weaning reduced hematological parameters in individuals fed low-nitrogen diets. Body length was also affected, with harvest mice born to mothers fed low- and high-nitrogen diets having shorter lengths. Splenocyte cellular activity was greater in offspring born to mothers on high-nitrogen diets in both species.

* Corresponding author. Present address: Department of Biology, University of Nebraska, 6001 Dodge Street, Omaha, Nebraska 68182; e-mail: gulywhumpr@sbcglobal.net.
† Present address: Department of Animal and Dairy Sciences, Mississippi State University, Mississippi State, Mississippi 39762.

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Introduction

Immune function is the primary line of defense against infectious agents and is a major physiological mechanism that regulates individual survival (Lochmiller 1996; Lochmiller and Deerenberg 2000). The traditional ecological view of immune function is one of energetic trade-offs (Lochmiller and Deerenberg 2000; Rådberg et al. 2002). However, alternative hypotheses about the cost of immunity have been proposed, which include costs associated with damage from infection (Rådberg et al. 1998; Westneat and Birkhead 1998) and decreased antioxidant protection (Hõrak et al. 2006). Although the hypothesis of energetic trade-offs has support, the complex nature of the immune system demands a complex explanation for the link between immunity and other physiologic processes.

There is evidence for an energetic trade-off between immunity, growth, maintenance, thermoregulation, and reproduction (Lochmiller and Deerenberg 2000; Norris and Evans 2000) that provides organisms with an "energetic choice" of which processes will be supported. Evidence supporting the energetic trade-off between immune function and other physiologic processes includes both direct and indirect sources. Measurements of direct metabolic costs associated with mounting an immune response have found a significant increase in metabolic rate after immune challenge (Demas et al. 1997; Eraud et al. 2005). Specifically, there is evidence that immune maintenance costs are minimal but that mounting an immune response is costly (Derting and Compton 2003). Therefore, to understand how individuals allocate energy among these processes, it is important to understand the costs associated with immunity and what role genetics, the environment, and nutritional state may play in determining the effectiveness of an individual's immune response (Klassing 1998; Derting and Compton 2003).

Recently, evidence has suggested that offspring may receive benefits from not only their own nutritional state but also their mother's prenatal condition. Maternal effects have been known to influence reproductive characteristics such as offspring and litter size (Dobson and Michener 1995), offspring sex ratio (Wiley and Clapham 1993; Boesch 1997; Dittus 1998; Sheldon and West 2004), and offspring growth rates (Tardif and Bales 2004). However, recent studies have also found evidence that a mother's condition may continue to affect offspring through their dispersal ability (Gaines and McClenaghan 1980) or survival (Kerr et al. 2007). Clearly, these maternal effects would be of great interest in regulating population dynamics and represent an important evolutionary link between the environment

and an individual's physiologic state, including their ability to mount an effective immune response.

The high proportion of low-quality forage in the habitat and diet of most wild herbivores creates an environment where malnutrition is a common occurrence (White 1978; Belovsky 1986). However, the nutritional landscape is variable both spatially and temporally, providing opportunities for individuals to secure patches of good habitat. Individuals, especially reproductive females, within good-quality habitats may experience a nutritional boost that may give offspring benefits derived from their mother's condition, as well as allowing them to develop within good-quality habitat. As a result, offspring development should be affected by two major factors: maternal condition during pregnancy and postweaning nutrition of the offspring.

We investigated the effects of maternal and offspring dietary N on the immune system and hematology of small mammals native to the tallgrass prairie of the southern Great Plains. We chose two of the most common species in the southern prairie, the hispid cotton rat (*Sigmodon hispidus*; Cameron and Spencer 1981) and the fulvous harvest mouse (*Reithrodontomys fulvescens*; Spencer and Cameron 1982), because of their dominant position by biomass and numbers within the ecosystem (Schetter et al. 1998; Brady and Slade 2001; Clark et al. 2005) and the difference in their diets. Cotton rats are primarily herbivores, consuming a mixture of monocots and dicots (Randolph et al. 1991), whereas the harvest mouse is an omnivore with a substantial insect component (Meserve 1976; Vasquez et al. 2004). Because of their wild diets, cotton rats and harvest mice should differ in their ability to adjust to various levels of dietary N during reproduction.

Our objectives were to determine the effects of maternal and postweaning offspring dietary N on the immune system and hematology of two rodent species that exhibit widely different diets and life-history strategies. We predicted that generalist species, such as the cotton rat, would be adapted to habitats with variable forage quality and should therefore exhibit more plastic physiology. As a result, we hypothesized that cotton rat offspring would be most influenced by maternal nitrogen intake. Specifically, female cotton rats with low-quality forage would still produce offspring, but those offspring would be of lower physiologic quality. Conversely, species that specialize in higher-quality habitats, such as harvest mice, would be limited in their variability with respect to maternal effects (i.e., would only reproduce when nutrition was acceptable) but could show variation due to postweaning offspring diet. This study will provide information about the link between maternal condition, offspring diet, and immune function.

Methods

This study is a portion of a larger project investigating the effects of dietary nitrogen on rodent reproduction (Parsons 2001). Captive colonies of cotton rats and harvest mice were formed using wild-caught individuals trapped at various sites in Payne County, Oklahoma. Research subjects were housed individually at the Laboratory Animal Resources facility at Oklahoma State University after capture and during pretrial and experimental periods. Specific methods for the housing of experimental animals are given by Parsons et al. (2005). All animals were allowed at least 2 wk to adjust to captivity before experimentation. All fieldwork was conducted under the auspices of an approved animal care and use protocol and met guidelines recommended by the American Society of Mammalogists (1998).

As part of the larger study, Parsons (2001) used seven isocaloric (17.3–18 kJ/g), pelleted experimental diets (Zeigler Brothers, Gardners, PA) formulated to represent a range of N levels. Each diet was formulated identically except for relative amounts of soybean meal and corn starch, which were varied to achieve ratios of 6%, 8%, 10%, 12%, 14%, 16%, and 20% crude protein (0.96%–3.2% N). These levels of dietary N reflect those consumed by free-ranging cotton rats (Randolph et al. 1991; Cameron and Eshelman 1996; Randolph and Cameron 2001). Although wild harvest mice are somewhat insectivorous and likely encounter higher levels of dietary N in certain foodstuffs (Kincaid and Cameron 1982; Stancampiano and Caire 1995), we believed that the range of experimental diets was sufficient to simulate the variability of N found in natural forage. Specific to this study, the seven experimental diets were grouped into one of three categories for the purpose of analysis: low (1.08%–1.90%), medium (2.01%–2.50%), and high (2.80%–3.67%) nitrogen in crude protein. These categories correspond roughly to a crude protein content of 6%–10% (low), 12%–14% (medium), and 16%–20% (high). However, because of variation in the N content of experimental diets, the actual N content was used to place experimental diets into the appropriate category (for a full description of experimental diets, see Parsons et al. 2005).

