

environmental statement: proposed use of steel shot for hunting waterfowl in the United States. U.S. Gov. Print. Off., Washington, D.C. 276pp.

WALDRON, H.A. 1966. The anemia of lead poisoning: a review. *Brit. J. Industrial Med.* 23:83-100.

WOBESER, G.A. 1981. Lead and other metals. Pages 151-159 *In Diseases of Wild Waterfowl*. Plenum Press, New York. 300pp.

**Glen Sanderson:** The next report is "BLOOD PROTOPORPHYRIN FOR DETECTING LEAD EXPOSURE IN CANVASBACKS," by Christian Franson, research veterinarian with the Patuxent Wildlife Research Center.

## BLOOD PROTOPORPHYRIN FOR DETECTING LEAD EXPOSURE IN CANVASBACKS

**J. CHRISTIAN FRANSON<sup>1</sup>,** U.S. Fish and Wildlife Service, Patuxent Wildlife Research Center, Laurel, MD 20708

**G. MICHAEL HARAMIS,** U.S. Fish and Wildlife Service, Patuxent Wildlife Research Center, Laurel, MD 20708

**MATTHEW C. PERRY,** U.S. Fish and Wildlife Service, Patuxent Wildlife Research Center, Laurel, MD 20708

**JOHN F. MOORE,** U.S. Fish and Wildlife Service, Patuxent Wildlife Research Center, Laurel, MD 20708

Methods for determining lead exposure in live wild waterfowl require inexpensive analytical procedures that permit large sample sizes and sampling techniques that are easily performed under field conditions and that are not traumatic to the birds. Blood lead analysis and  $\delta$ -aminolevulinic acid dehydratase (ALAD) assay have been used (Dieter et al. 1976), but these procedures are relatively expensive and time consuming and they require considerable equipment, reagents and training. Ingested shot can be detected by fluoroscopy (Bellrose 1959), however, it requires bringing expensive portable equipment to the field or transporting birds to a laboratory. Other disadvantages involve extensive handling of birds and possible human exposure to radiation. Blood protoporphyrin (PP) determination is a new and simple

method for evaluating lead exposure and requires only a small blood sample.

Human exposure to lead results in elevated erythrocyte PP (Waldron and Stofen 1974), which binds with zinc and can be measured fluorometrically (Blumberg et al. 1977). Barrett and Karstad (1971) reported erythrocyte fluorescence in lead poisoned mallards (*Anas platyrhynchos*) and Canada geese (*Branta canadensis*). Roscoe et al. (1979) observed that erythrocyte fluorescence in mallards was due to metal-free PP, and so they modified a human hematofluorometer for use on duck blood. This screening method for lead exposure in waterfowl requires only a small unprocessed blood sample. It also is inexpensive, rapid, and requires very little training.

In 1983 we initiated a study to determine the value of blood PP for detecting lead exposure in canvasbacks (*Aythya valisineria*). Our objectives were to: (1) monitor blood lead and PP concentrations in penned canvasbacks that were administered lead shot; (2) estimate the length of time that blood PP remains elevated after shot ingestion; (3) determine the stability of blood PP under refrigeration (4°C) and at room temperature (20°C); and (4) evaluate the blood PP technique under field conditions in the Chesapeake Bay area.

## METHODS

The 17 canvasbacks (9 males and 8 females) used in the lead shot study were hatched at the Patuxent Wildlife Research Center from eggs collected in Manitoba in 1978. Ducks were housed in 5, 1.0 × 6.0 m outdoor pens with 3 or 4 birds per pen. Each pen was provided with a central 1.0 × 1.0 m water area about 0.5 m deep. Ducks were fed developer pellets (Beacon Feeds, Cayuga, NY 13034) *ad libitum* (partial analysis: 2,293 kcal/kg ME, 14% protein, 2.75% fat and <7.0% fiber). (Mention of commercial suppliers or trade names does not constitute endorsement by the Federal government).

On 4 January 1983 all ducks were radiographed to ensure the absence of ingested shot or other metal items. The birds were then bled by jugular venipuncture using 2 cc sodium heparinized Vacutainers® (Becton-Dickinson, Rutherford, NJ 07070). Packed cell volume (PCV), blood lead, and blood PP were determined for each sample. A stomach tube was passed into the proventriculus and each bird was dosed with 1 No. 4 lead shot (190-220 mg) pellet. Ducks were bled on post-dosing days 1, 3, and 6, and then weekly through day 49, and biweekly through day 77. Ducks were radiographed on the same schedule until the lead shot was either eroded or passed, or the duck died.

