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Sources of variation in innate immunity in great tit nestlings living along a metal pollution gradient: an individual-based approach

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Highlights

- Innate immunity of wild great tits along a metal pollution gradient was studied.
- Metals were already present in the blood of 14-days old nestlings.
- Strongest effects of pollution were observed for lysis.
- The effects of metals on innate immunity were only detected at the individual level.
- This likely relates to high heterogeneity in exposure along the pollution gradient.

Abstract

Excessive deposition of metals in the environment is a well-known example of pollution worldwide. Chronic exposure of organisms to metals can have a detrimental effect on reproduction, behaviour, health and survival, due to the negative effects on components of the immune system. However, little is known about the effects of chronic sublethal metal exposure on immunity, especially for wildlife. In our study, we examined the constitutive innate immunity of great tit (*Parus major*) nestlings (N = 234) living in four populations along a metal pollution gradient. For each nestling, we determined the individual metal concentrations (lead, cadmium, arsenic) present in the red blood cells and measured four different innate immune parameters (agglutination, lysis, haptoglobin concentrations and nitric oxide concentrations) to investigate the relationship between metal exposure and immunological condition. While we found significant differences in endogenous metal concentrations among populations with the highest concentrations closest to the pollution source, we did not observe corresponding patterns in our immune measures. However, when evaluating relationships between metal concentrations and immune parameters at the individual level, we found negative effects of lead and, to a lesser extent, arsenic and cadmium on lysis. In addition, high arsenic concentrations appear to elicit inflammation, as reflected by elevated haptoglobin concentrations. Thus despite the lack of a geographic association between pollution and immunity, this type of association was present at the individual level at a very early life stage. The high variation in metal concentrations and immune measures observed within populations indicates a high level of heterogeneity along an existing pollution gradient. Interestingly, we also found substantial within nest variation, for which the sources remain unclear, and which highlights the need of an individual-based approach.

Keywords: Constitutive innate immunity, Nestlings, Natural antibodies, Complement, Nitric oxide, Acute phase proteins.

1. Introduction

Large amounts of diverse chemical compounds are introduced into the environment via human activities, posing a great risk for wildlife. An important source of contamination affecting many areas around the world is the excessive deposition of metals. They are released into the environment via a range of human activities such as mining, metallurgy, vehicular traffic and pesticides use, but also via natural phenomena such as plate tectonics, forest fires and natural erosion (Bichet et al., 2013; Burger, 2008; Pagliara and Stabili, 2012). Metals contaminate the air, soil and water, enter the food chain, accumulate in organisms (Dedourge-Geffard et al., 2009; Jaspers et al., 2004; Nolet et al., 1994; Roggeman et al., 2013) and can have detrimental effects on among others condition, reproduction, breeding performance and survival in a large range of animals (Brasso and Cristol, 2008; Eeva et al., 2005a; Eeva et al., 2012; Fair et al., 2003; Gorissen et al., 2005; Janssens et al., 2003b; Janssens et al., 2003c; Moron et al., 2014; Pedersen and Saether, 1999; Scheuhammer, 1987; Witeska et al., 2014). Further, a wide body of research has showed that metals can also have genotoxic effects such as for example DNA damage in wildlife (Pastor et al., 2001; Sebbio et al., 2014).

These negative effects may be mediated via the effects of metals on immune function (Borowska and Pyza, 2011; Das et al., 2008; Day et al., 2007; McMurry et al., 1995; Pagliara and Stabili, 2012; Tersago et al., 2004). As the immune system is critical for defence against pathogens and other threats, immunosuppressive effects of contaminant exposure probably reduce fitness (Norris and Evans, 2000). In the case of birds, immunotoxic effects of metal

pollution have been demonstrated, with the most convincing evidence originating from studies under controlled laboratory conditions or experimental feeding studies. Here they found a suppression of total antibody production, a decrease in lymphocyte proliferation, a decline in circulating white blood cells and a suppression of natural, humoral and cell-mediated immune responses (Kenow et al., 2007; Lewis et al., 2013; Rocke and Samuel, 1991; Trust et al., 1990; Vodela et al., 1997; Youssef et al., 1996). Fewer studies have investigated the immunological effects of free-living birds in their natural environment where the pollution is in fact occurring. However, the results of these studies are not consistent since some find negative effects of metal pollution on cell-mediated or humoral immunity, while others find no effect on cell-mediated immunity or immunocompetence in general (Baos et al., 2006; Elbert and Anderson, 1998; Fair et al., 2003; Hawley et al., 2009; Snoeijs et al., 2004; Wayland et al., 2003). Why studies under natural conditions tend to differ remains unclear. It may relate to a higher variance, e.g. in contaminant concentrations. In particular since high doses of metals are generally toxic to most organisms, while in some cases low doses may favour biological responses (= hormesis) (Bartlett and Smith, 2003; Eeva et al., 2005a; Nain and Smits, 2011). Furthermore, the effect that a contaminant will have on organisms also depends on the time course of exposure. Birds that are chronically exposed to sublethal metal contamination will probably experience immunological effects (Snoeijs et al., 2004), potentially due to accumulation (Yu et al., 2011). Finally, not only the duration but also the timing of the exposure is relevant. Early development is a particularly critical and relevant period, where any effect may have long-lasting consequences (reviewed in Lindstrom 1990). Potentially toxic concentrations of total arsenic (As), cadmium (Cd) and lead (Pb) can indeed already be found in the excrement of 15-day old nestlings (Janssens et al., 2003a) and in eggshells and egg content (Dauwe et al., 1999), indicating early life exposure. Nestlings are at a vulnerable developmental stage as their immune system continues to develop while they

grow (Fair and Ricklefs, 2002). While the effects of metal pollution during early development are expected to broadly affect an organism's physiology, quantifying immunological effects can be methodologically and logistically challenging (Horrocks, 2011; Matson et al., 2012; Matson et al., 2005).

