IMMUNOLOGICAL ASSOCIATIONS WITH DENSITY AND SURVIVAL IN WILD POPULATIONS OF COTTON RATS AND PRAIRIE VOLES

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PREFACE

The following thesis chapters are part of a three year research project conducted in partial fulfillment of the Masters of Science degree requirements at Oklahoma State University. Chapters one and three are formatted for submission to the journal Oikos, and chapter two is formatted for submission to the Journal of Mammalogy. The chapters are complete as written and do not require supplemental material for support.

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CHAPTER I

THE WINTER IMMUNOENHANCEMENT HYPOTHESIS: ASSOCIATIONS AMONG IMMUNITY, DENSITY, AND SURVIVAL IN PRAIRIE VOLE (MICROTUS OCHROGASTER) POPULATIONS

Variations in immune function have been documented across seasons in several mammalian species. Such variation has been hypothesized to reflect the interactions of hosts with their seasonally changing environment. Seasonal variations in photoperiod, temperature, and population density have been shown to modulate immune responsiveness of a host in laboratory studies. To examine these associations under natural conditions, we monitored three populations of prairie voles (Microtus ochrogaster) for temporal variations in selected immunological parameters, population density, and survival rate from winter 1996 to spring 1997. Immunological function of selected immune parameters showed seasonal and temporal variations. The spontaneous and IL-2 stimulated T-cell proliferative responsiveness of prairie voles peaked in winter and declined in spring. Relative organ mass, hemolytic complement activity, and PHA-hypersensitivity varied temporally but showed no seasonal trend. Population density and survival rate of all three prairie vole populations varied temporally. Survival rates were lowest between spring and fall 1996, which coincided with a population crash on all sites. The highest rate of survival was between winter and spring 1997, when all populations demonstrated a prominent increase in density. To examine the association of immunocompetence with population density and rate of survival, a stepwise multiple

regression procedure was used. Relative spleen mass, IL-2 stimulated T-cell proliferation, and PHA-hypersensitivity were selected for inclusion in a model that explained a significant amount of variability in population density, while IL-2 stimulated T-cell proliferation and relative thymus gland mass explained a significant amount of variability in survival rate. The results indicate that seasonal environmental changes can enhance immune responsiveness of a host, and may counteract the immunoenhancing effects of photoperiod in wild populations of prairie voles. Our results also support an association between immune function and demography in wild populations. Intrinsic and extrinsic explanations for the immunological variations are discussed, along with the immunological association with population density and survival.

The logical relationship between an individual's immune system and their chances of survival in the wild has been suggested to play a mechanistic role in population regulation (Mihok et al. 1985, Dobrowolska and Adamczewska-Andrzejewska 1991, Lochmiller 1996). However, a direct link between immune function and survivorship has been demonstrated in few studies (Bradley et al 1980, Bradley 1987, Sams et al. 1996), partly because of the technical difficulty in studying the fate of dying animals (Mihok et al. 1988). In comparison, the relationship between the immune system and physiological stress has been examined extensively in the laboratory (Monjan and Collector 1977, Plotnikoff et al. 1991). Several factors, including nutrition (Jose and Good 1973, Watson 1984, Latshaw 1991, Klurfeld 1993), temperature (Kelley et al.1982, Blecha and Kelley 1981, Dabbert et al. 1997), behavior (Barnard et al. 1996, Sorci et al. 1997), social environment (Gross 1972, Nelson et al. 1996), and photoperiod (Nelson and Blom 1994) have been demonstrated to modulate immune responsiveness of a host.

Although rarely documented in wild rodent species, most ecologists would probably agree that temporal changes in immunity, disease prevalence, and mortality rates occur in populations, and are thought to reflect the interactions of hosts with their seasonally changing environment. Alterations in humoral (Sealander and Bickerstaff 1967, Dobrowolska et al. 1974, Dobrowolska and Adamczewska-Andrzejewska 1991) and cell-mediated (McIntyre and Cole 1969, Shiffrine et al. 1980, Lochmiller et al. 1994) immunity, variations in morphology of lymphoid tissues (Sealander and Bickerstaff 1967, Shivatcheva and Hadjioloff 1987, Lochmiller and Ditchkoff 1998), and fluctuations in lymphocyte subpopulations (Davis and Lochmiller 1995) occur across seasons in a few mammalian species. Because immune system alterations of this nature have the distinct

possibility of altering the prevalence of diseases in the population (Andrews et al. 1972, Geller and Christian 1982, Descoteaux and Mihok 1986), its relevance in explaining both annual and multi-annual fluctuations in populations has been widely noted (Mihok et al. 1985, Dobrowolska and Adamczewska-Andrzejewska 1991, Lochmiller 1996).

Demas and Nelson (1996) hypothesized that many small mammals preparing to enter harsh winter conditions have evolved a life-history strategy whereby reproduction is suppressed while enhancing immune system function via photoperiodic cues (herein referred to as the "winter immunoenhancement hypothesis"). Their laboratory studies on the prairie vole (Microtus ochrogaster) and other small mammals have provided general support for this hypothesis (Nelson and Blom 1994, Nelson et al. 1995, Demas and Nelson 1996, Demas et al. 1997). However, immune function of wild animals in their natural environments often appears to be depressed in winter (Nelson et al. 1995). Nelson and Demas (1997) hypothesized that winter stressors present under natural field conditions may counteract the short-day enhancement of immune function. Their laboratory experiments with prairie voles and deer mice (Peromyscus maniculatus) have shown that photoperiod, temperature, and population density interact to varying degrees to suppress or enhance immune system function depending on the severity and combination of stressors (Nelson et al. 1996, Demas and Nelson 1996). As a result, animals experiencing a mild winter should be subjected to less stress, and possibly maintain a normal, enhanced immune function (Demas and Nelson 1996). Consequently, populations during mild winters should have higher over-winter rates of survival, possibly leading to higher densities. Although these are intriguing hypotheses, there have

been no definitive studies designed to examine these immune associations in wild mammalian populations.

The prairie vole is a small, herbivorous microtine rodent whose populations are characterized by dramatic seasonal and multi-annual fluctuations throughout their geographical range (Stalling 1990, Getz et al. 1997). It is not uncommon for a prairie vole population to undergo a multi-fold increase or decline in a single year (Getz et al. 1997). The objective of this study was to examine the winter immunoenhancement hypothesis under field conditions by monitoring variations in cellular and humoral immunity across seasons in replicated populations of prairie voles. We hypothesized that prairie voles would show temporal variations in measures of immunological function, and that these variations in immunity would be associated with changes in population density and survival rate as hypothesized by Nelson et al. (1996).

Study area

Research was conducted on the John H. Nelson Environmental Study Area, University of Kansas, located 14 km north-northeast of Lawrence, Kansas, USA. Study areas were located in upland open-field habitats dominated by grasses such as brome (Bromus inermis), dropseeds (Sporobolus sp.), fescues (Festuca sp.), and blue grass (Poa pratensis). Climate varies seasonally as mean monthly temperature ranges from -1.7 °C in January to 26.1 °C in July, while mean monthly precipitation is lowest in January (2.9 cm) and highest in June (12.9 cm; Atmospheric Science Library 1990).

Materials and methods

Experimental design and demography

Three populations of prairie voles were monitored in early fall (late September to early October), winter (January to early March), and spring (May to early June) from winter 1996 to spring 1997 (5 seasons total). All three study areas (herein referred to as Coolgrass, Weather, and Orchard) harbored resident populations of prairie voles. To monitor demographic changes on the Coolgrass and Orchard study areas, 8 x 8 census grids with 10-m spacing between trap stations were established. For the Weather study area, a 4 x 10 and 2 x 12 census grid with 10-m spacing was established to fit the unique topography of this location. All three study areas were separated by approximately 1 km. Live trapping was used to assess population size in the fall, winter and spring by mark-recapture techniques. Sherman live traps (Sherman Traps Inc., Tallahassee, FL) were pre-baited with rolled oats two days prior to trapping followed by three consecutive nights of trapping. Cotton bedding was added to the traps during winter for added insulation.

Size of each population was calculated using program CAPTURE (Otis et al. 1978) and estimators converted to density after adjusting for grid size. The CAPTURE model M_o was identified as the best estimator of population size for the majority of data sets and was the most conservative by estimating size closest to the minimum known alive estimator. Survival rates for all populations were determined by program MARK (White 1997) using variable time intervals that were associated with the months between trapping seasons. Sixteen different models examining variations in survival rates (ϕ) and

capture probability (p) associated with study area population, time, and the interaction were examined. The MARK model $\phi(t)p(.)$ was selected as the best model with the lowest QAICc, and a satisfactory goodness-of-fit ($\chi^2 = 12.24$, $P = 0.9845$). This model predicts that survival rates do not differ between populations but should change with time, $\phi(t)$, and capture probability is constant across populations and time, $p(.)$. Because the model determined that probability of capture was constant, survival rates could be predicted for the period between spring 1997 and fall 1997 (White 1997).

Experimental animals

Experimental animals were removed using Sherman traps from areas > 100 m from population census grids; no marked animals were found on removal areas. We attempted to collect six male and six female adult animals (> 30 g; Gaines and Rose 1976) from each removal area per season. Trapping success was low on some occasions because of reductions in density or weather factors, so sample sizes were reduced. All animals were transported to the laboratory and housed in polycarbonate cages with wire lids and hardwood shavings for bedding at 20 ± 1 °C in an approved animal care facility. Food (Purina 5001, St. Louis, MO) and tap water were provided ad libitum. Within 48 hours of capture, immune function was assessed using a battery of selected tests described below.

Phytohemagglutinin hypersensitivity response

In vivo cell-mediated immunity was indexed in each animal using a hypersensitivity reaction as described by Williams et al. (1979). An intradermal injection of 50 µl of phytohemagglutinin (PHA; Sigma, St. Louis, MO; 2.5 mg / ml phosphobuffered saline (PBS)) was administered to one shaved hip 24 hours prior to termination; the opposite hip was challenged with an equal amount of sterile PBS to serve as the control. Double skin-fold thickness was measured to the nearest 0.001 inches with a pressure-sensitive micrometer. The PHA-hypersensitivity cell-mediated immune response was expressed as the percent increase in double skin-fold thickness of the stimulated side corrected for the control.

Morphology

Following the measurements for 24 hour PHA-hypersensitivity, prairie voles were anesthetized by metophane inhalation (Methoxyfluane, Pitman-Moore, Mundelein, IL). Body mass and reproductive status of females (pregnant, lactating, and vaginal perforation), and males (scrotal or non-scrotal) were recorded. A blood sample was obtained from the retro-orbital sinus plexus using heparinized-microhematocrit capillary tubes and Vacutainer serum separation tubes (Becton Dickinson Co., Rutherford, NJ). Whole coagulated blood was centrifuged (12 min., 2400 rpm), serum decanted into cryostorage vials, and stored at - 80 °C for future analysis. Animals were euthanized via cervical dislocation.

The spleen was removed aseptically as previously described by Lochmiller et al. (1998) for eventual harvest of immune cells. Paired adrenal glands, thymus, and eyes

were removed, cleared of adherent fat, and weighed to the nearest 0.1 mg; weight was expressed as mg of organ / g body mass. Eyeballs were placed in 10 % buffered formalin for 2 weeks and then lenses were removed and dried to obtain a dry lens weight to the nearest 0.1 mg for use as an index of age.

Hemolytic complement activity

A component of the innate, non-specific immune system was assessed by measuring complement activity. Hemolytic complement activity in serum was determined by a slight modification of the methods of DeWaal et al. (1988) as described by Sams et al. (1996). Briefly, 5 µl of serum diluted 1:80 in vernal buffer was serially diluted two-fold in a 96-well, round bottom, microtiter plate. Twenty-five µl of washed sheep red blood cells (0.6 % SRBC in vernal buffer, Colorado Serum Co., Denver CO), and 25 µl of a rabbit-anti-SRBC antibody (1/40 in vernal buffer, Nordic Immunological Laboratories, Capistrano Beach, CA) were added to each well. Plates were vortexed and incubated for 1.5 h at 37 °C and centrifuged for 5 min at 500 rpm. Absorbance (414 nm) was measured in a Titertek Multiscan II plate reader (Flow Laboratories, Inc., Mclean, VI). Hemolytic complement activity was expressed as CH_{50} units / ml serum, where 1 CH_{50} unit equals the amount of serum required to lyse 50% of the SRBC in culture (Kabat and Mayer 1961).

Cytokine-induced T-cell proliferative response

Spleens were processed aseptically and a single-cell suspension prepared according to the methods of Lochmiller et al. (1998). The ability of T-cells to respond to the cytokine interleukin-2 (IL-2) was measured using a lymphoproliferative assay. Splenocytes were stimulated with IL-2 (40 U / ml culture, recombinant human IL-2; Boehinger Mannheim, Germany) as described by Dabbert and Lochmiller (1995). Briefly, IL-2 (10 µl) was added to a 90-µl splenocyte suspension (final concentration of 500,000 cells / well in a supplemented medium RPMI-S) in 96-well microtiter plates in triplicate; 10 µl RPMI-S medium was substituted for IL-2 in unstimulated control wells. The RPMI-S medium was prepared by the addition of 1.0 % sodium pyruvate (100 mM solution, Sigma), 1.0 % penicillin-streptomycin solution (Sigma P-0781), 100 µl 2-mercaptoethanol (50 µM solution, Sigma), and 10 % horse serum to RPMI-1650 medium (Sigma).

After 54 hrs at 37 °C and 5% CO_2, ^{3}H-thymidine (1 µCi / well) was added to each well and incubated for another 18 hrs. Cells were harvested using a PhD Cell Harvester (Cambridge Tech Inc., Watertown, PA) onto glass-fiber filter strips (Cambridge). The amount of radioactivity as disintegrations per minute (dpm), incorporated into DNA of proliferating T-cells was measured in triplicate cultures using a liquid scintillation counter (Packard Instruments, Meriden, CT). The lymphoproliferative response of IL-2 stimulated T-cells was corrected for spontaneous proliferation by subtracting dpm of control wells.

Statistical analysis

Data were examined for homoscedasticity (Levene's test, Steel and Torrie 1980) and normality (PROC UNIVARIATE, SAS Institute Inc. 1990); data failing to meet these assumptions were transformed prior to further analysis (Zar 1984). To test the winter immunoenhancement hypothesis, we examined for temporal variations in measures of immunological function by analysis of covariance (PROC GLM, SAS Institute Inc. 1990) with sex, study area, and sampling period as main factor effects. Eye lens weight (herein referred to as age) as a index of age (Askaner and Hansson 1967), and reproductive status of individuals were treated as covariates to examine the influence of age and reproductive status on immune response parameters. Multiple comparisons of significant main effects and interactions ($P < 0.05$) were conducted using least squares means and least squares means option SLICE (SAS Institute Inc. 1990).

The increased secretion of sex hormones during reproductively active periods of the year has been hypothesized to influence immune responsiveness of individuals (Grossman 1985). To test this during the breeding season, we conducted an analysis of variance with reproductive status (active versus inactive) and sampling period as main effects. Reproductively active and inactive male (scrotal versus non-scrotal) and female (pregnant, lactating, or vagina perforate versus non-pregnant, non-lactating, or vagina perforate closed) prairie voles were present during all sampling periods, and therefore all periods were used in the analysis. Male and females were analyzed separately because of the opposite effects androgen and estrogen can have on immunological function (Grossman 1985).

To test the hypothesis that the level of immunocompetence is associated with population density and rate of survival, we used stepwise multiple regression (PROC

REG, SAS Institute Inc. 1990). Observations for this regression analysis consisted of the mean for each immune parameter in a population for each sampling period. Statistical significance for immunological parameters entering and remaining in the regression model was set at $P < 0.15$.

Results

Population assessments

Population densities at all three study areas were similar and varied temporally (Fig. 1A). The Coolgrass population showed the greatest amplitude in density across sampling periods, with peak densities in spring 1996 and a nadir in winter 1997. Estimates of survival rate did not differ ($P > 0.05$) among populations, but fluctuated temporally (Fig. 1B). Highest rates of survival occurred between winter and spring in 1996 and 1997, and lowest survival for the periods between spring and fall 1996 and 1997. Survival rates in populations were lowest between spring and fall 1996, which coincided with a population crash on all sites. The highest rate of survival was between winter and spring 1997 when all populations demonstrated a prominent increase in density (Fig. 1).

Morphometrics

Body mass of prairie voles did not differ ($P > 0.05$) between sexes or among populations, but did vary across sampling periods ($P = 0.001$; Table 1). Body mass was greatest in winter 1996 and 1997, and remained high in spring 1996. Age ($P = 0.001$) and reproductive status ($P = 0.001$) were significant covariates that influenced body mass of prairie voles. Body mass was influenced by reproductive status in both males ($P = 0.006$)

and females ($P = 0.001$). Body mass of scrotal males averaged 39.3 ± 1.3 g ($n = 40$) while non-scrotal male mass was 38.2 ± 1.9 g ($n = 19$). Body mass of reproductively active females averaged 44.5 ± 1.7 g ($n = 26$) compared to 36.5 ± 1.1 g ($n = 55$) for non-active females.

