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Do trace metals select for darker birds in urban areas?

An experimental exposure to lead and zinc

Running title: trace metals and plumage melanin selection

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ABSTRACT

Trace metals from anthropogenic activities are involved in numerous health impairments and may therefore select for detoxification mechanisms or a higher tolerance. Melanin, responsible for the black and red colourations of teguments, plays a role in metal ions chelation and its synthesis is positively linked to immunity, to antioxidant capacity and to stress resistance due to pleiotropic effects. Therefore, we expected darker birds to 1) store higher amounts of metals into their feathers, 2) maintain lower metal concentrations in blood and 3) suffer less from metal exposure. We exposed feral pigeons (*Columba livia*) exhibiting various plumage darkness levels to low but chronic concentrations of zinc and/or lead, two of the most abundant metals in urban areas. First, we found negative and positive effects of lead and zinc respectively on birds' condition and reproductive parameters. Then, we observed positive relationships between plumage darkness and both zinc and lead concentrations in feathers. Interestingly, though darker adults did not maintain lower metal concentrations in blood and did not have higher fitness parameters, darker juveniles exhibited a higher survival rate than paler ones when exposed to lead. Our results show that melanin-based plumage colouration does modulate lead effects on birds' fitness parameters but that the relationship between metals, melanin, and fitness is more complex than expected and thus stress the need for more studies.

INTRODUCTION

Current human activities generate considerable environmental disturbances, such as light, noise and chemical pollutions. Like other chemical pollutions, trace metals emissions are of particularly timely concern, given their implication in several human diseases (reviewed in Jarup, 2003) and their noxious effects on wildlife (Hsu *et al.*, 2006). In birds, trace metals negatively affect immunity in great tits and zebra finches (Snoeijs *et al.*, 2004, 2005) and learning abilities in young herring gulls (Burger & Gochfeld, 2004). In addition, high levels of trace metals in the environment correlate with reproduction impairments (e.g. higher nest desertion, hatching failure and mortality; Eeva & Lehikoinen, 1996; Janssens *et al.*, 2003; Eeva *et al.*, 2009), and oxidative damages (Berglund *et al.*, 2007) in passerine birds.

Because concentrations of trace metals are higher in the cities than in rural areas (Azimi *et al.*, 2003; Roux & Marra, 2007), trace metals likely have ecological and evolutionary consequences on urban wildlife, though the exact levels organisms experience remain unknown and most probably depend on the taxonomic group considered. Because of their toxicity, trace metals may select for detoxification mechanisms (such as higher elimination rate of ingested metals) or for higher resistance to their toxic effects (such as higher oxidative stress resistance). Environments polluted with trace metals thus represent exciting opportunities to study ongoing evolutionary mechanisms in the wild.

Interestingly, highly melanic plumage may be advantageous in environments polluted with trace metals (i.e. darker individuals may suffer less in these habitats), both through direct and indirect effects of melanogenesis. First, melanin is composed of negatively-charged free carboxyl, hydroxyl and amine functions known to bind metal ions in vitro (Larsson & Tjälve, 1978; Liu *et al.*, 2004; Bridelli & Crippa, 2007). For this reason, metal chelation is suggested as one of the main biological functions of melanin (McGraw, 2003; Hong & Simon, 2007;

Chatelain *et al.*, 2014). In birds, metal transfer from the bloodstream into melanic feathers during their growth could represent an efficient detoxification mechanism through metal sequestration and elimination during moulting, a hypothesis already proposed for keratin in feathers (Burger, 1993). Consequently, more melanic feathers would be able to store higher amounts of metals, a hypothesis supported by a positive correlation between concentrations of some metals in feathers and melanin-based plumage colouration in feral pigeons, white-tailed eagles and barn owls (Niecke *et al.*, 1999, 2003; Chatelain *et al.*, 2014). In habitats where metals indeed have noxious effects, highly melanic birds would thus have a better detoxification ability than paler birds by lowering their circulating metal burden. Although this detoxification mechanism could represent a significant driver of melanin-based plumage colouration polymorphism maintenance, it has been poorly investigated. Positive correlations have been shown between melanin-based plumage colouration and concentrations of some metals (zinc, calcium and manganese; Niecke *et al.*, 1999, 2003; Zduniak *et al.*, 2014), while no such link has been demonstrated for highly toxic metals such as lead and cadmium, maybe because of the correlative nature of the studies (Gochfeld *et al.*, 1991; Chatelain *et al.*, 2014). Then, to the best of our knowledge, no study compared metal concentrations in feathers between differently melanin-coloured birds in controlled environmental conditions (ie. under the same metal exposure).

