POTENTIAL ROLE OF AMINO ACID MALNUTRITION AND CLIMATIC STRESSORS IN THE MODULATION OF IMMUNE FUNCTION IN BOBWHITE QUAIL POPULATIONS

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CHAPTER I

Proliferative Responses of Splenocytes from Wild and Domestic Northern Bobwhites (*Colinus virginianus*) to T- and B-Cell Mitogens

ABSTRACT: Baseline information on the functional responses expected for assays used to assess immunocompetence in the Northern bobwhite (Colinus virginianus) are largely unavailable. Our primary objective was to develop an in vitro lymphoproliferative response assay for assessing cell-mediated immunocompetence in the Northern bobwhite. Culture conditions were optimized for domestic Northern bobwhites and field tested on splenocytes from wild-caught quail. Results indicated that increasing cell concentration and media volume in culture, as well as decreasing concentrations of serum in media, improved splenocyte responses to Con A stimulation. Optimum culture conditions were attained with 1 million cells per well cultured in 200 μl of AIM-V serum-free media for 72 hours. Five μg Con A or 2.5 μg STM per well provided maximum stimulation as measured by ^{3}H-thymidine incorporation. Stimulation indices of splenocyte cultures of wild-caught Northern bobwhites to 5 μg Con A were approximately 4-fold greater than levels observed for domestic quail ($P = 0.0055$). Alternatively, stimulation indices of splenocyte cultures obtained from wild-caught and domestic Northern bobwhites to 2.5 μg STM per well were not different ($P = 0.3938$).

ABBREVIATIONS

Con A, concanavalin A; cpm, counts per minute; CS, chicken serum; PHA, phytohaemagglutinin; PWM, pokeweed mitogen; RPMI-S, supplemented RPMI 1640 media; STM, Salmonella typhimurium;

INTRODUCTION

Information about functional aspects of the avian immune system is extremely limited, especially for wild species (Lochmiller and Dabbert, 1993). Northern bobwhites (Colinus virginianus) are widely distributed in the wild and are propagated on game farms throughout much of the continental United States. Methods of testing Northern bobwhite resistance to specific pathogens are available (Bucholz and Fairbrother, 1992). In comparison, baseline information on functional assays for assessing immunocompetence of the Northern bobwhite is largely unavailable (Lochmiller et al., 1994; Grasman, 1992). Hence, our current ability to identify lesions in specific arms of the immune system of the Northern bobwhite has been hampered by this lack of information. Our objective was to develop an in vitro lymphoproliferative response assay for assessing cell-mediated immunocompetence of Northern bobwhite splenocyte cultures. Previously, only low to moderate lymphoproliferative responses have been observed in Northern bobwhite splenocyte cultures (Lochmiller et al., 1994).

MATERIALS AND METHODS

Northern bobwhites

Domestic birds were obtained from the El Reno Gamebird Farm (El Reno, Oklahoma, USA). All wild birds were trapped or harvested from Payne County, Oklahoma, USA.

Mitogens

Mitogens used were concanavalin A (Con A) (Sigma Chemicals, St. Louis, MO) and Salmonella typhimurium mitogen (STM) (RIBI Immunochem Research Inc., Hamilton, MT).

Splenocyte culture and transformation assays

Spleens were aseptically removed and placed in room temperature RPMI 1640 medium (RPMI-S) supplemented with 0.3 g/L L-glutamine, 1.0% Na pyruvate (100mM), 1.0% non-essential amino acids (10mM), 1.0% penicillin (10,000 U/ml)-streptomycin (10 mg/ml) and 1 to 5% (depending upon experiment) normal chicken serum (CS) (all reagents from Sigma Chemical, St. Louis, MO). Spleens were subsequently dissociated in sterile glass-on-glass tissue homogenizers containing 5 ml of RPMI-S. After mixing, debris were allowed to settle for 5 minutes and then the cell suspension was decanted into 16 x 125 mm polystyrene tubes (Becton Dickinson Labware, Lincoln Park, NJ). Cells were pelleted by centrifugation at 10 C and 275 xg for 7 minutes, the supernatant decanted, and the cell pellet resuspended in 5 ml RPMI-S (this wash step was repeated once). Viable cells were counted with a hemacytometer using trypan blue exclusion. Cell concentrations were then adjusted to either 200,000 or 1,000,000 cells/90 μl in either RPMI-S or AIM-V serum free culture media (Gibco Laboratories, Grand Island, NY). Aliquots (90 μl) of the cell suspension were added to triplicate or quadruplicate (dependent on availability of cells) wells of a 96-well, polystyrene, flat-bottom tissue culture plate (Corning Glass Works, Corning, NY). The

mitogens Con A or STM were mixed in RPMI-S to provide the target concentration in a 10 μl volume. Mitogen (10 μl volume) was added to each set of wells requiring stimulation, while unstimulated control wells (blanks) received 10 μl of RPMI-S without mitogen. Cultures were incubated for 24 to 72 hours at 40.5 C in an Auto Flow, CO_2 water-jacketed incubator (Nuaire, Inc., Plymouth, MN) with 5% CO_2-95% air.

Lymphocyte proliferation was assessed by cellular incorporation of ^{3}H-thymidine. One μCi of ^{3}H-thymidine (ICN Biomedicals, Inc., Irvine, CA) in 10 μl volume was added to each well 18 hours before termination of each culture. Cell cultures were harvested onto glass fiber filters (Cambridge Technology, Inc., Watertown, MA) using a PHD Cell Harvester (Cambridge Technology, Inc., Watertown, MA). Betamax ES scintillant (ICN Biomedicals, Inc., Irvine, CA) was added (2.5 ml) to each vial containing an air-dried glass-fiber filter. Activity (^{3}H-thymidine) of filters was measured as counts per minute (cpm) using a liquid scintillation counter (Beckman Instruments, Fullerton, CA) and data expressed as mean $\pm$ standard error. Stimulation indices for both assay systems were calculated as ratios of cpm in stimulated cultures to unstimulated controls.

Experimental Design

Several experiments were conducted to develop a functional assay which was well-suited for use with both wild-caught and domestic quail. Initial experiments were designed to

determine the appropriate culture conditions for Northern bobwhite splenocytes that would maximize proliferative responsiveness and provide stimulation indices in excess of those derived from colorimetric MTT assay systems (Lochmiller et al., 1994; MTT assay system). Initial experiments tested the appropriate length of culture and cell concentration by culturing 2 x 10^5 and 1 x 10^6 cells per well (triplicate) for 24, 48, and 72 hours, using 100 μl total volume of RPMI-S containing 5% CS and 5 μg Con A per well. A subsequent experiment to examine the influence of volume of media on responsiveness was determined in quadruplicate cultures of 1 x 10^6 cells per well in RPMI-S containing 5% CS and 5 μg Con A for both 48-and-72 hour cultures containing either 100 or 200 μl volumes. The influence of CS concentration in culture media on proliferative responsiveness was determined in quadruplicate wells containing 5 μg Con A and 200 μl volumes of either 1, 3, or 5% CS in RPMI-S, as well as AIM-V serum free media. Optimum concentrations of Con A and STM were determined in cultures of 1 x 10^6 cells per well (quadruplicate) in 200 μl of AIM-V media containing either 1, 5, 20, or 50 μg Con A (48 and 72 hours), or 2.5 or 5 μg STM (72 hours) per well.

Suitability of the assay for both wild-caught and domestic Northern bobwhites was determined using harvested or trapped wild birds whose proliferative responses to 5 μg Con A and 2.5 μg STM were compared to those of domestic birds. Culture conditions consisted of 1 x 10^6 cells per well in

200 μl of AIM-V media for 72 hours. Appropriate dilutions of mitogens were prepared in RPMI-S (1% CS) for all culture wells and all washes were performed using RPMI-S (1% CS).

Statisitical analysis

All cpm data were $\log_{10}$ transformed and subsequently judged normal ($P \geq 0.05$) and to have homogeneous variances ($P \geq 0.05$) according to Shapiro-Wilks and Bartlett Box-F tests, respectively (Norusis, 1990). Cell number, culture volume, serum concentration of media, and concentration of Con A were individually tested as main effects in 2-factor analyses of variance with hours of culture being the second factor in each analysis (Norusis, 1990). Means which differed (alpha = 0.05) from one another were identified using Student-Newman-Keuls pair-wise comparisons (Norusis, 1990). A Mann-Whitney U-test was used to detect differences in stimulation indices between samples of domestic and wild Northern bobwhites (Norusis, 1990).

RESULTS

Under initial culture conditions, cell number interacted with hours of culture ($P \leq 0.001$) thus data for the first experiment are reported at individual levels of cell number and hours of culture. When cultured for either 24 or 48 hours, 1 x 10^6 cells per well yielded the highest cpm ($P \leq 0.001$) compared to those containing only 2 x 10^5 cells per well (Fig. 1a). Response in cpm of cells cultured for 72 hours, however, did not differ ($P = 0.39$) between cell numbers. When cells were cultured at 1 x 10^6 cells per

well, response in cpm was highest at 48 hours of culture and lowest at 72 hours of culture ($P < 0.001$). Response in cpm of cells cultured at a concentration of 2×10^5 cells per well did not differ ($P = 0.171$) among hours of culture (Fig. 1a). Cultures of 1×10^6 cells per well in 200 μl total volume responded as much as 10-fold higher ($P < 0.001$) than those cultured in 100 μl total volume (Fig. 1b). Response in cpm at these culture conditions was higher ($P = 0.002$) at 48 than 72 hours (Fig. 1b). Proliferative response was higher ($P = 0.015$) for cells cultured in either AIM-V serum free media or RPMI-S containing 1% CS than those cultured in RPMI-S containing 5% CS (Fig. 1c). Proliferative responses at these culture conditions did not differ ($P = 0.648$) between cells cultured for 48 or 72 hours (Fig. 1c).

All subsequent experiments were performed with AIM-V serum free media. Concentrations of either 1 or 5 μg of Con A per well gave higher ($P = 0.003$) cpm responses as compared to 20 μg of Con A per well (Fig. 1d). Cells cultured with 50 μg of Con A per well yielded the lowest ($P = 0.003$) response in cpm (Fig. 1d). Proliferative responses at these culture conditions were higher ($P = 0.004$) for cells cultured for 48 as compared to 72 hours (Fig. 1d). Variation in responsiveness among animals, however, was lowest (CV = 17%) for the response of 5 μg Con A per well after 72 hours of culture (Fig. 1d) compared to other concentrations of Con A (CV $\geq$ 51%, excluding 50 μg/well which appeared to be toxic). Concentrations of 2.5 and 5 μg STM per well elicited similar

mean lymphoproliferative responses (26.2±20.0 and 27.2±20.6 x 10^3 cpm, respectively). Mean cpm ± SE of unstimulated controls was 4,079±1,407.

Stimulation indices of splenocyte cultures to 5 μg Con A per well for wild-caught Northern bobwhites were approximately 4-fold higher than domestic birds ($P = 0.0055$; Fig. 2). Alternatively, stimulation indices of splenocyte cultures obtained from wild-caught and domestic Northern bobwhites to 2.5 μg STM per well were not different ($P = 0.3938$; Fig. 2).

DISCUSSION

Our experiments constitute the first report of Northern bobwhite splenocyte responses to mitogenic stimulation as measured by cellular incorporation of ^{3}H-thymidine. Previous experiments using an MTT colorimetric assay system to measure mitogen-induced lymphoproliferation in Northern bobwhites only obtained stimulation indices of < 2 and did not detect a dose-response to Con A or STM (Lochmiller et al., 1994). The MTT assay system provides lower stimulation indices than ^{3}H-thymidine incorporation and may be less sensitive (Bounous et al., 1992; Wemme et al., 1992). This assay system is a measure of the metabolic rate of all cell types over the last 3 hours of an assay, while ^{3}H-thymidine incorporation measures the number of cells producing DNA during the last 18 hours of an assay (Mosmann, 1983). Both the shorter measurement period and a potential bias created

by different numbers of metabolically active cells such as macrophages may cause the lower sensitivity of MTT reduction. Our experiments detected a dose response for Con A, thus ^{3}H-thymidine incorporation appears to be a more sensitive assay system for measurement of mitogenic responsiveness of Northern bobwhite splenocytes.

Increasing cell concentrations and culture volume, and decreasing culture concentrations of serum, improved splenocyte responses to Con A stimulation. Five μg Con A per well (25 μg/ml) cultured for 72 hours appeared to give the most consistent and repeatable responses with the lowest variability among cultures. This concentration of Con A is comparable to optimum concentrations previously suggested for use in poultry systems (10 μg/ml, Baecher-Steppan, 1989; 32 μg/ml, Hovi et al., 1978). Proliferative responses of cultured splenocytes to the B-cell mitogen STM were similar for both low and high concentrations; we used the lower concentration (2.5 μg/well) for all subsequent experiments.

The STM concentration used in our experiment is half the concentration of Salmonella typhimurium lipopolysaccharide previously used as a mitogen for poultry splenocytes (Corrier et al., 1991). The lower stimulation indices observed for splenocytes cultured with a B-cell mitogen (bacterium or bacterial endotoxin) as compared to a T-cell mitogen is consistent with previous observations in poultry (Fritsche, et al., 1991; Baecher-Steppan et al., 1989; Hovi et al., 1978; Tufveson and Alm, 1975). Explanations for the

lower response of Galliform splenocytes to B-cell mitogens is unclear, but may be related to their apparent resistance to bacterial endotoxin (Adler and DaMassa, 1978), a reduced number of appropriate receptors for these mitogens, and/or the effects of suppressor T-cells (Tufveson and Alm, 1975).

Cell concentrations of 1×10^6 cells/well are analogous to those suggested by Baecher-Steppen et al. (1989) and Hovi et al. (1978) for poultry. Presence of CS inhibited mitogenic responses of Northern bobwhite splenocytes to Con A. Turkey peripheral blood leukocytes stimulated with Con A respond similarly in the presence of CS (Sharma and Belzer, 1992). Fetal bovine serum may inhibit the response of chicken splenocytes to PHA (Hovi et al., 1978). In some instances, heterologous serum has been shown to actually stimulate splenocyte populations in poultry (Kirchner and Oppenheim, 1972).

