EFFECT OF METAL(LOID)S ON BIOMARKERS IN BLOOD OF WHITE STORK (Ciconia ciconia) NESTLINGS FROM CONTINENTAL CROATIA

Bjedov, Dora

University of Zagreb

Faculty of Science

Dora Bjedov

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DOCTORAL DISSERTATION

Supervisors:

Mirna Velki, Ph.D., associate professor

Alma Mikuška, Ph.D., assistant professor

Zagreb, 2023.

This doctoral dissertation was made at the Department of biology, Josip Juraj Strossmayer University of Osijek, under the supervision of Mirna Velki, Ph.D., assoc. prof. and Alma Mikuška, Ph.D., assist. prof., as a part of the Doctoral programme of Biology at the University of Zagreb, Faculty of Science, Department of Biology.

SUPERVISOR INFORMATION

Velki, Mirna

Mirna Velki, Ph.D., is an associate professor at the Department of Biology, Josip Juraj Strossmayer University of Osijek. She was born in Osijek, Croatia, on 13th January 1987. She graduated from the Department of Biology, Josip Juraj Strossmayer University of Osijek in 2010 and became a Master in Biology. She finished doctoral studies at the Faculty of Science, University of Zagreb in 2014 and obtained PhD in natural sciences, scientific field of biology. Her main focus of research is ecotoxicology, biomarker responses in aquatic and terrestrial organisms, adverse effects of pesticides and other environmental pollutants and assessment of the mode of action of pollutants. She is the author and co-author of 45 scientific papers and 4 book chapters. She participated in 37 national and international conferences. She mentored 14 bachelor and master theses.

Mikuška, Alma

Alma Mikuška, Ph.D., is an assistant professor at the Department of Biology, University of Josip Juraj Strossmayer of Osijek. She was born in Zenica, Bosnia and Herzegovina, on August 5th, 1974. She graduated in Biology and Chemistry at the Faculty of Pedagogy, University of Josip Juraj Strossmayer in Osijek in 2000. She received her master's degree in 2007 at the Graduate Interdisciplinary Scientific Study "Nature and Environmental Protection" in Osijek, and her doctorate in 2010 at the Faculty of Science, University of Zagreb. At the Department of Biology, Josip Juraj Strossmayer University of Osijek she lectures courses related to the biology and ecology of vertebrates, as well as conservation biology. The special focus of her research is the biology and ecology of the birds, especially marsh birds from the heron family, white storks, spoonbills and white-tailed eagles. She is the author and co-author of 35 scientific and professional articles and 6 books. She participated in 35 national and international conferences. She mentored 40 bachelor and master theses and one doctorate.

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The road to excellence started on a literal dirt road. During one sunny day in the field, an interesting conversation with Tibor sparked my curiosity. His casual comment about the need to analyse heavy metal in the Croatian bird population inspired a million ideas for me. Now the metaphorical dirt road start. With no project, no money, and no supervisors. Before the beginning, I was at a dead end. This is where, on another sunny day, I walked up to professor Mikuška, and pitched my idea. The following day, I went to professor Velki, introduced myself and pitched my idea. Still, no projects, no money, but with two potential supervisors. After many brainstorming sessions, ideas are forming, financial support from student projects is coming, the first fieldwork is conducted, lab work is performed, and papers are published. My supervisors have orchestrated the perfect plan and confirmed a very known general concept – teamwork makes the dream work. All their input, advice, leadership, guidance and patience helped me develop critical thinking and a scientific mind.

May many sunny days be ahead of us.

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University of Zagreb
Faculty of Science
Department of Biology

Doctoral dissertation

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Dora Bjedov

Department of biology, Faculty of Science, University of Zagreb, Rooseveltov trg 6, Zagreb

Abstract

Apex bird species are proven to be relevant bioindicators for assessing environmental pollutants. To achieve this, the goal of this work was to compare biomarker responses in two fractions of white stork (*Ciconia ciconia*) nestling blood; establish novel biomarker approaches (esterase, fluorescent dyes, metallothioneins), apply previously analysed biomarkers (glutathione-dependent enzymes); investigate the differences in biomarker responses related to metal(loid)s in white stork nestling's blood from Croatia. Results indicate differences between the fractions in biomarker response based on variability. The pollutant impact from the surrounding metal, petroleum, and agricultural industry appears to affect the biomarkers responses/levels in white stork nestlings, which are often seen as early-warning signals. Observed increased metal(loid) levels at the landfill and agricultural areas might cause adverse effects on the nestlings. This first-time metal(loid) analyses in Croatian white stork nestlings indicate the necessity of biomonitoring and local assessments of pollution impact to prevent detrimental effects on the environment.

Keywords: *blood, birds, apex predator, biomarker, toxic metals, pollution impact*

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Alma Mikuška, Ph.D., assist. prof.

Reviewers: 1. Mirjana Pavlica, Ph.D., full prof.
2. Željka Lončarić, Ph.D., assist. prof.
3. Henner Hollert, Ph.D., full prof.

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Doktorska disertacija

UČINAK METAL(OID)A NA BIOMARKERE U KRVI PTIĆA BIJELE RODE (*Ciconia ciconia*) NA PODRUČJU KONTINENTALNE HRVATSKE

Dora Bjedov

Biološki odsjek, Prirodoslovno - matematički fakultet, Sveučilište u Zagrebu, Rooseveltov trg 6, Zagreb

Sažetak

Bijela roda izvrsna je modelna vrsta za praćenje zagađenja u okolišu. U ovom doktorskom radu provedeno je istraživanje odgovora biomarkera i koncentracije metal(oid)a u krvi ptića bijele rode (*Ciconia ciconia*) s područja Hrvatske. Ciljevi rada su: usporediti odgovore biomarkera u dvije frakcije krvi, uspostaviti nove protokole za mjerenje biomarkera (aktivnost esteraze, fluorescentne boje za detekciju oksidativnog stresa i razine metalotioneina), primijeniti prethodno analizirane biomarkere (glutation-ovisne enzime) i istražiti razlike u odgovorima biomarkera vezanih za metal(oid)e. Rezultati ukazuju na razlike između frakcija u odgovoru biomarkera. Moguć je utjecaj zagađivala iz okolne metalne, naftne i poljoprivredne industrije na biomarkere u ptićima bijele rode, koji se često smatraju ranim znakovima upozorenja. Povećane razine metal(loid)a na odlagalištima i poljoprivrednim površinama mogu uzrokovati štetne učinke na ptiće. Ovo je prvo istraživanje metal(loid)a u ptićima bijele rode s područja Hrvatske te ukazuju na neophodan monitoring za procjenu utjecaja zagađenja kako bi se spriječili štetni učinci.

Ključne riječi: *krv, ptice, vršni predator, biomarkeri, toksični metali, utjecaj zagađenja*

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Mentori: izv. prof. dr. sc. Mirna Velki
doc. dr. sc. Alma Mikuška

Povjerenstvo: 1. prof. dr. sc. Mirjana Pavlica, trajno zvanje
2. doc. dr. sc. Željka Lončarić
3. prof. dr. sc. Henner Hollert, trajno zvanje

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Adult white stork from the landfill Jakuševec

1. Introduction

1. Introduction

1.1. Biology and ecology of the white stork (*Ciconia ciconia* L. 1758)

The white stork, *Ciconia ciconia* L. 1758 is a large bird with long red legs and a long red beak, it is 100 – 110 cm tall, with a wingspan of 195 – 210 cm, and weighs 2.3 – 4.5 kg. Sexual dimorphism in body size is slight, adult males are about 12.5% larger than females. The plumage is white, with black tips and a black edge of the wings. In adults, the beak is bright red, while young birds have a dark grey beak, becoming bright red as they grow up (Fig. 1). It is a long-lived species, as the longest recorded lifespan is 39 years according to the EURING Longevity list (EURING, 2010). Storks rarely vocalize, however, they communicate to each other with loud beak clattering (rapid opening and closing of the beak) and low hissing sounds – recognizable sounds during courtship rituals and greeting the nesting partner. White storks coexist and forage in different natural habitats and habitats created by human activities – in open agricultural areas, grasslands, and pastures, especially in wet areas or those located near floodplains and rivers. For example, they tend to walk behind ploughing tractors and catch prey that was left after ploughing, or they walk behind lawnmowers and hunt grasshoppers and other insects that are left without shelter after mowing. Moreover, they forage in parallel with cattle in the pastures – the cattle expose numerous small animals in the pasture by moving. The shores of rivers and shallow waters are a rich source of aquatic animals that white storks feed on (BirdLife, 2023; Dumbović et al., 2010; Mikuska, 2013) In particular, their diet mostly comprises various invertebrates (grasshoppers, beetles, earthworms, crustaceans), amphibians, fish, snakes, lizards, small mammals (voles, mice, rats, shrews), and occasionally trash from landfills (Blanco, 1996; Kruszyk & Ciach, 2010; Tortosa et al., 2002).

The white stork is a migratory bird species breeding in Europe and winters in Africa. Reproduction begins with building a new nest or repairing an old nest, and mating. Courtship consists of a series of bows, head tilting left-right, spreading wings and clucking the beak. Every year, most pairs return to their old nests. The nest is a large pile of branches interwoven with lumps of mud, manure and similar material. Storks cover the centre of the nest (bed) with twigs and grass, but also with paper, rags and similar waste. Since white storks add material to the nest every year, very old nests can be up to 5 m high, > 2 m in diameter and weigh up to one ton. The nest is built and repaired by both the male and the female, and the one that returns first from Africa in the spring begins is usually a male. The female lays 1 – 7 eggs in the nest, usually 3 – 5 eggs. Laying on the eggs lasts 33 – 34 days, and the male and female lay on them

alternately. They also take care of the birds together. For the first 2 – 3 weeks, one of the parents is always with the nestlings, they warm them, protect them from the scorching sun and feed them intensively. Food and water are brought to the nestlings in a crop, *ingluvies* – a special expansion of the oesophagus. The nestlings' feathers are fully grown when they are 58 – 64 days old, and soon they begin to fly and become independent. Most of the juvenile birds that survive their first migration to Africa and return after 4 years, when they become sexually mature, will nest in the area where they were hatched, within a radius of about 25 km around the parental nest (BirdLife, 2020; Dumbović et al., 2010; Mikuska, 2013). The primary threat to the white stork populations are habitat loss. The drainage of wet meadows robs white storks of their basic food supply. In the countries of Central Eastern Europe, the intensification of agriculture, particularly as a result of EU accession, is a major threat to white storks.

Figure 1. Adult white stork *C. ciconia* breeding pair in Davor, Croatia.
(photos taken by Dora Bjedov).

In Croatia, the white stork is a regular breeding species in the continental part and a passage migrant. The breeding population is estimated at 1100 to 1300 pairs (Kralj et al., 2013). Croatian white stork populations breed near rivers Sava, Drava, Dunav, Lonja, Česma, and the valleys that are frequently flooded by their respective rivers. Their richest feeding grounds are the flooded pastures along the Sava River after the flood recedes in spring and early summer: in the numerous puddles and shallows, they abundantly hunt trapped fish, amphibians and reptiles. The white stork is an important species in the ecosystem, as a charismatic and umbrella

species. Protecting white stork indirectly protects many other species in the ecosystem, also known as the umbrella effect.

According to the Nature Protection Act (Official Gazette 70/05, Official Gazette 139/08) and the Ordinance on declaring wild taxa as protected and strictly protected (Official Gazette 99/09), the white stork is a strictly protected species in Croatia. Furthermore, the species is protected by international agreements as well: the Convention on the Protection of European Wild Species and Natural Habitats (Bern Convention), the Convention on the Protection of Migratory Species of Wild Animals (Bonn Convention) and the Convention on International Trade in Endangered Species of Wild Animals and Plants (CITES). The white stork is protected by Annex I of the EU Birds Directive (Directive 79/409/EEC) on the protection of birds (Dumbović et al., 2010; Kralj et al., 2013; Mikuska, 2013).

The white stork is a flagship species whose conservation helps the conservation of other species and their respective habitat (Bowen-Jones & Entwistle, 2002). A variety of ecological aspects make them suitable bioindicators of changes in the environment. Due to being remarkably adapted for cohabiting with humans, white storks are often exposed to pollutants and are prone to accumulate them. For instance, when people or other secondary carnivores in the food web consume polluted fish, meat or produce, they are exposed to levels of pollutants significantly higher than those in the surrounding environment (water, air, or soil). This may lead to a high occurrence of possible (sub)lethal effects. As apex predators, analysing stork biomarker responses and the pollutant levels could demonstrate disruption in the environmental processes, as well as changes from the apex predators to the lower trophic levels (Baos et al., 2012; Goutner & Furness, 1998; Smits et al., 2005).

1.2. White stork nestlings as model organisms

Biomonitoring is usually applicable when it comes to charismatic species, such as the white stork, and it is common to use them as bioindicators to predict positive and/or negative effects on the population and ecosystem levels. White storks are good for assessing environmental changes, for example, their change in behaviour (Balmori, 2005), morphological characteristics (Jovani & Blas, 2004), or physiological response (Kulczykowska et al., 2007) can be used to research the possible cause and effect association (or lack thereof) between the individual and (a)biotic stressor from the environment. When considering the age of the stork suitable for

sampling (adult, subadult, juvenile, fledgling, nestling), adults have been previously used for research (Maia et al., 2017). However, when it comes to monitoring the local environment in terms of pollutant assessment, nestlings are more appropriate than other age groups and are easier to sample (de la Casa-Resino et al., 2014; Fig. 2). This is due to the behaviour of nestlings – residing in the nest, fed on local food sources foraged by their parents; is a positive key feature, making them suitable sentinels of pollutant analysis. Since sources of pollutants in nestlings originate from a local environment, primarily exposure via food, the white stork nestlings have been previously used in biomonitoring studies and will continue to be the bioindicators of environmental change. Furthermore, nestlings' health status is influenced by their dietary habits, i.e. food quality foraged by their parent depends on the quality of the environment, and can affect overall fitness. In other words, pollutant concentration in the food reflects pollutants concentration in the nestling body (Burger, 1993; Furness, 1993; Janssens et al., 2002). Changes in the physiology caused by elevated pollutant levels can affect behaviour, cell metabolism, neuronal activity, etc. For the purpose of evaluating those changes, biomarker analysis is utilized for the evaluation of a pollutant's impact on non-target avian species as well as for ecological risk assessment, and thus provides useful information regarding local pollution and the effect it has on the environment (Furness, 1993; Tkachenko & Kurhaluk, 2012).

Figure 2. White stork *C. ciconia* nestlings in the nest (left) and on the ground, prior to blood sampling (right). (photos taken by Dora Bjedov)

White storks can be used in multidisciplinary research, and one individual can be used for research in the assessment of multiple environmental stressors and their subsequent effect, e.g., metalloid and heavy metal levels, independent biomarker activities and levels in different matrices. Wide spatial ranges of white stork in Croatia provide a possibility of a natural experiment setup by comparing values between unpolluted and differently polluted areas, e.g., spatial comparison of heavy metal and metalloid concentrations, and their effect on biomarker activities and levels. Considering this, white storks could be used to observe various independent stressors (biotic and abiotic) in the ecosystem and thus relate it to humans (Kamiński et al., 2008; Orłowski et al., 2006; Pineda-Pampliega et al., 2021).

When working with threatened and endangered species, non-destructive and non-invasive methods should be considered to minimize the environmental impact, and stress, and help conserve avian biodiversity. Non-destructive sampling methods include blood sampling and feather plucking, and therefore birds feel mild pain and distress. On the other hand, non-invasive sampling methods include cutting feathers, collecting shed feathers, regurgitated pellets or addled eggs, therefore birds do not feel any kind of pain (Espin et al., 2016). Blood and blood fractions (plasma, serum, red blood cells) are suitable to be a matrix applied for the determination of various pollutants, e.g., heavy metals, metalloids, and emerging and legacy persistent organic pollutants (Jenssen et al., 1994), therefore blood sampling could be a non-destructive method to perform large scale monitoring research of pollutants in a substantial number of individual birds.

In this dissertation, sampling was performed during the regular ringing scheme and monitoring of breeding success in Croatia (Fig. 3). Appropriate steps were applied to reduce stress for the nestlings, as recommended by Espin et al. (2016), nestlings were taken from their nest, and lowered on the ground in a bag. Each nestling was put on its back, and its head was covered to avoid further stress. Sampling was performed in the morning to avoid heat stress and to avoid the disturbance of feeding habits. Taking into account white stork nestlings are relatively heavy birds (3 – 5 kg), between 5 and 10 mL of blood is allowed to sample without harming the birds (Espin et al., 2016). To avoid unnecessary risk, we sampled 4 mL from the brachial vein.

Figure 3. White stork *C. ciconia* nestling sampling. Preparation of the syringe, alcohol pads and nestling ring reading (left); blood sampling from the wing brachial vein (right). (photos taken by Dora Bjedov)

1.3. Overview of biomarker application in avian ecotoxicology

Application of biomarker measurement gives insight into the physiological response to stressors in apex predators and provides information on early warning signs of possible environmental pollution (Badry et al., 2019, 2020). Selection of proper biomarkers in regard to spatial variation of environmental pollution is crucial for the adequate assessment and for that purpose following set of biomarkers have been analysed in this thesis: acetylcholinesterase activity, carboxylesterase activity, glutathione S-transferase, glutathione reductase, reactive oxygen species concentration, glutathione concentration, and metallothionein concentration.

1.3.1. Acetylcholinesterase (AChE) activity

Acetylcholinesterase (AChE) is a cholinergic enzyme, part of a serine esterase superfamily with an active site comprising of the catalytic triad: serine 203, histidine 447 and glutamate 334. The main function of AChE is hydrolysing neurotransmitter acetylcholine (ACh) in postsynaptic junctions. The interaction between AChE and the ACh is based on the substrate being hydrolysed and inactivated, thereby controlling the amount of ACh at the synapse site (Soreq & Seidman, 2001; Trang & Khandhar, 2022). AChE is primarily found in the central and peripheral nervous system, muscles, and hematopoietic cells (Quinn, 1987).

The activity of AChE is primarily used in biomonitoring studies regarding pesticide exposure. Although AChE activity was not evaluated in white stork nestlings for the purpose of pollutant monitoring so far, AChE activity is widely analysed in other avian species for different purposes. For example, change in AChE activity was determined in a white-crowned sparrow, *Zonotrichia leucophrys gambelli* for the purpose of determining the effects of daily photoperiods (Russell, 1968). Inhibition of AChE activity was analysed after administration of lethal and sublethal doses of five carbamate pesticides (aldicarb, oxamyl, methiocarb, thiofanox, and pirimicarb) to Japanese quail, *Coturnix coturnix japonica* (Westlake et al., 1981). Changes in the captive starling's, *Sturnus vulgaris* behaviour were associated with AChE activity inhibition subsequent to exposure to sublethal acute doses of the organophosphate (chlorfenvinphos) (Hart, 1993). Tully et al. (2003) report the reference ranges of AChE activity measured with two different assays, in the Hispaniolan Amazon parrot, *Amazona ventralis*. Dose-response inhibition of AChE activity in the brain and pancreas of rose-ringed parakeet, *Psittacula krameri* borealis following exposure to quinalphos (Anam & Maitra, 1995). Gard & Hooper (1993) compared AChE activity in eastern bluebirds, *Sialia sialis*, and starlings, *S. vulgaris* in regard to age-dependent changes. Furthermore, plasma cholinesterases were characterised in the grey heron, *Ardea cinerea*, white stork, *C. ciconia* and northern gannet, *Morus bassanus* from a nature reserve in Portugal to establish basal activities (Santos et al., 2012).

1.3.2. Carboxylesterase (CES) activity

Carboxylesterase (CES) is a non-specific esterase, an ubiquitous enzyme with the main function of hydrolysing carboxylic acid esters to their acid and alcohol, a detoxification mechanism for various xenobiotics (Potter & Wadkins, 2006; Redinbo & Potter, 2005). In addition to catalysing hydrolysis, CES participate in phase I metabolism of xenobiotics to convert lipophilic drugs or toxins into more polar chemicals by biotransformation of the parent compound – adding or exposing a polar functional group. The resulting products, carboxylates, are then conjugated by other enzymes to increase solubility and are promptly excreted (Yan, 2014).

The inhibition of CES activity has been recommended for the biomonitoring of wild bird fauna subsequent to organophosphate and carbamate exposure as a non-destructive biomarker

(Bartkowiak & Wilson 1995). To understand the inhibition or induction of CES activity, basal values should be established in healthy individuals. For that purpose, Sogorb et al. (2007) measured CES activity in 19 different bird species: white stork, *C. ciconia*, black stork, *Ciconia nigra*, Montagu's Harriers, *Circus pygargus*, peregrine falcon, *Falco peregrinus*, eagle owl, *Bubo bubo*, common buzzard, *Buteo buteo*, great bustard, *Otis tarda*, booted eagle, *Hieraaetus pennatus*, Bonelli's eagle, *Aquila fasciata*, short-toed eagle, *Circaetus gallicus*, Spanish imperial eagle, *Aquila adalberti*, black-backed gull, *Larus fuscus*, black vulture, *Aegypius monachus*, griffon vultures, *Gyps fulvus*, Egyptian vulture, *Neophron percnopterus*, northern goshawk, *Accipiter gentilis*, barn owl, *Tyto alba*, black kite, *Milvus migrans*, red kite, *Milvus milvus* and tawny owl, *Strix aluco*. For validation of the CES activity as a proposed biomarker of pesticide exposure, Bartkowiak & Wilson (1995) exposed pigeons, *Columba livia*, American kestrels, *Falco sparverius*, red-tailed hawks, *Buteo jamaicensis*, Swainson's hawks, *Buteo swainsoni*, Cooper's hawks, *Accipiter cooperii*, and red-shouldered hawks, *Buteo lineatus* to organophosphate methidathion and parathion. Results show the bimodal distribution in CES activity (Bartkowiak & Wilson, 1995). An often observed unpredictability of CES activity suggests more biomarker analysis and/or tissue should be incorporated when assessing and discussing pesticide exposure (Bartkowiak & Wilson, 1995; Morcillo et al., 2018). To additionally confirm CES as a potential biomarker, CES enzymatic activity was determined in the muscle and liver of yellow-legged gulls, *Larus michahellis*, in order to detect the exposure to anticholinesterase insecticides e.g., organophosphates and carbamates (Morcillo et al., 2018).

1.3.3. Glutathione S-transferase (GST) activity

Glutathione S-transferase (GST) is a phase II metabolic isozyme with the main function to catalyse the conjugation of the reduced glutathione to xenobiotic substrates, as a detoxification mechanism. The activity of GST requires a continuous supply of reduced glutathione from the enzymes γ-glutamyl cysteine synthetase and glutathione synthetase, as well as the action of transporters to remove glutathione conjugates from the cell (Csiszár et al., 2016; Hayes et al., 2005; Hayes & Pulford, 1995). Furthermore, the role of GST is catalysing the nucleophilic attack by reduced glutathione on electrophilic nitrogen, sulphur or carbon atoms in order to detoxify nonpolar xenobiotic substrates, thus suppressing their interaction

with vital cellular compounds such as proteins, DNA and RNA (Leaver & George, 1998; Strange et al., 2000).

Although there is no research regarding GST activity in avian plasma, GST activity has been analysed in homogenised blood of white stork nestlings, *C. ciconia*, red blood cells in Eurasian eagle-owl, *B. bubo* and griffon vulture, *G. fulvus* for the purpose of assessing oxidative stress caused by metal pollution and evaluating physiological conditions due to environmental stress (de la Casa-Resino et al., 2015; Espín et al., 2014a; Espín et al., 2014b; Oropesa, Gravato, Guilhermino, et al., 2013). For the purpose of comparison with chicken, *Gallus gallus domesticus* and rat, *Rattus norvegicus*, GST activity was determined in ostrich, *Struthio camelus* (Amsallem-Holtzman & Ben-Zvi, 1997). Detection of GST activity in plasma reflects *de novo* synthesis in the liver (Nijhoff et al., 1995). Considering this, older storks lose the function to regulate physiological homeostasis and depletion of blood enzymatic antioxidants (Kregel & Zhang, 2007; Martin & Grotewiel, 2006; Vleck et al., 2007). Seeing as that is a consequence of ageing, the suitable age group for pollutant biomonitoring are to be nestlings.

1.3.4. Glutathione reductase (GR) activity

Glutathione reductase (GR) is an enzyme with the main function to catalyse NADPH-dependent reduction of oxidised glutathione (GSSG) to reduced glutathione (GSH). Reduction of GSSG is an essential reaction for the preservation of glutathione levels since glutathione has a primary function in processes considering oxidation and reduction of certain compounds, as well as cell detoxification (Carlberg & Mannervik, 1985). GR is an enzyme that generally accumulates in cell regions with high reactive oxygen species production, e.g., high electron flux (Couto et al., 2016).

The activity of GR was previously assessed in homogenised blood cells, in adult and juvenile white storks *C. ciconia* for determining baseline values of anti-oxidant parameters, as well as investigating the differences related to age (Oropesa, et al., 2013). Kamiński, et al. (2009a,b) investigated interrelationships among macroelements, microelements and toxic heavy metals and several molecular biomarkers, GR activity included, in the blood of white stork nestlings. To confirm that white stork nestlings reflect the degree of environmental

pollution, Tkachenko & Kurhaluk (2014) analysed GR activity in blood. Apart from the white stork, GR activity was investigated in broiler chickens *G. gallus domesticus* to evaluate the effects of feeding graded levels of peroxidised poultry fat on blood and hepatic enzymatic biomarkers (Upton et al., 2009).

1.3.5. Reactive oxygen species (ROS) concentration

Oxidative processes that produce reactive oxygen species (ROS) are normal for cellular metabolism and the immune defence of organisms. ROS production is balanced with a variety of enzymatic and non-enzymatic antioxidant systems. Oxidative stress is caused by exposure to reactive oxygen intermediates, e.g., superoxide anion ($O2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet}$), which can react with biomolecules and subsequently damage DNA, RNA, proteins, and cell membrane (Halliwell & Gutteridge, 2015; Sullivan & Chandel, 2014).

Levels of ROS in white stork *C. ciconia* blood have not been previously evaluated on nestlings. Delhaye et al. (2016) investigated the production of superoxide by mitochondrial electron transport chains in live red blood cells (RBC), in 21 species of adult birds for the purpose of investigating the relationship between maximum lifespan and RBC mitochondrial ROS production, among other variables. Destructive methods were utilised as liver samples from double-crested cormorant, *Phalacrocorax auritus*, chicken, *G. gallus domesticus*, and herring gull, *Larus argentatus*, were used to evaluate H_2O_2 and $O_2^{\bullet-}$ formation (Schlezinger et al., 2000). Effects of ROS have been analysed indirectly, for example, mitochondrial ROS generation and induction of the ubiquitin-proteasome system in heat-stressed chickens, or by antioxidant enzymatic systems (Furukawa et al., 2016).

1.3.6. Glutathione (GSH) concentration

Reduced glutathione (GSH) is an antioxidant, with the function of preventing possible damage by ROS, free radicals, peroxides, and heavy metals to biomolecules. Glutathione exists in reduced (GSH) and oxidized (GSSG) states. The intracellular GSSG:GSH ratio can be used as an indication of increased cellular oxidative stress (Jones, 2002; Lu, 2009).

Determination of GSH has been examined in experimental avian models, but not in wild birds for the purpose of environmental pollution evaluation. Pineda-Pampliega et al. (2021) analysed GSH levels in blood of the white stork nestlings for the purpose of evaluating the effects of foraging on landfills on white stork nestling physiology. Regarding experimental laboratory research, Fernie et al. (2005) analysed GSH in American kestrels, *Falco vespertinus* artificially incubated, following an experimental dose of flame retardants polybrominated diphenyl ethers (PBDEs). The effect of permethrin on GSH concentration and GST activity was investigated in serum and liver of poultry birds (Ezeji et al., 2012). Furthermore, blood GSH was analysed as a part of intracellular antioxidant assessment during zebra finch, *Taeniopygia guttata* development (Romero-Haro & Alonso-Alvarez, 2015).

1.3.7. Metallothionein (MT) concentration

MTs are cysteine-rich proteins, mostly associated with a number of roles, e.g., removal of toxic metals, as a metal chaperone and in homeostasis of metal ions. MTs are usually induced by metals silver, copper, and cadmium, although not exclusively – other metals may provoke a change in the concentration (Babula et al., 2012). Localisation and MT inducibility by metals are tissue-specific, (Elliott et al., 1992), in addition to being the most sensitive to elevated Cd concentrations (Scheuhammer & Templeton, 1990).

Although MTs have never been analysed in white storks, they have been measured in the blood, liver and kidney of terrestrial bird species – white-tailed eagles, *Haliaeetus albicilla* (Marcinekova et al., 2019), great tits, *Parus major* (Vanparys et al., 2008), ringed turtle doves, *Streptopelia risoria*, (Scheuhammer & Templeton, 1990), and aquatic bird species – Atlantic puffin, *Fratercula arctica*, double-crested cormorant, *P. auritus*, herring gull, *L. argentatus*, Leach's storm-petrels, *Oceanodroma leucorhoa* (Elliott et al., 1992), Indian spot-billed duck, *Anas poecilorhyncha*, mallard, *Anas platyrhynchos*, great cormorant, *Phalacrocorax carbo* (Nam et al., 2005). As mentioned previously, MTs are induced by a variety of metals therefore MTs are frequently analysed in parallel with cadmium, copper and zinc as shown in surf scooter, *Melanitta perspicillata* and white-winged scooter, *Melanitta fusca* (Barjaktarovic et al., 2002).

1.4. Overview of metalloids and heavy metal in avian ecotoxicology

Heavy metals and metalloids are naturally occurring elements with high atomic weight and density higher than 5 g cm^{-3}. Metal(loid) compounds are essential micro- and macronutrients for organisms. They can be found in both aquatic and terrestrial systems as well as in the atmosphere. In addition to being ubiquitous, they have the ability to concentrate, magnify and accumulate through food webs resulting in species at the top of food chains, apex predators, and accumulating toxic levels of metals (Koivula & Eeva, 2010). Their various applications have resulted in broad environmental distribution, as well as increasing concerns due to their potential effect on the biota and subsequently on public health. The toxicity of heavy metals and metalloids depends on numerous variables such as age, gender, genetics and nutritional status of the exposed individual, chemical species, route of exposure and the dose. Because of their high level of toxicity, arsenic, cadmium, lead, and mercury rank among the priority metals that are of public health significance. These metallic elements are considered systemic toxicants that are known to induce multiple organ damage, even at lower levels of exposure. Heavy metals or metalloids, their fate and effect are usually investigated individually (de Francisco et al., 2003; Krone, 2018; Martínez-López et al., 2005; Mateo & Hoffman, 2001). Wild bird populations are usually under the influence of multiple pollutants simultaneously, as well as other stress factors e.g., parasites, infections, weather, and hunger; therefore all variables must be taken into account when interpreting results. However, due to various factors e.g., bioavailability, bioaccumulation and biomagnification, it is not plausible to estimate the potential effect of the analysed in the nestlings. After the environmental exposure of nestlings to heavy metals and metalloids, molecular (ir)reversible changes manifest very quickly. Measurable changes, i.e., effects of heavy metals and metalloids, could be detected with the application of biomarkers. One effect of heavy metals and metalloids is the induction of oxidative stress also known as metal-induced oxidative stress (Koivula & Eeva, 2010; Nuran Ercal et al., 2005). Birds have developed physiological defence mechanisms in response to heavy metal and metalloid exposure. An important defence mechanism against toxic pollutants, e.g., heavy metals and metalloids, is an antioxidant system – and its ability to react, detoxify and remove harmful chemical compounds.

1.4.1. Arsenic (As)

Arsenic (As) is a ubiquitous metalloid, and its presence is detected at low levels in virtually all environments. In addition to volcanic eruptions and soil erosion, As pollution occurs as a result of anthropogenic activities (Tchounwou et al., 2012). Several As-containing compounds are produced industrially and have been applied to manufacture products with agricultural applications such as pesticides, sheep dips, wood preservatives, and dyes. Since the beginning of the 20th century, As compounds have been used in medicine, both human and veterinary, to treat a variety of conditions including syphilis, yaws, amoebic dysentery, and trypanosomiasis (Tchounwou et al., 2012). The toxic effect of As is mediated through the inhibition of mitochondrial enzymes that cause cellular respiration to be impaired. In order to achieve this, oxidative phosphorylation is uncoupled. In a variety of biochemical reactions, As is able to substitute phosphorus for sulfhydryl groups, leading to its toxicity (Tchounwou et al., 2003).

In relation to effect and monitoring, As has been analysed and its effect was assessed in white stork, *C. ciconia* for the purpose of investigating adrenocortical response (Baos et al., 2006a), genotoxic effects (Baos et al., 2006b), influence after a mining accident (Gómez et al., 2004), effect of breeding near a landfill (de la Casa-Resino et al., 2014) and oxidative stress (de la Casa-Resino et al., 2015). Concerning other avian species, As has been shown to cause lethal or sublethal effects and affect the reproductive system. It primarily accumulates in hepatic and kidney tissue, with the highest concentration in avian fauna on top of the food chain e.g. birds of prey sparrow hawk, *A. nisus*, barn owl, *T. alba*, kestrel, *F. tinnunculus* (Eisler, 2004; Erry et al., 1999; Stohs & Bagchi, 1995; Valko et al., 2005).

In Croatia, monitoring of As levels using avian indicator species has only been performed previously through the analysis of grey heron, *A. cinerea* feathers (Bjedov et al., 2020).

1.4.2. Selenium (Se)

Selenium (Se) is a naturally occurring element found in Earth's crust. Trace levels of Se are necessary for cell function in biota, e.g. a principal component in antioxidant enzymes peroxidases (mainly GPx) and thioredoxin reductases (TrxR). However, Se deficiency in poultry and livestock can occur and dietary corrections must be made by enriching the diet with

Se. When birds are exposed to a high Se diet, Se concentration is reflected in levels in blood, eggs, and liver, and in time, in muscle. On the other hand, when birds are exposed to a low Se diet (or migrating from areas with high Se to areas with low Se concentration), blood, eggs, and liver easily adapt (Ohlendorf & Heinz, 2011). Considering the physiological impact, Se has dual properties. Although it is a dietary requirement, high doses of Se can bioaccumulate and induce adverse effects (Wilber, 1980). It is a non-metal according to the Periodic table of elements, however, it is usually referred to as a metalloid in ecotoxicological research. Moreover, it is usually included in the ecotoxicological analysis due to its ability to interact with Hg. The widespread association of Se with mercury is antagonistic, meaning Se has protective properties and could alleviate adverse effects caused by mercury. This has been researched in both birds and mammals (Ganther et al., 1972). However, Se can be toxic in high concentrations, and synergistic or additive reactions with mercury are possible, resulting in more prominent mercury toxicity (Sukra et al., 1976). Reactions between Se and mercury include the formation of Se-methylmercury and Se-mercury complexes, methylmercury demethylation, and mercury-induced Se deficiency (Khan & Wang, 2009).

The white stork has been used as a model organism in only a handful of studies. Supplement of dietary Se was assessed in nestlings of white stork, *C. ciconia* in regard to telomere shortening (Pineda-Pampliega et al., 2020). To examine the effect of breeding near landfills, (de la Casa-Resino et al., 2014) analysed blood Se levels. Concerning other avian species and recent literature, Se has been analysed for the purpose of investigating the correlation with heavy metals, primarily Hg in eggs, liver, kidneys and feathers (Ackerman et al., 2016; Vizuete et al., 2019).

In Croatia, monitoring of Se levels using avian indicator species has only been performed previously through the analysis of grey heron, *A. cinerea* feathers (Bjedov et al., 2020).

1.4.3. Cadmium (Cd)

Cadmium (Cd) is a heavy metal widely distributed in the earth's crust. The major industrial applications of Cd include the production of alloys, pigments, and batteries. The use has shown considerable growth in recent years and therefore Cd has become an issue of significant environmental concern. The main routes of exposure to Cd are via inhalation or

ingestion. Dermal absorption is possible, but very rare. The distribution route is primarily the circulatory system; therefore, blood levels reflect acute exposure to Cd (Tchounwou et al., 2012). Toxicity includes decreasing the concentrations of ACh, serotonin and norepinephrine. Moreover, Cd affects signal response in the cell. For example, oxidative stress induces the formation of inositol polyphosphate, increases cellular cytosolic calcium (Ca) concentration, blocks Ca channels (Suszkiw et al., 1984), binds to proteins, interferes with DNA repair (Abshire et al., 1996), activates protein degradation, induces expression of MTs (Hwua & Yang, 1998), GST, heme oxygenases, heat-shock proteins, and DNA polymerase β (Landolph, 1994).

Regarding research related to Cd exposure and effect, Cd is primarily nephrotoxic to birds, as concluded in fulmar petrel, *Fulmaris glacialis* and Manx shearwater, *Puffinus puffinus* (Nicholson et al., 1983). Scheuhammer (1987) investigated Cd effects on the activity of δ-aminolevulinic acid dehydratase (δ-ALAD) in blood obtained by decapitation of captive ring doves, *S. risoria*. On the other hand, non-destructive methods have been used on white stork, *C. ciconia* regarding Cd monitoring and their effect on oxidative status (Baos et al., 2006b; de la Casa-Resino et al., 2014; Kamiński et al., 2007) and endocrine assessment (Baos et al., 2006a) Moreover, white storks were used as a model species for Cd assessment in feathers (Orłowski et al., 2006). Considering other avian species, White et al. (1978) investigated histopathological alterations subsequent to dietary chronic exposure to Cd in mallard, *A. platyrhynchos*. Their research showed kidney lesions, gonad alterations, atrophied testicles, complete stop of spermatogenesis. The transfer of dietary Cd to the egg was assessed and found to be very low. Exposure to high Cd levels resulted in an increase in Cd concentration of the egg, reduced egg production and eggshell thickness (Leach et al., 1979).