A total of 18 cotton rats (eight low, five medium, five high) and 28 harvest mice (10 low, nine medium, nine high) mothers successfully produced a total of 93 and 100 offspring, respectively, and were used in this study. Mean offspring per mother was 5.2 ± 0.3 (cotton rat) and 3.7 ± 0.2 (harvest mouse). Numbers of offspring composing specific mother/offspring dietary combinations are shown in Table 1. Offspring were divided as described above and randomly assigned to an offspring diet.

Females of reproductive age were randomly selected and paired with sexually mature males for breeding. Each pair was housed in the same cage until it was evident that the female was pregnant. At that point, pairs were separated and females randomly assigned to one of the three experimental diets in a manner that ensured equal sample sizes. We attempted to place each female on her assigned ration at the beginning of the third trimester, using changes in body mass as a guide. We chose that time period because nutritional requirements of early gestation differ little from those of a nonpregnant condition, but they increase substantially after about 60% of the gestation period (Robbins 1993). Gestation periods for cotton rats and harvest mice are 27 and 21 d, respectively (Parsons 2001). Each female stayed on her designated diet from the third trimester

Table 1: Number of offspring in each mother/offspring diet used in this study

Species and Mother Diet Nitrogen Level	Offspring Diet Nitrogen Level		
	Low	Medium	High
Harvest mouse:			
Low (10)	7	13	13
Medium (9)	10	12	14
High (9)	9	8	14
Cotton rat:			
Low (8)	9	18	15
Medium (5)	5	10	7
High (5)	6	14	9

Note. Numbers in parentheses for mother diet indicate the number of mothers in each category that produced the associated offspring.

of gestation through weaning. Food and water were provided ad lib. throughout the experimental period, and cotton was provided for nesting.

The first day that young were observed in the nest was designated as the date of parturition. Offspring remained with their mothers until weaning (~18–21 d postparturition), at which time they were separated and placed individually into wire-topped plastic cages. Each individual offspring was randomly assigned to one of the experimental diets so that a maximum range of diets was represented in each litter. Weaned juveniles were supplied with food, in the form of their experimental diet, and water ad lib. for the duration of the experiment.

Hematology

Offspring from harvest mice and cotton rats were killed at 59–74 and 63–78 d postweaning, respectively. Individuals were killed by cervical dislocation after exposure to methoxyfluorane (Mallinckrodt Veterinary, Mundelein, IL) anesthesia. Epididymides, testes, uteri, and ovaries were removed, trimmed of fat, blotted dry, and weighed (g). Blood was obtained from the retro-orbital sinus plexus of anesthetized rats with heparinized capillary tubes. A 40-μL sample of blood was placed into 10 mL of Serano diluent, mixed, and counted on an automated cell counter standardized for cotton rat blood. The concentration of white blood cells (WBC), red blood cells (RBC), and hemoglobin (HGB), the hematocrit (HCT), the mean corpuscular volume (MCV), the mean corpuscular hemoglobin concentration (MCHC), and the number of platelets (PLT) were determined with a cell counter (Serano System 9000, Allentown, PA). Alterations in hematological parameters can indicate various states of health, including anemia (decreased RBC, HCT, HGB), reduced antibody or innate immunity (low WBC), lack of clotting (low PLT), and thrombosis (high PLT). Levels of circulating serum proteins indicate a general level of health, with higher circulating proteins associated with individuals in better condition.

Whole-blood smears were prepared by placing one drop of blood at the tip of a microscope slide and smearing the blood sample across the slide using a second slide. Blood smears were allowed to dry for 24 h, fixed with methanol, and stained using a combination of eosin and thiazine stain. The proportion of lymphocytes, neutrophils. monocytes, eosinophils, and basophils in 100 observed cells was counted using oil immersion (× 1,000) on a light microscope.

Blood was also placed into a heparinized serum separation tube and centrifuged at 15°C, 1,200 *g* for 10 min. Serum was placed into tubes and stored at −70°C until it was used for serum protein and immunoglobulin analysis. Total serum protein concentrations were determined using a standard Biuret method and compared against a human serum standard (Kingsley 1942). Total immunoglobulin concentrations were measured using an ammonium sulfate, sodium chloride precipitation assay (Bradford 1976). Absorbance of both total protein and total immunoglobulin solutions was read on a spectrophotometer at 550 nm (total protein) and 555 nm (total immunoglobulins) after standardization with human serum protein.

Spleens were removed aseptically, weighed, and placed in sterile petri dishes containing RPMI 1640 culture medium supplemented with 10% horse serum (RPMI-HS, pH 7.2), 1.025% L-glutamate (200 mM), and 1.0% penicillin-streptomycin (10,000 U/mL–10 mg/mL). Spleens were dissociated into individual cells by homogenization using a sterile glass tissue grinder with 5 mL of RPMI-HS medium. Cell solutions were allowed to settle for 7 min, the supernatant was decanted, and cells were washed by centrifugation (8 min, 10°C, 220 *g*) and resuspended in 5 mL of fresh RPMI-HS medium. Erythrocytes were lysed by washing cells in 5 mL of Tris-buffered ammonium chloride (0.83%, pH 7.2), followed by a wash with 5 mL RPMI-HS. Cells were counted using an automated cell counter, adjusted to a final concentration of 500,000 cells/90 μL, and maintained in RPMI-HS.

Paired popliteal nodes were removed from individuals, weighed, and placed into 3 mL of RPMI-HS. Popliteal nodes were homogenized, and the concentration of white blood cells and platelets found in popliteal nodes was measured by placing a 20-μL sample of the homogenate into 10 mL of Serano diluent for use in an automated cell counter.

MTT Assay

Proliferation assays measure the ability of immune cells involved in cell-mediated immunity—specifically, splenocytes/lymphocytes—to respond to growth signals. After preparation of splenocyte solution, 90 μL of adjusted spleen cell suspension was added in triplicate to appropriately labeled wells in a 96-well flat-bottomed plate. Cells were incubated at 37°C for 12 h in a CO_2 incubator. After incubation, 10 μL of 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) working solution (5 mg/mL phosphate-buffered solution [PBS]) was added to each well and returned to the incubator for 3 h. An acidic isopropanol solution (0.04 M HCl in absolute isopropyl alcohol) was added to stop the reaction. The amount of tetrazolium salt transformed by cells to a dark blue formazan was

measured by agitating culture trays for 1–2 min and then reading the light transmission through the cell solutions in a spectrophotometer set with dual peaks of 570 and 630 nm.

