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BIOMARKERS OF LIPID AND PROTEIN OXIDATION IN THE BRAIN OF PIGEONS *COLUMBA LIVIA* F. *URBANA* INHABITING AREAS WITH VARIOUS ANTHROPOPRESSURE (NORTHERN POLAND)

ABSTRACT

The mechanisms of functioning of organisms in anthropogenically modified habitats are an important issue of ecotoxicology and ecophysiology. Birds are sensitive indicators of the impact of various factors, as evidenced by the levels of pollutants such as heavy metals in their bodies, especially in their feathers. The aim of the study was to analyze the level of oxidative stress biomarkers using substances reactive to 2-thiobarbituric acid (TBARS) as biomarkers of lipid peroxidation and ketone and aldehyde derivatives as biomarkers of oxidatively modified proteins in the brain tissue of pigeons *Columba livia f. urbana* inhabited two sites of different exposure to lead in Pomerania (N Poland). We studied birds from two environments (Słupsk and Szpęgawa), which differ in the content of metals in the soil. Statistically significant higher levels of chemical elements were found in the area of Szpęgawa compared to the results obtained from Słupsk soils. Thus we classified this area as polluted, compared to the unpolluted area of Słupsk. Pigeons from polluted areas were characterized by statistically significant higher levels of Si and Pb in feathers. Data showed that pigeons accustomed to lead-contaminated areas (Szpęgawa) had statistically higher levels of TBARS and carbonyl derivatives of oxidatively modified proteins in brain tissue than pigeons from unpolluted region (Słupsk). Environmental lead pollution related to anthropogenic pressure caused a significant increase in the oxidation of free radicals and modification of protein structures in the brain of birds, which may lead to dysfunction of appropriate physiological processes in organisms.

Key words: anthropogenically changed environments, lead, oxidative stress, biomonitoring, feathers, brain, birds

INTRODUCTION

The extensive development of human activity has led to disturbances in the ecological conditions of natural ecosystems (Bassi *et al.* 2021). The majority of heavy metals in the ecosystems are derived from their content in soil-forming rocks. These elements are also replenished by human activities. Pollution of soils near industrial centres is mainly due to emissions of harmful compounds from industrial plants and transport. Cd, Pb, Zn, Hg, and Cu are widely used in industry and are active environmental pollutants (Hou *et al.* 2019; Walter 2023). Combustion of coal, oil shale, and oil is the dominant and more intensive source of pollution than metallurgical production. Up to 5 billion tonnes of fuels are burned each year. Almost all metals can be found in coal and oil ash, sometimes in concentrations that economically justify their extraction from the ash. The scale of fossil fuel use is such that the combustion of coal, oil shale, and oil is the dominant and more intensive source of pollution than metallurgical production (Pain *et al.* 2019).

The mechanisms of functioning of organisms in anthropogenically modified habitats are an important issue of ecotoxicology and ecophysiology. Birds are sensitive indicators of the impact of various factors, as evidenced by the levels of contaminants (especially heavy metals) in their bodies, particularly in feathers (Nam and Lee 2006; Kozák *et al.* 2022). The attachment of pigeons *Columba livia* f. *urbana* to their nesting sites creates conditions for long-term monitoring of the state of ecology of their habitats (Janiga and Zemberyová 1998).

The effects of lead exposure on two pigeon populations from N Poland have been investigated previously, e.g. Kurhaluk *et al.* (2021) and Tkachenko *et al.* (2021). We investigated the effects of lead accumulated in soils and in feathers of pigeons on the biomarkers of oxidative stress and antioxidant defense in various tissues. In this study, we look at the effects of lead exposure on one of the most sensitive system of organism, i.e. the brain tissue. It is known that the negative effects of lead on the nervous system are largely related to the disturbances in the formation of myelin (Monje 2018). Myelin surrounds the processes of nerve cells, isolating them from external influences. This is important for more reliable and faster transmission of signals through the nervous system. It is necessary to note the negative effect of lead on the synthesis of neurotransmitters as chemical messengers of signals between neurons and from neurons to the effector cells, which enable the association of individual neurons in the brain and ensure the successful fulfilment of all its numerous and vital functions (Monje 2018).

