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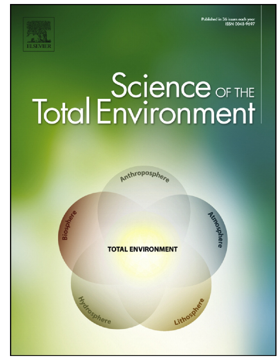

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Relationship between blood lead levels and physiological stress in mute swans *(Cygnus olor)* in municipal beaches of the southern Baltic

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Abstract

Lead is one of the non-essential metals that can become a serious environmental threat to the human population and wildlife causing various toxic impairments and pathologies. Waterfowl are especially sensitive to lead exposure as they stay in areas with a high risk of lead pollution due to hunting and fishing pressures. This study aims to determine recent blood lead levels in 45 live mute swans in the southern Baltic, in the Gulf of Gdańsk and to test the hypothesis that birds with elevated lead levels have a higher physiological stress. Mean concentration of lead in blood was 0.239 μg/g (range 0.028–0.675 μg/g). Almost half of the individuals examined in this study had increased blood Pb levels above the threshold level (0.23 μg/g), however none of them showed behavioural signs of Pb poisoning. Although the dominant food of mute swans staying at municipal beaches is bread delivered by people, which has low lead levels, lead was found in all blood samples taken from mute swans. In the study area, another possible source of lead poisoning, other than from ammunition, is connected with various anthropogenic activities, such as municipal sewage works. Moreover, poor diet results in greater susceptibility to absorption of lead and this may have an additional influence on elevated lead levels in blood of these birds. The Pb level was increased with heterophils to lymphocytes (H/L) ratio, which is used as a measure of longer-term physiological stress. The mean lead level in blood was higher in young birds than in adults, which may be a consequence of adults forcing them to take less calorific food from water plants containing elevated levels of lead in the area studied. However, it is difficult to disentangle this from other factors that may influence sensitivity to lead poisoning.

Keywords: lead (Pb), Poisoning, Waterfowl, Physiological stress

1. Introduction

Intense industrialization and urbanization result in pollution with non-essential metals that becomes a serious environmental problem for the human population (Imperato et al., 2003; Kachenko and Singh, 2006; Khan et al., 2008; Yaylali-Abanuz, 2011) and as well for the wildlife (Pastor et al., 2001; Moroń et al., 2014; Witeska et al., 2014; Vermeulen et al., 2015). Lead (Pb) is one of these metals which has become widely distributed in the environment, as it has been mined by mankind for 6 000 years (Hernberg, 2000; Binkowski et al., 2019). Since it is not degradable like organic compounds, this environmentally persistent contaminant may be transferred up the food chains (Muir et al., 1992, Butt et al., 2018). Lead remains in the body for a long time and interacts with proteins causing various impairments and pathologies (Goering, 1993; Kirberger et al., 2013) including neurological (Jones and Orosz, 1996; Douglas-Stroebel et al., 2004), haematological (Mitchell et al., 2001; Binkowski and Sawicka-Kapusta, 2015), renal (Hoffman et al., 2000; Binkowski et al., 2013), reproductive (Buerger et al., 1986) and immunological (Trust et al., 1990; Vallverdú-Coll et al., 2019) disorders. Various components of the ecosystem are differently influenced by lead, but waterfowl is a group which is probably influenced the most due to specific lead poisoning from ammunition and fishing gear (Pain et al., 2019).

Lead shots from ammunition and fishing sinkers have been recognized as a common cause of lead poisoning of wild waterfowl in many areas of the world (Blus et al., 1999; Franson et al., 2003; Sidor et al., 2003; Finkelstein et al., 2012). Although in some countries the use of lead fishing tackle and lead shots have been banned, still about 40,000 tons of lead shots and bullets as well as 2,000 - 6,000 tons of fishing weights were sold and used yearly in 15 European Countries (European Commission, 2004). Moreover, lead is introduced into the environment also from ore and the processing of metals, leaded aviation gasoline as well as from manufacturing batteries and natural fuel combustion (Pacyna and Pacyna 2001; Carr et al., 2011; Beyer et al., 2013; Gottesfeld et al., 2018).

