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Inter- and Intraclutch Variability in Heavy Metals in Feathers of Great Tit Nestlings (*Parus major*) Along a Pollution Gradient

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Abstract. Heavy metal (silver, arsenic, cadmium, copper, mercury, lead, and zinc) concentrations were analyzed in feathers of nestling great tits (*Parus major*) collected along a pollution gradient. Differences in metal concentrations along the gradient and inter- and intraclutch variability were investigated. In the immediate vicinity of the pollution source, feathers of nestling great tits contained significantly higher concentrations of silver, arsenic, mercury, and lead than at the sites further along the gradient. The concentrations of copper and zinc, two essential metals, were significantly lower at the second most polluted site. There was no significant difference in cadmium concentrations among sites. Most metals, except cadmium, were significantly positively correlated with each other. There was a significant amount of interclutch variability in feather metal concentrations, and they differed significantly among sites. The amount of inter- and intraclutch variability did not differ significantly, although intraclutch variability of most metals was markedly high. Our study indicates that feathers of great tit nestlings could be used as bioindicators of metal pollution, but attention should be paid in designing representative sampling procedures.

Contamination of the environment with heavy metals is a worldwide problem. In Belgium, several highly industrialized regions are polluted by a variety of heavy metals (VMM 1999). Monitoring the abundance and the environmental consequences of those heavy metals adequately requires the use of bioindicators (Burger 1993). The importance and relevance of birds as bioindicators for terrestrial and aquatic pollution, especially for heavy metals, has often been emphasized (Burger 1993; Furness 1993). Most monitoring studies to date, however, have focused on raptors (Denneman and Douben 1993; Jager *et al.* 1996; Garcia Fernández *et al.* 1997) and seabirds (Furness *et al.* 1990; Burger *et al.* 1992) due to their position at the top of the food chain and the spatial integration of their extended home ranges. Although passerines, such as great tits (*Parus major*), have been used frequently in ecological and behavioral research, it is only recently that they are being used in biomonitoring and ecotoxicological studies (Eeva and Lehikoinen 1995; Llacuna *et al.* 1995; Dauwe *et al.* 1999, 2000; Eens *et al.* 1999). Nevertheless, they possess several characteristics that make them suitable for monitoring point source contamination, *i.e.*, (1) they are primarily insectivorous and high on the food chain, (2) they are often found in large densities and are nonmigratory in many populations, (3) they use nest boxes and are therefore easy to study (Cramp and Perrins 1993; Dauwe *et al.* 1999, 2000; Eens *et al.* 1999).

Often monitoring studies examine concentrations of heavy metals solely in adult birds, because metals might accumulate with the age of the organism (van Straalen and Ernst 1991; Burger 1993). Nestling birds have been used to a much lesser extent, although they are potentially good biomonitors for terrestrial point-source pollution (Burger and Gochfeld 1993; Burger 1996). Developing nestling chicks are often fed food items from within a few dozen meters of the nest, making clear that the heavy metal content in those chicks is derived from local sources and can be used to identify local pollution in the foraging area (Burger 1993; Cramp and Perrins 1993; Furness 1993; Naef-Daenzer and Keller 1999). Heavy metal concentrations in nestling birds are also likely to be less variable than in adults because dietary specializations of adults are averaged between the two parents and because the metal burden is obtained only from the narrow period of nestling growth and from foods from near the breeding site (Furness 1993).

