PHENOTYPIC VARIATION OF AND EFFECT OF

DIETARY PROTEIN ON SELECTED MEASURES

OF IMMUNOCOMPETENCE IN THE COTTON

RAT (<u>SIGMODON HISPIDUS</u>)

By

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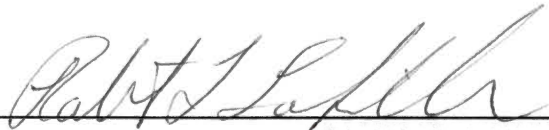
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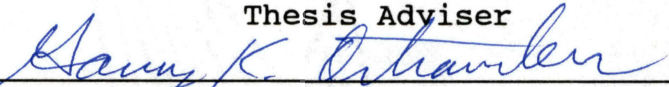DOCTOR OF PHILOSOPHY
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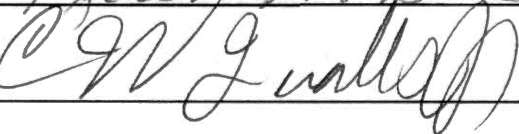OF IMMUNOCOMPETENCE IN THE COTTON

RAT (<u>SIGMODON HISPIDUS</u>)

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PREFACE

The study of immune function in wild mammalian populations has been limited. The immunocompetence of wild mammals undoubtedly contributes to their survival and thus to the dynamics of the population as a whole. We selected the hispid cotton rat, <u>Sigmodon</u> <u>hispidus</u>, as a model for the development of immunoassays which are applicable to outbred wild populations and for the assessment of the effects of various extrinsic factors (nutrition and captivity) and intrinsic factors (age, gender) on selected measures of immunocompetence. This thesis is comprised of six manuscripts formatted for submission to Journal of Experimental Zoology (Chapter I), Journal of Mammalogy (Chapters II and IV), Developmental and Comparative Immunology (Chapter III), and Canadian Journal of Zoology (Chapters V). The manuscripts are complete as written and need no supporting material.

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CHAPTER I

VARIABILITY IN PRIMARY ANTIBODY RESPONSIVENESS TO SHEEP

ERYTHROCYTES IN THE COTTON RAT (<u>SIGMODON</u> <u>HISPIDUS</u>)

ABSTRACT

Variation in humoral immune response to sheep erythrocytes (SRBC) was studied in 44 captive (conventional outbred colony) and 38 wild-caught cotton rats (Sigmodon hispidus). Primary antibody response to an intraperitoneal injection of a 10% suspension of SRBC was measured using the hemolytic plaque assay. Responses ranged from 0 to 3880 plaque-forming cells (PFC)/10^6 splenocytes. Subadult cotton rats (60 - 99g body weight) had significantly higher PFC/10^6 splenocytes and PFC/mg spleen than did adults and tended to have higher total PFC/spleen. There were no significant differences between juveniles and adults or subadults in primary antibody response to SRBC. Captive cotton rats had significantly greater numbers of PFC/10^6 splenocytes and PFC/mg spleen than did wild-caught animals while total PFC/spleen did not differ. The age-related differences in humoral immune response appear to be a natural tendency towards immune depression with senescence as described previously although occurring in the cotton rat at a younger age than in laboratory rodents. Differences between wild and captive animals may reflect historical differences in nutrition.

INTRODUCTION

Genetic control of immunity has been well established for both humoral and cell mediated responses in inbred populations of a variety of mammals. This control appears

to be polygenic in nature and involves the major histocompatibility genes (Klein,'86). Extrapolation of this concept to outbred populations implies the existence of a range of abilities to respond to antigenic challenge and this is supported by the literature. Biozzi et al. ('75), starting with a randomly bred population of albino mice, were able to select for the ability to generate hemagglutinating antibody titers to sheep erythrocytes (SRBC) and, after six generations, generated a population of high responders and a population of low responders. Likewise, Siegel and Gross ('80) were able to produce high and low responder populations of chickens to SRBC using directional selection on an outbred population.

Although the above studies were based on outbred populations, there is a paucity of information available on the distribution of immune responsiveness in wild populations. Shonnard et al. ('79) demonstrated that a dimorphism exists in the ability of a wild population of Norway rats to generate an antibody response to poly($Glu^{52}Lys^{33}Tyr^{15}$) but little else has been published. The objective of our study was to elucidate the degree of variability in primary antibody response to a T-dependent antigen (SRBC) in populations of both wild and captive cotton rats (Sigmodon hispidus). We were particularly interested in the relationships among age, sex, captivity, and immunologic responsiveness.

MATERIAL AND METHODS

<u>Animals</u>

A total of 83 cotton rats was used in this study.
Cotton rats were classified as either wild (used within 3
wks of capture from the wild) or captive (≥ 3 wks in
captivity or born in captivity). All wild animals were
captured from tallgrass prairie habitats approximately 5-8
km west of Stillwater, OK. Cotton rats were collected
intermittently from October 1988 to May 1989 using Sherman
live-traps baited with rolled oats. On arrival at the
laboratory animal facility, cotton rats were housed in
either wire-mesh bottom hanging cages or polypropylene boxes
with wood shavings and cotton for bedding. Commercially
available rabbit chow (pelleted; crude protein = 15%) and
water were provided ad libitum and the animal room was
maintained on a 14:10 light:dark cycle with ambient
temperature (25°). Captive cotton rats born in the
laboratory animal facility resulted from in-house breedings
between randomly selected males and females. Offspring were
weaned at 3 wks of age and housed as described for adults.
Age class designations were determined on the basis of body
weight: adults (≥ 100g), subadults (60 - 99), and juveniles
(< 60g) (Odum,'55).

<u>Immunization</u>

Five days prior to sacrifice, each cotton rat was
injected intraperitoneally with 0.2 ml of a 10% suspension

of SRBC (Colorado Serum Company, Denver, CO) in phosphate
buffered saline, pH 7.4 (PBS). Sham control animals (4)
received intraperitoneal injections of PBS only and control
animals (5) were not injected.

Necropsy

At the time of sacrifice, each cotton rat was
anesthetized with an intramuscular injection of ketamine
hydrochloride (Aveco, Fort Dodge, IA)) at 50 mg/kg body
weight, weighed, and bled from the retro-orbital plexus.
Animals were sacrificed by cervical dislocation while
anesthetized, spleen and thymus gland removed, and wet
weights determined to the nearest 0.1 mg. The spleen was
hemisected and splenocytes gently expressed in a glass-on-
glass tissue homogenizer containing 5 mls of PBS with 5%
fetal calf serum (Sigma Chem. Co., F4135, St.Louis, MO; PBS-
F). The resulting cell suspension was placed on ice for 10
min to allow sedimentation of the large tissue fragments.
Cells were then decanted and centrifuged at 250 g for 7 min
at 10°C. The resulting cell pellet was resuspended in Tris-
ammonium chloride (0.83%) to lyse red blood cells and, after
2 min, the cell suspension was underlayed with 1.0 ml of
fetal calf serum (100%). Following a room temperature
incubation for 7 min, the cell suspension was centrifuged as
before. The cells were resuspended and washed twice in 5
mls of PBS-F with final resuspension in 5 mls of PBS-F.

Splenocytes were counted using the Trypan Blue exclusion procedure and a hemacytometer.

SRBC Plaque Assay

The plaque assay used was adapted from Cunningham and Szenberg ('68). The splenocyte preparations were adjusted to a final concentration of 4.8×10^6 cells/ml and either 50 μl, 100 μl, or 200 μl aliquots were diluted to 220 μl with PBS-F in 12 x 75 mm culture tubes. Eighty μl of a 25% suspension of SRBC in PBS-F and 100 μl of 25% guinea pig serum (Bioproducts for Science, Indianapolis, IN) in PBS-F were added to each tube and the mixture vortexed. Incubation chambers were prepared as described by Marbrook ('80) and loaded with 125 μl of the resulting mixture. Incubation chambers were sealed with a molten mixture of Vaseline and paraffin (1:1 w/w) and incubated at 37°C for 2 hr. The resulting plaques were counted using a dissecting microscope.

Hemagglutination Assay

Cotton rat sera to be assayed for hemagglutination titers were heat inactivated at 56°C for 30 min. Fifty μl of each serum were serially diluted in 96-well microtiter plates containing 50 μl PBS in each well. Fifty μl of a 1% suspension of SRBC in PBS were added to each well and the plates incubated at 37°C for one hr. The hemagglutination

titer was defined as the reciprocal of the highest dilution of serum resulting in visible hemagglutination.

Statistical Analysis

The effects of sex, age, and captivity of immunized animals on antibody response to SRBC and lymphoid organ measurements were determined by analysis of variance using SYSTAT (Wilkinson,'89). Analysis of the raw data using Bartlett's chi-square test of homogeneity of variances indicated that group distribution frequencies were heterogeneous so statistical analyses were performed on ranked data (Conover and Iman,'81). The chi-square contingency test was used to determine independence of sex, age class, and captivity status and percentage of high responders (Steel and Torrie,'80). Correlation between hemagglutinating antibody titers and PFC response was analyzed using a Pearson correlation matrix (Steel and Torrie,'80).

RESULTS

The only significant ($P < 0.05$) differences between sexes were for spleen weight (males, 129.5 mg ± 14.9 (SE); females, 104.1 mg ± 13.7) and body weight (males, 85.4 gm ± 8.3; females, 75.5 gm ± 4.5). However, since spleen to body weight ratios (mg spleen/gm body weight) did not differ significantly ($P = 0.133$) between males and females, sex was

not considered as a source of variation in subsequent statistical analyses.

Distribution of PFC Response

We considered any animal having a primary antibody response greater than or equal to 90 plaque-forming cells (PFC)/10^6 splenocytes to be a high responder and animals with plaque counts < 90 PFC/10^6 splenocytes were considered low responders. Although somewhat arbitrary, we observed a natural break in the frequency distribution of responsiveness at 90 PFC/10^6 splenocytes. There was no significant difference in response between sham control and uninjected control animals so these were combined into a single group of control animals which were considered non-responders. A total of 74 cotton rats were immunized with SRBC and of these 51% were classified as low responder (Table 1). The mean PFC response for the low responders was not significantly (P > 0.05) different from unimmunized controls; 16 out of 38 low responders had PFC counts less than the control mean of 9.0/10^6 splenocytes. The frequency distribution of high responders was significantly (P = 0.052) affected by age class, where 62.1% of subadults and 26.3% of adults were high responders (Fig.1). Frequency of high responders was also influenced significantly (P = 0.03) by captivity status with 59.1% of captive animals and 33.3% of wild-caught animals being high responders (Fig.1).

Age Effects on PFC Response

Primary antibody response to SRBC when expressed as PFC/10^6 splenocytes (P < 0.03) and PFC/mg spleen (P < 0.03) differed significantly among age classes (Table 2). Differences among age classes in total PFC/spleen approached significance (P < 0.07). In all cases adults demonstrated a reduced capacity to respond to SRBC compared to juveniles and subadults. Subadults and juveniles tended to be statistically different from each other only in total plaque-forming cell production (P = 0.072). Mean PFC/10^6 splenocytes tended to be lower in adults than juveniles (P = 0.072).

Absolute and relative weight of the thymus gland (mg/g body weight) decreased (P < 0.001) with older age classes due to the natural atrophy of this organ (Fig. 2) (Miller,'63). Absolute weight of spleen increased significantly (P < 0.001) with older age classes but age-related differences were absent when spleen weights were expressed relative to body weight. Total splenocyte yield was significantly lower (P < 0.001) in juveniles than either subadults or adults. Relative weight of the spleen in juveniles was significantly lower than subadults (P < 0.001) but neither juvenile or subadult relative weights differed from adults (P = 0.151 and P = 0.061, respectively).

Effect of Captivity on PFC Response

Captive animals had significantly greater numbers of PFC/10^6 splenocytes (P < 0.02) and PFC/mg spleen (P < 0.02)

than did wild-caught cotton rats (Table 2). There was no difference (P = 0.382) in total PFC production between captive and wild- caught cotton rats (Table 2).

Thymus weight did not differ (P > 0.05) between captive and wild-caught animals (overall mean = 40.6 mg). Absolute (P < 0.001) and relative (P < 0.001) weights of the spleen were greater in wild-caught than captive cotton rats (Fig.3). Total splenocyte production was significantly greater (P < 0.001) in wild-caught than captive cotton rats. Differences in relative splenocyte production (cells/mg spleen) were not significantly (P > 0.05) different due to captivity status.

Hemagglutinating Antibody Production

Interestingly, we were unable to correlate serum antibody production, as measured by hemagglutination titers against SRBC, to PFC/10^6 splenocytes (r = 0.103), PFC/mg spleen (r = 0.097), or total PFC/spleen (r = 0.082)(Table 2). This may be due to a time lag between peak splenic plaque-forming cell response and peak serum antibody titers (Fig. 4). Temporal changes in primary serum hemagglutination titers in cotton rats peaked 7 days after challenge and titers began to decline slightly 9 days after challenge. Plaque-forming cell assays were always performed on day 5 after challenge.

DISCUSSION

Information on the development and function of the immune system, including variability in immune responsiveness within populations, is extremely limited for wild mammalian species. We are unaware of any previous reports which document the distribution of and contributing factors to humoral immune responsiveness in a wild rodent population. As reported in earlier work with inbred populations, immune response is polygenically controlled (Biozzi et al.,'79). The Ir, or immune response, genes are polymorphic loci located in the major histocompatibility complex which dictate an animal's ability to recognize simple T-dependent antigenic epitopes in an all-or-none fashion (Klein '86). Other gene loci located outside the MHC are also involved in immune response to complex antigens. Covelli et al. ('89) determined in white mice that control of primary antibody response to SRBC is mediated by 5-8 independent loci within and outside of the MHC. They also demonstrated that although SRBC was the selection antigen, the resulting high and low responding populations showed similar responsiveness to unrelated antigens (Biozzi et al.,'79). Our findings support the implication that wild populations, under the influence of multiple genes controlling immune response, will exhibit a broad range of abilities to respond to SRBC antigen. While almost 49% of the cotton rats in our sample were classified as high responders, with a wide range of responses (92 -3880

$PFC/10^6$ splenocytes), 22% were unable to produce a measurable primary immune response when compared to unimmunized controls.

Extrinsic environmental and intrinsic physiological factors may also influence immunologic responsiveness in animals, above and beyond genetic control. In cotton rats, ability to respond to SRBC immunization was not influenced by sex, but was apparently influenced by both age and captivity.

The literature is replete with references on the effects of aging on immunity (Goidl,'87; Hazzard and Soban,'88). Senescence in many mammals is accompanied by depressed immune system function (Goidl,'87). Typically, diminished ability to respond immunologically to antigen in laboratory rodents is not observed until the rodents are 2-3 yrs old (Goidl,'87). Our results indicate that adult cotton rats, none of which were older than 18 months of age, have reduced responsiveness to antigenic challenge. This was associated with concomitant reductions in thymic mass and a tendency towards reduced splenocyte production (per mg spleen basis) in the adult age class. This may be a direct effect of the aging process which has been shown to manifest itself as a combination of reduced T-helper function (Segre and Segre,'76), increased anti-idiotype suppression (Klinman,'81), and decreased frequency of antigen responsive splenic B-cells (Zharhary,'86). Our juvenile rats also showed a tendency to reduced immune response although not as

pronounced as that seen in the adults. This is likely due to the immunological immaturity of very young cotton rats and an aging scheme which allows inclusion of cotton rats with more mature immune systems with the very young cotton rats. It has been demonstrated in Swiss-L laboratory mice that adult levels of immunocompetence are not achieved until 4-6 wks of age (Spear and Edelman,'74).

Cotton rats maintained for extended periods in the laboratory had greater primary PFC responses to antigenic challenge than wild-caught animals. Differences between captive and wild-caught animals were apparent for all 3 age classes, and were especially prominent for the juveniles. This apparent captivity effect may be, in part, due to differences in nutritional status. Cotton rats consume a herbivorous diet in the wild and deficiencies in protein and other essential nutrients are probably common during development (White,'78). In comparison, cotton rats maintained in our captive colony were fed a complete, high quality protein ration ad libitum and typically gained additional body weight. Nutritional status has been shown to greatly impact immunological competence in many mammalian species (Watson,'84; Gershwin et al.,'85) including inbred rats and mice, domestic animals, and humans. In particular, dietary protein restriction can result in decreased thymus weight and, to a lesser extent, decreased spleen weight in mice (Bell et al.,'76). Kenney et al. ('68) reported similar effects of protein restriction in rats, where

reduced spleen weights were also related to reduced antibody titers to SRBC. Adoptive transfer experiments suggest that depressed antibody responsiveness associated with dietary protein restriction may be due to impaired accessory cell function (Price,'78). Another plausible explanation for the low responsiveness of wild cotton rats is cross reactivity of this specific antigen (SRBC) with environmental antigens such as those of parasitic origin encountered in the wild to which the animal has developed tolerance (Hraba,'68).

Disease resistance is undoubtedly a key factor in the regulation of wildlife populations. Biozzi et al. ('84) found that SRBC-selected high and low responder groups differed in their response to a variety of immunological challenges and that these differences correlated with ability of macrophages to process antigen. The high responders had a longer life span and a lower incidence of spontaneous tumor formation while the low responders had a stronger resistance to helminthic and bacterial infections (Biozzi et al.,'79; Biozzi et al.,'84). It is tempting to speculate that a wild population should exhibit a phenotypic distribution of immunological responsiveness that would optimize survival. If true, it is not difficult to see why low responsiveness to SRBC challenge might be the dominant phenotype in some wild cotton rat populations. Considering that successful reproduction in an r-selected species such as the cotton rat is dependent on survival of the neonates (MacArthur and Wilson,'67), resistance to bacterial and

parasitic infection would be desirable. Resistance to
spontaneous tumors, which in laboratory rats and mice occur
in adults (Covelli et al.,'89), is probably not as important
in a species for which the estimated average life span in
the wild is 6 months (Cameron and Spencer,'81). Additional
studies of the immune system of the cotton rat should
provide further insight into the mechanisms whereby
environment influences the regulation of populations.

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Table 1. Mean (± SE) and range of PFC responses (plaque-forming cells/10^6 splenocytes) to sheep erythrocytes in cotton rats. Mean PFC responses with different superscripts are significantly different at $P < 0.05$.

Response	PFC	Range	N
Unimmunized	$9 ± 3^a$	0 - 21	9
Low responders	$19 ± 3^a$	0 - 69	38
High responders	$766 ± 142^b$	92 - 3880	36

Table 2. Age-class- and captivity-related effects on antibody response to sheep red blood cell immunization in cotton rats (mean ± SE).

