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Abstract

Bacteria are fundamental associates of animals, and recent studies have highlighted their major role in host behaviour, immunity or reproductive investment. Thus, any environmental factor modifying bacterial community may affect host fitness. In birds, trace metals emitted by anthropogenic activities accumulate onto the plumage where they may alter bacterial community and ultimately affect bird fitness. Although trace metals are current major environmental issues in urban habitats, their effects on feather bacterial community have never been investigated. Here, we supplemented feral pigeons (*Columba livia*), an emblematic urban species, with zinc and/or lead in drinking and bath water. As expected, lead and zinc supplementations modified plumage bacterial communitycomposition. Zinc decreased bacterial load, while lead decreased bacterial richness andthe frequency of preening behaviour in birds, known to regulate feather bacteria. Our results demonstrate for the first time the effects of common urban trace metals on plumage bacterial community and shed light on one of the mechanisms by which trace metals can affect bird fitness. Further studies are now needed to investigate how this effectmodulates avianlife history traits known to depend on plumage bacterial community.

Key words:urban ecology,birds, bacteria

INTRODUCTION

Bacteria successfully colonize numerous and various habitats, including animal body parts that are in direct contact with the surroundings such as skin, feathers or fur, and the digestive tract. The normal flora, also called the microbiota, lives in symbiosis with its host, fulfilling essential functions for host metabolism, such as cellulose degradation, vitamins synthesis(Hill 1997)and inhibition of pathogens proliferation (Olsson et al. 1992, Oh et al. 2006, Balcázar et al. 2007).Recent studies have also revealed that bacteria may have surprising effects such as shaping host behaviour and investment in reproduction (Ezenwa et al. 2012, Jacob et al. 2015). However, we have just made the first steps in discovering the diversity and functions of these bacterial ecosystems, and it is now necessary to evaluate the factors that regulate microbiota.

The microbiota is influenced by numerous interconnected factors, including host behaviour,genotype and physiology(Mueller et al. 2006, Frank et al. 2011, Rosenthal et al. 2011, Hildebrand et al. 2013, Leclaire et al. 2014a),as well as its environment(Dotterud et al. 2008, Burkholder et al. 2008, Ruiz-de-Castañeda et al. 2011).Trace metals emitted by anthropogenic activities have well-known detrimental effects on animals (Jarup 2003, Hsu et al. 2006, Berglund et al. 2007, Eeva et al. 2009) andare thus of major environmental concernsin most urban environments(Azimi et al. 2005, Scheifler et al. 2006, Roux and Marra 2007, Kekkonen et al. 2012). Some trace metals,such as lead, cadmium, zinc, copper, chrome and nickel, are known to have toxic effects onenvironmental microbial communities(Babich and Stotzky 1978, Giller et al. 1998). In contrast, although tracemetals can be naturally ingested or inhaled by animals, and deposited on integuments, their effects on animal microbiota have been poorly investigated (but seeHojberg et al. 2005, Vahjen et al. 2010, Breton et al. 2013, Liu et al. 2014).

Plumage is a key point of interaction between birds and the microbial world, and, like other integuments, is highly colonized by bacteria(Burtt Jr and Ichida 1999, Muza et al. 2000, Shawkey and Hill 2004, Whitaker et al. 2005). Although several studies have shown that keratinolytic bacteria can alter feather structure *in vitro*(Burtt Jr and Ichida 1999, Shawkey et al. 2007), the *in vivo* effects of feather bacteria on their host have been scarcely studied.A few correlative and experimental studies in captive and free-living birds have shown, however, that feather bacteria mayinfluence sexual signalization such as feather colouration or plumage condition (Shawkey et al. 2007, Gunderson et al. 2009, Kilgas et al. 2012, Leclaire et al. 2014b; but see Jacob et al. 2014), and affect bird immune system (Leclaire et al. 2015) and investment in reproduction (Jacob et al. 2015). Any change in feather microbiota is thus likely to have a strong impact on bird fitness. The plumage and the uropygial gland secretions spread onto it can accumulatemetals(Pilastro et al. 1993, Frantz et al. 2012), which can affectplumage bacterial community by direct contact. Ingested metalsalso circulate in the bloodstream and accumulate in organs and bones (Pattee 1984, Dauwe et al. 2002, Scheifler et al. 2006, Kekkonen et al. 2012, Reid et al. 2012), and may induce noxious (Redig et al. 1991, Snoeijs et al. 2004, Dauwe et al. 2005, Eeva et al. 2009)or beneficial (Mertz 1981, Prasad 1998)effects on bird physiology;therefore, metals can affect bird ability to regulate their microbiota(Piault et al. 2008, Moreno-Rueda 2010, Leclaire et al. 2014b). Whatever the exact underlying mechanisms, further studies are clearly needed to evaluate the effects of trace metals on feather bacteria.

