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RESEARCH ARTICLE

Sex ratio of White Stork *Ciconia ciconia* in different environments of Poland

Piotr Kamiński · Ewa Grochowska · Sławomir Mroczkowski · Leszek Jerzak · Mariusz Kasprzak · Beata Koim-Puchowska · Alina Woźniak · Olaf Ciebiera · Damian Markulak

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Abstract The aim of this study was to analyze the variation in sex ratio of White Stork *Ciconia ciconia* chicks from differentiated Poland environments. We took under a consideration the impact of Cd and Pb for establish differences among sex ratio in chicks. We also study multiplex PCR employment for establish gender considerations. We collected blood samples via venipuncture of brachial vein of chicks during 2006–2008 breeding seasons at the Odra meadows (SW-Poland; control), which were compared with those from suburbs (SW-Poland), and from copper smelter (S-Poland; polluted) and from swamps near Baltic Sea. We found differences among sex ratio in White Stork chicks from types of environment. Male participation in sex structure is importantly higher in each type of environment excluded suburban areas. Differences in White Stork sex ratio according to the degree of environmental degradation expressed by Cd and Pb and sex-environment-metal interactions testify about the impact of these metals upon sex ratios in storks. Simultaneously, as a result of multiplex PCR, 18S ribosome gene, which served as internal control of PCR, was amplified in male and female storks. It means that it is possible to use primers designed for chicken in order to replicate this fragment of genome in White Stork. Moreover, the use of Oriental White Stork *Ciconia boyciana* W- chromosome specific primers makes it possible to determine the sex of *C. ciconia* chicks. Many factors make sex ratio of White Stork changes in subsequent breeding seasons,

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which depend significantly on specific environmental parameters that shape individual detailed defense mechanisms.

Keywords White Stork · *Ciconia ciconia* · Sex ratio · Sex determination · Environmental stress · Pb and Cd impact · Poland

Introduction

During the last couple of years, there are considerable significant changes in the European population of the White Stork *Ciconia ciconia*, accompanied by a rapid decrease of reproduction success and an increase of mortality (Daniluk et al. 2006; Peterson and Jakubiec 2006). We thus try to find the reasons of theses facts and possibilities to stop this process. So, we continue our field studies and try to find any correctness partially in forthcoming breeding seasons. The results of the present study are part of our long-term investigations of White Stork (Kamiński et al. 2009). We hypothesize that there is a slow but systematic change of definite predispositions toward control immunoglobulin synthesis and other resistance products by the immunological system during postnatal development. This view is based on our own previous studies and suppositions on hole-nesting passerines (Gorzelski et al. 1995; Pinowski et al. 1995a, b; Romanowski et al. 1995; Kamiński and Matus 1998). Therefore, we should necessarily expand our research about these important processes in genes. The process of inheriting acquired physiological reactions (answers) is important as a type of answer to environmental stress. This process proceeds slowly, but the information encoded in genes is probably transferred in genetic material and inherited in the next generations. In accordance with this, we can thus deal with situation that in certain longest time intervals, the state of population can even exert considerable decrease (in time periods, but it must not necessarily be dangerous for the population. Individuals of such populations may be sick but still alive and can transfer changed genetic information to their offspring, which must not necessarily be sick already in the next breeding season. So, the important question is: to what degree can we form adaptation capabilities of White Storks arriving to breed in Europe and are these possibilities successful or not?

There is a significant increase in studies of genetic determinations and ecology of bird populations following the discovery of extensive polymorphism at protein coding loci (Parkin 1995). Analysis of the respective variation allowed extensive comparative studies on the role of ecophysiological moulding of the genetic structure of bird populations. However, studies of the genetic contribution of certain individuals remain obscure. There is insufficient variation to permit recognition of individuality and a consequent measurement of mating success or total reproductive output. Nevertheless, DNA techniques begin to change these results, and hypervariable minisatellite DNA permits the recognition of individuality in many species. Simple inheritance allows the unequivocal assignment of parenthood to most individuals within the population. This allows studying enzyme polymorphism in population and genetic ecology (Parkin 1995). The role of DNA in ecophysiological studies is thus obvious.

Identifying the sex of White Stork nestlings from external morphological characters is impossible, but molecular genetic methods provide a rapid, simple, and repeatable test, which requires just a small amount of blood. There were already established molecular tests to determine sex in different bird species, including the Oriental White Stork *Ciconia boyciana*, but very little information on whether these methods are useful for studying White Storks is available.

