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EFFECTS OF ACUTE THERMAL STRESS ON THE IMMUNE SYSTEM OF THE NORTHERN BOBWHITE (*COLINUS VIRGINIANUS*)

C. B. DABBERT,[1,3] R. L. LOCHMILLER,[1] AND R. G. TEETER[2]

[1]*Department of Zoology, Oklahoma State University, Stillwater, Oklahoma 74078, USA; and*
[2]*Department of Animal Science, Oklahoma State University, Stillwater, Oklahoma 74078, USA*

ABSTRACT.—We tested the hypothesis that exposure of Northern Bobwhites (*Colinus virginianus*) to acute temperature stressors impairs their immune-system function and resistance to disease. Birds were randomly assigned to either a thermoneutral (constant 21°C), cold-stress (cycled from 3.6 to −20°C over 24 h), or heat-stress (cycled from 30.8 to 39.0°C over 24 h) treatment for four consecutive days. Immunocompetence of each bird was evaluated on day 5 using a panel of assays, including a measure of resistance to a *Pasteurella multocida* Type 3 challenge. Neither thermal stressor had an influence on spleen mass or measures of cell-mediated and humoral immunity. Disease resistance of Northern Bobwhites to *P. multocida* was not influenced by heat stress but increased following cold stress. Cold stress may have increased the 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 was compromised during cold stress. *Received 20 February 1996, accepted 26 September 1996.*

THE PHYSIOLOGICAL TOLERANCES of oganisms are strong determinants of the environmental conditions that they may inhabit (Root and Schneider 1995). Physiological stress that results from adverse climatic conditions may have a profound influence on the distribution and population size of a species if the stress reduces fitness or survival (Root 1988, Wiens 1989). Adverse climatic conditions may occur as random events throughout a species' range, or as annual seasonal changes, which frequently are greatest in magnitude at the margin of a species' range (Hoffman and Parsons 1991).

Northern Bobwhite (*Colinus virginianus*) populations are very unstable at the northern margin of their range (Rosene 1969), where annual overwinter declines in population size are well documented (Roseberry and Klimstra 1984). In contrast, bobwhite populations appear to be more stable in the southern latitudes (Rosene 1969). However, bobwhite populations occasionally fluctuate widely across seasons and years, even in warmer climates (Lehmann 1984). Although interrelationships between rainfall and recruitment have been examined (Wood et al. 1986, Guthery and Koerth 1992), the influence of temperature extremes on bobwhite population fluctuations is unresolved.

Reports of acute die-offs during especially cold weather (Roseberry and Klimstra 1984), coupled with studies of poultry that demonstrate a link between cold stress and immunity (Regnier and Kelly 1981), suggest that altered disease resistance is a factor in explaining many of the observed demographic patterns in bobwhites. Northern Bobwhites frequently are exposed to extreme cold in much of their northern range (Stanford 1971, Roseberry and Klimstra 1984), and wind-chill equivalencies below freezing occur as far south as Texas (Lehmann 1984). Likewise, extreme increases in temperature can occur during the nesting season throughout the bobwhite's range (Lehmann 1984). Exposure of poultry to comparable temperature stressors (El-Halawani et al. 1973) reduces humoral (Henken et al. 1983) and cell-mediated (Regnier and Kelly 1981) immunity, leading to altered host-resistance to infectious pathogens (Reece et al. 1992). Previous investigators have examined the relationship between climatic conditions and physiological tolerance in birds by assessing metabolic responses (Hayworth and Weathers 1984, Root 1988). However, data examining the effect of

[3] Present address: Department of Range, Wildlife and Fisheries Management, Texas Tech University, Lubbock, Texas 79409, USA.

E-mail: c7dcb@ttacs.ttu.edu

temperature stress on immunocompetence in birds are unavailable. We tested the hypothesis that exposure to acute temperature stressors impairs immune-system function and resistance to disease in Northern Bobwhites.

METHODS

Maintenance of study animals.—Adult male Northern Bobwhites, which were produced from random matings, were obtained from the El Reno Gamebird Farm (El Reno, Oklahoma). Birds were housed in groups of four per cage (91 × 71 × 27 cm) under a 12L : 12D photoperiod. Birds were acclimated to 21°C for one week prior to initiation of each experiment. Commercial gamebird feed 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 and heat stress) were used to compare responses of bobwhites to acute thermal stress. For each experiment, birds were weighed and randomly assigned to replicate thermal-stress or thermoneutral cages with four birds per cage (Hurlbert 1984, Maguire and Williams 1987). The cold-stress experiment was repeated twice during consecutive weeks using five replicate cold-stressor cages and five replicate thermoneutral cages per repetition ($n = 80$). The heat-stress experiment was repeated three times during consecutive weeks using five replicate heat-stressor cages and five 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 ± SE of 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 stable for 4 h before decreasing (for 4 h) back to 30.8 ± 0.5°C for the remaining 12 h of each 24-h period.