In Croatia, monitoring of Cd levels using avian indicator species has only been performed previously through the analysis of grey heron feathers, *A. cinerea* (Bjedov et al., 2020).

1.4.4. Mercury (Hg)

Mercury (Hg) is an ubiquitous heavy metal and environmental pollutant which may induce severe alterations in the tissues, potentially causing adverse health effects. In nature, three forms can be found: elemental, inorganic and organic Hg and all forms are toxic. The

application of Hg is mainly in the electrical industry, dentistry, nuclear reactors, and wood processing antifungal agents (Clarkson et al., 2003). Usually, Hg exposure is via ingestion, for example via dental amalgams and fish. Environmental Hg occurs in its elemental form (Hg^0), as inorganic mercury (Hg salts, i.e. $HgCl_2$) or as organic mercury (MeHg). The toxic effect of Hg depends on the chemical form as well as the route of exposure with the MeHg being the most toxic form. Hg is easily absorbed via the lungs and tissues in the mouth. The distribution path is through the circulatory system and can subsequently pass through the plasmalemma, including the blood-brain barrier. Toxicity effects of Hg usually manifest as kidney lesions, studied on common starlings, *S. vulgaris* (Nicholson & Osborn, 1984). In addition to nephrotoxicity, Hg causes reproductive impairment – delayed testicular development and reduced mating attempts, egg hatchability, clutch size and more eggs laid outside the nest (Wolfe et al., 1998). The molecular mechanism of Hg toxicity includes reaction with cysteine residues of proteins, depletion of cellular antioxidants and thus inducing oxidative stress by accumulating ROS (Valko et al., 2005). For this purpose, continuous monitoring of Hg pollution is warranted. Hg monitoring has been previously conducted in white storks, *C. ciconia* as an indicator species (de la Casa-Resino et al., 2014; Goutner & Furness, 1998; Maia et al., 2017; Pérez-López et al., 2016).

In Croatia, monitoring of Hg levels using avian indicator species has only been performed previously through the analysis of white-tailed eagle, *H. albicilla* nestling feathers (Bjedov et al., 2021b).

1.4.5. Lead (Pb)

Lead (Pb) is a naturally occurring metal in the environment and is present in the earth's crust. Anthropogenic activities e.g. burning of fossil fuels, mining, and manufacturing contribute to the increase of high Pb levels. Moreover, Pb has wide applications in industry, agriculture and domestic appliances. For example, Pb is used in battery production, ammunition, Pb-based paint, (crystal) glass, and sheet lead used for radiation protection (Tchounwou et al., 2012 and references therein). Since the 1970s, Pb exposure has significantly decreased due to the elimination of Pb in gasoline, residential paints, food, drink, and plumbing and drainage systems. Acute exposure to Pb is neurotoxic, nephrotoxic and gastrotoxic, i.e. can cause detrimental effects on the blood, blood pressure, central nervous system, kidneys, and

vitamin D metabolism. On the other hand, chronic exposure has an effect on the neural and reproductive systems (Apostoli et al., 1998; Kaul et al., 1999; Tchounwou et al., 2012). Mechanisms of toxicity are via reaction with biomolecules and interference in regard to their primary function. Pb has the ability to mimic or inhibit Ca homeostasis (competing for binding sites, enzyme inhibition, altering Ca transport), meaning Pb has a high affinity for skeletal tissue where it is frequently incorporated in preference to Ca. Pb binds to proteins altering their configuration and diminishing their activities namely with amide and sulfhydryl groups (Flora et al., 2007). Pb toxicity indirectly causes cell damage due to an increase in ROS formation. Research showed the occurrence of oxidative stress, namely cell peroxidation and high MDA levels which correlated with the Pb levels in the blood. Other oxidative stress biomarkers have shown higher activity as well, including blood SOD and GPx (Hermes-Lima et al., 2008; Jiun & Hsien, 1994; Tchounwou et al., 2012).

Pb effects, toxicity and distribution have been evaluated in various bird species. For the purpose of pollutant monitoring, Pb was analysed in nestlings of white stork, *C. ciconia* (Baos et al., 2006a; de la Casa-Resino et al., 2014; Kamiński et al., 2007, 2008; Maia et al., 2017; Pérez-López et al., 2016). In regard to other avian species but the same purpose, Hutton & Goodman, (1980) used different tissues of the feral pigeon, *C. livia* f. *domestica* and Burger et al. (1992) assessed cattle egret, *Bubulcus ibis* feathers. Effects of chronic Pb ingestion on the reproductive system, include assessment in different tissues of ringed turtle dove, *S. risoria* (Kendall & Scanlon, 1981), decreased egg production in Japanese quails, *C. japonica*, leghorns, *G. gallus domesticus* (Edens & Garlich, 1983) and overall reproductive success in starlings, *S. vulgaris* (Grue et al., 1986). Oxidative stress, pathology and alteration in behaviour induced with intentional poisoning, by exposure to dietary Pb were investigated in mallards, *A. platyrhynchos* (Mateo et al., 2003). Other analysed physiological effects include haematocrit, haemoglobin levels ALAD activity, and creatine phosphokinase (CPK) following an oral dose of Pb in nestling American kestrel, *F. sparverius* (Hoffman et al., 1985).

In Croatia, monitoring of Pb levels using avian indicator species has only been performed previously through the analysis of grey heron feathers, *A. cinerea* (Bjedov et al., 2020).

1.5. Overview of biomarker and pollutant analysis in white stork

The white stork has been used as a model species in ecotoxicological research (Table 1). For example, different types of compounds – metalloids, heavy metals, trace elements, legacy and emerging persistent organic pollutants have been evaluated in white stork. Seeing as many countries across the globe participate in regular monitoring and ringing scheme, the majority of the conducted studies use nestlings. Furthermore, only a handful of studies analyse biomarkers and/or pollutants in organs, meaning non-destructive methods – blood sampling, is traditionally implemented on this charming species. A complete research overview on white stork regarding biomarker measurement and pollutant analysis is shown in Table 1.

Table 1. Literature overview of biomarker measurements as well as pollutant analysis in model species white stork *C. ciconia*.

Reference	*Age group*	*Biomarker*	*(Toxic) Compounds*	*Matrix*	*Purpose of the measurement*
Kamiński et al., 2007	Nestling	SOD, CAT, CP, TBARS	Ca, Mg, Fe, Na, K, Pb, Zn, Cd	Blood	Assessment of the impact of elements on oxidative stress biomarkers
Kamiński, et al., 2009b	Nestling	TBARS, SOD, CAT, CP, GPx, GR	Na, K, Ca, Mg, Fe, Zn, Cu, Mn, Co, Pb, Cd	Blood	Assessment of the impact of Na, K, Ca, Mg, and Fe, microelements Zn, Cu, Mn, and Co, and toxic heavy metals Pb and Cd
Pineda-Pampliega et al., 2021	Nestling	TWCC, H:L ratio, albumin, ALT, AP, ASP, bilirubin, Chl, CK, creatinine, ferritin, glucose, transferrin, triglycerides, uric acid, MDA, tGSH, GSSG, GPx, metHb	Ca, Mg, P	Blood	Evaluation of the effects of foraging on landfills on white stork nestling physiology
Kamiński et al., 2009a	Nestling	SOD, CAT, CP, GPx, GR, MDA	Na, K, Ca, Mg, Fe, Zn, Cu, Mn, Co, Pb, Cd	Blood	Assessment the impact of elements on oxidative stress biomarkers
Kulczykowska et al., 2007	Nestling	T4, Mel		Blood	To determine ED biomarker response in regard to pollution
Smits et al., 2007	Nestling	Ca:P Ratio	P	Blood	Bone metabolism disruption due do mine spill
de la Casa-Resino et al., 2015	Nestling	GST, GSH, MDA	Pb, Hg, As	Blood	Determination of oxidative status in regard to metal pollution associated with metal pollution
Lashev et al., 2005	>1 year	RBC, Thrombocytes, WBC		Blood	Determination of the haematological parameters in white storks reared in captivity
Smits et al., 2005	Fledging	tAP, hepatic AP, kidney AP	Ca, P	Blood, liver, kidney	Assessment of skeletal pathology associated with heavy metal pollution

Pineda-Pampliega et al., 2020	Nestling	Tocopherol, MDA, TAC		Blood	The effect of antioxidant supplementation on telomeres
Baos et al., 2006a	Nestling	Corticosterone, T3, T4	Pb, Zn, Cu, Cd, As	Blood	Assessment of stress and hormone status affected by heavy metal and metalloids
Kamiński et al., 2008	Nestling		Ca, Mg, Fe, Zn	Blood	Chemical elements from polluted areas
Tkachenko & Kurhaluk, 2012	Nestling	TBARS, PC, ALT, AST, LDH, Lactate, Pyruvate		Blood	Assessment of pollution-induced oxidative stress and biochemical parameter alterations
Baos et al., 2006b	Nestling	T-cell-mediated immune response	Pb, Zn, Cu, Cd, As	Blood	Relationships between T-cell-mediated immune response and metal(loid) concentrations
Baos et al., 2006c	Nestling	DNA damage		Blood	Genotoxic effects of heavy metals and arsenic
Maia et al., 2017	Fledging, adult		Pb, Hg, As, Ni, Fe, Zn, Cu, Se, Mn, Cr, Co, Cd	Blood	Assessment of basal values of metals and metalloids
Puerta et al., 1989	Nestling	Hb, RCN, MCV, MCHb, MCHbC, proteins, TG, Chl, Chl-HDL, urea, uric acid, AP, AST, ALT, WCN		Blood	Report on haematology and blood chemistry
de la Casa-Resino et al., 2014	Nestling		Cd, Pb, Hg, Fe, Zn, Se, As	Blood	Assessment on element levels for white storks Breeding near a landfill
Oropesa et al., 2013	Adult, juvenile	ChEs		Blood	Characterization of plasma cholinesterase and its in vitro inhibition by pesticides
Montesinos et al., 1997	Nestling	Packed cell volume, Hb, RBC, MCV, MCHb, MCHbC, tromvocytes, WBC, heterophils, lymphocytes, monocytes, eosinophils, basophils, H:L ratio		Blood	Report on haematological and plasma biochemical reference intervals
Pastor et al., 2001	Nestling	Lymphocytes, DNA damage		Blood	Assessment of genotoxic damage after the Doñana Ecological Disaster

Tkachenko & Kurhaluk, 2013	Nestling	TBARS, SOD, CAT, CP, GR, GPx, TAC		Blood	Assessment of pollution-related changes in oxidative stress and antioxidant defence profile
Piedra et al., 2018	Adult		Hg	Liver, kidney, muscle	Assessment of propolis and bee pollen preparation effect on the concentration of mercury
Höfle et al., 2017	Nestling	Oxidative stress		Blood	Assessment of physiological adaptation to the exploitation of landfill sites for foraging
Goutner et al., 2011	Nestling		Hg	Feather	Assessment of mercury in relation to age, brood Size, and hatching Order
Orłowski et al., 2006	Nestling		Cr, Cu, Ni, Pb, Zn	Feather	Methodological implications for further ecotoxicological studies
Gómez et al., 2004	Adult		Cu, Pb, As, Cd	Liver, muscle, kidney	Assessment of a mine tailing accident influence near Doñana National Park
Hernández et al., 1988	N/A		α-HCH, γ-HCH, aldrin, dieldrin, heptachlor, heptachlor epoxide, p,p'-DDE, p,p'-DDT, p,p'-TDE, dichlorobenzophenone, PCBs, Hg, Pb, Cd, Cu, Zn	Eggs	Determination of the levels of organochlorine pollutants and heavy metals
Marinov et al., 2019	Adult		As, Cr, Mn, Ni, Pb, Zn	Feather	Assessment of heavy metal accumulation in the feathers
Meharg et al., 2002	Nestling		Pb, Pb isotope ratio	Blood	Examination of source identification of lead contamination in a marshland ecosystem

Abbreviations: AChE – acetylcholinesterase; ALT – alanine aminotransferase; AP – alkaline phosphatase; ASP – aspartate aminotransferase; AST – aspartate aminotransferase; CAT – catalase; CES – carboxylesterase; ChEs – cholinesterase; Chl – cholesterol; Chl-HDL – cholesterol-high density lipoprotein; CK – creatine kinase; CP; GPx – glutathione peroxidase, GR – glutathione reductase; GSH – reduced glutathione; GSSG – oxidised glutathione; GST – glutathione S-transferase; H:L – heterophils and lymphocytes ratio; Hb – haemoglobin; LDH – lactate dehydrogenase; MCHb – mean cell Hb; MCHbC – mean cell Hb concentration; MCV – mean cell volume; MDA – malondialdehyde; Mel – melatonin; MetHb – methaemoglobin; PC – carbonyl proteins; RBC – red blood cells; RCN – red cell number; ROS – reactive oxygen species; SOD – superoxide dismutase; T3 – triiodothyronine; T4 – thyroxine; TAC – total antioxidant capacity; TBARS – thiobarbituric acid reactive substances; TG – triglycerides; TWCC – total white cell; WBC – white blood cells; WCN – white cell number; As – arsenic; Ca – calcium; Cd – cadmium; Co – cobalt; Cr – chromium; Cu – copper; Fe – iron; Hg – mercury; K – potassium; Mg – magnesium; Mn – manganese; Na – sodium; Ni – nickel; p'p,-DDE – p,p′-dichlorodiphenyldichloroethylene; p'p'-DDT – p,p'-dichlorodiphenyltrichloroethane; p'p,-TDE – p,p'-dichlorodiphenyldichloroethane; Pb – lead; Se – selenium; Zn – zinc; α-HCH – α-Hexachlorocyclohexane; γ-HCH–γ-Hexachlorocyclohexane

1.6. Statistical analyses

All statistical analyses were performed using R version 4.0.0, and SPSS (version 24). Shapiro-Wilk test was used to test the data distribution. The normality of the data distribution was confirmed for differences between the biomarker response between plasma and S9, as well as biomarker response in regard to sampling areas. To compare the difference between the means of the biomarker response in plasma and S9, Welch's *t*-test was used as unequal variances were confirmed with the F-test. For enzymatic (AChE, CES, GST and GR) and non-enzymatic (ROS, GSH, MT) biomarkers, parametric tests were used. Linear mixed model (LMM) was performed with *nlme* function in order to avoid pseudoreplication since more than one white stork nestling was sampled from the same nest. The LMM was constructed using a variable *nest* as a random effect, and variables *sampling site* and *biomarker response* as fixed effects. One–way analysis of variance (ANOVA) was performed to identify the significant differences between the sampling locations, following an application of the *emmeans* function for *post hoc* analysis to determine differences in biomarker response between the study areas. Heavy metal and metalloid data did not follow a normal distribution; therefore non-parametric tests were used. Since more than one nestling was sampled from each nest, to avoid pseudoreplication, the median value was calculated for each nest. Furthermore, Kruskal-Wallis test was used to test for differences in heavy metal and metalloid concentration between the study areas followed by Dunn's *post hoc* test. To test the association between enzymatic and non-enzymatic biomarker response with heavy metal and metalloid concentration, Spearman's rank correlation coefficient (two-tailed) was applied. Response variability in plasma and S9 for each parameter was calculated by dividing the standard deviation of the obtained data by the mean of the obtained data. Enzyme activity was presented as specific enzyme activity, calculated from changes in absorbance. Fluorescence is presented as relative fluorescence units (RFU), calculated from an increase in fluorescence over time. Enzymatic and non-enzymatic biomarker results are expressed as mean and standard deviation (± *SD*) with a vertical bar plot. Due to non-parametric test application, heavy metal and metalloid results are expressed with box and whiskers plots. The plot starts with minimum values, and the box begins with the first quantile (Q1), followed by the median and third quantile (Q3), ending with maximum values. Distant observations (outliers) are represented with circles. The level of statistical significance was 0.05 (*p*-value).

1.7. Objectives and hypotheses

The objectives of the present dissertation are the optimization and application of protocols that will enable the determination of biomarker responses in the blood of white storks from different areas in Croatia. Comparison of biomarker responses and correlation with the concentration of analysed heavy metals and metalloids in the blood of white stork nestlings will allow the assessment of their bioaccumulation and effects on white stork nestlings.

To achieve the objectives, nine hypotheses were defined:

1) There is a significant difference in the response of the measured biomarkers between the two blood fractions: plasma and blood cell homogenate (S9).

2) There is no significant difference in the response of the measured biomarkers between the sexes of white stork nestlings.

3) Enzymes acetylcholinesterase and carboxylesterase measured in the blood of white stork nestlings from the intensive agriculture areas will show a decrease in the activity.

4) Biomarkers measured in the blood of white stork nestlings will show a change in the activity of the enzymes glutathione S-transferase and glutathione reductase and in the concentrations of metallothionein, glutathione and reactive oxygen species in regard to sampling areas.

5) There is no significant difference in the concentration of analysed heavy metals and metalloids between the sexes of white stork nestlings.

6) Concentrations of analysed heavy metals and metalloids will be increased in the blood of white stork nestlings in areas of intensive agriculture, hunting, and metal mechanical engineering industry.

7) There will be correlation between the measured biomarkers and the concentration of analysed heavy metals and metalloids in the blood of white stork nestlings.

8) There will be correlation between the concentrations of mercury and selenium in the blood of white stork nestlings.

9) There will be correlation between the concentration of metallothionein and the concentration of analysed heavy metals and metalloids in the blood of white stork nestlings.

White stork nestlings from Kopačevo

2. Research articles

2.1. Application of Non-Destructive Methods: Biomarker Assays in Blood of White Stork (*Ciconia ciconia*) Nestlings

animals

MDPI

Article

Application of Non-Destructive Methods: Biomarker Assays in Blood of White Stork (*Ciconia ciconia*) Nestlings

Dora Bjedov [1,†], Alma Mikuška [1,†], Carina Lackmann [2,3], Lidija Begović [1], Tibor Mikuška [4] and Mirna Velki [1,*]

[1] Department of Biology, Josip Juraj Strossmayer University of Osijek, 31000 Osijek, Croatia; dora.bjedov@gmail.com (D.B.); amikuska@biologija.unios.hr (A.M.); lbegovic@biologija.unios.hr (L.B.)
[2] Department of Evolutionary Ecology and Environmental Toxicology, Goethe University Frankfurt, 60438 Frankfurt am Main, Germany; lackmann@bio.uni-frankfurt.de
[3] Department of Ecosystem Analysis, Institute for Environmental Research, ABBt-Aachen Biology and Biotechnology, RWTH Aachen University, 52074 Aachen, Germany
[4] Croatian Society for Birds and Nature Protection, 31000 Osijek, Croatia; tibor.kopacki.rit@gmail.com
* Correspondence: mirna.velki@gmail.com or mvelki@biologija.unios.hr; Tel.: +385-(0)31-399-935
† Share first authorship on this work.

Abstract: White stork (*Ciconia ciconia*) nestlings can provide quantitative information on the quality of the surrounding environment by indicating the presence of pollutants, as they depend on locally foraged food. This study represents the first comparison of biomarkers in two fractions of white stork nestling blood: plasma and S9 (the post-mitochondrial fraction). The aim of this study was to evaluate acetylcholinesterase (AChE), carboxylesterase (CES), glutathione S-transferase (GST), and glutathione reductase (GR), as well as to establish a novel fluorescence-based method for glutathione (GSH) and reactive oxygen species (ROS) detection in plasma and S9. Considering the enzymatic biomarkers, lower variability in plasma was detected only for AChE, as CES, GST, and GR had lower variability in S9. Enzyme activity was higher in plasma for AChE, CES, and GST, while GR had higher activity in S9. Regarding the fluorescence-based method, lower variability was detected in plasma for GSH and ROS, although higher GSH detection was reported in S9, and higher ROS was detected in plasma. The present study indicated valuable differences by successfully establishing protocols for biomarker measurement in plasma and S9 based on variability, enzyme activity, and fluorescence. For a better understanding of the environmental effects on nestlings' physiological condition, biomarkers can be measured in plasma and S9.

Keywords: non-destructive sampling; apex bird species; plasma; S9; biomarkers

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1. Introduction

The white stork (*Ciconia ciconia*) is a large migratory bird species breeding in Europe and wintering in Africa, associated with open wet grasslands and agriculture habitats [1]. However, in recent years, studies show that a high percentage of white storks also stay in south-western Europe during winter [2,3]. As an apex bird species with opportunistic feeding habits, their diet mostly comprises of various invertebrates (grasshopper, beetles, earthworms, and crustaceans), amphibians, fish, snakes, lizards, small mammals (voles, mice, rats, and shrews), and, occasionally, trash from landfills [4–9]. White stork nestlings are fed on local food sources, foraged by their parents, making them suitable bioindicators and sentinels of contaminants in a local environment [10,11]. A decline in the breeding population of storks is related to decreasing availability of grasslands and wetlands and increase in anthropogenic activities, especially intensive agriculture [12].

Various chemicals are used in agriculture with potential accumulation of toxins in apex bird predators, such as the white stork [12]. Apex bird species are non-target organisms to pesticide exposure in environments such as wetlands or agricultural ponds [13].

Their health status is influenced by their dietary habits, and their body content reflects pollutant concentrations in the food [14–16]. Nestling physiological condition is the ability to maintain a stable homeostasis. This condition can be affected by local pollution. Changes in the physiological condition can affect behaviour, cell metabolism, neuronal activity, etc., and therefore can provide useful information regarding local pollution and the effect it has on the environment [15]. Biomarker analysis is utilized for evaluation of a pollutant's impact on non-target avian species as well as for ecological risk assessment [12]. Enzyme activity measurements in blood can provide valuable information regarding environmental impact on wildlife [17,18]. Enzymatic activity can be altered with various stressors and can provide an early warning sign of pollution [13]. Although some studies still use destructive sampling, such as capturing birds in traps and decapitation [19–22], blood sampling, if done correctly, is a simple, non-destructive method for laboratory analysis [23] and should be utilized over destructive methods. Non-invasive (e.g., collecting shed feathers [24] and collection of addled eggs [25]) and non-destructive (e.g., blood sampling [26,27]) methods should be employed for the purpose of animal welfare, to minimize the environmental impact on birds, thus helping conserve avian biodiversity, especially when working with near threatened and critically endangered species. For optimal assessment, biomarkers in blood often need to be measured in parallel; therefore, it is recommended to draw a maximum amount of blood at once [28]. Various biomarkers can be measurement in blood, such as antioxidant enzymes, mixed-function oxidases, hormones, corticosteroids [17,18], or environmental contaminants (e.g., lead [29,30]). So far, in avian blood, oxidative stress and esterase biomarkers have been analysed for the purpose of assessing organophosphate and carbamate exposure, air and heavy metal pollution indicators, and genotoxic damage [12,31–35].

Avian blood has diverse implementations in ecotoxicology; however, no data is available assessing the esterase and oxidative stress biomarkers in white stork nestlings in two blood fractions. For this purpose, the main goals of the study were to:

1. Optimize protocols for measurement of the following biomarkers in the collected blood samples, as well as adjust for the microplate reader: acetylcholinesterase, carboxylesterase, glutathione S-transferase and glutathione reductase activities, reactive oxygen species and glutathione levels, as well as total protein content.
2. Determine the basal activities of the measured biomarkers in the blood of white stork nestlings from Croatia.
3. Determine the sex of the white stork nestlings from the sampled blood.

Optimization of biomarker protocols and sex determination in white stork nestlings' blood will be useful for the purpose of obtaining information from small blood volume, and will enable application of these biomarkers in future research, which will improve the ecotoxicological investigations of birds without the need for destructive sampling. Application of biomarker measurement gives insight into the physiological response to stressors in apex predators and will provide information on early warning signs of possible environmental pollution.

2. Materials and Methods

2.1. Field Procedure and Blood Extraction

Fieldwork was performed during the 2020 breeding season in villages along Drava river in north-eastern Croatia. The area is influenced by industry near Osijek as well as intensive agrochemical use in the surrounding area. Blood samples were taken from 16 nestlings in 7 nests. Protocols for monitoring the white stork population in Croatia [36,37] were used for finding and approaching nests. All nests were accessed with a telescopic crane. Nestlings were captured in their nest, placed in a bag, and lowered onto the ground. Each nestling was put on its back, and its head covered with a cloth to avoid additional stress. The beak was measured for age determination and all nestlings were between 6 and 8 weeks old. All sampling procedures were done between 08:00 a.m. and 12:00 p.m. to avoid heat stress and to avoid disturbing feeding habits. Morphometric

measurements were taken (beak measurement to determine the order of the hatching), and blood samples were collected. A sterile 5 mL syringe and 0.8 mm (20 gauge) needle were used to puncture the brachial vein and approximately 4 mL of blood was drawn and transferred to lithium heparin collection tubes. Blood was stored under cold and dark conditions until centrifugation within 6–8 h. The study was conducted under the permit of The Ministry of Environment and Energy of the Republic of Croatia (Classification code: UP/I-612-07/20-48/130; Registry number: 517-05-1-1-20-4).

2.2. Sample Preparation

Blood was centrifuged at 3000× *g* for 10 min at 4 °C. The supernatant (plasma) was transferred to the new sterile tube and kept at –80 °C until further analysis. The pellet was dissolved with a 5 mL 0.1 M phosphate buffer (pH 7.2) and a sonicator was used for cell disruption at 30% strength for 2 min. Samples were subsequently centrifuged at 9000× *g* for 20 min at 4 °C to obtain the post-mitochondrial supernatant (S9). The S9 fraction was kept at –80 °C until further analysis. All measurements were performed in both types of samples: plasma and S9.

2.3. Chemicals

In the present study, the following chemicals (analytical grade) were used: acetonitrile (C_2H_3N, CAS 75-05-8, 41.053 g mol^{-1}), β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (β-NADPH) ($C_{21}H_{26}N_7Na_4O_{17}P_3$ x H_2O, CAS 2646-71-1 (anhydrous), 833.35 g mol^{-1} (anhydrous basis)), CellTracker™ Green CMFDA Dye ($C_{25}H_{17}ClO_7$, CAS 136832-63-8, 464.86 g mol^{-1}) (ThermoFisher Scientific, Waltham, MA, USA), 1-chloro-2,4-dinitrobenzene (CDNB) ($C_6H_3ClN_2O_4$, CAS 97-00-7, 202.55 g mol^{-1}), CM-H_2DCFDA ($C_{27}H_{19}Cl_3O_8$, CAS 1219794-09-8, 577.8013 g mol^{-1}) (ThermoFisher Scientific, Waltham, MA, USA), (2-Mercaptoethyl) trimethylammonium iodide acetate (acetylthiocholine iodide) ($CH_3COSCH_2CH_2N(CH_3)_3I$, CAS 1866-15-5, 289.18 g mol^{-1}), disodium hydrogen phosphate (NaH_2PO_4, CAS 7558-79-4, 141.957 g mol^{-1}), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) ([-$SC_6H_3(NO_2)CO_2H]_2$, CAS 69-78-3, 396.35 g mol $^{-1}$), glutathione disulfide (GSSG, $C_{20}H_{32}N_6O_{12}S_2$, CAS 27025-41-8, 612.6 g mol^{-1}), *p*-nitrophenyl acetate ($C_8H_7NO_4$, CAS 830-03-5, 181.147 g mol^{-1}), (2*S*)-2-amino-4-{[(1*R*)-1-[(carboxymethyl)carbamoyl]-2-sulfanylethyl]carbamoyl}butanoic acid (glutathione (GSH)) ($C_{10}H_{17}N_3O_6S$,CAS 70-18-8, 307.32 g mol^{-1}), and sodium dihydrogen phosphate dihydrate (NaH_2PO_4 × $2H_2O$, CAS 13472-35-0, 156.006 g mol^{-1}). For protein concentration measurements, the Pierce™ BCA Protein Assay Kit (Pierce Biotechnology, Waltham, MA, USA) was used.

2.4. Enzymatic Biomarkers

All biomarker measurements were adjusted for the Tecan Spark 10 M microplate reader (Tecan Trading AG, Männedorf, Switzerland). The plasma and S9 samples as well as blanks were measured in triplicate. Enzyme activity was calculated from the obtained changes in the measured absorbance and expressed as specific enzyme activity.

2.4.1. Protocol for Measurement of Acetylcholinesterase (AChE) Activity

The activity of AChE in the plasma and S9 samples was determined according to the method of Ellman et al. [38]. For the plasma samples, the reaction mixture contained 5 µL plasma diluted 5x with phosphate buffer (0.1 M, pH 7.2), 180 µL phosphate buffer (0.1 M, pH 7.2), 10 µL DTNB (1.6 mM, prepared with phosphate buffer (0.1 M, pH 7.2)), and 10 µL acetylthiocholine iodide (156 mM, prepared with distilled water). Increase in absorbance was measured for 5 min at 412 nm. For the S9 samples, the reaction mixture contained 25 µL S9 diluted 10x with phosphate buffer (0.1 M, pH 7.2), 180 µL phosphate buffer (0.1 M, pH 7.2), 10 µL DTNB (1.6 mM prepared with phosphate buffer (0.1 M, pH 7.2)), and 10 µL acetylthiocholine iodide (156 mM, prepared with distilled water). Increase in absorbance was measured for 10 min at 412 nm. Blank measurements of the plasma and S9 were performed in parallel containing 180 µL phosphate buffer, 10 µL DTNB, and 10 µL

acetylthiocholine iodide (all prepared in the same way as described previously). Specific enzyme activity was calculated with the extinction coefficient (ε) = 13.6 × 10^3 M^{-1} cm^{-1}.

2.4.2. Protocol for Measurement of Carboxylesterase (CES) Activity

The activity of carboxylesterase in plasma and S9 was determined according to the Hosokawa and Satoh method [39]. For the plasma samples, the reaction mixture contained 10 µL plasma and 150 µL *p*-nitrophenyl acetate (1 mM, dissolved in acetonitrile, diluted with distilled water). Increase in absorbance was measured for 4 min at 405 nm. For the S9 samples, the reaction mixture contained 20 µL S9 10x diluted with phosphate buffer (0.1 M, pH 7.2) and 150 µL *p*-nitrophenyl acetate (1 mM, prepared in acetonitrile, diluted with distilled water). Blank measurements of the plasma and S9 were performed in parallel containing 150 µL *p*-nitrophenyl acetate (prepared in the same way as described previously). Increase in absorbance was measured for 5 min at 405 nm. Specific enzyme activity was calculated with ε = 16.4 × 10^3 M^{-1} cm^{-1}.

2.4.3. Protocol for Measurement of Glutathione S-Transferase (GST) Activity

The activity of glutathione S-transferase in plasma and S9 was determined following the Habig and Jakoby method [40]. For the plasma samples, the reaction mixture contained 5 µL plasma, 160 µL CDNB (1 mM, dissolved in 96% ethanol and diluted with phosphate buffer (0.1 M, pH 7.2)), and 40 µL GSH (25 mM, prepared in distilled water). Increase in absorbance was measured for 2 min at 340 nm. For the S9 samples, the reaction mixture contained 20 µL S9 homogenate diluted 10x with phosphate buffer (0.1 M, pH 7.2), 160 µL CDNB (1 mM, dissolved in 96% ethanol and diluted with 0.1 M, pH 7.2 phosphate buffer), and 40 µL GSH (25 mM, prepared in distilled water). Blank measurements of the plasma and S9 were performed in parallel containing 160 µL CDNB and 40 µL GSH (all prepared in the same way as described previously). Increase in absorbance was measured for 5 min at 340 nm. For the plasma measurement, the first minute was needed for stabilization and was omitted from the calculations. Specific enzyme activity was calculated with ε = 9.6 × 10^3 M^{-1} cm^{-1}.

2.4.4. Protocol for Measurement of Glutathione Reductase (GR) Activity

The activity of glutathione reductase in plasma and S9 was determined using the Habig and Jakoby protocol [40]. For the plasma samples, the reaction mixture contained 20 µL plasma, 100 µL phosphate buffer (0.1 M, pH 7.2), 100 µL GSSG (2 mM, prepared in phosphate buffer (0.1 M, pH 7.2)), and 10 µL β–NADPH (1 mM, prepared in phosphate buffer (0.1 M, pH 7.2)). Decrease in absorbance was measured for 10 min at 340 nm. For the S9 samples, the reaction mixture contained 10 µL S9, 100 µL phosphate buffer (0.1 M, pH 7.2), 100 µL GSSG (2 mM, prepared in phosphate buffer (0.1 M, pH 7.2)), and 10 µL reduced β–NADPH (1 mM, prepared in phosphate buffer (0.1 M, pH 7.2)). Blank measurements for plasma and S9 were performed in parallel containing 100 µL phosphate buffer, 100 µL GSSG, and 10 µL reduced β–NADPH (all prepared in the same way as described previously). Decrease in absorbance was measured for 10 min at 340 nm. Specific enzyme activity was calculated with ε = 6.22 × 10^3 M^{-1} cm^{-1}.

2.5. Fluorescent Dyes Protocols

Detection of GSH and ROS using the fluorescent dyes was conducted based on the protocol previously developed for zebrafish larvae [41] and adjusted here for avian plasma and S9 samples. Measurements were conducted using the Tecan Spark 10 M microplate reader with the following settings: excitation wavelength—485 nm; emission wavelength—530 nm; and gain—50. Each plasma, S9, blank, and positive control sample was performed in parallel and measured in triplicate.

2.5.1. CellTracker™ Green CMFDA (GSH) Dye

For the plasma samples, the reaction mixture contained 2 µL plasma, 90 µL phosphate buffer (0.1 M, pH 7.2), and 5 µL CellTracker™ Green CMFDA (9.78 µM, prepared in DMSO). Fluorescence was measured every 5 min for 60 min. For the S9 samples, the reaction mixture contained 2 µL S9, 90 µL phosphate buffer (0.1 M, pH 7.2) and 5 µL CellTracker™ Green CMFDA (9.78 µM, prepared in DMSO). Fluorescence was measured every 5 min for 60 min. The blank reaction mixture contained 90 µL phosphate buffer and 5 µL CellTracker™ Green CMFDA (prepared in the same way as described previously) and the positive control reaction mixture contained 2 µL GSH (25 mM, prepared in distilled water), 90 µL phosphate buffer, and 5 µL CellTracker™ Green CMFDA (all prepared in the same way as described previously) for both the plasma and S9 samples. The first 30 min were used for calculations due to the optimal linear increase for plasma and S9.

2.5.2. CM-H_2DCFDA (ROS) Dye

For plasma samples, the reaction mixture contained 10 µL plasma, 90 µL phosphate buffer (0.1 M, pH 7.2), and 10 µL CM-H_2DCFDA dye (7.87 µM, prepared in DMSO). Fluorescence was measured every 5 min for 30 min. For the S9 samples, the reaction mixture contained 10 µL S9, 90 µL phosphate buffer (0.1 M, pH 7.2), and 5 µL CM-H_2DCFDA dye (7.87 µM, prepared in DMSO). Fluorescence was measured every 5 min for 120 min. The blank reaction mixture contained 90 µL phosphate buffer and 5 µL CM-H_2DCFDA dye (prepared in the same way as described previously), and the positive control reaction mixture contained 2 µL H_2O_2 (0.019 M, prepared in distilled water), 90 µL phosphate buffer, and 5 µL CM-H_2DCFDA dye (prepared the same way as described previously) for both plasma and S9.

2.6. Protein Quantification Assay

Protein quantification was performed using the Pierce™ BCA Protein Assay Kit and measurements were performed using the Tecan Spark 10 M microplate reader. The working solution was prepared as described in the protocol provided in the kit, with bovine serum albumin as a standard. Each plasma, S9, blank, and standard sample was performed in parallel and measured in triplicate. For the plasma samples, the reaction mixture contained 2.5 µL diluted plasma (5x with phosphate buffer, 0.1 M, pH 7.2), 22.5 µL phosphate buffer (0.1 M, pH 7.2), and 200 µL working solution. For the S9 samples, the reaction mixture contained 2.5 µL diluted S9 (10x diluted with phosphate buffer, 0.1 M, pH 7.2), 22.5 µL phosphate buffer (0.1 M, pH 7.2), and 200 µL working solution. The microplate with reaction mixture was shaken for 30 s in Tecan Spark 10 M microplate reader, incubated at room temperature for 2 h, and the protein concentration was determined at 562 nm.

2.7. Sex Determination

DNA was isolated using an extraction buffer containing 10 mM EDTA, 10 mM Tris-Cl (pH 8.0), 100 mM NaCl, 2% sodium dodecyl sulphate (SDS, Carl Roth GmbH, Karlsruhe, Germany), and ultrapure water in final concentrations. In a sterile tube, 125 µL S9, 360 µL extraction buffer, 10 µL proteinase K (10 mg mL^{-1} stock concentration), and 16 µL 1 M dithiothreitol (DTT, Carl Roth GmbH) were added. Following incubation on a thermo-shaker for 30 min, 56 °C at 1000 rpm, 200 µL 3 M sodium acetate (Carl Roth GmbH) was added, vortexed, and incubated for 5 min on ice. The samples were centrifuged for 10 min at 16,000× *g*, at 4 °C, after which the supernatant was transferred to a new tube. Ice-cold isopropanol was added to the supernatant 1:1 (v:v) for DNA precipitation. The samples were briefly shaken and then incubated for 30 min at –20 °C. Afterwards, the samples were centrifuged at 18,000× *g* for 20 min at 4 °C, the supernatant was discarded, and the pellet was washed with 1 mL 70% ethanol. Samples were centrifuged at 18,000× *g*, at 4 °C, for 90 s and the supernatant was discarded. DNA was air-dried and dissolved in 10 µL of nuclease-free water, vortexed, and centrifuged. For DNA quantification, a NanoPhotometer (Implen GmbH, München, Germany) was used. For the sex-specific *CHD*

gene [42], the amplification and visualising PCR products protocol by Begović et al. [43] was followed.

