

Effects of Chronic Dietary Cadmium on Hepatic Glutathione Levels and Glutathione Peroxidase Activity in Starlings (*Sturnus vulgaris*)

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Abstract. The effects of chronic exposure to dietary cadmium on the levels of hepatic glutathione (GSH) and on the activity of the glutathione peroxidase enzymes (GSH-Px) were studied for the first time in starlings (*Sturnus vulgaris*). Thirty-three individuals (17 females and 16 males) were divided into three groups: One represented the untreated control and two were respectively fed with diets containing 10 and 50 ppm cadmium chloride (CdCl₂). The total duration of treatment was 22 weeks. The three groups respectively accumulated mean hepatic Cd residues of 2.29, 75.71, and 208.49 ppm. Hepatic GSH increased in the treated groups respectively 24% and 52% in comparison to controls. Total GSH-Px activity in the liver was inhibited in the group fed with 50 ppm, due to inhibition of the selenium-dependent fraction of the enzyme, while the selenium-independent fraction did not change significantly. During the treatment, after 14 weeks of exposure to cadmium, the 50 ppm-treated group showed a 47% decrease of the activity of the selenium-dependent GSH-Px and a 50% increase of the somatic liver index in comparison with controls.

Although in polluted areas organisms are commonly exposed to chronic low levels of contaminants, few data are available about the long-term effects of this exposure. Among heavy metals, cadmium (Cd) is recognized as one of the most toxic environmental and industrial pollutants (Friberg *et al.* 1986). Its toxic effects on biological systems include lipid peroxidation, observed both *in vitro* and *in vivo* at high dosage (Hussain *et al.* 1987). More recently, similar results were found even at a very low dosage (Manca *et al.* 1991). Cd toxicity through lipid peroxidation could involve free radical production (Hirano *et al.* 1997; Abe *et al.* 1998). Cd is known to induce DNA damages (Mikhailova *et al.* 1997; Hurna *et al.* 1997; Misra *et al.* 1998). Oxygen radical scavengers such as superoxide dismutase, catalase, vitamin E and glutathione (GSH) are protective against Cd-induced oxidative damage in rats (Sarkar *et al.* 1997).

Another common cellular defense mechanism against lipid peroxidation is the enzyme system of glutathione peroxidases

(GSH-Px), both selenium-dependent (Se GSH-Px, EC 1.11.1.9) and selenium-independent (non-Se GSH-Px, EC 2.5.1.18). When orally administered, Cd causes an inhibition of Se GSH-Px and an increased activity of the non-Se GSH-Px (Jamall and Smith 1985; Nehru and Bansal 1997). Cd is known to accumulate through the food chain, and, among vertebrates, very high concentrations of Cd have been detected in kidneys of marine birds, even in nonindustrialized areas (Muirhead and Furness 1988; Marcovecchio *et al.* 1989). In birds, chronic exposure to this metal is known to increase susceptibility to disease or other stresses (Di Giulio and Scanlon 1985), to reduce reproductive success (White *et al.* 1978; Vodela *et al.* 1997a, 1997b), and to cause histopathologic and oxidative damage (Nicholson *et al.* 1983; Prasada Rao *et al.* 1989). Nevertheless, data on biochemical effects of chronic exposure to dietary Cd in this group of vertebrates are limited (Scheuhammer 1987; Bokori *et al.* 1995a, 1995b, 1996).

The purpose of this work is to study the effects of chronic Cd intake in birds by dietary administration of two different Cd concentrations under controlled conditions to starlings (*Sturnus vulgaris*). This species was chosen because, among those which easily adapt to caging and laboratory conditions, it possesses some interesting biological and ecological features suitable for bioaccumulation studies (Lower and Kendall 1990; Pilastro *et al.* 1993a, 1993b; Vogiatzis and Loumbourdis 1998). The aim of the research was to evaluate the effect of chronic exposure to dietary Cd on the hepatic GSH-Px activity and on the levels of GSH.