Our assay is well-suited for use in both domestic and wild-caught Northern bobwhites. Thus, this assay system can be used to evaluate cell-mediated immunity of Northern bobwhites held in the laboratory or birds captured or harvested from the field. Differences in responsiveness of splenocytes of wild and domestic Northern bobwhites is expected. Lochmiller et al. (1993) found a similar heterogeneity of responses between captive and wild-caught cotton rats to PWM and Con A. This heterogeneity could be due to a multitude of factors including nutrition, environmental stress, and exposure to antigens (Lochmiller

and Dabbert, 1993). It may also be caused by differences in the relative polymorphism of major histocompatibility genes of our sampled populations (Lochmiller and Dabbert, 1993).

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Figure 1a. Effects of cell number and culture time on the lymphoproliferative response (mean cpm ± SE) of Northern bobwhite splenocytes to 5 μg of Con A per well. Either 2 x 10^5 or 1 x 10^6 cells per well were cultured in 100 μl of RPMI-S containing 5% CS for 24, 48, or 72 hours. Mean cpm ± SE of unstimulated cultures containing 1 x 10^6 cells was 4,132±339, 1017±137, and 421±95 for 24, 48, and 72 hour cultures, respectively. Mean cpm ± SE of unstimulated cultures containing 2 x 10^5 cells was 483±46, 381±61, 265±71 for 24, 48, and 72 hour cultures, respectively.

b. Effects of culture volume (100 or 200 μl) on the lymphoproliferative response (mean cpm ± SE) of Northern bobwhite splenocytes to 5 μg Con A per well. One million cells per well were cultured in RPMI-S containing 5% CS for 48 and 72 hours. Mean cpm ± SE of unstimulated cultures containing 100 μl was 1049±134 and 330±120 for 48 and 72 hour cultures, respectively. Mean cpm ± SE of unstimulated cultures containing 200 μl was 1733±125 and 363±18 for 48 and 72 hour cultures, respectively.

c. Effects of serum concentration (0, 1, 3, or 5% CS) on the lymphoproliferative response (mean cpm ± SE) of Northern bobwhite splenocytes to 5 μg Con A per well. One

million cells per well were cultured in RPMI-S or AIM-V serum free media with 200 μl total volume for 48 and 72 hours. Mean cpm $\pm$ SE of unstimulated cultures incubated 48 hours was 864$\pm$98, 1172$\pm$182, and 1267$\pm$284, when RPMI-S contained either 5, 3, or 1% CS, respectively, and 1163$\pm$277 for cells cultured in AIM-V serum-free media. Mean cpm $\pm$ SE of unstiumlated cultures incubated for 72 hours was 833$\pm$33, 859$\pm$142, 848$\pm$122, when RPMI-S contained either 5, 3, or 1% CS, respectively, and 1243$\pm$94 for cells cultured in AIM-V serum-free media

d. Effects of concentration of Con A (1, 5, 20 or 50 μg per well) on the lymphoproliferative response (mean cpm $\pm$ SE) of Northern bobwhite splenocytes. One million cells per well were cultured in 200 μl of AIM-V serum free media for 48 and 72 hours. Mean cpm $\pm$ SE of unstimulated cultures incubated for 48 or 72 hours was 268$\pm$11 and 433$\pm$79, respectively.

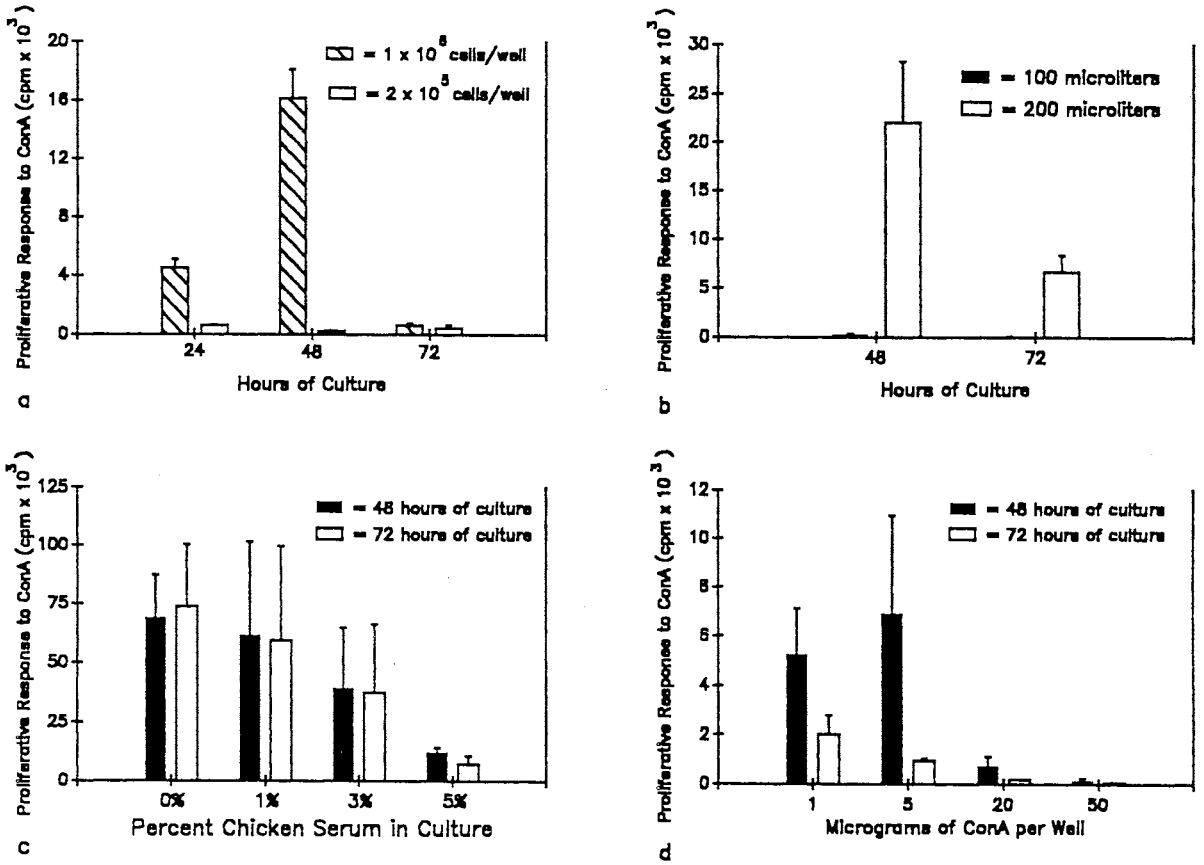
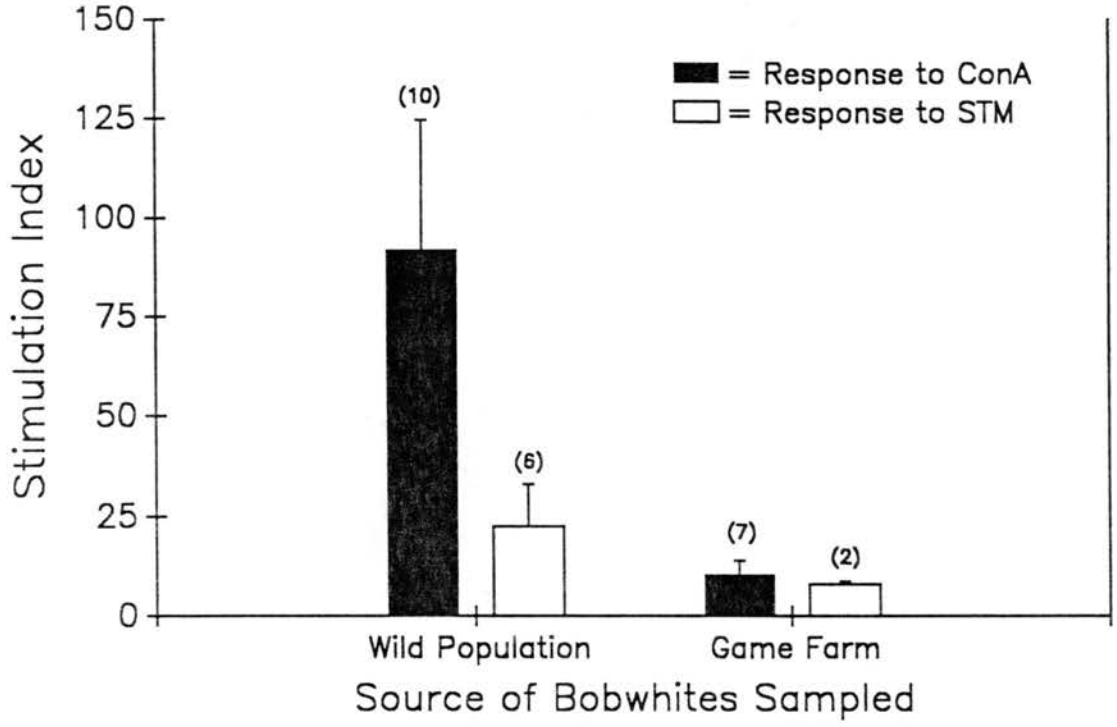

Proliferative Response to ConA (cpm x 10³)
20
16
12
8
4
0
= 1 x 10⁶ cells/well
= 2 x 10⁵ cells/well
24
48
72
Hours of Culture
a
Proliferative Response to ConA (cpm x 10³)
30
25
20
15
10
5
0
= 100 microliters
= 200 microliters
48
72
Hours of Culture
b
Proliferative Response to ConA (cpm x 10³)
125
100
75
50
25
0
= 48 hours of culture
= 72 hours of culture
0%
1%
3%
5%
Percent Chicken Serum in Culture
c
Proliferative Response to ConA (cpm x 10³)
12
10
8
6
4
2
0
= 48 hours of culture
= 72 hours of culture
1
5
20
50
Micrograms of ConA per Well
d

Figure 2. Comparison of mean stimulation indices (cpm stimulated culture/cpm unstimulated culture) of wild-caught and domestic Northern bobwhite splenocytes in response to either 5 μg Con A or 2.5 μg STM per well. One million cells per well were cultured in 200 μl of AIM-V serum free media for 72 hours. Numbers in parentheses indicate sample sizes.


150
125
100
75
50
25
0
Stimulation Index
(10)
(6)
(7)
(2)
= Response to ConA
= Response to STM
Wild Population
Game Farm
Source of Bobwhites Sampled

CHAPTER II

A Pathogenic Challenge Model For Adult Northern Bobwhite (*Colinus virginianus*) Using a Vaccine Strain of *Pasteurella multocida* Type 3

SUMMARY. A pathogenic challenge model causing approximately 50% mortality was developed in adult Northern bobwhite (*Colinus virginianus*) using Avichol^{R}, a live vaccine containing the Clemson University (CU) strain of *Pasteurella multocida* Type 3. A dose of 2,300 or 3,000 colony-forming units (CFU) of Avichol^{R} injected intramuscularly resulted in 30 to 75% mortality, whereas a dose of 230 CFU or less resulted in no mortality, and 58,720 CFU or more resulted in death in all birds challenged. Primary and secondary vaccination of Northern bobwhite with a formalinized anaculture of Avichol^{R} derived *P. multocida* resulted in protection against challenge in three separate experiments. Dexamethasone treatment of birds during vaccination resulted in decreased protection against challenge exposure.

Wild populations of Northern bobwhite (*Colinus virginianus*) often undergo annual demographic fluctuations that appear to be related to periods of environmental stress (i.e., drought, temperature extremes) (4,11). We have been examining the effects of various environmental stressors on immune function in this upland gamebird. Information about the Northern bobwhite immune system is extremely limited (6,8,12), and few methods are available for directly testing host resistance to specific pathogens (5).

Our primary objective was to develop a pathogenic challenge model that would permit testing of the integrity of Northern bobwhite resistance to *Pasteurella multocida* following exposure to immunomodulating factors. *P. multocida* is an appropriate pathogen, because environmental stressors have been suggested as factors modulating Northern bobwhite resistance to this bacterium (2). We chose Avichol^{R} (Schering-Plough Animal Health Corporation, Omaha, Neb.), a live vaccine containing the Clemson University (CU) strain of *P. multocida* Type 3 developed for use as a vaccine in poultry, as a pathogen, because it is easy to use and causes high mortality in Northern bobwhite (7). We also examined the ability of a formalinized culture to protect Northern bobwhites against Avichol^{R} and the sensitivity of this protection to effects of dexamethasone, an immunomodulating agent.

MATERIALS AND METHODS

Adult Northern bobwhites obtained from El Reno Gamebird Farm (El Reno, Okla.) were housed in brooders (91.4 x 71.1 x 27.9 cm) and provided quail starter feed (A&M Feed Mill, Stillwater, Okla.) and water *ad libitum*.

A series of experimental exposure trials were used to determine the dose of Avichol^{R} appropriate to cause approximately 50% mortality in adult Northern bobwhites. Individual birds were injected intramuscularly (i.m.) in the thigh with five fold serial dilutions providing 1.5 x 10^{6} to 23 colony-forming units (CFU) in a 50-μl volume. Bacterial cell counts (colony-forming units) were estimated photometrically and confirmed by a spot-plate-counting technique. Birds were observed daily for one week after challenge (14). Results from the first experiments indicated that a 50% lethal challenge dose was 2,000-3,000 CFU. This was verified in a second series of trials, in which birds were challenged with 2,300 ($\underline{n}$ = 12) and 3,000 ($\underline{n}$ = 24) CFU as previously described.

An anaculture(bacterin-toxoid preparation) was prepared from Avichol^{R} to explore its efficacy in protecting Northern bobwhite against challenge. The CU strain of *P. multocida* was grown in tryptic-soy broth in a shaker incubator at 37 C for 24 hours, at which time bacterial cell counts were determined. Bacteria were killed by adding formalin directly to the broth culture to a final concentration of 0.3%, followed by incubation for another 24 hours.