2.8. Data Analysis

Data analyses were performed using GraphPad Prism software version 8.4.3 [44]. Normality of the data was confirmed with a Shapiro–Wilk test. To compare the difference between the means of the biomarker response in plasma and S9, Welch's *t*-test was used as unequal variances were confirmed with the F-test. The level of statistical significance (p) was 0.05. Response variability in plasma and S9 for each parameter was calculated by dividing the standard deviation of the obtained data with the mean of the obtained data. All results are expressed as the mean ± SD and presented as bar plots.

3. Results and Discussion

3.1. Sex Determination

Sex was determined from S9 using the *CHD* gene. Sex-typing showed 8 males and 8 females (Figure S1). There were no statistical differences in biomarker response regarding sex. Various volumes of S9 were used, and the optimal protocol was determined based on DNA quantity and quality, as shown in Table S1. DNA quality was determined from $A_{260/280}$ and $A_{260/230}$, indicating purity [45–47]. The average $A_{260/280}$ was 1.99 ± 0.06. A ratio of ≥ 1.8 is accepted and considered uncontaminated DNA [48]. A ratio of ≤1.6 may indicate presence of protein, phenols, or other impurities absorbing at 280 nm [49]. The average $A_{260/230}$ was 2.02 ± 0.18. A ratio of 2.00–2.20 is considered uncontaminated DNA. If $A_{260/230}$ is lower, salt, lipid, protein, phenol, guanidinium chloride, or EDTA contamination is suspected [50,51]. If the two samples have the same $A_{260/280}$, but different $A_{260/230}$, this may be due to different sample concentrations [52]. During blood sampling, blood coagulation is possible, decreasing the sample concentration. During the sonication process, there are less available cells, as the samples do not have equal homogeneity; therefore, the DNA yield will be lower. Although coagulated samples cannot be used for enzyme assays, they can be used for DNA analysis, e.g., sex determination or DNA methylation [53].

3.2. Enzymatic Biomarkers

3.2.1. Overview of the Results

Results of the enzymatic biomarkers and fluorescent dyes analysed in plasma and S9 of white stork nestlings are presented in Table 1. Enzymatic biomarkers were analysed in either plasma or S9; however, when measuring several parameters in blood, there are certain limitations due to sample volume. Therefore, the enzymatic response in plasma and S9 samples was investigated. In case of a limited sample volume, the results of this study will help in deciding which biomarker should be chosen for measurement in which sample type. Enzymatic biomarkers from blood could be used to identify changes in biomarker response regarding geographical differences, weather conditions, environmental pollution gradient, age differences (nestlings, fledglings, juvenile, and adults), and clutch and brood size. Furthermore, results of the study could be implemented and help in the monitoring of the white stork population health status in the future.

Table 1. Results (sample size (n), mean ± SD, and variability) of the enzymatic parameters and fluorescent dyes measured in plasma and S9 of white stork (*C. ciconia*) nestlings.

Parameter	*n*	Plasma			S9		
-	-	Mean	SD	Variability (%)	Mean	SD	Variability (%)
AChE [nmol min^{-1} $mg_{PROT}{}^{-1}$]	16	14.79	5.12	34.60	3.13	1.26	40.21
CES [nmol min^{-1} $mg_{PROT}{}^{-1}$]	16	21.53	9.59	44.54	5.85	1.96	33.53
GST [nmol min^{-1} $mg_{PROT}{}^{-1}$]	16	18.26	7.84	42.93	14.41	2.94	20.37

Table 1. *Cont.*

Parameter	*n*	Plasma			S9		
-	-	Mean	SD	Variability (%)	Mean	SD	Variability (%)
GR [pmol min^{-1} mg$_{PROT}$$^{-1}$]	16	98.11	65.67	66.94	840.55	235.42	28.01
CellTracker^{TM} Green CMFDA (RFU)	16	7246.07	1571.19	21.68	24683.10	7603.60	30.80
CM-H$_2$DCFDA (RFU)	16	76.29	5.09	6.68	33.04	11.55	34.94

SD: standard deviation; AChE: acetylcholinesterase; CES: carboxylesterase; GST: glutathione S-transferase; GR: glutathione reductase; CellTracker^{TM} Green CMFDA: dye for glutathione detection; CM-H$_2$DCFDA: dye for ROS detection; RFU: relative fluorescence unit.

3.2.2. Acetylcholinesterase and Carboxylesterase Activity

An increase in absorbance for acetylcholinesterase (AChE) plasma and S9 (Figure S2) were observed for 5- and 10-min periods, respectively. Different sample concentrations and measurement times were used and determined based on a linear absorbance increase and $R^2 \geq 0.95$. Due to high AChE activity, plasma and S9 were diluted prior to measurement. Plasma samples were diluted 5 times, whereas the S9 samples were diluted 10 times because avian erythrocytes contain haemoglobin that interferes in the absorbance spectrum 400–415 nm. To obtain satisfactory results, the S9 samples had to be more diluted and the measurement times were prolonged, to reduce the haemoglobin influence on the assay, as shown in AChE activity in rat erythrocytes [54].

The results of AChE activity in plasma and S9 are shown in Figure 1. Significantly higher specific AChE activity was reported in plasma than in S9 ($p < 0.0001$). However, lower variability among samples was observed in plasma than S9 (Table 1). AChE, as a transmitter hydrolysing acetylcholine, is primarily found in the central and peripheral nervous system as well as muscular system [55]. There is no data available for AChE activity in the blood of white stork nestlings. However, blood AChE histochemistry was assessed [56], and AChE activity was analysed for the purpose of determining the effects of daily photoperiods, a behaviour biomarker of organophosphate (OP) exposure, to establish the basal levels, compare the response to organophosphate and carbamate exposure, and compare the age-dependent changes in plasma [19–21,57–60]. Furthermore, plasma cholinesterases were characterised to establish the basal activities [34,61]. There is wide variation in AChE activity interspecies [57] and between matrices, pointing out the need to determine the basal AChE activity in plasma and S9 in white stork nestlings. Lower AChE activity in S9 may be due to AChE localization—bound to erythrocyte membranes [62,63] that are destroyed with sonication and centrifugation. After S9 preparation, the pellet containing cell membranes is usually discarded.

An increase in carboxylesterase (CES) absorbance (OD) was observed for plasma and S9 samples (Figure S3) for 2- and 5-min time periods, respectively. Different sample concentrations and measurement times were tested, and the final values used are based on a linear absorbance increase and $R^2 \geq 0.95$. In the S9 samples, the measurement time was prolonged due to a high haemoglobin concentration, interfering with the assay [54]. Nevertheless, a linear increase could be observed.

The results of CES activity in plasma and S9 are shown in Figure 2. Significantly higher specific CES activity was reported in plasma than in S9 ($p < 0.0001$). Moreover, higher variability among samples was observed in plasma than S9 (Table 1). CES is a ubiquitous enzyme, with the main function being the hydrolysation of carboxylic acid esters to acid and alcohol, a detoxification mechanism for various xenobiotics [64,65]. CES activity has previously been measured in blood of pigeons (*Columba livia*) and several bird of prey species for the purpose of evaluating CES activity as a potential biomarker of OP exposure [31]. CES and cholinesterase activity was determined in the muscle and liver of yellow-legged gull (*Larus michahellis*) for the purpose of monitoring environmental pollution [66]. Furthermore, CES activity was measured in blood of white storks (*C. ciconia*), black storks (*Ciconia nigra*), vultures, and diurnal and nocturnal predatory birds

for the purpose of evaluating CES activity as a potential biomarker of OP and carbamate exposure [67]. Specific CES activity was higher in plasma than in S9, due to low esterase activity in avian erythrocytes [68].

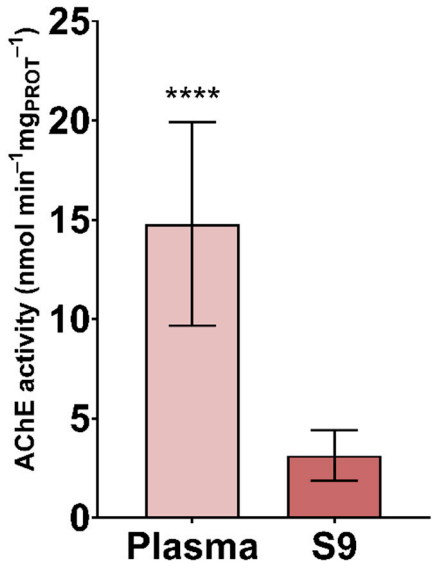

Figure 1. Specific activity of acetylcholinesterase (AChE) in plasma and S9 (nmol min^{-1} mg$_{PROT}$$^{-1}$) of white stork (*C. ciconia*) nestlings (n = 16), presented as the mean ± SD. Statistical difference is indicated with **** (Welch's t-test, $p < 0.0001$).

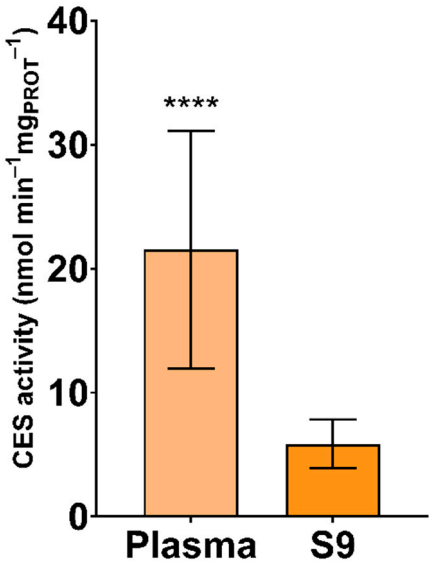

Figure 2. Specific activity of carboxylesterase (CES) in plasma and S9 (nmol min^{-1} mg $_{PROT}$$^{-1}$) of white stork (*C. ciconia*) nestlings (n = 16), presented as the mean ± SD. Statistical difference is indicated with **** (Welch's t-test, $p < 0.0001$).

AChE and CES have possible applications in avian species for environmental biomonitoring, as exposure biomarkers to diverse environmental pollutants. AChE is usually regarded as a destructive biomarker, since it is analysed in brain tissue [69], which is not suitable for endangered species, making this non-destructive evaluation essential. Although CES is usually analysed in serum, and therefore is considered a non-destructive biomarker (e.g., [70]), certain limitations exist, e.g., the blood volume that could be taken without harming the bird. Due to esterase's variability between avian species, it is impor-

tant to determine the basal activity for each species, as well as to determine activity in plasma and S9.

3.2.3. Glutathione S-Transferase and Glutathione Reductase Activity

The increase in glutathione S-transferase (GST) absorbance (OD) was observed for plasma and S9 samples (Figure S4) for 1- and 5-min time periods, respectively. Different sample concentrations and measurement times were tested, and the final values used are based on a linear absorbance increase and $R^2 \geq 0.95$. For S9, due to haemoglobin interference [54], the measurement was prolonged.

The results of GST activity in plasma and S9 are shown in Figure 3. There was no statistical difference between specific GST activity in plasma and S9, although higher variability among samples was observed in plasma than S9 (Table 1). GST is an enzyme catalysing GSH to xenobiotic substrate conjugates, as a detoxification mechanism [71,72]. As shown in Figure 3, specific GST activity was similar in plasma and S9 of white stork nestlings due to the enzyme distribution in these two blood fractions. Since GST's primary function is xenobiotic metabolism, it can be found intra- and extracellular [73]. Plasma GST detection and its activity reflects de novo synthesis in the liver [74]. So far, GST has been analysed in the blood of various avian species for the purpose of assessing oxidative stress caused by metal pollution and persistent organic pollutants as well as evaluating physiological conditions due to environmental stress [35,75–85]. When comparing GST activity in S9 between nestling, juvenile, and adult storks, Oropesa et al. [35] reports 877.72 nmol min^{-1} $mg_{PROT}{}^{-1}$ in juveniles, and 964.61 nmol min^{-1} $mg_{PROT}{}^{-1}$ in adults, considerably higher than reported in this study for nestlings.

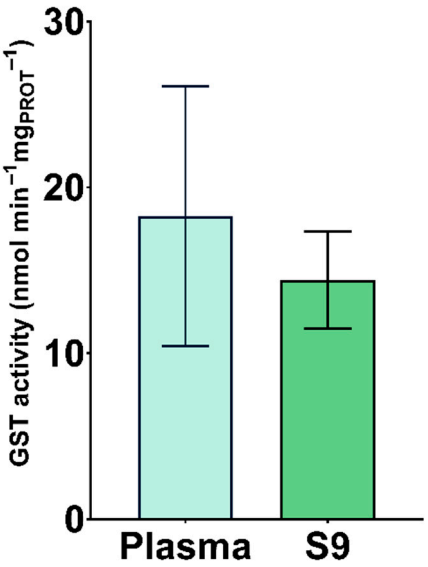

Figure 3. Specific activity of glutathione S-transferase (GST) in plasma and S9 (nmol min^{-1} mg $_{PROT}{}^{-1}$) of white stork (*C. ciconia*) nestlings (n = 16), presented as the mean ± SD.

A decrease in glutathione reductase (GR) absorbance (OD) was observed in plasma and S9 (Figure S5) for the 10-min time period. Different sample concentrations and measurement times were tested, and the final values used are based on a linear absorbance increase and $R^2 \geq 0.95$.

The results of GR activity in plasma and S9 are shown in Figure 4. Significantly higher specific GR activity was reported in S9 than in plasma ($p < 0.0001$). Furthermore, higher variability among samples was observed in plasma samples compared to S9 samples (Table 1). GR is an enzyme catalysing the NADPH-dependent reduction of GSSG to GSH. GSSG reduction is an essential reaction for the preservation of GSH levels, since GSH has a primary function in processes regarding oxidation and reduction, as well

as cellular detoxification [86]. GR has been measured in avian blood for the purpose of assessing ecophysiological determination and antioxidant defences as a response to environmental pollution, in addition to evaluating the effect of oxidized fat and selenium on GR activity [35,78,79,83,84,87–91]. Oropesa et al. [35] reports that the GR activity in S9 of juvenile and adult storks (*C. ciconia*) is substantially lower (410 pmol min^{-1} $mg_{PROT}{}^{-1}$ and 380 pmol min^{-1} $mg_{PROT}{}^{-1}$, respectively) than reported in this study for white stork nestlings. This could be due to production of free radicals and depletion of antioxidant defences, both related to aging and age-related diseases [92,93]. Considering that older storks loose function to regulate physiological homeostasis and depletion of some blood enzymatic antioxidants, as a consequence of aging [94–96], nestlings might be a more suitable age group for biomonitoring assessments. As shown in Figure 4, higher GR activity was found in S9 than plasma. That being said, GR is a cellular enzyme that accumulates in cellular regions with high electron flux, resulting in high ROS production [97].

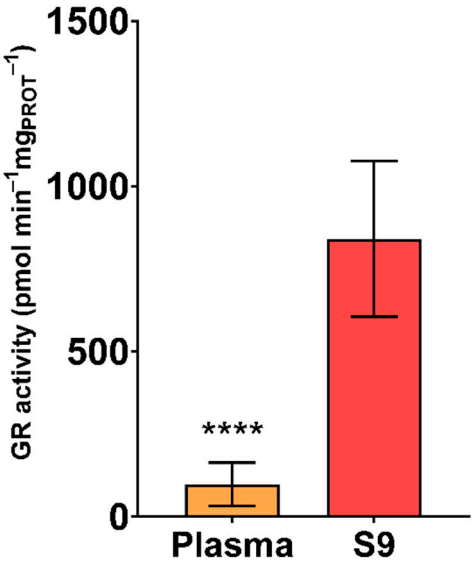

Figure 4. Specific activity of glutathione reductase (GR) in plasma and S9 (pmol min^{-1} $mg_{PROT}{}^{-1}$) of white stork (*C. ciconia*) nestlings (n = 16), presented as the mean ± SD. Statistical difference is indicated with **** (Welch's t-test, $p < 0.0001$).

Measuring oxidative stress parameters in blood has certain restrictions, e.g., fieldwork limitations and small sample volumes. Our work demonstrates that oxidative stress biomarker measurements could be performed by using either plasma or S9 if there is limitation of the sample volume. When interpreting the results, it is also important to take into account that oxidative stress might not originate in the circulation system but in other tissue; therefore, it is necessary to analyse several biomarkers in different matrices for a broad view of the physiological condition. For this purpose, we evaluated GST and GR in two blood fractions, giving insight into their activity in plasma and S9.

3.3. Fluorescent Dyes

The fluorescence-based assay for GSH detection has been successfully established in avian plasma and S9, confirmed by a positive control in which a substrate (GSH) was added resulting in 17 times higher fluorescence detection in the positive control than the blanks for plasma, and 19 times higher fluorescence detection in the positive control than blanks in S9. Furthermore, the fluorescence-based assay for ROS detection was also successfully established in avian plasma and S9, confirmed by a positive control in which a substrate (H_2O_2) was added, resulting in 12 times higher fluorescence detection in the positive control than blanks for plasma, and 3 times higher fluorescence detection in the positive control than blanks in S9.

CellTracker™ Green CMFDA dye was used for GSH detection. Different sample concentrations and measurement times were tested, and the final values used are based on a linear fluorescence increase and $R^2 \geq 0.95$ (Figure S6). Increase in fluorescence (RFU) was observed in plasma for 60 min. Fluorescence (RFU) in S9 was measured for 120 min, and an optimal linear increase was observed in the first 30 min, after which GSH saturation was observed, resulting in a stagnation line.

The results of fluorescent GSH detection in plasma and S9 for 30 min are shown in Figure 5. Significantly higher GSH fluorescence was reported in S9 than in plasma ($p < 0.0001$). When comparing the variability between responses in these two types of samples, it can be observed that lower variability was observed in plasma compared to S9 (Table 1). Until now, the CellTracker™ Green CMFDA dye for GSH detection was not used in avian blood. However, it was used in zebrafish (*Danio rerio*) embryo and larvae, as well as mouse (*Mus musculus*) embryonic fibroblasts for the purpose of assessing cytotoxicity, apoptosis, and oxidative stress caused by pesticides and silver nanoparticles [41,98,99]. Higher GSH detection was observed in S9, as shown in Figure 5. As S9 contains cellular and subcellular fractions, it was rich with GSH. Most of the GSH is found in the cytoplasm, mitochondria, nucleus, and peroxisomes [100]. Extracellular concentrations of GSH are low [101,102], as shown in Figure 5. In case of smaller sample sizes, usage of plasma for GSH detection is recommended due to the observed lower variability.

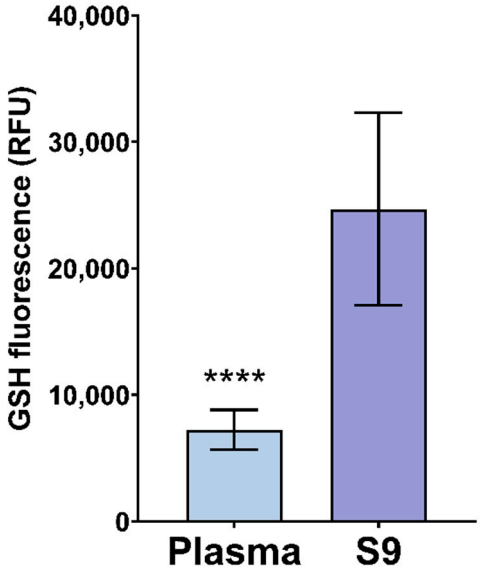

Figure 5. Relative fluorescence (RFU) of reduced glutathione (GSH) in plasma and S9 of white stork (*C. ciconia*) nestlings ($n = 16$), presented as the mean ± SD. Statistical difference is indicated with **** (Welch's *t*-test, $p < 0.0001$).

CM-H_2DCFDA dye was used for ROS detection. Different sample concentrations and measurement times were tested, and the final values used were determined based on a linear fluorescence increase and $R^2 \geq 0.95$ (Figure S7). An increase in fluorescence (RFU) was observed in plasma and S9 for 30- and 120-min time periods, respectively. For plasma, an optimal linear increase was observed for 10 min, after which ROS saturation was observed. In the S9 samples, a linear increase was observed for 120 min.

The results of using a fluorescent dye for measuring ROS detection in plasma and S9 for 10 min are shown in Figure 6. Significantly higher ROS fluorescence was reported in plasma than in S9 ($p < 0.0001$). Lower variability among samples was observed in plasma than S9 (Table 1). Until now, CM-H_2DCFDA dye was not used in avian blood for ROS detection. However, it was used in zebrafish (*D. rerio*) for the purpose of detecting oxidative stress induced by pesticide exposures [41,99]. Avian erythrocytes have functional mitochondria in terms of ROS production and respiratory activity [103]. Higher ROS

detection was observed in plasma compared to S9, as shown in Figure 6. This may be due to extracellular ROS production, induced by external sources (e.g., drugs, pollutants, and radiation) [104]. In case of small sample sizes, using plasma for ROS detection is recommended due to the observed lower variability.

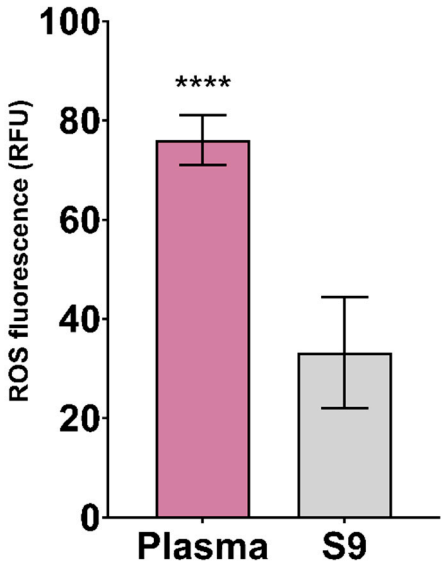

Figure 6. Relative fluorescence (RFU) of the reactive oxygen species (ROS) in plasma and S9 of white stork (*C. ciconia*) nestlings (n = 16), presented as the mean ± SD. Statistical difference is indicated with **** (Welch's t-test, $p < 0.0001$).

Fluorescence-based oxidative stress detection in blood is a simple, non-destructive method for ROS and GSH detection. Fluorescent detection of GSH and ROS production have never been reported in white stork nestlings. Moreover, to the best of our knowledge, the fluorescent dyes CellTracker^{TM} Green CMFDA and CM-H_2DCFDA have not been used in avian blood before. However, fluorescent dyes have been successfully used in other model organisms for the purpose of evaluating pesticide exposure and oxidative stress response [41,99]. Fluorescent dyes for GSH and ROS can be used in both plasma and S9 of white stork nestlings for the purpose of evaluating oxidative stress.

4. Conclusions

The blood sampling of white stork nestlings is a non-destructive method that can be easily obtained when performed in parallel with ringing. The present study successfully used enzymatic (AChE, CES, GR, and GST) and non-enzymatic (GSH and ROS) biomarkers for determining the basal values in white stork chicks. Fluorescent-based assays, as a novel method for oxidative stress detection in birds, were developed in this study. To get better overall insight into oxidative stress, using enzymatic antioxidants and fluorescence-based oxidative stress detection in two blood fractions will give a better overview of a nestling's physiological condition. The established protocols can be expanded to other avian species as well. Assessment of the relationship between the biomarkers in the two blood fractions is paramount in order to understand the usefulness of both plasma and S9. This research indicated valuable differences in enzyme activity and oxidative stress detection with fluorescent-based probes for the first time in plasma and S9. Responses for each biomarker in the two blood fractions provide useful information in case of a small sample volume as well as providing overall information about physiological condition. Therefore, we conclude that both plasma and S9 can be used for biomarker analysis.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ani11082341/s1, Table S1. DNA concentration; Figure S1. Sex determination results; Figure S2. AChE absorbance; Figure S3. CES absorbance; Figure S4. GST absorbance; Figure S5. GR absorbance; Figure S6. CellTracker^{TM} Green CMFDA dye for GSH detection; Figure S7. CM-H_2DCFDA dye for ROS detection.

Author Contributions: Conceptualization: D.B., A.M., and M.V.; investigations: D.B., A.M., C.L., L.B., and T.M.; data analysis and curation: D.B., A.M., and M.V.; writing: D.B. and A.M.; review and editing: D.B., A.M., C.L., L.B., T.M., M.V. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Samples and data were collected according to Institute of Ornithology, Croatian Academy of Science, protocols, under the supervision of certified ringer/researcher. Samples and data were collected as part of routine White Stork ringing and monitoring scheme in Croatia. All procedures were conducted in accordance with the Croatian Nature Protection Act (Official Gazette no. 80/13, 15/18 and 14/19) and approved by Croatian Ministry of Economy and Sustainable Development (reference no: 517-05-1-1-20-4 from 27 August 2020). No extra animal discomfort was caused for sample collection for the purpose of this study.

Data Availability Statement: All data was included in the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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2.2. Blood biomarkers in white stork (*Ciconia ciconia*) nestlings show different responses in several areas of Croatia

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Blood biomarkers in white stork (*Ciconia ciconia*) nestlings show different responses in several areas of Croatia

Dora Bjedov[1] | **Mirna Velki**[1] | **Carina Lackmann**[2] | **Lidija Begović**[1] | **Tibor Mikuška**[3] | **Luka Jurinović**[4] | **Alma Mikuška**[1]

[1]Department of Biology, Josip Juraj Strossmayer University of Osijek, Osijek, Croatia

[2]Department of Evolutionary Ecology and Environmental Toxicology, Goethe University Frankfurt, Frankfurt am Main, Germany

[3]Croatian Society for Birds and Nature Protection, Osijek, Croatia

[4]Poultry Centre, Croatian Veterinary Institute, Zagreb, Croatia

Correspondence
Alma Mikuška, Department of Biology, Josip Juraj Strossmayer University of Osijek, Cara Hadrijana 8/A, 31000 Osijek, Croatia.
Email: amikuska@biologija.unios.hr

Funding information
Alexander von Humboldt-Stiftung; Josip Juraj Strossmayer University of Osijek; Deutsche Bundesstiftung Umwelt

Abstract

White stork nestlings can provide quantitative data on the quality of the environment, as they are dependent on their parents that provide locally foraged food. Blood was sampled from the brachial vein (n = 109) and the sampling was performed in parallel with ringing during breeding season 2020 from five areas in eastern Croatia: Lonjsko polje, Jelas polje, Slavonski Brod–east, Podunavlje, and Donje Podravlje. In the present study, for the first time in Croatia, the following enzymatic biomarkers were assessed in white stork nestlings: activities of acetylcholinesterase (AChE), carboxylesterase (CES), glutathione S-transferase (GST), and glutathione reductase (GR), as well as nonenzymatic biomarkers: levels of glutathione (GSH) and reactive oxygen species (ROS). All endpoints were measured in two blood fractions: plasma and a postmitochondrial fraction (S9). Nestlings from Podunavlje and Donje Podravlje, areas known for intensive agriculture, showed lower AChE and CES activity when compared to the other investigated areas, indicating the presence of inhibitory xenobiotics. Higher oxidative stress was observed in Slavonski Brod–east, an area surrounded by metal and engineering industry, and Podunavlje compared to the other sampling areas. Hence, this study shows the impact of pollutants from the surrounding metal, petroleum, and agricultural industry might have on the biomarkers in white stork nestlings, which are often seen as early-warning signals.

KEYWORDS
white stork, bioindicators, oxidative stress, spatial variation, Croatia

1 | INTRODUCTION

The white stork (*Ciconia ciconia*) is a migratory and breeding species in Europe, frequently populating habitats abundant with prey such as various invertebrates (grasshoppers, beetles, earthworms) and vertebrates (fish, frogs, snakes, lizards, and rodents) (BirdLife, 2021; Kosicki et al., 2006). White storks occasionally forage trash from landfills (BirdLife, 2021; Blanco, 1996; del Hoyo et al., 1992; Kruszyk & Ciach, 2010; Pineda-Pampliega et al., 2021; Tortosa et al., 2002). Although the conservation status is considered to be of least concern and the population is globally increasing (BirdLife, 2021), a population decline is locally observed in breeding areas of continental Croatia due to the habitat destruction and/or alteration, conversion of wetlands, and foraging areas, as well as excessive agriculture and pesticide usage (Mikuška et al., 2017).

In continental Croatia, the white stork breeds in rural areas surrounded by intensively managed agricultural fields, mosaic areas consisting of different agricultural lands and grasslands (Radović & Tepić,

Dora Bjedov and Mirna Velki contributed equally to this study.

2009). Since agriculture is one of the most important economic sectors in continental Croatia (Bašić et al., 2007), white storks are unintentionally exposed to the various pesticide classes through the habitats they forage (Parsons et al., 2000). With the use of pesticides in continental Croatia (Romić et al., 2015), for example, organophosphate (OP) and carbamate (CB), insecticides which are extensively used to control pests and disease vectors at agricultural lands (Hill, 2002), there is a high possibility for pesticide exposure. Apex predators, such as white storks, could ingest contaminated prey and subsequently suffer secondary poisoning (Baudrot et al., 2020). As their nestlings reside in the nest and are completely dependent on the local food sources foraged by their parents, they are suitable bioindicators and sentinels of contaminants in the local environment (Blázquez et al., 2006; Lewis & Pomeroy., 2017). Their health status is influenced by food quality. Thus, their body content and physiological conditions can reflect pollutant concentrations in their food and provide useful information regarding local pollution and the pollutant effects on the environment (Burger, 1993; Furness, 1993; Marsili et al., 1996). Hence, avian blood has various applications in ecotoxicology, for example, for measurements of enzyme activities. Esterase activities measured in the blood, for example, have been widely used in biomonitoring as biomarkers of OP and CB exposure, as both pesticide classes are known to inhibit esterase activities (Bartkowiak & Wilson, 1995; Thompson & Walker, 1988).

For the purpose of pesticide biomonitoring, the range of cholinesterase activity in juvenile and adult white storks has been established through various studies (Oropesa, Gravato, Sánchez, et al., 2013; Santos et al., 2012). The acetylcholinesterase (AChE) inhibition, which leads to acetylcholine accumulation in synapses resulting in nerve dysfunction (Peakall, 1992), has been observed in common quail (*Coturnix coturnix*) after exposure to OP chlorpyrifos (Soler-Rodriguez et al., 1998), as well as in chaffinch (*Fringilla coelebs*) and coal tit (*Periparus ater*) after exposure to OP fenitrothion (Hamilton et al., 1981). Effects of sublethal acute doses of OP chlorfenvinphos were experimentally investigated on common starlings (*Sturnus vulgaris*) and different changes in behavior, depending on the level of AChE inhibition, were observed (Hart, 1993). AChE is a molecular target for OP and CB pesticides and resulting inhibitions lead to the inability to hydrolyze acetylcholine (Lionetto et al., 2012), consequently leading to lethargy and reduced activity (Grue & Shipley, 1984; Hart, 1993; Radvanyi et al., 1986; Rattner & Franson, 1984).

Another important commonly studied esterase is carboxylesterase (CES). It plays an important role in drug biotransformation, as environmental pollutants or lipophilic chemicals have been shown to induce CES activity (Satoh & Hosokawa, 2006). CES belongs to the group of Phase I xenobiotic metabolism enzymes (Sogorb & Vilanova, 2002). When exposed to various xenobiotics such as pesticides, pharmaceuticals, and veterinary drugs, they hydrolyze ester-containing compounds (Hatfield & Potter, 2011; Satoh & Hosokawa, 2006). Studies on rats (*Rattus norvegicus domestica*) and three-spined stickleback (*Gasterosteus aculeatus*) have shown that CES is a more sensitive molecular target than AChE when exposed to OP and CB pesticides (Gupta & Dettbarn, 1993; Wogram et al., 2001). Pesticide effects on avian CES activity are very well documented (Barata et al., 2010; Cordi et al., 1997; Parker & Goldstein, 2000). Plasma CES inhibition has also been observed in common starling (*S. vulgaris*) nestlings, by experimentally dosing them with OP and CB, where it was shown that both pesticide classes affect AChE and CES activity differently (Parker & Goldstein, 2000).

Pesticides and other pollutants, for example, heavy metals, can cause adverse effects by inducing reactive oxygen species (ROS) overproduction leading to oxidative damage (Ćupić Miladinović et al., 2018; Hoshi et al., 2014; Koivula et al., 2011; Nuran Ercal et al., 2005). Oxidative stress occurs due to excessive ROS production and/or antioxidant depletion, subsequently affecting reproduction, longevity, and immune response (Costantini, 2008). However, the cells contain different antioxidant mechanisms with the main function of detoxifying oxidized compounds (Sies, 1997). Oxidative stress in the blood of storks was previously assessed by Oropesa, Gravato, Guilhermino et al. (2013) for the purpose of assessing the impact of continuous exposure to pollution. For evaluating avian physiological changes, heavy metals were measured in ambient air, feathers, and blood, as well as oxidative stress biomarkers and hematological parameters (Elarabany & El-Batrawy, 2019; Tkachenko & Kurhaluk, 2012).

In our previous study (Bjedov et al., 2021), we have shown that blood sampled from white stork nestlings during a regular ringing scheme can be used for the measurement of different biomarkers in two types of blood samples. Considering the information that can be obtained from biomarker responses, such as health status or potential exposure to harmful substances, the aim of this study was to investigate biomarker responses to environmental pollutants in avian blood. To our knowledge, this is the first study assessing these biomarkers in blood sampled from stork nestlings in Croatia. Biomarkers of effect: AChE and CES; and oxidative stress biomarkers: glutathione S-transferase (GST), glutathione reductase (GR), glutathione (GSH), and ROS were assessed in two types of white stork nestlings' blood samples obtained from five areas in continental Croatia. Our main hypotheses, regarding the sampling locations, were (1) nestlings located in proximity to intensive agriculture and fertilizer use would have reduced AChE and CES activity due to the pesticide pollution and (2) nestlings located in the vicinity of the metal and engineering industry would have altered activities of GST and GR, as well as GSH and ROS levels due to the heavy metal pollution.