Waterfowl are especially sensitive to lead exposure as aquatic ecosystems have been identified as areas with a high risk of lead pollution, mainly due to hunting and fishing pressures. They are considered as ideal indicators of metal pollution, because they occupy a relatively high trophic position in the aquatic food chains, have a fast metabolic rate, are long-lived, quite common and widespread (Hollamby et al., 2006). That is why they are very frequently used as bioindicators of bioavailability and magnification of metal pollutants (e.g. Mateo and Guitart 2003; Kalisińska et al., 2004; Binkowski and Meissner, 2013; Grajewska et al., 2015; Szumiło-Pilarska et al., 2016). The mute swan *(Cygnus olor)* is a large waterbird weighing 4–16 kg (Wieloch et al., 2004) which let the possibility to obtain large quantities of blood from one individual. Hence, recently it is becoming a model species for ecotoxicological and haematological studies (e.g. Perrins et al., 2003; Kelly and Kelly 2004; O'Connell et al., 2009; Dolka et al., 2014; Binkowski et al., 2016; Trzeciak and Meissner, 2018; Kucharska et al., 2019).

Measurement of physiological stress has a great importance in ecological studies and also in conservation physiology when assessing the status and future fate of natural populations (Wasser et al. 1997; Chávez-Zichinelli et al., 2010; Dantzer et al., 2014; Włodarczyk et al. 2018). There are different methods for assessing physiological stress in vertebrates (Wasser et al., 1997; Moreno et al., 2002; Romero, 2004, Davis et al., 2008). As blood may be used for non-destructive biomonitoring and obtaining leucogram is not expensive and rather easy, a heterophils to lymphocytes ratio is increasingly being used as a biomarker of physiological stress (e.g. Vleck et al., 2000; Plischke et al., 2010; Banbura et al., 2013; Włodarczyk et al., 2018), since the adrenal and leukocyte responses to stress are tightly linked and are similar across different vertebrate taxa (Gross and Siegel 1983; Davis et al., 2008; Quillfeldt et al., 2008). Lead poisoning in birds usually causes a wide range of sub-lethal impacts that affect their physiology (Lumeij, 1985; Buerger et al., 1986; Trust et al., 1990; Binkowski et al., 2013). However, papers showing a relationship between non-essential metals level and the magnitude of physiological stress are still sparse (Eeva et al., 2005; Plautz et al., 2011; Cid et al., 2018; Markowski et al., 2019).

This study aims to determine recent blood lead levels in mute swans in the Gulf of Gdańsk in the southern Baltic, which is the most important wintering site of this species in Poland, gathering in some seasons about 5% of the north-western and central European population (Meissner and Rydzkowski, 2010; Meissner et al., 2016). Data obtained in this study provide baseline information for comparison with future studies to monitor changes in lead pollution in this species (O'Connell et al., 2009). This study also sets out to test the hypothesis that mute swans with elevated lead levels have a higher physiological stress, which could reduce their health status and may have negative effects on the birds' breeding performance and survival (Buerger et al., 1986; Tavecchia et al., 2001; Eeva et al., 2005; Ferreyra et al., 2015).

2. Material and methods

2.1. Sample collection

The study was conducted in the Gulf of Gdańsk on the southern Baltic coast in the municipal beaches of three neighbouring cities Gdańsk, Sopot and Gdynia, where several hundreds of mute swans stay during their non-breeding period (Meissner and Ciopcińska, 2007). The data were collected between October and April in years 2012-2015. Birds were captured in the morning hours by hand after attracting them with food. No more than four birds were caught during a given day and each bird was sampled only once. In total, samples of blood were collected from 45 birds. As at night, mute swans showed no activity (Meissner and Ciopcińska, 2007) and it was assumed that catching them in the morning cannot introduce biases towards hungry or satiated birds. Mute swans were sexed according to cloaca examination (Brown and Brown, 2002) and aged by plumage characteristics and colour of the bill (Baker, 2016). As the majority of birds (88%) had been ringed previously from other projects, their history, i.e. the date and the age at ringing were known (data provided by Polish Bird Ringing Centre). Two age groups were distinguished: young birds (up to the third year of life) and adults (older than three years), as the mute swan starts to breed not earlier than in the third or fourth year of their life (Coleman et al., 2001).

In each bird caught, the following biometric measurements were taken: forearm length, head length, tarsus plus toe length with ruler with stop and fourth primary length with ruler with pin. All these measurements were taken to the nearest 1 mm following a methodology developed for mute swan studies (Mathiasson, 2005) or standard procedures used in bird ringing stations (Busse and Meissner, 2015). The body mass of the captured birds was determined on a spring balance (Voltcraft HS-30) with an accuracy of 20 g.

