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White Stork (*Ciconia ciconia*) Nestlings Affected by Agricultural Practices? Assessment of Integrated Biomarker Responses

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Abstract: The present study assessed the effect of agricultural practices on biomarker response in white stork (*Ciconia ciconia*) nestlings from continental Croatia. During the breeding season of 2022, blood was sampled (n = 54) from the landfill Jakuševec and the agricultural areas Baranja and Srijem. Different patterns of biomarker response in regard to the sampling site were detected. Results demonstrate the presence of inhibitory pollutants from the landfill Jakuševec and agricultural area Baranja, which is reflected in lower cholinesterase (ChE) activity. Oxidative stress biomarkers show different responses concerning study sites. Glutathione reductase (GR) activity was higher in Baranja and Srijem, suggesting a different mixture of pollutants inducing an antioxidative response. Leachate from the landfill Jakuševec is suspected to affect the white stork nestlings by elevating the concentrations of reactive oxygen species (ROS). An environmental combination of pollutants appears to induce oxidative stress in white stork nestlings. In addition to agricultural practices, white stork nestlings may also be under environmental pressure from the surrounding pollution. Further research is warranted to include additional chemical analysis to associate the environmental concentrations with the potential adverse effects in apex predators, such as the white stork.

Keywords: blood; esterases; oxidative stress; birds; apex predator; pollution impact; agrochemicals; landfill

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

Academic Editor: Fernando P. Carvalho

Received: 2 April 2023
Revised: 5 May 2023
Accepted: 10 May 2023
Published: 12 May 2023

1. Introduction

Agricultural practices have formed the environment in the world and significantly affected the variety of bird populations [1]. Nearly two thousand bird species are directly impacted by specific types of biological resource use and agriculture. The largest impact on birds is from annual and perennial crop production [2]. Over the past century, the use of organochlorine pesticides, e.g., DDT, aldrin, dieldrin, and heptachlor, have significantly affected the reproduction of apex birds [3–6]. Industrialized agriculture, i.e., increased farming practices, started to dominate, resulting in manufactured, human-maintained, and anthropogenically oriented ecosystems [7]. The formation of man-made deserts is the repercussion of the enduring mismanagement of natural resources under the duress of uncontrolled livestock and human population. That said, land misuse may drive ecosystems beyond their degree of plasticity, resulting in irreversible changes [8]. Due to this, bird species richness and abundance have significantly decreased—the driving factors being

extensive cultivation and crop monoculture [9]. These factors are perceived as triggers for a decline in bird species richness and abundance, and the highest impact on bird communities can be seen at the local magnitude [10,11]. In addition to agricultural and industrial sources, today, pollution impacts at least 225 threatened bird species. Over the past century, the use of organochlorine pesticides, e.g., DDT, aldrin, dieldrin, and heptachlor, have significantly affected the decline of birds of prey populations [2–5]. Chronic ingestion of organochlorine pesticides in birds results in infertility and thinning of eggshells, which finally leads to a decline in breeding success and a decline in bird populations. Today, nearly half a century after the DDT ban, agrochemicals remain a major threat to wild birds [2]. The breeding success of different bird species was previously studied in agricultural landscapes [12] and landfills [13]. Promising and interesting in this respect is the white stork.

The white stork (*Ciconia ciconia*) is a breeding species distributed in the western Palearctic, associated with agriculture and arable (farmland) lands. They appear to be well-adapted to agricultural habitats. Although according to BirdLife [14], the global white stork population is the Least concern (LC), a decrease has been observed locally [15]. This inconsistency in population trend represents an unexpected issue warranting an investigation into its causes. Potential drivers could be land use in lowlands, i.e., cropland and grasslands. The drainage of wet meadows robs storks of their basic food supply. In the countries of Central and Eastern Europe, the intensification of agriculture is a major threat to storks [15]. These land use changes introduce diverse technologies, for instance, selective breeding and biofortification, as well as the application of different agrochemicals (pesticides and fertilisers) for the purpose of ecosystem management for the agricultural sector. All these practices, directly and indirectly, affect the environment [15–18].

When considering agricultural practices, several factors influence the use of arable lands by farmland birds, including white storks [18]. For example, a frequently studied factor is foraging efficiency [7,10,18,19]. Domesticated animals prevent succession, enriching the environment with small mammals and invertebrates—suitable prey for the white stork. That being said, this factor is considered to have a positive effect on the white stork population, and farmland birds in general [19]. On the other hand, agricultural practices include pesticide and fertilizer use as well, which is often associated with negative effects on birds [16,17]. Namely, the main effects of pesticides operating through the food chain are depleting the prey abundance and reducing the vegetation needed for herbivorous bird species and their prey [16]. An additional concern is a potential effect that arises after ingestion of polluted prey since pesticide exposure can affect homeostasis and cause physiological changes at different magnitudes [17,20].

For appropriate risk assessment, the exposure to environmental pollutants can be estimated in white storks, and farmland birds in general, using biomarkers in the blood [21–23]. Their nestlings could ingest polluted prey foraged by their parents and suffer secondary poisoning, which can be reflected in pollutant accumulation and consequential effects on biomarker responses in the blood. Biomarkers indicate normal biological homeostasis as well as disruption. For that purpose, they are an essential part of biomonitoring [24]. When assessing the magnitude of pesticide (also other pollutants) exposure, esterases are traditionally the biomarkers of choice [25]. The inhibition of cholinesterase is regarded as a suitable indicator of pesticide exposure and has been analysed in white stork blood [26]. Carboxylesterase activity is recommended as a biomarker of pesticide exposure in birds as shown in red-tailed hawks (*Buteo jamaicensis*), Swainson's hawks (*Buteo swainsoni*), Cooper's hawks (*Accipiter cooperii*), and red-shouldered hawks (*Buteo lineatus*) [25]. An additional effect of pollutant exposure includes an increase in oxidative stress biomarker response [27,28]. Oxidative stress is a result of excessive reactive oxygen species production and/or depletion of antioxidants [29]. These effects can be measured by analysing a battery of enzymatic and non-enzymatic biomarkers. The white stork has been previously used as a sentinel species for biomonitoring assessment as several oxidative stress biomarkers have been evaluated. In particular, glutathione-dependent enzymes have been analysed in white

stork nestlings' blood: glutathione S-transferase activity [22,23], glutathione reductase activity [30], glutathione peroxidase activity [31] and the concentration of glutathione [22,23].

In Croatia, the white stork is a regular breeding species in continental rural areas surrounded by intensively managed, mosaic agricultural land [32]. The last national census indicated breeding of over 1841 pairs with breeding pair densities from 0.5–12.4 pairs/100 km^2 [33,34]. In the present research, non-destructive sampling was performed, i.e., blood sampling, which is relevant for longitudinal (seasonal) biomonitoring and has practical application. Blood, as a potential matrix for assessment, is an easily accessible biological tissue, especially during ringing and this approach is beneficial to reduce the stress of each nestling [35]. The main objective of the present study was to assess the potential toxic pressure of environmental pollutants on the biomarker response in regard to different sampling areas. Namely, two sampling areas are known for distinctive agriculture, horticulture, and farming, while the landfill Jakuševec is an artificial habitat under human management. The second objective was to explore the biomarker response between the sexes of white stork nestlings, seeing as male white stork nestlings are approximately 5% heavier and larger [36].

2. Materials and Methods

2.1. Study Site

White stork breeding status has been monitored for more than 70 years, and the ringing scheme is performed annually [34]. White stork nestlings were sampled from nests located at the landfill Jakuševec and the agricultural areas Baranja and Srijem. The landfill Jakuševec has been a breeding site for white storks since 2012 (*Jurinović, pers. obs.*; Figure 1) and it is the only site in Croatia where breeding white storks are highly dependent on a landfill as the main foraging site. It is located in eastern Zagreb, proximal to the Sava River. The landfill is a disposal site for non-hazardous waste and a source of environmental pollutants, including biological and anthropogenic waste [37]. Srijem and Baranja incorporate different habitats: flood plains, extensively farmed meadows, pastures, and cultivated landscapes with nutrient-rich small water bodies near the Danube River (Figure 1). Although both areas are located in the proximity of the Danube floodplain, the surrounding area of Baranja is surrounded by large-scale cultivation of orchards, vineyards, maize agriculture, and cereal crops, while Srijem is covered by large- and small-scale cereal agriculture. Overall, intensive agriculture, horticulture, and farming, as well as the subsequent pesticide and fertiliser use might be the pollution source for both Baranja and Srijem [38]. During the breeding season, white storks forage within the radius of cca. 5 km from the nest. Within the single settlement, distances among occupied nests were from 10–500 m, while distances among different settlements with breeding storks were from 2–8 km [39,40].

