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No experimental evidence of an adaptive antioxidative response induced by trace metals exposure in feral pigeons

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Abstract

Trace metals produced by anthropogenic activities in particular in urban environments, such as lead and zinc, can induce oxidative damage in exposed individuals. Therefore, trace metals could act as a selective pressure for higher resistance to oxidative damage by favouring individuals able to plastically produce antioxidants once exposed to metal to counterbalance the oxidative damage production. In this study, we experimentally challenge this hypothesis in 69 feral pigeons (*Columba livia*) originating from an urban environment by exposing a subset of them to zinc (n = 18), lead (n = 17) or zinc and lead (n = 16) during 14 weeks to see if exposed individuals showed an increased antioxidant production compared to controls (n = 18). Our results reported that zinc exposure induced oxidative stress by increasing oxidative damage but failed to detect a compensatory production of antioxidants in exposed individuals. However, our results report lower oxidative damage for reproducing individuals, which is consistent with an oxidative shielding phenomenon occurring prior to reproduction in males to protect the sperm, and during egg laying in females to protect offspring. In conclusion, our study does not support an adaptive antioxidative response in individuals exposed to trace metals, but it does partly support the oxidative shielding hypothesis.

Keywords: oxidative stress, trace metals, urban pollution, oxidative shielding, feral pigeon, colour polymorphism, eumelanin

Introduction

Urbanization constitutes a novel constraint for living organisms that drastically changes the environmental variables and generally has a negative impact on biodiversity (Johnson and Munshi-South 2017). However, several species called urban exploiters are found in urban areas because they can cope with the constraints of urbanization such as pollutions and human proximity. In this context, one of the blooming topics in urban ecology is to understand how species adapt to survive in cities (Johnson and Munshi-South 2017). Trace metal pollutants are known to be linked to urban environments. Concentrations of trace metals are higher in urban environments compared to rural ones, thus urban exploiters have to face high chronic exposure to trace metals (Azimi et al. 2003, Roux and Marra 2007, Sánchez-Virosta et al. 2021). Once absorbed, trace metals often occur as divalent cations that can cause oxidative damage (Nuran et al. 2001, Schilderman et al. 1997, Valko et al. 2005). Indeed, most trace metals are harmful pro-oxidants that can interfere with metabolism by altering antioxidant enzymatic activities and increasing reactive oxygen species (ROS) levels (Koivula et al. 2011). Free radicals are naturally produced during cell metabolism. These ROS can cause molecular and cellular damage, but are necessary for several cellular functions. In consequence, ROS production may entail fitness costs (Alonso-Alvarez et al. 2017). Since antioxidants can reduce internal levels of ROS, keeping up the redox balance, trace metals could act as a selective pressure for higher resistance to oxidative damage. This selective pressure would favour individuals able to plastically produce antioxidants once exposed to metal to counterbalance ROS production.

During reproduction, metabolism demands are higher and oxidative stress may occur (Alonso-Alvarez et al. 2004, Costantini et al. 2014). However, several studies failed to observe a link between reproduction and an increase in oxidative damage. A recent idea to explain the absence of a relation between reproduction and oxidative damage is that reproducing individuals can produce higher antioxidants to prevent oxidative damage (the so-called oxidative shielding) and protect their offspring and/or gametes (Birch et al. 2024, Blount et al. 2016, Vitikainen et al. 2016). This hypothesis predicts to observe a higher production of antioxidants in reproducing individuals compared to non-reproducing ones. Similarly, the adaptation to cope with metal exposure in an urban environment would involve reducing oxidative damage by plastically increasing antioxidant production once individuals are exposed to trace metals. In this study, we experimentally tested this hypothesis in an urban exploiter: the feral pigeon (*Columba livia*). If this hypothesis is true, we expected to observe, in trace metal exposed individuals, a higher production of antioxidants as compared to non-exposed individuals, maintaining the oxidative damages at similar levels in the two groups. Alternatively, if our hypothesis is wrong, we should observe a higher oxidative damage in trace metal-exposed individuals as compared to non-exposed individuals.