<sup>1</sup> Present Address: National Wildlife Health Laboratory, 6006 Schroeder Road, Madison, WI 53711.

Blood samples were placed on ice and transported within 1 hour to the laboratory where PCV was determined by the microhematocrit method. Since Roscoe et al. (1979) reported maximum blood PP levels occurred in samples which were oxygenated and refrigerated for 48 hours, we stoppered the Vacutainers® and stored them at 4°C. Two days later blood PP was measured with a hematofluorometer (AVIV® Biomedical Inc., Lakewood, NJ 08701) modified according to Roscoe et al. (1979). To determine when PP returned to pre-dose concentrations, a curve of the form  $b_0 + b_1(\text{day}) + b_2(1/\text{day})$  was fit to the data for each duck. The mean number of days required for the line to cross the pre-dose value was calculated from the individual curves. Similarly, a curve described by  $b_0 + b_1(1/\text{day}) + b_2(1/\text{day})^2$  was used to estimate how long blood lead concentrations remained elevated after lead exposure.

For comparison with hematofluorometer results, an ethyl acetone-acetic acid-HCL extraction method followed by photofluorometric assay for PP (Chisolm and Brown 1975) was conducted on 32 blood samples at the John F. Kennedy Institute, Baltimore, Maryland. Qualitative identification of proportions of zinc-bound and free PP was determined using fluorescence spectra for 10 samples (Chisolm and Brown 1979).

We tested the stability of PP at different temperatures with 16 heparinized blood samples at a wide range of PP concentrations (16 to 1,151 µg/dl at 48 hours). The samples were divided between 2 Vacutainers®, oxygenated by removing stoppers for several minutes, and stored under refrigeration (4°C) or at room temperature (20°C) for 45 days, or until clotting occurred. PP was measured daily with the hematofluorometer for 4 days, then less frequently for the remainder of the period.

Blood lead was determined by a method similar to that described by Hinderberger et al. (1981). One ml of concentrated nitric acid was added to the blood prior to placement in boiling water for 1 hour. After addition of 0.75 ml of 30% hydrogen peroxide, the sample was returned to the boiling water for another hour before dilution to 10 ml with 1% ammonium phosphate. Analysis was done with a Perkin-Elmer Zeeman 5000 atomic absorption spectrophotometer (AAS) equipped with a graphite furnace and utilizing the Zeeman effect for background correction. Furnace conditions were: wavelength, 217 nm; slit, 0.7nm; mode, peak area; drying temperature/time/ramp, 250°C/40 sec/30 sec; charring temperature/time/ramp, 950°C/30 sec/20 sec; atomization temp/time, 2,200°C/5 sec.

Samples were run using maximum power heating, a pyrolytically-coated graphite tube, a L'vov platform, argon flow of 50 ml/min during atomization, and a 3 sec integration time. Quantitation was done by comparing

sample peak areas as computed with a Perkin-Elmer data station with those of standards containing 10% nitric acid and 1% ammonium phosphate. Ten empty Vacutainer® tubes were rinsed with nitric acid and the acid was analyzed for lead by the same methods.

Two captive canvasbacks died during the study, and their livers were prepared and dry ashed according to Haseltine et al. (1981). Analysis was done with a Perkin-Elmer 5000 AAS equipped with a deuterium arc background corrector. Except for a 217.0 nm lead line wavelength, standard conditions recommended by the manufacturer were used.

The lower limits of reportable residues were 0.05 ppm for blood lead and 0.10 ppm for liver lead (wet weight). Recoveries averaged 96% from lead-fortified mallard blood and 87% from lead-fortified chicken liver; residues were not corrected.

During January and February of 1983 and 1984, 522 wild canvasbacks were bled at banding sites near Gibson Island, Maryland, on the Chesapeake Bay and Potomac River near Dahlgren, Virginia. Ducks were caught in wire traps baited with corn (Haramis et al. 1982) and transferred to modified poultry crates where they were held for 1 to 4 hours before bleeding. Exceptions occurred for several groups of birds which were caught in early evening and held overnight before sampling. Blood collection, handling, and analysis followed methods described earlier for the dosing experiment.

