

IMMUNE AND GROWTH RESPONSE OF WESTERN BLUEBIRDS AND ASH-THROATED FLYCATCHERS TO SOIL CONTAMINANTS

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Abstract. During the past two decades, a critical need has developed to determine how exposure to contaminants in the environment affects individual and population processes. In this study, the immunocompetence of Western Bluebirds (*Sialia mexicana*) and Ash-throated Flycatchers (*Myiarchus cinerascens*) was studied on a landscape–soil contaminant gradient at Los Alamos National Laboratory (LANL) in New Mexico during 1997–1999. A variety of contaminants (heavy metals, chemicals, insecticides, polychlorinated biphenyls, and radioactive isotopes) range across different spatial scales and concentrations on LANL land. The two species have similar life-history traits, except that the Ash-throated Flycatcher has a faster rate of development and fledges 4–5 days earlier than the bluebird. The number of active nest boxes increased from 1997 to 1998 and 1999 for the Western Bluebird, but not for the Ash-throated Flycatcher. Survival of nestling flycatchers was lower in areas within 60 m of a potential contaminant release site, with a higher survivorship function associated with boxes farther away. The two species did not differ in their response to antigens, and there was no difference between locations for immunocompetence for either species. Flycatcher nestlings had a higher average cell-mediated response than bluebird nestlings, as predicted by the faster rate of development of the flycatchers. Phytohemagglutinin response varied between locations for both species. The cell-mediated effects were dynamic in that, in general, the same locations showed similar patterns for each year. Hematocrits steadily increased with age for both species and varied between locations for the bluebird, but not the flycatcher.

Key words: antigen; Ash-throated Flycatcher; contaminants; immunocompetence; *Myiarchus cinerascens*; nestling mortality; *Sialia mexicana*; Western Bluebird.

INTRODUCTION

During the past two decades, a critical need has developed to determine how exposure to contaminants in the environment affects both individuals and populations. As Nyholm (1995a) points out, evaluation of the impact of contaminants should be based on knowledge of contaminant-related health risks in organisms naturally occurring in the ecosystems affected. Often risks cannot be evaluated because contaminants in the environment have not been characterized. Amounts, mixes, and even the types of contaminants are often poorly known in areas proposed for environmental remediation. In addition, available data are often limited to laboratory studies involving single toxicants. Areas designated for remediation may pose uncertainties concerning the potential impacts to the fauna occurring naturally in these environments. Rather than focusing on specific contaminants, an alternative approach is to compare the health of sensitive organisms between areas having suspected contaminant impacts and control

areas lacking a history of activities that would lead one to suspect contamination.

Because the immune system plays a pivotal role in defending an animal against attack by pathogens and parasites (Wakelin 1996, Roitt et al. 1998), impairment of immune system responsiveness is likely to reduce fitness (Norris and Evans 1999). Norris and Evans (1999) define immunocompetence as the “ability of a host to prevent or control infection by pathogens and parasites.” Møller and Erritzøe (2000) showed that reduced immunocompetence might make birds more susceptible to predation. Immunocompetence in birds has also been recently shown to be predictive of post-fledgling survival (Saino et al. 1997, Soler et al. 1999, Christe et al. 2001). Kjetil et al. (2002) found that the intensity of parasitic nematodes increases with organochlorine levels in the Glaucous Gull (*Larus hyperboreus*).

Many contaminants depress one or several components of the immune system, although immunity may respond variably to environmental contaminants. Several studies have investigated the effects of contaminants on the avian immune response in the laboratory (Trust et al. 1990, Fairbrother et al. 1994, Grasman and Scanlon 1995, Smits and Williams 1999, Smits and Bortolotti 2001). Recently, there have been only a few

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studies that have investigated the effect on immunocompetence in wild birds of post-hatching exposure to contaminants in the environment (Smits et al. 2000, Grasman and Fox 2001). Most of the studies have found immunosuppressive effects of contaminants that include mine tailings, heavy metals (Sharma and Dugyala 1996), radionuclides (Camplani et al. 1999), and pesticides (Pruett 1994). However, Smits and Bortolotti (2001) found immunosuppression in male American Kestrels (*Falco sparverius*) and immunostimulation in females. Smits et al. (2003) also found immunostimulation of the cell-mediated response in relation to polychlorinated biphenyl (PCB) exposure in Kestrels. Bishop et al. (1998) also found an increased blastogenic response to mitogen in Tree Swallows (*Tachycineta bicolor*) exposed to apple orchard pesticides. Thus, it is important to investigate both immunosuppression and immunostimulation, and make predictions based on the types of contaminants of potential exposure.

Two basic types of techniques are available for assessing immunocompetence. First, monitoring techniques provide a measure of an individual's health and the status of its immune system at the time it was sampled. Second, challenge techniques expose a component of the immune system, usually the humoral or cell-mediated component, to a novel antigen and quantify the subsequent immune response (Norris and Evans 1999). This study investigated both the humoral and cell-mediated responses of immune function using both types of quantification methods. It is important to measure both major components of the immune response because contaminants may differentially affect the various aspects of the immune system.

This study was conducted at Los Alamos National Laboratory (LANL) in north-central New Mexico, USA. LANL was established in 1943 as a part of the United States Army's project to develop a nuclear weapon. Many activities and operations at the laboratory have involved or produced liquids, solids, and gases that contain radioactive or nonradioactive hazardous materials. Contaminants, including heavy metals, chemicals, PCBs, and radioactive isotopes, range in soil concentrations across LANL land. This diversity and locations of contaminants on LANL make traditional ecological risk assessments difficult, if not impossible.

Wildlife at LANL may be exposed to various contaminants that could affect individuals by reducing reproduction or survival. Contaminants may not only cause extinction of populations directly exposed to them, but they may also increase the risk of extinction of species across landscapes (Maurer and Holt 1996). For example, contaminants may produce population sinks that draw individuals from source populations in contaminant-free areas and thereby adversely affect the long-term viability of those source populations. Thus, it is important to have biologically relevant information

concerning ecological risks faced by migratory birds that breed on LANL land.

Two common migratory cavity-nesting bird species that are present on LANL are Western Bluebirds (*Sialia mexicana*; WEBL) and Ash-throated Flycatchers (*Myiarchus cinerascens*; ATFL). Both species nest in existing nest cavities (boxes), and are mostly ground insectivores during the breeding season. The two species have similar life-history traits, except that the ATFL has a faster rate of development and fledges four to five days earlier than the bluebird at a similar size (~30 g), and the ATFL has a significantly higher field metabolic rate. ATFL nestlings require ~22% more energy per day than WEBLs (Mock et al. 1991). Insectivores may be at greater risk than non-insectivores due to the greater accumulation of contaminants (Klemons et al. 2000).

WEBLs forage over short distances (50 to 100 m) from their nests during the breeding season and exhibit strong site tenacity (J. M. Fair, *unpublished data*). Because bluebirds forage within small areas, they tend to integrate food chain effects from a localized area in a similar manner as Tree Swallows, which seem to effectively monitor local pollution (St. Louis et al. 1990, Wayland et al. 1998). In a similar cavity-nesting species, the Pied Flycatcher (*Ficedula hypoleuca*), Nyholm (1995b) found that nestlings accumulated non-essential metals that reflected interannual variation in deposition in the environment. In contrast with the WEBL, the ATFL foraging area, both aerially and on the ground, is not known.