Second, both the pleiotropic effect of the gene coding for melanin synthesis (POMC) and its linkage disequilibrium with various loci result in correlations between variation in melanin-based plumage colouration and several biological traits, including immunity, antioxidant capacity and stress resistance (Ducrest *et al.*, 2008; Mckinnon & Pierotti, 2010). Indeed, darker pigeons exhibit both a lower endoparasite intensity and a greater cellular immune response than paler pigeons (Jacquin *et al.*, 2011). Moreover, eumelanin level in the barn owl positively correlates with resistance to oxidative stress (Ducrest *et al.*, 2008; Roulin *et al.*,

2011) and to physiological stress (corticosterone synthesis; Almasi *et al.*, 2010, 2012)**.** Therefore, melanin-based plumage colouration may shape birds' tolerance to trace metals.
Both direct (metal binding) and indirect (resistance to parasites, oxidative stress and physiological stress) associations between biological traits and plumage melanism may favour darker birds in environments polluted with trace metals. Accordingly, previous studies observed a higher frequency of darker feral pigeons in European cities (Obukhova, 2007; Jacquin *et al.*, 2013a), where environmental concentrations of metals are the highest (Azimi *et al.*, 2005; Scheifler *et al.*, 2006; Roux & Marra, 2007; Kekkonen *et al.*, 2012). However, there is no experimental evidence for fitness advantages of being more melanistic in habitats polluted with trace metals. Still, the existence of such melanin-based plumage polymorphism in cities raises questions about its evolutionary maintenance, which could result from either a transitory polymorphism (i.e. the hypothesized selective pressure induced by trace metals is ongoing), some imperfect linkage disequilibrium between loci involved in melanogenesis and other biological traits under selection or the co-occurrence of antagonistic selective pressures.

Partly due to large emissions by anthropogenic activities (beginning during the Roman Empire and increasing with the Industrial Revolution of the 19th century Nriagu, 1996), lead is the most abundant toxic metal in the environment (Azimi *et al.*, 2005; Roux & Marra, 2007). Although lead used in gasoline drastically diminished since the 70s (Jarup, 2003), it remains of high ecological importance due to its accumulation into the soil (Roux & Marra, 2007) and to the negative biological effects of a chronic exposure, even at low levels (Patrick, 2006). In addition, zinc is the most abundant metal in the environment (Azimi *et al.*, 2005). While it may induce harmful effects at high concentrations (Greenberg & Briemberg, 2004; Bozym *et al.*, 2010), it is overall an essential nutrient (Mertz, 1981; Prasad, 1998, 2009), also able to compensate the negative effects induced by other traces metals (Chichovska & Anguelov, 2006; Prasanthi *et al.*, 2006, 2010). Therefore, lead and zinc likely induce the strongest (negative or positive) effects on urban wildlife. While lead and zinc effects on condition and reproductive success may greatly modulate birds' fitness and population dynamics, to the best of our knowledge, no experimental study demonstrated such effects and the previous correlative studies cannot exclude confounding urban factors also known to impair birds' reproduction (Halfwerk *et al.*, 2011; Dominoni *et al.*, 2013).

In this work, we chronically exposed feral pigeons (*Columba livia*) to lead and/or zinc in experimentally controlled concentrations inferred from previous measures in urban areas. The feral pigeon is a highly polymorphic bird species with respect to its melanin-based plumage colouration and experiences an extended moulting period (Johnston & Janiga, 1995). First, we evaluated the effects of such exposures on lead and zinc concentrations in feathers and in blood, and on bird condition and reproductive parameters. Then, we investigated whether the ability of feathers to store zinc and lead depends on their melanin-based colouration and, as a consequence, whether melanistic birds maintain lower lead concentrations in blood; because zinc concentration in blood is under strict homeostatic regulation in eukaryotes (Gaither & Eide, 2001), no relationship was expected between plumage colouration and blood levels of zinc. Finally, we tested whether melanin-based plumage colouration could be advantageous in environments polluted with metals by investigating the interaction between plumage colouration and metal exposure on birds' condition and reproductive parameters.

MATERIALS AND METHODS

Biological model

Free-living feral pigeons are considered to originate from the continuous reproduction between wild, synanthropic and domesticated pigeons (Johnston & Janiga, 1995). Contrasting artificial selective pressures on various phenotypic traits linked to past domestication has generated polymorphism in the degree of melanin-based plumage colouration. Free-living feral pigeons have then evolved in natural environments in close proximity with human populations for centuries and maintained one of the highest melanin-based plumage colouration polymorphism amongst birds. This polymorphism involves melanin type (eumelanin and pheomelanin, respectively responsible for the black and reddish colour of teguments) and melanin degree (i.e. the area of pigmentation; see below). Both parameters of melanin-based plumage colouration in feral pigeons are highly heritable (heritability of melanin degree: 0.82±0.12; Jacquin *et al.*, 2013b).