## RESULTS

Shot retention in the dosing study was 100% 3 days post-exposure with only 1 duck passing its shot between days 3 and 6. Retention was 65% on day 13, 18% on day 20, and on day 27 only 1 duck still retained lead shot. Two canvasbacks died during the experiment, 1 at day 13 and the other at day 15 post-dosing. The liver lead concentrations of these birds were 33 and 30 ppm (wet weight), respectively.

Means for pre-dose PP and PCV were 20 µg/dl and 49%, respectively, with no sex differences (Student's t-test,  $P > 0.05$ ;  $t = 0.84$ , 15 df (PCV) and  $t = 1.09$ , 14 df (PP)). Lead was detected in pre-dose blood samples from 12 of 17 ducks (mean = 0.09 ppm, range = 0.06-0.14 ppm). Blood lead reached a maximum concentration of 7.4 ppm 3 days post-exposure, and PP peaked at 652 µg/dl on day 6 (Fig 1). Mean PP returned to pre-dose concentration 42 days after lead shot administration (95% C.I. = 39-46 days) and blood lead was elevated for about 48 days (95% C.I. = 46-49 days).

Although blood PP concentrations were elevated in the lead-dosed canvasbacks when samples were analyzed

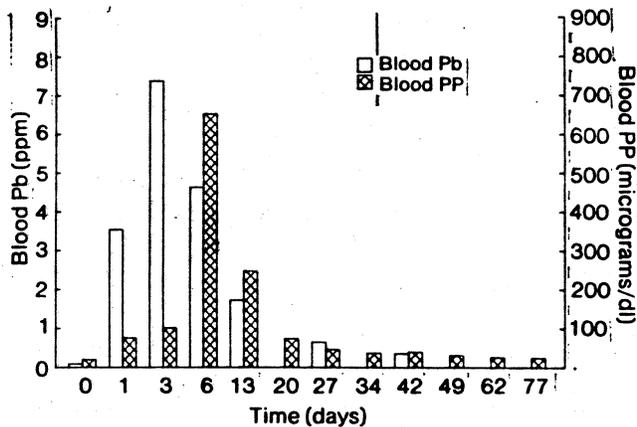


Fig. 1. Mean blood lead (Pb) and protoporphyrin (PP) in 17 canvasbacks dosed with one No. 4 lead shot.

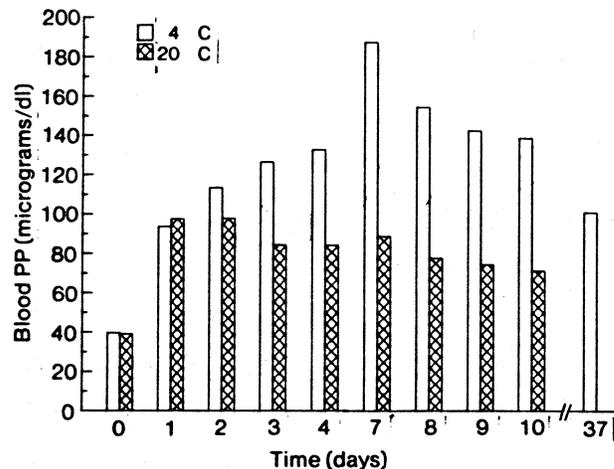


Fig. 2. Mean protoporphyrin (PP) over time in 10 canvasback blood samples held at 4°C or 20°C.

on the day of collection, they were even greater when oxygenated and refrigerated for 48 hours. For example, samples collected and read on day 6 post-dosing had a mean PP concentration of 93  $\mu\text{g}/\text{dl}$ . When oxygenated and refrigerated for 2 days, however, the same set of samples had a mean PP concentration of 652  $\mu\text{g}/\text{dl}$ .

When held at 20°C, the average clotting time for blood samples was 11 days post-collection, compared with 40 days for samples kept at 4°C. Fig 2 represents mean *in vitro* change in PP over time for 10 canvasback blood samples which remained unclotted for either 10 days at 20°C, or 37 days at 4°C. When blood samples were oxygenated and stored at 20°C, maximum PP was observed 2 days after collection. For refrigerated samples (4°C), PP peaked at 7 days post-collection. PP values on day 2 were similar for both temperatures. PP values for unexposed birds did not rise above 40  $\mu\text{g}/\text{dl}$  regardless of storage time.

