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# Lead isotope ratio measurements as indicators for the source of lead poisoning in Mute swans (*Cygnus olor*) wintering in Puck Bay (northern Poland)

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**Keywords:** Pb; isotope; ICP-MS; poisoning; pellet; Mute swan

**Capsule:** Lead shotgun pellets available today on the Polish market were not the main source of lead in the blood of the Mute swans wintering in northern Poland.

## Abstract

Lead (Pb) poisoning is most commonly linked amongst anthropogenically-caused deaths in waterfowl and this is often associated with hunting and fishing activities. However, the exact identification of the source may be difficult with commonly-used techniques. We have studied isotope ratios using Inductively-Coupled Plasma Mass Spectrometry (ICP-MS) to investigate the source of Pb in the blood of Mute swans (*Cygnus olor*) (n=49) wintering in northern Poland. We compared the values from blood and ammunition pellets available on the Polish market. The mean Pb concentrations found was 0.2406 µg/g (w/w) and nearly half of the blood specimens had elevated Pb levels (higher than the cited 0.23 µg/g w/w threshold of poisoning). Only the mean 208/206 Pb isotope ratio was similar in blood and pellet samples. Mean ratios of isotopes 206/204, 206/207 and 208/207 in swans' blood and in pellets differed significantly. Moreover, coefficients of variation were higher in blood samples than in pellets. These discrepancies and significant differences in abundance of <sup>204</sup>Pb and <sup>207</sup>Pb isotopes in both materials indicated that pellets available today on the Polish market were not the source of Pb in the blood of mute swans wintering in northern Poland.

## Introduction

Bird lead (Pb) poisoning is an important environmental concern since the late 1800's (Calvert, 1876). Most often it is connected with fishing and hunting (Clark and Scheuhammer, 2003; Franson and Pain, 2011; Guitart et al., 1994; Mateo et al., 1997). Waterfowl species and their habitats are generally most affected by these activities (Friend and Franson, 1999; Kimmel and Tranel, 2007). The spent hunting Pb pellets and fishing sinkers lying on the foraging area of birds may be mistaken by them and ingested as gastroliths. After the ingestion, with the help of the grinding mechanisms of the gizzard and in its acidic environment, Pb particles dissolve easily and get into the bloodstream causing poisoning (DeMichele 1984; Pain, 1990). This phenomenon has been a significant source of mortality for many years among various species of swans in different parts of the world (Blus et al., 1989; Ely and Franson, 2014; Nakade et al., 2005; Perrins et al., 2003). It was estimated globally that until the middle of 1990s, 10,000 swans from 14 different countries died as a result of Pb poisoning caused by ammunition pellets deposited in wetland habitats (Blus, 1994).

The verification of the source of Pb poisoning is difficult. Apart from ammunition and fishing sinkers, other sources of Pb exposure are from air, water and soil deposition of Pb-based paints, large-scale mining and Pb smelting activities (Blus et al., 1991; Finkelstein et al., 2003; Henny, 2003; Svanberg et al., 2006; Thomas et al., 2009). In order to verify the connection of poisoned waterfowl to hunting and fishing activities, a post-mortem evaluation using X-ray analysis is needed and this may not be always possible to carry out, especially for *in vivo* studies, as well as, it might be inefficient in the case of long time after the ingestion (Binkowski and Sawicka-Kapusta, 2015; Plouzeau et al., 2011). An establishment of Pb isotope ratios in bird tissues or feathers may be a practicable solution to the above-mentioned restrictions (e.g. Church et al., 2006; Scheuhammer and Templeton, 1998; Scheuhammer et al., 2003; Lambertucci et al., 2011).

Due to the fact that three (206, 207, 208) of the four Pb isotopes come from radioactive decay of uranium and thorium, their abundance ratios may vary in different deposits. As a result of this, the isotopes and their ratios (between themselves, including the 204 isotope) may be applied to the recognition of the Pb ore which was used to produce the material (Scheuhammer and Templeton, 1998; Scheuhammer et al., 2003; Thomas et al., 2009). Knowing these isotopic ratios in various materials, it is possible to compare them with values found in biological samples to recognize the possible pollutant connection (Maddaloni et al., 1998; Scheuhammer et al., 2003; Svanberg et al., 2006).