The feral pigeon (*Columba livia*) is an emblematic urban bird living in high density populations,with potentially elevated plumage bacteria transmission between individuals (reviewed in Archie and Theis 2011). Mate choice is crucial for this species which mate for

life (Johnston and Janiga 1995). In this species, bacterial load affects immunity(Leclaire et al. 2015), as well as feather condition and colouration(Leclaire et al. 2014b)which are two ofthe main criteria used in mate choice (Johnston and Janiga 1995). Consequently, trace metals, besides their direct effects on bird physiology, may further impact pigeon reproduction and fitness through their effects on plumage bacterial load and composition. Here we investigated the effects of an experimental exposure to lead and/or zinc on two traits that can affect each other: plumage bacteria composition and frequency of preening in the feral pigeon. Preening is a costly antimicrobial behaviour that can be directly influenced by feather bacterial load (Leclaire et al. 2014b)and bird health status (Piault et al. 2008, Moreno-Rueda 2010), which are both potentially affected by trace metals.

METHODS

Subjects and housing

Ninety six(48 males and 48 females)free-living adult feral pigeons (*Columba livia*) were caught during winter 2013 (February/March) in several pigeons' flocks within the Parisian agglomeration. Birds were all considered as adults because of their well-formed caruncle, the absence of juvenile plumage and the presence of iridescent neck feathers (Johnston and Janiga 1995). Pigeons werekeptin 8 outdooraviaries(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). They were evenly distributed among aviaries according to their flock, sexand plumage eumelanin level(see below) in such a way that there was no confounding effect between aviaries and these variables (i.e. no statistically significant link between aviary and flock: $Chi^2=71.09$, df=70, P=0.441; sex: 6

males and 6 females per aviary; or plumage eumelanin level: $F_{1,80}$=0.38, P=0.537). Birds were fed *ad libitum* with a mix of maize, wheat and peas and water was provided in a trough.The aviaries were enriched with a bowlofwater used forbathing and with branchesasperches. Birds were individually identified with a numbered plastic ring.Birds were genetically sexed following the protocol described byGriffiths et al. (1998). Before onset of treatment, birds were kept 2 to 7 weeks for acclimation. Alongside this experiment, we measured the effects of treatments (see below) on bird body mass condition, reproduction and immunity. At the end of the breeding season, 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'éducationnationale, de l'enseignementsupérieur et de la recherche" (authorization N_00093.02) and the "Direction Départementale des Services Vétérinaires de Seine et-Marne" (authorization N_ 77-05).

Measurement of plumage colouration

At capture, birds were first categorised as eumelanic (grey to black pigmented) or pheomelanic (red pigmented) which define their melanin type. Then, eumelanic birds were individually photographed in order to measure their eumelanin level. Eumelanin level was calculated as the percentage of black on the wing surface (number of black pixels/number of white pixels x 100) using the Gimp image retouching and editing software. This measure has been shown to be a reliable and repeatable estimation of melanin concentration in pigeons (Jacquin et al. 2011). In eumelanic pigeons, plumage eumelanin level ranged from 4.2 to 95.9%. Because of the small amount of pheomelanic birds (14 over 96) the measure of a pheomelanin level was not relevant.