In feral pigeons, the degree of melanism varies continuously from pure white to full black individuals depending on the level of eumelanin in feathers. Darker individuals are found in higher proportions in urban compared to rural environments (Jacquin et al. 2012, Jacquin et al. 2013). Moreover, melanin level was linked to the capacity to store trace metals in feathers (Chatelain et al. 2014). Taken together, these results suggest a possible mechanism where darker individuals are selected through their capacity to handle trace metals better than paler ones. This potential mechanism could occur via oxidative stress, where darker individuals would show lower oxidative damage when exposed to trace metals compared to paler individuals (Chatelain et al. 2016a). Therefore, the eumelanin-based colouration may indirectly modulate the oxidative damages impaired by trace metal exposure and could also modulate the antioxidative response.

To test these predictions, we experimentally exposed urban feral pigeons to trace metals, zinc and lead, alone or in interaction at concentrations observed in urban environments (Chatelain et al. 2016a). Then, 7 and 14 weeks after the beginning of the treatment, the levels of oxidative damage and antioxidants were compared between metal-exposed groups (zinc, lead, zinc+lead) and the control group. We also examined whether the levels of oxidative damage and antioxidants are linked to eumelanin-based colouration (Chatelain et al. 2016a). We expected oxidative damage to be more pronounced in pale individuals than in dark individuals when exposed to trace metals. Therefore, we hypothesized that the antioxidative response would mainly occur in pale individuals. Finally, we investigated whether the oxidative shielding occurred in pigeons by comparing the production of oxidative damage~~s~~ and antioxidants between reproducing and non-reproducing individuals.

Materials and methods

Capture and housing

We captured a total of 69 adult feral pigeons between January 29th and February 4th, in the center of Paris (France) in 2019. Individuals were first sexed visually based on morphological criteria (size, appearance of the caruncle and iridescent reflection of the neck). We confirmed or corrected sex by behavioural observation specific to each sex (such as "bow-coo" for males, (Fabricius and Jansson 1963)). Birds were transferred to the CEREEP (Centre d'Ecologie Expérimentale et Prédictive-Ecotron Ile-de-France, Saint-Pierre-le-Nemours, France) and placed in eight outdoor aviaries (3.1 × 2.0 × 2.4m). They were identified individually with coloured rings. We distributed individuals among aviaries and treatments to balance sex, mass and level of melanism (see below). With a total of 38 males and 31 females, we placed 9 pigeons in 6 aviaries (5 males and 4 females), 8 pigeons in one aviary (4 males and 4 females) and 7 pigeons in one aviary (4 males and 3 females). They were fed *ad libitum* with a mixture of corn, wheat and peas. The aviaries contained a drinking-trough and a basin used as a bath, as well as a nesting area and bamboo perches. Birds were released at capture site at the end of the experiment. All experiments were carried out in strict accordance with the recommendations of the 'European Convention for the Protection of vertebrate Animals used for Experimental and Other Scientific

Purposes’ and were conducted under the authorizations of the French authority (authorization APAFIS#17554-201811161046635v2).

Treatment

Treatment started a week after capture and lasted 14 weeks. We implemented 4 different supplementation treatments (one by aviary): a lead treatment (10 ppm in tap water; Sigma-Aldrich, St. Louis, MO, USA; n = 17); a zinc treatment (100 ppm in tap water; n = 18), a lead and zinc treatment (10 ppm of lead and 100 ppm of zinc in tap water; n = 16) and a non-metal control treatment (tap water only; n=18). We chose these concentrations in order to better mimic the concentrations to which pigeons are exposed in urban areas based on the concentrations found in the city of Paris (Chatelain et al. 2016b). Indeed, the effectiveness of this treatment has been previously demonstrated by increasing lead and zinc concentrations circulating in the blood (Chatelain et al. 2016b). There were 2 aviaries per treatment. Treated water was added to drinking-troughs and baths. Pigeons had unlimited access to water which was replaced twice a week.