We focused on nestlings because they represent a vulnerable stage of the life cycle and offer the potential for interaction between growth, immune response, and responses to contaminants. Exposure of developing birds to multiple environmental stresses such as food limitation, pathogens, cold exposure, or contaminants may have several physiological consequences including decreased immunocompetence. In addition, mounting an immune response to foreign antigens may constitute a cost for developing birds and result in decreased growth, perhaps with long-term consequences for fitness (Gebhardt-Henrich and Richner 1998). Immune responses to nonpathogenic antigens, such as vaccines, have been shown to impair growth performance and developmental stability in domestic poultry (Klasing et al. 1987) and Japanese Quail (*Coturnix coturnix japonica*; Fair et al. 1999).

Since contaminants can cause either immunosuppression or immunostimulation, interpreting the results from a field study with a variety of contaminants can be difficult. Therefore, areas with contaminants with known immunosuppressive effects might have lowered immune response, and areas with pesticides and PCBs might have higher than normal immune response. The areas at LANL offer locations exclusively with both types of contamination for testing these predictions.

In this study, we evaluated the magnitude and sources of ecological risk to cavity-nesting birds in an area of concern in New Mexico from various partly characterized contaminants released into the environment. These risks were evaluated in terms of ecological and physiological costs measured with regard to several life-history traits for individuals and populations. The objectives of this study were to (1) complete an immunological comparison of two species with similar life-history traits; (2) establish the relationship between the responsiveness of the immune system to potential exposure to partially characterized soil contaminants, several hematological parameters, and growth; and (3) delineate areas of concern for environmental restoration at LANL for birds in respect to soil contaminants.

STUDY AREA AND METHODS

This study was conducted at Los Alamos National Laboratory (LANL), Los Alamos County, and U.S. Forest Service land in north-central New Mexico, USA. LANL is situated on the Pajarito Plateau and consists of a series of relatively narrow mesas separated by deep, steep-sided canyons that decline east-southeast from the Jemez Mountains down to the Rio Grande. In general, Los Alamos has a temperate montane climate with four distinct seasons. Annual precipitation is 47.6 cm.

During the winter of 1997, a total of 450 bluebird boxes were established at LANL in contaminated and reference areas. Nest boxes were placed ~2 m off the ground on trees and spaced ~50–75 m apart in open ponderosa pine forest of the canyons and piñon-juniper woodland on the plateau mesas. Using a soil contamination database maintained by LANL, potentially contaminated sites were identified based on proximity to potential release sites (PRSs). All nestbox locations were marked using a non-differentially corrected geographic information system (GPS; Garmin GPS III Personal Navigator, Olathe, Kansas, USA) with real-time FM differential correction. Locations were checked for accuracy, and contaminant data were accessed using ArcView (ESRI 1996) with locations of PRSs. Distances from PRSs for all nest boxes were normally distributed ($W = 0.90$, Shapiro-Wilk test).

Given that soil characterization at LANL is underway but incomplete, estimates of the nature and extent of any contaminants could not be reliably established. Therefore, as a surrogate measure, we used the distance from release sites to evaluate the impacts of PRSs on the birds. The main study design included a similar number of boxes located near potentially contaminated areas and farther away in reference areas (Fig. 1). This gave a bimodal distribution with a large number of boxes near a PRS (<300 m), a large number further away (>1300 m), and a very small number of boxes in between. Sampling across canyons provided a gradient in the number and size of PRSs. Thus, three distance categories could be clearly delineated. All nest

boxes located within 300 m from a PRS were categorized as potentially contaminated, and those greater than 1300 m were classified as reference areas. Locations within 60 m of a PRS were considered to be within a foraging distance for bluebirds (J. M. Fair, *unpublished data*). For using distance to PRS as a continuous variable, a nonlinear regression model was selected specifying an intercept (PROC NLIN, SAS Institute 1987). All categories were determined using the clumped distributions of the box distances to a PRS.

Survival and growth

Starting in May 1997, nest boxes were visited every week, and nests with eggs were considered active and subsequently visited every 2 d until the first eggs hatched (day = 0). After day 0, nests were visited again on day 3, 5, 10, and 15 for measurements. Nestlings were measured on day 5, 10, and 15. The length of the left and right ninth primary was measured using the flattened wing method (Svensson 1984) with a ruler to the nearest mm. The left and right tarsi were measured with digital calipers to the nearest 0.1 mm. All birds were weighed using a digital balance to the nearest 0.01 g.

Comparisons of WEBL and ATFL nestling survival rates were analyzed using procedure LIFETEST (SAS Institute 1987), which allows for right censoring of data points and is more robust than the Wilcoxon statistic in detecting differences between groups later in time (Allison 1995). However, for comparisons of survival between antigen treatments, where treatment survivorship curves crossed each other and invalidated the model (Hosmer and Lemeshow 1999), treatments were compared to each other. The assumption of Mayfield (1975) that each death occurred midway through the interval between nest visits was used. All analyses were completed on nestlings censored at a fixed time on the earliest fledging age, day 15.

The sex of WEBL nestlings that were 15 days or older was determined by plumage color identification. The sex of younger nestlings was determined by polymerase chain reaction (PCR) amplification of a CHD gene (Griffiths et al. 1996). This gene is present in two genomic copies where one is Z-linked and occurs in both sexes and the other is W-linked and occurs only in females (Sheldon et al. 1998). On days 10 and 15 of age, blood (~70 μ L) was collected from the brachial vein of the wing in both heparinized and non-heparinized microcapillary tubes, which were kept at room temperature. At the time of bleeding, one drop (6 μ L) whole blood was also added to 600 mL of cell lysis solution (Purgene, Gentra Systems, Minneapolis, Minnesota, USA) in a microcentrifuge tube that was then stored at room temperature until sex determination. Each nestling was handled for <5 min in accordance with the *Guidelines for the Use of Wild Birds in Research* (Gaunt and Oring 1997). Nestlings were individually taken from the box to save handling time. The animal care and use com-