Materials and Methods

Animals and Treatment

Thirty-three starlings (17 females and 16 males) were randomly assigned to three treatment groups (A, B, and C), roughly maintaining an equal sex distribution. Group A (n = 13) and B (n = 12) were fed a normal diet supplemented with cadmium chloride (CdCl₂). CdCl₂ was dissolved in ethanol and evenly nebulized over dry pellet food (Faeda Mangimi, Vicenza, Italy). The final Cd concentrations in food were, respectively, 10.27 µg/g (SE = 0.47, n = 14) and 55.23 µg/g (SE 1.63, n = 23), as detected by dry weight analysis (Pilastro *et al.* 1993a, 1993b). Group C (n = 8) was used as control and did not receive any

added Cd in the diet. Cd concentration in control diet was lower than 1 ppm, as stated by the manufacturer. All animals were individually housed, with water and food provided *ad libitum*. Every 2 weeks, food consumption during a 24-h period was measured on eight randomly chosen birds. The treatment lasted for a period variable from 9 to 22 weeks. Eight birds (three from group A, three from group B, and two from group C) were humanely sacrificed by instant decapitation 9 weeks after the beginning of treatment, eight after 14 weeks, and the remaining birds after 22 weeks. Body and liver weights were immediately recorded. The liver was frozen in liquid nitrogen and stored at -20°C until further analysis. The average somatic liver index (SLI, liver weight/body weight \times 100) was determined for each group.

Atomic Absorption Spectrophotometry

Fractions of wet liver tissue (approximately 0.5 g) were lyophilized, weighed, and digested in 2 ml of concentrated HNO_3 in pressurized Teflon containers at 160°C for 3 h. After cooling at room temperature, samples were diluted to 10 ml with Milli-Q deionized water. Cd was measured by atomic absorption spectrophotometry with graphite furnace (Perkin-Elmer 4000, Perkin Elmer, Norwalk, CT, USA) and deuterium background correction. Standards in the range of 0–1 $\mu\text{g/g}$ were prepared daily from a stock solution of 1,000 $\mu\text{g/g}$ Cd in 0.1 N HNO_3 . A blank was run every eight samples with the same procedure. All the labware was precleaned in a HNO_3 : HCl solution (1 M, 1:3) for 24 h and repeatedly rinsed in deionized water.

Enzyme Analysis

Aliquots of minced liver were homogenized in 1:4 w/v cold isotonic buffer (0.01 M KH_2PO_4 , 1 mM EDTA, 1.15% KCl, pH 7.4). Levels of glutathione (γ -glutamyl cysteinylglycine, GSH) were determined on 200 μl of homogenate by the method by Anderson (1985) and expressed as mg/kg fresh weight. For each sample, the remaining homogenate was centrifuged first at 19,000 *g* for 30 min and then at 78,000 *g* for 90 min. The supernatant was then assayed for total GSH-Px activity, according to Gunzler *et al.* (1987), using cumene hydroperoxide as a substrate. The activity of the non-Se GSH-Px was estimated as the difference between the total GSH-Px activity and the activity of the selenium-dependent fraction, measured with H_2O_2 as a substrate (Jamall and Smith 1985). Protein concentration was determined according to Lowry *et al.* (1951). Enzyme analyses could not be performed on two individuals from group A and one from group B.

Statistical Analysis

One-way analysis of variance, multiple range test (LSD) for significant differences between means ($p < 0.05$), and Spearman rank correlation analysis were performed according to Sokal and Rohlf (1995).

Results

During the treatment the average daily food consumption of starlings was 18.6 g of dry weight (SE = 0.51, $n = 53$). No significant differences were found between sexes and among treatment groups.

Among birds undergoing treatments of different length (respectively 9, 14, and 22 weeks) for most parameters no significant differences were found, therefore the data collected from birds undergoing the same treatment were pooled regard-

less of its length. The only exceptions were the activity of Se GSH-Px and the values of somatic liver index (SLI), on which within-group analyses were additionally performed.