2.2. Sampling Procedure and Blood Preparation

The present research was performed 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). Sampling was conducted during the breeding season of 2022 on 6–8 weeks old white stork nestlings (n = 54). From the landfill Jakuševec, 10 nestlings were sampled from 10 nests. Regarding the agricultural areas, 24 nestlings from 10 nests were sampled from Baranja and 20 nestlings from 13 different nests were sampled in Srijem. Approximately 4 mL of blood was collected with a 0.8 mm needle and sterile 5 mL syringe from the brachial vein and transferred to lithium heparin tubes. Samples were stored under cold and dark conditions for 6–8 h. Prior to centrifugation to obtain the supernatant—plasma (3000× g, 10 min, 4 °C)—200 µL of whole blood was transferred in a sterile tube for molecular sex determination. Plasma samples were transferred to sterile tubes and stored at −80 °C until biomarker analysis. Afterwards, pellets (blood cells) were prepared as described in detail in Bjedov et al. [21]. Briefly, the blood cells were suspended in a 5 mL phosphate buffer (0.1 M, pH 7.2), sonicated, and centrifuged (9000× g, 20 min, 4 °C) to acquire the blood cell homogenate—post mitochondrial fraction (S9). S9 samples

were kept at −80 °C until further biomarker analysis. All biomarkers were analysed in both types of samples—plasma and S9.

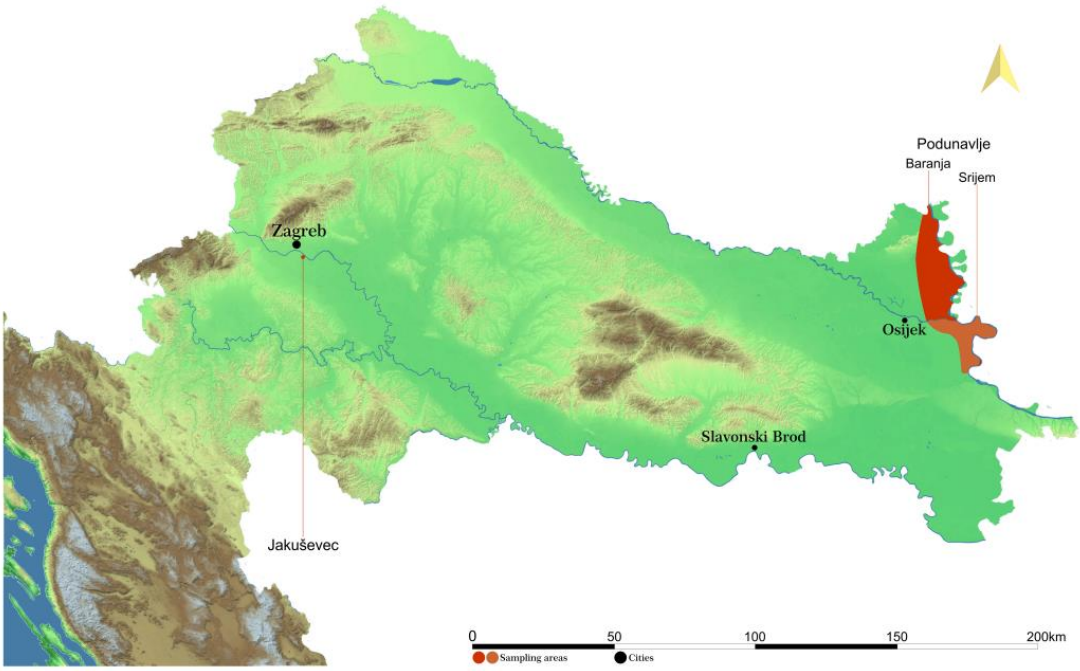

Figure 1. Sampling areas during the breeding season of 2022 in continental Croatia: landfill Jakuševec and agricultural areas Baranja and Srijem.

2.3. Chemicals

In the present research, the following analytical grade chemicals were used for biomarker measurement: 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 disulphide (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, Waltham, MA, USA), 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}). For molecular sex determination, the following analytical grade chemicals were used: 2–amino–2–(hydroxymethyl)–1,3–propanediol (Trizma® base) ($C_4H_{11}NO_3$, CAS 77–86–1, 121.14 g mol^{-1}), sodium dodecyl sulphate (SDS, $C_{12}H_{25}NaO_4S$, CAS 151–21–3, 288.38 g mol^{-1}), ethylenediaminetetraacetic acid disodium salt dihydrate, (EDTA, $C_{10}H_{14}N_2Na_2O_8 \times 2\ H_2O$, CAS 6381–92–6, 372.24 g mol^{-1}), sodium chloride (NaCl, CAS 7647–14–5, 58.44 g mol^{-1}), proteinase K (solution 20 mg mL^{-1}, CAS 39450–01–6), 1,4–Dithiothreitol (DTT, $C_4H_{10}O_2S_2$, CAS 3483–12–3, 154.2 g mol^{-1}), sodium acetate anhydrous ($NaCH_3COO$, CAS 127–09–3, 82.03 g mol^{-1}), acetic acid ($C_2H_4O_2$, CAS 64–19–7, 60.05 g mol^{-1}), Fast Gene Agarose (Nippon Genetics EUROPE), EmeraldAmp MAX PCR Master Mix (Takara), primers (Metabion), Midori Green Advance DNA stain (Nippon Genetics EUROPE), Fast gene 50 bp DNA ladder RTU (Nippon Genetics EUROPE). For the protein concentration assay, the Pierce™ BCA Protein Assay Kit was used.

2.4. Esterase Activity

Esterase activity was analysed as described in detail in Bjedov et al. [21]. Briefly, cholinesterase (ChE) activity was determined with DTNB (1.6 mM) and phosphate buffer (0.1 M, pH 7.2) [41]. Using the *p*–nitrophenyl acetate (1 mM), carboxylesterase (CES) activity was measured according to the protocol developed by Hosokawa and Satoh [42].

2.5. Oxidative Stress Biomarkers

Samples were analysed according to the protocol described in Bjedov et al. [21] In brief, the activity of glutathione S–transferase (GST) activity was analysed using Habig and Jakoby's [43] protocol with CDNB (1 mM) and GSH (25 mM). Glutathione reductase (GR) activity was measured with GSSG (2 mM), β–NADPH (1 mM), and phosphate buffer (0.1 M, pH 7.2) [43]. Detection of glutathione (GSH) was performed using CellTracker™ Green fluorescent dye (9.78 µM). For the detection of reactive oxygen species (ROS), fluorescent dye CM–H_2DCFDA (7.87 µM) was applied. Samples and negative and positive controls as well as blanks were performed in parallel and measured in triplicates using Tecan Spark 10M microplate reader with the following settings: 485 nm (excitation wavelength), 530 nm (emission wavelength), and 50 (gain).

2.6. Protein Content

Protein content determination was performed with a Pierce™ BCA Protein Assay Kit. The concentration of the proteins was performed with the Tecan Spark 10M microplate reader. With the combination of the protocol provided in the kit and the protocol described in Bjedov et al. [21], the working solution was prepared. Bovine serum albumin was used as a reference standard.

2.7. Molecular Sex Determination

Molecular sex determination was performed from whole blood samples, according to the protocol as described in Bjedov et al. [21], based on *CHD* gene amplification [44]. In brief, DNA was isolated from blood with extraction buffer Tris-NaCl-EDTA buffer (EDTA (10 mM), Tris-Cl (10 mM, pH 8.0), NaCl (100 mM), 2% SDS), proteinase K (10 mg mL^{-1} stock concentration), and DTT (1 M). After the incubation, NaOAc was added and samples were incubated on ice. Samples were centrifuged, and isopropanol was added, following additional centrifugation. A supernatant containing genomic DNA was obtained and DNA was quantified using a NanoPhotometer. Amplification of the *CHD* gene and visualisation of PCR products were performed according to the protocol of Begović et al. [45].

2.8. Data Analysis

Data analysis was performed using R version 4.2.3. In the preliminary investigation of the data, potential outliers were visually inspected with a Cleveland dot plot and subsequently investigated with the Grubbs test (package *outliers* [46]). A Shapiro-Wilk test was used to test the normality of the data distribution [47]. For normally distributed datasets, linear mixed-effect modelling was performed, using the *lme* function (package *nlme* [48]).

To identify the spatial differences in enzymatic and non-enzymatic biomarker response, the linear mixed-effects model (LMM) for nested random effects was used. The LMM was constructed using the variables *sampling location* and *biomarker response* as fixed effects and the variable *nest* as a random effect. An analysis of variance (ANOVA; [49]) was performed on LMM, and *post-hoc* comparisons were performed using the estimated marginal means (least-squares means) *emmeans* function, adjusted by the Tukey method for multiple comparisons (package *emmeans;* [50]).

To determine sex differences in enzymatic and non-enzymatic biomarker response, an LMM was created with the variables *biomarker response* and *sex* as fixed effects, and *nest* as a random effect. An ANOVA was performed on this LMM, and a *post-hoc* test was carried out using the *emmeans* function, adjusted by the Tukey method for multiple comparisons.