After four consecutive days of temperature treatment, birds were weighed and their immunocompetence within three replicate cages per treatment was assessed using *in vivo* and *in vitro* assays. Birds within the remaining two replicate cages per treatment repetition were used to assess 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 in the right pectoralis after 96 h of temperature treatment. Blood was collected into heparinized capillary tubes via ulnar venipuncture for a thin-film blood smear. Serum was obtained by collecting blood via jugular venipuncture; sera were stored at −70°C until analyzed.

Blood smears were stained using Diff Quik (Baxter Healthcare, Miami, Florida) and observed using 1,000 × magnification (oil-immersion). 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-immersion fields (Janes et al. 1994). Thrombocytes similarly were quantified in 10 oil-immersion fields (Fairbrother and O'Loughlin 1990).

Humoral immunity.—Humoral immunity is an organism's antibody response to pathogens. Antibody response to a T-dependent (both T-and B-cells must recognize antigen) 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]) in the right pectoralis. Concurrently, antibody response to a T-independent (antigen activates B-cells without T-cell help) antigen was assessed by measuring serum antibody titers following a primary intramuscular injection in the left pectoralis of 0.1 ml of a 1:10 dilution of *Brucella abortus* antigen (Difco Laboratories, Detroit, Michigan) in PBS containing 5% alhydrogel as an adjuvant. Antigens were administered two days prior to the start of the four-day experimental trial. Antibody titers to SRBC were measured using a microhemagglutination assay (Wegmann and Smithies 1966); titers to *Brucella abortus* antigen were measured using a bacterial agglutination assay (McCorkle and Glick 1980).

Cell-mediated immunity.—Cell-mediated immunity is characterized as the localized reaction of lymphocytes and phagocytes (cells) to pathogens. Cell-mediated immunity was assessed by measuring *in vivo* responses to an intradermal injection of phytohemagglutinin (PHA) into the "wing web," or patagium (Cheng and Lamont 1988). PHA is a plant lectin that causes dermal infiltration by lymphocytes and phagocytes. The subsequent inflammation can be measured as an index of cell-mediated immunity. 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 (± 0.002 mm) was measured immediately before and 24 h after injection using a pressure-sensitive dial gauge. Cell-mediated immune response (i.e. wing-web index) was calculated as the difference in wing-web thickness at sites injected with PHA versus sites injected with PBS (control).

Anesthetized birds were euthanized by cervical dislocation. The spleen of each bird was removed aseptically, weighed, homogenized, and total cellularity manually determined using a hemacytometer. For cold-stress experiments, proliferative responses

of splenocytes cultured with 5 μg/well of the plant lectin Concanavalin A (Con A; a T-cell stimulator) and 2.5 μg/well of *Salmonella typhimurium* mitogen (a B-cell stimulator) were measured in 96-well flat-bottom plates. One million cells per well were cultured in 200 μL of AIM-V serum-free media for 72 h at 40.5°C with 5% CO_2/95% air (Dabbert and Lochmiller 1995). Proliferative responses were assessed using ^{3}H-thymidine (1 μcurie) incorporation into cells during the last 18 h of culture. Stimulation indices for both mitogens were calculated as ratios of counts per minute 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 in the thigh muscle with 3,000 colony-forming units (CFU) of Avichol® (Schering-Plough Animal Health Corporation, Omaha, Nebraska), a vaccine strain of *Pasteurella multocida* Type 3, in 100 μL of PBS (Dabbert et al. 1995). Survival was monitored for one week.

Triiodothyronine and thyroxine determination.—Serum triiodothyronine (T_3) and thyroxine (T_4) concentrations of cold-stressed birds were determined colorimetrically using CEDIA™ homogeneous enzyme immunoassay kits and recommended procedures of the manufacturer. The assay was adapted for use on a Cobas Mira® wet-chemistry analyzer.

Statistical analyses.—For each trial, differences between thermal-stress and thermoneutral treatment groups in food intake, body mass change, 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 variances for all dependent variables (Norusis 1990). All variables had homogeneous variances after transformation (either log, square-root, or sine transformations).