Table 1 summarizes the Cd content of liver and kidneys, the body weight, the liver weight, and SLI for all treatment groups. Even if a progressive Cd accumulation was observed with increased Cd uptake, birds belonging to group A accumulated proportionally more Cd than those belonging to group B ($F = 35.3$, $p < 0.0001$, $n = 25$). A small amount of the metal was present also in control birds (2.29 ppm). No significant differences were found in liver and body weight among groups, but SLI increased in Cd-fed birds (more significantly in group B), in comparison to controls ($F = 3.9$, $p = 0.03$).

Figure 1 shows the hepatic levels of Cd and GSH. Cd was measured by atomic absorption spectrophotometry and expressed in mg/kg dry weight. GSH was expressed as mg/kg fresh weight. GSH concentration significantly increased in the Cd-fed groups and was positively correlated with Cd concentration (mg/kg of dry weight) ($r_s = 0.60$, $p < 0.001$, $n = 33$).

Figure 2 shows the total activity of GSH peroxidase and those of the Se GSH-Px and non-Se GSH-Px (GSH transferase), all expressed in nmol/mg protein/min. Total GSH-Px activity decreased only in group B ($F = 4.95$, $p = 0.014$, $n = 30$). In the same group the Se GSH-Px decreased about 50% in comparison to controls ($F = 20.6$, $p < 0.0001$), while the non-Se GSH-Px did not change significantly among groups ($F = 0.5$, $p = 0.61$). Thus, the decrease of total GSH-Px activity seemed due to the inhibition of the selenium-dependent fraction of the enzyme. Figure 3 shows a time-dependent effect observed in group B, in which the decrease in activity of Se GSH-Px and the increase in SLI were especially pronounced after 14 and 22 weeks of treatment (respectively, $F = 4.93$, $p = 0.03$, $n = 11$, and $F = 9.51$, $p < 0.01$, $n = 12$).

Discussion

Although no specific markers of physiological stress were measured in the present study, the starlings undergoing chronic dietary Cd administration did not show any remarkable stress symptoms, such as changes in feeding and molting habits, and no deaths were recorded during the treatment. This species apparently tolerates chronic Cd exposure, similar to what previously reported in other birds (White *et al.* 1978; Mayack *et al.* 1981), mammals (Groten *et al.* 1994; Kamiyama *et al.* 1995), and amphibians (Vogiatzis and Loumbourdis 1998). In starlings, Cd accumulation was not proportionally dose-dependent because birds treated with the higher Cd diet (group B) accumulated a lower amount of the metal in the liver in comparison to group A. In experiments of dietary oral administration in acute dosage other species of birds, such as the Japanese quail (Scheuhammer 1987) and of mammals (Lehman and Klaassen 1986; Good and Klaassen 1989; Ohta *et al.* 1989) show a clear dose-dependent Cd accumulation, but the experiments we conducted on starlings concerned chronic dietary exposure. The modality of Cd administration (acute or chronic) is known to affect the fractional absorption of the metal in the gastrointestinal tract of mice (Lind *et al.* 1997), but no data are presently available for birds in this respect. A possible explanation for our results is that a higher Cd concentration in the diet could induce a higher Cd accumulation in kidneys, resulting in tubule degeneration and metal leakage, as previously observed

Table 1. Values of chemical and somatic parameters in Cd-fed starlings

	Control	Group A	Group B
Cd content in liver ($\mu\text{g/g}$ dry weight)	2.29 ^a (0.60)	75.71 ^b (9.21)	208.49 ^c (12.90)
Cd content in kidney ($\mu\text{g/g}$ dry weight)	5.87 (0.99)	116.03 (12.21)	308.93 (21.1)
Body weight (g)	74.50 ^a (2.50)	74.20 ^a (2.00)	71.40 ^a (2.90)
Liver weight (g)	1.90 ^a (0.08)	2.16 ^a (0.06)	2.30 ^a (0.17)
SLI	2.56 ^a (0.09)	2.91 ^{ab} (0.05)	3.21 ^b (0.25)

The values after 9, 14, and 22 weeks of treatment are pooled. Data are expressed as arithmetic means (standard errors within brackets). Different superscripts within rows indicate statistically significant differences between means ($n = 36$; $p < 0.05$). SLI: somatic liver index (liver weight/body weight) $\times 100$

