Project Number: BB-JXR-1985

POST-MORTEM PB DETECTION IN BIRDS

A Major Qualifying Project Report

submitted to the Faculty

of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

by

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Date: February 28, 2007

Approved:

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Abstract

One objective of this study was to determine the accuracy of the LeadCare® System in testing the lead (Pb) content of post-mortem body fluids in birds. Birds brought to the Tufts Wildlife Clinic that died or were euthanized were frozen then thawed before necropsy. Post-mortem fluid tests were comparable to pre-mortem blood samples. Another objective was to compile the Pb levels of all patients tested from 2002-2006. Elevated Pb levels were detected in species not typically associated with Pb exposure.

Acknowledgements

We would like to thank the following people and organizations for their help throughout this project:

Mark Pokras, D.V.M.
Jill Rulfs, Ph.D.
Karen Donahue CVT
Robin Shearer
Scott and Debbie McCullough
Diane and Doug Tarbet
Theodore Crusberg, Ph.D.

Tufts Wildlife Clinic
Worcester Polytechnic Institute

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Chapter 1 Background

A significant amount of research has been done on the effects of lead on avian species around the world. It has become an important environmental concern not only because lead (Pb) is toxic to animals, but also because of the implications lead in the environment has for humans. It has been shown that Pb poisoning in children has behavioral side-effects along with possible mental retardation over a long period of exposure (Gasana and Chamorro, 2002). This background chapter provides a brief overview of Pb in the environment, the extent of Pb toxicity in birds, physiological effects in birds, and various methods of blood Pb testing.

Lead in the Environment

Unlike for other toxic materials, no evidence of a mechanism has been noted to date of re-absorption of Pb by the environment. It may react with certain salts to form other Pb compounds (De Francisco, Troya, and Aguera, 2003). However, these compounds are then ingested by wildlife; they do not disappear from the environment. In 1990, $3x10^6$ tons of Pb were used in production of items such as batteries and cable sheathing (Miller *et al.,* 1990; Global Pbnet.org). Although many policies have been put in place in order to phase out the use of Pb, particularly from gasoline and house paint, it remains a large public health issue (Gasana and Chamorro, 2002).

One reason to study animals with Pb poisoning is to be able to identify sites where they ingested Pb to locate potential areas of Pb toxicity for humans. The U.S. Fish and Wildlife Service recommend the use of study samples from Pb-poisoned birds and marking their locations of origin (Friend, 1985). In this way problem areas that are identified can be studied more thoroughly in order to begin environmental rehabilitation procedures.

Extent of Lead Poisoning in Birds

Lead, particularly within the past 20 to 30 years, has been a significant concern among veterinarians and conservationists within the bird populations in America. Many birds are dying from Pb toxicity, including taxa not typically associated with having elevated Pb levels such as raptors and songbirds. This is currently not widely recognized as an environmental issue. Many of these birds are not consuming Pb through Pb shot and fishing weights, but through their

normal diet. It is estimated that 3,000,000 birds die in America from Pb poisoning every year (De Francisco *et al.*, 2003).

According to a study completed in western Canada from 1986-96, 10% of the 372 eagles tested for Pb had levels high enough to be considered Pb poisoning (Wayland *et al.,* 1998). Five percent more had levels that were elevated. Of the 10% with Pb poisoning, 28% of the golden eagles (*Aquila chrysaetos*) tested had high (lethal) Pb levels, and 13 % of the bald eagles (*Haliaeetus leucocephalus*) tested had high Pb levels. These were mainly in adult birds. Birds were collected from wildlife agencies in the area and from raptor rehabilitation organizations over the 10 year period. The authors took blood samples from the birds that were alive, and tissue samples from necropsies.

A recent study in England identified a high percentage of mute swans (*Cygnus Olor*) with Pb in their body (Perrins, Cousquer, and Waine, 2003). However, sources of Pb such as fishing weights were not found in the bird. Out of 1276 birds tested, over 60% had elevated Pb levels (1.21umol/L[24.85ug/dL]). This is an enormous percent, and Pb shot or Pb fishing weights probably does not account for all of this.

Physiological Effects of Lead

There are numerous physiological changes recognized in birds with high Pb levels. Burger and Gochfeld (1993) completed an analysis of behavioral side-affects after herring gull nestlings (*Larus argentatus*) were given small doses of lead. The authors looked for changes in behavioral development compared to the control group. They found that the chicks with Pb in their diet tended to not differentiate between individuals as parents and that they also were slower to move towards food (Burger & Gochfeld, 1993). These behavioral symptoms are very significant in young birds and could easily lead to the chick's death in the wild.

Other physiological symptoms include problems with normal organ function, severe organ damage, and general emaciation and anemia (Mateo *et al.,* 2003). One organ affected by Pb is the gizzard which may not function properly if it contains Pb fishing weights or Pb shot. Both the kidneys and the liver can have severe damage due to Pb toxicity. These organs function to filter Pb out of the system. Emaciation can be caused by a number of factors including problems with the digestive tract which leads to less intake of food, and neurological effects because the animal has difficulties finding and ingesting food.

Studies have also discovered mechanisms for some of the molecular effects of Pb poisoning. It has been found that Pb inhibits blood δ-aminolevulinic acid dehydratase (ALAD) activity while it elevates protoporphyrin (PP) concentration (Henny *et al.,* 1991). In order to sustain levels of hemoglobin in erythrocytes, ALAD is required in the biosynthetic pathway of heme (Mamet *et al.,* 2001). Without heme synthesis, due to the inhibition of ALAD, anemia develops. This is a common symptom in Pb poisoning. By inhibiting the enzyme ALAD, Pb is also elevating PP levels because ALAD takes the iron from PP in heme synthesis. Without ALAD activity, PP accumulates in the erythrocyte because there is no feedback mechanism in place (Mamet *et al.* 2001).

Blood Lead Testing

Since 1942, lead blood level testing has evolved to be more precise and use smaller volumes of blood (Searle *et al.,* 1973). Today, there are a variety of test methods available with which to monitor blood Pb levels in wildlife, including atomic absorption spectroscopy, graphite furnace atomic absorption spectrometry, aminolevulinic acid or protoporphyrin blood levels, and anodic stripping voltammetry. These methods vary in price and difficulty of use, as well as in their prevalence of use in the veterinary community.

Two indirect methods of determining blood Pb concentrations are available. Exposure to Pb causes an increase in the presence of urinary δ-aminolevulinic acid (ALA). Therefore, after being mixed with a number of reagents that separate the ALA molecules from other molecules, urine samples can be tested for their absorbency at 553 nm and compared to a standard curve to ascertain the Pb concentration. This method requires 15+ minutes of preparation and procedure, as well as access to a spectrophotometer (Tomokuni and Ogata, 1972). Similarly, because Pb exposure increases the concentration of protoporphyrins (PP), the PP concentration can also reveal Pb concentration. However, a 1979 study of Pb poisoned mallards showed that raised PP levels could not be determined until two days after the blood had been drawn (Roscoe and Nielsen, 1979). The two-day wait, in addition to the need for a spectrophotometer, suggests that testing for PP levels is an impractical method for clinical use.

Atomic absorption spectroscopy (AAS) was first introduced in 1955 (Rains, 1969). Due to such characteristics as its high sensitivity and versatility with a number of elements, AAS became a major analytical technique in less than 10 years (Lewis, 1969). In the case of urine

tests, AAS was an improvement on an earlier method that utilized colormetric determination and was often inaccurate (Willis, 1962). The AAS method requires converting a sample, such as blood or urine, into atomic vapor by use of a flame and then analyzing the resulting vapor for light absorbency at a wavelength typical of the element being tested (Walsh, 1969). These wavelengths change according to the materials used, such as the lamp or cathode through which the sample travels (Rains, 1969). In graphite furnace atomic absorption spectrometry (GFAAS), the flame used for AAS is replaced with a constant-temperature furnace. This method eliminates the nonuniformities caused by combustion that sometimes interfere with the accuracy of a reading (Woodriff *et al*., 1977).

The atomic absorption methods remained the most commonly used until the anodic stripping voltammetry (ASV) method was developed. This method was first recommended for use with blood and urine in 1970. Using as little as 50 μl of blood, it works by concentrating the Pb ions of a sample onto a carbon electrode and then reversing the current, which strips the Pb ions from the electrode and creates a peak current that is proportional to the concentration of Pb in the sample (Searle *et al.*, 1973). The 1973 study by Searle et. al compared determined Pb concentrations in blood and urine samples of Pb-poisoned and normal patients using the AAS and ASV methods. Of 224 samples analyzed, the ASV results correlated well with the AAS results, with no significant deviation between the two. Additionally, advantages of ASV over AAS were noted, including increased sensitivity and lower cost. For clinical use, ASV seemed to be the more practical method.

In 1976, a rapid micromethod was developed for ASV that used hydrochloric acid to lyse blood cells, rather than perchloric acid, and it ultimately eliminated a step that could decrease the accuracy of the ASV method (Morrell and Gridhar, 1976). Elimination of this step also made the micromethod easier to perform and less time-consuming. The idea of the micromethod most likely became the basis for the development of the LeadCare® Analyzer, a clinical machine for testing blood Pb levels.

The LeadCare® device, manufactured by Environmental Science Associates (ESA Inc), needs only 50 μL of blood to give a reading within three minutes. It uses anodic stripping voltammetry with a hydrochloric acid reagent to determine the concentration of Pb in the sample. Results are accurate at a minimum level of 1.6 μg/dl and will give a numerical result for levels up to 65 μg/dl. Levels above that are given only as "high." (LeadCare® User Manual). A 2003

study compared blood Pb results from blood samples of various wild bird species tested with the LeadCare® device and with GFAAS. Results showed that, compared to GFAAS, the LeadCare® analyzer was statistically accurate for purposes of clinical diagnosis (Reinhagen and DeNezzo, 2003). Unlike the ALA, PP, AAS or GFAAS methods, the LeadCare® device is practical for use in a clinical laboratory where a diagnosis of Pb toxicity needs to be made as soon as possible in order for treatment to begin.

Due to its low cost and portability, the LeadCare® device has become a common testing method for blood Pb screening not only in pediatric offices, but in veterinary clinics, as well. The Wildlife Clinic at the Tufts Cummings School of Veterinary Medicine, the San Diego Zoo, and the Falcon Medical Research Hospital in Saudi Arabia, are three examples of veterinary clinics that employ this device. Daily medical care at these facilities has included frequent blood level testing of avian species.

Chapter 2 Project Description

There were two primary objectives for this project: to determine the ability of the LeadCare® device to detect lead in post-mortem body fluids and to analyze archival data for trends in patients from the Tufts Wildlife Clinic tested positive for lead. Each objective had several goals:

- Detecting Lead in Post-Mortem Fluids
    - Test for the effect of shelf or refrigeration storage on blood and fluid samples, before and after being mixed with the LeadCare® reagent.
    - Test pre-mortem blood samples for lead concentration using the LeadCare® device.
    - Obtain and test post-mortem fluids for presence of lead.
    - Compare test results of pre- and post-mortem samples.
- Analyzing Archival Lead Test Results
    - Compile four years of patient and lead test data into a spreadsheet.
    - Display locations of the origins of patients that tested positive on a map.

The methods used to complete these objectives and achieve the goals are explained in detail in the next chapter.

Chapter 3 Methods

The experimental methods we used for our study are described in this chapter. Protocols include those for obtaining fresh blood samples and post-mortem body fluids, for determining the effects of storage on samples, and for developing an archival analysis with a geographic distribution. Additionally, a total of nine blood samples were used to test for storage accuracy. Seven post-mortem fluid samples taken from birds were tested for Pb content. There were 302 patients who had been tested for Pb since 2002 with files available for analysis.

