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Melatonin and thyroxine response to pollution in white stork nestlings (*Ciconia ciconia*): Aspects of rhythmicity and age

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Abstract

There is growing evidence that ubiquitous environmental contaminants may interfere with vertebrate endocrine systems. The selected endocrine biomarkers are used to indicate the condition of free-ranging populations of wildlife, including avian species. The aim of this study was to determine the impact of environment quality on serum thyroxine (T4) and melatonin (Mel) in white stork nestlings (*Ciconia ciconia*) living in different locations: small villages in natural areas surrounded by forests and crop fields, near the city and near the copper smelter. We extended our analyses to examine the hormones' day–night changes in conjunction with chicks' age. Total serum T4 and Mel was measured by RIA. T4 level, as a decisive measure of thyroid hormone productivity, was significantly lower in the nestlings exposed to pollutants from the copper smelter. Mel, as a well-known scavenger of free radicals, was elevated in the nestlings in the area near the copper smelter. This study indicates that alteration in T4 and Mel levels could be a useful marker of exposure of nestling wild storks to different toxic substances in field studies. Mel is postulated to be a susceptible defensive molecule as a protective mechanism for organisms.

Keywords: Biomarkers; Endocrine disruption; Melatonin; Metals; Pollution; Rhythm; Stork; Thyroxine

1. Introduction

There is growing evidence that ubiquitous environmental contaminants may interfere with vertebrate endocrine systems. Among them are any number of organic agricultural and industrial pollutants and heavy metals. Metal-mediated formation of free radicals and oxidative stress result in DNA damage and deterioration of biological macromolecules (Valko et al., 2005). Over the past decade the selected endocrine biomarkers have been used to track the condition of free-ranging populations of wildlife, including avian species (Scanes and McNabb, 2003; Baos et al., 2006; Jenssen, 2006; Mora et al., 2006). Recently, the studies of exposure to various environmental pollutants point towards a susceptible endocrine organ and their products, i.e. thyroid gland and thyroid hormones — thyroxine (T4) and triiodothyronine (T3) (Rolland, 2000; Zoeller, 2005; Boas et al., 2006; Mori et al., 2006). Nowadays it is known that thyroid disruption can be caused by a variety of chemicals and is accomplished by variety of mechanisms interfering at different levels of the hypothalamic-pituitary-thyroid axis (McNabb, 2005; Boas et al., 2006; Zoeller, 2006). Normal thyroid gland function is essential for avian development and metabolism (McNabb, 2006). The impairment of thyroid function may have tremendous consequences for growth, thermoregulation, cognitive capabilities and immune function, especially in developing individuals of long-distance migrants, such as the white storks.

Melatonin (*N*-acetyl-5-methoxytryptamine; Mel), produced by pineal gland, retina and GIT (gastrointestinal tract) in vertebrates, is known as highly sensitive and effective scavenger of

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hydroxyl and peroxyl free radicals in cells (Reiter et al., 2000; Sofic et al., 2005; Tan et al., 2007). Moreover, experimental studies showed a response of Mel in animals exposed to many different contaminants and a preventive action of Mel pretreatment in the metal-mediated cell cytotoxicity and oxidative stress (Tan et al., 2002, 2007). As such, Mel can be considered as an indicator of physiological condition of organism exposed to polluting substances in environment and provide a useful tool in many ecotoxicological investigations.

The aim of this study was to determine the impact of environment quality on selected hormones' levels in the wild white stork. We investigated two hormones: thyroxine and melatonin in animals living in polluted and unpolluted environments. Since the response to contaminants in developing organisms is of particular concern, we have focused on nestlings. In our study, we have also considered other factors affecting endocrine status, such as a time of the day and age of nestlings, in order to distinguish the changes particularly associated with quality of environment.