Most of the previously published papers concerning Pb isotopes in birds studied bones as the tissue of the final deposition where the metal reaches the highest concentrations. However, Pb in bones accumulates for the animals' whole life, so its burden reflects the lifelong exposure of the bird to numerous sources (Scheuhammer, 1987). Taking into account the fact that many scientists have reported that isotopic ratios overlap in lots of materials, bone seems not to be the best choice for this analysis. Tissues with faster Pb turnover should be more appropriate. The one which shows recent exposure (including a couple of previous weeks) is blood, already used in waterfowl biomonitoring studies, including cases of Pb poisoning (Binkowski and Meissner, 2013; Delves and Campbell, 1988; Ely and Franson, 2014; Gomez-Ramirez et al., 2011; Pain, 1989; Tsuji et al., 2008). Moreover, we included all the isotopes because previous studies by co-workers showed that, in many cases, their values overlapped among tissue, pellets and soil samples (Meharg et al., 2002; Scheuhammer and Templeton, 1998).

The aim of our research was to identify Pb concentrations in wild Mute swans wintering in Puck Bay, one of the most important sites for non-breeding concentrations of waterfowl in the Baltic (Durinck et al., 1994). To find if its source were lead pellets from ammunition, we compared the Pb isotopic ratios (204/206, 206/207, 208/206, 208/207) found in blood with values noted for pellets from ammunition available on the Polish market. Finally, we tried to answer the question if the method of isotope ratio measurement in bird blood may be useful in the regular monitoring of Pb poisoning.

## **Materials and method**

Samples of blood were collected from 49 wild Mute swans between 31<sup>st</sup> October 2012 and 25<sup>th</sup> April 2013 in municipal Baltic coasts of three neighbouring cities (Gdansk, Sopot and Gdynia) in northern Poland (Figure 1). About 0.5 mL of blood was collected from the metatarsal vein into plastic tubes and frozen (-18°C). Then, all the samples were transported to the laboratory in the Institute of Biology (Pedagogical University of Cracow).

Simultaneously, lead shot cartridges (caliber 12 which is the most common in waterfowl hunting, according to members of the Polish Hunting Association) available in Poland manufacturers (n=25) were purchased and disassembled.

### *Blood samples preparation*

After defrosting, each sample was weighed and transferred into the open mineralizer tube (Velp Scientifica DK-20) where 2 mL of ultrapure nitric acid was added (Ultranal 65%, POCH). Over three hours, samples were mineralized at high temperatures (up to 160°C),

transferred to tubes, diluted up to 10 mL with ultrapure water (18.2 MΩ·cm at 25°C, Direct-Q 3, Merck-Millipore) and stored at 5°C.

### *Pellet samples preparation*

From each cartridge, two pellets were taken. Each of them was placed in a tube and 2 mL of nitric acid (Ultranal 65%, POCH) was added. Then, the samples were treated according to the same protocol as the blood samples. Afterwards, the pellet samples were additionally diluted with ultrapure water to fit the working range of the Pb analysis method.

### *Lead concentration and isotopic analyses*

All the Pb analyses were carried out by ICP-MS (Agilent 7700 series) in the laboratories of Kingston University, London. Pb concentrations were measured as ppb in the solution and then recalculated to µg/g w/w (wet weight) of the sample. Four isotopic ratios: 206/204, 206/207, 208/206 and 208/207 were chosen for evaluation. We studied the given relationship only once without treating the inverse relationship in addition. These isotopes ratios were calculated as the ratios of raw counts given by the spectrometer (expressed finally in %).

The main standard solution was SRM Pb(NO<sub>3</sub>)<sub>2</sub> 1000 mg/L ICP grade (VWR) which was used to prepare a five points calibration curve. The main parameters of the method were as follows: RF power 1550 V, Helium carrier gas flow 1.15 L/min, nitrogen make-up gas 1.2 mL/min, peripump 0.1 rps, torch H 0.2 mm and torch V 0 mm. The tuning solutions used were 1 µg/L Li, Mg, Y, Ce, Ti, and Co intended for short-term use. Limits of the method were calculated according to the protocol of Fleming et al. (1997) and were as follows: limit of detection 0.024 µg/L (ppb) and limit of quantification 0.037 µg/L (ppb).