Blood Pb Testing

Peripheral venous blood samples were taken from live animals through a vein and were placed in lithium heparin tubes. Less than 1mL was taken from each animal. Blood was drawn from all animals to be euthanized including species other than birds. Using the provided 50µL pipette and 200µL pipette tips, 50µL of the sample was taken from the heparin tube and placed in the LeadCare® reagent tube. (This amount needed to be precise in order to obtain an accurate reading.) The mixture was swirled in the reagent tube in order to thoroughly mix. The sample was allowed to sit for 2 to 3 minutes in order to complete the acid reaction. According to the LeadCare® manual, the reagent tube could then be placed in the fridge for up to seven days before testing or it could be tested at that time. When ready to test, 50µL of the mixture from the reagent tube was placed in the well on the LeadCare® sensor strip. The machine took three minutes to read the sample. Readings were given in micrograms per deciliter (µg/dL). Each result was recorded along with the date, patient species, patient ID, and who tested the sample. For more detailed instructions written specifically for use of the LeadCare® device, please see Appendix D.

Effects of Storage on Accuracy of Samples

The project schedule did not allow for constant vigilance over blood samples taken from subjects. For this reason, the effects of various storage methods were tested for their accuracy. A set of nine pre-mortem samples (already mixed with heparin to prevent clotting) were tested for lead concentration immediately after the blood was drawn, and after 2-7 days in the refrigerator, with and without having been mixed with the reagent before being placed in the

refrigerator. Samples were also tested for accuracy without being placed in a refrigerator, to determine how long samples could sit at room temperature and still yield accurate results.

Obtaining Post-Mortem Fluids

Post-mortem cadavers were frozen at -20°F and then thawed at room temperature for 3-5 days to allow fluids to collect in the body cavity. Birds were then dissected according to a standard avian necropsy protocol. The bird was placed in a prone position on the exam table, wings pulled back, exposing the ventral tract. Using a surgical scalpel, an incision was made along the sternum. The layers of skin and muscle were separated and peeled away from the sternum. The sternum was removed in order to access the organs beneath it. Once the body cavity was opened, attempts were made to collect body fluids to test for Pb concentration. Photographs of open cavities were taken and labeled according to the location where fluid was found and/or for which it was searched. These pictures can be found in Appendix B. Fluids were mainly found cranial to the liver and around the heart or below the organs in the dorsal area of the body cavity after opening. These fluids were tested using the same protocol for blood samples above.

Archival Analysis

Since the purchase of the LeadCare®System in 2002 by Tufts Wildlife Clinic, over 300 blood samples from various species of animals have been tested for lead. The records kept included the patient species, patient ID, date tested, and test results. These were all placed into a spreadsheet. Along with all data we also examined the patient records and recorded the locations where each animal was found and any symptoms the patient exhibited upon arrival. This information is located in Appendix C.

Map Creation

In order to display specific geographic origins of patients that tested positive for Pb, we used Google Earth ©. This free program allowed us to search global satellite imagery for specific locations, and then place and display customized markers as data points.

Maps were constructed using geographic origins as recorded in patient files as data points. Origins were organized into folders by Pb level. For example, all results between 0 and 1.4

μg/dl were in one folder because these data points were potentially inaccurate. Next, all results from 1.41 to 9.9 were combined into one folder and could be displayed together and separately from the other data groups. Remaining folders were classified as 10 – 19.9, 20 – 29.9, 30 – 39.9, 40 – 49.9, 50 – 59.9, and > 60 μg/dl. The last folder included any result that registered as "HI."

The town of origin was entered as "XXXX, MA" unless the patient was brought to the clinic from a different state. Most records did not have origins more specific than the town. Subsequently, markers could not be placed on particular roads or bodies of water. The marker was placed at the point the program chose for the city. If more than one marker was at the same location in the same folder, one of them would be shifted slightly to avoid overlap. Therefore, the position of the data marker only corresponds to the town of origin. Markers were also colored to distinguish levels from each other when displaying more than one folder at a time.

Once the folders had been created, they were displayed by clicking the check mark on and off in the box next to the folder. All of the folders could be displayed simultaneously, or one could be displayed independently without interference from the others.

Chapter 4 Results

This chapter presents the results of all experiments and analysis done in this study. The effects of storage on sample accuracy, the comparison of accuracy between pre- and post-mortem samples, and the collective data from the archival analysis are included. The results of the retrospective study contain the distribution of Pb levels, species, symptoms, and geographic origins of the patients.

I. Pre-mortem vs. Post-mortem

Storage Accuracy of LeadCare® Reagent

The first study completed involved the testing of the storage accuracy of the mixed reagent from the LeadCare® system. Refer to Methods for a description of mixing procedure. Nine samples were tested the day they were mixed and then saved and re-tested 6-7 days later. The results are located in Table 4.1 below.

Table 4.1: Storage accuracy of Pb mixed blood samples

Test Sample	***Pb Result 1st Test (µg/dL)***	***Pb Result 2nd Test (µg/dL)***	***Difference (ug/dL)***	Fridge length (days)
1	6.5	5.2	-1.3	7
2	34.8	33.6	-1.2	7
3	14.8	13.4	-0.9	7
4	24.0	25.2	1.2	6
5	7.8	5.7	-2.1	6
6	7.3	8.1	0.8	7
7	2.8	2.2	-0.6	7
8	3.1	2.6	-0.5	7
9	6.1	7.0	0.9	7
		Average Difference	-0.4	
		Standard Deviation (σ)	±1.2	

The average difference from 0 days to 7 days in the fridge was -0.4 µg/dL. Three out of 9 samples increased with an average of 1.0 µg/dL. Six out of 9 samples decreased with an average of -1.1µg/dL. The LeadCare® system claims the machine has a variability of ±3.0µg/dL. Therefore, the effects of lengthier storage times on sample results proved to be insignificant in terms of true differences in values. The standard deviation of ±1.2 µg/dL falls well within this

range. A more detailed chart of original data is located in Appendix A.1. Figure 4.1 demonstrates a graphical representation of the differences in sample tests. The dashed line represents the first test and the solid line shows the value of the second test. There is almost no difference between the two.

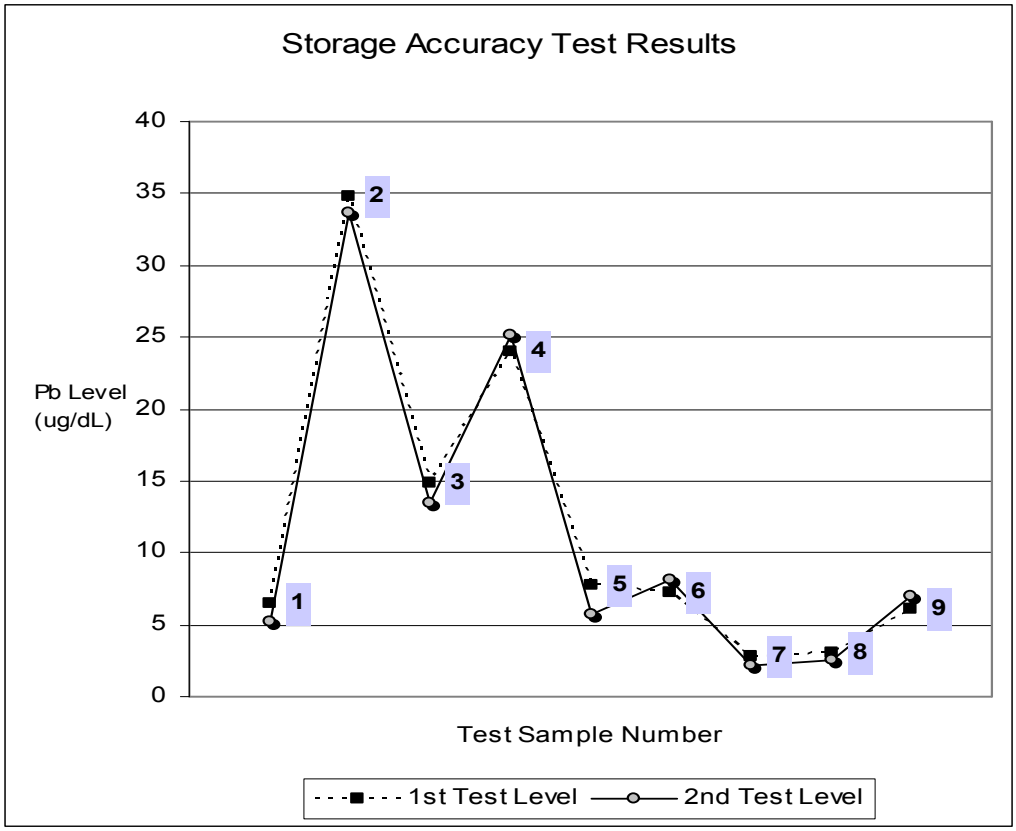

Figure 4.1. Storage accuracy results

Pre-mortem vs. Post-mortem Results

One of the main goals of this study was to examine the suitability of the LeadCare® test for analyzing post-mortem avian body fluids. Throughout our 4 month study, 13 cadavers were saved, however, only 7 samples were able to make it to the point of extracting post-mortem body fluids. The 7 samples received are detailed in Table 4.2, including both pre and post-mortem values. More detailed information is located in Appendix A.3.

Table 4.2. Results of pre and post-mortem Pb testing

Sample Number	*Pre Pb Level (ug/dL)*	*Post Pb Level (ug/dL)*
1	65	65
2	3	5.7
3	9.2	4.7
4	30	9.4
5	9.4	10
6	5.8	4.7
7	1.5	2

Figure 4.2 below is a graphical representation of the pre and post mortem Pb test results of each individual. The pre-mortem blood Pb level is represented by the solid line, and the post-mortem body fluid Pb level is represented by the dashed line. In many of the samples the difference was very small.

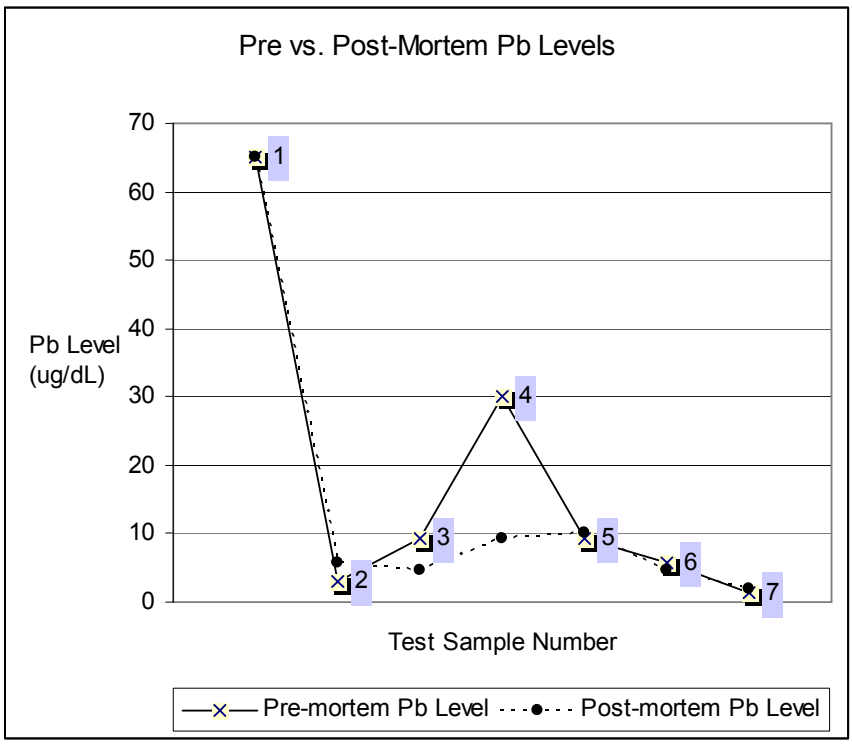

Figure 4.2. Pre and post mortem results

The average difference from pre to post-mortem samples was -3.2µg/dL. The standard deviation of the difference between pre and post-mortem Pb levels of the seven samples was ±8.0µg/dL. This high value was due to one outlier. The standard deviation without the outlier was ±2.4µg/dL. This data along with deviations is located in Table 4.3.