Anaculture sterility was tested by inoculating duplicate tubes of thioglycollate broth and thioglycollate broth containing 10% horse serum incubated at 24 and 37 C for one week. Killed bacteria were adjusted to 2 x 10^9 CFU/ml in a solution of 1:1 (v/v) anaculture and alhydrogel (Superfos Biosector a/s, Vedbaek, Denmark).

Six birds were injected i.m. with 0.5 ml of bacteria in the right pectoral muscle; six unvaccinated control birds were injected in the same manner with phosphate-buffered saline (PBS). Ten days post-vaccination, all 12 birds were challenged as previously described, except the challenge dose was increased to 12,000 CFU of Avichol^R, as this was the smallest dose that caused 100% mortality. Survival was monitored for one week. Differences in mortality between vaccinated and unvaccinated birds were tested using a Z statistical test for binomial proportions (15).

In a subsequent experiment, 20 adult hens received a primary vaccination as previously described and a secondary vaccination 7 days later. Thirty-one days post-secondary vaccination all 20 vaccinated birds and four unvaccinated controls were challenged with 12,000 CFU of Avichol^R as previously described. Survival was again monitored for one week. Differences in mortality between vaccinated and unvaccinated birds were tested using a Z statistical test for binomial proportions (15).

Sensitivity of vaccinal immunity to host immunosuppression was examined in Northern bobwhite. The birds were weighed

and assigned to one of two experimental groups (control or dexamethasone; n = 6 birds per group) according to body mass to equilibrate mean body masses among groups (13). Adult birds (n = 12) were vaccinated once with the prepared anaculture as previously described. Birds were injected i.m. in the left pectoral muscle with either 0.5 ml of PBS (control) or 1 mg of dexamethasone (0.5 ml injectable; Schering-Plough) on days 0, 3, 6, and 9 post-vaccination. All birds were challenged with 12,000 CFU of Avichol^R on day 10 and monitored for one week as previously described. Differences in mortality among birds treated with PBS and dexamethasone were tested using a Z statistical test for binomial proportions (15).

RESULTS

Unvaccinated adult Northern bobwhite challenged with 230 CFU or less of Avichol^R showed no clinical signs of disease and suffered no mortality. Doses of Avichol^R of 58,720 CFU or greater caused mortality in all birds challenged. A dose of 2,300 CFU of Avichol^R caused a mean mortality of 42% in two replicate challenges. Mortality from the challenge dose of 3,000 CFU caused a mean mortality of 54% in three replicate experiments.

Birds that received a primary vaccination with the prepared anaculture had generally lower mortality (Z = 1.15; P = 0.13) than unvaccinated birds when challenged with 12,000 CFU of Avichol^R (Table 1). Birds that were vaccinated twice had lower mortality (Z = 3.41; P = 0.0003)

than controls when challenged with 12,000 CFU of Avichol^R 31 days postvaccination (Table 1).

Dexamethasone reduced resistance of vaccinated birds to challenge with Avichol^R. Birds vaccinated and treated with dexamethasone had 50% more mortality (Z = 2.0; P = 0.02) upon challenge than birds vaccinated and treated with PBS (Table 1). In all cases except two, mortality occurred within 48 hours of challenge. All birds showing clinical signs of *P. multocida* infection died.

DISCUSSION

Our data indicate that Avichol^R, a strain of *P. multocida* considered avirulent to chickens, is highly virulent to Northern bobwhites when administered i.m.. This virulence is consistent with a previous report of the effects of an avirulent strain of *P. multocida* on Northern bobwhites (7). Vaccination of Northern bobwhites with an anaculture made from Avichol^R improved bobwhite resistance to challenge with the live bacterium, even though the birds were dosed with concentrations of bacteria that caused 67-100% mortality in unvaccinated birds. When current results are compared with results of earlier studies using chickens (9) and turkeys (3) vaccinated with bacterin with aluminum hydroxide adjuvant and challenged in the nasal cleft with virulent *P. multocida*, the vaccinated Northern bobwhites challenged with Avichol^R had slightly higher and more variable mortality rates among experiments even though all experiments used the same challenge dose of bacteria. These differences in

mortality are likely caused by intramuscular challenge, which appears to be more virulent than nasal cleft challenge (15).

Dexamethasone, at the dose (5.6 mg/kg-body weight) used, has been shown to reduce *in vivo* measures of humoral (i.e., antibody response) and cell-mediated (i.e., phytohemagglutinin-P skin response) immune function of mallards (*Anas platyrhynchos*) (17). Similar pharmacologic actions were observed in this study using Northern bobwhite in which dexamethasone reduced the protective ability of the bacterin in challenge experiments.

Mechanisms of the virulence of *P. multocida* in birds is poorly understood (16). Resistance of birds to *P. multocida* is thought to involve both cellular and humoral immunity as controlled by the avian Major Histocompatability Complex (10). The pharmacological mode of action of dexamethasone on the resistance of Northern bobwhite vaccinated with anaculture is also unclear, but it may result from lymphocyte lysis (1) or dysfunctional interleukin secretion or synthesis (18).

As marketed, Avichol^{R} can be easily processed for use without facilities or media required to grow *P. multocida*. A dose of 3,000 CFU of Avichol^{R} administered i.m. can be used as a suitable pathogenic challenge model for determining the effects of immunomodulating factors on the resistance of adult Northern bobwhite to bacterial challenge. Birds can be vaccinated with formalin-killed

broth cultures of Avichol^{R} to evaluate bird development of vaccinal immunity, and dexamethasone can be used as a positive control for immunosuppression.

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Table 1. Comparison of mortality among vaccinated once or twice, adult Northern bobwhite treated with either phosphate-buffered saline or dexamethasone, and challenged with 12,000 colony-forming units of Avichol^{R}.

Group	No. dead / total	Percent mortality
Experiment		
Primary vaccination[A]		
Unvaccinated	4/6	67
Vaccinated	2/6	33
Secondary vaccination[B]		
Unvaccinated	4/4	100
Vaccinated	5/20	25
Immunosuppressant		
Vaccinated/PBS	0/6	0
Vaccinated/Dexamethasone[C]	3/6	50

[A]Vaccinated once only.

[B]Secondary vaccination 7 days after primary vaccination.

[C]1 mg on days 0, 3, 6, and 9 postvaccination.

CHAPTER III

Survival implications of acute thermal stress in a warm-adapted avian species, the Northern bobwhite, *Colinus virginianus*

Summary

1. We tested the hypothesis that exposure of Northern bobwhite to acute temperature stressors impairs immune system function and host resistance to disease. We postulated that Northern bobwhite are better adapted to deal with extremes in heat than cold conditions that may occur throughout their geographic range.

2. Birds were randomly assigned to either a thermo-neutral (constant 21 °C), cold-stress (cycled from 3.6 to -20 °C during a 24 h period), or heat-stress (cycled from 30.8 °C to 39.0 °C during a 24 h period) treatment for 4 consecutive days. Immunocompetence of each bird was evaluated on day 5 using a panel of assays, including measuring host resistance to a *Pasteurella multocida* Type 3 challenge.

3. Neither thermal stressor had an influence on spleen weight or measures of cell-mediated and humoral immunity.

4. Disease resistance of Northern bobwhite to challenge with *P. multocida* was not influenced by heat stress but increased following cold stress. Cold stress may have increased the numbers or activity of phagocytic leukocytes, which are important in resistance of birds to bacterial pathogens. A concomitant decrease in lymphocyte numbers suggested that resistance to viral pathogens may have been compromised during cold stress.

Introduction

Physiological tolerances of a species are strong determinants of the environmental conditions which it may inhabit (Wiens 1989). Physiological stress resulting from adverse climatic conditions may strongly influence distribution and population size of a species if the stress results in reduced fitness or survival (Wiens 1989). Adverse climatic conditions may occur as random events throughout a species' range or as annual seasonal changes, which are frequently greatest in magnitude at the margin of a species' range (Hoffman & Parsons 1991).

Northern bobwhite (*Colinus virginianus*) populations display these patterns, being very unstable at their Northern margin (Rosene 1969) where annual over-winter declines in population size have been well documented (Roseberry 1962). In contrast, their populations appear to be more stable in the southern latitudes (Rosene 1969), which is thought to be closer to the suspected evolutionary origin of this species (Johnsgard 1973). However, Northern bobwhite populations occasionally fluctuate widely across seasons and years even in these warmer climates (Lehmann 1984). Although interrelationships between rainfall and recruitment previously have been examined (Guthery & Koerth 1992; Wood, Guthery & Koerth 1986), the role of temperature extremes in this phenomenon remains unresolved.

Reports of acute die-offs during especially cold weather events (Roseberry 1962; Stanford 1971) coupled with studies

of poultry demonstrating a link between cold-stress and immunity (Regnier & Kelly 1981) suggested to us that altered disease resistance may be a factor in explaining many of these observed demographic patterns in this species adapted to warm climates. Northern bobwhite are frequently exposed to extreme cold in much of their Northern range (Gerstell 1939; Roseberry 1962; Stanford 1971), and wind-chill equivalencies below freezing have been documented even as far as south Texas (Lehmann 1984). Likewise, extreme elevations in temperature can occur during nesting throughout their range (Lehmann 1984). It has been shown that exposure of poultry to comparable temperature stressors (El-Halawani et al. 1973) results in the reduction of humoral (Henken, Nieuwland & Wensink 1983) and cell-mediated (Regnier & Kelly 1981) immunity leading to altered host resistance to infectious pathogens (Reece, Howes & Frazier 1992).

We tested the hypothesis that exposure of Northern bobwhite to acute temperature stressors impairs immune system function and host resistance to disease. We postulated that Northern bobwhite are better adapted to deal with extremes in heat than cold conditions that may occur throughout their geographic range.

Materials and methods

MAINTENANCE OF ANIMALS

Adult male Northern bobwhite were obtained from the El Reno Gamebird Farm (El Reno, OK, USA) and housed in groups of 4

per cage (91 x 71 x 27 cm) under a 12L:12D photoperiod. Commercial gamebird feed (A&M Feed Mill, Stillwater, OK, USA) and water were provided *ad libitum*. Daily food intake per cage was determined during cold-stress trials by measuring the difference in weight between uneaten food and food provided the previous day.

EXPERIMENTAL DESIGN

Two separate experimental trials (cold stress, heat stress) were used to compare responses of Northern bobwhite to acute thermal stress. For each experiment, birds were weighed and randomly assigned to replicate thermal-stress or thermoneutral cages with 4 birds per cage replicate (Hurlbert 1984; Maguire & Williams 1987). The cold-stress experimental trial was repeated twice using 5 replicate cold-stressor cages and 5 replicate thermoneutral cages per repetition(n = 80). The heat-stress experimental trial was repeated three times using 5 replicate heat-stressor cages and 5 replicate thermoneutral cages per repetition (n = 120). Birds assigned to each thermoneutral treatment were maintained in an environmental chamber at a constant 21 °C; cold-stressed birds were housed in an environmental chamber that cycled from 3.6 ± 1.3 °C (18 h period) to -20 ± 4.5 °C (6 h period) during a 24 h period. Heat-stressed birds were housed in an environmental chamber that cycled from 30.8 ± 0.5 °C to 39.0 ± 0.5 °C during a 4 h period, where the temperature remained for 4 h before decreasing (4 h) back to 30.8 ± 0.5 °C for the remaining 12 h of each 24 h period.

After 4 consecutive days of temperature treatment, birds were weighed, and immunocompetence within 3 replicate cages per treatment repetition was assessed using *in vivo* and *in vitro* assays. Birds within the remaining two replicate cages per treatment repetition were used to assess host resistance to a pathogenic challenge of avian cholera.

BLEEDING PROTOCOL AND LEUKOCYTE COUNTS

Birds were anesthetized with an intramuscular injection of 5 mg/kg ketamine hydrochloride (Fort Dodge Laboratories, Fort Dodge, IA, USA) in the right pectoralis. Blood was collected into heparinized-microhematocrit tubes via brachial venipuncture for a thin-film blood smear. Serum was obtained by collecting blood via jugular venipuncture, and sera was stored at -70 °C until analyzed.

Blood smears were stained using Diff Quik (Baxter Healthcare Corp, Miami, FL, USA) and observed slides under 1,000x magnification (oil-emersion). A total of 100 leukocytes was classified according to the criteria of Dein (1984) to provide the percentage of heterophils, eosinophils, monocytes, lymphocytes, and basophils. A relative total leukocyte count was obtained by enumerating leukocytes in 50 oil-emersion fields (OEF) (Janes, Bower & Anthony 1994). Thrombocytes were similarly quantified in 10 OEF (Fairbrother & O'Loughlin 1990).

HUMORAL IMMUNITY

Humoral immunity to a T-dependent antigen was assessed by measuring serum antibody titers following a primary

intramuscular injection of 0.5 ml of a 40% sheep red blood cell (SRBC) suspension (in phosphate-buffered saline (PBS); Colorado Serum Co., Denver, CO, USA) in the right pectoralis. Concurrently, humoral immmunity to a T-independent antigen was assessed by measuring serum antibody titers fowllowing a primary intramuscular injection of 0.1 ml of a 1:10 dilution of *Brucella abortus* antigen (BA) (Difco Laboratories, Detroit, MI, USA) in the left pectoralis in PBS containing 5% alhydrogel as an adjuvant (Superfos Biosector a/s, Vedbaek, Denmark). Antigens were administered 2 days prior to the start of the 4-day experimental trial. Antibody titers to SRBC were measured using a microhemagglutination assay (Wegmann & Smithies 1966); titers to BA were measured using a bacterial agglutination assay (McCorkle & Glick 1980).

CELL-MEDIATED IMMUNITY

Cell-mediated immunity was assessed by measuring *in vivo* wing-web responses to an intradermal injection of the T-cell mitogen phytohemagglutinin (PHA) (Sigma Chemical Co., St. Louis, MO, USA) (Cheng & Lamont 1988). Birds were injected in the right wing web with 0.5 mg of PHA (dissolved in 0.1 ml of PBS) and in the left wing web (control) with 0.1 ml of PBS after 72 h of temperature treatment. Wing-web thickness was measured using a pressure-sensitive dial gauge to the nearest 0.002 mm immediately before and 24 h after injection. Cell-mediated immune response (wing-web index)

was calculated as the difference in wing-web thickness between the PHA and PBS (control) injected sites.

