PIXE analysis of Florida alligator plasma

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Abstract

Over the past several years, there has been an alarming increase in the death rate of some Florida alligators and a large decrease in the hatch rate of eggs. Several studies have been conducted to try to find a cause. These studies have ruled out toxins in the environment and high levels of toxic metals (mercury, for example). A previous study using Particle Induced X-ray Emission (PIXE) analysis suggested that there may be a difference in the level of selenium present in healthy alligators, compared with sick ones. The present study will investigate levels of copper, zinc, selenium, bromine, and rubidium in plasma from seven alligators kept in an isolated environment for a year. Although this study did show significant variations between different alligators and over time, it did not show any significant differences between the living alligators and the dead ones. Despite the fact that the results here show that there is no correlation between the alligators that die and the levels of elements in their bloodstream, there were some interesting findings about the differences between individuals and the range of elemental levels over the course of a year.

Introduction

There has been an unexplained increase in the mortality rate and decrease in the hatch rate of alligators from Lake Griffin, Florida, since 1997. In the past six years, almost 400 alligators have died, and the hatch rate has fallen to about 10%, although it did rebound back to about 30-45% (whereas the normal hatch rate is around 80%). These alligators all appear to be in “good to excellent physical appearance and condition” and “do not demonstrate any consistent sign of gross organ or tissue abnormality” [1]. Additionally, there have been no consistent differences in a wide range of blood-borne chemicals and microbes between the healthy and sick alligators [1]. The only symptoms they seem to have in common are signs of neurological impairment such as “depressed clinical responses, reduced nerve conduction velocities, and axonal degeneration of specific foci in the mid brain” [1]. Two previous studies have investigated the levels of heavy metals in the alligator. In the first study, alligator liver samples were sent to the University of Pennsylvania’s toxicology lab for analysis of heavy metals. The results showed no significant difference in the levels of toxic metals between the healthy and the unhealthy alligators [2]. The second study was conducted at the University of Florida and dealt with PIXE analysis of liver and kidney samples of 16 alligators, 8 from Lake Griffin and 8 from Lake Woodruff. The results from this study matched the results from the previous study, except that the researchers found a significant difference in the level of kidney selenium between the sick and healthy alligators. However, matching statistics were not found when the levels of selenium in the kidney were examined [3]. One of the problems of the previous studies was the lack of controls in the experiment. All of the alligators were wild, and no background information was available on food, duration of sickness, etc.

For the present study, seven wild alligators, both male and female, were captured between July and August 2002, four from Lake Woodruff and three from Lake Griffin. These alligators have been kept in a facility that monitors and controls air and water temperature, as well as diet (amount, type, and length between feedings). Blood was collected from the alligators at regular intervals and spun in a centrifuge to extract the plasma for our analysis. When the alligators were initially captured, they all appeared to be healthy. Over the course of the year, however, three of the alligators died, two from Lake Griffin and one from Lake Woodruff. We will study the levels of five elements, Br, Cu, Rb, Se, and Zn, to determine if there were any significant changes in these levels throughout the year.

PIXE was chosen for this project because it has the ability to simultaneously detect a wide range of elements that are present in levels that are several orders of magnitude smaller than is necessary for other techniques. Additionally, PIXE provides relatively precise measurements from very small amounts of material. The PIXE process uses a beam of high-energy particles (in this case protons) to bombard a target. When these protons impact the atoms that make up the sample, they can knock out the electrons in the low-energy shells of the atom. When this happens, the electrons in the high-energy shells drop down to fill in the gaps. The process of an electron dropping from a high energy to a low energy state releases electromagnetic radiation in the x-ray spectrum. The x-rays are then collected by the detector. The energy of the emitted x-rays is proportional to the square of the atomic number of the element that emits them, and the intensities of these characteristic x-rays are proportional to the concentration of the element in the sample [4]. Using these x-ray energies, one can then determine the concentrations of different elements in the sample.

Experimental

Samples were prepared by mixing 100 μL of Y_2O_3 in a 5% nitric acid solution with 500μL of alligator plasma to obtain levels of Yttrium around 200 ppm as an internal standard of measurement. Target backings were made using Isopore^{tm} 0.1 μm thick pore membrane filters attached to nylon rings. Finally, 100 μL of plasma solution was dropped on the backings and allowed to dry overnight. The samples were then bombarded with a 2.5 MeV proton beam from the University of Florida's National Electrostatics Corporation (NEC) 5 SDH Peletron tandem accelerator. The beam was passed through a thin gold foil before striking the target to evenly distribute the intensity of the beam over as much of the sample as possible (about 5mm in diameter). Total beam intensity was kept between 40 and 70 nA, and total charge accumulated ranged from 14 to 60 μC. The characteristic x-rays passed through an absorber to filter out the low-Z elements and were detected using a 30mm^2 x 3mm thick Kevex Si(Li) detector located inside the vacuum chamber making an angle of 135° with the incident beam direction. The signal from the detector was then amplified with a Kevex 4525P amplifier/pulse processor. The resolution for the detector/processor combination is 180 eV for the 6.4 keV Mn K_α line. The data were finally analyzed using Robwin. A typical spectrum is shown in Fig 1.