Table 4.3. Standard deviations of differences between pre and post-mortem test results

Patient Number	***Difference (pre-post, ug/dL)***	***Deviation from σ_1***	Deviation from σ_2
1	0	8.0	2.4
2	2.7	5.3	0.3
3	-4.5	3.5	2.1
4	-20.6	12.6	-*
5	0.6	7.4	1.8
6	-1.1	6.9	1.3
7	0.5	7.5	1.9
Average difference:	-3.2		
Standard deviation of all 7 (σ_1):	±8.0		
Standard deviation w/out 4 (σ_2):	±2.4		

*Note: This sample was inaccurate and the fluid obtained contained bile from the digestive system.

II. Retrospective Analyses

An analysis of all previous Pb tests recorded at the clinic was completed. This encompassed all tests since the purchase of the test kit in 2002. A number of items were analyzed including:

- Animal tested (i.e. aquatics, raptors)
    - Species tested (i.e. Canada goose, Common loon)
- Blood Pb result distribution
- Geographic location of animal tested
    - Different severities of Pb levels at different locations
- Symptoms of animal tested

Type of Animal Tested

A total of 302 animals have been tested for Pb at the Wildlife Clinic since 2002. Of these, 52.6% were aquatic birds with a total of 159 tested. Raptors such as hawks and falcons formed

16.9% of the animals tested. Pigeons, doves, and other birds, comprised 15.9% of the animals tested. These values are represented in Figure 4.3 below and can be located in Appendix C.2. There were also a number of other species of animals such as a raccoon and a llama. A complete list of species tested can be found in Appendices C.7.

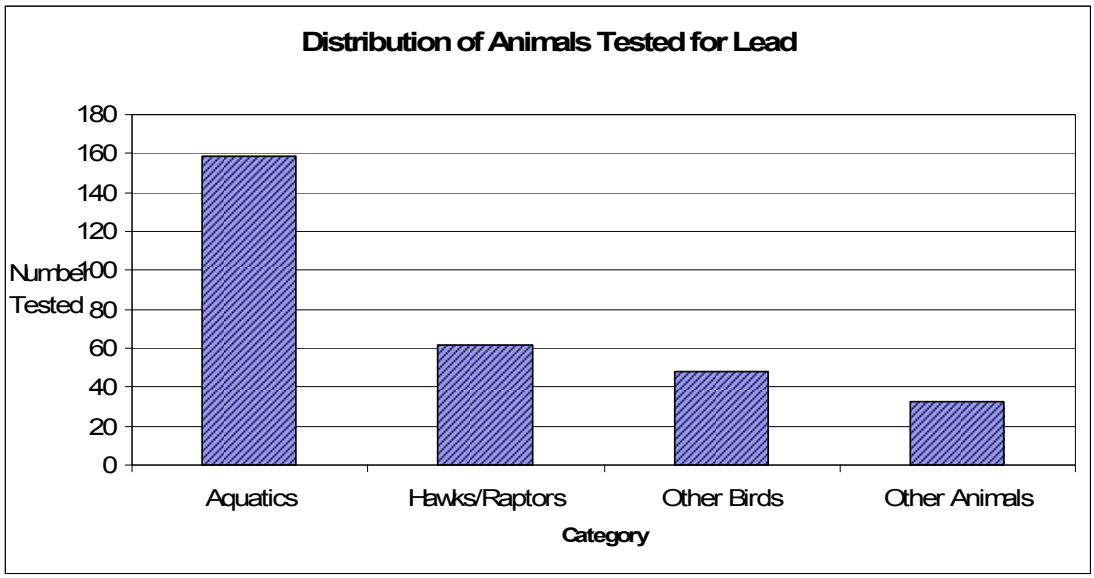

Figure 4.3. Bar graph of taxa distribution of animals tested for Pb

Blood Test Result Distribution

The variation in the distribution of the Pb level of the animals tested was also examined. As can be seen in Figure 4.4, most (68.9%) fell below the 10.0µg/dL range. This 10.0 µg/dL value is important to note because this is the level at which humans are treated for Pb toxicity. A large percentage fell above that range at 31.1%. Below 10.0µg/dL, the levels were split into two sets. This was done because the LeadCare® System is not accurate below 1.4µg/dL. Therefore, we assumed those tests were not accurate and the actual Pb level was impossible to determine at such low values.

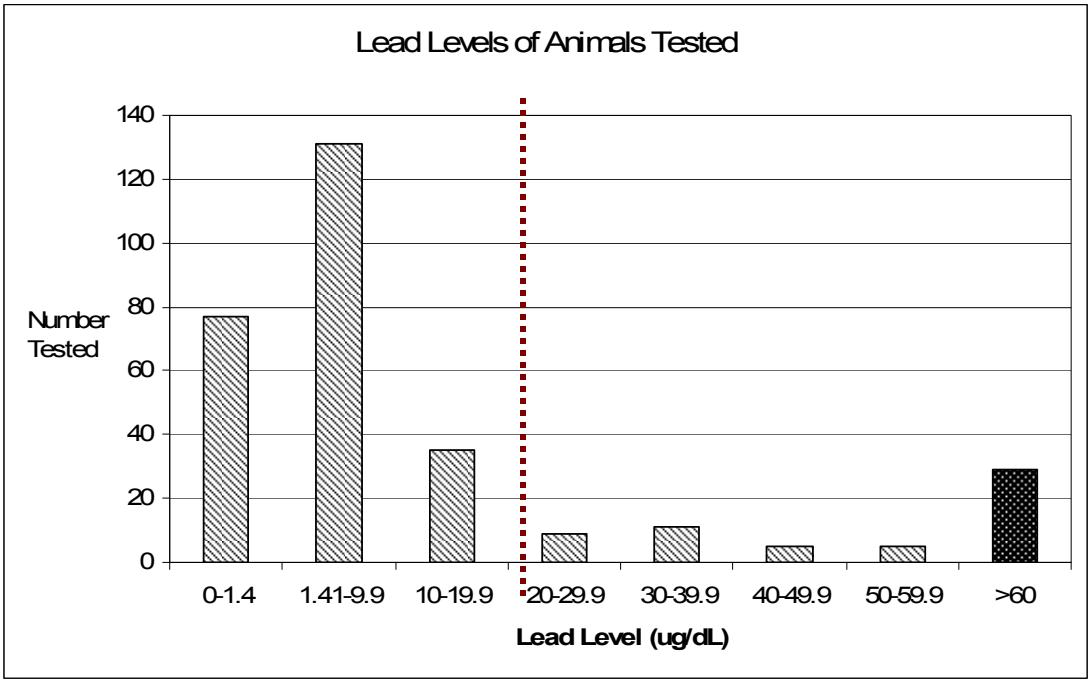

Figure 4.4. Distribution of Pb levels of all animals tested

Most animals with Pb levels at or above 20.0µg/dL at the Wildlife Clinic are given chelation treatment. Of all animals tested for Pb toxicity at the clinic, 19.5% were above this clinical cutoff. This cutoff is noted in the chart (Fig. 4.4) as a dashed line. This 19.5% value included 59 animals in the past four years at the clinic that had been tested for Pb toxicity. The data for this chart are located in Appendix C.1.

Aquatics Distribution

Each group of animals was examined in more detail to show a distribution of their Pb levels. Most of the groups had the majority of animals tested with Pb levels below 10.0µg/dL. The second highest was usually a reading of "HI". In this case, the students or veterinarians accurately diagnosed the symptoms of the animal as lead poisoning.

Of waterfowl tested, 63.7% had Pb levels below 10.0µg/dL. There was also a high percentage (21.4%) over 20.0µg/dL. The percentage with "HI" levels was 9.4%. A graphic display of these data is located in Figure 4.5. The original data can be found in Appendix C.3.

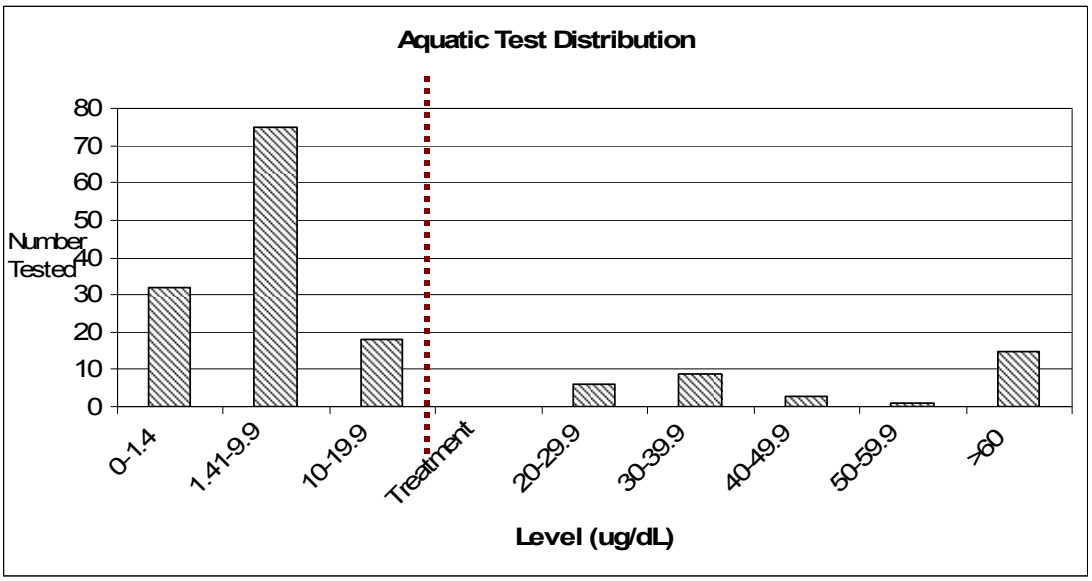

Figure 4.5. Distribution of test results of aquatic bird species

Hawks/Raptors Distribution

Of hawks tested, 74.5% had Pb levels below 10.0µg/dL. There was a much lower percentage (5.9%) of birds with levels over 20.0µg/dL with only one bird having a level over 50.0µg/dL and none with "HI" levels. A graphical display of this data is located in Figure 4.6. The original data can be found in Appendix C.4.