2. Materials and methods

2.1. Animals and sampling

Studies were performed in 2005 in free-living white stork nestlings (*Ciconia ciconia*) in colonies in the West of Poland: in the village of Kłopot (Village 1; 52°06′ N; 14°42′ E), near the city of Zielona Góra (City; 51°57′ N; 15°30′ E) and in the area affected by the copper smelter in Głogów (Smelter; 51°40′ N; 16°05′ E). In 2006, sampling was repeated in the village of Kłopot, near the copper smelter in Głogów and in a new location in the North of Poland: in the village of Cecenowo (Village 2; 54°38′ N; 17°33′ E). White storks are abundant in Poland, where they spend the breeding period (March–August). All storks breed in open nest on roofs, at the top of trees, or pylons. Chicks hatch after approximately one month of incubation, but depend on both parents for food and protection during next 70–80 days. The diet of the nestlings consists mainly of earthworms (Pinowski et al., 1991; according to our analysis of nestling vomit in the field). Our studies were carried out during their nesting season, between 23rd and 27th June in 2005 and between 26th June and 04th July in 2006. Most colonies were located in small villages Kłopot (Village 1) and Cecenowo (Village 2), far from industry, in natural areas surrounded by forests and crop fields; the others were situated 10 km from the city of Zielona Góra (City; 120,000 inhabitants; no heavy industry), or 15 km from the apparent source of pollution, i.e. a copper smelter in Głogów (Smelter).

Blood samples (5 mL each) were collected from nestlings via veni-puncture of the brachial vein. In 2005, in selected nests, sampling was conducted twice a day: during the day (10–12 am) and at night (11 pm–1 am); the rest in 2005 and all in 2006 were sampled only during the day (10–12 am). Blood samples were taken from 48 individuals from 31 nests (Village 1, 2 and City) and 32 individuals from 15 nests (Smelter). Each chick was retrieved from the nest and placed into individual ventilated cotton sacks. Nestling white storks displayed very little response to handling, including that at night. Body mass (to the nearest 50 g) was determined by using a spring balance. The age was estimated by measurement of the beak length according to Kania (1988). The age of chicks and their weight are presented in Table 1. Storks were manually restrained during sampling. No differentiation was made between sexes, as the birds do not exhibit sexual dimorphism. Blood was collected by means of 22G × 1¼″ needles and immediately transferred to test tubes (1.5 mL). In the field, the samples were stored in ice in the thermal cooler bag. After 4 h at room temperature when blood clotted, serum was collected by centrifugation at 18,700 *g*, then stored at −70 °C.

The experiments complied with the Guidelines of the European Union Council and the current laws in Poland, according to the Ethical Commission (permission number: 05/2005).

2.2. Analytical methods

Serum Mel was assayed using total melatonin RIA kit (IBL, Hamburg), with preceding extraction procedure. Solid phase extraction (SPE) of melatonin was carried out on Octadecyl C_{18} Speedisk Column, 10 μm (J.T. Baker, USA) according to a procedure described previously (Kulczykowska and Iuvone, 1998). The columns were conditioned with three 1-mL portions of methanol followed by three 1-mL portions of HPLC-grade water. The 100-μL serum samples were aspirated through columns, then washed with 1 mL of 10% methanol in HPLC-grade water. The samples were eluted with two 300-μL portions of methanol, dried under air and held at −70 °C prior to analysis. Before RIA procedure, samples were resuspended in Dulbecco's Phosphate Buffered Saline containing 0.01% Thimerosal (Sigma-Aldrich, USA). All samples in duplicate were counted in a Wallac Wizard γ-counter. The detection limit was 3.0 pg per mL of serum. The intra- and inter-assay coefficients of variation were 8.1% and 14.9%, respectively.

Total serum T4 was measured by total thyroxine RIA-gnost kit (Schering, France). The assay was performed using tubes coated with antibodies and 8-anilino-1-sulfonic acid (ANSA) as a displacement reagent. All samples in duplicate were counted in a Wallac Wizard γ-counter. The detection limit was 2.5 ng per mL of serum. The intra-assay coefficient of variation was 5.6%. The inter-assay variation was not determined, because all samples were measured in the same assay.

The metals, Zn, Mg and Cd are frequent pollutants in the surroundings of a factory complex like a copper smelter. Levels of these elements in serum were measured by Atomic Absorption Spectrometry (AAS) with the Perkin-Elmer Instrument (type RW 0683/3 PYC). Zn and Mg were examined by flame method and Cd by graphite tube method (Weltz, 1985).

Table 1
The mean age and weight of nestlings from Smelter and other locations (Village 1, Village 2, City)

Location	Age (days)	Mass (g)	*n*
Smelter	35±1	2710±113	32
Others	32±1	2520±89	48

Mean values±SEM; *n* — number of storks per group.