Relative weights of the spleen, thymus gland, and paired adrenals were similar ($P > 0.05$) between sexes and among populations. However, relative weights of the spleen, thymus gland, and paired adrenals demonstrated strong temporal fluctuations ($P = 0.001$, 0.006, and 0.001, respectively; Table 1). Relative spleen mass reached a consistent peak in springs 1996 and 1997. Relative thymus gland mass was greatest in fall and winter 1996 and reached a low in spring 1996. Relative mass of paired adrenals was greater in winter 1996 compared to all other sampling periods, which were similar in mass. Age was a covariate that influenced relative mass of the thymus gland ($P = 0.001$), but not the spleen or paired adrenals ($P > 0.05$).

Reproductive status of females affected ($P = 0.049$) relative mass of the thymus gland, which was greatest in non-active females (Fig. 2A). Reproductive status did not influence the relative mass of the spleen or paired adrenals in females ($P > 0.05$). Relative mass of paired adrenal glands in male prairie voles was influenced by reproductive status as indicated by a reproductive status by sampling period interaction ($P = 0.038$). Relative mass of paired adrenal glands of scrotal males, but not non-scrotal males, differed across sampling periods ($P = 0.001$), with a high in winter 1996 and a nadir in fall 1996. All other sampling periods were similar (Fig. 3). Relative mass of

paired adrenals was smaller in scrotal males compared to non-scrotal males in fall 1996 ($P = 0.004$; Fig. 3).

Phytohemagglutinin hypersensitivity response

The PHA-hypersensitivity index (%) of in vivo cell-mediated immunity varied across sampling periods ($P = 0.001$; Fig. 4) and among vole populations ($P = 0.029$). Cell-mediated immune responses were greater in winter and spring 1996 compared to all other sampling periods. Prairie voles from the Coolgrass population (175.7 ± 12.6 %, $n = 48$) mounted a greater ($P < 0.05$) cell-mediated immune response compared to those from the Weather (132.7 ±10.2 %, $n = 47$) and Orchard (128.6 ± 12.2 %, $n = 37$) populations. Gender and reproductive status had no apparent influence on cell-mediated immune responses of male and female voles ($P > 0.05$).

Non-specific immunity

Hemolytic complement activity, as a measure of innate humoral immunity, varied across sampling periods ($P = 0.001$; Fig. 5), but was similar between sexes and among populations ($P > 0.05$). A peak in complement activity was observed in spring 1996, with lows in fall 1996 and spring 1997. Intermediate levels of complement activity were evident during winter collections (Fig. 5). Complement activity increased two-fold from fall to winter 1997, followed by a significant spring decline, as predicted by the winter immunoenhancement hypothesis.

Reproductive status was a significant covariate ($P = 0.045$) of hemolytic complement activity in prairie voles. Separate analysis of males and females indicated that reproductive status of males had little influence on complement activity ($P > 0.05$); however, reproductively active female voles had reduced hemolytic complement activity compared to non-reproductively active females ($P = 0.015$; Fig. 2B).

Cytokine-induced T-cell proliferative response

The ability of unstimulated splenocytes to spontaneously proliferate in culture varied across sampling periods ($P = 0.001$), with peaks in winter followed by spring declines as predicted by the winter immunoenhancement hypothesis (Fig. 6). The spontaneous proliferative response of females was influenced by reproductive activity as evidenced by a reproductive status by sampling period interaction ($P = 0.036$). The unstimulated proliferative responsiveness of splenocytes of both reproductively active and inactive female prairie voles differed across sampling periods ($P = 0.001$ and 0.025, respectively). Reproductively active and inactive females differed in winter 1997 ($P = 0.008$), where reproductively active females had three-fold higher levels of spontaneous proliferation compared to non-active females (Fig. 2C). There were no differences in spontaneous proliferation between sexes or among populations ($P > 0.05$).

Interleukin-2 responsiveness of T-cells differed significantly across sampling periods ($P = 0.001$), with levels in winter 1997 being two-fold greater than during other sampling periods (Fig. 6). Winters 1996 and 1997 peaks were followed by spring declines as predicted by the winter immunoenhancement hypothesis. There was no

difference between reproductively active and inactive males or females in their ability to respond to IL-2 ($\underline{P} > 0.05$).

Relationships to demography

A robust examination of the relationships among immunity and demography was hampered by a lack of differences among populations for density and survival rate. The stepwise regression procedure selected the mean values (for a population at each sampling period) for relative spleen mass, IL-2 induced T-cell proliferative response, and PHA-hypersensitivity for inclusion in a model that explained a significant amount of the variation in population density across seasons and among populations ($r^2 = 0.857$, $\underline{P} = 0.001$; Table 2). Relative spleen mass and IL-2 responsiveness of T-cells collectively explained over 70 % of the variation in population density. Mean values for IL-2 responsiveness of T-cells and relative thymus mass were selected as the best predictors for explaining the variation in rates of survival across sampling periods and among populations ($r^2 = 0.842$, $\underline{P} = 0.001$; Table 2). In particular, the IL-2 responsiveness of T-cells explained 79 % of the variation in survival rate of populations.

Discussion

Animals residing in highly seasonal environments have evolved a myriad of physiological adaptations for maintaining fitness. Nelson et al. (1995) and Nelson and Demas (1996) reviewed many of these physiological adaptations and noted that predictable cues such as photoperiod allow individuals time to develop these seasonal adaptations for coping with future environmental change. It has been hypothesized

(Demas and Nelson 1996) that small mammals preparing to enter harsh winter conditions should enhance immune function using short-day photoperiodic cues. Although several laboratory studies have provided support for this winter immunoenhancement hypothesis (Nelson and Blom 1994, Nelson et al. 1995, Demas and Nelson 1996, Demas et al. 1997), its presence under natural field conditions has not been explored. Because immune function in wild species is sometimes depressed in winter (Nelson et al. 1995), Nelson and Demas (1997) hypothesized that winter stressors present under natural field conditions could counteract the short-day enhancement of immune function.

The pattern of immune organ development and function observed in prairie voles from replicated populations in this study generally provided support for the winter immunoenhancement hypothesis as described by Demas and Nelson (1996). Additionally, there was evidence that natural stressors present under field conditions often counteract the winter enhancement of immune function in prairie voles as predicted by Demas and Nelson (1997). If seasonal changes in photoperiod are a consistent cue for winter adaptation, we would predict that animals in the wild should increase their body mass and levels of immunocompetence before and attempt to maintain it through winter. As winter progresses and nutritional and climatic stressors take their toll on the host, we predict declines in these parameters during late winter or early spring. The body mass and T-cell proliferative responsiveness of adult animals in this study showed such a trend.

Body mass changes suggested that adults were increasing their body reserves in preparation for winter. Seasonal increases in body mass among small mammals in winter are not uncommon, and has been previously reported in voles (Anderson and Rauch 1984). Anderson and Rauch (1984) hypothesized that increases in fat reserves were

necessary to offset seasonal changes in temperature. The T-cell proliferative responsiveness of splenocytes to IL-2 and proliferation of unstimulated splenocytes in this study also supported the winter immunoenhancement hypothesis. Responses increased from fall to winter sampling periods and winter peaks were consistently followed by spring declines. However, the increased response in winter also could be explained by assuming that individuals of poor immunological condition were removed from the population earlier in winter by succumbing to environmental stressors (Andrews et al. 1972), which left a higher percentage of immunocompetent individuals when winter sampling occurred. The decreased responses of T-cells to IL-2 in spring could be due to reductions in body nutrients for those individuals surviving over winter (Anderson and Rauch 1984), or alternatively, could reflect an endocrine-induced suppression from the initiation of breeding (Grossman 1985). Although there was an apparent decline in T-cell proliferative responsiveness from winter to spring, this did not have a significant impact on overall cell-mediated immunity, as measured by the in vivo PHA-hypersensitivity index. This suggests that environmental stressors were not severe enough to suppress immunity completely by spring 1996 and 1997. The immune system is a highly redundant, interactive physiological process that maintains immunocompetence through such stressful periods (Kelley 1985).

Temporal variation in relative thymus mass and hemolytic complement activity suggested that natural stressors may have suppressed the predicted winter immunoenhancement. Such natural stressors can increase corticosteroid secretion (Moberg 1985), which can have significant immunosuppressing effects in some species (Khansari et al. 1990). In our study, paired adrenal mass showed the same trend as the

PHA-hypersensitivity response, with highest values in winter and spring 1996, however, hemolytic complement activity and relative spleen and thymus gland masses showed no such trend with adrenal mass. The lack of a similar trend may be partially explained by the observation that prairie voles are a relatively corticosteroid-resistant species (Klein et al. 1996). The potential for corticosteroid-independent mechanisms of immune system regulation are possible as similar variations in immune function have been seen in laboratory studies of adrenalectomized animals (Keller et al. 1983).

Variations in immunocompetence, like those associated with photoperiod and natural stressors, also are associated with gender in some rodent species, but was not observed in prairie voles in this study. Males of many species generally exhibit reduced immune responses in comparison to their female counterparts (Zuk and McKean 1996). This suppression has been attributed to the level of circulating testosterone in males (Grossman 1985). Prairie voles are monogamous (Stalling 1990) and have lower circulating concentration of testosterone than many other species examined (Klein and Nelson 1998). The lack of reproductive status affecting the immune response of males in this study, and the lower levels of circulating testosterone found in prairie voles in comparison to other arvicoline rodents (Klein and Nelson 1998) may explain these apparent inconsistencies with other rodent species.

This study provides further evidence for a trade-off between reproduction and immunity (a foundation of evolutionary theory; Stearns 1992). This trade-off is most pronounced during the winter when nutritional constraints are probably most limiting in the habitat of prairie vole populations (Bronson 1989). Reproduction maybe too costly (24 % of females were reproductively active in winter, in comparison to 41 % in spring

and 33 % in fall seasons), and resources are allocated to immunocompetence as predicted by the winter immunoenhancement hypothesis (Demas and Nelson 1996). During the breeding season, suppressed immunity in reproductively active female prairie voles could be the result of a combination of endocrine adjustments and reallocation of limited nutrient resources when physiological demands are maximum. Reduced levels of immunocompetence in reproductively active females was in general associated with increased secretion of progesterone during the breeding season (Grossman 1985). Reallocation of resources from immunity to reproductive effort has been proposed to explain observed reductions in antibody responsiveness (Deerenberg et al. 1997) and health (Ots and Horak 1996) in birds. We suggest similar trade-offs comprise an important component of prairie vole life history.

Nelson et al. (1996) observed that population density of prairie voles in the laboratory influenced responses of the humoral immune system. Regression analysis in this study demonstrated an association of density and survival rate with parameters of immunity in prairie voles as predicted by Nelson et al. (1996). Relative spleen mass, Il-2 stimulated T-cell proliferation, and PHA-hypersensitivity responses accounted for a substantial amount of the variation in population density across seasons and among populations. Although an association of immunity with demography was indicated, we can not establish a cause-effect relationship. The lack of differences in density and survival rates among populations within a season precluded a strong test of this hypothesis. However, these findings fail to dispute Nelson's et al. (1996) hypothesis that there is an association of density with immunity. Given these observations and recent theories of population regulation incorporating explicit immunological mechanisms

(Mihok et al. 1985, Lochmiller 1996), more robust tests of this hypothesis are clearly warranted. Because season has a powerful regulatory role in determining level of immunocompetence in prairie voles in the wild, such a test of this hypothesis would necessitate exploring the association of immunity with demography across seasons. The key ingredient to a robust test of this hypothesis would be to compare high-density populations to replicated low-density populations with each season. Alternatively, following fewer populations across an entire multi-annual cycle may provide a similar robust test of this hypothesis.

Our results support the contentions of others (Sheldon and Verhulst 1996, Lochmiller et al. 1994, Demas and Nelson 1996) that seasonal life-history adjustments are responsive to environmental constraints and involves alterations in immune system function. Central to this concept is the assumption that both reproduction and immunocompetence are costly to the host in terms of nutrients and hence survival. Reproduction is energetically and nutritionally costly to prairie voles (Bronson 1989); however, costs associated with maintenance of a competent immune system or combating an immune challenge are poorly understood in laboratory models and speculative in wild animals. The limited studies that do exist suggest that these fitness costs can be significant in a host (Booth et al. 1993, McCracken et al. 1995), especially during sepsis or injury (Chiolero et al. 1997). Future studies combining measurements of metabolism and immunity could provide important insights into life-history evaluation of this species.

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Table 1. Temporal variations in morphological parameters of prairie voles from winter 1996 to spring 1997. Relative organ masses are presented as mg of organ per g of body mass. Superscripts that differ signify statistical significance at $P < 0.05$ across sampling periods.

	Winter 1996			Spring 1996			Fall 1996			Winter 1997			Spring 1997		
Parameter	x	se	n	x	se	n	x	se	n	x	se	n	x	se	n
Body mass (g)	42.64^{a}	1.05	36	41.77^{ab}	1.83	23	35.29^{b}	1.72	25	41.3^{ab}	1.07	28	33.13^{c}	1.87	28
Spleen (mg/g)	2.24^{a}	0.13	36	3.49^{b}	0.36	23	2.58^{ab}	0.25	25	3.07^{ab}	0.47	28	3.45^{b}	0.45	27
Thymus (mg/g)	0.42^{ac}	0.04	33	0.19^{b}	0.04	22	0.51^{a}	0.09	25	0.21^{b}	0.02	27	0.30^{bc}	0.04	28
Adrenals (mg/g)	0.67^{a}	0.03	36	0.64^{b}	0.16	23	0.44^{b}	0.04	25	0.44^{b}	0.02	28	0.49^{b}	0.02	27

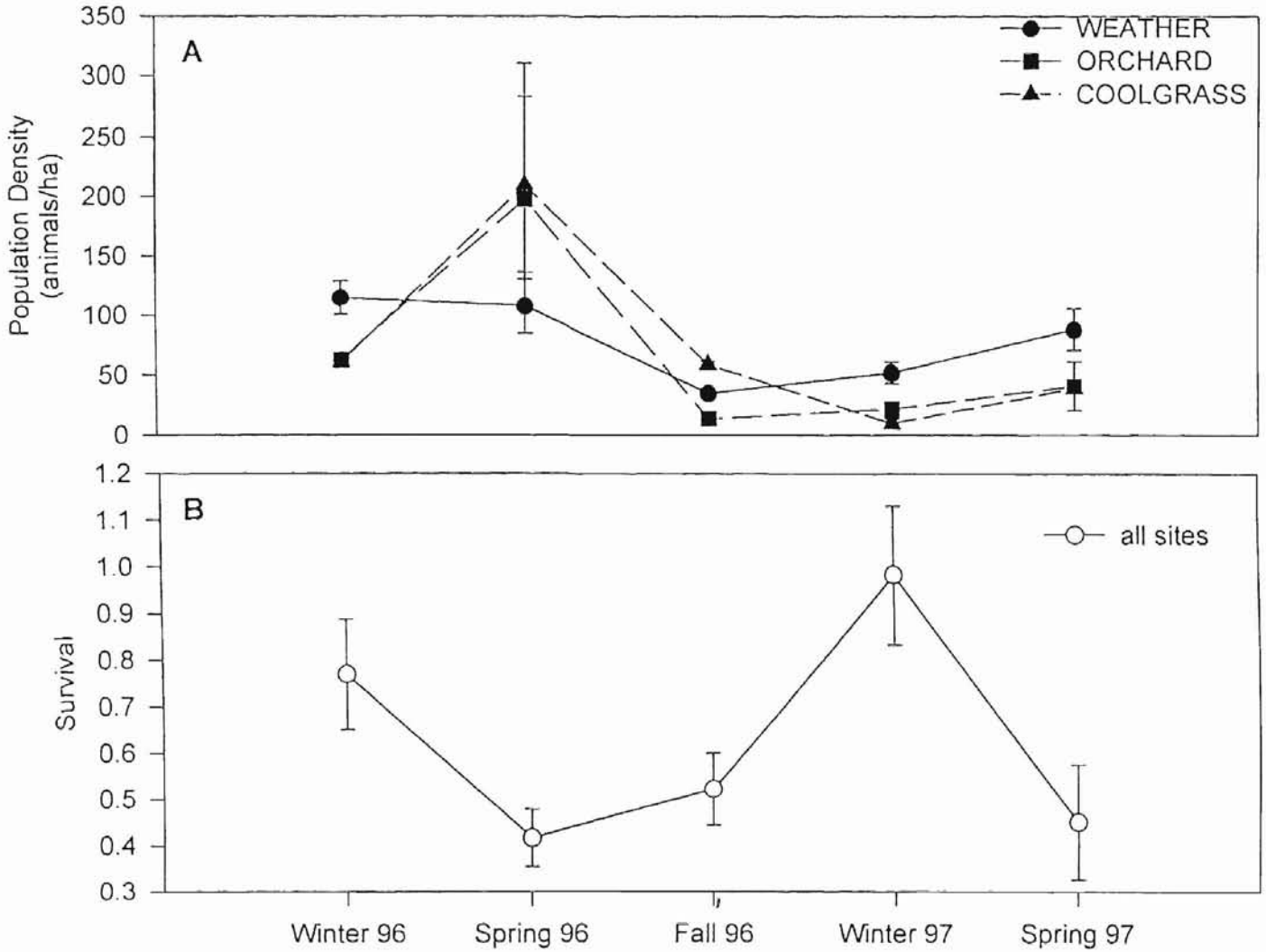
Table 2. Independent immune variables selected by stepwise-multivariate regression for predicting density and survival rate of prairie vole populations on the John H. Nelson Environmental Study Area, Lawrence, Kansas, 1996 through 1997 (n = 15).