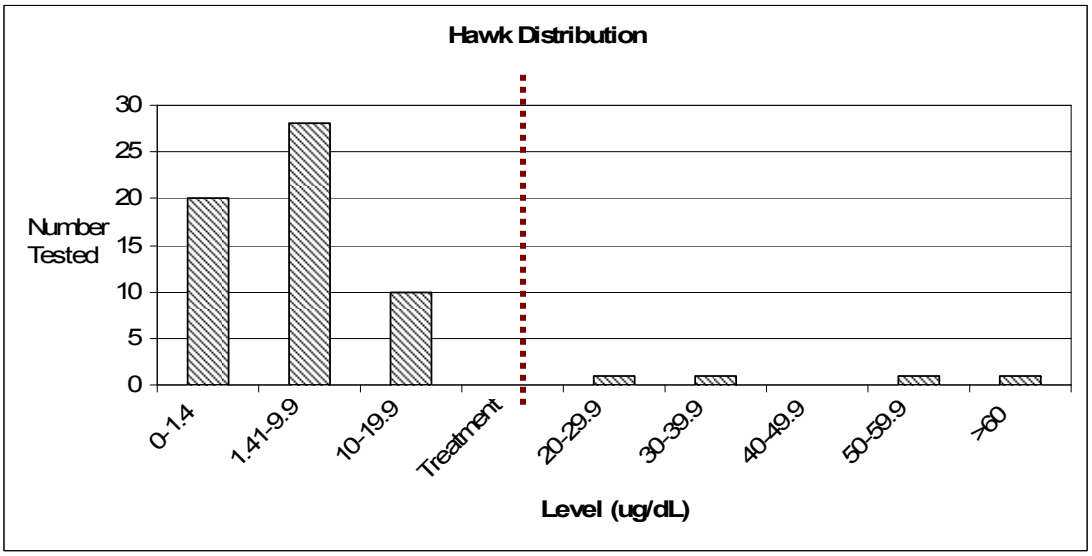

Figure 4.6. Distribution of test results of land predators

Other Birds Distribution

Only 45.8% of other land birds tested had Pb levels below 10.0µg/dL. A much higher percentage, 41.7%, had levels above 20.0µg/dL. A graphical display of this data is located in Figure 4.7. The original data can be found in Appendix C.5.

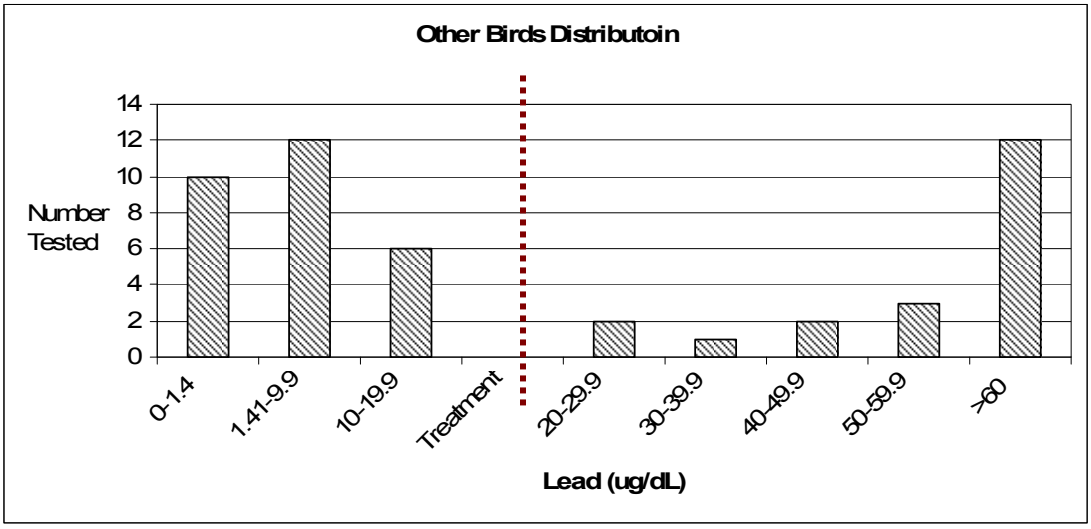

Figure 4.7 Distribution of test results of other birds

Other Animal Species Distribution

Out of the 33 other animal species tested, only 2 had levels above 10.0µg/dL. However, 48.8% of patients tested had positive tests, meaning their blood Pb level was above 1.4µg/dL. This demonstrates that some Pb did exist in their bodies. A graphical display of this data is located in Figure 4.8. The original data can be found in Appendix C.6.

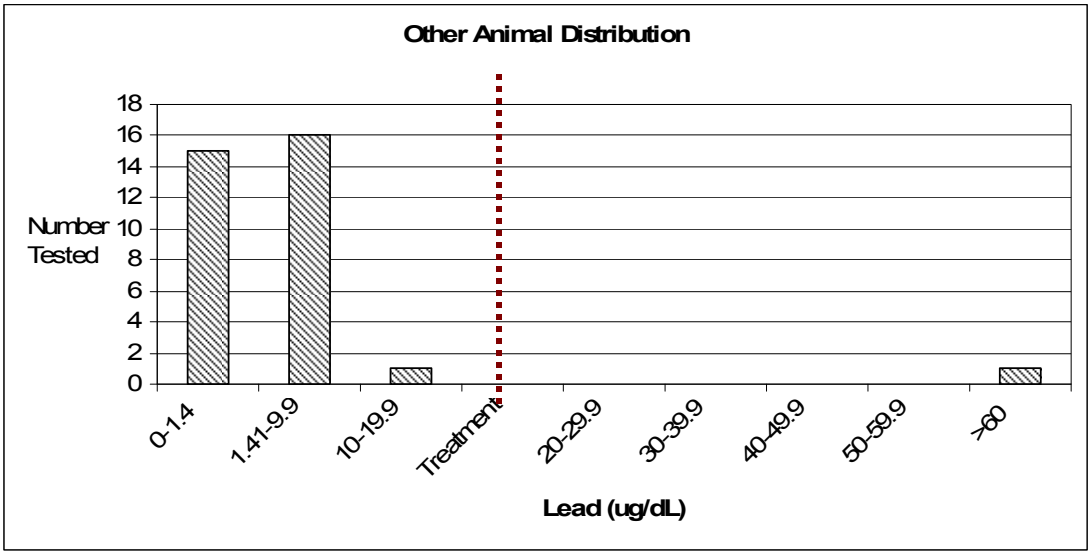

Figure 4.8. Distribution of test results for other animal species

Retrospective Patient Outcome

Out of 188 patients with available results, less than half lived to be transferred or released. Only 11.7% died in house, but 46.3% were euthanized while in the hospital. Patient results include animals with all levels of Pb intoxication, but the majority of which had levels lowers than 20 μg/dL. Figure 4.9 below displays patient results in bar graph form. Figure 4.10 is more specific, breaking results down by Pb level range.

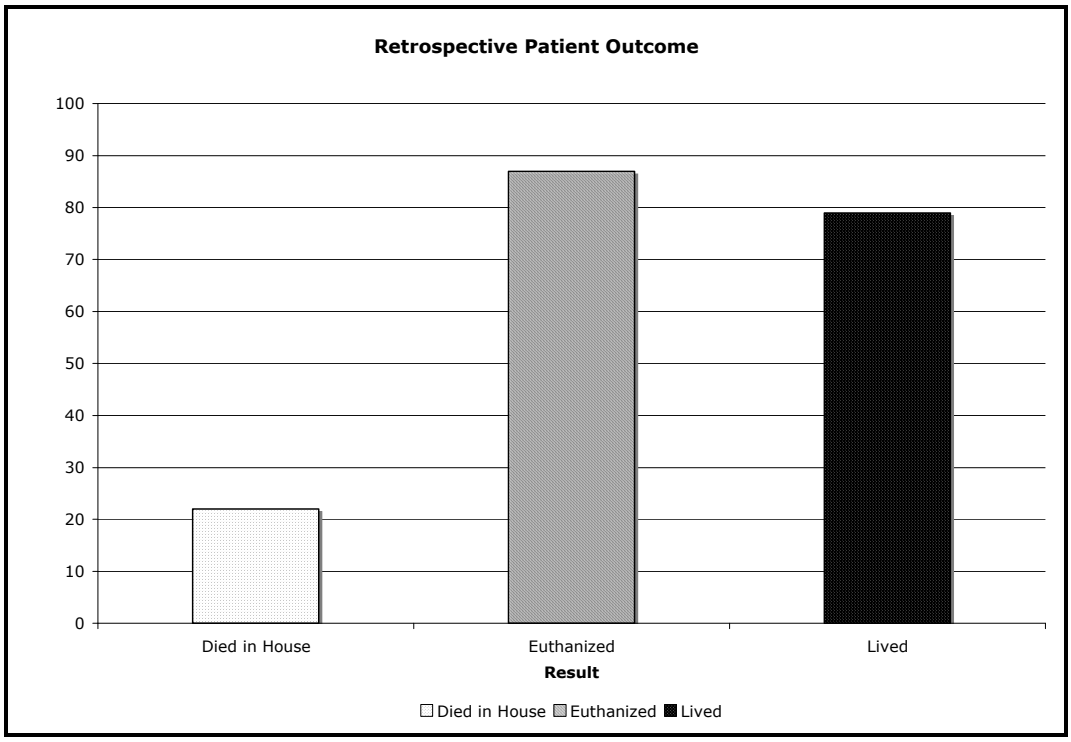

Figure 4.9. General outcome of patients tested for Pb

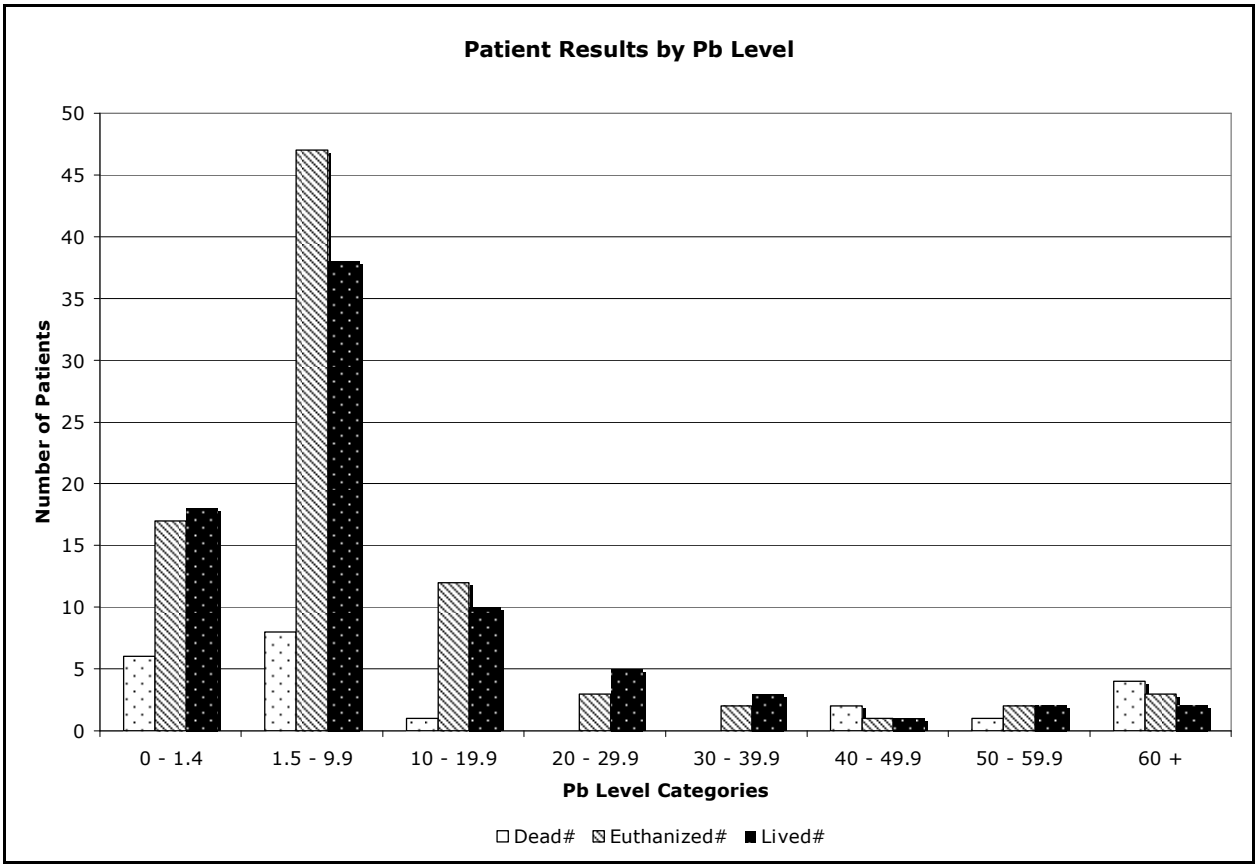

Figure 4.10. Outcome of patients tested for Pb according to level

Patient Origins

Records showed that there were 91 different origin points for patients. These points were towns or cities within Massachusetts. Only 39 of these points (43%) were origins for more than 1 patient. Points with significant numbers of patients were Boston with 6, Westboro with 5 and Worcester with 17. The remaining 36 points had from 2 - 4 patients. The map below (Fig. 4.11) shows the geographic range of origins with >1 patient. The point farthest west is Springfield, MA, and the farthest southeast is West Barnstable, MA. Labels for the three cities are positioned just directly to the right of their origin. A cross indicates a point of origin for two or more patients.