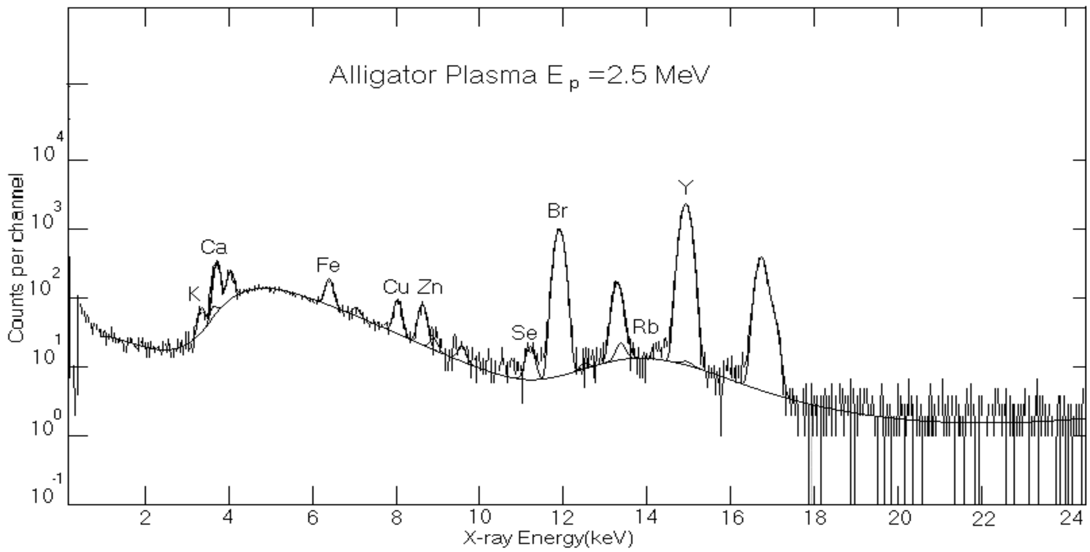

FIG 1. A typical PIXE spectrum. The line through the data represents the fit provided by Robwin.

After analysis by Robwin, the raw data were entered into an Excel spreadsheet to calculate the concentration of each element present in the plasma. The results were then analyzed to detect any changes or trends in the elemental levels in the alligator blood throughout the year.

Analysis

Four different plasma samples were taken from each alligator: one at the time of capture, one from November 2002, one from March 2003 and one from June 2003. However, we are missing two data points for June 2003 because two of the alligators died before that sample was taken. We analyzed the data three times per element using Excel's Analysis of Variations function (ANOVA). This function takes sets of data arranged in adjacent rows and columns, calculates the sums, means, and variances of each group, and then calculates the F-value, the P-value, and the F-critical value. For this study, we took a P-value of less than 0.01 (i.e., 1 chance in 100 that the differences are due to chance) to reject our null hypothesis, that there is no difference in measured values of metals among alligators, nor is there a difference in these values between different months. The first analysis, found in Table I, used the single variable ANOVA function to compare the living alligators to the dead ones, and shows that there is no significant difference in the levels of the elements analyzed between the two groups.

TABLE I. Surviving versus dead alligators

Element	P-value
Bromine	**0.95**
Copper	**0.89**
Rubidium	**0.53**
Selenium	**0.32**
Zinc	**0.12**

The second and third analyses used the ANOVA function for two variables to compare the values among the alligators, the values of each alligator over time, and both among different alligators at different times. The second analysis, compared the differences among alligators and between months for all the animals from which we had four plasma samples, and shows significant differences in at least two categories for all elements except rubidium.

TABLE II. July, November , March, all seven gators

Element	P-values		
	Alligator	Month	Interaction
Bromine	<0.001	<0.001	<0.001
Copper	<0.001	<0.001	<0.001
Rubidium	**0.051**	**0.018**	**0.38**
Selenium	<0.001	**0.225**	<0.001
Zinc	<0.001	<0.001	**0.42**

The third analysis, Table III, analyzed all of the alligators, leaving off the June '03 data point. This table shows significant differences in all categories for all elements except rubidium, which shows differences most likely due to chance in all of the categories.