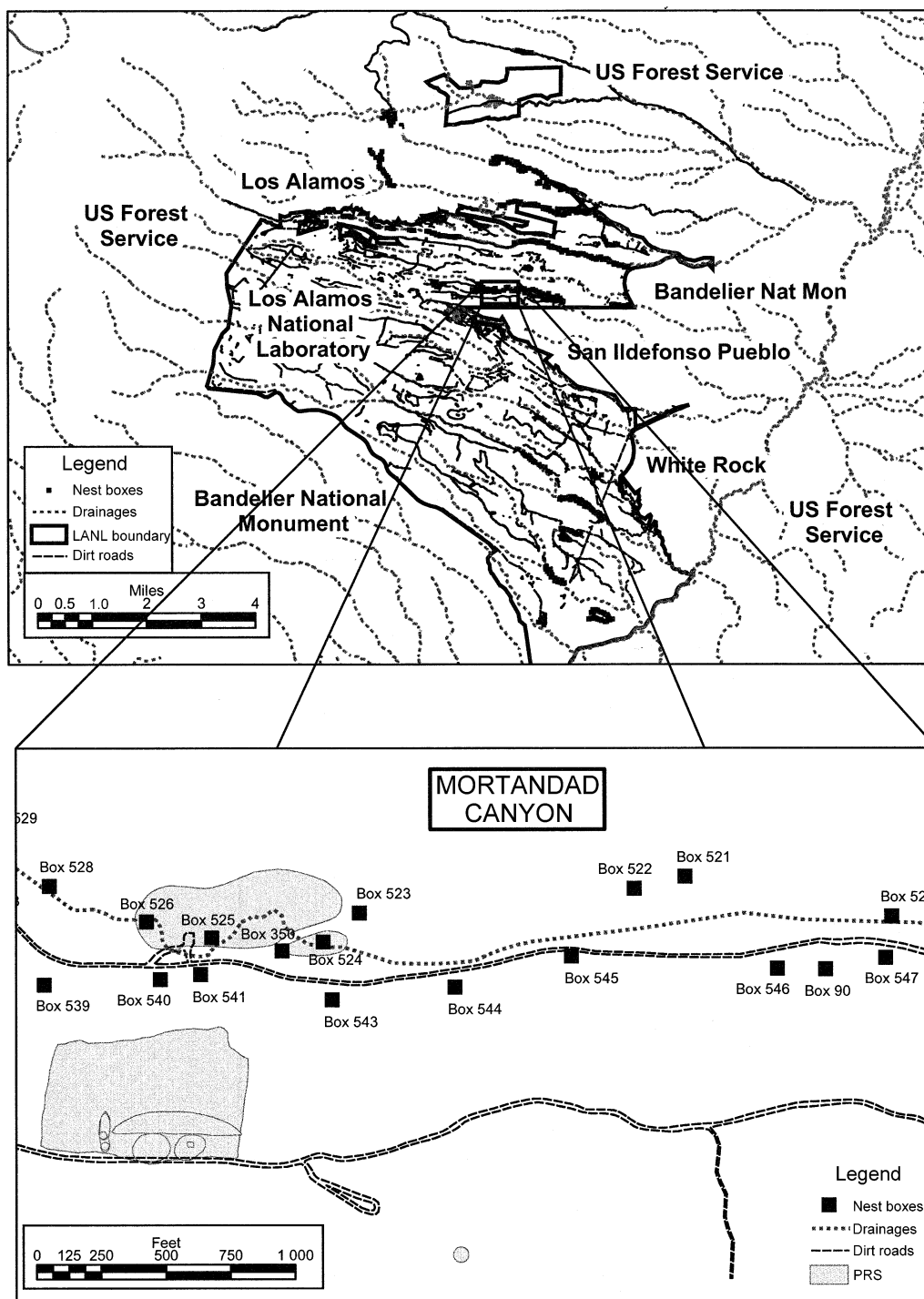


FIG. 1. Study area at Los Alamos National Laboratory (LANL) land and nest box monitoring locations for 1997–1999 and example of Mortandad Canyon with nest box placement surrounding potential release sites (PRSs).

mittees of both LANL and the University of Missouri-St. Louis approved all protocols.

Antibody response

Nestlings were injected with sheep red blood cells (SRBC; Sigma Company, St. Louis, Missouri, USA)

to measure immune response to a T-cell dependent antigen, and with killed Newcastle disease virus vaccine (NDV; Fort Dodge Animal Health, Overland Park, Kansas, USA), and *Mycoplasma sonoviae* vaccine (MYCO; Fort Dodge Animal Health) to measure response to T-cell independent antigens. On day 5, birds within a

brood were randomly assigned to be inoculated intraperitoneally (IP) with either (1) 0.075 mL of 10% SRBC solution in phosphate buffer, (2) 0.075 mL vaccine of NDV, (3) 0.075 mL MYCO, or (4) a 0.075 mL dummy oil immersion. To obtain plasma, blood collected on days 10 and 15 in heparinized tubes was spun for 10 min in a microcapillary centrifuge within 1 h of collection. Non-heparinized tubes were centrifuged 12 to 15 h after collection to obtain serum. Plasma and serum were maintained at 4°C until analysis. Antibody responses to NDV and MYCO were measured by a monoclonal antibody blocking enzyme linked immunoassay (ELISA [Svanova Biotech AB, Uppsala, Sweden]; Czifra et al. 1996). Optical density (OD) was determined with an EL-312 microtiter plate photometer (Bio-Tek Instruments, Winooski, Vermont, USA).

Serum samples were heat-inactivated at 56°C for 30 min, and agglutination of antibodies to SRBC antigens in sera was serially titrated. Antibody titers were determined with modified microtiter techniques (Wegmann and Smithies 1966). Titers are expressed as the \log_2 of the reciprocal of the highest dilution showing agglutination (Brugh 1977).

Cell-mediated response

Cell-mediated immunity was measured using the dermal phytohemagglutinin (PHA; (Sigma Chemical Company, St. Louis, Missouri, USA) reaction in the wing web. PHA injected for localized in vivo inflammatory response in birds has long been used to measure cell-mediated immunity (Stadecker et al. 1977, Lamont and Smyth 1984) and has been determined to not impose additional stress or survival cost (Merino et al. 1999, Smits et al. 1999). Birds were inoculated intradermally on day 15 in the wing web with either 0.05 mL (1.0 mg/mL) PHA in phosphate-buffered saline (PBS; right side) or 0.05 mL PBS only (left side). The amount of swelling in the wing web 24 h after inoculation was measured by a pocket dial gage micrometer (L. S. Starrett, Anthol, Massachusetts, USA) to the nearest 0.001 mm, and the same person made all before and after measurements. Repeatability of PHA measurements using the left wings of WEBLs and ATFLs in 2001 with wing webs not injected with PBS (post 24 h) was found to be high ($S = 1746$, $P = 0.68$, Wilcoxon signed-ranks test). PHA was the variable with the smallest sample size for the ATFL ($n = 10$), and this gave an estimated power of ~ 0.83 based on the recorded variance and means of comparison of species. A PHA index (Fair et al. 1999) was computed as the thickness of the PHA-inoculated wing web minus the thickness of the opposite wing web and standardized by the average wing thickness before inoculation, i.e.,

$$\text{PHA Index} = \frac{\text{postPHA} - \text{postPBS}}{\left(\frac{\text{prePHA} + \text{prePBS}}{2} \right)}$$

Hematology

Hematocrits of blood collected in heparinized capillary tubes were determined directly on a microhematocrit reader within 2 h of collection. Total plasma protein was estimated using a refractometer (Model RHC-200, Westover Scientific, Woodinville, Washington, USA). Although Lumeij and de Bruijne (1985) found the refractometric method unreliable for determining plasma protein concentrations in pigeons, refractometers are commonly used in clinical practice and have been shown to be reliable for mammalian plasma (Schalm et al. 1975) and American Kestrels (Dawson and Bortolotti 1997a).

