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From wetlands to landfills: white stork (*Ciconia ciconia* L., 1758) as a reliable bioindicator of ecosystem health

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White storks (*Ciconia ciconia* L., 1758) and their nestlings have emerged as valuable bioindicators of environmental pollution, particularly in ecosystems affected by human activities. This review explores the role of white storks in biomonitoring, focusing on the use of biomarkers and pollutant analysis to understand the physiological consequences of environmental stressors. Key biomarkers, such as oxidative stress markers, immune responses, and hormonal alterations provide insight into the effects of pollutants like heavy metals, pesticides, and other toxic compounds. The biomarkers are typically measured in matrices such as blood, feathers, eggs, and tissues, each offering unique advantages in assessing pollutant exposure. However, ethical concerns regarding wildlife monitoring and the potential harm caused by invasive sampling techniques call for non-invasive methods. Future research should explore novel non-invasive techniques and employ long-term monitoring programmes to understand the cumulative effects of pollution. Despite challenges such as biological variability and environmental factors, white storks remain reliable indicators of ecological change and pollutant burden, providing critical data that can guide pollution management policies, inform conservation strategies, and protect both wildlife and human health from current environmental threats.

KEY WORDS: ecotoxicological monitoring; environmental pollutant bioaccumulation; non-invasive biomarker techniques; oxidative stress biomarkers; pollutant-induced physiological responses

BIOMONITORING AND THE IMPORTANCE OF BIOINDICATORS IN POLLUTION ASSESSMENT

As industrialisation, agriculture, and urbanisation continue to introduce pollutants into natural environments, new effective methods are needed to assess and monitor environmental health (1). Biomonitoring, which involves the use of living organisms to detect and measure environmental changes, has become a fundamental approach in ecotoxicological studies (2). The use of bioindicators is crucial in assessing environmental pollution, as these species can accumulate pollutants from their surroundings and provide measurable biological responses (3). Unlike direct chemical analyses of water, soil, or air, which offer only a snapshot of pollution levels, bioindicators accumulate pollutants over time, offering a more comprehensive assessment of long-term environmental exposure (4). By analysing pollutant accumulation and biological responses in such bioindicators, biomonitoring provides critical insights into pollution levels and their effects on the environment (5, 6). This process requires reliable species that can serve as early warning systems, reflecting both direct and indirect impacts of pollution. Birds, in particular, are widely used in biomonitoring due to their sensitivity to environmental stressors, broad distribution, and position in the food chain (6–17). Avian species occupy diverse habitats, have well-documented life histories, and often exhibit clear physiological and behavioural responses to pollutants. Among these, the white stork (*Ciconia ciconia*) has proven to be a valuable sentinel bioindicator due to its exposure to pollutants across different landscapes, including wetlands, agricultural areas, and landfills.

This review highlights their suitability and advocates their broader use in future research. A single individual can provide data on pollutant exposure, including heavy metals and metalloids, while independent biomarker analyses can evaluate their physiological effects across different biological matrices. The wide spatial distribution of white stork populations allows researchers to compare pollutant levels and biomarker responses across varying levels of environmental pollution. Furthermore, by conducting spatial comparisons of heavy metal and metalloid concentrations, researchers can assess the extent of pollution and its ecological effects (18–20).

WHITE STORKS AND NESTLINGS AS BIOINDICATORS

Ecological role and habitat of white storks

The white stork is recognised as an umbrella species, a species that benefits numerous other species within the same ecosystem.

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Due to its close relationship with human settlements and agricultural landscapes, the white stork is particularly exposed to environmental pollutants (21). As a long-distance migratory species and a top predator within its ecological niche, it serves as a valuable bioindicator of ecosystem health.

White storks primarily build their nests in urban environments, often on the roofs of houses, electric pylons, stacks of straw and other structures created by humans. They mainly forage in open habitats such as grassy meadows, agricultural fields, pastures, steppes, savannahs, and shallow wetlands (22, 23), avoiding tall grass or shrubs and forests. For the past few decades, their foraging areas have also included landfills (18, 24), which has likely shifted the migration behaviour of Iberian populations. In Portugal, the white stork has become resident species (25). Foraging areas are often located within a few kilometres of their nests (26, 27), and their breeding success highly depends on the bounty of the foraging sites (28).

White storks are carnivores whose diet is highly varied and opportunistic ranging from small mammals (voles, mice, shrews, and young rats) to large insects (beetles, grasshoppers, crickets, and locusts) (22, 23), amphibians, reptiles (snakes and lizards) (23), earthworms, fish, molluscs and crustaceans when available, and the eggs and nestlings of ground-nesting birds (29). This kind of dietary flexibility allows the species to thrive in different environments and adapt to seasonal and regional prey availability.

Yet such a versatile diet is also the source of pollutants, including heavy metals, persistent organic pollutants (POPs), and other toxic substances (6, 18). Further up the food chain, these pollutants can reach levels far exceeding those found in the surrounding environment (air, water, or soil). Such accumulation increases the risk of sublethal and lethal effects, which can have cascading consequences throughout the food web. Monitoring biomarker responses and pollutant levels in white storks can provide crucial insights into environmental changes, pollution sources, and overall ecosystem stability (30–32), as they enable the assessment of several environmental stressors at the same time.

Advantages of using white stork nestlings for biomonitoring

A major advantage of using the white stork in (eco)toxicological studies is the widespread participation of countries in monitoring and ringing programmes. As part of the European Union for Bird Ringing (EURING) programme, white stork nestlings are individually marked with coded rings to track their movement across breeding and wintering grounds to better understand their migration patterns and population trends (33–35). Over 69,000 white storks were ringed in this way in Europe (35) and 2,096 in Croatia between 2002 and 2009 (34).