So far, only few studies investigated the relation of genetic changes, chromosomes, and lymphocytes of precocial birds, depending on environmental degradation or in areas with high background radiation. For example, Pastor et al. (2004) clearly showed that White Storks and Black Kites *Milvus migrans* which hatched in contaminated areas up to 4 years after a toxic accident still suffered an at least 2- to 10-fold increase in genetic damage, as compared to individuals from unpolluted areas. These results indicate that a toxic spill can affect wildlife for as long as 4 years and that attempts to clean up the dangerous waste were ineffective as far as DNA damage is concerned. Simultaneously, Pastor et al. (2001a) have studied a single cell gel electrophoresis which was used as a genotoxicity test in White Storks from an area heavily contaminated after an ecological disaster, occurring as a consequence of a massive toxic spillage of acid waste rich in heavy metals. Because this protected area is an important breeding and wintering site for many endangered bird species, the analysis of DNA damage is of special interest. The abovementioned results show that White Storks which hatched in a polluted area 1 year after a toxic spill bear a high burden of genetic damage as compared to control individuals. Moreover, investigations by Pastor et al. (2001b) indicate that the suitability of the comet assay as a biomarker for genotoxic analysis as part of environmental biomonitoring has been recently validated in studies using different sentinel organisms (fish, amphibians, rodents, mollusks). Birds preying on a variety of invertebrates and vertebrates in marshlands are appropriate for evaluating potential deleterious effects of toxic spills on wildlife (Benito et al. 1999; Pastor et al. 2001a; Meharg et al. 2002; Gomez et al. 2004). Studies on wetland birds high in the aquatic trophic chain and sampled within a few months after a toxic spill have shown the accumulation of heavy metals (14 months after a mine waste spill, blood samples from Storks and Kites collected in the neighboring area and from control birds at reference areas were examined by fluorescence image analysis after lymphocyte isolation and by subsequent alkaline single cell gel electrophoresis, known as comet assay). These results indicate that exposed birds had a significantly increased level

of genotoxic damage as compared to controls from unpolluted areas (Jovanovic and Atkins 1969; Pastor et al. 2001b; Roslik and Kryukov 2001; Meharg et al. 2002).

Parallel to rare studies on storks, there are investigations about genetic changes and karyological studies in the blood or other type of material from the birds of prey. Several genetic aspects of reproduction in rapacious birds were analyzed by Schmutz and Oliphanf (1987) in falcons and by Padilla et al. (1999) in eagles. Griffiths et al. (1998) tested DNA to determine sex of many birds. On the other hand, McGraw and Parker (2006) suggest that the production of circulating lipoproteins critically regulates the development of a colorful sexually selected trait in zebra finches (*Taeniopygia guttata*). Nevertheless, there is still a lack of papers dealing with changes in genes correlating with ecophysiological responses. Only Barker et al. (2002) studied taxonomic and biogeographic implications of nuclear DNA sequence data in passerines.

The aim of this paper was to analyze the sex ratio and gender determinations of White Stork nestlings from disturbed environments of Poland. We considered the impact of the toxic heavy metals Cd and Pb and established the sex ratio differences in different types of environment, i.e., male and female participation in sex structure. We also analyzed the differences in sex ratio in relation to the degree of environmental degradation, as expressed by Cd and Pb. Therefore, we can make statements about the impact of these metals on sex structure in the White Stork. Thus, we combine factors for sex comparison of White Stork chicks between environments against Cd and Pb level and for determination sex-environment-element interactions at determined significance level. Simultaneously, we analyze the differences in sex ratio according to the degree of environmental degradation expressed by Cd and Pb. We can thus suppose about the impact of these metals upon sex structure in White Stork. The aim of our study was also to analyze the possibilities of multiplex PCR employment for determination the changes in sex of White Stork. As a result of multiplex PCR, 18S ribosome gene, which served as an internal control of PCR, was amplified in male and female storks. Therefore, it is possible to use primers developed for studies on domestic chicken to replicate this genome fragment in the White Stork. Also, primers specific for the Oriental White Stork *Ciconia boyciana* W chromosome can be used to determine the sex of the White Stork. This is an attempt to verify the use of nucleotide sequences characteristic for domestic chicken and Oriental White Stork to detect the White Stork sex, which proved successful. Simultaneously, employing the 18S ribosome gene, which is a conservative sequence in birds and can be correctly amplified in the White Stork, might be convenient to simplify and utilize molecular test for sex detecting in a wide range of wild birds.