Data analysis

The Statistical Analysis System (SAS Institute 1987) was used for all statistical analyses, and assumptions for parametric statistics were examined. Growth and physiological parameters were compared among antigen treatments using repeated-measures analysis of variance (ANOVA) models. Means for each treatment (antigens, locations) were compared with Duncan's Multiple Range Test. Data not normally distributed or having unequal variances were compared with Kruskal-Wallis nonparametric tests.

Growth trajectories were compared statistically among treatments using ANCOVA with age as the covariate. Growth curves were also fit to a variety of nonlinear models, and the best fitting nonlinear model selected according to Akaike's information criterion (AIC: Akaike 1977) (PROC NLIN). Growth parameters were then analyzed for each individual bird to estimate parameters. Other variables measured at each bleeding, such as hematocrit and white blood cell count, were analyzed for antigen and sex effects using repeated-measures ANOVA. Due to the fact that the hemagglutination responses are count data, antibody responses to SRBC were compared using the categorical procedure of PROC CATMOD in SAS (SAS Institute 1987). Regression analysis to test for associations between hematocrit and plasma protein for each age was used.

RESULTS

Survival

The number of active boxes increased from 1997 to 1998 and 1999 for the WEBL, but not for the ATFL (Table 1). Predators of nestlings included bull snakes (*Pituophis melanoleucus*), piñon mice (*Peromyscus truei*), Colorado chipmunk (*Eutamias quadrivittatus*), and raccoons (*Procyon lotor*) (J. M. Fair, *personal observation*). Predation for both species increased in 1999, and the mortalities (predation, died in nest, known fledged, and alive at fledgling) did not differ between the two species ($\chi^2_3 = 3.93$, $P = 0.27$; Fig. 2). The mortalities also did not differ between areas within a foraging distance to a PRS, contaminated areas, and reference areas ($\chi^2_6 = 7.13$, $P = 0.31$).

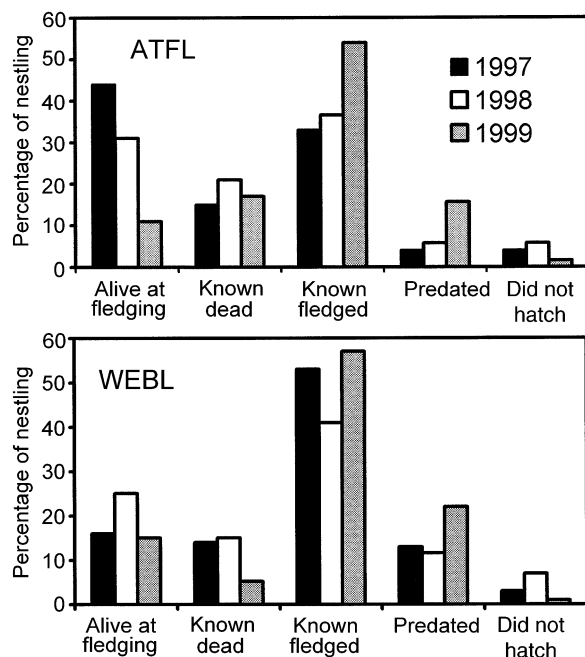


FIG. 2. Mortality factors for Ash-throated Flycatchers (ATFL) and Western Bluebirds (WEBL) at LANL during 1997–1999.

There was no difference in survival rates censored to age 15 d between the two species ($\chi^2_1 = 0.81$, $P = 0.36$, log-rank test). Survival was not related to hatch date, clutch size, or brood size for either species (PROC PHREG; SAS Institute 1987). However, survival was related to the distance from a PRS, with a lower survivorship function associated with boxes located within foraging distance to a PRS for the ATFL ($\chi^2_1 = 5.58$, $P = 0.018$). This was also evident in comparing survival between boxes located closer than 308 m to contaminated sites (mean = 0.71 ± 0.20 [mean ± 1 SE]) and in reference sites (mean = 0.87 ± 0.09) ($\chi^2_1 = 6.2$, $P = 0.01$; Fig. 3). Survival of WEBL nests did not differ between contaminated sites (mean = 0.86 ± 0.09) and reference sites (mean = 0.85 ± 0.11) ($\chi^2_1 = 1.1$, $P = 0.30$). Hatching success of eggs was not correlated with elevation, hatch date, distance to nearest PRS, the type of PRS, or between areas closer than 308 m or farther away for either species (Fair and Myers 2002).

Growth

The logistic model best fit the growth curves of both bird species and both had similar asymptotic body masses (Table 2). ATFLs weighed more than WEBLs on day 5 ($F_{1,484} = 3.96$, $P = 0.047$), but not on day 10 ($F_{1,473} = 0.87$, $P = 0.35$), and by day 15, bluebirds weighed more ($F_{1,389} = 11.19$, $P = 0.0009$). ATFL nestlings had longer wings throughout the development period ($F_{6,63} = 12.65$, $P = 0.0004$, repeated-measures ANOVA). This was also the case with tarsus length

($F_{6,60} = 88.39$, $P < 0.0001$). The three growth variables (body mass, wing and tarsus length) differed between years in both species, but exhibited no particular pattern. Body masses of WEBL nestlings did not differ between sexes at day 15 ($F_{2,333} = 2.43$, $P = 0.09$), but males were heavier by 15 d in the ATFL ($F_{2,66} = 4.83$, $P = 0.01$). For both species, body mass at 15 d increased with hatch date (for ATFL $F_{14,54} = 2.58$, $P = 0.0065$, $r^2 = 0.40$; for WEBL $F_{4,289} = 4.35$, $P < 0.0001$, $r^2 = 0.41$).

Body mass at 15 d did not differ between antigen treatments in WEBL ($F_{5,330} = 1.99$, $P = 0.08$) or ATFL ($F_{4,64} = 2.39$, $P = 0.06$); however, control nestlings without antigen injection weighed significantly less in both species (Duncan's Multiple Range Test). In the WEBL, body mass at 15 d varied with respect to location ($F_{14,321} = 2.26$, $P = 0.006$), but this was not true of the ATFL ($F_{4,64} = 2.25$, $P = 0.07$). WEBLs closer to PRSs weighed more at 15 d than farther away ($F_{1,334} = 8.14$, $P = 0.005$), but this effect was not present in the ATFL ($F_{1,67} = 0.64$, $P = 0.43$). Phenotypic variation in body mass was approximately the same for the WEBL and ATFL (coefficient of variation 10.6% and 9.4%, respectively).