2 | MATERIALS AND METHODS

2.1 | Sampling areas

Blood samples were obtained from white stork chicks during the regular ringing scheme in June and July 2020. In total, 109 nestlings were sampled in five different areas along large rivers (Sava, Danube, and Drava) and their floodplain areas: Lonjsko polje, Jelas polje, Slavonski Brod–east, Podunavlje, and Donje Podravlje (Figure 1). The Nature Park Lonjsko polje is located in the central part of the Sava River and represents the largest protected area along the whole river

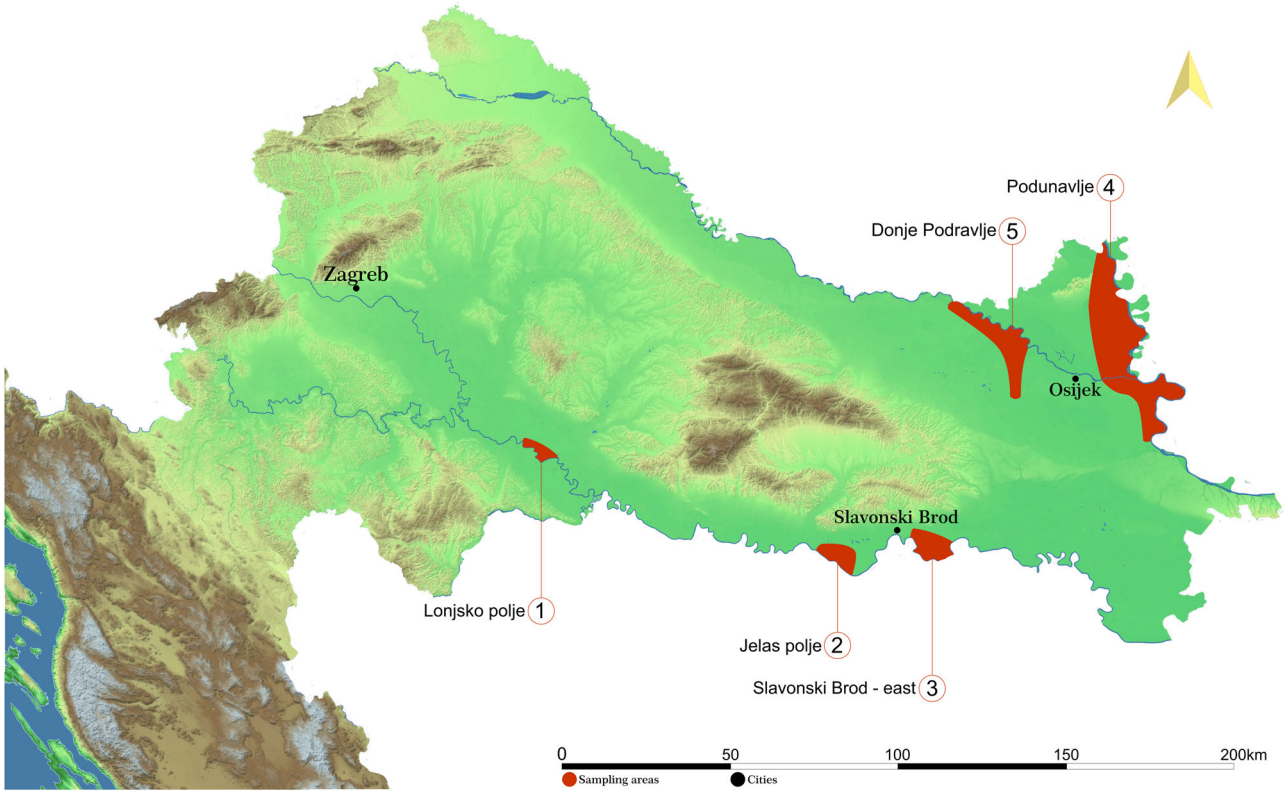

FIGURE 1 Sampling areas of white stork (*Ciconia ciconia*) nestlings

(Schwarz, 2016). The area is covered by floodplain forests (67%), wet meadows and grasslands (10%), small-scale agriculture (12%), and small settlements (12%) (Javna ustanova Park prirode Lonjsko polje, 2008). Lacking any major industry and large-scale agriculture, this area with the least contamination potential and thus serves as a control in this study. Jelas polje is situated at the lower part of the middle course of Sava River, west of Slavonski Brod and downstream of Lonjsko polje (Schwarz, 2016). This area contains alluvial forests (40%), large fishponds (13%), wet meadows and grasslands (10%), small- (20%) and large- (7%) scale agriculture, and small settlements (10%). The intensive use of pesticides could be considered a major source of pollution (Romić et al., 2015). The third area, Slavonski Brod–east lies along the Sava River just downstream from a city known for its highly developed industry of metal engineering. Across Slavonski Brod is an oil refinery located in Bosanski Brod (Gvozdić et al., 2011). This area is covered by alluvial forests (20%), small- and large-scale agriculture (40%), and small villages (20%), several large pastures (20%) that are regularly flooded by Sava still exist (Schwarz, 2016). Podunavlje region is situated along the Danube River and it is a large floodplain. Settlements with stork nests are situated at the border of the former floodplain and surrounded by large-scale cereal agriculture. Donje Podravlje is situated along the Drava river and the research area covers a large- and small-scale orchard and corn agriculture. The intensive use of pesticides may be the major source of pollution for both Podunavlje and Donje Podravlje sampling areas (Romić et al., 2015). Based on agricultural land use, analysis of water and atmospheric pollutants (Gvozdić et al., 2011; Musić et al., 2020; Romić et al., 2015; Vađić et al., 2021), the gradation of pollution in these sites was assumed, from Lonjsko polje as the least polluted to Slavonski Brod–east as the most polluted site. According to Romić et al. (2015) Jelas polje, Podunavlje, and Donje Podravlje would be the sites with intermediate levels of pollution (Podunavlje being the most polluted and Donje Podravlje least polluted) in relation to the cover of large-scale intensive agriculture.

2.2 | Blood extraction and sample preparation

To avoid disturbing dietary habits, as well as heat stress, sampling was conducted between 08:00 and 12:00 am. All nestlings were between 6 and 8 weeks old. Nondestructive methods were used to obtain the blood samples and two blood fractions were prepared for efficient evaluation of nestlings' condition (Bjedov et al., 2021). Blood samples were collected with a sterile 5 mL syringe and 0.8 mm needle. Approximately 4 mL of blood was collected from the brachial vein and transferred to the lithium heparin collection tube. Blood samples were kept under cold and dark conditions for 6–8 h, after which they were centrifuged at 3000*g* for 10 min at 4°C. Plasma (supernatant) aliquots were distributed to the new sterile 2 mL tubes and stored at −80°C until analysis. Pellets were prepared as described in Bjedov et al. (2021). In brief, the pellet was

suspended in 5 mL of 0.1 M sodium phosphate buffer (pH 7.2) and sonicated for cell disruption, following centrifugation at 9000*g* for 20 min at 4°C to acquire the postmitochondrial fraction (S9). S9 samples were kept at −80°C until further analysis. All biomarker analyses were performed in both types of samples. The study was conducted under the permit of The Ministry of Environment and Energy of the Republic of Croatia (Classification code: UP/I-612-07/20-48/130; Registry number: 517-05-1-1-20-4).

2.3 | Chemicals

In the present study following chemicals (analytical grade) were used: acetonitrile (C_2H_3N, CAS 75-05-8, 41.053 g mol^{-1}), β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (β-NADPH) ($C_{21}H_{26}N_7Na_4O_{17}P_3$ x H_2O, CAS 2646-71-1 [anhydrous], 833.35 g mol^{-1} [anhydrous basis]), CellTracker™ Green CMFDA Dye ($C_{25}H_{17}ClO_7$, CAS 136832-63-8, 464.86 g mol^{-1}) (ThermoFisher Scientific), 1-chloro-2,4-dinitrobenzene (CDNB) ($C_6H_3ClN_2O_4$, CAS 97-00-7, 202.55 g mol^{-1}), CM-H_2DCFDA ($C_{27}H_{19}Cl_3O_8$, CAS 1219794-09-8, 577.8013 g mol^{-1}) (ThermoFisher Scientific), (2-Mercaptoethyl) trimethylammonium iodide acetate (acetylthiocholine iodide) ($CH_3COSCH_2CH_2N(CH_3)_3I$, CAS 1866-15-5, 289.18 g mol^{-1}), disodium hydrogen phosphate (NaH_2PO_4, CAS 7558-79-4, 141.957 g mol^{-1}), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) ($[-SC_6H_3(NO_2)CO_2H]_2$, CAS 69-78-3, 396.35 g mol^{-1}), glutathione disulfide (GSSG, $C_{20}H_{32}N_6O_{12}S_2$, CAS 27025-41-8, 612.6 g mol^{-1}), *p*-nitrophenyl acetate ($C_8H_7NO_4$, CAS 830-03-5, 181.147 g mol^{-1}), (2 *S*)−2-amino-4-{[(1 *R*)−1-[(carboxymethyl) carbamoyl]−2-sulfanylethyl]carbamoyl}butanoic acid (GSH) ($C_{10}H_{17}N_3O_6S$, CAS 70-18-8, 307.32 g mol^{-1}), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \times 2H_2O$, CAS 13472-35-0, 156.006 g mol^{-1}). The Pierce™ BCA Protein Assay Kit was used for protein concentration measurements.

2.4 | Biomarker analysis

All samples were analyzed both in plasma and S9, as described in detail in Bjedov et al. (2021). The AChE activity was analyzed according to Ellman et al. (1961), the reaction mixture contained sample, DTNB, and acetylthiocholine iodide. For CES analysis, the protocol by Hosokawa and Satoh (2001), with *p*-nitrophenyl acetate as a substrate, was applied. GST activity was determined according to Habig and Jakoby (1981), the reaction mixture contained sample, CDNB, and GSH. GR activity was analyzed using Habig and Jakoby (1981), with the following reagents: sodium phosphate buffer, GSSG, and reduced β-NADPH. The GSH concentration was assessed using CellTracker™ Green CMFDA and ROS levels were measured using CM-H_2DCFDA fluorescent dye. Protein content was determined using the Pierce™ BCA Protein Assay Kit. All biomarker measurements, samples, and blanks were measured in triplicates using the Tecan Spark 10 M microplate reader.

2.5 | Sex determination

Sex was determined from S9 according to Begović et al. (2017) with modifications described in Bjedov et al. (2021), based on *CHD* gene amplification (Fridolfsson & Ellegren, 1999).

2.6 | Data analysis

All statistical analyses were performed using R version 4.0.0 and GraphPad Prism 8.4.3. Outliers were explored using Cleveland dot plot and box plots and detected using Grubb's test. A Shapiro–Wilk test, QQ-plots, and histograms confirmed the normality of the data. A linear mixed model was performed with *nlme* function to avoid pseudoreplication as more than one nestling was sampled from the same nest. One-way analysis of variance (ANOVA) was subsequently performed using ANOVA to determine the significant differences between the areas. To identify differences in biomarker responses between the sampling areas, post hoc analysis was used with function *emmeans*. The level of statistical significance was 0.05 (*p* value). Enzyme activity is presented as specific enzyme activity, quantified from the changes in absorbance. Fluorescence is presented as the relative fluorescence units (RFU), quantified from the fluorescence increase over time. All results are expressed as mean and standard deviation (±*SD*) with a vertical bar plot.

3 | RESULTS

3.1 | Overview of the results

Results of sex determination in white stork nestlings' blood are shown in Table SI1, and there were no statistical differences in biomarker responses regarding sex (*data not shown*). Average activities of enzymatic biomarkers (AChE, CES, GST, and GR) and levels of nonenzymatic biomarkers (GSH and ROS) in both plasma and S9 are shown in Table SI2.

3.2 | Biomarkers of effect

Significant differences in measured AChE and CES plasma activities between the sampling areas were observed (Table SI2, Figure 2). The plasma AChE activity was significantly higher in Slavonski Brod–east compared to Podunavlje and Donje Podravlje. Higher AChE activity was also found in Lonjsko polje when compared to Donje Podravlje. Regarding the plasma CES, the measurements showed that in nestlings from Slavonski Brod–east CES activity was significantly higher compared to all other sampling areas.

Measurements of AChE activity in S9 samples showed no statistical differences between different sampling areas (Table SI2, Figure 2). Regarding the S9 CES, the activity was significantly higher

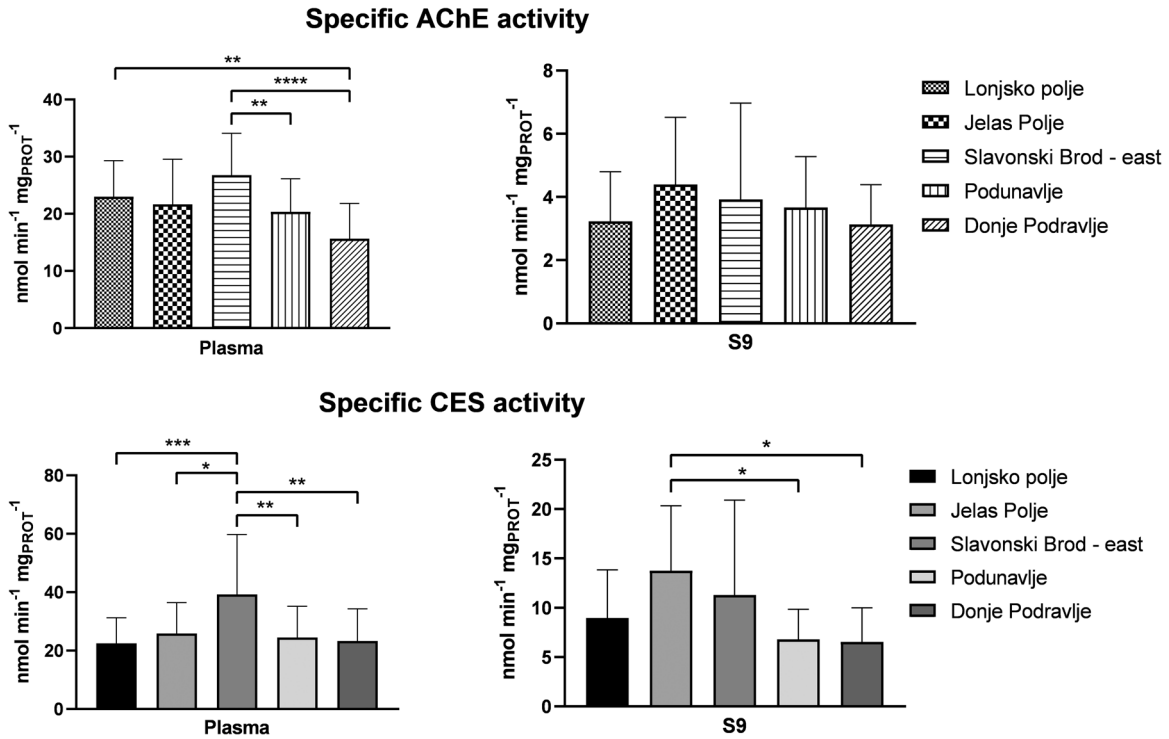

FIGURE 2 Specific acetylcholinesterase (AChE) and carboxylesterase (CES) activity (nmol min^{-1} $mg_{PROT}{}^{-1}$) in two blood fractions: plasma and S9 in white stork (*Ciconia ciconia*) nestlings from Lonjsko polje, Jelas polje, Slavonski Brod–east, Podunavlje and Donje Podravlje in continental Croatia. Values are presented as mean ± *SD*. Lines and asterisks represent significant differences between areas *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$), ****($p < 0.0001$)

in Jelas polje than in Podunavlje and Donje Podravlje (Table SI2, Figure 2).

3.3 | Enzymatic oxidative stress

No significant difference in plasma GST activity was observed between the studied sites (Table SI2, Figure 3). Regarding the plasma GR, the measurements showed that in nestlings from Slavonski Brod–east GR activity was significantly higher compared to all other sampling areas. Measurements of GST and GR activities in S9 samples showed no statistical differences between different sampling areas (Table SI2, Figure 3). However, for S9 GR activity, a trend similar to plasma GR activity was observed (highest activity measured at Slavonski Brod–east sampling site).

3.4 | Nonenzymatic oxidative stress

Results of fluorescent GSH and ROS detection in plasma showed multiple significant differences among the studied areas (Table SI2, Figure 4). The highest GSH levels were recorded in Podunavlje and the lowest in Donje Podravlje. The highest ROS levels were detected in Slavonski Brod–east and the lowest in Lonjsko polje. The respective observed significant differences between sites are shown in Table SI2 and Figure 4.

Regarding the GSH and ROS levels in S9, similar as for other biomarkers, fewer differences were observed between studied sites (Table SI2, Figure 4). Measured GSH and ROS levels showed similar patterns to plasma GSH and ROS levels, however a significant difference in GSH was observed only between Jelas polje and Donje Podravlje, and a significant difference in ROS was observed only between Lonjsko polje and Slavonski Brod–east (Table SI2, Figure 4).

4 | DISCUSSION

4.1 | Biomarkers of effect

Results of plasma AChE and CES activities showed different responses between sites indicating the influence of pollutants present in the environment on these biomarkers. Namely, lower plasma AChE activities were observed in Donje Podravlje and Podunavlje sites (Figure 2), areas associated with large-scale intensive agriculture and consequently higher pesticide use, compared to sites along the Sava River. These results suggest the presence of inhibitory pollutants related to pesticide pollution. Regarding the plasma CES results, activity measured in Slavonski Brod–east was significantly higher compared to all other sites (Figure 2). This could be due to the proximity of the chemical and metal industry in Slavonski Brod and Bosanski Brod since some environmental pollutants, such as lipophilic

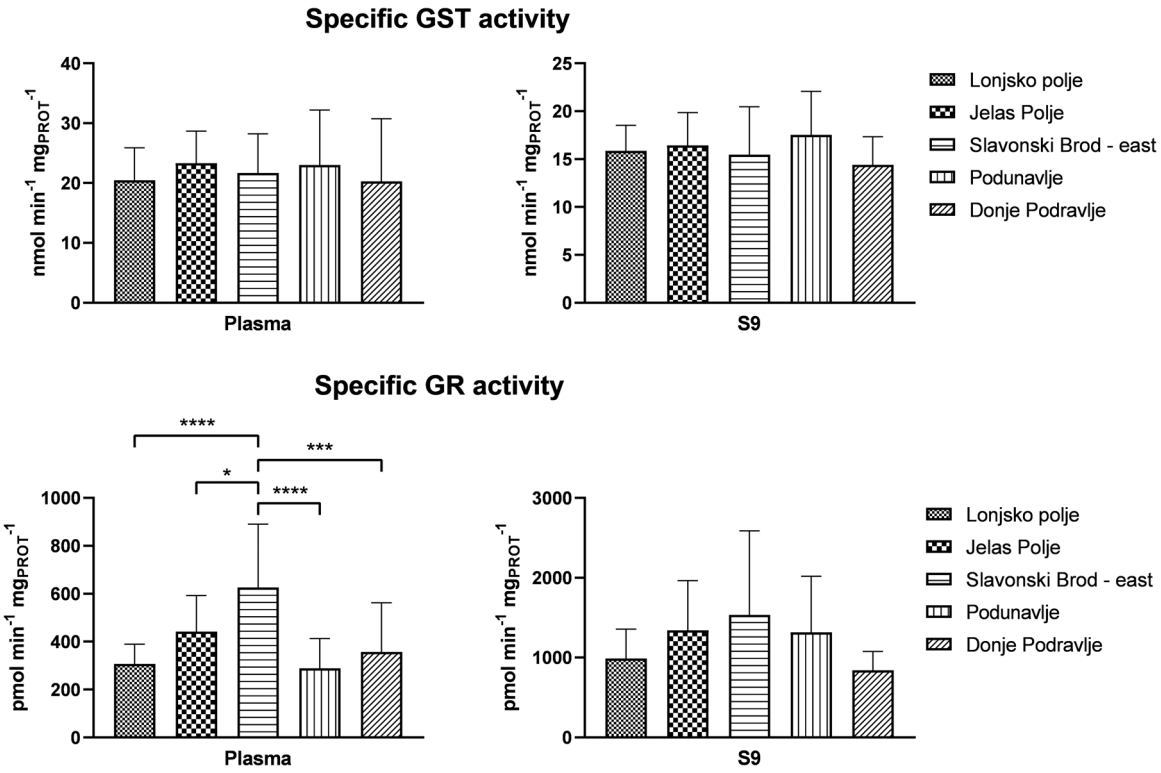

FIGURE 3 Specific glutathione S-transferase (GST) (nmol min^{-1} $mg_{PROT}{}^{-1}$) and glutathione reductase (GR) activity (pmol min^{-1} $mg_{PROT}{}^{-1}$) in two blood fractions: plasma and S9 in white stork (*Ciconia ciconia*) nestlings from Lonjsko polje, Jelas polje, Slavonski Brod–east, Podunavlje and Donje Podravlje in continental Croatia. Values are presented as mean ± *SD*. Lines and asterisks represent significant differences between areas *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$), ****($p < 0.0001$)

drugs or metal ions and detergents, are known to cause the CES induction (Satoh & Hosokawa, 2006; Zhang et al., 2009).

When compared to the plasma samples, AChE and CES activities measured in S9 samples were substantially lower. AChE activity in S9 was lower probably due to the AChE localization, as they are bound to erythrocyte membranes (Abdollahi et al., 1995; Stedman & Stedman, 1935; Tinoco-Ojanguren & Halperin, 1998), which are discarded during the sonication process. Measured lower S9 CES activity is most likely due to the low esterase activity in erythrocytes (Stedman & Stedman, 1935). In addition to lower activities, the comparison of S9 results between sites showed no (in case of AChE) or fewer (in case of CES) significant differences in responses. This indicates that for assessment of the pollutant effects on these biomarkers, plasma samples might be more suitable.

Observed results of lower AChE and CES activities from Podunavlje and Donje Podravlje might be due to the presence of various fertilizers and pesticides (Lazarus et al., 2021; Romić et al., 2015). Cereal crops are cultivated in Podunavlje providing pollutant sources since cereals are heavily treated with herbicides (hormone-type herbicides, urea herbicides) and fungicides (triazoles, imidazoles, benzimidazoles) (Romić et al., 2015). Moreover, Donje Podravlje is an area known for planting orchards and corn, maintaining them with the use of herbicides (aminophosphates, chloroacetamides, and triazines) and fungicides (pyrimidine fungicides, dithiocarbamates, inorganic fungicide) consequently resulting in lower AChE activity in Podunavlje and Donje Podravlje (Romić et al., 2015). Higher concentrations of herbicides in eastern Croatia were detected in water and soil. The concentration of triazine herbicides were exceeding the maximum allowable concentration in drinking water and in the plow soil layer (Sraka et al., 2007). As herbicides such as atrazine and simazine are known to inhibit AChE and CES (Mit et al., 2021; Mladenović et al., 2018), these findings may explain significantly lower activities in Podunavlje and Donje Podravlje sites. These areas are known for the extensive practice of intensive agriculture and farming leading to pesticide and fertilizer pollution (Romić et al., 2015).

4.2 | Enzymatic oxidative stress

Measurements of GST activity in both types of samples showed no significant differences between different sampling areas even though slightly higher GST values could be observed in Jelas polje and Podunavlje sites (Figure 3). GST is a metabolic Phase II enzyme, directly involved in detoxification mechanisms by catalyzing the conjugation of GSH to xenobiotics, and indirectly involved in oxidative stress mechanisms (Isaksson et al., 2009; Leaver & George, 1998). Considering the obtained results, from the GST response at different sites no conclusions could be made about the presence of stressors in the environment.

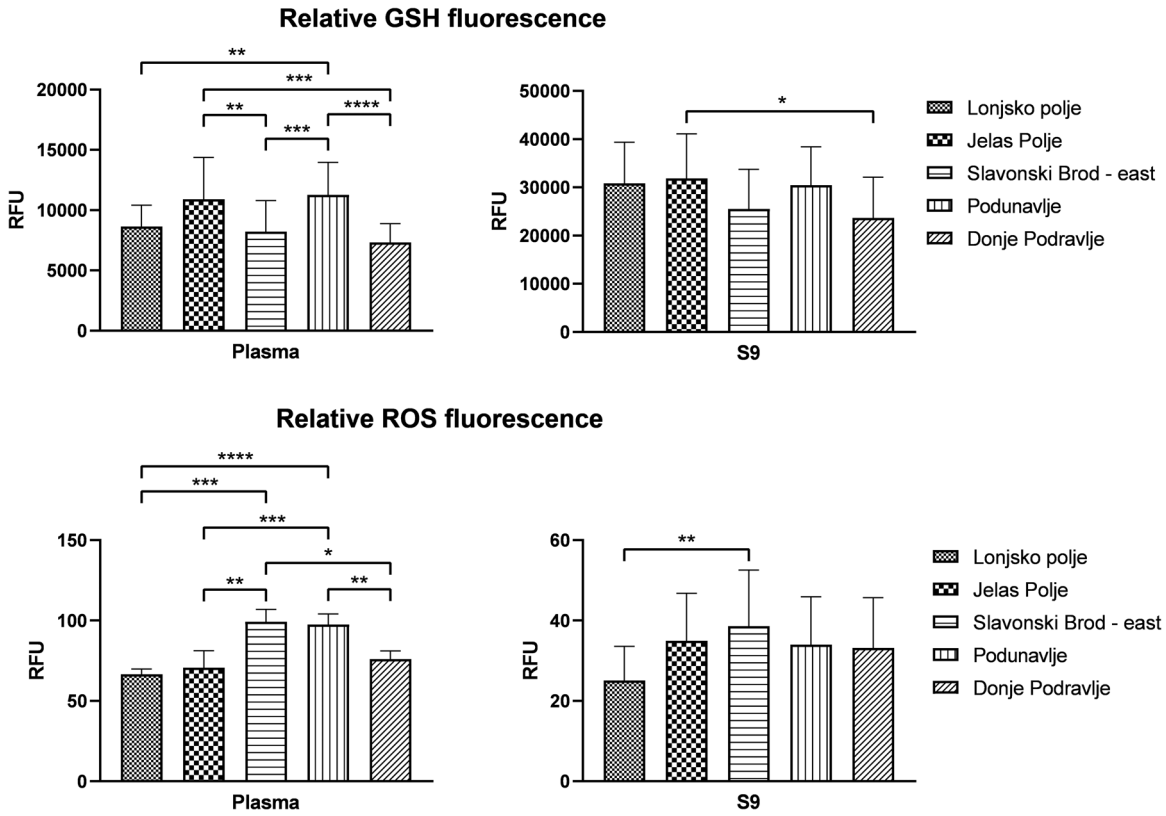

FIGURE 4 Glutathione (GSH) and reactive oxygen species (ROS) relative fluorescence (RFU) in two blood fractions: plasma and S9 in white stork (*Ciconia ciconia*) nestlings from Lonjsko polje, Jelas polje, Slavonski Brod–east, Podunavlje and Donje Podravlje in continental Croatia. Values are presented as mean ± *SD*. Lines and asterisks represent significant differences between areas *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$), ****($p < 0.0001$)

Response of GR proved to be more sensitive to the existing environmental pollution compared to GST. When comparing specific GR activities between plasma and S9, higher activity was recorded in S9, probably due to the GR accumulating near regions with elevated ROS production, for example, cellular electron flux (Couto et al., 2016). However, plasma appears to be a more suitable fraction for the assessment of this biomarker since in plasma more statistical differences between the sites were recorded. As presumed in our hypotheses, the highest GR activity was recorded in Slavonski Brod–east (Figure 3) indicating the presence of environmental pollutants causing elevated oxidative stress related to the metal and engineering industry in the cities of Slavonski Brod and Bosanski Brod. Namely, heavy metals (Berglund et al., 2007), crude oil (Pritsos et al., 2017), petroleum (Custer et al., 2000), and POPs (e.g., pesticides) (Abbasi et al., 2017; Ćupić Miladinović et al., 2018) are known to cause an increase in the oxidative stress in birds, although results vary among species. Similar results were reported by Abbasi et al. (2017) who recorded higher GST and GR activities in the blood of spotted owlet (*Athene brama*), presumably in response to the above threshold concentrations of *p,p′*-DDE and BDE-100, a known organochlorine pesticide and polybrominated diphenyl ether, respectively. GR was also analyzed in the white stork nestlings from Poland (Kamiński, Kurhalyuk, Jerzak, et al., 2009; Kamiński, Kurhalyuk, Kasprzak, et al., 2009), however, a decrease in GR activity at polluted sites was observed. Due to a complex antioxidative response and multiple factors affecting the antioxidant enzymes, both increase (Berglund et al., 2007) and decrease (Kamiński, Kurhalyuk, Jerzak, et al., 2009) in GR activity can occur as the result of oxidative stress.

4.3 | Nonenzymatic oxidative stress

Measurement of plasma GSH showed higher GSH levels in Jelas polje and Podunavlje when compared to the other three sites (Figure 4). High levels of GSH are due to the presence of environmental pollutants and/or increased oxidative stress. Both of the areas are used for intensive agriculture and hunting activities. This could indicate an undetected nonpoint source of pollution such as the spread of pollutants from farming (Romić et al., 2015) or traces of lead ammunition (Bilandžić et al., 2009; Lazarus et al., 2008). In Spain, increased GSH levels were recorded in the blood of white stork nestlings from a colony located nearby a landfill and were associated with heavy metal pollution (de la Casa-Resino et al., 2015). Although feeding at landfills increases GSH levels and antioxidants values in the blood, it also increases the overall fitness of the white stork nestlings (Pineda-Pampliega et al., 2021). Patterns of GR activity (Figure 3) are

consistent with the pattern of GSH levels (Figure 4) among sites. These results may be due to the GR supplying the cells with GSH by GSSG reduction (Carlberg & Mannervik, 1985).

Regarding the plasma ROS measurements, the highest ROS levels were detected in Slavonski Brod–east and Jelas polje (-Figure 4). ROS overproduction by mitochondria (Holzerová & Prokisch, 2015), can lead to cellular damage, especially lipids, proteins, and DNA (Halliwell & Gutteridge, 2015; Monaghan et al., 2009). It is important to notice that, similar to other biomarkers, a comparison of S9 results between the sites for both GSH and ROS showed fewer significant differences in responses. In the case of GSH, a similar pattern of relative GSH fluorescence was observed in both plasma and S9 (Figure 4), but higher GSH levels were recorded in S9 when comparing between the fractions because the cell is richer with GSH than plasma (Lu, 2009; Wu et al., 2004). When comparing ROS levels between the fractions, higher levels in plasma could be due to the external pollutants affecting the ROS production and leading to increase in extracellular ROS (Lee et al., 2013). Considering the obtained results, it can be again concluded that plasma samples might be more suitable for the assessment of both GSH and ROS levels in biomonitoring of pollution effects.

Overall, oxidative stress is higher in nestlings residing near high-intensity industry and agriculture: Slavonski Brod–east and Podunavlje, respectively. The highest ROS production was detected in Slavonski Brod–east, corroborating our hypothesis of pollution coming from metal and petrol industries. According to the annual report of the Ministry of Economy and Sustainable Development of the Republic of Croatia (Vađić et al., 2021) high levels of lead (Pb), cadmium (Cd), arsenic (As), and nickel (Ni) were detected in aerosols proximal to Slavonski Brod. Špirić et al. (2014) also report higher air mercury (Hg) concentrations in the area around Slavonski Brod, while Zuliani et al. (2019) report higher Hg and methylmercury (Me-Hg) in fish muscle tissue. Metal industry in Slavonski Brod and oil refinery in Bosanski Brod could be the potential sources since petroleum refinery effluents are the major source of aquatic pollution. Refinery effluents usually consist of heavy metals and polycyclic aromatic hydrocarbons (PAHs) that can cause oxidative stress by increasing the production of ROS (Custer et al., 2000; Koivula & Eeva, 2010; Singh & Shikha, 2019). According to the annual report on Water Quality Status of Croatian Surface Waters (Musić et al., 2020) higher concentration on fluoranthene, a PAH pollutant and known carcinogen on the Candidate List of Substances of Very High Concern due to its persistent and bioaccumulative properties (Lotufo, 1998), was recorded in the vicinity of the sampled areas. Moreover, downstream from Slavonski Brod–east, the Sava measuring station records an increasing trend of Ni, Pb, Cd, and Hg from 2017 (Musić et al., 2020), as well as in fish muscle tissue. Concerning the Podunavlje site, according to the Croatian Waters annual report from 2019, a high concentration of perfluorooctane sulfonate (PFOS) and its derivatives were recorded in Podunavlje surface waters (Musić et al., 2020). PFOS is a known organic pollutant causing liver damage and affecting lipid metabolism in birds (Jacobsen et al., 2018; Peden-Adams et al., 2009), and therefore, possibly contributing to the high oxidative stress observed in Podunavlje nestlings.

5 | CONCLUSION

This study represents the first analysis of several biomarkers in the two blood fractions (plasma and S9) for the purpose of evaluating biomarkers of effect and oxidative stress in white stork nestlings from five areas in Croatia. It has been demonstrated that the nestlings from Podunavlje and Donje Podravlje, areas known for extensive agriculture, and thus pesticide and fertilizer usage, appear to be affected, as lower AChE and CES activities were observed compared to the other sampling areas. Occurrence of oxidative stress, indicated by changes in the activity of GR and levels of GSH and ROS, was recorded in Slavonski Brod–east and Podunavlje, areas known for the metal and petroleum industry as well as pesticide application. In addition, the usage of two types of samples for measurements enabled the assessment of the suitability of the sample type for a particular biomarker measurement indicating plasma being more adequate for chosen endpoints. Reported results suggest that the white stork nestlings may be affected by the pollutants from their surrounding environment. However, more research is needed for the systematic chemical biomonitoring and evaluation of the harmful effects on avian apex predators in Croatia to actually relate these results to the chemical analysis of pollutant concentrations in the environment.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization: Dora Bjedov, Alma Mikuška, and Mirna Velki. *Investigations*: Dora Bjedov, Alma Mikuška, Mirna Velki, Luka Jurinović Tibor Mikuška, Carina Lackmann, and Lidija Begović. *Data analysis and curation*: Dora Bjedov, Alma Mikuška, and Mirna Velki. *Writing*: Dora Bjedov. Review and editing: all. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

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2.3. Heavy metal(loid) effect on multi-biomarker responses in apex predator: Novel assays in the monitoring of white stork nestlings

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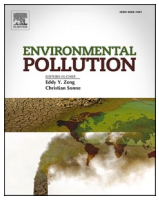

Heavy metal(loid) effect on multi-biomarker responses in apex predator: Novel assays in the monitoring of white stork nestlings☆

Dora Bjedov [a], Mirna Velki [b], Leontina Toth [c], Vlatka Filipović Marijić [d], Tibor Mikuška [e], Luka Jurinović [f], Sandra Ečimović [b], Nataša Turić [c], Zdenko Lončarić [g], Sara Šariri [d], Yasir Al Marsoomi [h], Alma Mikuška [b,*]

[a] Croatian Institute for Biodiversity, BIOTA Ltd., Maksimirska cesta 129/5, 10000, Zagreb, Croatia
[b] Department of Biology, Josip Juraj Strossmayer University of Osijek, Cara Hadrijana 8/A, 31000, Osijek, Croatia
[c] Teaching Institute of Public Health Osijek-baranja County, Franje Krežme 1, 31000, Osijek, Croatia
[d] Division for Marine and Environmental Research, Ruđer Bošković Institute, Bijenička 54, 10000, Zagreb, Croatia
[e] Croatian Society for Birds and Nature Protection, Ivana Gundulića 19/A, 31000, Osijek, Croatia
[f] Poultry Centre, Croatian Veterinary Institute, Heinzelova 55, 10000, Zagreb, Croatia
[g] Department of Agroecology and Environment Protection, Faculty of Agrobiotechnical Sciences Osijek, Josip Juraj Strossmayer University of Osijek, Vladimira Preloga 1, 31000, Osijek, Croatia
[h] HOGENT University of Applied Sciences and Arts, Geraard de Duivelstraat 5, 9000, Gent, Belgium

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ABSTRACT

The goal of the present study was to investigate differences in biomarker responses related to metal(loid)s in white stork (*Ciconia ciconia*) nestling's blood from continental Croatia. To achieve this, a battery of biomarkers that can be affected by environmental pollutants, including metal(loid)s, was assessed (esterase activity, fluorescence–based oxidative stress biomarkers, metallothionein levels, glutathione–dependent enzyme activity). The research was conducted during the white stork breeding season in diverse areas (a landfill, industrial and agricultural sites, and an unpolluted area). White storks' nestlings near the landfill exhibited reduced carboxylesterase (CES) activity, elevated glutathione (GSH) concentration, as well as high Pb content in the blood. Increased As and Hg concentrations in blood were attributable to environmental contamination in agricultural area and an assumed unpolluted area, respectively. Furthermore, agricultural practices appeared to affect CES activity, as well as elevate Se levels. In addition to the successful implementation of biomarkers, present research showed that agricultural areas and a landfill are areas with increased metal(loid) levels possibly causing adverse effects on the white storks. This first–time heavy metal and metalloid analyses in the white stork nestlings from Croatia point to the necessary monitoring and future assessments of pollution impact to prevent irreversible adverse effects.

1. Introduction

Levels of heavy metals and metalloids have been increasing in the environment due to the growing intensity of anthropogenic activities (Markowski et al., 2013). Heavy metals and metalloids are transferred through food webs into biota from the environment as a result of bioaccumulation, increasing their concentration with every trophic level (biomagnification) and consequently causing adverse effects on all trophic levels and possibly on human health (Ali and Khan, 2019). Heavy metals (cadmium, mercury, lead) and metalloids (arsenic, selenium) are usually associated with hazardous effects on organisms (Baos et al., 2006). Cadmium, lead and arsenic can be waste products of industry and insecticides, pesticides and preservatives are common sources of arsenic in the environment (Ali et al., 2019), while potential (anthropogenic) sources of mercury are the combustion of fossil fuels as well as the smelting activities (AMAP/UNEP, 2019). Occasionally combustion of fossil fuels and sewage sludge are common sources of selenium that pollute the environment (Ohlendorf and Heinz, 2011).

The effects of heavy metals and metalloids can be assessed by measuring the responses of different biomarkers (Tkachenko and

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* Corresponding author.
E-mail address: amikuska@biologija.unios.hr (A. Mikuška).

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Kurhaluk, 2012). Increased heavy metal and metalloid levels are known to elevate cellular reactive oxygen species (ROS) causing metal–related oxidative stress (Espín et al., 2014; Koivula et al., 2011; Koivula and Eeva, 2010), and lead, arsenic and methylmercury are known to cause an adverse effect on the neural system (Karri et al., 2018). Moreover, elements such as cadmium, zinc and copper are known to induce metallothioneins (Ruttkay-Nedecky et al., 2013). Metallothioneins are proteins binding metals that participate in antioxidative defence (Betteridge, 2000; Ruttkay-Nedecky et al., 2013), therefore can be used for the assessment of heavy metal and metalloid effects.

Birds, especially apex predator species, have been used for biomonitoring environmental pollution (Badry et al., 2020; Mateo et al., 2000, 2003; Strum et al., 2008). The white stork (*Ciconia ciconia*) is an apex bird species with opportunistic feeding habits, which diet mostly comprises invertebrates, fish, amphibians, reptiles, small mammals, and waste from landfills (Kruszyk and Ciach, 2010; Pineda-Pampliega et al., 2021; Tortosa et al., 2002). White storks are breeding in habitats frequently associated with urban areas (BirdLife, 2022). Their nestlings can be used as sentinels of local pollution since exposure to pollutants is primarily via the food, foraged by their parents in the vicinity of the nest (Dauwe et al., 2004; Markowski et al., 2013). Foraging in polluted habitats may lead to secondary poisoning with metal(loid)s (Baudrot et al., 2020); white stork blood can be used for assessing acute exposure to these compounds and their effect (Kurhalyuk et al., 2006; Kamiński et al., 2009a; Kamiński et al., 2009b; Tkachenko and Kurhaluk, 2012, 2014).

