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EFFECTS OF INGESTED LEAD ON ANTIBODY PRODUCTION IN MALLARDS (*ANAS PLATYRHYNCHOS*)

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ABSTRACT: We examined the effects of lead ingestion on in vitro and in vivo indices of immune function in mallards (*Anas platyrhynchos*). Twenty-four mallard drakes were randomly divided into three groups (I, II, III), then assigned to treatment or control subgroups ($n = 4$). On day 0, all treatment birds were dosed orally with one number 4 lead shot ($\bar{x} = 0.2$ g). We challenged all individuals in each group with washed sheep red blood cells (SRBC) injected intraperitoneally on days 0 (Group I), 7 (Group II) or 14 (Group III), and collected blood for analyses 7 and 8 days after SRBC challenge. We measured and compared blood lead concentrations, in vitro lymphocyte transformation responses to phytohemagglutinin A and lipopolysaccharide, and hemagglutination titers to SRBC. Mean blood lead concentrations were elevated ($P \leq 0.04$) in treatment birds at each sampling period. Large individual variability in lymphocyte stimulation responses precluded further analysis of those data. Hemagglutination titers to SRBC were lower ($P < 0.0001$) in lead-poisoned ducks than in controls, suggesting that ingested lead may have immunosuppressive effects on mallards.

Key words: *Anas platyrhynchos*, mallard, lead, immunosuppression, hemagglutination, lymphocyte transformation, intoxication, experimental study.

INTRODUCTION

Ingested spent lead shot causes direct toxicity to ducks and geese (Bellrose, 1959; Eisler, 1988), and accounts for 2 to 3% of the nonhunting mortality in waterfowl populations in North America (Bellrose, 1959; Stout and Cornwell, 1976). Moreover, insidious sublethal effects of lead may contribute to waterfowl mortality proximately ascribed to other causes. Altered metabolic processes (Finley et al., 1976), impaired gastrointestinal and neurophysiological functions (Mautino, 1984), and suppressed immune responses (Vos, 1977) could compromise waterfowl that ingest lead, thereby increasing susceptibility to exposure, malnutrition, predation or disease. Because sublethal effects of lead intoxication are difficult to quantify in wild waterfowl populations, the magnitude of these effects remains largely inestimable (Wobeser, 1984).

Although disease may be responsible for nearly 90% of all nonhunting waterfowl mortality (Stout and Cornwell, 1976), factors that increase disease susceptibility in waterfowl are poorly understood. Immunosuppression secondary to lead intoxication has been hypothesized as one possible contributor to lowered disease resistance in waterfowl (Franson, 1984; Wobeser, 1984). However, lead-induced immunosuppression in waterfowl has not been documented, and investigations of the relationship between lead poisoning and disease in waterfowl have provided equivocal results. We conducted an experiment to examine changes in immune responses in drake mallards (*Anas platyrhynchos*) after ingesting lead shot. Our study tested hypotheses that ingestion of one number 4 lead shot would (1) inhibit lymphocyte transformation by mitogenic stimulation and (2) depress antibody production to sheep red blood cells (SRBC).

MATERIALS AND METHODS

Our experiment was conducted from 12 February to 7 March 1989 at the Colorado Division of Wildlife's Foothills Wildlife Research Facility (Fort Collins, Colorado 80526, USA; 40°35′N, 105°10′W). Pen-raised, wild strain adult mallard drakes ($n = 24$) were randomly divided into

three equal groups (I, II, III), then randomly assigned to either control or treatment subgroups (n = 4 drakes/subgroup). We housed subgroups in 3 m × 4 m outdoor isolation pens, and located corresponding control and treatment subgroups in separate but adjoining pens. Ducks were fed a mixture (about 1:1 by weight) of field corn and commercial chow (20% Lay Pellets, Ranchway, Inc., Fort Collins, Colorado 80521, USA; guaranteed analysis: crude protein ≥ 20.0%, crude fat ≥ 2.0%, crude fiber ≤ 10.5%, calcium 3.0 to 3.5%, phosphorus ≥ 1.0%, iodine ≥ 0.00023%) provided ad libitum throughout the study. Grit from natural sources was available in all pens. Water was provided from a single source, flow-through well system. To equilibrate handling stress, we handled control and treatment birds similarly.