Treatments

Two weeks before the onset of treatment, aviaries were divided into 4 metal-exposure treatments; this means that there were 2 aviaries per treatment with 12 pigeons each (24 pigeons in total per treatment). For each treatment, the two aviaries were purposely spatially separated from one another.Aviaries were in direct contact along a linear transect and numbered from 1 to 8 (*lead* (1), *zinc* (2), *control* (3), *lead+zinc* (4), *lead* (5), *control* (6), *zinc*(7), *lead+zinc* (8)). Side-by-side aviaries were separated by wire mesh.Treatments consisted of water supplemented with lead (*lead* group; 1ppm lead acetate; aviaries 1 and 5), zinc (*zinc* group; 10 ppm zinc sulphate; aviaries 2 and 7), lead and zinc (*lead+zinc* group; 1ppm lead acetate and 10ppm zinc sulphate; aviaries 4 and 8), or control(*control group;* tap water with no metal added; aviaries 3 and 6). We chose these concentrations based on both lead blood concentrations measured in urban birds (ranging from 0,053 to 0,264ppm; Roux and Marra 2007) and the gastrointestinal absorption rate of lead in zebra finches (<10%)calculated from (Dauwe et al. 2002). Zinc concentrations were approximated using the zinc/lead concentration ratio in the environment and in bird feathers (on average, zinc was 10 times more concentrated than lead;Azimi et al. 2005, Frantz et al. 2012, Chatelain et al. 2014).Drinking troughs and baths were filled with the corresponding treated water every other day. Our supplementation treatments were validated by measuring lead and zinc concentrations in blood and feathers of the birds. Blood was sampled 10 weeks after the start of the experiment. Moreover, the fifth secondary remige of each bird was removed a first time and the regrowth feather was used for metal measurements. Both blood and feathers were digested using a previouslydescribed protocol (Chatelain et al. 2014) and lead and zinc concentrations were measured by mass spectrometry (ICP-MS) and by optic emission spectrometry (ICP-OES) respectively. Validity of analytical methods was checked by means

of a standard biological reference material (TMDA-64.2. Environment Canada). Lead and zinc concentrations in blood were the highest among birds exposed to lead *(lead* and *lead+zinc* groups) and birds exposed to zinc (*zinc* and *lead+zinc*groups) respectively (Table 1). These results ensured that metals added to water were ingested by the birds. In feathers, while lead concentrations were significantly the highest among birds exposed to lead *(lead* and *lead+zinc* groups), the increase in zinc concentration among birds exposed to zinc (*zinc* and *lead+zinc* groups) was not significant (Table 1).Zinc and lead measured in the feathers were respectively 80 and 1.5 times less concentrated than the ones measured in feathers of urban pigeons (Nam et al. 2004, Adout et al. 2007, Hoff Brait and AntoniosiFilho 2011, Frantz et al. 2012, Chatelain et al. 2014), suggesting that our experimental exposure corresponded to the lower range of urban exposure.

Measurement of plumage bacterial load

Plumage bacterial load was measured 20 weeks after onset of treatment.4 hours after renewing the water of the bowls used for bathing, 10 birds (5 males and 5 females) were randomly sampled in each treatment. Each sampled birdwas caught with a net that hadbeen previously sprayed with 70% ethanol. Then, a whole flora agar slide (Hygialim, 3026091, Plate Count Agar +triphenyltetrazoliumchloride+Neutralizing)was put flat against the back of the bird for 10 seconds. The slides were then incubated at 37°C for 24h.Feather bacterial load was expressed as the number of bacterial colonies per slide.

Molecular analysis of plumage bacterial communities

Fifteen weeks after the start of the experiment, 91 adults were caught with a net previously sprayed with 70% ethanol (n.b. 5 pigeons died for unknown reasonsbefore this measure, 2 from the *zinc* group, 2 from the *lead+zinc* group and 1 from the *control* group). After washing

her hands with alcohol, the experimenter cut a clump (10 feathers on average) of back feathers with sterilized scissors and pliers, avoiding the outermost feathers. The feathers were immediately placed in sterile 2ml plastic tubes and stored at -20°C until analysis.

We extracted DNA using the QiagenDNeasy® Blood and Tissue Kit and the standard protocol designed for the purification of total DNA from Gram-positive bacteria (Qiagen, Venlo, Netherlands; July 2006).