Study area

We collected blood samples from White Stork nestlings in the village of Kłopot adjacent to the Odra meadows (52° 07′ 56.3″ N, 14° 42′ 10.4″ E; SW Poland (see Tryjanowski et al. 2005)), which served as controls from an unspoiled environment (termed "Odra meadows" or "controls"). Comparisons were made with suburban sites: four villages about 20 km away from Zielona Góra (51° 56′ 26.1″ N, 15° 30′ 38.9″ E; 100, 000 inhabitants, SW Poland), mainly Czarna (51° 54′ 43.9″ N, 15° 42′ 01.1″ E) and Czarnowo (52° 02′ 03.7″ N, 14° 57′ 24.7″ E). In the villages near Zielona Góra, samples were collected at a distance of several kilometers from the city boundary. We called these areas "suburbs." We also took blood samples near Głogów (51° 39′ 32.6″ N, 16° 04′ 49.9″ E; S Poland) where a copper smelter is situated (polluted area). This factory produced copper and lead from lead fields, and, therefore, we termed these areas "polluted." The last study site were swamps near the Baltic Sea and close to Słupsk (54° 38′ 34.5″ N, 17° 32′ 31″ S; N Poland), hereafter called "swamps."

Material and methods

A total of 87, 165, and 104 of White Stork nestlings from 36, 39, and 48 nests were studied in the breeding seasons 2006, 2007, and 2008, respectively. The age of the nestlings varied from 14 up to 62 days. As far as the study sites are concerned, 84 individuals from the Odra meadows, 48 from swamps, 85 from suburbs, and 53 from polluted areas were investigated. To eliminate influences of any diurnal rhythm, we handled nestlings always between 10:00 a.m. and 12:00 a.m.

Venous blood was taken for basic and genetic analyses, and we collected blood samples by puncturing the brachial vein of nestlings. These were taken out of the nest and placed into individual ventilated cotton sacks. Blood (5 ml) was collected using 5 ml syringe washed up with ethylenediaminetetraacetic acid (EDTA). Samples were kept in a chilled cooler, transported to the laboratory, and centrifugated. Plasma samples were then frozen at −20 °C and stored until analyses. Behavioral observations as well as on-site examinations suggested that all nestlings were physically healthy.

Four adult birds (two males and two females), the sex of which was determined by veterinarians (by thorough visual inspection of genital area and also by laparoscopy in order to confirm), served as controls in molecular tests. Blood samples were collected in EDTA-treated plastic tubes and stored in temperature of +4 °C. High molecular mass DNA was extracted from the whole volume of blood using a MasterPure™ DNA Purification Kit for Blood (Epicentre Technologies), following the manufacturer's instructions. To obtain DNA of good quality and high quantity, we took 180 μl instead of 360 μl, as recommended by the producer (Epicentre Technologies). To determine

the genetic sex of young White Storks, the multiplex PCR method was applied. Two sets of primer were used: primers specific for W chromosome were described by Itoh et al. (1997) for amplification of the female specific sequence on W chromosome in the Oriental White Stork *C. boyciana*. The second set of primers, described by Clinton et al. (2001), was used to replicate the 18S ribosome gene, which serves as a positive control of PCR reaction. The amplification was performed in 20 μl reaction volume containing approximately 50 ng of genomic DNA, 8 pmol of each primer, 200 μM of each dNTP, 2.0 mM $MgCl_2$ and 1.75 U *Taq* polymerase (Fermentas, Vilnius, Lithuania) in 1-fold reaction buffer with KCl. Temperature profile of the reaction was as follows: denaturation at 94 °C for 2 min, followed by 35 amplification cycles of 80 s at 94 °C, 90 s at 58 °C, 60 s at 72 °C, and the final extension of 5 min at 72 °C. Amplicons were separated in 2.5 % agarose gel in 1-fold TBE buffer (10×TBE: 0.89 M Tris, 0.89 M boric acid, 0.02 M EDTA, pH 8.0) on 120 V during 60 min and stained with ethidium bromine.

Cd and Pb concentrations (μg kg^{-1}; ppm dw) were determined applying the ICP-MS method by ICP-MS AGILENT 7500CE. These were made in relation to analysis of reference materials. Parallel measurements were made in blind trials.