White stork nestlings are considered optimal bioindicators due to their vulnerability to environmental pollutants during early development and their potential for broader application in future studies (6, 18, 36–38). As they reside in the nest and rely on locally sourced food provided by their parents, nestlings are particularly suitable for evaluating pollutant exposure from their immediate surroundings. Since pollutants primarily accumulate through dietary intake, pollutant concentrations in nestlings serve as a direct indicator of environmental pollution. In addition, the health of nestlings is closely tied to parental foraging success, which depends on the health of their habitat. Increased pollutant concentrations may disrupt physiological processes and affect behavioural patterns, cellular metabolism, and neuronal activity (39–41).

As ecotoxicological research progresses, nestlings will continue to be favoured for large-scale monitoring efforts, but further research of age- and tissue-specific responses will enhance our understanding of pollutant dynamics. By using white stork nestlings as bioindicators, researchers can gain valuable insights into the ecological risks posed by environmental pollution and develop more effective conservation and pollution management strategies. Through these efforts, the white stork nestlings continue to serve as a critical sentinel species for evaluating the health of ecosystems and the impacts of environmental stressors on wildlife populations.

Advantages of white stork geographical distribution

In Europe, the white stork breeds from Portugal and Spain in the west, across central Europe and southern Sweden and Finland, to Ukraine and Russia in the east (29). In Asia, it breeds in Turkey, Caucasus, Iraq, Iran, Israel, Jordan, Lebanon, Syria. Smaller breeding populations winter in Kazakhstan, Kyrgyzstan, Uzbekistan, Tajikistan, western China, India, Nepal, and Bangladesh (29). In Africa, resident populations are found in Morocco, Algeria, and Tunisia in the Mediterranean north, as well as in South Africa.

The European breeding population is divided into two migratory flyways: western, which migrates across the Strait of Gibraltar and winters in West Africa, and eastern, which crosses the Bosporus and Middle East and winters in eastern and south Africa (29, 35).

Research conducted in Western and Central Europe has provided valuable insights into heavy metal and metalloid accumulation, while studies in North Africa and the Middle East have expanded our understanding of pollutant exposure in more arid and industrially developing regions (42). Additionally, long-term monitoring programs in Eastern Europe have traced temporal trends in POPs, highlighting the impact of historical and emerging pollutants on wildlife. The species' broad geographic distribution makes it particularly relevant for assessing global pollution trends, as it integrates pollution from diverse environments, ranging from pristine wetlands to heavily anthropogenic landscapes such as agricultural fields and landfills (18). By analysing pollutant burdens and biomarker responses in white storks from different regions, researchers can better understand spatial variations in environmental pollution and track changes over time.

BIOMARKERS FOR ENVIRONMENTAL POLLUTION MONITORING

Overview of biomarkers analysed in birds

Biomarkers serve as early warning signs of pollution-induced biochemical and cellular changes before significant ecological consequences occur (43, 44). Given the spatial and temporal variability in pollutant distribution, selecting a robust set of biomarkers is essential for accurately assessing pollution effects across different ecosystems and exposure scenarios (45, 46).

Acetylcholinesterase and carboxylesterase activities are widely used enzymatic biomarkers to assess the neurotoxic effects of organophosphate and carbamate pesticides (47). Glutathione *S*-transferase and glutathione reductase activities are crucial in evaluating phase II detoxification processes and antioxidant defence mechanisms, which help mitigate oxidative damage induced by heavy metals and POPs (48). Reactive oxygen species and glutathione levels provide insights into oxidative stress dynamics and serve as indicators of cellular damage and adaptive responses (49). Metallothioneins, a class of cysteine-rich proteins, are specifically induced in response to metal exposure (50). By integrating these biomarkers, ecotoxicological studies can achieve a more comprehensive assessment of pollutant impact on apex predators such as the white stork. This multi-biomarker approach not only enhances our ability to detect early physiological disruptions but also aids in understanding the broader ecological risks posed by environmental pollutants.

Acetylcholinesterase activity

Acetylcholinesterase (AChE) is a key cholinergic enzyme belonging to the serine esterase superfamily. Its active site is defined by a catalytic triad, which includes serine 203, histidine 447, and glutamate 334. The primary function of AChE is to catalyse the hydrolysis of the neurotransmitter acetylcholine (ACh) at postsynaptic junctions, control its concentration at the synapse, and prevent prolonged or excessive neural stimulation. In other words, AChE plays a crucial role in the modulation of cholinergic signalling and neurotransmission (51, 52). It is mostly found in the central and peripheral nervous systems, muscle tissue, and hematopoietic cells, where it helps to regulate synaptic activity and muscular function (53).

In environmental and toxicological research, AChE activity often serves as a biomarker of pesticide exposure, particularly in studies assessing the impact of organophosphates and carbamates. It has been studied extensively in numerous avian species to understand a variety of physiological and ecological effects. Changes in AChE activity in the white-crowned sparrow (*Zonotrichia leucophrys gambelli*) helped to understand the influence of daily photoperiods on enzyme function (54). Its inhibition in Japanese quail (*Coturnix coturnix japonica*) helped to understand the effects of exposure to lethal and sublethal doses of carbamate pesticides aldicarb, methiocarb, oxamyl, pirimicarb, and thiofanox (55) as well as behavioural changes in captive common starlings (*Sturnus vulgaris*) after exposure to sublethal doses of the organophosphate chlorfenvinphos (56). Some studies aimed at determining reference ranges for AChE activity, such as those in the Hispaniolan Amazon parrot (*Amazona ventralis*) (57). Dose-dependent inhibition of AChE activity was also investigated in the brain and pancreas of the rose-ringed parakeet (*Psittacula krameri borealis*) following exposure to the organophosphate quinalphos (58). AChE activities were compared between eastern bluebirds (*Sialia sialis*) and common starlings to establish age-related changes (59). Similar studies were conducted in the white stork, grey heron (*Ardea cinerea*), and northern gannet (*Morus bassanus*) in a nature reserve in Portugal to establish baseline AChE activity levels in plasma cholinesterases as reference for comparative studies of wildlife in the region (60).