Hypersensitivity

Hypersensitivity tests measure in vivo immune response to a foreign agent. This represents only cell-mediated, not humoral, immune response. In vivo cell-mediated immune response was measured by hypersensitivity reaction to injections of PBS (pH 7.2) and phytohemagglutinin (PHA-P), as described by Wilson et al. (2003). Individuals were given an intradermal injection of 100 μL (cotton rats) or 25 μL (harvest mice) of PHA-P (stimulant; 2.5 mg/mL PBS) on one rump and a PBS-only injection (control) on the opposite rump. Skin-fold thickness (24 h postinfection) was measured to the nearest 0.001 in using a micrometer. The difference between the initial and 24-h post-injection skin-fold thickness was calculated for PBS and PHA-P, and the ratio of PHA-P to PBS reactivity was used as an index of immune response. The PBS-only injection functioned as a sham injection to control for the effects of the act of injecting the individual.

Statistical Analyses

Comparisons involving organ size are confounded by problems associated with scaling. To account for scaling by body size, organ weights were analyzed using residual variation. A regression of organ mass versus body mass was made using PROC REG, and the residual variation values were exported to a new data set. Residuals were then used for comparing treatment types with ANOVA.

Analysis of immune effects was performed using a split-plot design, with maternal diet (low, medium, high) as the whole-plot factor and offspring diet (low, medium, high) as the subplot factor. Due to the presence of randomly assigned mothers (random effects) and fixed offspring (fixed effects), PROC MIXED was used to calculate *F*-test values, with Satterthwaite's approximation used to calculate the degrees of freedom for the error term (SAS Institute 2000). If there were no significant interactions, differences in the main effects were compared using the PDIFF option in the LSMEANS statement. Differences in terms with significant interactions were compared using the SLICE option in LSMEANS. Comparisons using LSMEANS were performed using a Bonferroni adjustment to reduce the likelihood of generating Type I errors. All data are presented as mean ± standard error, and all differences were considered significant at the $\alpha = 0.05$ level.

Results

A total of 28 female harvest mice and 18 cotton rats successfully produced offspring for use in this experiment. Litter size differed for each species, with harvest mice producing litters of 1–5 offspring (3.7 ± 0.2 offspring), whereas cotton rats had litters of 3–8 (5.22 ± 0.3 offspring).

Body length in harvest mice was lower ($F_{2,90} = 4.85$, $P = 0.01$) for offspring born to mothers on high- (71.22 ± 0.93 mm) and low-nitrogen (70.38 ± 0.82 mm) diets compared with those born to mothers on medium-nitrogen diets (73.77 ± 0.78 mm; Fig. 1). No difference in body length was observed in cotton rats ($F_{2,81} = 2.38$, $P = 0.06$). For both harvest mice and cotton rats, kidney mass was greatest when exposed to high-nitrogen diets; however, in harvest mice, maternal diet ($F_{2,89} = 3.08$, $P = 0.05$) was the influential factor, whereas in cotton rats it was offspring diet ($F_{2,82} = 8.34$, $P = 0.0005$). In harvest mice, kidney mass was significantly greater for offspring reared from mothers on high-nitrogen diets (Fig. 2). Similarly, kidney mass also differed in cotton rats; however, these differences were due to offspring diet instead of maternal diet (Fig. 2). In addition, liver mass also showed a difference ($F_{2,82} = 3.00$, $P = 0.05$) due to offspring diet, with lower liver mass in cotton rat offspring reared on low-nitrogen diets (3.04 ± 0.14 g) compared with offspring fed medium- (3.35 ± 0.08 g) or high-nitrogen (3.40 ± 0.11 g) diets. No maternal effects were observed in cotton rats for these organs. However, maternal effects were observed in the mass of spleens in cotton rats ($F_{2,84} = 5.70$, $P = 0.005$; Fig. 3*A*). In addition, the number of splenocytes within the spleen was also affected by maternal diet ($F_{2,82} = 10.06$, $P = 0.0001$; Fig. 3*B*).

Reproductive organs also showed a dietary effect in both harvest mice and cotton rats. There were no differences detected for uterus size in either harvest mice or cotton rats. However, ovary mass showed an effect from maternal diet in harvest mice ($F_{2,40} = 3.32$, $P = 0.05$), with offspring born to mothers on a low-nitrogen diet having significantly lower ovary mass (0.0029 ± 0.0004 g) than offspring from mothers fed medium- (0.0052 ± 0.0006 g) or high-nitrogen (0.0048 ± 0.0011 g) diets. No differences in ovary mass were detected for cotton rats. Testes mass showed a maternal diet effect in both harvest mice

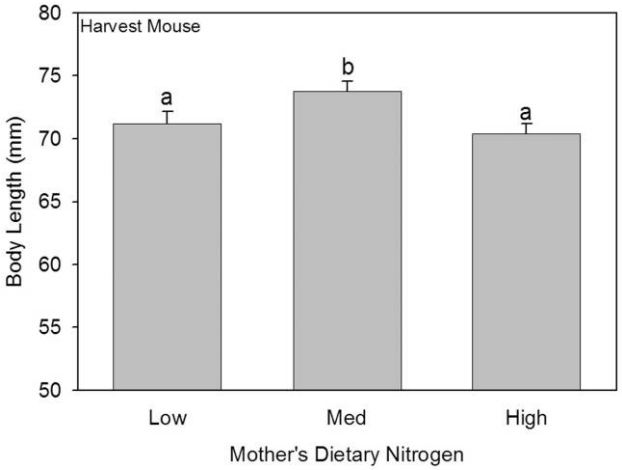

Figure 1. Effects of maternal dietary nitrogen on body length of harvest mouse (*Reithrodontomys fulvescens*) offspring born to mothers fed a diet low (6%–10%), medium (12%–14%), or high (16%–20%) in nitrogen during their third trimester of pregnancy. Significance is indicated by different letters.