	Age class			P	Captivity		P
	Juvenile (n=26)	Subadult (n=29)	Adult (n=19)		Captive (n=44)	Wild-caught (n=30)	
PFC[a]/10^6 splenocytes	454 ± 135	478 ± 152	139 ± 102	0.029	527 ± 125	171 ± 65	0.011
Total PFC	13745 ± 7367	18903 ± 5631	5812 ± 3349	0.069	14771 ± 4172	12203 ± 6230	0.382
PFC/mg spleen	162.9 ± 74.7	239.6 ± 69.2	48.9 ± 35.9	0.028	208.5 ± 54.1	98.1 ± 54.9	0.046
Hemagglutinating titer ($\log_2$)	7 ± 2	11 ± 3	14 ± 5	0.562	8 ± 2	14 ± 3	0.116

[a] PFC: plaque forming cell

Figure 1. Percentage of high responders to SRBC immunization by sex, age class, and captivity. Numbers over each bar indicate sample size (n).
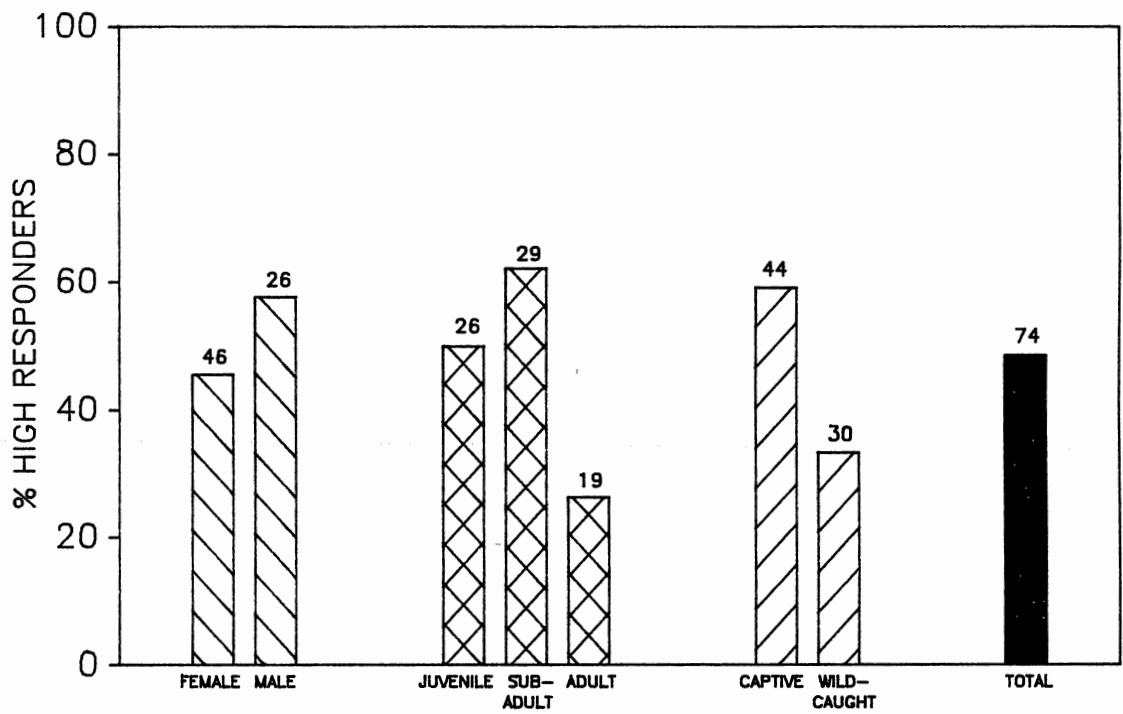
Figure 2. Age-related changes in thymus (A), spleen (B), and splenocyte (C) measurements in cotton rats (mean ± SE). Statistically significant differences ($P < 0.05$) among age classes for a given measurement are indicated by different superscripts over the standard error bars.
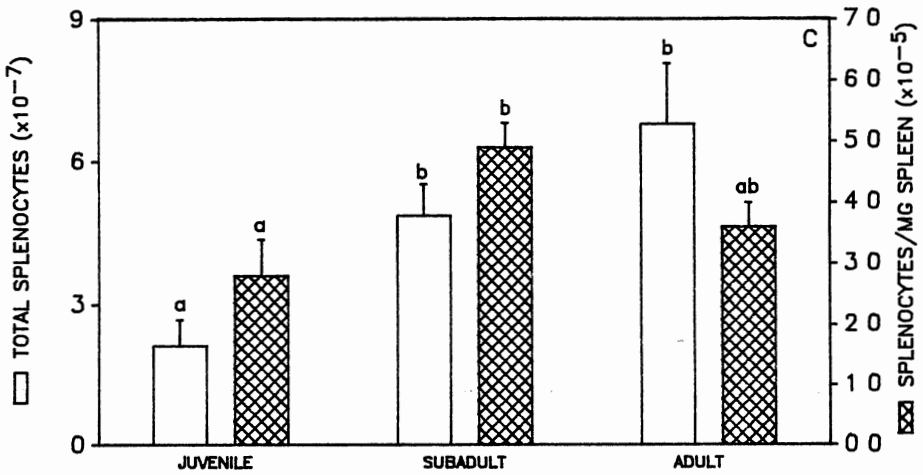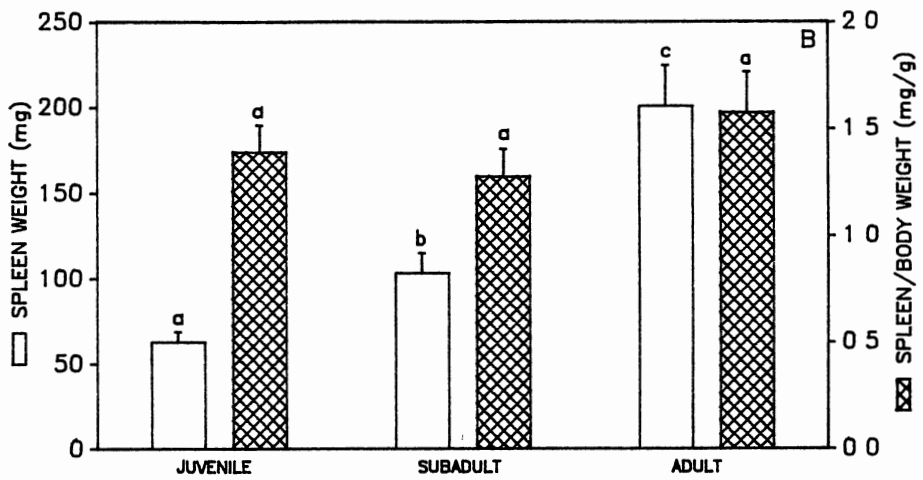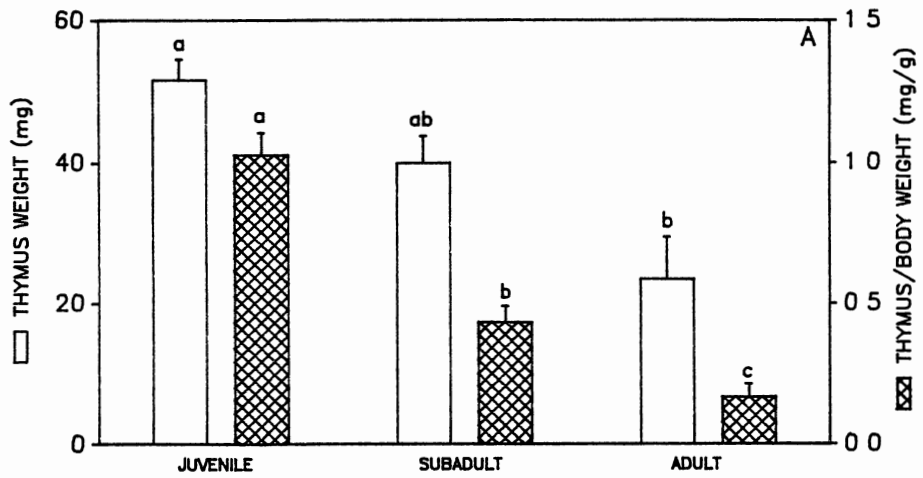
Figure 3. Captivity-related changes in spleen (A) and
splenocyte (B) measurements in cotton rats (mean ± SE).
Statistically significant differences (P < 0.05) between
captive and wild-caught animals for a given measurement are
indicated by different superscripts over the standard error
bars.
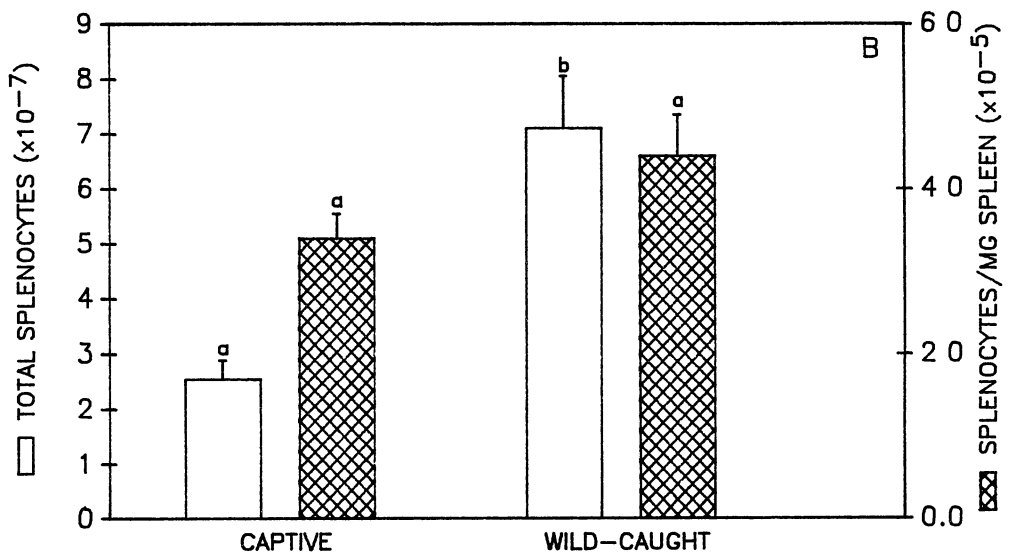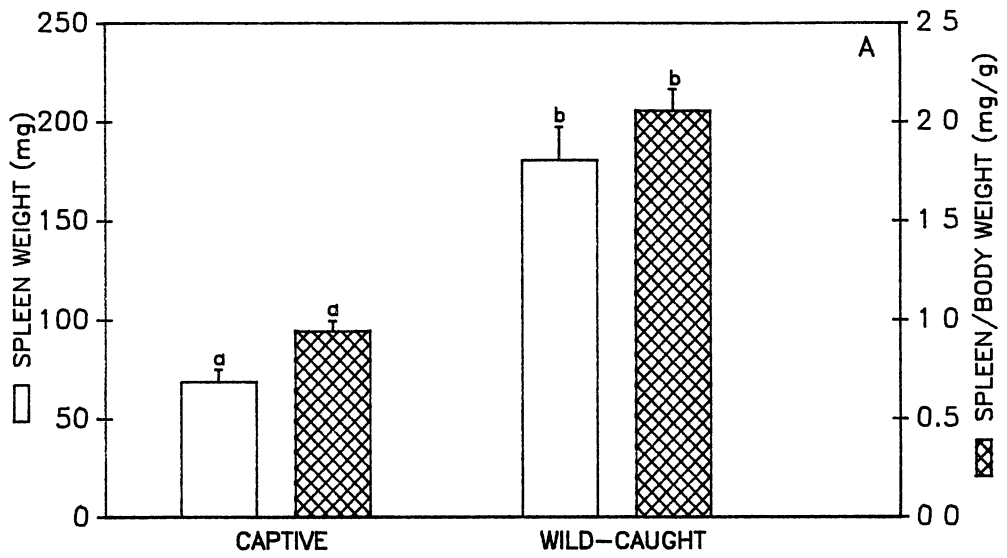
Figure 4. Relationship between days post immunization with SRBC and serum hemagglutinating antibody levels in six cotton rats.
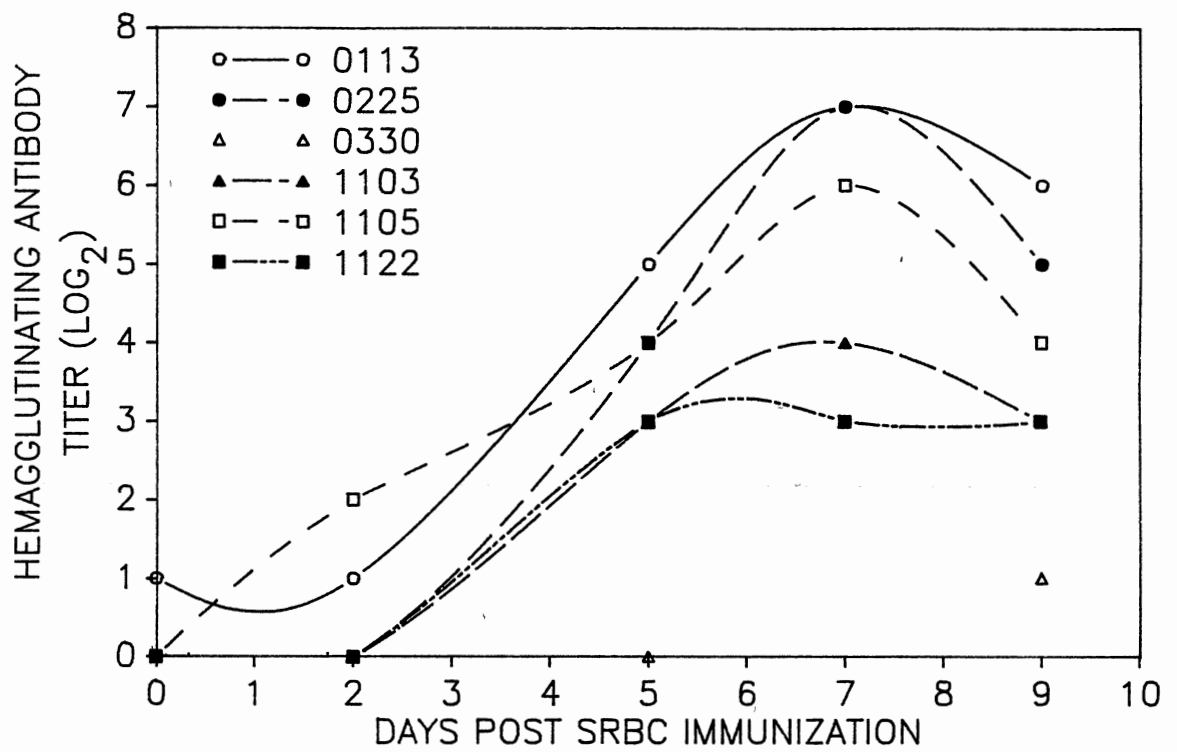
CHAPTER II

PHENOTYPIC VARIATION OF DELAYED-TYPE HYPERSENSITIVITY

RESPONSE IN <u>SIGMODON</u> <u>HISPIDUS</u>

The mechanisms regulating the fluctuation of mammalian populations are not well understood although many intrinsic and extrinsic factors have been suggested as influences for this regulation (Flowerdew, 1987). Immunocompetence is an intrinsic factor which certainly has the potential to be important in population dynamics. The role of immunocompetence in population regulation of wildlife can best be elucidated by identifying normal levels of immunocompetence and correlating changes with alterations in population density. The successful assessment of immune function of individuals within a wild population requires the development of valid immunoassays for measuring both cell-mediated and humoral aspects of immune function. Assessments of immune function can also be facilitated by the existence of data regarding the "normal" phenotypic distribution of responses within a population.

This report details the adaptation of an assay to measure delayed-type hypersensitivity responsiveness in the cotton rat (<u>Sigmodon</u> <u>hispidus</u>). Delayed-type hypersensitivity is a manifestation of a series of in vivo events which includes both antigen specific T-lymphocyte response and antigen independent responses mediated by a variety of cell types. Specifically we describe the degree of phenotypic variation of responses observed in a population as well as the relationship of delayed-type hypersensitivity response to other measures of immunocompetence.

All cotton rats used in this study were born in our
laboratory animal facility and housed in wire-mesh bottom
hanging cages or polypropylene boxes with wood shavings and
cotton for bedding. Commercially available rodent chow
(Purina 5001, St. Louis, MO; crude protein = 24%) and water
were provided ad libitum and the animal room was maintained
on a 14L:10D cycle with ambient temperature (25°C). Age
class designations were determined on the basis of body
weight (Odum, 1955): adults ($\geq$100 g), subadults (60-99 g),
and juveniles (<60 g).

Delayed-type hypersensitivity (DTH) response was
assayed using the procedure described by Jones (1983). On
day 0, each animal was sensitized with 100 μl of 3%
oxazolone in absolute ethanol applied percutaneously to a
small shaved area on the abdomen. The animals were
challenged on day 7 with 25 μl of 3% oxazolone in absolute
ethanol applied to each surface of the left ear; vehicle
only (absolute ethanol) was applied to the right ear. On
day 8, each cotton rat was anesthetized with an
intramuscular injection of ketamine hydrochloride (Aveco,
Fort Dodge, IA)) at 50 mg/kg body weight, weighed, and bled
from the retro-orbital plexus to collect whole blood.
Animals were euthanized by cervical dislocation while
anesthetized and both ears were removed at the base and
weighed to the nearest 0.1 mg. The DTH response was
calculated as absolute difference in ear weights and as a
relative percent increase in weight of the left ear

(experimental) over the right ear (control). The spleens were aseptically removed into pre-tared 15 x 60 mm sterile Petri dishes containing 5.0 mls RPMI-S consisting of RPMI-1640 supplemented with L-glutamine (2.05 mM), sodium pyruvate (1 mM), non-essential amino acids (1 mM), 2-mercaptoethanol ($2x10^{-5}$ M), penicillin (100,000 u/L), streptomycin (100 mg/L), and normal horse serum (10%). The thymus was also removed and both organs weighed to the nearest 0.1 mg. Total peripheral leukocyte (WBC) counts were determined by diluting an aliquot of whole blood in 2% glacial acetic acid and quantifying with a hemacytometer.

Lymphoproliferation was measured by cellular reduction of tetrazolium salt (Mosmann, 1983). The spleen was hemisected and splenocytes gently expressed in a glass-on-glass tissue homogenizer containing 5 mls of ice-cold RPMI-S. The resulting cell suspension was placed on ice for 10 min to allow sedimentation of the large tissue fragments. The cell suspension was transferred to a sterile 16 x 125 mm culture tube and centrifuged at 275 x$\underline{g}$ for 10 min at 20°C. The cells were washed 3 times in 5 mls of medium with centrifugation as above. Splenocyte numbers were determined by diluting an aliquot of the suspension in Trypan blue and counting on a hemacytometer. Splenocyte concentrations were adjusted to 0.5 x 10^6 cells/0.09 mls and 0.09 ml aliquots were cultured in 96-well plates with 0.01 ml of concanavalin A (Canavalia ensiformis; Con A) at 2.5, 5.0, 10.0, and 20.0 µg/ml culture. Aliquots of cells in the absence of mitogen

were cultured as unstimulated controls. After 69 hrs of incubation at 37°C with 5% CO_2, 10 μl of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; 5 mg/ml in PBS) was added to each well. At 72 hrs, 160 μl of acid-isopropanol (0.04 M HCl in isopropanol) was added to each well and absorbance at 570 and 630 nm was quantified using a Titertek Multiskan Plus microtiter plate reader. Response was calculated as the difference between absorbances of mitogen-stimulated cultures and control cultures.

Differences in DTH response, morphology, and lymphoproliferative response due to age and sex of cotton rats were examined by analysis of variance (SAS, 1985). Duncan's multiple range test was used to compare treatment means when main effects were significant. Correlations between measures of DTH and other morphological and functional measures of immunocompetence were made using Pearson correlation analysis.