Despite its widespread application in various avian species, however, AChE activity has been measured but in a handful of studies on white stork nestlings for the purpose of pollutant monitoring. A recent research in Croatia (6, 13, 14, 61) revealed significant variations linked to environmental pollution with agricultural and industrial pollutants, including heavy metals and pesticides, all of which inhibited AChE activity. These studies highlight the value of white stork nestlings as bioindicators and the need for continued monitoring of pollution impacts on the white stork and ecosystems.

Carboxylesterase activity

Carboxylesterase (CES) is a non-specific esterase enzyme widely distributed in various tissues and organisms to catalyse the hydrolysis of carboxylic acid esters into their corresponding acids and alcohols. It is involved in the phase I metabolism of xenobiotics as part of an essential detoxification mechanism of environmental pollutants and pharmaceutical compounds (62, 63). They contribute to the biotransformation of lipophilic drugs or toxins by introducing or exposing polar functional groups, thereby converting these compounds into more hydrophilic forms. The resulting carboxylate products are subsequently conjugated by other enzymes to increase their solubility, facilitating their excretion from the organism (64). Given its role in detoxification, CES activity is increasingly utilised in biomonitoring studies to assess the effects of pesticide exposure. In this context, CES has been proposed as a non-destructive biomarker for wild bird species exposed to organophosphates and carbamates as an AChE alternative or complementary marker of chemical exposure (65). Establishing baseline values of CES activity in healthy individuals is crucial to understanding the potential inhibition or induction of this enzyme in response to environmental pollutants. To this end, Sogorb et al. (66) measured baseline CES activity across 19 bird species, including the white stork, Montagu's harrier (*Circus pygargus*), black stork (*Ciconia nigra*), peregrine falcon (*Falco peregrinus*), common buzzard (*Buteo buteo*), eagle owl (*Bubo bubo*), great bustard (*Otis tarda*), Bonelli's eagle (*Aquila fasciata*), booted eagle (*Hieraaetus pennatus*), short-toed eagle (*Circaetus gallicus*), black-backed gull (*Larus fuscus*), Spanish imperial eagle (*Aquila adalberti*), black vulture (*Aegypius monachus*), Egyptian vulture (*Neophron percnopterus*), griffon vulture (*Gyps fulvus*), northern goshawk (*Accipiter gentilis*), black kite (*Milvus migrans*), barn owl (*Tyto alba*), tawny owl (*Strix aluco*), and red kite (*Milvus milvus*). These measurements improved the reliability of CES as a biomarker for environmental exposure. Investigating the effects of organophosphates methidathion and parathion on CES activity in several bird species, including rock pigeons (*Columba livia*), red-tailed hawks (*Buteo jamaicensis*), American kestrels (*Falco sparverius*), Swainson's hawks (*Buteo swainsoni*), red-shouldered hawks (*Buteo lineatus*), and Cooper's hawks (*Accipiter cooperii*), Bartkowiak and Wilson (65) demonstrated a bimodal distribution of CES activity and variability in enzymatic response across individuals and species. This variability underscores the limitations of CES activity as a sole biomarker of pesticide exposure, considering that CES activity varies with species, age, and environmental conditions. For this reason and the sake of a more comprehensive assessment of pesticide exposure, these authors (65) and Morcillo et al. (67) have recommended that additional biomarker analyses and/or tissue sampling should be incorporated into monitoring studies. In a study assessing CES enzymatic activity in the muscle and liver tissues of the yellow-legged gull (*Larus michahellis*), Morcillo et al. (67) focused on detecting exposure to anticholinesterase insecticides and demonstrated that CES activity could effectively reflect environmental exposure to these toxicants.

CES activity has also been investigated in a limited number of studies on white stork nestlings. Research conducted in Croatia has revealed regional variations, with significantly lower levels observed in areas impacted by agricultural and industrial pollution, suggesting the presence of inhibitory xenobiotics (6, 13, 14, 61). These findings contribute to the growing body of evidence supporting CES as a sensitive and reliable biomarker for monitoring the effects of anthropogenic chemicals, pesticides in particular, on wild bird species, provided that they are combined with other biomarkers.

Glutathione *S*-transferase activity

Glutathione *S*-transferase (GST) is an important phase II detoxifying enzyme that plays a central role in the metabolism of xenobiotics. Its primary function is to catalyse the nucleophilic attack of reduced glutathione on electrophilic centres such as nitrogen, sulphur, or carbon atoms in xenobiotic substrates. This reaction effectively neutralises the harmful effects of nonpolar xenobiotics by transforming them into more water-soluble conjugates, which can then be excreted from the body more easily. To prevent the interaction of toxic molecules with cellular proteins, DNA, and RNA and protect cells from oxidative damage (68, 69) the activity of GST requires a consistent supply of reduced glutathione, which is synthesised by gamma-glutamylcysteine synthetase and glutathione synthetase. Furthermore, detoxification requires transport proteins to export the resulting glutathione conjugates from the cells (70–72).

The enzyme has been studied extensively in various bird species, including white stork nestlings, griffon vultures, and eagle owls to assess physiological responses to environmental stressors, including oxidative stress caused by metal pollution (73–77). Research in Croatia has demonstrated regional variations in GST activity, with differences in enzymatic responses linked to environmental pollution from agricultural and industrial sources (6, 13, 14, 61). However, birds show a noticeable decline in their ability to regulate physiological homeostasis as they age. Older white storks experience a depletion of enzymatic antioxidants in the blood (78–80), which renders the application of GST as a biomarker for environmental pollution unreliable. For this reason, nestlings are the most sensitive age group for pollutant biomonitoring.

Glutathione reductase activity

Glutathione reductase (GR) catalyses the NADPH-dependent reduction of oxidised glutathione (GSSG) to its reduced form, GSH. This reaction is essential for maintaining the cellular glutathione pool and for protecting cells from oxidative damage and toxic substances (81, 82).