Lead has been shown to affect lipid metabolism in animals. It is associated with impaired cholesterol kinetics, which can have serious implications for energy status, and synthesis of steroidal adrenal and sex hormones, ultimately leading to impaired maturation of sex products and reduced survival of individuals (Pain *et al.* 2019). In animals, lead exposure disrupts the ratio of serum glycoprotein and lipoprotein fractions, causing an increase in triglycerides, total cholesterol, and low-density lipoprotein cholesterol and a decrease in high-density lipoprotein cholesterol. This leads to atherosclerosis of the blood vessels (Zhao *et al.* 2023).

Lead induces oxidative stress, which is associated with a sharp increase in the reactive oxygen species (ROS) in animal cells (Raine *et al.* 2015). As a result, the intensity of lipid peroxidation increases, the levels of damaged proteins also increase, and membrane structure and function are altered (Kurhaluk *et al.* 2022). At the same time, the damage to the mitochondrial membranes, e.g. disrupts energy budget of cell metabolism (Koivula *et al.* 2011). It needs to be highlighted that lead may bind to the sulfhydryl groups of proteins, which leads to adverse effects related to the change and/or disruption of their functional role, including the incorrect functioning of enzymes, transcription factors, stress proteins, etc. (Walter 2023).

The aim of the present study was to analyze the levels of biomarkers of oxidative stress using 2-thiobarbituric acid reactive substances (TBARS) as biomarkers of lipid peroxidation, and ketonic and aldehydic derivatives as biomarkers of oxidatively modified proteins in the brain tissue of pigeons *Columba livia* f. *urbana* habituated in two sites of different lead exposure in the Pomeranian region (N Poland).

MATERIAL AND METHODS

Study area and characteristics of groups

We studied pigeons *Columba livia* f. *urbana* from two localities (Słupsk and Szpęgawa). The first is Szpęgawa (N 54°05′44.4″, E 18°43′15.7″), a village in the Pomeranian Voivodeship near the Voivodeship road No. 224 in Northern Poland. Szpęgawa is situated about 120 km East of Słupsk (Photo 1). Słupsk (N 54°27′57.681″, E 17°1′50.366″) is a city of 90 thousand inhabitants located in the central part of Pomerania. The pigeon colony from Słupsk, numbering over 300-400 individuals for many years, was analyzed in 2007-2008 in order to conduct a series of ecophysiological studies (Hetmański 2011).

The Stanisławie junction of the A1 motorway is located near Szpęgawa. Its importance as the A1 motorway in Poland is due to the fact that it is part of the international route E75 of the Trans-European Transport Corridor. It is the only Polish highway

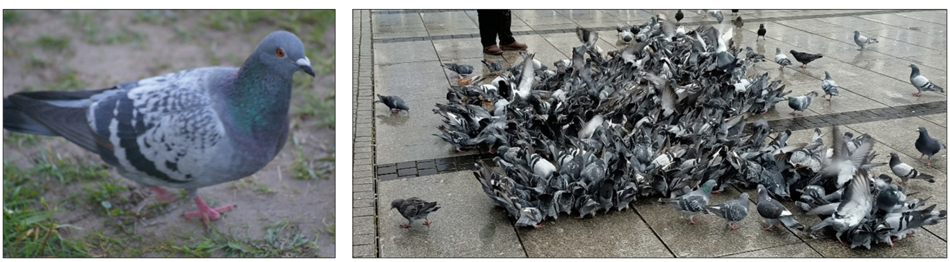

Photo 1. Birds from two localities – Szpęgawa (A) and Słupsk (B). Photo by Tomasz Hetmański

with a southern route. The A1 motorway is characterized by heavy traffic. Currently, it connects Gdańsk, Gdynia and Sopot, a large urban center, with the Czech border (D1 motorway). One of the problems with such highways is that they can be accessed from narrow, single-lane roads. Nearby areas, such as the village of Szpęgawa and the Stanisławie road junction, are strongly affected by emissions of pollutants into the atmosphere. This is also related to the increase in the volume of freight traffic and the popularity of car tourism, which is associated with the emission of so-called aerosol mixture of fuels and combustion products into the atmosphere. In the village there are farms located on agricultural land.