The central goal of this study was to investigate the relationship between metal exposure and immunological condition in great tit (*Parus major*) nestlings living along a pollution gradient that related to distance from a smelter. We assessed several aspects of innate immune function, which generally is non-specific and serves as an initial line of defence against invading pathogens. Natural antibodies (NAbs) and complement activity are two interrelated non-cellular components of innate immunity. Present in immunologically naïve individuals, NAbs broadly recognize and bind to antigens, a process which can result in activation of the complement cascade, which ends with the lysis of foreign cells (Boes, 2000; Matson et al., 2005; Murphy et al., 2012; Ochsenbein and Zinkernagel, 2000). Another aspect of innate immunity are acute phase proteins (APPs). APPs are typically synthesized by hepatocytes in response to cytokines released by macrophages in the presence of bacteria (Coon et al., 2011; Cray et al., 2009; Murphy et al., 2012; Owen-Ashley and Wingfield, 2007). APPs have several antimicrobial functions, such as activating the complement cascade and opsonizing bacteria (Murphy et al., 2012). The APP haptoglobin (Hp) circulates in the blood at low concentrations that rise significantly in response to an acute infection, trauma or inflammation (Cray et al., 2009; Matson et al., 2012; Murata et al., 2004; Quaye, 2008). Nitric oxide (NO) is a multifunctional signaling molecule, which acts as a vasodilator, neurotransmitter and a modulator of inflammatory processes. Assessing nitric oxide can provide useful information on individual variation in work load, physiological condition and health state (Bichet et al., 2013; Bourgeon et al., 2007; Sild and Horak, 2009). We focused on nestlings as they enable investigation of the impacts of pollution early in life, which, as pointed out above, is a life

history stage where any change to the developmental trajectory may have significant fitness consequences. We first explored these relationships among the populations along the pollution gradient and we expect that there will be negative relations between contamination and immune parameters. But given the possibility of small-scale variation in contamination (Fritsch et al., 2011), we also expect that this small-scale variation is reflected in our immune measures. In order to investigate this, we determined the individual metal concentrations and four parameters of immunity for each nestling, which enabled us to investigate whether immune parameters can vary with metal concentrations on the individual level. The latter has, as yet, rarely been taken into account.

2. Material and methods

2.1 Study sites

Our study was conducted in four different great tit populations in a well-established pollution gradient near a non-ferrous smelter south of Antwerp (Hoboken), Belgium. Common pollutants in this area are lead, cadmium and arsenic (Table 1) (Dauwe et al., 1999; Geens et al., 2010; Janssens et al., 2001; VMM, 2011). Each study site is located at a different distance east of the smelter with Umicore located near the smelter (0-350 m), Fort 8 located 400-600 m to the east of the smelter, Fort 7 located 2500 m to the east and finally Fort 4 located 8500 m eastwards of the factory (Figure 1). All study sites have a similar habitat type, which can be classified as deciduous park area (Janssens et al., 2001) and traffic intensity and urbanization are high and comparable in all study sites. Great tits living at these different sites breed in nest boxes with approximately 30 to 60 nest boxes per study site and similar nest box densities.

2.2 Data sampling

During the breeding season of 2012 (March – May), we checked nest boxes every other day to determine the laying date, clutch size, start of incubation and hatch day. When nestlings were 14 days old (hatch day = day 1), a blood sample (± 150 µL) was obtained by puncturing the brachial vein with a 27 gauge needle and collecting the blood with a Microvette CB 300 lithium-heparin tube (Sarstedt). The collected blood was then stored under cool conditions and centrifuged at 7000 rpm for 10 minutes upon returning to the lab on the day of collection. Resulting plasma samples were stored at -80°C until use in immunological assays. Red blood cells were stored at -20°C until sex determination and metal analyses were performed. All blood was sampled immediately after taking nestlings out of the nest (within several minutes), to minimize the potential effects of stress on baseline immune functions (Buehler et al., 2008; Matson et al., 2006; Millet et al., 2007). After blood collection, the nestlings were weighed (0.1 g) using a digital balance (Kern TCB 200-1).

2.3 Sex determination

DNA was isolated from red blood cells (approximately 1 µL of packed red blood cells) using Chelex 100 resin (Bio-Rad Laboratories) (Walsh et al., 1991). For the molecular sex determination, 1.5 µL of the extracted DNA was used for polymerase chain reaction (PCR) amplifications according to Griffiths et al. (1998). PCR products were separated on agarose gels showing a single Z-band for males and Z- and W- bands for females.