The activity of GR has been studied in homogenised blood cells of both adult and juvenile white storks to establish baseline levels of antioxidant enzymes and to investigate potential age-related variations in these parameters (76). This research highlighted the role of GR in the antioxidant defence system and provided valuable insights into the physiological adaptation of birds to environmental stressors. Further studies focused on GR activity in relation to environmental pollutants. Kamiński et al. (83, 84) provided important evidence that GR activity, along with other biomarkers, can be indicative of the level of environmental pollution affecting wildlife. Tkachenko and Kurhaluk (38) confirmed that white stork nestlings are sensitive and valuable bioindicators of environmental stress caused by pollutants. Studies in Croatia have shown that GR activity varies across regions, with increased levels detected in areas affected by environmental pollutants (6, 13, 14, 61). In addition to studies in wild birds, Upton et al. (85) have shown that GR activity in the blood and liver of broiler chickens (*Gallus gallus domesticus*) fed varying levels of peroxidised poultry fat reflects the effects of diet-induced oxidative stress on enzymatic biomarkers.

Glutathione concentration

In experimental settings, GSH levels have been evaluated in various avian species as part of studies examining the effects of environmental toxins. Fernie et al. (86) assessed GSH levels in artificially incubated American kestrels exposed to polybrominated diphenyl ethers (PBDEs), whereas Ezeji et al. (87) explored the effects of permethrin on GSH levels in serum and liver samples of poultry species. Both studies highlighted the importance of GSH as a sensitive biomarker of pollutant-induced oxidative damage. A study of GSH levels in the blood of Zebra finches (*Taeniopygia guttata*) by Romero-Haro and Alonso-Alvarez (88), in turn, has provided valuable insights into the role of GSH in mitigating oxidative stress during early development. While GSH levels have been studied extensively in experimental avian models, their application as an indicator of environmental pollution in wild bird populations remains limited.

Pineda-Pampliega et al. (18) assessed GSH concentrations in the blood of white stork nestlings to investigate the cumulative physiological effects of foraging on landfills, which are often associated with exposure to pollutants and potential nutritional stressors. Our group investigated non-destructive methods with fluorescent dyes to detect GSH and found that they can be reliable for environmental pollutant monitoring in white stork nestlings (6, 13, 14, 61).

In addition to GSH concentrations, oxidative stress can be measured as the ratio between the oxidised and reduced glutathione forms (GSSG and GSH, respectively). The balance between the two is crucial for maintaining cellular redox homeostasis. A higher ratio indicates higher stress and impaired antioxidant defences (87, 88). Oropesa et al. (76) compared the GSH/GSSG ratio between fledgling and adult white storks only to discover that the age groups did not differ. This finding suggests that the ratio can serve to establish baseline values for different age groups, which are crucial for consideration in environmental research studies.

Reactive oxygen species concentration

Reactive oxygen species (ROS), such as the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($HO^{\bullet}$), are highly reactive intermediates capable of interacting with biomolecules and causing significant damage to DNA, RNA, proteins, and cell membranes. These interactions can lead to cellular dysfunction and contribute to the development of various diseases and ageing processes (49, 89).

Delhaye et al. (90) investigated the production of superoxide anions by mitochondrial electron transport chains in red blood cells (RBCs) across 21 bird species to elucidate the relationship between ROS levels and lifespan. Schlezinger et al. (91) used destructive methods to measure H_2O_2 and $O_2^{\bullet -}$ formation in the liver of double-crested cormorant (*Phalacrocorax auritus*), herring gull (*Larus argentatus*), and chicken to better understand the underlying oxidative stress mechanisms. As a non-destructive alternative, our group employed fluorescent dyes to detect ROS in white stork nestlings (6, 13, 14, 61). Our findings support the use of ROS detection as a sensitive biomarker for assessing pollution exposure in avian species and highlight the complexity of ROS production and its potential to affect various physiological and metabolic pathways.

Metallothionein concentration

Metallothioneins (MTs) are cysteine-rich, low-molecular-weight proteins that play a crucial role in metal homeostasis, detoxification, and cellular protection against oxidative stress. These proteins function primarily as metal chelators, binding and sequestering essential and non-essential metal ions such as zinc (Zn), copper (Cu), and cadmium (Cd). By facilitating metal ion transport and storage, they also mitigate heavy metal accumulation and ensuing toxicity (92). The most potent inducers of MT expression are silver (Ag), Cu, and Cd, but metals such as mercury (Hg) and lead (Pb) can also trigger changes in MT levels, depending on species-specific and tissue-specific sensitivities (92). MT induction is particularly pronounced in tissues directly involved in metal detoxification and storage, such as the liver, kidney, and blood (93). The degree of MT induction varies with tissues, reflecting localised metal accumulation and physiological demands (94).

Studies in white stork nestling plasma showed no significant variations in MT levels across different sampling sites, despite varying pollution sources (6). This suggests that MT expression in white stork nestlings may not be as responsive to environmental pollutants as other biomarkers. Research in other bird species, terrestrial and marine (93–98) shows mixed findings. In the great tit (*Parus major*), for example, liver and kidney MT levels were reported to vary with heavy metal pollution (96), but Marcinekova et al. (95) reported no significant variations in blood MT levels across several species from differently polluted areas, perhaps because the metal levels were in the range of background levels and therefore not elevated enough to induce response. In fact, the main difference in MT response between these studies may be tissue-specific rather than species-specific. Future studies on white storks could benefit from analysing MT levels in tissues other than blood plasma. That being said, we are against euthanising the bird for MT analysis, and alternative methods should be explored to better understand heavy metal exposure and its physiological effects in white storks.

BIOMONITORING METHODS INVOLVING WHITE STORKS

Non-destructive and non-invasive sampling methods

When working with species of conservation concern, the use of non-destructive and non-invasive sampling methods is essential to minimise stress and ensure ethical research practices. Non-destructive techniques, such as blood sampling and feather plucking, cause minimal distress to birds, while non-invasive approaches, including collecting shed feathers, regurgitated pellets, and unhatched eggs, eliminate pain and discomfort entirely (99).