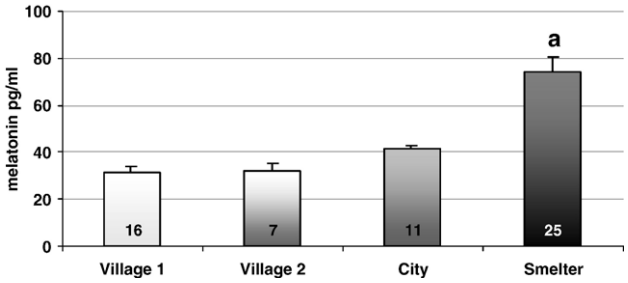

Fig. 1. Serum melatonin concentrations of white stork nestlings from four locations: Village 1, Village 2, City, and Smelter. Values are means±SEM. Numbers of birds are given in bars. a, significantly different vs. Village 1, Village 2 and City (ANOVA followed by post-hoc RIR Tukey's test; $F=17.45$, $P<0.000000$).

Calibration curves were constructed using Merck standards. The detection limits for Zn, Mg and Cd were in the low mg/L range.

2.3. Statistical analysis

Values are presented as means±standard error of the mean (SEM). Significant deference between means for paired sample studies were identified using paired Student *t*-test. The un-paired data were evaluated by un-paired *t*-test. For multiple comparisons, the one-way ANOVA followed by post-hoc RIR Tukey's test was used (STATISTICA program). Regression analysis was performed using STATISTICA program. Significance was taken at $P<0.05$.

3. Results

Serum Mel concentration measured in samples collected during the day from nestlings near the copper smelter (Smelter) was significantly higher than that measured in chicks in the villages (Village 1 and Village 2) and near the city (City) (Fig. 1). On the other hand, T4 concentrations in serum collected from nestlings near the copper smelter were the lowest (Fig. 2). The mean age and mean weight of nestlings in polluted and other locations did not differ significantly (Table 1).

Mel and T4 concentrations in samples collected twice a day in two locations: Village 1 and City are summarized in Table 2. Serum Mel concentration measured at night was significantly higher than that measured during the day in both groups.

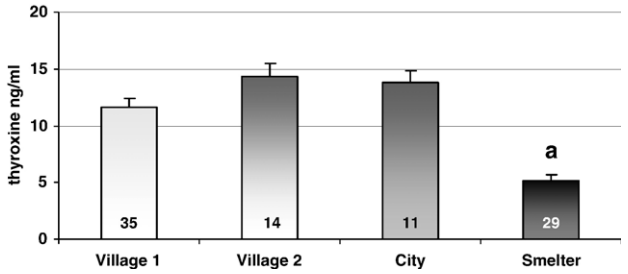

Fig. 2. Serum thyroxine concentrations of white stork nestlings from four locations: Village 1, Village 2, City, and Smelter. Values are means±SEM. Numbers of birds are given in bars. a, significant different vs. Village 1, Village 2 and City (ANOVA followed by post-hoc RIR Tukey's test; $F=25.46$, $P<0.000000$).

Table 2
Serum Mel and T4 concentrations in samples taken during the day and at night from white storks in 11 nests in the village of Kłopot (Village 1) and near the city of Zielona Góra (City)

Mel (pg/mL)	Day	Night	*n*
Village 1	33.1±2.7	80.6±5.0[a]	12
City	41.5±1.2	70.6±3.8[ab]	11
T4 (ng/mL)			
Village 1	12.0±1.7	14.5±0.8	12
City	13.8±1.1	9.5±0.7	11

Natural lighting regime (16L:8D). Mean values±SEM; *n* — number of storks per group.
[a]$P<0.0001$, for comparison of corresponding day and night values (paired Student *t*-test).
[b]$P<0.01$, for comparison of day–day and night–night values in two locations (un-paired Student *t*-test).

Moreover, the night Mel values between the two locations differed markedly. However, no day–night differences in T4 values in any locations were observed.

In the City group, night Mel concentration and night increases of Mel concentration (ΔMel) correlated positively with age of the chicks (Fig. 3). The City group was exceptionally diversified in terms of nestlings' age (19–42 days), so that such examination was feasible. No correlation was observed either between age and day Mel concentration or between age and T4 concentration.

Levels of metals in serum samples of nestlings from polluted and unpolluted locations are shown in Table 3. The significantly higher levels of Zn, Mg and Cd were observed in chicks from polluted area near the copper smelter.