Subjects and Housing

Ninety six (48 males and 48 females genetically sexed) free-living adult feral pigeons (*Columba livia*) exhibiting various melanin-based plumage colourations were caught during winter 2013 (February/March) in several pigeon flocks within Paris (Supplementary material). Birds were all adults as indicated by reliable morphological criteria such as well-formed caruncle, absence of juvenile plumage and presence of iridescent neck feathers (Johnston & Janiga, 1995). The birds were immediately transferred in 8 outdoor aviaries (3.10 m x 2.00 m x 2.40 m) at the CEREEP field station (Centre d'Ecologie Expérimentale et Prédictive-Ecotron Ile-de-France, UMS 3194, Ecole Normale Supérieure, Saint-Pierre-lès-Nemours, France). Birds were fed *ad libitum* with a mix of maize, wheat and peas. The aviaries were enriched 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, all birds were released back to the wild at their site of capture. 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 “Ministère de l’éducation nationale, de l’enseignement supérieur et de la recherche” (authorization N_00093.02) and the “Direction Départementale des Services Vétérinaires de Seine et-Marne” (authorization N_ 77-05).

Plumage colouration measurement

At their capture, birds were first categorised as eumelanic (grey to black pigmented; 37 males and 45 females) or pheomelanic (red pigmented; 11 males and 3 females), which defines what we called their melanin type. Pheomelanic birds are usually in low frequencies in cities worldwide (Obukhova, 2007), and are particularly scarce in Paris (about 3%; based on personal data on 2074 pigeons captured over 5 years); despite the particularly strong capture effort provided to capture pheomelanic pigeons, their amount remained small (14 out of 96). Then, birds were individually photographed to precisely quantify their eumelanin or pheomelanin level. Eumelanin or pheomelanin level was calculated as the percentage of black or red on the wing surface of birds respectively (number of black pixels/number of white pixels x 100) using the Gimp image retouching and editing software, which is a reliable and repeatable estimation of melanin concentration (Jacquin *et al.*, 2011). At the end of the experiment, fledglings born during the experiment were also photographed to assess their eumelanin or pheomelanin level. The percentage of pigmented surface did not significantly differ between eumelanic and pheomelanic birds ($F_{1,94}$=0.27, P=0.606).

Treatments

Two weeks before the start of the experiment, the birds were distributed in the aviaries in order to equilibrate both sex (6 females and 6 males per aviaries) and plumage colouration ($F_{1,94}$<0.01, P=0.974). However, because of their lower number (n=14), pheomelanic individuals were split in 6 aviaries only. Importantly, because birds’ precise location origin in

Paris is likely to affect their previous exposure to trace metals and consequently their initial (prior to the experiment) trace metal body concentrations (Frantz *et al.*, 2012), birds were randomly distributed in the aviaries according to their flock (Chi²=71.09, df=70, P=0.441). This randomization should also avoid any other correlation between birds' history (e.g. age) and aviary. The aviaries were then randomly assigned to one of the 4 following metal exposure treatments: exposed to lead only (*lead* group; 1ppm lead acetate in tap water, Sigma-Aldrich), exposed to zinc only (*zinc* group; 10ppm zinc sulphate in tap water, Prolabo), exposed to both lead and zinc (*lead+zinc* group; 1ppm lead acetate and 10ppm zinc sulphate in tap water) or control (*control* group; tap water without any metal addition). This resulted in 2 aviaries with 12 pigeons each (24 pigeons in total) per treatment. We chose these concentrations based on both lead concentrations in blood measured in feral pigeons (ranging from 110 to 154ppb; personal data on feral pigeons captured in 2009 in Paris) and the gastrointestinal absorption rate of lead in zebra finches (<10%) calculated from Dauwe *et al*. (2002). Zinc concentrations were approximated using zinc/lead concentrations ratio in the environment and bird feathers in Paris (Azimi *et al.*, 2005; Frantz *et al.*, 2012). Drinking troughs and baths were filled with the corresponding treated water every other day, miming part of birds' exposure to trace metals in the wild (i.e. through ingestion and deposition onto the plumage).

Scaled mass index

From the start to the 20th week of the experiment, all adults were captured once a week to be weighed to the nearest gram with a Pesola Newton scale. Scaled mass index was calculated according to the method described by Peig & Green (2009, 2010). Briefly, scaled mass index was calculated using $\widehat{M_i} = M_i \left[\frac{L_0}{L_i}\right]^{b_{SMA}}$ where M_i and L_i are the body mass and tarsus length of individual i respectively; b_{SMA} is the scaling exponent estimated by the Standardized Major

Axis regression of M on L; L_0 is the arithmetic mean of tarsus length in the study population; and $\widehat{M_i}$ is the predicted body mass for individual i when tarsus length is standardized to L_0. Scaled mass index is a better indicator of the relative size of energy reserves and of other body components than the ordinary least squares (OLS) regression of body mass against size (Peig & Green, 2009), broadly used amongst ecologists.