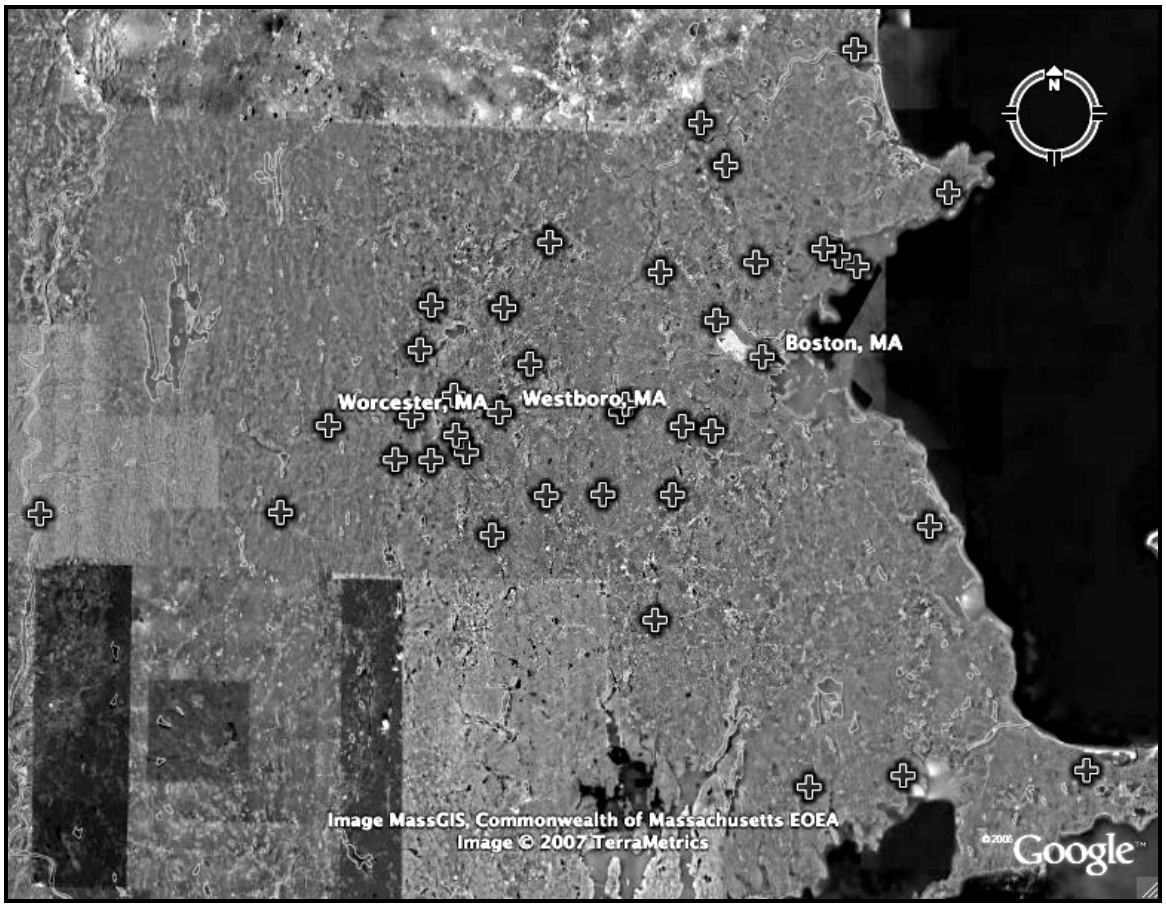

Figure 4.11. Map of origins with >2 patients

When all of the origins are viewed, the geographic range extends as far south as Nantucket. However, the majority of all patients originated from an area between Boston and

Worcester, as can be seen in Figure 4.12 below. In Figure 4.12, a dark cross indicates a point of origin for one or more patients.

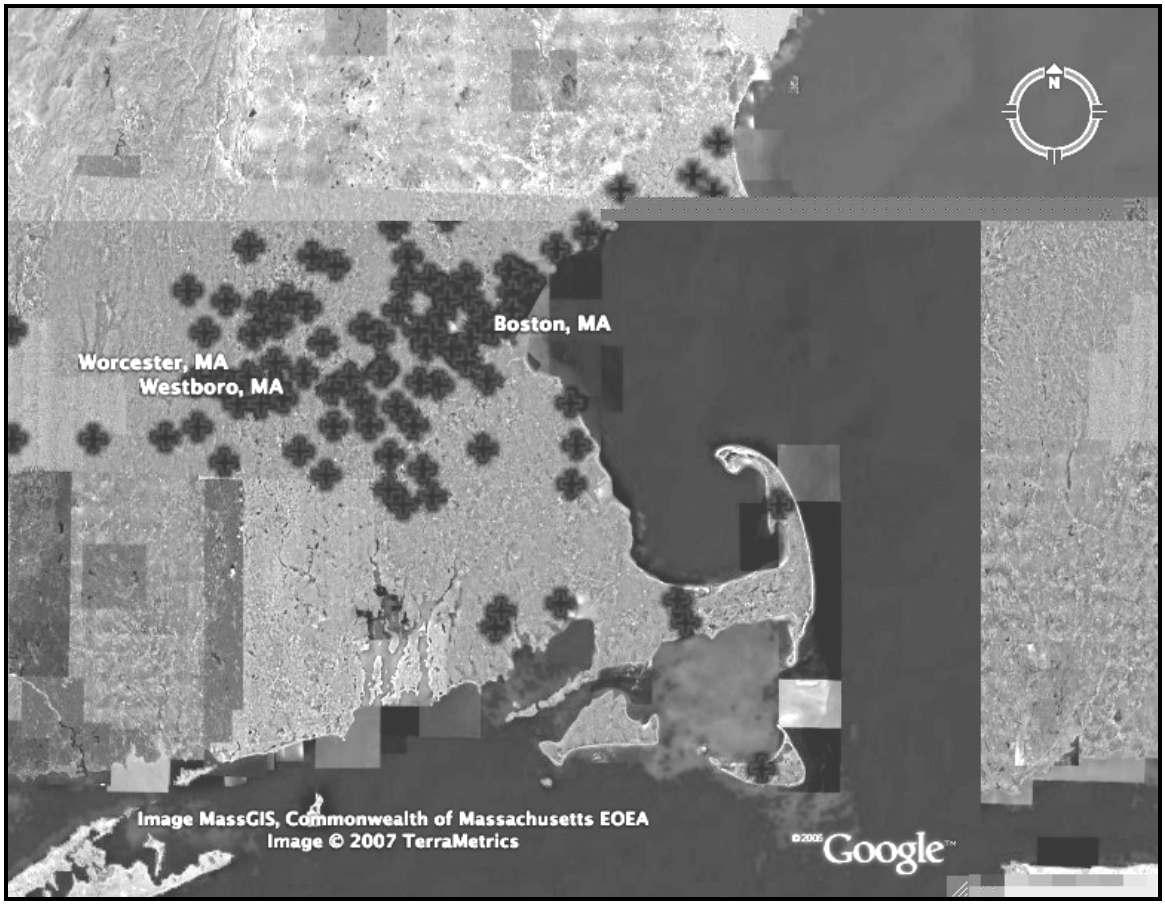

Figure 4.12. Map of all origins of patients

Patient Symptoms

Of all of the available files, 189 listed symptoms of the patients. The symptoms diagnosed for each patient were placed into one of five categories:

- Emaciation and/or dehydration
- Fractures, wounds, and/or trauma
- Depression (including quietness or an inability to stand or fly)
- Neurological (such as tremors)
- The presence of lead objects (such as fishing gear or shot)

Most patients had more than one of these symptoms. Very few exhibited no symptoms. Emaciation and/or dehydration and fractures, etc., were the two most commonly found

symptoms, with 51% and 48% of patients exhibiting such symptoms respectively. The third most common symptom, depression, was observed about half as often in only 23% of patients. Much less frequently observed were neurological symptoms (6%) and the presence of Pb objects (4%). Figure 4.13 displays these results.

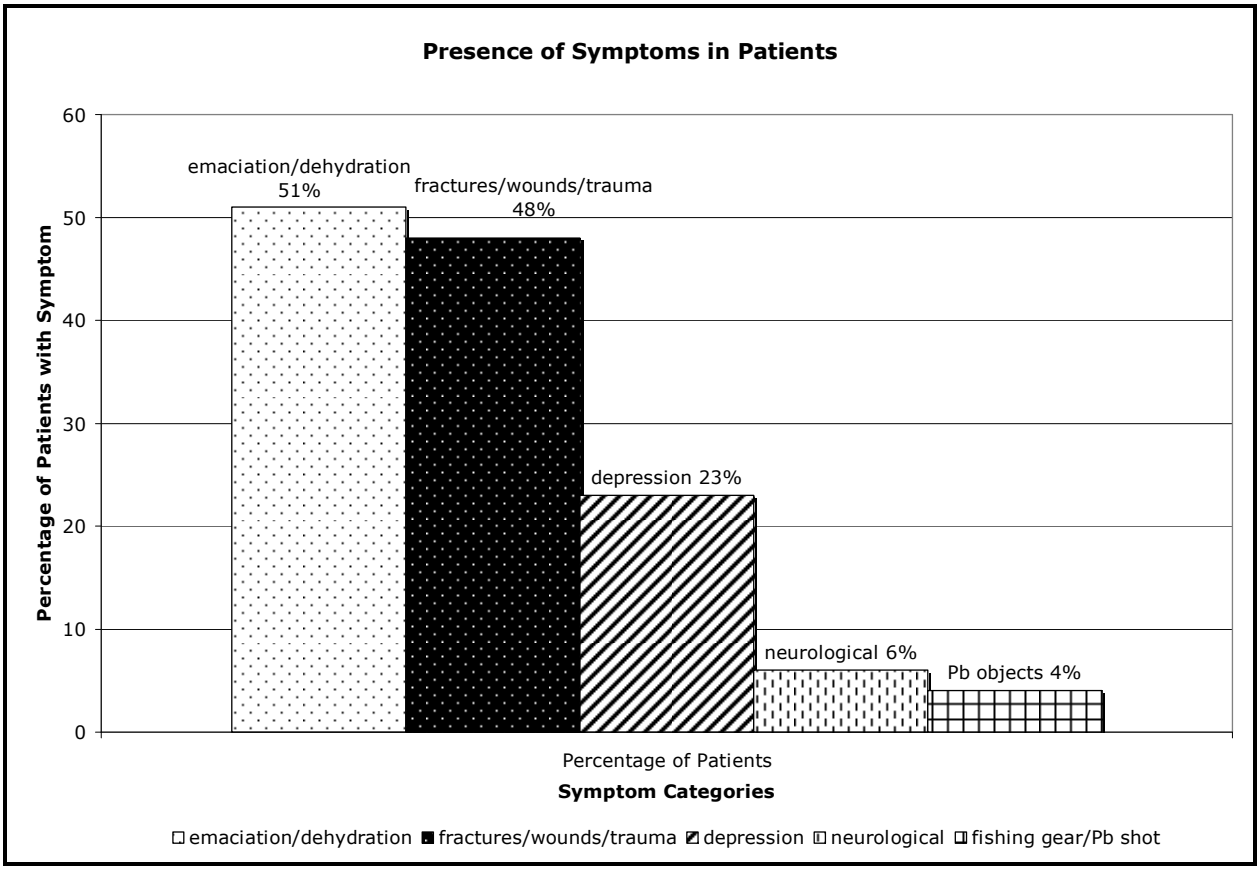

Figure 4.13. Distribution of symptoms in patients tested for Pb toxicity

Chapter 5 Discussion

The LeadCare® System is the one used by Tufts Wildlife clinic for their tests. This test is meant for use with children, however it has been proven accurate in avian Pb testing by Reinhagen & DeNezzo (2003). They compared results between the LeadCare® System and the graphite furnace atomic absorption spectrophotometry method and found the two methods to be statistically equal. Therefore this system was deemed accurate and appropriate for other experimentation. This project studied the limitations of the LeadCare® System in testing of body fluids as opposed to blood samples.