2.4 Immunological assays

We sampled multiple nests per site (Figure 1, Umicore = 13, Fort 8 = 14, Fort 7 = 15, Fort 4 = 16), of which 4 nestlings per nest were selected for immunological assays. Specifically, we selected the two heaviest and the two lightest individuals, as this enabled us to investigate whether immune responses are influenced by body weight. For every individual (N = 234) we

measured four immune indices and endogenous metal concentrations using the assays as described below.

Haemolysis-haemagglutination assay: To assess the levels of circulating natural antibodies and the activity of the complement system, we used the haemolysis-haemagglutination assay as developed by Matson et al. (2005), with several minor alterations. This assay is based on the interaction of avian plasma samples and rabbit red blood cells, which results in agglutination (HA) and lysis (HL) of the cells. Agglutination indicates the interaction between NAbs in plasma and antigens present on the rabbit erythrocytes. Lysis reflects the interaction of complement and NAbs. Here, all the plates were treated with a blocking solution consisting of milk powder and Dulbecco's phosphate buffered saline (PBS) and subsequently washed three times using a PBS – TWEEN 20 solution before use. Further, we based the serial dilution (1:2) on 15 µL of plasma and 15 µL of Dulbecco's phosphate buffered saline (PBS). We scored haemagglutination and haemolysis titers blindly from randomized ordered digitized images of individual samples. Half scores were assigned to wells that showed intermediate agglutination or lysis. All samples were scored twice on different days, in order to assess score repeatability.

Haptoglobin assay: To quantify concentrations of haptoglobin (mg/mL) in the plasma samples, we followed the manufacturer's instructions of a commercially available colourimetric assay (PHASE Haptoglobin assay, Tridelta Development Ltd). In each plate, a standard curve and an among-plate standard were run in duplicate (Matson et al., 2012).

Nitric oxide assay: To quantify the concentrations of nitric oxide (mmol/L), we used the spectrophotometric assay based on the reduction of nitrate to nitrite by copper-coated cadmium granules (Sild and Horak, 2009). The assay consists of three main steps: deproteinization, nitrate reduction, and a Griess reaction. We ran a standard curve and an

among-plate standard in duplicate in each plate. Absorbance was recorded at 542 nm using a Molecular Devices VersaMax Tunable Microplate Reader.

2.5 Metal analysis

We used red blood cells to determine individual arsenic, lead and cadmium concentrations for all sampled nestlings as they represent the main pollutants in our study areas (Boshoff et al., 2014; Geens et al., 2010; Tersago et al., 2004; Vermeulen et al., 2009). Although arsenic can occur in two forms, with the inorganic form being highly toxic and the organic form being less harmful to health, the chosen digestion method resulted in total arsenic (sum of organic and inorganic compounds) measurements for each sample. Samples were weighed with a precision of 0.0001 g prior to analysis, so that the exact weight of red blood cells was known. Red blood cells were digested using HNO_3 (69%) and H_2O_2 (27%) in an open microwave digestion method including successive digesting steps at 100, 150 and 200 W (Blust et al., 1988; Roggeman et al., 2013). After digestion, samples were weighed again and samples were diluted with Milli-Q water to obtain 3-6% acid. Finally, concentrations of Pb, Cd and As were measured using an inductively coupled plasma-mass spectrometer (ICP-MS; Agilent 7500) and all metal concentrations were calculated on a fresh weight basis. For each batch of randomly assembled samples, four blanks and four reference samples (mussel tissue, Standard Reference Material 2976, National Institute of Standards and Technology) were included. The values found (As: 13.6 ± 0.66 µg/g; Cd: 0.77 ± 0.03 µg/g and Pb: 1.04 ± 0.05 µg/g) are very close to the certified values (As: 13.3 ± 1.8 µg/g; Cd: 0.82 ± 0.16 µg/g and Pb: 1.19 ± 0.18 µg/g) and recoveries were within 87 – 98% of the certified values. When the concentration of a pollutant was below the LOD, we used a value of LOD/2 for further calculations (Bervoets et al., 2004; Custer et al., 2000).

2.6 Statistical analysis

To construct all models, we used the lmer function imbedded in the package lme4 in R (Bates, Maechler et al. 2013). Starting from a model containing all independent variables of interest and their interactions, we used the backward elimination procedure for model reduction. Fixed effects were tested using the Maximum Likelihood method, while random effects were tested using the Reduced Maximum Likelihood method. Decisions related to keeping parameters in the model, were based on a significance level of 5%. When significant associations were found, we used differences in least square means (difflsmeans in the package lmerTest (Kuznetsova, Brockhoff et al. 2013)) to explore these effects further. All model residuals were checked for deviations of model assumptions using Q-Q plots and Shapiro-Wilk normality tests. Values deviating from the mean plus or minus three times the standard deviation were discarded from final analysis (13 cases in total). All statistical analyses were performed in R 2.15.3 (R development core team 2013-03-01 release; www.r-project.org).

To assess differences in metal contamination among the populations, we constructed a linear mixed model with metal concentration as dependent variable, population as fixed factor and the random factor nest nested in population. Data of metal concentrations were square root or log transformed when necessary.