There were no inter-annual differences in hormones' concentration among the same locations sampled in 2005 and 2006, thus the data were combined. However, the data for Villages 1 and 2 were presented separately thereby to show that hormones' levels studied in two consecutive years in nestlings

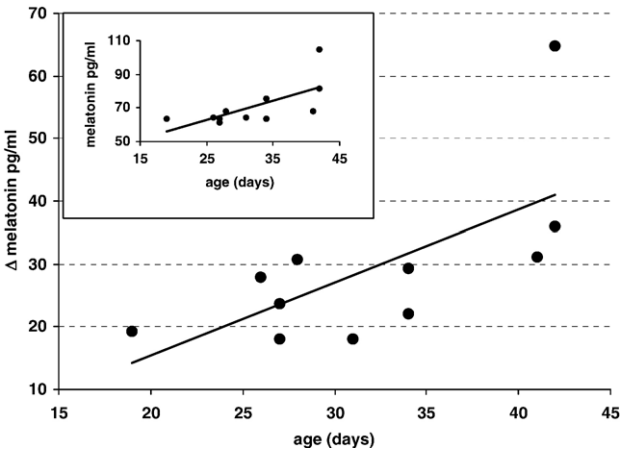

Fig. 3. Relationship between melatonin night increases (Δ) and age of white stork nestlings from the City group. A regression line was drawn according to the equation: $y=1.16x-7.91$ ($r=0.66$, $P<0.05$). Inside: relationship between serum melatonin concentrations at night and age of white stork nestlings from the City group. A regression line was drawn according to the equation: $y=1.16x+33.68$ ($r=0.68$, $P<0.05$).

Table 3
Levels of essential metals Zn, Mg and Cd in serum samples of white stork chicks from Village 1 and Smelter locations

Location	Zn (mg/L)	Mg (mg/L)	Cd (mg/L)
Village 1	9.38±1.27	1469.2±87.1	2.57±0.16
Smelter	16.60±2.22	2932.6±203.0	5.06±0.03
P	<0.01	<0.000001	<0.000001
n	16	14	14

Mean values±SEM; *n* — number of storks per group; *P*, un-paired Student *t*-test.

from environments of comparable qualities, even though of distant locations, were similar.

4. Discussion

This study provides new information on two hormones T4 and Mel response in stork nestlings in relation to environment's contamination. The location of fieldwork nearby a point source of heavy metals (copper smelter), near the city and in small villages situated far from industry, in natural areas surrounded by forests and crop fields, enables to analyze the response of free-living chicks to environments of different quality. The City and Village 1 and Village 2 locations are not exposed to any organic agricultural pollutants, as pesticides, or industrial toxic compounds. Thus, in this study, we have focused on heavy metals as the main contaminants in the Smelter location.

Elevated heavy metal concentrations in the arable layer of soils, which are reported in the area close to copper smelter, result from current and former deposition of pollutants (Koszyk and Szerszeń, 1988; Greinert, 2001). It should be noted, that the diet of white stork nestlings consists mainly of earthworms, thus the components of soil result in chemical composition of blood. In this study, we can indeed observe significantly higher serum levels of Zn, Mg and Cd in chicks from an area near the copper smelter, compared to those measured in samples taken in an unpolluted region. Birds can tolerate both Zn and Mg in relatively wide range, but this is not the case of extremely hazardous cadmium. The Zn, Mg, and Cd values measured in this study in Smelter group were significantly higher than those reported elsewhere and can be considered as toxic (Bowerman et al., 1994; Benito et al., 1999).

In our study, an effect of pollutants on two sensitive hormonal systems in nestlings: one associated with Mel, an important free radicals' scavenger and the second associated with T4, hormone representing thyroid gland function has been shown. It can be expected that these disruptions, in turn, can strongly affect survival and general fitness of ready-to-migrate young white storks. The similar hormones' levels studied in two consecutive years in nestlings from environments of comparable qualities (Villages 1 and 2), even though of distant locations, pointed to a usefulness of both hormones as bioindicators.