Anesthetized birds were euthanized by cervical dislocation. The spleen of each bird was aseptically removed, weighed, homogenized, and total cellularity manually determined using a hemacytometer. For cold stress experiments, blastogenic responses of splenocytes cultured with the plant lectin Concanavalin A (Con A) (5 μg/well; Sigma Chemical Co., St. Louis, MO, USA) and *Salmonella typhimurium* mitogen (STM)(2.5 μg/well; Ribi Immunochem Research Inc., Hamilton, MT, USA) were measured in 96-well flat-bottom plates. One million cells per well were cultured in 200 μl of AIM-V serum-free media (Gibco Laboratories, Grand Island, NY, USA) for 72 h at 40.5 °C with 5% CO_2-95% air (Dabbert & Lochmiller 1995). Proliferative responses were assessed using ^{3}H-thymidine (1 μcurie; ICN Biomedicals, Inc., Irvine, CA, USA) incorporation into cells during the last 18 h of culture. Stimulation indices for both mitogens were calculated as ratios of cpm in stimulated cultures to unstimulated control cultures.

PATHOGENIC CHALLENGE PROTOCOL

All birds were maintained at 21 °C during the pathogenic challenge assay. Each bird was injected intramuscularly in the thigh muscle with 3,000 CFU of Avichol^R (Schering-Plough Animal Health Corp., Omaha, NE, USA), a vaccine strain of *Pasteurella multocida* Type 3, in 100 μl of PBS (Dabbert et

al. 1995). Survival was monitored over a 1-week period postchallenge.

TRIIODOTHYRONINE AND THYROXINE DETERMINATION

Serum triiodothyronine (T_3) and thyroxine (T_4) concentrations of cold-stressed birds were colorimetrically determined using a CEDIA^{TM} homogeneous enzyme immunoassay kits (Microgenics Corp., Concord, CA, USA) and recommended procedures of the manufacturer. The assay was adapted for use on a Cobas Mira^{R} wet chemistry analyzer (Roche Diagnostics Systems Inc., Montclair, NJ, USA).

BODY MOISTURE DETERMINATION

Northern bobwhite carcasses were dried to constant weight by lyophilization using a Labconco Freeze Dryer System (Labconco Corp., Kansas City, MO, USA). Body moisture content was determined by subtracting dried body weight from original body weight and dividing the resulting value by original body weight.

STATISTICAL ANALYSIS

For each trial, differences between thermal-stress and thermoneutral treatment groups in food intake, body weight change and moisture content, hormone and hematological parameters, spleen mass and cellularity, and measures of humoral and cell-mediated immunity were tested using an analysis of variance with a split-plot design with repetition (Norusis 1990). A Bartlett-Box F-test was used to test for homogeneity of sample variances for all dependent variables (Norusis 1990). All variables had

homogeneous variances after log transformation and square-root transformation of relative total basophil and eosinophil counts, respectively, for cold-stress experimental trials; and square-root transformation and sine transformation of relative percent monocytes and relative total heterophil counts, respectively, for heat-stress experimental trials.

Difference in mortality after *P. multocida* challenge between temperature treatments was tested using a Z-test for binomial proportions (Ott 1988). Statistical significance was indicated at $P < 0.05$, and all values are reported as mean $\pm$ standard error.

Results

HEAT-STRESS

Body weight averaged 182 $\pm$ 1 g ($n = 72$) at the start of the experiment and did not differ between treatment groups ($P = 0.74$). Heat-stress had no measureable effect on body weight change or moisture content, spleen mass or cellularity, PHA wing-web index, microhemagglutination or bacterial agglutination titers, or hematological parameters ($P \geq 0.06$; Table 1). Mortality rate after challenge with 3,000 CFU of *P. multocida* was not different ($Z = 0.89$; $P = 0.19$) between birds subjected to heat-stress (42%; $n = 24$) and those maintained at thermoneutrality (54%; $n = 24$). Mortality occurred within 48 h of challenge in all cases; all birds showing signs of *P. multocida* infection died.

COLD-STRESS

Body weight averaged 216 ± 15 g (n = 48) at the start of the experiment and did not differ between treatment groups (P = 0.40). Temperature treatment influenced (P = 0.003) body weight; cold-stressed birds lost an average of 9.1 ± 0.9 g compared to thermoneutral birds that gained an average of 2.4 ± 1.6 g over the 4-day trial. Body moisture content (66.10 ± 0.29%) of cold-stressed birds was greater (P = 0.002) than thermo-neutral birds (64.67 ± 0.22%). Food intake of cold-stressed birds exceeded (P = 0.037) that of thermo-neutral birds only on day three of exposure; intake was not different ($P \geq 0.113$) on days one, two, and four (Fig. 1).

Serum T_4 concentrations were 27% lower (P = 0.009) in cold-stressed birds compared to birds maintained at thermoneutrality, but serum T_3 concentrations were similar between treatments (Fig. 2). Cold stress had no measureable effect on spleen weights, PHA wing-web indices, splenocyte proliferation indices, microhemagglutination titers, and bacterial agglutination titers of Northern bobwhite (Table 2). Total splenocyte counts and relative total peripheral blood leukocyte counts of Northern bobwhite subjected to cold stress were ≥ 30% lower ($P \leq 0.017$) than those in the thermo-neutral treatment (Fig. 3); total thrombocyte counts were not affected by treatment (Table 2). Cold stress tended to increase (P = 0.053) the relative percentage of monocytes but decreased (P = 0.037) the relative percentage

of heterophils in peripheral blood smears (Fig. 4). Percentages of other peripheral blood leukocytes and thrombocytes were not affected ($P \geq 0.482$) by temperature treatment (Fig. 4). Because of reduced total peripheral blood leukocyte counts, relative total lymphocyte and heterophil counts were markedly decreased ($P \leq 0.022$) by cold stress treatment; total counts of other leukocytes were not affected ($P \geq 0.246$) by treatment (Fig. 4). The heterophil/lymphocyte ratio was similar between treatments ($P = 0.198$; Table 2).

Mortality rate from a challenge with 3,000 CFU of *P. multocida* was about 50% lower for cold-stressed birds compared to those in the thermoneutral group ($Z = 2.9352$; $P < 0.0017$). In all cases, mortality occurred within 48 h of challenge, and all birds showing signs of *P. multocida* infection eventually died.

Discussion

Exposure of Northern bobwhite to a cycling heat stressor that was only 4.8 °C below the upper mean lethal temperature for this species (Case & Robel 1974) had no effect on host immunocompetence, including resistance to a *P. multocida* challenge. Heat stress has been suggested to increase susceptibility of Northern bobwhite to *P. multocida* infection (Bermudez, Munger & Ley 1991). Northern bobwhite appeared to be well adapted to heat stress and used panting as a heat dissipating mechanism during our experiment. Performance of Northern bobwhite contrasts with many poultry

strains, that experience immune dysfunction after exposure to 36 °C (Regnier & Kelley 1981).

In comparison, exposure of Northern bobwhite to a cycling cold stressor induced a variety of physiological alterations that appeared to have both positive and negative consequences on host survival. Exposure of birds to extreme cold (e.g., 0 °C) can elevate existence metabolism 3-fold over basal metabolic rate (Robbins 1983). Body weight loss concomitant with increased body moisture content (inversely proportional to fat) in cold-stressed birds suggested to us that fat reserves were partly used to fuel these increased metabolic demands. It is noteworthy that these weight changes occurred despite *ad libitum* availability of a high quality food source. In the wild, climatic factors (e.g., snow cover) that reduce food availability can be expected to exacerbate the rate of fat loss during periods of extreme cold. Loss of $\geq$ 20% body weight is usually lethal to Northern bobwhite (Robel, Bisset & Clement 1979). Considering that birds in our study lost an average of 4% body weight in only 4 days, long periods of extreme cold temperatures could reduce the probability of survival of this warm-adapted species in the wild.

Physiological adjustments of birds to cold temperatures are complex and typically involve adjustments in thyroid metabolism (Dawson, Carey & Vant't Hof 1992), such as elevation of serum T_3 concentrations and declines in serum T_4 concentrations (Cogburn & Freeman 1987). Although

Northern bobwhite in our study showed declines in T_4 levels, T_3 levels remained unchanged. Thyroid hormones are capable of influencing immune system activity during cold exposure. For example, low (0.1 ppm) *in vivo* doses of T_3 will enhance production of IL-2-like activity in birds, but higher (1.0 ppm) doses are immunosuppressive (Chandratilleke, Scanes & Marsh 1994). Administration of both growth hormone and either T_3 or T_4 can decrease proliferative responses of lymphocytes in poultry (Marsh & Scanes 1994). Cold-stress, however, did not modify splenocyte proliferative responses of Northern bobwhite in our study.

Stress-induced increases in resistance of birds to challenge with bacterial pathogens is a commonly observed phenomenon. For example, cold stress, feed restriction, corticosteroid injection, social stress, and handling stress can increase resistance of poultry to *Staphylococcus aureus*, *Escherichia coli*, or *P. multocida* infections (Gross & Siegel 1965; Simensen & Olson 1981; Mutalib, Riddell & Osborne 1983; Juskiewicz 1967). The immune system of Northern bobwhite responded to *P. multocida* challenge after cold stress in a similar fashion. Stress-induced increases in resistance of bird species to bacterial pathogens has been attributed to increased numbers and/or activity of phagocytic leukocytes (Siegel 1980). It is likely that cold-stress initiated similar physiological alterations of immune cells of Northern bobwhite, especially given the increased percentage of monocytes observed in these birds. Although

cold stress appeared to increase immunoreactivity to *P. multocida* without changing responses to standard functional assays of cell-mediated and humoral immunity, it is doubtful that large fluctuations in leukocyte populations are entirely beneficial. How Northern bobwhite might respond to other pathogenic organisms after acute cold-stress remains unclear. Decreases in lymphocyte numbers of cold-stressed birds may indicate that resistance to viral pathogens (fowl pox or quail bronchitis) is compromised (Gross 1962); however, this has not been demonstrated experimentally.

Our study also illustrates the complex nature of interactions between environmental stressors and immune system function and the difficulty in predicting environmental effects on immmunocompetence of wild bird species from research using domesticated avian models. Disparate immunological reactions of Northern bobwhite, a warm-adapted species (Johnsgard 1973), to heat- and cold-stressors supports our hypothesis that Northern bobwhite are better adapted to deal with extremes in heat than cold conditions that may occur throughout their geographic range. The validity of directly extrapolating physiological responses of poultry to Northern bobwhite is unclear, given their disparate evolutionary life histories. Even within poultry, response to thermal stressors differs dramatically among genetic breeds (Regnier & Kelley 1981; Spinu & Degen 1993).

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Table 1. Comparison of the physiological status of Northern bobwhite subjected to either a 4-day cycling heat stressor or thermoneutral temperature

Parameter	Heat-stress[a]	Thermoneutral[a]
Body mass[b]	182.2±1.8	182.5±1.4
Percent body moisture	64.3±0.3	64.8±0.5
Spleen mass[c]	82.2±8.3	78.2±5.1
Total splenocyte count[d]	62.9±9.5	64.4±6.1
Phytohemagglutinin wing-web index[e]	53.3±3.3	45.7±1.7
Anti-sheep red blood cell titer[f]	7.5±0.5	7.4±0.5
Anti-*Brucella abortus* antigen titer[f]	7.8±0.6	7.7±0.4
Differential leukocyte counts (%)		
Lymphocytes	15.8±1.4	14.7±2.1
Heterophils	66.8±1.7	66.8±1.8
Monocytes	14.6±0.7	16.5±1.6
Eosinophils	1.1±0.2	1.1±0.3
Basophils	1.2±0.2	1.0±0.2
Total leukocyte count[g]	114.7±6.0	107.8±11.1
Total thrombocyte count[h]	29.4±2.2	27.2±3.4

[a] n = 36 birds per treatment
[b] g
[c] mg
[d] cells x 10^6
[e] difference in swelling between PHA and PBS injected wing-webs
[f] $\log_2$
[g] number per 50 oil-emersion fields
[h] number per 4 oil-emersion fields

Table 2. Comparison of the physiological status of Northern bobwhite subjected to either a 4 day cycling cold stressor or thermoneutral temperature

Parameter	Cold-stress[a]	Thermoneutral[a]
Spleen mass[b]	64.1±2.25	67.8±5.5
Phytohemagglutinin wing-web index[c]	30.1±2.3	34.4±2.5
Concanavalin A stimulation index[d]	7.9±1.7	7.3±2.6
Salmonella typhimurium mitogen stimulation index[d]	2.9±0.6	4.5±0.8
Anti-sheep red blood cell titer[e]	5.2±0.4	5.3±0.3
Anti-*Brucella abortus* antigen titer[e]	7.9±0.2	7.7±0.4
Total thrombocyte count[f]	28.9±6.1	26.1±3.2

[a]n = 24 birds per treatment
[b]mg
[c]difference in swelling between PHA and PBS injected wing-webs
[d]ratio of cpm of stimulated/unstimulated controls
[e]$\log_2$
[f]number per 4 oil-emersion fields

Fig. 1. Mean ± SE for daily ad libitum food consumption by 4 adult Northern bobwhites . Thermo-neutral birds (n = 10) were maintained at a constant 21 C; temperature of cold-stressed birds (n = 10) was cycled from 3.6 C (18 h) to an average temperature of -20 C (6 h) during a 24 h period.