Blood samples and their components (plasma, serum, and red blood cells) are particularly useful for monitoring environmental pollutants such as heavy metals, metalloids, and POPs (100). Given its suitability for large-scale environmental monitoring, blood sampling is a reliable method for evaluating pollutant exposure across a significant number of individual birds.

Feathers have also been widely used to assess metal pollution (11, 15, 16). Their main advantage is that they reflect long-term pollutant exposure. Goutner et al. (42), for example, reported that feather Hg levels increase significantly with age (42). Orłowski et al. (20), in contrast, reported a negative correlation between feather length and levels of chromium (Cr), Cu, and Zn, suggesting that the best tissue for non-invasive analysis are breast feathers of the same length.

Eggs, in turn, have been utilised to investigate pollutant transfer from adult females to their offspring. Hernández et al. (101) identified the presence of POPs such as hexachlorocyclohexane (HCH), aldrin, dieldrin, and polychlorinated biphenyls (PCBs), as well as Hg, Pb, Cd, Cu, and Zn. Besides pollutant transfer, these findings highlight the implications for embryonic development and reproductive success.

Invasive methods and tissue-specific analysis

Liver and kidney analyses have proven particularly valuable for assessing heavy metal and organic pollutant accumulation. Following up the impact of a mine tailing accident near Doñana National Park two years later, Gómez et al. (102) observed an increase in Cu, Cd, Pb, Zn, and arsenic (As) levels in the liver, muscle, and kidneys of white storks, which confirmed the species as a sensitive and reliable bioindicator for the monitoring of these elements. Similarly, Piedra et al. (103) measured Hg concentrations in the liver, kidney, and muscle and investigated the potential of propolis and bee pollen supplementation to mitigate Hg bioaccumulation effects in white storks. Liver and kidney responded well to supplementation but muscles did not (103). These studies emphasise the importance of organ analysis in evaluating chronic exposure to pollutants, as these tissues serve as primary sites for metal detoxification and storage.

BENEFITS OF USING A MULTI-BIOMARKER APPROACH AND INTEGRATION WITH POLLUTANT ANALYSIS

Unlike single-biomarker studies, which may capture only specific toxic effects or exposure pathways, a multi-biomarker approach allows researchers to evaluate a wide spectrum of physiological responses, offering a more holistic understanding of pollution effects. By analysing multiple biomarkers simultaneously, ecotoxicologists can detect early biological disruptions, assess pollutant interactions, and differentiate between acute and chronic exposure effects, especially if the multi-biomarker approach, with endpoints measured at different levels of biological organisation, is integrated with the assessment of pollutant levels in the environment. Integrated approach helps to distinguish between different pollutant classes, evaluate cumulative and synergistic effects of multiple stressors, and improve the accuracy of ecological risk assessments. Furthermore, determining several biomarkers across different tissues (e.g., blood, liver, feathers) can provide a more detailed picture of exposure history and pollutant metabolism, helping to establish long-term pollution trends. While blood provides real-time exposure data, the liver and kidney offer insights into bioaccumulation, feathers reflect historical pollution, and eggs reveal generational transfer risks.

Integrated approach is particularly valuable for species like the white stork, which inhabits diverse environments and is exposed to a wide range of pollutants. Several studies using a panel of biomarkers in stork nestlings (6, 13, 14, 18, 73, 104, 105) (Table 1) have shown the advantages of such an approach in determining the connection between specific pollutants and the observed harmful effects.

By measuring biomarkers such as oxidative stress indicators, hormone levels, immune responses, and genotoxicity markers alongside pollutant concentrations (e.g., Pb, Cd, Zn, and Hg), researchers can capture both the physiological and biochemical effects of pollution and a fuller picture of how environmental pollutants affect these birds. Better understanding of the complex interactions between environmental pollutants and biological systems, in turn, can inform more effective conservation and management strategies for these birds in polluted habitats.

Metal exposure effects on oxidative stress and immune response

Several studies (36, 84, 104) focus on biomarkers like catalase (CAT), superoxide dismutase (SOD), thiobarbituric acid reactive substances (TBARS), and glutathione peroxidase (GPx), which are indicative of oxidative stress. Combining these biomarkers with heavy metal measurements (e.g., Pb, Cd, Hg) can reveal how metal pollution exacerbates oxidative damage. Kamiński et al. (83) have shown that Cd and Pb trigger an imbalance between the production of ROS and antioxidant defence systems in nestlings. Baos et al. (105, 106) have linked exposure to metals (Pb, Zn, Cu, and As) with disruptions in immune functions, such as T-cell-mediated immune responses and corticosterone levels. The measurement of immune biomarkers along with metal concentrations thus provides an insight into how metal exposure may suppress immune functions and increase susceptibility to diseases in white storks.

Genotoxicity and pollutant source identification

Genotoxicity markers, such as DNA damage and the T-cell response in studies by Baos et al. (107) and Pastor et al. (108), help identify the genetic consequences of exposure to metals (Pb and As). When these biomarkers are measured alongside metal concentrations, it is possible to detect the extent of genetic damage in white storks and understand whether pollution from specific sources (e.g., industrial pollution, landfills) plays a significant role in such genetic alterations. This can also provide a clearer picture of how long-term exposure to pollutants can affect the reproductive success and health of the population.

Endocrine disruption and hormonal imbalance

Hormones such as corticosterone, thyroxine (T4), and triiodothyronine (T3) serve as critical indicators of stress and metabolic responses. Kamiński et al. (19) and Baos et al. (105) measured hormone levels alongside heavy metal levels in the blood, and the latter reported changes in adrenocortical stress response to exposure to sublethal Pb concentrations in white stork nestlings, concluding that the hypothalamus–pituitary–adrenal (HPA) axis may be a reliable indicator of metal exposure. These combined data help identify how pollution disrupts endocrine functions and whether it leads to developmental or behavioural changes in white stork nestlings. The correlation between pollutant concentrations and hormonal changes can also help determine the extent of environmental stress caused by pollutants.