Reproductive success measurements

Breeding success. A week after the start of the treatments six nest boxes per aviary were opened to allow birds to mate and breed (a box per couple). A bird was considered as reproducing when it had laid (female) or incubated (female and male) at least one egg during the breeding season. Overall, 52 pigeons (25 eumelanic and 3 pheomelanic females and 17 eumelanic and 7 pheomelanic males) successfully reproduced.

Eggs' quality measurement. Feral pigeons commonly produce two-egg clutches, one to 6 times a year. The day it had been laid, the egg was removed from the nest, weighed and measured (3 measures of eggs' maximum length and maximum width were taken, and then averaged). Egg volume was calculated as $V = 0.4866 \times Length \times (Width^2)$ (Paillisson *et al.*, 2007). Eggs of the first, third and fifth clutches were put back in the nest to allow incubation (n=83) whereas the others were frozen (n=65). Shell, albumen and yolk of frozen eggs were separated, then weighed to the nearest µg (eggshells were previously oven-dried). Dried shell thickness was measured to the nearest µm with a specimeter (Mitutoya 0-1mm).

Hatching success. The hatch was considered as successful when the chick was completely cleared from its shell and alive. 52 eggs successfully hatched out of 83.

Nestlings' growth measurement. Each one-day-old hatchling was weighed and measured (3 measures of tarsus and wing length were taken, and then averaged; n=52). Weight, tarsus and wing measures were reiterated every day until 25 days old (note that nestlings' body mass slow down at 16 days old on average; Johnston & Janiga 1995). Growth (for body mass, tarsus and wing length) was calculated as $W = \frac{A}{1+\exp(-k(t-t_i))}$, where W=morphological measure (body mass, tarsus length or wing length), A=asymptote (final body mass, tarsus length or wing length at the end of growth), k=growth rate constant t=age and t_i =the inflexion point of the curve (Newbrey & Reed, 2009; Jacquin *et al.*, 2012). Therefore we characterized nestlings' growth by its growth rate (k) and its age of slowing growth (t_i). Only the growth of nestlings which successfully fledged was calculated (n=41). Three months after the birds stopped growing, their weight, tarsus length and wing length were measured to assess their scaled mass index (n=40).

Fledging success. Chick was considered as successfully fledged when it was found outside the parental nest and was able to fly and to feed by itself.

Juveniles' condition. At the end of the experiment, while the younger bird was 3 months old and the older was 6 months old, we measured juvenile haematocrit, corresponding to the erythrocyte volume fraction of a blood sample (n=40). It is expected to be an indicator of general health state (Cooper, 1975; Averbeck, 1992). In addition, the number of leukocytes per 10000 erythrocytes was counted from blood smear. Slides were fixed with methanol during 5 minutes and coloured with GIEMSA (diluted 1:20) during 45 minutes. We identified heterophils, eosinophils, lymphocytes and monocytes. Because glucocorticoid decreases the number of circulating lymphocytes while it stimulates the influx of heterophils from the bone marrow, leukocyte profiles are suitable for identifying some physiological stress (Davis *et al.*, 2008). Therefore, we calculated the heterophils/lymphocytes ratio. We also considered the total number of white blood cells (total number of leukocytes per 10000 erythrocytes) that is suggested to be an indication of birds' immunity (Davis *et al.*, 2008).

Laying date was measured to unsure that trace metals effects on reproductive parameters would not be due to cumulative effects but was not considered as a reproductive success measurement.

Metal quantitative analyses

In blood. 10 weeks after the start of the experiment, 50µl of blood were collected from the brachial vein of each 96 adult pigeons and were immediately frozen until analyzes. Prior to metal measurement, blood was defrosted and vortexed. Then, 200mg (±0.1 mg) were digested with 1ml HNO_3 solution (68%) during 24h at 80°C.

In feathers. 13 weeks after the start of the experiment, a secondary remige (the 5th) was removed a first time. Once the regrown feather finished its development and was devascularizated, it was plucked off and conserved in an individual plastic bag. Feathers were washed vigorously with 0.25M NaOH solution, rinsed energetically 3 times in ultrapure water (Milli-Q purified) to remove external contamination (Scheifler *et al.*, 2006; Frantz *et al.*, 2012), left 1h in ultrapure water and dried 12h at 80°C to dry mass. Barbs were removed from the rachis, weighed to the nearest 0.1 mg and digested following the method described above.

The product of digestion was transferred into plastic tubes and water was added to reach a final volume of 8ml; then, each sample was diluted by 2.5. Total lead and zinc concentrations were determined in all of the 96 feather samples and 48 blood samples (6 females and 6 males amongst each of the four treatments) by mass spectrometry (quadrupole ICP-MS, XSerie II) and optical emission spectrometry (ICP-OES, JY 2000) respectively.