The body mass of birds used in this study was in the range of that given in the literature for the non-breeding period (7.47–11.73 kg for females, and 8.06–12.03 kg for males) (Wieloch et al., 2004). Moreover, before capture each bird was observed and no individual showed clinical signs of any disease. Thus, it was assumed that blood samples were obtained from mute swans that were in good condition. The capture of birds and blood collection were allowed based on a permit obtained from the Local Ethics Committee in Gdańsk (no. 61/2012) and a bird ringing license issued by Ministry of the Environment to M. Trzeciak and W. Meissner.

Blood was collected from the metatarsal vein and blood smears were made on microscope slides by the pull-wedge method (Clark et al., 2009) for further leukocyte profile and lead measurement protocols.

2.2. Index of body condition

To check if the lead level in blood is related to the amount of energetic stores, the scaled mass index (SMI) (Peig and Green, 2009) was used as a proxy of body condition. It was calculated as:

$$SMI = BM\left[\frac{FL_0}{FL}\right]^b,$$

where BM and FL are body mass and forearm length of an individual, FL_0 is the arithmetic mean value of forearm length of the whole mute swan sample and b is the scaling exponent estimated from the standardized major axis regression of body mass and forearm length, calculated by dividing the slope of the ordinary linear square regression of lnBM and lnFL by Pearson's correlation coefficient (Peig and Green, 2009). Among all linear body measurements, the forearm length was used to calculate SMI, because correlation coefficients between body mass and forearm length ($r=0.66$, $p<0.001$) were the strongest. Moreover, another study showed that amongst others this measurement was a reasonable measure of variation in mute swan body size (Coleman and Coleman, 2002).

2.3. Measurement of leukocyte profiles

Smear samples were air-dried and stained with a commercial version of the May-Grunwald and Giemsa (MGG) technique (Rapi Hem, Aqua–med, Łódź, Poland). The remaining blood (ca. 1 mL) was frozen at -20°C until Pb analysis was conducted. These samples were then examined to obtain counts of lymphocytes, heterophils, monocytes, basophils and eosinophils, which were identified according to the criteria of Hawkey and Dennett (1989). The percentage of each type of leukocyte was determined by counting a total of 100 white blood cells, viewed under a compound microscope (Leica DM 500) with 10x ocular and 100x oil immersion lenses. Obtained cell counts were used for calculation of the relative proportion of heterophils to lymphocytes (H/L ratio), as a measure of longer-term physiological stress. The numbers of monocytes, basophils and eosinophils were very low. This is typical for birds and indicates a lack of infection (Davis et al., 2008). The investigator examining the blood samples (M. Trzeciak) was unaware of the sex, age or biometrics of the birds during analyses.

2.4. Lead concentration analysis

Samples of blood after defrosting were weighed with an accuracy to 0.1 mg (WPA, Radwag, Poland) and transferred into vessels of the open mineralization system (DK-20, Velp Scientifica, Italy), where 2 mL of ultrapure nitric acid was added (Ultranal 65%, POCH, Poland). Over 3 hours, samples were heated at temperatures reaching 160°C and finally transferred into volumetric flasks, where they were diluted up to 10 mL with ultrapure water (resistivity at 25°C 18.2 MΩ cm; Direct-Q 3, Merck-Millipore, USA).

Lead levels were measured by ICP-MS (7700 series, Agilent, USA) as µg/L in solutions and then recalculated to µg/g (of wet weight) of the samples. The standard solution SRM $Pb(NO_3)_2$ 1000 mg/L (ICP grade, VWR, USA) was used to prepare a five points calibration curve. The main parameters of the method were as follows: RF power 1550 V, He carrier gas flow 1.15 L/min, N_2 makeup 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 mg/L Li, Mg, Y, Ce, Ti, and Co and intended for short-term use.

The limit of lead detection (LoD; 0.024 µg/L for the solutions, 0.6 ng/g for the samples) and limit of quantification (LoQ; 0.037 µg/L for the solutions, 0.9 ng/g for the samples) were calculated according to the protocol of Fleming et al. (1997). The validity of the analysis was made based on the certified reference material (ERM CE195, Bovine blood, IRMM, Belgium; recovery 99.2%, RSD 1.1%, n=10). We ran also the analyses of reagent blanks, fully repeated and parallel samples, as well as spikes (SRM $Pb(NO_3)_2$, CertiPUR, Merck, Germany). All these results and recoveries were satisfactory (blanks below LoD, recoveries between 90 and 110%).