Different LMM output candidates were identified for each analysed biomarker, and the selection procedure was performed using Akaike's Information Criterion corrected for small sample sizes (AICc). Candidate models were reconstructed to test the hypothesis regarding the variation in biomarker response using the *aictab* function (package *AICcmodavg* [51]). In addition, the *null* model was included, meaning no effect, and the model with the most parsimony was defined based on the AICc value and AICc weight (w_i) [52]. The variables *biomarker response*, *sampling location,* and *sex* were fixed factors, while the variable *nest* was a random variable.

Data regarding specific ChE activity in S9 did not follow normal data distribution; therefore, non-parametric tests were applied. The median for each nest was calculated and the Kruskal-Wallis test was performed. Dunn's *post-hoc* test corrected for multiple comparisons was used to specify spatial differences. The null hypothesis was rejected at $\alpha = 0.05$ throughout the study.

3. Results

Results showed a significant difference in biomarker responses. A complete overview of descriptive statistics regarding esterase activity (ChE and CES) and oxidative stress biomarker response (GST, GR, GSH, and ROS) is shown in Table 1, and detailed outputs of statistical analyses are shown in Table S1. To simulate reference values, a table was constructed with the previously analysed biomarker responses [22,23] to compare with the values from the present study, in relation to the year of sample collection (Table S3).

Table 1. Descriptive statistics of esterase activity and oxidative stress biomarkers from two blood fractions (plasma and S9) analysed in white stork (*Ciconia ciconia*) nestlings from landfill Jakuševec and agricultural areas Baranja and Srijem.

		Jakuševec		**Baranja**		**Srijem**	
		Plasma	**S9**	**Plasma**	**S9**	**Plasma**	**S9**
ChE ($nmol\ min^{-1}\ mg_{PROT}{}^{-1}$)	*n*	*10*	*10*	*24*	*24*	*19*	*20*
	min.	16.22	3.29	19.61	2.65	21.20	2.37
	max.	51.51	7.00	37.56	8.02	47.47	10.03
	range	35.29	3.70	17.95	5.36	26.26	7.66
	mean	27.31	4.76	27.83	4.63	32.49	4.22
	SD	10.02	1.42	5.43	1.50	7.97	1.88
CES ($nmol\ min^{-1}\ mg_{PROT}{}^{-1}$)	*n*	*10*	*10*	*24*	*24*	*19*	*20*
	min.	10.22	6.67	17.78	6.38	19.69	6.64
	max.	62.77	12.84	53.44	10.65	67.80	11.42
	range	52.55	6.18	35.66	4.28	48.11	4.78
	mean	32.89	8.31	37.33	8.25	36.07	8.55
	SD	16.43	1.73	9.52	1.16	11.68	1.29
GST ($nmol\ min^{-1}\ mg_{PROT}{}^{-1}$)	*n*	*10*	*10*	*24*	*24*	*17*	*19*
	min.	9.87	8.30	9.39	8.60	9.30	7.06
	max.	17.22	17.90	18.77	25.22	17.49	25.52
	range	7.35	9.61	9.38	16.62	8.19	18.46
	mean	13.17	13.25	13.67	14.64	12.65	13.94
	SD	2.63	3.65	2.31	3.74	2.18	5.02
GR ($pmol\ min^{-1}\ mg_{PROT}{}^{-1}$)	*n*	*10*	*10*	*24*	*23*	*19*	*20*
	min.	141.50	535.40	158.30	479.50	144.50	556.40
	max.	364.70	1534.00	605.80	1543.00	431.60	1623.00
	range	223.20	998.20	447.50	1064.00	287.10	1067.00
	mean	266.30	923.00	290.40	845.90	234.40	1028.00
	SD	79.16	285.20	97.18	265.40	72.17	250.20

Table 1. *Cont.*

		Jakuševec		Baranja		Srijem	
		Plasma	**S9**	**Plasma**	**S9**	**Plasma**	**S9**
GSH (RFU)	*n*	*10*	*10*	*24*	*23*	*18*	*17*
	min.	2062	17,294	2940	17,990	2012	15,363
	max.	6306	23,564	7426	25,407	4571	24,685
	range	4244	6270	4486	7418	2558	9323
	mean	4097	21,066	4055	21,955	3509	21,097
	SD	1116	1987	1026	1749	585	2752
ROS (RFU)	*n*	*10*	*10*	*24*	*24*	*19*	*20*
	min.	90.33	26.67	92.33	21.33	97.67	21.33
	max.	137.30	80.67	137.70	72.00	133.00	71.67
	range	47.00	54.00	45.33	50.67	35.33	50.33
	mean	118.50	52.13	111.80	39.19	118.50	38.65
	SD	14.11	16.84	10.50	13.98	8.22	17.77

ChE—cholinesterase; CES—carboxylesterase; GST—glutathione S-transferase; GR—glutathione reductase; GSH—glutathione; ROS—reactive oxygen species; RFU—relative fluorescence unit; *n*—number of nestlings; min.—minimum value; max.—maximum value; *SD*—standard deviation.

Results of esterase activity are shown in Figure 2 and Table 1. The difference in ChE activity was detected only in plasma, and not in S9 (Figure 2, Table 1). Specifically, changes in plasma ChE activity were observed in nestling from Srijem compared to Jakuševec ($p = 0.01$). Significant differences between the study sites regarding CES activity in plasma and S9 were not observed (Figure 2, Table 1).

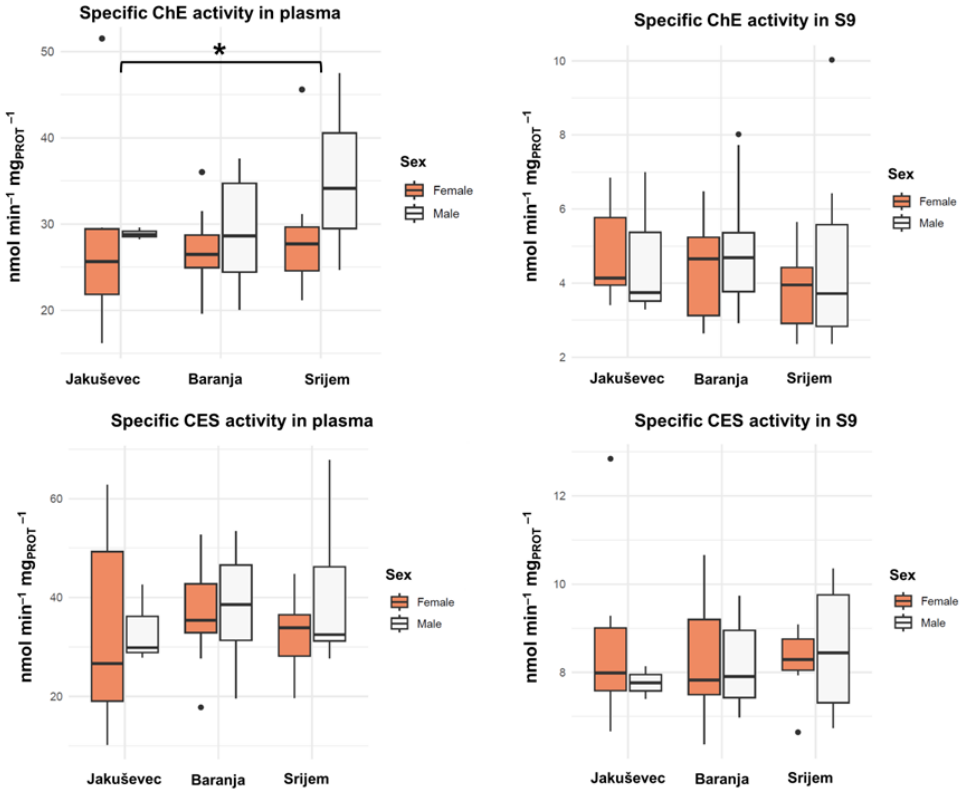

Figure 2. Biomarker responses between sexes: specific cholinesterase (ChE) and carboxylesterase (CES) activity in white stork (*Ciconia ciconia*) nestlings' blood—plasma and S9 from landfill Jakuševec, and agricultural areas Baranja and Srijem. Data are shown with a boxplot: the box represents values in the 25th and 75th percentile with the median as a central line. Black dots represent outliers. Orange boxplots represent females and bone-white boxplots represent males. Significant differences (LMM, ANOVA followed by Tukey *post-hoc*) between the study sites are marked with * ($p < 0.05$).

Results of enzymatic oxidative stress biomarkers are shown in Figure 3, Table 1. There were no statistically significant differences in GST activity in both plasma and S9 (Figure 3, Table 1). Significant differences were detected in GR activity between the study sites (Figure 3, Table 1). Plasma GR activity was significantly higher in nestlings from Baranja compared to Srijem ($p = 0.04$). Similar GR activity in S9 was observed between all sampling sites (Figure 3, Table 1).