To test the relationship between metal pollution and immune parameters of birds living at the different populations, we constructed four linear mixed models (one for each immune parameter) containing the immune parameter (agglutination, lysis, haptoglobin or nitric oxide) as dependent variable, sex and population as fixed factors, weight, clutch size and hatch day (Julian day) were included as covariates and nest was included as a random factor (nested in population). Data for agglutination, lysis, haptoglobin and nitric oxide were not normally distributed so we used log10(x+1) transformations for agglutination and lysis data, a square root transformation for haptoglobin data and a log10 transformation for nitric oxide data to

meet model assumptions. We used Spearman's rank correlations to quantify repeatability between the two agglutination scores ($\rho = 0.91$, $P < 0.0001$) and between the two lysis scores ($\rho = 0.97$, $P < 0.0001$).

To test the relationship between immune measures and metal concentrations on an individual level, we constructed several random slope models (using different slopes for the relation between immune measures and metals in the different areas) with the immune parameter (agglutination, lysis, haptoglobin or nitric oxide) as dependent variable, weight and metal concentration (lead, cadmium or arsenic) as covariates and nest as a random factor. To meet model assumptions, data for agglutination and lysis were log10(x+1) transformed; data for nitric oxide were log10 transformed.

In order to assess within and among population and nest variation in contamination and immunity we constructed several linear mixed models containing either the immune parameter (agglutination, lysis, haptoglobin or nitric oxide) or metal concentration (lead, cadmium or arsenic) as dependent variable, weight as covariate and population and nest as random factors (nest nested in population).

3. Results

3.1 Metal concentrations in red blood cells of nestlings

All metals differed significantly among the populations. Concentrations were highest in the populations closest to the pollution source (Figure 2). Reference samples for metal analysis showed recovery values between 87 and 98% for all measured metals, suggesting a successful and reliable digestion and measurement.

3.2 Immunity along the pollution gradient

There were no significant differences in agglutination scores among the populations in the pollution gradient (P= 0.96, Table 2). There was also no significant effect of body weight (χ^2 = 1.6, *df* = 1, P = 0.21), clutch size (χ^2 = 0.14, *df* = 1, P= 0.71), hatch day (χ^2 = 0.61, *df* = 1, P=0.44) or sex (χ^2 = 0.58, *df* = 1, P= 0.44) on agglutination scores of nestlings.

There were significant differences in lysis scores among the populations (P= 0.02, Table 2). Based on the differences in least square means, lysis scores at Fort 8 were always significantly lower than values at the other populations (vs. UM, P= 0.002; vs. Fort 7, P= 0.03; vs. Fort 4, P= 0.03). There was a significant effect of nestling body weight on lysis scores (χ^2 = 8.22, *df* = 1, P= 0.004) with a higher lysis score for heavier nestlings (β = 0.02). We found no significant effect of clutch size (χ^2 = 1.64, *df* = 1, P= 0.20), hatch day (χ^2 = 1.04, *df* = 1, P= 0.31) or sex (χ^2 = 0.03, *df* = 1, P= 0.87) on lysis scores of nestlings.

For haptoglobin, there were no significant differences among the populations (P= 0.10, Table 2). Haptoglobin concentrations did not vary significantly with clutch sizes (χ^2 = 0.03, *df* = 1, P= 0.87), however, there was a non-significant tendency for haptoglobin concentrations to vary with hatch day (χ^2 = 3.77, *df* = 1, P= 0.05) and sex (χ^2 = 3.26, *df* = 1, P= 0.07). Nestling body weight had a significant effect on lysis scores (χ^2 = 5.12, *df* = 1, P= 0.02), with heavier nestlings having higher haptoglobin concentrations (β = 0.007).

There were no significant differences in nitric oxide concentrations among the populations (P= 0.35, Table 2). Further, we found no effect of weight (χ^2 = 0.65, *df* = 1, P= 0.42), clutch size (χ^2 = 0.29, *df* = 1, P= 0.59), hatch day (χ^2 = 0.96, *df* = 1, P= 0.33) or sex (χ^2 = 1.67, *df* = 1, P= 0.20) on plasma NO concentrations.

3.3 Effects of metals on immune parameters at the individual level

Based on the results of the 234 sampled nestlings, we found no significant effect of As (β = -0.11, P = 0.85) or Cd (β = -0.77, P = 0.22) on agglutination scores (Table 3). There was, however, a non-significant tendency that Pb positively varied with nestling agglutination scores (β = 0.20, P = 0.07, Table 3). We found a significant negative relationship between lysis scores and Pb (β = -0.19, P = 0.02, Table 3) and a non-significant tendency for As (β = -0.68, P = 0.09, Table 3) and Cd (β = -0.78, P = 0.09, Table 3). We found a significant positive relationship between haptoglobin concentrations and As (β = 1.35, P = 0.003, Table 3), but not Cd (β = 0.91, P = 0.13, Table 3) or Pb (β = 0.14, P = 0.12, Table 3). We also found no significant relationships between metal concentrations and nitric oxide (As: β = -0.49, P = 0.30; Cd: β = -0.37, P = 0.53; Pb: β = -0.04, P = 0.69, Table 3).

3.4 Within and among population and nest variation

Within-nest variation was greater than among-nest variation (= within population) for all measured metals (Pb: within 0.045, among 0.032; Cd: within 0.031, among 0.010; As: within 0.033, among 0.011). We also uncovered greater within-population variation than among-population variation for Cd (within 0.040, among 0.001) and As (within 0.044, among 0.004) but not for Pb (within 0.077, among 0.084). Furthermore, within-nest variation was greater than among-nest variation for all measured immune parameters (HA: within 0.044, among 0.002; HL: within 0.017, among 0.004; Hp: within 0.013, among 0.003; NOx: within 0.016, 0.008). Similarly within-population variation was greater than among-population variation in all measured immune parameters (HA: within 0.046, among < 0.001; HL: within 0.021, among 0.001; Hp: within 0.015, among < 0.001; NOx: within 0.024, among < 0.001).