In the present study the concentration of heavy metals (silver, arsenic, cadmium, copper, mercury, lead, and zinc) in the outermost tail feathers of 15-day-old nestling great tits was examined along a pollution gradient. Feathers can serve as a useful and noninvasive tissue for metal analysis because heavy metals, such as lead and mercury, are sequestered in the sulfhydryl groups of the keratin as the feather grows. Once the feather growth is completed, the blood supply atrophies and the metal content in the feather remains extremely resistant to further change (Appelquist *et al.* 1984; Burger 1993). Feather metal concentrations were expected to be higher in the immediate vicinity of the pollution source. In a previous study, we found that heavy metal and selenium concentrations in adult feathers increased significantly toward the pollution source

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(Janssens *et al.* 2001). Silver, arsenic, cadmium, cobalt, copper, mercury, nickel, lead, selenium, and zinc concentrations in the tail feathers of great tits near the industrial plant were on average 2–40 times higher than those at a reference area, the differences among sites being highly significant (Janssens *et al.* 2001). One of the main objectives of this study was to determine the amount of inter-and intraclutch variability in metal concentrations in nestling feathers. Examining the variability in metal levels is a key factor in the design of sampling procedures to assess the incidence of such pollutants (Becker 1989; Morera *et al.* 1997).

Material and Methods

Study Sites and Data Sampling

The study was carried out in the vicinity of the nonferro industrial plant situated in the south of Antwerp, Belgium (see Figure 1). The metallurgic factory is the most extensively heavy metal air-emitting point source in Flanders. Pollution originates mainly from dust from ore piles blown up by the wind (Verbruggen 1994). Lead, cadmium, arsenic, copper, and zinc are especially common pollutants in this area, and they form an exponentially decreasing pollution gradient away from the factory complex (VMM 1999; Janssens *et al.* 2001).

In 1997 and 1998 we established four study sites, each with an average surface area of approximately 15 ha and 30–50 great tit nest boxes, along a pollution gradient away from the pollution source. The existing information about pollutant concentrations in the air and rain water was used in planning the location of the study sites (VMM 1997). Special attention was paid in selecting study sites so that they would represent a similar habitat type (deciduous park areas).

The first site (called UM) is located in the immediate vicinity of the pollution source (0–400 m). Previous studies have shown that concentrations of lead and cadmium in the feathers and eggs of tits from this site are among the highest reported in literature (Dauwe *et al.* 1999; Janssens *et al.* 2001). The second study area (Fort 8) is situated 400–600 m to the east of the pollution source, and the third (Fort 7) and fourth areas (UIA) are respectively 2,500 and 4,000 m eastward from the factory (see Figure 1).

Nest boxes were checked daily during the breeding season of 1999 to gather breeding data. All young were marked with aluminum rings and measured when precisely 15 days of age. The mean weight of the 15-day-old nestlings was 16.7 ± 0.1 g (range 13.9–18.9 g). From each nestling we collected the two outermost tail feathers. Feathers were put in sterile, metal-free plastic eppendorf tubes and stored at −20°C until further analysis.

Sample Preparation and Metal Analysis

Feathers were washed vigorously in deionized water alternated with (1 mol L^{-1}) acetone (95%) to remove external contamination (Gochfeld *et al.* 1996). This procedure was repeated two times. Samples were then put in 4-ml polypropylene metal-free vials and dried in an oven at 60°C for 24 h. Dry weight was determined on a Mettler H54 balance to the nearest 0.1 mg. Subsequently, a 1:1 mixture (100 μl) of HNO_3 (70%) and H_2O_2 (30%) was added to the dried samples to begin digestion. The destruction was completed with the microwave procedure described by Blust *et al.* (1988). After microwave destruction samples were diluted with 1 ml ultrapure deionized water and stored at −20°C until metal analysis.

We measured silver, arsenic, cadmium, copper, mercury, lead, and zinc in the feather samples with an axial inductively coupled plasma-mass spectrophotometer (ICP-MS, Varian Liberty series II) equipped with a microconcentric Groove nebulizer. All metal concentrations are expressed in μg g^{-1} (ppm) on a dry-weight basis. All samples were run in triplicate in batches that included blanks, a standard calibration curve, and certified reference material of the Community Bureau of Reference (*i.e.*, bovine liver, CRM 185). Accepted recoveries ranged from 94–107%.