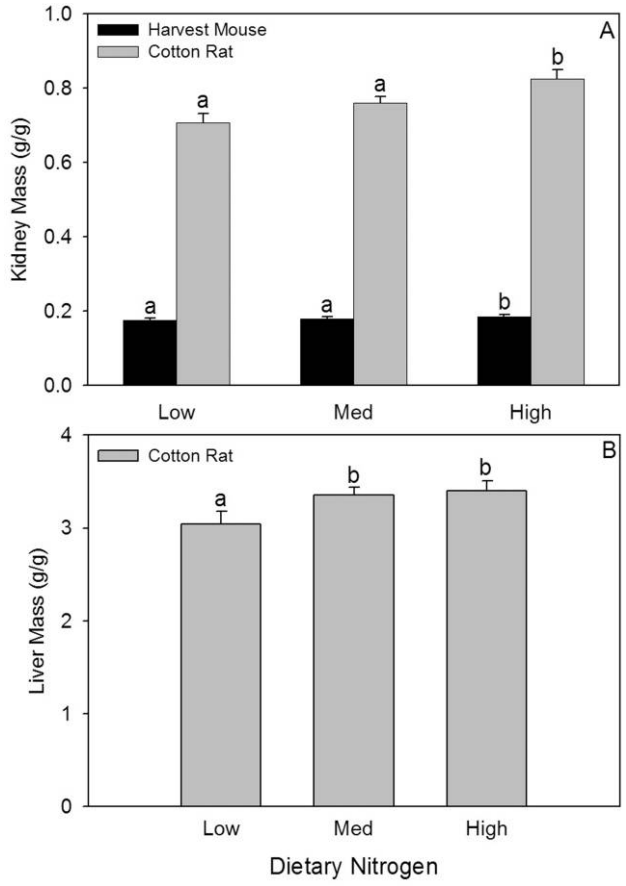

Figure 2. *A*, Effects of maternal (harvest mice) or offspring (cotton rat) dietary nitrogen on kidney mass (g/g body mass) of mother or of offspring fed a postweaning diet low (6%–10%), medium (12%–14%), or high (16%–20%) in nitrogen. *B*, Effects of offspring dietary nitrogen on liver mass (g/g body mass) of cotton rats. Significance is indicated by different letters.

($F_{2,38} = 3.32$, $P = 0.05$; Fig. 4*B*) and cotton rats ($F_{2,30} = 3.16$, $P = 0.05$; Fig. 4*B*).

Measures of the activity of immune function within harvest mice and cotton rats showed a significant maternal effect within both species for cellular metabolism of splenocytes ($F_{2,78} = 5.21$, $P = 0.008$ and $F_{2,80} = 4.79$, $P = 0.01$, respectively). Offspring from both species born to mothers on high-nitrogen diets showed increased splenocyte metabolism (Fig. 5*A*). However, tests of cell-mediated immune response (delayed-type hypersensitivity response) showed only a maternal effect within cotton rats ($F_{2,84} = 4.34$, $P = 0.02$; Fig. 5*B*). Cotton rat offspring born to mothers on low- and high-nitrogen diets had lower responsiveness than offspring born to mothers on medium-nitrogen diets.

Hematological parameters showed different treatment effects for each species, with maternal diet predominantly affecting cotton rats and offspring diet predominantly affecting harvest mice (Table 2). Within harvest mice, offspring reared on low-nitrogen diets showed the only significant differences. MCV ($F_{2,84} = 8.41$, $P = 0.0005$), MCH ($F_{2,72} = 4.36$, $P = 0.02$), and serum total proteins ($F_{2,46} = 2.94$, $P = 0.06$) were lower in offspring on a low-nitrogen diet (Table 2). Cotton rats showed a difference in WBC due to maternal diet ($F_{2,80} = 3.41$, $P = 0.04$), with offspring reared from mothers on medium-nitrogen diets having higher WBC than offspring fed either low- or high-nitrogen diets (Table 2). Cotton rats also showed a difference in MCV ($F_{2,82} = 14.10$, $P < 0.0001$) and MCH ($F_{2,77} = 7.03$, $P = 0.002$); however, the differences were due to maternal diet, with offspring born to mothers fed a high-nitrogen diet having higher MCV and MCH (Table 2). Within cotton rats, an offspring effect was also observed for MCV ($F_{2,82} = 10.13$, $P = 0.0001$) and MCH ($F_{2,77} = 4.99$, $P < 0.0009$), with no significant interactions between mother and offspring diet. With respect to the effect of offspring diet, MCV of offspring reared on low-nitrogen diets, regardless of maternal diet, had significantly lower corpuscle volume compared with offspring reared on medium- or high-nitrogen diets (Table 2). An identical pattern was observed for MCH, with offspring reared on low-nitrogen diets having lower MCH than offspring on low- or medium-nitrogen diets (Table 2). Additional maternal effects were observed in serum protein and immunoglobulin levels, with off-

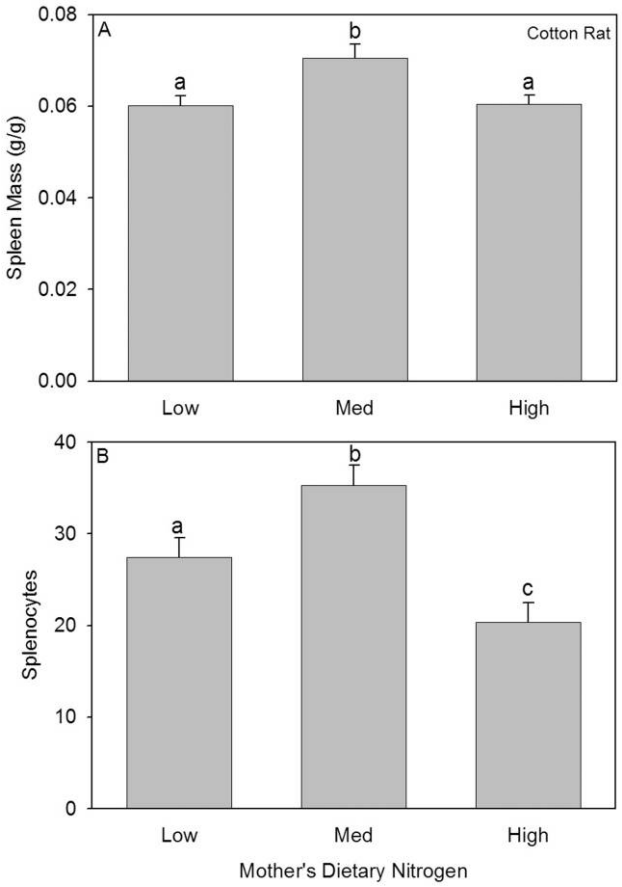

Figure 3. Effects of maternal dietary nitrogen on (*A*) spleen mass (g/g body mass) and (*B*) concentration of splenocytes in cotton rats (*Sigmodon hispidus*) born to mothers fed a diet low (6%–10%), medium (12%–14%), or high (16%–20%) in nitrogen during their third trimester of pregnancy. Significance is indicated by different letters.