Our previous results showed the different responses of blood biomarkers depending on the breeding area (Bjedov et al., 2022). Furthermore, we analysed the response of biomarkers in different blood fractions. With this, we showed that different blood fractions can be applied in the biomonitoring of biomarker response which is important for reducing blood volume necessary for analysis (Bjedov et al., 2021). In the present study, for the first time in Croatia, levels of heavy metals and metalloids in the blood of white stork nestlings were assessed. In addition, biomarker responses in white stork nestling's from differently polluted areas were evaluated. Specifically, our main goals were to:

1. Assess the heavy metal and metalloid concentrations in white stork nestlings for each sampling area. Spatial variation in metal(loid) levels is anticipated due to the presence of different pollutants in the diverse sampling areas (i.e. industrial, agricultural, and landfill areas).
2. Analyse the activities of cholinesterase, carboxylesterase, glutathione S–transferase, and glutathione reductase, as well as the levels of glutathione, reactive oxygen species and metallothionein between differently polluted study areas – landfill, agricultural areas, industrial areas and protected areas (Nature Park). Spatial variation in biomarker response is expected due to the presence of different pollutants in sampling areas, in particular, reduced esterase activity in agricultural areas, and change in oxidative stress biomarkers in industry areas and landfill Jakuševec.
3. Investigate the interaction between the biomarker response and concentration of heavy metals and metalloids. We hypothesise elevated metal(loid)s could increase oxidative stress biomarkers. Correlation between mercury and selenium is expected since it is known that selenium has a high affinity for mercury and vice versa.

2. Material & methods

2.1. Study areas

Blood samples from 106 white stork nestlings, aged 6–8 weeks, were collected during ringing in 2021. Nests were located in seven areas (Fig. 1). Jakuševec landfill is non–hazardous waste, situated in eastern Zagreb, on the right bank of the Sava River (Fig. 1). It is the largest landfill in Croatia and among the largest in this region of Europe (Opačak and Wang, 2019). Jakuševec is a source of compounds both from biological and anthropogenic waste (Barčić and Ivančić, 2010). White storks are breeding on the Jakuševec landfill since 2012 (Jurinović, *pers. obs.*). Nature Park Lonjsko polje is the largest protected area along the Sava River (Fig. 1). This area is mostly covered by floodplain woodlands, followed by wet meadows and grasslands, as well as small–scale agriculture, and villages (Gugić et al., 2008). Lonjsko polje is an area near the Sava river lacking industries, and extensive

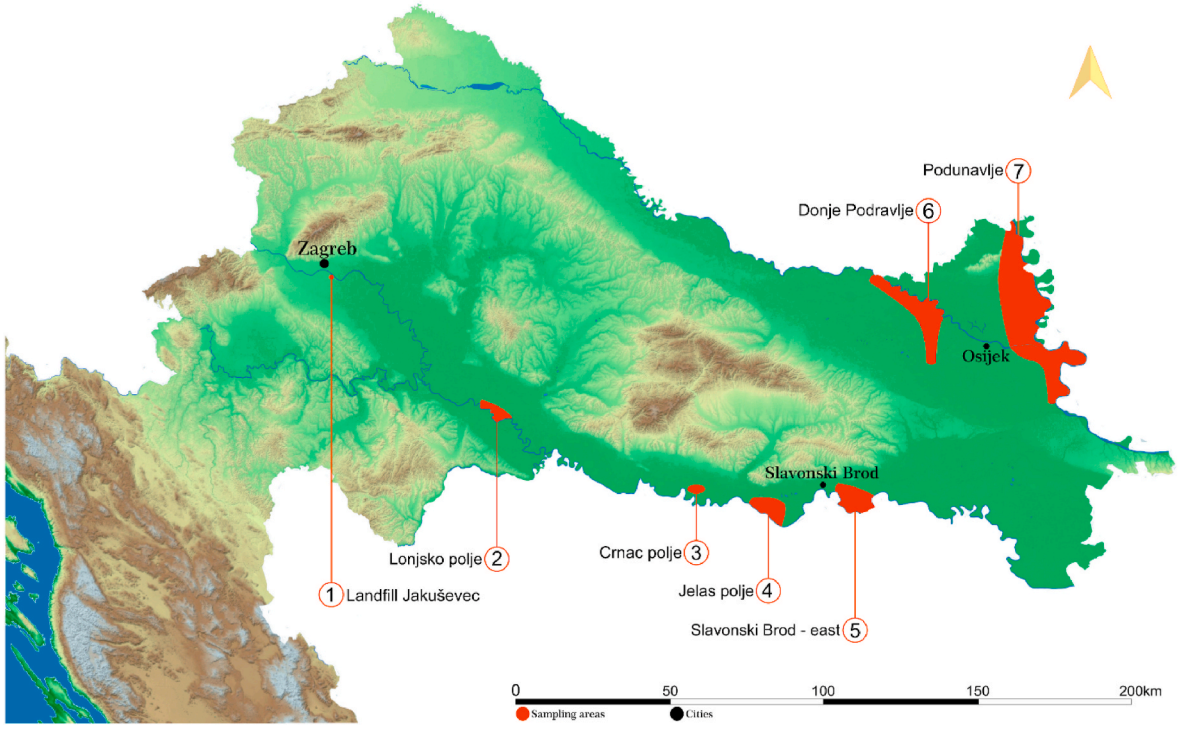

Fig. 1. Blood sampling areas of white stork (*Ciconia ciconia*) nestlings.

agriculture and has the lowest risk for contamination. However, it is surrounded by the viticulture and wine industry. In addition, upstream of Lonjsko polje is the oil, gas, and petrochemical industry in Sisak which might contribute to pollution sources (Smital and Ahel, 2015). Crnac polje area is a former floodplain that has been ameliorated and turned into fertile soil suitable for agriculture, a potential source of pollutants, and also an area of hunting ground for game animals and birds (Fig. 1). Jelas polje area is covered with alluvial woodlands, vast fish farming, wet meadows and grasslands, small–scale and large–scale agriculture and villages (Fig. 1; Schwarz, 2016). A possible major source of pollution could be the extensive use of pesticides (Romić et al., 2015). Slavonski Brod – east area is located directly downstream from a city notable for its thriving metal engineering industry (Fig. 1). Furthermore, additional sources of pollution may arise from the oil refinery in Bosanski Brod, on the other side of the Sava (Gvozdić et al., 2011). The area is covered by both small– and large–scale agriculture, alluvial forests and large grasslands, regularly flooded by the Sava River, and small villages (Schwarz, 2016). Podunavlje is a large floodplain area located near the Danube River (Fig. 1). White stork nests are in the proximity of the former floodplain border and are surrounded by large–scale cultivation of cereal crops therefore possibly contributing to the pollution. Donje Podravlje is located along the Drava River and the sampling area covers large– and small–scale fruit plantations as well as maize agriculture (Fig. 1). The extensive use of pesticides could be the pollution source for both Donje Podravlje and Podunavlje areas (Romić et al., 2015). Based on industrial activities, analysis of water and atmospheric pollutants (Barčić and Ivančić, 2010; Gvozdić et al., 2011; Musić et al., 2020; Romić et al., 2015; Vađić et al., 2021) the gradation of pollution was presumed, Jakuševec and Slavonski Brod – east as the most polluted to Nature Park Lonjsko polje as the least polluted sampling area. Therefore, Crnac polje, Jelas polje, Podunavlje, and Donje Podravlje would be considered the areas with transitional levels of heavy metal and metalloid pollution.

2.2. Study species

The annual census (monitoring) of the white stork breeding population and their breeding success is regular in Croatia, and nestlings are ringed for more than 70 years (Kralj et al., 2013). From 2020, along with ringing, we are taking blood samples from nestlings for the purpose of biomonitoring. In our previous work, blood was sampled during the regular ringing scheme. In this way, the first–time ringing scheme of white storks is connected with the monitoring of environmental pollution in Croatia (Bjedov et al., 2021, 2022). This approach is favourable for birds, due to reduced stress to both parents and the nestlings by visiting nests only once during the breeding season.

2.3. Sampling procedure

This research was conducted under the permit of The Ministry of Environment and Energy of the Republic of Croatia (Classification code: UP/I–612–07/20–48/130; Registry number: 517–05–1–1–20–4). The chemicals used in this research are listed in Supplementary Information (SI). Blood samples were collected and the preparation of blood fraction was performed according to the Bjedov et al. (2021) protocol. In brief, approximately 4 mL of blood per nestling was transferred to lithium heparin tubes, kept in dark and at 4 °C until laboratory analysis. Prior to centrifugation, 1 mL of whole blood was separated for heavy metal and metalloid analysis. Plasma (the supernatant) was transferred to the new sterile tube and kept at −80 °C until further analysis. The pellet was dissolved and cell lysis was performed using the sonicator. Samples were subsequently centrifuged in order to obtain the post-mitochondrial supernatant (S9) which was kept at −80 °C until laboratory assays.

2.4. Metalloid and heavy metal analysis

Blood samples were analysed for arsenic (As), selenium (Se), cadmium (Cd), mercury (Hg) and lead (Pb). Inductively coupled plasma–mass spectrometry (ICP–MS, Agilent Technologies 7900) was applied to determine metalloid and heavy metal concentrations in breeding seasons 2020 and 2021. Data for metalloid and heavy metal concentrations from the 2020 breeding season were part of our unpublished preliminary study, therefore we use them for comparison with the results from the breeding season 2021. The methods are described in detail in SI. In brief, prior to analysis, blood samples were diluted, vortexed and homogenized. The limit of detection (LOD) was determined as 3*SD of the reagent blank control. LOD for As, Se, Cd, and Pb was < 0.03 μg L^{-1} and for Hg was < 0.007 μg L^{-1}. The limit of quantification (LOQ) was determined as 10*SD of the standard solution. LOQ for As, Se, Cd, and Pb was < 0.1 μg L^{-1}, and for Hg was < 0.02 μg L^{-1}.

2.5. Enzymatic biomarker assays

Enzyme assays were performed in both plasma and S9 with the Tecan Spark 10 M microplate reader (Tecan Trading AG, Männedorf, Switzerland). The plasma and S9 samples, as well as blank controls, were measured in three technical replicas. Enzyme activity was calculated from the changes in absorbance and was expressed as specific enzyme activity. All biomarker assays were analysed according to the protocol previously described in detail by Bjedov et al. (2021). Briefly, the activity of cholinesterase (ChE) was determined with the following compounds: acetylthiocholine iodide, 5,5′–dithiobis (2–nitrobenzoic acid) (DTNB) and sodium phosphate buffer (Ellman et al., 1961). Carboxylesterase (CES) activity was analysed using *p*–nitrophenyl acetate (Hosokawa and Satoh, 2001). Glutathione S–transferase (GST) activity was measured based on Habig and Jakoby's (1981) protocol with the chemicals 1–chloro–2,4–dinitrobenzene (CDNB) and glutathione (GSH). The activity of glutathione reductase (GR) was determined with the following reagents: oxidised glutathione (GSSG), β–Nicotinamide adenine dinucleotide (β–NADPH) and sodium phosphate buffer (Habig and Jakoby, 1981).

2.6. Non–enzymatic biomarker assays

2.6.1. Reactive oxygen species and glutathione concentration

Fluorescent dyes were used for the detection of reactive oxygen species (ROS) and glutathione (GSH). Measurements were performed using the Tecan Spark 10 M microplate reader with the following settings: excitation wavelength 485 nm, emission wavelength 530 nm, and gain 50. Plasma and S9 samples, blank, negative and positive control were performed in parallel and measured in three technical replicas. Protocols are described in Bjedov et al. (2021). Fluorescence of ROS and GSH were detected using CM–H_2DCFDA and CellTracker™ Green CMFDA fluorescent dye, respectively.

2.6.2. Metallothionein concentration

The concentration of metallothionein (MT) proteins was determined in plasma. The quantification of MT concentration in the heat–treated samples was performed by differential pulse voltammetry following the methodology of the modified Brdička procedure (Raspor et al., 2001; Mijošek et al., 2018). All samples and MT standards were heat–treated and analysed in two technical replicas and a detailed description can be found in SI.

2.7. Protein concentration

Pierce™ BCA Protein Assay Kit was used for protein quantification and analysis was performed in plasma and S9 using the Tecan Spark 10 M microplate reader. With the protocol provided in the kit as well as the protocol described in Bjedov et al. (2021), the working solution was

prepared, with bovine serum albumin as a standard.

2.8. Statistical analysis

All statistical analyses were performed using R version 4.0.0, and SPSS (version 24). Shapiro–Wilk test was used to test the data distribution. Heavy metal and metalloid data did not follow a normal distribution, therefore non–parametric tests were used. Since more than one nestling was sampled from each nest, to avoid pseudoreplication, the median value was calculated for each nest. Furthermore, Kruskal–Wallis test was used to test for differences in heavy metal and metalloid concentration between the study areas followed by Dunn's *post hoc* test. Considering a normal distribution of enzymatic (ChE, CES, GST and GR) and non–enzymatic (ROS, GSH, MT) biomarkers, parametric tests were used. Linear mixed effect modelling was performed, using the *lme*–function (*nlme* package). A linear mixed–effect model was created to avoid introducing pseudoreplication since the nestlings from the same nest (the clustering effect) could affect the outcome. In order to assess the differences in analysed biomarkers between the study areas, a linear mixed model (LMM) was constructed. The model was constructed using *biomarker response* and *sampling area* as fixed, and *nest* as a random variable. One–way analysis of variance (ANOVA) was performed on LMM, and *post hoc emmeans*–function was subsequently performed to uncover specific differences in biomarker response between the study areas. To test the association between enzymatic and non–enzymatic biomarker response with heavy metal and metalloid concentration, Spearman's rank correlation coefficient (two–tailed) was applied. Enzyme activity was presented as specific enzyme activity, calculated from changes in absorbance. Fluorescence is presented as relative fluorescence units (RFU), calculated from an increase in fluorescence over time. Enzymatic and non–enzymatic biomarker results are expressed as mean and standard deviation (± *SD*) with a vertical bar plot. Due to non–parametric test application, heavy metal and metalloid results are expressed with box and whiskers plots. The level of statistical significance was 0.05 (*p*–value).

3. Results

Heavy metals and metalloids were detected and quantified in all analysed blood samples. Metalloid results are shown in Fig. 2, Table SI1, and differ significantly among the sampling areas. The concentration of As from 2020 did not show any significant variation (Fig. SI1). On the other hand, the median As concentration analysed in 2021 was significantly higher in Podunavlje, compared to Lonjsko polje (Fig. 2A, Table SI1). Regarding Se, similar levels were recorded at all sampling areas in 2020 (Fig. SI1), while in 2021, significant differences were observed between sampling areas (Fig. 2B, Table SI1). The significantly highest median concentration was recorded in Crnac polje in comparison with Jakuševec and Donje Podravlje (Fig. 2B, Table SI1). Se levels in Lonjsko polje were significantly higher compared to Jakuševec and Donje Podravlje (Fig. 2B, Table SI1).

Heavy metal results are shown in Fig. 3, Table SI1, and differ significantly among the sampling areas. Similar Cd values were recorded between all sampling areas in 2020 and 2021, therefore no significant changes were observed (Fig. 3A, Table SI1, Fig. SI2). Blood Hg was significantly higher in Lonjsko polje compared to Podunavlje in 2020, and with Crnac polje and Donje Podravlje in 2021 (Fig. 3B, Table SI2). The concentration of Pb was similar at all sampling areas in 2020, contrary to the 2021 season where the level of Pb was the highest in Jakuševec compared to Crnac polje, Jelas polje and Podunavlje (Fig. 3C, Table SI1, Fig. SI2).

The summary of descriptive statistics regarding all the biomarkers analysed in this study is listed in Table SI2.

No significant changes in ChE activity were observed in plasma or S9 between the sampling areas (Fig. 4, Table SI2). Regarding CES, plasma activity was significantly higher in Jakuševec compared to Jelas polje and Donje Podravlje, while activity in Crnac polje was significantly higher compared to Donje Podravlje. In S9, CES activity was the highest in Slavonski Brod – east compared to Jakuševec and Podunavlje (Fig. 4, Table SI2).

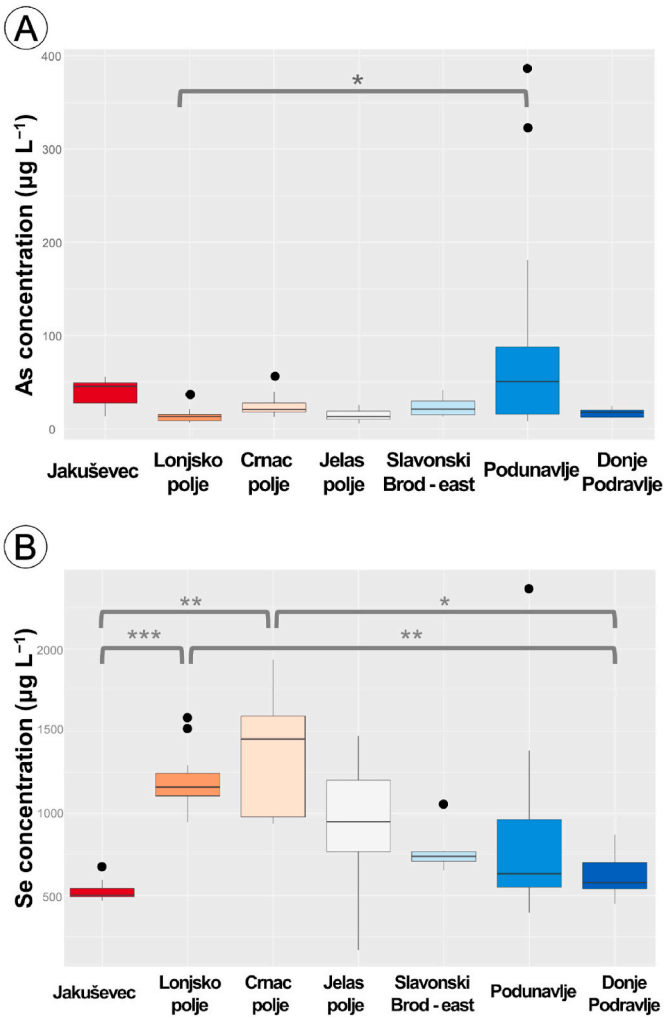

Fig. 2. Metalloids A) arsenic (As) and B) selenium (Se) concentrations (µg L^{-1}) in whole blood from white stork (*Ciconia ciconia*) nestlings sampled from Jakuševec, Lonjsko polje, Crnac polje, Jelas polje, Slavonski Brod – east, Podunavlje and Donje Podravlje areas. Horizontal lines represent minimum and maximum values, the box represents values in the 25th and 75th percentile with the median as a central line. Significantly different observations (outliers) are represented as a black dot. Significant differences between the study areas are noted with lines and asterisks: * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

GST and GR measurements in plasma and S9 showed no statistical differences between the sampling areas (Fig. 5, Table SI2).

Significant changes in GSH fluorescence were observed only in plasma, with the highest values in Jakuševec compared to Podunavlje (Fig. 6, Table SI2). ROS fluorescence has similar average values for all sampling areas; therefore, no significant changes were detected in both plasma and S9 fractions (Fig. 6, Table SI2).

Results of metallothionein concentration analysed in plasma are presented in Table SI2. Due to similar responses regarding the sample areas, no significant changes were observed.

The relationship between the biomarkers (ChE, CES, GST, GR, GSH, ROS), heavy metal(loid)s, as well as biomarker and heavy metal(loid) concentrations, were assessed to evaluate the potential effect caused by these elements. Overall correlations were weak to moderate.

Regarding the correlation between analysed biomarkers, response in plasma ChE activity showed a moderate positive correlation with ChE

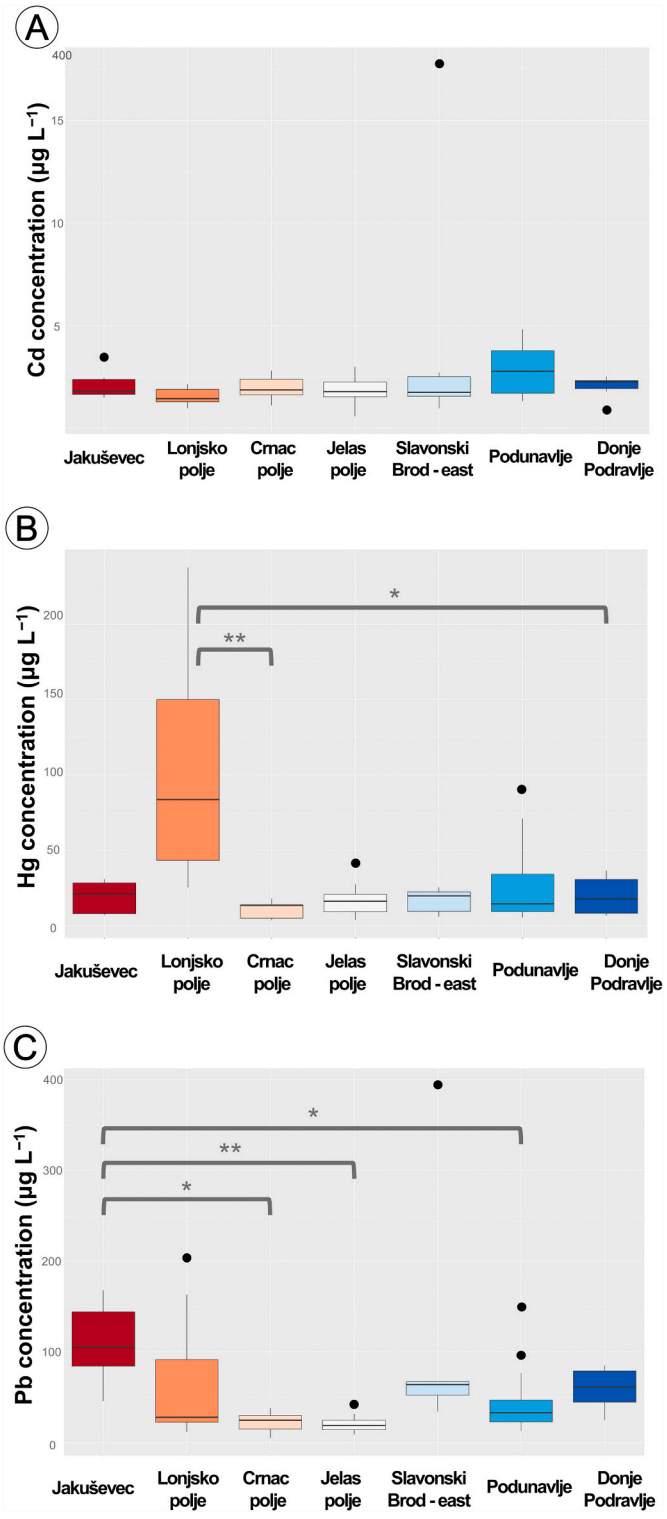

Fig. 3. Heavy metal A) cadmium (Cd), B) mercury (Hg), and C) lead (Pb) concentrations (µg L^{-1}) in whole blood from white stork (*Ciconia ciconia*) nestlings sampled from Jakuševec, Lonjsko polje, Crnac polje, Jelas polje, Slavonski Brod – east, Podunavlje and Donje Podravlje areas. Horizontal lines represent minimum and maximum values, the box represents values in the 25th and 75th percentile with the median as a central line. Significantly different observations (outliers) are represented as a black dot. Significant differences between the study areas are noted with lines and asterisks: * ($p < 0.05$), ** ($p < 0.01$).

activity in S9. Furthermore, S9 ChE activity showed a moderate positive correlation with GR activity and ROS levels in S9 (Table 1). Plasma CES activity had a medium positive correlation with GR and GST in plasma (Table 1). A moderate positive correlation in plasma was observed between the activities of GR and GST (Table 1). A relatively strong negative correlation was detected between GST and GSH in S9, while a moderate positive association was recorded between GST and ROS in S9 (Table 1).

As for the relationship in regard to metal(loid) and biomarker response, a moderate negative relationship was recorded between the As concentration and CES activity in S9 (Table 1). Enzymatic biomarkers analysed in S9, ChE and GST, showed a medium negative association with Se concentrations, while a moderate positive correlation was recorded between the concentration of GSH in S9 and Se (Table 1). Regarding Cd, two positive correlations were observed with ChE and GR in plasma, moderate and relatively strong, respectively (Table 1). Only one significant negative correlation was recorded between Hg and GR in S9 (Table 1). One medium negative association was observed between Pb and GSH in S9.

In regard to relationships between the metal(loid)s, only one significant was detected, a strong negative correlation between Pb and Se concentrations (Table 1).

When investigating the relationships between the MT and heavy metal(loid) concentration, only low, non–significant correlations were detected. Weak negative correlations were detected for Cd and Pb, and weak positive correlations were observed with As, Se and Hg (Table 2). On the other hand, results showed a relatively strong negative correlation between Pb and Se (Table 1, Table 2).

4. Discussion

4.1. Metalloids and heavy metals: spatial variation and the possible effect

As is a metalloid, toxic in high concentrations (Hughes, 2002), and was detected in all analysed blood samples (Fig. 2A, Table SI1). The highest As levels were observed in Podunavlje, but they were only statistically significant compared to Lonjsko polje (Fig. 2A, Table SI1). These results correspond with high As concentration in water and soil from Podunavlje since the groundwater As levels are elevated in eastern Croatia (Podunavlje area), reported in Ćavar et al. (2005), Habuda-Stanić et al. (2007), Romić et al. (2011), Ujević et al. (2010) and Vidosavljevic et al. (2022). In Podunavlje, three nestlings had higher or equal As concentrations (Fig. 2A, Table SI1) than the maximum As levels reported by Benito et al. (1999), regarding white storks feeding in the area around Doñana National Park subsequent to the toxic spill from the Aznalcóllar mine. Moreover, de la Casa-Resino et al. (2014) report lower As levels in colonies associated with landfills compared to their reference colony. In the present study, a colony situated in landfill Jakuševec showed a higher median As concentration than reported by de la Casa-Resino et al. (2014; Fig. 2A, Table SI1). The effects of As on birds were previously investigated (Eisler, 2004 and references therein), however, there are no studies evaluating threshold levels causing adverse effects. According to Burger and Gochfeld (1997a), the reference value for uncontaminated areas is 20 µg L^{-1}. These results indicate As contamination based on average values in Podunavlje, Jakuševec, Crnac polje and Slavonski Brod – east (Fig. 2A, Table SI1). Overall, it cannot be concluded if there is any risk of adverse effects for the white stork nestlings. Due to continuous high As levels in Podunavlje, chronic exposure and sublethal effect are possible.

The concentration of Se in the blood is a good indicator of acute exposure via ingestion (Ohlendorf and Heinz, 2011). The highest Se levels were recorded in agricultural areas near the Sava River: Crnac polje followed by Lonjsko polje compared to landfill Jakuševec and Donje Podravlje (Fig. 2B, Table SI1). Eastern continental Croatia is deficient in Se (Džomba et al., 2014; Vukšić and Šperanda, 2016), however, various agricultural practices affect Se levels in the

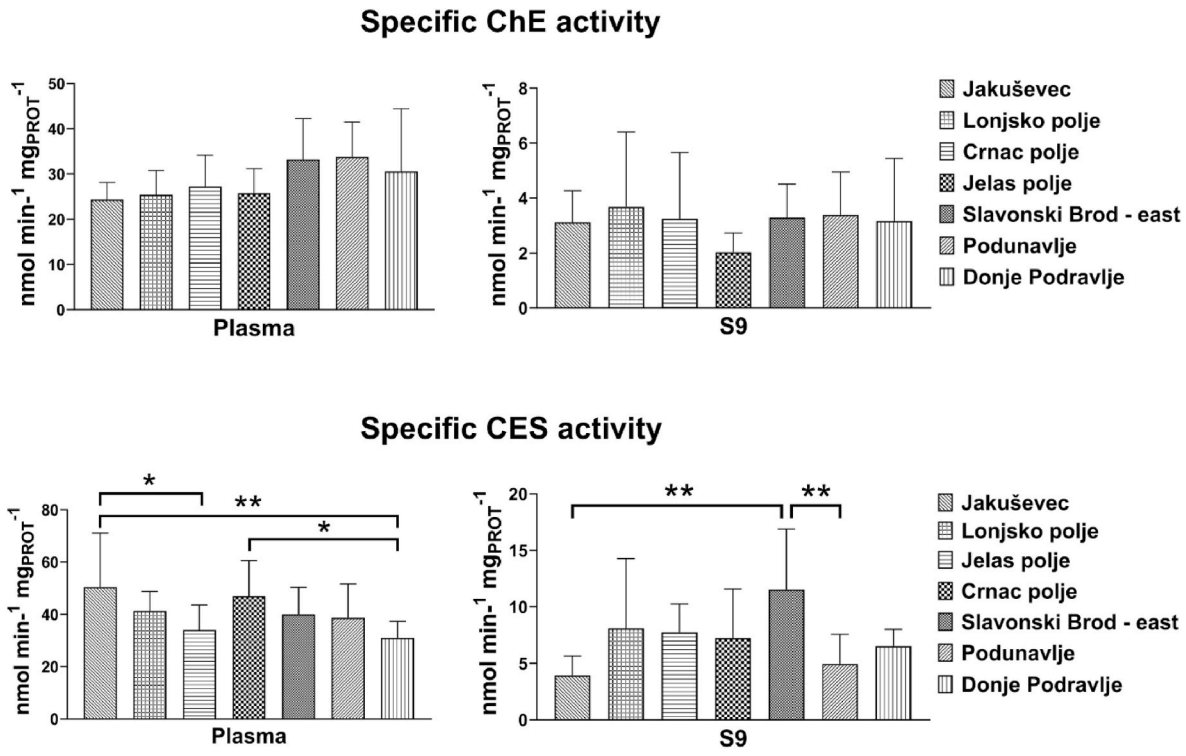

Fig. 4. Specific cholinesterase (ChE) and carboxylesterase (CES) activity (nmol min^{-1} mg^{-1}_{PROT}) in plasma and S9 from white stork (*Ciconia ciconia*) nestlings sampled from Jakuševec, Lonjsko polje, Crnac polje, Jelas polje, Slavonski Brod – east, Podunavlje and Donje Podravlje areas. Data is presented as mean ± *SD*. Significant differences between the study areas are noted with lines and asterisks: * ($p < 0.05$), ** ($p < 0.01$).

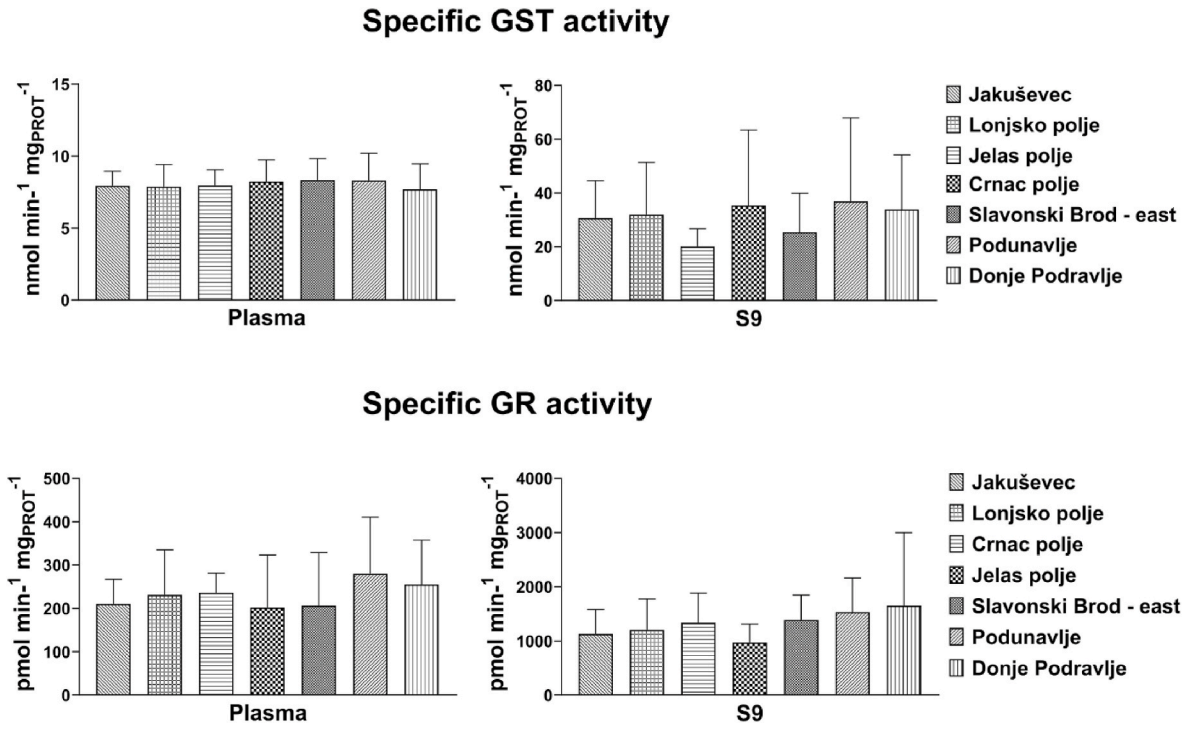

Fig. 5. Specific glutathione S–transferase (GST) activity (nmol min^{-1} mg^{-1}_{PROT}) and glutathione reductase (GR) activity (pmol min^{-1} mg^{-1}_{PROT}) in plasma and S9 from white stork (*Ciconia ciconia*) nestlings from sampling areas: Jakuševec, Lonjsko polje, Crnac polje, Jelas polje, Slavonski Brod – east, Podunavlje and Donje Podravlje areas. Data is presented as mean ± *SD*.

environment. According to Ohlendorf and Heinz (2011), the geochemical background concentration of blood Se is considered 100–400 μg L^{-1}. In view of this, median blood Se levels in white stork nestlings were above the threshold levels in all sampling areas, (Fig. 2B, Table SI1). Only 2% of the nestlings had Se levels < 400 μg L^{-1} (Table SI1). Furthermore, 63% of the nestlings had Se levels of 400–1000 μg L^{-1}

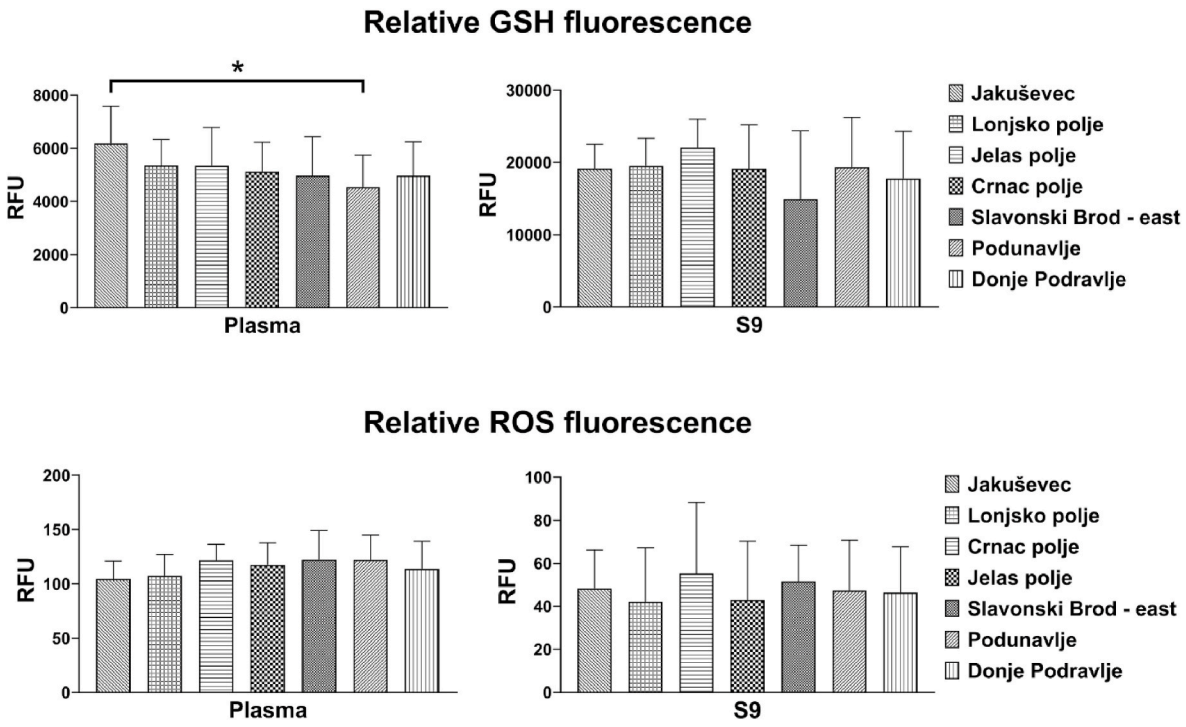

Fig. 6. Relative glutathione (GSH) and reactive oxygen species (ROS) fluorescence (RFU) in plasma and S9 from white stork (*Ciconia ciconia*) nestlings sampled from Jakuševec, Lonjsko polje, Crnac polje, Jelas polje, Slavonski Brod – east, Podunavlje and Donje Podravlje areas. Data is presented as mean ± *SD*. Significant differences between the study areas are noted with lines and asterisks: * ($p < 0.05$).

indicating possible adverse effects, followed by 35% of nestlings with Se levels > 1000 μg L^{-1} (Table SI1). Comparing blood Se levels in nestling avian species from Doñana National Park following a toxic spill from the Aznalcóllar mine, Benito et al. (1999), report that mean Se concentration was in the range of assumed geochemical background levels. Although all analysed blood Se was below irreversible physiological and/or lethal concentrations in this study, according to the United States Department of the Interior (1998) concentration of 1000 μg L^{-1} Se in the blood is a threshold warranting additional investigation.