The threshold lead level in blood above which can be treated as resulting in poisoning in these birds was set at 0.23 µg/g (originally 0.25 µg/mL) (Mudge, 1983; Friend, 1985). This level has also been used in other studies (Pain, 1990; Friend , 1999; Binkowski et al., 2016).

2.5. Statistical analysis

To assess the possible variation of lead levels in mute swan blood over time, the samples obtained were assigned into three groups according to the period of sampling: autumn (October-December), winter (January-February) and spring (March-April). These periods correspond to the phenology of this species. In autumn, mute swans move towards wintering grounds and their numbers gradually increase until January. In winter, their numbers usually reach a seasonal maximum and in spring the birds start to move towards breeding areas (Kieckbusch, 2010; Bordjan, 2012; Meissner et al., 2016, 2018).

A generalized linear model (GLZ in Statistica software) with a logarithm link function and normal error distribution (McCullagh and Nelder, 1983) was used to account simultaneously for the effects of all independent variables. The lead levels determined in mute swan blood was linked to sex, age class, period and H/L ratio. The Wald χ^2 statistic was used to test for significant differences between groups. A stepwise backward selection was applied to include significant variables ($p < 0.05$) into the model. The H/L ratios were arcsine transformed to fit normality (Zar, 1996). All statistical procedures were performed using Statistica 13.1 software (Dell Inc.).

3. Results

Lead was found in all blood samples taken from mute swans (there were no concentrations found below LoD and LoQ). Mean concentration of lead in blood was 0.239 µg/g with a range 0.028–0.675 µg/g (see Table A1 for mean values of all measured parameters). The frequency distribution of lead concentration in mute swans' blood (Fig. 1) significantly differs from normal distribution (Shapiro-Wilk test, $W=0.885$, $p<0.001$) being rightward skewed (skewness=1.51). Twenty-one (i.e. 47%) of birds had higher lead levels in blood than the threshold level 0.23 µg/g (Fig. 1, Mudge 1983; Friend 1985).

The backward stepwise GLZ procedure selected a model with the H/L ratio, scaled mass index (SMI), period and age class which had significant influence on the lead level in mute swan blood. The

Pb level was higher in young birds than in adults and increased with H/L ratio and scaled mass index (Table 1, Fig. 2). Birds caught in spring had lower lead level than in those captured in autumn and winter (Table 1, A1, Fig. 3). Sex had no significant effects on Pb level (GLZ stepwise procedure, Score Statistic = 0.002, p=0.963).

4. Discussion

Waterfowl are most frequently affected by lead poisoning (Haig, et al., 2014; Pain, et al. 2019) and swans are particularly vulnerable to being affected by direct ingestion of spent gunshot (Pain et al., 2015). There are different sources of lead release into the environment, but ammunition is now regarded as the major source of lead contamination (European Commission, 2004). However, results of previous work showed that pellets available on the Polish market were not the source of lead in the blood of mute swans wintering in the Gulf of Gdańsk. Probably, the other sources connected with various anthropogenic activities such as municipal sewage works play locally a greater role as a cause of lead poisoning (Binkowski et al., 2016). An additional source of lead poisoning may be lead found in the sediment (Beyer et al., 2000). There are no data on lead concentration in sandy municipal beaches in the study area, but there is no mining, smelting or other heavy industrial activity aimed at lead processing in this area. Very high lead levels in the waterfowl leading even to deaths may be caused by ingestion of contaminated sediment, which was however reported from areas subjected to severe contamination from lead mining and smelting for many years (Blus et al. 1993, Beyer et al., 2000). The blood lead levels found in this study should be treated as elevated, according to the threshold mentioned (Mudge, 1983; Friend, 1985), as well as to comparisons to the data of free-living birds available in the literature, where mean concentrations such as 0.125 µg/g in Mallards (free-living; Binkowski and Meissner, 2013) and 0.029 µg/g in Coots (free-living; Binkowski and Sawicka-Kapusta, 2015) are presented. However, in the British Isles, where lead poisoning of waterfowl has been thoroughly studied, the levels in mute swans were even higher than those noted by us and usually averaged around 1 µg/g with a range 0.096 to 3.472 µg/g (Perrins et al., 2003; Simpson et al., 1979; O'Halloran et al., 1988). In these and in the present study, lead was found in all blood samples taken from mute swans. Almost half of individuals examined in this study had increased blood Pb levels above the threshold level, however none of them showed behavioural signs of lead poisoning which might have occurred due to chronic and very high lead exposure (Roscoe et al., 1979; Dumonceaux and Harrison, 1994). Right-skewed frequency distribution of lead concentration in the birds' blood and also in other tissues such as liver, kidney, wing coverts and shaft of flight feathers is common due to elevated levels or even levels typical for acute poisoning in the sample (e.g. Garcia-Fernandez et al., 1997; Gangoso et al., 2009; Jenni et al., 2015). Although mute swans staying along municipal beaches face limited feeding conditions and feed almost exclusively on bread delivered by people (Meissner and Ciopcińska, 2007), which has low lead levels, lead was found in all blood samples taken from mute swans. As susceptibility to absorption of lead in case of poor diet is greater (Trost, 1981; O'Halloran et al., 1991), this may have additional influence on elevated lead levels in blood of these birds.

