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# Trace Metals Affect Early Maternal Transfer of Immune Components in the Feral Pigeon

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## ABSTRACT

Maternal early transfers of immune components influence eggs' hatching probability and nestlings' survival. They depend on females' own immunity and, because they are costly, on their physiological state. Therefore, trace metals, whether toxic and immunosuppressive (e.g., lead, cadmium, etc.) or necessary and immunostimulant (e.g., zinc, copper, iron, etc.), are likely to affect the amount of immune components transferred into the eggs. It may also vary with plumage eumelanin level, which is known to be linked to immunity, to transfer of antibodies, and to metal detoxification. In feral pigeons (*Columba livia*) injected with an antigen and experimentally exposed to lead and/or zinc (two highly abundant trace metals in urban areas), we measured specific antibody transfer and concentrations of two antimicrobial proteins (lysozyme and ovotransferrin) in eggs. As expected, lead had negative effects on specific antibody transfer, while zinc positively affected lysozyme egg concentrations. Moreover, eggs from lead-exposed females exhibited higher ovotransferrin concentrations; because it binds metal ions, ovotransferrin may enable egg detoxification and embryo protection. Finally, eggs' lysozyme concentrations increased with plumage darkness of females not exposed to zinc, while the relation was opposite among zinc-exposed females, suggesting that benefits and costs of plumage melanism depend on trace metal environmental levels. Overall, our study underlines the potential ecotoxicological effects of trace metals on maternal transfers of immune components and the role of plumage melanism in modulating these effects.

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## Introduction

At birth, offspring are immunologically naive, as they do not produce their own antibodies (Dibner et al. 1998; Mauck et al. 2005) and they are consequently highly vulnerable to parasites. Specific antibodies transferred into egg yolk may increase offspring's ability to cope with parasites and may have consequences for offspring survival (e.g., *Borrelia burgdorferi* s.l.; Gasparini et al. 2006; Boulinier and Staszewski 2008; Hasselquist and Nilsson 2009). Furthermore, antimicrobial proteins, such as lysozyme and ovotransferrin, are also transferred into egg albumen and are consequently part of the egg's innate immune system. Lysozyme catalyzes the lysis of gram-positive bacteria by hydrolyzing the peptidoglycan in their cell walls (Pellegrini et al. 1992; Masschalck and Michiels 2003), while ovotransferrin inhibits microbes' growth by chelating metallic ions necessary for their development ($Fe^{3+}$, $Cu^{2+}$, $Zn^{2+}$; Valenti et al. 1983, 1985). It has been shown that the transfer of such proteins may enhance offspring survival by decreasing egg-hatching failure (Saino et al. 2002*a*).

The amount of immune components in eggs depends both on the female's production (Saino et al. 2002*a*; Jacquin et al. 2013) and on the proportion allocated to eggs. Females might thus face trade-offs relative to their potential investment in the transfer of maternal antibodies (Gasparini et al. 2007; Boulinier and Staszewski 2008; Ismail et al. 2015). Therefore, any external environmental factor that may influence the physiological state of the female (e.g., immunity, nutritional status, etc.) should impact the transfer of immune substances into the eggs.

Trace metals (lead, zinc, cadmium, etc.) are particularly abundant in urban areas (Azimi et al. 2003; Roux and Marra 2007) and may have detrimental effects on bird physiology and reproductive parameters (e.g., heme synthesis, humoral immunity, oxidative stress, clutch size; Redig et al. 1991; Snoeijs et al. 2004; Dauwe et al. 2005; Valko et al. 2005; Berglund et al. 2007; Eeva et al. 2009). Therefore, trace metal exposure may entail significant costs for females and consequently reduce the investment of immune components into eggs (Saino et al. 2002*b*; Hargitai et al. 2006). Alternatively, mothers may adjust their investment into the egg, in response to the detrimental effects induced by metal exposure, to prepare offspring for their future environment (Gasparini et al. 2007).