The concentration of Cd in the blood can reflect recent exposure via diet, and therefore is a good matrix for Cd exposure assessment (Martínez-López et al., 2005). Blood Cd concentration in wild birds is usually < 50 μg L^{-1} (Finkelstein et al., 2007; Thompson and Dowding, 1999; Tsipoura et al., 2008; Wayland et al., 2005). Analysed Cd levels in white stork nestlings were similar in all study areas with the highest average concentration in Podunavlje (Fig. 3A, Table SI1), and considerably below the threshold for adverse effects. de la Casa-Resino et al. (2014) observed lower levels in white stork nestlings in colony breeding near a landfill than in this study (Fig. 3A, Table SI1).

Environmental exposure to Hg is extensively studied in birds (Burger and Gochfeld, 1997b; Goodale et al., 2008; Jackson et al., 2011; Lavoie et al., 2014; Weech et al., 2006; Whitney and Cristol, 2018). Hg is known for bioaccumulation and biomagnification in aquatic environments, posing a significant health risk for piscivorous birds, therefore the lowest observed adverse effect regarding Hg level in aquatic birds is 300 μg L^{-1} (Ackerman et al., 2014). Considering white storks are omnivorous and forage in both terrestrial and aquatic environments, the threshold for adverse effects could be lower for the nestlings. All observed levels are below the threshold for detrimental effects. de la Casa-Resino et al. (2014) report lower Hg concentrations in colonies associated with landfills, compared to nestlings from Nature Park Lonjsko polje. Significantly higher concentration was observed in white stork nestlings residing in Nature Park Lonjsko polje compared to Crnac polje and Donje Podravlje (Fig. 3B, Table SI1). Lonjsko polje appears to have continuously elevated Hg concentrations, seeing as the nestlings from 2020 had significantly higher Hg levels compared to Podunavlje (Fig. SI2). Upstream of Lonjsko polje, Halamić et al. (2003) report higher sediment Hg levels than the geochemical background. Moreover, the surrounding area is known for its adjacent petrochemical industry in Sisak (Smital and Ahel, 2015), viticulture, and the use of pesticides and fertilisers (Zrinšćak et al., 2011), possibly contributing to elevated Hg concentrations. These results are potentially concerning and warranting future monitoring seeing as Hg is a persistent, toxic pollutant.

Pb was significantly higher in nestlings from Jakuševec, compared to Crnac polje, Jelas polje and Podunavlje (Fig. 3C, Table SI1). Average Pb levels reported by Pérez-López et al. (2016) were lower than in presumably contaminated areas in Croatia: landfill Jakuševec, Slavonski Brod – east and Donje Podravlje. According to Benito et al. (1999), Pb concentration in the blood of nestling white storks feeding in the area around Doñana National Park subsequent to the toxic spill from the Aznalcóllar mine was lower than reported in Jakuševec (Fig. 3C, Table SI1). The suggested level of Pb in blood–inducing subclinical poisoning is > 200 μg L^{-1} (Descalzo et al., 2021; Pain et al., 2019). Two nestlings had high Pb levels, possibly inducing the subclinical effects, nestling from Slavonski Brod – east and nestling from Lonjsko polje (Fig. 3C, Table SI1). Interestingly, the nestling from Slavonski Brod – east with the highest Pb level in blood reported in this study, had the highest Cd levels as well (Fig. 3C, Table SI1). The landfill Jakuševec could be a source of groundwater Pb contamination since the contamination is known to migrate and subsequently pollute the surrounding environment (Levin et al., 2021; Vongdala et al., 2018). Moreover, the metallurgic industry in Slavonski Brod – east, and (illegal) hunting in Donje Podravlje could be a source of high Pb concentrations.

4.2. Enzymatic biomarker response at differently polluted areas

Although ChE is frequently associated with the nervous system, it is characterised in haematopoietic cells as well (Lawson and Barr, 1987), subsequently leading to variations in ChE activity between blood fractions: plasma and S9 (Fig. 4, Table SI2). ChE is usually detected in

Table 1
Results of Spearman rank correlation (r_s) between (enzymatic and non–enzymatic) biomarkers and metalloids and heavy metals analysed in plasma and S9 of white stork (*Ciconia ciconia*) nestlings.

		ChE plasma	ChE S9	CES plasma	CES S9	GR plasma	GR S9	GST plasma	GST S9	GSH plasma	GSH S9	ROS plasma	ROS S9	As	Se	Cd	Hg	Pb
ChE plasma	r_s	1.000																
ChE S9	r_s	**0.324** *	1.000															
CES plasma	r_s	−0.078	−0.059	1.000														
CES S9	r_s	−0.062	−0.263	−0.158	1.000													
GR plasma	r_s	0.154	−0.050	**0.311** *	0.152	1.000												
GR S9	r_s	0.134	**0.327** *	−0.188	0.018	−0.011	1.000											
GST plasma	r_s	0.261	0.092	**0.292** *	−0.032	**0.305** *	−0.122	1.000										
GST S9	r_s	−0.127	0.148	−0.097	−0.073	0.156	0.134	−0.104	1.000									
GSH plasma	r_s	−0.149	0.049	0.155	0.034	0.049	−0.110	0.142	0.093	1.000								
GSH S9	r_s	0.144	−0.215	0.005	0.224	−0.101	−0.077	−0.110	**−0.416** **	−0.100	1.000							
ROS plasma	r_s	0.246	−0.208	0.090	0.010	−0.008	−0.080	−0.186	−0.263	−0.083	0.228	1.000						
ROS S9	r_s	0.137	**0.444** **	−0.009	−0.146	−0.150	0.115	−0.058	**0.357** *	−0.038	−0.223	−0.124	1.000					
As	r_s	0.156	0.096	0.130	**−0.350** *	−0.045	0.226	0.129	0.236	−0.126	−0.150	0.037	0.161	1.000				
Se	r_s	0.001	**−0.297** *	0.127	0.168	−0.025	−0.176	−0.073	**−0.357** *	−0.088	**0.380** **	0.267	−0.261	−0.106	1.000			
Cd	r_s	**0.317** *	0.008	−0.082	0.021	**0.421** **	−0.058	0.066	−0.086	0.027	0.155	0.157	−0.078	0.127	−0.060	1.000		
Hg	r_s	−0.090	−0.016	0.199	0.033	0.015	**−0.348** *	−0.064	−0.144	−0.114	−0.199	0.010	−0.129	−0.131	0.216	−0.036	1.000	
Pb	r_s	−0.100	0.190	−0.081	−0.175	−0.037	0.128	−0.031	0.139	0.058	**−0.297** *	−0.118	0.176	0.023	**−0.580** **	0.071	0.003	1.000

Significant relationships are bolded and noted with * ($p < 0.05$), ** ($p < 0.01$).

Table 2
Results of Spearman rank correlation (r_s) between metallothionein (MT) and heavy metals and metalloids in the blood of white stork (*Ciconia ciconia*) nestlings.

		MT	As	Se	Cd	Hg	Pb
MT	r_s	1.000					
As	r_s	0.086	1.000				
Se	r_s	0.015	0.162	1.000			
Cd	r_s	−0.108	0.148	0.029	1.000		
Hg	r_s	0.06	−0.096	0.156	0.007	1.000	
Pb	r_s	−0.084	0.048	**−0.434** **	0.07	−0.017	1.000

A significant relationship is bolded and noted with ** ($p < 0.01$).

plasma, and bound on the cell membrane (Stedman and Stedman, 1935), hence the lower activity in S9. ChE activity in plasma and S9 did not show any differences between sampling areas indicating similar exposure, or lack thereof, to pesticides and/or other environmental pollutants (Fig. 4, Table SI2). A decrease in ChE activity from white stork nestlings associated with agriculture and farming areas was expected. However, due to a lack of significant spatial variation, results did not support this assumption. Changes in ChE activity, namely inhibition, is used as a potential biomarker of pesticide exposure. Cholinesterase activity was characterised and paraoxon–methyl, carbofuran, and carbaryl induced–inhibition were researched in the blood white stork by Oropesa et al. (2013a,b). Low cholinesterase activity was observed in pigeons (*Columba livia*) and raptor birds (*Falco sparverius, Buteo swainsoni, Accipiter cooperi, Buteo lineatus*) after exposure to parathion and methidathion, both organophosphate pesticides (Bartkowiak and Wilson, 1995). Exposure to pesticides can occur in non–agricultural areas as well, e.g., golf courses (Rainwater et al., 1995), suburban environments (Décarie et al., 1993), and active dredge spoils (Zinkl et al., 1981).

CES activity is lower in avian erythrocytes (S9) due to different enzyme localisation (Stedman and Stedman, 1935). Significant differences between the study areas were recorded in both plasma and S9 (Fig. 4, Table SI2). In plasma, a significant decrease in CES activity was observed in Donje Podravlje and Crnac polje, compared to Jakuševec and Jelas polje (Fig. 4, Table SI2). The lowest CES activity in S9 was recorded in Jakuševec and Podunavlje compared to Slavonski Brod – east (Fig. 4, Table SI2). Crnac polje, Podunavlje and Donje Podravlje are areas associated with agricultural and farming practices (Romić et al., 2015). That being said, CES is frequently used as a potential biomarker of exposure to various pesticides, most commonly organophosphorus insecticides (Sanchez-Hernandez and Sanchez, 2002; Thompson, 1991; Thompson, 1999; Thompson and Walker, 1988, 2020). In our previous study (Bjedov et al., 2022), CES activity in S9 corresponds with low activity in Podunavlje. Surprisingly, in contrast to our hypothesis, CES inhibition in nestlings' blood from landfill Jakuševec was observed (Fig. 4, Table SI2). This may indicate exposure to a combination of pollutants such as flame retardants, pharmaceuticals, plasticizers, nanomaterials and pesticides (Ramakrishnan et al., 2015).

Considering GST enzyme is involved in the intracellular GSH cycle and synthesis (Hayes et al., 2005), higher specific GST activity in S9 is expected when comparing fractions (Fig. 5, Table SI2). Specific GST activity showed no statistical difference between the study areas, in both plasma and S9 (Fig. 4, Table SI2). Regarding these results, no conclusion could be made about the pollutant effect on GST activity. In our previous study (Bjedov et al., 2022) similar results in GST activity were observed, as significant differences between the differently polluted areas were not detected.

GR is an enzyme with the main function of maintaining and supplying the cell with GSH. Since GR is mostly located intracellular – near the regions with high cellular electron flux generating ROS (Couto et al., 2016), specific GR activity was higher in S9, compared to plasma (Fig. 5, Table SI2). No significant variations were detected between the sampling areas, suggesting all nestlings had similar exposure, below the threshold for significant change in GR activity (Fig. 5, Table SI2). In our previous work, the highest GR activity was in Slavonski Brod – east for both fractions (Bjedov et al., 2022). The difference in GR response between the years could be the result of different prey compositions seeing as different prey accumulate pollutants at different rates (Ali and Khan, 2019; Pinzone et al., 2019). GR activity was analysed for the purpose of determining antioxidative status in breeding white storks from polluted areas (Kamiński et al., 2009a; Kamiński et al., 2009b; Oropesa et al., 2013a,b; Tkachenko and Kurhaluk, 2012, 2014). After acute dietary exposure to diclofenac and Pb, both individually and in combination, GR showed no significant changes in activity (Osičková et al., 2014).

4.3. Non–enzymatic biomarker response at differently polluted areas

GSH is primarily localised intracellularly (Lu, 2009), hence the difference in GSH levels between the plasma and S9 (Fig. 6, Table SI2). GSH concentration is significantly higher in nestlings from landfill Jakuševec compared to Podunavlje (Fig. 6, Table SI2). Higher GSH level in white storks foraging near landfills have been previously documented (Oropesa et al., 2013a,b; Pineda-Pampliega et al., 2021) and appears to be a sign of good physiological condition (Pineda-Pampliega et al., 2021). Moreover, an increase in GSH levels could be induced by high Se and Pb concentrations, as shown in domestic duck – Shaoxing duck (Ji et al., 2006). These results may be due to high Pb levels in Jakuševec compared to sampling areas Crnac polje, Jelas polje and Podunavlje (Fig. 3, Table SI1).

Although generally associated with mitochondria and electron flux, ROS may be generated by plasma membrane (Lee et al., 2017) or induced by external sources, namely pollutants and/or drugs (Lee et al., 2013), resulting in higher fluorescence detection of extracellular ROS in plasma compared to S9 (Fig. 6, Table SI2). Significant spatial variations were not observed regarding ROS concentration (Fig. 6, Table SI2). Therefore, no conclusion could be made about the effect of metal(loid)s and/or other pollutants on ROS concentration in the studied areas.

MTs are cysteine–rich proteins, mostly induced by Cd, zinc (Zn) and copper (Cu) (Babula et al., 2012). The lack of significant spatial variation in MT concentration may be indicative of low heavy metal and metalloid levels to induce a response (Table SI2). Furthermore, MTs localisation and inducibility by metals are tissue–specific, and MT induction is mostly correlated with Cd levels in the kidney (Elliott et al., 1992). The type of tissue used in this study (blood) could elucidate the lack of MT induction. Blood is always preferred over destructive sampling, however, MT analyses in the blood may not be suitable indicators of (heavy) metal(loid) pollution in white stork nestlings. Although MTs have not been previously analysed in white storks, they have been measured in the blood, liver and kidney of terrestrial bird species (white–tailed eagles, *Haliaeetus albicilla*, (Marcinekova, 2019), great tits, *Parus major*, (Vanparys et al., 2008), ringed turtle doves, *Streptopelia risoria* (Scheuhammer and Templeton, 1990),), and aquatic bird species (Leach's storm petrels, *Oceanodroma leucorhoa*, Atlantic puffin, *Fratercula arctica*, herring gull, *Larus argentatus*, double crested cormorant, *Phalacrocorax auritus* (Elliott et al., 1992), mallard, *Anas platyrhynchos*, spot–billed duck, *Anas poecilorhyncha*, great cormorant, *Phalacrocorax carbo* (Nam et al., 2005)).

4.4. Interactions between biomarkers, metalloids and heavy metals

A positive relationship between ChE and ROS in S9 is shown in Table 1. Although not investigated in birds, our findings are supported by Yamchuen et al. (2014) with research on human blood, showing an increase in ChE activity with the generation of ROS oxidised low–density lipoprotein–treated cells. Additionally, a negative correlation was observed between ChE in S9 and Se (Table 1). The positive roles of Se in organisms are very well researched, such as neuroprotective properties in Sprague–Dawley rats, *Rattus norvegicus* (Sharma et al., 2014). On the other hand, too high Se concentration can cause neurotoxicity and inhibit ChE activity as shown in fish, *Oreochromis mossambicus* (Gopi

et al., 2021). A significant positive relationship was recorded between ChE and Cd (Table 1). To our knowledge, the effects of Cd on ChE activity were not investigated in birds, however, its effect is known for other vertebrate groups. According to Jebali et al. (2006), Cd neurotoxicity is well documented in fish, *Seriola dumerili*, as exposure to lower concentrations of $CdCl_2$ increases ChE activity, while exposure to higher $CdCl_2$ levels significantly decreases ChE activity. These results correspond with the positive relationship observed between plasma ChE and Cd (Table 1), since the highest plasma ChE activity and Cd levels were recorded in Slavonski Brod – east and Podunavlje (Figs. 3 and 4, Tables SI1 and SI2). Moreover, an increase in ChE activity with exposure to increasing concentration of Cd was investigated on red swamp crayfish, *Procambarus clarkii* (Devi and Fingerman, 1995). Acute seven–day exposure to Cd can cause a significant increase in ChE activity (Pretto et al., 2010). Our results indicate nestlings in Slavonski Brod – east and Podunavlje were exposed to higher Cd concentration prior to blood sampling, thus elevating ChE activity in plasma.

CES activity in S9 negatively correlates with As levels. Possible synergistic effects of increased As levels, and pesticide use known for the Podunavlje area, could affect CES by reducing its activity in S9, as shown in Fig. 3. Investigation on CES activity affected by metalloids and pesticides was evaluated by (Lajmanovich et al., 2019) on *Rhinella arenarum* tadpoles. Synergistic effects of glyphosate and As caused inhibition of CES activity, corroborating our results.

A positive correlation was observed between GSH–dependent enzymes: GR and GST activity in plasma (Table 1). GR enzyme catalyses the conversion of oxidised GSH (GSSG) to reduced – GSH, and GST enzyme detoxifies xenobiotics using GSH as a substrate (Csiszár et al., 2016). Furthermore, heavy metals are known to induce changes in GR activity. A significant positive relationship was detected between plasma GR activity and Cd concentration (Table 1). The increase in GR activity after Cd treatment is well–researched (Sheweita, 1998). Exposure to environmentally relevant Cd concentrations induced GR activity in fish, *Australoheros facetus* (Crupkin and Menone, 2013), corroborating our results. Ensibi and Daly Yahia (2017) show an increase in GR activity after 48 h $CdCl_2$ exposure, followed by a significant decrease after 72 h. These opposing results appear to be due to different Cd concentrations. Moreover, a significant negative correlation was observed between GR activity in S9 and Hg levels (Table 1). Inhibition in GR activity was studied *in vitro* and *in vivo*, on the rat, *R. norvegicus* and quail, *C. coturnix* erythrocytes, as a dose–dependent response to Hg (Mykkanen and Ganther, 1974).

Significant correlations have been observed regarding GSH concentrations – negative with Pb, and positive with Se (Table 1). Although the negative correlation between GSH and Pb levels appears to be conflicting compared to other research, the negative association between blood Pb and GSH/GSSG was previously documented (Vacchi-Suzzi et al., 2018). However, the detrimental effects of Pb can be alleviated with Se (Huang et al., 2018, 2019; Kasperczyk et al., 2004; LeBoeuf et al., 1985; Özkan-Yilmaz et al., 2014), hence the high GSH and Pb concentration at Jakuševec (Figs. 3C and 6, Tables SI1 and SI2).

A significant negative correlation was detected between Se and Pb levels (Table 1, Table 2). The antagonistic effect of Se on Pb has been studied on chicken (*G. gallus domesticus*). Higher Se levels can alleviate Pb content, and subsequently protect against Pb toxicity (Xu et al., 2016).

5. Conclusion

The present study provides the first–time heavy metal and metalloid analyses and their interaction with biomarkers measured in two blood fractions of the white stork nestlings from Croatia. Our results indicate changes in analysed multibiomarker response in regard to differently polluted areas and subsequently demonstrate white stork nestlings could serve as bioindicator species providing useful information regarding heavy metal and metalloid effects on biomarker response. Further research involving different bird species as well as birds from other guilds living and/or breeding in metal–polluted areas is required to determine the extent to which the physiological response of wild birds is affected. Moreover, continuous monitoring of biomarkers, heavy metals and metalloids in nestling white storks could ascertain the long–term effects on their survival and overall fitness.

Author contributions

Conceptualization – Dora Bjedov, Mirna Velki, Alma Mikuška, Tibor Mikuska; Investigation – Dora Bjedov, Leontina Toth, Sara Šariri, Yassir Al Marsoomi, Luka Jurinović, Tibor Mikuska, Zdenko Lončarić; Resources – Tibor Mikuska, Vlatka Filipović Marijić, Sandra Ečimović, Nataša Turić, Luka Jurinović, Zdenko Lončarić; Data Curation – Dora Bjedov, Mirna Velki, Alma Mikuška; Writing - Original Draft – Dora Bjedov; Writing - Review & Editing – All; Supervision – Mirna Velki, Alma Mikuška; Funding acquisition – Mirna Velki, Alma Mikuška, Vlatka Filipović Marijić, Sandra Ečimović, Nataša Turić, Zdenko Lončarić.

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Declaration of competing interest

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.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.121398.

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Adult and nestling white stork from Osijek

3. Discussion

3. Discussion

3.1. Establishment of the protocols, response variability and sex differences

The use of birds as biomonitors of environmental quality has been widely valued and recognised as an important tool for monitoring schemes for the purpose of environmental management and assessment. Birds are recognized as excellent bioindicators of ecosystem health because they react fast to environmental change. Their physiological and/or behavioural change, as well as population status and breeding success, is easy to observe (Evers et al., 2008). One of the objectives of monitoring is to use the appropriate sentinel birds to acquire data to analyse the levels of environmental pollutants, identify the pollutants at every level of the food web, determine the harmful effects on the individual birds and their population, and lastly estimate potential risks to human health. Most suitable birds for such assessment are apex predators as they are able to accumulate a variety of pollutants over time and space, most specifically bird nestlings, which are easily captured and sampled, especially their blood, contributing to the use of non-destructive methods. Sampling methods are diverse, and selection should be based on the aim of the study and at the same time considering animal welfare above all. Although some studies still use destructive sampling, meaning capturing birds in traps and decapitating them (Anam & Maitra, 1995; Maitra et al., 1994; Soliman et al., 2020), alternative methods can be utilised for the same purpose. For optimal approach for the individual as well as biomarker assessment, the destructive methods of tissue sampling should be avoided. Blood sampling, if done correctly, is a simple method for laboratory analysis (Schmoll et al., 2004) and should be utilized over destructive methods. An additional advantage regarding blood analyses is the opportunity to measure various biomarkers, and thus relate concentrations of pollutants to biological effects.

For a better assessment of environmental pollutant effects on white stork nestlings, a battery of biomarkers and sex determination was optimised in white stork nestlings' blood. The results of the present dissertation will be useful for the purpose of obtaining information in the case of a small volume sample. The establishment of biomarker protocols for white stork blood will enable the application of these biomarkers in future research/biomonitoring which can contribute to the ecotoxicological investigations of birds without the need for destructive sampling. Successfully established protocols were used to determine the activities of AChE, CES, GST, and GR, as well as levels of GSH and ROS. In addition, their respective variability between the plasma and post-mitochondrial supernatant (S9) was assessed.

All biomarkers showed significantly different activities/levels between plasma and S9. Results confirmed the hypothesis that there is a significant difference in the response of the measured biomarkers between the two blood fractions: plasma and blood cell homogenate (S9), except in the case of GST. Lower variability in plasma was shown in the case of AChE activity, and levels of GSH and ROS, while CES activity exhibited lower variability in S9. The application of assays can be expanded to other avian species and will be useful in the determination of suitable fractions to measure each biomarker in the case of a small blood sample.

Protocols were established to determine the sex for each nestling and biomarker response. The white stork is a species exhibiting sexual dimorphism in both the adult and nestling stage. Although not very apparent, male nestlings are approximately 5% heavier and larger (Tryjanowski et al., 2011). When comparing biomarker response and heavy metal(loid) concentration between the sexes, no significant differences were detected. This may be due to sex hormones not being expressed enough to exhibit strong sexual dimorphism in nestlings (such as it is in adults). Therefore, these results confirm two hypotheses: there is no significant difference in the response of the measured biomarkers between the sexes of white stork nestlings, and there is no significant difference in the concentration of analysed heavy metals and metalloids between the sexes of white stork nestlings. Jerzak et al. (2010) evaluated blood chemistry parameters (protein, uric acid, cholesterol, high-density lipoprotein cholesterol and aspartate aminotransferase) in white stork nestlings, and significant differences between the sexes were recorded. Although interesting results, to date there is a lack of a clear explanation why blood parameters may differ between sexes in early developmental stages.

3.2. Biomarker response at differently polluted areas

Analysed biomarker responses were compared between the seven differently polluted areas: landfill Jakuševec, Lonjsko polje Nature park, Crnac polje, Jelas polje, Slavonski Brod – east, Podunavlje and Donje Podravlje. Each sampling area was chosen for its respective pollution, therefore the pollution gradation was based on agricultural land use, analysis of water and atmospheric pollutants (Gvozdić et al., 2011; Musić et al., 2020; Romić et al., 2015; Vađić et al., 2021). Based on this, Lonjsko polje was assumed as the least polluted and Jakuševec and Slavonski Brod – east as the most polluted areas. Jelas polje, Podunavlje, and Donje Podravlje were rated as the sites with intermediate levels of pollution (Podunavlje being the most polluted and Donje Podravlje being least polluted) in relation to the cover of large-scale intensive

agriculture. Namely, cereal crops are cultivated in Podunavlje providing various pollutant sources since cereals are heavily treated with herbicides and fungicides (Romić et al., 2015). Donje Podravlje is covered with planting orchards and corn, maintaining them with the use of herbicides and fungicides. That being said, the results of AChE and CES response showed a significant reduction in activity, confirming the third hypothesis that enzymes AChE and CES measured in the blood of white stork nestlings from the intensive agriculture areas might show a decrease in the activity. Blood AChE activity was previously analysed in white stork, *C. ciconia* assessing pesticide induced-inhibition by Oropesa et al. (2013). Changes in AChE and CES activity, namely inhibition, is used as a potential biomarker of pesticide exposure, most commonly organophosphorus insecticides (Sanchez-Hernandez and Sanchez, 2002; Thompson, 1991; Thompson, 1999; Thompson and Walker, 1988, 2020). Reduced AChE activity was observed in pigeons, *C. livia*, American kestrel, *F. sparverius*, Swainson's hawk, *B. swainsoni*, Cooper's hawk, *A. cooperi*, and Red-shouldered hawk, *B. lineatus* after exposure to organophosphate pesticides (Bartkowiak and Wilson, 1995). Overall, the results of lower AChE and CES activities from Podunavlje and Donje Podravlje appear to be in response to the presence of various pollutants, e.g. fertilizers and pesticides (Lazarus et al., 2021; Romić et al., 2015). Exposure to pesticides can occur in non-agricultural areas as well, e.g. golf courses (Rainwater et al., 1995), suburban environments (Décarie et al., 1993) and active dredge spoils (Zinkl et al., 1981). That being said, AChE activity measured in white stork nestlings from landfill Jakuševec was the lowest, but not significantly compared to other sampling locations. These results might indicate the beginning of exposure, meaning the concentration has not reached the threshold for a significant change. On the other hand, it is possible that the nestlings are continuously exposed to low concentrations of pollutants resulting in chronic exposure. The above-mentioned results reflect that there is a possibility of leachate from landfill Jakuševec since a study of ambient air shows traces of persistent organic pollutants, e.g., organochlorine pesticides (DDT and metabolites), polychlorinated biphenyls (PCB congeners) (Herceg Romanić & Krauthacker, 2000), polycyclic aromatic hydrocarbons (PAHs) (Ahel et al., 2000) and polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs) (Krauthacker et al., 2006), and by consuming local prey, nestlings are exposed. On the other hand, other compounds are also known to cause inhibition of AChE activity, e.g., quaternary ammonium cations (Changeux, 1966; Coleman & Eley, 1962; Colović et al., 2013). Quaternary ammonium compounds could be found in landfills as they are frequently used in disinfectants, surfactants, electrolytic capacitors, contraception, fabric softeners, and antistatic agents (Zhang et al., 2015).

Response of GST showed no significant spatial change, meaning nestlings were exposed to a similar concentration and/or mixture of pollutants exhibiting similar GST activity. It is possible the pollutant concentrations in the environment, and subsequently in white stork nestlings, is not high enough to induce a response. Other biomarker responses differ significantly in regard to the sampling area compared to GST activity, concluding GST is a resilient enzyme when it comes to environmental pollution. The fourth hypothesis, GST measured in the blood of white stork nestlings will show a change in the activity in regard to sampling areas, is rejected. Although not significant compared to other sampling locations, when observing trends in GST response, the highest activity was recorded in Slavonski Brod – east (plasma) and Podunavlje (S9), areas known for heavy industry and agricultural practices. High GST activities have been associated with resistance to insecticides (Ranson and Hemingway, 2005). On the other hand, GST activity showed a significant decrease in the serum and liver of chickens *G. gallus domesticus* regarding dietary exposure to insecticides (Ezeji et al., 2012). Metalloids (As) and pesticides (malathion) are known to cause an increase in GST activity, both individually and in combination, an effect shown in chicken, *G. gallus domesticus* liver (Naraharisetti et al., 2009).

The activity of GR appears to be more sensitive to environmental pollutants compared to GST. As presumed in the fourth hypothesis, spatial significant differences in GR activity were detected. Therefore, the hypothesis that GR measured in the blood of white stork nestlings will show a change in the activity with regard to sampling areas is confirmed. Namely, the highest GR activity was recorded in Slavonski Brod – east indicating the presence of pollutants in the surrounding environment causing increased oxidative stress related to the metal and engineering industry proximal to the cities of Slavonski Brod and Bosanski Brod. When compared with other studies conducted on white storks, GR activity was analysed in the *C. ciconia* nestlings from Poland. Surprisingly, a decrease in GR activity at polluted sites was detected (Kamiński, et al., 2009a,b). Following acute dietary exposure to diclofenac and Pb, both individually and in combination, GR showed no significant changes in activity (Osičková et al., 2014). The antioxidative response is a complex process and there are multiple variables affecting the antioxidant enzymes, both increases (Berglund et al., 2007) and decreases (Kamiński, et al., 2009a,b) in GR activity can occur as the result of oxidative stress.

The potential occurrence of oxidative stress, reflected in ROS concentration, is higher in nestlings residing near high-intensity industry and agriculture: Slavonski Brod – east and Podunavlje, respectively. The concentration of ROS measured in the blood of white stork nestlings shows a change in the activity with regard to sampling areas corroborating our fourth hypothesis. Nestlings' physiology appears to be affected as they are exposed to pollutants whose source is from metal and petrol industries (Slavonski Brod – east) and agricultural practices (Podunavlje). According to the annual report of the Ministry of Economy and Sustainable Development of the Republic of Croatia (Vađić et al., 2021) high levels of Pb, Cd, As, and nickel (Ni) were detected in aerosols close to Slavonski Brod. Additionally, the metallurgic industry and oil refinery in Slavonski Brod and Bosanski Brod, respectively, could be the potential sources since petroleum refinery effluents are the major source of aquatic pollution. Refinery effluents are usually mixtures of heavy metals and PAHs that may cause increased production of ROS resulting in oxidative stress (Custer et al., 2000; Koivula & Eeva, 2010; Singh & Shikha, 2019).

Measurement of GSH levels confirmed the fourth hypothesis, the concentration of GSH in the blood of white stork nestlings shows a change in the activity in regard to sampling areas. In particular, the highest GSH levels in Jelas polje, Podunavlje and landfill Jakuševec. High levels of GSH are due to the presence of environmental pollutants and/or induction of oxidative stress. For example, an increase in GSH levels could be induced by high Se and Pb concentrations. The areas Jelas polje and Podunavlje are used for intensive agriculture (Se biofortification) and (il)legal hunting activities (with Pb ammunition), indicating a possible undetected non-point source of pollution such as the spread of pollutants from farming (Romić et al., 2015) or traces of lead ammunition (Bilandžić et al., 2009; Lazarus et al., 2008). High levels of GSH in white storks, *C. ciconia* breeding in proximity to landfill Jakuševec indicate the good physiological condition and may be associated with foraging at the landfill (Pineda-Pampliega et al., 2021).

MTs as cysteine-rich proteins are usually induced by metals Cd, zinc (Zn) and copper (Cu) (Babula et al., 2012). Results obtained in the present dissertation lack a significant spatial difference in MT concentration. Based on the results the fourth hypothesis, MT measured in the blood of white stork nestlings will show a change in the concentration with regard to sampling areas, is rejected. The lack of change in MT concentration in regard to sampling areas could be explained by the very low heavy metal and metalloid exposure levels to induce a response in white stork nestlings and a type of tissue used in the present study.

That being said, MTs inducibility by metals are tissue-specific and is usually correlated with Cd concentration in the kidney (Elliott et al., 1992). Although blood is always preferred over destructive sampling, measurement of MT concentration in the blood may not be a suitable indicator of heavy metal and metalloid pollution in white stork nestlings compared to other tissue. The level of MTs has not been previously analysed in white storks, but they have been measured in other avian species. Namely, MTs have been characterised in the blood, liver and kidney of terrestrial and aquatic bird species. Along a pollution (metal) gradient, Vanparys et al. (2008) analysed heavy metals and MTs (along with other biomarkers) to confirm metal accumulation and subsequent biomarker responses following a dose-dependent reaction. The study implemented measuring the concentration of metal(loid)s from the environment and associating them with MTs in a passerine great tit, *P. major*, for the purpose of biomonitoring. Potential MT induction was assessed for potential influence by sex and age (biological factors) in white-tailed eagles, *H. albicilla* (Marcinekova, 2019). The mean basal concentration of MTs in white-tailed eagles, *H. albicilla* plasma was 7.73 ± 0.27 ng mL^{-1}, significantly lower than reported in this 0.44 – 0.48 mg mL^{-1} (mean concentration range in regard to sampling area), indicating that (basal) levels of MTs are species-specific. Related research performed MTs assessment on different organs, most commonly kidney and liver, in ringed turtle doves, *S. risoria* (Scheuhammer & Templeton, 1990), mallard, *A. platyrhynchos*, spot-billed duck, *A. poecilorhyncha*, and great cormorant, *P. carbo* (Nam et al., 2005), and Leach's storm petrels, *O. leucorhoa*, Atlantic puffin, *F. arctica*, herring gull, *L. argentatus*, double-crested cormorant, *P. auritus*, (Elliott et al., 1992).

3.3. Metalloids and heavy metals: spatial variation and the possible effect

Analysed metalloids and heavy metals show spatial variation, thus confirming the sixth hypothesis that the concentration will be increased in the blood of white stork nestlings in areas of intensive agriculture, hunting, and the metal mechanical engineering industry. The highest analysed As levels in blood were observed in Podunavlje. These results are supported by continuously high As concentrations in water and soil from Podunavlje surrounding area since the groundwater As levels are elevated in eastern Croatia (Ćavar et al., 2005; Habuda-Stanić et al., 2007; Romić et al., 2011; Ujević et al., 2010; Vidosavljevic et al., 2022). When comparing to As levels analysed in S9, the highest concentration, although not significant compared to other sampling locations, was recorded in Jelas polje.

This is probably due to (il)legal hunting, considering Pb shots contain metal alloy As and leach to the local soil and groundwater (Nelson, 1977). The effects of As on birds were investigated by Eisler (2004, and references therein). The reference value for unpolluted areas is 20.00 µg L^{-1} (Burger & Gochfeld, 1997a), however, research assessing marginal levels causing detrimental effects is lacking. Benito et al. (1999) reported maximum As concentration (181.00 µg L^{-1}) in white storks, *C. ciconia* feeding in the area surrounded by Doñana National Park following a toxic spill from the Aznalcóllar mine. In the present dissertation, three nestlings had higher or equal As concentrations (388.00 µg L^{-1}, 181.00 µg L^{-1}, 326.00 µg L^{-1}) from Podunavlje. de la Casa-Resino et al. (2014) report lower As levels in two colonies near a landfill (23.04 µg L^{-1}, 22.48 µg L^{-1}) compared to their control colony (2.34 µg L^{-1}). In our study, a colony situated in the Jakuševec landfill showed a higher median As concentration (44.45 µg L^{-1}) than reported by de la Casa-Resino (2014). These results indicate As contamination based on average values in Podunavlje, Jakuševec, Crnac polje and Slavonski Brod – east. Although conclusions cannot be made whether the white stork, *C. ciconia* nestlings are at risk of detrimental effects, chronic exposure and subsequent sublethal effect are highly likely.

A clear gradient of Se concentration is reflected in agricultural areas along the Sava River. The highest recorded blood Se concentration was measured in Crnac polje (median 1452.00 µg L^{-1}) followed by Lonjsko polje (median 1160.00 µg L^{-1}) and Jelas polje (median 950.00 µg L^{-1}). The results are conflicted in regard with the statement that eastern continental Croatia is deficient in Se (Džomba et al., 2014; Vukšić & Šperanda, 2016). However, various agricultural practices, e.g., Se biofortification, can affect Se levels in the soil and subsequently in the environment. Analyses of the blood Se levels are generally accepted indicator of acute exposure via ingestion (Ohlendorf & Heinz, 2011). Assumed geochemical background concentration of blood Se in birds is considered 100 – 400 µg L^{-1} (Ohlendorf & Heinz, 2011). That being said, if compared to the threshold levels of 100 µg L^{-1}, median blood Se levels in all white stork, *C. ciconia* nestlings were above the threshold. Only 2% of the nestlings had Se levels in the range that is considered the assumed geochemical background in Jelas polje and Podunavlje, respectively. Furthermore, 63% of the nestlings had Se concentration in the range < 1000 µg L^{-1} indicating possible adverse effects, followed by 35% of nestlings with Se levels in the range 1004.00 – 2370.00 µg L^{-1}. One nestling from Podunavlje had a blood Se concentration of 2370.00 µg L^{-1}. The United States Department of the Interior (1998) warrants an additional assessment for individuals exceeding the blood Se concentration of 1000 µg L^{-1}.

Comparing blood Se levels in nestling avian species from Doñana National Park following a toxic spill from the Aznalcóllar mine, Benito et al. (1999) report a mean Se concentration of 382.00 µg L^{-1}. Considering all results, further assessment and exploration of the particular effects are needed, although blood Se appears to be below levels for irreversible physiological and/or lethal effects.