Heavy metals are considered as environmental stressors that could produce physiological stress in animals (Davis et al., 2008). An experiment with captive mute swans fed a diet heavily contaminated with lead showed that these birds had a body mass decrease, suffered nephrosis and had reduced haematocrit level and haemoglobin concentration (Day et al., 2003). In our study, birds with higher lead levels in blood had a higher H/L ratio, which indicates a higher immune response. Hence, physiological stress was significantly higher in those individuals. Some other studies also demonstrated that heavy metal pollution increases the heterophils/lymphocytes (H/L) ratio in birds (Eeva et al., 2005; Plautz et al, 2011; Cid et al., 2018). As the enhanced immune function is biologically costly (Lochmiller and Deerenberg, 2000), higher cellular immune responses may thus have negative effects on the birds' breeding performance and survival (Vallverdú-Coll et al., 2019).

In mute swans examined in this study, higher lead levels were associated with a higher condition index, which is in contrast to the results obtained for different wildfowl by other authors (Sanderson and Bellrose, 1986; Hohman et al., 1990; Ferreyra et al., 2015). In general, the effect of lead poisoning on body mass is unclear as body mass decrease was detected mainly in wild birds exposed to very high concentrations of lead (Day et al., 2003; Friend 1999) or those that had lead shot in their gizzard (Hohman et al., 1990). It seems that larger birds, such as the mute swan, show a higher tolerance to lead than smaller ones (Williams et al., 2017). The effect of lead poisoning on body mass may vary considerably among species (Martinez-Haro et al., 2011; Newth et al., 2016; Cid et al., 2018) under the influence of different conditions, including diet (Grasman and Scanlon, 1995; Schueuhammer, 1997; Day et al., 2003; Martinez-Haro et al., 2011).

The mean lead level in blood was higher in young birds than in adults. On the municipal beaches, mute swans form dense flocks and aggressively beg for food (mainly bread) delivered by people (Keane and O'Halloran, 1992; Józkowicz and Górska-Kłęk, 1996; Meissner and Ciopcińska, 2007). When the competition for food increased, mute swans showed more agonistic behaviour towards their flock mates with juvenile swans being victims in most aggressive intraspecific interactions (Meissner and Ciopcińska, 2007). These juveniles are often pushed to forage in suboptimal sites, where food is not clumped, but scattered over a large area (Milinski et al., 1995). As a result, juveniles usually stay on the edges of the flock (Józkowicz and Górska-Kłęk, 1996) and may be forced to take less calorific food from water plants and algae more often than adults. In the Gulf of Gdańsk, lead concentrations in algae are decreasing, but still elevated (Żbikowski et al., 2006). Therefore, feeding on plants by young birds in a higher proportion compared to adults can cause higher levels of lead in their blood. However, it is difficult to disentangle this from other factors that may influence sensitivity to lead poisoning (Pain et al., 2019). Complex physiological processes regulate exposure and toxicity risk to lead and there is conspicuous variation in tolerance to lead

(Hoffman et al., 1981; Haig et al., 2014). No significant difference was found in blood lead levels between mute swan males and females in this and other studies (O'Halloran et al., 1991; O'Connell et al., 2009). However, O'Connell et al. (2009) showed that only in rural areas around Cork (Ireland) females had higher Pb level than males. The diet and feeding ecology of both sexes are similar, but males reveal higher winter site fidelity than females (Wieloch et al., 2004), which may potentially lead to sexual differences in lead levels in blood.