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Interestingly, transfer of antibodies from female feral pigeons to their young increases with female plumage darkness (Jacquin et al. 2013), potentially due to genetic links between melanogenesis and immunity (Ducrest et al. 2008). Thus, plumage melanism may affect the transfer of immune components. Moreover, because of melanin's property to bind metal ions (Hong and Simon 2007), darker birds are able to store higher amounts of trace metals in their feathers (Chatelain et al. 2014), which may enable darker birds to maintain lower blood metal concentrations. Therefore, plumage melanism could modulate the impacts of trace metals on maternal transfers of immune components.

To examine whether and how trace metal exposure may shape early maternal transfer of immune components, we chronically exposed feral pigeons (*Columba livia*) to concentrations of zinc and/or lead representative of the natural environmental range measured in urban areas. Lead and zinc are among the most abundant metals in the atmosphere and in soil of urban areas (Azimi et al. 2005; Maas et al. 2010). Lead is a well-known toxic metal (Jarup 2003; Patrick 2006) and an immunosuppressor (i.e., lead decreases humoral immune response and increases hemosporidian parasite intensity; Trust et al. 1990; Snoeijs et al. 2004, 2005; Gasparini et al. 2014), while zinc has beneficial effects on immunity (i.e., zinc is essential for the development and function of cell-mediating innate immunity—neutrophils, natural killer cells, macrophages, T- and B-cells—and decreases Chlamydiaceae prevalence and hemosporidian parasite intensity; Prasad 1998, 2009; Smith 2003; Gasparini et al. 2014). Therefore, both lead and zinc may modulate immune components' transfer by affecting both females' immunity and females' reproductive investment. We estimated early maternal transfer of immune components: specific antibodies (anti–keyhole limpet hemocyanin [KLH] antibodies), lysozymes, and ovotransferrins. We expected lead and zinc exposure to respectively reduce and increase immune component transfer, associated with costs and benefits on females. Opposite results may suggest a strategic maternal response.

## Methods

### *Subjects and Housing*

Free-living feral pigeons (*Columba livia*) were caught in February and March 2014 from several pigeon flocks within the Parisian agglomeration. A sample of 144 pigeons was chosen in such a way as to best equilibrate sex ratio (72 males and 72 females, sexed using discriminant function analysis; Dechaume-Moncharmont et al. 2011) and eumelanin-based plumage coloration degree. The plumage eumelanic coloration was estimated according to the method described by Chatelain et al. (2014). Pigeons were kept in 12 outdoor aviaries (3.10 m × 2 m × 2.40 m) at the Centre d'Ecologie Expérimentale et Prédictive-Ecotron Ile-de-France field station (Unité Mixte de Service 3194, Ecole Normale Supérieure, Saint-Pierre-lès-Nemours, France). They were evenly distributed among aviaries according to their gender, flock, and eumelanic plumage coloration intensity in such a way that there were no confounding effects between aviaries and these variables (gender: 6 males and 6 females per aviary; flock: $\chi^2 = 202.19$, df = 176, $P = 0.085$; plumage coloration intensity: $F = 0.13$, df = 1, 144, $P = 0.721$). They were fed ad lib. with a mix of maize, wheat, and peas. The aviaries were provided with a bowl of water used for bathing and with branches as perches. Birds were individually identified with a numbered plastic ring. At the end of the experiment (i.e., after 9 mo of captivity), birds were released back to the wild at their site of capture.

### *Treatments*

The aviaries were randomly assigned to one of the four following metal exposure treatments: exposed to lead only (lead group; 10 ppm lead acetate, Sigma-Aldrich, St. Louis, MO), exposed to zinc only (zinc group; 100 ppm zinc sulphate, Prolabo, Paris), exposed to both lead and zinc (lead+zinc group; 10 ppm lead acetate and 100 ppm zinc sulphate), or control (control group; tap water with no added metal). Consequently, there were 3 aviaries with 12 pigeons each (36 pigeons in total) per treatment. Metals were diluted in tap water. We chose these concentrations based on lead blood concentrations measured in urban birds (ranging from 0.053 to 0.264 ppm; Roux and Marra 2007), the gastrointestinal absorption rate of lead in zebra finches (<10%) calculated from Dauwe et al. (2002), and previous supplementation experiments in feral pigeons (Chatelain et al. 2015). Drinking troughs and baths were filled with the corresponding treated water every other day.

