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Authors: Rocke, Tonie E., and Samuel, Michael D.

Source: Journal of Wildlife Diseases, 27(1) : 1-9

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-27.1.1

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Journal of Wildlife Diseases, 27(1), 1991, pp. 1–9

EFFECTS OF LEAD SHOT INGESTION ON SELECTED CELLS OF THE MALLARD IMMUNE SYSTEM

Tonie E. Rocke and Michael D. Samuel
U. S. Fish and Wildlife Service, National Wildlife Health Research Center,
6006 Schroeder Road, Madison, Wisconsin 53711, USA

ABSTRACT: The immunologic effects of lead were measured in game-farm mallards (*Anas platyrhynchos*) that ingested lead shot while foraging naturally, mallards intubated with lead shot, and unexposed controls. Circulating white blood cells (WBC) declined significantly in male mallards exposed to lead by either natural ingestion or intubation, but not females. Spleen plaque-forming cell (SPFC) counts were significantly lower in mallards intubated with lead pellets compared to controls. Declines in WBC and SPFC means with increasing tissue lead concentrations provide further evidence that lead exposure reduced immunologic cell numbers. Hormonal activity and diet may have influenced the immunologic effects of lead exposure in this study.

Key words: Lead shot, immunotoxicology, hematology, mallards, *Anas platyrhynchos*, experimental study.

INTRODUCTION

Lead poisoning mortality of wild waterfowl ingesting spent lead shot is well documented (Sanderson and Bellrose, 1986), but far less information is available on sublethal effects of lead ingestion. Increased susceptibility to infectious disease is postulated to be a sublethal effect of lead exposure in waterfowl (Franson, 1986). This hypothesis is supported by field observations of epizootics, such as avian cholera, that closely follow or are concurrent with waterfowl losses from lead poisoning [Franson, 1986; U.S. Fish and Wildlife Service, National Wildlife Health Research Center (NWHRC), unpubl. data]. Further circumstantial evidence for this hypothesis is provided by recurrent observations of waterfowl with elevated tissue lead concentrations and simultaneous infections of aspergillosis or coccidiosis (NWHRC, unpubl. data). It is unknown if these findings are coincidental or if they indicate an association between waterfowl disease susceptibility and lead exposure.

Many laboratory studies on rats and mice, and epidemiological studies of humans, have demonstrated that lead adversely affects resistance to disease (Selye et al., 1966; Hemphill et al., 1971; Cook et al., 1975; Gainer, 1977) and alters various immunologic functions (Koller, 1973; Koller and Kovacic, 1974; Luster et al., 1978; Faith et al., 1979; Blakley and Archer, 1981). Similar studies in birds have been complicated by the greater difficulty of evaluating immunologic competency of birds in comparison to mammals. In the few laboratory studies that have been conducted, dosing has most commonly been accomplished by intraperitoneal or intravenous injections of lead acetate. Administration of lead acetate to domestic chickens (*Gallus domesticus*) caused a 1,000-fold increase in susceptibility to endotoxin in one study (Truscott, 1970). In contrast, Vengris and Maré (1974) found that lead acetate had no effect on interferon induction or antibody production to Newcastle disease virus by chickens.

Findings of these and other experiments on the immunotoxicology of lead in birds have been contradictory, generating criticism that routes of exposure and dosages administered are not environmentally relevant. To avoid these problems, we examined the immunologic effects of lead shot "naturally" ingested by waterfowl feeding in a marsh setting. Selected immunologic parameters were measured in game-farm mallards confined to enclosures on a hunted wetland at the Sacramento National Wildlife Refuge (SNWR; Willows, California 95988, USA; 39°20′N,

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122°20'W). Immunologic effects were also measured in mallards that had been intubated with lead pellets and exhibited signs of acute lead poisoning.