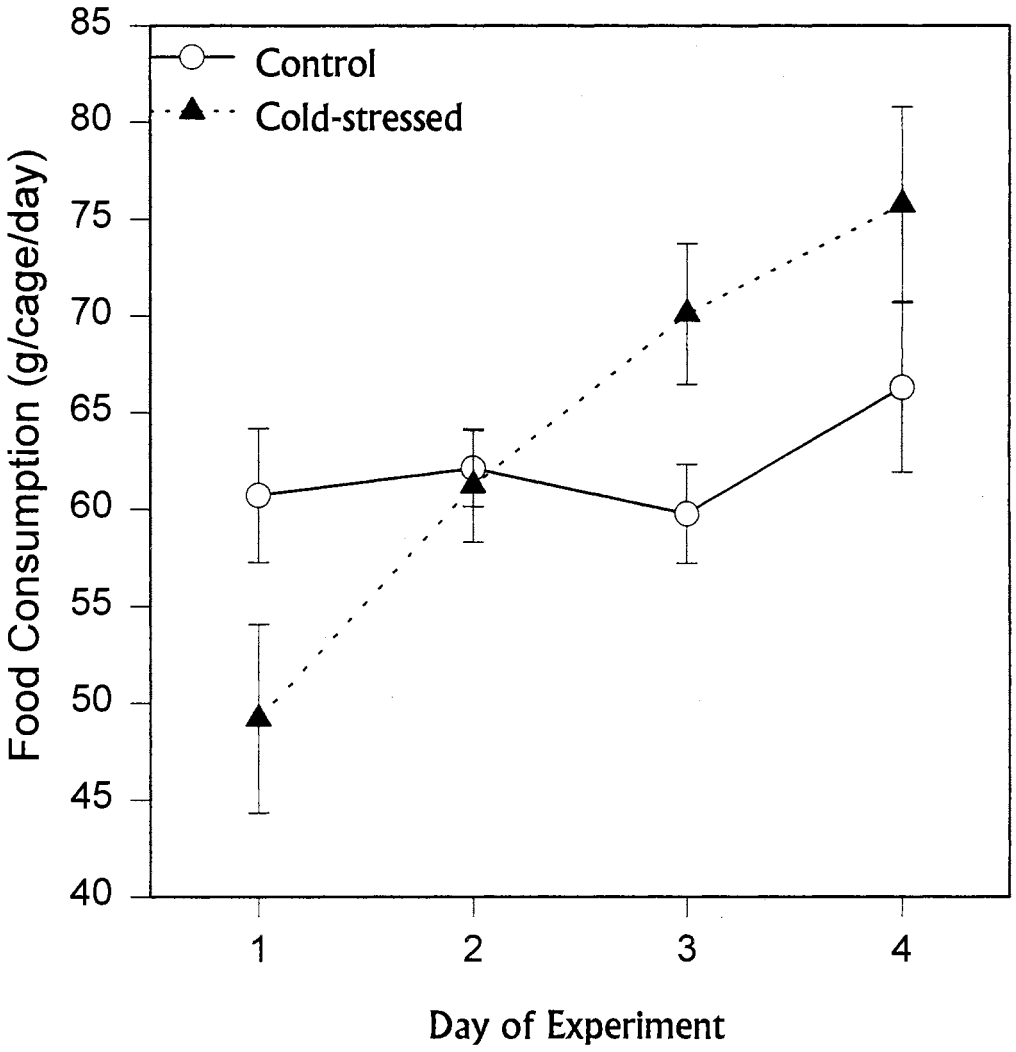

Control
Cold-stressed
Food Consumption (g/cage/day)
85
80
75
70
65
60
55
50
45
40
1
2
3
4
Day of Experiment

Fig. 2. Mean ± SE for serum T_3 and T_4 concentrations of adult Northern bobwhites after 4 days of temperature treatment. Thermo-neutral birds (n = 40) were maintained at a constant 21 C; temperature of cold-stressed birds (n = 40) was cycled from 3.6 C (18 h) to an average temperature of -20 C (6 h) during a 24 h period. Significant difference ($P < 0.055$) between temperature treatments (*) is indicated

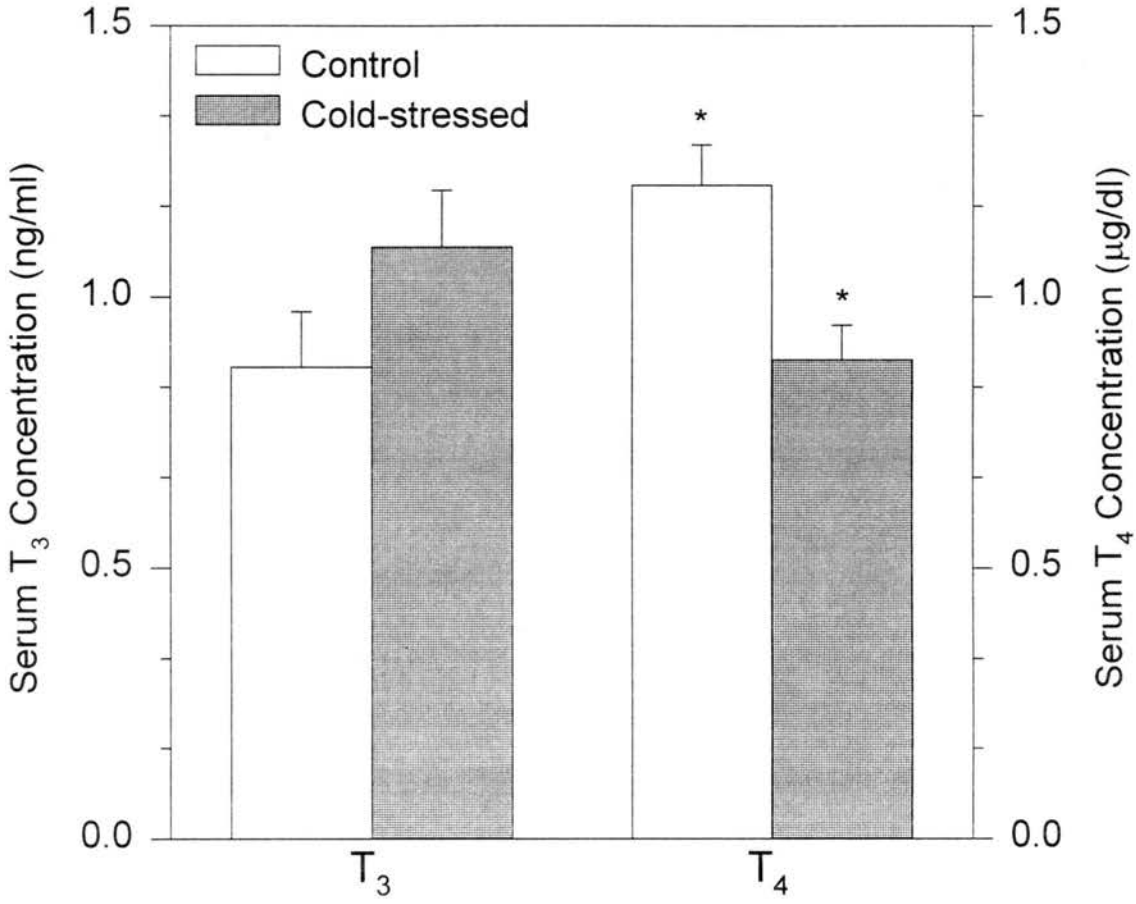

1.5
1.0
0.5
0.0
Control
Cold-stressed
*
*
Serum T_3 Concentration (ng/ml)
Serum T_4 Concentration (μg/dl)
T_3
T_4

Fig. 3. Mean ± SE for total splenocyte count and total peripheral blood leukocyte count of adult Northern bobwhites after 4 days of temperature treatment. Thermo-neutral birds (n = 40) were maintained at a constant 21 C; temperature of cold-stressed birds (n = 40) was cycled from 3.6 C (18 h) to an average temperature of -20 C (6 h) during a 24 h period. Significant difference ($P < 0.055$) between temperature treatments (*) is indicated

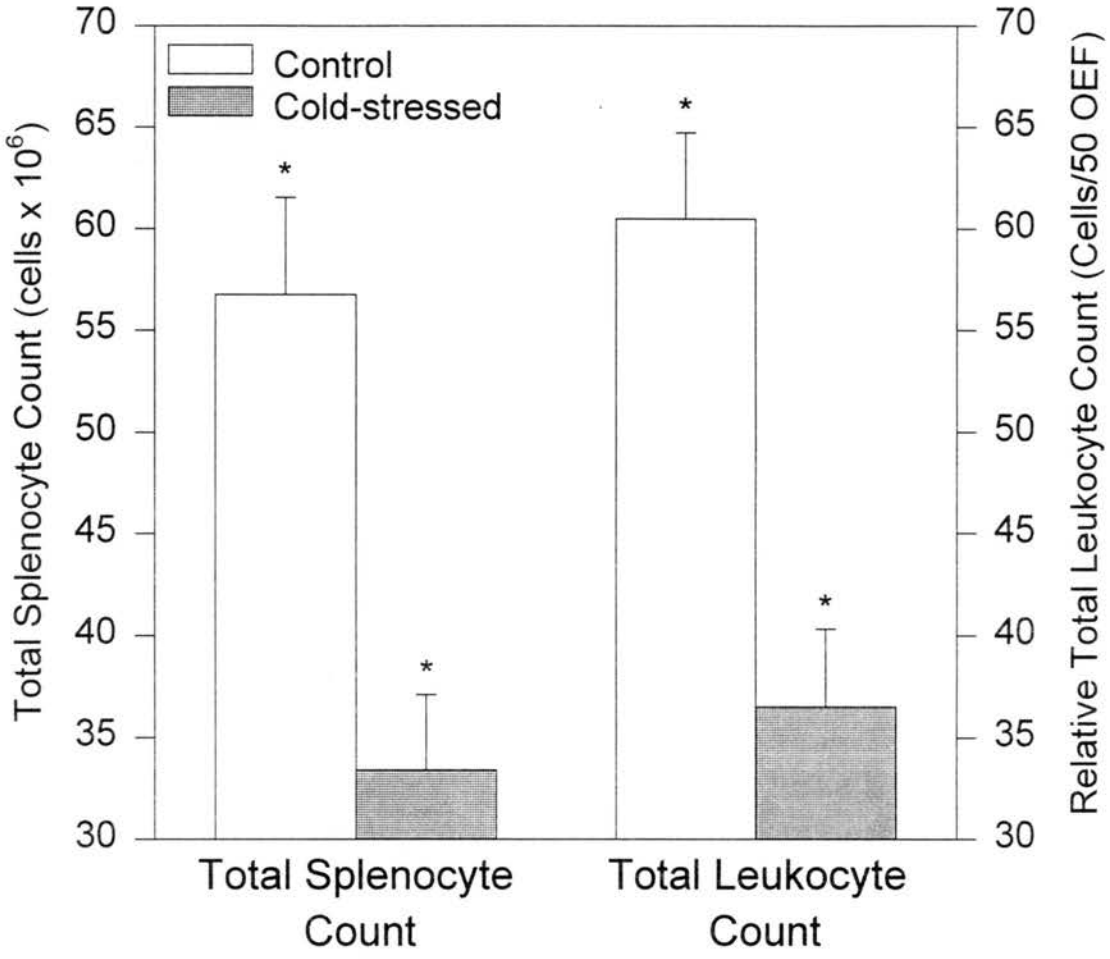
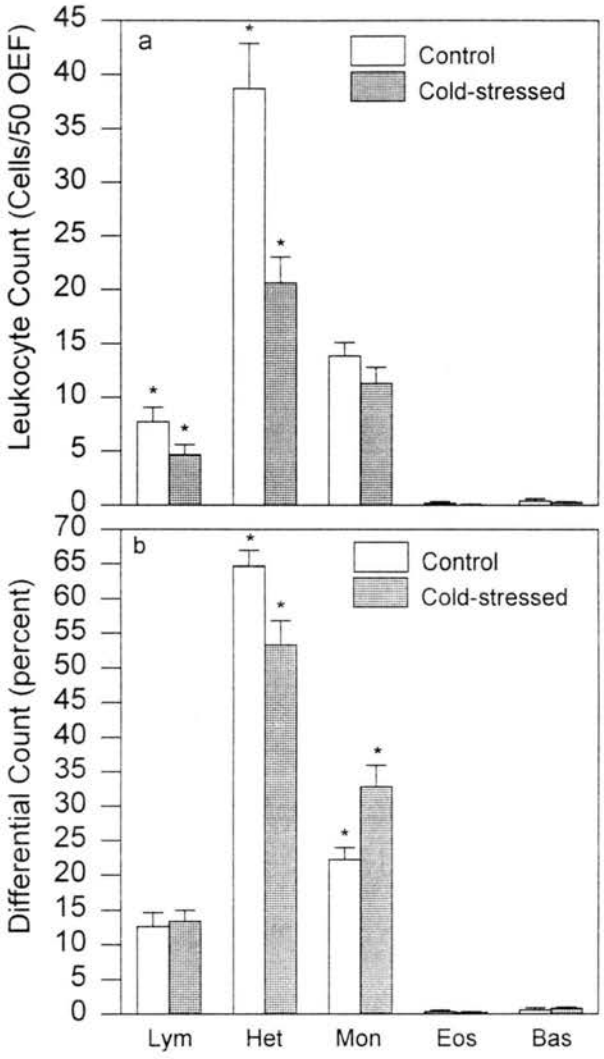

Control
Cold-stressed
Total Splenocyte Count (cells x 10^6)
Relative Total Leukocyte Count (Cells/50 OEF)
70
65
60
55
50
45
40
35
30
*
Total Splenocyte Count
Total Leukocyte Count

Fig. 4a,b. Mean $\pm$ SE for numbers (a) and relative percentages (b) of leukocytes of adult Northern bobwhites after 4 days of temperature treatment. Thermo-neutral birds (n = 40) were maintained at a constant 21 C; temperature of cold-stressed birds (n = 40) was cycled from 3.6 C (18 h) to an average temperature of -20 C (6 h) during a 24 h period. Significant difference ($P < 0.055$) between temperature treatments (*) is indicated


a
Control
Cold-stressed
Leukocyte Count (Cells/50 OEF)
45
40
35
30
25
20
15
10
5
0
b
Control
Cold-stressed
Differential Count (percent)
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
Lym
Het
Mon
Eos
Bas

CHAPTER IV

Thermal Stress Influences Clinical Chemistry Values of Northern Bobwhite (*Colinus virginianus*)

Abstract. Values of serum biochemistry were determined for 120 adult male Northern bobwhite (*Colinus virginianus*) that were either maintained at thermoneutrality or exposed to thermal stresss. Thermal stress did not affect serum aspartate aminotransferase or albumin concentrations. Lacate dehydrogenase and uric acid in serum of cold-stressed birds were greater than concentrations of serum from thermoneutral or heat-stressed birds. Serum triglyceride concentrations of both cold- and heat-stressed birds were higher than concentrations from birds maintained at thermoneutrality. Serum total protein was greater in cold-stressed birds than in heat-stressed birds.