A similar supplementation protocol was used in a previous experiment, and its efficiency was validated by measuring lead and zinc concentrations in birds' blood and feathers (Chatelain et al. 2015). Metal concentrations to which the birds were exposed were 10 times lower than the ones used in this study. Hence, we are confident that metals added to water were indeed ingested by the birds.

### *Reproduction*

Two weeks after the start of the metal treatments, six nest boxes per aviary were opened to allow birds to mate and breed. Females produced two-egg clutches one to three times during the experiment. For each clutch laid between April 11 and August 30, we randomly collected one of the two eggs and kept them frozen at −20°C until analysis (i.e., specific antibodies, lysozymes, and ovotransferrin level measurements).

### *Measurement of Specific Antibody Transfer*

To estimate maternal transfer of specific antibodies into the egg, 20 d after the start of the metal treatments, we subcutaneously injected females ($n = 72$) with 50 $\mu$g of a KLH solution (hemocyanin from *Megathura crenulata*; Sigma-Aldrich). Then we took a blood sample the day the mother laid the first egg of the two-egg clutch, centrifuged it, and kept the plasma frozen until analyses. Collected eggs were dissected, and eggshell, yolk, and albumen were separated one from another. Once unfrozen, the yolk was blended, diluted 1:1 in

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phosphate-buffered saline, and homogenized for 1 min with a vortex. Chloroform was then added 1 : 1 and homogenized for 1 min with a vortex. After centrifugation (6 min at 10,000 *g*), the supernatant was used for antibody assays (Jacquin et al. 2013). Anti-KLH antibody concentrations in mother plasma and egg yolk extractions were measured using a sandwich enzyme-linked immunosorbent assay, following the method described by Jacquin et al. (2013).

### *Measurement of Lysozyme Transfer*

Albumens were unfrozen and homogenized with a vortex. Each well of a 96-well microplate was filled with 9.5 $\mu$L of albumen or lysozyme standard (lysozyme from chicken egg white; L6876, Sigma-Aldrich). The standards were prepared in phosphate-buffered saline (9 g $L^{-1}$, pH 6.3) for a standard curve ranging from 12.5 to 200 $\mu$g $mL^{-1}$ (4°C); 250 $\mu$L of micrococcus solution (*Micrococcus lysodeikticus* ATCC 4698, M3770, Sigma-Aldrich; $DO_{450\ nm} = 1$) was added in all the wells, and the microplate was left to incubate for 10 min at 26°C. Absorbance at 450 nm was recorded ($t = 10$).

### *Measurement of Ovotransferrin Transfer*

Ovotransferrin transfer was measured following an adapted protocol from Shawkey et al. (2008) and Horrocks et al. (2011). Albumens were thawed and homogenized with a vortex. Each well of a 96-well microplate was filled with 24 $\mu$L of albumen or ovotransferrin standard (conalbumin from chicken egg white; C0755, Sigma-Aldrich). The standards were prepared in reagent 1 (300 mM Tris, 150 mM sodium hydrogen carbonate, 4.2 g $L^{-1}$ Triton X-100, pH = 8.4) for a standard curve ranging from 1 to 80 mg $mL^{-1}$. Then 150 $\mu$L of reagent 1 containing a 1 : 32 dilution of iron-standard solution (2,000 mg $L^{-1}$) was added in all the wells. The plate was shaken for 10 s and incubated for 5 min at 37°C. Following incubation, initial absorbance was recorded at 570 and 660 nm. Then 25 $\mu$L of reagent 2 (50 mM Tris, 32.6 mM L-ascorbic acid, 10 mM Ferrozine, pH = 4) was added to each well. The plate was shaken again for 10 s and incubated for 5 min at 37°C. Finally, 25 $\mu$L of reagent 3 (600 mM citric acid, 25.6 mM Thioura) was added to each well. The plate was shaken for 3 s, and absorbance was recorded a first time ($t_0$) and a second time after 6 min ($t_6$).