MATERIALS AND METHODS

Immunologic tests were conducted on four treatment groups: mallards that "naturally" ingested lead pellets (field-exposed), mallards administered lead pellets by gavage (lead-intubated), unexposed controls, and mallards treated with a known immunosuppressant, cyclophosphamide (CY-treated). Birds in all treatment groups were one-year-old game-farm mallards of both sexes (Wild Wings of Oneka, Hugo, Minnesota 55038, USA), wing-clipped to prevent flight and tagged with patagial markers and leg bands. All birds were handled and held similarly in wetland enclosures constructed of game bird netting at SNWR prior to inclusion in the study.

Mallards in the field-exposed group were placed in a 4-acre enclosure on a hunted wetland at SNWR for 80 days (February to April, 1986) prior to testing. This wetland had a lead shot density of 931,000 (±66,933 SE) pellets per acre in the top 10 cm of sediment (NWHRC, unpubl. data). Habitat within the enclosure consisted primarily of open water with scattered submergent vegetation flooded to a mean depth of 13.2 cm. Rice, scratch grains, and river-run gravel were provided to supplement natural food sources and grit in the marsh. A 2 ml blood sample was collected from all birds prior to confinement in the enclosure to ensure no previous lead exposure. At the end of 80 days, 63 surviving mallards were captured and removed from the enclosure for immunologic testing. They were moved to an outdoor holding pen (6.1 m × 9.2 m) constructed of game bird netting with a gravel bottom, where they were held with all the other experimental groups throughout the testing period. Rice, scratch grains and water were provided *ad libitum* to all birds during confinement in the holding pen.

Mallards from another 4-acre marsh enclosure on a non-hunted wetland were used for the other treatment groups. One group of 16 was captured 14 days prior to immunologic testing, removed from the enclosure, and intubated with lead shot. Each received two Number 4 lead shot (Remington Arms Company, 1800 Washington Rd., Pittsburgh, Pennsylvania 15201, USA) by gavage. Prior to lead exposure, 2 ml of blood were withdrawn from the jugular vein of all birds for lead analysis. This group of birds was kept in the holding pen to prevent additional lead exposure before immunologic testing began.

A control group of 20 mallards was captured, removed from the non-hunted enclosure, and placed in the holding pen on the same day as the field-exposed group. Twelve of these birds were used as unexposed controls; the other eight were treated with CY.

Because of the large number of birds, 4 consecutive days were required to complete blood collection and initiate immunologic tests. To reduce the potential effect of daily variation in assay procedures, "test groups" with 25% of the birds from each treatment group were randomly selected for testing on each of the 4 days. The holding pen was divided in four equal sections with game bird netting and each test group was placed within a section.

To begin testing, 3 ml of blood were withdrawn from the jugular vein of each bird; 1 ml was placed in a heparinized glass evacuated blood tube (Vacutainer®, Becton-Dickinson, Rutherford, New Jersey 07070, USA) and frozen for lead analysis. The remainder was collected in a Ca++-EDTA coated glass blood tube (Vacutainer®, Becton-Dickinson, Rutherford, New Jersey 07070, USA) for determining numbers of circulating white blood cells (WBC) and packed cell volume (PCV). After blood collection, each bird was sensitized to sheep red blood cells (SRBC) to stimulate antibody-producing cells. Sheep red blood cells, collected and prepared as described by Burgess (1981), were diluted in physiologic saline (20% suspensions) and inoculated into ducks by intravenous injection (1 ml).

Numbers of WBC were determined with the Eosinophil Unopette® Method (Becton-Dickinson, Test #5877, Rutherford, New Jersey 07070, USA) as described by Dein (1984). Eosinophil counts were performed within several hours of blood collection. Blood smears were prepared and stained with Wright's for differential cell counts; 100 cells were counted. Packed cell volume was determined using microhematocrit capillary tubes. All counts were performed by the same individual.

Four days post SRBC inoculation, experimental birds were euthanized with CO_2. Spleens were removed, weighed, and emulsified. The direct spleen plaque-forming cell (SPFC) assay was used to measure the number of splenic lymphocytes producing antibody to SRBC. SPFC assays were conducted using the Cunningham modification (Cunningham and Szenberg, 1968) of the Jerne Nordin assay (Jerne and Nordin, 1963) adapted to mallards by Sauch and Hinsdill (Burgess, 1981). The number of SPFC were expressed both per gram of spleen and per 10^6 viable cells, and these data were log-transformed for statistical analyses. Spleen cell viability was determined by trypan blue dye ex-

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clusion. To ensure consistency between samples, each step of the SPFC assay was performed by the same individual.