Statistical Analysis

We used SPSS for Windows (SPSS 1999) and SAS (SAS 1989) statistical software to perform statistical analysis. According to Shapiro-Wilk's W-tests all data sets had normal distributions. Heavy metal concentrations in feathers were tested for mean differences among sites using a one-way ANOVA followed by a multiple comparison test (Tukey). A Pearson correlation was used to test correlations among metal concentrations. We further used a likelihood ratio test to investigate the presence and amount of inter- and intraclutch variability. Differences in the amount of inter- and intraclutch variability were compared using a paired *t* test.

A significance level of 0.05 was chosen for all statistical tests. However, when carrying out multiple comparisons (*i.e.*, when calculating correlation coefficient values among metals), α was adjusted using a Bonferroni correction (Sokal and Rohlf 1981) to correct for the increased probability of type I errors. Values given are arithmetic means ± SE.

Results

Differences Among Sites

In total, we collected feathers from 386 nestlings from 52 nests. On average we collected feathers of 7.67 ± 0.33 young per nest (range 3–13). The average weight of the two outermost tail feathers was 0.008 ± 0.0001 g. All measured elements (silver, arsenic, cadmium, copper, mercury, lead, and zinc) were detected in the feathers of great tit nestlings. Moreover, we found significant differences in metal concentrations among sites for six out of seven metals (Table 1).

Great tit nestlings from the UM site, the site closest to the pollution source, had significantly higher concentrations of silver, arsenic, mercury, and lead in their feathers than did nestlings at the other three sites (Table 1). Mercury and lead concentrations in feathers of nestlings near the pollution source were on average 9–10 times higher than 4 km further east. Arsenic concentrations were 27 times higher at the most polluted site. Although cadmium concentrations were four times higher at the UM site than at the UIA site, the difference was not significant (Table 1). The concentrations of the essential metals, copper and zinc, were significantly lower at the second most polluted site compared to the other sites along the gradient.

Correlation Among Metals

We also examined correlations among metal concentrations in the feathers of great tit nestlings. Arsenic, mercury, and lead were significantly positively correlated with most other metals (Table 2). Only cadmium concentrations were poorly corre-