A seasonal difference in lead levels in mute swan blood was found also in Ireland with highest values in winter (O'Halloran et al., 1991) or in autumn and spring (O'Connell et al., 2009), but the latter studies were based on small sample sizes. In the Gulf of Gdańsk, lead levels in blood were higher in autumn and winter than in spring. It should be noted that mute swans start to move towards breeding sites in late February or early March (Wieloch et al., 2004) and only nonbreeding individuals remain in spring in the study area.

5. Conclusions

Almost half of individuals examined (47%) had Pb blood level above the threshold. However, none of the specimens revealed behavioural symptoms of poisoning. Analysis of leukocyte profiles showed that birds with higher lead levels in blood had also a higher heterophils to lymphocytes ratio, which indicates increased physiological stress. As the enhanced immune function is biologically costly, this may have negative effects on the birds' breeding performance and survival of the population sampled on the southern Baltic.

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Declaration of Competing Interest

The authors report no conflict of interest.

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Appendix

Table A1. Mean and standard deviation of lead levels in blood, H/L ratio (not arcsine transformed) and scaled mass index (SMI) in juvenile and adult mute swans in prescribed periods. Data from age and sex classes were pooled.

Parameter	Autumn N=12	Winter N=16	Spring N=17	All periods N=45
Pb level [µg/g]	0.261 (0.156)	0.235 (0.135)	0.228 (0.073)	0.239 (0.120)
H/L ratio	0.210 (0.074)	0.271 (0.100)	0.314 (0.136)	0.271 (0.115)
SMI	10.456 (1.036)	8.410 (1.142)	8.616 (1.228)	9.033 (1.422)

FIGURES

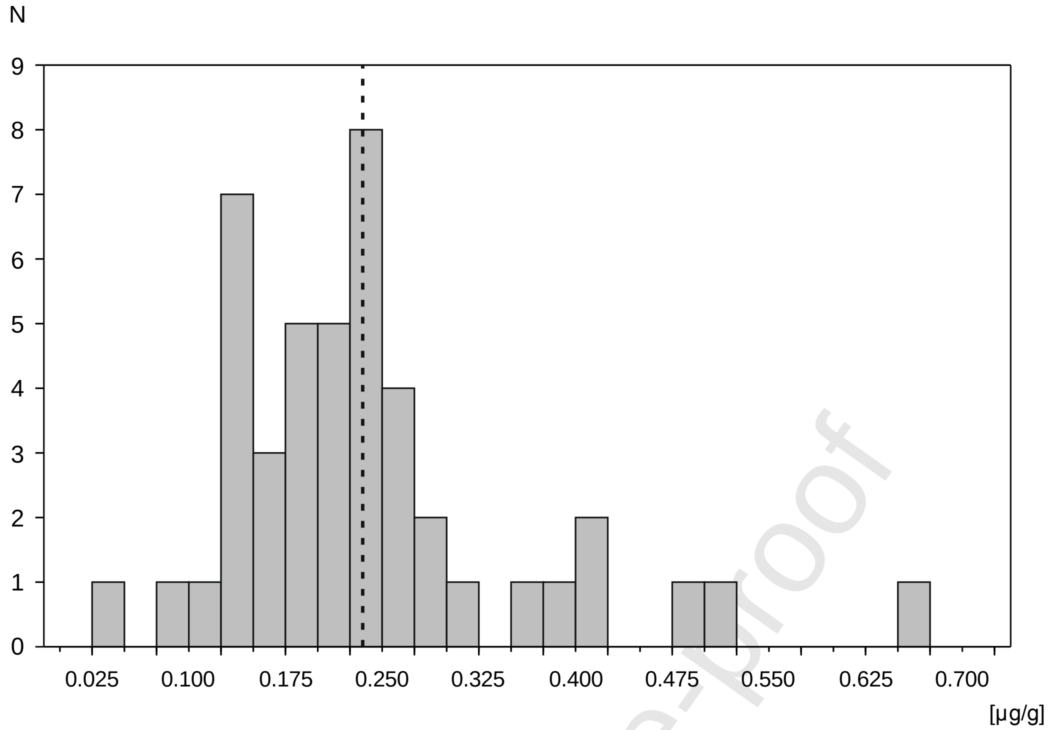


Figure 1. Frequency distribution of lead concentrations in blood of mute swans caught on the Polish Baltic coast. The threshold level (0.25 µg/mL ~ 0.23 µg/g) according to Mudge (1983) and Friend (1985) is shown as a vertical dashed line.