Statistical analysis Statistical analyses of data were performed using Statistica 9.1 software (StatSoft Inc. 2010). All results are given as arithmetic means±S.D in tables. Percentage participation of White Stork *Ciconia ciconia* sexes in differentiated Poland environments is displayed in figures. Differences in sex ratio of White Stork chicks from different environments were examined by one-way ANOVA test, followed by a multiple range test ($P<0.05$) and RID Tukey test (test of a reasonably important difference for bumpy numerical force of attempt). A significance level of $\alpha=0.05$ was chosen for all statistical tests. Data not normally distributed were log transformed. We invoked the GLM procedure. To analyze the overall differences in cadmium and lead concentrations between male and female white stork from four different study sites, multivariate analysis of variance (MANOVA) was used. We thus answered the question: does the type of environment and sex of White Stork chicks affect the concentration of lead and cadmium in the blood of birds? Heavy metal concentration were used as a multiple dependent variable with gender and environment as factors. To examine differences among environments, we used RIR Tukey test (test of a reasonably important difference for bumpy numerical force of attempt) Zar (1999). We answered the question: between which groups did statistically important differences occur?

This study was done in accordance with the Guidelines of the European Union Council and the current laws in Poland according to the Ethical Commission (05/2005). Necessary permits from the Local Committee for Animal Research in Gdansk and from the Ministry of Nature Conservation were obtained: LKE Gdansk 20/05 and DLOPiK-op/ogiz-4200/III-21/3706/07 /jr, respectively.

Results

No significant differences in sex ratio were found between years (calculated as the percentage of male chicks in each nest; ANOVA, $F_{2.85}=0.04$, $P=0.96$). There were significant differences in the sex ratio between chicks from different types of environment. The proportion of males was significantly higher everywhere (Odra meadows: $P<0.001$; swamps near Baltic Sea: $P<0.005$; polluted areas: $P<0.001$), except in the suburban areas where females prevailed ($P<0.01$) (Fig. 1). However, male and female participation is similar in studied environments, and it is expressed by domination of both sex in the Odra meadows and suburban areas (Figs. 2 and 3).

There are significant differences among the mean values of blood lead levels in male and female White Stork ($F=10.713$, $P=0.001$) and depending on the type of environment ($F=36.896$, $P=0.000$) (Tables 1 and 2). We found significant interactions between gender (sex) and the type of environment ($F=6.123$, $P=0.001$), i.e., both gender and the environment are important in terms of the concentration of lead in stork's blood. On the other hand, we did not indicate differences in the concentrations of cadmium depending on gender ($F=0.533$, $P=0.466$) and interaction between gender and the type of environment in the case of Cd ($F=2.192$, $P=0.091$) (Table 2). However, the type of environment influence the concentration of Cd ($F=6.035$, $P=0.001$) (Table 2).

We determined differences (RIR Tukey test) in female and male intoxication by Pb (range 1.29 to 6.45 ppm dw) and in female intoxication by Cd (range 2.01 to 3.57 ppm dw) among disturbed environments. Lead concentration was higher in female White Stork chicks from polluted area, as compared to other groups (gender) from other studied environments ($P<0.001$), as well as compared with males from polluted area ($P=0.002$) (Tables 1 and 3). Cadmium concentration was higher in the females from polluted area, as compared with

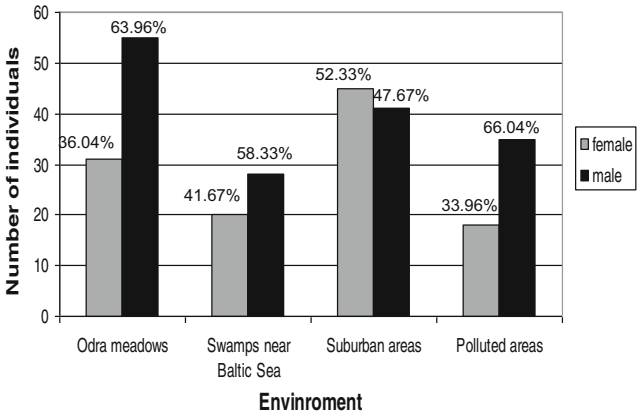

Fig. 1 Proportion of female and male White Stork *Ciconia ciconia* chicks in different environments of Poland (difference at Odra meadows $P<0.001$, swamps near Baltic Sea $P<0.05$, suburban areas $P<0.01$, and polluted areas $P<0.001$)