Oxidative stress

Blood was obtained by puncturing the brachial vein on the left wing. Blood samples were taken three times for each pigeon: just before the beginning of trace metal supplementation in the water (Week 0, January 29th), at week 7 (March 21st, 2019) and at week 14 (May 7th, 2019). Plasma was collected after samples were centrifuged 5 minutes (10 000 rpm), and held in a freezer at -20 °C. We analysed two oxidative stress markers: the plasma reactive oxygen metabolites concentration (ROM) and the total antioxidant capacity of the plasma (OXY). These markers were measured using two kits, which function on colorimetric determination of ROM concentrations or of antioxidant capacity. For both kits, the samples were randomly assigned to

96-well plates, mixing the reagent with 4 µl of individual plasma resulted in a coloured mixture readable with a spectrophotometer. ROM plasma concentration was measured using the d-ROM test (kit MC0001, Diacron International, Groseto, Italy). The test mainly measures the hydroperoxides that are products of the peroxidation of macromolecules (mainly lipids) with ROS. These markers are the first formed during the chain reaction induced by ROS. Hydroperoxides are relatively stable chemical species but can potentially generate new radicals, they are considered as markers of oxidative stress. The ROMs are expressed in mM of H_2O_2 equivalents (Costantini et al. 2007, Vassalle et al. 2008). The OXY test (OXY-adsorbent test, Diacron, Italy) quantifies the total antioxidant capacity of the plasma against the oxidizing action of hypochlorous acid (HClO). The measurements are expressed in µmol of HClO consumed by 1 mL of sample (µmol HClO / mL).

Reproductive status

A total of 18 pigeons (8 from the control group, 6 from the lead treatment, 2 from zinc treatment and 2 from lead and zinc treatment) formed 9 pairs that laid at least one egg. Pigeons are indeterminate egg-laying birds (Kennedy 1991), meaning that females can continue to produce eggs after removing a first clutch. Laying started at week 6. From then, we collected eggs twice a week and parents were identified by observing rings of individuals on nests. During the 14 weeks experiment, two pairs laid 1 egg, three laid 3 eggs, two pairs laid 4 eggs and two pairs laid 5 eggs. An individual was considered as a “reproducing individual” when it produces at least one egg. We then used this binary variable for statistical analyses (reproducing versus non-reproducing individuals).

Melanism level

Melanism level was estimated via plumage colouration. The upper surface of the right wing was photographed for each pigeon under the same light source with a colour chart in the picture for calibration. Eumelanin-based colouration was estimated as the percentage of black pixels of a delimited part of the wing, which comprises all feathers of a wing except primaries and secondaries. This score was calculated using the Gimp image software (v 2.10). This measurement is repeatable between individuals and is considered a good relative estimate of eumelanin level (Jacquin et al. 2011).

Statistical analyses

Analyses were performed with JMP®, version 16. Three individuals died during the experiment, thus we had a total of 66 individuals with oxidative stress measures. ROM were log-transformed to better converge with a normal distribution. To explain variations in oxidative variables using the three repeated measures per individual, the two models used Log10[ROM] and OXY as response variables and tested the effect of zinc coded as unexposed (0) or exposed (1), the effect of lead coded as unexposed (0) or exposed (1), time (three categorical occurrences: Week 0, 7 and 14), sex coded as male (0) or female (1), reproduction (has produced eggs or not) and eumelanin level (continuous variable). We included the following biological meaningful interactions without saturating the models: time*lead, time*zinc, lead*zinc, time*zinc*lead, melanin*zinc, melanin*lead, melanin*lead*time, melanin*zinc*time, time*reproduction time*sex, reproduction*sex and time*reproduction*sex. We also added aviary and pigeon ID as random variables to take into account for pseudoreplication.

The two models were linear mixed-model ANOVA with restricted maximum likelihood (REML). The method of best-fitted model selection (based on AIC) was done by removing non-significant interactions in a step-wise manner. Based on AIC, the aviary random factor was not retained in the final models. Statistic outputs were produced using ANOVA type III. Post-hoc t-tests were performed to interpret significant interactions between two factors.

Results

Antioxidative response induced by trace metal exposure

Our results show a significant interaction between zinc exposure and time on ROM plasma concentrations (Table 1). Post-hoc tests revealed that, at week 14, pigeons exposed to zinc had significantly higher ROM concentration than non-exposed pigeons (t-test, $t_{61.7} = 2.25$, $p = 0.03$, Figure 1). Such a difference was not observed in either week 0 (t-test, $t_{61.1} = 0.13$, $p = 0.90$) or week 7 (t-test, $t_{62.7} = 0.16$, $p = 0.87$, Figure 1). Then, lead exposure had no effect on ROM (alone or in interaction, Table 1). Finally, we did not detect any effects of lead and zinc exposure alone or in interaction on the total antioxidant capacity OXY (Table 1).