4. Discussion

We explored the possible harmful effects of metals on several aspects of constitutive innate immunity in 14 day old great tit nestlings along a pollution gradient. We show that although

there is a gradient in heavy metals along the different populations, there is no gradient in immune parameters for nestlings inhabiting the different populations. However, when looking at the individual contamination levels and the individual immune measurements, there is evidence for a relationship between metal pollution and nestling immunity. This highlights the need to consider heterogeneity in exposure, which necessitates an individual based approach.

4.1 Among population differences in metal concentrations and immunity

We indeed found significant among population differences in red blood cell metal concentrations for all measured metals. Endogenous concentrations in the red blood cells of great tits were highest near the smelter. These results confirm the outcome of previous studies, some of which were carried out more than 10 years ago (Dauwe et al., 1999; Dauwe et al., 2006; Geens et al., 2010; Janssens et al., 2001; Janssens et al., 2002; Janssens et al., 2003a; Jaspers et al., 2004). Interestingly, we can show that contamination can already be traced at a very young age, as in 14 day old nestling great tits. It is generally assumed that older birds have higher metal concentrations compared to nestlings or young birds since they have been exposed for longer periods and to different sources (food, water, inhalation of airborne contaminants and via ingestion when cleaning their feathers (Dauwe et al., 2004)) and thus have higher chances to bioaccumulate contaminants (Burger et al., 2009; Costa et al., 2013; Cui et al., 2013; Hogstad, 1996). Despite the higher concentrations of several metals found in adults, evidence suggests that nestlings may become polluted via the egg and/or the food supplied by their parents. There is evidence for both pathways since high concentrations of metals can be found in eggs (Dauwe et al., 1999; Gochfeld and Burger, 1998) and in the excrement of 15-day old nestlings (Janssens et al., 2003a).

In short these results support the idea that nestlings can be considered as suitable bioindicators to assess local pollution and its effects on wildlife.

Moreover, growing nestlings are thought to be more sensitive to the negative effects of contaminants (Scheuhammer, 1987) as the nestling phase represents a vulnerable stage of the life cycle in which their immune system continues to develop while they are also investing in growth (Fair and Ricklefs, 2002). Disturbing the development of an individual has direct consequences which probably has long-lasting effects on fitness (Lindstrom, 1999).

When comparing populations in terms of immunity along the pollution gradient, we found no differences in agglutination scores (= NAb activity), haptoglobin concentrations or nitric oxide concentrations among the populations. We did find differences in lysis scores (= activity of the complement system) among populations with birds living at one specific site (Fort 8) having significantly lower lysis scores compared to birds living at all other sites. This is probably related to factors other than pollution. The exact causes for these lower lysis scores remain unclear especially since we selected populations based on similar habitat types. However, we cannot rule out that there are small differences in certain environmental factors such as habitat quality (food abundance), predation risk, ectoparasite load or human disturbance driving this effect. As with metal pollution, maternal transfer or other parental effects may influence immune function in nestlings (Hasselquist and Nilsson, 2009). Since any parental effects may not simply be direct, but rather might interact with parental metal pollution levels, further study is required.

Our failure to find a gradient in immune measures despite the gradient in metal pollution may suggest that elevated levels of metals do not have negative effects on immune function. However, such a conclusion contrasts with previous research in both nestlings and adults (Baos et al., 2006; Elbert and Anderson, 1998; Fair et al., 2003; Hawley et al., 2009; Snoeijs et al., 2004). The short time frame of the current study may play a role: although metals are already accumulated in the blood, they may not yet exert effects on immune traits.

4.2 Relationship between metals and immune parameters on an individual level

Despite the above mentioned lack of among population differences in immune function along a pollution gradient, we did find evidence of immune parameters varying with metal concentrations on the individual level. These associations differed among metals and immune traits.

Among the different immune traits measured, the strongest effects of pollution were observed for lysis. Lysis varied negatively with Pb concentrations. A previous study performed on the same populations as the current study showed that humoral immune responsiveness was affected, with adult great tits farthest away from the smelter having significantly higher antibody titers (Snoeijs et al., 2004). Our study provides further evidence on the negative relationship between Pb and immunity by showing that innate immunity is also affected. Concerning the other two metals As and Cd, which are both known as immunotoxicants (As: Guardiola et al. 2013; Kozul et al. 2009; Lage et al. 2006; Cd: Li et al. 2010), we found similar non-significant tendencies. Overall, effects of contamination on agglutination and nitric oxide concentrations appeared to be limited. Presently, it remains unclear why certain immune measures are affected by metal pollution and others are not.