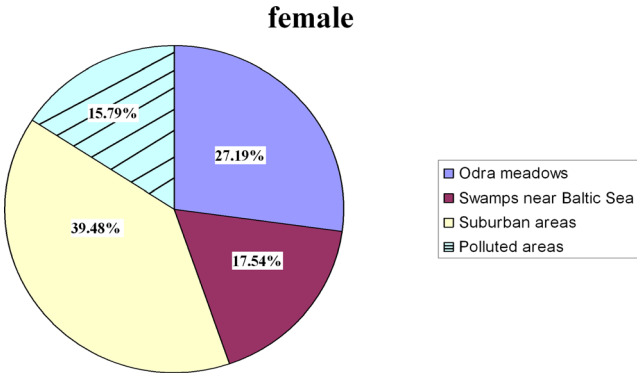

Fig. 2 Proportion of female White Stork *Ciconia ciconia* chicks in different environments of Poland

females from Odra meadows (P=0.004) and from the swamps near Baltic Sea (P=0.02) (Tables 1 and 3).

Our results of the electrophoresis and the measurements by the spectrophotometer indicated that MasterPure™ DNA Purification Kit for Blood (Epicentre Technologies) enables isolation of DNA of good quality and high concentration ranging from 300 up to 1000 ng μl^{-1}. As a result of multiplex PCR, 18S ribosome gene, which served as an internal control of PCR, was amplified in male and female storks. It means that it is possible to use primers designed for domestic to replicate this fragment of genome in White Stork. The respective fragment in domestic chicken is 256 bp, similar to that of the White Stork. Employing 18S ribosome gene, which is a conservative sequence in birds and can correctly been amplified in the White Stork, might be convenient to simplify and utilize molecular tests for determining sex in a wide range of wild birds. Primers specific for the Oriental White Stork *C. boyciana* W chromosome enable to determine the sex of *C. ciconia* chicks.

Discussion

White Stork is a bioindicator for large-scale environmental research. The main threats to its breeding population include natural factors, i.e., weather, human impact, groundwater level, drainage, transformation of grassland into arable fields, networks of electric wires changes in roofing types, and agricultural impact (Papi et al. 1997; Johst et al. 2001; Moritzi et al. 2001; Sasvári and Hegyi 2001; Tortosa et al. 2003; Jovani and Tella 2004; Schaub et al. 2004; Tryjanowski et al. 2004; Denac 2006; Kaługa 2006; Profus 2006; Saether et al. 2006). Storks have been associated with anthropopression, and changes in agricultural and industrial management caused serious fluctuations in the numbers of this species and even its permanent retreat from considerable parts of areas. A decrease in White Stork population size was noted during the last years in Europe (Daniluk et al. 2006; Peterson and Jakubiec 2006). Storks are under pressure of changing landscape (Rubacha and Jerzak 2006); however, in Poland, it was rather stable (Daniluk et al. 2006). The Poland White Stork populations are generally moderately abundant, although in some parts, they are infrequent (Tomiałojć and Stawarczyk 2003).

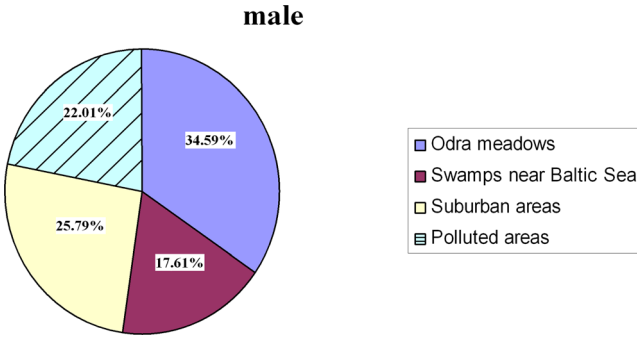

Fig. 3 Proportion of male White Stork *Ciconia ciconia* chicks in different environments of Poland

The changes of White Stork population were assumed by Lack (1973) who had taken an attempt for analysis of the mechanisms regulating the number of population. Changes of population dynamics, number, fecundity, and mortality are the results of processes, among which the environmental impact and disturbed chemicals are most significant. Storks can remain still below environmental capacity (Jakubiec 1985) and so environmental conditions do not always reflect their demands.