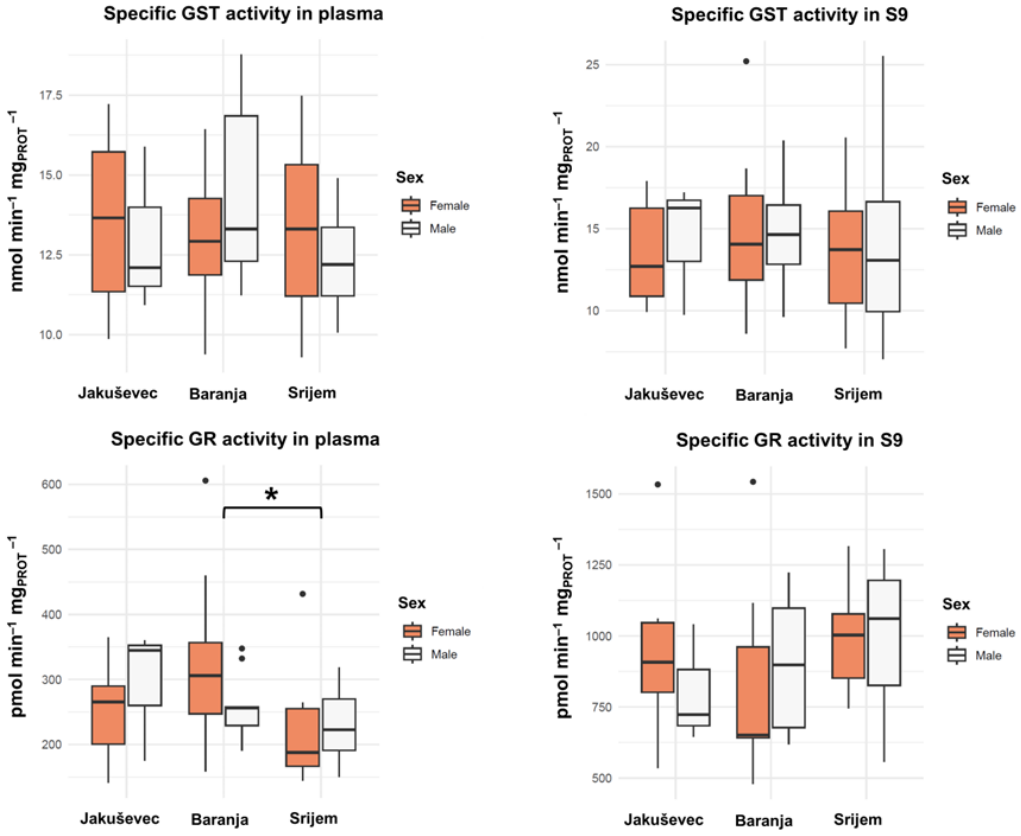

Figure 3. Biomarker responses between sexes: specific glutathione S-transferase (GST) and glutathione reductase (GR) activity in white stork (*Ciconia ciconia*) nestlings' blood—plasma and S9 from landfill Jakuševec, and agricultural areas Baranja and Srijem. Data are shown with a boxplot: the box represents values in the 25th and 75th percentile with the median as a central line. Black dots represent outliers. Orange boxplots represent females and bone-white boxplots represent males. Significant differences (LMM, ANOVA followed by Tukey *post-hoc*) between the study sites are marked with * ($p < 0.05$).

Results of fluorescent non-enzymatic oxidative stress biomarkers are shown in Figure 4 and Table 1. No changes in plasma or S9 GSH concentration in regard to sampling areas were observed. In addition, similar responses regarding ROS levels were not detected in both plasma and S9 (Figure 4, Table 1).

An overview of candidate model output and their succession based on the AICc value and w_i is shown in Table 2. Although there were no significant differences in biomarker response between sexes (Figures 2–4), several models included explanatory variables *sampling location* and *sex*. Variation in the biomarker responses CES activity in plasma, GST activity in plasma and S9, GR activity in S9, GSH concentration in S9, and ROS concentration in plasma and S9 was best elucidated by sampling location and association to sex. On the other hand, the biomarker responses ChE activity in plasma and S9, CES activity in S9, GR activity in plasma, and GSH concentration in plasma are best explained by spatial variability.

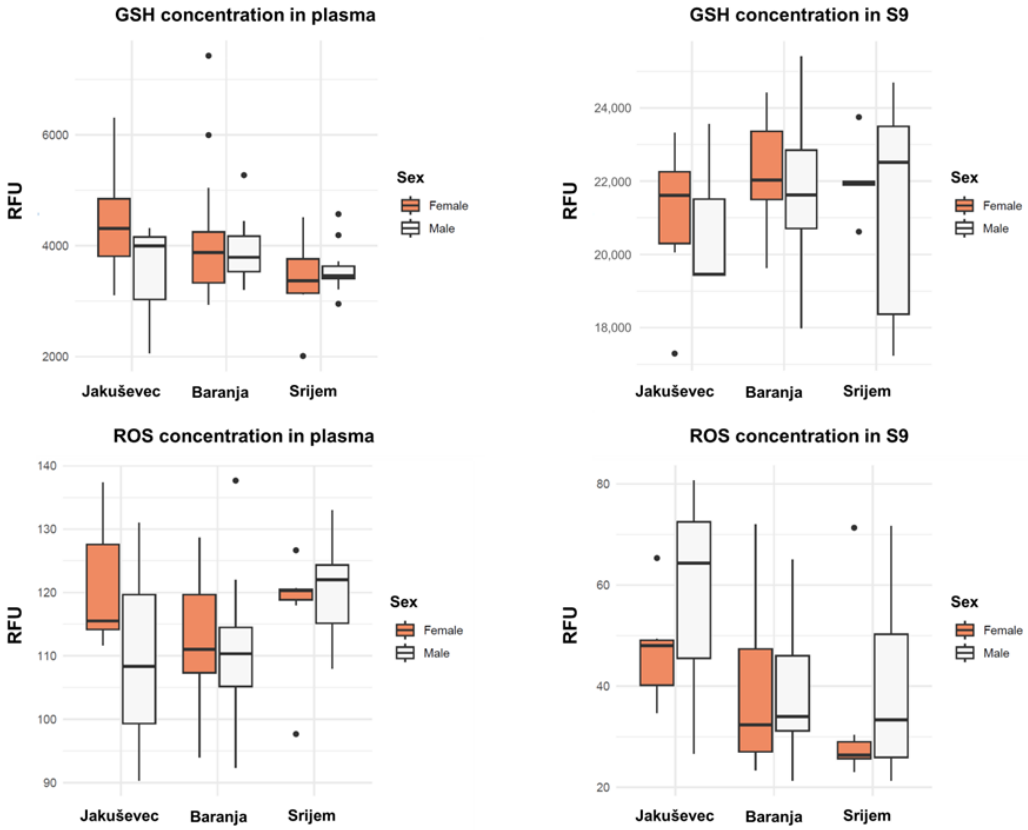

Figure 4. Relative fluorescence of glutathione (GSH) and reactive oxygen species (ROS) concentration between sexes in white stork (*Ciconia ciconia*) nestlings' blood—plasma and S9 from landfill Jakuševec, and agricultural areas Baranja and Srijem. Data are shown with a boxplot: the box represents values in the 25th and 75th percentile with the median as a central line. Black dots represent outliers. Orange boxplots represent females and bone-white boxplots represent males.

Table 2. Model selection outputs for individual candidate linear mixed effect models (variable nest as a random factor) potentially interpreting the observed enzymatic and non-enzymatic biomarker response in the blood of white stork (*Ciconia ciconia*) nestlings' blood—plasma and S9 from landfill Jakuševec and agricultural areas Baranja and Srijem.

Candidate Models	K	AICc	Δ_i	w_i	Res. LL
ChE Plasma ~ sampling location + sex	6	336.87	0.00	0.57	−161.48
ChE Plasma ~ sampling location	5	337.54	0.67	0.41	−168.10
ChE Plasma ~ null	3	343.76	6.89	0.02	−168.63
ChE S9 ~ null	3	195.07	0.00	0.61	−94.29
ChE S9 ~ sampling location	5	195.94	0.87	0.39	−92.33
ChE S9 ~ sampling location + sex	6	209.58	14.51	0.00	−97.86
CES Plasma ~ sampling location + sex	8	381.89	0.00	1.00	−181.23
CES Plasma ~ sampling location	5	393.29	11.40	0.00	−190.99
CES Plasma ~ null	3	398.61	16.73	0.00	−196.06
CES S9 ~ null	3	173.55	0.00	0.76	−83.53
CES S9 ~ sampling location	5	175.87	2.31	0.24	−82.30
CES S9 ~ sampling location + sex	6	336.87	163.32	0.00	−161.48
GST Plasma ~ sampling location + sex	6	234.15	0.00	0.67	−110.10
GST Plasma ~ null	3	236.81	2.66	0.18	−115.15
GST Plasma ~ sampling location	5	237.04	2.89	0.16	−112.85
GST S9 ~ sampling location + sex	6	292.95	0.00	0.50	−139.52
GST S9 ~ sampling location	5	294.22	1.27	1.27	−141.46
GST S9 ~ null	3	294.54	1.60	0.23	−144.02