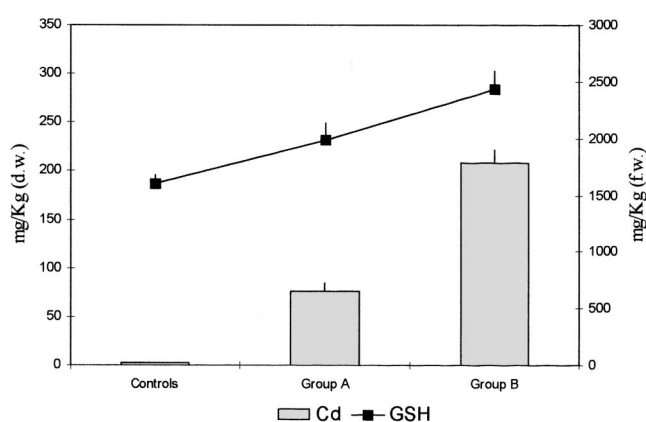


Fig. 1. Levels of hepatic cadmium (Cd) and glutathione (GSH) in starlings (*Sturnus vulgaris*) fed on a diet supplemented with different amounts of Cd: control (0 ppm), group A (10 ppm), and group B (50 ppm). The values after 9, 14, and 22 weeks of treatment are pooled. Data are expressed as arithmetic means. Bars represent standard errors. The scale on the left refers to Cd values (represented by histograms), while the scale on the right refers to GSH values (represented by lines)

in mallard ducks (White *et al.* 1978; Prasada Rao *et al.* 1989) and rats (Min *et al.* 1996). In our case, however, this is unlikely because, according to the data reported in Table 1, the liver/kidney ratio of Cd accumulation in starlings is approximately the same in the two treatment groups, on the contrary of what would be expected in the case of tubular damage followed by Cd leakage. Also, Cd concentration detected in kidneys of the treated starlings is apparently below the limit known to cause kidney damage in birds (Scheuhammer 1987).

An alternative explanation for the nonproportional Cd accumulation involves a direct cytotoxic and clastogenic effect of the metal on intestinal mucosa, due to Cd-mediated lipid peroxidation events (Karmakar *et al.* 1998; Sarkar *et al.* 1997, 1998). Thus, the cytotoxic effects of chronic Cd administration could negatively affect intestinal absorption in the starlings from group B. Traces of other metals, such as lead, copper, zinc, and iron, could also contribute in impairing intestinal Cd absorption (Prasada Rao *et al.* 1989; Sugawara *et al.* 1996; Crowe and Morgan 1997).

Since concentration of Cd in control food did not exceed 1 $\mu\text{g/g}$, the limited amount of Cd detected in liver and kidneys of

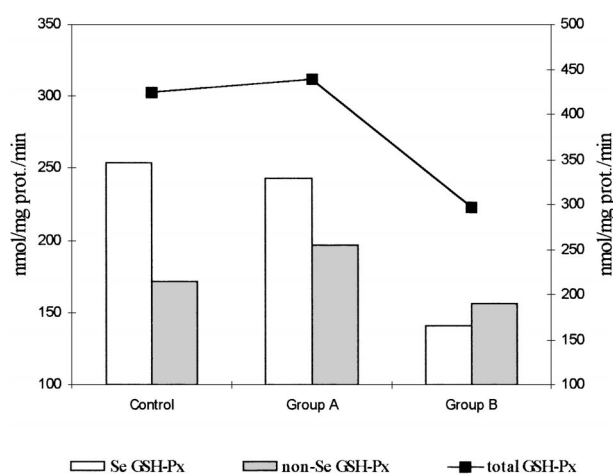


Fig. 2. Activities of total GSH peroxidase (total GSH-Px), selenium dependent (Se GSH-Px) and non-selenium dependent (non-Se GSH-Px) peroxidase for the three starling groups (control, A and B). Data are expressed as arithmetic means. Bars represent standard errors. Within each group, values are pooled regardless of the period of treatment. The scale on the right refers to total GSH-Px values