A total of 115 cotton rats was sampled for this study. There were 61 adults, 42 sub-adults, and 12 juveniles; 59 animals were males. Relative DTH responses (percent increase of experimental ear weight over control) ranged from 0 to 87.8% with an overall mean of 21.1 ± 1.5% (SE). There was no measurable hypersensitivity response in 18% of the cotton rats and 74% of the responses were less than 30% (Fig. 1). It is known that DTH response to oxazolone (Fachet and Andó, 1978), as well as other immunological

responses (Benacerraf and McDevitt, 1972; Lubet and Kettman, 1978), is controlled in part by genes associated with the major histocompatibility complex. The genes in this complex are characterized by remarkable polymorphism (Klein, 1986) and it is not unexpected to find considerable variation in measures of immune response in a natural population. This is supported by the finding that in guinea pigs sensitized to 2,4-dinitrophenyl conjugates of poly-L-lysine, DTH response was strain-dependent with 100% of strain II guinea pigs responding, 40% of Hartley strain responding, and no responders in the strain XIII animals (Levine et al., 1963).

The DTH response requires T-lymphocytes which elicit the response through antigen-specific recognition and control the response through suppression. Absence of a measurable response, as seen in 21 of the cotton rats in this study, can be the result of absence of T-cells which recognize the antigen or preferential antigen-specific stimulation of the suppressor T-cells which results in premature cessation of the reaction (Turk, 1980). Immune unresponsiveness can also be non-specific where T-cell function in general is abrogated but this seems unlikely in this study in the absence of significant correlation of DTH response with other measures of immunocompetence such as peripheral blood leukocyte counts, thymus weights, and lymphoproliferative responses to in vitro stimulation with a T-cell specific mitogen (Table 1).

Absolute DTH responses were significantly (P = 0.0001) influenced by age class with adults having significantly (P < 0.05) greater responses than subadults or juveniles whereas relative DTH responses were independent (P = 0.2550) of age class (Table 2). In contrast, thymus weight (P = 0.0001), spleen weight (P = 0.0033), and splenic cellularity (P = 0.0230) were all significantly influenced by age class (Table 2). Age class effects were also noted for lymphoproliferative response to in vitro mitogenic challenge at the higher concentrations of Con A (P = 0.0254 for 10.0 μg/ml and P = 0.0097 for 20.0 μg/ml). Total peripheral leukocyte counts were unaffected by age class (P = 0.3048). It has been reported that the ability to mount a DTH response is reduced in premature or small-for-age neonatal guinea pigs (Uhr, 1960) and human infants under 2 months of age (Uhr, et al., 1960). A reduction in DTH response with advanced age has also been noted for humans (Wells and Smith, 1936) and guinea pigs (Baer and Bowser, 1963). While juvenile cotton rats had a lower mean DTH response (12.9 ± 10.3% ($\underline{SD}$)) than did adults or sub-adults (21.6 ± 14.1% and 22.8 ± 19.3%, respectively), the youngest animal sampled was 35 days old which may have contributed to the failure to see a significant reduction in DTH for this age class.

Correlation analysis showed that none of the morphological or functional measures of immunocompetence were significantly correlated with relative DTH response (Table 1) and only spleen weight and total splenocytes were

significantly correlated with absolute DTH response (Table 1). This latter correlation was a reflection of age group effect since spleen weight, total splenocytes, and absolute DTH response increased significantly with age class. This probably has no functional significance since these correlations did not exist for the DTH expressed as a percent. These data suggest that quantitative differences in morphological measures of immunocompetence and in vitro responsiveness do not necessarily translate into differences in ability to mount a complex in vivo response to antigen. It has been suggested that delayed-type hypersensitivity is the biological manifestation of cell-mediated immunity and that it is of considerable biological significance in terms of its role in immune surveillance against tumors and other foreign antigens such as microbial pathogens (Dvorak, 1974). The DTH response assay provides wildlife immunologists with a useful tool for measuring in vivo cell-mediated immune response and for examining the influence of various intrinsic and extrinsic factors on immune function.

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Table 1.--<u>Overall</u> <u>means</u> <u>of</u> <u>morphological</u> <u>and</u> <u>functional</u> <u>measures</u> <u>of</u>

<u>immunocompetence</u> <u>in</u> Sigmodon hispidus <u>and</u> <u>Pearson</u> <u>correlation</u>

<u>coefficients</u> <u>with</u> <u>delayed-type</u> <u>hypersensitivity</u> <u>measured</u> <u>as</u> <u>a</u> <u>relative</u>

<u>percent</u> <u>increase</u> <u>of</u> <u>experimental</u> <u>over</u> <u>control</u> <u>(DTH-%)</u> <u>and</u> <u>an</u> <u>absolute</u>

<u>difference</u> <u>in</u> <u>ear</u> <u>weights</u> <u>(DTH-D)</u>.

Immune parameter	Overall		Correlation analysis (r)	
	Mean	SE	DTH %	DTH-D
Thymus (mg)	50.0	2.0	0.049	−0.155
Spleen (mg)	118.6	5.1	0.172	0.219 *b
Total splenocytes (x 10^7)	8.670	0.490	0.136	0.198 *
Total peripheral leukocytes (x10^3/mm^3)	7.302	0.350	0.140	0.137
Lymphoproliferative response to Con A [a]				
unstimulated	0.378	0.015	0.040	−0.018
2.5 µg/ml	0.154	0.010	−0.032	−0.098
5.0 µg/ml	0.210	0.010	0.008	0.053
10.0 µg/ml	0.202	0.010	0.056	0.088
20.0 µg/ml	0.195	0.010	0.086	0.129

* $p < 0.05$

[a] Absorbance at 570/630 nm for unstimulated cultures and difference in

absorbance at 570/630 nm between stimulated and unstimulated cultures

for stimulated cultures

Table 2.--<u>Means</u> <u>of</u> <u>morphological</u> <u>and</u> <u>functional</u> <u>measures</u> <u>of</u>

<u>immunocompetence</u> <u>for</u> <u>three</u> <u>age</u> <u>classes</u> <u>of</u> Sigmodon hispidus.

| | Age class | | | | | |
| | Adults | | Subadults | | Juveniles | |
Immune parameter	Mean	SE	Mean	SE	Mean	SE
Hypersensitivity						
Percent	21.6	1.8	22.8	3.0	12.9	3.0
Absolute difference (mg)	51.8[a]	5.1	28.1[b]	3.8	14.5[b]	3.3
Thymus (mg)	39.1	2.1	59.7	3.0	71.2	6.6
Spleen (mg)	133.8	7.3	104.6	7.3	90.4	12.6
Total splenocytes ($x10^7$)	9.430	0.610	8.648	0.939	4.883	0.694
Total peripheral						
leukocytes ($x10^3/mm^3$)	7.732	0.443	7.046	0.672	6.017	0.754
Lymphoproliferative						
response to Con A [a]						
Unstimulated	0.377	0.018	0.355	0.022	0.466	0.076
2.5 μg/ml	0.155	0.012	0.166	0.017	0.101	0.037
5.0 μg/ml	0.230	0.011	0.188	0.018	0.181	0.029
10.0 μg/ml	0.229	0.011	0.169	0.020	0.184	0.028
20.0 μg/ml	0.225	0.011	0.156	0.021	0.174	0.030

[a] Absorbance at 570/630 nm for unstimulated cultures and difference in

absorbance at 570/630 nm between stimulated and unstimulated cultures

for stimulated cultures

Figure 1. Frequency distribution (expressed as a percentage

of the total number sampled) of relative delayed-type

hypersensitivity responses ın <u>Sigmodon</u> <u>hispidus</u> (n = 115).

Delayed—type hypersensitivity response
(relative % increase of experimental over control)
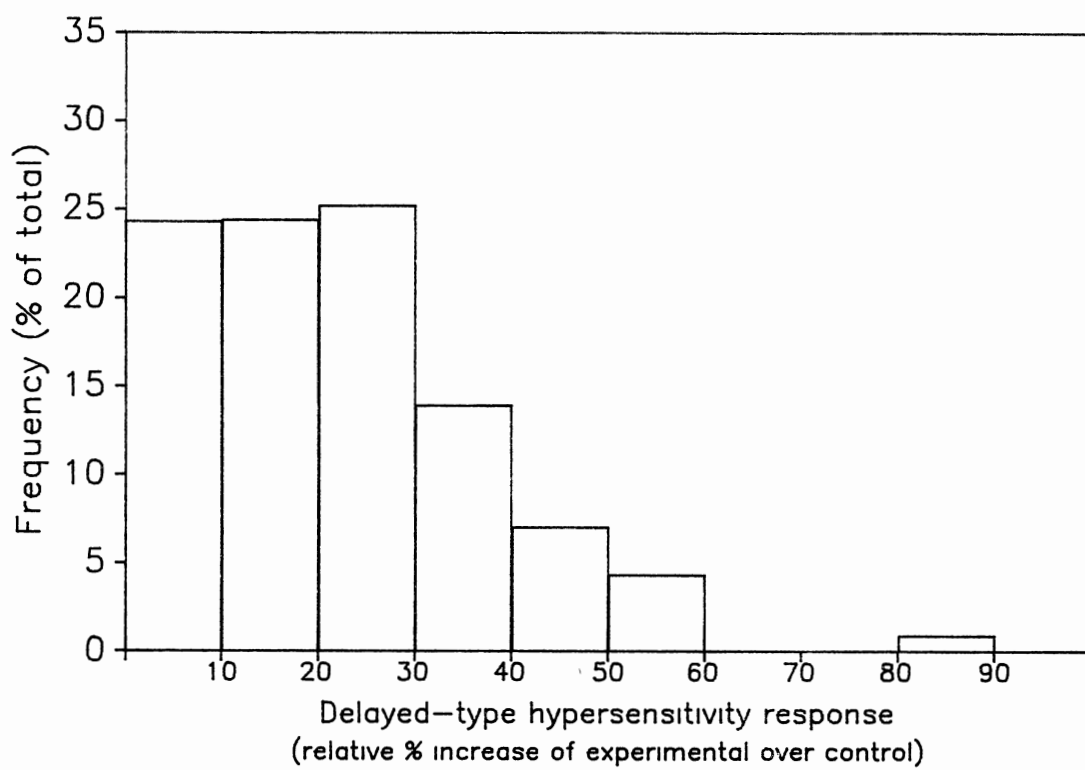
CHAPTER III

SPONTANEOUS LYTIC ACTIVITY AGAINST HETEROLOGOUS ERYTHROCYTES IN COTTON RAT (SIGMODON HISPIDUS)

ABSTRACT

We examined the sera of 188 hispid cotton rats
(<u>Sigmodon</u> <u>hispidus</u>) for naturally occurring hemolytic
activity against heterologous erythrocytes. Ninety-two
percent of the sera examined lysed sheep erythrocytes with a
mean titer ($\log_2$) of 2.1 ± 0.1 (<u>SE</u>). All 16 sera tested
against chicken erythrocytes showed hemolytic activity while
only 44% of the same sera could lyse bovine erythrocytes.
No hemolytic activity was present in cotton rat sera against
erythrocytes from other rodent species (<u>Neotoma</u> <u>floridana</u>,
<u>Microtus</u> <u>pinetorum</u>). Hemolytic activity of serum from the
cotton rat was heat labile and appeared to be mediated
through the classical complement pathway. The protective
nature of this hemolytic factor is unclear but it is
probably directed at a more biologically relevant molecule.
These observations, along with other reports of naturally
occurring target-specific factors in serum of cotton rats,
may reflect the importance of innate protective mechanisms
to small mammal populations in the wild.

INTRODUCTION

Natural, or innate immunity, is defined as that
physiological protection which is constitutively present and
independent of previous exposure to an antigen. Generally,
this refers to non-specific mechanisms, such as protective
barriers of the skin, cellular responses (movement toward
and activity at local sites of inflammation) of neutrophils

and macrophages to antigens, and activation of the alternate pathway of complement. Hemolytic activity of serum can be mediated, in some species, by direct activation of the alternate pathway of complement by heterologous erythrocytes. Little attention has been given, however, to the constitutive production of antibodies or other target-specific molecules, in the absence of exposure to the targets, as an innate protective mechanism.

The study of natural agglutinins in serum for heterologous erythrocytes in the laboratory mouse has shown that the presence of these natural agglutinins is strain dependent (1). It has also been demonstrated that hemolytic activity of serum for heterologous erythrocytes occurs only rarely and at very low levels in the laboratory mouse (1). Examples of other naturally occurring antibodies, or other target-specific molecules, are the presence of agglutinins specific for African trypanosomes and an antihemorrhagic factor which neutralizes rattlesnake venom in sera from a variety of mammalian species (2,3).

In this study, we describe a serum factor which has lytic activity for a variety of heterologous erythrocytes and which is detectable in 93% of sera tested from the hispid cotton rat, _Sigmodon hispidus_. A cursory examination of sera from other mammalian species for hemolytic activity and assays designed to elucidate the nature of the hemolytic activity are described as well.

MATERIALS AND METHODS

Of the 188 cotton rats sampled, 176 outbred animals were born and raised in our conventional laboratory animal facility at Oklahoma State University. The remainder of the cotton rats were live-trapped using rolled oats as bait in tallgrass prairie habitat in Payne County, Oklahoma. Additionally, sera from four other species of wild rodents (white-footed mouse, Peromyscus leucopus; hispid pocket mouse, Perognathus hispidus; short-tailed grasshopper mouse, Onychomys leucogaster; and Eastern woodrat, Neotoma floridana) from grass and brush dominated rangeland in central Oklahoma, cottontail rabbits (Sylvilagus floridanus), and white-tailed deer (Odocoileus virginianus) were collected. Sera samples were also obtained from laboratory rodents, Mus musculus (CD-1) and Rattus rattus (Sprague-Dawley), housed in our laboratory animal facility. Animals maintained in the laboratory were housed in polypropylene cages with wood shavings for bedding. Food (Purina Rodent Chow, St. Louis, MO) and water were provided ad libitum and a 14L:10D light cycle was maintained. Animals were classified into four age groups based on age in days: <75, 75-150, 150-300, and >300.

All sera collected was tested for hemolytic activity against sheep erythrocytes (SRBC; Colorado Serum Co., Denver, CO) and selected sera were also tested against bovine (BRBC; Colorado Serum Co., Denver, CO) and chicken erythrocytes (CRBC; Colorado Serum Co., Denver, CO).

Additionally, a limited number of cotton rat sera were tested against erythrocytes collected in the laboratory from Eastern woodrats (N. floridana) and pine voles (Microtus pinetorum).

Hemolysis of heterologous erythrocytes was measured using a modification of the microhemagglutination assay (4). Serial two-fold dilutions of the sera in phosphate buffered saline (PBS), pH 7.4, were made in 96-well microtiter plates. Fifty μl of a 1% suspension of washed erythrocytes in PBS was added to 50 μl of diluted serum. In some instances, 25 μl of 25% guinea pig serum (GPS; Bioproducts for Science, Indianapolis, IN) in PBS was added to increase the sensitivity of the assay. The reaction mixtures were incubated at 37°C for 1 hr. The lytic titer was defined as the $\log_2$ of the inverse of the highest serum dilution causing complete lysis of the erythrocytes as determined by visual observation.

Heat stability of the lytic activity was determined by heat inactivation of the sera at 56°C for 30 min. Fourteen cotton rat sera samples were assayed for hemolytic activity against SRBC (13 sera samples) and CRBC (3 of those tested against SRBC and 1 additional sample) in the presence of ethylene glycol-bis-β-aminoethylether N,N,N',N' tetraacetic acid (EGTA) which chelates the calcium required for classical complement activation. The hemolytic assay described above was performed with PBS replaced by 0.1 M EGTA in veronal buffered saline (5) containing 0.1% gelatin

and magnesium at various concentrations ranging from 2 to 20 mM and without added calcium (VBS). Sera from nine cotton rats were pooled for treatment with carrageenan which precipitates the first component of the classical complement pathway. Equal volumes of pooled sera and carrageenan at various concentrations (0.8, 1.6, and 3.2 mg/ml in PBS) were incubated at 4°C for 20 min followed by centrifugation at 1200 x g for 20 min. The supernatants were collected and assayed, along with untreated pooled sera, for lytic activity against SRBC using the modified microhemagglutination assay.

The effects of age group, captivity, and the addition of GPS to the assay on the hemolytic titers of cotton rats and differences among mean hemolytic titers of the various species sampled were tested using a one-way analysis of variance (6); Duncan's multiple comparison of means test was used when significant differences were found (7). Differences in frequency distribution of hemolytic response between wild-caught and captive cotton rats and between those responses measured with or without added GPS were determined using Chi-square analysis (7).

RESULTS AND DISCUSSION

Of the nine mammalian species tested, only four (S. hispidus, P. leucopus, O. leucogaster, and S. floridanus) possessed serum hemolytic activity for SRBC (Table 1). Hemolytic activity for SRBC differed significantly (P =

0.0001) among species with S. hispidus and P. leucopus having higher titers than all other species tested ($P <$ 0.05). Only two of the species tested had measurable hemolytic activity against BRBC and there was no significant difference among species ($P = 0.204$). Sera from all but 3 species (N. floridana, M. musculus, and O. virginianus) had hemolytic activity against CRBC with P. leucopus having a significantly ($P < 0.05$) higher titer than the other mammalian species (Table 1). Cotton rat sera showed no hemolytic activity against erythrocytes from related rodent species: Eastern woodrat (N. floridana) and pine vole (Microtus pinetorum).

The overall mean hemolytic activity of cotton rat sera was 2.1 ± 0.1 (SE) for SRBC, 0.8 ± 0.2 for BRBC, and 2.6 ± 0.2 for CRBC. Extrinsic additions of complement in the form of GPS had a significant ($P = 0.0001$) effect on the hemolytic activity of cotton rat sera with the mean titer for those with added complement being higher than those without (2.3 ± 0.1 (SE) and 1.3 ± 0.2, respectively). The frequency distributions (Fig. 1), which were significantly different ($X^2 = 27.62$, $P < 0.001$), suggest that addition of guinea pig complement shifts the activity approximately 1 hemolytic activity unit.

There was no difference in hemolytic activity of serum between captive and wild-caught cotton rats for SRBC ($P = 0.132$), BRBC ($P = 0.327$), or CRBC ($P = 0.325$). Due to the shift in hemolytic activity from the addition of GPS, the

effect of age was analyzed separately for the two groups.
For both the animals without added GPS and those which were
assayed in the presence of GPS, age group contributed
significantly ($\underline{P}$ = 0.0132 and $\underline{P}$ = 0.0215, respectively) to
hemolytic activity against SRBC (Table 2). Hemolytic
activities of animals between 150 and 300 days of age were
higher ($\underline{P}$ < 0.05) and animals under 75 days of age were
lower ($\underline{P}$ < 0.05) than other age groups. Hemolytic activity
of cotton rat sera against BRBC and CRBC was not
significantly influenced by age ($\underline{P}$ = 0.823 and $\underline{P}$ = 0.878,
respectively) although only animals older than 150 days were
represented in this analysis.

It is difficult to assess the biological significance
of the enhanced hemolytic activity in the 150 to 300 days of
age group since the average life span of the cotton rat in
the wild is approximately 6 months (8). The presence of the
lower activities in the youngest animals is probably of
greater importance. This may reflect either lower numbers
or immaturity of cells synthesizing the hemolytic molecule.
If the hemolytic molecule is actually an antibody directed
towards an antigen which cross-reacts with SRBC, the younger
animals may not have had sufficient opportunity to encounter
the antigen.