The efficiency of the analysis was checked against the certified reference material for total Pb (ERM CE195, IRMM, Belgium; recovery 99.2%, RSD 1.1%, n=10). Additionally, we carried out the analyses of reagent blanks, fully repeated and parallel samples, as well as, control solutions (SRM 981, NIST) and spikes (SRM Pb(NO<sub>3</sub>)<sub>2</sub> CertiPUR, Merck). All these results and recoveries were satisfactory.

### *Statistical analyses*

Initially, the results of Pb isotopes' concentration were visualized on graphs to find the potential outliers. On that basis, the data of two birds (#3 and #16) were excluded from all the further analyses; however, both are marked in the figures to show the whole variation of isotope ratios. Testing for differences between two coefficients of variation was conducted according to the procedure described by Zar (1996). The Cochran-Cox test (instead of t-test) was used for checking difference between two means, when homogeneity of variance assumption was violated. Pearson correlation coefficients were calculated to show relationships between isotopic ratios in pellets and in swans' blood. As the blood samples were taken through the whole wintering period and some of them probably concerned individuals who had just arrived, while others might be from swans who had stayed for more than one month in the study area. To check for possible differences among samples taken in different periods, the data were divided into three groups: early wintering period (October-

December), mid-wintering (January and February) and late wintering period (March and April).

All statistical procedures were performed using Statistica 10 software (StatSoft Inc. 2011). Significance level was set to 0.05.

## Results

Lead was found in all the studied samples. Mean concentration in swans' blood was 0.2406 µg/g w/w (SD=0.1215), range 0.0643 - 0.6750 µg/g w/w.

There were no differences in mean isotopic ratios among samples collected in early, mid and late wintering period (one-way ANOVA,  $p > 0.30$  in all cases). Hence, data from these periods were combined.

We found all the isotopes studied in all the samples of blood and pellets examined. The highest abundance (over 50%) in both materials was noted for the isotope  $^{208}\text{Pb}$ , while the lowest (less than 2%) for  $^{204}\text{Pb}$ . The mean abundance of the isotope  $^{208}\text{Pb}$  and  $^{206}\text{Pb}$  did not differ significantly between blood and pellets, while  $^{204}\text{Pb}$  was more abundant in swans' blood and  $^{207}\text{Pb}$  in pellets (Table 1).

Visualization of the data showed generally bigger isotopic variation in blood (Figure 2 - Figure 5). Only in the case of 206/204 ratio was the difference between coefficients of variation in blood and pellets not statistically significant ( $Z=1.86$ ;  $p=0.063$ ). In other cases this coefficient was significantly higher in blood samples than in pellets (206/207:  $Z=10.11$ ,  $p < 0.0001$ ; 208/206:  $Z=6.50$ ,  $p < 0.0001$  and 208/207:  $Z=2.75$ ,  $p=0.006$ ).

Although the mean 206/207 and 208/207 isotopic ratios differed statistically between blood and pellet samples, the difference between the means was only 0.9% and 0.4% respectively (Table 2, Figure 2, Figure 3 and Figure 5). In the case of 208/206 ratio, five birds (#13, #30, #42, #44 and #49) had higher values than others, but comparable between themselves, ratio values (Figure 3 and Figure 4). After excluding these birds from the statistical analysis, the ratios in both materials examined were not statistically different (Table 2). The 206/204 ratio differed significantly between blood and pellet samples and the differences between the means was substantial, being 12.3% lower in blood than in pellets (Table 2).

The correlation analysis between the isotopic ratios (between 206/204 and 208/207, as they include all the isotopes studied without the repetitions) showed the significant positive relationship in pellets ( $r=0.68$ ,  $n=45$ ,  $p < 0.001$ ) and lack of a relationship in the case of blood ( $r=0.02$ ,  $n=42$ ,  $p=0.89$ ; Figure 5).