To characterize the bacterial communities present in each sample,we performed automated ribosomal intergenic spacer analyses(ARISA; Ranjard et al. 2000). This DNA fingerprinting method isbased on the amplification of the internal transcribed spacer (ITS)region lying between the 16S and 23S ribosomal RNA genes in theribosomal operon. The ITS region is extremely variable, in bothsequence and length, for different bacterial species. Therefore, theDNA amplification profile obtained with ARISA allows straightforwardestimation of bacterial diversity, avoiding biases inherent inclassical culture-based techniques (Ranjard et al. 2000).We amplified the ITS using the FAM (6-carboxyfluorescein)-labeled primer S-D-Bact-1522-b-S-20 (5′-[6FAM] TGCGGCTGGATCCCCTCCTT-3′) and the unlabeled primer L-D-Bact-132-a-A-18 (5′-CCGGGTTTCCCCATTCGG-3′)(Ranjard et al. 2000). We performed the PCR amplification in 10 μL mixturescontaining 200 μM each deoxynucleotide triphosphate, 0.20 μM each primer, 1.25 units of Perfect*Taq* DNA polymerase, 1× PCR buffer(5 Prime, GmbH, Hamburg, Germany), and 1μL DNA extract, using the followingprotocol: initial denaturation at 94 °C for 3 min, 40 cyclesconsisting of denaturation at 94 °C for 30 s, annealing at 55 °Cfor 45 s, elongation at 72 °C for 1 min, and a final elongation at72 °C for 10 min.We then mixed 1 μL of the PCR products with 15μL of highly deionized formamide and 0.2μL of Genescan 1200 LIZ size standard (Applied Biosystems, Foster City, CA). The mixtures were separated with a 24-capillary 3500XL DNA Analyzer (Applied Biosystems) using POP-7 polymer and the manufacturer's default electrophoresis

run settings. Data analysis and genotyping were performed with GeneMapper software (Applied Biosystems). For each sample, the sequencer produced an ARISA profile in which each peak corresponds to 1 phylotype or operational taxonomic unit (OTU). In the various samples, the sequencer detected ITS fragments ranging in size from 300 to 950base pairs.

For each individual, we calculated bacterial richness as the number of different OTUs. Because the probability to detect an OTU is likely affected by the amount of feathers used for DNA extraction, feathers were dried overnight and weighted to the nearest mg after DNA extraction. We estimated bacterial community dissimilarities between individuals using Jaccard distance based on presence/absence of OTUs.

Observations of preening behaviour

An observer recorded a total of 95 independent behaviouralsessions of 5 minutes each. The observer remainedoutside the aviary and waited a few minutes before starting her observations not to influence bird behaviour.The observed birds were chosen randomly but the observer switchedto a newtreatment for each new session to have a similar number of observations forall treatments: 25 observations (corresponding to 21 different individuals) among the *control* group, 29 observations (24 individuals)among the *lead* group, 22 observations (20 individuals) among the *zinc* group and 19 observations (16 individuals) alongthe *lead+zinc* group. The behaviour recording was performed with the JWatcher software. We recorded the time birds spent preening;during this behaviour, the plumagemay be spread with preen secretions(Mardon et al. 2011), which have antimicrobial and antiparasite properties (e.g. in feral pigeons, house sparrows, eastern bluebirds and hoopoes; Moyer et al. 2003, Shawkey et al. 2007, Ruiz-Rodriguez et al. 2009, Waite et al. 2012, Czirják et al. 2013). Because preening is costly (Piault et al. 2008, Moreno-Rueda 2010), the time allocated to preening has been shown to beadjusted to bacterial load (Leclaire et al.

2014b),and to reflect birds'health status. For instance, juvenile apapanes (*Himationesanguinea*) experimentally infected with *Plasmodium relictum* spent less time preening (Yorinks and Atkinson 2000).

Statistical analyses

Statistical analyseswere performedusingR (R.3.0.2; R Development Core Team). Final models were retained based on their AIC.

To test the effects of metal exposure on the composition of bacterial communities, we performed a PERMANOVA with 5000 permutations (i.e. nonparametric multivariate analysis of variance, Adonis function, VEGAN package in R(Oksanen et al. 2007), based on Jaccard distance for OTU presence/absence data. Zinc exposure, lead exposure, sex and their interactions were included as explanatory variables. Because spatial proximity may influence bacterial communitysimilarity between individuals, we added the aviary as a covariate. Then, we ran similar analyses between each pair of treatments.Finally, we tested the differences of bacterial communities between aviaries among each metal treatment.