The hemolytic activity was heat labile suggesting a
complement pathway mechanism since activity was eliminated
from all sera by a heat inactivation procedure usually used
to eliminate complement activity from serum. It has been

reported that erythrocytes from some species possess an antigen which can induce the alternate complement pathway in heterologous species. For example, rabbit erythrocytes are spontaneously lysed by normal human sera (5), human erythrocytes activate the alternate complement pathway in bovine serum (9), and equine erythrocytes activate the avian analog to mammalian alternate complement (10, 11). To determine if the lytic activity in cotton rat sera was mediated by activation of the alternate pathway, lysis was measured in the presence of EGTA which selectively chelates calcium ions which are required by the classical complement pathway but not necessary for activation of the alternate pathway (12). Hemolytic activity was completely absent in the presence of EGTA and magnesium. Additionally, all lytic activity was effectively removed from a pooled cotton rat sera after treatment with several concentrations of carrageenan to precipitate the first component of the classical complement pathway (13). All lytic activity was removed from the resulting supernatants by this treatment.

These data indicate that hemolytic activity in cotton rat sera occurs naturally in the absence of exposure to the antigen, is restricted to erythrocytes of certain species, and is mediated by the classical complement pathway which implies that it is antibody mediated. While not restricted to cotton rats, this hemolytic activity is not found in all mammalian species and only occurs at the magnitude seen in cotton rats in one other wild rodent species tested, P.

<u>leucopus</u>. It has been reported (14) that six species of non-human primates have natural hemolytic activity for sheep and bovine erythrocytes which is not mediated by alternate complement pathway activation but appears to be mediated by natural antibodies against the Forsmann antigen (15).

The protective nature, if any, of this hemolytic factor in the cotton rat is unclear. It is likely that the specificity of this lysis is actually directed toward an antigen which shares epitopes with some erythrocyte antigens and which is more biologically relevant, possibly pathogenic, to the cotton rat. Naturally produced protective serum components have been identified in cotton rat serum. Antibodies which recognize and mediate lysis of <u>Trypanosoma</u> <u>vivax</u> are commonly found in the sera of cotton rats who have never been exposed to the antigen (16, 17). Additionally, cotton rats have been reported to have a naturally occurring antihemorrhagic factor in their serum which neutralizes rattlesnake venom (18). This antihemorrhagic factor does not appear to be an antibody but, nevertheless, is another example of a naturally occurring serum component with recognition specificity.

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Table 1. Mean (± SE) hemolytic activity (log_2 of highest 2-fold dilution of serum giving complete lysis) of sera from 8 mammalian species against erythrocytes from sheep (SRBC), cattle (BRBC), or chickens (CRBC). Separate means are given for cotton rat sera analyzed with and without added guinea pig serum (GPS).

Common name	Scientific name	SRBC			BRBC			CRBC		
		Mean	SE	n	Mean	SE	n	Mean	SE	n
Cotton rat (no GPS)	Sigmodon hispidus	1.3	0.2	37	0.8	0.2	16	2.6	0.2	16
Cotton rat (with GPS)		2.3	0.1	151						
White-footed mouse	Peromyscus leucopus	2.3	0.3	14	0.3	0.2	11	4.3	0.2	10
Hispid pocket mouse	Perognathus hispidus	0		7	0		2	1.0		2
Short-tailed grasshopper mouse	Onychomys leucogaster	1.5	0.6	4	0		3	2.7	0.3	3
Eastern woodrat	Neotoma floridana	0		3	0		3	0		3
CD-1 mouse	Mus musculus	0		4	0		2	0		2
Cottontail rabbit	Sylvilagus floridanus	0.2	0.2	4	0		4	1.7	0.3	3
White-tailed deer	Odocoileus virginianus	0		4	0		4	0		3

Table 2. Age-related differences in hemolytic activity ($\log_2$ of highest 2-fold dilution of serum giving complete lysis) against sheep erythrocytes in serum from cotton rats analyzed with or without the addition of guinea pig serum (GPS).

Age group	Hemolytic activity					
	Without GPS			With GPS		
(days)	Mean	SE	n	Mean	SE	n
0 - 74	0		1	2.0	0.2	33
75 - 150	0.7	0.2	12	2.3	0.1	93
151 - 300	2.0	0.4	4	3.4	0.7	5
> 300	1.7	0.3	7	2.2	0.4	13

Fig. 1 Frequency distribution of hemolytic activity against sheep erythrocytes in cotton rat sera assayed with or without exogenous additions of guinea pig complement.
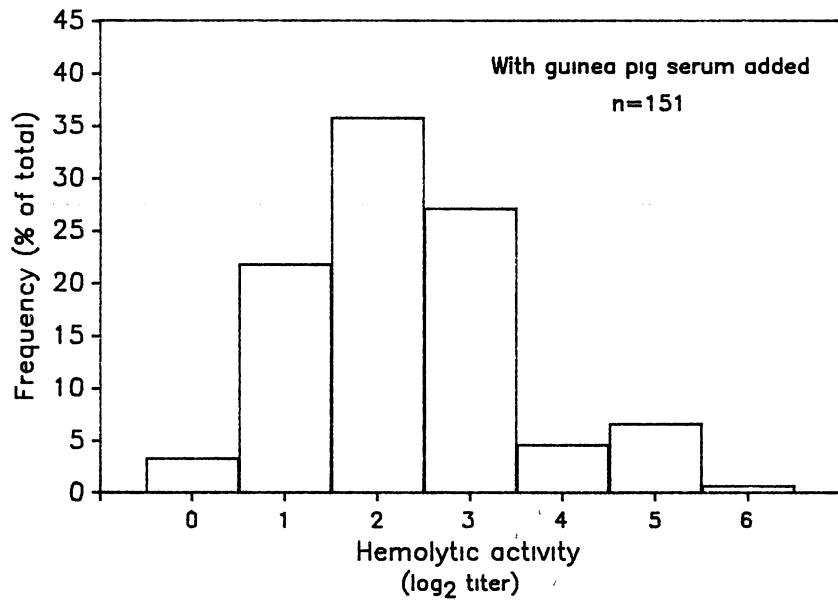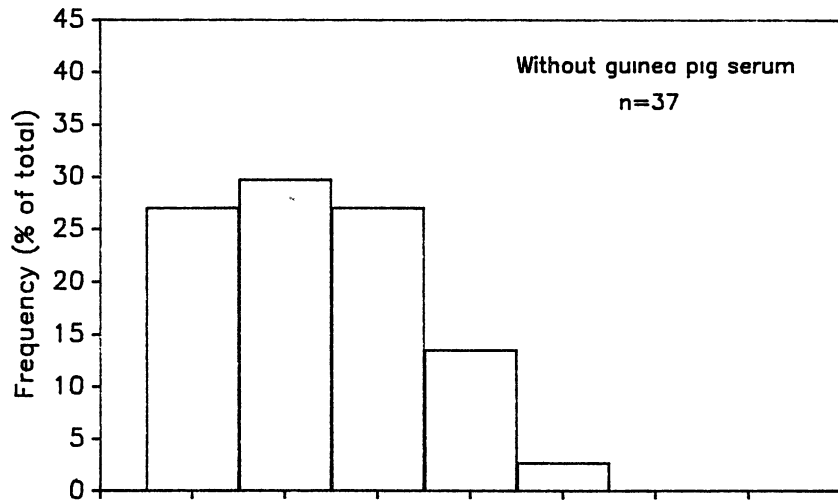
CHAPTER IV

TEMPORAL VARIATION IN HUMORAL AND CELL-MEDIATED IMMUNE

RESPONSE IN SIGMODON HISPIDUS

ABSTRACT.--Unpredictable temporal and spatial
fluctuations in density of small mammal populations are
well documented. Although the mechanisms regulating
this phenomenon are poorly understood both extrinsic
and intrinsic factors have been suggested as components
to such mechanisms. Reduced immunocompetence may
contribute to rapid decreases in population size. We
examined temporal variations in selected measures of
immunocompetence of cotton rats (Sigmodon hispidus) for
a population in central Oklahoma. A total of 310
hispid cotton rats was collected from August 1989 to
May 1991 using conventional livetrap methods. Ability
of splenocytes to produce specific antibody in response
to a single injection of sheep erythrocytes and to
proliferate in response to in vitro exposure to
mitogens were measured to assess immunocompetence.
Morphological and hematological data were collected and
reproductive status of each animal was recorded.
Statistically significant differences in all variables
except total serum protein existed among the 10
collections. Demographics and reproductive status
measurements indicated that this population was
undergoing an expected annual rhythm with peak
reproductive activity occurring in late summer and
early fall. The temporal profiles associated with
measurements of immune function, however, were not
correlated with seasonality and were only moderately

similar to each other. Changes over time in the average immunocompetence of this population probably reflect a shift in genotypic polymorphism. This sort of genetic shift may be a primary factor in predisposing populations to rapid changes in density.

It has been well documented that small mammal populations fluctuate. Sometimes these fluctuations are regular and fairly predictable. Goertz (1964) reported that cotton rat (<u>Sigmodon</u> <u>hispidus</u>) populations experience an annual cycle with numbers declining in winter following a summer-fall peak. In many instances, however, these fluctuations are irregular and do not correlate with any measurable environmental influences. Crashes such as these have been described for various cotton rat populations distinct both geographically and temporally (Odum, 1955; Haines, 1963; Goertz, 1964; Fleharty, 1972).

Extrinsic factors such as weather, predation, vegetative cover, disease agents, and food availability have been postulated as regulation mechanisms for population size. It has been suggested that these factors may only exacerbate a rapid change in animal density in a population already primed to crash through some intrinsic mechanism. Christian (1950) proposed that dramatic changes in population numbers are mediated by the adreno-pituitary axis resulting in stress exhaustion as outlined by Selye (1946). Chitty (1958, 1960) and Krebs and Meyers (1974) proposed that regulation is intrinsically controlled by shifts in the

genetic polymorphism of a population with respect to behavior and reproduction.

We propose that immunological competence may also be an important component of this model. Immune response is under polygenic control (Benacerraf and McDevitt, 1972) and may be regulated by 10 or more genes (Biozzi et al., 1979). Changes in phenotypic expression of immunocompetence over time could provide a measure of the shift in genetic polymorphism in a population. This study was designed to assess the degree of temporal variation in selected measures of immune function in a discrete population of cotton rats over the annual cycle. Measures of humoral, or antibody-mediated, immune function (primary antibody response to sheep erythrocytes) and cell-mediated immunity (in vitro lymphoproliferation of splenocytes in response to mitogenic challenge) were included as well as an assessment of morphological indicators of immune function.

MATERIALS AND METHODS

Animals.--All animals were captured from tallgrass prairie habitat 5-8 km west of Stillwater in central Oklahoma. Composition of vegetation in this habitat type has been described previously (Ewing et al., 1984). Cotton rats were collected every 6 to 10 weeks from August 1989 to May 1991 using Sherman live-traps baited with rolled oats. On arrival at the laboratory animal facility, cotton rats were housed in either wire-mesh bottom hanging cages or polypropylene boxes with wood shavings and cotton bedding.

Commercially available rodent chow (Purina 5001, St. Louis, MO; crude protein = 24%) and water were provided ad libitum and the animal room was maintained on a 14L:10D cycle with ambient temperature (25°C). Age class designations were determined on the basis of body weight (Odum, 1955): adults (≥100 g), subadults (60-99 g), and juveniles (<60 g). All animals were acclimated to captivity for 1 to 2 weeks before assessing immunocompetence.

Necropsy--Each cotton rat was anesthetized with an intramuscular injection of ketamine hydrochloride (Aveco, Fort Dodge, IA; 50 mg/kg body weight), weighed, and bled from the retro-orbital plexus to collect whole blood for hematology (in EDTA-K_3) and serum at termination. Animals were euthanized by cervical dislocation while anesthetized. Spleens were aseptically removed into pre-tared 15 x 60 mm sterile Petri dishes containing 5.0 ml RPMI-S consisting of RPMI-1640 supplemented with L-glutamine (2.05 mM), sodium pyruvate (1 mM), non-essential amino acids (1 mM), 2-mercaptoethanol (2×10^{-5} M), penicillin (100,000 u/L), streptomycin (100 mg/L), and normal horse serum (10%). The thymus was also removed and both organs were weighed to the nearest 0.1 mg. Position of the testes in males and scarring of the uterus, presence or absence of fetuses, and presence or absence of a perforate vulva for each female were recorded.

Hematology--Total peripheral leukocyte (WBC) counts were determined by diluting an aliquot of whole blood in 2%

acetic acid and counting using a hemacytometer. Packed cell volumes (PCV) were analyzed using the microcapillary tube method and expressed as a percentage. Total concentrations of protein in sera samples were measured against a human sera standard (SeraChem Clinical Chemistry Control) using the biuret protein procedure (Kingsley, 1942).

Lymphoproliferative response to in vitro mitogenic stimulation--Lymphoproliferation was measured by cellular reduction of tetrazolium salt (Mosmann, 1983). The spleen was hemisected and splenocytes gently expressed in a glass-on-glass tissue homogenizer containing 5 ml of ice-cold RPMI-S. The resulting cell suspension was placed on ice for 10 min to allow sedimentation of the large tissue fragments, transferred to a sterile 16 x 125 mm culture tube, and centrifuged at 275 xg for 10 min at 20°C. Cells were washed 3 times in 5 ml of medium with centrifugation as above. Splenocyte numbers were determined by diluting an aliquot of the suspension in Trypan blue and counting on a hemacytometer. Splenocyte concentrations were adjusted to 0.5×10^6 cells/0.09 ml and 0.09 ml aliquots were cultured in 96-well plates with 0.01 ml of mitogen. Two mitogens, Concanavalin A (Con A; 5.0 μg/ml culture) and pokeweed extract (PWM; 0.625 μg/ml culture), were used. Aliquots of cells in the absence of mitogen were cultured as unstimulated controls. After 69 hrs of incubation at 37°C in 5% CO_2, 10 μl of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; 5 mg/ml in PBS) was added to

each culture. At 72 hrs, 160 μl of acid-isopropanol (0.04 M

HCl in isopropanol) was added to each culture and absorbance

at 570 and 630 nm was quantified using a Titertek Multiskan

Plus microtiter plate reader. Response was calculated as a

stimulation index representing the ratio of absorbances of

mitogen-stimulated cultures to control cultures.

Hemolytic plaque assay--Primary antibody response to

heterologous erythrocytes was determined using a procedure

described by Cunningham and Szenberg (1968). Five days

prior to termination, each cotton rat was injected

intraperitoneally with 0.2 ml of a 10% suspension of sheep

erythrocytes (Colorado Serum Company, Denver, CO; SRBC) in

phosphate buffered saline, pH 7.4 (PBS). At termination,

splenocyte suspensions were centrifuged at 275 x$\underline{g}$ for 7 min

at 10°C and the resulting pellet resuspended in Tris-

ammonium chloride to lyse red blood cells. After 2 min, the

cell suspension was underlaid with 1.0 ml of ice-cold fetal

calf serum (FCS) and, following a room temperature

incubation for 7 min, centrifuged as above. The cells were

resuspended and washed twice in 5 ml of PBS with 5% FCS

(PBS-F) with final resuspension in 2 ml of PBS-F containing

0.5% gelatin (PBS-F-G). Splenocytes were counted using the

Trypan blue exclusion procedure and a hemacytometer.

Splenocytes were adjusted to a final concentration of 9.6 x

10^6 cells/ml and 50 μl and 200 μl aliquots were diluted to

220 μl with PBS-F-G in 12 x 75 mm culture tubes. Eighty μl

of a 25% suspension of SRBC in PBS-F-G and 100 μl of 25%

guinea pig serum (Bioproducts for Science, Indianapolis, IN) in PBS-F-G were added and the mixtures vortexed. Incubation chambers were prepared (Marbrook, 1980) and loaded with 125 μl of reaction mixture. Chambers were sealed with a molten mixture of Vaseline and paraffin (1:1) and incubated at 37°C for 2 hr. The resulting plaques of anti-SRBC antibody-secreting splenocytes were counted using a dissecting microscope. High responders were defined as animals with greater than or equal to 90 plaque-forming cells (PFC)/10^6 splenocytes based on a natural break in the distribution of response for normal cotton rats.

Statistical analysis--Differences in morphological and functional measures of immunocompetence due to season of capture, age, and sex of cotton rats were examined using a three-way analysis of variance (SAS, 1985). Since analysis of the raw data using Bartlett's chi-square test of homogeneity of variances indicated that group variances were heterogeneous, statistical analysis of plaque production was performed on logarithmically transformed (total plaques) or arcsine transformed (PFC/10^6 splenocytes) data (Steel and Torrie, 1980). Duncan's multiple range test was used to compare treatment means when main effects were significant ($\underline{P} < 0.05$).

RESULTS

A total of 310 cotton rats were collected from August 1989 to May 1991. We made no attempt to select particular age or sex classes to return to the laboratory for analyses;

trapping proceeded until the desired number of animals was collected. Males represented 57% of all cotton rats captured and age group distribution varied with adults ranging from 18.4% of total captures in February 1991 to 90.1% of total captures in May 1990 (Fig. 1). Overall, adults represented 50% of all animals captured. Subadults comprised 9.1% (May 1990) to 76.3% (February 1991) of animals captured in a sampling period (Fig. 1) and represented 32% of all animals captured. Juveniles were captured less frequently and none were trapped in May 1990 (Fig. 1), but reached a peak in August 1990 when they represented a majority (35.3%) of all animal captures. Reproductive status (% pregnant and scrotal; Fig. 1) also differed seasonally (P = 0.0001 for both males and females) and among age classes (P = 0.0007 for females and P = 0.0280 for males). Of all animals captured, 56.5% of males were scrotal and 20.3% of females were pregnant.

Packed cell volume varied significantly (P = 0.0001) over seasons, ranging from 34.1% in December 1989 to 42.5% in May 1990 (Fig. 2). Peak PCV means occurred in October 1989 and May 1990 and a nadir in the temporal profile occurred in December 1989 and February 1990. Mean PCV remained fairly stable from July 1990 to May 1991. Males had a significantly higher (P < 0.05) mean PCV than females (40.1 ± 0.3% ($\underline{SE}$) vs 38.2 ± 0.3%). Total peripheral leukocyte (cells x $10^3/mm^3$) counts differed significantly among seasons (P = 0.0001) and age classes (P = 0.0001) but

there was no significant ($\underline{P}$ = 0.3233) season by age class interaction (Fig. 2). Animals caught in December 1989, July 1990, and February 1991 had significantly ($\underline{P}$ < 0.05) lower mean WBC counts than animals caught at other times. The three age classes had mean WBC counts which were significantly ($\underline{P}$ < 0.05) different from each other (adults, 8.336 ± 0.281; subadults, 6.091 ± 0.362; juveniles, 4.763 ± 0.365). Concentration of total proteins in the serum was the only parameter in this study that did not vary temporally ($\underline{P}$ = 0.1433); the overall mean was 6.34 ± 0.06 g/dl. Juveniles (5.97 ± 0.10 g/dl) had significantly ($\underline{P}$ = 0.0155) lower concentrations of total protein in serum than adults (6.48 ± 0.09 g/dl) or subadults (6.37 ± 0.09 g/dl).