The acute phase protein haptoglobin correlates positively with As concentrations. Higher endogenous As concentrations found in nestling red blood cells may function as inflammatory stimuli which cause haptoglobin concentrations to rise. Although haptoglobin concentrations are higher in nestlings with higher endogenous As concentrations, concentrations found in nestlings ranged from 0.14 mg/mL to 0.21 mg/mL which is believed to still be within the normal range for great tit nestlings (own observations show that haptoglobin concentrations can go up to 0.97 mg/mL). Since As is accepted as essential for growth, development and functioning of animals, it may be that there is a continuum for As in which the element is

essential at a certain level of intake and toxic at another level (Chandra and Dayton, 1982). The positive effect of As on haptoglobin concentrations could be explained by the possibility that As concentrations near the outer extreme of the continuum are already high enough to stimulate inflammation and raise haptoglobin concentrations. Metals can cause oxidative stress by increasing the amount of reactive oxygen species (Koivula and Eeva, 2010) and since haptoglobin is known to be an antioxidant due to its hemoglobin-binding abilities (Jelena et al., 2013; Quaye, 2008), animals may up regulate their haptoglobin concentrations in order to cope with these higher metal concentrations.

Thus, we found evidence for relationships between metal pollution and nestling immunity. However, not all components of the innate immune system measured here were affected in the same way. Future experimental work is required to further explore the potential causes of these differences.

4.3 Within population and within nest variation

Although we found clear evidence for a (still existing) pollution gradient, we did not find evidence for a similar gradient in immune measures. When exploring within and among nest variation and within and among population variation, we found that there was more variation in metal concentrations and immune measures within nests and populations than between nests and populations. Only for Pb variation in metal concentrations was higher between populations than within populations. These results suggest that there is high heterogeneity on the individual level within sites and even within nests. This heterogeneity implies that even in the most polluted area there will be individuals who are not or much less contaminated compared to others. This high heterogeneity urges an individual-based approach and should be acknowledged in future studies.

The high heterogeneity within populations, that is between nests, may be due to certain environmental aspects such as small-scale differences in metal contamination or spatial differences in for example contamination of food items. Other potentially important aspects that could explain variation may be differences in, for example, habitat quality (food abundance), which may be reflected in differences in clutch size or hatch day. However, we did not find significant effects of clutch size or hatch day on any of our immune measures. Yet, within population differences may result from territory differences in prey species which, in turn, differ in contamination. In general nestlings get exposed to contaminants via the air, nest materials and via contact with their parents, but the most likely way is via their food. Several studies have indeed shown that food samples (e.g caterpillars, beetles, spiders) along polluted areas contain considerable amounts of metals (Dauwe et al., 2004; Eeva et al., 2005b; Fritsch et al., 2012).

The high heterogeneity within nests supports the hypothesis of a high level of variation in the contamination of food items, even on a smaller scale such as within breeding territories. Based on own video recordings of parental feeding rates and literature, we know that he main invertebrate prey of nestling great tits in all populations are Lepidoptera larvae (Dauwe et al., 2004). It could be that the contamination in the population is clustered which may lead to certain prey items that are highly polluted and others that are not. Thus, since the exposure of organisms in polluted ecosystems varies over space and is dependent of the spatial heterogeneity of contaminant levels and the availability of these contaminants (Marinussen and vanderZee, 1996; Smith et al., 2007), it is very plausible that there is high variation in contamination within and between prey species of birds. This may be related to differences in habitats or differences in landscape composition within bird home-ranges, since they may modulate exposure and change the level of accumulation (Fritsch et al., 2012; Vermeulen et al., 2009).

Another possible explanation for the high heterogeneity within nests may be individual variation in contamination due to, for example, individual variation in digestion or metabolism of the nestlings. The gastrointestinal tract harbors a large and diverse microbial community which metabolizes xenobiotics very extensively (Sousa et al., 2008; Van de Wiele et al., 2010), which may vary between individuals. But little is known about this topic, despite the recent increase in interest (Arumugam et al., 2011).

Finally, factors related to condition may play a role in explaining the high variation within nests (but see above for a lack of effect on hatch day and clutch size). Palacios et al. (2009) suggested that natural antibodies and complement are relatively condition-independent. This lack of an association between body weight and natural antibodies may be explained by the fact that they are less sensitive to short-term variations in nutritional status, environmental conditions or stress levels compared to acquired antibody responses (Deerenberg et al., 1997; Matson et al., 2005; Palacios et al., 2009). Our results partially support these results as we also did not find an effect of weight on agglutination and nitric oxide concentrations.

5. Conclusions

In this study we show that endogenous concentrations of Pb, Cd and As in the blood of nestlings were highest in populations closest to the pollution source. Thus, metal pollution is already a concern for very young birds. However, there was a high heterogeneity in blood concentrations of metals across populations and nests. This heterogeneity indicates that an individual-based approach is required to analyze the effects of metals on immunity. Indeed, the consequences of metals in the blood were only detected on the individual level. We found that lysis scores were negatively affected by Pb. Further, elevated As concentrations appeared to induce inflammation responses as reflected in elevated haptoglobin concentrations. Most

previous studies focused on either acquired cell-mediated or humoral immunity but with this study, we report additional effects of metal pollution on the innate branches of the immune system. This appears highly relevant, in particular at very early life stages as studied here. Neonates and juveniles depend strongly on innate immune defenses since adaptive defenses are still poorly developed at this age. Further research to evaluate metal levels in the environment and assess metal levels in food items together with the individual approach suggested here, is required to gain more insight into the causes and consequences of heterogeneity.