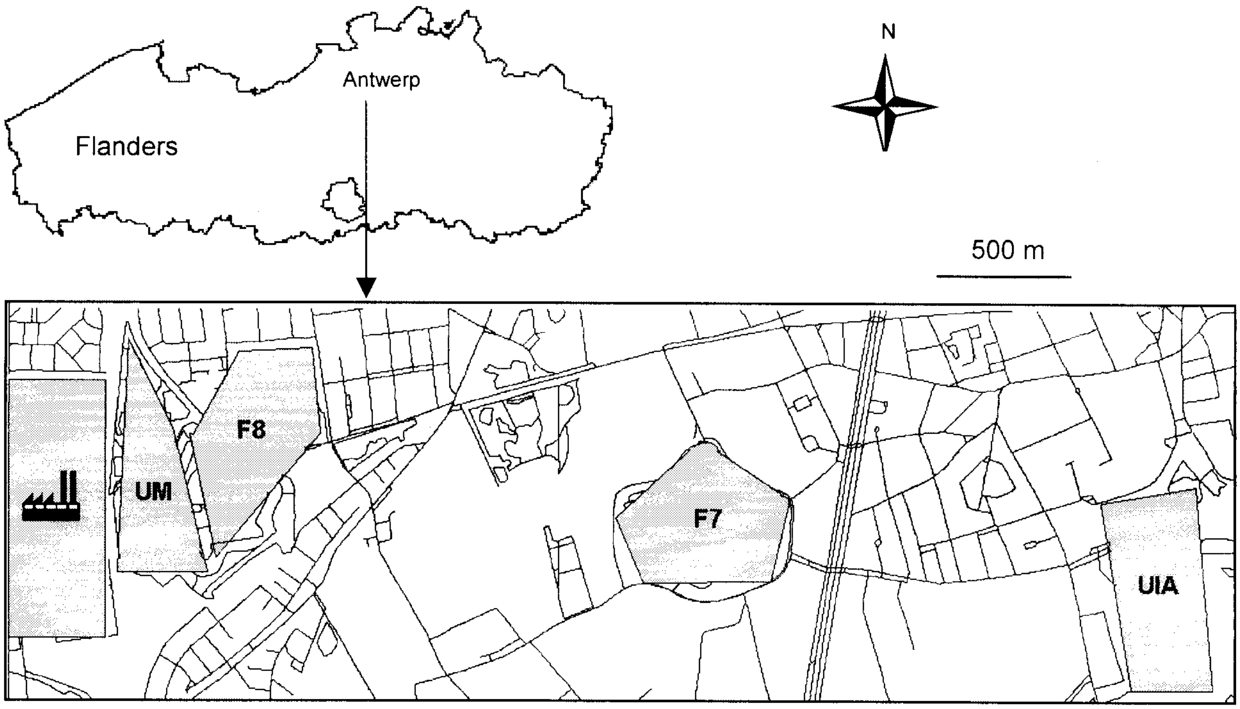

Fig. 1. Location of our study area near Antwerp, in Flanders (Belgium) and the four study sites: UM (Umicore), F8 (Fort 8), F7 (Fort 7), and UIA (Universitaire Instelling Antwerpen)

Table 1. Mean concentrations per nest (ppm dry weight ± SE) and ranges of heavy metals in the feathers of great tit nestlings along a pollution gradient

	UM (n = 13)		Fort 8 (n = 17)		Fort 7 (n = 12)		UIA (n = 10)		p
Ag	0.28 ± 0.1 (0.02–1.3)	A	0.04 ± 0.006 (0.0–0.09)	B	0.03 ± 0.01 (0.0–0.12)	B	0.06 ± 0.02 (0.0–0.14)	B	0.004
As	0.87 ± 0.27 (0.01–3.3)	A	0.13 ± 0.03 (0.0–0.3)	B	0.12 ± 0.06 (0.0–0.7)	B	0.03 ± 0.009 (0.0–0.08)	B	0.0002
Cd	0.05 ± 0.01 (0.0–0.14)		0.04 ± 0.01 (0.0–0.12)		0.02 ± 0.006 (0.0–0.08)		0.01 ± 0.004 (0.0–0.04)		0.1
Cu	3.05 ± 0.72 (0.5–9.1)	A	1.04 ± 0.19 (0.35–3.6)	B	3.32 ± 0.47 (0.8–7.7)	A	4.56 ± 0.74 (2.2–8.5)	A	0.0001
Hg	1.86 ± 0.51 (0.0–6.1)	A	0.45 ± 0.18 (0.0–2.5)	B	0.12 ± 0.05 (0.0–0.5)	B	0.21 ± 0.06 (0.0–0.6)	B	0.0002
Pb	4.24 ± 1.34 (0.3–14.2)	A	0.56 ± 0.11 (0.05–1.9)	B	0.48 ± 0.13 (0.0–1.7)	B	0.44 ± 0.07 (0.0–0.8)	B	0.0003
Zn	31.3 ± 5.0 (9.1–63.5)	A	16.5 ± 2.5 (5.2–46.1)	B	40.2 ± 2.6 (18.5–50.2)	A	43.35 ± 1.36 (37.6–51.4)	A	0.00001

The results of a one-way ANOVA applied to test for significant differences between the different study sites are also given. Significant differences among sites using a Tukey HSD test are shown by letters (A–B). Means followed by the same letter do not differ significantly from each other.

lated with other metal concentrations: correlations with silver, arsenic, copper, lead, and zinc were not significant (Table 2).