## Discussion

Tissues in which levels are used to diagnose Pb poisoning are blood, liver and bones (Binkowski and Sawicka-Kapusta, 2015; Pain, 1990). Moreover, other tissues such as feathers have been used for monitoring of Pb concentration in birds (e.g. Lambertucci et al., 2011; Scheuhammer and Templeton 1998). Among them, only the blood represents an *in vivo* image of the latest exposure, which is important due to the fact that Mute swans in this part of

Europe is a partly migratory species (Wieloch et al., 2004). There is a big discrepancy in the literature data in respect of establishing a threshold level that can be treated as poisoning in these birds. Some sources give a value of 0.5  $\mu\text{g/mL}$  ( $\sim 0.46 \mu\text{g/g w/w}$  – recalculated on the basis of the blood density). Some, however are more restrictive and treat an exact half of the mentioned values as a poisoning or at least exposure indicator (Mudge, 1983; Sanderson and Bellrose, 1986). Taking the threshold 0.25  $\mu\text{g/mL}$  ( $\sim 0.23 \mu\text{g/g w/w}$ ) into account, 45% of specimens examined had the elevated Pb level signalling poisoning and even the mean value for all the specimens studied was close to this threshold. For the 0.5  $\mu\text{g/mL}$  threshold, 8% of specimens examined were poisoned. However, we did not observe, during blood collection, any behavioral signs of Pb poisoning or symptoms of weakness which might be possible due to chronic and very high Pb exposure (Franson, 1986).

Birds wintering in the study area come from local sedentary and from migratory population (W. Meissner – unpublished data on ringed birds). Migratory Mute swans make rather short-distance movements which depend on winter severity, being longer in harsh seasons (Wieloch et al., 2004). According to recoveries of ringed birds, the farthest breeding site of Mute swans wintering in the municipal beaches of Gdansk, Gdynia and Sopot was localized in Latvia, about 550 km from the study area (WRG KULING ringing recoveries database). Short-distance migrants may arrive to wintering grounds directly or in a few flights interrupted by stops at suitable habitats. Hence, the Pb poisoning noted in a wintering place, may have origins in another area. It has been previously mentioned that blood is the tissue of fastest Pb turnover, so this narrows the results to about a month of prior exposure (Pain, 1989) and automatically decreases the range of exposure areas. However, it is impossible without the GPS tracking of particular individuals to point out the exact area of possible exposure. A high variation of isotope ratios in blood and the presence of outliers suggest that Mute swans wintering in the study area differed in origin or behave differently exploiting different food sources.

The Pb 206/207 ratio is used the most often in research for the recognition of the Pb source in tissues of birds (e.g. Scheuhammer and Templeton, 1998, Scheuhammer et al., 2003, Svanberg et al., 2006). Both of these isotopes are linked to the time when uranium and thorium were mixed in ores, so the oldest ores have the lowest amounts of them (Scheuhammer and Templeton, 1998). In our study, the range of the ratio 206/207 in blood completely overlaps with values noted for pellets. Although the statistical difference between means occurred, it reached only 0.9%, so we can suspect the potential Pb flux from pellets to birds. However, differences in  $^{204}\text{Pb}$  and  $^{207}\text{Pb}$  abundance in pellets and swans' blood and the much higher 206/204 ratio in blood indicate that pellets available today on the Polish market were not the main source of lead in the blood of the swans studied. This was supported by a lack of correlation between 206/204 and 208/207 in blood, when such a relationship was quite strong in the case of pellets and larger 206/207, 208/206 and 208/207 isotopic variation in blood than in pellets. Swans whose samples were outliers were probably exposed to different sources of lead. The group of five birds, which had visibly different, (but comparable between themselves) 208/206 ratio values (2.3-2.4) might have the same origin or use the same stopover site before arriving to the wintering ground, which were different from other birds.