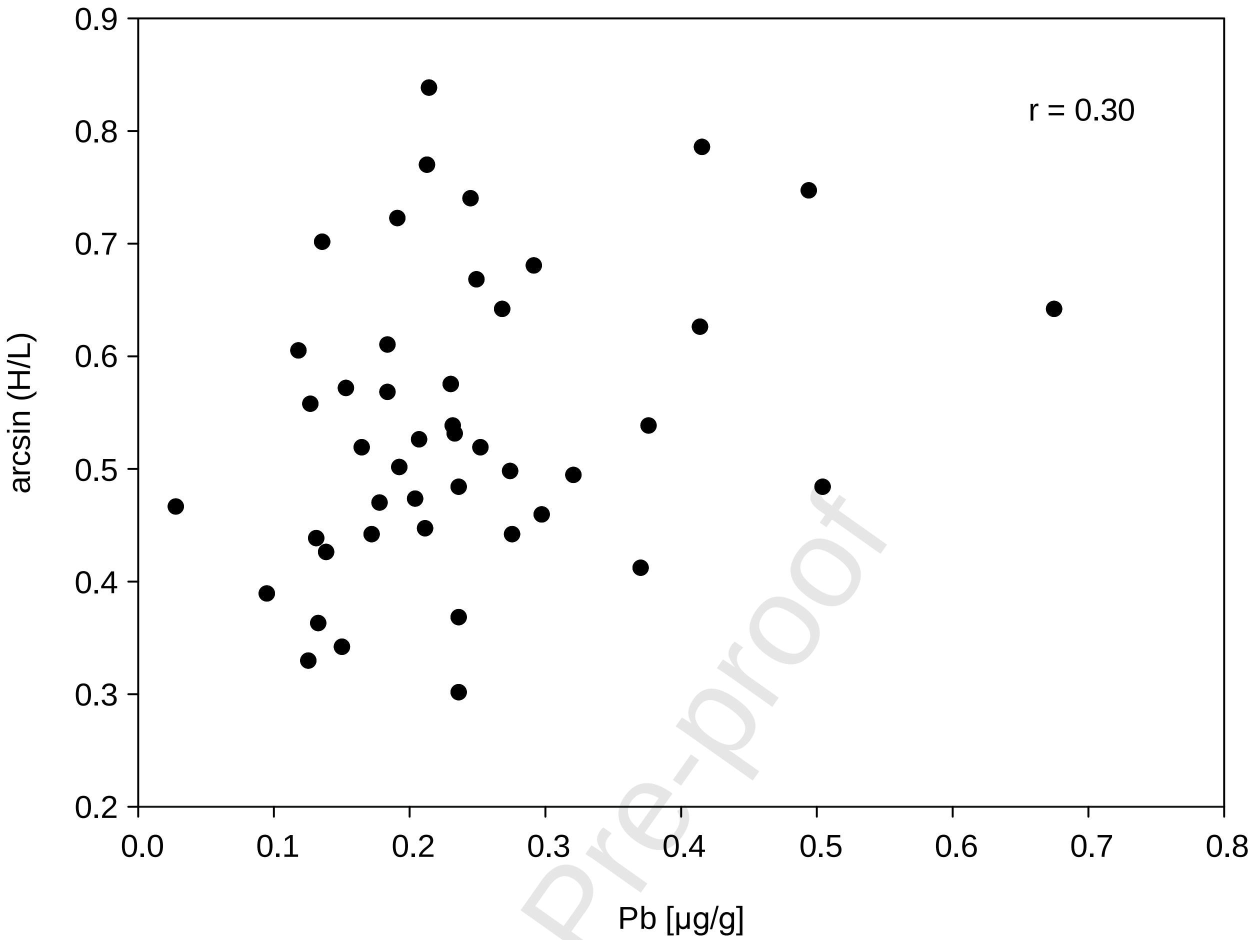


Figure 2. Relationship between lead levels in blood and heterophils to lymphocytes (H/L) ratio in mute swans studied. The correlation coefficient is statistically significant at p = 0.005.