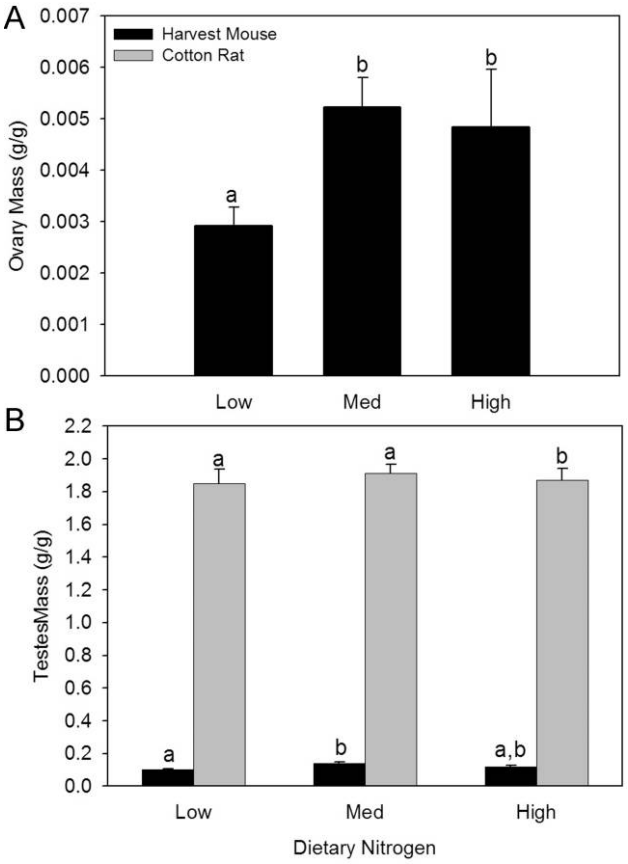

Figure 4. Effects of maternal dietary nitrogen on (*A*) ovary mass (g/g body mass) and (*B*) testes mass (g/g body mass) born to mothers fed a diet low (6%–10%), medium (12%–14%), or high (16%–20%) in nitrogen during their third trimester of pregnancy. Significance is indicated by different letters.

spring born to mothers on high-nitrogen diets having higher levels of total serum immunoglobulins ($F_{2,49} = 6.91$, $P = 0.002$) and proportionately more as the percentage of serum protein that are immunoglobulins ($F_{2,49} = 5.92$, $P = 0.005$). Finally, a maternal effect was observed for the number of thymic platelets ($F_{2,69} = 4.34$, $P = 0.02$), with offspring born to mothers on medium-nitrogen diets having more platelets than offspring whose mothers had low- or high-nitrogen diets.

Discussion

In this study, offspring from both cotton rats and harvest mice showed alterations in the development of their immune system as a result of changes in maternal and/or offspring dietary nitrogen levels. Although individual parameters differed in their response to nitrogen levels, there was a clear trend in the relative importance of maternal and offspring nutrition for the observed differences. Specifically, harvest mice were most affected by changes in offspring diet, whereas cotton rats showed more maternal diet effects.

Harvest mouse offspring did not show any alterations to their organ morphology and showed only minor (<5%) reduction in overall body length. In addition, harvest mice showed no alterations in their innate immunity or cellularity. The only alterations observed were to the functioning of the immune system, with a 27.5% increase in splenocyte metabolism and a 9.5% increase in total serum protein and decreases of 5.5% and 2.5% in MCV and MCHC, respectively.

Cotton rats in this study had a trend toward maternal effects, including a 14% reduction in spleen size; reductions in splenocyte counts, innate immune function, thymic platelet levels, and corpuscular volume and hemoglobin content; and increased splenocyte activity and serum immunoglobulin levels. However, offspring diet also contributed to lower organ mass, with kidneys and liver being 9.3% and 7.0% lower in offspring fed a low-nitrogen diet. Previous studies on the effects of protein restriction on immune development and function show similar effects. Davis et al. (1995) found that weanling cotton rats fed moderate- or low-protein diets had reduced numbers of splenocyte subpopulations (B and T cells). In addition, a low-protein offspring diet adversely affected the development of numerous immune organs, resulting in reduced spleen, thymus, and lymph nodes. Davis et al. (1995) also found that

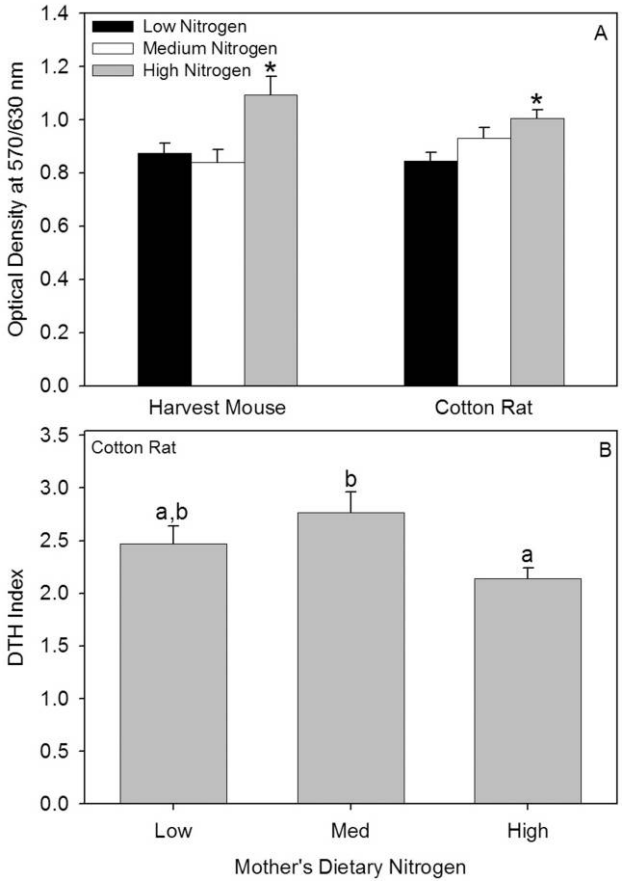

Figure 5. Effects of maternal dietary nitrogen on (*A*) the cellular proliferation index of splenocytes from both cotton rats (*Sigmodon hispidus*) and harvest mice (*Reithrodontomys fulvescens*) and (*B*) in vivo cell-mediated hypersensitivity (delayed-type hypersensitivity) in cotton rats. Significance is indicated by different letters.