Oxidative shielding

Our results show a significant three-way interaction between sex, reproduction and time on reactive oxygen species concentration ROM (Table 1). First, post-hoc tests revealed that, at week 0, reproducing male pigeons had significantly lower ROM concentration than non-reproducing male pigeons (t-test, $t_{19.8} = 3.65$, $p = 0.002$, Figure 2A). Such a difference was not observed for males at week 7 (t-test, $t_{12.7} = 0.22$, $p = 0.83$) or at week 14 (t-test, $t_{10.8} = 0.29$, $p = 0.78$). Second, post-hoc tests revealed that, at week 14, reproducing female pigeons had significantly lower ROM concentration than non-reproducing female pigeons (t-test, $t_{20.3} = 1.97$, $p = 0.03$, Figure 2B). Such a difference was not observed for females at week 0 (t-test, $t_{16.6} = 0.10$, $p = 0.92$) or at week 7 (t-test, $t_{13.2} = 0.88$, $p = 0.40$). Finally, we did not detect any effect of reproduction alone or in interaction on the total antioxidant capacity OXY (Table 1).

Antioxidative response, oxidative shielding and eumelanin-based colouration

We detected no effect of eumelanin-based colouration alone or in interaction on ROM or on the total antioxidant capacity OXY (Table 1).

Discussion

Antioxidative response induced by trace metal exposure

In the context of an antioxidative response, a higher production of antioxidants is expected in individuals exposed to trace metal than in non-exposed individuals. The antioxidative production would thus help to maintain oxidative damages at similar levels in both groups. Contrary to these predictions, we observed no effect of zinc or lead exposure on antioxidant production, but also an increase of oxidative damage in pigeons when exposed to zinc (Figure 1). Therefore, our results suggest no antioxidative response in pigeons towards trace metal exposure, and confirm previous studies on the toxicity of zinc via the oxidative stress (Koivula and Eeva 2010) and the weak toxicity of lead via the oxidative stress (Rainio et al. 2015). As zinc is a redox-inactive metal, it has been suggested that this compound depletes the major antioxidants of cells, contributing to higher stress levels (Koivula and Eeva 2010). Our results are not in agreement with this mechanism as we did not detect any negative effect of zinc on antioxidants production. Nevertheless, one can imagine that the depletion may occur by preventing the *de novo* synthesis of antioxidants leading to the temporary stabilization of their concentration over our experiment. Alternatively, as oxidative damage and antioxidant response may differ among tissues, our measures in blood plasma may not be generalized to the entire organism (Costantini 2019). The effect of zinc exposure on oxidative damage could also be indirect. Individuals exposed to zinc would invest more in the immune system (Chatelain et al. 2016b) which is known to induce oxidative damages (Costantini 2022, Costantini et al. 2014). Finally, the failure to detect the antioxidative response might be due to the duration of metal exposure which would be too short to create sufficient oxidative stress to induce antioxidants production. It is possible that short-term chronical metal exposure may be detoxified by the pigeons by accumulating metals into several tissues such as the feathers (Frantz et al. 2012). A longer-term experimental study, which would lead to potential saturation of detoxifying tissues with metals, would be necessary to truly falsify the antioxidative response hypothesis.