Our present study indicates differences in the sex ratio of White Stork chicks from different types of environment. In general, the proportion of males is considerably higher in each type of environment. We can assume from these results that differences in the sex ratio of White Stork nestlings depending on the degree of environmental degradation, as expressed by toxic heavy metals and the interaction of sex, environment, and toxic metals, show the impact of these metals on sex ratios. We can explain these dependencies either by environmental determination of the condition of storks or by ecophysiological reactions to environmental stressors (Kamiński et al. 2009). On the other hand, we know that the sex ratio may even differ between populations of the same species (Fernandes et al. 2006; Kekkonen et al. 2008). As Tryjanowski et al. (2011) suggest, in intensively used farmland, where environmental conditions for White Stork are worse and density and breeding success are lower (Tryjanowski and Kuźniak 2002; Olsson 2006; Saether et al. 2006), the sex ratio may be more skewed toward females. If male chicks are produced more often, they are older and, therefore, have a higher chance of survival (at least on the nestling stage) resulting in a male skew ratio in the local population. Simultaneously, we do not know the sex ratio of adult storks in our study areas. Moreover, to our knowledge, there are no good population data on the sex ratio of breeding White Storks, but some suggestions indicate that there are more male than female adults (Creutz 1985; Chernetsov et al. 2006; Olsson 2006;

Table 1 Intoxication of female and male White Stork *Ciconia ciconia* chicks by the heavy metals Cd and Pb (ppm dw) in different Polish environments (descriptive statistics)

	Cd (ppm dw)			Pb (ppm dw)		
	Mean±SD	Min–Max	*N*	Mean±SD	Min–Max	*N*
Females						
Odra meadows	2.01±0.88	0.77–4.27	30	1.29±0.75	0.07–2.30	18
Swamps near Baltic Sea	2.19±0.61	1.26–4.53	19	2.38±1.31	0.07–4.22	19
Suburban areas	2.87±1.36	0.74–5.15	39	2.23±1.14	0.51–4.65	28
Polluted areas	3.57±1.60	0.83–5.62	18	6.45±3.65	1.15–10.70	11
Males						
Odra meadows	2.35±0.99	0.77–4.65	49	1.40±0.65	0.12–2.40	29
Swamps near Baltic Sea	2.50±1.36	1.15–7.74	25	1.64±0.98	0.09–4.11	25
Suburban areas	2.85±1.40	0.69–5.13	40	2.33±1.14	0.03–5.30	28
Polluted areas	2.79±1.38	1.01–5.25	35	3.75±2.60	0.30–10.30	26

Tryjanowski et al. 2011). Therefore, females, as the less frequent sex, may be more successful in finding partners of greater fitness to breed with. This may explain the situation in a Spanish White Stork population, where younger and weaker chicks become the best breeders in the future (Aguirre and Vergara 2007; Tryjanowski et al. 2011). However, no sex bias in hatching order or body mass could be detected (see Tryjanowski et al. 2011).

We should emphasize that modifications of original protocol of DNA extraction supplied by manufacturer of MasterPure™ DNA Purification Kit for Blood (Epicentre Technologies) enable to extract genomic DNA from only half of the blood volume recommended by producer. Our results of electrophoresis and the measurements with the spectrophotometer indicated that this kit enables an isolation of DNA of good quality and high concentration ranging from 300 up to 1000 ng μl^{-1}. As a result of the multiplex PCR, one or two bands, depending on the sex, were obtained. 18S ribosome gene, which served as an internal control of PCR, was amplified in both sexes. This indicates that primers developed for domestic chicken can be used to replicate this fragment of genome in the White Stork. This fragment is 256 bp long in the chicken and of similar length in White Storks. Primers specific to the Oriental White Stork *C. boyciana* W chromosome (Itoh et al. 1997) can be used to determine the sex of White Stork chicks.

Very little is known about the use of nucleotide sequences characteristic for domestic chickens and the Oriental White Stork for detecting White Stork sex. Our study attempts to verify the use of nucleotide sequences characteristic for chicken and the Oriental White Stork in order to detect White Stork sex. We proved that it is possible to detect the sex of White Stork nestlings using these sequences. Employing 18S ribosome gene, which is a conservative sequence in birds and can be amplified correctly in the case of the White Stork, might be convenient to simplify and utilize molecular test for detecting sex in a wide range of wild birds. E.g., Itoh et al. (2001) reported two sets of control primer. However, 18S ribosome gene primers should be tested in a wide array of birds because it is supposed that these primers could replace different control primer depending on specific species. On the other hand, it must be emphasized that, based on the ideas on the low rate of chromosomal and molecular evolution in birds (Prager and Wilson 1975; Tegelström et al. 1983), chromosomal rearrangements and their fixation rate should be examined in the White Stork. Such study could