I. Pre-mortem vs. Post-mortem

Due to time limitations, samples needed to be saved for testing up to a week after they were taken. Therefore storage accuracy was tested before the pre to post-mortem testing could begin.

Storage Accuracy of LeadCare® Reagent

Through this accuracy experiment, it was determined that the LeadCare® Manual recommendations were correct saving blood samples from birds. This was equivalent to less than 24 hours of blood sitting at room temperature in heparin tubes, mixed with the reagent and sitting at room temperature for less than 48 hours after this, or mixed with the reagent and placed in the refrigerator for up to 7 days. Every test completed fell within the accuracy range of the machine (±3.0µg/dL).

Pre-mortem vs. post-mortem

According to the statistical tests completed, the LeadCare® Test was accurate in determining post-mortem body fluid levels in avian species. There was one outlier, Test #4, that was far outside of the ±3.0µg/dL range of the test. This was due to a difficulty in receiving fluids from the body. In attempting to obtain body fluids, bile was taken with other general fluid and therefore this did not give an accurate reading of the Pb level in the bird. Without this outlier, the standard deviation from the average was ±2.4µg/dL, which is within the accuracy range of the machine.

In the clinic, an animal with a Pb level above 20.0ug/dL is usually diagnosed as having lead toxicity and subsequently undergoes chelation treatment. In the tests, only the outlier described above would have changed the classification of the bird's Pb level from when it was alive to after death. All of the other tests remained in the same category as noted in the charts (i.e. 1.4 to 9.9µg/dL, 10 to 19.9µg/dL, etc.). Also, none of the other tests switched from a classification requiring treatment (above 20.0µg/dL) to a level lower than 20.0µg/dL and not requiring treatment.

This demonstrates that this Pb test can accurately be used in the field to test deceased birds for Pb levels in their body. A value obtained would be an accurate estimate of the Pb level of the bird in its blood before it died. Potential implications of this include the ability to determine environmental areas of concern for high Pb levels.

Future work with this project can include a field study of those birds obtained by the Seabird Ecological Assessment Network (SEANET) in Massachusetts and brought to the clinic for post-mortem analysis. SEANET notes the location the bird was found, and therefore a determination of Pb levels in the environment in that location can be inferred. A potential further study would involve testing the soil Pb content in the area.

By analyzing pre vs. post-mortem Pb levels in birds we were able to determine the accuracy of the LeadCare® Test with body fluids as opposed to blood. Now that this accuracy has been determined, this can be used on a broader scale in testing and conducting a solely post-mortem analysis. Results from these tests can then be used to determine areas of environmental Pb concern in the country.

II. Retrospective Analyses

There were limitations on the retrospective study of patients who were tested for Pb. Our results were based only on the charts that were archived at the wildlife clinic, and so we did not have data for patients who had their blood tested but who were being treated at other facilities. Furthermore, we did not have complete results for every patient due to missing information in the chart such as location of origin and symptoms.

Type of Animal Tested

Throughout our analysis of the different species of animals tested for Pb toxicity, it was very interesting to note the variety of species that had positive results (>1.4ug/dL). It was also interesting to note the variety that tested above a level of 10.0ug/dL (the treatment cutoff in humans). There were also a number of hawks that had Pb levels above 10.0ug/dL including bald eagles, red-tailed hawks, turkey vultures, among others. Those tested with a "HI" Pb level included a squirrel, a great horned owl, pigeons, geese, swans, American crow, loons, and a gannet. Although many of these are waterfowl and had this high level due to ingested lead shot or lead fishing weights, the others had not.

From this, questions can be raised as to where these animals are ingesting Pb, and why they are ingesting a large amount. Is there an environmental sink for Pb in Massachusetts from remnants of the leaded gasoline era, or is this a new issue that needs to be addressed?

These are important discussions that need to be addressed in Massachusetts and around the country. If Pb is clearly being ingested by animals in the environment, then that same Pb could pose a threat to humans.

Blood Test Result Distribution

Along with the wide distribution of species tested positive for Pb, there was a high percentage of those who tested above 10.0ug/dL. A total of 31.1% of all animals tested at the wildlife clinic were at a level that would require treatment in humans, particularly children. A note of interest is that not all of these animals were tested because they exhibited symptoms of Pb toxicity. Some were tested for various research projects, as a last resort for a possible cause of the symptoms health decline, or as one of the many blood tests that are completed in a thorough analysis of a patient when it arrives.

According to the Wildlife Clinic's standards 19.5% of the patients were at a clinical Pb toxicosis level and were then eligible for treatment as being above 20.0ug/dL. This is also a very high percentage and demonstrates a clear problem of Pb in Massachusetts.

Patient Outcome

The results of patient outcomes (died, euthanized, lived) were used to make conclusions about survival rates for patients that were tested for Pb levels. We estimate that the clinic

survival rate (42%) of these patients is comparable to the overall survival rate for all patients treated at the clinic. However, amongst the patients whose results were in the higher brackets (40 μg/dl and up) only 27.7% of patients survived and were relocated or released. In these categories, there is also an increase in the percentage of animals that died in house before they could be treated or given euthanasia, from 11.7% to 38.8%. Therefore, the patients with higher levels of lead toxicity had a reduced survival rate as compared to those with lower levels. We anticipate that a larger survey of Pb toxicity in wildlife would give similar results.

Patient Origins

There were very few locations (cities or towns) that were the origin for more than 4 patients. Of the three, Worcester (17 patients) and Boston (6) are considered to be major urban areas. Westboro (5) is a suburban area. Both Worcester and Westboro are located in close proximity to the clinic, so it's possible that more patients did not come in from more distant areas, like Boston, because they were treated at other locations. Nonetheless, the origins of Pb tested patients span a wide geographical range. Lead toxicity, therefore, is not confined to one specific location or type of location. Sources of lead vary. For instance, it can be from highway runoff or fishing gear, hence why it is possible for an animal to become toxic in urban, suburban and rural areas. The majority of patients were brought in from the area between Worcester and Boston, which also agrees with the possibility that people are bringing animals in for treatment because they live close to the clinic. This area of Massachusetts is also varied in terrain, but contains many small lakes and rivers where waterfowl are likely to live and people are likely to fish or hunt.

Patient Symptoms

Patient files were reviewed for the symptoms that accompanied patients who were tested for Pb levels. Unexpectedly, fishing gear and lead shot were found in only 4% of patients. This indicates that lead poisoned wildlife are getting lead from other sources besides solid lead objects. In fact, lead objects were found in more patients with lower lead levels (< 10) than the higher levels. (Yet, this could also be due to the much greater proportion of cases on file between 1.5 and 9.9 μg/dl.) Overall, the most common symptom was emaciation and/or dehydration, followed by some type of wound, fracture and/or trauma. At 6%, very few patients also showed

neurologic symptoms, the effect that it most frequently associated with lead toxicity. Depression was the third most common symptom at 23%. Combinations of these symptoms may be an indicator of the presence of lead in a patient, although a few patients exhibited no symptoms at all, meaning that there is no exact formula for determining if a patient should be tested for lead poisoning.

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Appendices

Appendix A: Storage and Post-mortem Data

Table A.1. Storage Accuracy – Original Data

Date	Patient Name	Patient ID Number	Test Results	Difference	Fridge length
9/1/2006	Herring Gull	W061061	6.5ug/dL		5 days
9/3/2006	"	"	5.2ug/dL	-1.3	7 days
8/31/2006	Turkey Vulture	W061084	34.8ug/dL		
9/6/2006	"	"	33.6ug/dL	-1.2	7 days
9/7/2006	"	"	14.8ug/dL		New sample - chelated
9/12/2006	"	"	13.4ug/dL	-0.9	7 days
9/12/2006	Rock Dove	W061124	24ug/dL		
9/18/2006	"	"	25.2ug/dL	1.2	6 days
9/16/2006	Red-tailed Hawk	W061183	7.8ug/dL		
9/22/2006	"	"	5.7ug/dL	-2.1	6 days
9/18/2006	Snapper	W061186	7.3ug/dL		
9/24/2006	"	"	8.1ug/dL	0.8	7 days
9/18/2006	Pigeon	W061180	2.8ug/dL		
9/24/2006	"	"	2.2ug/dL	-0.6	7 days
9/20/2006	Rock Dove	W061124	3.1ug/dL		
9/27/2006	"	"	2.6ug/dL	-0.5	7 days
9/22/2006	Herring Gull	W061210	6.1ug/dL		
9/29/2006	"	"	7.0 ug/dL	0.9	7 days

Table A.2. Storage Accuracy Standard Deviation Difference

Table 3.2. The difference from the standard deviation.