We used in the study the majority of ammunition manufacturers available in Poland. However, most of them were produced abroad. Nowadays, there is an almost unlimited access to various types of ammunition and their manufacturers, including companies from Europe and USA. The potential variability of Pb isotopes in pellets may be even higher. In some areas, hunting was banned 40 years ago, but the number of pellets laying on the ground is high and still comparable to places where hunting is active (Mateo et al., 1997; Mateo et al., 1998). Taking this into account, isotopic ratio studies should include the data for presently and previously available ammunition. Additionally, it should be pointed out that, where the nature of soil is acidic, it is possible that old layers of pellets may slowly dissolve and constitute the local soil Pb background, thus influencing the whole ecosystem, including water, plants and animals (Thomas et al., 2009). In this case, the distinction between Pb from pellets and from soils is impossible, which constitutes a serious impediment in studies on sources of Pb in animal tissues, even in blood, which has a quite rapid Pb turnover. Another problem concerns difficulties in the interpretation of results obtained from migratory bird populations. Although using blood samples allows an assessment of Pb sources for about a month prior, the lack of data on migratory schedule between breeding and wintering sites makes the interpretation of obtained results problematic. Even in wintering grounds, some wildfowl species make regular movements up to 40-50 km using different foraging areas during the day and at night (Cox and Afton, 1996; Jorde et al., 1983; Link et al., 2011), and this should also be taken into account in studies designed to recognise sources of Pb pollution. Hence, isotopic measurement seems to be good method for use in biomonitoring studies, but in the case of migratory populations of birds, a more detailed interpretation of obtained results is difficult due to unknown origin and migration schedule of individuals from a given population.

## **Conclusions**

Since the discrepancies in isotopic abundance and ratios in blood and pellets were significant, we conclude that pellets available today on the Polish market were not the main source of Pb in the blood of the Mute swans studied. Isotopic measurement has been shown to be a good method for use in biomonitoring studies, but in the case of migratory populations of birds, interpretation of the obtained results is more difficult due to unknown origin and bird migration schedule. There is also a need to include in a further study an isotopic measurement of pellets, bullets, and fishing sinkers (in the wider geographical area; of new and possibly older production), as well as environmental and bird samples (from the given locality). Such attempts will verify if an isotopic ratio analysis of a full range of potential Pb sources will give a clearer conclusion.

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## Tables

Table 1. Mean lead isotope abundances (%  $\pm$  SD) in comparison to their total sum using t-test (t) and Cochran-Cox test (t').

	<sup>204</sup> Pb	<sup>206</sup> Pb	<sup>207</sup> Pb	<sup>208</sup> Pb
Swans blood	1.829% $\pm$ 1.516	24.530% $\pm$ 0.719	21.131% $\pm$ 0.801	52.509% $\pm$ 1.348
Pellets	1.359% $\pm$ 0.029	24.662% $\pm$ 0.353	21.569% $\pm$ 0.740	52.409% $\pm$ 0.641
Test results	t'=2.70, p=0.009	t'=1.18, p=0.244	t=2.83, p=0.006	t'=0.47, p=0.638
Difference	25.68%	-0.53%	-2.08%	0.19%

Table 2. Comparisons of the mean Pb isotope ratios in blood and pellets (values min – max in brackets). Cochran-Cox test was used.

Ratio	Pellets			Swans			Test results		Difference between means
	Mean	SD	N	Mean	SD	N	t'	p	
206/204	18.191 (17.901 - 18.601)	0.159	45	16.198 (13.928 - 18.291)	1.073	47	12.59	<0.001	-12.3%
206/207	1.151 (1.133 - 1.173)	0.009	45	1.157 (1.123 - 1.178)	0.012	47	2.96	0.004	0.9%
208/206	2.129 (2.105 - 2.150)	0.011	45	2.130 (2.090 - 2.264)	0.026	43	0.26	0.795	0.0%
208/207	2.449 (2.429 - 2.475)	0.011	45	2.4458 (2.414 - 2.488)	0.015	42	2.97	0.004	-0.4%