Table 2 Results of multivariate analysis of variance for total characteristics of mean concentrations of lead, and cadmium as a multiple dependent variable in relation to sex of White Stork *Ciconia ciconia* chicks and type of environment as quality factors

	Cd (ppm dw)		Pb (ppm dw)	
	P	*F*	*P*	*F*
Sex	0.533	0.466	10.713	*0.001****
Environment	6.035	*0.001****	36.896	*0.000****
Sex × environment	2.192	0.091	6.123	*0.001****

****P* means the significance of values set in italics

Table 3 Differences between type of environment (A—Odra meadows, B—swamps near Baltic Sea, C—suburban areas, D—polluted areas) and toxic metals Cd and Pb (ppm dw) in White Stork *Ciconia ciconia* chicks in Poland

	Differences
Cd (ppm dw)	DF: AF***; BF***
Pb (ppm dw)	DF: AF***; BF***; CF***; AM***; CM***; DM**; BM***

RID Tukey test; test of reasonably important difference for bumpy numerical force of attempt

**P*<0.05; ***P*<0.01; ****P*<0.001

provide important data on the genetic predisposition in this bird species.

Mechanisms responsible for environmental stressors in birds, stick already at molecular level, first of all. Thus, we should also consider that, as must be concluded from the last studies (Pastor et al. 2001a, b, 2004; Møller 2005; Wang et al. 2005; Baos et al. 2006), environmental stressors, including toxic metals and disturbed free radicals, can change epigenetic patterns through DNA modifications and thereby effect gene activation and cell phenotype. Oxidative stress and antioxidant defense systems profiles gene expression related to oxidative stress. Glutathione peroxidases (GPx) and peroxiredoxins (TPx) and genes involved in reactive oxygen species (ROS) metabolism, such as oxidative stress responsive genes and genes involved in superoxide metabolism like superoxide dismutases (SOD) and catalases (CAT), can easily and reliably analyze the expression of genes related to oxidative stress (Pastor et al. 2001a, b, 2004; Møller 2005; Wang et al. 2005; Baos et al. 2006). These results indicate that birds exposed to disturbed environments have a significantly increased level of genotoxic damage compared with control animals from unpolluted areas (Pastor et al. 2001a, b; Meharg et al. 2002). Lead plays a considerable role among different factors in the environmental modification of genetic material of birds. E.g., White Storks fed in contaminated waters, resulting from acid mining-sludge spillage at the eastern flank of the Guadalquivir marshes (Doñana, SW Spain) where sludge was rich in a variety of toxic metals as well as in organic pollutants like aromatic amines and amino acids. The Storks did not show elevated blood levels of metals immediately after the accident, but nestling blood collected in the following year showed a higher degree of genotoxic damage compared to controls (Meharg et al. 2002). In that study, Pb isotope analysis was used to assess if storks had ingested sludge-derived contaminants. Sludge Pb isotope ratio was distinct from that of Doñana sediments. The stork blood Pb isotope ratios exactly matched that of the sludge. Therefore, storks had ingested sludge-derived contaminants. A detailed study of lead contamination along these areas was also conducted to investigate the lead level typical for the White Stork colony in relation to the spatial contamination of storks' habitat (Meharg et al. 2002).

Generally, the results of our study and of other authors (Pastor et al. 2001a, b, 2004; Meharg et al. 2002; Møller 2005; Wang et al. 2005; Baos et al. 2006) show that White Stork nestlings are affected by toxic heavy metals and may satisfactorily explain observed DNA damage. E.g., Baos et al. (2006) reported high levels of genetic damage in waterbirds from the Cota Doñana (SW Spain) after a mining accident as compared to control areas. Also, potential relationships between DNA damage and metals (Zn, Pb, As, Cu, Cd) pollution have been reported. Correlations between the abovementioned metals and genetic damage varied throughout the years within species, with some metals having a significant relevance. The results by Baos et al. (2006) suggest that White Stork chicks are affected in part by heavy metals, which alone, however, can0-not satisfactorily explain the observed DNA damage. However, their studies and the results of the present paper indicate that White Storks exposed to environmental stress have a significantly higher level of genotoxic damage compared with control birds from unpolluted locations. This clearly shows that species-specific differences must be carefully considered when establishing schemes for pollution monitoring and highlights the need for including an appropriate time scale in studies of the effects of pollutants in the wild.