As with body mass, male and female WEBLs had similar tarsus lengths ($F_{2,312} = 2.31$, $P = 0.10$), but male ATFLs had longer tarsi ($F_{2,62} = 4.0$, $P = 0.02$). Tarsus lengths were the same for all antigen treatments for the WEBL ($F_{5,309} = 0.71$, $P = 0.61$) and the ATFL ($F_{4,60} = 0.41$, $P = 0.80$). Tarsus length was longer farther away from PRSs in the WEBL ($F_{1,313} = 5.0$, $P = 0.03$), but not the ATFL ($F_{1,63} = 2.66$, $P = 0.11$). ATFLs had slightly higher phenotypic variation in tarsus length than bluebirds (12.0% vs. 8.3%, respectively). Tarsus length at 15 d did vary from location to location for WEBL ($F_{14,300} = 0.03$, $P = 0.01$), but did not vary for the ATFL ($F_{4,60} = 0.60$, $P = 0.66$). For the WEBL, Mortandad Canyon had significantly smaller tarsus lengths than all other locations ($n = 15$ locations). Tarsus length for the WEBL also increased as distance to PRS increased ($F_{3,78} = 3.22$, $P = 0.04$, PROC NLIN).

Wing length measured to the tip of the ninth primary was similar for both sexes in the WEBL and in the ATFL. Wing length and hatch date were unrelated in the 15-d-old WEBLs ($F_{1,337} = 0.17$, $P = 0.68$), but there was a positive association for the ATFL ($F_{1,67} =$

TABLE 1. Nest box summary of Western Bluebirds (WEBL) and Ash-throated Flycatchers (ATFL) that were analyzed for this study during 1997–1999.

Year	Western Bluebird		Ash-throated Flycatcher	
	Total nests	Total nestlings	Total nests	Total nestlings
1997	22	83	18	54
1998	81	216	16	48
1999	73	267	19	53

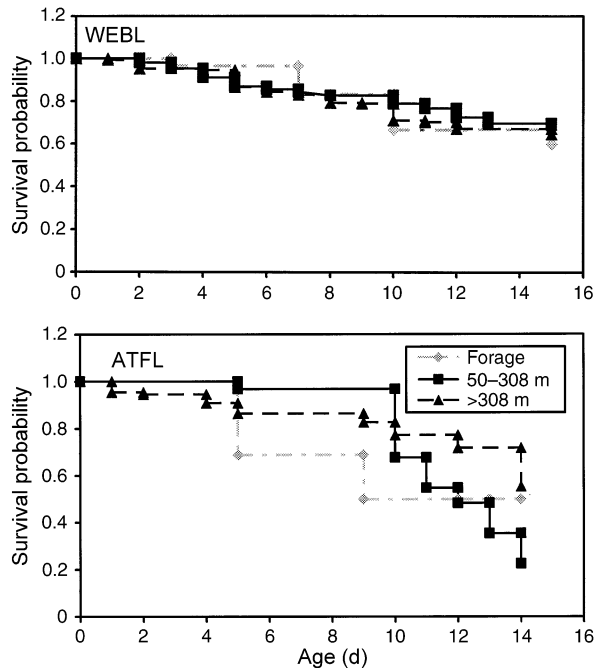


FIG. 3. Survival distribution functions for Western Bluebirds (WEBL) and Ash-throated Flycatchers (ATFL) for 1997–1999 in relation to distance from potential release sites (PRSs).

7.15, $P = 0.009$, $r^2 = 0.10$). Wing length did not differ among antigen treatments in either the WEBL ($F_{5,333} = 1.10$, $P = 0.36$) or the ATFL ($F_{4,64} = 0.09$, $P = 0.99$), although WEBLs that did not receive any antigen were smaller. ATFL wing lengths on day 15 did not differ among locations ($F_{4,64} = 2.01$, $P = 0.10$); however, wing length did vary between locations for the WEBL ($F_{14,334} = 4.68$, $P < 0.001$). Wing length ranged from 61.3 mm at the cemetery location ($n = 28$) to 48.5 mm at the DP Canyon site ($n = 10$). There were no differences in wing length between sites close to PRSs and those at a distance for the WEBL ($F_{1,337} = 0.59$, $P = 0.44$) or the ATFL ($F_{1,67} = 3.8$, $P = 0.06$). Wing length had less phenotypic variation in ATFLs than WEBLs (4.0% vs. 10.0%, respectively). There was a slight positive relationship with wing length and PHA response for 15-d-old WEBLs ($F_{1,184} = 3.46$, $P = 0.06$, $r^2 = 0.02$), suggesting that birds with longer wing

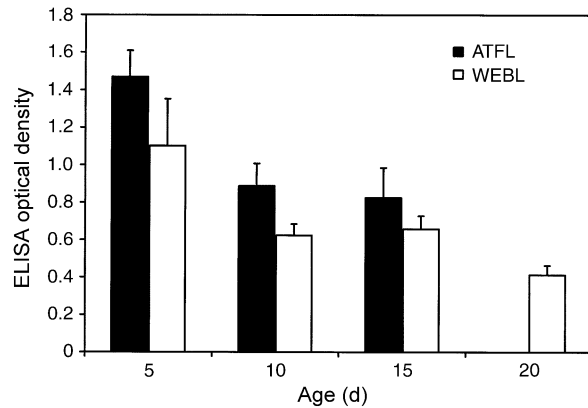


FIG. 4. Mean ELISA optical density (+1 SD) for Newcastle disease virus (NDV) antibodies for Ash-throated Flycatchers (ATFL) and Western Bluebirds (WEBL) for 1997–1999 over time (days).

lengths may possess a more developed cell-mediated response.

Antibody response

Humoral response determined by hemagglutination and antibody production demonstrated a high level of phenotypic variation at age 15 d for both species, amounting to ~50% of the mean for the SRBC response and 50% and 82% of the mean for the NDV and MYCO responses, respectively. Antibodies to NDV and MYCO were measured by ELISA OD for all three years for 5-, 10-, and 15-d-old nestlings. Nestlings of both species exhibited a higher response to the NDV than MYCO ($F_{1,97} = 3.93$, $P < 0.05$, ANOVA) at age 15 d. There were no differences 5 or 10 d post-injection of NDV (10- and 15-d-old nestlings) between species ($F_{1,96} = 0.01$, $P = 0.90$, and $F_{1,95} = 1.51$, $P = 0.22$, respectively; Fig. 4). There were also no differences in antibody responses of 10- and 15-d-old nestlings between locations ($F_{12,96} = 0.43$, $P = 0.95$, and $F_{13,95} = 0.52$, $P = 0.90$, respectively). However, NDV and MYCO ELISA ODs at 15 d varied between the three years (Table 3). NDV ELISA OD decreased steadily (antibody concentration increased) after antigen challenge, with the 20-d-old WEBLs (15 d post-injection) exhibiting the highest antibody response and 15 d being the next highest ($F_{3,262} = 6.09$, $P = 0.0005$). For

TABLE 2. Logistic growth curve parameters for Western Bluebirds (WEBL) and Ash-throated Flycatchers (ATFL) during 1997–1999.

Year	Western Bluebird				Ash-throated Flycatcher			
	<i>n</i>	<i>A</i>	<i>K</i>	<i>r</i> ²	<i>n</i>	<i>A</i>	<i>K</i>	<i>r</i> ²
1997	174	26.24	2.33	0.76	115	26.60	2.65	0.84
1998	374	25.57	2.42	0.84	90	24.32	1.94	0.85
1999	590	27.40	2.66	0.86	117	24.99	1.93	0.87

Note: Abbreviations are: *n*, total number of nestlings; *A*, asymptotic body mass (g); *K*, growth rate; and *r*², coefficient of determination.