To investigate more precisely the effect of spatial proximity on bacterial communities' similarities, we compared a matrix of bacterial Jaccard distances between individuals to a matrix of spatial distances (scored as 0 for individuals inhabiting the same aviary, to 7 for the most distant individuals), considering a matrix of treatment membership (scored as 0 for individuals submitted to the treatment and 1 for individuals of different treatments) using partial mantel test with 5000 permutations.

We graphically represented similarities between individuals using a constrained redundancy analysis (RDA function in R) based on the Jaccard distance matrix.

We also tested plumage bacterial richness using a generalized linear model for Poisson distribution with zinc exposure, lead exposure, sex and their interactions as explanatory variables, the weight of feathers used for the analysis as a covariate and aviary as random effect.

To test the effects of metal exposure on plumage bacterial load, we performed Wilcoxon tests because our sample size was low (n=10 per treatment). First, we tested the effects of zinc and theeffects of lead in two different tests;then, we performed Wilcoxon tests between each pair of treatments to test the effects of the interaction between zinc and lead exposure.

Finally, we investigated the amount of time birds allocated to preening by performing a generalized linear mixed model for Poisson distribution with zinc exposure, lead exposure, sex and their interactions as explicative variables and bird identity and aviary as random effects.

All the previously described models were performed on the totality of the birds, whatever their plumage colouration (i.e. eumelanic and pheomelanic birds). The same models were performed on eumelanic birds only (i.e. excluding pheomelanic pigeons). In these models, plumage eumelanin level and its interaction with the other considered parameters were added as explanatory variables.

RESULTS

The composition of bacterial communities depended on the interaction between lead and zinc exposure($F_{1,82}$=3.47, P<0.001; Fig. 1) and on aviaries($F_{1,82}$=3.91, P<0.001).Each pairwise test between metal exposureswas significant (Table 2). The composition of bacterial communities differed significantly between aviaries among each metal treatment but was less dissimilar among the*lead* group(zinc-exposure: $F_{1,19}$=3.48, P<0.001, lead-exposure: $F_{1,21}$=1.70, P=0.018, zinc and lead-exposure: $F_{1,19}$=4.48, P<0.001, control: $F_{1,20}$=7.59, P<0.001). Moreover, there was a highly significant positive correlation between bacterial distance and spatial distance (r=0.30, P<0.001; Fig. 2).

Plumage bacterial richness depended on the interaction between zinc and lead-exposure (Chi^2=9.09, df=80, P=0.003; Fig. 3): the*lead* group had lower bacterial richness than the*control* group (Chi^2=5.58, df=42, P=0.018) and the*zinc*+*lead* group (Chi^2=12.63, df=41, P<0.001). Moreover, the*lead*+*zinc* group tended to have higher bacterial richness than the*zinc* group (Chi^2=3.63, df=40, P=0.057).

Plumage bacterial load was significantly lower amongbirds exposed to zinc (*zinc* and *lead*+*zinc* groups)than amongthe others (*lead* and *control* groups;W=280.5, P=0.029; Fig.4). Although there was no significant difference between each pair of treatments (P>0.067), the*zinc* grouptended to have lower plumage bacterial loads than the*control* group (W=29, P=0.072) and the*lead* group(W=25, P=0.067). Lead didnot significantly affect plumage bacterial load (W=181, P=0.623).

Finally, the time birds spent preening depended on the interaction between zinc and leadexposure (Chi^2=3.97, df=92, P=0.04). We performed partial models to compare each pair of treatments. Although there was no significant difference between each pair of treatments

(P>0.101), our results suggest that time spent preening was shorter among the*lead* group than among the other groups (*control*: 65.00±15.18s, *lead*: 34.21±9.69s, *zinc*: 53.86±13.79s, *lead*+*zinc*: 60.84±15.30s).

Sex and plumage eumelanin level were retained in none of the tested models.