To verify that immunologic assays could detect lowered immune responsiveness, 8 mallards were treated with a known immunosuppressant. CY (Cytoxan®, Mead Johnson Co., Evansville, Indiana 47721, USA) was administered daily in doses of 30 mg/kg body weight by intramuscular injection for 3 days after SRBC inoculation.

Liver, ulna, and femur were collected from each bird at necropsy and frozen at −20 C until analyzed for lead. Tissues were then ashed in a muffle furnace and digested in nitric and hydrochloric acids. Liver, blood, and bone lead concentrations were determined by atomic absorption spectrophotometry (Perkin-Elmer Model 2380; Perkin-Elmer Analytical Instruments, Norwalk, Connecticut 06856, USA) using methods described by Fernandez and Hilligoss (1982). Lower limits of detection for this assay were 0.02 mcg/ml for blood and 0.22 for liver (NWHRC, unpubl.). Blood lead concentration ≥0.2 ppm and liver lead concentration ≥2.0 ppm (wet weight basis) were considered elevated and evidence of exposure to lead (Friend, 1985). We considered birds exposed to lead if either blood or liver lead concentrations were elevated at the time of immunologic testing.

Data were analyzed using the Statistical Analysis System (SAS Institute, Inc., 1987). All variables were examined using two-way analysis of variance (ANOVA) to detect treatment, sex, and interaction effects using individual birds as the experimental units. Dependent variables with no detectable sex or interaction effects were reanalyzed using one-way ANOVA (pooled across sex categories). *A priori* contrasts between each treated group and the unexposed controls were then made regardless of the overall significance of the ANOVAs (Kirk, 1982). Contrasts between treated and control groups for two-way ANOVAs tested males and females separately. Trend analysis (Kirk, 1982) was conducted to evaluate increases or decreases in the dependent variables related to treatment group means. Robust regression using iteratively reweighted least squares (Beaton and Tukey, 1974) was employed to test individual birds for significant relationships between their blood lead or liver lead concentrations and other dependent variables.

RESULTS

Of the 63 mallards tested in the field-exposed group, 55 had evidence of lead exposure (blood lead concentration ≥0.2 ppm or liver lead concentration ≥2.0 ppm) and were included in the subsequent analyses. None of the field-exposed mallards exhibited clinical signs of lead poisoning when immunological parameters were measured, even though blood or liver lead concentrations were elevated. Mean lead concentrations of all tissues in the field-exposed group were significantly higher ($P \leq 0.05$) in both males and females compared to controls (Table 1). Females in the field-exposed group had significantly higher concentrations ($P = 0.03$) of lead in their femur than males (Table 1).

Mean spleen weight ($P = 0.04$) and total circulating WBC ($P = 0.001$) were significantly depressed in male field-exposed mallards compared to control males (Table 2). However, there was no corresponding decrease ($P \geq 0.12$) in either of these parameters for female mallards in the field-exposed group when compared with control females. Differential white cell counts of field-exposed male mallards revealed that heterophils ($P = 0.001$) and, to a lesser extent, lymphocytes and monocytes ($P = 0.06$) were the cell types most affected. Mean PCV ($P \geq 0.50$), mean eosinophils ($P \geq 0.25$) and mean basophils ($P \geq 0.08$) of field-exposed mallards were similar to control values for both males and females (Table 2).

Three of the 16 lead-intubated mallards died from lead poisoning before SPFC numbers were measured, and most of the surviving birds exhibited clinical signs of lead poisoning (emaciation, lethargy, anemia, bile-stained feces; Wobeser, 1981). All of the lead-intubated birds had elevated blood lead concentrations (Table 1); 12 of the surviving 13 were within the range known to be toxic (≥0.5 ppm; Friend, 1985). Male mallards had significantly higher liver lead concentrations than female mallards ($P = 0.04$), whereas females had greater ($P = 0.03$) concentrations of lead in their femur.