Average Difference	**-0.4**
Standard Deviation (σ)	**1.2**
Sample	**Deviation from σ**
1	0.1
2	0.0
3	0.3
4	0.0
5	0.9
6	0.4
7	0.6
8	0.7
9	0.3

Table A.3. Pre-Post mortem results – Original Data

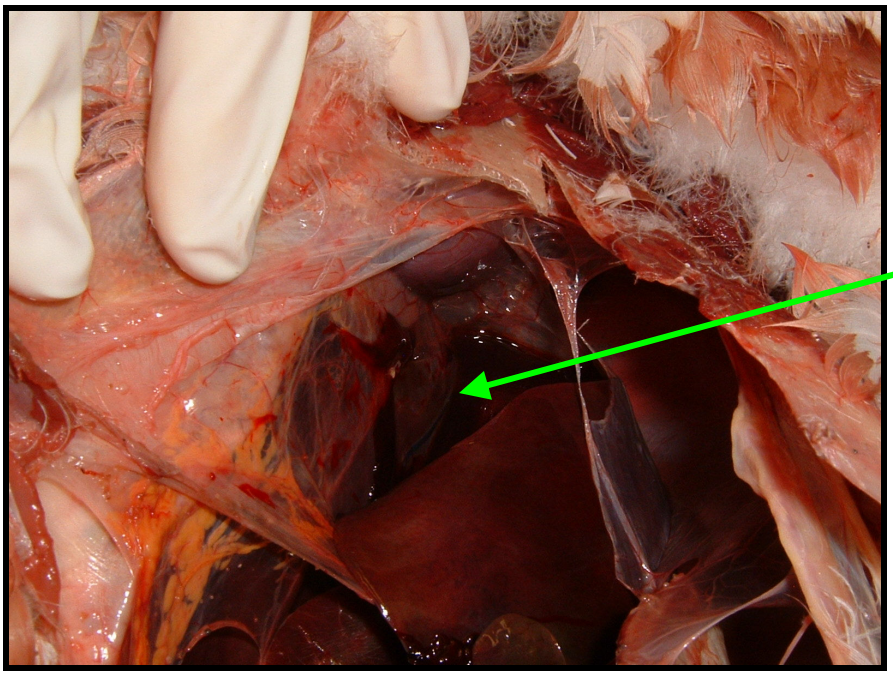
Date	Patient Name	Patient ID Number	Test Results (ug/dL)	Pre/Post	Other Information
11/5/2006	Mute Swan	W061353	HI	Pre	
11/10/2006	"	"	HI	Post	Tested twice, once gizzard, once liver
11/21/2006	Canada Goose	W061372	3	Pre	
11/29/2006	"	"	5.7	Post	
11/21/2006	Ring-necked Pheasant	W061379	11.2	Pre	
11/28/2006	"	"	9.2	Post	
12/5/2006	"	"	2.7	Pre	1st sample
12/5/2006	"	"	4.7	Post	2nd sample
1/17/2006	Mallard	W061411	30	Pre	Blood pulled 12/18/06 and in fridge un-mixed
1/24/2006	"	"	9.4	Post	Sample contained bile, not accurate
1/24/2007	American Crow #1	W060025	9.4	Pre	
1/31/2007	"	"	10	Post	
1/24/2007	American Crow #2	W060023	5.8	Pre	
1/31/2007	"	"	4.7	Post	1st time
1/31/2007	"	"	4.6	Post	2nd time
1/24/2007	Barred Owl	W060029	1.5	Pre	
1/31/2007	"	"	2	Post	

Table A.4. Differences and Standard Deviations with pre-post mortem results

Patient Number	Difference (pre-post): ug/dL
1	0
2	2.7
3	-4.5
4	-20.6
5	0.6
6	-1.1
7	0.5
Average difference:	-3.2
Standard deviation of all 7 (σ_1):	7.98
Standard deviation w/out 4 (σ_2):	2.40

Appendix B: Photographs of Necropsies

Figure B.1. Mute Swan W061353



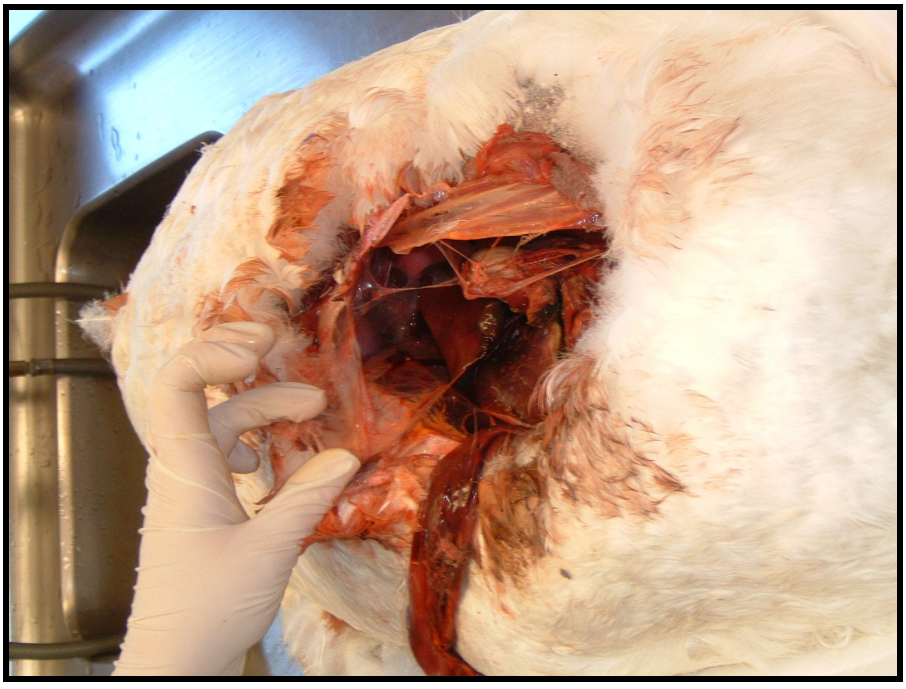


Figure B.2. Canada Goose W061372

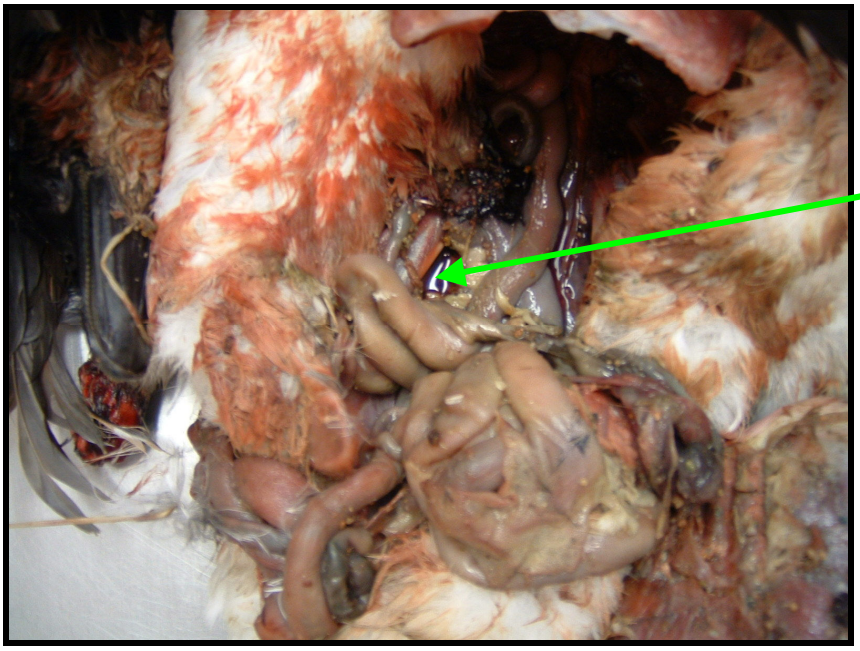


Figure B.3. Mallard W061411

Figure B.4. American Crow W070025

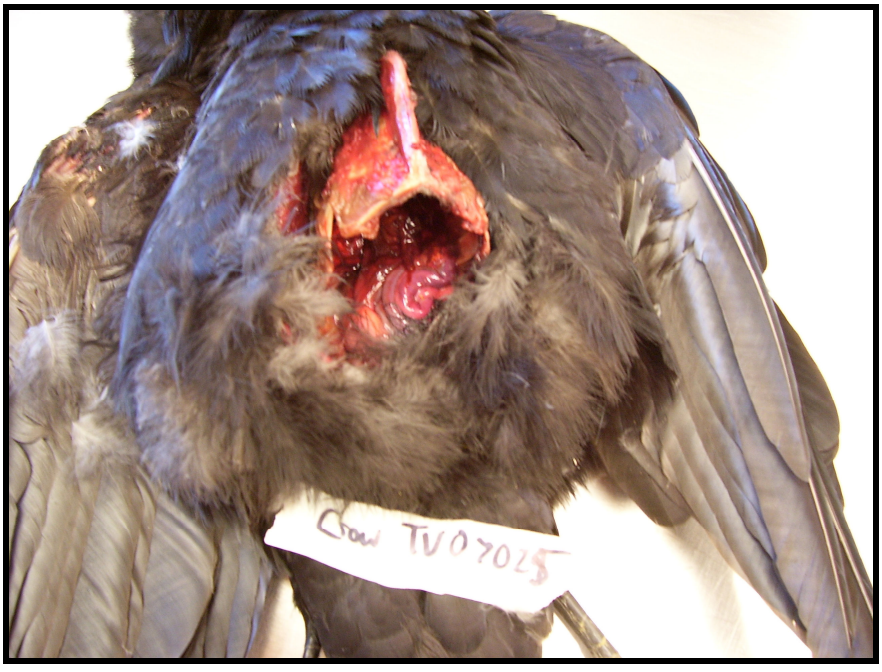


Figure B.5. American Crow W070023

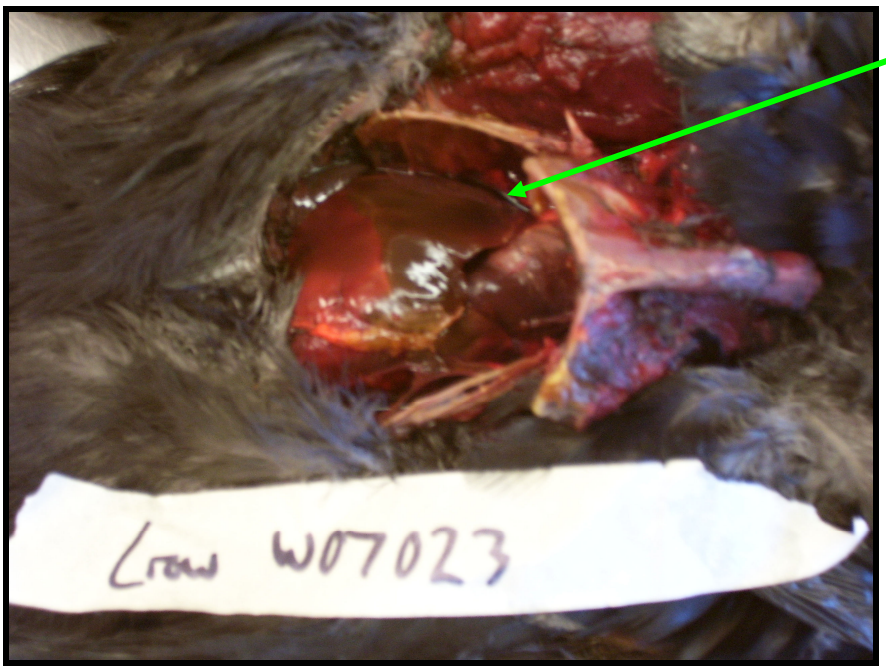
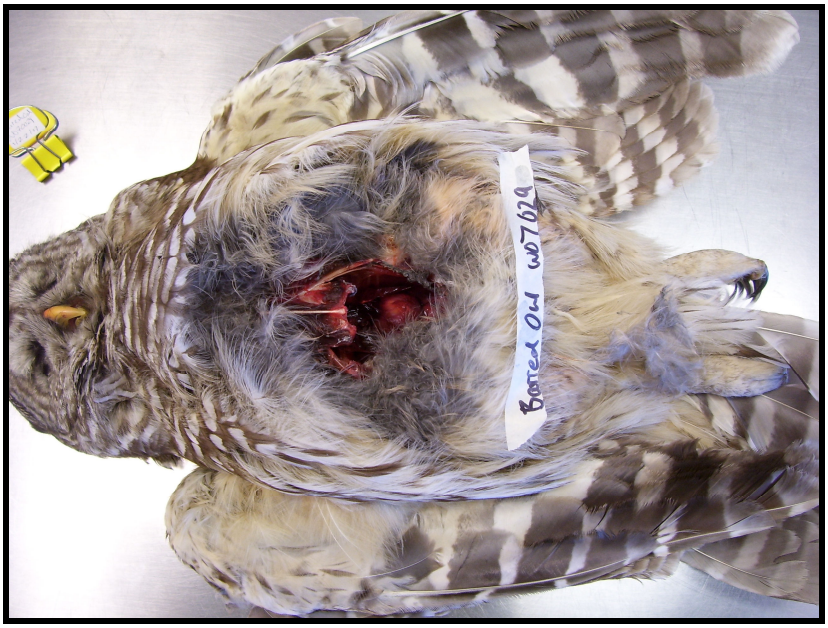
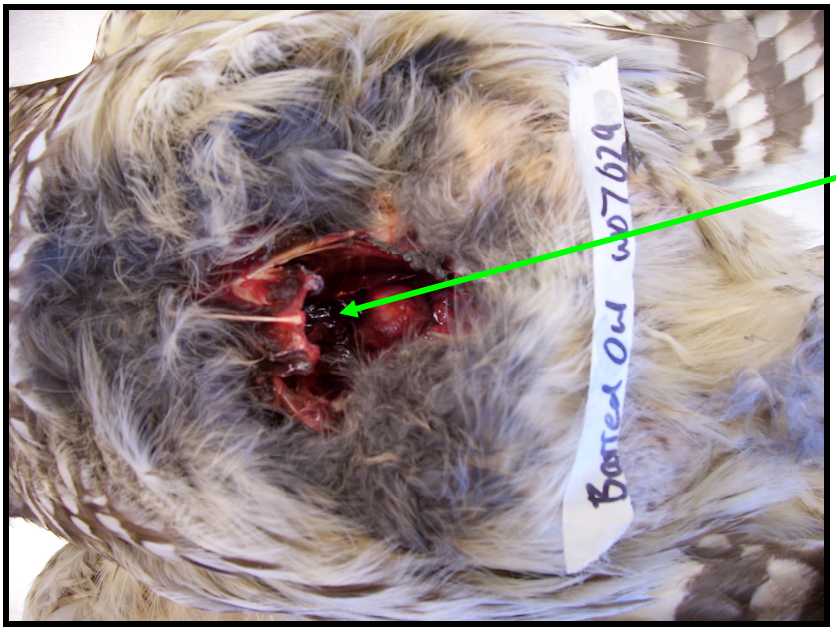