Assessing health status through blood chemistry

Blood chemistry, including markers such as albumin, glucose, and triglycerides (18), provides insights into the overall health of white storks. When these are measured alongside metal concentrations [e.g., iron (Fe), Zn, and Pb], it allows for a more comprehensive assessment of how pollutants affect vital biological processes, including metabolism, nutrition, and organ function. For instance, heavy metal exposure might affect the liver and kidney's ability to detoxify and regulate metabolic processes, which would show up as alterations in blood chemistry markers.

Long-term ecological impacts

Combining data on biomarkers and pollutant concentrations can help to evaluate the long-term effects of pollution on the survival and reproductive success of white storks. For example, female storks exposed to a mine spill during development exhibited early reproductive ageing compared to unexposed storks following an initial phase of unusually high productivity in their early breeding years (30). Chronic exposure or environmental disasters can lead to long-term effects across multiple generations in wildlife, especially when they affect nestlings or fledglings. Long-term studies can therefore help researchers predict how chronic exposure to pollutants might impact white storks' ability to thrive in polluted environments and whether these effects persist across generations.

CASE STUDIES IN WHITE STORKS AND THEIR APPLICATION IN ECOTOXICOLOGICAL RESEARCH

Table 1 lists the key case studies in white storks with valuable insights into the effects of pollutants on various biomarkers. Most involve nestlings, given their sensitivity to environmental pollutants. Biomarkers such as oxidative stress indicators (e.g., SOD, CAT, malondialdehyde) and immune responses (e.g., T-cell activity, DNA damage) are commonly analysed to assess the impact of pollutants like Pb, Cd, Hg, and As.

Studies like those by Kamiński et al. (83, 104) and Tkachenko and Kurhaluk (37) focus on oxidative stress biomarkers in blood. Nestlings from polluted regions showed higher activity of key enzymes, including SOD, CAT, GPx, and GR, suggesting a physiological response to mitigate the oxidative stress caused by pollution (83). Furthermore, heavy metal pollution, particularly with Cd, was found to interact with essential elements like calcium (Ca) and magnesium (Mg) and to affect SOD and CAT activity, as well as carbonyl protein (CP) and TBARS levels. No interactions were found in unpolluted environments but varied in polluted areas, where Ca-enzyme interactions were consistently positive during the day, while Mg-enzyme interactions shifted from positive during the day to negative at night (104).

In addition to oxidative stress, nestlings from polluted areas exhibited greater metabolic stress, as evidenced by elevated protein oxidation (carbonyl derivatives) and alanine and aspartate aminotransferase activities. TBARS and protein carbonyl levels correlated strongly, confirming the connection between oxidative stress and metabolic disruption in polluted environments (37).

Besides oxidative stress, Baos et al. (105, 107) reported that exposure to sublethal levels of Pb compromised the HPA axis function regulating key behavioural and metabolic processes necessary for survival (105). An assessment of the genotoxic effects of heavy metals and As in white stork nestlings following a mining accident revealed a correlation between As exposure and genetic damage, which varied across species and time (107). This variability suggests that species-specific differences and temporal variations should be carefully considered when designing pollution monitoring programmes and evaluating the ecological impact of pollutants.

Other biomarkers like cholinesterase (ChEs) and GST were analysed to examine neurotoxicity and oxidative status, respectively. Heavy metals (Pb, Cd, Zn, Cu, As, Hg) dominated the research, with studies often linking these to changes in biochemical parameters.

Studies involving adults, such as those by Gómez et al. (102) and Maia et al. (109), assessed baseline metal concentrations and the broader effects of environmental pollution over time. Maia et al. (109) reported lower Ni, Cu, Se, and Hg and higher Pb concentrations in fledglings compared to adults (109). Contrary to the assumption that Pb levels would be higher in adults than juveniles, it is possible that blood Pb concentration reflects immediate dietary intake. The differences we observed between the age groups may be explained by feeding behaviour (110), and this factor should be considered when interpreting the results. None of the studies found gender-dependent differences in metal concentrations in either nestlings (6), fledglings, or adult white storks (109).

Table 1 Summary of studies on biomarker measurements and pollutant analysis in white storks (*Ciconia ciconia*)