Introduction

Serum clinical chemistry has been used to diagnose health and nutritional status of birds (Rodriguez et al. 1987, Ferrer et al. 1992). Serum metabolites have been reported for quail of the species *Colinus* (Mcrae and Dimmick 1982, Gee et al. 1982),to provide normal values. Life history events can alter serum metabolite concentrations of Northern bobwhite (*C. virginianus*; Mcrae and Dimmick 1992), but potential impacts of abiotic factors such as climate have not been investigated.

Accurate interpretation of clinical chemistry values is dependent on understanding influences of abiotic and biotic factors. Northern bobwhite are frequently exposed to snow, wind, and extreme cold in much of their Northern range (Stanford 1971). Extreme elevations in temperature also can occur during the nesting season and throughout the range of Northern bobwhite (Lehmann 1984). Thermal stress can alter serum metabolite concentrations of domestic poultry (Arad et al. 1984). Our study provides data about the effects of thermal stress on clinical chemistry values of Northern bobwhite.

Materials and Methods

Adult male Northern bobwhite were obtained from the El Reno Gamebird Farm (El Reno, OK, USA) and housed in groups of 4 per cage (91 x 71 x 27 cm) under a 12L:12D photoperiod. Commercial gamebird feed (A&M Feed Mill, Stillwater, OK, USA) and water were provided *ad libitum*.

Two separate experimental trials (cold stress, heat stress) were used to compare responses of Northern bobwhite to acute thermal stress. For each experiment, birds were weighed and randomly assigned to replicate thermal-stress or thermoneutral cages with 4 birds per cage replicate (Hurlbert 1984; Maguire & Williams 1987). The cold-stress experimental trial was repeated twice using 3 replicate cold-stressor cages and 3 replicate thermoneutral cages per repetition (n = 48). The heat-stress experimental trial was repeated three times using 3 replicate heat-stressor cages and 3 replicate thermoneutral cages per repetition (n = 72). Birds assigned to each thermoneutral treatment were maintained in an environmental chamber at a constant 21 °C; cold-stressed birds were housed in an environmental chamber that cycled from 3.6 °C (18 h period) to -20 °C (6 h period) during a 24 h period. Heat-stressed birds were housed in an environmental chamber that cycled from 29.4 °C to 38.9 °C during a 4 h period, where the temperature remained for 4 h before decreasing (4 h) back to 29.4 °C for the remaining 12 h of each 24 h period.

After 4 consecutive days of temperature treatment birds were anesthetized with an intramuscular injection of 5 mg/kg ketamine hydrochloride (Fort Dodge Laboratories, Fort Dodge, IA, USA) in the right pectoralis. Sera was obtained by collecting blood via jugular venipuncture and were stored at -70 °C until analyzed. Serum concentrations of lactate dehydrogenase (LD), aspartate aminotransferase (AST),

triglycerides, uric acid, total protein, and albumin were determined using Roche kits and a Cobas Mira^{R} wet chemistry analyzer (Roche Diagnostics Systems Inc., Montclair, NJ).

STATISTICAL ANALYSIS

Differences among cold-stress, heat-stress, and thermoneutral treatment groups in were tested using a one-way analysis of variance with repetition (Norusis 1990). A Bartlett-Box F-test was used to test for homogeneity of sample variances for all dependent variables (Norusis 1990). All variables had homogeneous variances after log transformation of serum triglycerides and uric acid values, and rank transformation of LD and AST values. Differences among treatment means were tested by using the least significant difference test (Norusis 1990). Statistical significance was indicated at $P < 0.05$, and all values are reported as mean ± standard error.

Results (Table 1)

Thermal stress did not affect ($P \geq 0.09$) serum AST or albumin concentrations. The concentration of LD and uric acid in serum of cold-stressed birds was greater ($P \leq 0.03$) than concentrations of serum from thermoneutral or heat-stressed birds. Serum triglyceride concentrations of both cold- and heat-stressed birds were higher ($P \leq 0.002$) than concentrations from birds maintained at thermoneutrality. Serum total protein concentration of cold-stressed birds was greater than ($P = 0.02$) concentrations in serum of heat-stressed birds.

Discussion

Our data indicate that exposure of Northern bobwhite to acute thermal stress results in altered concentrations of serum metabolites. Social stress can increase serum triglyceride concentrations of domestic poultry (Okwusidi et al. 1991). It appears that Northern bobwhite responded similarly after exposure to thermal stress. Cold temperatures (e.g. 0 °C) can cause existence metabolism of birds to elevate 3-fold over basal metabolic rate (Robbins 1983). Elevated concentrations of LD in serum of Northern bobwhite that were cold-stressed probably resulted from increased metabolism. Protein catabolism and high protein diets can elevate concentrations of uric acid in birds (Campbell and Coles 1986). Elevated concentrations of uric acid in serum of Northern bobwhite exposed to cold stress may have been caused by increased food intake and/or muscle catabolism. Impact of exposure of birds to thermal stressors should be considered when interpreting clinical chemistry values.

Acknowledgments. This study was funded by the Grand National Quail Foundation, Enid, Oklahoma, and the National Sciences Foundation (BSR-8657043 and IBN-9318066). The Oklahoma Cooperative Fish and Wildlife Research Unit greatly assisted us by providing vehicles and laboratory facilities. We are

grateful to the Oklahoma Department of Wildlife Conservation for donations of Northern bobwhite.

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Table 1. Mean (± SE) of clinical chemistry values of Northern bobwhite maintained at thermoneutrality or exposed to thermally stressful temperatures.

	Thermo-neutral	Heat-stress	Cold-stress
LD (IU/l)	569 ± 62[a]	514 ± 23[a]	798 ± 113[b]
AST (IU/l)	529 ± 38	456 ± 9	567 ± 42
Triglyceride (mg/dl)	91 ± 3[a]	108 ± 7[b]	122 ± 11[b]
Uric acid (mg/dl)	6.5 ± 0.3[a]	6.3 ± 0.3[a]	10.0 ± 0.9[b]
Total protein (g/dl)	4.0 ± 0.1[ab]	3.8 ± 0.1[b]	4.2 ± 0.1[a]
Albumin (mg/dl)	1.7 ± 0.1	1.6 ± 0.1	1.8 ± 0.1

[a-b]Means within rows with no common superscript are significantly different, $P < 0.05$.

CHAPTER V

Examination of the Dietary Methionine Requirements of Breeding Northern bobwhite, *Colinus virginianus*

ABSTRACT Adult Northern bobwhite were used to test the hypothesis that dietary methionine levels recommended by the NRC for breeding quail are excessive for wild bobwhite. We tested the hypothesis by comparing immunocompetence, reproductive performance, and chick viability of Northern bobwhite hens fed diets containing low (0.31%), moderate (0.39%), or high (0.47%) concentrations of methionine. Chick viability was determined by assessing immunocompetence, including evaluating the ability of hens to passively transfer immunity to their chicks. Hens were fed the experimental diets for 6 weeks on an *ad libitum* basis. After six weeks, methionine treatment had no measurable effect ($P \geq 0.20$) on hen phytohemagglutinin wing-web indices, organ weights, or serum anti-*Pasteurella multocida* titer indices. Mean egg weight, percent egg production, total cumulative egg production, yolk weight, yolk volume, and percent fertile and percent hatch of fertile eggs did not differ ($P \geq 0.12$) among diet treatments. Amount of albumen in eggs produced by hens fed the high-methionine diet averaged 0.27 g more ($P = 0.003$) than eggs of hens fed the low methionine diet. Anti-*P. multocida* titer of yolks from eggs in week 6 were not different ($P = 0.36$) between birds fed the high and the low methionine diets. Mortality rate of chicks after challenge with 23 colony-forming units of *P. multocida* was not different ($P \geq 0.05$) among diets. Chicks hatched from eggs

laid by vaccinated hens during weeks 2 and 3, however, had lower ($P < 0.05$) mortality than chicks of unvaccinated hens.

INTRODUCTION

Northern bobwhite (*Colinus virginianus*) populations display wide fluctuations (by 50%) from one season to the next (Lehmann 1953). Although population fluctuations often appear to be related to rainfall (Kiel 1976), the cause of this pattern has not been determined (Wood et al. 1986; Cain et al. 1982). Recent investigations have failed to associate water, protein, or energy availability (potentially caused by drought) to population fluctuations (Koerth and Guthery 1990; Wood et al. 1986), possibly because nutrient requirements of quail in the wild have not been clearly established (NRC 1994; Murphy 1994).

Bird requirements for protein actually reflect an overall requirement for nitrogen and approximately 10 essential amino acids (NRC 1994). Consequently, a diet can be relatively high in protein content, but deficient in individual amino acid nutrients. For example, Boren (1992) observed that quail diets in central Oklahoma contained adequate levels of crude protein but were deficient (relative to tentative NRC requirements) in all essential amino acids except valine, histidine, and threonine in May, and all essential amino acids in February. Diets of Northern bobwhite were especially low in methionine (0.29% dry weight) during the breeding season (May; Boren 1992), satisfying only 64% of the recommended levels for breeding Japanese quail (*Coturnix coturnix*; NRC 1994). Similar levels of methionine deficiency have been shown to greatly

reduce fecundity in domestic poultry as a result of reductions in clutch size, egg weight, fertility, hatchability, embryo development, and chick immunocompetence (Klasing and Barnes 1988; Muramatsu et al. 1987; Gilbert and Pearson 1983). Given that such drastic declines in similar fitness measures have not been observed in wild populations of Northern bobwhite, we hypothesized that dietary methionine levels recommended by NRC (1994) for breeding quail are considerably above those required by wild bobwhite. We tested this hypothesis by comparing reproductive performance and chick viability of Northern bobwhite hens fed diets containing low (level compared to that observed in wild breeding bird diets), moderate, and high concentrations of methionine. Chick viability was determined by assessing immunocompetence, including evaluating the ability of hens to passively transfer immunity to their chicks.

MATERIALS AND METHODS

Maintenance of Animals

Adult Northern bobwhite from an outbred colony (El Reno Gamebird Farm, El Reno, OK, USA) were weighed and randomly assigned to replicate cages (Hurlbert 1984; Maguire and Williams 1987) in groups of 4 females and 1 male per cage (91 x 71 x 27 cm). Birds were housed under a 18L:6D photoperiod with food and water provided on an *ad libitum* basis.

Experimental Design

Three isonitrogenous experimental diets were formulated to provide methionine levels of 0.31 (Low), 0.39 (Medium), and 0.47% (High). DL-methionine replaced corn in the low methionine diet to yield a high methionine diet (Table 1). The low and high methionine diets were mixed 1:1 to produce the medium methionine diet. Daily food intake per cage was measured as the difference in weight between uneaten food and food provided the previous day. Daily intake of methionine was calculated by multiplying average daily feed intake by methionine content of the respective experimental diet.

All birds were fed the high methionine diet for a 1 week acclimation period before being switched to their respective experimental diets; they were fed the experimental diets for 2 additional weeks before initiating the experiment to provide a period for amino acid depletion (Harms and Ivey 1992). Relationships among methionine requirement, reproduction, and immunocompetence were evaluated over the subsequent 6 week experimental trial.

Females were weighed 1, 2 and 6 weeks after start of the experimental trial. Eggs were collected daily from each cage. One-half the eggs laid in each cage collected on days 2 to 6, of weeks 1, 2, 3, and 5 were incubated as a group in plastic trays in a Sportsmaster incubator® (G.Q.F. Manufacturing, Savannah, GA 31402)

until they hatched. Eggs were stored at 10 °C in a humidified chamber before placement into the incubator as a group on day six of weeks 1, 2, 3, and 5. Egg fertility and hatchability were determined for each experimental group. After hatching, chicks were leg-banded and placed in a single floor brooder as described by Wilson and Dugan (1987). Chicks were weighed at hatching and 10-days of age. Chicks were given water and a quail starter feed (A&M Mills, Stillwater, OK 74078) on an *ad libitum* basis.

Egg Composition Analysis

Eggs produced on the last 3 days of each week were weighed. Eggs not placed in the incubator were carefully opened with scissors and the contents separated into shell, yolk, and albumen. Wet weight of yolk and albumen were recorded. Yolk volume was determined by water displacement. Yolks were diluted 1:1 (v/v) with phosphate-buffered saline (PBS). This solution was homogenized with a vortexer and then centrifuged at 2500 rpm for 15 minutes. The yolk supernatant was stored at -20 °C until analyzed. Yolks that ruptured before dilution were not included.

Concentration of immunoglobulin Y (IgY) in yolk and lysozyme in albumen samples of birds fed the high and low methionine diets were determined for eggs that were not incubated from week 3 and 6. The IgY concentration of yolk supernatants from eggs collected the last 4 days of weeks 3 and 6 were processed through the gammaYolk™ separation kit (Pharmacia Biotech, Piscataway, NJ 08854) according to the

manufacturers instructions. Yolk supernatant (300 µl) was processed to obtain ≥ 90% pure IgY. Samples were resuspended in their original 300 µl volume, and IgY concentration of duplicate samples determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA 94547) and a Serachem® (human) level 1 standard (Fisher Scientific Co., Pittsburgh, PA 15219). Yolk supernatants also were assayed for concentration of anti-*Pasteurella multocida* antibody.