Table 2: Hematological parameters of harvest mouse and cotton rat offspring

	Harvest Mouse							Cotton Rat						
		Low-N Diet		Medium-N Diet		High-N Diet			Low-N Diet		Medium-N Diet		High-N Diet	
Parameter	Factor	Mean	SE	Mean	SE	Mean	SE	Factor	Mean	SE	Mean	SE	Mean	SE
Thymic white blood cells ($\times 10^6/mm^3$)		9.69	1.00	11.34	.93	9.65	.76		19.36	2.49	24.61	2.16	24.55	2.73
Thymic platelets ($\times 10^3/mL$)		31.48	4.70	39.00	4.17	30.67	3.03	Mother	73.60^A	12.24	113.71^B	10.84	69.93^A	13.78
White blood cells ($\times 10^3/mL$)		4.43	.35	4.14	.28	4.43	.32	Mother	11.95^A	1.56	12.91^B	1.68	11.39^A	1.16
Red blood cells ($\times 10^6/mL$)		11.89	.32	11.22	.37	11.46	.21		7.04	.19	7.06	.12	6.84	.09
Hemoglobin (g/dL)		15.42	.38	15.73	.21	15.84	.21		13.70	.35	13.77	.36	13.93	.18
Hematocrit (%)		44.92	1.15	45.70	.79	45.52	.74		43.14	1.48	43.40	.67	42.24	.53
Mean corpuscle volume (fL)	Offspring	37.85^A	.32	39.6^B	.44	39.80^B	.28	Mother	61.23^A	.42	62.10^A	.40	59.04^B	.43
								Offspring	59.21^A	.73	61.57^B	.40	61.84^B	.34
Mean corpuscle hemoglobin (pg)	Offspring	13.22^A	.14	13.41^B	.11	13.27^B	.13	Mother	20.08^A	.70	20.27^A	.16	19.35^B	.19
								Offspring	19.49^A	.31	20.12^B	.15	20.28^B	.13
Mean corpuscle hemoglobin content (%)		34.32	.27	34.05	.17	34.02	.16		32.94	.17	32.61	.12	32.77	.14
Platelets ($\times 10^3/mL$)		619.81	34.76	647.17	25.86	639.43	21.76		526.93	19.85	529.06	17.71	509.82	17.32
Serum total proteins (g/dL)	Offspring	5.32^A	.21	4.86^B	.11	4.96^B	.07		5.50	.06	5.69	.07	5.77	.08
Serum total immunoglobulins (g/dL)		.63	.06	.54	.16	.64	.02	Mother	$.92^A$	.03	$.86^A$	.02	1.11^B	.02
Immunoglobulins (%)		11.26	2.22	11.31	3.46	13.31	.47	Mother	16.15^A	.59	15.22^A	.46	19.74^B	1.13
Lymphocytes (%)		88.91	2.17	88.27	1.62	85.06	1.36		68.60	5.82	68.92	2.28	67.71	3.05
Neutrophils (%)		10.73	2.03	10.93	1.63	14.47	1.33		27.50	5.79	28.52	2.27	29.76	3.00
Monocytes (%)		.27	.14	.80	.33	.35	.12		2.30	.54	1.76	.30	1.76	.33
Eosinophils (%)		.00	.00	.00	.00	.00	.00		.20	.13	.36	.13	.35	.17
Basophils (%)		.09	.09	.00	.00	.12	.08		.60	.50	.52	.21	.35	.19

Note. Offspring were born to mothers fed a diet low, medium, or high in nitrogen. After weaning, offspring from each mother were separated and themselves were fed a diet low, medium, or high in nitrogen. Observed differences among the offspring were the result of either maternal diet (mother) or offspring diet (offspring). Superscripts with different letters indicate statistical differences.

protein restriction reduced hemoglobin levels and corpuscle volume, as was observed in this study. It is important to note that this study is the first to include both maternal and offspring dietary effects on immune function in either cotton rats or harvest mice.

There are few studies investigating the effects of excess nitrogen in the diets of wild herbivores. However, this lack may be of growing concern because current and future fertilizer use and climatological changes may increasingly favor high-nitrogen plants. Our results indicate that, contrary to our a priori assumptions, at higher dietary nitrogen, individuals may face increasing energetic demands from the excretion of excess nitrogenous compounds, which may lead to decreased immune function. Although we did not measure metabolic rate or nitrogen excretion during this study, a concurrent study from the same lab measured nitrogen excretion in the same species (Parsons 2001; Clark et al. 2005). Parsons (2001) found that changes in liver and gut morphology were associated with both maternal and offspring dietary nitrogen. Clark et al. (2005), using results from Parsons (2001), found that increasing dietary nitrogen is associated with increased urinary nitrogen output and that with increasing dietary nitrogen, harvest mice increase nitrogen output faster than cotton rats. This provides indirect support for the hypothesis that the energy needed to eliminate excess nitrogen may reduce energy available for immunity.

Our results showed that in both harvest mice and cotton rats, kidney size increased with dietary nitrogen (maternal diet for harvest mice, offspring diet for cotton rats). Liver size also increased in cotton rats with increased dietary nitrogen (again, offspring diet). Similarly, Derting and Hornung (2003) found a positive correlation between dietary nitrogen and dry kidney mass in white-footed mice. Although our results are from a laboratory study, wild rodents face increased dietary nitrogen through alterations in habitat quality due to seasonal anthropogenic factors, such as grazing (Mellado and Olvera 2008). Animals exposed to high-nitrogen diets during times of additional stress (i.e., dehydration) may face increased energetic and water demands from increased kidney activity (Hewitt et al. 1981). Increased energetic demands associated with high-nitrogen diets may be responsible for our observed decrease in immune and hematological parameters in individuals on high-nitrogen diets. However, the hypothesis that individuals are facing an energetic cost to either increased or limited nitrogen is one plausible explanation among many, and it is not clear whether this cost would be sufficient to impose an energetic trade-off with other systems. Clearly, there is a great need to study the details of energetic demands among many species in various ecological systems.

Parsons (2001) found that harvest mice fed nitrogen-restricted diets do not alter litter size or offspring mass until they reach a critical level of dietary nitrogen (1.3% N) where they stop reproduction altogether. Cessation of reproduction under low-nitrogen diets (Parsons 2001) and the lack of maternal effects observed in this study suggests that there is a high nitrogen requirement for successful reproduction in harvest mice. In addition, the relative stability of harvest mouse offspring under varying conditions of maternal and offspring dietary nitrogen supports the hypothesis that harvest mice are less able to adjust for a poor-quality diet during pregnancy and will reproduce only when they have sufficient dietary nitrogen to produce "normal" offspring. This is likely a response to the high-protein diets that harvest mice typically have (Meserve 1976; Vazquez al. 2004).

Studies have shown that cotton rats are able to breed under a wide variety of conditions (Cameron and Eshelman 1996; Lochmiller et al. 2000). Although cotton rats are able produce young under suboptimal conditions, the immune system of these young may be compromised (McMurry et al. 1994*a*, 1994*b*), and the date of first estrus may be delayed (Cameron and Eshelman 1996). Some evidence suggests that the presence of high-protein forage (dicots, insects, and seeds) allows cotton rats to maximize their growth potential, resulting in high-density populations (Shetter et al. 1998). Cameron and Eshelman (1996) also found that cotton rats raised on a diet mimicking a strictly monocot diet (i.e., 4% protein) had lower body mass, growth rate, and lower tissue protein levels. However, some deficiencies may be overcome if sufficient nutrition is consumed later in life (Lochmiller et al. 2000), leading to a bet-hedging strategy for cotton rat reproduction.

Rodents living in the wild must obtain enough nutrients to support maintenance, growth, and reproduction. As a result, they are faced with making trade-offs about where to establish their home range, how much time to spend foraging, and when to reproduce. Many of these decisions are based on the amount of forage present in their environment and will have an impact not only on their growth but also on the development of their offspring's immune system. Kincaid and Cameron (1982) found that although female cotton rats were found in monocot-dominated habitat during most of the year, they switched to patches with more dicots during lactation, presumably in an effort to increase protein intake during an energetically demanding period. This shift in forage quality also would aid in preventing the immune and morphological effects observed in this study. This is reinforced by Eshelman and Cameron's (1996) observation that cotton rats would inhabit patches with low cover as long as they were supplemented with high-protein (15% N) food. Given the negative impact of high-nitrogen diets observed in this study, it is unclear whether cotton rats would continue to inhabit such patches if the protein was increased to the high levels (16%–20% N) used in our study.