Inter- and Intraclutch Variability

There was a significant amount of interclutch variability for all considered elements (likelihood ratio test, $\chi^2 > 10.83$, $p < 0.001$ for all considered metals). Furthermore, the interclutch variability differed significantly among sites, with the largest amount of variation at the most polluted site (likelihood ratio test, $\chi^2 > 16.27$, $p < 0.001$ for all considered metals). The overall amount of intra- and interclutch variability is given in Table 3. There was no significant difference between the intra- and interclutch variability for the seven metals (paired *t* test, $t_6 = 0.92$, $p = 0.4$). For six metals (silver, arsenic, cadmium, copper, and zinc), levels of intraclutch variability were lower than the levels of interclutch variability. However, levels of

Table 2. Correlation matrix of metal concentrations (given are Pearson correlation coefficients, n = 386)

	Ag	As	Cd	Cu	Hg	Pb	Zn
Ag	—	0.91***	0.1^{NS}	0.37**	0.53***	0.81***	0.41***
As		—	0.12^{NS}	0.28*	0.54***	0.72***	0.34*
Cd			—	-0.1^{NS}	0.46**	0.19^{NS}	-0.18^{NS}
Cu				—	0.07^{NS}	0.3*	0.79***
Hg					—	0.8***	0.14^{NS}
Pb						—	0.38**
Zn							—

***$p < 0.0001$.
**$p < 0.0083$ (Bonferroni-correction).
*$p < 0.05$.
NS not significant.
To correct for the increased probability of type I error due to multiple testing a significance level of $\alpha = 0.0083$ was used.

Table 3. The total amount of inter- and intraclutch variation of heavy metal concentrations in nestling great tit feathers

	Interclutch Variation	Intraclutch Variation
Ag	0.0227	0.0054
As	0.194	0.113
Cd	0.00124	0.00123
Cu	3.52	0.897
Hg	0.84	4.116
Pb	4.029	15.81
Zn	237.8	57.68

Variances are covariance parameter estimates (REML) of a likelihood ratio test to investigate the presence and amount of inter- and intraclutch variability.

intraclutch variability for lead and mercury were markedly higher than the interclutch variability.

Levels of intra- and interclutch variability were also studied for each study site separately (see Table 4). We found no significant difference between the intra- and interclutch variability for the seven metals in each of the four study sites (paired *t* test, $p > 0.3$ for all considered study sites). However, at every site, levels of intraclutch variability for lead and mercury were higher than the interclutch variability. Levels of intraclutch variability for arsenic were higher than the interclutch variability at F8, F7, and UIA.

Discussion

The first objective of our study was to compare metal concentrations in the outermost tail feathers of nestling tits among four populations located at different distances from an important emission source. Because great tit nestlings are fed food items from within a limited parental foraging area and metal contamination will be obtained in a clearly defined time period, metal levels in nestling passerines may closely reflect local pollution levels (Furness 1993). We therefore expected concentrations of heavy metals in nestling feathers to be considerably higher in the immediate vicinity of the factory complex and to decrease gradually away from the pollution source. According to our expectations, metal concentrations in the feathers of nestling tits were significantly higher near the factory complex for silver, arsenic, mercury, and lead. However, we found no significant difference in cadmium concentrations along the gradient. This metal showed no clear correlation with environmental cadmium exposure, which is not too surprising given that cadmium is generally tightly bound to metallothioneins in the kidney and is therefore not generally available to be incorporated into feathers (Elliot and Scheuhammer 1997). Also, the concentrations of two essential metals, copper and zinc, were not significantly higher near the pollution source. Moreover, we found significantly lower copper and zinc concentrations in the second most polluted site (F8) compared to the other three sites. This was remarkable because we found elevated copper and zinc concentrations in adult great tit feathers at the most polluted site (UM) compared to the lesser polluted UIA site 4 km further (Janssens *et al.* 2001). Also, the zinc and copper deposition at the most polluted site is considerably higher than those at the other three sites (VMM 1999). Homeostatic mechanisms (*e.g.*, excretion via the excrements) are known to keep concentrations of essential metals, such as copper and zinc, physiologically adequate (Burger 1993). Also, physiological changes induced by pollution may have an influence on the uptake and the accumulation of copper and zinc (DiGuilio and Scanlon 1985; Blus *et al.* 1995; Elliott and Scheuhammer 1997; Heinz *et al.* 1999). It seems doubtful that nestling great tits in this study did not accumulate higher concentrations of metals considering the differences in metal contamination among the sites. We suggest that the outermost tail feathers of 15-day-old great tit nestlings cannot be used as biomonitor for zinc and copper because they may not reflect adequately the body burden of the nestlings.