For the study of suburban pigeons, a farm was selected in the area with coordinates of N 54°05′44.4″, E 18°43′15.7″. This old farm was built after World War II. In the 1980s, loose slag (sludge), waste from metallurgical production, was used to harden the soil on the farm. The farm is also a place for breeding city pigeons from the population of Słupsk pigeons.

Ethics Statement

The experiments were conducted by the Guidelines of the European Union Council and the current laws in Poland. Adult pigeons, at least 1 year old, were used in the study. Sexual maturation in these birds begins at the age of 3 months (Hetmański 2007; Hetmański 2011). The study was conducted with the approval of the Bioethics Committee (licence number 44/2012).

Tissue samples

Brain tissue from 17 pigeons living in Słupsk and 14 pigeons living in Szpęgawa were used for the study. The brains of birds were removed after decapitation. One pigeon was used for each preparation. Briefly, the brain tissues were excised, weighed and washed in ice-cold buffer. The minced tissue was rinsed with cold isolation buffer to remove blood and homogenized in a homogenizer H500 (POL-EKO, Poland). Brain homogenates were centrifuged at 3,000 g for 15 minutes at 4°C. The isolation buffer contained 180 mM KCl, 10 mM HEPES, 10 mM EGTA and 0.5% of bovine serum albumin; the pH was adjusted to 7.3 with KOH. The suspension was then used for analysis. The Bradford method (1976) with bovine serum albumin as standard was used for protein quantification. The absorbance was recorded at 595 nm.

Chemical elements concentration

Concentration of chemical elements from which pigeons collect gastroliths was determined in surface soil samples taken from both sites. In Słupsk, 4 soil samples were taken (each sample was analyzed in triplicate) from the Old Market Square, where the largest flock of pigeons is located. In Szpęgawa, 4 soil samples were collected (the analysis of each sample was carried out in three repetitions) from the area between the farm buildings, where the birds receive food and water. Feather samples were also collected from 5 adult birds (at least 1 year old) at each site. Contour feathers were taken from the birds' backs.

Soil samples were taken from a depth of 1-3 cm. Samples were then aggregated and air-dried before storage and analysis. Each soil and feather sample was analyzed in three series. Between different readings, the soil sample was thoroughly mixed in the same bag. The results of three readings were averaged. The analysis of the concentration of chemical elements in feather and soil samples was carried out using an X-ray fluorescence analyzer (XRF) at the Faculty of Physics of the Pomeranian University in Słupsk. An XRF analyzer model Sci Sps X-200 from Sci Sps, Inc. was used to determine the concentration of chemical elements in samples. The analyzer is designed to test elements in various samples such as soil, alloys, precious metals and some others.