Figure B.6. Barred Owl W070029





Appendix C: Archival Analyses

Table C.1. Total distribution of all animals tested for lead since 2002

Pb Level	Number Tested
0-1.4	77
1.41-9.9	131
10-19.9	35
20-29.9	9
30-39.9	11
40-49.9	5
50-59.9	5
>60	29
Total:	302

Table C.2. Distribution of type of animal tested

Category	Totals
Aquatics	159
Raptors	62
Other Birds	48
Other Animals	33
Total:	302

Table C.3. Aquatics distribution

Aquatics:	
Pb Level (ug/dL)	# Tested
0-1.4	32
1.41-9.9	75
10-19.9	18
20-29.9	6
30-39.9	9
40-49.9	3
50-59.9	1
>60	15
Total:	159

Table C.4. Raptor distribution

Raptors:	
Pb Level (ug/dL)	# Tested
0-1.4	14
1.41-9.9	24
10-19.9	10
20-29.9	1
30-39.9	1
40-49.9	0
50-59.9	1
>60	0
Total	51

Table C.5. Other bird distribution

Other Birds:	
Pb Level (ug/dL)	# Tested
0-1.4	10
1.41-9.9	12
10-19.9	6
20-29.9	2
30-39.9	1
40-49.9	2
50-59.9	3
>60	12
Total	48

Table C.6. Other animal distribution

Other Animals:	
Pb Level (ug/dL)	# Tested
0-1.4	15
1.41-9.9	16
10-19.9	1
20-29.9	0
30-39.9	0
40-49.9	0
50-59.9	0
>60	1
Total	33

Table C.7. List of animal species tested (Common names)

Species Key			
Aquatics	Hawks/Raptors	Other Birds	Other Animals
Canada Goose	Peregrine	Mourning Dove	Snapping Turtle
Mallard	Red-tailed Hawk	Raven	Squirrel, Gray
Domestic Duck	Red-shouldered Hawk	Crow, American	Gray Fox
Swan	Bald Eagle	Turkey	Porcupine
Herring Gull	Golden Eagle	Pigeon	Llama
Common Loon	Condor, Andean	Ring-necked Pheasant	Canine
Gannet, Northern	Turkey Vulture	Dove	Canada Lynx
Greater Black-backed Gull	Broad-winged Hawk	Night Hawk	Fisher
Ring-billed Gull	Osprey	Himalayan Monal	Cottontail Rabbit
Muskovy	Goshawk	Racing Pigeon	Bobcat
Great Blue Heron	Cooper's Hawk	Flicker	Raccoon
Roseate Tern	Barbary Falcon	Wood Pecker	Other*
Common Merganser	Sharp-shinned Hawk		
Double-crested Cormorant	Great Horned Owl		
Black Scoter	Barred Owl		
Eider, Common	Screech Owl		
Black Duck			
Laughing Gull			
Other*			

* The “other” in these categories denotes that in the write-up of the Pb result, either the location that the bird came from (i.e. SEANET) or a client name from the hospital (i.e. “Fluffy” McCullough) was used. These names are descriptive enough in order to place the animal into the broader species category, but not specific enough to place them in a species category.

Table C.8. List of all origin locations from Pb tests (only Massachusetts)

Andover	Dedham	Melrose	Shrewsbury
Arlington	Dorchester	Methuen	Spencer
Ashland	Fitchburg	Millbury	Springfield
Attleboro	Framingham	Milford	Sterling
Auburn	Gloucester	Nahant	Sturbridge
Ayer	Grafton	Nantucket	Sudbury
Bedford	Hatfield	Natick	Swampscott
Bellingham	Holliston	New Marlboro	Tewksbury
Billerica	Hubbardston	Newton	Uxbridge
Blackstone	Hyde Park	North Attleboro	Wakefield
Bolton	Lancaster	North Grafton	Walpole
Boylston	Leicester	Norton	Waltham
Brockton	Lincoln	Peabody	Watertown
Brookline	Littleton	Plainville	Webster
Burlington	Lowell	Princeton	Wellfleet
Byfield	Manchester	Providence	West Boylston
Cape Cod Wildlife	Mansfield	Quincy	Westboro
Center (W.	Marblehead	Revere	Winthrop
Barnstable)	Marion	Rowley	Woburn
Centerville	Marlboro	Roxbury	Worcester
Charlton	Marshfield	Rutland	Wrentham
Cherry Valley	Maynard	Salem	
Cochituate	Medfield	Salisbury	
Dartmouth	Medway	Sharon	

Table C.9. Original counts of patient outcomes

Range	# Dead	# Euthanized	# Lived	
0 - 1.4	6	17	18	
1.5 - 9.9	8	47	38	
10 - 19.9	1	12	10	
20 - 29.9	0	3	5	
30 - 39.9	0	2	3	
40 - 49.9	2	1	1	
50 - 59.9	1	2	2	
60 +	4	3	2	
total	22	87	79	188
	11.70%	46.30%	42%	

Table C.10. Original count of symptoms

Symptoms	Emaciation/ dehydration	Fractures/ wounds/trauma	Depression	Neurologic	Fishing gear/ Pb shot
Percentage of Patients	51	48	23	6	4

Appendix D: LeadCare® System Manual

Retrieved from online manual at: http://www.woongbee.com/POCT/leadcare.htm

LeadCare® Childhood Blood Lead Testing

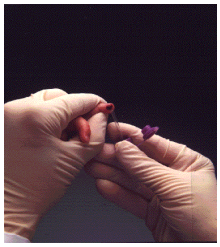
The LeadCare System is for the determination of lead in whole blood. When you test young patients for lead levels, you want fast, accurate, inexpensive results. You want the LeadCare system, a simple, foolproof way to perform blood lead measurements using a finger stick or venous sample. No more waiting days for expensive lab analyses. You get quantitative blood lead results equivalent to those reported by outside laboratories in just three minutes.

A LeadCare system analysis costs far less than you'd pay an outside laboratory, and it qualifies for reimbursement as a quantitative blood lead. You'll also cut your staff's result-tracking and administrative time. You'll save your patients days of possibly needless worry plus the time-consuming inconvenience and cost of a return visit. Blood lead measurement couldn't be easier.

LeadCare is easy and safe to use. The hand-held analyzer is portable and requires neither manual calibration nor refrigeration. Its unique gold electrode sensor contains no mercury or other toxic materials. The point-of-care LeadCare system was developed by ESA and Andcare with a grant from the CDC. It's the diagnostic tool which makes sense medically and economically.

Fast! *Easy as 1- 2- 3*

STEP ONE



Draw a capillary or venous blood sample using EDTA or heparin as anticoagulants.

STEP TWO

Using the pipette provided with the kit, dispense 50 µl, about two drops of blood, into the reagent and mix.

STEP THREE

Transfer it to the sensor strip. Press the button. Just three minutes later, you have your result.

Accuracy

LeadCare System vs. Atomic Absorption Spectroscopy performed at a major lead outreach and referral clinic/hospital
Number of Samples: 112
Slope: 1.07
Intercept: -0.57 µg/dl
Correlation coefficient: 0.97

Portable

Power source: 9V battery or AC adapter
Dimensions: 7.7 in x 4.2 in x 2.5 in. (19.6cm x 10.7cm x 6.4cm)
Weight: 14 oz

Specification

Test method: Electrochemical with disposable sensors
Blood lead level range: 1.4 - 65 µg/dl
Blood sample volume: 50 µl
Test time: 3 minutes
Calibration: Electronic calibration button
Classification: Moderately complex under CLIA guidelines. Suitable for use in physician's office laboratory.

Theory of Anodic Strippimg Voltammetry (ASV)

ASV Method

Anodic Stripping Voltammetry is a highly precise, virtually interference-free method.

1. Whole blood is added to the reagent solution (Fig. 1),
2. Any lead present is released from the blood components (Fig. 2).
3. Now any lead in the reagent solution is concentrated (plated) onto a thin-film electrode during the plating step of the analysis cycle (Fig. 3).
4. The plated lead is removed from the electrode by applying a stripping current (Fig. 4) and the amount of lead is measured by integration of the electrical current released during this rapid electrochemical step.

Anodic Stripping Voltammetry

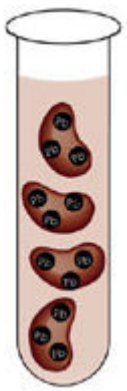


Figure 1

Figure 2

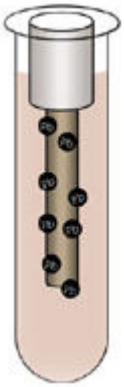


Figure 3

Figure 4

The current released during the stripping step, is a directly proportional to the amount of lead present in the blood sample.

Accurate Results

LeadCare® System vs. Atomic Absorption Spectroscopy performed at a major lead outreach and referral clinic/hospital

Number of Samples	**112**
Slope	**1.07**
Intercept	**-0.57 µg/dl**
Correlation Coefficient	**0.97**

Method Correlation

Results from the Model 3010B Lead Analyzer have shown close correlation with the widely accepted graphite furnace methodologies. This is further supported by results from numerous proficiency surveys.

The Model 3010B provides the sensitivity you need for the detection of blood lead in childhood lead screening, industrial hygiene and occupational health monitoring programs.

The LeadCare system operates by fundamentally the same principal but uses a single-use electrode contained on a disposable slide.