Blood is a good matrix for assessing immediate Cd exposure via contaminated prey (Martínez-López et al., 2005). In healthy wild birds, the concentration of blood Cd is usually below 50 µg L^{-1} (Finkelstein et al., 2007; Thompson & Dowding, 1999; Tsipoura et al., 2008; Wayland et al., 2005). Analysed Cd concentrations in white stork, *C. ciconia* nestlings were substantially below the presumed threshold for adverse effect (50 µg L^{-1}). Similar Cd concentrations were detected in all sampling areas with the highest median concentration in Podunavlje (2.77 µg L^{-1}). Spatial variability is absent indicating similar exposure, or lack thereof to Cd. Surprisingly, one nestling from Slavonski Brod – east had a blood Cd level of 17.70 µg L^{-1} indicating exposure via contaminated prey prior to sampling, however, the value is still considerably below the level for adverse effects. Expecting high metalloid and heavy metal pollution at a landfill, white storks, *C. ciconia* breeding near landfills appear to have generally lower Cd concentration in blood (0.49 µg L^{-1} reported by de la Casa-Resino et al. 2014; 1.80 µg L^{-1} reported in this dissertation).

Hg is known for bioaccumulation and biomagnification in aquatic environments, therefore environmental exposure to Hg is broadly researched in avian species (Burger & Gochfeld, 1997b; Goodale et al., 2008; Jackson et al., 2011; Lavoie et al., 2014; Weech et al., 2006; Whitney & Cristol, 2018). Since Hg exposure is posing a significant health risk in aquatic ecosystems, i.e., for piscivorous birds, the lowest observed adverse effect regarding blood Hg level in aquatic birds is 300 µg L^{-1} (Ackerman et al., 2014). Considering white storks are omnivorous and forage in both terrestrial and aquatic environments, the threshold for adverse effects possibly differs for the white stork, *C. ciconia* nestlings. All observed Hg concentrations are below the assumed level for adverse effects. Nestlings residing close to the landfill were anticipated to have high Hg concentration in blood, however recorded median is 21.00 µg L^{-1}, similar to in landfill colonies (24.36 µg L^{-1}, 53.03 µg L^{-1}) reported by de la Casa-Resino et al. (2014). Significantly higher concentrations of Hg in blood were observed in white stork, *C. ciconia* nestlings residing in Lonjsko polje Nature park (median 83.80 µg L^{-1}).

The maximum detected Hg concentration in nestling from Lonjsko polje Nature park is 238.00 µg L^{-1}. Lonjsko polje Nature park appears to have perpetually increased Hg concentrations, as shown in results from 2020. Upstream of Lonjsko polje Nature park, Halamić et al. (2003) reported higher sediment Hg concentration than the geochemical background. Moreover, the surrounding area is known for viticulture, and the use of pesticides and fertilisers as well (Zrinšćak et al., 2011), possibly contributing to increased Hg concentrations. These results are potentially concerning due to the bioaccumulative and biomagnifying properties of Hg in the ecosystem. Future monitoring is necessary seeing as Hg is a persistent, toxic pollutant.

As expected, white stork, *C. ciconia* breeding near landfill Jakuševec have significantly higher Pb content, reflected in the nestlings' blood. In previous years, presumed contamination was recorded in Slavonski Brod – east and Donje Podravlje. Proposed levels of Pb concentration causing blood-inducing sub-clinical poisoning is above 200 µg L^{-1} (Descalzo et al., 2021; Pain et al., 2019). The concentration of Pb in blood, possibly inducing the sub-clinical effects, was detected in two nestlings 393.00 µg L^{-1} and 205.00 µg L^{-1} from Slavonski Brod – east and Lonjsko polje, respectively. Interestingly, the nestling from Slavonski Brod – east with the highest Pb concentration in blood (393.00 µg L^{-1}), had the highest Cd concentration as well (17.70 µg L^{-1}) indicating immediate exposure to these pollutants prior to sampling. Areas with presumed high Pb concentrations are Jakuševec, Slavonski Brod – east and Donje Podravlje. The landfill Jakuševec could potentially pollute the groundwater with Pb since the pollution is known to migrate and subsequently enter the surrounding environment (Levin et al., 2021; Vongdala et al., 2018). Moreover, the metallurgic industry in Slavonski Brod – east, and (illegal) hunting in Donje Podravlje could be a source of analysed Pb concentrations. Average Pb levels reported by Pérez-López et al. (2016) were lower than in presumably contaminated areas. Moreover, according to Benito et al. (1999), Pb concentration in the blood of nestling white storks, *C. ciconia* foraging in the surrounding area of Doñana National Park following a toxic spill from the Aznalcóllar mine was lower (71.00 µg L^{-1}) than reported in Jakuševec (105.00 µg L^{-1}).

3.4. Interactions between biomarker response and metal(loid) concentrations

The correlation between the biomarker responses and metal(loid)s, was evaluated in order to assess the possible effect. Significant correlations were confirmed, and the seventh hypothesis, that there will be a correlation between the measured biomarkers and the

concentration of analysed heavy metals and metalloids in the blood of white stork nestlings, is corroborated.

Significant correlations were recorded between esterases and metal(loid)s. These associations and/or mechanisms are documented in other vertebrates, but not in avian species. Firstly, a positive correlation was detected between AChE activity and Cd concentration. Cd is known for its neurotoxicity, and different concentrations provoke different responses. For example, exposure to relatively low levels of cadmium chloride, $CdCl_2$ (50 μg kg^{-1}) elevates the AChE activity. On the other hand, exposure to relatively high $CdCl_2$ concentrations (100 μg kg^{-1}, 250 μg kg^{-1}) inhibits AChE activity in fish, *Seriola dumerili* (Jebali et al., 2006). An increase in AChE activity with exposure to increasing concentration of Cd was explored on silver catfish, *Rhamdia quelen* (Pretto et al., 2010) and red swamp crayfish, *Procambarus clarkii* (Devi & Fingerman, 1995). Secondly, a negative correlation was observed between CES activity and As concentration. CES activity is lower and As concentration is higher in Podunavlje, this may indicate synergistic effects of As and pesticides, meaning higher As concentration in blood is reducing CES activity. Research conducted on metalloids (As) and pesticides (glyphosate) effect on CES activity in *Rhinella arenarum* tadpoles shows inhibition of CES activity (Lajmanovich et al., 2019), thus supporting the results.

Significant correlations have been recorded between oxidative stress biomarkers and metal(loid)s. A positive correlation was observed between GR activity and Cd concentration. The increase in GR activity after $CdCl_2$ treatment (2 mg kg^{-1}) has been researched in mice. In particular, 1 h after exposure, $CdCl_2$ had no effect on GR activity, however, 24 h and 72 h after exposure, $CdCl_2$ significantly increased GR activity by 56% and 34%, respectively (Sheweita, 1998). Exposure to environmentally relevant Cd concentrations induced GR activity in fish, *Australoheros facetus* (Crupkin & Menone, 2013), corroborating the results. On the other hand, Ensibi & Daly Yahia (2017) report conflicting results when exposing copepod *Centropages ponticus* to $CdCl_2$ (0.20 μg L^{-1}, 0.40 μg L^{-1}). A significant increase in GR activity after 48 h $CdCl_2$ exposure was recorded, followed by a significant decrease after 72 h (Ensibi & Daly Yahia 2017). These opposing results appear to be due to different dosing to Cd concentrations. Sheweita (1998) injected mice intraperitoneally, while Ensibi & Daly Yahia (2017) exposed copepods in sea water (via ingestion and contact exposure). A negative correlation was observed between GR activity and Hg concentration. Mechanism of GR activity inhibition with Hg level increase and vice versa is documented in mammals and birds. GR activity inhibition

was studied as a dose-dependent response to Hg both *in vitro* and *in vivo* on erythrocytes in rats, *R. norvegicus* and quails, *Coturnix coturnix* (Mykkanen & Ganther, 1974).

Significant correlations have been observed regarding GSH concentrations – negative with Pb, and positive with Se. This is supported by the high GSH levels and Pb concentration at landfill Jakuševec. The negative association between blood Pb and GSH/GSSG was previously documented. Increased exposure levels to Pb are associated with increased oxidative stress in the blood of workers exposed at occupational levels (Kasperczyk et al., 2004; Vacchi-Suzzi et al., 2018). Exposing mice to 3.00 mg kg^{-1} and 6.00 mg kg^{-1} of dietary Se caused a dose-dependent reaction in hepatic GSH levels (LeBoeuf et al., 1985). That being said, the possible adverse effects of elevated Pb can be alleviated with Se. Mitigative effect of Se on Pb-induced cell apoptosis has been assessed along the endoplasmic reticulum (ER) pathway in chicken *G. gallus domesticus* testes (Huang et al., 2018).

Regarding element-element interactions, a positive correlation can be observed between Se and Hg. Since the correlation is not significant, the eighth hypothesis, stating the correlation between Se and Hg levels in the blood of white stork nestlings, is rejected. Antagonistic interaction between Hg and Se is very well known. Se protection against Hg toxicity was recorded in numerous avian species (Beijer & Jernelöv, 1978; Cuvin-Aralar & Furness, 1991; Koeman et al., 1975; Lucia et al., 2010). Animal tissue with high Se concentration often accumulates high concentrations of Hg as well, and a strong positive correlation can be seen, especially in aquatic birds (Scheuhammer et al., 1998). Furthermore, an absence of a significant correlation between the concentration of metallothionein and the concentration of analysed metalloids and heavy metals in the blood of white stork nestlings was observed. For that reason, the ninth hypothesis, assuming a correlation between the MTs and the metal(loid)s level in the blood of white stork nestlings, is rejected. Surprisingly, a significant negative correlation was detected between Se and Pb levels. The antagonistic effect of Se on Pb has been studied on chicken, *G. gallus domesticus*. Since high concentrations of Pb can cause severe oxidative stress, higher Se levels can alleviate Pb content, and subsequently protect against Pb toxicity (Xu et al., 2016). Moreover, different concentrations of Pb can affect ATP generation and mitochondrial apoptosis, causing kidney cell apoptosis in the chicken, *G. gallus domesticus*. As previously said, Pb can increase oxidative stress and promote the mitochondrial apoptotic pathway. For that purpose, Jin et al. (2017) demonstrated the antagonistic function of Se against Pb-induced apoptosis in the chicken, *G. gallus domesticus* kidney cells.

White stork nestling from Petrijevci

4. Conclusion

4. Conclusion

The present thesis provides a first-time analysis of heavy metal and metalloid concentrations and their interaction with biomarkers in white stork nestlings' blood from Croatia. The obtained results indicate the presence of pollutants in the sampled areas and demonstrate white stork nestlings could provide useful information as a bioindicator species for biomonitoring. Based on these results, the following conclusions could be made:

Blood sampling was successfully associated with an already established census and ringing scheme of white stork nestlings. Protocols for enzymatic and non-enzymatic biomarkers were implemented and fluorescent-based dyes were established for the first time as a novel detection of ROS and GSH in birds.

For a better overview, the response of the biomarkers was successfully analysed in two blood fractions – plasma and S9. It was determined that both fractions can be used in biomarker analyses. Significant differences between the activities and levels in fractions are present, meaning the biomarker response in plasma does not always correspond to the biomarker response in S9.

Sex is not a significant variable when investigating the difference in biomarker response and metal(loid) levels in the blood of white stork nestlings.

Lower AChE and CES activities were observed in white stork nestlings from Podunavlje and Donje Podravlje compared to the other study areas. Podunavlje and Donje Podravlje are areas known for extensive agriculture, horticulture and farming, therefore nestlings might be affected by pesticide and fertiliser use.

White stork nestlings from Slavonski Brod – east and Podunavlje exhibited increased GR activity and ROS and GSH concentrations which may be an indication of oxidative stress. Observed responses may be explained by the fact that the study areas are known for the metal and petroleum industry, as well as agriculture, horticulture and farming with the application of pesticides and fertilisers, respectively.

The highest observed As concentration measured in white stork nestlings from Podunavlje indicate a potential sublethal effect due to chronic exposure.

Alarming Se concentration measured in the blood of white stork nestlings residing in Crnac polje indicates possible adverse physiological effects.

Lonjsko polje Nature park appears to have a continuously elevated concentration of Hg. All observed blood Hg concentrations were below the threshold for adverse effects in white stork nestlings.

The highest Pb concentration was recorded in Jakuševec, however, all Pb levels at the landfill were below concentration for adverse effects. A nestling from Slavonski Brod – east and a nestling from Lonjsko polje could exhibit the subclinical effects.

The white stork nestlings were shown to be a good indicator species for the monitoring of environmental heavy metal and metalloid effects, via measurement of a battery of biomarkers in two blood fractions – plasma and S9. Overall results show it is necessary to carry out regular monitoring schemes and analyse biomarker responses and/or chemical levels to estimate the impact of pollutants through model species to prevent harmful effects on the ecosystem. Furthermore, conducting research using the white stork nestlings provides information for a timely reaction in the case of possible pollution impact on human health.

Photo by Aissa Salhi

5. References

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6. Curriculum vitae

Dora Bjedov, MSc, was born on March 9th 1995 in Osijek. In 2019, she received the academic title Master of Science in Biology at the Department of biology, Josip Juraj Strossmayer University of Osijek. She is currently working as expert for ornithofauna at BIOTA Ltd. Her main area of expertise includes avian ecology, physiology and toxicology. During her studies, she volunteered at Allwetterzoo Münster, Germany, working with rainbow lorikeets (*Trichoglossus moluccanus*) and was vice president of the Biology student association ZOA, as well as the head of the Ornithology section. She did two traineeships as a part of Erasmus+, one in Trondheim, Norway, working at Norwegian University of Science and Technology (NTNU) on a project: Urban development, shipping and tourism impacts on marine ecosystem in Tromsø: Investigation of cocktail effects using bivalves as sentinel species; and other in Roskilde, Denmark, working at Aarhus University on a project: ECOSTRESS – Ecological consequences of environmentally persistent pollutants in a marine sentinel species: A multi-stressor approach. She received eCOST scholarship, working on "Assessment of existing capacity and knowledge to detect pan-European spatial and temporal trends in the exposure of raptors to legacy organochlorines" at Aarhus University in Roskilde as a part of a project ERBFacility – European Raptor Biomonitoring Facility. She has published seven original scientific articles and presented data in 20 international and domestic congresses and symposiums.

List of publications

Bjedov, **D**., Velki, M., Kovačić, L. S., Begović, L., Lešić, I., Jurinović, L., Mikuska, T., Sudarić Bogojević, M., Ečimović, S. & Mikuška, A. (2023). White Stork (*Ciconia ciconia*) Nestlings Affected by Agricultural Practices? Assessment of Integrated Biomarker Responses. *Agriculture, 13*(5), 1045. https://doi.org/10.3390/agriculture13051045

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Active conference participation

Bjedov, **D**., Velki, M., Mikuska, T., Jurinović, L., Turić, N., Toth, L., Ečimović, S., Lončarić, Ž. & Mikuška, A. (2022). Metal and metalloid concentrations in the blood of white stork (*Ciconia ciconia*) nestlings from continental Croatia. 14th Croatian Biological Congress with International Participation. October 2022. Pula, Croatia.

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7. Extended abstract *Prošireni sažetak*

Ptići bijele rode (*Ciconia ciconia*) mogu pružiti kvantitativne informacije o kvaliteti okoliša ukazujući na prisutnost zagađivala, budući da ovise o lokalnoj hrani koju im donose roditelji. Kako bi se procijenila zagađenost okoliša i utjecaj na ptiće, krv je uzorkovana iz brahijalne vene, a uzorkovanje je provedeno paralelno s prstenovanjem tijekom gnijezdeće sezone 2020. i 2021. s područja kontinentalne Hrvatske: odlagalište otpada Jakuševec, Park prirode Lonjsko polje, Crnac polje, Jelas polje, Slavonski Brod – istok, Podunavlje i Donje Podravlje. Ciljevi ovog istraživanja bili su usporediti odgovor biomarkera u dvije frakcije krvi (plazma i S9) ptića bijele rode; uspostaviti nove protokole za mjerenje biomarkera (aktivnost esteraze, fluorescentne boje za detekciju oksidativnog stresa, razine metalotioneina), primijeniti prethodno analizirane biomarkere (glutation-ovisne enzime); istražiti razlike u odgovorima biomarkera vezanih za metal(loide) u krvi ptića bijele rode iz Hrvatske. Protokol za mjerenje biomarkera uspješno je primijenjen u plazmi i S9 i utvrđene su razlike na temelju varijabilnosti, aktivnosti enzima i fluorescencije. Protokol ukazuje na razlike u odgovoru biomarkera i koncentraciji metal(oid)a u odnosu na područje uzorkovanja. Rezultati prikazuju moguć utjecaj zagađivala iz okolne metalne, naftne i poljoprivredne industrije na odgovor biomarkera u ptićima, koji se često smatraju ranim znakovima upozorenja. Naime, ptići iz Podunavlja i Donjeg Podravlja, područja poznatih po intenzivnoj poljoprivredi, pokazuju nižu aktivnost acetilkolinesteraze (AChE) i karboksilesteraze (CES) u usporedbi s ostalim istraživanim područjima, što ukazuje na prisutnost inhibitornih ksenobiotika. Promjene u odgovoru biomarkera oksidativnog stresa uočene su u ptićima iz Slavonskog Broda – istok, području okruženom metalurgijom i strojarskom industrijom, te u ptićima iz Podunavlja u usporedbi s drugim područjima uzorkovanja. Povećane razine metal(loid)a na odlagalištima i poljoprivrednim površinama mogu uzrokovati štetne učinke na ptiće. Ptići iz Jakuševca pokazuju smanjenu aktivnost CES, povišenu koncentraciju glutationa (GSH), kao i visok sadržaj olova (Pb) u krvi. Povećane koncentracije arsena (As) u Podunavlju i žive (Hg) u Lonjskom polju mogu se pripisati zagađenju okoliša u poljoprivrednom području odnosno pretpostavljenom nezagađenom području. Nadalje, čini se da poljoprivreda utječe na aktivnost CES, kao i povećavanje razina selena (Se). Uz uspješnu primjenu biomarkera, ovo istraživanje pokazalo je da su poljoprivredne površine i odlagalište otpada područja s povišenim razinama metal(oid)a koji mogu imati štetne učinke na bijele rode i njihove ptiće. Ovo je prva analiza teških metala i metaloida u ptićima bijele rode s područja Hrvatske te ukazuje na neophodno praćenje i buduću procjenu utjecaja zagađenja kako bi se spriječili štetni učinci.

APPENDIX I. *Bibliometric information*

NACIONALNA
I SVEUČILIŠNA
KNJIŽNICA
U ZAGREBU

Ulica Hrvatske bratske zajednice 4
p. p. 550
10 000 Zagreb
Hrvatska
T +385 1 616 40 40
F +385 1 616 41 86
www.nsk.hr

Maja Mihalić, mag.bibl.

Nacionalna i sveučilišna knjižnica u Zagrebu
e-mail: mmihalic@nsk.hr
tel. +385 1 616 4153

Zagreb, 13.3.2023.

Predmet: **Potvrda o zastupljenosti i citiranosti radova znanstvenika/ce** [i]

Dora Bjedov

Ustanova: Biološki odsjek, Prirodoslovno-matematički fakultet, Sveučilište u Zagrebu
Područje/grana znanosti: Prirodne znanosti
Svrha zahtjeva: Napredovanje u viša znanstveno-nastavna zvanja

Zastupljenost radova:

- Web of Science Core Collection
  - Science Citation Index Expanded (SCI-EXPANDED) --1955-present: **3 + 1 rad u tijeku indeksiranja**
- Ostali relevantni izvori:
  - Zoological Records: **1**
  - ProQuest Environmental Science Database: **1**

Citiranost radova:

- Web of Science® *(from All Databases, Citation Report):*
  Broj citata: 5; h-indeks: 1
- Scopus *(View citation overview):*
  Broj citata: 8; h-indeks: 1
- Google Scholar:
  Broj citata: 16; h-indeks: 2

Prilog: Ispis bibliografskih zapisa radova iz navedenih elektroničkih izvora

[i] Nacionalna i sveučilišna knjižnica u Zagrebu izdaje odgovarajuće potvrde prema Pravilniku o uvjetima za izbor u znanstvena zvanja (NN 28/2017) i Pravilniku o izmjenama i dopunama Pravilnika o uvjetima za izbor u znanstvena zvanja (NN 111/2022) u skladu s dostupnim elektroničkim izvorima i bazama podataka dostupnim na Portalu elektroničkih izvora za hrvatsku akademsku i znanstvenu zajednicu. U prikazu zastupljenosti radova provedena je deduplikacija, što znači da se pojedini rad prikazuje u samo jednom, relevantnijem izvoru.

NACIONALNA I SVEUČILIŠNA KNJIŽNICA U ZAGREBU
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ZAGREB OIB: 84838770814

NACIONALNA
I SVEUČILIŠNA
KNJIŽNICA
U ZAGREBU

Ulica Hrvatske bratske zajednice 4
p. p. 550
10 000 Zagreb
Hrvatska
T +385 1 616 40 40
F +385 1 616 41 86
www.nsk.hr

Maja Mihalić, mag.bibl.

Nacionalna i sveučilišna knjižnica u Zagrebu
e-mail: mmihalic@nsk.hr
tel. +385 1 616 4153

Zagreb, 13.3.2023.

Predmet: **Potvrda o statusu časopisa s indeksiranim radovima u relevantnim bazama podataka WoSCC (JCR) i Scopus (SJR)**

Record 1 of 4

Title: Heavy metal(loid) effect on multi-biomarker responses in apex predator: Novel assays in the monitoring of white stork nestlings.

Author(s): Bjedov, Dora; Velki, Mirna; Toth, Leontina; Marijic, Vlatka Filipovic; Mikuska, Tibor; Jurinovic, Luka; Ecimovic, Sandra; Turic, Natasa; Loncaric, Zdenko; Sariri, Sara; Al Marsoomi, Yasir; Mikuska, Alma

Source: Environmental pollution **Published:** 2023

WoSCC – JIF *(u tijeku indeksiranja)*:
ENVIRONMENTAL SCIENCES 2021 Q1
JIF 2021 9.988

WoSCC – JCI[ii] *(u tijeku indeksiranja)*:
ENVIRONMENTAL SCIENCES 2021 Q1
JCI 2021 1.61

Scopus *(u tijeku indeksiranja)*:
Health, Toxicology and Mutagenesis 2021 Q1
Medicine (miscellaneous) 2021 Q1
Pollution 2021 Q1
Toxicology 2021 Q1
SJR 2021 1.954

Record 2 of 4

Title: Blood biomarkers in white stork (Ciconia ciconia) nestlings show different responses in several areas of Croatia

Author(s): Bjedov, D (Bjedov, Dora); Velki, M (Velki, Mirna); Lackmann, C (Lackmann, Carina); Begovic, L (Begovic, Lidija); Mikuska, T (Mikuska, Tibor); Jurinovic, L (Jurinovic, Luka); Mikuska, A (Mikuska, Alma)

Source: JOURNAL OF EXPERIMENTAL ZOOLOGY PART A-ECOLOGICAL AND INTEGRATIVE PHYSIOLOGY **Volume:** 337 **Issue:** 5 **Pages:** 547-558 **DOI:** 10.1002/jez.2588 **Early Access Date:** FEB 2022 **Published:** JUN 2022

[ii] **Journal Citation Indicator (JCI) i JCI Quartile** - novi indikatori (lipanj 2021.) dostupni putem InCites Journal Citation Reports (JCR) platforme. JCI i JCI Quartile dostupni su za sve časopise u sklopu Web of Science Core Collection-a od 2017. godine pa nadalje, što znači po prvi puta i za časopise u sklopu indeksa A&HCI (Arts and Humanities Citation Index) i ESCI (Emerging Sources Citation Index).

NACIONALNA I SVEUČILIŠNA KNJIŽNICA U ZAGREBU
Hrvatske bratske zajednice 4
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NACIONALNA
I SVEUČILIŠNA
KNJIŽNICA
U ZAGREBU

Ulica Hrvatske bratske zajednice 4
p. p. 550
10 000 Zagreb
Hrvatska
T +385 1 616 40 40
F +385 1 616 41 86
www.nsk.hr

WoSCC – JIF:
ZOOLOGY 2021 Q1
JIF 2021 2.693

WoSCC – JCI:
ZOOLOGY 2021 Q1
JCI 2021 1.20

Scopus:
Animal Science and Zoology 2021 Q1
Ecology, Evolution, Behavior and Systematics 2021 Q1
Genetics 2021 Q2
Molecular Biology 2021 Q3
Physiology 2021 Q2
SJR 2021 0.782

Record 3 of 4

Title: Application of Non-Destructive Methods: Biomarker Assays in Blood of White Stork (Ciconia ciconia) Nestlings

Author(s): Bjedov, D (Bjedov, Dora); Mikuska, A (Mikuska, Alma); Lackmann, C (Lackmann, Carina); Begovic, L (Begovic, Lidija); Mikuska, T (Mikuska, Tibor); Velki, M (Velki, Mirna)

Source: ANIMALS **Volume:** 11 **Issue:** 8 **Article Number:** 2341 **DOI:** 10.3390/ani11082341 **Published:** AUG 2021

WoSCC – JIF:
AGRICULTURE, DAIRY & ANIMAL SCIENCE 2021 Q1
VETERINARY SCIENCES 2021 Q1
JIF 2021 3.231

WoSCC – JCI:
AGRICULTURE, DAIRY & ANIMAL SCIENCE 2021 Q1
VETERINARY SCIENCES 2021 Q1
JCI 2021 1.34

Scopus:
Animal Science and Zoology 2021 Q1
Veterinary (miscellaneous) 2021 Q1
SJR 2021 0.610

APPENDIX II. *Hypotheses and expected scientific contribution*

Hypothesis	Published article confirming/rejecting the hypothesis
1) There is a significant difference in the response of the measured biomarkers between the two blood fractions: plasma and blood cell homogenate (S9).	Bjedov, D., Mikuška, A., Lackmann, C., Begović, L., Mikuška, T., & Velki, M. (2021). Application of non-destructive methods: Biomarker assays in blood of white stork (*Ciconia ciconia*) nestlings. *Animals*, *11*(8). https://doi.org/10.3390/ani11082341
2) There is no significant difference in the response of the measured biomarkers between the sexes of white stork nestlings.	Bjedov, D., Mikuška, A., Lackmann, C., Begović, L., Mikuška, T., & Velki, M. (2021). Application of non-destructive methods: Biomarker assays in blood of white stork (*Ciconia ciconia*) nestlings. *Animals*, *11*(8). https://doi.org/10.3390/ani11082341
3) Enzymes acetylcholinesterase and carboxylesterase measured in the blood of white stork nestlings from the intensive agriculture areas will show a decrease in the activity.	Bjedov, D., Velki, M., Lackmann, C., Begović, L., Mikuška, T., Jurinović, L., & Mikuška, A. (2022). Blood biomarkers in white stork (*Ciconia ciconia*) nestlings show different responses in several areas of Croatia. *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology*, *337*(5), 547–558. https://doi.org/10.1002/jez.2588
4) Biomarkers measured in the blood of white stork nestlings will show a change in the activity of the enzymes glutathione S-transferase and glutathione reductase and in the concentrations of metallothionein, glutathione and reactive oxygen species in regard to sampling areas.	Bjedov, D., Velki, M., Lackmann, C., Begović, L., Mikuška, T., Jurinović, L., & Mikuška, A. (2022). Blood biomarkers in white stork (*Ciconia ciconia*) nestlings show different responses in several areas of Croatia. *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology*, *337*(5), 547–558. https://doi.org/10.1002/jez.2588

5) There is no significant difference in the concentration of analysed heavy metals and metalloids between the sexes of white stork nestlings.	Bjedov, D., Velki, M., Toth, L., Filipović Marijić, V., Mikuška, T., Jurinović, L., Ečimović, S., Turić, N., Lončarić, Z., Šariri, S., Al Marsoomi, Y., & Mikuška, A. (2023). Heavy metal(loid) effect on multi-biomarker responses in apex predator: Novel assays in the monitoring of white stork nestlings. *Environmental Pollution*, 121398. https://doi.org/10.1016/j.envpol.2023.121398
6) Concentrations of analysed heavy metals and metalloids will be increased in the blood of white stork nestlings in areas of intensive agriculture, hunting, and metal mechanical engineering industry.	Bjedov, D., Velki, M., Toth, L., Filipović Marijić, V., Mikuška, T., Jurinović, L., Ečimović, S., Turić, N., Lončarić, Z., Šariri, S., Al Marsoomi, Y., & Mikuška, A. (2023). Heavy metal(loid) effect on multi-biomarker responses in apex predator: Novel assays in the monitoring of white stork nestlings. *Environmental Pollution*, 121398. https://doi.org/10.1016/j.envpol.2023.121398
7) There will be correlation between the measured biomarkers and the concentration of analysed heavy metals and metalloids in the blood of white stork nestlings.	Bjedov, D., Velki, M., Toth, L., Filipović Marijić, V., Mikuška, T., Jurinović, L., Ečimović, S., Turić, N., Lončarić, Z., Šariri, S., Al Marsoomi, Y., & Mikuška, A. (2023). Heavy metal(loid) effect on multi-biomarker responses in apex predator: Novel assays in the monitoring of white stork nestlings. *Environmental Pollution*, 121398. https://doi.org/10.1016/j.envpol.2023.121398
8) There will be correlation between the concentrations of mercury and selenium in the blood of white stork nestlings.	Bjedov, D., Velki, M., Toth, L., Filipović Marijić, V., Mikuška, T., Jurinović, L., Ečimović, S., Turić, N., Lončarić, Z., Šariri, S., Al Marsoomi, Y., & Mikuška, A. (2023). Heavy metal(loid) effect on multi-biomarker responses in apex predator: Novel assays in the monitoring of white stork nestlings. *Environmental Pollution*, 121398. https://doi.org/10.1016/j.envpol.2023.121398

9) There will be correlation between the concentration of metallothionein and the concentration of analysed heavy metals and metalloids in the blood of white stork nestlings.	Bjedov, D., Velki, M., Toth, L., Filipović Marijić, V., Mikuška, T., Jurinović, L., Ečimović, S., Turić, N., Lončarić, Z., Šariri, S., Al Marsoomi, Y., & Mikuška, A. (2023). Heavy metal(loid) effect on multi-biomarker responses in apex predator: Novel assays in the monitoring of white stork nestlings. *Environmental Pollution*, 121398. https://doi.org/10.1016/j.envpol.2023.121398

Expected scientific contribution	Published article reflecting scientific contribution
The results obtained by this study will enable the application of non-destructive methods and optimization of protocols for measuring biomarkers in the blood of nestlings, which will allow assessing the impact of environmental pollutants without adverse effects on the population of this protected species. Biomarkers will also be measured in two blood fractions, which will provide information on the variability of biomarker responses in each sample type, and will assess which fraction is more appropriate for measuring each biomarker.	Bjedov, D., Mikuška, A., Lackmann, C., Begović, L., Mikuška, T., & Velki, M. (2021). Application of non-destructive methods: Biomarker assays in blood of white stork (*Ciconia ciconia*) nestlings. *Animals*, *11*(8). https://doi.org/10.3390/ani11082341 Bjedov, D., Velki, M., Lackmann, C., Begović, L., Mikuška, T., Jurinović, L., & Mikuška, A. (2022). Blood biomarkers in white stork (*Ciconia ciconia*) nestlings show different responses in several areas of Croatia. *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology*, *337*(5), 547–558. https://doi.org/10.1002/jez.2588
Given that this will be the first study of this type in Croatia, the scientific importance of this study is reflected in the association of measured biomarkers and concentrations of metals and metalloids in birds' blood and the results will ultimately serve as indicators of local environmental pollution by heavy metals and metalloids that can affect this strictly protected species – the white stork.	Bjedov, D., Velki, M., Lackmann, C., Begović, L., Mikuška, T., Jurinović, L., & Mikuška, A. (2022). Blood biomarkers in white stork (*Ciconia ciconia*) nestlings show different responses in several areas of Croatia. *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology*, *337*(5), 547–558. https://doi.org/10.1002/jez.2588 Bjedov, D., Velki, M., Toth, L., Filipović Marijić, V., Mikuška, T., Jurinović, L., Ečimović, S., Turić, N., Lončarić, Z., Šariri, S., Al Marsoomi, Y., & Mikuška, A. (2023). Heavy metal(loid) effect on multi-biomarker responses in apex predator: Novel assays in the monitoring of white stork nestlings. *Environmental Pollution*, 121398. https://doi.org/10.1016/j.envpol.2023.121398

APPENDIX III. *Supplementary information*

Application of non-destructive methods: biomarker assays in blood of the white stork (*Ciconia ciconia*) nestlings

Dora Bjedov[1Δ], Alma Mikuška[1Δ], Carina Lackmann[2,3], Lidija Begović[1], Tibor Mikuška[4], Mirna Velki[1*]

[1]Department of Biology, Josip Juraj Strossmayer University of Osijek, Cara Hadrijana 8/A, 31000 Osijek, Croatia

[2]Department of Evolutionary Ecology and Environmental Toxicology, Goethe University Frankfurt, Max-von-Laue-Str. 13, 60438 Frankfurt/Main, Germany

[3]Department of Ecosystem Analysis, Institute for Environmental Research, ABBt – Aachen Biology and Biotechnology, RWTH Aachen University, Worringerweg 1, 52074 Aachen, Germany

[4]Croatian Society for Birds and Nature Protection, Ivana Gundulića 19/A, 31000, Osijek, Croatia

*Correspondence: mirna.velki@gmail.com ; mvelki@biologija.unios.hr ; Tel.: +385 (0)31 399 935

[Δ]Share first authorship on this work.

Number of pages: 5

Number of tables: 1

Number of figures: 7

Contents:

Table S1 Quantitative [ng μL^{-1}] and qualitative results of DNA isolation in S9 of white stork (*C. ciconia*) nestlings.

Sample	DNA [ng μL^{-1}]	$A_{260/280}$	$A_{260/230}$
Nestling 1	2673.80	1.976	2.104
Nestling 2	1363.90	1.999	1.703
Nestling 3	502.10	1.958	2.130
Nestling 4	604.15	2.017	2.005
Nestling 5	1118.50	2.028	2.116
Nestling 6	1380.30	2.084	2.089
Nestling 7	759.10	2.076	2.197
Nestling 8	598.65	2.099	2.156
Nestling 9	202.50	1.961	2.269
Nestling 10	180.00	1.981	1.900
Nestling 11	212.85	1.886	2.137
Nestling 12	431.60	1.975	2.150
Nestling 13	713.00	1.958	1.733
Nestling 14	814.00	2.033	1.835
Nestling 15	1344.80	1.987	1.913
Nestling 16	419.15	1.906	2.133

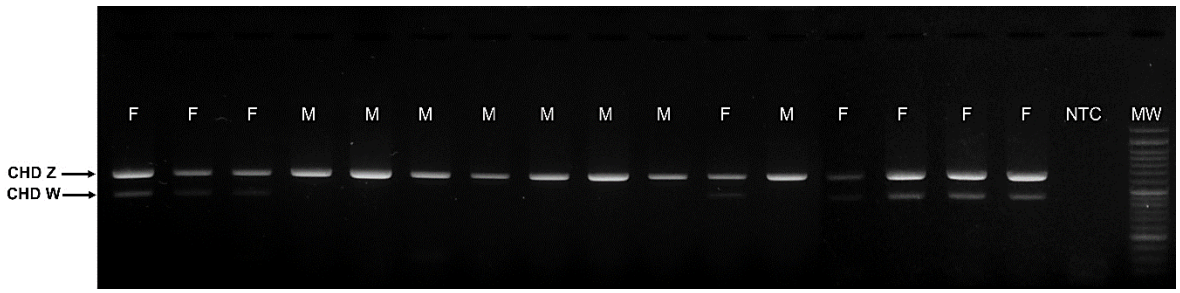

Figure S1 Results of molecular sex-typing. Females (F) have both CHD Z and CHD W fragments with 600 and 450 bp, respectively. Males (M) have only CHD Z fragment, 600 bp. NTC is negative control with no DNA template, and MW is molecular marker.

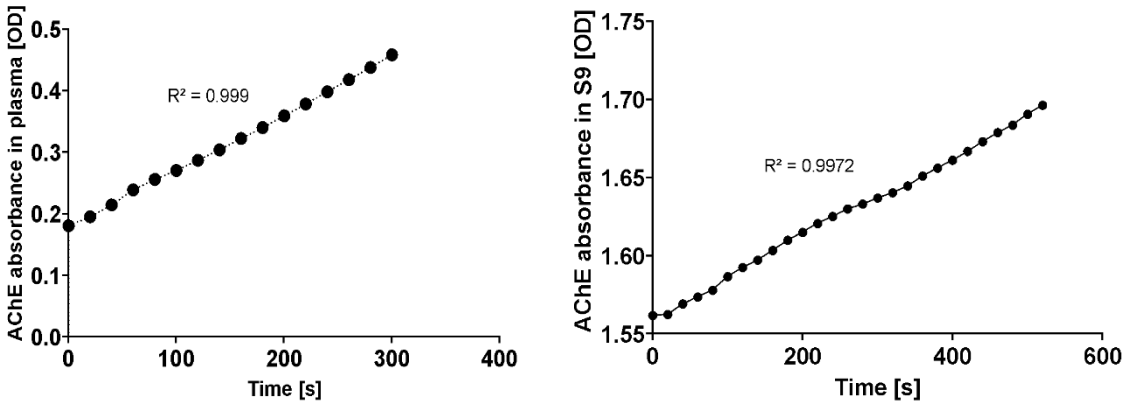

Figure S2 Increase in absorbance [OD] observed during measurement of acetylcholinesterase (AChE) activity in white stork (*C. ciconia*) nestlings. Plasma samples were measured for 5 min and S9 for 10 min (R-squared value of linear trendline is given in the figure).