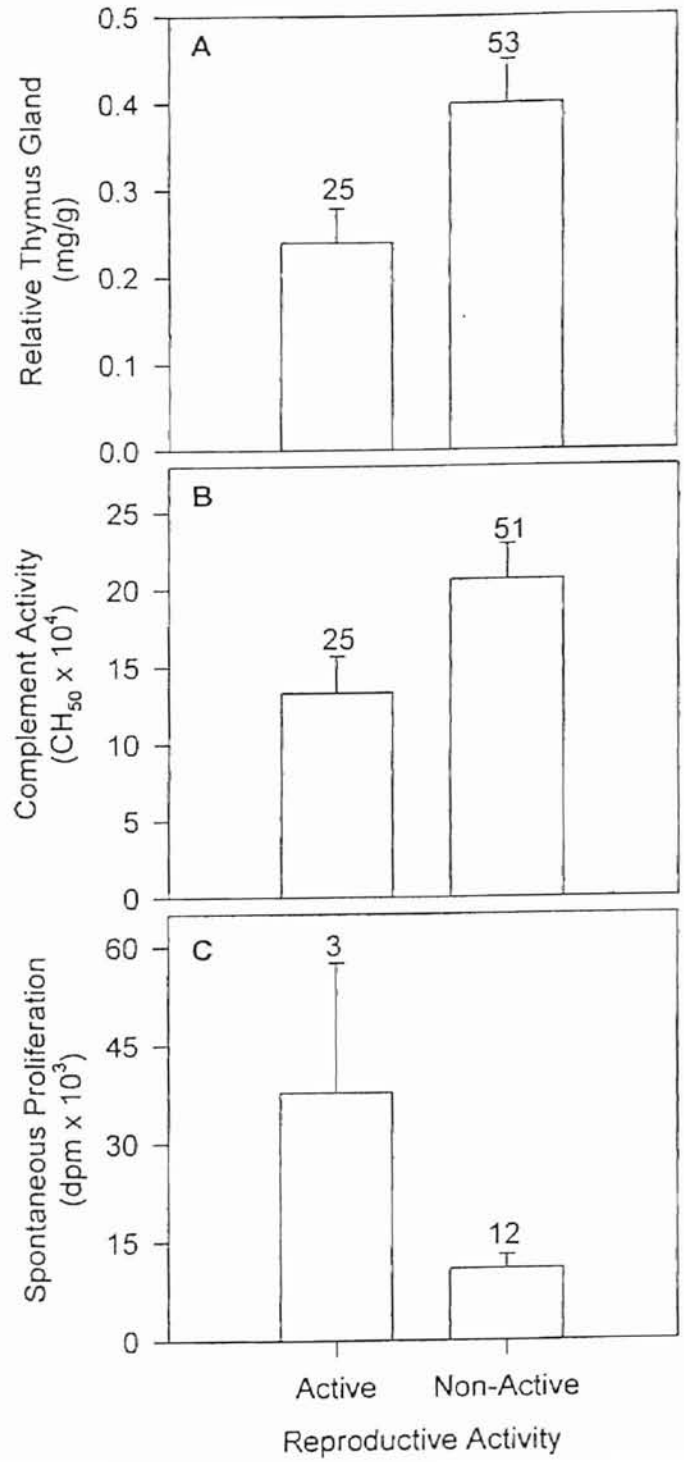
Parameter	Variables	Estimate	SE	Partial r^2	F	P-value
Density						
	Intercept	-64.26	25.41		6.39	0.035
	Spleen (mg/g)	1086.09	202.79	0.3928	28.68	0.001
	IL-2 stimulation (dpm)	-0.01	0.01	0.3474	9.32	0.016
	PHA-hypersensitivity (%)	0.25	0.10	0.1171	6.56	0.034
Survival rate						
	Intercept	0.02	0.13		0.02	0.089
	IL-2 stimulation (dpm)	0.01	0.01	0.7963	47.09	0.001
	Thymus gland (mg/g)	12.19	7.57	0.0455	2.59	0.142

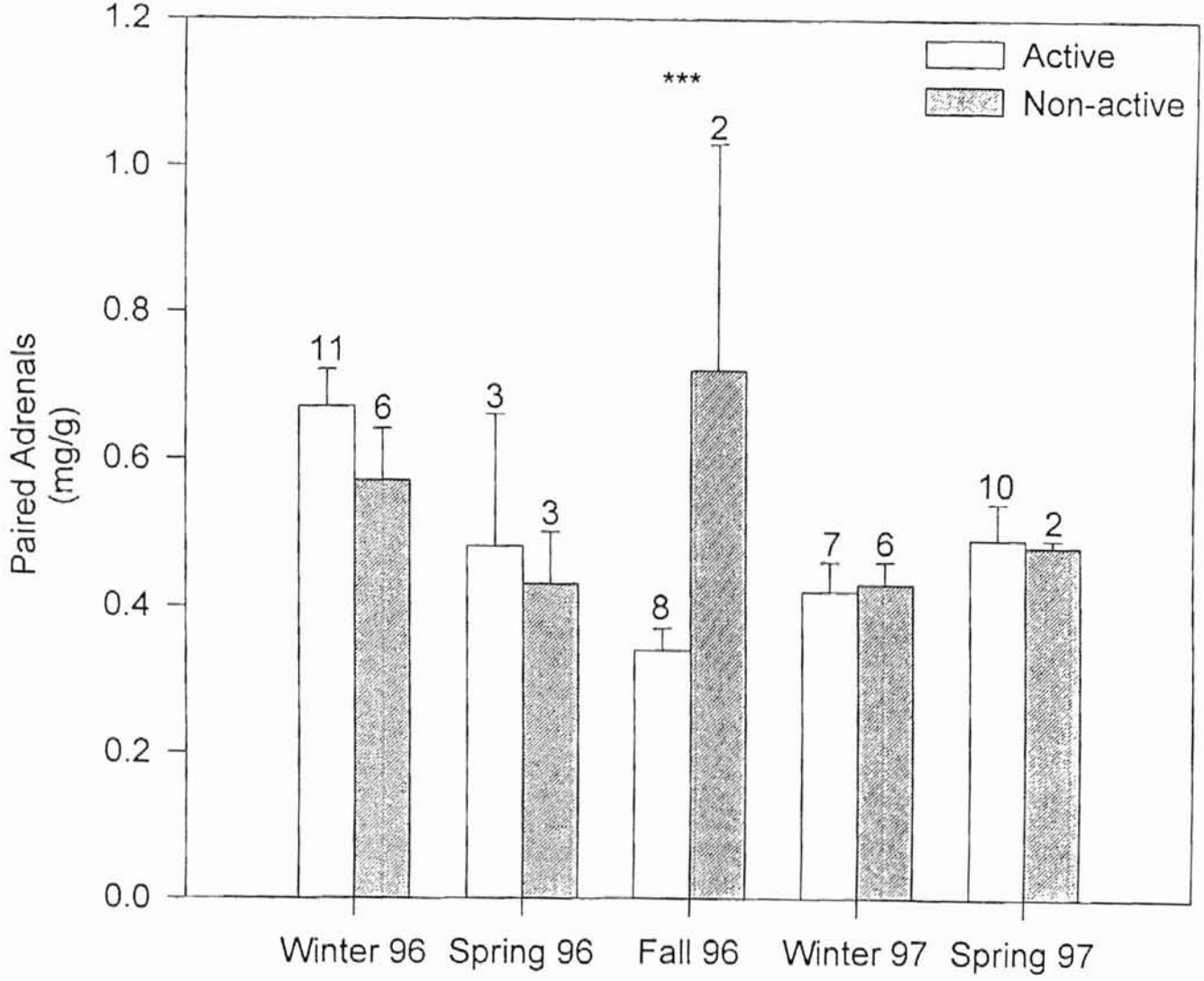
List of Figures

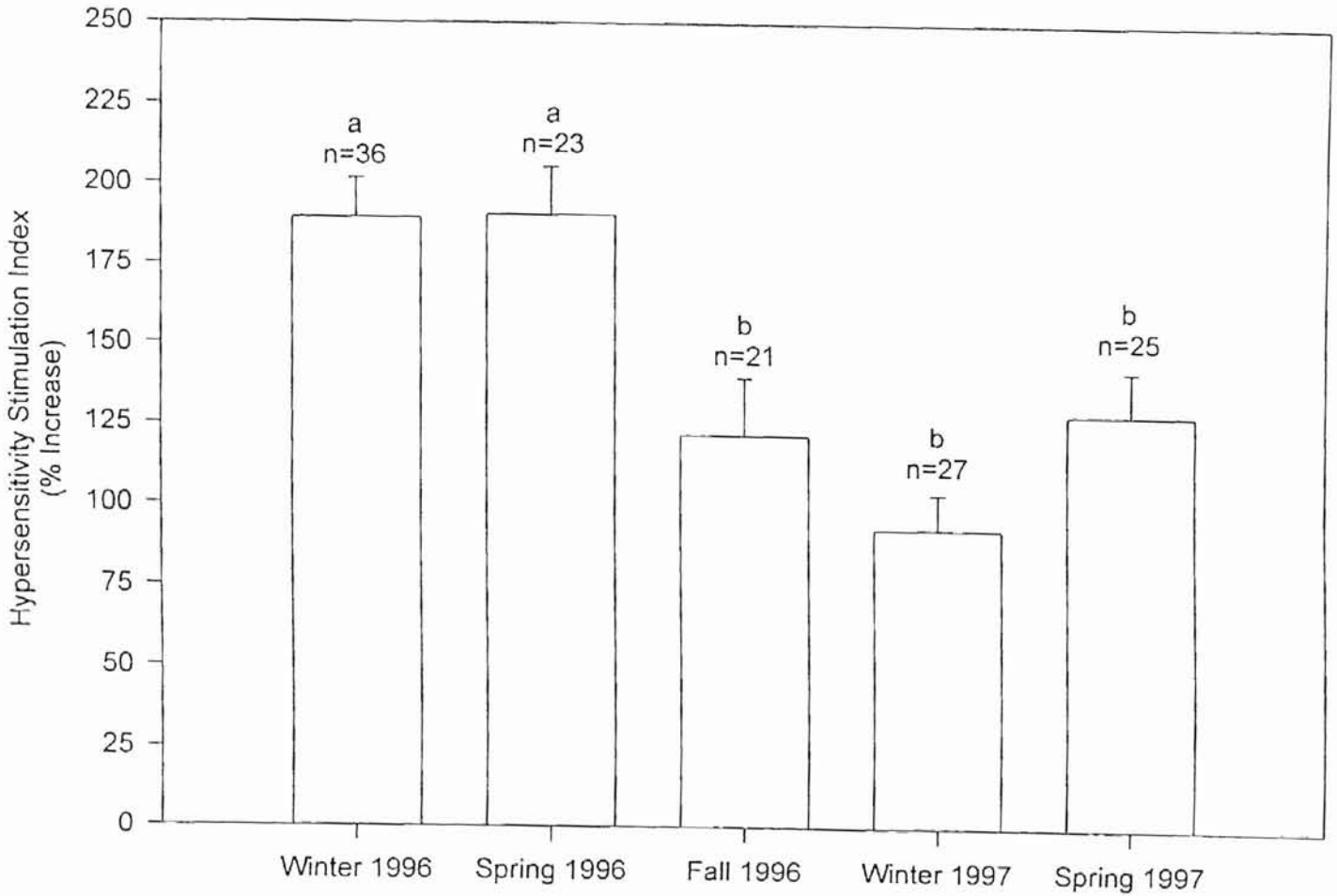
Figure 5. Temporal variations in hemolytic complement activity (mean ± SE) of prairie voles from winter 1996 to spring 1997. Letters that differ above SE bars signify statistical differences at $\underline{P} < 0.05$.

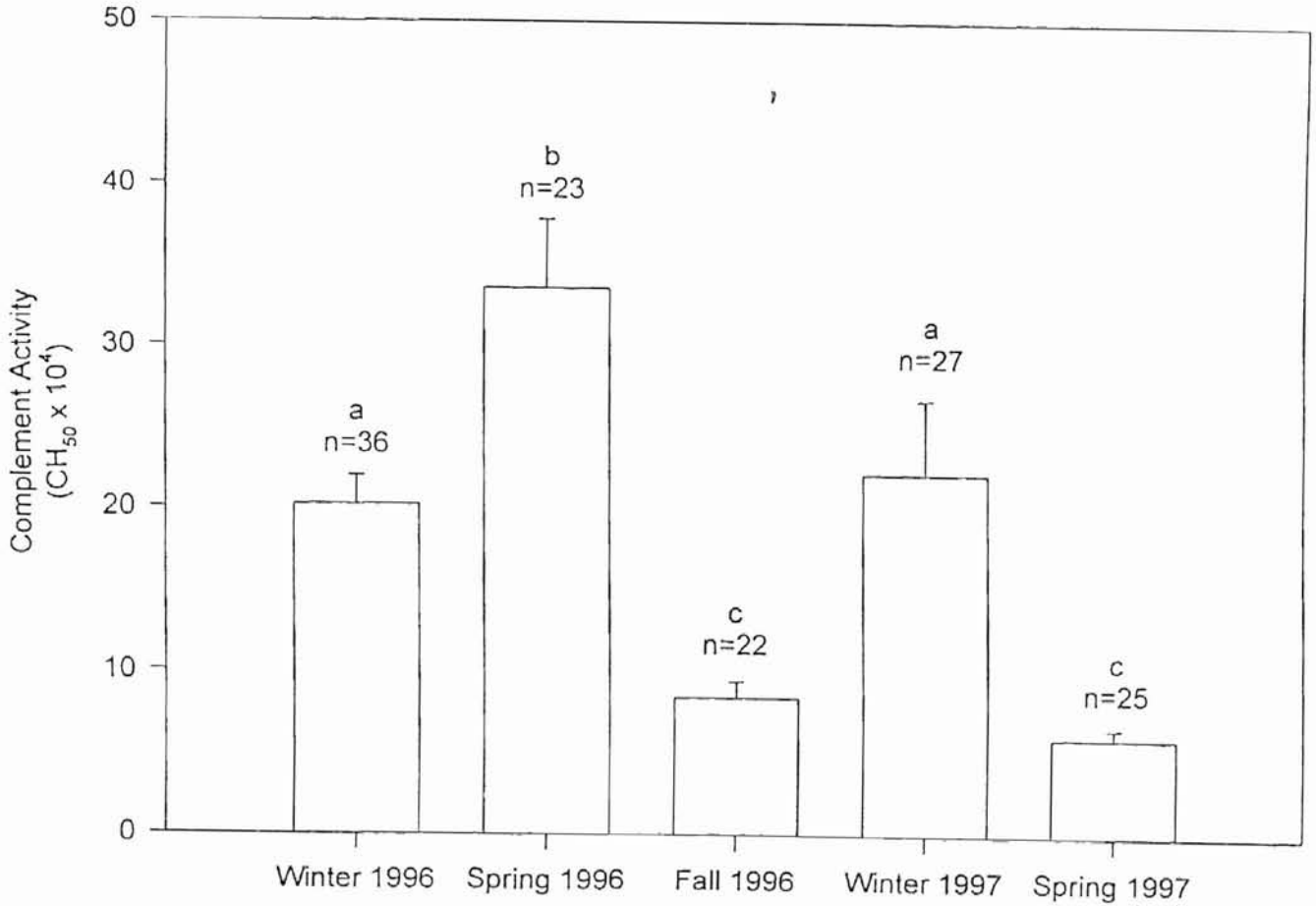
Figure 6. Seasonal variations in spontaneous (no fill) and IL-2 stimulated (solid fill) T-cell proliferation (mean ± SE) of prairie voles from winter 1996 to spring 1997. Letters that differ above SE bars signify statistical significance at $\underline{P} < 0.05$.