Statistical analyses

To distinguish the respective effects of lead and/or zinc exposure treatments on the variables measured, we binary coded (absence/presence) the exposure to lead on one hand and the exposure to zinc on the other (table 1). First, we tested for the existence of correlations between the explanatory variables considered (lead exposure, zinc exposure, plumage melanin type and plumage eumelanin level among adults and among juveniles separately). Colinearity between eumelanin level and metal exposure among juveniles did not allow to include metal exposure and juvenile eumelanin level in the same model (see results). There was no significant relation between adult eumelanin level and metal exposure whatever the adult group (all adults or adults that bred, produced hatchlings or fledglings successfully).

For each dependent variable listed in table 1, we performed three successive models:

- in models 1, we tested ecotoxicological effects of lead exposure, zinc exposure and their interaction in all birds whatever their plumage melanin type (pheomelanic and eumelanic, thus not included in the model);
- in models 2, we tested the effects of melanin type (pheomelanic vs. eumelanic), lead and zinc exposures and their interactions in all birds; note that the interaction between zinc exposure and lead exposure was not tested because no pheomelanic birds have been exposed to both zinc and lead;
- in models 3, we tested the effects of plumage eumelanin level in interaction with lead and zinc exposure in eumelanic birds only (note that the number of pheomelanic pigeons was too low to test the effect of pheomelanin level).

First, we investigated metal concentrations in feathers using linear mixed models. Age (adult vs. juvenile) was added into the models when testing the effects of metals alone (model 1) and of melanin type (model 2). However, there were too few pheomelanic juveniles (N=4) to test the interaction between melanin type and age. When testing the effects of eumelanin level (model 3), colinearity issues (see above) did not allow us to test the effects of metal exposure and eumelanin level in the same model, so that we performed separate models for adults and juveniles. In juveniles, we first tested the effect of eumelanin level alone; when significant, we then tested the effect of lead exposure, zinc exposure and their interaction, and compared the two models using their AIC. Lead is undetectable in the feathers after one year of captivity in our study site (Chatelain *et al.*, 2014). Therefore, lead detected in the feathers of adult not experimentally exposed to this metal arose mostly from birds' original environment prior to our experiment, which could conceal the potential link between plumage colouration and lead concentrations in feathers. Thus, we performed additional models investigating lead concentrations in feathers according to plumage colouration in birds exposed to lead (*lead* and *lead+zinc* groups), both in adults and juveniles, which exhibited significantly higher lead feather concentrations (see results).

Second, we investigated metal concentrations in blood using linear mixed models.

Third, we performed linear mixed models with time (expressed as the number of weeks after the beginning of the experiment) and its interactions with the other variables listed above as fixed effects to explain adult scaled mass index variation along time; individual was added as a random effect.

Finally, we investigated the effects of metal exposure on reproductive parameters using linear mixed models (egg quality, nestling growth and scaled mass index at 3 days of age) or general linear mixed models for binomial distribution (breeding success, hatchling success, fledging success, juvenile total white blood cell and heterophils/lymphocytes ratio); mother identity was added as random effect. When testing the effect of plumage colouration (parental or juveniles), plumage colouration was included as melanin type (model 2) or eumelanin level (model 3) of both parents for egg quality and reproductive success (breeding, hatchling, fledging), or eumelanin level of juvenile (model 3) for juvenile growth, scaled mass index and physiological state (the effect of melanin type was not tested for these variables because pheomelanic juveniles were rare (4 birds out of 40). When testing the effects of juvenile eumelanin level (model 3) and to take colinearity issues into account (see above), we first tested the effect of eumelanin level alone; if significant, we then tested the effect of lead exposure, zinc exposure and their interaction, and compared the two models using the AIC (i.e. when performing linear mixed models). In all performed models, the aviary was added as random effect. There was no significant relationship between laying date and trace metal exposure, suggesting that even though cumulative effect might exist, it would not be significantly different between the treatments. Therefore, laying date was not added as random effect in the models.

Statistical analyses were performed using R software (version 3.0.2) and a type 3 approach was used to take the unbalance of the design into account. We did not correct p-values for multiple testing as suggested by Moran (2003), García (2004) and Nakagawa (2004).

RESULTS

Trace metal concentrations in feathers

Among all pigeons (pheomelanic and eumelanic), zinc concentrations in feathers were higher in birds exposed to zinc (*zinc* and *lead+zinc* groups; $F_{1,104}$=4.25, P=0.042; table 1) and in juveniles ($F_{1,104}$=6.64, P=0.011; 91.36ppm±1.68 and 99.27ppm±2.28 in adults and in juveniles respectively). Moreover, lead concentrations in feathers were higher in birds exposed to lead (*lead* and *lead+zinc* groups) than in the others (*zinc* and *control* groups; $F_{1,105}$=15.09, P<0.001; table 1).