While the extraction method provided PP values about 3 times higher than those read on the hematofluorometer (Fig 3), the correlation between methods was highly significant ( $r^2 = 0.92$ ,  $P < 0.0001$ ). Fluorescence spectra from 3 canvasback blood samples are shown in Fig 4. A small amount of zinc PP (emission wavelength = 593 nm) was present in blood from unexposed ducks, but the major component was free PP (emission maxima at 635 and 710 nm). With increased PP concentration, relative intensity of the zinc PP peak decreased.

Of 522 wild canvasbacks sampled, 9 (1.7%) had blood PP concentrations higher than 40  $\mu\text{g}/\text{dl}$ , and of these, lead was detected in 5 (mean = 0.40 ppm, range = 0.06-1.5 ppm). Blood PP concentrations were 31-40  $\mu\text{g}/\text{dl}$  in 48 samples (9.2%), with lead detected in 4 of 12

samples from this group (mean = 0.08 ppm, range = 0.07-0.11 ppm). In 18 random samples from the remaining 465 ducks (PP  $\leq 30 \mu\text{g}/\text{dl}$ ), blood lead was detected in 9 of the samples with a mean of 0.14 ppm and a range of 0.05-0.31 ppm. Trace amounts of lead were detected in 2 of 10 empty Vacutainers®.

There were no differences in hematocrits between birds held for 1-4 hours (PCV = 53.8%) and those held overnight (PCV = 53.2%; Student's t-test,  $P > 0.05$ ).

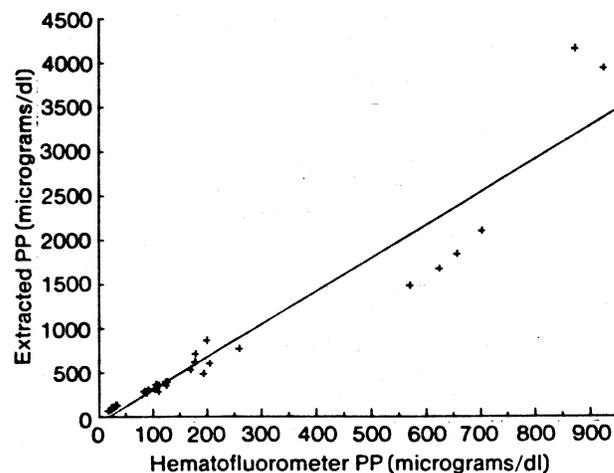


Fig. 3. Correlation between blood protoporphyrin (PP) concentrations determined on the hematofluorometer and measured microphotofluorometrically in acidified acetone extracts of blood.  $y = -67.01 + 3.711x$  ( $P = 0.0001$ ,  $r^2 = 0.919$ ).