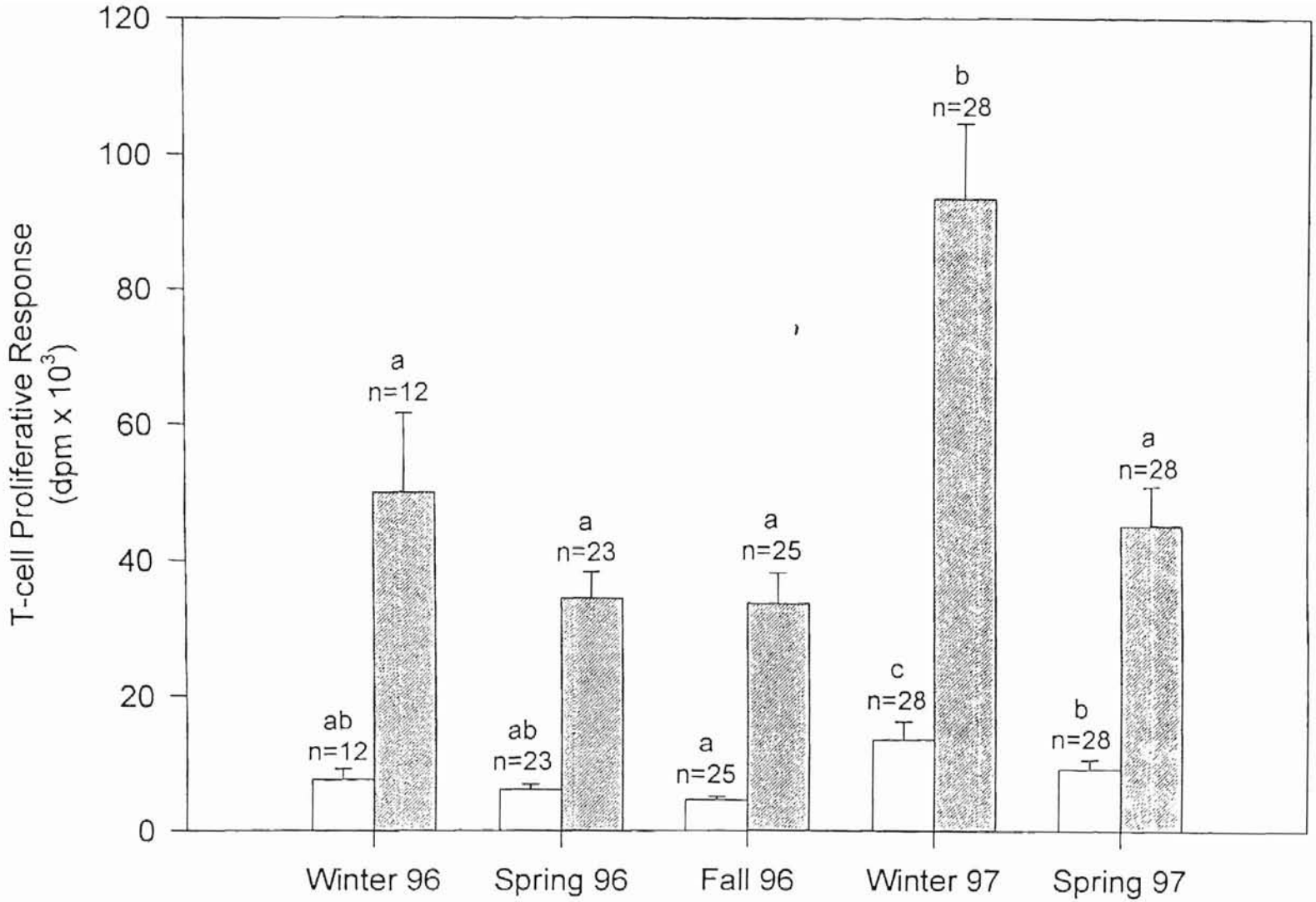

A
B
WEATHER
ORCHARD
COOLGRASS
all sites
Population Density
(animals/ha)
Survival
350
300
250
200
150
100
50
0
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
Winter 96
Spring 96
Fall 96
Winter 97
Spring 97


A
B
C
Relative Thymus Gland (mg/g)
Complement Activity (CH_{50} x 10^4)
Spontaneous Proliferation (dpm x 10^3)
0.5
0.4
0.3
0.2
0.1
0.0
25
53
25
51
60
45
30
15
0
3
12
Active
Non-Active
Reproductive Activity


Active
Non-active
***
Paired Adrenals (mg/g)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
11
6
3
3
8
2
7
6
10
2
Winter 96
Spring 96
Fall 96
Winter 97
Spring 97


Hypersensitivity Stimulation Index
(% Increase)
250
225
200
175
150
125
100
75
50
25
0
a
n=36
a
n=23
b
n=21
b
n=27
b
n=25
Winter 1996
Spring 1996
Fall 1996
Winter 1997
Spring 1997


Complement Activity
(CH_{50} x 10^4)
50
40
30
20
10
0
a
n=36
b
n=23
c
n=22
a
n=27
c
n=25
Winter 1996
Spring 1996
Fall 1996
Winter 1997
Spring 1997


T-cell Proliferative Response
(dpm x 10^3)
120
100
80
60
40
20
0
ab
n=12
a
n=12
ab
n=23
a
n=23
a
n=25
a
n=25
c
n=28
b
n=28
b
n=28
a
n=28
Winter 96
Spring 96
Fall 96
Winter 97
Spring 97

CHAPTER II

HOST RESISTANCE TO A PATHOGENIC CHALLENGE LACKS SEASONAL SPECIFICITY IN PRAIRIE VOLE (MICROTUS OCHROGASTER) AND COTTON RAT (SIGMODON HISPIDUS) POPULATIONS

Numerous studies examining the relationships between immunological function and seasonal environmental changes have documented a clear seasonal rhythm in selected measures of immunocompetence. We tested the hypothesis that host resistance to a pathogenic challenge would demonstrate seasonal variations in cotton rat and prairie vole populations using a Listeria monocytogenes host-resistance assay. Wild cotton rats and prairie voles were live-trapped in spring, early fall, and winter from spring 1996 to spring 1997. Animals were returned to the laboratory and inoculated intraperitoneally with a challenge dose (LD_{50}) of Listeria and monitored for mortality for 120 hours post-challenge. We found that host resistance to Listeria lacked significant seasonal variations in populations of cotton rats and prairie voles, two species with clear seasonal rhythms in many individual measures of immune system function. Prairie voles were more susceptible than cotton rat to a Listeria challenge in spring 1997. Our results indicate that although seasonal variations in selected immune parameters may exist in these host species, overall resistance to an immune challenge with Listeria varied little across seasons. Because resistance to Listeria involves both innate and adaptive immune responses, it appeared unlikely that seasonal environmental stressors were capable of reducing both innate and adaptive responses to a point of decreasing host resistance.

Key words: season, immunity, host-resistance assay, Listeria monocytogenes, Microtus ochrogaster, prairie vole, Sigmodon hispidus, cotton rat

Temporal variations in immunity (Dobrowoska and Adamczewska-Andrzejewska 1991, Lochmiller et al. 1994), disease prevalence (Geller and Christian 1982, Descoteaux and Mihok 1986), and mortality rates (Wood 1970, Dickman and Braithwaite 1992) have been documented in several wild mammalian populations. Such variations have been hypothesized to reflect the interactions of hosts with their seasonally changing environment (Nelson and Demas 1996) and breeding season (Dickman and Braithwaite 1992). Demas and Nelson (1996) hypothesized that seasonal changes in immune function of many small mammals are associated with photoperiodic cues. Their laboratory studies on prairie voles (Microtus ochrogaster) and other small mammals have provided general support for this hypothesis as they have documented (Nelson and Blom 1994, Demas and Nelson 1996, Demas et al. 1997) the enhancement of several individual measures of immune function in association with short-day lengths (hereafter referred to as the winter immunoenhancement hypothesis). However, Nelson and Demas (1997) hypothesized that winter stressors present under natural field conditions could counteract the short-day enhancement of immune function. The gonadal steroid-induced suppression of immune function during breeding seasons (Pung et al. 1984, Grossman 1885, Olsen and Kovacs 1996, Klein and Nelson 1998) may reflect an evolutionary trade-off between reproduction and immunity (Sheldon and Verhulst 1996).

Although seasonal variations in individual immunological parameters have provided support for an association between seasonally changing environments (intrinsic and extrinsic) and immunological health, the immune system is multi-faceted (Janeway and Travers 1994) and alterations in individual immune parameters may not reflect actual alterations in overall immunocompetence. A better evaluation of overall immunocompetence may be derived through the use of a host-resistance assay in which both innate and adaptive immune responses are challenged within the host using virulent organisms (Bradley and Morahan 1982). Inducing innate and adaptive immune responses to a pathogenic challenge could provide a more robust evaluation of the significance of seasonal-induced changes in individual measures of immunocompetence.

The prairie vole and cotton rat (Sigmodon hispidus) are small herbivorous rodents of the Great Plains and have been the focus of considerable research on how the environment influences immunity (Lochmiller et al. 1994, Klein et al. 1996, Nelson et al. 1996, Klein and Nelson 1998, Lochmiller and Ditchkoff 1998). We examined the winter immunoenhancement hypothesis under field conditions where variations in immunocompetence were monitored seasonally using a whole organism assay. Survival following a Listeria monocytogenes challenge was the endpoint. Given that several individual measures of immune system function change seasonally in these two species (Lochmiller et al. 1994, Nelson et al. 1996, Lochmiller and Ditchkoff 1998), we hypothesized that prairie voles and cotton rats would demonstrate temporal variations in resistance to a bacterial pathogen. We predicted that resistance in winter would be high and resistance during the breeding season would be low compared to other seasons of the year.

MATERIALS AND METHODS

Experimental design. - Experimental animals were live-trapped in spring (May to early June), early fall (late September to early October), and winter (January to early March) from spring 1996 to spring 1997 using Sherman traps (Sherman Traps Inc., Tallahassee, FL) baited with rolled oats. Cotton was added to the traps during winter for added insulation. Prairie voles were collected in upland open-field habitats on the John H. Nelson Environmental Study Area, University of Kansas, located 14 km north-northeast of Lawrence, Kansas, USA. Cotton rats were collected on tall grass prairie habitats in central Oklahoma, USA. We attempted to collect at least 18 adult males and 18 adult females (prairie voles > 30 g, Gaines and Rose 1976; cotton rats > 90 g, Odum 1955) per season. On some occasions, trapping success was low because of reductions in population density or climatic factors, so sample sizes were reduced. All animals were transported to the laboratory and housed in polycarbonate cages with wire lids and hardwood shavings for bedding at 20 ± 1 °C in an approved animal care facility. Food (Purina 5001, St. Louis, MO) and tap water were provided ad libitum. Within 24 hours of capture, immune function was assessed using a host-resistance assay.

Host-resistance assay. - Listeria monocytogenes, an opportunistic gram-positive bacterium which resides intracellularly in infected individuals (Portnoy et al. 1992), was chosen as the pathogenic agent because both innate and cell-mediated responses are necessary for clearance of infection (Czuprynski 1992). Listeria (ATCC # 13932, American Type Culture Collection, Rockville, Maryland) was cultured and colony forming units (CFU's) calculated as described by Gerhart et al. (1994) and Quinn et al.

(1994). Briefly, Listeria was cultured for 16 hours at 36.5 °C and 5 % CO_2 on Trypone Soy Blood Agar (TSBA) plates. Bacteria were harvested into sterile phosphobuffered saline (PBS), and an approximate count of CFU's was made from a 25 % bacterial solution (750 µl PBS and 250 µl of harvested solution) using a spectrophotometer at 650 nm. Actual counts of CFU were obtained from 6 bacterial aliquots (10 µl) of the harvested solution serially diluted (up to 10^{-7}) and cultured on TSBA plates for 16 hours. Colony forming units were calculated as the number of colonies cultured per 6 aliquots multiplied by a dilution factor (10^{-7}) per 10 µl. Colony-forming units for all sampling periods ranged from 3.15×10^8 to 1.23×10^9 and were considered to be equal.

Animals were challenged intraperitoneally with an approximate challenge dose of Listeria that killed 50 % of laboratory animals in preliminary trials (LD_{50}). Prairie voles received a 100 µl dosage ($x = 6.21 \times 10^8$ CFU) while cotton rats were challenged with 500 µl ($x = 8.39 \times 10^8$ CFU). The endpoint of the assay was the mortality rate at 120 hours post-challenge.

Statistical analysis. - Frequency of resistant (survivors) and susceptible (dead) individuals for each species was compared across seasons using Chi-square analysis (PROC CATMOD, SAS Institute Inc. 1990). Cotton rats were analyzed in a 4 (season) x 2 (susceptible and resistant) matrix; prairie voles were analyzed in a 3 x 2 matrix, because sample size was insufficient in spring 1996 to conduct the experiment. No differences in mortality rate were seen between sexes for cotton rats ($\chi^2 = 0.54$, $P = 0.461$) or prairie voles ($\chi^2 = 0.11$, $P = 0.735$), so males and females were analyzed together.

To test whether species differed in their resistance to bacterial challenge, a Chi-square analysis (PROC CATMOD; SAS Institute Inc. 1990) was conducted with species and season as main factor effects. A significant interaction ($\chi^2 = 6.71$, $\underline{P} = 0.035$) between the main effects prohibited pooling data across seasons for each species. Resistance to bacterial challenge between species was therefore examined separately for each season.

RESULTS

The percentage of resistant cotton rats in the population was similar across seasons ($\chi^2 = 6.72$, $\underline{P} = 0.082$; Table 1). Overall, about 56.2 ± 5.9 (SE) % of adult cotton rats were resistant to the Listeria monocytogenes bacterial challenge. Similarly, the percentage of resistant prairie voles in the population did not differ across seasons ($\chi^2 = 2.21$, $\underline{P} = 0.331$; Table 1), and the overall percentage of resistant prairie voles in the population averaged 39.0 ± 7.4 (SE) %. Interspecific differences were only apparent in spring 1997 ($\chi^2 = 7.70$, $\underline{P} = 0.006$), when cotton rats demonstrated a 2-fold greater resistance to bacterial challenge than prairie voles (Table 1).

DISCUSSION

Animals residing in highly seasonal environments have evolved a myriad of physiological adaptations for maintaining fitness. Nelson et al. (1995) and Nelson and Demas (1996) reviewed many of these characteristic physiological adaptations and noted that predictable cues, such as photoperiod, allow individuals time to develop seasonal adaptations for coping with future environmental change. It has been hypothesized (Demas and Nelson 1996) that small mammals preparing to enter harsh winter conditions

enhance their immune systems (the winter immunoenhancement hypothesis) via short-day photoperiodic cues. Several laboratory (Nelson and Blom 1994, Nelson et al. 1995, Demas and Nelson 1996, Demas et al. 1997) and field (Lochmiller et al. 1994, Lochmiller and Ditchkoff 1998) studies have provided general support for this hypothesis. However, Nelson and Demas (1997) hypothesized that winter stressors present under natural field conditions could counteract the short-day enhancement of immune function. Furthermore, trade-offs between reproduction and immunity could provide a situation where immune function is suppressed during the breeding season (Sheldon and Verhulst 1996).

Variations in individual immune parameters, although useful in documenting physiological changes, may not accurately indicate alterations in actual resistance to disease challenge within a host (Bradley and Morahan 1982). The immune response is a highly integrated and redundant physiological system where resistance to pathogenic challenges often involves both innate and adaptive immune responses (Janeway and Travers 1994). The results of this study indicated that although seasonal variations in immune parameters may exist, overall resistance to Listeria challenge varied little across seasons in these two rodent species. Because of the potential for natural stressors to counteract the short-day enhancement of immune function, and the immunosuppressive function of androgen and progesterone secreted by reproductively-active individuals during the breeding season (all but 3 animals were reproductively-active during both the spring and fall), it is possible that host resistance to Listeria challenge was somewhat suppressed in all seasons.

Resistance to Listeria is mainly a T-cell dependent response (Kaufmann et al. 1982, Kaufmann et al. 1986, Sasaki et al. 1990), but adaptive cell-mediated responses do not play a significant role until approximately 3 to 4 days after infection (Rosenthal and Snyder 1985, Luster et al. 1988). Ingestion by neutrophils (Conlan 1997) and macrophages are the primary mechanism for clearance of Listeria during the first three days following infection (Rosenthal and Snyder 1985, Luster et al. 1988). Therefore, T-cell dependent responses were probably only significant in clearance of infection after 48 to 72 hours in this study. Because individuals often up-regulate their immune function for future challenge, a reserve capacity exists and therefore, a depression beyond a critical point must occur if it is to result in altered host resistance (Luster et al. 1988). Sex hormones and environmental factors can alter cellular immunity (Pung et al. 1984, Rifte et al. 1991, Wettstein et al. 1990), although it is probably unlikely that these conditions are severe enough to decrease cellular immunity past a critical threshold level needed to alter host resistance.

Host resistance models, much like the Listeria model used in our experiment, have been used widely to assess alterations in immune function (Van Loveren 1995). Resistance to individual pathogens relies on various immune system functions, if a different pathogenic model had been used, the results of our study may have differed. In general, bacterial pathogens are useful for examining both cell-mediated and humoral deficiencies (Bradley and Morahan 1982). However, past studies have shown that some host species are resistant to various bacterial secretions and infections (Van Loveren 1995, Dabbert et al. 1994), and other pathogenic agents such as viruses, protozoans, or fungi do not assess host resistance to the same degree as Listeria (Van Loveren 1995).

Administration of a lower dosage (LD_{10-30}) of Listeria (Luster et al. 1988), may have revealed different results. However, higher doses such as the one administered in our study are more useful in monitoring the immunoenhancement of individuals (Luster et al. 1988), as hypothesized by Demas and Nelson (1996). It would be expected that only individuals with an enhanced immune system would be able to resist such a high bacterial challenge.