An XRF (X-ray fluorescence) analyzer generates an X-ray beam that can be used to irradiate a sample. The interaction of X-ray quanta with the analyzed sample causes characteristic X-ray emission from chemical elements contained in the sample. Analysis were performed with a Rh target (50 kV, 600 μA) and poly-capillary optics providing a 25 μm spot size. The X-ray fluorescence signal was collected using two XFlash® silicon drift detectors. They provide a high spectral resolution of 135 eV measured at full width at half maximum, FWHM, with a K-α line of 5.95 Mn. Detectors record X-ray fluorescence spectra, i.e. X-ray fluorescence, containing information about the presence of chemical elements and their concentrations. K and L series X-ray fluorescence are commonly used to identify chemical elements because they provide the best results. The detectors have an active area of 30 mm^2 and are placed at an angle of 45° to the X-ray beam. Analysis were performed in vacuum (20 mbar) using a sampling step of 20 μm and a dwell time of 10 ms. The device is factory calibrated with 37 standard elements, including all measurable pathfinders. X-ray fluorescence hyperspectral data were processed using PyMca 5.1.3 (Solé *et al*. 2007) and Datamuncher (Alfeld and Janssens 2015). The device software uses either standard methods, such as the basic parameters of the spectra of given elements (this is the method we used in our measurements), or user-generated empirical calibration curves to relate X-ray spectrum to elemental concentrations.

Biochemical assays

2-Thiobarbituric acid reactive substances (TBARS) assay

The degree of lipid peroxidation was determined by quantifying the concentration of 2-thiobarbituric acid reactive substances (TBARS) using the Kamyshnikov method (2004). This method is based on the reaction of the lipid peroxidation degradation product, malondialdehyde (MDA), with 2-thiobarbituric acid (TBA) at high temperature and acidity, producing a colored adduct that is measured spectrophotometrically.

The nmol of MDA per mg of protein was calculated using an extinction coefficient of $1.56 \cdot 10^5$ mM^{-1} cm^{-1}.

Protein carbonyl derivative assay

The determination of oxidative modification of carbonyl derivatives of proteins (OMP) was based on spectrophotometric measurement of the level of aldehyde (AD) and ketone derivatives (KD) in tissue samples. The rate of oxidative degradation of proteins was estimated from the reaction of the resulting carbonyl derivatives of amino acid reactions with 2,4-dinitrophenylhydrazine (DNFH), as described by Levine *et al.* (1990) with some modifications. DNFH was used to determine the carbonyl content of soluble and insoluble proteins. Carbonyl groups were determined spectrophotometrically from the difference in absorbance at 370 nm (aldehydic derivatives, OMP_{370}) and 430 nm (ketonic derivatives, OMP_{430}).

Statistical analysis

Results were expressed as arithmetic means ± S.D. Significant differences between means were measured using the multiple range test, where at least $p < 0.05$. Normally distributed data were log-transformed. Tests for basic statistical analysis (significance of regression slope, analysis of variance for significance between locations) were performed using STATISTICA 13.3 (TIBCO Software Inc., USA) with 95% confidence intervals ($\alpha = 0.05$) to determine the significance of differences between types of regions and enzyme activity in the brain tissue of birds from various regions (Stanisz 2006, 2007).

RESULTS

Chemical elements concentration

In the case of some chemical elements, their concentrations in the soils of both regions were statistically significant, as we have already written about in our previous publications (Kurhaluk *et al.* 2021; Tkachenko *et al.* 2021). We would like to emphasize this point because it allowed us to determine our model of the relative predominance of lead contaminants. The metal content in the soils of the studied areas differed significantly, i.e. in Szpęgawa, a statistically significantly higher level of metals was observed, compared to the results obtained from the Słupsk soil. This allowed the area to be classified as polluted (polluted area), as the content of metals except Si, Ni and Cu was statistically higher compared to the data from the Słupsk area. In soil samples from Szpęgawa, the content of Al was 121%, Ti – 23%, Mn – 242%, Fe – 15.5% and Pb – 543.5% more than in Słupsk (unpolluted area). The lead content in the soil from Szpęgawa was five times higher. The content of metals such as Zn, Zr and Si was much higher in Słupsk than in Szpęgawa. The content of elements in pigeon feathers was ambiguous. Pigeons from polluted areas were characterized by statistically significantly higher contents of

Si and Pb in feathers and lower contents of Fe, Cu and Zn, compared to pigeons from the Słupsk area.