Among all pigeons (pheomelanic or eumelanic), zinc concentrations in feathers were higher in eumelanic birds than in pheomelanic ones ($F_{1,104}$=25.64, P<0.001; 96.26 ppm±1.15 in eumelanic birds and 72.54 ppm±5.13 in pheomelanic birds). Melanin type was not retained in the final model fitted for lead concentrations in feathers (among all birds and among birds exposed to lead).

Among eumelanic adults, zinc concentrations in feathers increased with eumelanin level ($F_{1,63}$=11.21, P<0.001; Fig. 1). Among eumelanic adults exposed to lead (*lead* and *lead+zinc* groups), lead concentrations in feathers increased with plumage eumelanin level ($F_{1,33}$=5.12, P=0.030; Fig. 2). Among eumelanic juveniles, eumelanin level was not retained for the models fitted for zinc and for lead concentrations in feathers among all juveniles and juveniles exposed to lead.

Trace metal concentrations in blood

Among all adults (pheomelanic and eumelanic), lead concentrations in blood tended to depend on the interaction between zinc exposure and lead exposure ($F_{3,43}$=3.64, P=0.063; table 1): birds exposed to zinc only (*zinc* group) exhibited lower lead concentrations in blood than birds exposed to both lead and zinc (*lead+zinc* group; $F_{1,21}$=10.79, P=0.004), birds exposed to lead only (*lead* group; $F_{1,21}$=6.04, P=0.023) and controls (*control* group; $F_{1,21}$=4.18, P=0.054). None of the considered variables were retained in the models fitted for zinc concentrations in blood.

Scaled mass index variation

Among all adults (pheomelanic and eumelanic), scaled mass index depended on the interaction between time, zinc exposure and lead exposure ($F_{1,96}$=8.93, P=0.003): scaled mass index decreased along time in *lead* ($F_{1,24}$=52.39, P<0.001), *control* ($F_{1,24}$=28.11, P<0.001) and *lead+zinc* groups ($F_{1,24}$=57.92, P<0.001) while time was not retained in the final model fitted for scaled mass index in *zinc* group.

Among eumelanic adults, scaled mass index depended on the interaction between time, zinc exposure, lead exposure and eumelanin level ($F_{1,82}$=19.29, P<0.001; Fig. 3): scaled mass index decreased along time in *control* ($F_{1,20}$=35.64, P<0.001) and *lead+zinc* group ($F_{1,24}$=57.92, P<0.001). In *lead* group, scaled mass index depended on the interaction between time and eumelanin level ($F_{1,19}$=40.02, P<0.001), with scaled mass index decreasing along time among the darkest birds only ($F_{1,9}$=87.10, P<0.001). Neither time nor eumelanic level was retained in the final model fitted for scaled mass index among *zinc* group.

Melanin type was not retained in the model fitted for scaled mass index.

Reproductive success

None of the variables considered was retained in the final model fitted for birds' breeding success.

Among all parents (pheomelanic and eumelanic), metal exposure was not retained in the models fitted for egg weight and volume, albumen or eggshell weight. However, yolk was heavier in eggs from parents exposed to zinc (*zinc* and *lead+zinc* groups) than from the others (*control* and *lead* groups; $F_{1,53}$=7.36, P=0.007; mean±se 4.22g±0.08 and 3.89g±0.09 respectively; table 1). Moreover, eggshell was thicker in eggs from parents exposed to zinc (*zinc* and *lead+zinc* groups; $F_{1,62}$=5.18, P=0.023; mean±se 0.49mm±0.01 and 0.47mm±0.00 respectively; table 1) while it was thinner in eggs from parents exposed to lead (*lead* and *lead+zinc* groups; $F_{1,62}$=8.24, P=0.004; mean±se 0.47mm±0.00 and 0.49mm±0.01 respectively; table 1).

None of the variables considered was retained in the model fitted for hatching success.

Among all parents, nestlings of parents exposed to lead (*lead* and *lead+zinc* groups) were significantly lighter than the other ones (*control* and *zinc* groups; $F_{1,52}$=4.17, P=0.041; mean±se 14.94g±0.72 and 17.20g±0.67 respectively; table 1). None of the variables considered was retained in the models fitted for tarsus and wing length of one-day-old chick. With regard to nestling growth, none of the variables considered was retained in the models fitted for weight, tarsus and wing growth rate. However, the age at which weight and tarsus growth slowed down depended on the interaction between lead and zinc exposure ($F_{1,24}$=5.53, P=0.019; and $F_{1,41}$=9.66, P=0.002; Fig. 4). Indeed, growth slowed down earlier in juveniles of parents exposed to lead only (*lead* group) than in juveniles of parents exposed to both lead and zinc (*lead+zinc* group; $F_{1,24}$=5.53, P=0.019 and $F_{1,24}$=6.01, P=0.014 respectively) and of controls parents (*control* group; $F_{1,25}$=11.46, P=0.002 and $F_{1,25}$=19.52, P<0.001 respectively). Zinc and lead exposure was not retained in the models fitted for the age at which wing growth slowed down, and melanin type and eumelanin level were not retained in any of the models fitted for nestlings' growth.