All ducks were weighed 2 days prior to dosing, and heparinized blood (about 1 ml) was collected from each bird via jugular venipuncture for estimating pretreatment blood lead levels. On day 0, one number 4 lead shot ($\bar{x}$ = 0.2 g) was introduced into the esophagus of each treatment bird using a modified tuberculin syringe. We observed all ducks daily for signs of lead poisoning (Wobeser, 1981).

Our experiment was designed to sample both in vitro and in vivo indices of immune function at 7-day intervals after lead ingestion. Mallards in groups, I, II, and III were challenged with a 20% solution of washed sheep red blood cells (SRBC) (2 ml, injected intraperitoneally (IP)) on days 0, 7 and 14, respectively. Previous pilot studies with mallards revealed that hemagglutination titers to SRBC peaked 7 to 9 days after IP challenge (K. A. Trust, unpubl.). Therefore, we collected samples 7 and 8 days after SRBC challenge: group I birds were bled on days 7 and 8, group II on days 14 and 15, and group III on days 21 and 22. Sampling was conducted over 2 days to minimize risks to treatment birds arising from acute blood loss. We collected 6 ml of blood into heparinized syringes at the onset of each sampling period; about 1 ml was placed into heparinized tubes and stored at −20 C for lead analysis, and the remaining 5 ml were stored at 25 C for ≤1 hr until processing for lymphocyte transformation could begin. One day later, we collected an additional 3 ml of blood; about 1 ml serum was harvested at 24 hr and frozen until agglutination titers were run.

We prepared blood samples for lead determination using the methods of Adrian (1971). All samples were analyzed for lead using atomic absorbance spectrophotometry (Video 22 Dual Channel AA/AE Spectrophotometer, Instrumenation Laboratory, Andover, Massachusetts 01810, USA; 217.0 nm, air-acetylene flame, Smith-Hieftje background correction) in a single assay run. We expressed blood lead concentrations in ppm.

Lymphocyte transformation tests were conducted using methods of Higgins and Teoh (1988) modified by results of our own pilot trials (K. A. Trust and I. M. Orme, unpubl.). Blood aliquots (5 ml) were mixed 1:1 by volume with sterile phosphate buffered saline (PBS). This mixture was then layered over 5 ml Lympholyte-M (Accurate Chemical and Scientific Corp., Westbury, New York 11590, USA) and centrifuged (500 g, 20 C, for 20 min). Lymphocyte layers were collected and washed twice with culture medium containing RPMI 1640 (Gibco Laboratories, Life Technology, Inc., Grand Island, New York 14072, USA), sodium bicarbonate, penicillin/streptomycin (100 U/1,000 μg/ml), and 10% chicken serum. After washing, lymphocytes were resuspended in 10 ml medium and counted using a hemocytometer. Final concentrations of cell suspensions were then adjusted to achieve a uniform 8 × 10^5 cells/culture.

We used two mitogens at four final concentrations (0, 1.25, 2.5, 5 μg/ml) for lymphocyte stimulation assays; lipopolysaccharide (LPS) (Sigma Chemical Company, Saint Louis, Missouri 63178, USA) tested B-lymphocyte responses, and phytohaemagglutinin (PHA) (Sigma Chemical Company) tested T-lymphocyte responses. For each sample, we added triplicate 100 μl aliquots of lymphocyte suspensions to 96-well microtiter plates, then added 10 μl of mitogen solutions to achieve desired final concentrations. Cultures were incubated in a humid atmosphere containing 5% CO_2 at 37 C for 48 hr. After 48 hr, we pulsed all wells with 0.5 μCi [^{3}H] thymidine (ICN Biomedicals, Inc., Costa Mesa, California 92626, USA) for 6 hr, then harvested cells onto glass fiber strips for liquid scintillation counting. Results were expressed as mean counts per min (cpm) for triplicate samples.