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Table 1: Range of mean annual metal concentrations in atmospheric particulate matter (PM_{10} ng/m³) with their reference values as set by the European guide line (EU) and mean annual metal depositions (µg/m².day) with their reference values as set by the Flemish regulations concerning environmental permits (VLAREM II) in the area surrounding the smelter (information taken from VMM, 2011).

	PM_{10} (ng/m³)	Reference value EU (ng/m³)		Deposition (µg/m².day)	Reference value VLAREM II (µg/m².day)
Pb	80 - 258	500	**Pb**	314	250
Cd	1.3 - 2.9	5	**Cd**	3.9	20
As	9.6 - 44	6	**As**	24	no reference value set

Table 2: Overview of the measured immune parameters for each of the four populations along the pollution gradient. Populations shown, are ranked from most polluted (UM) to least polluted site (Fort 4). Values for agglutination, lysis and nitric oxide are raw data while values reported for haptoglobin are square root transformed. All values shown are mean values and their SE. Values with different letters are significantly different as determined by differences in least square means.

	Umicore		Fort 8		Fort 7		Fort 4		Chi^2	df	P value
Agglutination score	3.92 ± 0.38		3.90 ± 0.33		3.85 ± 0.34		3.85 ± 0.31		0.30	3	0.96
Lysis score	1.72 ± 0.12	A	1.24 ± 0.10	B	1.60 ± 0.09	A	1.64 ± 0.11	A	10.33	3	0.02
Haptoglobin (mg/mL)	0.18 ± 0.02		0.16 ± 0.02		0.14 ± 0.01		0.14 ± 0.02		6.35	3	0.10
Nitric oxide (mmol/L)	0.0075 ± 0.0004		0.0084 ± 0.0004		0.0091 ± 0.0005		0.0087 ± 0.0005		3.26	3	0.35

Table 3: Results of the mixed models used to investigate the effects of metals on the different immune parameters on an individual level. Significant effects are marked in bold, trends (> 0.05 < 0.1) are marked in italics.

	Agglutination				Lysis				Haptoglobin				Nitric oxide		
	Chi²	df	P value		Chi²	df	P value		Chi²	df	P value		Chi²	df	P value
Pb				**Pb**				**Pb**				**Pb**			
weight	0.71	1	0.40	**weight**	5.83	1	**0.02**	**weight**	1.59	1	0.21	**weight**	0.67	1	0.41
Pb	3.31	1	*0.07*	**Pb**	5.55	1	**0.02**	**Pb**	2.43	1	0.12	**Pb**	0.16	1	0.69
Cd				**Cd**				**Cd**				**Cd**			
weight	0.08	1	0.77	**weight**	3.98	1	**0.05**	**weight**	1.77	1	0.18	**weight**	0.92	1	0.34
Cd	1.48	1	0.22	**Cd**	2.81	1	*0.09*	**Cd**	2.27	1	0.13	**Cd**	0.40	1	0.53
As				**As**				**As**				**As**			
weight	0.43	1	0.51	**weight**	4.83	1	**0.03**	**weight**	2.85	1	*0.09*	**weight**	1.14	1	0.29
As	0.04	1	0.85	**As**	2.89	1	*0.09*	**As**	8.90	1	**0.003**	**As**	1.08	1	0.30

Figure 1: The location of the pollution source and the selected study sites (Umicore, Fort 8, Fort 7 and Fort 4) along the pollution gradient in the South of Antwerp (Hoboken), Belgium.

Figure 2: Mean concentrations (µg/g fresh weight ± SE) of metals with highest concentrations closest to the pollution source. Different letters above bars, represent significant differences between populations. Brackets show a trend (> 0.05 < 0.1) towards differences in metal concentrations between populations. Bars sharing the same letters are not significantly different as determined by differences in least square means.

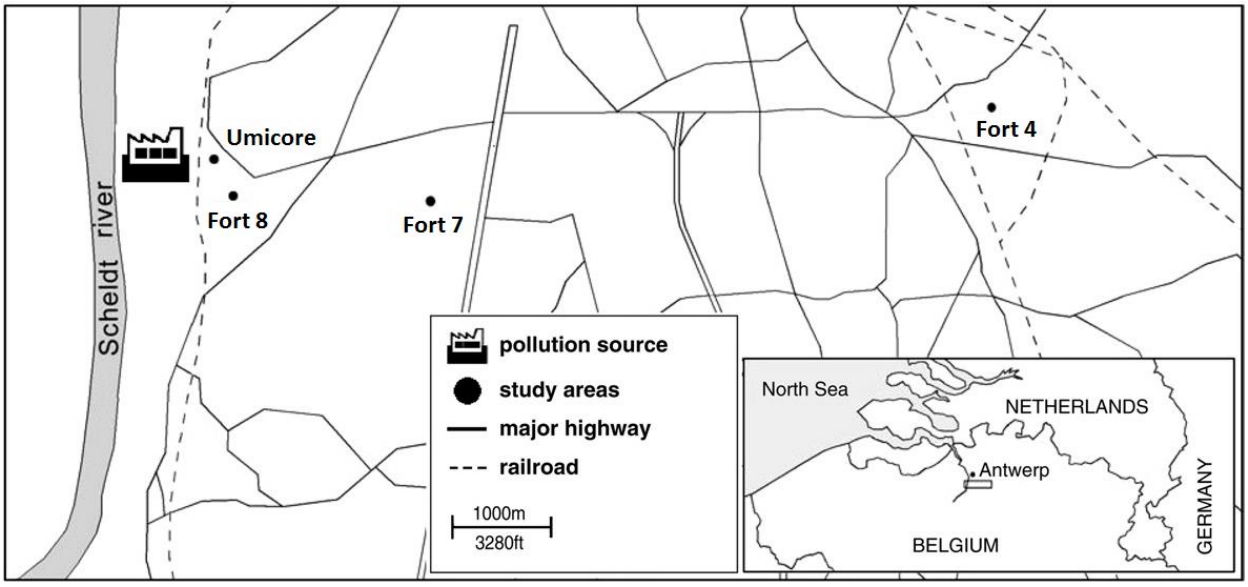
Scheldt river
Umicore
Fort 8
Fort 7
Fort 4
pollution source
study areas
major highway
railroad
1000m
3280ft
North Sea
NETHERLANDS
Antwerp
BELGIUM
GERMANY