DISCUSSION

As expected,the composition of plumage bacterial communityvaried with metal exposure. The exposure to lead alone appears to induce the strongest effect. Plumage bacterial composition was more similar amongst the two aviaries hosting birds exposed to lead only than expected if considering a spatial effect only. In addition, the plumage bacterial compositionsof birds exposed to lead only were the most distant from the communities of the other treatments.Moreover, birds of the *lead* grouphad reduced plumage bacterial richness compared to birds of the *control*group. These results suggest that lead may select for lead-tolerant plumage bacteria. To the best of our knowledge, our study is the first to showthat lead exposure has effects on plumage bacterial community. It is consistent with a previous study showing that lead alters the intestinal microbiome of mice (Breton et al. 2013).Moreover, birds of the *lead* grouptended to preen less frequently than birds of the *control* group. Lead exposure decreases bird immunity (unpublished results) and reproductive success (but there is no effect of metal exposure on bird breeding success;Chatelain et al. in press). Lead, by decreasing bird condition,may affectbird ability to preen, a costly behaviour that helps to regulate feather microbiota(Piault et al. 2008, Moreno-Rueda 2010, Leclaire et al. 2014b). Because preening is adjusted to feather bacterial load(Leclaire et al. 2014b), the change in

preening frequency observed in our study may also non-exclusively result from the change in feather bacterial community caused by trace metal exposure. Whatever the mechanism underlyingthe differences in preening frequency,the tendency of lead to reduce bird control on its plumage bacterial communitymay change the dominant status of bacteria species and therefore induce the proliferation of species that were previously sensitive to preen secretions. High-throughput DNA sequencing would help identifying lead-tolerant bacteria species and therefore inferring their potential pathogenicity and propensity to degrade feathers. More analyses should also be conducted to identify the proximal mechanisms involved in lead toxicity. For instance, *in vitro* exposure of feathers to these metals would allow us to disentanglethe direct and indirect effects that may induce these metals.

Like lead exposure, zinc exposure had toxic effects on the plumage bacterial community with birds exposed to zinc exhibiting lower bacterial load than control birds. Similarly, high doses of zinc decrease bacterial load and change bacterial community in the gastrointestinal tract of piglets (Hojberg et al. 2005, Vahjen et al. 2010), and inhibit bacterial growth in sludge and sediment (Cabrero et al. 1998, Vega-López et al. 2007).Zinc is known to be essential to several metabolic functions of bacteria (Sugarman 1983). At high concentrations, zinc can however reduce protein and ATP content, interact with nucleic acids and enzyme active sites, decrease membrane health and eventually lead to cell necrosis (Martinez-Tabche and Gutierr 2000, Vega-López et al. 2007). Although the concentration of zinc we used is within the natural range found in cities, it maybe high enough to negatively affect feather bacteria and to decreasebacterial load. Zinc may also affect feather bacterial loadindirectly through its immunostimulating effect (Smith 2003). In feral pigeons, zinc has a positive effect on the production of specific antibodies (unpublished data), and might,therefore, increase the bactericidal capacity of uropygial secretions.

Bacterial community composition, bacterial richness and the time birds spent preening depended on the interaction between lead and zinc exposure. More precisely, the toxic effects of lead exposure was not detectedin birds exposed to both lead and zinc, suggesting that zinc may compensate lead toxicity. Zinc is known to reduce the absorption and retention of ingested lead (Cerklewski and Forbes 1976, El-Gazzar et al. 1978, Prasanthi et al. 2010), which may therefore reduce the negative effects of lead on bird condition. In addition, the negative effect of zinc exposure on bacterial load would be higher in birds exposed to zinc only than in birds exposed to both lead and zinc, suggesting again an interaction between lead and zinc exposure.