Age group	Biomarker	Compounds	Matrix	Purpose of the measurement	Ref.
Nestling	AChE, CES, GST, GR, GSH, ROS		Blood	Assess spatial variability in biomarker response in regard to agricultural pollution	(14)
Nestling	AChE, CES, GST, GR, GSH, ROS, MT	As, Se, Cd, Hg, Pb	Blood	Evaluate the differences in biomarker responses related to metal(loid)s from Croatia	(6)
Nestling	AChE, CES, GST, GR, GSH, ROS		Blood	Assess spatial variability in biomarker response in regard to environmental pollution	(13)
Nestling	AChE, CES, GST, GR, GSH, ROS		Blood	Establish novel biomarker detection and analysis in bird plasma and blood cell homogenate (S9)	(61)
Nestling	CAT, SOD, TBARS, CP	Ca, Fe, Mg, K, Na, Zn, Pb, Cd	Blood	Investigation of element impact on oxidative stress biomarkers	(104)
Nestling	SOD, TBARS, CAT, GR, CP, GPx	Na, Ca, K, Mg, Zn, Fe, Cu, Co, Mn, Pb, Cd	Blood	Investigation of element impact on oxidative stress biomarkers	(84)
Nestling	TWCC, albumin, H/L ratio, ALT, ASP, AP, bilirubin, creatinine, Chl, ferritin, CK, glucose, triglycerides, transferrin, uric acid, metHb, MDA, GSSG, tGSH, GPx	Ca, P, Mg	Blood	Investigation of the effects of foraging on landfills on physiological parameters	(18)
Nestling	SOD, CP, CAT, GPx, MDA, GR	Na, Ca, K, Mg, Zn, Fe, Cu, Co, Mn, Cd, Pb	Blood	Evaluation of the impact of elements on oxidative stress biomarkers	(83)
Nestling	T4, Mel		Blood	To determine ED biomarker response regarding pollution	(115)
Nestling	Ca:P Ratio	P	Blood	Disruption of bone metabolism due to mine spill	(116)
Nestling	GST, MDA, GSH	Pb, As, Hg	Blood	Investigation of oxidative status associated with metal pollution	(73)
>1 year	RBC, WBC, Thrombocytes		Blood	Haematological parameters evaluation in White storks reared in captivity	(117)
Fledging	tAP, kidney AP, hepatic AP	P, Ca	Blood, liver, kidney	Skeletal pathology assessment associated with heavy metal pollution	(32)
Nestling	MDA, tocopherol, TAC		Blood	Antioxidant supplementation effect on telomeres	(118)
Nestling	T3, T4, corticosterone	Pb, Cu, Cd, Zn, As	Blood	Investigation of stress and hormone status affected by heavy metal and metalloids	(105)
Nestling		Ca, Fe, Mg, Zn	Blood	Investigation of chemical elements from polluted areas	(19)
Nestling	TBARS, AST, ALT, PC, LDH, Pyruvate, Lactate		Blood	Pollution-induced oxidative stress and biochemical parameter alterations assessment	(37)
Nestling	T-cell-mediated immune response	Cu, Cd, Pb, Zn, As	Blood	Investigation of relationships between T-cell-mediated immune response and metal levels	(106)
Nestling	DNA damage		Blood	Genotoxic effects of heavy metals and arsenic	(107)
Fledging, adult		Pb, Hg, As, Ni, Fe, Zn, Cu, Se, Mn, Cr, Co, Cd	Blood	Assessment of basal values of metals and metalloids	(109)
Nestling	Hb, RCN, MCHb, MCHbC, MCV, TG, Chl, proteins, urea, uric acid, Chl-HDL, AP, AST, ALT, WCN		Blood	A report on haematology and blood chemistry	(119)
Nestling		Cd, Hg, Fe, Pb, Zn, As, Se	Blood	Evaluation on element levels in White storks breeding near a landfill	(120)

Table 1 continued

Age group	Biomarker	Compounds	Matrix	Purpose of the measurement	Ref.
Adult, juvenile	ChEs		Blood	Characterisation of plasma cholinesterase and its *in vitro* inhibition by pesticides	(121)
Nestling	Packed cell volume, RBC, MCHb, MCV, MCHbC Hb, trombocytes, WBC, lymphocytes, heterophils, eosinophils, monocytes, basophils, H/L ratio		Blood	Report on reference intervals for haematological and plasma biochemical parameters	(122)
Nestling	Lymphocytes, DNA damage		Blood	Assessment of genotoxic damage after the Doñana Ecological Disaster	(108)
Nestling	TBARS, CAT, SOD, GR, CP, TAC, GPx		Blood	Pollution-related assessment of changes in oxidative stress and antioxidant defence profile	(36)
Adult		Hg	Liver, kidney, muscle	Investigation of propolis and bee pollen preparation effect on the concentration of Hg	(103)
Nestling	Oxidative stress		Blood	Evaluation of physiological adaptation to the exploitation of landfill sites for foraging	(123)
Nestling		Hg	Feathers	Investigation of Hg in relation to age, brood size, and hatching order	(42)
Nestling		Cr, Ni, Cu, Zn, Pb	Feathers	Methodological implications for further ecotoxicological studies	(20)
Adult		Cu, As, Pb, Cd	Liver, muscle, kidney	Evaluation of a mine tailing accident influence near Doñana National Park	(102)
		Dichlorobenzophenone, alpha-HCH, gamma-HCH, dieldrin, aldrin, heptachlor, heptachlor epoxide, *p,p'*-TDE, *p,p'*-DDE, *p,p'*-DDT, PCBs, Pb, Hg, Cu, Zn, Cd	Eggs	Assessment of the levels of organochlorine pollutants and heavy metals	(101)
Adult		As, Mn, Cr, Pb, Ni, Zn	Feathers	Determination of heavy metal accumulation in the feathers	(124)
Nestling		Pb, Pb isotope ratio	Blood	Assessment of source identification of Pb contamination in a marshland ecosystem	(125)
Nestling	Lymphocytes		Blood	A 4 year follow-up analysis of genotoxic damage after the massive spillage of toxic waste at Doñana National Park	(126)

AChE – acetylcholinesterase; ALT – alanine aminotransferase; AP – alkaline phosphatase; alpha-HCH – alpha-hexachlorocyclohexane; ASP – aspartate aminotransferase; AST – aspartate aminotransferase; CAT – catalase; CES – carboxylesterase; ChEs – cholinesterase; Chl – cholesterol; Chl-HDL – cholesterol-high density lipoprotein; CK – creatine kinase; CP – carbonyl proteins; gamma-HCH – gamma-hexachlorocyclohexane; 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

LIMITATIONS AND CHALLENGES IN USING WHITE STORKS AS BIOINDICATORS

Environmental and ecological factors

Various environmental factors can complicate the interpretation of biomarker data and pollutant concentrations. Habitat loss, driven by urbanisation and agriculture, reduces the availability of foraging areas, which can affect the white storks' health and behaviour (21). Similarly, climate change can alter migration patterns, breeding success, and food availability, leading to variations in exposure to pollutants (18, 111). Agricultural intensification and the associated increase in pesticide use also pose challenges, as it may lead to direct chemical exposure, potentially interfering with biomarker measurements and confounding the results. These external factors create variability in the ecological conditions of different populations of white storks, making it difficult to distinguish the effects of specific pollutants from other environmental stressors.