Thymus weights varied significantly among seasons ($\underline{P}$ = 0.0001) and age classes ($\underline{P}$ = 0.0001), and there was also a significant ($\underline{P}$ = 0.0001) season by age class interaction. Thymus weights tended to remain stable for juveniles (overall mean = 48.6 ± 2.1 mg) with the only significant difference being that animals in May 1991 had heavier thymuses than those caught in August 1989 and February 1991. Adults and subadult thymus weight had a bimodal distribution with subadults lagging the adults by a few months which seems to reflect the natural age-related thymic atrophy (Fig. 3). The lowest mean thymus weights for subadults occurred in May 1990 and February 1991 when a large proportion of those animals were approaching the 100g body weight cutoff for classification as an adult. Likewise,

adults had the heaviest thymuses in December 1989 and
November 1990 when, undoubtedly, many of these animals had
just attained the 100g body weight criterion for
classification as adults. Additionally, females had
significantly ($\underline{P}$ = 0.0495) heavier thymuses than males (42.0
± 1.6 vs 33.7 ± 1.6 mg). Spleen weights differed
significantly ($\underline{P}$ = 0.0001) among age classes (adults, 287.1
± 8.6; subadults, 161.4 ± 6.1; juveniles, 108.9 ± 6.7 mg)
and season of collection ($\underline{P}$ = 0.0010; Fig. 4); there was no
season by age class interaction ($\underline{P}$ = 0.1420). The only
remarkable deviation from consistency of spleen weight
occurred in February 1991 when average spleen weight was
significantly lower than all other months except August 1990
(Fig. 4). Relative total splenocyte yield (expressed on a
per mg spleen basis) showed significant ($\underline{P}$ = 0.0001)
seasonal variation which was bimodal with a significant peak
in February 1990 and significant nadirs in July 1990 and May
1991 (Fig. 4). There were no differences in relative total
splenocytes due to age class ($\underline{P}$ = 0.5501) or sex ($\underline{P}$ =
0.8322).

Humoral immune function was measured by enumerating the
number of splenocytes which were secreting antibody specific
for sheep erythrocytes. Both total plaque-forming cells
(TPFC) and PFC/10^6 splenocytes showed significant ($\underline{P}$ =
0.0001) seasonal variation (Fig. 5). The temporal profile
was bimodal featuring peaks in February 1990 and 1991 with a
nadir in November 1990. Additionally, juveniles (2372 ±

644) had significantly ($\underline{P}$ = 0.0344) lower TPFC than subadults (6853 ± 1512) or adults (7425 ± 2167). Total plaque production was significantly correlated with body weight, thymus weight, and spleen weight and cellularity while relative plaque production (per 10^6 splenocytes) was correlated only with PCV and total serum protein (Table 1). The percent of high responders (Table 2) ranged from 6.1% in May 1990 to 29.6% in February 1990 but there was no significant seasonal effect ($\underline{X}^2$ = 12.396, $\underline{P}$ = 0.192)..

Cell-mediated immune function was assessed by measuring lymphoproliferative responses of splenocytes following in vitro mitogenic challenge with Con A, a T-cell specific mitogen, and PWM, a mitogen which stimulates both T- and B-lymphocytes. Cotton rat splenocytes varied significantly ($\underline{P}$ = 0.0001) over time in their ability to respond in vitro to Con A or PWM (Fig. 6). The response to PWM was greater than Con A for all seasons except December 1989 and May 1991, although the two profiles were very similar. Lymphoproliferation of unstimulated cells also varied significantly ($\underline{P}$ = 0.0001) over time (Fig. 6) and this profile seemed to indicate an inverse relationship between the level of unstimulated lymphoproliferation and the subsequent ability of the cells to respond to in vitro stimulation. Lymphoproliferation of splenocytes, either unstimulated ($\underline{P}$ = 0.2282) or in response to Con A ($\underline{P}$ = 0.4425) or PWM ($\underline{P}$ = 0.3133), was unaffected by age class. Ability of unstimulated splenocytes to proliferate in

culture was positively correlated with body weight and negatively correlated with splenocyte yield, WBC count, PCV, and total serum proteins (Table 1). Spleen weight was negatively correlated to ability of splenocytes to proliferate in response to mitogenic stimulation while WBC counts were positively correlated to mitogenic response (Table 1). Additionally, response to PWM was also positively correlated with PCV and total serum proteins (Table 1).

DISCUSSION

Since periodic fluctuations in mammalian populations were first described by Elton (1924), many mechanisms for population regulation have been postulated and tested. There are at least two levels of regulation; one of which mediates a regular annual fluctuation commonly seen in many mammalian populations (Flowerdew, 1987). This appears to be regulated by a combination of extrinsic factors such as weather, food availability, and predation and intrinsic factors which mediate a seasonal cessation of breeding activity. This form of regulation has been described in cotton rat populations (Fleharty et al., 1972) and is supported by observed changes in reproductive activity in the current study. The second form of population regulation is responsible for increases and crashes which take population numbers far beyond normal annual boundaries. The mechanisms involved here are not well elucidated but probably involve both extrinsic and intrinsic factors.

Although discrete populations of wild mammals were once considered to be fairly homogeneous with respect to genetic variability (Krebs and Myers, 1974), it is now known that these populations can exhibit significant genetic polymorphism. Temporal changes in genetic polymorphism as inferred by changes in particular allele frequencies have been described for a variety of mammals (Smith et al., 1978). Tamarin and Krebs (1969), for example, showed that allele frequency for the transferrin locus varied over time in fluctuating populations of <u>Microtus</u> sp. Chitty (1958, 1960) has proposed a mechanism of self regulation of population density which is based on this genetic polymorphism. His theory suggests that this polymorphism has evolved to offset the effects on the population of interactions between individuals but that exposure to a combination of intrinsic and extrinsic factors could override the normal equilibrium. Ford (1964) described studies demonstrating selective advantages of up to 30% per generation so it is not difficult to see how a population could rapidly shift to a more homogeneous genetic profile.

Benacerraf and McDevitt (1972) showed that immune function is under the control of the Ir genes of the major histocompatibility complex (MHC). Biozzi et al. (1979) determined that antibody response in laboratory mice to complex antigens such as sheep erythrocytes was controlled by at least 10 different loci some of which were not in the MHC. It is our contention that if a shift in the genetic

polymorphism of a population involves loci closely linked to those controlling immune response this could alter the resistance of animals to selected diseases, perhaps even mediated by organisms normally tolerated by immunocompetent individuals.

Our data suggest that this population of cotton rats has shifted at least phenotypically, and probably genotypically, over the 22 month sampling period with respect to immunocompetence. Although none of the profiles are temporally identical, there are some common features. First, lower packed cell volumes and peripheral leukocyte counts as well as relative splenocyte yield and lymphoproliferative responses occurred in December of 1989. February 1990 was also characterized by low PCV but elevated humoral (PFC and TPFC) and cell-mediated (lymphoproliferative response to Con A and PWM) immune responses. These results coincide with a considerable increase in relative total splenocyte yields in February 1990. Goertz (1965) showed that, for wild cotton rats in Payne County, Oklahoma, spleens were heavier in summer and fall than in winter and spring. There was little variation in spleen weights from cotton rats in this study and no seasonality was evident.

While it is tempting to suggest that these results reflect an obligatory response to winter conditions, there was little similarity in the performance of rats from February 1990 to those from February 1991 nor was there a

comparable response seen between rats from December 1989 and November 1990. In contrast to the lack of seasonal correlation in this study, Dubach (1975) showed that temporal changes in genetic variability of a population of _Peromyscus maniculatus_ was correlated with seasonal changes. Goertz (1964) showed that cotton rat numbers seem to be sensitive to onset of severe weather in the winter but Fleharty et al. (1972) suggested that severe climatic changes only augment a decline already in progress which is mediated by other mechanisms.

The involvement of disease in population regulation is unclear. Some believe that disease may act as a secondary influence in that animals in very high densities increase the opportunity of spread of disease (Christian, 1950). Stoddard (1931) and Davis (1958) have suggested that disease is an important cause of periodic declines in cotton rat populations. In contrast, Christian (1950) claims that disease or parasitism is not a primary causative factor in population crashes as evidenced by several studies of microtine populations where pathologic lesions could not be detected. These studies acknowledged the possibility of viral pathogens which could not be detected but did not consider this very likely. Additionally, Findlay and Middleton (1934) found _Toxoplasma_ cysts in the brains of over half of the rodents examined after a die-off of _Microtus agrestis_ but dismissed this as probable cause because this organism was known to have a widespread

distribution in the population. Hamilton (1937) could not find pathologic lesions in <u>Microtus</u> <u>pennsylvanicus</u> examined during a boom and crash but did note an abundance of external parasites. Newson (1962) reported that in voles there was a seasonal variation in reticulocyte count, hemoglobin, and spleen weight which was independent of a seasonal fluctuation of ectoparasites. However, in neither of these latter two studies, was an attempt made to quantify blood-borne opportunistic pathogens possibly transmitted by the ectoparasites. A common assumption of most studies of this type is equal probability of capture for healthy and diseased animals. Such studies also fail to examine neonates which are immunologically less developed and more susceptible to disease than adults.

While it is difficult to extrapolate the results of this study to actual disease resistance, it is unlikely that the temporal fluctuations seen for the measures of immunocompetence used in this study would occur in the absence of any impact on capacity to resist disease. Significant temporal changes in immunological phenotype for a wild population of cotton rats has been demonstrated in this study and we are continuing to monitor this population to correlate immunocompetence with future changes in population densities.

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Table 2.--Pearson correlation coefficients between selected measurements of immunocompetence and other morphological and hematological measurements in cotton rats.

	Lymphoproliferation			Plaque-forming cells	
	Con A[a]	PWM[b]	Unstimulated	Total	per 10^6 SC[c]
Body weight (g)	-0.003	0.0386	0.0010**	0.201**	-0.070
Thymus (mg)	0.013	-0.060	0.098	-0.150*	-0.045
Spleen (mg)	-0.202**	-0.242**	0.081	0.228**	-0.041
Total splenocytes ($x10^7$)	0.051	0.050	-0.200**	0.295**	0.012
Peripheral leukocytes ($x10^3/mm^3$)	0.155*	0.209**	-0.254**	0.059	-0.039
Packed cell volume (%)	0.058	0.170*	-0.206**	-0.117	-0.152*
Total serum proteins (g/dl)	0.153	0.266**	-0.267**	-0.056	-0.169*

[a] Concanavalin A, 5 μg/ml
[b] Pokeweed mitogen, 0.625 μg/ml
[c] SC = splenocytes
* $P < 0.05$ ** $P < 0.01$

Table 2.--<u>Temporal</u> <u>variation</u> <u>in</u> <u>antibody</u> <u>response</u> <u>(plaque-</u>
<u>forming</u> <u>cells/10</u>^{6} <u>splenocytes)</u> <u>to</u> <u>sheep</u> <u>erythrocytes</u> <u>in</u>
<u>cotton</u> <u>rats</u> <u>caught</u> <u>from</u> <u>August</u> <u>1989</u> <u>to</u> <u>May</u> <u>1991.</u> <u>High</u>
<u>responders</u> <u>are</u> <u>defined</u> <u>as</u> <u>those</u> <u>with</u> <u>≥90</u> <u>plaque-forming</u>
<u>cells/10</u>^{6} <u>splenocytes.</u>

Date of capture	High responders (%)
August 1989	16.1
October 1989	14.3
December 1989	25.0
February 1990	29.6
May 1990	6.1
July 1990	14.7
August 1990	11.8
November 1990	6.3
February 1991	15.8
May 1991	8.3

Fig. 1.--Age class distribution (number of each age class
caught in a given season) and reproductive status (%
pregnant females and scrotal males) of cotton rats caught in
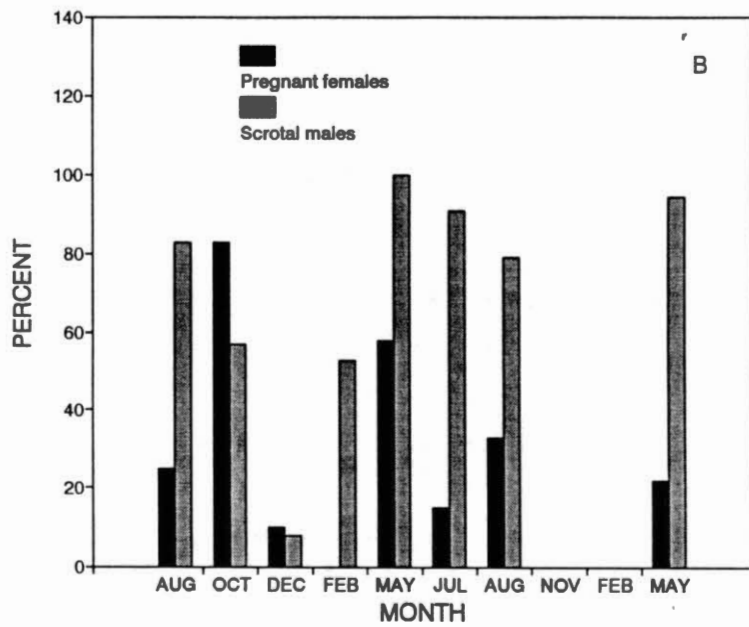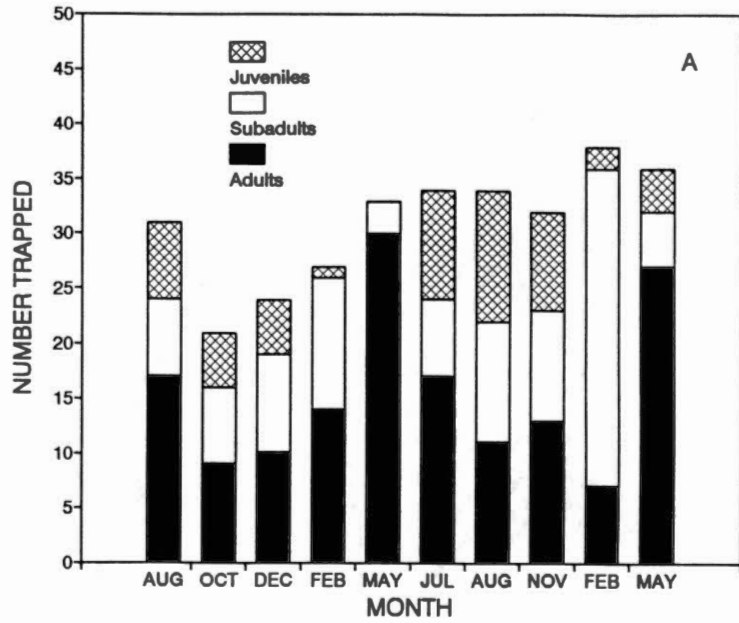Payne County, Oklahoma, from August 1989 to May 1991.

Fig. 2.--Temporal changes in average packed cell volume (A) and total peripheral leukocyte counts (B) for cotton rats caught from October 1989 to May 1991. Vertical lines represent $\underline{SE}$. Points with no superscripts in common indicated significantly different means ($\underline{P}$ < 0.05).
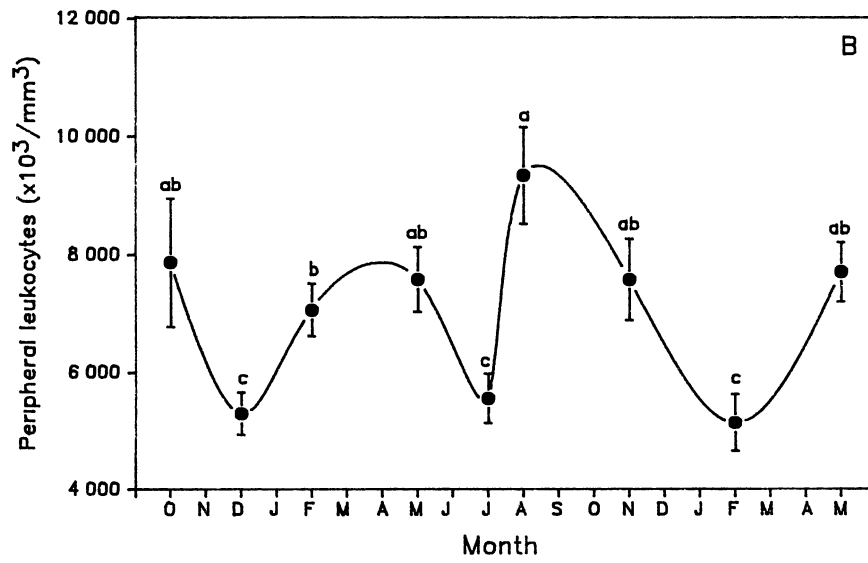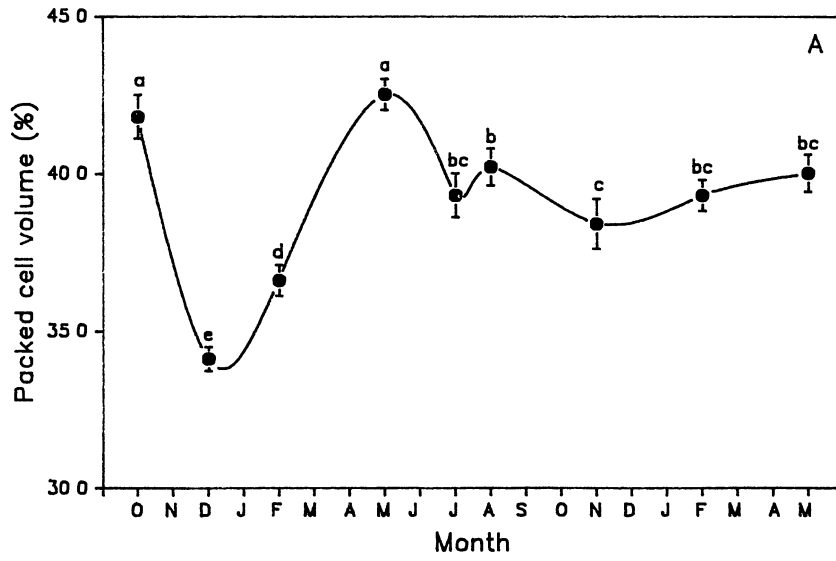
Fig. 3.--Temporal changes in thymus weight for juvenile, subadult, and adult cotton rats caught from August 1989 to May 1991. Vertical lines represent $\underline{SE}$. Points with no superscripts in common indicated significantly different means ($\underline{P}$ < 0.05).
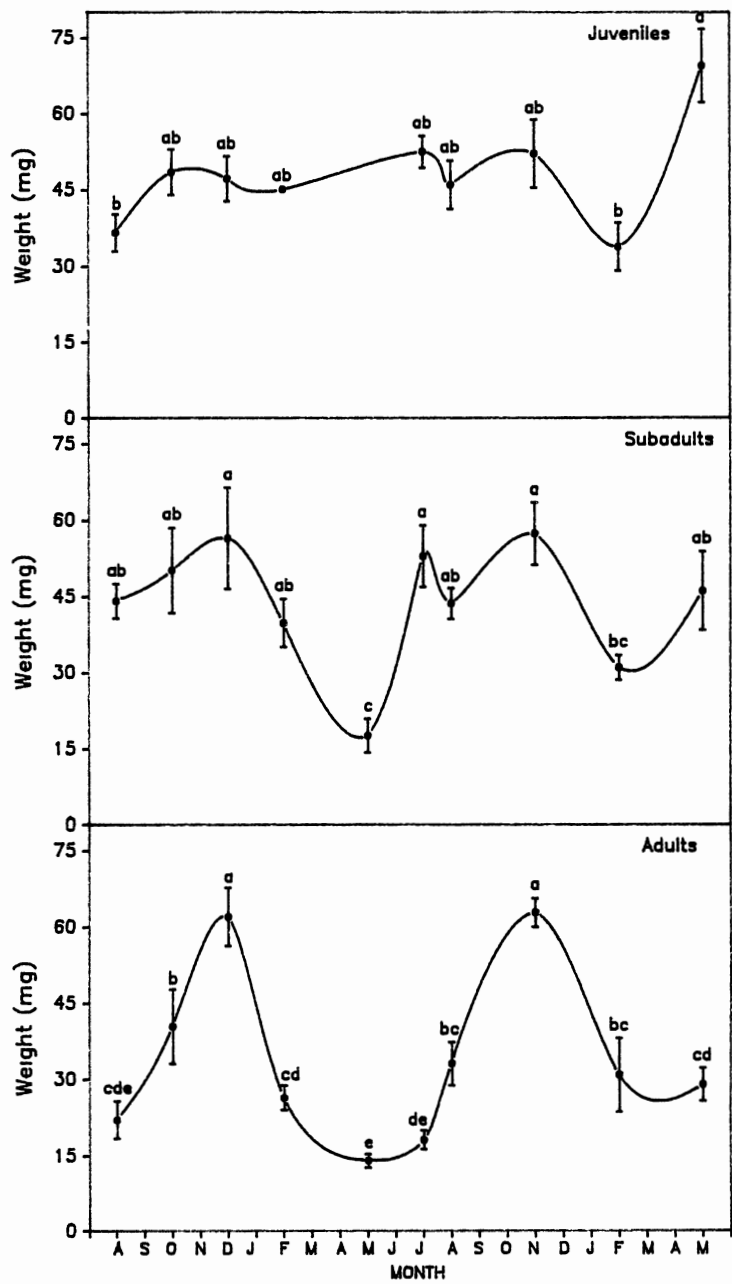
Fig. 4.--Temporal changes in spleen weight (A) and splenocytes per mg spleen (B) for cotton rats caught from August 1989 to May 1991. Vertical lines represent $\underline{SE}$. Points with no superscripts in common indicated significantly different means ($\underline{P} < 0.05$).
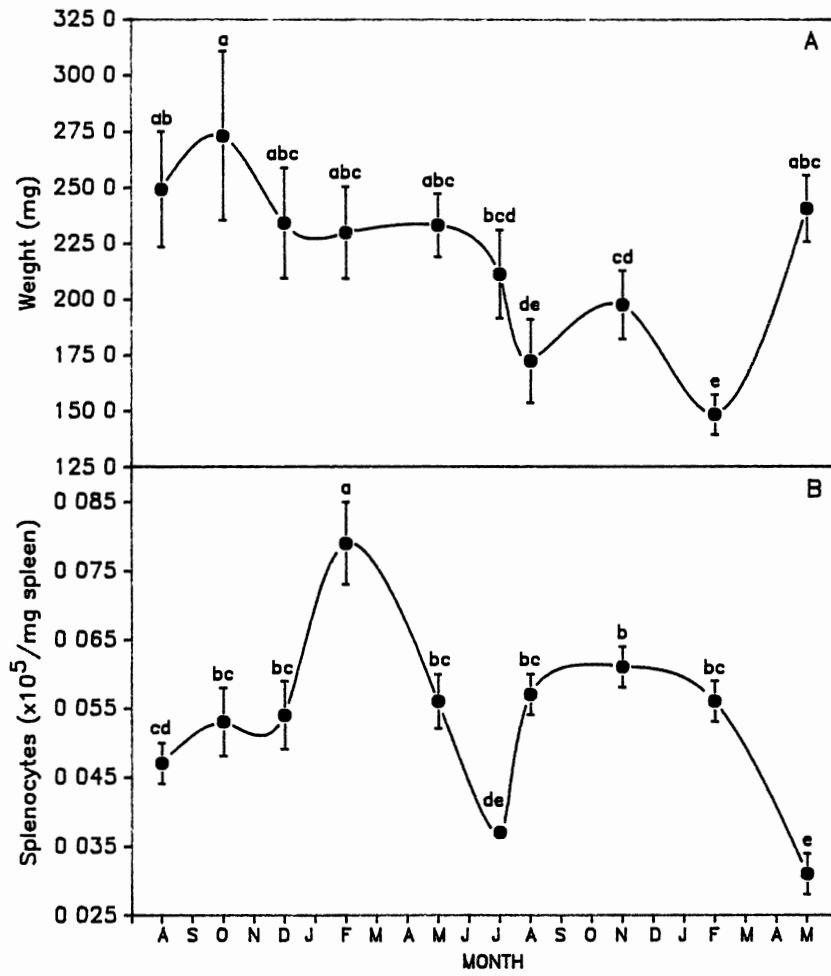
Fig. 5.--Temporal changes in plaque-forming cells/10^6 splenocytes (A) and total plaque-forming cells (B) for cotton rats caught from August 1989 to May 1991. Vertical lines represent SE. Points with no superscripts in common indicated significantly different means (P < 0.05).