Differences in host resistance to pathogenic challenge among species are common in the literature (Van Loveren 1995), and probably occur due to interspecific genetic differences (Bradley and Morahan 1982). However, it is unlikely that genetic differences between the cotton rat and prairie voles were the cause of the significant difference in host resistance in spring 1997. If the cotton rat was genetically more resistant to Listeria challenge, we would expect differences between species to persist across all seasons. Furthermore, it is unlikely that gonadal hormone-induced immunosuppression facilitated reduced resistance to Listeria challenge in prairie voles as only one individual, for both prairie voles and cotton rats, was reproductively inactive during spring 1997. Therefore, we suspect regional environmental factors (although not monitored), specific for the different latitudes, were the most likely cause of observed differences in host resistance between species in spring 1997.

In conclusion, our results indicate no seasonal variation in host resistance to Listeria challenge. Seasonal variation in individual immunological parameters has been previously reported in small mammals (Lochmiller et al. 1994, Nelson and Blom 1994, Nelson et al. 1995, Demas and Nelson 1996, Demas et al. 1997, Lochmiller and Ditchkoff 1998). However, the immune system is a multi-faceted physiological system,

and resistance to Listeria involves both innate and adaptive cellular responses. Therefore, it is unlikely that environmental conditions, capable of altering a few individual immune parameters, would be severe enough to reduce cellular immunity to a level that alters host resistance to Listeria. Future studies of host-resistance in wild animals incorporating other pathogens that trigger alternative immune responses and various challenge dosages should provide better insight into potential seasonal immunological trade-offs in these and other rodent species.

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Table 1. Mortality rate of cotton rats and prairie voles in a Listeria monocytogenes host resistance assay from 1996 to 1997. Bacterial dosage received by each individual is reported in colony forming units (CFU). Asterisks signify significant differences in mortality rate between species at $P < 0.05$.

			Individuals		
	Period	CFU	Dead	Alive	% Mortality
Cotton rat					
	Spring 1996	5.63×10^8	18	23	43
	Fall 1996	5.01×10^8	19	29	40
	Winter 1997	1.23×10^9	24	16	60
	Spring 1997	1.06×10^9	12	26	32*
Prairie vole					
	Fall 1996	1.13×10^9	11	8	58
	Winter 1997	4.17×10^8	9	9	50
	Spring 1997	3.15×10^8	12	4	75*

CHAPTER III

VARIATIONS IN IMMUNOCOMPETENCE: ASSOCIATIONS AMONG IMMUNITY, DENSITY AND SURVIVAL RATE IN COTTON RAT (SIGMODON HISPIDUS) POPULATIONS

Interpopulation differences in mortality and disease prevalence have been documented in a few mammalian species, and it has been proposed that these differences are associated with immune function. Habitat-specific differences have been documented to affect the density of animals that can be supported on a particular site. Furthermore, habitat-specific differences such as population density, food quality and quantity have been shown to modulate immune responsiveness of a host in laboratory studies. To examine these association under natural conditions, we monitored four populations of cotton rats (Sigmodon hispidus) for temporal and interpopulation differences in selected immunological parameters, population density, and survival rate from fall 1995 to spring 1997. Immunological function of selected immune parameters showed both temporal and interpopulation variations. Hemolytic complement and PHA-hypersensitivity varied temporally but showed no differences among populations. Relative thymus gland and spleen mass varied temporally and among populations with individuals from the Gypsum population having larger relative organ masses than Railroad, Tantara, or 51c populations. Differences among populations in total splenic yield occurred in autumn 1995 and 1996, and winter 1996 and 1997, and was positively correlated with population density in winter 1996. Relative splenic yield also showed population differences in fall

1995 and winter 1996 and 1997, but was not correlated with population density in any season. Gamma globulin concentration differed among populations in all seasons, but was only correlated (positively) with population density in fall 1996 and spring 1997. Spontaneous proliferation of splenocytes and pokeweed-stimulated lymphocyte proliferative responsiveness was different among populations in winter 1996 and 1997, but neither showed a correlation with density. Population density was in general highest on Gypsum and Railroad study areas in comparison to Tantara and 51c study areas. Survival rates did not differ among populations, but fluctuated with sampling period. No selected immune parameters were capable of predicting population density as high or low in a logistic regression model. However, relative spleen mass, hemolytic complement activity, gamma globulin concentration, and relative splenic yield were selected for inclusion in a model that explained a significant amount of variability in population density across seasons, while PHA-hypersensitivity, total splenic yield, relative spleen mass, and gamma globulin concentration explained a significant amount of variability in survival rate. Our results indicate that both density-dependence and environmental stochasticity are likely to be important considerations in the dynamics of cotton rat populations. The results also support an association between immune function and population density and survival in the wild. Intrinsic and extrinsic explanations for the immunological variations are discussed, along with the immunological association with population density and survival.

Numerous studies on mammalian population dynamics have provided evidence that changes in extrinsic (Lack 1954, Pearson 1966, Tast and Kalela 1971, Freeland 1974, Birney et al. 1976, Hansson 1979, Erlinge et al. 1983, Keith 1983, Sinclair et al. 1985) and intrinsic (Christian 1950, Chitty 1967, Lidicker 1975, Tamarin 1980, Hestbeck 1982, Boonstra 1994) environments can cause fluctuations in population numbers. Nearly all the authors suggested that numerical fluctuations in these populations occurred as a result of individuals responding to density dependent factors through altered survival and reproduction. Because of the logical relationship between the immune system of an individual and its chance of survival in the wild, immune function has been suggested to play a mechanistic role in population regulation (Mihok et al. 1985, Dobrowolska and Adamczewska-Andrzejewska 1991, Lochmiller 1996).

Most ecologists would probably agree that temporal changes in immunity, disease, and mortality rates occur in populations, and these changes are thought to reflect the interactions of hosts with their seasonally changing environment. Such changes may help to explain annual and multi-annual fluctuations; however, these temporal variations can not explain differences in population density and survival among populations in the same season. Nelson et al. (1996) hypothesized that changes in population density can alter the immune responsiveness of small mammals. Laboratory (Nelson et al. 1996) and field (Dobrowolska et al. 1974, Geller and Christian 1982, Dobrowolska 1983, Wolk and Kozlowski 1989, Dobrowolska and Adamczewska-Andrzejewska 1991) studies have provided support for this hypothesis. Differences in habitat quality, particularly food quality, may also explain differences among populations as they have been proposed to regulate populations through density dependent mortality, and reproduction (Sinclair et

al. 1985, Flowerdew 1987). Schetter (1996) found that levels of essential amino acids during peek breeding seasons may dictate the ultimate density of cotton rats (Sigmodon hispidus) that can be supported in a particular habitat. Furthermore, a deficiency or imbalance of essential amino acids in the diets of small mammals often produces profound depressions in immunocompetence (Jose and Good 1973). Although numerous studies have provided support for an association between density and immunocompetence, no definitive study has been designed to examine interpopulation differences in immunocompetence of wild mammalian species.

The objective of this study was to examine the relationship between population density and immune function in replicated wild populations of cotton rats. The cotton rat is the dominant rodent species of central Oklahoma (Goertz 1964), and their populations vary by year (Odum 1955) and habitat (McMurry et al. 1994). It is not uncommon for cotton rat populations from different habitats to differ 5-fold in population density (Schetter 1996). We hypothesized that cotton rats would show temporal variations in measures of immunological function within populations and that interpopulation differences would be associated with population density as hypothesized by Nelson et al. (1996).

Study areas

Four populations of cotton rats residing in tallgrass prairie habitats were selected for study. Two research areas, termed Gypsum and Railroad, were located in southwestern Oklahoma, USA. Johnsongrass (Sorghum halapense) was the dominant grass species on these sites, while the forbs western ragweed (Ambrosia psilostachya), white sage

(Artemisia ludoviciana), and the legume prairie acacia (Acacia angustissima) were present. Two research areas, termed Tantara and 51c, were located in northcentral Oklahoma, USA. Herbaceous ground cover was dominated by the grasses little bluestem (Schizachyrium scoparium), big bluestem (Andropogon gerardii), tall dropseed (Sporobolus asper), and indiangrass (Sorghastrum nutans), while the forbs western ragweed, white sage, goldenrod (Solidago spp.), and sercia lespedeza (Lespedeza cuneata) were present. Smooth sumac (Rhus glabra) was present on all research areas.

Climate varies seasonally in both southwestern and northcentral Oklahoma. Low mean monthly temperature for both southwestern and northcentral regions occurs in January (3.6 °C and 2.4 °C, respectively) while highs occur in July (28.0 °C and 27.8 °C, respectively; Ruffner 1985). Mean monthly precipitation also varies seasonally with lowest precipitation in January for southwestern and northcentral regions (2.11 cm and 1.98 cm, respectively) and highest precipitation in May (11.98 cm and 11.58 cm, respectively; Ruffner 1985).

Materials and methods

Experimental design and demography

Populations of cotton rats were monitored in early fall (late September to early October), winter (January to early March), and spring (May to early June) from fall 1995 to spring 1997 (6 seasons total). To monitor demographic changes on the Tantara and 51c areas, 8 x 8 census grids with 10-m spacing were established. For the Gypsum and Railroad areas, a 4 x 16 census grid with 10-m spacing, and a 4 x 8 and a 2 x 16 census grid with

10-m spacing were established, respectively, to fit the unique topography. Trapping to assess population size was conducted for four consecutive nights in the fall, winter, and spring by mark-recapture techniques. Sherman live traps (Sherman Traps Inc., Tallahassee, FL) baited with rolled oats were used, and cotton bedding was added to the traps during winter for added insulation.

Size of each population was calculated using program CAPTURE (Otis et al.1978), and estimators converted to density after adjusting for grid size. The CAPTURE model M_o was identified as the best estimator of population size for the majority of data sets and was the most conservative by estimating population size closest to the minimum known alive estimator. Survival rates for all populations were determined by program MARK (White 1997) using variable time intervals that were associated with the months between trapping seasons. Sixteen different models examining variations in survival rates (ϕ) and capture probability (p) associated with study area population, time, and the interaction were examined. The MARK model $\phi(t)p(t)$ was selected as the best model with the lowest QAICc, and satisfactory goodness-of-fit ($\chi^2 = 7.38$, $P = 0.598$). This model predicts that both survival rates ($\phi(t)$) and capture probability ($p(t)$) should not differ between populations but should change with sampling period.

Experimental animals

Experimental animals were removed using Sherman traps from areas > 100 m from population census grids. No marked animals were found on removal grids. We attempted

to collect six male and six female adult animals (cotton rats > 99 g; Odum 1955) from each removal area per season. Trapping success was low on some occasions because of reductions in density or weather factors, so sample sizes were reduced. Also, because the weight of adult cotton rats are often reduced in winter (Odum 1955) the largest individuals present were selected. All animals were housed in polycarbonate cages with wire lids and hardwood shavings for bedding at 20 ± 1 °C in an approved animal care facility. Food (Purina 5001, St. Louis, MO) and tap water were provided ad libitum.

Phytohemagglutinin hypersensitivity response

In vivo cell-mediated immunity was indexed using a hypersensitivity reaction as described by Williams et al. (1979). An intradermal injection of 100 µl of phytohemagglutinin (PHA; Sigma, St. Louis, MO; 2.5 mg / ml phosphobuffered saline (PBS)) was administered to one shaved hip 24 hours prior to termination; the opposite hip was challenged with an equal amount of sterile PBS to serve as the control. Double skin fold thickness was measured to the nearest 0.001 inches with a pressure-sensitive micrometer. The PHA-hypersensitivity cell-mediated immune response was expressed as the percent increase in double skin-fold thickness of the stimulated side corrected for the control.

Morphology

Following the measurements for 24-hour PHA-hypersensitivity, cotton rats were anesthetized by metophane inhalation (Methoxyfluane, Pitman-Moore, Mundelein, IL).

Body mass and reproductive status of females (pregnant, lactating, and vaginal perforation), and males (scrotal or non-scrotal) were recorded. A blood sample was obtained from the retro-orbital sinus plexus using heparinized-microhematocrit capillary tubes and Vacutainer serum separation tubes (Becton Dickinson Co., Rutherford, NJ). Whole coagulated blood was centrifuged (12 min, 2400 rpm), serum decanted into cryostorage vials and stored at -80 °C for future analysis. Animals were euthanized via cervical dislocation.

The spleen was removed aseptically and cellularity assessed by preparing and enumerating a single cell suspension (Lochmiller et al. 1998). Splenic cellularity was expressed as total and relative (splenocytes / mg spleen) splenocyte counts. Thymus gland was removed, cleared of adherent fat, and weighed to the nearest 0.1 mg. Weight was expressed as mg of organ / g body mass. Eyeballs were removed and placed in 10 % buffered formalin for 2 weeks, and then lenses were removed and dried to obtain a dry lens weight to the nearest 0.1 mg for use as an index of age.

Non-specific immunity

A component of the innate, non-specific immune system was assessed by measuring complement activity. Hemolytic complement activity in serum was determined by a slight modification of the methods of DeWaal et al. (1988) as described by Sams et al. (1996). Briefly, 5 µl of serum diluted 1:80 in vernal buffer was serially diluted two-fold in a 96-well, round bottom, microtiter plate. Twenty-five µl of washed sheep red blood cells (0.6 % SRBC in vernal buffer, Colorado Serum Co., Denver CO), and 25 µl of a

rabbit-anti-SRBC antibody (1/40 in vernal buffer, Nordic Immunological Laboratories, Capistrano Beach, CA) were added to each well. Plates were vortexed and incubated for 1.5 h at 37 °C and centrifuged for 5 min at 500 rpm. Absorbance (414 nm) was measured in a Titertek Multiscan II plate reader (Flow Laboratories, Inc., McLean, VA). Hemolytic complement activity was expressed as CH_{50} units / ml serum, where 1 CH_{50} unit equals the amount of serum required to lyse 50 % of the SRBC in culture (Kabat and Mayer 1961).

Immunoglobulin concentration was assessed by measuring serum gammaglobulins as described by Bradford (1976). Gamma globulin concentration was determined using a spectrophotometer in an ammonium-sulfate sodium-chloride precipitation assay and expressed as g / dl.

Mitogen-induced lymphoproliferative response

The ability of lymphocytes to respond to the mitogen pokeweed (Phytolacca americana) was measured using a lymphoproliferative assay. Splenocytes were stimulated with pokeweed mitogen (1.25 µg / ml culture PWM; Sigma) as described by Dabbert and Lochmiller (1995). Briefly, pokeweed (10 µl) was added to a 90-µl splenocyte suspension (final concentration of 500,000 cells / well in a supplemented medium RPMI-S) in 96-well microtiter plates in triplicate; 10 µl RPMI-S medium was substituted for pokeweed in unstimulated control wells. The RPMI-S medium was prepared by the addition of 1.0 % sodium pyruvate (100 mM solution, Sigma), 1.0 % penicillin-

streptomycin solution (Sigma P-0781), 100 µl 2-mercaptoethanol (50 µM solution, Sigma), and 10 % horse serum to RPMI-1650 medium (Sigma).

After 54 hrs at 37 °C, 5% CO_2, ^{3}H-thymidine (1 µCi / well) was added to each well and incubated for another 18 hrs. Cells were harvested using a PhD Cell Harvester (Cambridge Tech Inc., Watertown, PA) onto glass-fiber filter strips (Cambridge). The amount of radioactivity, as disintegration per minute (dpm), incorporated into DNA of proliferating lymphocytes was measured in triplicate cultures using a liquid scintillation counter (Packard Instruments, Meriden, CT). The lymphoproliferative response of pokeweed-stimulated lymphocytes was corrected for spontaneous proliferation by subtracting dpm of control wells.

Statistical analysis

Data were examined for homoscedasticity (Levene's test, Steel and Torrie 1980) and normality (PROC UNIVARIATE, SAS Institute Inc. 1990). Data failing to meet these assumptions were transformed prior to further analysis (Zar 1984). To test the hypothesis that cotton rats would demonstrate temporal and interpopulation variations in immunological function, we analyzed selected immunological parameters by analysis of covariance (PROC GLM, SAS Institute Inc. 1990) with study area and sampling period as main factor effects. Gender was removed as a main factor effect in the analysis because it was significant in less than 3% of the cases. Eye lens weight (herein referred to as age) as a index of age (Askaner and Hansson 1967), and reproductive status of individuals were treated as covariates to examine the influence of age and reproductive

status on immune response parameters. Multiple comparisons of significant main effects and interactions ($P < 0.05$) were conducted using least squares means and least square means option SLICE (SAS Institute Inc. 1985).

The increased secretion of sex hormones during periods of reproductive activity has been hypothesized to influence immune responsiveness of individuals (Grossman 1985). To test this hypothesis during the breeding season, we conducted an analysis of variance with reproductive status (active versus inactive) and sampling period (spring versus autumn) as main factor effects. Reproductively active and inactive male (scrotal versus non-scrotal) and female (pregnant, lactating, or vagina perforate versus non-pregnant, non-lactating, or vagina perforate closed) cotton rats were present only during spring and autumn, and therefore only these periods were used in the analysis. Male and females were analyzed separately because of the opposite effects androgens and estrogens can have on immunological function (Grossman 1985).