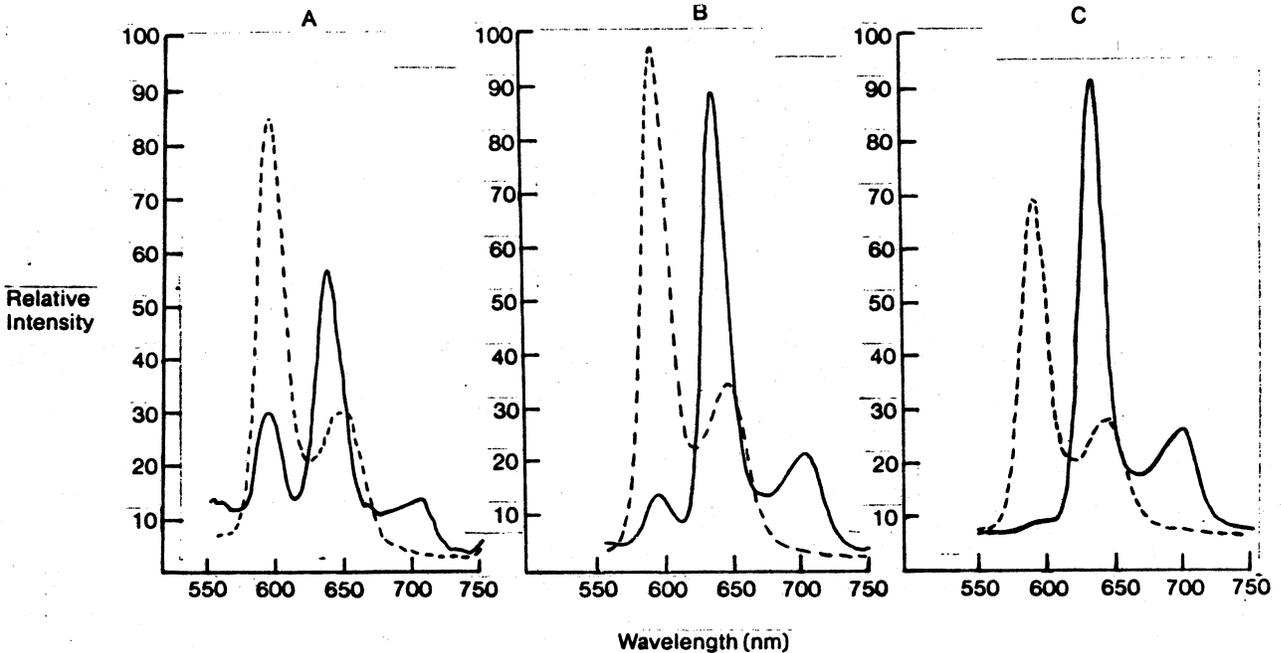


Fig. 4. Fluorescence spectra from 3 canvasback blood samples with hematofluorometer readings of 23 ug/dl(A), 103 ug/dl(B), and 571

ug/dl(C). Dotted line = standard of zinc protoporphyrin (590 nm) and free protoporphyrin (635 nm).

## DISCUSSION

According to Roscoe et al. (1979), blood PP concentrations  $>40 \mu\text{g/dl}$  in mallards are indicative of ingestion of the equivalent of at least 1 lead shot pellet 2 days to 1 month before testing. Our results with canvasbacks agree closely with Roscoe's findings. We detected elevated PP 1 day post-dosing and PP remained above pre-dose concentrations for up to 42 days thereafter. Although peak blood lead and PP concentrations were reached at similar times after dosing for both species, concentrations for canvasbacks were considerably higher in our study than reported for mallards by Roscoe et al. (1979). They reported maximum lead and PP concentrations of 1.30 ppm and  $129 \mu\text{g/dl}$ , respectively, whereas peak values in our canvasbacks were 7.4 ppm and  $652 \mu\text{g/dl}$ , respectively. A combination of factors probably contributed to these differences, including variable sensitivities of methods and instrumentation, slight differences in sampling times, and species variability.

In contrast to Roscoe et al. (1979), we observed that PP values for lead poisoned canvasbacks were elevated on the day of blood collection. Oxygenation and refrigeration of samples for 2 days resulted in considerably higher readings, but in blood samples collected and ana-

lyzed 6 days post-dosing, PP concentrations were  $>4$  times control values.

Minor differences occurred in PP readings between samples that were refrigerated and those stored at room temperature for 2 days before analysis (Fig 2). This suggests that PP determination may be useful even if refrigeration is unavailable. Although none of the blood samples held at room temperature clotted within 2 days of collection, it should be noted that mean clotting time was 11 days. Although PP concentrations in refrigerated samples continued increasing for 7 days, we do not advocate holding blood this long. The added increase in PP compared with analysis at 2 days probably does not justify risking loss of the sample to clotting. Also, if a duck is carrying ingested lead shot, the PP value after 2 days of refrigeration will be much higher than  $40 \mu\text{g/dl}$ , making it unnecessary to hold the blood longer.

Although the extraction method yielded PP concentrations about 3 times higher than hematofluorometer readings, we have confidence in our hematofluorometer results because of the close correlation between the 2 methods. Part of the reason for the disparity in the measurements is that the extraction method measures total PP, including zinc-bound PP, while the hematofluorometer measures only free PP. Since some zinc PP occurs in canvasback blood, one would expect a higher

value with the extraction method. In any case, when using the hematofluorometer to estimate lead poisoning, it is necessary only to demonstrate PP concentrations above some predetermined threshold in a given laboratory for a given species. It is not necessary to obtain the absolute value for PP unless one is attempting to relate blood PP concentrations to clinical signs of toxicosis.

We determined blood lead levels for all samples from wild canvasbacks with PP concentrations in excess of 40 µg/dl. Using 40 µg/dl PP as an indicator of lead shot exposure from 1 day to 1 month before testing, we determined that 1.7% of the study population had been exposed to lead shot. While only about half of these samples contained detectable quantities of lead in the blood (>0.05 ppm), Dieter et al. (1976) reported 16 of 95 (17%) canvasbacks trapped on the Potomac River near Chesapeake Bay in 1974 showed significant lead exposure. Blood from these ducks exhibited ≥50% reduction in delta-aminolevulinic acid dehydratase (ALAD) activity, an enzyme sensitive to lead, and a mean blood lead concentration of 0.263 ppm.