Other studies have also reported detectable concentrations of heavy metals in nestling birds (Burger and Gochfeld 1993; Goutner and Furness 1997; Sepúlveda *et al.* 1999; Spahn and Sherry 1999). However, comparing our feather metal concentrations with other studies is difficult because most monitoring studies have focused mainly on seabirds and herons. Gochfeld (1980) found mercury levels in feathers of common tern chicks (*Stema hirundo*) up to 2.6 ppm, whereas feathers of little (*Egretta garzetta*) and great egret (*Ardea albus*) nestlings sequestered up to 3.3 ppm and 16 ppm, respectively (Goutner and Furness 1997; Sepúlveda *et al.* 1999). Burger (1993) reported lead and cadmium concentrations in feathers of nestling common terns of respectively 1.5 and 0.1 ppm; feathers of roseate tern (*Sterna dougallii*) and black skimmer (*Rynchops niger*)

Table 4. The amount of inter- and intraclutch variation of heavy metal concentrations at the different study sites

	UM		F8		F7		UIA	
	Inter	Intra	Inter	Intra	Inter	Intra	Inter	Intra
Ag	0.072	0.016	0.0005	0.002	0.0004	0.003	0.003	0.002
As	0.5	0.31	0.006	0.072	0.025	0.04	0.0001	0.005
Cd	0.002	0.001	0.0015	0.0014	0.0003	0.002	0.0001	0.0003
Cu	5.62	0.94	0.48	0.55	1.53	0.92	4.22	1.53
Hg	1.82	10.5	0.03	4.31	0.003	0.21	0.0015	0.12
Pb	9.02	59.13	0.001	2.19	0.09	0.96	0.012	0.25
Zn	269.8	32.3	95.3	59.2	58.96	74.02	11.6	61.9

Variances are covariance parameter estimates (REML) of a likelihood ratio test to investigate the presence and amount of inter- and intraclutch variability.

chicks accumulated up to 4.1 and 0.6 ppm, respectively (Burger and Gochfeld 1993). On average 1.01 ppm lead and 0.47 ppm cadmium was found in the feathers of little blue heron (*Egretta caerulea*) chicks (Spahn and Sherry 1999). The cadmium and mercury concentrations found in great tit nestlings in our study are markedly lower than those reported in other studies. Lead concentrations however, were comparable or even higher in our study. In a study conducted in 1998 at the most polluted study area, great tit nestlings were found to accumulate up to 4.38 ppm lead and 0.007 ppm cadmium in their feathers (Dauwe *et al.* 2000). The cadmium concentrations found in our study were somewhat higher, namely, 0.047 ppm (see Table 1). This might be a result of limited sample size in the earlier study and the fact that only three young per nest were sampled (Dauwe *et al.* 2000). Our study showed that although the inter- and intraclutch variability did not differ significantly, some metal levels showed a great amount of intraclutch variability.

Metals are considered a contributing factor influencing avian survival and reproduction (Eeva and Lehikoinen 2000; Larison *et al.* 2000). Accumulation of high concentrations of heavy metals, such as lead and cadmium, in specific organs may cause pathological changes or dysfunctions (Scheuhammer 1987). We found that toward the factory complex, female great tits interrupted their laying sequence more often, and hatching success was significantly decreased compared to the less polluted study areas (Janssens *et al.* unpublished data). However, we found no significant differences in nestling condition along the pollution gradient (unpublished data).