Biological variability and methodological limitations

Another challenge is the biological variability among individual white storks and nestlings. Differences in age, sex, genetics, and nutritional status can influence the response to pollutants, resulting in variability in biomarker levels (112, 113). For example, oxidative stress indicators may naturally be elevated due to the bird's age or health status, independent of pollutant exposure (76). Besides, methodological limitations can blur the assessment and interpretation of pollutant levels and biomarkers. Variations in sampling techniques, such as blood collection or tissue analysis, may lead to inconsistencies in results across studies. Furthermore, biomarkers may not fully capture the heterogeneity of pollutant exposure owed to spatial and temporal variabilities in pollutant concentrations, that is, more refined analytical techniques may be required to detect subtle environmental effects.

Ethical and practical considerations in biomonitoring

The use of white storks in biomonitoring efforts also raises important ethical concerns. Wildlife studies inherently involve the risk of disturbing the animals, especially when handling birds for sample collection. For example, capturing and tagging storks, drawing blood, or collecting feathers could lead to stress or injury to the birds, which raises concerns about animal welfare. Minimising harm during these procedures is essential, and guidelines must be followed to ensure that the storks' health and well-being are not compromised (114). Moreover, non-invasive sampling techniques, such as the collection of feathers, addled eggs or eggshells, are preferred where possible to reduce the stress on the birds.

The practical application of white storks as bioindicators is further challenged by practical considerations, including the logistics of accessing white stork populations, ensuring proper sample preservation, and securing adequate funding for long-term monitoring programmes. The key to maintaining these programmes is balancing the need for comprehensive data with ethical responsibility.

Handling white stork specimens typically requires the involvement of ornithologists with the appropriate permits, which may pose organisational challenges in extensive monitoring programmes. On the other hand, white storks are large birds that may injure untrained individuals who handle them. Since white storks nest on difficult-to-reach structures (roofs, chimneys, electric pylons) sample collection may be challenging and involve obtaining access to private or company property. Finally, the white stork is a protected species (29), and any manipulation of specimens requires special permits.

CONCLUSIONS

Despite challenges such as biological variability and environmental factors, white storks remain reliable indicators of ecological change and pollutant burden. Biomarkers such as oxidative stress indicators, immune responses, and hormonal changes provide valuable insights into the physiological effects of pollutants like heavy metals (Pb, Hg, Cd), pesticides, and toxic compounds on white storks. Various matrices used for analysis, including blood, feathers, eggs, and tissues, allow for comprehensive assessments of pollutant exposure and accumulation. By reflecting the effects of pollutants on biomarker levels, white storks serve as a proxy for evaluating environmental health, particularly in ecosystems affected by industrial activities, agricultural practices, and urbanisation.

Future directions in ecotoxicological research

Future research in ecotoxicology should focus on genomic, epigenetic, and metabolomic biomarkers to provide deeper insights into long-term effects on white stork health and reproduction. Non-invasive techniques, such as the use of feathers and eggs for pollutant analysis, should be more frequently employed to minimise the adverse effects of biomonitoring on wildlife. Through long-term effects, developmental patterns, and causal relations over time, longitudinal monitoring programmes would provide a better understanding of chronic exposure and cumulative pollution impacts on avian species, particularly in relation to climate change and emerging environmental stressors. Advancing the integration of remote sensing technology and data modelling could also enhance the ability to predict future trends and provide early warning signs of ecosystem degradation.

Implications for nature conservation and human health

Using white storks as bioindicators has important nature conservation implications. Their role in monitoring pollutant levels directly contributes to better pollution management strategies and policies aimed at protecting wildlife from harmful pollutants.

Furthermore, understanding the health of white stork populations can inform broader ecological assessments, influencing decisions on habitat restoration, land use planning, and species protection.

Given that white storks often forage in areas where humans also obtain food, they can also provide valuable insights into potential risks for human health by identifying areas with high pollutant levels, especially in regions vulnerable to heavy industrialisation or agricultural expansion.

The data derived from stork biomonitoring can also inform regulatory decision-making on pollutant emissions and ecosystem protection laws and ultimately support the preservation of biodiversity and ecosystem services essential for human and wildlife well-being.

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Od livade do odlagališta: bijela roda (*Ciconia ciconia* L., 1758) kao pouzdan bioindikator zdravlja ekosustava

Bijele rode (*Ciconia ciconia* L., 1758) i njihovi ptići postali su vrijedni bioindikatori zagađenosti okoliša, osobito u ekosustavima pod utjecajem antropogenih aktivnosti. U ovom se preglednom radu istražuje uloga bijelih roda u biomonitoringu, s naglaskom na korištenje biomarkera i analizu zagađivača kako bi se razumjeli fiziološki učinci okolišnih stresora. Ključni biomarkeri, poput markera oksidacijskog stresa, imunosnih odgovora i hormonskih promjena, pružaju uvid u učinke zagađivala kao što su teški metali, pesticidi i drugi toksični spojevi. Biomarkeri se obično mjere u matricama poput krvi, pera, jaja i tkiva, pri čemu svaka od njih nudi jedinstvene prednosti u procjeni izloženosti zagađivalima. Međutim, etička pitanja u vezi s praćenjem divljih životinja i potencijalna šteta prouzročena invazivnim tehnikama uzorkovanja zahtijevaju primjenu neinvazivnih metoda. Buduća istraživanja trebaju istražiti nove neinvazivne tehnike i provoditi dugoročne programe praćenja kako bi se razumjeli kumulativni učinci zagađenosti. Unatoč izazovima kao što su biološka varijabilnost i okolišni čimbenici, bijele rode su pouzdani pokazatelji ekoloških promjena i opterećenja okoliša zagađivalima, pružajući ključne informacije koje mogu usmjeriti politike upravljanja zagađenošću i strategije očuvanja te zaštititi divlje životinje, pa tako i ljude, od okolišnih prijetnji.

KLJUČNE RIJEČI: bioakumulacija okolišnih zagađivala; ekotoksikološki monitoring; fiziološki odgovori izazvani zagađivalima; markeri oksidacijskog stresa; neinvazivne tehnike