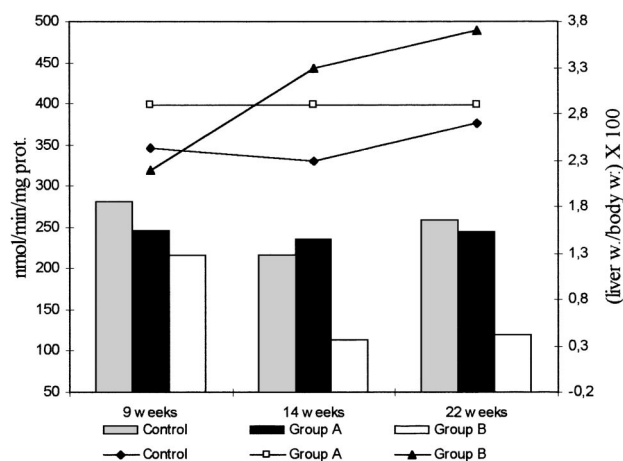


Fig. 3. Activity of Se GSH-Px and somatic liver index (SLI) after 9, 14, and 22 weeks for the three starling groups (control, A and B). The scale on the left refers to SeGSH-Px values (histograms), while the scale on the right refers to SLI values (lines)

control birds could be explained by dust contamination, although birds were kept in separate cages with controls above the others.

Our results show that chronic Cd administration in starlings yields a positively correlated increase in GSH levels. GSH is known to protect cells from oxidative damage through its oxidation as GSSG via Se-dependent GSH-Px (Ochi *et al.* 1988; Bagchi *et al.* 1997; Hatcher *et al.* 1997; Sarkar *et al.* 1997). Lipid peroxidation and other Cd-induced clastogenic effects can be counteracted by cell mechanisms that increase GSH levels (Liu *et al.* 1995; Gong and Hart 1997), possibly by enhanced synthesis of its precursors (Gill *et al.* 1989; Shimizu and Morita 1990; Pinamonti *et al.* 1994; Abe *et al.* 1998). In normal conditions 95% of total GSH is in the reduced form (Anderson 1985). Presumably, the above percentage could be

modified in treated starlings by the heavy oxidative stress induced by Cd administration. Although our methods do not discriminate between the reduced and oxidized form of glutathione, measurements of total GSH levels nevertheless allow us to detect the increase in GSH synthesis, which could be interpreted as a immediate cell defense against Cd-induced oxidative damage.

In starlings, the decrease in GSH Px activity at the higher Cd concentration is apparently due only to the decrease of the Se-dependent fraction, according to what has been observed in other species after chronic Cd exposures (Gill *et al.* 1989; Iszard *et al.* 1995; Nehru and Bansal 1997). These results have been interpreted as related to the formation of Cd-Se complexes (Gambhir and Nath 1992; Wahba *et al.* 1993; Jamba *et al.* 1997), especially Cd selenide (Sidhu *et al.* 1993), which could lower the selenium availability required by the Se GSH-Px. No inhibition of Se GSH-Px is actually observed when adequate dietary supplementation of selenium is provided (Meyer *et al.* 1982; Jamall and Smith 1985), and selenium administration is known to exert a protective effect against Cd-induced free radical damage in several organisms (Sidhu *et al.* 1993; Nehru and Bansal 1997; Padmaja *et al.* 1997). As stated by the manufacturer, in the pellet food administered to starlings the concentration of selenium was below the detection limit (1 ppm).

The diet containing about 50 ppm Cd, in comparison to 10 ppm, seems to trigger in starlings severe alterations at the hepatic level. This finding is also supported by the corresponding increase in SLI and could suggest the existence of some threshold limit in Cd tolerance between 10 and 50 ppm.

Previous studies conducted on Cd accumulation in feathers (Pilastro *et al.* 1993a) showed that starlings could be employed as biomonitors of environmental Cd pollution. Some ecological aspects of starlings, such as the large area of distribution, the omnivorous diet, the easy identification and the high number of individuals (Lower and Kendall 1990; Vogiatzis and Loubourdis 1998), make this species suitable for environmental biomonitoring programs. The effects of the metal on enzyme activity and the good correlation between the amount of metal ingested and the parameters examined encourages further investigations in this sense.

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