Agglutination titers were measured from heat-inactivated (56 C for 1 hr) sera. We determined antibody titers to SRBC with modified microtiter techniques: Two-fold serial dilutions were constructed using 50 μl serum in microtiter plates, 50 μl of 1% SRBC was added to each well, and plates were incubated at 37 C in 5% CO_2 for 3 hr. Titers were expressed as an inverse of the highest dilution showing hemagglutination.

Because ours was not a terminal experiment, we began therapy on all lead-dosed ducks by injecting 35 mg/kg EDTA in 5% dextrose intramuscularly after collecting samples on day 22. Treatment consisted of an alternating regime (5 consecutive days of treatment followed by 5 days without EDTA) over 25 days.

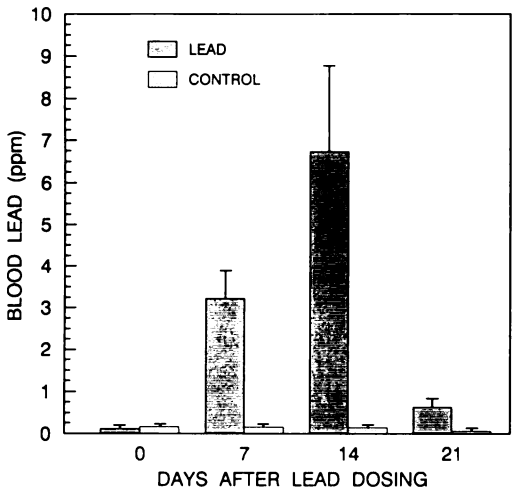

FIGURE 1. Mean blood lead levels (ppm) were elevated ($P < 0.0002$) in mallards 7 to 21 days after ingestion of one number 4 lead shot. Vertical lines are one standard error of mean observations.

We analyzed blood lead, body mass, and hemagglutination data using analysis of variance (PROC GLM) for a two-way factorial design, with treatment and time as main effects (SAS, 1987).

RESULTS

Ingestion of one number 4 lead shot affected blood lead concentrations but not body mass of treated ducks. Pretreatment blood lead levels and body mass did not differ between control and treatment groups ($P \geq 0.36$). Blood lead concentrations were elevated ($P < 0.0002$) in treated ducks 7 to 21 days after ingestion of one number 4 lead shot, although levels declined between days 14 and 21 (treatment X time $P = 0.04$) (Fig. 1). Control lead concentrations remained below detection limits ($\bar{x} \pm SE = 0.11 \pm 0.01$ ppm), and did not change over time ($P = 0.20$). Body mass did not differ between groups before ($P = 0.68$) or after ($P = 0.16$) treatment.

Three lead-dosed drakes died during this experiment. Two of these (from Group I) died on day 15, and a third was euthanized 2 wk after the study ended. All exhibited some combination of clinical signs of lead poisoning prior to death, and gross lesions were compatible with lead intoxication.

Overall, our attempts to measure in vitro immune responses using lymphocyte stimulation assays proved unrewarding. All assays performed on day 7 failed. Transformation data from days 14 and 21 were highly variable (Table 1). This variability, exacerbated by small sample sizes, precluded analysis and reliable interpretation of lymphocyte transformation data. Subjectively, stimulation responses to PHA appeared similar for lead-treated and control ducks, and responses tended to decrease with increasing PHA levels (Table 1A). We observed little or no stimulation of mallard lymphocytes in response to LPS, and in some groups LPS appeared to slightly depress stimulation responses (Table 1B).

Ingestion of one number 4 lead shot reduced agglutination titers to SRBC in all treatment birds ($P < 0.0001$; Fig. 2). Treatment effects were uniform across sampling periods (time $P = 0.97$, treatment × time $P = 0.43$).