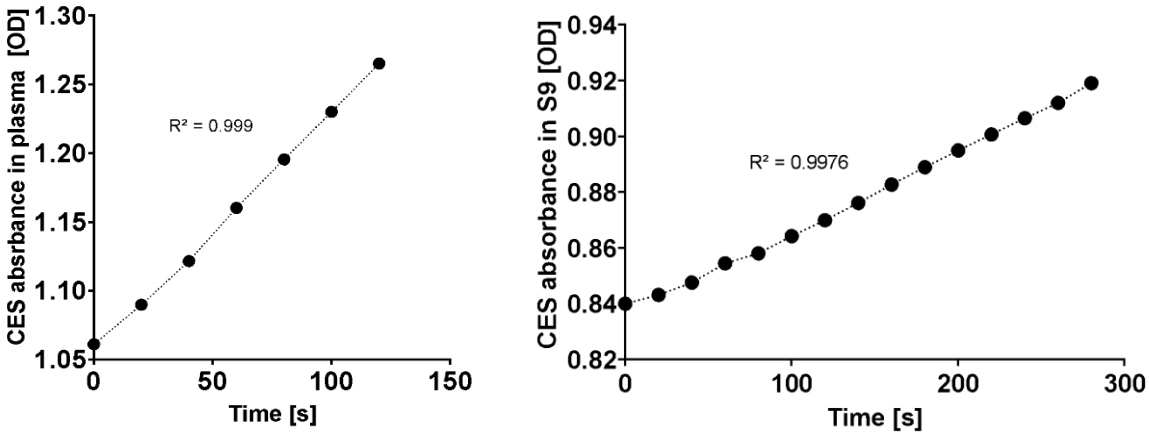

Figure S3 Increase in absorbance [OD] observed during measurement of carboxylesterase (CES) activity in white stork (*C. ciconia*) nestlings. Plasma samples were measured for 2 min and S9 for 5 min (R-squared value of linear trendline is given in the figure).

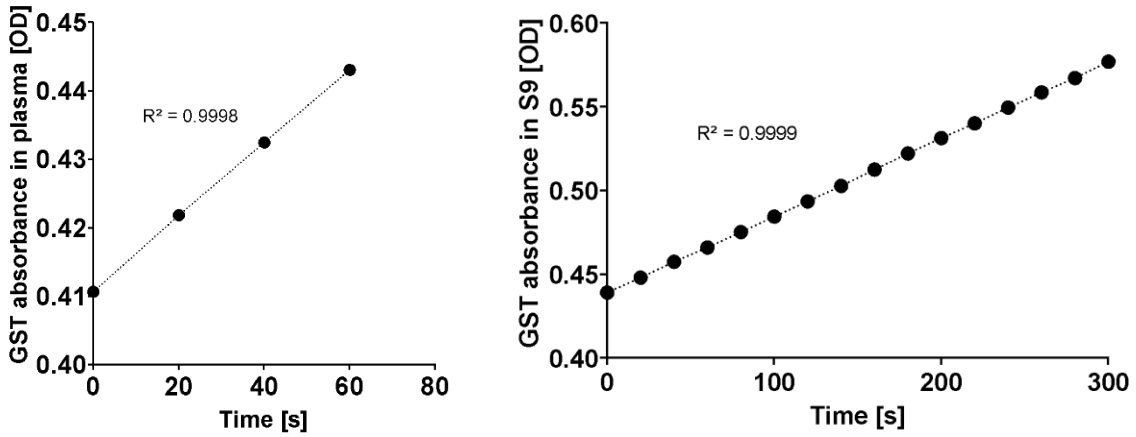

Figure S4 Increase in absorbance [OD] observed during measurement of glutathione S-transferase (GST) activity in white stork (*C. ciconia*) nestlings. Plasma samples were measured for 1 min and S9 for 5 min (R-squared value of linear trendline is given in the figure).

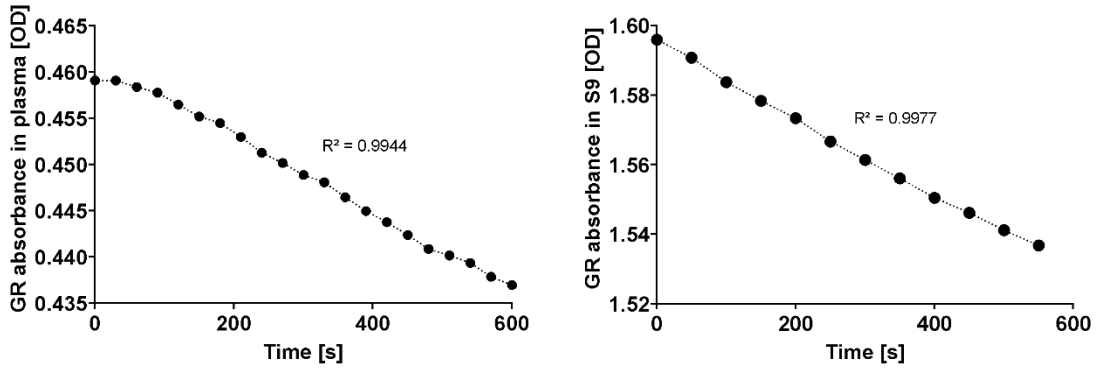

Figure S5 Decrease in absorbance [OD] observed during measurement of glutathione reductase (GR) activity in white stork (*C. ciconia*) nestlings. Plasma and S9 samples were measured for 10 min (R-squared value of linear trendline is given in the figure).

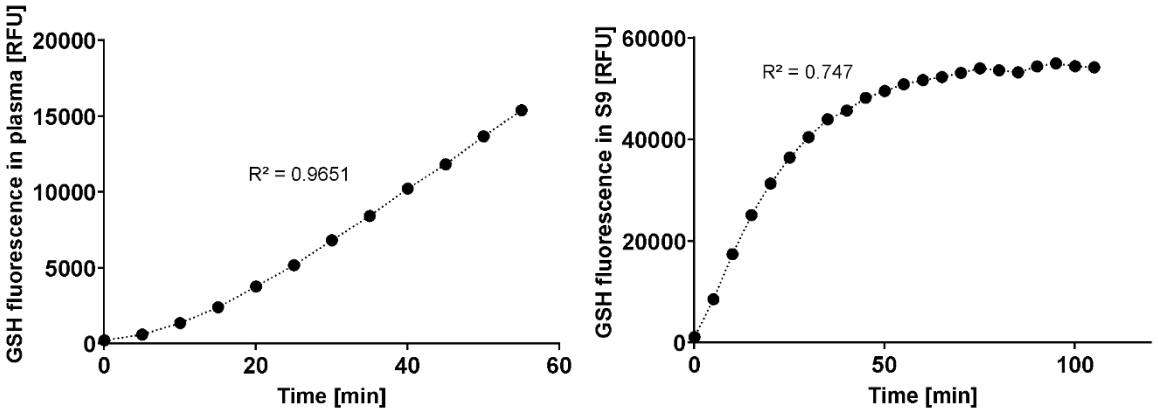

Figure S6 CellTracker^{TM} Green CMFDA dye was used for GSH detection. Increase in fluorescence [RFU] observed during measurement of glutathione (GSH) activity in white stork (*C. ciconia*) nestlings. Plasma samples were measured for 60 min and S9 for 120 min (R-squared value of linear trendline is given in the figure).

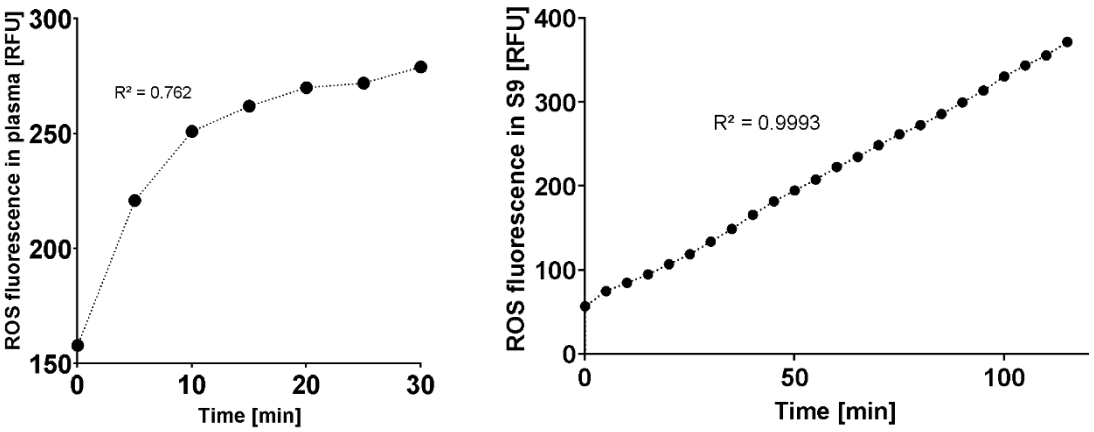

Figure S7 CM-H_2DCFDA dye was used for ROS detection. Increase in fluorescence [RFU] observed during measurement of reactive oxygen species (ROS) activity in white stork (*C. ciconia*) nestlings. Plasma samples were measured for 30 min and S9 for 120 min (R-squared value of linear trendline is given in the figure).

APPENDIX IV. *Supplementary information*

Blood biomarkers in white stork (*Ciconia ciconia*) nestlings show different responses at several locations in continental Croatia

Dora Bjedov[1], Mirna Velki[1], Carina Lackmann[2], Lidija Begović[1], Tibor Mikuška[3], Luka Jurinović[4], Alma Mikuška[1]*

[1]Department of Biology, Josip Juraj Strossmayer University of Osijek, Cara Hadrijana 8/A, 31000 Osijek, Croatia

[2]Department of Evolutionary Ecology and Environmental Toxicology, Goethe University Frankfurt, Max-von-Laue-Str. 13, 60438 Frankfurt/Main, Germany

[3]Croatian Society for Birds and Nature Protection, Ivana Gundulića 19/A, 31000

Osijek, Croatia

[4]Croatian Veterinary Institute, Poultry Centre, Heinzelova 55, 10000 Zagreb, Croatia

*Corresponding author:

Alma Mikuška, PhD, assistant professor

Josip Juraj Strossmayer University of Osijek

Department of Biology

Cara Hadrijana 8/A, 31 000 Osijek

Croatia

E-mail: amikuska@biologija.unios.hr

Table SI1. Results of sex determination in white stork (*Ciconia ciconia*) nestlings' S9 from five sampling locations: Lonjsko polje, Jelas polje, Slavonski Brod - east, Podunavlje and Donje Podravlje.

	Lonjsko polje	**Jelas polje**	**Slavonski Brod - east**	**Podunavlje**	**Donje Podravlje**
Males	13	13	13	12	9
Females	8	3	15	13	9

Table SI2. Descriptive statistics of biomarker analysis in white stork (*Ciconia ciconia*) nestlings. Data is presented as number of nestlings (*n*), mean and standard deviation (± *SD*) for each parameter in both plasma and S9 from five sampling locations: Lonjsko polje, Jelas polje, Slavonski Brod - east, Podunavlje and Donje Podravlje.

Parameter	Sample type		Location				
			Lonjsko polje	**Jelas polje**	**Slavonski Brod - east**	**Podunavlje**	**Donje Podravlje**
AChE (nmol min^{-1} mg$_{PROT}^{-1}$)	Plasma	*n*	*21*	*16*	*29*	*25*	*18*
		mean	22.98	21.63	26.73	20.35	15.63
		± *SD*	± 6.32	± 7.92	± 7.34	± 5.75	± 6.17
	S9	*n*	*20*	*14*	*27*	*23*	*16*
		mean	3.23	4.40	3.92	3.67	3.13
		± *SD*	± 1.57	± 2.13	± 3.05	± 1.61	± 1.26
CES (nmol min^{-1} mg$_{PROT}^{-1}$)	Plasma	*n*	*21*	*16*	*29*	*25*	*18*
		mean	22.51	25.85	39.26	24.51	23.29
		± *SD*	± 8.70	± 10.55	± 20.47	± 10.64	± 11.01
	S9	*n*	*19*	*15*	*27*	*25*	*17*
		mean	8.97	13.75	11.31	6.81	6.55
		± *SD*	± 4.87	± 6.60	± 9.60	± 3.04	± 3.45
GST (nmol min^{-1} mg$_{PROT}^{-1}$)	Plasma	*n*	*21*	*16*	*29*	*25*	*18*
		mean	20.47	23.33	21.70	23.01	20.29
		± *SD*	± 5.42	± 5.36	± 6.51	± 9.15	± 10.45
	S9	*n*	*20*	*15*	*27*	*23*	*16*
		mean	15.87	16.43	15.46	17.54	14.41
		± *SD*	± 2.66	± 3.44	± 5.01	± 4.54	± 2.94
GR (pmol min^{-1} mg$_{PROT}^{-1}$)	Plasma	*n*	*20*	*16*	*27*	*23*	*16*
		mean	307.46	442.47	626.37	289.16	357.36
		± *SD*	± 82.02	± 150.65	± 264.31	± 123.81	± 205.12
	S9	*n*	*20*	*15*	*27*	*24*	*16*
		mean	988.56	1341.82	1536.43	1316.99	840.55
		± *SD*	± 366.90	± 621.61	± 1051.75	± 701.54	± 235.42
GSH (RFU)	Plasma	*n*	*20*	*16*	*29*	*24*	*17*
		mean	8647	10909	8219	11282	7323
		± *SD*	± 1756	± 3463	± 2568	± 2668	± 1554
	S9	*n*	*21*	*16*	*29*	*25*	*17*
		mean	30870	31842	25518	30492	23687
		± *SD*	± 8479	± 9273	± 8224	± 7885	± 8430
ROS (RFU)	Plasma	*n*	*20*	*16*	*29*	*23*	*17*
		mean	67	71	99	98	76
		± *SD*	± 3	± 10	± 8	± 6	± 5
	S9	*n*	*21*	*16*	*29*	*25*	*18*
		mean	25	35	39	34	33
		± *SD*	± 9	± 12	± 14	± 12	± 12

APPENDIX V. *Supplementary information*

Heavy metal(loid) effect on multi-biomarker responses in apex predator: novel assays in the monitoring of white stork nestlings

Dora Bjedov[1], Mirna Velki[2], Leontina Toth[3], Vlatka Filipović Marijić[4], Tibor Mikuška[5], Luka Jurinović[6], Sandra Ečimović[2], Nataša Turić[3], Zdenko Lončarić[7], Sara Šariri[4], Yasir Al Marsoomi[8], Alma Mikuška[2]*

[1]Croatian Institute for Biodiversity, BIOTA Ltd., Maksimirska cesta 129/5, 10000 Zagreb, Croatia

[2]Department of Biology, Josip Juraj Strossmayer University of Osijek, Cara Hadrijana 8/A, 31000 Osijek, Croatia

[3]Teaching Institute of Public Health Osijek-baranja County, Franje Krežme 1, 31000 Osijek, Croatia

[4]Division for Marine and Environmental Research, Ruđer Bošković Institute, Bijenička 54, 10000 Zagreb, Croatia

[5]Croatian Society for Birds and Nature Protection, Ivana Gundulića 19/A, 31000 Osijek, Croatia

[6]Poultry Centre, Croatian Veterinary Institute, Heinzelova 55, 10000 Zagreb, Croatia

[7]Department of Agroecology and Environment Protection, Faculty of Agrobiotechnical Sciences Osijek, Josip Juraj Strossmayer University of Osijek, Vladimira Preloga 1, 31000 Osijek, Croatia

[8]HOGENT University of Applied Sciences and Arts, Geraard de Duivelstraat 5, 9000 Gent, Belgium

*Corresponding author

Alma Mikuška, PhD, assistant professor

Josip Juraj Strossmayer University of Osijek

Department of Biology

Cara Hadrijana 8/A, 31 000 Osijek

Croatia

E-mail: amikuska@biologija.unios.hr

Chemical analysis

Chemicals

For the protein concentration assay, the Pierce™ BCA Protein Assay Kit was used. In the present research, the following analytical grade chemicals were used: 5,5'–dithiobis–(2–nitrobenzoic acid) (DTNB) ($[–SC_6H_3(NO_2)CO_2H]_2$, CAS 69–78–3, 396.35 g $mol^{–1}$), (2–Mercaptoethyl) trimethylammonium iodide acetate (acetylthiocholine iodide) ($CH_3COSCH_2CH_2N(CH_3)_3I$, CAS 1866–15–5, 289.18 g $mol^{–1}$), acetonitrile (C_2H_3N, CAS 75–05–8, 41.05 g $mol^{–1}$), *p*–nitrophenyl acetate ($C_8H_7NO_4$, CAS 830–03–5, 181.15 g $mol^{–1}$), 1–chloro–2,4–dinitrobenzene (CDNB) ($C_6H_3ClN_2O_4$, CAS 97–00–7, 202.55 g $mol^{–1}$), β–nicotinamide adenine dinucleotide 2′–phosphate reduced tetrasodium salt hydrate (β–NADPH) ($C_{21}H_{26}N_7Na_4O_{17}P_3 \times H_2O$, CAS 2646–71–1 (anhydrous), 833.35 g $mol^{–1}$ (anhydrous basis)), glutathione disulfide (GSSG, $C_{20}H_{32}N_6O_{12}S_2$, CAS 27025–41–8, 612.60 g $mol^{–1}$), dimethyl sulphoxide (DMSO) (C_2H_6OS, CAS 67–68–5, 78.13 g $mol^{–1}$), CellTracker™ Green CMFDA Dye ($C_{25}H_{17}ClO_7$, CAS 136832–63–8, 464.86 g $mol^{–1}$) (ThermoFisher Scientific), CM–H_2DCFDA ($C_{27}H_{19}Cl_3O_8$, CAS 1219794–09–8, 577.80 g $mol^{–1}$) (ThermoFisher Scientific), disodium hydrogen phosphate (NaH_2PO_4, CAS 7558–79–4, 141.96 g $mol^{–1}$), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \times 2H_2O$, CAS 13472–35–0, 156.01 g $mol^{–1}$), sodium chloride (NaCl, CAS 7647–14–5, 54.40 g $mol^{–1}$), ammonium chloride (NH_4Cl, CAS 12125–02–9, 53.49 g $mol^{–1}$), ammonium hydroxide (NH_4OH, p.a. 25%, CAS 1336–21–6, 17.3 g $mol^{–1}$), hexamine cobalt (III) chloride ($[Co(NH_3)]_6Cl_3$, CAS 10534–89–1, 267.48 g $mol^{–1}$), ethylenediaminetetraacetic acid, (EDTA, $C_{10}H_{16}N_2O_8$, CAS 60–00–4, 292.24 g $mol^{–1}$), calcium chloride ($CaCl_2$, CAS 10043–52–4, 110.98 g $mol^{–1}$), ammonium hydroxide (NH_4OH, CAS 1336–21–6, 35.05 g $mol^{–1}$) nitric acid (HNO_3, CAS 7697–37–2, 63.01 g $mol^{–1}$), Seronorm™ Trace Elements Whole Blood (three levels, references 201505, 201605, and 201705).

Metalloids and heavy metals concentration

In 2020, a preliminary investigation of heavy metal and metalloid levels were analysed in S9. S9 samples were prepared with a 3:1 mixture of HNO_3 (Trace metal grade, Fischer) and H_2O_2 (Primar–trace metal grade, Fischer) to a final volume of 8 mL for digestion in a microwave oven (CEM Mars 6, USA). The concentrations of heavy metals and metalloids were measured using ICP–MS (Agilent 7500a Inductively Coupled Plasma Mass Spectrometer).

All the S9 samples were measured in duplicate with NIST® SRM® 1567b Wheat flour (Sigma-Aldrich) as the certified standard reference materials. The limit of detection (LOD) for the analysed elements was as follows: 0.070098962 μg L^{-1} for As, 0.168084451 μg L^{-1} for Se, 0.003114409 μg L^{-1} for Cd, 0.003842494 μg L^{-1} for Hg, and 0.243 μg L^{-1} for Pb. The limit of quantification (LOQ) for the analysed elements was: 0.233 μg L^{-1} for arsenic, 0.5 μg L^{-1} for Se, 0.01 μg L^{-1} for cadmium, 0.0128 μg L^{-1} for Hg, and 0.81 μg L^{-1} for Pb. Considering the analysed matrix being homogenised blood cells – S9, samples were standardised per mg of protein. Analyses of heavy metal and metalloid concentration in S9 were conducted at the Faculty of Agrobiotechnical Sciences Osijek, Croatia.

In 2021, whole blood samples were used to analyse heavy metal and metalloid concentrations. Prior to analysis, chemical equipment was thoroughly rinsed and soaked overnight in a 10% HNO_3 bath followed by washing and rinsing with milli–Q water. Whole blood samples were vortexed, and 250 μL was transferred into a PE tube, diluted with a mixture of 2% NH_4OH (*w:v*), 0.25% H_4EDTA (*w:v*), 7.5 g L^{-1} NaCl, and 0.5 g L^{-1} $CaCl_2$ to the final volume of 12.5 mL. The dilution factor was 1:50. The samples were homogenised, transferred to the measuring vessel and then analysed in three technical replicas. The linearity range of the standard solution for As, Se, Cd and Pb were 0 – 100 μg L^{-1} (0, 0.1, 1, 10, 50, 100 g L^{-1}, standard curve $R^2 > 0.9999$) and for Hg was 0 – 2 μg L^{-1} (0, 0.02, 0.5, 1, 1.5, 2 μg L^{-1}, standard curve $R^2 = 0.998$). Analytical recoveries were determined from the certified standard reference materials (Seronorm™ Trace Elements Whole Blood references 201505, 201605, and 201705) analysed in parallel with the samples. The range of recovery rates (in view of the concentrations in the reference material) ranged between 90% for Hg, 93% for Cd and Se, and 120 % for As. For the quantification of As, Se, Cd, Hg and Pb concentration, Germanium (^{72}Ge), Indium (^{115}In), and Bismuth (^{209}Bi) was used as internal standard, respectively. Analyses of heavy metal and metalloid concentration in whole blood were conducted at the Teaching Institute of Public Health Osijek-baranja County, Osijek, Croatia.

Metallothioneins concentration

Plasma samples were purified by separation of MTs from cytosolic proteins, by heat treatment in order to denature the high molecular weight proteins. Prior to heat treatment, plasma was first 2–folds diluted with 0.9% NaCl solution to avoid the co–precipitation of MT with high molecular mass proteins.

Samples were heated directly at 100 °C in pre–warmed Dri–block DB–3D (Techne Ltd., UK) for 15 min, and subsequently incubated on ice, in the refrigerator at 4 °C for 30 min. The coagulated proteins are then precipitated using pre–cooled benchtop centrifuge (Heraeus Biofuge Fresco, Kendro Laboratory Products, SAD) for 13000 *g*, (30 min, at 4°C). Approximately 130 – 150 μL of MT–rich supernatant was isolated. To perform MT measurements, 797 VA computrace (Metrohm, Switzerland) with three–electrode complex (Hanging mercury (Hg) drop electrode as working electrode, Ag/AgCl/saturated KCl as reference electrode, and a platinum counter electrode) was used. The samples were analysed in a 10 mL electrolyte solution containing 5 mL of 2 M NH_4Cl/NH_4OH and 5 mL of 1.2×10^{-3} M $Co(NH_3)6Cl_3$ (pH 9.5), the temperature was regulated to 20 °C using HAAKE K10 (Germany), and the electrochemical cell was purged with pure nitrogen. The following parameters were applied for the measurement of MT in the blood: start potential = –0.90 V, end potential = –1.65 V, voltage pulse amplitude = 0.02502 V, duration of the voltage pulse application = 0.057 s, Hg drop size = 4, duration of one step potential = 0.2 s, scan rate = 0.0130 V. MT concentrations were determined using a calibration curve obtained by the commercially available standard rabbit liver MT–95–L, 5 mg mL^{-1} (IKZUS Proteomics, USA). Prior to the calibration, a 19.6 μg mL^{-1} standard working solution was prepared and a linear calibration curve was constructed by four additions of the MT standard. The analyses of plasma metallothionein levels were conducted at Ruđer Bošković Institute, Zagreb, Croatia.

Table SI1. Descriptive statistics of metalloids arsenic (As) and selenium (Se), as well as heavy metals cadmium (Cd), mercury (Hg), and lead (Pb) analysed in whole blood from white stork (*Ciconia ciconia*) nestlings from season 2021, presented as number of nestlings (*n*), minimum (min), 25% percentile, median, 75% percentile, maximum (max) and range from seven sampling locations: landfill Jakuševec, Lonjsko polje, Crnac polje, Jelas polje, Slavonski Brod – east, Podunavlje and Donje Podravlje.

		Jakuševec	**Lonjsko polje**	**Crnac polje**	**Jelas polje**	**Slavonski Brod – east**	**Podunavlje**	**Donje Podravlje**
	n	*8*	*11*	*13*	*9*	*6*	*27*	*12*
As	Min	13.30	6.38	12.80	5.74	12.80	8.15	11.50
(μg L^{-1})	25% Percentile	21.43	8.65	17.15	9.77	14.23	14.70	11.95
	Median	45.55	13.30	20.90	13.20	21.30	50.50	17.70
	75% Percentile	52.15	16.50	33.55	22.15	33.23	94.60	20.73
	Max	55.90	37.30	55.60	25.70	41.40	388.00	24.40
	Range	42.60	30.92	42.80	19.96	28.60	379.90	12.90
Se	Min	468.00	946.00	936.00	169.00	654.00	396.00	451.00
(μg L^{-1})	25% Percentile	482.00	1096.00	977.50	678.50	690.80	546.00	536.30
	Median	503.00	1160.00	1452.00	950.00	739.50	634.00	579.00
	75% Percentile	578.50	1293.00	1594.00	1245.00	843.50	991.00	811.30
	Max	690.00	1581.00	1934.00	1470.00	1058.00	2370.00	869.00
	Range	222.00	635.00	998.00	1301.00	404.00	1974.00	418.00
Cd	Min	1.48	0.95	1.10	0.57	0.95	1.31	0.95
(μg L^{-1})	25% Percentile	1.59	1.24	1.62	1.40	1.38	1.66	1.90
	Median	1.80	1.44	1.86	1.78	1.74	2.77	2.26
	75% Percentile	2.42	1.98	2.44	2.32	6.46	3.85	2.34
	Max	3.52	2.13	2.80	2.98	17.70	4.82	2.51
	Range	2.04	1.18	1.70	2.41	16.75	3.51	1.56
Hg	Min	6.74	25.20	3.44	3.57	5.80	5.24	6.40
(μg L^{-1})	25% Percentile	7.66	41.50	4.76	8.50	6.25	8.78	7.68
	Median	21.00	83.80	13.40	16.20	19.75	14.30	17.60
	75% Percentile	28.35	156.00	14.15	23.90	23.30	34.80	31.03
	Max	30.80	238.00	17.90	40.90	25.40	90.40	36.50
	Range	24.06	212.80	14.46	37.33	19.60	85.16	30.10
Pb	Min	45.60	12.00	5.11	9.04	34.00	12.80	24.50
(μg L^{-1})	25% Percentile	72.88	20.30	14.85	12.90	45.40	21.70	43.03
	Median	105.00	28.10	24.70	18.90	64.05	33.00	61.30
	75% Percentile	154.00	122.00	30.30	28.10	148.80	48.80	81.03
	Max	168.00	205.00	38.10	42.20	393.00	149.00	85.10
	Range	122.40	193.00	32.99	33.16	359.00	136.20	60.60

Table SI2. Descriptive statistics of biomarker analysis in white stork (*Ciconia ciconia*) nestlings, presented as number of nestlings (*n*), mean and standard deviation (± SD) in plasma and S9 from seven sampling locations: landfill Jakuševec, Lonjsko polje, Crnac polje, Jelas polje, Slavonski Brod – east, Podunavlje and Donje Podravlje.

			Jakuševec	Lonjsko polje	Crnac polje	Jelas polje	Slavonski Brod – east	Podunavlje	Donje Podravlje
AChE	Plasma	*n*	*8*	*12*	*18*	*11*	*10*	*29*	*17*
(nmol min^{-1} mg_{PROT}^{-1})		Mean	24.36	25.39	27.21	25.74	33.21	33.81	30.55
		SD	3.73	5.35	6.90	5.44	9.07	7.67	13.84
	S9	*n*	*8*	*12*	*17*	*11*	*7*	*27*	*15*
		Mean	3.12	3.68	3.25	2.03	3.29	3.39	3.16
		SD	1.15	2.73	2.41	0.70	1.22	1.56	2.29
CES	Plasma	*n*	*8*	*12*	*18*	*11*	*9*	*30*	*17*
(nmol min^{-1} mg_{PROT}^{-1})		Mean	50.45	41.29	34.03	46.97	39.94	38.71	30.98
		SD	20.63	7.44	9.59	13.61	10.44	12.87	6.34
	S9	*n*	*7*	*9*	*16*	*10*	*7*	*24*	*11*
		Mean	3.92	8.09	7.23	7.74	11.51	4.94	6.51
		SD	1.72	6.19	4.34	2.51	5.37	2.62	1.49
GST	Plasma	*n*	*7*	*11*	*15*	*11*	*8*	*30*	*16*
(nmol min^{-1} mg_{PROT}^{-1})		Mean	7.93	7.88	8.22	7.97	8.34	8.30	7.70
		SD	1.01	1.53	1.50	1.08	1.47	1.89	1.76
	S9	*n*	*8*	*11*	*16*	*10*	*7*	*27*	*16*
		Mean	30.63	32.02	35.33	20.08	25.40	36.88	33.89
		SD	13.93	19.37	28.02	6.62	14.52	30.97	20.26
GR	Plasma	*n*	*8*	*8*	*12*	*8*	*8*	*24*	*12*
(pmol min^{-1} mg_{PROT}^{-1})		Mean	209.90	231.10	202.20	236.10	206.20	280.10	255.10
		SD	57.35	103.90	120.90	45.35	122.70	130.50	102.80
	S9	*n*	*8*	*12*	*17*	*10*	*6*	*27*	*15*
		Mean	1130.00	1205.00	1334.00	968.40	1386.00	1533.00	1649.00
		SD	451.60	566.10	548.40	344.50	459.40	626.80	1348.00
GSH	Plasma	*n*	*8*	*12*	*18*	*11*	*10*	*29*	*17*
(RFU)		Mean	6180	5356	5120	5347	4967	4532	4966
		SD	1399	976	1100	1444	1470	1205	1278
	S9	*n*	*8*	*12*	*18*	*10*	*10*	*30*	*17*
		Mean	19122	19514	19080	22048	14893	19329	17749
		SD	3373	3809	6130	3924	9457	6865	6519
ROS	Plasma	*n*	*14*	*18*	*24*	*15*	*15*	*36*	*23*
(RFU)		Mean	104.40	107.30	117.30	121.60	122.10	122.00	113.60
		SD	16.39	19.55	20.22	14.60	26.93	22.91	25.46
	S9	*n*	*8*	*12*	*18*	*11*	*10*	*30*	*16*
		Mean	48.21	42.11	55.28	43.00	51.63	47.36	46.40
		SD	17.97	25.10	32.96	27.23	16.79	23.39	21.31
Mt	Plasma	*n*	*5*	*7*	*11*	*9*	*8*	*19*	*11*
(mg mL^{-1})		Mean	0.44	0.46	0.45	0.45	0.46	0.47	0.48
		SD	0.09	0.03	0.05	0.08	0.08	0.07	0.12

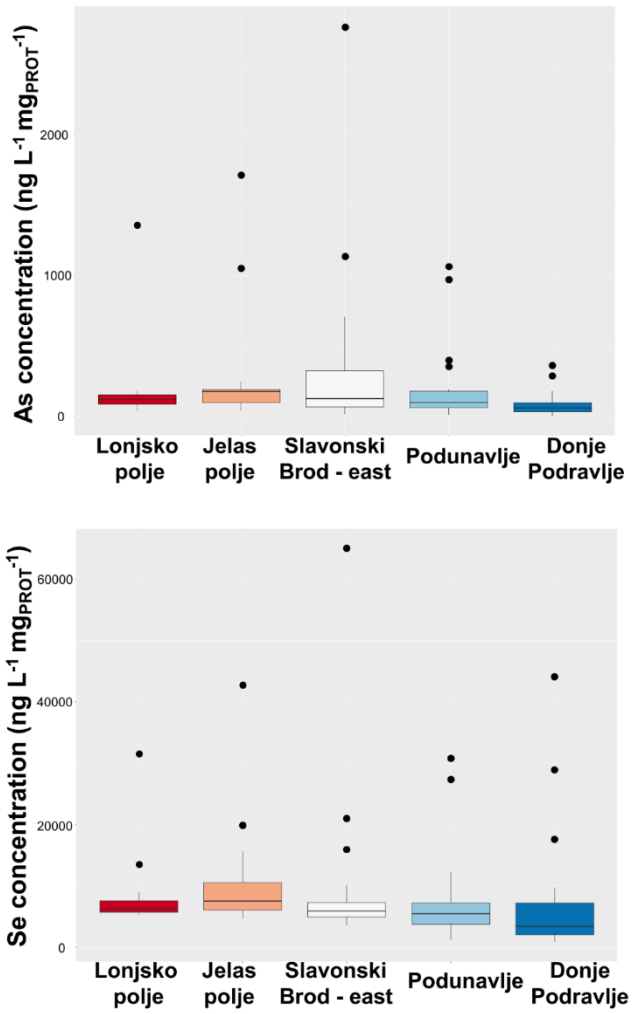

Figure SI1. Arsenic (As) and selenium (Se) concentrations (ng L^{-1} mg_{PROT}^{-1}) in S9 from white stork (*Ciconia ciconia*) nestlings sampled in 2020 from Lonjsko polje, Jelas polje, Slavonski Brod – east, Podunavlje and Donje Podravlje. Horizontal lines represent minimum and maximum values, the box represents values in 25th and 75th percentile with median as a central line. Significantly different observations (outliers) are represented as a black dot.

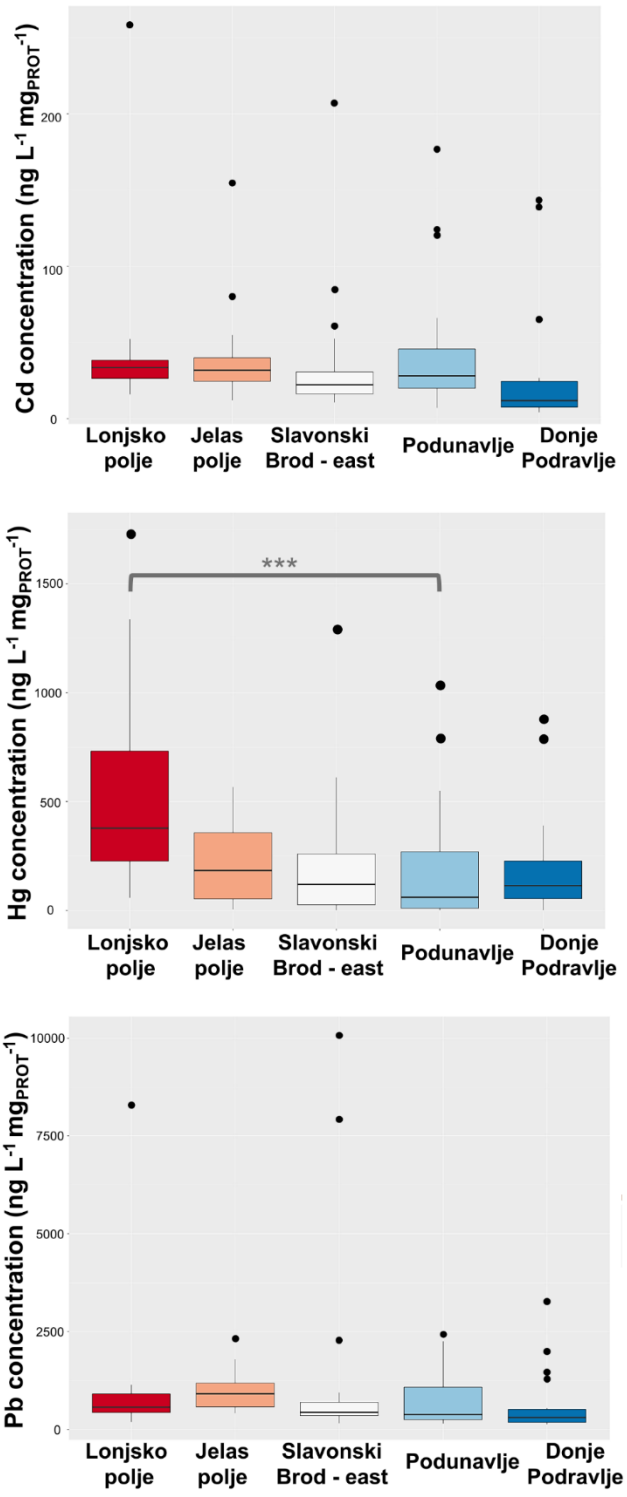

Figure SI2. Cadmium (Cd), mercury (Hg) and lead (Pb) concentrations (ng L^{-1} mg_{PROT}^{-1}) in S9 from white stork (*Ciconia ciconia*) nestlings sampled in 2020 from Lonjsko polje, Jelas polje, Slavonski Brod – east, Podunavlje and Donje Podravlje. Horizontal lines represent minimum and maximum values, the box represents values in 25th and 75th percentile with median as a central line. Different observations (outliers) are represented as a black dot. Statistical significance is presented with * ($p < 0.05$).