Like the field-exposed mallards, significant depressions in mean spleen weight ($P = 0.05$), total circulating WBC ($P = 0.001$), heterophils ($P = 0.006$), lympho-

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TABLE 1. Mean lead concentrations (ppm) in tissues of mallards that naturally ingested lead pellets (field-exposed), mallards intubated with lead pellets (lead-intubated), and control (unexposed) mallards.

Parameter	Sex	Unexposed mallards	Field-exposed mallards**	Lead-intubated mallards**
Number of birds	Male	6	31	11
	Female	6	24	5
Blood lead	Male	0.09 (0.04)[a]	1.40 (0.7)	3.47 (1.38)
		0.05–0.17[b]	0.13–8.80	1.07–6.82
	Female	0.05 (0.04)	1.21 (0.78)	4.15 (3.56)
		0.00–0.13	0.16–7.21	0.23–10.59
Liver lead	Male	0.13 (0.08)	6.40 (4.92)	32.16 (11.60)*
		0.05–0.29	0.07–62.60	2.26–51.24
	Female	0.87 (0.72)	2.32 (1.10)	13.85 (7.96)
		0.00–1.90	0.26–8.80	4.42–26.23
Ulna lead	Male	4.30 (2.72)	22.06 (12.22)	63.18 (20.80)
		0.44–8.35	1.96–170.51	13.80–106.79
	Female	8.20 (6.64)	34.59 (24.98)	96.32 (35.62)
		0.00–20.49	0.71–190.65	45.45–128.59
Femur lead	Male	9.38 (4.88)	38.64 (19.18)*	114.78 (28.96)*
		0.70–17.23	4.68–182.43	45.78–180.63
	Female	30.51 (27.08)	131.13 (90.94)	577.07 (156.34)
		1.01–64.59	2.01–837.42	428.75–765.89

[a] Number in parentheses equals two standard errors.

[b] Range.

* Significantly different from female mallards in the same treatment group at $P \leq 0.05$.

** Mean lead concentrations for all tissues of field-exposed and lead-intubated groups were significantly different from unexposed mallards.

cytes ($P = 0.02$) and monocytes ($P = 0.01$) were noted in male mallards intubated with lead compared to control males (Table 2). But these parameters did not differ significantly ($P \geq 0.20$) between lead-intubated females and control females (Table 2). Mean PCV of lead-intubated males was greatly reduced ($P = 0.001$) compared to controls, but was not statistically different ($P = 0.33$) between lead-intubated and control females.

Mean tissue lead concentrations increased between control, field-exposed, and lead-intubated groups (Table 1). These trends occurred for blood lead ($P \leq 0.001$), ulna lead ($P \leq 0.001$), liver lead ($P \leq 0.001$), and femur lead ($P \leq 0.001$) with the highest tissue lead concentrations found in lead-intubated mallards. Negative trends were found for mean spleen weight ($P = 0.05$), WBC ($P \leq 0.001$), PCV ($P = 0.002$), heterophils ($P = 0.002$), lymphocytes ($P = 0.01$), and monocytes ($P = 0.004$) of male mallards (Table 2). However, no significant ($P \geq 0.20$) changes were found for mean eosinophils and basophils in males or spleen weight, PCV, and any of the white blood cells in females. Robust regression analysis indicated that PCV values of individual male ducks were also negatively related ($P \leq 0.01$) to their blood lead and liver lead concentrations.

Plots of mean white blood cells (total WBC, heterophils, lymphocytes and monocytes) and spleen weights with mean blood lead and liver lead for males suggested a nonlinear pattern. A $\log_{10}$ transformation was applied to the lead concentrations to linearize the relationship. Robust regression indicated a significant ($P \leq 0.05$) negative relationship between monocytes and both blood and liver lead concentrations of individual male mallards, but no significant correlations ($P \geq 0.05$) were detected between tissue lead and spleen

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TABLE 2. Mean number of white blood cells, spleen weight, and packed cell volume of mallards that naturally ingested lead pellets (field-exposed), mallards intubated with lead pellets (lead-intubated), and controls (unexposed).