To test the hypothesis that the level of immunocompetence was associated with population density or rate of survival, we used logistic regression and stepwise multiple regression (PROC LOGISTIC and PROC REG, respectively, SAS Institute Inc. 1985). Logistic regression was used to assess density dependence of immunocompetence across seasons, with populations Gypsum and Railroad classified as high density and Tantara and 51c classified as low density based on demographic results. Stepwise multiple regression was used to assess the relationship between selected parameters of immunity to population density and survival across seasons and among populations. Observations for these regression analyses consisted of each population mean for each immune parameter during each sampling period. Statistical significance for immunological

parameters entering and remaining in the model was set at $P < 0.15$. Associations between immune function and population density within a period were assessed using a Pearson correlation analysis (PROC CORR, SAS Institute Inc. 1985) with statistical significance set at $P < 0.05$.

Results

Population assessment

Population density of all four study areas demonstrated general seasonal, yearly, and study area variation (Fig. 1A). Overall, population density was greatest on Gypsum and Railroad study areas. Gypsum and Railroad populations also demonstrated the greatest fluctuation between sampling periods with yearly highs in fall and subsequent lows in winter and spring (Fig. 1A). Tantara and 51c populations demonstrated a similar trend in variation with each other, but less variation in population density between sampling periods in comparison to Gypsum and Railroad (Fig. 1A). Estimates of survival rates did not differ among populations ($P > 0.05$) but fluctuated temporally with period of sampling (Fig. 1B). Survival rate was highest for the periods between fall 1995 and spring 1996, and was consistently lower and relatively constant for the remainder of the study (Fig. 1B).

Phytohemagglutinin hypersensitivity response

The PHA-hypersensitivity index (%) of in vivo cell-mediated immunity varied across sampling periods ($P = 0.001$), and was influenced by the covariate age ($P = 0.003$). Cell-mediated immune responses were lowest in fall 1995 and increased across seasons to a

high in winter 1997 (Fig. 2). The cell-mediated hypersensitivity response did not vary among populations and reproductive condition($P > 0.05$).

Morphology

Body mass of cotton rats was influenced by the covariate age ($P < 0.001$) and exhibited a population-by-sampling period interaction ($P = 0.002$). Body mass on all study areas varied ($P < 0.001$) across sampling periods. Variations in body mass between populations within a period occurred during fall 1996 ($P = 0.033$), and winters 1996 ($P = 0.003$) and 1997 ($P < 0.001$; Fig. 3). No significant correlation was found between body mass and population density within a season ($P > 0.05$). Reproductive status of male and female cotton rats, analyzed separately, demonstrated no significant effects on body mass ($P > 0.05$).

Relative thymus gland mass differed across periods ($P = 0.023$) with individuals in fall 1995 and winters 1996 and 1997 having larger thymus glands than individuals in the other periods (Fig. 4A). Relative thymus gland mass also differed among populations ($P = 0.017$) with individuals from the Gypsum population having larger thymus glands than individuals from Railroad, Tantara, and 51c populations (Fig. 5A). Variations in relative spleen mass ($P < 0.001$) occurred across sampling periods with individuals in fall 1995 having larger spleens, and individuals in winter 1997 having smaller spleens than individuals in the other periods (Fig. 4B). Differences in relative spleen mass among populations ($P = 0.007$) occurred, with individuals from the Gypsum population having greater ($P < 0.05$) spleen masses than individuals from Railroad, Tantara, or 51c populations (Fig. 5B). Age was a covariate that influenced relative thymus gland ($P <$

0.001) and spleen mass ($P = 0.028$). No differences in relative organ mass were seen from reproductively active and inactive male or female cotton rats ($P > 0.05$).

Total ($P < 0.001$) and relative splenic cellularity ($P < 0.001$) exhibited a population-by-sampling period interactions. Total splenic cellularity of all four populations varied across sampling periods ($P<0.001$; Fig. 6A). Differences among populations within a period were present in falls 1995 and 1996, and winters 1996 and 1997 (Fig. 6A). Total splenic cellularity was positively correlated with population density in winter 1996 ($r = 0.990$, $P = 0.010$), but not in other periods. Temporal variations in relative splenic cellullarity occurred for all populations ($P < 0.010$), and differences among populations within a period were present in fall 1995 ($P < 0.05$), and winters 1996 ($P < 0.001$) and 1997 ($P < 0.01$; Fig. 6B). No significant correlation was found for relative splenic cellularity and population density in any season. Age was a significant covariate that influenced relative splenic cellularity of cotton rats ($P = 0.005$), but had no influence on total splenic cellularity ($P > 0.05$). Reproductive status of male and female cotton rats had no affect on splenic cellularity ($P > 0.05$)

Non-specific immunity

Hemolytic complement activity, as a measure of innate immunity, was similar among populations ($P > 0.05$), but differed across sampling period ($P = 0.001$). Complement activity showed distinct seasonal trends with lows in fall and highs in spring of each year (Fig. 7). Yearly variations (about 3-fold) were present for each season (Fig. 6). There was no significant effect of reproductive status on hemolytic complement activity for male or female cotton rats ($P > 0.05$).

Concentrations of Gamma globulins, as a measure of total immunoglobulin concentration, exhibited a population-by-sampling period interaction ($P = 0.006$). Differences in gamma globulin concentration occurred for all sampling periods ($P < 0.05$; Fig. 8). However, the ordering of gamma globulins among populations differed across seasons ($P < 0.001$). Population density within a period was positively correlated with gamma globulin concentration in fall 1996 ($r = 0.971$, $P = 0.029$) and in spring 1997 ($r = 0.951$, $P = 0.049$). No differences were seen in gamma globulin concentration for reproductively active and inactive males or females ($P > 0.05$).

Mitogen-induced lymphoproliferative response

The ability of unstimulated lymphocytes to spontaneously proliferate in culture varied by a population-by-sampling period interaction ($P < 0.001$). Intra-population variation across sampling periods occurred for all populations ($P < 0.05$; Fig. 9A). Differences among populations within a sampling period occurred during winters 1996 and 1997 (Fig. 9A). No correlation was found between spontaneous splenocyte proliferation and population density within a period. No difference in spontaneous proliferation of splenocytes were seen for males or females due to reproductive status ($P > 0.05$).

Lymphocyte responsiveness to pokeweed stimulation also displayed a population-by-sampling period interaction ($P < 0.001$). Lymphocyte responsiveness to the mitogen pokeweed demonstrated temporal variations in the Railroad ($P = 0.023$), Tantara ($P < 0.001$), and 51c ($P = 0.011$) populations, but not for the Gypsum population ($P = 0.065$; Fig. 9B). Variations in pokeweed-induced lymphocyte proliferation among populations within a period occurred in winters 1996 and 1997 ($P < 0.001$; Fig. 9B), but showed no

correlation with population density. No difference in stimulated proliferation of lymphocytes were seen for males or females due to reproductive status ($P > 0.05$).

Relationships to demography

Mean response levels for immune parameters in populations were used in a logistic and stepwise multiple regression analysis to explore whether immune function was associated with changes in population density and survival rate across sampling periods. No immunological parameters were selected that could accurately predict population density, as high or low, in a logistic model at $P < 0.15$. However, the analysis may have been hampered by the population-by-sampling period interactions of certain immune parameters. Stepwise multiple regression procedure selected the mean values for relative spleen mass, hemolytic complement activity, gammaglobulin concentration, and relative splenic cellularity for inclusion in a model that explained a significant amount of the variation in population density across seasons and among populations ($r^2 = 0.6501$, $P < 0.001$; Table 1). Mean values of PHA-hypersensitivity response, total splenic cellularity, relative spleen mass, and gammaglobulin concentration were selected as the best predictors for explaining the variation in rates of survival across sampling periods and among populations ($r^2 = 0.7561$, $P < 0.001$; Table 1).

Discussion

Interpopulation differences in mortality and disease prevalence have been documented in a few mammalian species (Andrews et al. 1972, Gaines and Rose 1976, Dickman and Braithwaite 1992), and it has been proposed that these differences are associated with

immune function (Dobrowolska and Adamczewska-Andrzejewska 1991, Lochmiller 1996). Studies have shown that differences in food resources can alter population demography (Cole and Batzli 1979, Desy and Batzli 1989, Doonan and Slade 1995, Schetter 1996) and immune responsiveness (Watson 1984, Gershwin et al. 1985, Klurfeld 1993). Furthermore, an increase in population density, which is often associated with increased food resources in the wild, is associated with decreased immune function in small mammals (Dobrowolska et al. 1974, Dobrowolska 1983, Wolk and Kozlowski 1989, Dobrowolska and Adamczewska-Andrzejewska 1991, Nelson et al. 1996).

Variations in immune function of mammalian species have been documented across seasons, and is believed to be associated with individuals responding to naturally changing environmental conditions (Demas and Nelson 1996). The selected immune parameters examined in this study demonstrated the predicted temporal variations, however, gamma globulin concentration, splenocyte proliferation, and total and relative splenocyte yields also demonstrated interpopulation differences within seasons. Furthermore, relative mass of spleen and thymus gland also demonstrated overall differences among populations. Immunological variations among populations are likely due to individuals responding to habitat specific differences.

Quality of food resources available to herbivorous rodents, although not monitored in this study, have been documented to differ in habitats. Schetter (1996) found differences in levels of essential amino acids in forage on habitats harboring resident populations of cotton rats, while Cole and Batzli (1979) found differences in crude protein on habitats harboring resident populations of prairie voles (Microtus ochrogaster). Furthermore, Schetter (1996) and Cole and Batzli (1979) documented

highest population densities of cotton rats and prairie voles in habitats containing elevated levels of essential amino acids and crude protein, respectively. Laboratory studies have shown that deficiencies of essential amino acids (Jose and Good 1973) and levels of crude protein (Vestey and Lochmiller 1993) can decrease immune responsiveness of small mammals. Therefore, if population density and immune function are both positively associated with food quality than we would expect that the interpopulation differences in immune function in our study may be positively correlated with population density. Total splenocyte yield in winter 1996, and gammaglobulin concentration in fall 1996 and spring 1997 demonstrated such a correlation with density. However, relative splenic yield in winters 1996 and 1997, gammaglobulin concentration in fall 1995, spring 1996, and winters 1996 and 1997, along with splenocyte proliferation in all seasons showed no correlation with density, and implies that other habitat specific characteristics are important in regulating interpopulation differences in immunocompetence.

Increased population density has been shown to decrease immune function in some wild populations of small mammals (Dobrowolska et al. 1974, Dobrowolska 1983, Wolk and Kozlowski 1989, Dobrowolska and Adamczewska-Andrzejewska 1991). However, no negative correlations were demonstrated between population density and the selected immune parameters examined in this study. It is possible that potential differences in food quality of the sites, as indirectly assumed by the positive correlation between population density and immune function of certain immunological parameters, may have counteracted the density induced immunosuppression. Furthermore, increases in population density of small mammals have been documented to alter quality of food on

the habitat (Lindroth and Batzli 1986, Seldal et al. 1994). Increased herbivory by small mammals can increase secondary compounds (Lindroth and Batzli 1986, Seldal et al. 1994) which reduces the digestible protein of the forage (Seldal et al. 1994). Although the relationship with density induced declines in forage quality was not examined, Bergeron and Jodoin (1989) found significant relationships between incidence of hepatic and renal pathology and consumption of low quality forage in meadow voles (Microtus pennsylvanicus) from high density populations (Bergeron and Jodoin 1989), and provides indirect evidence that density dependent associations with food quality can suppress immune function of small mammals in the wild.

Population density of cotton rats have been documented to be limited by adequate quantities of food in their natural environment (Doonan and Slade 1995). Severe calorie restriction from food limitations has been shown to negatively impact the health of wild herbivorous mammals (Hart et al. 1985), and to specifically suppress both cell-mediated and humoral immune responses (Gershwin et al. 1985, Chandra 1981). We have no evidence of limitations in food quantities in this study. However, if such a limitation existed it may have counteracted the potential immunoenhancing benefits from potential increased food quality available in some habitats (although not measured) as indirectly assumed by positive, population density and immune parameter correlations.

Shifts in genetic polymorphism of immune regulatory (Ir) genes (Benacerraf and Germain 1978) could also have altered the immune responsiveness of individuals between populations. Shifts in genetic variability have been documented for small mammals with changes in population density (Baccus and Wolf 1989, Teska et al. 1990, Ritte et al. 1991) and the nutritional quality of the habitat (Wettstein et al. 1990).

Because genetic shifts that increase heterozygosity may be important mechanisms for maintaining high levels of major histocompatibility complex (MHC) polymorphism in natural populations (Ritte et al. 1991) the importance of genetic differences among population is understood. However, because of the lack of population differences in PHA-hypersensitivity reactions, a T-cell dependent response which relies on MHC presentation, even indirect assumptions about potential genetic difference among populations are unwarranted.

Interpopulation differences in immune function of this study were most pronounced during winter and fall seasons. Lack of interpopulation differences in immune function during the spring (except for gammaglobulins which were significant in all seasons) may be due to the initiation of breeding and the increased secretion of gonadal steroids. Gender differences in immune function and disease resistance are commonly reported (Bundy 1989, Zuk 1990), and thought to reflect the immunosuppressive nature of testosterone (Zuk 1996). Estrogens are generally less immunosuppressive than male androgens, however, female secreted progesterone has potent immunosuppressive qualities (Grossman 1985). Nearly all individuals in our study were sexually active in spring (90 %) and the potential for hormonal induced suppression of immune function may have masked immunological differences between populations. However, 88 % of individuals were sexually active in the fall, when interpopulation differences were present, and no seasonal (spring versus fall) differences were seen between reproductively active or inactive individuals. Therefore, gonadal steroid induced immunosuppression potentially causing the lack of interpopulation differences in immune function during spring appears unlikely. Lack of reproductively

active individuals in winter, however, may have removed a potential life history trade-off between reproductive potential and immune function (Sheldon and Verhulst 1996) and provided an opportunity for available energy to be allocated to immunity rather than reproduction and produced more evident interpopulation differences in immunity.

Nelson et al. (1996) observed in the laboratory that increases in population density of deer mice positively influenced humoral immune responses. The positive correlation between population density and immune function in this study provides some support for a density dependent-immunoenhancement in wild cotton rats. However, the lack of the logistic model to select any immune parameter as a predictor of high or low population density provides doubt to the association between density and immune function. However, the latter may have been inhibited by the temporal variations in immune function among the populations. The positive correlation between population density and immune function seen in a few parameters does indicate an association between immune function and population density, as proposed by Nelson et al. (1996). The regression analysis in this study also demonstrates an association between density and survival rate across seasons with certain parameters of immunity. Because of the temporal variations of population density, temporally variable immune parameters appears to be as important as interpopulation differences in immune function at predicting population density. Furthermore because survival does not show differences among populations it appears as if the temporal fluctuation of immunity is more important than population specific variations in immune function. This is indirectly supported by field studies on cotton rats in which supplemental food added to sites could increase population density of the areas

but could not prevent seasonal declines, and it was concluded, site specific characteristics could not offset the seasonal changes in population structure (Doonan and Slade1995).

Our results support the contentions of others (Doonan and Slade 1995) that both density dependent and environmental stochasticity are likely to be important considerations in the dynamics of cotton rat populations. Multifactored models have recently dominated the theories of population regulation (Batzli 1992) but maybe difficult to monitor in uncontrolled situations. Examination of immunological parameters which are responsive to changes in environmental stimuli and is logically associated with survival of wild animals in the wild is one way of predicting the influence of habitat specific alterations in the physiology of small mammals. Future research on interpopulation differences in immunocompetence of other species will provide more support for the relationship between immunocompetence and population regulation.

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Table 1. Independent immune variables selected by stepwise multiple regression for predicting density and survival rate of cotton rat populations in Oklahoma, 1995-1997 ($\underline{n} = 24$).