Neonatal and juvenile mortality is high among wild mammals (Clutton-Brock et al. 1987) and is dependent on maternal condition during gestation (Mech et al. 1991). As a result, it has been hypothesized that there is a direct link between the nutritional status of females during gestation, immune function in offspring, and offspring survival to maturity (Lochmiller and Dabbert 1993; Lochmiller 1996; Lochmiller and Deerenberg 2000). In mammals, the last trimester of gestation and preweaning growth represent the stages of development most susceptible to nutritional deficiencies because they are characterized by rapid cellular proliferation (Lochmiller et al. 1982). Laboratory-induced nutritional deficiencies that occur during

late gestation have been shown to cause immunosuppression similar to that observed in this study. Gebhart and Newberne (1974) showed that maternal protein restriction during late gestation reduced cell-mediated and humoral immunity in offspring, even though mothers and offspring were fed a normal diet after parturition. Similarly, serum complement levels were reduced by one-third in offspring born to mothers fed a diet consisting of 8% protein (Watson et al. 1976). In addition, malnutrition during development may continue to affect immune function into the F1 and F2 generations (Chandra 1975).

Although both harvest mice and cotton rats showed reductions in immunity or hematology, the stark difference in the factors leading to these changes (i.e. maternal vs. offspring) suggests that these two species differ in their ability to cope with a nutritionally poor diet. Cotton rats are able to adjust their reproduction to accommodate a diet poor in nitrogen, whereas harvest mice cannot. Parsons (2001) suggests that harvest mice use a different reproductive strategy than cotton rats. Whereas cotton rats will reproduce under almost any condition, producing suboptimal offspring, harvest mice will reproduce only under conditions where they can afford to produce optimally viable offspring. Clearly, understanding the difference in the relative importance of maternal and offspring nutrition is important to the conservation of wild mammals. This is especially relevant given recent anthropogenic increases in nitrogen deposition that may significantly alter the plant community and change the nutritional structure of the landscape (Tilman 1987; Carson and Barrett 1988; Vitousek 1994; Bobbink et al. 1998; Matson et al. 2002).

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Literature Cited

American Society of Mammalogists. 1998. Guidelines for the capture, handling, and care of mammals as approved by the American Society of Mammalogists. J Mammal 79:1416–1431.

Belovsky G.E. 1986. Optimal foraging and community structure implications for a guild of generalist grassland herbivores. Oecologia 70:35–52.

Bobbink R., M. Hornung, and J.G.M. Roelofs. 1998. The effects of air-borne nitrogen pollutants on species diversity in natural and semi-natural European vegetation. J Ecol 86:717–738.

Boesch C. 1997. Evidence for dominant wild female chimpanzees investing more in sons. Anim Behav 54:811–815.

Bradford M.M. 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principal of protein-dye binding. Anal Biochem 72:248–254.

Brady M.J. and N.A. Slade. 2001. Diversity of a grassland rodent community at varying temporal scales: the role of ecologically dominant species. J Mammal 82:974–983.

Cameron G.N. and B.D. Eshelman. 1996. Growth and reproduction of hispid cotton rats (*Sigmodon hispidus*) in response to naturally occurring levels of dietary protein. J Mammal 77:220–231.

Cameron G.N. and S.R. Spencer. 1981. *Sigmodon hispidus*. Mamm Species 158:1–9.

Carson W.P. and G.W. Barrett. 1988. Succession in old-field communities: effects of contrasting types of nutrient enrichment. Ecology 69:984–994.

Chandra R.K. 1975. Antibody formation in first and second generation offspring of nutritionally deprived rats. Science 190:289–290.

Clark J.E., E.C. Hellgren, J.L. Parsons, E.E. Jorgensen, D.M. Engle, and D.M. Leslie Jr. 2005. Nitrogen outputs from fecal and urine deposition of small mammals: implications for nitrogen cycling. Oecologia 144:447–455.

Clutton-Brock T.H., M. Major, S.D. Albon, and F.E. Guinness. 1987. Early development and population dynamics in red deer. I. Density dependant effects on juvenile survival. J Anim Ecol 56:53–67.

Davis R.L., R.L. Lochmiller, and W.D. Warde. 1995. Splenocyte subpopulations of weanling cotton rats (*Sigmodon hispidus*) are influenced by moderate protein intake. J Mammal 76: 912–924.

Demas G.E., V. Chefer, M.I. Talan, and R.J. Nelson. 1997. Metabolic costs of mounting an antigen-stimulated response in adult and aged C57BL/6J mice. Am J Physiol 42:R1631–R1637.

Derting T.L. and S. Compton. 2003. Immune response, not immune maintenance, is energetically costly in white-footed mice (*Peromyscus leucopus*). Physiol Biochem Zool 76:744–752.

Derting T.L. and C.A. Hornung. 2003. Energy demand, diet quality, and central processing organs in wild white-footed mice (*Peromyscus leucopus*). J Mammal 84:1381–1398.

Dittus W.P.J. 1998. Birth sex ratios in toque macaques and other mammals: integrating the effects of maternal condition and competition. Behav Ecol Sociobiol 44:149–160.

Dobson F.S. and G.R. Michener. 1995. Maternal traits and reproduction in Richardson's ground squirrels. Ecology 76: 851–862.

Eraud C., O. Duriez, O. Chastel, and B. Faivre. 2005. The energetic cost of humoral immunity in the collared dove, *Streptopelia decaocto*: is the magnitude sufficient to force energy-based trade-offs? Funct Ecol 19:110–118.

Eshelman B.D. and G.N. Cameron. 1996. Experimentally induced habitat shifts by hispid cotton rats (*Sigmodon hispidus*): response to protein supplementation. J Mammal 77: 232–239.

Gaines M.S. and L.R. McClenaghan Jr. 1980. Dispersal in small mammals. Annu Rev Ecol Syst 11:163–196.

Gebhart B.M. and P.M. Newberne. 1974. Nutritional and immunological responsiveness: thymus-derived cell function in the offspring of lipotrope and protein deficient rats. Immunology 26:489–495.

Hewitt S., J.F. Wheldrake, and R.V. Baudinette. 1981. Water balance and renal function in the Australian desert rodent *Notomys alexis*: the effect of diet on water turnover rate, glomerular filtration rate, renal plasma flow, and renal blood flow. Comp Biochem Physiol A 68:405–410.

Hõrak P., M. Zilmer, L. Saks, I. Ots, U. Karu, and K. Zilmer. 2006. Antioxidant protection, carotenoids, and the costs of immune challenge in greenfinches. J Exp Biol 209:4329–4338.