Yolk supernatants from eggs of Northern bobwhite fed either the high or low methionine diet and collected the last 3 days of week 6 were assayed for Anti-*P. multocida* specific antibodies. Anti-*P. multocida* specific antibodies in yolk supernatants were determined by the Avian Medicine Laboratory of the Poultry Diagnostic Center, University of Georgia, Athens, Georgia, using the Flockchek®:Anti-Pm(C) (Idexx, Portland, ME, 04101), a *Pasteurella multocida* antibody test kit for detection of antibody in chicken serum. Kits were used according to manufacturers recommendations with the modification that yolk supernatants were diluted 1:20 in PBS before analysis. Known negative samples from Northern bobwhite were included with each run. An anti-*P. multocida* titer index was calculated as optical density (OD) of yolk supernatant minus their respective manufacturer supplied negative controls.

Lysozyme concentration in albumen was determined using a modification of the technique described by Osserman and

Lawlor (1966). Briefly, a 0.5% *Micrococcus lysodeikticus* (Sigma Chemical Co., St. Louis, MO 63178-9916) solution in 1% agarose (pH 8.6) was poured into immunodiffusion plates. Previously diluted (1:50 in PBS) albumen samples (5 µl) were placed in wells cut into the plates, which were subsequently incubated in a humidified chamber at 37 °C for 4 hours. Diameters of cleared rings were measured to the nearest 0.1 mm. Lysozyme standards from hen egg white (Sigma Chemical Co., St. Louis, MO 63178-9916) were used to prepare a standard curve by plotting ring diameters against the log of standard concentrations; unknown albumen samples were compared to the resulting standard curve.

Cell-mediated Immunity

Cell-mediated immunity of adult females was assessed on day 2 of week seven by measuring in vivo wing-web responses to an intradermal injection of the T-cell mitogen phytohemagglutinin (PHA; Sigma Chemical Co., St. Louis, MO 63178-9916)) (Cheng and Lamont 1988). Twenty-four hours prior to this measurement, birds were injected in the right wing web with 0.5 mg of PHA (dissolved in 0.1 ml of PBS) and in the left wing web (control) with 0.1 ml of PBS. Wing-web thickness was measured using a pressure-sensitive dial gauge to the nearest 0.002 mm immediately before and 24 h after injection. Cell-mediated immune response (wing-web index) was calculated as the difference in swelling between the PHA and PBS (control) injected sites.

Pathogenic Challenge and Vaccination Protocol

An anaculture (bacterin-toxoid preparation) was prepared from Avichol® (Schering-Plough Animal Health Corporation, Omaha, NE 68103), a live vaccine containing the Clemson University (CU) strain of *Pasteurella multocida* Type 3 (Dabbert and Lochmiller 1995). This CU strain of *P. multocida* was grown in tryptic-soy broth in a shaker incubator at 37 °C for 24 hours at which time bacterial cell counts were determined. Bacteria were killed by adding formalin directly to the broth culture to a final concentration of 0.3%, followed by incubation for another 24 hours. Killed bacteria were adjusted to 2×10^9 colony-forming units (CFU)/ml in a solution of 1:1 (v/v) anaculture and alhydrogel (Superfos Biosector a/s, DK-2950 Vedbaek, Denmark). All 60 female northern bobwhite were injected intramuscularly with 0.5 ml of bacteria in the right pectoral muscle the first day of the 6-week experimental trial. Birds received an identical secondary vaccination 1 week after the primary vaccination. An additional cage of 4 females and 1 male were maintained on the high methionine diet throughout the experiment but were not vaccinated. These females and their eggs and progeny were used as negative vaccination controls throughout the experiment but not included in experimental diet analyses. Females were anesthetized with an intramuscular injection of 5 mg ketamine hydrochloride (Fort Dodge Laboratories, Fort Dodge, IA 50501) in the right pectoralis before blood collection or

euthanasia. Blood was collected into heparinized-microhematocrit tubes via brachial venipuncture immediately following measurement of wing webs, for packed-cell volume and prechallenge serum collection; sera was stored at -70 °C until analyzed. Prechallenge sera was assayed to determine anti-*P. multocida* antibody concentrations using the same kits and methodology as previously described. In this case, however, sera samples were diluted 1:100 in PBS before analysis. Known negative samples from Northern bobwhite were included with each run. An anti-*P. multocida* titer index was calculated as optical density of sera samples minus their respective manufacturer supplied negative controls.

All 60 vaccinated females and the four unvaccinated control females were injected intramuscularly in the thigh muscle with 12,000 CFU of Avichol® in 100 μl of PBS on day 2 of week seven. Survival was monitored for 1 week. Anesthetized hens surviving pathogenic challenge were bled by cardiac puncture into vacuum tubes for serum, and subsequently euthanised by cervical dislocation; sera was stored at -70 °C until analyzed. Liver, spleen, ovaries, oviduct, and adrenal gland of each female were removed, blotted dry, and weighed. Weights were standardized for body size as percentage of 6-week body weight. Serum triglycerides, uric acid, total proteins, and albumen were colorimetrically determined using a Cobas Mira® wet chemistry analyzer (Roche Diagnostic Systems Inc.,

Montclair, NJ 07042-5199) and recommended procedures of the manufacturer.

Chicks of vaccinated and unvaccinated hens were injected intramuscularly in the left thigh with 23 CFU of Avichol® in 100 μl PBS. Chicks were challenged at 10 days-of-age. Survival of chicks also was monitored for one week (Dabbert et al. 1995).

Statistical Analysis

Effect of dietary methionine concentration on differences in food intake, body weight, hematological parameters, organ weight, egg composition parameters, egg hatchability and fertility, and measures of humoral and cell-mediated immunity were tested using an ANOVA with a randomized block design. Week of experiment was included as a second factor in analyses of variables that were measured more than once. A Bartlett-Box F-test was used to test for homogeneity of sample variances for all dependent variables. PHA wing-web indices and optical density of postchallenge sera were log transformed; food intake per bird and percent egg fertility were rank transformed. Differences among treatment means were tested by using the least significant difference test (Norusis 1990). Statistical significance was indicated at $P < 0.05$, and all values are reported as mean ± standard error. Relationships among immune parameters of hens and postchallenge hen survival were evaluated using logistic regression. Differences between OD of prechallenge sera and known negative sera were tested using a separate variances

t-test. All statistical analyses were conducted using the Statistical Package for Social Sciences® (SPSS; Norusis 1990). Difference in mortality after *P. multocida* challenge between treatments was tested using a Z-test for binomial proportions (Ott 1988).

RESULTS

Body weight of hens averaged 214.7 ± 2.2 (SE) g (n = 60) at the start of the experiment and did not differ (P = 0.58) among diets. Methionine treatment had no influence on body weight (P = 0.79), which remained unchanged (P = 0.80) over the course of the study (Table 2). Food intake was similar (P = 0.46) across diets but increased (P < 0.001) during the last 3 weeks of the study (Fig. 1).

Methionine treatment had no measurable effect (P ≥ 0.20) on PHA wing-web indices, packed-cell volume, organ weights, or anti-*P. multocida* titer indices of prechallenge serum samples (Table 1). Mean anti-*P. multocida* titer index of prechallenge sera was ≥ 4-fold greater (P ≤ 0.002; Table 2) than mean anti-*P. multocida* titer index of known negative birds (7.6 ± 5.0). Mortality of vaccinated hens following Avichol® challenge did not differ (P ≥ 0.5) among dietary treatments; mortality of unvaccinated hens (100%; n = 4) was greater (P < 0.001) than mortality of vaccinated hens. Prechallenge mean anti-*P. multocida* titer index and PHA wing-web index were not related (P = 0.3) to postchallenge mortality of vaccinated hens (Table 2). Methionine treatment had no measurable effect (P≥ 0.20) on

postchallenge serum concentrations of triglycerides, uric acid, total protein, or albumen (Table 3).

Mean egg weight, percent egg production, total cumulative egg production, yolk weight, and yolk volume did not differ ($P \geq 0.3$) among diet treatments from weeks 1 to 6 (Table 4). Egg weight and yolk weight increased ($P \leq 0.003$) while percent egg production decreased ($P = 0.004$) over the six week feeding trial (Table 4). Yolk density was lower ($P = 0.005$) in birds fed the high ($X = 0.94 \pm 0.01$) methionine diet compared to those fed the low methionine diet ($X = 1.00 \pm 0.01$). Amount of albumen in eggs produced by hens fed the high-methionine diet averaged 0.27 g more ($P = 0.003$) than eggs of hens fed the low methionine diet (Table 4). Amount of albumen in eggs increased ($P < 0.001$) over the course of the 6-week trial (Table 4). Concentration of lysozyme in the albumen fraction and IgY in the yolk fraction of eggs did not differ ($P \geq 0.24$) across diets, but both decreased ($P \leq 0.02$) from 3 to 6 weeks (Table 4). Anti-*P. multocida* titer of yolk supernatants from eggs collected the last 3 days of week 6 were not different ($P = 0.36$) between birds fed the high ($X = 0.060 \pm 0.008$) and low ($X = 0.053 \pm 0.005$) methionine diets. Weight of chicks at hatch and at 10 days-of-age were not different ($P \geq 0.3$) among diets; however, weight of chicks at hatch and at 10 days-of-age increased ($P \leq 0.004$) slightly after week 2 (Fig. 2). Percent fertile ($X = 86.3 \pm 0.02$) and percent hatch of fertile ($x = 81.5 \pm 0.02$) eggs were not different ($P \geq 0.12$) among diets or

weeks. Chick mortality rate after challenge with 23 CFU of *P. multocida* was not different ($P \geq 0.05$) among diets. Chicks of eggs laid by vaccinated hens during weeks 2 and 3 had lower ($P < 0.05$) mortality than chicks of unvaccinated hens (Fig. 3).

DISCUSSION

Vaccination of Northern bobwhite hens with an anaculture made from Avichol® provided them protection from the live bacterium that normally causes 70 to 100% mortality in unvaccinated birds (Dabbert et al. 1995). This immunity was passively transferred to their progeny providing significant protection against subsequent challenge with the live bacterium. This passive transfer of immunity was not compromised by dietary methionine levels of 0.3%. Although hens fed the high methionine diet consumed an average 28 mg/day more methionine than those fed the low methionine diet, we observed no enhancement of Northern bobwhite reproductive fitness within our captive setting. Our data suggest that dietary methionine levels recommended by NRC (1994) for breeding quail are excessive for wild Northern bobwhite. It appears a dietary methionine concentration of 0.3% may be sufficient for wild Northern bobwhite to produce viable chicks.

The National Research Council (1994) recommends feeding diets containing 0.90% methionine + cystine and 24% crude protein for breeding Northern bobwhite to support maximum production. Increasing crude protein content of diets fed

to breeding Northern bobwhite hens from 12 to 18%, with a constant total sulfur amino acid content of 0.869%, results in increases of egg weight, production, and fertility (Aboul-Ela et al. 1992). However, no improvements in these parameters are observed by increasing dietary crude protein from 18 to 24% (Aboul-Ela et al. 1992). Our study suggests a diet containing 18% crude protein and a methionine + cystine concentration of 0.64% is marginally adequate for breeding Northern bobwhite. Other studies suggest this total sulfur amino acid concentration is close to the requirement. Total sulfur amino acid requirement of breeding Japanese quail has been estimated to be between 0.62 and 0.68% for diets containing 19 to 20% crude protein (Shim and Chen 1989; Shim and Lee 1988; Allen and Young 1980).

Studies with breeding Japanese quail suggest that when the total sulfur amino acid concentration is 0.62%, dietary methionine content must be between 0.3 and 0.39% to support maximum egg weight (Shim and Chen 1989). Reduced weight of albumen in eggs laid by Northern bobwhite fed the low methionine diet in our study suggests that dietary methionine concentration for normal egg composition is slightly greater than 0.31% and less than 0.39%. The reason we observed reduced albumen weight without concomitant reductions in yolk weight may be related to their disparate rates of synthesis. Yolk proteins are synthesized in the liver and are continuously deposited in the ovum until

ovulation. In contrast, albumen is synthesized in the magnum of the oviduct over a 3 to 4 hour window (Penz & Jensen 1991). The ecological significance of reduced albumen availability for wild hatchling Northern bobwhite is unclear. Because whole-body protein synthesis is reduced in bird embryos as albumen content of the egg is reduced (Muramatsu et al. 1990), Northern bobwhite chicks hatched from low-albumen-weight eggs may have a reduced probability of survival.

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Table 1. Ingredient and chemical composition (% DM) of high and low methionine diets. A medium methionine diet was made by mixing low and high diets in a 1:1 ratio.

Ingredients and Analyses	Low methionine	High methionine
Corn	65.27	65.11
Soybean meal (48%, CP)	25.85	25.85
Limestone	5.53	5.53
Dicalcium phosphate	1.30	1.30
Fat, animal & vegetable	0.96	0.96
Vitamin mix	0.50	0.50
Salt	0.35	0.35
Trace mineral mix	0.10	0.10
Lysine	0.08	0.08
Threonine	0.06	0.06
DL-Methionine	0.00	0.16
Analyzed content[a]		
Crude protein	17.8	17.9
Methionine	0.31	0.48

[a]Dietary crude protein content of the high and low methionine diets was determined by the Oklahoma State University Soil, Water, and Forages Analytical Laboratory (Stillwater, OK 74078). Methionine content of high and low methionine diets was determined by a commercial laboratory (Novus International, St. Louis, MO 63178).

Table 2. Influence of dietary methionine concentration on body weight, organ weight, and hematology and immunity parameters of breeding Northern bobwhite hens.

Parameter	High methionine	Medium methionine	Low methionine
Sample size	20	20	20
Body weight, g			
Week 2	212.6 ± 3.2	217.2 ± 3.2	212.4 ± 3.3
Week 3	217.3 ± 3.1	216.6 ± 3.3	213.3 ± 3.3
Week 8	214.8 ± 3.3	215.3 ± 6.3	208.8 ± 4.8
Phytohemagglutinin wing-web index[a]	66.4 ± 10.1	52.8 ± 3.9	58.6 ± 0.3
Packed-cell volume,%	35.3 ± 1.0	35.8 ± 1.5	35.1 ± 0.6
Anti-*Pasteurella multocida* titer index[b]	0.029 ± 0.010	0.027 ± 0.014	0.063 ± 0.012
Organ Weight, mg/g body weight			
Ovary	19.5 ± 2.9	22.6 ± 2.3	18.1 ± 1.6
Oviduct	22.5 ± 2.7	27.5 ± 1.7	23.7 ± 1.2
Spleen (x 10)	3.4 ± 0.4	3.7 ± 0.2	4.5 ± 0.6

Table 2. continued.