Table 2. *Cont.*

Candidate Models	K	AICc	Δ_i	w_i	Res. LL
GR Plasma ~ sampling location	5	332−89	0.00	1.00	−160.79
GR Plasma ~ null	3	344.04	11.16	0.00	−168.77
GR Plasma ~ sampling location + sex	6	578.28	245.40	0.00	−282.19
GR S9 ~ sampling location + sex	6	678.64	0.00	1.00	−332.36
GR S9 ~ sampling location	5	717.68	39.05	0.00	−353.20
GR S9 ~ null	3	738.77	60.13	0.00	−366.14
GSH Plasma ~ sampling location	5	794.85	0.00	0.94	−391.76
GSH Plasma ~ sampling location + sex	6	800.47	5.61	0.06	−393.28
GSH Plasma ~ null	3	820.31	25.45	0.00	−406.90
GSH S9 ~ sampling location + sex	6	817.38	0.00	1.00	−401.67
GSH S9 ~ sampling location	5	872.89	55.51	0.00	−430.76
GSH S9 ~ null	3	899.66	82.28	0.00	−446.57
ROS Plasma ~ sampling location + sex	6	377.38	0.00	1.00	−181.73
ROS Plasma ~ sampling location	5	393.67	16.30	0.00	−191.20
ROS Plasma ~ null	3	401.37	23.99	0.00	−197.44
ROS S9 ~ sampling location + sex	6	424.95	0.00	1.00	−206.54
ROS S9 ~ sampling location	5	445.28	20.33	0.00	−217.02
ROS S9 ~ null	3	456.63	31.68	0.00	−225.07

K—number of estimated parameters; AICc—Akaike's Information Criterion value corrected for small sample size; Δi—the difference in AICc value compared to that of the model with most parsimony; w_i—Akaike weight (model probability); Res. LL—restricted log-likelihood of each model.

Results of molecular sex determination showed 25 females and 26 males (Table S2). In particular, 6 nestlings were female and three nestlings were male from the landfill Jakuševec, 12 nestlings were female and 11 nestlings were male from Baranja, and 7 female nestlings and 12 male nestlings were recorded in Srijem. Regarding analysed biomarkers, there were no significant differences between the biomarker response between female and male nestlings (Table S2).

4. Discussion

The present study assessed the potential impact of foraging and breeding in the vicinity of the previously mentioned ecosystem management and landfill on white stork nestlings via biomarker measurements in blood. It is frequently used for environmental pollution monitoring and fitness assessment of the nestlings is reflected in haematological parameters and molecular biomarkers [53,54]. Moreover, the effect of current exposure to pollutants can be determined in blood, regardless of their subsequent accumulation in different tissue [55,56].

The impact of various pollutants can be detected on ChE activity in white stork nestlings' plasma (Figure 2, Table 1). The main difference between plasma and S9 ChE activity can be attributed to the fact that ChE is primarily found in the nervous system and muscular systems in the role of neurotransmitter hydrolysis [57]. Changes in ChE activity, namely inhibition, are traditionally used as a potential biomarker of organophosphate insecticide exposure [58–60]. For example, reduced ChE activity was observed in pigeons, *Columba livia*, American kestrels, *Falco sparverius*, Swainson's hawks, *B. swainsoni*, Cooper's hawks, *A. cooperi*, and Red-shouldered hawks, *B. lineatus* after exposure to organophosphate pesticides [25]. Surprisingly, the lowest observed ChE activity was recorded in white stork nestlings from the landfill Jakuševec. Exposure to pesticides can occur in non-agricultural areas as well. In addition, a mixture of different chemicals can also affect ChE activity. For example, research on ambient air in Jakuševec showed traces of persistent organic pollutants (POPs), e.g., organochlorine pesticides (dichlorodiphenyltrichloroethane, DDT and metabolites), polychlorinated biphenyls (PCB congeners) [61], polycyclic aromatic hydrocarbons (PAHs) [62] and polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs) [63]. Furthermore, in the Sava River (water measuring station Jankomir and Rugvica) adjacent to the Jakuševec landfill, Croatian Waters [64], reported elevated con-

centrations of tributyltin compounds (TBT), mercury (Hg), and Hg-compounds [64]. Hg neurotoxicity has been previously investigated in the common loon, *Gavia immer,* and bald eagle, *Haliaeetus leucocephalus* [65]. Other chemicals are also known to cause ChE activity inhibition, for example, quaternary ammonium cations [66–68]. Quaternary ammonium salts could be found in landfills as they are often used in medicine as disinfectants and surfactants as well as in common household items such as fabric softeners and shampoos [69]. Overall, it is possible that the white stork nestlings from the landfill Jakuševec are exposed to leachate consisting of a mixture of inhibitory agrochemicals and various pollutants.

Eastern Croatia is known for its agricultural practices, although some differences are present between the regions (Baranja and Srijem). For example, Baranja is more developed in the agricultural sense, with many orchards, vineyards, and cereal farming, and uses more pesticides than any other county in Croatia [38,70]. The above-mentioned agricultural activities may contribute to pesticide pollution, especially since the soil and plants are treated during the breeding season (April–June) and can subsequently affect the developing white storks [33]. According to Romić et al. [38], most commonly used pesticides contributing to environmental pollution are herbicides, fungicides, aminophosphonates, pyrimidines, and dithiocarbamates [38]. For instance, dithiocarbamate is a fungicide used for treating seeds that alters endocrine and metabolic activities, as shown in red munia, *Amandava amandava* [71]. These changes in agricultural practises may introduce the pollutant impact on oxidative stress in white stork nestlings' blood. Changes in GR activity are observed in the nestlings from Baranja, compared to nestlings from Srijem in plasma (Figure 3, Table 1). The differences between the plasma and S9 can be attributed to GR being a primarily cellular enzyme. GR usually accumulates in the regions within the cell that are associated with high electron flux [72]. Baranja and Srijem are areas influenced by the Danube flooding regime, introducing pollutant concentrations that affect the GR activity. In Baranja, Croatian Waters [59], reported a high concentration of polybrominated diphenyl ethers (PBDE; brominated diphenyl ethers −28, −47, −99, −100, −153, and −154), mercury (Hg), and Hg-compounds in the Danube (Batina measuring station) [59]. PBDE association with oxidative stress has been investigated in several bird species. Experimental dosing of American kestrels (*F. sparverius*) to PBDEs induced oxidative stress in the liver, particularly in females, as well as a decrease in plasma and hepatic retinol and plasma thyroxine concentrations [73]. PBDEs induced blood changes regarding oxidative stress on both biochemical levels and gene expression in spotted owlet (*Athene brama*) and black kite (*Milvus migrans*). On the other hand, several biomarkers of oxidative stress—thiobarbituric acid reactive substances (TBARS) GSH, GSSG and total sulfhydryl (TSH) concentration—do not appear to be affected by environmental PBDEs levels in tree swallow (*Tachycineta bicolor*) nestlings. Overall, PBDE can induce changes in GSH metabolism, increase oxidative stress biomarker response, and alter antioxidant concentrations, In addition, Hg-compounds, in particular methylmercury, are known to alter GSH metabolism in mallards, *Anas platyrhynchos* [74]. In previous research from Poland, GR activity was analysed in white stork nestlings, reporting significant changes in GR activity in regard to heavy metal concentrations [30]. Higher plasma GR activity in white stork nestlings from Baranja may be attributable to more intense agricultural practises as well as elevated levels of PBDE, Hg, and Hg compounds.

As a part of cell homeostasis, ROS are a by-product of metabolism and have advantages, e.g., a role in cell signalling, as well as disadvantages—the disbalance can lead to damaging biomolecules such as DNA, RNA, and proteins [75]. The difference between the plasma and S9 can be attributed to the sources of ROS. Plasma sources may be due to oxidases localised at the plasma membrane and generating ROS for cell signalling. In S9, a mitochondrial mechanism might be the source of cellular ROS [76]. As mentioned previously, the surrounding area of the landfill Jakuševec has elevated concentrations of POPs—DDT and its metabolites, PCB congeners, PAHs, PCDD/s, TBTs, Hg, and Hg compounds. POPs are known to affect cellular oxidative stress, both singularly and in combination [77]. On the other hand, PCDD/Ts and PCBs usually do not affect oxida-

tive stress [78]. However, an interplay between the compounds and other environmental pollutants is possible and thus may be affecting ROS concentrations in the cell.

Sexual dimorphism in adult white storks is present in body size, with adult males being about 12.5% larger than females. Even though it is not very apparent, male nestlings are approximately 5% heavier and larger [36]. We found sex to be a non-significant variable (Table S2). Furthermore, candidate model outputs from the present study stipulate that changes in plasma ChE activity and plasma GR activity may be explained by both spatial variability and sex (Table 2). There is a possibility that white stork nestlings are too immature at the time of blood sampling to manifest differences regarding accumulating pollutants to induce significant change. This may be due to sex hormones not being expressed enough to exhibit strong sexual dimorphism in nestlings. Tryjanowski et al. [36] confirmed significant variance in blood biochemical parameters with nestlings concerning sex. To our knowledge, there is a lack of an explanation to elucidate the reason there is a significant difference, or lack thereof, in biomarker responses analysed in blood between sexes in the early stages of white stork development.