Oxidative shielding

At first glance, no evidence of oxidative shielding was observed in response to reproduction in the feral pigeon. Specifically, there was no effect on the production of antioxidants that would maintain oxidative damage at consistent levels over time in reproducing individuals. However, we found contrasting results regarding oxidative damage in reproducing individuals between males and females (Figure 2). Reproducing males have significantly lower levels of oxidative damage at the beginning of the experiment (week 0) compared to non-reproducing males (Figure 2A). Conversely, non-reproducing females displayed elevated levels of oxidative damage at the end of the experiment compared to reproducing females (Figure 2B). These results are consistent with the oxidative shielding hypothesis, as reproducing individuals exhibited lower levels of oxidative damage than non-reproducing individuals during the relevant period of reproduction. In males, the relevant period for oxidative shielding could be at the beginning of the reproduction to protect sperm before the fertilization (Birch et al. 2024). In contrast, in females, the relevant period for oxidative shielding could be during eggs laying to protect offspring from intergenerational transmission of damage via the egg (Blount et al. 2016). As reported before and in disagreement with this hypothesis, we did not detect any increase in antioxidant production. Our inability to detect antioxidant production in reproducing individuals may have been because the antioxidants we measured were not good markers of antioxidant production in that particular case (to be measured in another tissue, see before). Alternatively to the oxidative shielding hypothesis, only better individuals can invest in the reproduction, which are the females that better cope with oxidative stress or that are less impacted by environmental factors across the time of the experiment that might have induced the oxidative damages observed in the non-reproducing females (such as parasites for instance through immune responses (Costantini 2022)). For males, as females started to lay eggs 6 weeks after the beginning of the experiment, the difference observed between reproducing and non-reproducing males may be explained by the mate choice occurring a few weeks before egg laying. As we caught pigeons from the natural conditions, pigeons had to create new mating pairs in the aviaries and the period of mate pairing could therefore have taken place at week 0. According to the sexual selection theory (Andersson and Simmons 2006), females would have chosen better males, which might be males with lower oxidative damage (Schantz et al. 1999).

Melanism and oxidative stress

Dark eumelanin pigeons are better able to detoxify trace metals as compared to paler ones (Chatelain et al. 2016a) and we therefore expected to detect the strongest effects of trace metals exposure on oxidative damages or shielding in paler individuals. Contrary to our expectations, eumelanin-based colouration did not affect oxidative damage or antioxidant capacity. The detoxication mechanism proposed here should mainly occur during moulting or juvenile growth in pigeons when feathers are growing (Chatelain et al. 2016a). It is possible that during the time lapse of our experiment, the accumulation of metals in feathers was not enough important to create different levels of metal detoxication between dark and pale eumelanic pigeons because pigeons were not moulting. However, alternative mechanisms would have expected correlations between melanin-based colouration and oxidative status, as reported earlier (Emaresi et al. 2016, Leclaire et al. 2019), such as (i) the biochemical role of antioxidants in melanogenesis (Galván and Alonso-Alvarez 2009, Galván and Solano 2009) or (ii) through pleiotropic effects of the melanocortin system (Ducrest et al. 2008, Roulin and Ducrest 2011). However, we did not detect such relationships in pigeons.

Conclusion

In conclusion, our study provides no support for an adaptive antioxidative response induced by trace metals exposure in feral pigeons. More longer-term experimental studies, which would lead to the potential saturation of detoxifying tissues with metals, involving measurements of antioxidant production in several tissues would be necessary to truly falsify this hypothesis. In addition, our results partly support the oxidative shielding hypothesis, suggesting that it occurs at different periods for males and females: during sperm production in males and during egg laying in females.

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Data Availability

The data used in this study are available on request from the corresponding author.

Conflict of interest

The authors declare that they have no conflict of interest with the content of this article.

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Table 1: Output of the final linear mixed models retained after model selection on Log10 [ROM] and OXY using three repeated measures (Week 0, 7 and 14) on each adult feral pigeon. Aviary and pigeon ID were initially included as random factors, then, aviary ID was finally removed based on AIC. ANOVA type III was applied. Non-significant interactions that **were removed** are not shown. P-value < 0.05 are highlighted in bold.

	Log10 [ROM]			**OXY**		
Effects	F	DF	P	F	DF	P
Lead	0.52	1,61.8	0.47	0.28	1,61.4	0.60
Zinc	1.99	1,61.0	0.16	2.52	1,60.2	0.12
Time (T0, T1 and T2)	5.16	2,122	**0.007**	1.63	2,128.5	0.20
Melanin based coloration	0.31	1,60.8	0.58	0.04	1,59.9	0.84
Sex	15.1	1,63.4	**0.0002**	0.00	1,61.8	0.97
Reproduction (yes or no)	1.43	1,62.5	0.24	0.44	1,61.9	0.51
Zinc * Time	3.36	2,121.2	**0.04**	_	_	_
Sex * Time	1.11	2,122.8	0.33	_	_	_
Sex * reproduction	0.05	1,62.7	0.82	_	_	_
Time * Reproduction	1.34	2,122.9	0.27	_	_	_
Sex*reproduction*Time	3.30	2,122.9	**0.04**	_	_	_