First, we corrected for initial differences in absorbance values by subtracting well-specific initial absorbance at 570 and 660 nm from $t_0$ and $t_6$ read at the corresponding wavelength. Then we determined the difference in absorbance: $\Delta A = A_{570-660}(t_6) - A_{570-660}(t_0)$.

### *Statistical Analyses*

Egg-laying date was not correlated with metal exposures and mother eumelanin level. A linear mixed-effects model was performed with log-transformed anti-KLH antibody level in egg as the dependent variable and exposure to zinc, exposure to lead, mother eumelanin level, log-transformed mother anti-KLH antibody level, and their interactions as the explanatory variables. Aviary number and maternal identity were added as random factors.

We performed a linear mixed-effects model with lysozyme or ovotransferrin concentration in egg as the dependent variable and exposure to zinc, exposure to lead, mother eumelanin level, and their interactions as the explanatory variables. Aviary number and maternal identity were added as random factors.

Table 1: Final linear mixed-effects model ANOVAs

| | Anti-KLH antibodies | | | Lysozymes | | | Ovotransferrins | | |
|---|---|---|---|---|---|---|---|---|---|
| | *F* | df | *P* | *F* | df | *P* | *F* | df | *P* |
| Zinc exposure | … | … | … | 7.44 | 1, 40 | .006** | 1.25 | 1, 49 | .263 |
| Lead exposure | *3.93* | *1, 38* | *.046** | .06 | 1, 40 | .814 | 2.41 | 1, 49 | .121 |
| Mother eumelanin level | … | … | … | 11.45 | 1, 40 | <.001*** | … | … | … |
| Mother antibody level | *2.67*/**14.21** | *1, 38*/**1, 38** | *.102*/**<.001**** | … | … | … | … | … | … |
| Zinc exposure × lead exposure | … | … | … | .42 | 1, 40 | .517 | 4.32 | 1, 49 | .037* |
| Zinc exposure × mother eumelanin level | … | … | … | 14.67 | 1, 40 | <.001*** | … | … | … |
| Lead exposure × mother antibody level | *1.90* | *1, 38* | *.169* | … | … | … | … | … | … |

Note. Log-transformed egg anti–keyhole limpet hemocyanin (KLH) level, lysozyme concentration, or ovotransferrin concentration was the dependent variable; zinc exposure, lead exposure, mother eumelanin level, mother anti-KLH antibody level when relevant, and their interactions were the explanatory variables; and aviary and mother identity were random factors. For anti-KLH antibody transfer, two models were equally parsimonious and are shown in italics (1) and bold (2).

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

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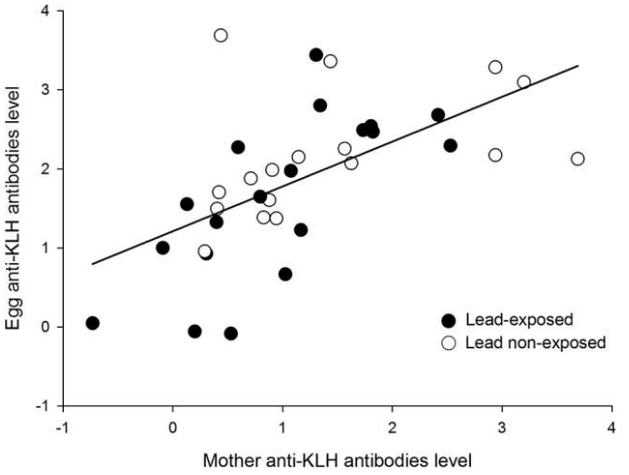



Figure 1. Log-transformed egg anti–keyhole limpet hemocyanin (KLH) antibody level according to log-transformed anti-KLH antibody level of females exposed (filled circles) and not exposed (open circles) to lead. The line represents the regression between log-transformed egg anti-KLH antibody level and log-transformed female anti-KLH antibodies, whatever lead exposure ($R^2 = 0.29$, $P < 0.001$).