TABLE 3. Repeated-measures ANOVA results for ELISA immunoassay optical density for Western Bluebird (WEBL) and Ash-throated Flycatcher (ATFL).

Source	Western Bluebird						Ash-throated Flycatcher					
	MYCO			NDV			MYCO			NDV		
	df	F	P	df	F	P	df	F	P	df	F	P
Year	2	493.5	<0.0001	2	160.34	<0.0001	2	50.44	<0.0001	2	55.3	<0.0001
Sex	2	0.00	0.999	2	0.57	0.568	2	1.79	0.197	2	6.69	0.005
Elevation	1	3.36	0.094	1	1.61	0.237	1	0.490	0.501	1	0.48	0.512
Hatch date	1	3.45	0.090	1	0.02	0.892	1	0.520	0.488	1	7.01	0.033
Distance to PRS	1	0.00	0.955	1	0.27	0.617	1	0.040	0.848	1	1.76	0.227
Contamination vs. uncontamination	1	0.66	0.419	1	0.14	0.686	1	5.07	0.037	1	0.08	0.776
Hematocrit	1	1.96	0.191	1	0.03	0.873	1	1.10	0.300	1	0.00	0.980
Plasma protein	1	0.99	0.344	1	1.56	0.247	1	1.34	0.253	1	1.06	0.309
Right wing	1	15.68	0.002	1	6.55	0.31	1	1.15	0.288	1	5.01	0.029
Right tarsus	1	1.10	0.321	1	3.12	0.115	1	1.68	0.201	1	0.03	0.900

Note: Abbreviations are: MYCO, *Mycoplasma sonoviae* vaccine; and NDV, Newcastle disease virus.

WEBLs at 15 d of age, MYCO ELISA OD decreased with hatch date (Table 3). There was also a positive relationship between ELISA OD and total plasma protein for both NDV- and MYCO-injected birds (Table 3).

Humoral antibody response to SRBC as estimated by hemagglutination was measured for all three years for 10- and 15-d-old nestlings. The two species did not differ in their humoral response to SRBC ($\chi^2_1 = 1.28$, $P = 0.30$, CATMOD). The response to SRBC also did not differ between years on day 15 for WEBL ($\chi^2_2 = 2.11$, $P = 0.10$) or ATFL ($\chi^2_2 = 0.06$, $P = 0.78$).

Cell-mediated response

Over all three years of the study, cell-mediated response to PHA was measured on 10 ATFLs and 197 WEBLs on day 15 and 16 of age. The ATFL usually fledged close to that age, and so it was more difficult to obtain PHA response measurements. The PHA index showed considerable phenotypic variation, ranging from 47% of the mean in the WEBLs to 69% in the ATFLs. ATFL nestlings had a higher average PHA index than WEBL nestlings ($F_{1,203} = 3.95$, $P = 0.048$), although this test had a power of 0.83 due to the small sample size. For the WEBL, PHA response was similar for all three years ($F_{2,184} = 0.49$, $P = 0.61$). For the ATFL, PHA response was also similar between years ($F_{1,6} = 0.87$, $P = 0.39$). There were two locations that contained different PHA responses for the WEBL; the cemetery had a higher PHA index (mean = 1.57, $n = 16$), and DP Canyon had a lower PHA index (0.81, $n = 10$). There was no relationship between boxes within foraging distance to a PRS, contaminated sites, and references sites for the WEBL ($F_{1,2} = 0.62$, $P = 0.54$), and there was no interaction between years. There was no relationship with PHA response and distance to a PRS. The ATFL did not have PHA measured in enough locations to be analyzed for locations.

Hematology

Hematocrit increased during nestling development in both species (Fig. 5) and differed only slightly between species at 10 d of age ($F_{1,420} = 4.84$, $P = 0.03$). There was little phenotypic variation between individuals compared to the immunoassays (mean = 14% per age period). Hematocrit did not differ between sexes for the WEBL ($F_{2,867} = 2.5$, $P = 0.08$), but the average hematocrit steadily increased from 35.4% on age 5 d to 45.6% on age 20 d ($F_{3,867} = 88.82$, $P < 0.0001$). The ATFL also exhibited an increase in hematocrit from 24.7% at 5 d of age to 44.7% at 15 d of age ($F_{2,273} = 53.37$, $P < 0.0001$). In the ATFL, however, males had lower hematocrits (average = 36.8%) than females (average = 40.2%; $F_{2,273} = 5.38$, $P = 0.0051$). There were no differences in the three years at 5, 10, or 15 d of age for either species. Hematocrit varied between locations in the WEBL ($F_{15,806} = 4.27$, $P < 0.0001$), but not in the ATFL ($F_{6,260} = 1.56$, $P = 0.17$). Hematocrits were not correlated with distance to PRS ($F_{2,80} = 0.01$, $P = 0.96$, PROC NLIN).

Total plasma proteins (grams per deciliter) increased during nestling development in the WEBL, and the two species differed at all ages (for 5 d $F_{1,211} = 67.85$, $P < 0.0001$; for 10 d $F_{1,363} = 123.9$, $P < 0.0001$; for 15 d $F_{1,312} = 53.23$, $P < 0.0001$; Fig. 5). Plasma protein varied between the years for both ATFL ($F_{2,227} = 6.95$, $P = 0.0015$, repeated-measures ANOVA) and WEBL ($F_{2,624} = 6.01$, $P = 0.0027$). Plasma protein did not differ between sexes for the WEBL ($F_{2,730} = 0.94$, $P = 0.39$), but the average plasma protein concentration slowly increased as age increased ($F_{3,730} = 79.45$, $P < 0.0001$). The ATFL showed an increase in protein and then a drop between the three measurement days ($F_{2,233} = 6.87$, $P = 0.0013$). As in hematocrit for the ATFLs, there was a pronounced difference in the sexes, with the males having a higher protein concentration (average = 4.6 g/dL) than the females (average = 4.0 g/dL; $F_{2,233} = 5.48$, $P = 0.0047$). There were no differ-