Differences 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 $\bar{x} \pm$ SE.

Results

Heat-stress.—Body mass averaged 182 ± 1 g ($n = 72$) at the start of the experiment and did not differ between treatment groups ($P = 0.74$). Heat stress had no significant effect on body mass, moisture content, spleen mass or cellularity, PHA wing-web index, microhemagglutination or bacterial agglutination titers, or hematological parameters ($P > 0.06$; Table 1). Mortality rates after challenge with 3,000 CFU of *P. multocida* did not differ ($Z = 0.89$; $P = 0.19$) 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.

TABLE 1. Physiological status of Northern Bobwhites subjected to either a 4-day cycling heat stressor or thermoneutral temperature ($n = 36$ birds per treatment). Values are $\bar{x} \pm$ SE.

Variable	Heat stress	Thermoneutral
Body mass (g)	182.2 ± 1.8	182.5 ± 1.4
Body moisture (%)	64.3 ± 0.3	64.8 ± 0.5
Spleen mass (mg)	82.2 ± 8.3	78.2 ± 5.1
Total splenocytes (no. cells × 10^6)	62.9 ± 9.5	64.4 ± 6.1
Wing-web index[a]	53.3 ± 3.3	45.7 ± 1.7
Anti-sheep red blood cell titer ($\log_2$)	7.5 ± 0.5	7.4 ± 0.5
Anti-*Brucella abortus* antigen titer ($\log_2$)	7.8 ± 0.6	7.7 ± 0.4
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[b]	114.7 ± 6.0	107.8 ± 11.1
Total thrombocyte count[c]	29.4 ± 2.2	27.2 ± 3.4

[a] Difference in thickness between webs injected with phosphate-buffered saline vs. phytohemagglutinin.
[b] No. per 50 oil-immersion fields.
[c] No. per 10 oil-immersion fields.

Cold stress.—Body mass 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 mass; cold-stressed birds lost an average of 9.1 ± 0.9 g, whereas thermoneutral birds gained an average of 2.4 ± 1.6 g over the four-day trial. Body-moisture content (66.10 ± 0.29%) of cold-stressed birds was higher ($P = 0.002$) than that of thermoneutral birds (64.67 ± 0.22%). Food intake of cold-stressed birds exceeded ($P = 0.037$) that of thermoneutral birds only on day 3 of exposure; intake was not different ($P > 0.113$) on days 1, 2, and 4 (Fig. 1).

Serum T_4 concentrations were 27% lower ($P = 0.009$) in cold-stressed birds than in birds maintained at thermoneutrality, but T_3 concentrations were similar between treatments (Fig. 2). Cold stress had no measurable effect on spleen masses, PHA wing-web indices, splenocyte proliferation indices, microhemagglutination titers, or bacterial agglutination titers (Table 2).

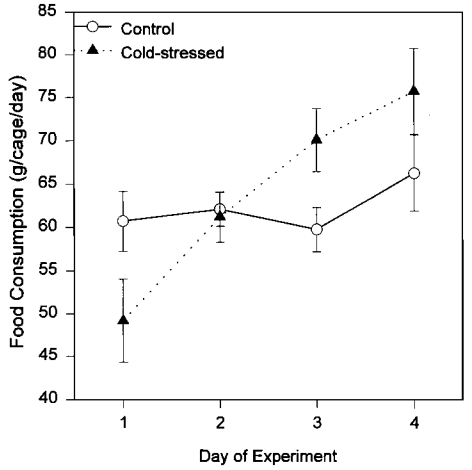

Fig. 1. Daily *ad libitum* food consumption ($\bar{x}$, SE) by four adult Northern Bobwhites. Thermoneutral birds ($n = 10$) were maintained at constant 21°C; temperature of cold-stressed birds ($n = 10$) was cycled from 3.6°C (18 h) to an average of −20°C (6 h) during a 24-h period.

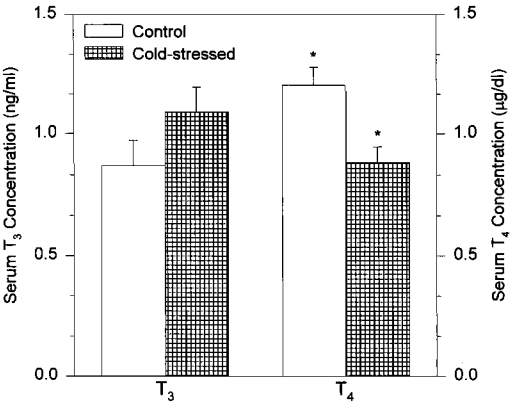

Fig. 2. Serum T_3 and T_4 concentrations ($\bar{x}$, SE) of adult Northern Bobwhites after four days of temperature treatment. Thermoneutral birds ($n = 40$) were maintained at constant 21°C; temperature of cold-stressed birds ($n = 40$) was cycled from 3.6°C (18 h) to an average of −20°C (6 h) during a 24-h period. Asterisks denote significant differences ($P < 0.055$) between temperature treatments.