Parameter	High methionine	Medium methionine	Low methionine
Liver	25.9 ± 0.8	34.3 ± 3.6	31.6 ± 1.8
Adrenal (x 100)	4.0 ± 0.7	5.2 ± 0.9	5.1 ± 0.4
Mortality after *Pasteurella multocida* challenge, %	15	16	20

[a]difference in swelling between PHA and PBS injected wing-webs

[b]anti-*P. multocida* titer index was calculated as optical density of sera samples minus their respective manufacturer supplied negative controls.

Table 3. Influence of dietary methionine concentration on clinical chemistry parameters of breeding Northern bobwhite hens.

Parameter	High methionine	Medium methionine	Low methionine
Serum triglycerides, mg/dl	711±137	656±167	882±153
Serum uric acid, mg/dl	9±0.6	7±1.1	7±0.9
Serum total protein, g/dl	5±0.1	6±0.1	5±0.2
Serum albumen, mg/dl	2±0.1	2±0.2	2±0.1

Table 4. Changes in Northern bobwhite egg production, composition, and chemical content over a six week feeding trial. Dietary methionine had no influence on these parameters except albumen weight; thus the data are pooled to present the means and standard errors.

	Week of feeding trial					
Parameter	1	2	3	4	5	6
Egg production, %	90±3[a]	90±2[a]	91±3[a]	82±3[ab]	78±3[b]	80±4[ab]
Cumulative total egg production	24±1[a]	47±1[b]	73±2[c]	96±2[d]	119±3[e]	43±4[f]
Egg Weight, g	8.6±0.1[a]	8.9±0.1[b]	9.2±0.1[bcd]	9.1±0.1[b]	9.3±0.1[d]	9.1±0.1[cd]
Yolk weight, g	3.3±0.1[a]	3.4±0.1[a]	3.5±0.1[b]	3.5±0.0[bc]	3.6±0.1[cd]	3.7±0.0[d]
Albumen weight, g	3.8±0.1[a]	3.8±0.1[a]	3.9±0.1[ab]	4.1±0.1[bc]	4.1±0.1[ab]	4.3±0.1[c]
Lysozyme concentration, mg/ml			5.3±0.7[a]			7.8±0.4[b]
IgY concentration, mg/ml			18.8±0.6[a]			6.0±0.6[b]

[a-f]Means within rows with no common superscript differ significantly

Fig. 1. Daily food consumption ($X \pm SE$) of breeding Northern bobwhite hens were fed isonitrogenous (ca. an 18% crude protein) diets that varied in methionine concentration. Diets were fed ad libitum for 6 weeks; no difference in feed intake was observed among methionine groups so all data were pooled across week of trial. Means with no common superscript differ significantly.

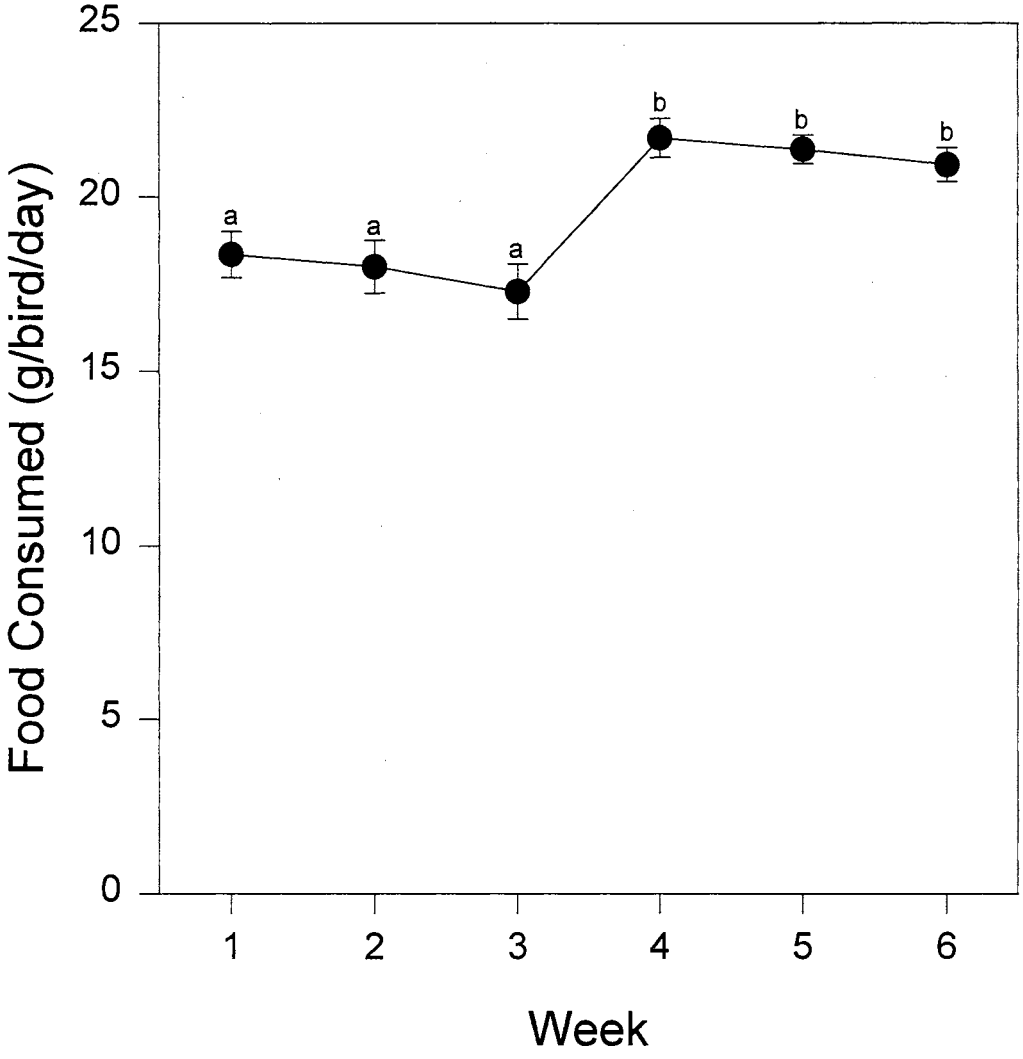

25
20
15
10
5
0
Food Consumed (g/bird/day)
a
a
a
b
b
b
1
2
3
4
5
6
Week

Fig. 2. Weight (X ± SE) of Northern bobwhite chicks at hatch and at 10 days-of-age of hens fed diets containing 3 different levels of dietary methionine. Weights were not different among diets so all data were pooled across week of lay. Means of the same age with no common superscript differ significantly.

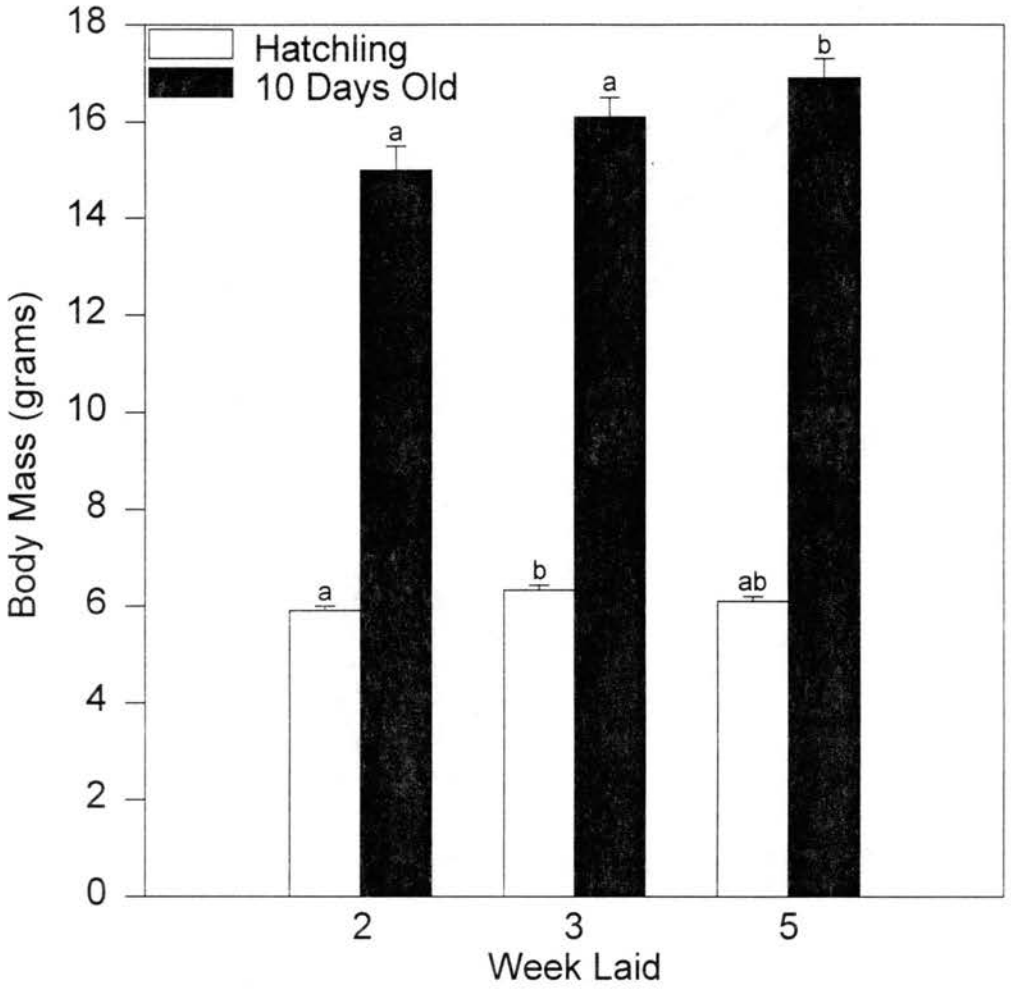
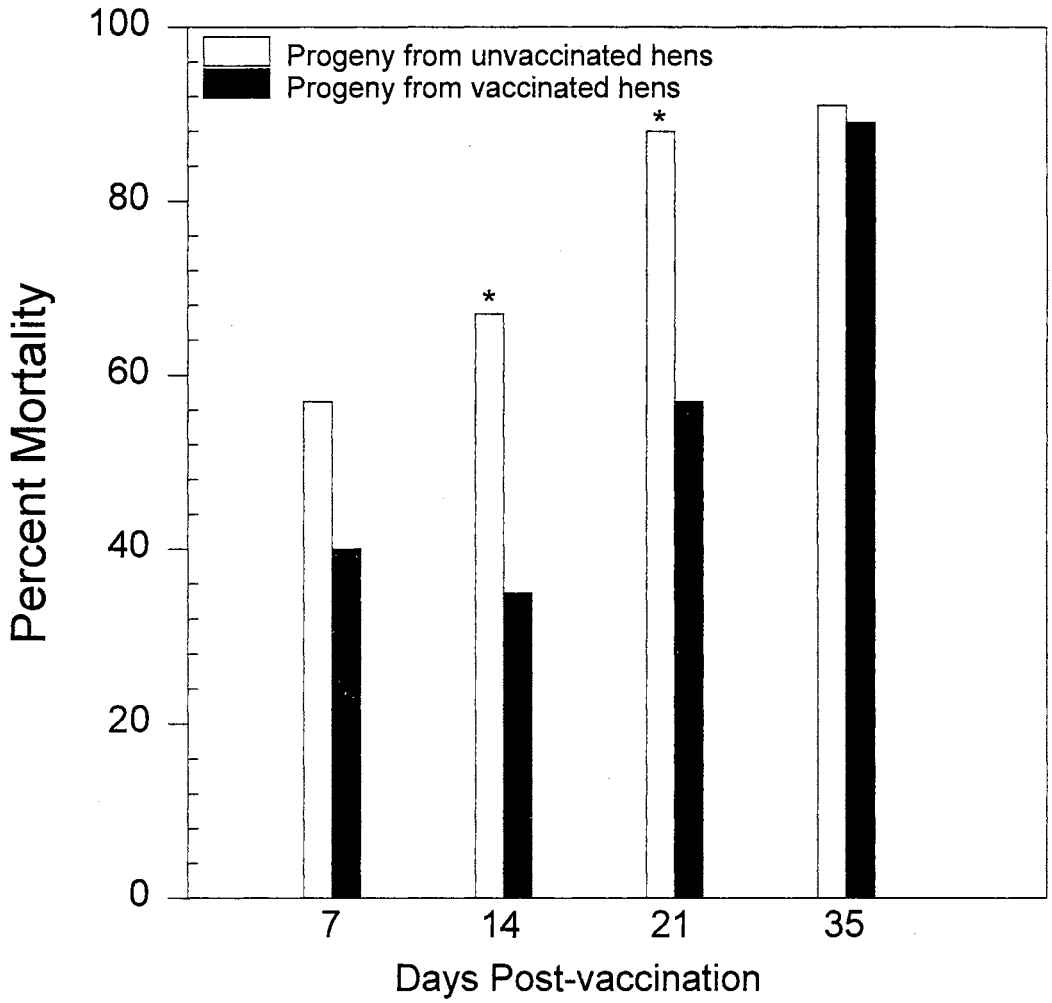

Hatchling
10 Days Old
Body Mass (grams)
0
2
4
6
8
10
12
14
16
18
a
a
b
a
b
ab
2
3
5
Week Laid

Fig.3. Percent mortality of 10 day-old progeny of vaccinated and unvaccinated Northern bobwhite hens, after challenge with 23 colony-forming units of *Pasteurella multocida*. * indicates significantly different mortality rate from progeny of vaccinated hens.


100
80
60
40
20
0
Percent Mortality
Progeny from unvaccinated hens
Progeny from vaccinated hens
*
*
7
14
21
35
Days Post-vaccination

VITA

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