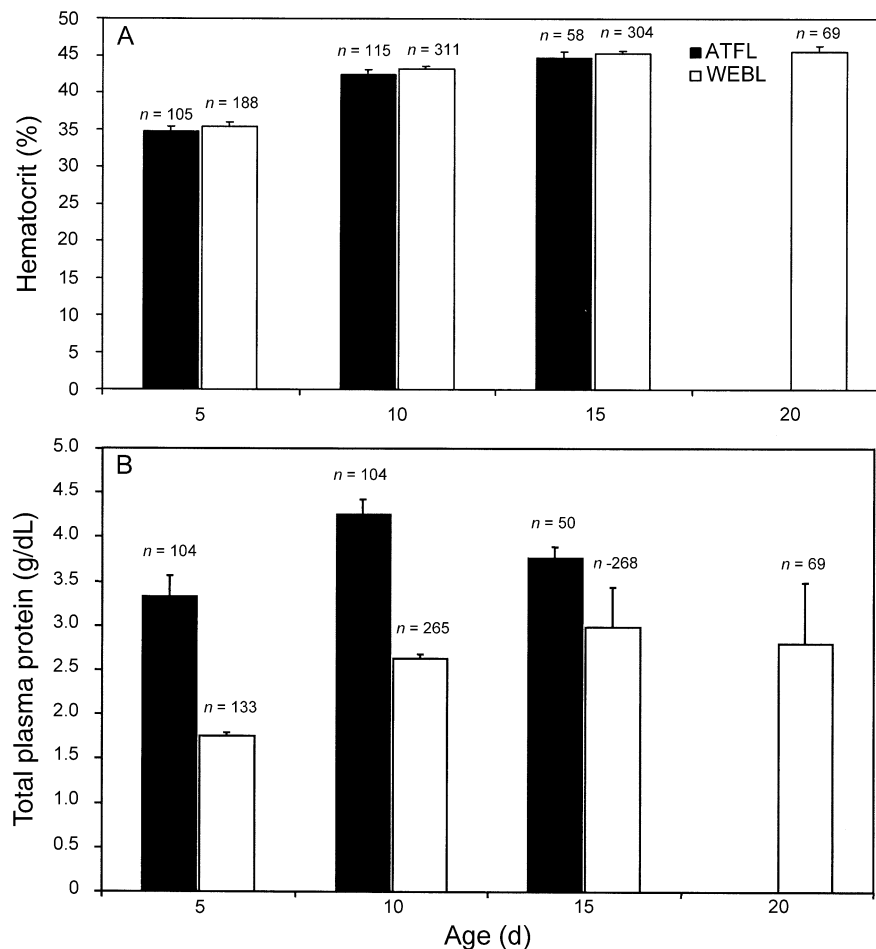


FIG. 5. (A) Mean hematocrit and (B) total plasma protein (means + 1 SD) for Ash-throated Flycatchers (ATFL) and Western Bluebirds (WEBL) for 1997–1999 over time (days).

ences between years for plasma protein on day 5 and 10, but 1998 had higher values at 15 d of age for both species ($F_{2,314} = 8.86$, $P = 0.0002$). Plasma protein differed between locations in the ATFL ($F_{6,227} = 4.96$, $P = 0.0002$) but not the WEBL ($F_{14,694} = 0.92$, $P = 0.53$; Tables 4 and 5). Plasma protein was not correlated with distance to PRS ($F_{3,77} = 1.86$, $P = 0.16$, PROC NLIN).

DISCUSSION

Survival and growth

Survival did not vary with year, species, hatch date, brood size, or clutch size. However, it did vary between locations, and was lower for ATFLs only in areas closer than 60 m to a PRS. Although survival did not differ between species, ATFLs had less variation in survival (11.5%) across all locations and years compared to the WEBL (20.5%), and ATFLs had lower survival in areas associated with contamination. Although survival varied with respect to contamination sites, none of the parameters measured with respect to growth or immune response varied in parallel. Thus, there may have been

effects, but the mechanisms are not clear. There were several causes of mortality in this study, from snake predation to abandonment. Specific predators such as snakes may also vary with contamination or habitat, although there was no relationship with mortality types and locations or contamination.

ATFLs weighed more on day 5, and by fledging age, WEBLs weighed more. This was not the case for the tarsus and wing length, where ATFLs had longer wings and tarsi throughout development. Clearly, wing development and bone structure are more important than body mass for successful fledging. It might be predicted that wing length, due to its importance and more rapid growth in the ATFL, would be more susceptible to stress in that species. However, the tarsus exhibited less phenotypic variation than body mass and its growth appears to be conservatively regulated. The tarsus in birds can't shrink, whereas mass can; thus it is hard to compare the two, particularly in the influence of similar percentage changes on survival, that is, the sensitivity of different parts of the life history to environmental perturbation. Due to the fact that these life-history traits

TABLE 4. Mean physiological and growth variables (with 1 SE in parentheses) for all occupied nest box locations (*n*) for 15-day-old WEBL for 1997, 1998, and 1999.

Locations	<i>n</i>	Hematocrit (%)	Plasma protein (g/dL)	Weight (g)	Right wing (mm)
A/W Mesa	3	44.81 (1.74)	3.5 (0.30)	27.26 (0.35)	54.5 (0.29)
Ancho Canyon	18	44.86 (2.09)	2.79 (0.08)	27.05 (0.39)	57.1 (0.98)
Bayo Canyon (contaminated)	47	45.65 (0.82)	2.93 (0.11)	26.97 (0.42)	56.52 (0.59)
Bayo Canyon	55	46.64 (0.73)	2.94 (0.11)	25.16 (0.26)	57.82 (0.84)
Cemetery	17	44.77 (1.03)	3.01 (0.26)	28.65 (0.57)	64.79 (1.41)
DP Canyon (contaminated)	10	39.45 (3.63)	2.58 (0.14)	24.4 (0.98)	48.45 (1.98)
Golf Course	12	47.13 (1.73)	3.13 (0.30)	26.86 (0.38)	54.47 (2.13)
LA Canyon (contaminated)	8	38.75 (1.42)	2.87 (0.16)	26.13 (0.85)	55.7 (0.72)
LA Canyon	19	44.02 (1.34)	3.37 (0.16)	24.27 (0.80)	56.6 (1.11)
Mortandad Canyon (contaminated)	5	44.09 (1.10)	3.12 (0.08)	26.87 (0.59)	60.1 (2.27)
Mortandad Canyon	10	43.38 (2.24)	2.73 (0.17)	25.60 (0.63)	55.65 (1.71)
Pueblo Canyon (contaminated)	19	46.67 (1.41)	3.29 (0.14)	25.44 (0.95)	56.13 (1.37)
Pueblo Canyon	12	50.85 (0.98)	3.36 (0.01)	26.55 (0.86)	54.08 (0.98)
Sandia Canyon (contaminated)	5	53.18 (3.72)	2.06 (0.56)	28.04 (0.80)	55.3 (0.94)
Water Canyon	23	42.07 (1.19)	2.80 (0.16)	26.50 (0.42)	53.06 (0.65)
TA-33 Mesa (contaminated)	13	44.50 (1.19)	...	24.7 (0.75)	57.13 (0.77)
TA-51 (contaminated)	6	46.83 (1.42)	2.88 (0.07)	28.5 (0.90)	61.25 (1.03)

result from such different processes and have such different consequences for fitness, comparisons between species for the same measurement should be meaningful, however, even if the consequences are unknown. Because wing length did not differ between locations and contaminated areas, wing length would not appear to be a sensitive predictor of environmental stress. However, for the WEBL tarsus length did vary among locations and was longer at locations farther away from PRSs. Tarsus length may be a better predictor of environmental stress, and it may be that most tarsus growth occurs early in development and most wing length growth occurs somewhat later.