Total splenocyte counts and relative total peripheral blood leukocyte counts of birds subjected to cold stress were >30% lower ($P < 0.017$) than those in the thermoneutral 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 decrease ($P = 0.037$) the relative percentage of heterophils in peripheral blood smears (Fig. 4). Percentages of other peripheral blood leukocytes and thrombocytes were not affected ($P > 0.482$) by temperature treatment (Fig. 4). Relative total lymphocyte and heterophil counts decreased ($P < 0.022$) after cold-stress treatment, whereas total counts of other leukocytes were not affected ($P > 0.246$; 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 lower (43.8%) for cold-stressed birds compared with those in the thermoneutral group (87.5%; $Z = 2.935$; $P < 0.0017$). 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 Bobwhites to a cycling heat stressor that was only 4.8°C below the upper mean lethal temperature for this species (Case and 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 Bobwhites to *P. multocida* infection (Bermudez et al. 1991). Bobwhites appeared to acclimate to heat stress and panted to dissipate heat during our experiments. Performance of bobwhites contrasts with many poultry strains that expe-

TABLE 2. Physiological status of Northern Bobwhites subjected to either a 4-day cycling cold stressor or thermoneutral temperature ($n = 24$ birds per treatment). Values are $\bar{x} \pm$ SE.

Variable	Cold stress	Thermoneutral
Spleen mass (mg)	64.1 ± 2.2	67.8 ± 5.5
Wing-web index[a]	30.1 ± 2.3	34.4 ± 2.5
Concanavalin A stimulation index[b]	7.9 ± 1.7	7.3 ± 2.6
Salmonella typhimurium mitogen index[b]	2.9 ± 0.6	4.5 ± 0.8
Anti-sheep red blood cell titer ($\log_2$)	5.2 ± 0.4	5.3 ± 0.3
Anti-*Brucella abortus* antigen titer ($\log_2$)	7.9 ± 0.2	7.7 ± 0.4
Total thrombocyte count[c]	28.9 ± 6.1	26.1 ± 3.2

[a] Difference in thickness between webs injected with phosphate-buffered saline vs. phytohemagglutinin.
[b] Ratio of stimulated to unstimulated cultures (counts per minute).
[c] No. per 10 oil-immersion fields.

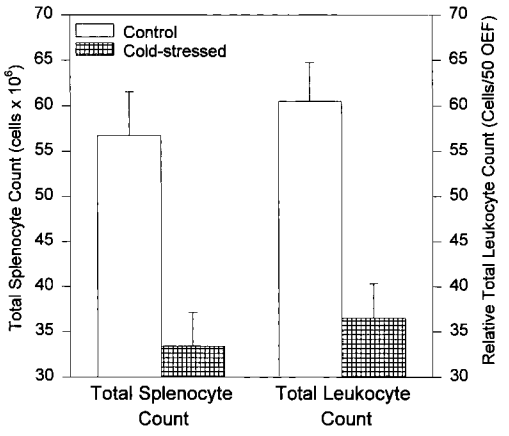

Fig. 3. Total splenocyte count and total peripheral blood leukocyte count (both $\bar{x}$, SE) of adult Northern Bobwhites after four days of temperature treatment. Thermoneutral birds ($n = 40$) were maintained at constant 21°C; temperature of cold-stressed birds ($n = 40$) was cycled from 3.6°C (18 h) to an average of −20°C (6 h) during a 24-h period. All differences between treatments were significant ($P < 0.055$).

rience immune dysfunction after exposure to 36°C (Regnier and Kelley 1981).

In contrast, exposure of bobwhites 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 three-fold over basal metabolic rate (Robbins 1983). Body mass loss concomitant with increased body-moisture content (inversely proportional to fat content) in cold-stressed birds suggested that fat reserves were partly used to fuel these increased metabolic demands. It is noteworthy that these mass changes occurred despite *ad libitum* availability of high-quality food. In the wild, climatic factors that reduce food availability (e.g. snow cover) could exacerbate the rate of fat loss during periods of extreme cold. Loss of >20% body mass usually is lethal to Northern Bobwhites (Robel et al. 1979). Considering that birds in our study lost an average of 4% of their body mass in only four days, long periods of extreme cold could reduce the survival of bobwhites in the wild.