Parameter	Sex	Unexposed mallards	Field-exposed mallards	Lead-intubated mallards
Number of birds	Male	6	31	11
	Female	6	24	5
Total white blood cells/cu mm	Male	39,659 (15,546)*	22,733 (3,264)*	20,103 (3,062)*
	Female	27,106 (6,934)	34,793 (5,420)	26,355 (8,052)
Heterophils	Male	18,764 (8,350)	8,941 (1,494)*	9,802 (2,848)*
	Female	15,529 (4,804)	17,248 (3,132)	10,843 (5,304)
Eosinophils	Male	2,422 (1,908)	1,650 (456)	1,505 (724)
	Female	1,520 (852)	1,962 (746)	1,475 (988)
Basophils	Male	108 (216)	627 (276)	473 (260)
	Female	868 (458)	714 (286)	791 (438)
Lymphocytes	Male	14,147 (9,772)	9,210 (1,550)**	7,009 (1,946)*
	Female	7,637 (1,866)	12,462 (2,720)	10,014 (4,386)
Monocytes	Male	4,197 (2,756)	2,290 (630)**	1,314 (652)*
	Female	1,601 (1,650)	2,588 (1,190)	3,300 (2,090)
% Packed cell volume	Male	44 (3)	45 (2)	32 (6)*
	Female	42 (2)	42 (2)	38 (7)
Spleen weight (g)	Male	0.9 (0.3)	0.7 (0.1)	0.6 (0.2)*
	Female	0.6 (0.1)	0.8 (0.1)	0.5 (0.1)

* Number in parentheses equals two standard errors.
* Significantly different from controls at $P \leq 0.05$.
** Significantly different from controls at $P = 0.06$.

weight, WBC, heterophils, or lymphocytes.

No sex effect was detected in SPFC means for either of the lead-exposed groups. In the field-exposed group, no differences ($P \geq 0.15$) in mean spleen cell viability or SPFC (Table 3) were detected between mallards that ingested lead and controls. For the lead-intubated group, numbers of SPFC (per gram and per 10^6 viable cells) were significantly lower than control values ($P = 0.03$), even though spleen cell viability ($P = 0.87$) was not altered (Table 3). A significant ($P = 0.04$) negative trend was found when SPFC means were ordered by treatment groups according to increasing tissue lead concentrations (Table 3). Regression of mean SPFC/10^6 cells against mean blood lead ($r^2 = 0.99$, $P = 0.03$) and liver lead ($r^2 = 0.99$, $P = 0.06$) concentrations (Fig. 1) demonstrated a negative relationship between lead exposure and antibody-forming cells. Robust regression analysis confirmed a significant ($P \leq 0.01$) negative relationship between the SPFC (per gram and per 10^6 cells) values and liver lead concentrations of individual birds; however, these regressions had poor predictive power due to the high variability of SPFC measurements between individual birds.

As expected, CY treatment resulted in significant depressions ($P \leq 0.01$) of both SPFC and WBC means compared to controls (Table 4). All white blood cell types were affected. Mean SPFC of male, CY-treated mallards were depressed to a greater extent than that of females ($P \leq 0.01$).

DISCUSSION

In the spring, when this study was conducted, female mallards may be less susceptible to immunologic effects of lead exposure than males. Male mallards exposed to lead by either natural ingestion or intubation had lowered WBC counts, but

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TABLE 3. Mean number of spleen plaque-forming cells (SPFC) measured in mallards that ingested lead while feeding naturally (field-exposed), mallards intubated with lead (lead-intubated), and controls (unexposed).