Immune response

Mock et al. (1991) predicted from its more rapid development rate and higher daily energy requirements (22% higher per day) that the ATFL would be more susceptible to nest failure than the WEBL. Although the adult mass of both species is similar (range 25–30 g) and nestlings fledge at a similar body mass (27–28 g), the age of fledging differs by 4 to 5 days (WEBLs in this study fledged at 17 to 22 days of age; ATFLs fledged at 14 to 16 days). Ricklefs (1973) proposed that biochemical and molecular constraints might limit the extent that tissues can both differentiate function-

ally, proliferate, and grow. The ATFL may become functionally mature at an earlier age to achieve sustained flight soon after its earlier fledging age, even though its growth curve resembles that of the WEBL. Does this maturation also include the immune system? T-cell-mediated response of the ATFL was stronger than the WEBL, suggesting that T-cell development and maturation may parallel that of other tissues and the development of the high field metabolic rate reported by Mock et al. (1991).

Young chicks may be sensitive to disease agents because of the relative immaturity of their immune systems. Pitcovski et al. (1987) point out that, although the ability to mount a humoral response to some antigens is present during first-week posthatch, the rate of maturation differs among antigens (Matsuda and Bito 1973). Even after some acquired immunity has developed, antibody titers are stronger for older birds (Peleg et al. 1985). Seto and Henderson (1967) showed that antibody responses of chickens were stronger and earlier for mouse erythrocytes than to sheep erythrocytes and did not reach substantial levels until four weeks of age. Due to sensitivity of the agglutination test that we used, the ATFLs and WEBLs had detectable antibodies even though the levels were extremely low.

TABLE 5. Mean physiological and growth variables (\pm 1 SE in parentheses) for all occupied nest box locations (*n*) for the 15-day-old ATFL for 1997, 1998, and 1999.

Locations	<i>n</i>	Hematocrit (%)	Plasma protein (g/dL)	Mass (g)	Right wing (mm)
Ancho Mesa	7	43.66 (1.80)	3.34 (0.36)	25.15 (0.44)	63.93 (1.77)
Bayo Canyon (contaminated)	11	46.42 (1.02)	3.18 (0.24)	24.05 (0.72)	69.04 (0.93)
Bayo Canyon	8	48.00 (1.69)	4.33 (0.26)	24.11 (0.43)	63.91 (1.37)
LA Canyon	17	43.54 (2.37)	4.15 (0.27)	25.35 (0.31)	67.86 (0.55)
Mortandad Canyon (contaminated)	3	48.44 (0.73)	3.8 (0.20)	24.75 (0.59)	68.17 (1.59)
Mortandad Canyon	8	43.60 (1.39)	3.20 (0.18)	26.15 (1.33)	68.44 (0.76)
TA-33 Mesa (contaminated)	4	39.62 (1.00)	4.27 (0.54)	26.16 (1.33)	66.25 (0.48)

The B-cell specific antigens (that were T-cell independent) elicited a similar response between the species. The ontogeny of B-cell development parallels serum immunoglobulin levels, which, for the first week in life, consist of IgG from maternal contribution (Apanius 1998). Thus, it appears that the T-cells that play a vital role in both humoral and cell-mediated responses are more abundant at fledging age in the ATFL than the WEBL. The impact of stress on the cell-mediated response can be severe, with a reduction in both delayed-type hypersensitivity and cytotoxicity (Kuby 1997). Defects in the humoral system primarily influence the immune response to infectious encapsulated bacteria, and defects in the cell-mediated system are associated with increased susceptibility to viral, protozoan, and fungal infections. Only one other published interspecific comparison of immunocompetence in birds found that immune responses were positively correlated (Møller et al. 2001).

The cell-mediated effects were dynamic in that the same locations in general showed similar patterns for each year. Each year the birds at the cemetery and golf course had relatively high PHA response and the longest wing lengths. These two areas are located close to each other and would be of concern for pesticide exposure, and this result would follow our previous prediction of stimulation of immune function. In contrast, the birds breeding in a site called DP Canyon had some of the lowest PHA responses as well as the shortest wing lengths for both years that had breeding birds. Sediment contamination of DP Canyon is primarily associated with historical releases of processed wastewater discharged directly into the canyon. The main contaminants of concern are the radionuclides americium-241, cesium-137, plutonium isotopes, and strontium-90. Other inorganic chemicals of concern include antimony, cadmium, copper, lead, and zinc, which have all been shown to have immunosuppressive effects (Sharma and Dugyala 1996). Likewise, birds exposed to radionuclides at Chernobyl have been found to have suppressed immune function (Camplani et al. 1999). Additionally, young beagle dogs exposed to plutonium-239 oxide showed a long-term decrease in the response to PHA (Davila et al. 1992).

Although PHA response represents only one aspect of the immune system, it might not only be critical to maintain a high cell-mediated response owing to its interconnectedness with the humoral response, but it proves to be one of the quickest, most feasible, and least variable method to assess at least T-cells. However, like Zuk and Johnson (1999), we would caution against adopting a unitary definition of immunocompetence because different aspects of the immune system may vary differently with respect to season and may be differentially affected by stressors.

Hematocrit showed no general year or species differences, while total plasma protein was considerably higher for the ATFL at all bleeding periods. For the

ATFL, there were significant differences in total plasma protein in the locations across the plateau. Although used throughout human and veterinary clinical medicine to determine anemia, there was less variation in hematocrits than would be useful in assessing bird condition. It has been suggested that healthy birds may maintain optimal hematocrit levels within a narrow range (Breuer et al. 1995). Both plasma protein and hematocrits may be only useful in identifying birds that are in critical condition, and this often can be determined by simple external examination (Dawson and Bortolotti 1997b).

Contaminants and biomarkers

Long-term life-history studies can be intensive, potentially expensive, and, in some cases, technologically impossible. Proactive studies to determine the sensitivities of various life-history traits to contaminants could save millions of dollars in the long run and potentially save not only a single species, but also an ecosystem, if the species of concern is a bioindicator. Life-history traits such as home range could have an impact on potential contaminant exposure. A future research priority may be to accurately measure the foraging ranges of these species to estimate contaminant exposure differences.

Monitoring networks across a landscape of various remediation concerns can offer useful information for decision making, as specific questions can then be asked regarding specific areas. In this project, specific areas of concern can now be monitored and hypotheses tested for environmental impacts of stressors of concern endemic to those locations. Also, once the broad stroke view of all of the areas is completed, as in this study, areas of concern can be pinpointed. Then a more focused and detailed study can be designed for that area using more specified contaminant data that can better determine the environmental causes of the perceived decrease in the trait or immunocompetence.

From this study, we can see that two similar species, which may otherwise belong to a single guild in a traditional ecological risk assessment, may respond differently to environmental perturbation. The more research that is completed to compare life-history and physiological trait variation of species in the wild, the more will be known about how much can be extrapolated to other species. Trait variation and extrapolation are critical when dealing with threatened and endangered species where data are minimal or lacking.

At this point, it is unknown whether ATFLs are more vulnerable to contaminant exposure or to poor food availability due to a contaminant-prey interaction. Continued research can lead us to a better understanding of the mechanisms behind this susceptibility, which will increase the precision and accuracy of environmental risk assessments.

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