Figure 1

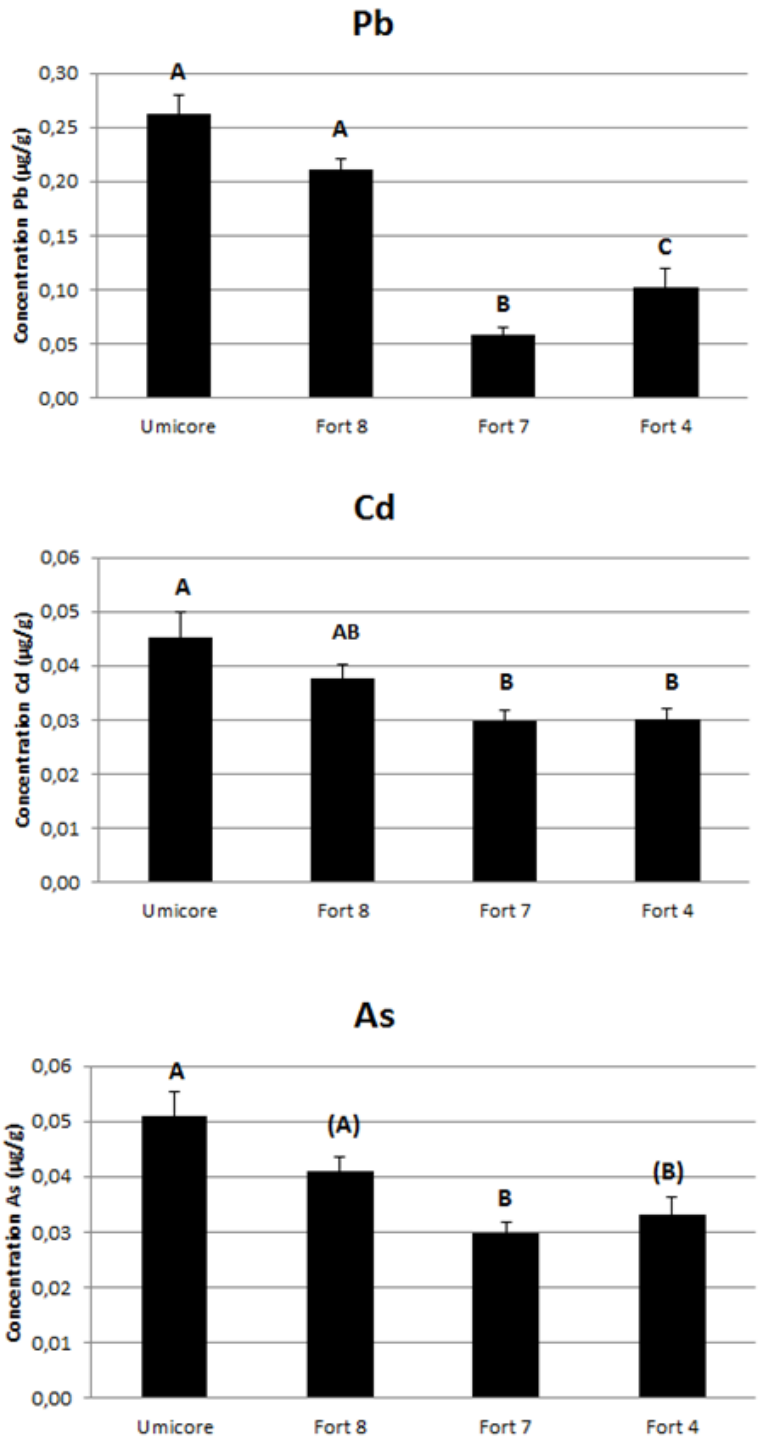
Pb
Concentration Pb (µg/g)
0,30
0,25
0,20
0,15
0,10
0,05
0,00
A
A
B
C
Umicore
Fort 8
Fort 7
Fort 4
Cd
Concentration Cd (µg/g)
0,06
0,05
0,04
0,03
0,02
0,01
0,00
A
AB
B
B
Umicore
Fort 8
Fort 7
Fort 4
As
Concentration As (µg/g)
0,06
0,05
0,04
0,03
0,02
0,01
0,00
A
(A)
B
(B)
Umicore
Fort 8
Fort 7
Fort 4

Figure 2