Statistical analyses were performed using R (ver. 3.0.2). We retained final models using the Akaike information criterion (AIC).

## Results

Two models were equally parsimonious (their AICs were not significantly different; 105.8 and 104.4, respectively). In the first one, anti-KLH antibody level was lower in eggs laid by lead-exposed mothers (lead and lead+zinc groups) than in the others (control and zinc groups; table 1; $7.38 \pm 1.61$ and $11.78 \pm 2.62$, respectively). In the second one, egg anti-KLH antibody level increased with the mother anti-KLH antibody level (table 1; fig. 1).

Lysozyme concentration depended on the interaction between zinc exposure and maternal eumelanin level (table 1; fig. 2): lysozyme concentration was negatively linked with maternal eumelanin level in egg from zinc-exposed mothers (zinc and lead+zinc groups; $F = 5.70$, df $= 1, 18$, $P = 0.017$), while it was positively linked with maternal eumelanin level in eggs from mothers not exposed to zinc (control and lead groups; $F = 4.42$, df $= 1, 22$, $P = 0.035$). Ovotransferrin concentration depended on the interaction between zinc exposure and lead exposure (table 1; fig. 3): eggs laid by mothers exposed to lead only (lead group) had higher ovotransferrin concentrations than eggs laid by mothers exposed to both lead and zinc (lead+zinc group; $F = 7.37$, df $= 1, 25$, $P = 0.007$).

## Discussion

Our aim was first to investigate the effects of chronic exposure to trace metals in concentrations encountered in urban areas on early maternal transfer (from mother to eggs) of components influencing egg immune components (specific antibodies, lysozymes, ovotransferrin, and metals) and, second, to test whether mother melanin-based plumage coloration modulates such effects. In accordance with our hypothesis, the transfer of specific antibodies (anti-KLH antibodies) was smaller in eggs from lead-exposed mothers (lead and lead+zinc groups) than in the other eggs. This result underlines the negative effects of lead on egg immunity and may explain the higher hatching failure observed in blue and great tit nests exposed to high concentrations of trace metals (Sens et al. 2003; Eeva et al. 2009). Moreover, nestlings are immunologically naive and fight off parasites with their innate immune system inherited from their mother (reviewed in Hasselquist and Nilsson 2009). Therefore, coping with parasites may be more costly for nestlings with low maternal antibodies and may explain the lower nestling growth rate and the higher nestling mortality also observed in blue tits, great tits, and feral pigeons exposed to trace metals (Eeva and Lehikoinen 1996; Eeva et al. 2009; Chatelain et al. 2015). Moreover, this result is in accordance with a previous study demonstrating a negative effect of lead exposure on the second humoral immune response (anti-KLH antibody level) in adult feral pigeons (Chatelain et al. 2016).

In addition, ovotransferrin concentration was higher in eggs laid by mothers exposed to lead only (lead group) than in eggs laid by mothers exposed to both lead and zinc (lead+zinc group). First, this result suggests that lead exposure increases ovotransferrin transfer and, second, that zinc exposure moderates the effect of lead exposure on ovotransferrin transfer due to its effects on lead absorption and lead binding (Cerklewski and Forbes 1976; El-Gazzar et al. 1978; Godwin 2001). Interestingly, ovotransferrin binds divalent ions including lead ($Pb^{2+}$; Pohanka et al. 2012). Higher ovotransferrin levels may therefore help detoxify lead potentially transferred into the egg