Lipid peroxidation and oxidatively modified proteins

The first stage of our biochemical research was to determine the level of lipid peroxidation in the brain tissue of pigeons living in the regions with different levels of lead exposure. These important biomarkers that are end products reacting with 2-thiobarbituric acid, namely malondialdehyde levels, are shown in Figure 1A. The data show that pigeons tamed in areas contaminated with lead (Szpęgawa) were characterized by a statistically higher level of TBARS (by 150.3%, $p < 0.05$) in the brain tissue than the pigeons from unpolluted areas.

In the second stage of the study, the oxidative modification of proteins was examined, assessed on the basis of the content of aldehyde derivatives (OMP AD) and ketone derivatives (OMP KD) in the brain tissue of pigeons from two habitats. We showed that lead-contaminated bird habitat was associated with statistically significant increases in AD OMP and KD OMP levels in this tissue (Figure 1B).

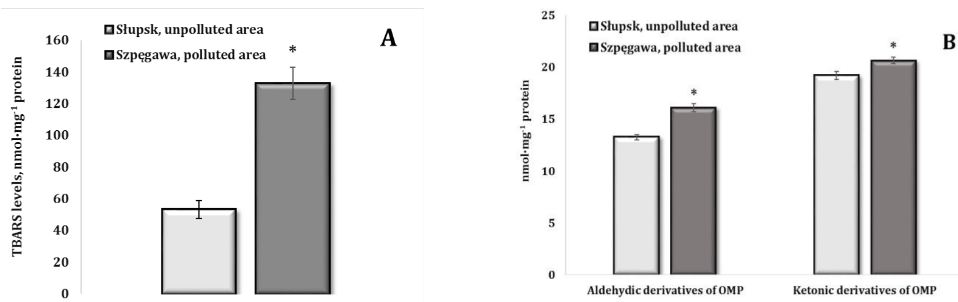

Figure 1. TBARS levels (A, nmol·mg^{-1} of protein), aldehydic and ketonic levels of oxidatively modified proteins (B, nmol·mg^{-1} of protein) in the brain tissue of pigeons *Columba livia* f. *urbana* living in the areas with different levels of pollution (Pomeranian Voivodeship, Northern Poland).
Results are expressed as arithmetic means ± S.D.
* changes were statistically significant at $p<0.05$ compared to the birds living in the unpolluted areas.

Therefore, anthropopressure-related environmental pollution with lead is associated with the induction of a significant intensification of free radical oxidation processes in the brain tissue of birds and a significant modification of protein structures in this tissue, which may cause functional disorders of physiological processes.

DISCUSSION

Many studies have shown the adverse effect of lead on the functioning of the redox system in various tissues. Our studies showed a high correlation between oxidative stress biomarkers, assessed by TBARS levels, and increased levels of aldehyde and ketone derivatives of oxidatively modified proteins in the brain tissue of pigeons accustomed to polluted, high-traffic areas. These data also confirm the relationships demonstrated by other authors that lead is one of the most dangerous chemical elements for all living organisms and has a polytropic, negative effect on them (Skalny *et al*. 2021). At the same time, the risk of lead is increased by the large number of possible sources of its entry into the living organism, as well as its ability to accumulate in tissues and organs and to maintain toxic properties for a long time, which has also been convincingly demonstrated in the works of many authors (Hou *et al*. 2018; Andrew *et al*. 2019).

Relationship between the content of heavy metals in pigeon feathers and the color of their plumage is considered by scientists to be an element of the adaptive response (Chatelain *et al*. 2014). For example, it is believed that pigeons have developed an adaptive response to the abundance of heavy metals by partially excreting them through their own feathers. This is because researchers have found that dark pigeons – those with more melanin pigment in their feathers – are more common in large cities than in suburbs and villages (Chatelain *et al*. 2016a, b). Scientists have proposed that melanin binds metal atoms, thanks to which pigeons with darker plumage have a better life in cities: they remove heavy metals from the body more effectively by simply transferring them to the feathers (Chatelain *et al*. 2016a, b). Similarly, birds can remove zinc, lead, or both, and their dark plumage gives them an evolutionary advantage in environments polluted with heavy metals. In reality, however, this problem is still not fully understood. It is uncertain whether urban pigeons' plumage is becoming darker due to the cleansing properties of melanin-rich feathers, as the genes that produce melanin are also linked to the regulation of stress hormones and the immune system. Thus, pigeon plumage color and the level of heavy metals in their environment may not be directly related. This may be part of a more complex adaptation because the stress levels and immune systems of urban animals differ from those of their wild counterparts (Mundy 2005).