Fig. 6.--Temporal changes in lymphoproliferation of splenocytes in response to in vitro challenge with either Concanavalin A or pokeweed mitogen (A) and unstimulated in vitro lymphoproliferation (B) for cotton rats caught from December 1989 to May 1991. Vertical lines represent SE. Points with no superscripts in common indicated significantly different means (P < 0.05).
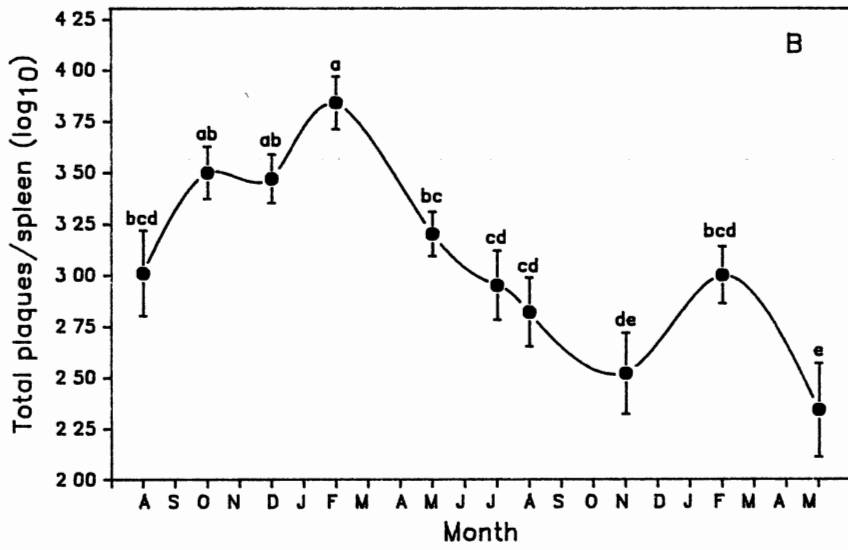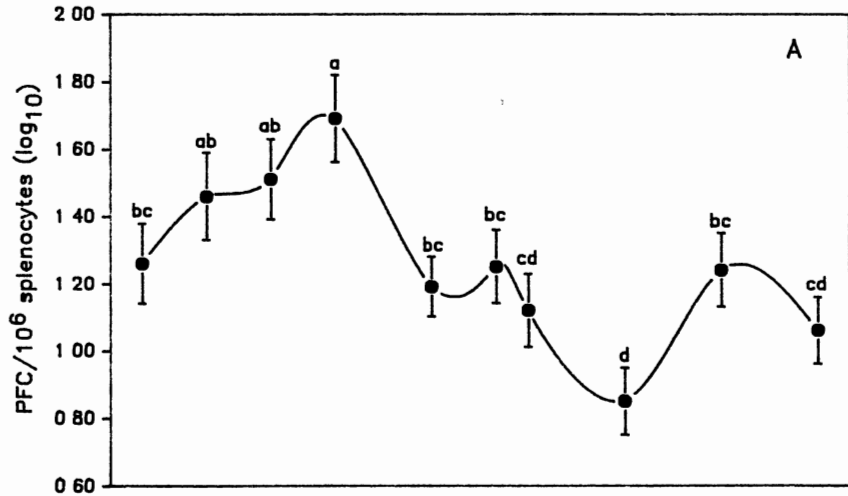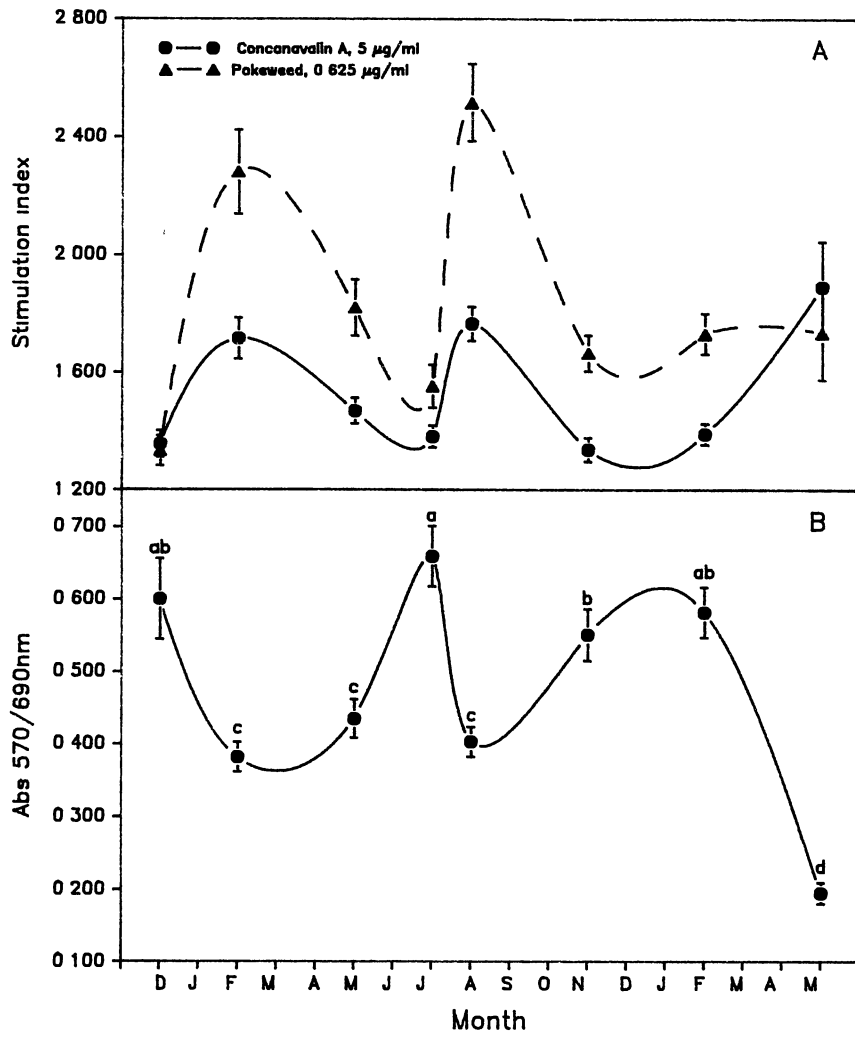
CHAPTER V

INFLUENCE OF DIETARY PROTEIN ON SELECTED MEASURES OF HUMORAL

AND CELLULAR IMMUNITY IN THE COTTON

RAT (<u>SIGMODON</u> <u>HISPIDUS</u>)

Abstract

Small mammal populations are subject to unpredictable but dramatic fluctuations in numbers. The mechanisms underlying this phenomenon are unclear but most probably include both extrinsic and intrinsic factors, such as nutrition and disease. To determine the effect of low dietary protein on the immunocompetence of small mammals, we conducted dietary protein feeding trials with cotton rats (Sigmodon hispidus) of different age classes (weanling, juvenile, subadult, and adult) randomly assigned to an isocaloric diet containing either 4% or 16% crude protein. Morphological and functional measures of both humoral and cell-mediated immunity were examined. Response of lymphoid organ morphology to low dietary protein was variable with the spleen being more sensitive to dietary protein than the thymus. No effect was seen on peripheral leukocyte counts and only adults had reduced relative splenocyte yields. Ability of splenic lymphocytes to respond to in vitro stimulation with mitogens was unaffected by dietary protein while capacity of weanling and subadult cotton rats fed 4% protein to mount a delayed-type hypersensitivity response was enhanced (P = 0.0099 and P = 0.0408, respectively). Nonspecific immunity, assessed by serum complement activity, was reduced (P = 0.001) in juveniles fed 4% protein compared to 16% but was unaffected (P = 0.142) by dietary protein in weanlings. Dietary protein did not influence the ability of subadults or adults to generate specific antibody in

response to exposure to heterologous erythrocytes. These data suggest that cotton rats are less sensitive to dietary protein than laboratory rodents, younger animals may be at a greater risk, and nutritional history may influence the response of a population to a reduction in available dietary protein. The results support the consideration of interactions between diet and immunity in small mammal population regulation.

Introduction

Mammalian populations are subject not only to regular seasonal shifts in numbers but also to unpredictable periods of exaggerated increases and declines in numbers. Many extrinsic factors, including climate, predation, and habitat alteration, have been suggested as mechanisms influencing the regulation of these fluctuations (Flowerdew 1987). Disease and nutrient availability have also been implicated as possible influences on population regulation but little is known about this relationship in wild populations.

It has been well documented in humans and laboratory rodents (Chandra and Newberne 1977; Watson 1984; Gershwin et al. 1985) that dietary protein malnutrition can effect both cellular and humoral mechanisms of immunity. Although some broad relationships seem to remain consistent, there is considerable variability among species and even, in some cases, within different strains of the same species (Cooper et al. 1974). There is little information in the literature

on immunological function in wild mammalian populations and, although it is possible that similar immunological alterations occur in response to dietary protein malnutrition, it is unlikely that these effects can be extrapolated from the existing literature. This study was designed to elucidate the effect of severe dietary protein restriction on both humoral and cell-mediated immunity in the hispid cotton rat (Sigmodon hispidus). We examined age-related influences of a high and low protein diet on selected measures of immunocompetence, including both specific (antibody production and delayed-type hypersensitivity response to exposure to antigen) and non-specific (serum complement activity and lymphoproliferative response to in vitro mitogenic challenge) immune responses.

Methods and materials

Animals and experimental design

Cotton rats in this study were housed under a 14L:10D light cycle in either polypropylene boxes with wood shavings for bedding or wire-bottom hanging cages. Cotton rats used in the first 3 feeding trials (n = 108) were born in our outbred captive colony and maintained on rodent chow (5001,Purina, St. Louis, MO; 24% CP) provided ad libitum until onset of trial. The remainder (n = 23) were trapped from tallgrass prairie habitat in central Oklahoma and placed on dietary trial (Trial 4) after a 1-week acclimation period in the laboratory. Known age juvenile and subadult

cotton rats from our captive colony were used in a series of 3 feeding trials (Trial 1: 18 days of age, weanlings; Trial 2: 31-34 days of age, juveniles; Trial 3: 56 days of age at onset, subadults) and adult cotton rats of wild-caught origin were used in Trial 4. Duration of the trials (21, 45, 42, and 35 days, respectively; Table 1) was variable, depending on subjective assessments of general condition of experimental subjects receiving the low protein diets.

For each trial, animals were randomly assigned within sexes to isocaloric experimental diets containing either 4 or 16% crude protein (United States Biochemical Corporation, Cleveland, OH 44128; Table 2) which was provided ad libitum. Food intake was monitored periodically during trials 2 and 4 to document that diets were palatable and to determine if animals fed a low protein diet would attempt to compensate by increasing consumption. A known weight of food was introduced into cages and uneaten portions weighed the following day. Initial and terminal body weights were obtained for each animal on trial. At termination of the trials, each animal was anesthetized with an intramuscular injection of ketamine hydrochloride (Aveco, Fort Dodge, IA 50501) at 50 mg/kg body weight, weighed, bled from the retro-orbital plexus to collect whole blood, and euthanized by cervical dislocation. Spleen and thymus were removed and weighed to the nearest 0.1 mg. Total peripheral leukocyte (WBC) counts were determined by diluting an aliquot of whole blood in 2% glacial acetic acid and counting cells with a

hemacytometer. Serum was collected by centrifugation and
stored at -20°C. Total concentrations of protein in sera
samples were determined using the biuret method (Kingsley
1942) and a human sera standard (SeraChem Clinical Chemistry
Control) and packed cell volumes (PCV) were analyzed using
the microcapillary tube method and expressed as a
percentage.

Serum hemolysis of sheep erythrocytes

Hemolysis of heterologous erythrocytes was measured
using a modification of the microhemagglutination assay
(Wegmann and Smithies 1966). Serial two-fold dilutions of
cotton rat sera in phosphate-buffered saline (PBS) were made
in 96-well microtiter plates. Fifty μl of a 1% suspension
of washed sheep erythrocytes (Colorado Serum Company,
Denver, CO; SRBC) in PBS was added to 50 μl of diluted serum
followed by 25 μl of 25% guinea pig serum (GPS; Bioproducts
for Science, Indianapolis, IN) in PBS. The reaction
mixtures were incubated at 37°C for 1 hr. Lytic titer was
defined as the $\log_2$ of the inverse of the highest serum
dilution resulting in complete lysis of the erythrocytes as
determined by visual observation.

Complement activity

Complement activity of serum was measured using a
modification of the assay described by Mayer (1961) and
expressed in CH_{50} units/ml where 1 CH_{50} unit is the amount

of complement giving lysis of 50% of cells. Briefly, a 1%
suspension of SRBC coated with rabbit anti-sheep erythrocyte
stroma (SRBC-A) was prepared by incubating a 1/100 dilution
of antibody (Nordic Immunological Laboratories, Capistrano
Beach, CA) with an equal volume of 10% SRBC for 15 min on
ice with constant rocking. The cells were then washed twice
and resuspended in veronal buffered saline (VBS) to a final
concentration of 1%. A 50 μl aliquot of the SRBC-A was
added to 200 μl of a 1/200 dilution of serum in VBS and the
mixture was incubated at 37°C for 30 min. After adding 750
μl VBS, the reaction mixtures were centrifuged and
absorbance of each supernatant at 414 nm was measured using
a Titertek Multiskan II. Percent lysis was calculated using
a standard curve generated with supernatants from lysis of
20% and 80% of the total cell volume. Percent lysis was
converted to CH_{50} units/ml using the equation,

$$CH_{50}/ml = \{[y/(y-100)]^{1/n}\}/x$$

where $1/n = 0.298$, x is volume of serum used in ml, and y is
percent hemolysis.

Delayed-type hypersensitivity

Delayed-type hypersensitivity (DTH) response was
assayed using the procedure described by Jones (1984).
Eight days prior to termination, each animal in trials 1, 2,
and 3 was sensitized with 100 μl of 3% oxazolone in absolute
ethanol applied percutaneously to a small shaved area on the
abdomen. The animals were challenged after 7 days with 25

μl of 3% oxazolone in absolute ethanol applied to each surface of the left ear; vehicle only (absolute ethanol) was applied to the right ear. Twenty-four hours later, at termination, both ears were removed at the base and weighed to the nearest 0.1 mg. Delayed-type hypersensitivity (DTH) response was calculated as absolute difference in ear weights and as a relative percent increase in weight of the left ear (experimental) over the right ear (control).