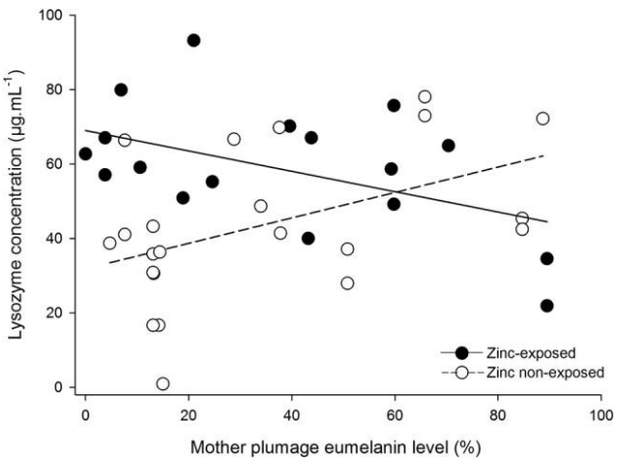



Figure 2. Egg lysozyme concentration ($\mu$g mL$^{-1}$) according to the plumage eumelanin level (%) of females exposed (filled circles, solid line; $R^2 = 0.65$, $P = 0.006$) and not exposed (open circles, dashed line; $R^2 = 0.82$, $P = 0.002$) to zinc.

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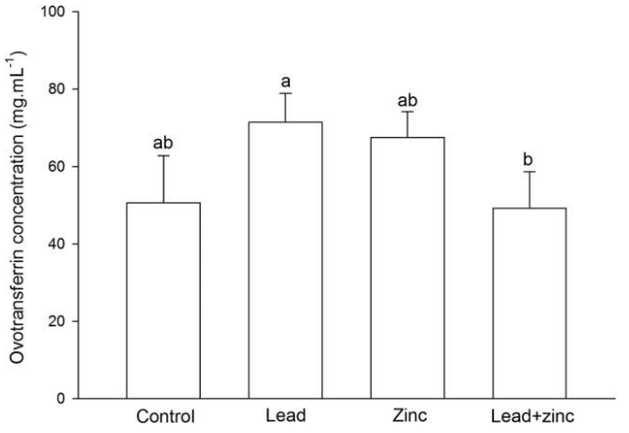



Figure 3. Egg ovotransferrin concentration (mg $mL^{-1}$) according to the exposure to lead and/or zinc. Bars with the same letter are not significantly different at $\alpha = 0.05$.

(Agusa et al. 2005) and may consequently reduce the noxious effects of lead on embryonic development. This result may suggest an adaptive strategy of maternal transfer of ovotransferrin. More studies are needed to understand whether ovotransferrin concentrations in eggs reflect egg bactericide capacity (Valenti et al. 1983, 1985), maternal inflammation and infection state (Horrocks et al. 2011), or egg potential for metal ion detoxification (Pohanka et al. 2012).

Contrary to previous observations in feral pigeons (Jacquin et al. 2013), females with darker plumage did not transfer higher amounts of specific antibodies than females with paler plumage. Maternal melanin-based plumage coloration had few effects on early maternal transfer of immune components; nonetheless, it significantly shaped the effects of trace metal exposure on lysozyme concentration. Among birds not exposed to zinc, lysozyme concentration increased with mother eumelanin level, suggesting that females with darker plumage transfer more lysozymes into their eggs than do females with paler plumage. However, we found the opposite correlation among zinc-exposed birds (i.e., lysozyme concentration decreased with increasing mother eumelanin level). This result suggests a beneficial effect of zinc on females with paler plumage. It is possible that zinc is more available for birds with paler plumage because birds with darker plumage transfer it into their feathers (Chatelain et al. 2014). As a consequence, females with darker plumage may transfer higher amounts of zinc into their feathers and therefore benefit less from zinc supplementation.

Our results demonstrate negative and positive effects of lead and zinc, respectively, on early maternal transfer of immune components that might explain the effects of trace metals on birds' reproduction observed *in natura* (Eeva and Lehikoinen 1996; Sens et al. 2003; Eeva et al. 2009). Because early maternal effects may greatly affect birds' reproductive success and, consequently, population functioning and dynamics, our study stresses the need to better understand the effects of trace metals on maternal investments. Future studies should investigate the biological significance of decreased or increased transfers of immune components, for instance, in juveniles' growth, survival, and immunity. Moreover, our results suggest that maternal eumelanin level increased lysozyme transfer but diminished the beneficial effects of zinc exposure, potentially because of zinc transfer into the feathers. Our study points out the need to investigate the costs and benefits of highly melanic plumage according to environmental concentrations of trace metals.

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