Parameter	Unexposed mallards	Field-exposed mallards	Lead-intubated mallards
Number of birds	12	55	13[a]
Log viable cells/gram spleen	8.79 (0.1)[b]	8.87 (0.1)	8.78 (0.1)
Log SPFC/gram spleen	6.67 (0.2)	6.65 (0.1)	6.22 (0.4)*
Log SPFC/10^6 spleen cells	3.87 (0.2)	3.78 (0.1)	3.43 (0.4)*

[a] Three additional birds died prior to testing.
[b] Number in parentheses equals two standard errors.
* Significantly different from controls at $P \leq 0.05$.

females appeared to be unaffected. Others have also noted that females were less affected by lead poisoning during prebreeding and breeding seasons (Bellrose, 1959; Jordan and Bellrose, 1951). Finley and Dieter (1978) reported that laying females had higher lead residues in bones with high medullary content (femur and sternum) than males or non-laying females. They attributed this to the mobilization of calcium for eggshell formation. We also observed that the females in our study stored more lead in their femurs and less lead in their livers than males. Physiologic differences in the absorption and accumulation of lead in tissues and bones may account for the divergence in immunologic effects between males and females. A difference in immunologic effects was also noted between male and female mallards treated with the known immunosuppressant, CY. Gonadotropic hormones are known to influence lymphoid tissue and function in mammals (White and Goldstein, 1972) and could possibly interact or interfere with the action of immunosuppressive agents.

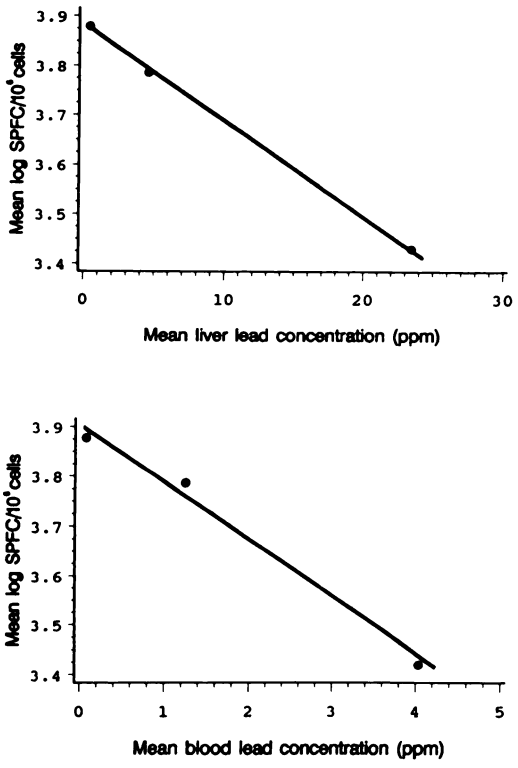


FIGURE 1. Linear regression of mean log spleen plaque-forming cells (SPFC) per million spleen cells versus mean liver lead concentration and mean blood lead concentration of unexposed mallards, field-exposed mallards, and lead-intubated mallards.

Mallards intubated with lead pellets showed more severe signs of lead poisoning and greater reductions in WBC counts and SPFC than mallards ingesting lead in the field. Lead-intubated birds also had higher blood and liver lead concentrations than the field-exposed group. Diet may have partially accounted for these differences. Diet has been shown to influence lead absorption and lead poisoning pathogenesis (Sanderson and Bellrose, 1986). Lead-intubated mallards were fed mostly small grains, which tend to increase the toxic effects of lead (Sanderson and Bellrose, 1986). In contrast, foods high in calcium and protein have been shown to ameliorate the effects of lead exposure (Koranda et al., 1979). In the Sacramento Valley, wild birds naturally shift to diets higher in pro-

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TABLE 4. Mean number of white blood cells and spleen plaque-forming cells (SPFC) of mallards treated with cyclophosphamide (CY-treated), and controls (unexposed).