Several factors may account for the 10-fold difference between the canvasback exposure rates reported by Dieter et al. in 1976 and those in our study. The most logical explanation is that fewer canvasbacks ingested lead shot in the Chesapeake Bay area in 1984 than in 1974. Since canvasback was the primary duck hunted on the Chesapeake Bay until 1972, when canvasback shooting was banned, it would seem logical that hunting and lead shot deposition have declined in the Bay since that time. Also, hunters were required to use steel shot in most Chesapeake Bay waters from 1976 to 1981. Even though lead shot is now permitted, many hunters continue to use steel shot, resulting in less lead being available to ducks.

Another factor which could contribute to the higher exposure rates reported in 1974 is that ALAD activity is affected by lead for a longer time than is PP activity. Thus, Dieter et al. (1976) may have detected lead exposure in canvasbacks which occurred prior to their arrival on the Chesapeake Bay. Dieter and Finley (1978) reported that ALAD was inhibited by approximately 50% in mallards 2 months after dosing with 1 No. 4 lead shot, and even after 3 months inhibition was still approximately 30%. Our data for canvasbacks indicate that PP returns to pre-dose concentrations approximately 42 days after lead shot exposure. Although ALAD is a more sensitive indicator of lead exposure, perhaps PP is a better measure of actual physiological damage because it is affected later in the hemoglobin synthesis pathway.

*Acknowledgements.* We thank E. Derleth and D. McAuley for assistance in trapping and bleeding ducks, and V. Hoffman, D. Imler, and K. Halama for technical help. J. Chisolm consulted on protoporphyrin determina-

tion and provided microphotofluorometric analyses and fluorescence spectra. P. Geissler provided statistical advice, and J. Grillo assisted with data analysis.

## LITERATURE CITED

- BARRETT, M.W. AND L.H. KARSTAD. 1971. A fluorescent erythrocyte test for lead poisoning in waterfowl. *J. Wildl. Manage.* 35:109-118.
- BELLROSE, F.C. 1959. Lead poisoning as a mortality factor in waterfowl populations. III. *Nat. Hist. Surv. Bull.* 27(3):235-238.
- BLUMBERG, W.E., J. EISINGER, A.A. LAMOLA AND D.M. ZUCKERMAN. 1977. Zinc protoporphyrin level in blood determined by a portable hematofluorometer: a screening device for lead poisoning. *J. Lab. Clin. Med.* 89:712-723.
- CHISOLM, J.J., JR. AND D.H. BROWN. 1975. Microscale photofluorometric determination of "free erythrocyte porphyrin" (protoporphyrin IX). *Clin. Chem.* 21:1669-1681.
- \_\_\_\_\_ and \_\_\_\_\_. 1979. Micromethod for zinc protoporphyrin in erythrocytes: including new data on the absorptivity of zinc protoporphyrin and new observations in neonates and sickle-cell disease. *Biochem. Med.* 22:214-237.
- DIETER, M.P. AND M.T. FINLEY. 1978. Erythrocyte delta-aminolevulinic acid dehydratase activity in mallard ducks; duration of inhibition after lead shot dosage. *J. Wildl. Manage.* 42:621-625.
- \_\_\_\_\_, M.C. PERRY AND B.M. MULHERN. 1976. Lead and PCBs in canvasback ducks: relationship between enzyme levels and residues in blood. *Arch. Environ. Contam. Toxicol.* 5:1-13.
- HARAMIS, G.M., E.L. DERLETH AND D.G. MCAULEY. 1982. Techniques for trapping, aging, and banding wintering canvasbacks. *J. Field Ornithol.* 53:342-351.
- HASELTINE, S.D., G.H. HEINZ, W.L. REICHEL AND J.F. MOORE. 1981. Organochlorine and metal residues of waterfowl nesting on islands in Lake Michigan off Door County, Wisconsin, 1977-78. *Pest. Monit. J.* 15:90-97.
- HINDERBERGER, E.J., M.L. KAISER AND S.R. KOIRTYOHANN. 1981. Furnace atomic absorption analysis of biological samples using the L'vov platform and matrix modification. *Atomic Spectroscopy* 2:1-7.
- ROSCOE, D.E., S.W. NIELSON, A.A. LAMOLA AND D. ZUCKERMAN. 1979. A simple, quantitative test for erythrocytic protoporphyrin in lead-poisoned ducks. *J. Wildl. Dis.* 15:127-136.