Such numerous and multidirectional effects of lead on cells lead to disruption of neurovegetative and digestive processes in animals, progression of vegetative dystonia and anemia, increase in the incidence of cardiovascular diseases, and reduced immunity and resistance of the body to stress factors (Burger and Gochfeld 2000, 2005; Kanstrup *et al*. 2019; Wang *et al*. 2022). Generally speaking, when lead accumulates in large amounts in the body of animals, it has a strong negative impact on various aspects of their life activity, reduces their functional adaptation capabilities, and in some cases even leads to their death (Kanstrup *et al*. 2019). Since animals are one of the intermediaries of the food chain, high lead concentrations in the body of wild birds reflect the direction of physiological regulatory processes during their poisoning and are of interest as bioindicators of the level of environmental pollution (Williams *et al*. 2018; Monclús *et al*. 2020).

High concentrations of lead inhibit mitochondrial respiration, disrupt protein synthesis processes and negatively affect the composition of microelements. Moreover, lead causes oxidative stress in cells, associated with a sharp increase in the amount of ROS, which is an important mechanism of the toxic effect of the metal (Patra *et al.* 2011; Lopes *et al.* 2016). Due to its variable valency, lead can initiate free radical processes, the intensity of which increases in the context of a slowdown in the activity of antioxidant enzymes (Adonaylo and Oteiza 1999; Patil *et al.* 2006). The observed activation of lipid peroxidation leads to disruption of the structure and integrity of cell membranes (Ayala *et al.* 2014). As a result, the metabolism is disturbed and many irregularities and changes in the functioning of the body occur. For example, damage to mitochondrial membranes disrupts tissue respiration and inhibits ATP synthesis. Damage to lysosomal membranes is associated with the release of hydrolytic enzymes and excessive intensification of hydrolytic processes. We have already demonstrated these dependencies in the course of oxidative processes (Kurhaluk *et al.* 2021; Tkachenko *et al.* 2021).

It is well known that the toxic effect of heavy metals in general, and lead in particular, is that they can mechanically clog the body, i.e. deposit on the walls of blood vessels, renal and hepatic ducts, reducing the filtration capacity of these organs. This leads to the accumulation of toxins and metabolic products from the body's cells, i.e. to self-poisoning of the body, because the liver is responsible for processing toxic substances that enter the body, and the kidneys are responsible for their removal from the body. Additionally, the accumulation of excessive amounts of lactic acid and ketoacids in cells due to disruption of redox processes leads to the development of acidosis, i.e. a shift of the acid-base balance towards increased acidity, which in turn leads to even greater acidification of the cell membrane permeability. The toxic effect of lead on the animal's body is also associated with its competitive relationship to certain elements, since it can replace mono- and divalent cations (sodium, calcium, magnesium, iron) in biologically important molecules, thereby disrupting various biological processes (Flora *et al.* 2012; Wani *et al.* 2015).

CONCLUSIONS

The study analyzed the level of biomarkers of lipid peroxidation and oxidatively modified proteins in the brain tissue of birds inhabiting areas with various degrees of anthropopressure. Environmental lead pollution related to anthropogenic pressure is associated with a significant increase in oxidative stress in the brain tissue of birds and a significant modification of lipid and protein structures in this tissue, which may lead to functional disorders of physiological processes.

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