Lymphoproliferative response

Lymphoproliferation was measured by cellular reduction of tetrazolium salt (Mosmann 1983). Spleens were aseptically removed into pre-tared 15 x 60 mm sterile Petri dishes containing 5.0 ml RPMI-S consisting of RPMI-1640 supplemented with L-glutamine (2.05 mM), sodium pyruvate (1 mM), non-essential amino acids (1 mM), 2-mercaptoethanol (2×10^{-5} M), penicillin (100,000 u/L), streptomycin (100 mg/L), and normal horse serum (10%). The spleen was hemisected and splenocytes gently expressed in a glass-on-glass tissue homogenizer containing 5 ml of ice-cold RPMI-S. The resulting cell suspension was placed on ice for 10 min to allow sedimentation of the large tissue fragments, transferred to a sterile 16 x 125 mm culture tube, and centrifuged at 275 x g for 10 min at 20°C. The cells were washed 3 times in 5 ml of medium with centrifugation as above. Splenocyte numbers were determined by diluting an aliquot of the suspension in Trypan blue and counting on a

hemacytometer. Splenocyte concentrations were adjusted to 0.5×10^6 cells/0.09 ml and 0.09 ml aliquots were cultured in 96-well plates with 0.01 ml of either concanavalin A (Con A, Canavalia ensiformis) at 2.5, 5.0, 10.0, and 20.0 μg/ml culture or pokeweed mitogen (PWM, Phytolacca americana) at 0.156, 0.313, 0.625, and 1.25 μg/ml culture. Aliquots of cells in the absence of mitogen were cultured as unstimulated controls. After 69 hrs of incubation at 37°C and 5% CO_2, 10 μl of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; 5 mg/ml in PBS) was added to each well. At 72 hrs, 160 μl of acid-isopropanol (0.04 M HCl in isopropanol) was added to each well and absorbance at 570 and 630 nm was quantified using a Titertek Multiskan Plus microtiter plate reader. Response was calculated as a stimulation index representing the ratio of absorbances of mitogen-stimulated cultures to unstimulated control cultures.

Hemolytic plaque assay

The plaque assay used was adapted from Cunningham and Szenberg (1968). Five days prior to sacrifice, each cotton rat was injected intraperitoneally with 0.2 ml of a 10% suspension of SRBC in PBS. Splenocyte suspensions were centrifuged at 250 x g for 7 min at 10°C. The cell pellet was resuspended in Tris-ammonium chloride (Tris/Cl) to lyse red blood cells, underlaid, after 2 min, with 1.0 ml of ice-cold fetal calf serum, and centrifuged as before following a

7 min incubation at room temperature. The cells were resuspended and washed twice in 5 ml of PBS containing 5% fetal calf serum (PBS-F) with final resuspension in 2 ml of PBS-F containing 0.5% gelatin (PBS-F-G). Splenocytes were counted using the Trypan Blue exclusion procedure and a hemacytometer.

Cells were adjusted to a final concentration of 9.6 x 10^6 cells/ml and 50 μl and 200 μl aliquots were diluted to 220 μl with PBS-F-G in 12 x 75 mm culture tubes. Eighty μl of a 25% suspension of SRBC in PBS-F-G and 100 μl of 25% GPS in PBS-F-G were added to each tube and the mixtures vortexed. Incubation chambers were prepared (Marbrook 1980), loaded with 125 μl of the reaction mixture, sealed with a molten mixture of Vaseline and paraffin (1:1), and incubated at 37°C for 2 hr. The resulting plaques of anti-SRBC antibody-secreting splenocytes (PFC) were counted using a dissecting microscope.

Statistical analyses

Differences in morphological and functional measures of immunocompetence and changes in body weight due to dietary protein were examined by a one-way analysis of variance (SAS 1985). Bartlett's test of homogeneity (Steel and Torrie 1980) showed that variances for total splenocyte yields, total peripheral leukocyte counts, and PFC counts were heterogeneous so, prior to statistical analysis, these data were transformed (square root transformation for splenocyte

and leukocyte counts, logarithmic transformation for total
PFC, and arcsine transformation for PFC/mg spleen and
PFC/10^6 splenocytes). Differences due to dietary protein in
the distribution of high (>90 PFC/10^6 splenocytes) and low
($\leq$ 90) response in the hemolytic plaque assay was examined
using chi-square analysis (Steel and Torrie 1980).

Results

Initial body weights did not differ significantly ($\underline{P}$ =
0.813, 0.630, 0.503, and 0.850 for trials 1-4, respectively)
within any trial (Table 1) while change in body weight over
the duration of the trial was significantly ($\underline{P}$ = 0.0001)
influenced by dietary protein for all trials. All cotton
rats fed 16% protein gained weight ranging from 25.3 to
144.6% increase over initial body weight with an overall
average increase of 71.4 ± 5.7% ($\underline{SE}$) (Table 1). Cotton rats
fed 4% protein either minimally gained (weanlings and
subadults) or lost (juveniles and adults) body weight during
trial with an average body weight change of 1.2 ± 0.1%
(Table 1). These animals were generally small and thin but
no gross signs of illness were observed. Palatability of
the experimental diets, assessed by determination of average
daily intake (g/g body weight) for juveniles and adults, was
unaffected ($\underline{P}$ = 0.318 and $\underline{P}$ = 0.120, respectively) by
percent protein (Table 1).

Packed cell volume (all trials) and total
concentrations of proteins in the sera (trials 1, 2, and 3)

were both significantly (P = 0.001) reduced in cotton rats fed 4% protein (Fig. 1). Although WBC counts tended (P = 0.0698) to be greater among adults fed a 16% protein diet compared to those fed a 4% protein diet, there was no significant difference in WBC counts due to diet for any trial (Fig. 1).

Development of both primary and secondary lymphoid organs of cotton rats was suppressed on protein-restricted diets. Absolute weight of the thymus was significantly (P = 0.0001 for trials 1 and 2 and P = 0.0029 for trial 3) lower in weanling, juvenile, and subadult cotton rats fed a 4% protein diet (Fig. 2). There were no significant differences in relative thymus weights due to dietary protein among the 4 age classes (Fig. 2) with an overall mean of 0.53 ± 0.03 mg. Absolute (P = 0.0001) and relative (P = 0.012 for trial 1, P = 0.0025 for trial 2, and P = 0.0001 for trial 4) spleen weights were significantly decreased in weanling, juvenile, and adult cotton rats fed 4% compared to 16% protein in the diet (Fig. 3). Spleen weight did not differ between diet groups for subadult animals.

Total splenocyte yield was significantly (P = 0.0001) lower for weanling, juvenile, and adult cotton rats fed 4% protein (Fig. 4). Splenocyte yield reflected proportional changes in spleen weight with the exception of the adult trial where cotton rats fed 4% protein had significantly (P = 0.0002) lower relative splenocyte yields (Fig. 4).

Non-specific immunity, assessed by serum complement activity in CH_{50} units/ml, was significantly ($\underline{P}$ = 0.001) reduced in juveniles fed 4% protein but was unaffected ($\underline{P}$ = 0.142) by diet for weanlings (Fig. 5). The ability of cotton rats to mount a specific humoral, or antibody-mediated, response was assessed for subadults and adults by titering serum antibody generated in response to antigen (SRBC) exposure and by enumerating the number of splenocytes producing specific antibody. Hemolytic activity of the serum against sheep erythrocytes (Table 3) was unaffected by diet for subadults ($\underline{P}$ = 0.915) and adults ($\underline{P}$ = 0.708). Diet did not affect the ability of subadults to generate specific-antibody-forming cells (PFC) when expressed as $PFC/10^6$ splenocytes ($\underline{P}$ = 0.568) or total PFC ($\underline{P}$ = 0.747), but animals fed 4% protein had significantly ($\underline{P}$ = 0.015) greater PFC/mg spleen (Table 3). Adults fed 4% protein had significantly ($\underline{P}$ = 0.013) higher total PFC while diet had no effect on $PFC/10^6$ splenocytes ($\underline{P}$ = 0.247) or PFC/mg spleen ($\underline{P}$ = 0.114; Table 3). Additionally, diet had no significant effect on the proportion of high responders (Table 3) for either subadults ($\underline{X}^2$ = 0.007, $\underline{P}$ = 0.936) or adults ($\underline{X}^2$ = 0.683, $\underline{P}$ = 0.408).

There was a significantly greater DTH response in weanling ($\underline{P}$ = 0.0099) and subadult ($\underline{P}$ = 0.0408) cotton rats fed 4% protein (Fig. 6); the DTH response of juveniles was unaffected by dietary protein ($\underline{P}$ = 0.1099). Dietary protein concentration had no statistically significant effect on

ability of splenocytes to respond to in vitro mitogenic challenge at the concentrations of mitogens used in this study (Fig. 7).

Discussion

Cotton rats consume an herbivorous diet where average levels of available dietary protein rarely exceed 16% and deficiencies are not uncommon (White 1978). It has also been shown that protein content does not influence dietary selection by cotton rats and that, in fact, they often preferentially select plant parts which are lower in protein than other available parts (Randolph et al. 1991). The 4% protein diet used in this study probably represents a severe dietary protein restriction and, since the average life span of the cotton rat in the wild is approximately 6 months (Cameron and Spencer 1981), the duration of the trials in this study can be defined as representing a chronic protein restriction.

It has been demonstrated that lymphoid tissue is sensitive to dietary protein. Bell, et al. (1976) showed that weanling mice fed a diet containing 4% protein had smaller thymuses and spleens than did weanling mice fed a diet containing 20% protein and this effect was more pronounced in the thymus than the spleen. Kenney et al. (1968) also demonstrated reduced spleen size in response to low dietary protein in laboratory rats. The effect of dietary protein restriction on the lymphoid tissue and

cellularity of the cotton rats in this study was variable
but the spleen appeared more sensitive than the thymus to
dietary protein restriction. This was contrary to expected
sensitivities based on studies in laboratory rodents where
the thymus is more susceptible to protein status (Bell et
al. 1976; Hook and Hutcheson 1976).

Lower thymus weights observed among cotton rats fed a
low protein diet were primarily a reflection of differences
in body weight. Suppressed development of the thymus was
not evident among adult cotton rats (trial 4) because these
animals were older and had already undergone significant
age-related thymic atrophy (Miller 1963). In contrast,
spleen weights were reduced to a greater extent than the
thymus in younger cotton rats (trials 1 and 2) fed the 4%
protein diet and this reduction was greater than that
attributed to suppressed body growth. Total splenocyte
yields were similarly affected by dietary protein in younger
cotton rats (trials 1 and 2) although this was primarily a
linear reflection of reduced spleen weights. Spleens from
adult cotton rats in this study were more sensitive to
dietary protein than those of the subadults which were
fairly resistant. This may be a reflection of differences
in age or nutritional history since the adults were most
likely exposed to lower available dietary protein prior to
trial than were the subadults.

The results of studies on the response of cell-mediated
immunity (CMI) to dietary protein restriction are

conflicting and the degree of discrepancy seems to be dependent on the measurement of CMI used. Many studies (Harland 1965; Smythe et al. 1971; Neumann et al. 1975; McMurray et al. 1981) with malnourished children have shown a reduced ability to mount a DTH response to a variety of antigens. Similar observations have been reported for laboratory animals. For example, Kramer and Good (1978) demonstrated that weanling guinea pigs fed a 3% protein diet for 28 days were unable to mount a DTH response to BCG, whereas those guinea pigs fed either 6% or 9% protein mounted a measurable, but reduced, DTH response compared to those given 27% protein.

The above studies are in contrast to observed DTH responses of cotton rats to protein restriction where elevated DTH responses characterized those on the 4% protein diet. Similar observations were made by Malave and Pocino (1981) who reported that laboratory mice moderately restricted in dietary protein mounted DTH responses to SRBC that were consistently higher than normally-fed counterparts. This may reflect an increased sensitivity to dietary protein of the suppressor subclass of T-lymphocytes which are primarily responsible for regulation of immune responsiveness as suggested by similar findings for IgM response to alloantigens (Malave and Layrisse 1976) and skin allograft rejection (Cooper et al. 1974).

Examination of the literature demonstrates that it is difficult to generalize about mitogenic responses of

lymphocytes to dietary protein restriction. Lymphoproliferation in response to in vitro challenge with phytohemagglutinin has been reported to increase (Cooper et al. 1974), decrease (Watson and Haffer 1980), or remain unchanged (Kramer and Good 1978) during malnutrition. Concentration of protein in the diet of cotton rats had no measurable impact on the ability of splenocytes to respond to in vitro challenge with either a T-cell specific mitogen (Con A) or a mitogen specific for both B- and T-cells (PWM). Maintenance of a normal lymphoproliferative response occurred despite substantially smaller spleen weights and cellularity; presumably, ability of lymphocytes in our system to undergo clonal expansion following non-specific stimulation is resistant to dietary protein deficiency.

Serum complement activity, one of several non-specific defense mechanisms against foreign antigen, has been reported to be reduced in response to dietary protein restriction in both humans and laboratory rodents (Touraine et al. 1982; Kenney et al. 1965). In this study, although juvenile cotton rats fed 4% protein showed significant reduction in complement activity, younger cotton rats failed to show a response. This may reflect imposition of protein restriction during a critical period for development of complement activity in juveniles since the levels of complement in juvenile cotton rats on protein sufficient diets were much higher than those of weanling cotton rats on the same diet.

Data available on the effect of dietary protein on the capacity to generate specific antibody in response to antigenic challenge are as conflicting as those for cell-mediated immune function. Hook and Hutcheson (1976) reported that PFC response to SRBC in mice was significantly reduced in mice fed 2 and 6% dietary protein. Increased PFC responses to alloantigens (Malave and Layrisse 1976) and increased serum antibody titers to Brucella abortus in conjunction with reduced PFC response to SRBC (Cooper et al. 1974) in mice fed low protein diets have also been observed. The only effect of dietary protein on the capacity of subadult and adult cotton rats to generate specific antibody in response to SRBC was on total PFC production which was significantly higher for adults in the 16% protein group.

The effects of dietary protein restriction on immunological function of cotton rats did not appear to be dependent on the stage of development at which the restriction was imposed as has been observed in other studies (Watson and Haffer 1980). Our data suggest that while individual cotton rats in their natural habitat may well be at risk immunologically when dietary protein is limited, as a population, they seem to be more resistant to nutritionally-induced immunosuppression than laboratory rodents. This no doubt reflects, in part, the outbred nature of cotton rat populations which implies genetic heterogeneity. Many aspects of immune function are known to be controlled by genes located in the major

histocompatibility complex which is characterized by a high degree of polymorphism (Klein 1986). It would seem likely then that a genetically heterogeneous population would be at a selective advantage given dietary protein restrictions over one which is more genetically homogeneous. The relationship between aberrations in measurements of immune function and actual disease resistance in the natural environment is not clear. Cooper et al. (1974) showed that laboratory mice fed 8% protein diets had higher resistance to pseudorabies than those fed 27% but lower resistance to infection with streptococci. Further studies on this relationship with regard to degree and duration of the protein restriction may yield important information regarding mechanisms involved in mammalian population regulation.

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Table 1 Experimental design, initial body weight (BWI), body weight change expressed as percent increase of terminal body weight over initial body weight (BWC), and food intake (g/g body weight/day) for 4 dietary protein feeding trials with cotton rats (_Sigmodon hispidus_).

	N		Age at onset	Duration	BWI (g) 4%		BWI (g) 16%		BWC (%) 4%		BWC (%) 16%		Food intake 4%		Food intake 16%	
Trial	4%	16%	(in days)	(days)	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1	21	20	18	21	37.0	1.0	36.9	1.3	4.6	2.9	84.0	4.9				
2	10	12	31 - 34	45	33.0	2.1	34.0	1.6	-1.8	2.1	144.6	10.4	0.136	0.014	0.110	0.004
3	24	21	56	42	81.3	3.2	84.4	3.1	3.5	4.2	44.1	2.8				
4	11	12	unknown [a]	35	108.9	5.6	110.4	5.8	-7.3	2.9	25.3	2.5	0.074	0.008	0.066	0.001

[a] wild-caught adults

Table 2 Diet formulations for the 2 isocaloric diets fed to cotton rats during experimental feeding trials. Diets were prepared commercially by United States Biochemical Corporation, Cleveland, OH, 44128.

	Composition (%)	
Ingredient	4% diet	16% diet
Casein-high nitrogen	4.00	16.00
Starch- corn	82.00	70.00
Cottonseed oil	10.00	10.00
Salt Mixture USP XIV	4.00	4.00

Table 3. Mean hemolytic titers (log_2), numbers of splenocytes secreting specific antibody (PFC) in response to a single injection of sheep erythrocytes, and percent high responders (>90 PFC/10^6 splenocytes) in subadult (trial 3) and adult (trial 4) cotton rats fed isocaloric diets containing 4 or 16% protein. Numbers in parentheses represent SE. Asterisk (*) indicates significantly ($P < 0.05$) different means within a trial.