Overall results indicate the possibility that agrochemicals are affecting the white stork nestlings; however, the cause for concern is not limited to modern agricultural practices. As one of the first modern synthetic insecticides in the 1940s, DDT and its metabolites are present in the environment today. DDT and its metabolites, as well as other POPs, are still current in the water, ambient air, and biota from continental Croatia, even though their use was banned or restricted in Croatia from the 1970s to the 1980s [79–82]. Considering POPs are chemical formulations that are highly persistent and have the ability to bioaccumulate, their effect might be interlaced with the effect of modern-day agrochemicals, causing synergistic effects in biota, and subsequently in apex predators, such as the white stork. For that purpose, there is a need to monitor the entire ecosystem at locations that are suspected of insufficient treatment and/or mitigation strategies to avoid secondary pollution sources.

5. Conclusions

The present study assessed several biomarker responses in two blood fractions in the white stork nestlings from continental Croatia. Results demonstrate the presence of inhibitory substances in the landfill Jakuševec and the agricultural area Baranja, which is reflected in lower ChE activity. Oxidative stress biomarkers show different responses in regard to study sites. GR activity in plasma was higher in Baranja, suggesting a different mixture of pollutants inducing an antioxidative response. Sex did not affect the biomarker response. Although we cannot conclude the range of pollutant effects, the effect of agricultural practices is possible. In addition, it appears white stork nestlings are generally under pressure from various pollutants in the surrounding environment making them suitable indicator species. Further research is warranted to include research on dietary aspects in terms of prey items as well as more sensitive biomarker endpoints as well as additional chemical analysis to associate the environmental concentrations to the potential adverse effects in apex predators, such as the white stork.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13051045/s1, Table S1: Detailed results of pairwise *post-hoc* statistical analysis of the adopted models, performed with *emmeans* package in R (version 4.2.3.). Fixed effects are variables *biomarker response* and *sampling location*, while random effect is the variable *nest*. *P* value was adjusted by Tukey method. Significant comparisons are bolded; Table S2: Results of molecular sex determination in white stork (*Ciconia ciconia*) nestlings' blood and biomarker response in females and males for each sampling location—Jakuševec, Baranja and Srijem; Table S3: Descriptive statistics of esterase activity and oxidative stress biomarkers from two blood fractions (plasma and S9) analysed in white stork (*Ciconia ciconia*) nestlings from landfill Jakuševec and agricultural areas Baranja and Srijem during breeding seasons 2020[a], 2021[b] and 2022[c].

Author Contributions: Conceptualization—D.B., M.V. and A.M; Investigation—D.B., L.S.K., I.L., L.J., T.M. and L.B.; Data Curation—D.B., M.V. and A.M.; Writing—Original Draft—D.B.; Writing—Review and Editing—All; Supervision—M.V. and A.M.; Funding acquisition—M.V., T.M., S.E., M.S.B., L.J. and A.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was financially and materially supported by the Department of Biology's projects Oxidative stress and metallothioneins as indicators of environmental pollution in the blood of white stork (*Ciconia ciconia*) nestlings (Grant number OZB-ZP2022-310528) and Wetland ornithofauna research: the presence of non-essential metals and anthropogenic particles in the diet of white storks and white-tailed eagles in Croatia (Grant number OZB-ZP2022-3105422). The Tecan Spark microplate reader was purchased with the Alexander von Humboldt Foundation equipment grant awarded to Mirna Velki.

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 Republic of 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 (Classification code: UP/I-612-07/20-48/130; Registry number: 517-05-1-1-20-4). No extra animal discomfort was caused during 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.

References

1. Sanderson, F.J.; Kloch, A.; Sachanowicz, K.; Donald, P.F. Predicting the effects of agricultural change on farmland bird populations in Poland. *Agric. Ecosyst. Environ.* **2009**, *129*, 37–42. [CrossRef]
2. Lees, A.C.; Haskell, L.; Allinson, T.; Bezeng, S.B.; Burfield, I.J.; Renjifo, L.M.; Rosenberg, K.V.; Viswanathan, A.; Butchart, S.H. State of the World's Birds. *Annu. Rev. Environ. Resour.* **2022**, *47*, 231–260. [CrossRef]
3. Ratcliffe, D.A. Studies of the recent breeding success of the peregrine, *Falco peregrinus*. *J. Reprod. Fertil. Suppl.* **1973**, *19*, 377–389. [PubMed]
4. Deweese, L.R.; McEwen, L.C.; Hensler, G.L.; Petersen, B.E. Organochlorine contaminants in passeriformes and other avian prey of the peregrine falcon in the western united states. *Environ. Toxicol. Chem.* **1986**, *5*, 675–693. [CrossRef]
5. Ratcliffe, D.A. Changes Attributable to Pesticides in Egg Breakage Frequency and Eggshell Thickness in Some British Birds. *J. Appl. Ecol.* **1970**, *7*, 67. [CrossRef]
6. Walker, C.H.; Hamilton, G.A.; Harrison, R.B. Organochlorine insecticide residues in wild birds in Britain. *J. Sci. Food Agric.* **1967**, *18*, 123–129. [CrossRef]
7. Kamiński, P.; Jerzak, L.; Kasprzak, M.; Kartanas, E.; Bocheński, M.; Hromada, M.; Baszyński, J.; Kozera, W.; Woźniak, A.; Ulrich, W. Do agricultural environments increase the reproductive success of White Stork *Ciconia ciconia* populations in South-Western Poland? *Sci. Total Environ.* **2020**, *702*, 134503. [CrossRef]
8. Le Houérou, H.N. Man-Made Deserts: Desertization Processes and Threats. *Arid. Land Res. Manag.* **2002**, *16*, 1–36. [CrossRef]
9. Azhar, B.; Puan, C.L.; Zakaria, M.; Hassan, N.; Arif, M. Effects of monoculture and polyculture practices in oil palm smallholdings on tropical farmland birds. *Basic Appl. Ecol.* **2014**, *15*, 336–346. [CrossRef]
10. Flohre, A.; Fischer, C.; Aavik, T.; Bengtsson, J.; Berendse, F.; Bommarco, R.; Ceryngier, P.; Clement, L.W.; Dennis, C.; Eggers, S.; et al. Agricultural intensification and biodiversity partitioning in European landscapes comparing plants, carabids, and birds. *Ecol. Appl.* **2011**, *21*, 1772–1781. [CrossRef]
11. Kirk, D.A.; Lindsay, K.E.; Brook, R.W. Risk of Agricultural Practices and Habitat Change to Farmland Birds. *Avian Conserv. Ecol.* **2011**, *6*, 5. [CrossRef]
12. Vander Haegen, W.M. Fragmention by agriculture influences reproductive success of birds in a shrubsteppe landscape. *Ecol. Appl.* **2007**, *17*, 934–947. [CrossRef] [PubMed]
13. Gilbert, N.I.; Correia, R.A.; Silva, J.P.; Pacheco, C.; Catry, I.; Atkinson, P.W.; Gill, J.A.; Franco, A.M.A. Are white storks addicted to junk food? Impacts of landfill use on the movement and behaviour of resident white storks (*Ciconia ciconia*) from a partially migratory population. *Mov. Ecol.* **2016**, *4*, 7. [CrossRef] [PubMed]
14. BirdLife. White Stork (Ciconia ciconia)—BirdLife Species Factsheet. 2023. Available online: http://datazone.birdlife.org/species/factsheet/white-stork-ciconia-ciconia (accessed on 9 March 2023).
15. Wuczyński, A.; Betleja, J.; Jerzak, L.; Król, W.; Mielczarek, P.; Profus, P.; Siekiera, A.; Siekiera, J.; Springer, S.; Sztwiertnia, H.; et al. Strong Declines of the White Stork *Ciconia ciconia* Population in South-Western Poland: A Differentiated Importance of Altitude and Land Use Changes. *Acta Ornithol.* **2022**, *5*, 255–271. [CrossRef]