Physiological adjustments of birds to cold temperatures are complex and typically involve adjustments in thyroid metabolism (Dawson et al. 1992), such as elevation of serum T_3 concentrations and declines in serum T_4 concentrations

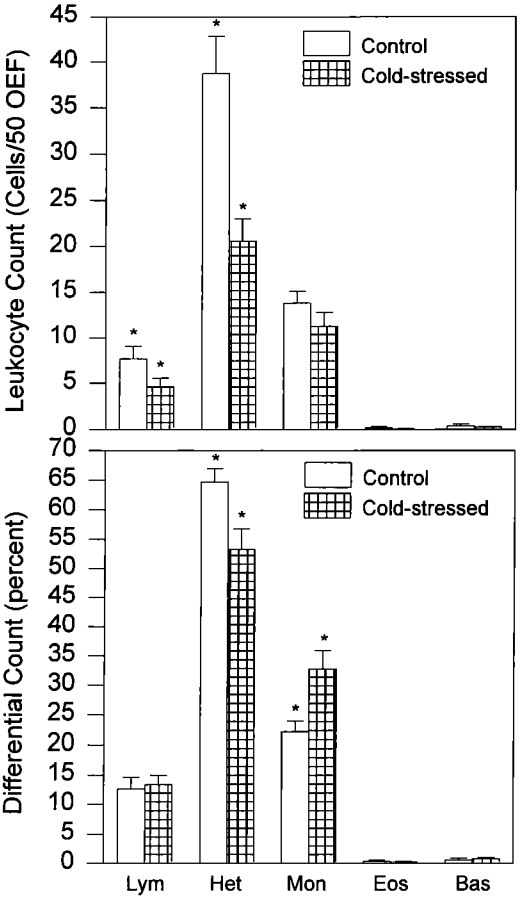

Fig. 4. Numbers (upper) and relative percentages (lower) of leukocytes ($\bar{x}$, SE) of adult Northern Bobwhites after four days of temperature treatment. Lym = lymphocytes; Het = heterophils; Mon = monocytes; Eos = eosinophils; Bas = basophils. Thermoneutral birds ($n = 40$) were maintained at constant 21°C; temperature of cold-stressed birds ($n = 40$) was cycled from 3.6°C (18 h) to an average of −20°C (6 h) during a 24-h period. Asterisks denote significant differences ($P < 0.055$) between temperature treatments.

(Cogburn and Freeman 1987). Although T_4 levels declined in Northern Bobwhites in our study, T_3 levels remained unchanged. Thyroid hormones can influence immune-system activity during exposure to cold. 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 et al. 1994). Administration of both growth hormone and either T_3 or T_4 can decrease proliferative responses of lymphocytes in poultry (Marsh and Scanes 1994). Cold stress,

however, did not modify splenocyte proliferative responses of bobwhites in our study.

Stress-induced increased resistance of birds to challenge with bacterial pathogens is a commonly observed phenomenon. For example, cold stress, food restriction, corticosteroid injection, social stress, and handling stress can increase resistance of poultry to *Staphylococcus aureus*, *Escherichia coli*, and *P. multocida* infections (Gross and Siegel 1965, Juskiewicz 1967, Simensen and Olson 1981, Mutalib et al. 1983). The immune system of bobwhites responded to *P. multocida* challenge after cold stress in a similar fashion. Cold stress can increase the phagocytic activity of mammalian leukocytes (Houstek and Holub 1994). Similarly, stress-induced increases in resistance of bird species to bacterial pathogens have 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 bobwhites, especially given the increased relative 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, large fluctuations in leukocyte populations probably are not entirely beneficial. How bobwhites 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 (e.g. fowl pox or quail bronchitis) is compromised (Gross 1962); however, this has not been demonstrated experimentally.

Our study illustrates the complex nature of interactions between environmental stressors and immune-system functions and the difficulty in predicting environmental effects on immunocompetence of wild birds based on research using domesticated subjects. The validity of directly extrapolating physiological responses of poultry to Northern Bobwhites is unclear, given their disparate life histories. Even within poultry, response to thermal stressors differs dramatically among breeds (Regnier and Kelley 1981, Spinu and Degen 1993).

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