Kerr T.D., S. Boutin, J.M. LaMontagne, A.G. McAdam, and M. Humphries. 2007. Persistent maternal effects on juvenile survival in North American red squirrels. Biol Lett 2007:289–291.

Kincaid W.B. and G.N. Cameron. 1982. Dietary variation in three sympatric rodents on the Texas coastal prairie. J Mammal 63:668–672.

Kingsley G.R. 1942. The direct Biuret method for determination of serum protein as applied to photoelectric and visual colorimetery. J Lab Clin Med 27:840–845.

Klassing K.C. 1998. Nutritional modulation of resistance to infectious diseases. Poult Sci 77:1119–1125.

Lochmiller R.L. 1996. Immunocompetence and animal population regulation. Oikos 76:594–602.

Lochmiller R.L. and C.B. Dabbert. 1993. Immunocompetence, environmental stress, and the regulation of animal populations. Trends Comp Biochem Physiol 1:823–855.

Lochmiller R.L. and C. Deerenberg. 2000. Trade-offs in evolutionary immunology: just what is the cost of immunity? Oikos 88:87–98.

Lochmiller R.L., S.S. Ditchkoff, and J.A. Sinclair. 2000. Developmental plasticity of postweaning cotton rats (*Sigmodon hispidus*) as an adaptation to nutritionally stochastic environments. Evol Ecol 14:127–142.

Lochmiller R.L., J.B. Whelan, and R.L. Kirkpatrick. 1982. Seaonal energy requirements of adult pine voles *Microtus pinetorum*. J Mammal 63:475–481.

Matson P., K.A. Lohse, and S.J. Hall. 2002. The globalization of nitrogen deposition: consequences for terrestrial ecosystems. Ambio 31:113–119.

McMurry S.T., R.L. Lochmiller, M.R. Vestey, and C.W. Qualls Jr. 1994*a*. Cellular immune responses of nutritionally stressed juvenile cotton rats (*Sigmodon hispidus*) during acute benzene exposure. Arch Environ Contam Toxicol 27: 14–19.

———. 1994*b*. Immunological responses of weanling cotton rats (*Sigmodon hispidus*) to acute benzene and cyclophosphamide exposure. Bull Environ Contam Toxicol 52:155–162.

Mech L.D., M.E. Nelson, and R.E. McRoberts. 1991. Effects of maternal and grandmaternal nutrition on deer mass and vulnerability to wolf predation. J Mammal 72:146–151.

Mellado M. and A. Olvera. 2008. Diets of prairie dogs (*Cynomys mexicanus*) co-existing with cattle or goats. Mamm Biol 73: 33–39.

Meserve P.L. 1976. Food relationships of a rodent fauna in a California coastal sage scrub community. J Mammal 57:300–319.

Norris K. and M.R. Evans. 2000. Ecological immunology: life history trade-offs and immune defense in birds. Behav Ecol 11:19–26.

Parsons J.L. 2001. Nitrogen Requirements of Southern Plains Small Mammals: Dietary Niche, Life History, and Environmental Implications. MS thesis. Oklahoma State University, Stillwater.

Parsons J.L., E.C. Hellgren, and E.E. Jorgensen. 2005. Neonatal growth and survival of rodents in response to variation in maternal dietary nitrogen: life-history strategy vs. dietary niche. Oikos 110:297–308.

Rådberg L., M. Grahn, D. Hasselquist, and E. Svensson. 1998. On the adaptive significance of stress-induced immunosuppression. Proc R Soc B 265:1637–1641.

Rådberg L., M. Vestberg, D. Hasselquist, R. Holmdahl, E. Svensson, and J.A. Nilsson. 2002. Basal metabolic rate and the evolution of the adaptive immune system. Proc R Soc B 269: 817–821.

Randolph J.C. and G.N. Cameron. 2001. Consequences of diet choice by a small generalist herbivore. Ecol Monogr 71:117–136.

Randolph J.C., G.N. Cameron, and J.A. Wrazen. 1991. Dietary choice of a generalist grassland herbivore, *Sigmodon hispidus*. J Mammal 72:300–313.

Robbins C.T. 1993. Wildlife Feeding and Nutrition. 2nd ed. Academic Press, San Diego, CA.

SAS Institute. 2000. SAS/STAT User's Guide. Release 8.1. SAS Institute, Cary, NC.

Schetter T.A., R.L. Lochmiller, D.M. Leslie Jr., D.M. Engle, and M.E. Payton. 1998. Examination of the nitrogen limitation hypothesis in non-cyclic populations of cotton rats (*Sigmodon hispidus*). J Anim Ecol 67:705–721.

Sheldon B.C. and S.A. West. 2004. Maternal dominance, maternal condition, and offspring sex ratio in ungulate mammals. Am Nat 163:40–54.

Spencer S.R. and G.N. Cameron. 1982. *Reithrodontomys fulvescens*. Mamm Species 174:1–9.

Stancampiano A.J. and W. Caire. 1995. Food habits of *Peromyscus* and *Reithrodontomys* in the Wichita Mountains Wildlife Refuge, Oklahoma. Proc Okla Acad Sci 75:45–49.

Tardif S.D. and K.L. Bales. 2004. Relations among birth con-

dition, maternal condition, and growth in captive common marmoset monkeys (*Callithrix jacchus*). Am J Primatol 62: 83–94.

Tilman D. 1987. Secondary succession and the pattern of plant dominance along experimental nitrogen gradients. Ecol Monogr 57:189–214.

Vazquez L.B., G.C. Cameron, and R.A. Medellín. 2004. Characteristics of diet of *Peromyscus aztecus* and *Reithrodontomys fulvescens* in montane western Mexico. J Mammal 85:196–205.

Vitousek P.M. 1994. Beyond global warming: ecology and global change. Ecology 75:1861–1876.

Watson R.R., M. Rister, and R.L. Baehner. 1976. Superoxide dismutase activity in polymorphonuclear leukocytes and alveolar macrophages of protein malnourished rats and guinea pigs. J Nutr 106:1801–1808.

Westneat D.F. and T.R. Birkhead. 1998. Alternative hypotheses linking the immune system and mate choice for good genes. Proc R Soc B 265:1065–1073.

White T.C.R. 1978. The importance of a relative shortage of food in animal ecology. Oecologia 33:71–86.

Wiley D.N. and P. J. Clapham. 1993. Does maternal condition affect the sex ratio offspring in humpback whales? Anim Behav 46:321–324.

Wilson J.A., R.L. Carlson, D.M. Janz, R.L. Lochmiller, J.L. Schroder, and N.T. Basta. 2003. Ecotoxicological risks associated with land treatment of petrochemical wastes. III. Immune function and hematology of cotton rats. J Toxicol Environ Health A 66:345–363.