WALDRON, H.A. AND D. STOFEN. 1974. Sub-clinical lead poisoning. Academic Press, New York. 224 pp.

## QUESTION AND ANSWER SESSION

**Bill Clark**, California Department of Fish and Game: Of the 17 ducks dosed with 1 No. 4 lead shot, you say 15 survived? All of them followed your curve up to above 40 units of protoporphyrin?

**Christian Franson**, U.S. Fish and Wildlife Service: Yes. All of them had more than 50 units of protoporphyrin in the blood. On the bar graph I showed you there was a mean of those birds and, at the peak, blood lead went to about 7.5 ppm.

**Glen Sanderson**, Session Leader Illinois Natural History Survey: The last paper in this Subsession is "HEAVY METAL CONCENTRATIONS OF DUCK TISSUES IN RELATION TO INGESTION OF SPENT SHOT," by Frank Fisher and Shelley Hall. Frank will present the paper.

## HEAVY METAL CONCENTRATIONS OF DUCK TISSUES IN RELATION TO INGESTION OF SPENT SHOT<sup>1</sup>

**FRANK M. FISHER, JR.**, Department of Biology, Rice University, P.O. Box 1892, Houston, TX 77251

**SHELLY L. HALL**, Department of Biology, Rice University, Houston, TX 77251

Lead poisoning of waterfowl from dissolution of ingested lead shot has been recognized as a major problem in wildlife management for almost a century. The accumulation of lead in liver, bones, and other tissues has been shown to correlate with the presence of lead shot in the gizzard (Longcore et al. 1974, Anderson 1975, White and Stendell 1977, Scanlon et al. 1980, Calle et al. 1982). The toxic effects of lead on waterfowl also have been extensively documented (Bellrose 1959). Lead

shot contains considerable amounts of antimony as a hardening agent and arsenic which aids in forming spherical shot. In addition, the shot is sometimes plated with nickel and copper to increase resistance to deformation. It is unknown whether these additional elements accumulate in tissues upon dissolution of lead shot in the gizzard, or if there is a toxic interaction of these elements that may affect waterfowl.

Other than measuring delta-aminolevulinic acid dehydratase activity there is no way to immediately assess the lead burden of an individual bird without sacrificing it for tissue analysis. Even detection of ingested shot by fluoroscopy indicates nothing about past exposure or whether the detected shot is steel or lead. Since arsenic exposure can be assessed by elemental analysis of hair and fingernails, we anticipated that elemental analysis of feathers could be used to assess ingestion of shot without sacrificing the bird. While the concentration of lead in liver tissue appears to be an indicator of short term exposure (Longcore et al. 1974), the amount in bones is considered to be more representative of long term exposure (White and Stendell 1977). If any of the other elements present in lead shot are accumulated by waterfowl, they too might be detected in bones. The purpose of this study was to: 1) determine if any of the additional components of lead or steel shot are accumulated upon shot ingestion as indicated by their concentrations in bones and feathers, and 2) determine the feasibility of heavy metal analysis of feathers to assess exposure to spent shot.

## MATERIALS AND METHODS

Specimens were collected during the 1981-82 and 1982-1983 hunting seasons from brackish marshes in Chambers County, Texas. Wings and gizzards were frozen until analysis. The presence of ingested shot in the gizzard was determined by X ray analysis (Torrex 150 X ray inspection system, Torr X ray Corp. 65-75 KV, 3 ma, 30 sec exposure), followed by visual inspection.

Tissues from specimens collected during the 1981-1982 season were prepared for inductively coupled plasma emission spectrometric analysis (ICP) following EPA guidelines (1979). Tissues were not ashed prior to acid digestion to avoid volatilization and decreased recovery of any of the elements (Dahlquist and Knoll 1978). An ulna from each specimen was dissected from the wing, broken in half and the marrow discarded. All tissue was scraped from the bone with a glass microscope slide. Wings showing evidence of shot damage were discarded. Three to six primary feathers were washed extensively in distilled water to remove as much con-

<sup>1</sup> Originally published in Bull. Environ. Contam. and Toxicol. 35:163-172.