In all parents, fledging success was lower in juveniles from pheomelanic fathers ($F_{1,59}$=8.13, P=0.004) and tended to be also lower among birds exposed to lead (*lead* and *lead+zinc* groups; $F_{1,59}$=3.62, P=0.057). Eumelanin level was not retained in the model fitted for fledging success.

In all juveniles, scaled mass index was smaller in lead groups (*lead* and *lead+zinc* groups) than in the other groups (*control* and *zinc* groups; $F_{1,40}$=6.43, P=0.011; mean±se 293.91±19.11 and 349.73±20.02 respectively; table 1). Moreover, the number of white blood cells was higher in zinc groups (*zinc* and *lead+zinc* groups) than in the other groups ($F_{1,40}$=5.14, P=0.023; 6.0%±0.5 and 4.8%±0.3 respectively; table 1). The number of heterophils among the number of lymphocytes depended on the interaction between lead and zinc exposure ($F_{1,37}$=5.79, P=0.016, P=0.016; table 1): it was higher in *lead* group than in *control* group ($F_{1,20}$=4.65, P=0.031) and *lead+zinc* group ($F_{1,19}$=4.01, P=0.046). Among eumelanic juveniles, eumelanin level was not retained in the model fitted for scaled mass index, the number of white blood cells and for the heterophils/lymphocytes ratio.

Finally, the plumage eumelanin level of surviving juveniles significantly depended on lead-exposure ($F_{3,30}$=6.69, P=0.015; Fig. 5), with eumelanin level being higher among juveniles exposed to lead only (*lead* group) than among juveniles from other groups (*zinc*, *control*,and *lead+zinc* groups).

DISCUSSION

To investigate whether and how trace metals may affect pigeons and may select for darker pigeons in urban areas, we tested whether 1) trace metals have ecotoxicological effects, 2) darker individuals store higher amounts of metals into their feathers, 3) darker individuals maintain lower metal levels in their blood and 4) darker individuals are more tolerant to the exposure to toxic metals. To this aim, we used an experimental approach; zinc and lead supplementations successfully increased zinc and lead concentrations in feathers respectively, while exposure to a given metal did not raise its concentration in the blood (table 1). Overall, metal concentrations in blood may not always reliably estimate recent exposure as they can result from numerous mechanisms (lead clearance from bones, lead and zinc transfer into organs, bones and feathers (Cosson, 1989; Gulson *et al.*, 1996; Kim *et al.*, 1998; Agusa *et al.*, 2005)).

First, our study consistently demonstrated detrimental effects of lead and beneficial effects of zinc on some of bird condition and reproductive success parameters (table 2). The scaled mass index decreased over the 20-week-long experiment in birds exposed to lead (*lead* and *lead+zinc* groups) and in *control* group, while remaining constant in *zinc* group. Eggshell was thinner in eggs from lead-exposed parents. This negative effect, previously observed in pied flycatchers (Eeva & Lehikoinen, 1995) and likely due to limitation of calcium deposition

(Clunies *et al.*, 1992; Simons, 1993), may impair eggshell permeability and resistance to impacts (King & Robinson, 1972). On the contrary, zinc increased eggshell thickness. Zinc also increased yolk mass, potentially elevating egg nutritive content (Noy & Sklan, 1998). Accordingly, previous work reported a positive link between plasma zinc and vitellogenin production (Mitchell & Carlisle, 1991). Since zinc exposure did not influence egg size nor one-day-old chick size, this extra yolk mass may have been allocated to other physiological traits, such as immunity (see Li *et al.*, 1998). Accordingly, juveniles exposed to zinc (*zinc* and *lead+zinc* groups) had a higher amount of white blood cells, an index of the immune system (Davis *et al.*, 2008), than the other groups (*lead* and *control* groups). In addition, lead-exposure induced lighter one-day-old chicks. Because lead did not affect egg total, yolk nor albumen mass, it may not alter maternal investment in eggs but may be maternally transferred into the eggs and affect embryonic development (Burger, 2002). Juveniles exposed to lead only (*lead* group) tended to have a lower fledging success, possibly a consequence of a poorer condition at hatching (Grant, 2008). Among fledglings, lead exposure also induced a shorter growth period of body mass and tarsus and a smaller scaled mass index at three months of age, which could impair their future survival or reproduction ("catch-up" hypothesis, see Criscuolo *et al.*, 2008). Consistently, juveniles exposed to lead only (*lead* group) had a higher number of heterophils/lymphocytes ratio, suggesting a higher stress hormone level (Davis *et al.*, 2008). Finally, zinc-exposure had protective effects against lead: when provided along with zinc, lead did not induce negative effects on several of the measured traits (table 1, Fig. 4). This protective effect may result from zinc ability to reduce the absorption and retention of ingested lead (Cerklewski & Forbes, 1976; El-Gazzar *et al.*, 1978), as suggested by lower lead concentrations in blood in birds exposed to zinc only (*zinc* group) than in the other birds (*contro*l, *lead zinc+lead* groups). The effects of trace metals observed on juveniles may be due to either direct effects on juveniles or to indirect effects through parental investment that further experimental studies should disentangle.