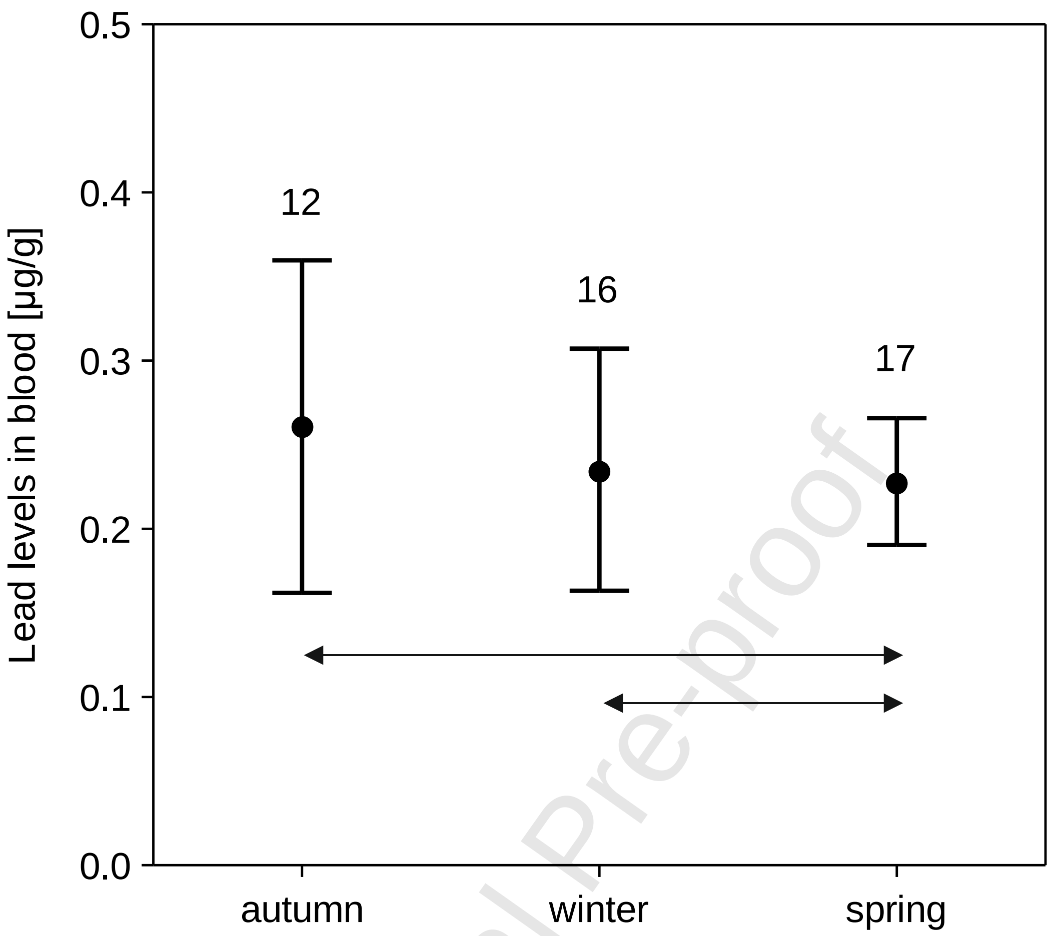


Figure 3. Mean lead levels in mute swan blood in autumn, winter and spring. Dots – mean values, vertical lines – 95% confidence level. Total sample sizes are given above. Arrow shows significant difference between two periods ($p<0.05$).

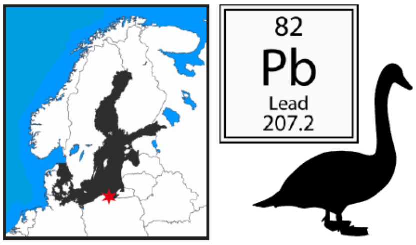
Table. 1. Effects of H/L ratio, scaled mass index (SMI), period and age on the lead levels in mute swan blood according to the selected GLZ model. Estimated regression coefficients are set to zero for baseline categories of variables (period: autumn and age: young).

Explanatory variable	Coefficient	SE	Wald χ^2	*P*
Constant	-3.519	0.627	31.46	<0.001
H/L ratio	1.810	0.512	12.46	0.019
SMI	0.122	0.057	4.59	0.032
Period (spring)	-0.247	0.098	6.43	0.011
Period (winter)	0.131	0.106	1.52	0.218
Age (young)	-0.176	0.069	6.53	0.011

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



Elevated heterophils to lymphocytes ratio in mute swans
with high Pb level in blood - a sign of long-term physiological stress

Graphical abstract

Highlights

- Pb blood level in mute swans staging in municipal beach was elevated
- The physiological stress was higher in individuals with higher Pb blood level
- The mean Pb blood level was higher in young birds than in adults
- Pb blood level in mute swan was higher in autumn and winter than in spring