DISCUSSION

Ingested lead shot impaired antibody production in mallards. Hemagglutination titers to SRBC were depressed in lead-treated ducks by day 7. Titers remained low throughout our experiment, despite waning blood lead levels in treated birds by day 21. This suggests that immunosuppressive effects might occur in mallards within a few days of ingesting a lead pellet, and that these effects could persist for at least 2 to 3 wk. Several studies on mammals support our findings that lead poisoning may depress antibody production: reduced antibodies to SRBC were found in lead-intoxicated mice (Koller and Kovacic, 1974; Blakley and Archer, 1981) and rats (Luster et al., 1978). In rabbits, lead acetate reduced antibody titers to pseudorabies virus almost 10-fold (Koller, 1973).

Specific effects of lead on in vitro B- and T-lymphocyte activity cannot be discerned from our inconclusive lymphocyte transformation data. In mammals, lead seems to impair macrophage and/or T-cell function rather than B-cell function (Lus-

TABLE 1. Mallard lymphocyte transformation responses to phytohemagglutinin A (PHA) and lipopolysaccharide (LPS).

Day	Treatment	Radioactivity (cpm) of cell cultures			
		0	1.25	2.5	5.0
Stimulated with PHA (μg/ml)					
14	Control	1,786 (673)*	8,925 (4,398)	1,437 (394)	681 (150)
	Lead	497 (204)	3,299 (1,400)	2,056 (666)	791 (250)
21	Control	421 (142)	8,545 (5,385)	2,345 (1,589)	410 (151)
	Lead	599 (194)	15,622 (9,225)	1,720 (1,366)	466 (172)
Stimulated with LPS (μg/ml)					
14	Control	1,314 (614)	733 (220)	824 (200)	703 (192)
	Lead	361 (172)	229 (52)	216 (50)	217 (61)
21	Control	374 (120)	375 (135)	551 (69)	377 (58)
	Lead	490 (172)	686 (246)	968 (253)	919 (313)

* Data presented as mean (standard error) for subgroups of ducks.

ter et al., 1978; Faith et al., 1979; Blakley and Archer, 1981). Either macrophage or T-cell effects could account for reduced hemagglutination titers to SRBC that we observed in vivo. Macrophage function was not explicitly tested here, but warrants examination in future studies. Depressed T-cell activity should have been detected in transformation responses to PHA. Although extreme variability in our data precluded rigorous interpretation, PHA seemed to equivalently stimulate treatment and control samples. In contrast, LPS generally failed to stimulate cell cultures, and instead appeared to slightly cytotoxic effects in some groups.

Interanimal and assay-related variability probably affected success of our in vitro experiment. Larger sample sizes and/or more elaborate experimental designs may be required to overcome individual variability in mallard lymphocyte transformation responses that we encountered. Similarly, improved methods may reduce assay-related variance. Two recent studies offer notable advances in adapting lymphocyte transformation assays to accommodate duck lymphocytes. Unfortunately, both lack rigor. Neither Higgins and Chung (1986) nor Higgins and Teoh (1988) reported sample sizes, estimates of variability, or statistical analyses for their lymphocyte transformation experiments; however, transformation responses under similar sets of conditions appear to vary widely among their experiments.

Our methods differed slightly from those recommended by Higgins and Teoh (1988). Of these differences, lower incubation temperatures (37 C versus 41.6 C) seemed most likely to influence results (Higgins and Teoh, 1988). The magnitudes of our average transformation responses resembled some of those reported by Higgins and Teoh (1988, Tables II, XI) for PHA doses ≤ 5 μg/ml, but were generally lower than theirs. Higher incubation temperatures and PHA doses (>10 μg/ml) may improve mallard lymphocyte transformation responses, and should be tested. Our experiences underscore the need to refine and standardize techniques for examining immune function in mallards and other wild waterfowl species. By reporting both failed and successful attempts to measure mallard immune responses here, we offer other workers a point of reference in beginning to improve upon our approaches.