**Figures**

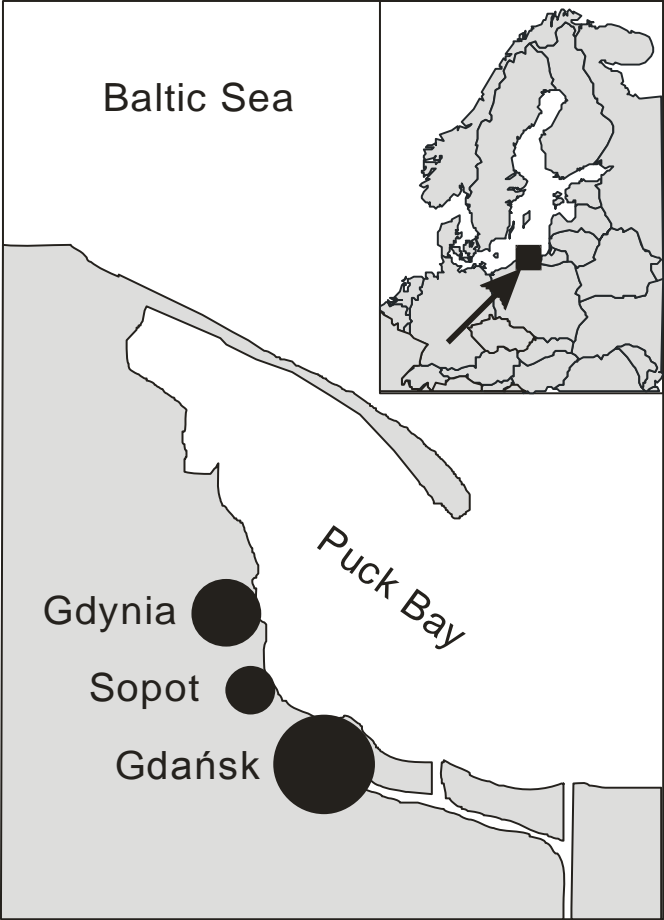


Figure 1. Localization of the study area.

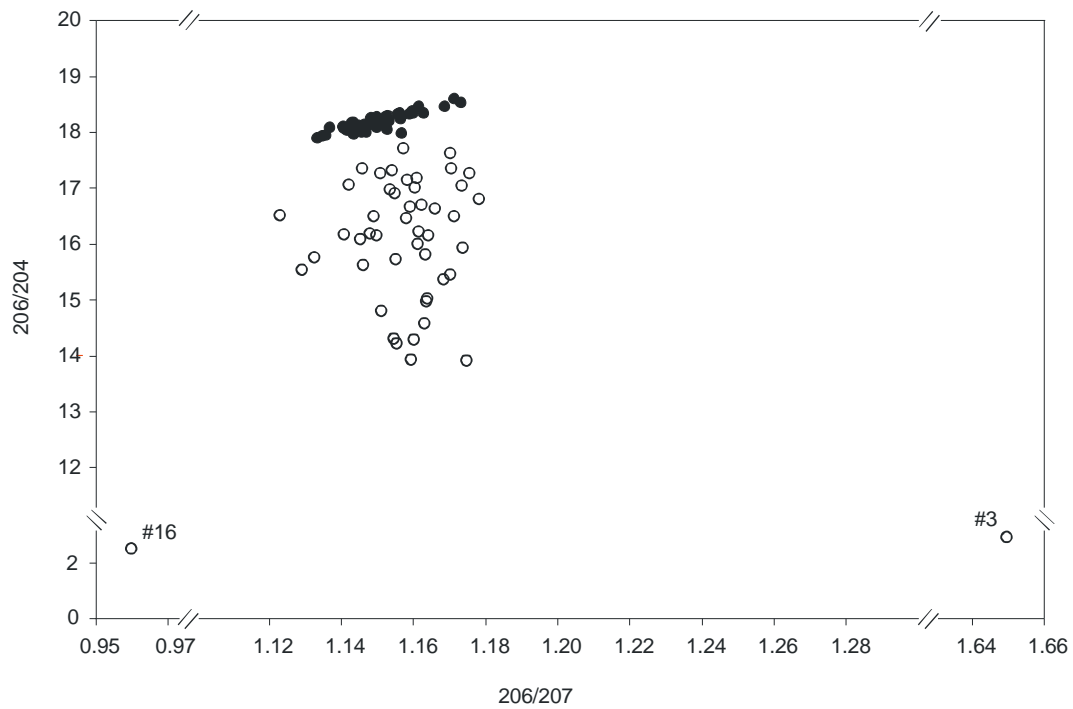


Figure 2. Correlagram of two Pb isotopic ratios: 206/207 and 206/204 in swan blood (white dots) and pellets (black dots). Samples of specimens #3 and #16 were excluded from all analyses as outliers.