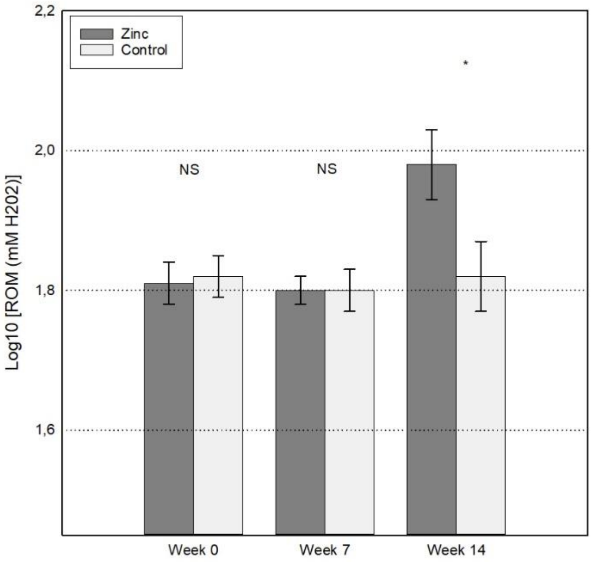

Figure 1. Oxidative damage (i.e Reactive Oxygen Metabolites concentrations, ROM expressed in Log10 [mM of H_20_2 equivalent]) for pigeons supplemented with zinc (dark grey, **N=34**) and control pigeons (**pale grey, N = 35**, mean ± SE; NS above bars indicate a non-significant difference and * a significant difference) across time (Week 0 (before experiment), Week 7 and Week 14).

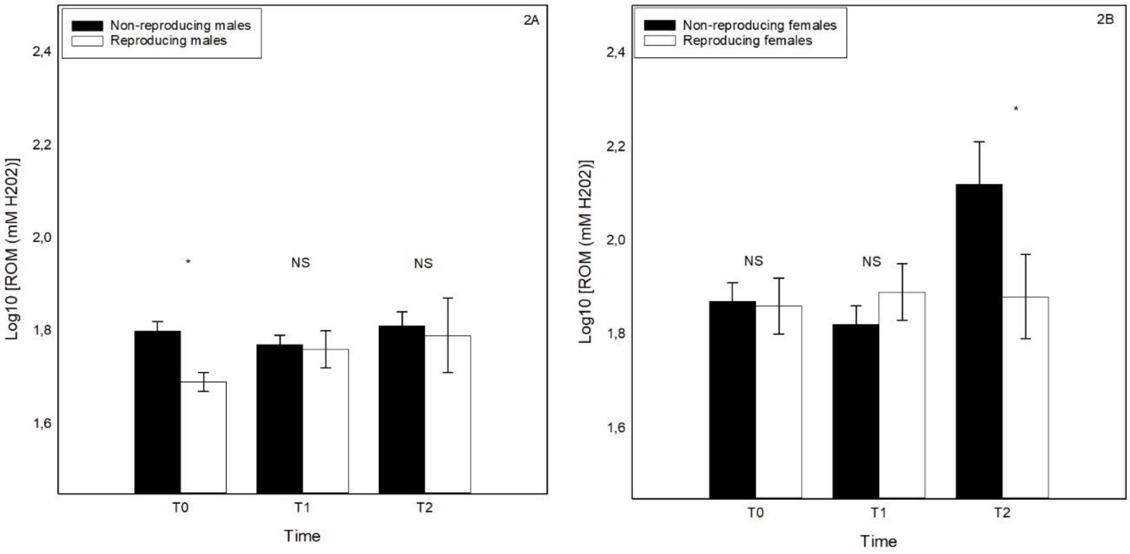

Figure 2: Oxidative damage (i.e Reactive Oxygen Metabolites concentrations, ROM expressed in Log10 [mM of H_20_2 equivalent]) for reproducing pigeons (dark grey) and non-reproducing pigeons (mean ± SE; NS above bars indicate a non-significant difference and * a significant difference between reproducing and non-reproducing pigeons) across time (Week 0 (before experiment), Week 7 and Week 14) for **2A**. males and **2B**. females.