In the case of the White Stork, our results show that individuals breeding in contaminated areas have substantial genetic damage as compared with control individuals. The resulting implications for future survival as well as for reproduction depend on various ecophysiological implications. We can conclude that the observed increased level of genotoxic damage is exclusively attributable to an environmental anthropogenic disturbance. However, intensive agriculture in surrounding areas is accompanied by the use of plaguicides, residues of organochlorine pesticides and polychlorinate biphenyls appeared to be one of the reasons of this situation (Hernández et al. 1988, 1999; Jiménez et al. 1999). Therefore, the observed damage of DNA in White Storks seems to be provoked by organic pollutants and heavy metals (Benito et al. 1999; Hernández et al. 1999). On the other hand, it is not easy to explain the increase of genotoxic damage exclusively by heavy metal contamination, mainly because respective levels have tended to decrease in all potentially involved compartments (sediments, diet, etc.). Therefore, an analysis of the relation between genotoxic damage of an individual and metal concentrations is essential to investigate this hypothesis (Pastor et al. 2004). Moreover, further investigation is needed on the similarity in genotoxic damage in nest mates, which could indicate a heritable genetic damage and/or a common diet, with implications for contaminant accumulation (Pastor et al. 2004). Taking into account the high level of genetic damage, sublethal effects like decreased lower reproductive success or lower survival rates could become apparent in the populations of White Stork. Thus, a long-term monitoring is needed to assess whether genotoxic damage detected in these birds might have any further effect on the population dynamics (Pastor et al. 2004).

Summarizing, the abovementioned studies indicate that although not all types of genotoxic exposures should be expected to result in DNA damage in mononuclear blood cells, DNA damage is abundant in bird cells and affected by lifestyle and many environmental factors, including diet, exercise, hypoxia, and sunlight (Pastor et al. 2001a, b, 2004; Møller 2005; Wang et al. 2005; Baos et al. 2006). According to Tryjanowski et al. (2011), the sex of White Stork chicks depends largely on the environmental conditions for adult females. With ample food supply and resulting good body condition of adult females,

more male nestlings are born. These, in better shape, have a higher survival rate, and adults have a better chance of finding a mate because females are in the majority. With a bad food supply and in unfavorable weather conditions, however, more female nestlings are born, which may be weaker but still will find a mate later in life (because males are in the majority now). It must be also emphasized that selection of a on sex ratio adjustment may be variable due to differences in the breeding ecology, sexual dimorphism, and life histories, and because biological variation in sex ratio adjustment and within species such variability in selection is less straightforward (Griffin et al. 2005). Therefore, the sex ratio of the White Stork can be manipulated by parents according to environmental conditions, food availability, or generally via a year effect, including weather conditions which influence the reproductive condition in this species (Tryjanowski et al. 2004, 2011). It can thus be concluded that reasons for changes in White Stork sex differentiation vary according to environmental variation. Many factors lead to a change of the sex ratio in White Storks in subsequent breeding seasons, depending significantly on specific environmental parameters that shape individual detailed defense mechanisms. This is a direct cause for the formation of a specific sex structure of storks. It can be assumed that these regularities are associated with changing environmental conditions (primarily food abundance) and weather conditions in different breeding seasons.

Conclusions

1. Differences in White Stork sex ratio according to the degree of environmental degradation, as expressed by Cd and Pb levels and an interaction of sex environment and metals, indicate the impact of toxic metals on the sex ratio in young storks.
2. The type of environment has a significant impact on the concentration of Pb and Cd in the blood of the White Stork.
3. Males from the most contaminated environments (polluted area) cumulate average of more than 40 % of Pb than females of the same environment.
4. Many factors determine changes of the sex ratio of White Storks in subsequent breeding seasons, depending significantly on specific environmental parameters that shape individual detailed defense mechanisms.
5. White Storks exposed to toxic heavy metals have a significantly increased level of genotoxic damage compared with control birds from unpolluted locations.
6. Primers developed for domestic chicken are appropriate to replicate the respective fragment of genome in the White Stork, and primers specific for the Oriental White Stork *C. boyciana* W chromosome can be used to determine the sex of White Stork chicks.
7. Employing the 18S ribosome gene, which is a conservative sequence in birds and can be correctly amplified for the White Stork, might be convenient to simplify and utilize molecular test for sex detecting in a wide range of wild birds.

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