Second, we predicted a positive relationship between plumage darkness and metal concentrations in feathers. Our prediction was validated for both zinc, confirming previous work (Chatelain *et al.*, 2014) and lead (table 2). Our results suggest that more melanic feathers would be able to store higher amounts of both zinc and lead. Although washed feathers may still have some amounts of trace metals on their surface, such residual concentrations likely do not correlate with plumage eumelanin level. Therefore, melanin contained in the plumage could play a significant role in metal detoxification. Interestingly, zinc concentrations in feathers were higher in eumelanic pigeons than in pheomelanic ones; such differences could be due to differences in chemical composition between melanin types (e.g. more carboxylic acid groups in eumelanin; Hong & Simon, 2007), to different physiological requirements of zinc (e.g. oxidative stress; Prasad, 2009) or to differences in immune responses (Chatelain et al. unpublished data).

Third, we did not find the negative correlation between plumage eumelanin level and metal concentrations in blood expected from our detoxification hypothesis (table 2). Metal concentration in blood does not reliably reflect recent exposure as it results from numerous mechanisms (Cosson, 1989; Gulson *et al.*, 1996; Kim *et al.*, 1998; Agusa *et al.*, 2005) that may hide detoxification process. Future work should investigate whether such potential detoxification through melanin lowers metal burden in organs. Although feral pigeons moult all over the year (Johnston & Janiga, 1995), metal detoxification may be more efficient when moulting is most intense (i.e. fall) and during juvenile growth (i.e. synchronized growth of all feathers).

Fourth, we did find a statistical interaction between plumage eumelanin level and metal exposure on scaled mass index (table 2). In birds exposed to lead only (*lead* group), paler birds maintained their initial condition over the course of the experiment, while darker birds lost weight. Note however that darker birds had an initial lower scaled mass index than the paler ones, so that we cannot distinguish whether this result was due to the effect of initial scaled mass index or to eumelanin level. In the latter case, this result may suggest a disadvantage of a more melanic plumage in environments polluted with lead; alternatively, it may also be the result of a trade-off between condition maintenance and other biological traits, such as parental investment, which would be in line with the higher survival rate of darker juveniles among birds exposed to lead only (*lead* group). Indeed, plumage eumelanin level of three-month-old juveniles was higher in *lead* group than in the other groups (table 2). This result was not due to different reproductive success (breeding, hatching and fledging success) between differently coloured adults. In addition, because melanin-based plumage colouration is highly heritable in feral pigeons (0.82±0.12; Jacquin *et al.*, 2013b), it is unlikely that a direct effect of lead on melanogenesis explains the higher plumage darkness of juveniles under lead-exposure. More likely, this result may reflect higher survival rate of darker juveniles when exposed to lead only as compared to paler ones. Indeed, fledging success tended to be lower under lead-exposure, which could be due to higher mortality in paler offspring. This hypothesis is in accordance with the higher survival rate of darker pigeon juveniles in a Parisian suburban environment (Récapet *et al.*, 2013) and with the higher frequency of darker pigeons observed in European cities (Obukhova, 2007).

In conclusion, we found several lines of evidence supporting the possibility that trace metals exert selective pressures on melanin-based plumage colouration (table 2). Indeed, darker birds stored higher amounts of zinc and lead in their feathers and likely had a higher juvenile survival under lead-exposure. However, scaled mass index of darker birds decreased over time when exposed to lead only. Moreover, bird sensitivity to trace metals did not depend on their plumage colouration for several traits. The lack of results may originate from the low beneficial effect of plumage melanin under metal exposure, from the experimental exposure to metal concentrations underestimating the natural range (zinc and lead in the feathers were respectively 80 and 1.5 times less concentrated than measured in wild feral pigeons (Nam *et al.*, 2004; Adout *et al.*, 2007; Brait & Filho, 2011; Frantz *et al.*, 2012; Chatelain *et al.*, 2014)), from high inter-individual variation (i.e. in trace metal exposure prior to the experiment) reducing the probability to detect some effects, or from ongoing selection ultimately favouring dark plumage along with a series of other traits. More experimental studies involving exposure to a cocktail of metals in their urban range of concentrations are needed to better understand how this new selective pressure may favour particular phenotypes, especially melanin-based colouration.

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