16. Boatman, N.D.; Brickle, N.W.; Hart, J.D.; Milsom, T.P.; Morris, A.J.; Murray, A.W.A.; Murray, K.A.; Robertson, P.A. Evidence for the indirect effects of pesticides on farmland birds. *Ibis* **2004**, *146*, 131–143. [CrossRef]
17. Fry, D.M. Reproductive effects in birds exposed to pesticides and industrial chemicals. *Environ. Health Perspect.* **1995**, *103*, 165–171.
18. Buckingham, D.L.; Peach, W.J.; Fox, D.S. Effects of agricultural management on the use of lowland grassland by foraging birds. *Agric. Ecosyst. Environ.* **2006**, *112*, 21–40. [CrossRef]
19. Zbyryt, A.; Sparks, T.H.; Tryjanowski, P. Foraging efficiency of white stork *Ciconia ciconia* significantly increases in pastures containing cows. *Acta Oecol.* **2020**, *104*, 103544. [CrossRef]
20. Grue, C.E.; Shipley, B.K. Sensitivity of nestling and adult starlings to dicrotophos; an organophosphate pesticide. *Environ. Res.* **1984**, *35*, 454–465. [CrossRef]
21. Bjedov, D.; Mikuška, A.; Lackmann, C.; Begović, L.; Mikuška, T.; Velki, M. Application of non-destructive methods: Biomarker assays in blood of white stork (*Ciconia ciconia*) nestlings. *Animals* **2021**, *11*, 2341. [CrossRef]
22. Bjedov, D.; Velki, M.; Lackmann, C.; Begović, L.; Mikuška, T.; Jurinović, L.; Mikuška, A. Blood biomarkers in white stork (*Ciconia ciconia*) nestlings show different responses in several areas of Croatia. *J. Exp. Zool. A Ecol. Integr. Physiol.* **2022**, *337*, 547–558. [CrossRef] [PubMed]
23. Bjedov, D.; Velki, M.; Toth, L.; Filipović Marijić, V.; Mikuška, T.; Jurinović, L.; Ečimović, S.; Turić, N.; Lončarić, Z.; Šariri, S.; et al. Heavy metal(loid) effect on multi-biomarker responses in apex predator: Novel assays in the monitoring of white stork nestlings. *Environ. Pollut.* **2023**, *324*, 121398. [CrossRef] [PubMed]
24. Ezeji, E.; Anyalogbu, E.; Ezejiofor, T.; Udensi, J. Determination of Reduced Glutathione and Glutathione S-transferase of Poultry Birds Exposed to Permethrin Insecticide. *Am. J. Biochem.* **2012**, *2*, 21–24. [CrossRef]
25. Bartkowiak, D.J.; Wilson, B.W. Avian plasma carboxylesterase activity as a potential biomarker of organophosphate pesticide exposure. *Environ. Toxicol. Chem.* **1995**, *14*, 2149–2153. [CrossRef]
26. Oropesa, A.L.; Gravato, C.; Sánchez, S.; Soler, F. Characterization of plasma cholinesterase from the White stork (*Ciconia ciconia*) and its in vitro inhibition by anticholinesterase pesticides. *Ecotoxicol. Environ. Saf.* **2013**, *97*, 131–138. [CrossRef]
27. Koivula, M.J.; Eeva, T. Metal-related oxidative stress in birds. *Environ. Pollut.* **2010**, *158*, 2359–2370. [CrossRef]
28. Osičková, J.; Band'ouchová, H.; Kováčová, V.; Král, J.; Novotný, L.; Ondráček, K.; Pohanka, M.; Sedláčková, J.; Škochová, H.; Vitula, F.; et al. Oxidative stress and liver damage in birds exposed to diclofenac and lead. *Acta Vet. Brno.* **2014**, *83*, 299–304. [CrossRef]
29. Puppel, K.; Kapusta, A.; Kuczyńska, B. The etiology of oxidative stress in the various species of animals, a review. *J. Sci. Food Agric.* **2015**, *95*, 2179–2184. [CrossRef]
30. Kamiński, P.; Kurhalyuk, N.; Kasprzak, M.; Jerzak, L.; Tkachenko, H.; Szady-Grad, M.; Klawe, J.J.; Koim, B. The impact of element-element interactions on antioxidant enzymatic activity in the blood of white stork (*Ciconia ciconia*) chicks. *Arch. Environ. Contam. Toxicol.* **2009**, *56*, 325–337. [CrossRef]
31. Kamiński, P.; Kurhalyuk, N.; Jerzak, L.; Kasprzak, M.; Tkachenko, H.; Klawe, J.J.; Szady-Grad, M.; Koim, B.; Wiśniewska, E. Ecophysiological determinations of antioxidant enzymes and lipoperoxidation in the blood of White Stork *Ciconia ciconia* from Poland. *Environ. Res.* **2009**, *109*, 29–39. [CrossRef]
32. Radović, A.; Tepić, N. Using Corine Land Cover habitat database for the analysis of breeding bird habitat: Case study of white storks (*Ciconia ciconia*) from northern Croatia. *Biol. Bratisl.* **2009**, *64*, 1212–1218. [CrossRef]
33. Mikuska, T. Distribution and population status of White Storks in Croatia. In Proceedings of the 12th Meeting of the European Stork Villages, Čigoč, Croatia, 25–27 June 2015.
34. Kralj, J.; Barišić, S.; Tutiš, V.; Ćiković, D. *Atlas Selidbe Ptica Hrvatske*; HAZU: Zagreb, Croatia, 2013.
35. Espin, S.; García-Fernández, A.; Herzke, D.; Shore, R.; van Hattum, B.; Martínez-López, E. Sampling and Contaminant Monitoring Protocol for Raptors. 2016. Available online: https://www.researchgate.net/publication/294885017_Sampling_and_contaminant_monitoring_protocol_for_raptors (accessed on 9 March 2023).
36. Tryjanowski, P.; Sparks, T.H.; Bochenski, M.; Dabert, M.; Kasprzak, M.; Kaminski, P.; Mroczkowski, S.; Wisniewska, E.; Jerzak, L. Do males hatch first and dominate sex ratios in White Stork *Ciconia ciconia* chicks? *J. Ornithol.* **2011**, *152*, 213–218. [CrossRef]
37. Barčić, D.; Ivančić, V. Impact of the Prudinec/Jakuševec landfill on environment pollution. *Sumar. Lis.* **2010**, *137*, 347–358.
38. Romić, D.; Husnjak, S.; Mesić, M.; Salajpal, K.; Barić, K.; Poljak, M.; Romić, M.; Konjačić, M.; Vnučec, I.; Bakić, H. Utjecaj Poljoprivrede na Onečišćenje Površinskih i Podzemnih Voda u Republici Hrvatskoj. Hrvatske vode. Elaborate/Study. 2015. Available online: https://www.bib.irb.hr/775043 (accessed on 9 March 2023).
39. Zurell, D.; Eggers, U.; Kaatz, M.; Rotics, S.; Sapir, N.; Wikelski, M.; Nathan, R.; Jeltsch, F. Individual-based modelling of resource competition to predict density-dependent population dynamics: A case study with white storks. *Oikos* **2015**, *124*, 319–330. [CrossRef]
40. Zurell, D.; von Wehrden, H.; Rotics, S.; Kaatz, M.; Groß, H.; Schlag, L.; Schäfer, M.; Sapir, N.; Turjeman, S.; Wikelski, M.; et al. Home Range Size and Resource Use of Breeding and Non-breeding White Storks Along a Land Use Gradient. *Front. Ecol. Evol.* **2018**, *6*, 79. [CrossRef]
41. Ellman, G.L.; Courtney, K.D.; Andres, V.; Featherstone, R.M. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **1961**, *7*, 88–95. [CrossRef]
42. Hosokawa, M.; Satoh, T. Measurement of Carboxylesterase (CES) Activities. *Curr. Protoc. Toxicol.* **2001**, *10*, 4.7.1–4.7.14. [CrossRef]
43. Habig, W.H.; Jakoby, W.B. Assays for Differentiation of Glutathione S-Transferases. *Methods Enzymol.* **1981**, *77*, 398–405.