Parameter	Sex	Unexposed mallards	CY-treated mallards
Number of birds	Male	6	4
	Female	6	4
Log viable cells/gram spleen	Male	8.85 (0.1)*	8.68 (0.1)
	Female	8.73 (0.1)	8.58 (0.1)
Log SPFC/gram spleen	Male	6.63 (0.3)	3.84 (0.5)*,**
	Female	6.69 (0.3)	5.21 (1.1)*
Log SPFC/10^6 cells	Male	3.84 (0.2)	1.16 (0.5)*,**
	Female	3.90 (0.3)	2.62 (1.0)*
Spleen weight (g)	Male	0.90 (0.4)	0.40 (0.1)*
	Female	0.60 (0.1)	0.20 (0.1)***
Total white blood cells/cu mm	Male	39,658 (15,546)	4,107 (2,772)*
	Female	27,106 (6,934)	4,739 (1,348)*

* Number in parentheses equals two standard errors.
* Significantly different from controls at $P \leq 0.05$.
** Significantly different from females at $P \leq 0.05$.
*** Significantly different from controls at $P = 0.06$.

tein in the spring (Miller, 1987), and our field-exposed mallards presumably made similar dietary shifts.

We have noted that lead-exposed mallards experienced higher rates of mortality in the fall than in the spring at the same study site at SNWR (NWHRC, unpubl. data). Jordan and Bellrose (1951) observed that females were less susceptible to the toxic effects of ingested lead during the spring breeding season. Seasonal changes in tissue lead accumulation, hormonal activity and diet of wild waterfowl could likewise influence the immunologic effects of lead exposure. If this study had been conducted in the fall, a greater decline in immunologic cell numbers might be expected, particularly in females.

Other studies on the immunologic effects of lead exposure in waterfowl have reached conflicting conclusions. Barga (1980) reported that subclinical lead poisoning of mallards by injection of lead acetate and oral dosing of lead pellets had little effect on the outcome of acute or persistent duck virus enteritis (DVE) infections, antibody production to DVE, or viral shedding. In a more recent study, however, Trust et al. (1990) found that hemagglutinating antibody titers to SRBC were suppressed in male mallards dosed with lead shot. In our study, the number of splenic antibody-forming cells (per 10^6 cells) of lead-intubated mallards was reduced nearly 60%; this reduction was strongly correlated to increasing liver lead concentrations. Depressions in SPFC counts were especially marked in mallards with high tissue concentrations of lead, but were not evident in field-exposed mallards. Depressions in WBC numbers of males occurred at much lower concentrations of tissue lead and were evident in both field-exposed and lead-intubated mallards. The declining trends evident in both WBC and SPFC means with increasing tissue lead concentrations provides support for the hypothesis that lead adversely affects the immune system of exposed waterfowl.

Unfortunately, the biological relevance of altered immunologic cell numbers in lead-poisoned birds is unknown. Information relating disease resistance to numbers of antibody-producing cells or white blood cells in mallards is lacking. We did not test the ability of these cells and others involved in disease resistance (such as macrophages, lymphocytes, natural killer cells) to perform their immunologic functions. In addition, the relationship between im-

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munologic function and disease resistance in lead-poisoned birds is obscured by many additional factors, including effects of lead on pathogens. For example, Wobeser (1986) observed that mallards heavily dosed with lead experienced lower mortality from avian cholera, even though lymphoid organs had atrophied. This finding was probably due to suppression of bacterial growth by the excessively high blood lead concentrations.

In summary, adult mallards, particularly males, exposed to lead pellets by ingestion and intubation in the spring of the year appeared to have lower numbers of certain immunologic cells; hence, they might be rendered more susceptible to some infectious agents. Like other pathologic effects of lead poisoning, immunotoxicity may be influenced by many factors, including physiologic condition, hormonal activity, and seasonal changes in diet.

ACKNOWLEDGMENTS

This research was supported by the U.S. Fish and Wildlife Service. Technical assistance was provided by F. Weekley, T. Eckhardt, J. Roitz, L. Sileo, and D. Goldberg. Lead concentrations were measured by M. Smith, National Wildlife Health Research Center. The authors are appreciative of the critical review provided by J. C. Franson, C. J. Brand, and A. Fairbrother.

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Received for publication 26 March 1990.

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