Thyroxine is the primary secretory product of the thyroid gland. It is established in mammals that T4 has little direct action, being mostly regarded as a precursor for triiodothyronine (T3), a well-documented biologically active form of the hormone. In birds, however, it is not so clear; although it has been generally assumed that T4 is primary a prohormone, there are many older data suggesting that T3 and T4 are equipotent (review: McNabb, 2000). We have studied T4 as a decisive measure of thyroid gland productivity. A significantly lower level of thyroxine has been shown in the nestlings exposed to pollutants from the copper smelter. Thyroid hormones (Ths) are crucial to maturation of many body systems in vertebrates, including those in birds: nervous and sensory, muscle and skeletal, integumentary (feathering) and controlling of heat production (McNabb, 2000). Therefore any drop in Ths may result in severe impairment of development. A series of studies has also demonstrated that exposure to heavy metals results in their accumulation in the brain impairing its function (Lahiri et al., 2004, 2005). Therefore low supply of T4 to the brain along with the direct effect of heavy metals to the brain might cause a neurobehavioral abnormality in fledglings and may have a serious consequence for readiness for migration of adults. Moreover, the toxic compounds which usually accumulate in body tissues, may have not only acute, but also delayed effects, which are hard to predict. Birds exposed to high levels of heavy metals may be at risk, especially during their migration, through impairment of many physiological functions, which depend on Ths. Thus, in stork nestlings, analysis of thyroid's hormones in field studies is of special importance.

Melatonin, as a powerful scavenger of hydroxyl and peroxyl free radicals, is an essential part of the defense mechanisms against free-radicals-induced oxidative stress along with enzymatic (i.e. superoxide dismutase, glutathione peroxidase and catalase) and non-enzymatic (such as Vitamins C, E and gluthatione, carotenoids) antioxidants (Reiter et al., 2000; Hardeland, 2005; Tan et al., 2007). Mel production can be considered as a protective action of the organism in response to oxidative stress (Hardeland, 2005; Tan et al., 2007). In fact, substantial amounts of melatonin are found in tissues and organs which are exposed to the environmental pollutants such as the gut and skin (Tan et al., 2007). In our study, a significantly higher serum Mel concentration has been shown in nestlings subjected to pollutants nearby in the copper smelter.

It is well established that there are three main sources of Mel in vertebrates, including birds: pineal gland, retina and GIT. In all species examined, serum melatonin concentration shows a diurnal rhythm, with the higher levels during the dark period (Binkley, 1988). The low level of circulating Mel in the light originates from the hormone's production by photoreceptive pineal gland and retina (incompletely inhibited by light) or by sources outside the photoreceptive organs, i.e. GIT. During the day, the main extrapineal and extraretinal origin of Mel is the small intestine (Bubenik and Pang, 1997; Van't Hof and Gwinner, 1999; Kulczykowska et al., 2006). In our study, in nestlings from the Smelter location, it is just the daytime Mel level that was elevated. In many vertebrate species, Mel appears to be involved in the process of feeding and digesting (Harlow and Weekley, 1986; Bubenik, 2002; Sjöblom, 2005). Studies of the impact of food-deprivation on melatonin in fish strongly suggest that synthesis of the indole in the GIT is stimulated by the presence of food (Mancera et al., 2006). However, to the authors' knowledge, there are no data on regulatory mechanism of Mel production in GIT. Thus, we can only speculate that the

toxic compounds present in the nestlings' diet may influence the Mel synthesis on-the-spot in GIT, which in turn affects the hormone's concentration in circulation.

The very significantly higher nocturnal serum Mel concentrations in all storks are consistent with the pattern established for other avian species (Pang et al., 1983). However, lower levels of Mel at night in the City group compared with that in Village 1, may be a result of artificial lighting working at night in the area near the city, which can decrease biosynthetic activity of the pineal gland and retina.

We have also reported a relationship between night Mel levels (and night increases of Mel) and age of nestlings. It may be taken as a clear indication of progressive development of timekeeping system in stork nestlings, a phenomenon known in other avian species (Zeman and Gwinner, 1993; Herichova et al., 2001). On the other hand, Mel is known to be involved in migratory orientation (Schneider et al., 1994), so that a steady increase of its production to reach the level sufficiently large to serve as a processor of geomagnetic information to organisms can be presumed.

In conclusion, health effects, including changes in hormonal balance may be attributed to combined exposures to many pollutants. The endocrine studies provide a useful tool for early warning and diagnosis of disruptive effects of pollutants to free-living animals. However, we should keep in mind the variations in hormone levels associated with age, gender, time, season, physiological status etc. Therefore in this study, in selected locations, we have examined the day and night hormones' levels and their changes during chick development. This study indicates that alterations in thyroid hormones and pineal melatonin levels could be useful biochemical markers of exposure of wild storks' nestlings to different toxic factors in field-tests. Moreover, it has been demonstrated that Mel serves as a sensitive, first-line defensive molecule in the protective mechanism of organisms.

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