44. Fridolfsson, A.K.; Ellegren, H. A Simple and Universal Method for Molecular Sexing of Non-Ratite Birds. *J. Avian Biol.* **1999**, *30*, 116–121. [CrossRef]
45. Begović, L.; Mihić, I.; Pospihalj, T.; Mikuška, T.; Mlinarić, S.; Mikuška, A. Evaluation of methods for molecular sex-typing of three heron species from different DNA sources. *Turk. J. Zool.* **2017**, *41*, 593–598. [CrossRef]
46. Grubbs, F. Sample criteria for testing outlying observations. *Ann. Math. Stat.* **1950**, *21*, 37–58. [CrossRef]
47. Shapiro, S.S.; Wilk, M.B. An analysis of variance test for normality (complete samples). *Biometrika* **1965**, *52*, 591–611. [CrossRef]
48. Searle, S.R.; Speed, F.M.; Milliken, G.A. Population marginal means in the linear model: An alternative to least squares means. *Am. Stat.* **1980**, *34*, 216–221.
49. Girden, E. *ANOVA: Repeated Measures*; Sage Publications, Inc.: New York, NY, USA, 1992.
50. Lenth, R.; Singmann, H.; Love, J.; Buerkner, P.; Herve, M. Emmeans: Estimated Marginal Means; aka Least-Squares Means. 2019. Available online: https://cran.microsoft.com/snapshot/2018-01-13/web/packages/emmeans/emmeans.pdf (accessed on 9 March 2023).
51. Mazerolle, M.J. *Package "AICcmodavg"*; R Package: Vienna, Austria, 2017.
52. Mazerolle, M.J. Model Selection and Multimodel Inference Using the AICcmodavg Package. R Vignette. 2020. Available online: https://mirror.marwan.ma/cran/web/packages/AICcmodavg/vignettes/AICcmodavg.pdf (accessed on 9 March 2023).
53. Bailly, J.; Faivre, B.; Bernard, N.; Sage, M.; Crini, N.; Driget, V.; Garnier, S.; Rieffel, D.; Scheifler, R. Multi-element analysis of blood samples in a passerine species: Excesses and deficiencies of trace elements in an urbanization study. *Front. Ecol. Evol.* **2017**, *5*, 6. [CrossRef]
54. Coeurdassier, M.; Fritsch, C.; Faivre, B.; Crini, N.; Scheifler, R. Partitioning of Cd and Pb in the blood of European blackbirds (*Turdus merula*) from a smelter contaminated site and use for biomonitoring. *Chemosphere* **2012**, *87*, 1368–1373. [CrossRef] [PubMed]
55. Fairbrother, A. Environmental Contaminants in Wildlife: Interpreting Tissue Concentrations. *J. Wildl. Dis.* **1997**, *33*, 383–384. [CrossRef]
56. Hribšek, I.; Jovičić, K.; Karadžić, B.; Skorić, S. Allocation of Metals and Trace Elements in Different Tissues of Piscivorous Species *Phalacrocorax carbo*. *Arch. Environ. Contam. Toxicol.* **2017**, *73*, 533–541.
57. Quinn, D.M. Acetylcholinesterase: Enzyme Structure, Reaction Dynamics, and Virtual Transition States. *Chem. Rev.* **1987**, *87*, 955–979. [CrossRef]
58. Sanchez-Hernandez, J.C.; Sanchez, B.M. Lizard cholinesterases as biomarkers of pesticide exposure: Enzymological characterization. *Environ. Toxicol. Chem.* **2002**, *21*, 2319–2325. [CrossRef]
59. Thompson, H. Serum "B" esterases as indicators of exposure to pesticides. In *Cholinesterase-Inhibiting Insecticides: Their Impact on Wildlife and the Environment*; Mineau, P., Ed.; Elsevier Science Publishing Company Inc.: New York, NY, USA, 1991; pp. 110–125.
60. Thompson, H.M. Esterases as markers of exposure to organophosphates and carbamates. *Ecotoxicology* **1999**, *8*, 369–384. [CrossRef]
61. Herceg Romanić, S.; Krauthacker, B. Organochlorine Pesticides and Polychlorinated Biphenyls in Ambient Air Collected in Zagreb, Croatia. *Bull. Environ. Contam. Toxicol.* **2000**, *64*, 811–816. [CrossRef] [PubMed]
62. Ahel, I.; Tepić, N.; Britvić, S.; Ahel, M. Assessment of organic pollution in the Jakuševec landfill (Croatia) by biomarker and chemical methods. *Fresenius Environ. Bull.* **2000**, *9*, 726–733.
63. Krauthacker, B.; Herceg Romanić, S.; Wilken, M.; Milanović, Z. PCDD/Fs in ambient air collected in Zagreb, Croatia. *Chemosphere* **2006**, *62*, 1829–1837. [CrossRef]
64. Musić, V.; Šikoronja, M.; Tomas, D.; Varat, M. Izvješće o Stanju Površinskih Voda u 2019. godini. 2020. Available online: https://voda.hr/sites/default/files/2022-05/izvjesce_o_stanju_povrsinskih_voda_u_2020._godini_0.pdf (accessed on 9 March 2023).
65. Scheuhammer, A.M.; Basu, N.; Burgess, N.M.; Elliott, J.E.; Campbell, G.D.; Wayland, M.; Champoux, L.; Rodrigue, J. Relationships among mercury, selenium, and neurochemical parameters in common loons (*Gavia immer*) and bald eagles (*Haliaeetus leucocephalus*). *Ecotoxicology* **2008**, *17*, 93–101. [CrossRef] [PubMed]
66. Changeux, J.P. Responses of Acetylcholinesterase from *Torpedo marmorata* to Salts and Curarizing Drugs. *Mol. Pharmacol.* **1966**, *2*, 369–392.
67. Coleman, M.H.; Eley, D.D. The inhibition of acetylcholinesterase by quaternary ammonium ions. *Biochim. Biophys. Acta* **1962**, *58*, 231–238. [CrossRef] [PubMed]
68. Colović, M.B.; Krstić, D.Z.; Lazarević-Pašti, T.D.; Bondžić, A.M.; Vasić, V.M. Acetylcholinesterase inhibitors: Pharmacology and toxicology. *Curr. Neuropharmacol.* **2013**, *11*, 315–335. [CrossRef] [PubMed]
69. Ramakrishnan, A.; Blaney, L.; Kao, J.; Tyagi, R.D.; Zhang, T.C.; Surampalli, R.Y. Emerging contaminants in landfill leachate and their sustainable management. *Environ. Earth Sci.* **2015**, *73*, 1357–1368. [CrossRef]
70. Bašić, F.; Bogunović, M.; Božić, M.; Husnjak, S.; Jurić, I.; Kisić, I.; Mesić, M.; Mirošević, M.; Romić, D.; Žugec, I. Regionalisation of Croatian agriculture. *Agric. Conspec. Sci.* **2007**, *72*, 27–38.
71. Pandey, S.P.; Mohanty, B. The neonicotinoid pesticide imidacloprid and the dithiocarbamate fungicide mancozeb disrupt the pituitary-thyroid axis of a wildlife bird. *Chemosphere* **2015**, *122*, 227–234. [CrossRef]
72. Couto, N.; Wood, J.; Barber, J. The role of glutathione reductase and related enzymes on cellular redox homoeostasis network. *Free Radic. Biol. Med.* **2016**, *95*, 27–42. [CrossRef]
73. Fernie, K.J.; Shutt, J.L.; Mayne, G.; Hoffman, D.; Letcher, R.J.; Drouillard, K.G.; Ritchie, I.J. Exposure to Polybrominated Diphenyl Ethers (PBDEs): Changes in Thyroid, Vitamin A, Glutathione Homeostasis, and Oxidative Stress in American Kestrels (*Falco sparverius*). *Toxicol. Sci.* **2005**, *88*, 375–383. [CrossRef] [PubMed]

74. Hoffman, D.J.; Heinz, G.H. Effects of mercury and selenium on glutathione metabolism and oxidative stress in mallard ducks. *Environ. Toxicol. Chem.* **1998**, *17*, 161–616. [CrossRef]
75. Pizzino, G.; Irrera, N.; Cucinotta, M.; Pallio, G.; Mannino, F.; Arcoraci, V.; Squadrito, F.; Altavilla, D.; Bitto, A. Oxidative Stress: Harms and Benefits for Human Health. *Oxid. Med. Cell Longev.* **2017**, *2017*, 8416763. [CrossRef] [PubMed]
76. Stier, A.; Bize, P.; Schull, Q.; Zoll, J.; Singh, F.; Geny, B.; Gros, F.; Royer, C.; Massemin, S.; Criscuolo, F. Avian erythrocytes have functional mitochondria; opening novel perspectives for birds as animal models in the study of ageing. *Front. Zool.* **2013**, *10*, 33. [CrossRef]
77. Abbasi, N.A.; Arukwe, A.; Jaspers, V.L.B.; Eulaers, I.; Mennillo, E.; Ibor, O.R.; Frantz, A.; Covaci, A.; Malik, R.N. Oxidative stress responses in relationship to persistent organic pollutant levels in feathers and blood of two predatory bird species from Pakistan. *Sci. Total Environ.* **2017**, *580*, 26–33. [CrossRef]
78. Hilscherova, K.; Blankenship, A.; Kannan, K.; Nie, M.; Williams, L.L.; Coady, K.; Bursian, S. Oxidative stress in laboratory-incubated double-crested cormorant eggs collected from the Great Lakes. *Arch. Environ. Contam. Toxicol.* **2003**, *45*, 533–546. [CrossRef]
79. Herceg Romanić, S.; Marenjak, T.S.; Klinčić, D.; Janicki, Z.; Srebočan, E.; Konjević, D. Organochlorine compounds in red deer (*Cervus elaphus* L.) and fallow deer (*Dama dama* L.) from inland and coastal Croatia. *Environ. Monit. Assess.* **2012**, *184*, 5173–5180. [CrossRef]
80. Klinčić, D.; Herceg Romanić, S.; Matek Sarić, M.; Grzunov, J.; Dukić, B. Polychlorinated biphenyls and organochlorine pesticides in human milk samples from two regions in Croatia. *Environ. Toxicol. Pharmacol.* **2014**, *37*, 543–552. [CrossRef]
81. Bosnir, J.; Puntarić, D.; Smit, Z.; Klarić, M.; Grgić, M.; Kosanović, L.M. Organochlorine pesticides in freshwater fish from the Zagreb area. *Arh. Hig. Rada Toksikol.* **2007**, *58*, 187–193. [CrossRef]
82. Lazarus, M.; Tariba Lovaković, B.; Orct, T.; Sekovanić, A.; Bilandžić, N.; Đokić, M.; Kolanović, B.S.; Varenina, I.; Jurič, A.; Lugomer, M.D.; et al. Difference in pesticides; trace metal(loid)s and drug residues between certified organic and conventional honeys from Croatia. *Chemosphere* **2021**, *266*, 128954. [CrossRef] [PubMed]