Lead apparently increases susceptibility to disease in many mammalian species (Vos, 1977). Our data suggest a similar link could exist between lead and disease in waterfowl, but other studies on disease resistance in experimentally lead-poisoned avian species have produced equivocal re-

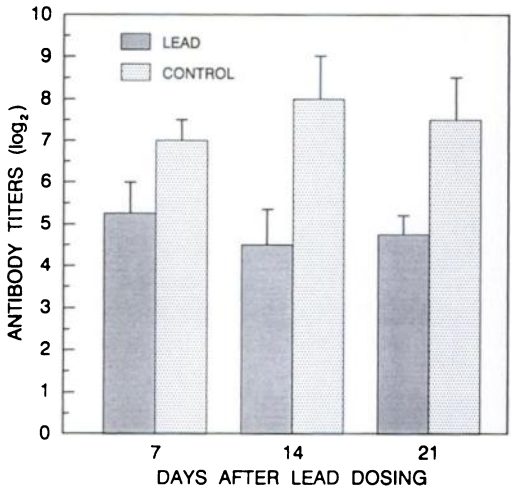

FIGURE 2. Hemagglutination titers to sheep red blood cells were lower ($P < 0.0001$) in lead-poisoned ducks than in controls at each sampling period. Bars are means of log_2 (titers); vertical lines are one standard error of mean observations.

sults. Although Truscott (1970) demonstrated increased susceptibility to *Escherichia coli* endotoxin in young chickens simultaneously dosed with lead acetate, results of two subsequent studies contradict his findings. Vengris and Maré (1973) failed to show that lead adversely affected antibody or interferon production in chickens. Similarly, Wobeser (1984) could not demonstrate increased susceptibility to *Pasteurella multocida* in lead-poisoned mallards, although he speculated that well-nourished ducks in his experiment may have tolerated blood lead levels that were bactericidal. Here, depressed in vivo antibody production to SRBC occurred after challenge with lead doses 75 to 90% lower than those used by Wobeser (1984). Unfortunately, the latter two experiments are otherwise difficult to compare: in addition to diet differences, Wobeser (1984) did not measure blood lead or antibody levels in treated ducks, and we did not challenge our birds with *P. multocida*.

Available data cannot reconcile the apparent contradiction between depressed antibody production (our study) and unaltered disease resistance (Wobeser, 1984) in lead-poisoned mallards. Carefully controlled experiments will be needed to resolve these discrepancies and describe relationships between lead intoxication and disease in waterfowl. Because quality and quantity of diet (Sobel et al., 1940; Jordan, 1968) and grit (Jordan, 1952; Longcore et al., 1974) affect absorption and/or excretion rates for lead, these should be manipulated to simulate natural environmental and body conditions experienced by wild mallards. Variability in responses to lead attributed to age- and sex-specific metabolism (Jordan and Bellrose, 1950, 1951) should also be considered in designing experiments. Realistic exposure levels and routes for both lead and pathogens should be incorporated into future studies to represent natural processes. Moreover, response variables and techniques for their measurement should be standardized to allow comparison among experiments.

Current federal guidelines mandate the elimination of lead shot for all waterfowl hunting beginning in 1991, but with spent lead pellets exceeding 40,500/ha in some areas (U.S. Fish and Wildlife Service, 1987), lead will probably persist in wetlands well into the 21st century. Depending upon soil and water characteristics, Jorgensen and Willems (1987) estimated the environmental half-life for spent lead shot to be 40 to 70 yr, and suggested 100 to 300 yr may be required for complete degradation. The consequences of this environmental persistence are illustrated by recent increases in lead-related waterfowl mortalities attributable to drought conditions: receding water levels made available lead pellets that were formerly submerged beyond the reach of most waterfowl. Some areas reporting increased prevalence of lead poisoning have enforced nontoxic shot regulations for several years (M. R. Szymczak, pers. comm.). It follows that acute and chronic lead poisoning, as well as sublethal effects of ingested lead, will continue to plague waterfowl populations for some time.

Our results indicate ingested lead shot

depresses some aspect of antibody production to an innocuous antigen (SRBC) in captive mallard drakes. Similar effects in wild mallards might increase individual susceptibility to pathogens. The potential interactions of sublethal lead intoxication and susceptibility to disease in waterfowl warrant further investigation.

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