Measurement	Subadults		Adults	
	4%	16%	4%	16%
Hemolytic titer	6.0 (0.6)	6.1 (0.5)	3.6 (0.5)	3.8 (0.2)
Total PFC	31263 (16313)	12111 (4254)	2610 (1503)	16219* (9891)
PFC/10^6 splenocytes	288 (134)	139 (40)	130 (84)	98 (41)
PFC/mg spleen	287 (161)	87 (29)	28 (17)	57 (29)
Percent high responders	42.8	41.7	20.0	33.3

Fig. 1 Mean (± SE) of packed cell volume (a), total serum proteins (b), and peripheral blood leukocyte counts (c) in cotton rats fed either 4% or 16% protein. An asterisk (*) indicates a significant ($P < 0.05$) difference in means within a trial.
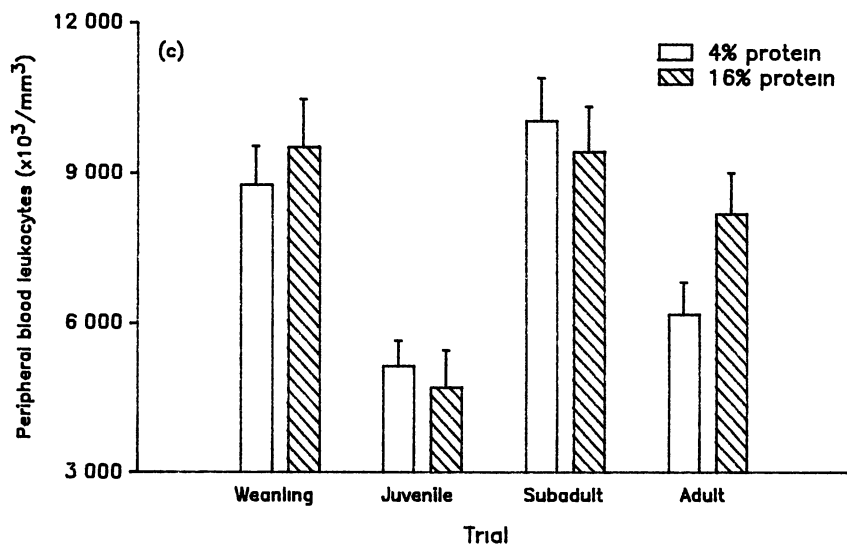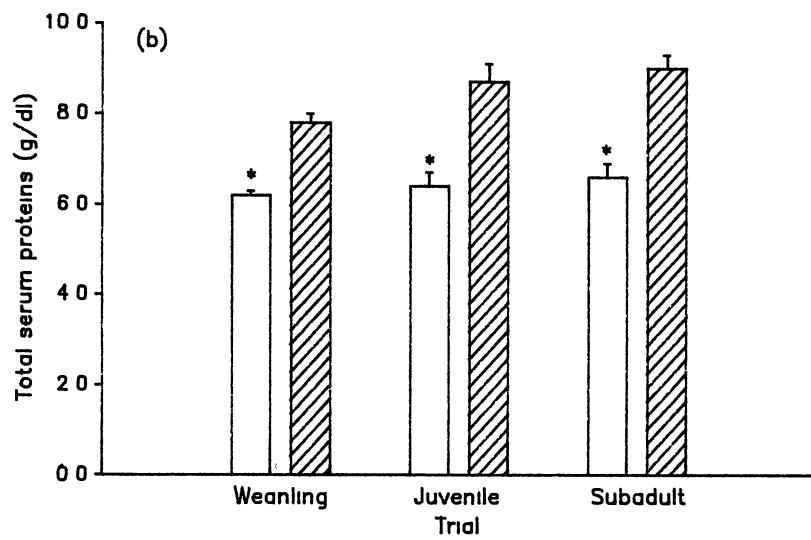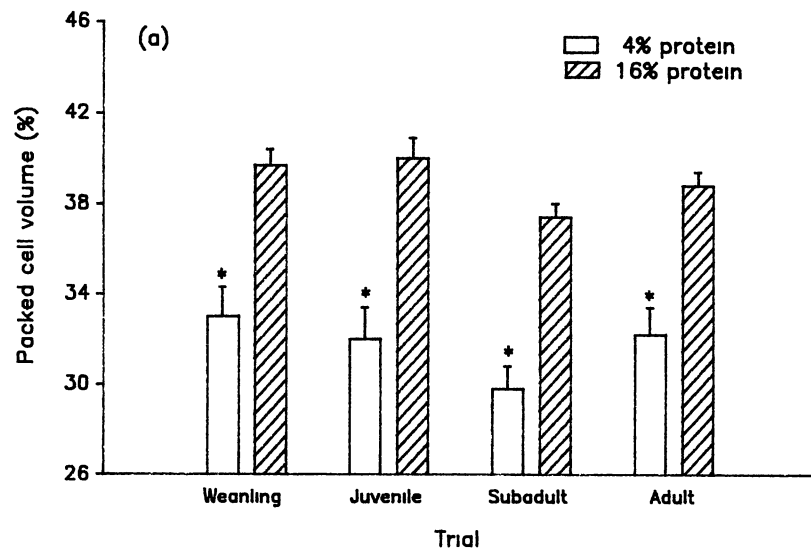
Fig. 2 Mean (± SE) of absolute (a) and relative (b) thymus weight in cotton rats fed either 4% or 16% protein. An asterisk (*) indicates a significant ($P < 0.05$) difference in means within a trial.
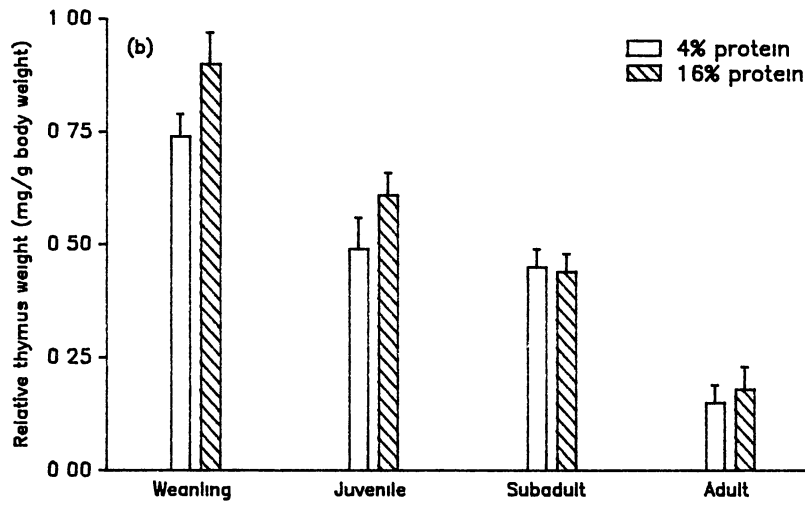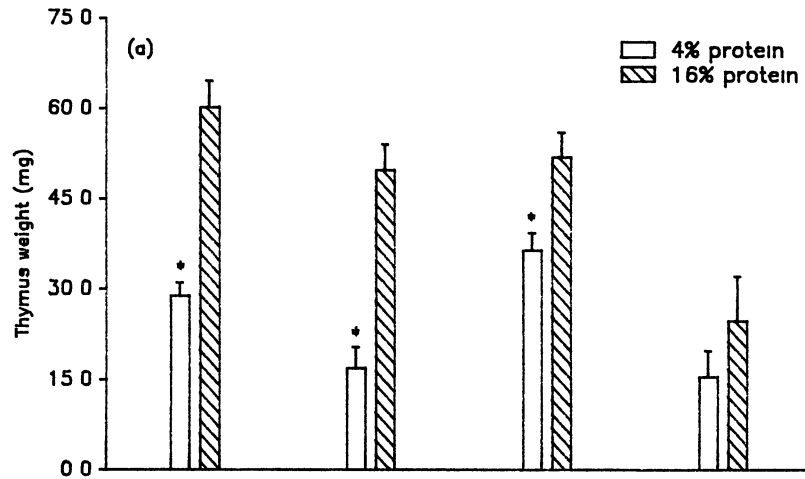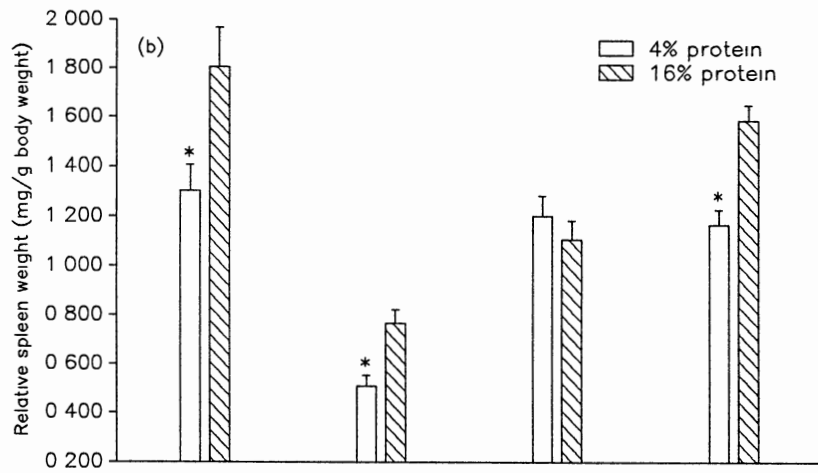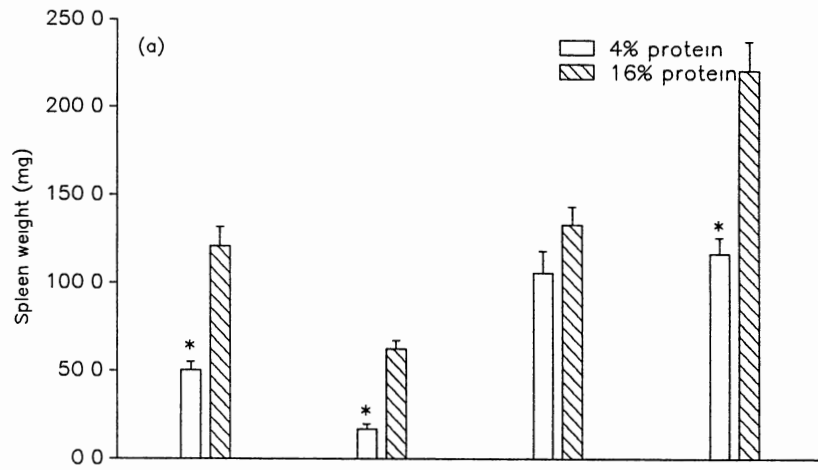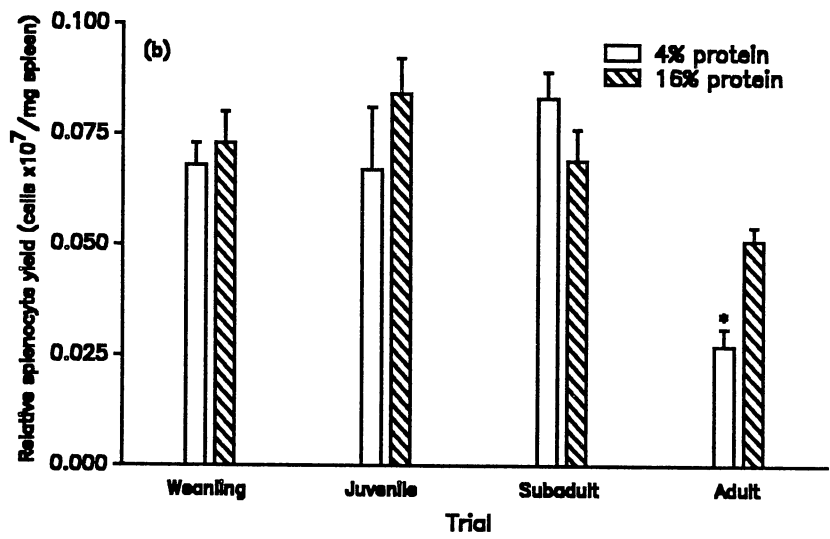
Fig. 3 Mean (± SE) of absolute (a) and relative (b) spleen
weight in cotton rats fed either 4% or 16% protein. An
asterisk (*) indicates a significant ($P < 0.05$) difference
in means within a trial.

(a)

4% protein
16% protein

Spleen weight (mg)

(b)

4% protein
16% protein

Relative spleen weight (mg/g body weight)

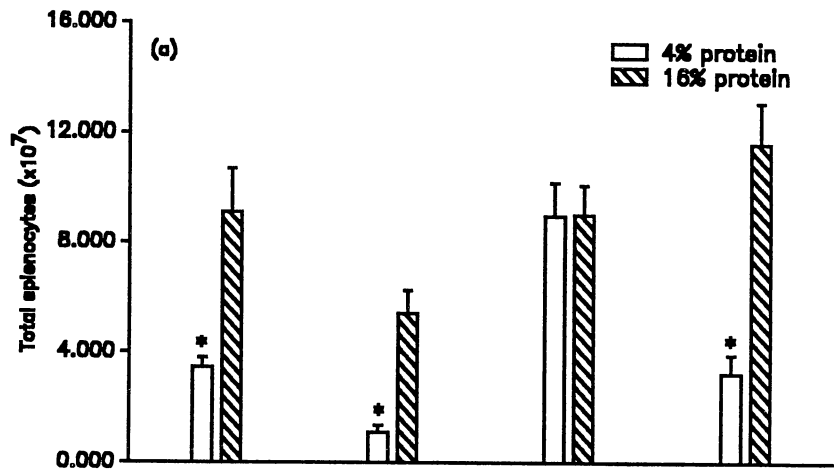Fig. 4 Mean (± SE) of absolute (a) and relative (b) splenocyte yield in cotton rats fed either 4% or 16% protein. An asterisk (*) indicates a significant ($P < 0.05$) difference in means within a trial.

Fig. 5 Mean (± SE) of complement activity (CH_{50} units/ml serum) in weanling and juvenile cotton rats fed either 4% or 16% protein. An asterisk (*) indicates a significant ($\underline{P} < 0.05$) difference in means within a trial.
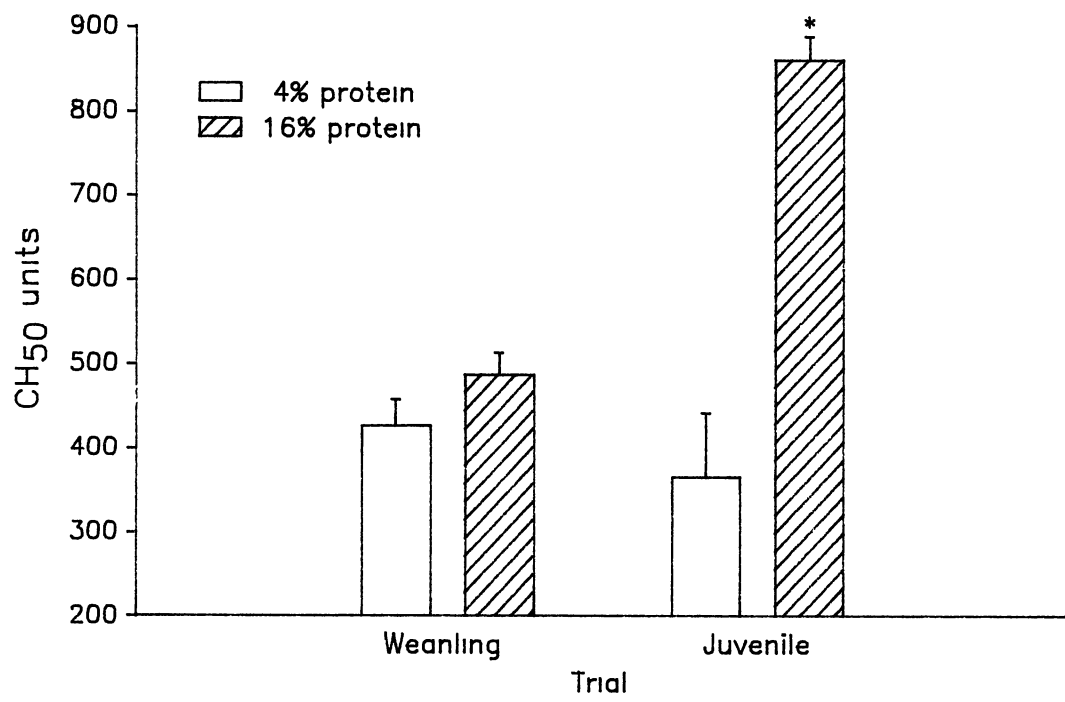
Fig. 6 Mean (± SE) delayed-type hypersensitivity response expressed as percent increase in weight of ear receiving antigen over weight of ear receiving vehicle only in weanling, juvenile, and subadult cotton rats fed either 4% or 16% protein. An asterisk (*) indicates a significant (P < 0.05) difference in means within a trial.
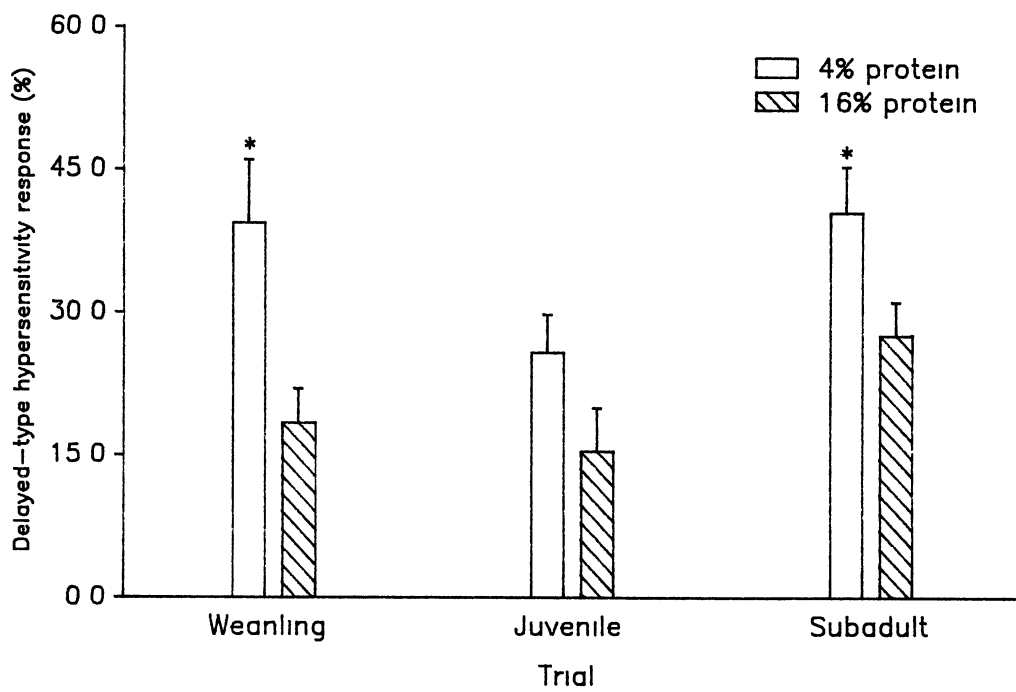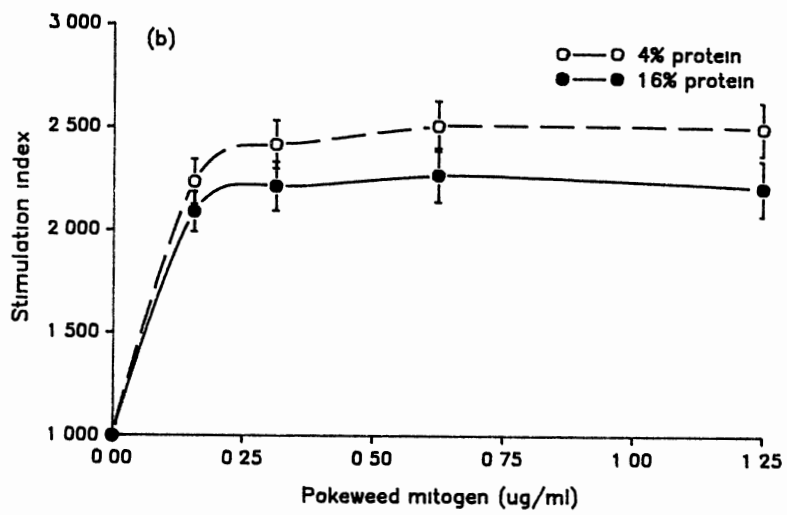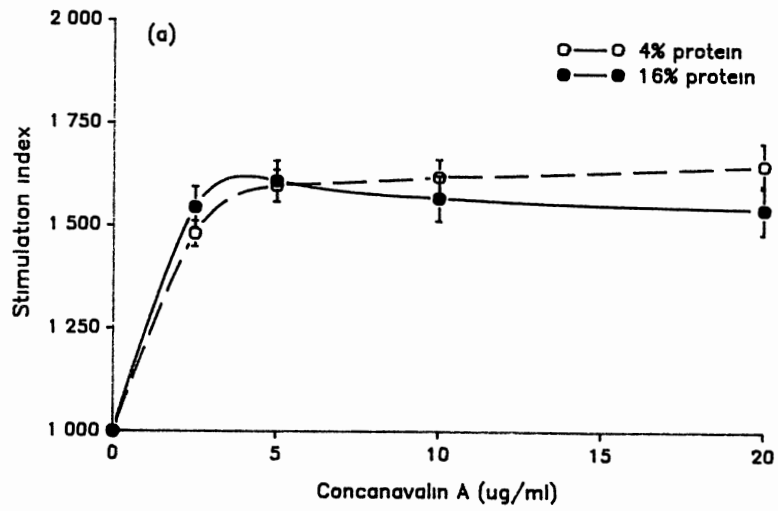
Fig. 7 Mean (± SE) stimulation index (ratio of absorbance at 570/630nm of stimulated cultures to unstimulated control cultures) in response to concanavalin A (a) or pokeweed (b) of splenocytes from cotton rats fed either 4% or 16% protein.

(a) Stimulation index vs Concanavalin A (ug/ml)
○——○ 4% protein
●——● 16% protein

(b) Stimulation index vs Pokeweed mitogen (ug/ml)
○——○ 4% protein
●——● 16% protein

VITA

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