One of the main objectives of this study was to investigate the amount of inter- and intraclutch variability in metal concentrations in nestling feathers as a potential source of bias in the assessment of pollution levels. Studying the variability in metal concentrations is important in designing sampling procedures to assess the occurrence of such pollutants (Becker 1989; Morera *et al.* 1997). Nestling birds are considered good biomonitors for heavy metal pollution because the metal concentrations in their body tend to reflect the local contaminant levels closely (Burger 1993). Moreover, Furness (1993) stated that pollutant levels in nestling birds are likely to be less variable, and as a result, chick sample size need not be particularly large to obtain reliable results. Our results showed indeed that although for some metals there is a considerable amount of intraclutch variability, heavy metal concentrations in nestling feathers are less variable than those in feathers of adult great tits. In 1999, mercury concentrations in feathers of adult great tits at the most polluted area showed a considerable amount of variation, *i.e.*, 5.75 ± 17.06 μg/g (mean ± SD, see Janssens *et al.* 2001), whereas for nestling birds this variation was markedly lower (1.86 ± 1.83 μg/g, see Table 1).

We found a significant amount of interclutch variation, which differed among sites and among metals. At the most polluted site, there was considerably more variation in metal levels among the different clutches. For most metals, we observed a lower variation in the feather concentrations among nestling tits of the same clutch than among different clutches within the same locality. However, the overall amount of inter- and intraclutch variability did not differ significantly. We also looked at the levels of intra- and interclutch variability for each study site separately (see Table 4) because there were large differences in metal levels among the four sites. Again, we found no differences between the inter- and intraclutch variability. However, at the two most polluted sites lead and mercury concentrations showed a great amount of intraclutch variability, and for the other metals the level of intraclutch variability was lower than the interclutch variability. At the other two sites, we found that for almost all metals the level on intraclutch variability was higher than the interclutch variability.

Nestling tits of the same age may differ in size and also in the development of their tail feathers. Some 15-day-old chicks have already well-developed feathers, but in others the feathers consist mainly of shaft. Because the feather shaft contains significantly lower metal levels than the vane (Goede and de Bruin 1984), this can account for some of the intraclutch variability. Sanpera *et al.* (1997) and Morera *et al.* (1997) found that mercury concentrations in eggs of seabirds differed according to the laying sequence, with first eggs having higher concentrations than the following ones. Concentrations in hatched young from one nest may still reflect the intraclutch variability of metal levels in the eggs. However, Nyholm (1998) found that the concentrations of toxic metals in the eggs do not influence lead concentrations in blood or liver of the fully grown pied flycatcher nestlings. Goutner *et al.* (2001) studied the relationships between mercury content of heron chick body-feathers and nestling age, hatching order (seniors–juniors), and growth parameters. Mercury levels were not significantly correlated with chick age, and most of the variability in mercury (90%) was attributable to differential prey selection and/or foraging habitat and patch utilization by parents. Morphological measurements corrected for chick age could not explain variability in mercury concentrations (Goutner *et al.* 2001).

In conclusion, this study suggests that using feathers of nestling great tits as bioindicators is a powerful method to evaluate the presence of heavy metals in the environment. Great tits are ubiquitous and abundant, which permits sampling from almost any area in Europe. They readily nest in man-made nest boxes, so breeding populations can rapidly be established and easily monitored. These features make them suitable as biomonitors for point-source contamination. The use of nestlings has also several advantages. They are restricted to their nest and will be fed food items collected in the immediate vicinity of the nest box. Therefore the heavy metal contamination will originate from a restricted area around the nest. Moreover, contamination will be accumulated in the short period of nestling growth (Furness 1993). Nestling great tits can easily be monitored, and they are relatively insensitive to disturbances at the nest. This allows the set-up of a network of volunteers that can monitor great tit nestlings with little interference to the population. Chick data are also normally distributed, and metal levels in adult birds tend to show large skews. Statistical analysis of chick data is therefore often easier. However, the fact that there is a considerable amount of intra-clutch variability in metal concentrations needs to be taken in account both when designing sampling strategies and when performing any analysis and interpretation of the results.

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