TABLE III. July, November, March, Jun. Five living alligators			
Element	**P-values**		
	Alligator	Month	Interaction
Bromine	<0.001	<0.001	<0.001
Copper	<0.001	<0.001	<0.001
Rubidium	**0.012**	**0.03**	**0.45**
Selenium	0.003	0.003	<0.001
Zinc	<0.001	<0.001	<0.001

Since the differences in all the elements between the living and the dead alligators are most likely due to chance, we can treat all the alligators as coming from the same population. After this analysis, we took an average of each month's level of elements over all the alligators and plotted them in Figure 2. The vertical bars represent 1 standard error.

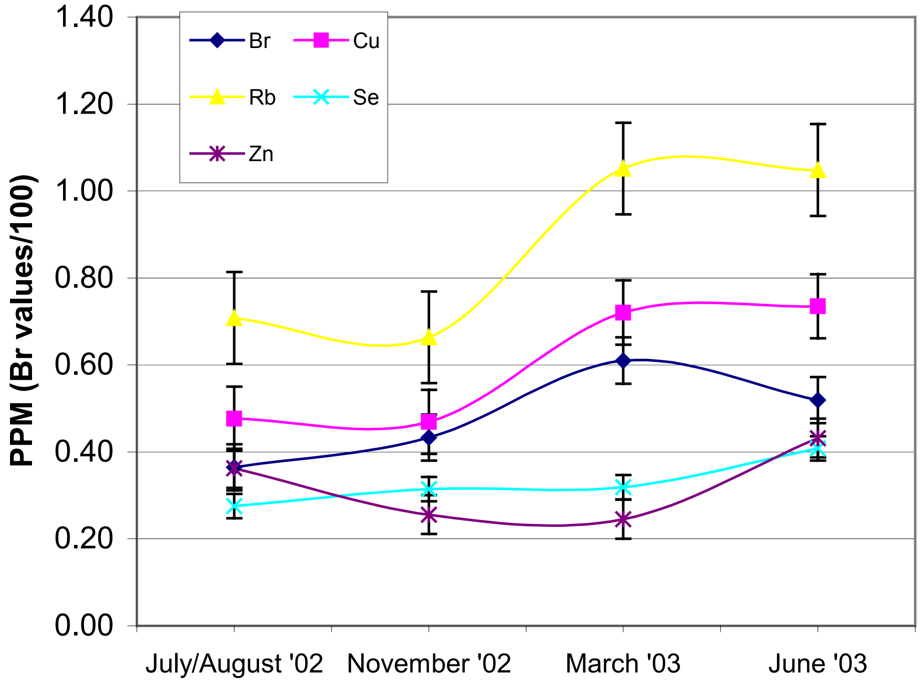

FIG 2. Elemental levels averaged for each month over all of the alligators, with trend lines added.

Discussion

While this study has failed to show a significant difference in the levels of different elements carried in blood plasma between living and dead alligators, it does reveal several interesting facts. First of all, the fact that there are no significant differences between living and dead alligators (Table I) is interesting in itself. This tells us that we need to look elsewhere for the cause of death in these animals. Secondly, preliminary data from previous experiments had suggested that there was a change in the levels of selenium between healthy alligators and sick ones [3]. This study has shown that there is no significant difference between the two groups regarding the levels of selenium in their plasma. Third, the study shows that there is a significant difference in elemental levels between individual alligators. However, this is not surprising because the alligators are individual living creatures, and this type of change is common within an individual system. Since measurements of the levels of these elements in alligator plasma have never been done, there is no way of knowing whether or not these levels are normal. We can only look at trends. Fourth, in all elements except rubidium there is a significant difference from month to month. Because of the nature of their captivity, these changes cannot be due to changes in diet or changes in environment. The only variable that was not controlled was the alligators' body weights. From the time they were captured until about November, they were eating well and gained 10-15% of their original body weight. Around that time, when the ambient temperature dropped below 20°C, until April, the alligators ate nothing at all and continued to eat poorly through May and June, and consequently lost most of the weight they originally gained. The remaining alligators have only begun eating well again in July, and are now regaining the weight. It may be that the general increase in levels of all of the elements in the plasma between November and June is due to the metabolism of body tissues and the release of metals and compounds into the bloodstream during that period.

The newest theory as to why the alligators are dying is that concerns a lack of thiamine (vitamin B1). Thiamine deficiency produces symptoms in humans similar to those the alligators are experiencing. Unfortunately, PIXE analysis can only detect elements, not compounds, so it would be ineffective in either proving or disproving that theory. However, since PIXE is very good at picking out small amounts of elements, future analysis of alligator and other biological samples would be useful in creating standards of measurement for safe levels of elements present in blood and other tissues.

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