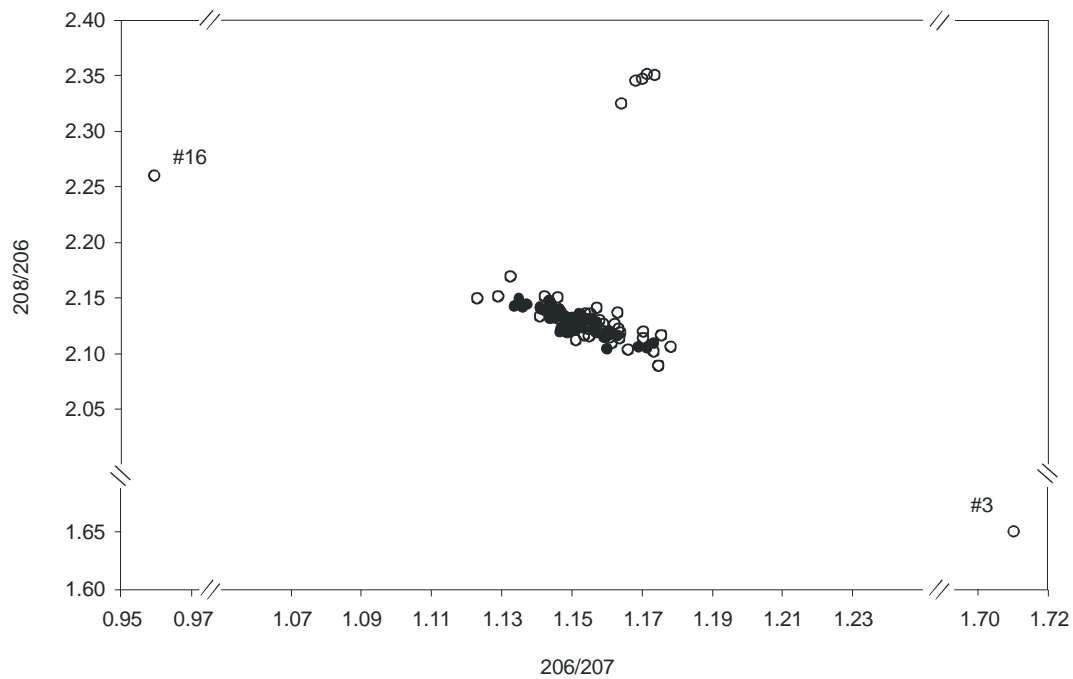


Figure 3. Correlagram of two Pb isotopic ratios: 206/207 and 208/206 in swan blood (white dots) and pellets (black dots). Samples of specimens #3, #16 and #13, #30, #42, #44, #49 (group of five points at the top) were excluded from the analysis as outliers.