Parameter	Variables	Estimate	SE	Partial $\underline{r}^2$	$\underline{F}$	$\underline{P}$-value
Density						
	Intercept	-284.64	79.70		12.75	0.002
	Spleen (mg/g)	143.35	26.71	0.2479	28.80	0.001
	Hemolytic complement (CH_{50})	-0.01	0.01	0.2220	20.94	0.001
	Gammaglobulin (g/dl)	81.73	32.06	0.1135	6.50	0.020
	Relative splenic cellularity (cells/mg)	125.29	65.81	0.0667	3.62	0.072
Survival rate						
	Intercept	0.48	0.40		1.43	0.250
	PHA-hypersensitivity (%)	-0.01	0.01	0.3328	2.84	0.112
	Splenic cellularity (cells x 10^6)	-0.01	0.01	0.2094	19.67	0.001
	Spleen (mg/g)	0.33	0.09	0.1425	13.14	0.003
	Gammaglobulin (g/dl)	0.23	0.11	0.0714	4.39	0.053

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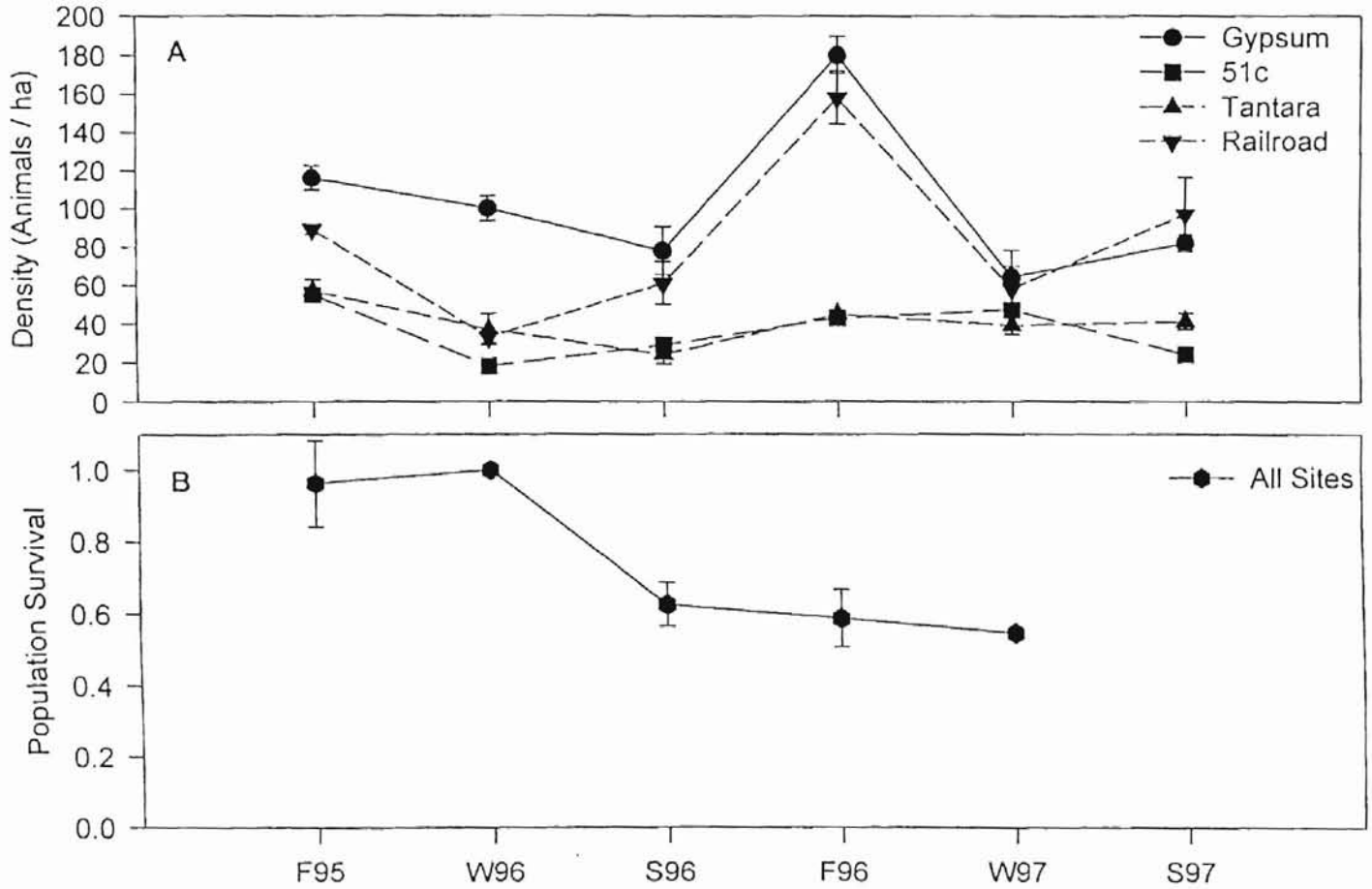
Figure 6. Variation in total (A) and relative (B) splenic cellularity (mean ± SE) in four populations of cotton rats from fall 1995 to spring 1997. Intrasampling period differences are shown with asterisks where * signifies $\underline{P} < 0.05$, ** is $\underline{P} < 0.01$, and *** is $\underline{P} < 0.001$. Numbers above SE bars refer to sample size.

Figure 7. Temporal variations in hemolytic complement activity (mean ± SE) of cotton rats from fall 1995 to spring 1997. Letters that differ above SE bars signify statistical differences at $\underline{P} < 0.05$.

Figure 8. Variation in gamma globulin concentration (mean ± SE) in four populations of cotton rats from fall 1995 to spring 1997. Intrasampling period differences are shown with asterisks where * signifies $\underline{P} < 0.05$, ** is $\underline{P} < 0.01$, and *** is $\underline{P} < 0.001$. Numbers above SE bars refer to sample size.

Figure 9. Variation in unstimulated (A) and pokeweed-stimulated (B) lymphoproliferation of splenocytes (mean ± SE) in four populations of cotton rats from fall 1995 to spring 1997. Intrasampling period differences are shown with asterisks where * signifies $\underline{P} < 0.05$, ** is $\underline{P} < 0.01$, and *** is $\underline{P} < 0.001$. Numbers above SE bars refer to sample size.


A
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40
20
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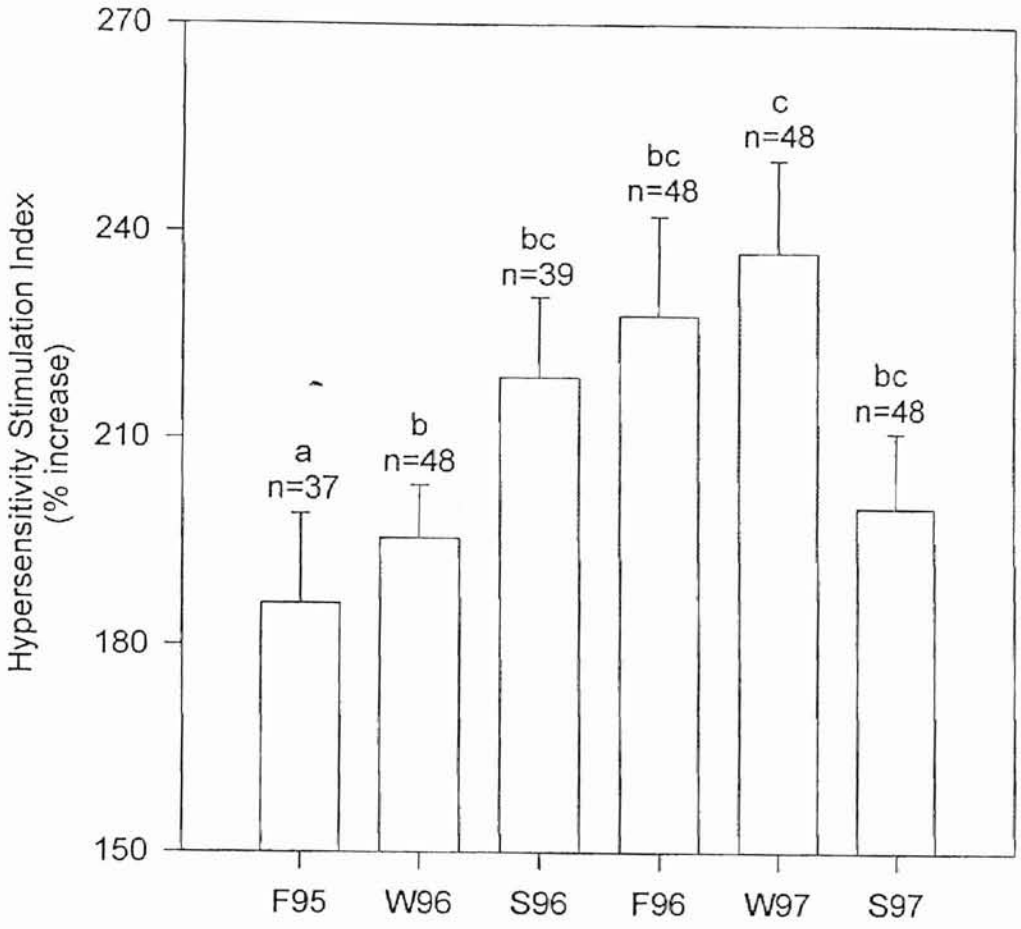
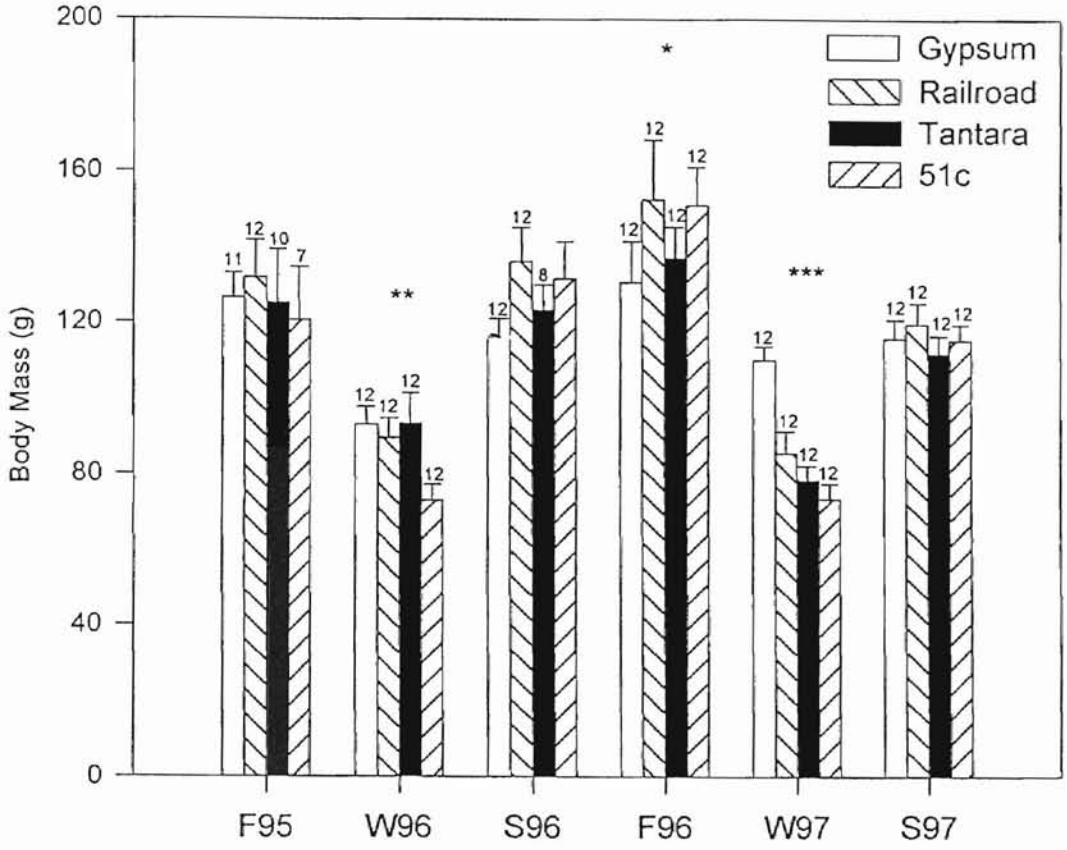
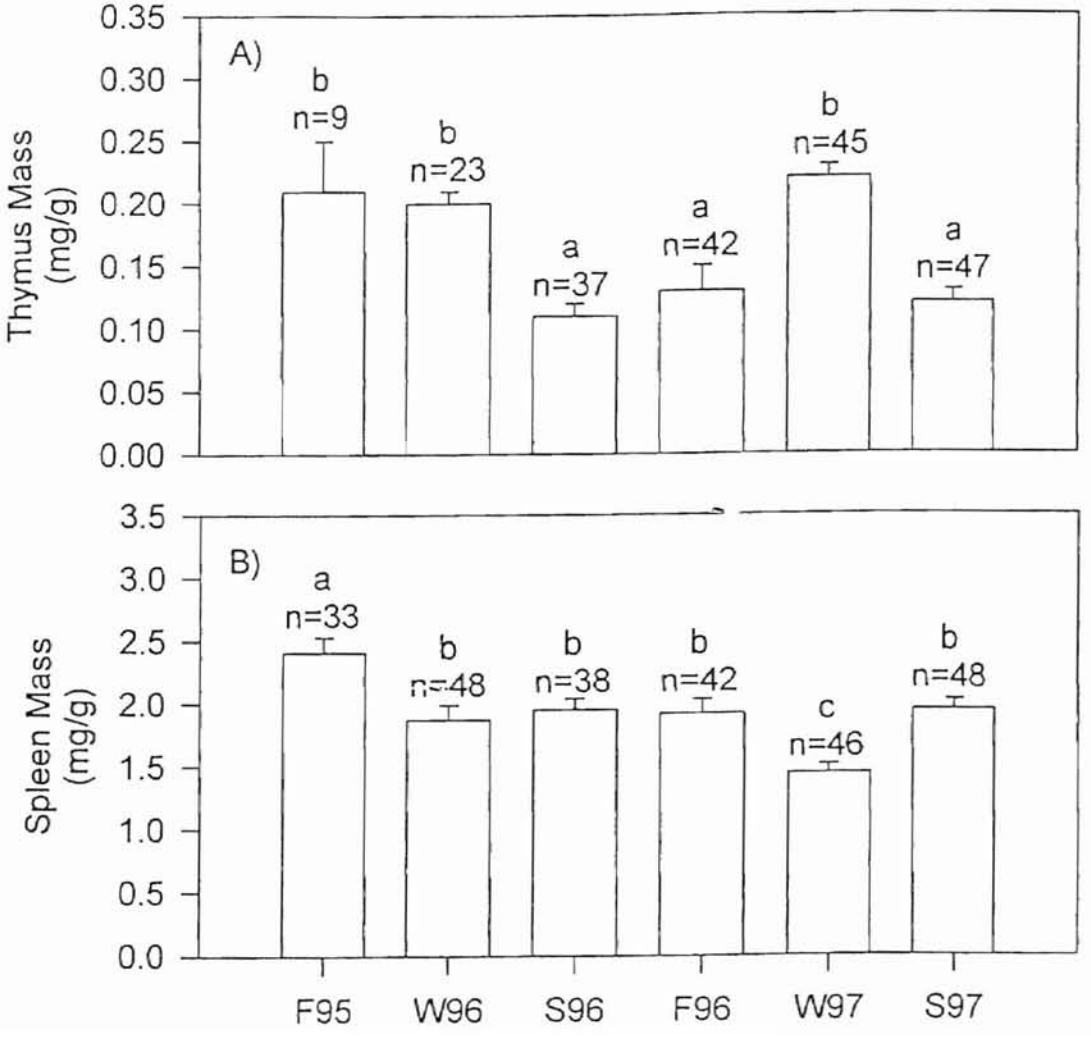
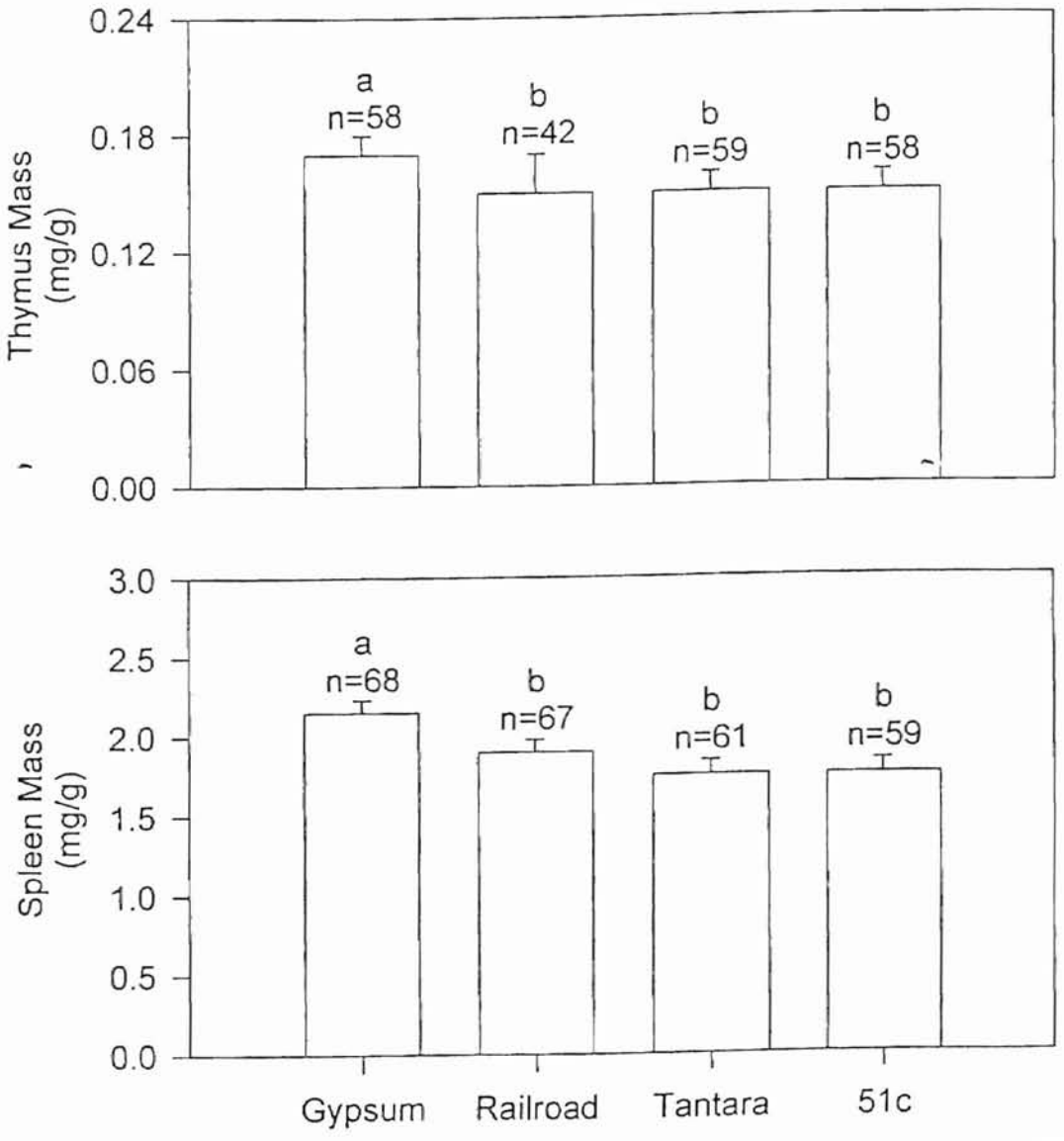
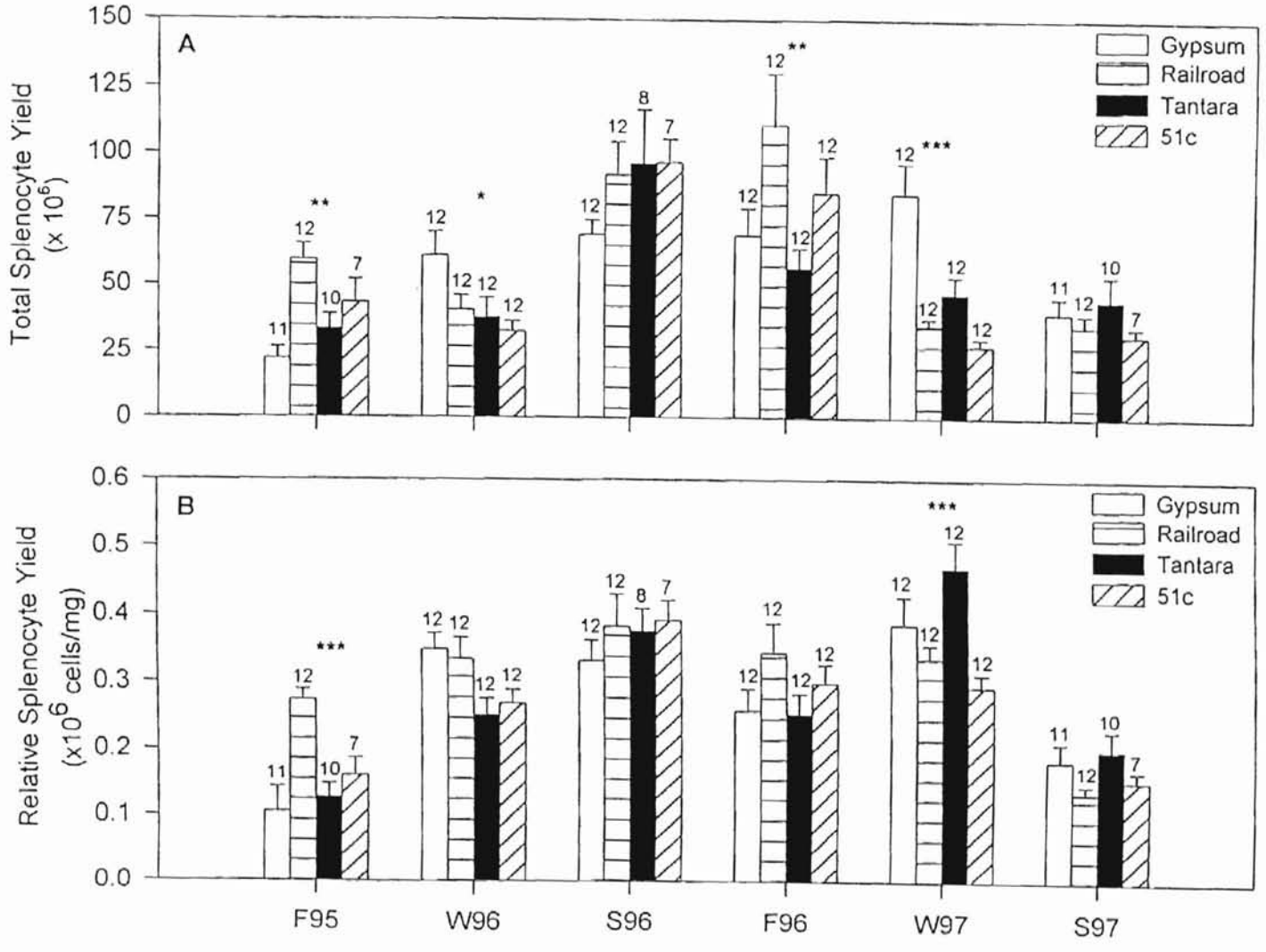
Gypsum
51c
Tantara
Railroad
B
Population Survival
1.0
0.8
0.6
0.4
0.2
0.0
All Sites
F95
W96
S96
F96
W97
S97