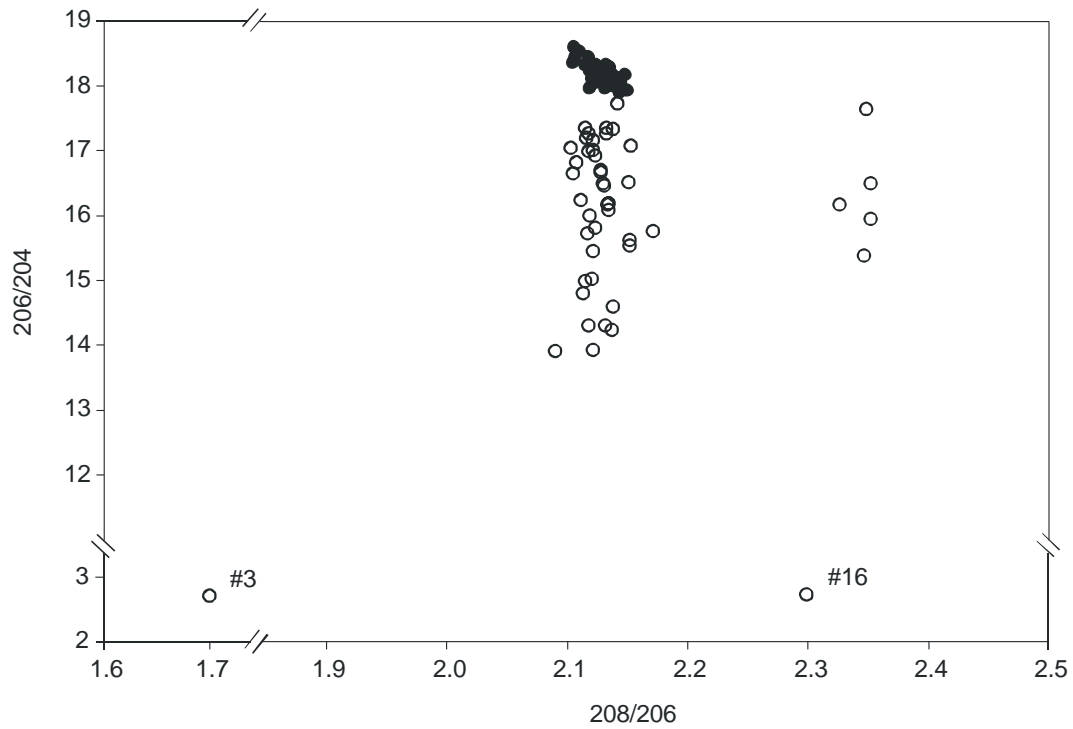


Figure 4. Correlogram of two Pb isotopic ratios: 208/206 and 206/204 in swan blood (white dots) and pellets (black dots). Samples of specimens #3, #16 and #13, #30, #42, #44, #49 (group of five points on the right) were excluded from the analysis as outliers.

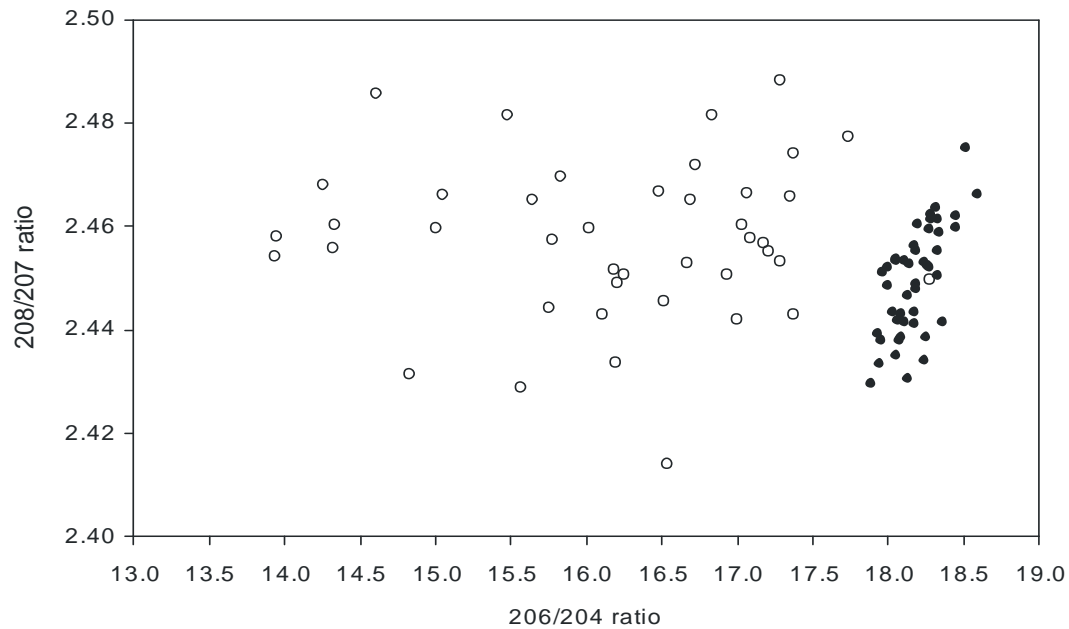


Figure 5. Scatter plot of relationship between 208/207 and 206/204 isotopic ratios in swans' blood (white dots,  $r=0.02$ ,  $n=42$ ,  $p=0.89$ ) and pellets (black dots,  $r=0.68$ ,  $n=45$ ,  $p<0.001$ ). Two outliers (#3 and #16) were omitted.