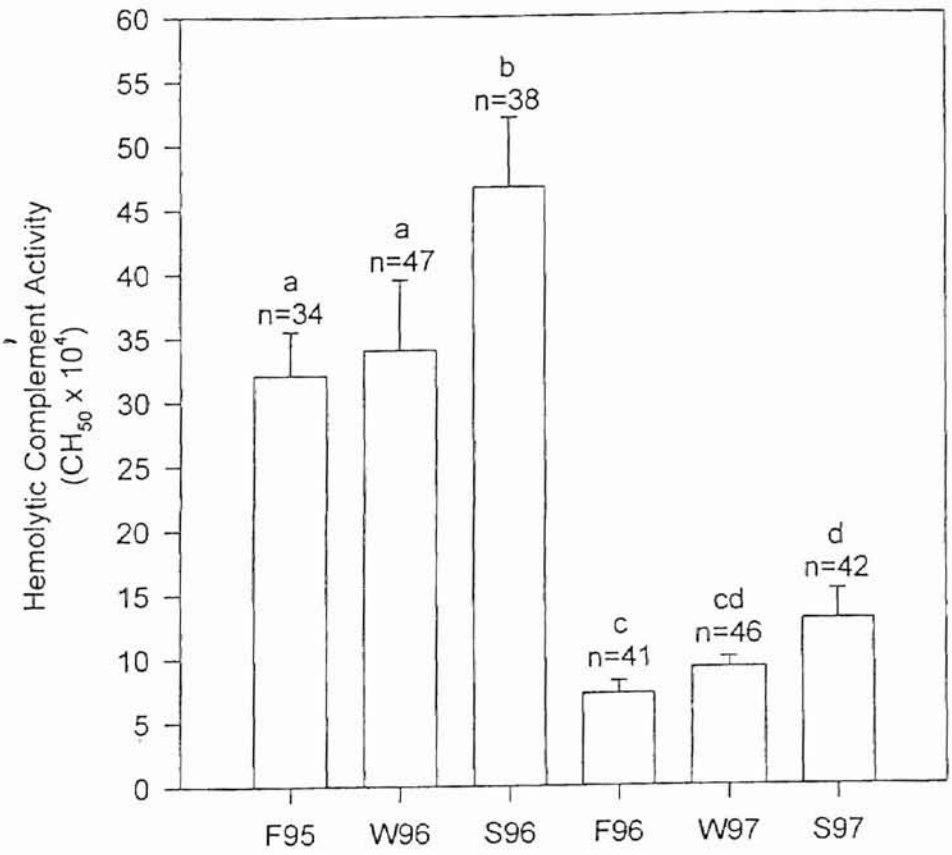

270
240
210
180
150
Hypersensitivity Stimulation Index
(% increase)
a
n=37
b
n=48
bc
n=39
bc
n=48
c
n=48
bc
n=48
F95
W96
S96
F96
W97
S97

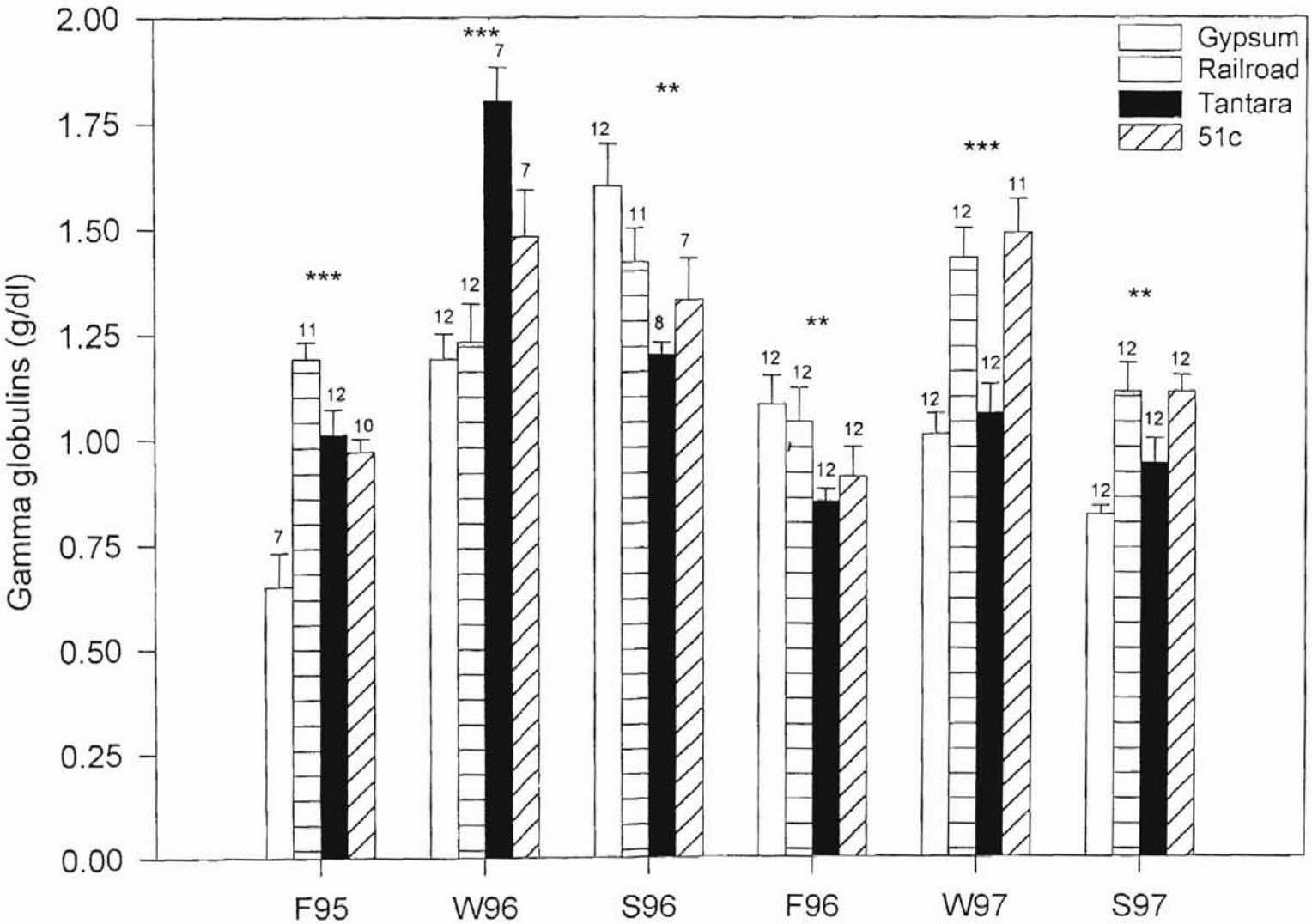

Body Mass (g)
200
160
120
80
40
0
Gypsum
Railroad
Tantara
51c
F95
W96
S96
F96
W97
S97
*
**
***

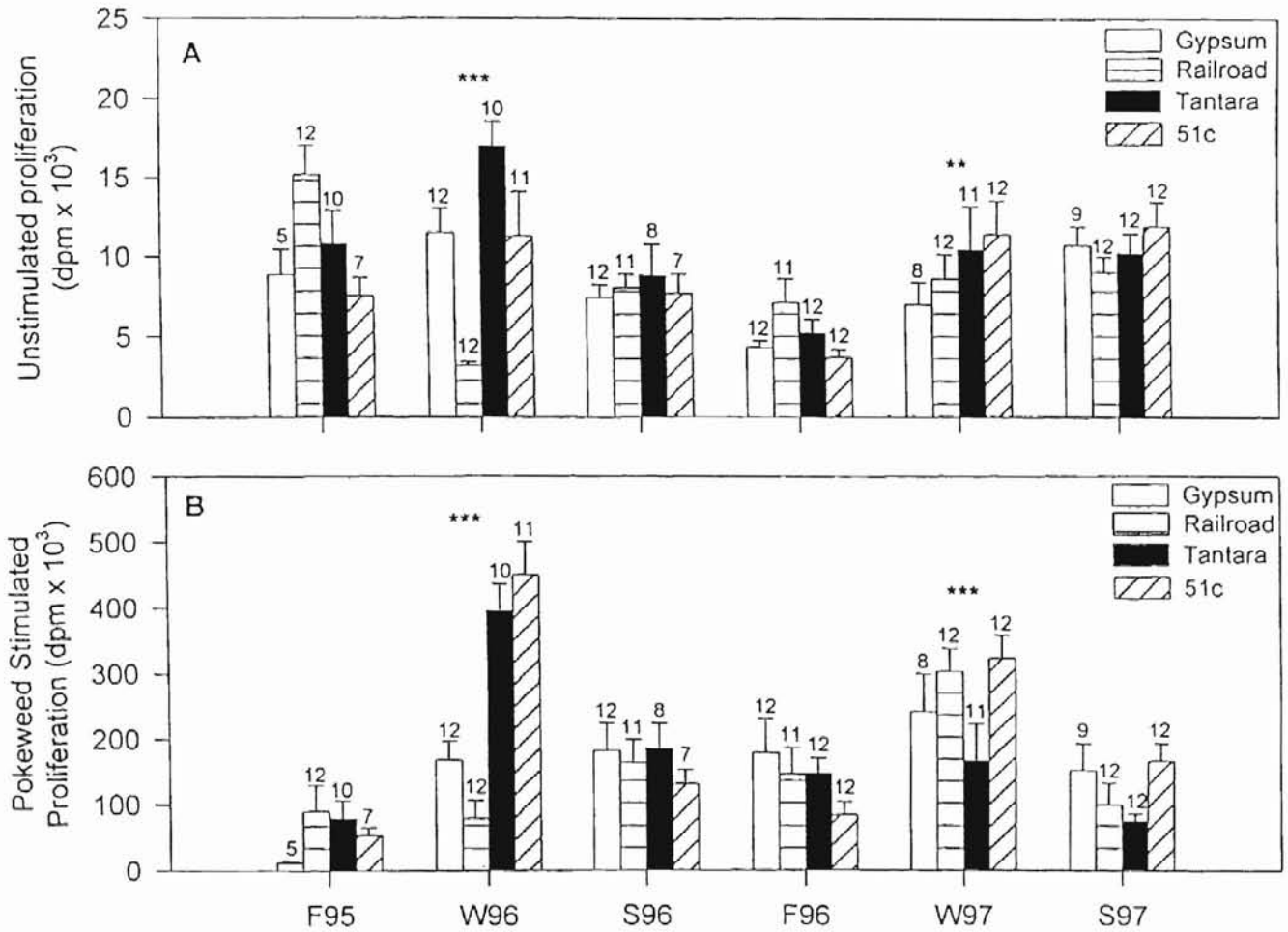

A)
Thymus Mass (mg/g)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
b n=9
b n=23
a n=37
a n=42
b n=45
a n=47
B)
Spleen Mass (mg/g)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a n=33
b n=48
b n=38
b n=42
c n=46
b n=48
F95
W96
S96
F96
W97
S97


Thymus Mass (mg/g)
0.24
0.18
0.12
0.06
0.00
a n=58
b n=42
b n=59
b n=58
Spleen Mass (mg/g)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a n=68
b n=67
b n=61
b n=59
Gypsum
Railroad
Tantara
51c


A
B
Total Splenocyte Yield
(x 10^6)
Relative Splenocyte Yield
(x10^6 cells/mg)
Gypsum
Railroad
Tantara
51c
F95
W96
S96
F96
W97
S97


Hemolytic Complement Activity
(CH_{50} x 10^4)
60
55
50
45
40
35
30
25
20
15
10
5
0
a
n=34
a
n=47
b
n=38
c
n=41
cd
n=46
d
n=42
F95
W96
S96
F96
W97
S97


Gamma globulins (g/dl)
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Gypsum
Railroad
Tantara
51c
F95
W96
S96
F96
W97
S97


A
B
Gypsum
Railroad
Tantara
51c
Unstimulated proliferation (dpm x 10³)
Pokeweed Stimulated Proliferation (dpm x 10³)
***
**
F95
W96
S96
F96
W97
S97

VITA

John A. Sinclair

Candidate for the Degree of

Master of Science

Thesis: IMMUNOLOGICAL ASSOCIATIONS WITH DENSITY AND SURVIVAL IN WILD POPULATIONS OF COTTON RATS AND PRAIRIE VOLES

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