Our results showed a strong effect of spatial proximity on bird plumage bacterial community, with birds in closer aviaries showing more similar bacterial communities. In accordance with other studies (Bisson et al. 2007, 2009, Saag et al. 2011), they point out the relatively small spatial scale transmission of plumage bacteria. While bacteria are likely transmitted through close contacts(Kulkarni and Heeb 2007) and reciprocal delousing, some bacteria may be able to survive on non-feather substrates and, therefore, be transmitted through bathwater, perches, soil and the grids separating the aviaries(Bisson et al. 2007). Because pigeons live in high density but have limited movements within their local environment (Frantz et al. 2012), the plumage bacterial communityof wild pigeons may, therefore,greatly vary between populations, which may lead to local coevolution and co-adaptation between the host and its bacterial community.The strong effect of spatial proximity on plumage bacterial community detected in our study may have decreased and increased bacterial community similarities between and within aviaries respectively; ideally, future studies should increase the number of replicates (aviaries) or house pigeons in individual and spatially distant cages.

Our experimental exposure of feral pigeons to naturallyoccurring concentrations of lead and/or zinc highlights, for the first time, the effects of some trace metals commonly encountered in urban areas on plumage bacterial community.The birds used in our study were captured in Paris, andhad therefore been previously exposed to trace metals in their natural urban habitat. Consequently, plumage bacterial communities at the start of the experiment might havealready been shaped by pastmetal exposure. Because there was no significant correlation between bird capture site (i.e. pigeon flock) and aviary, the potential initial differences in bacterial community between the birds would, however, have reduced the power of our analysis, and the significant differences between treatments observed in our study are therefore conservative.

Although our knowledge on plumage bacterial community composition and function is scarce, feather bacteria seem to play a role in bird immunity, reproduction and feather colouration and condition (Clayton 1999, Shawkey et al. 2007, Gunderson et al. 2009, Leclaire et al. 2014, 2015, Jacob et al. 2015 but see Jacob et al. 2014). Through their effects on plumage bacteria, trace metals may, for instance,affect thermoregulation and visual signalspotentially involved in dominant status assessment and mate choice (Wolf 2000, Hill and McGraw 2006). Future studies should now investigate if and how the changes in plumage bacterial communities induced by trace metals affect bird fitness.

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FIGURE LEGENDS

Fig. 1 Constrained redundancy analysis (RDA function in R) on bacterial community dissimilarities (estimated by Jaccard distances) between *lead*, *zinc*, *lead and zinc* and *control*groups. Both aviaries of a same treatment are distinguished using either filled or empty circles.

Fig. 2 Mean ± SE plumage bacterial community dissimilarities(estimated by Jaccard distance) in dyads of pigeons according to the spatial distance between them (0 means that the individuals belonged to the same aviary, 1 means that they were in side-by-side aviaries, 2 means that 1 aviary was between them, etc.).

Fig. 3 Mean ± SE plumage bacterial richness (number of different OTUs) according to metal exposure. a and b were significantly different (p-value<0.05) while ab was not different from a or from b.

Fig. 4 Mean ± SE plumage bacterial load (number of bacterial colonies per slide) according to metal exposure. Tests were performed for each pair of treatment. a and b tended to be different (p-value<0.072) while ab was not different from a or from b.

Table 1. Lead blood and feathers concentrations (mean±se, ppb) in lead-exposed and lead non-exposed birds, zinc blood and feathers concentrations (mean±se, ppm) in zinc-exposed and zinc non-exposed birds and ANOVAs with lead or zinc concentrations in blood or feathers as dependent variable and zinc or lead exposure as explanatory variable.

		Lead exposed	Lead non-exposed	F	P
Lead	Blood	55.49±6.54	35.73±6.77	4.47	0.040
	Feathers	402.31±35.68	255.81±49.75	19.61	<0.001
		Zinc exposed	Zinc non-exposed	F	P
Zinc	Blood	4.69±0.15	4.20±0.18	5.52	0.022
	Feathers	96.62±2.15	92.31±2.25	2.13	0.149

Table 2. PERMANOVAs with 5000 permutations based on Jaccard distance for OTU presence/absence data with the metal exposure as the explicative variable. Bacterial communities' similarities were compared between each pair of metal treatment.

	Control	Zinc-exposure	Lead-exposure
Zinc-exposure	$F_{1,40}$=2.74, P=0.002	-	-
Lead-exposure	$F_{1,42}$=3.44, P<0.001	$F_{1,41}$=3.16, P<0.001	-
Zinc and lead-exposure	$F_{1,40}$=4.40, P<0.001	$F_{1,39}$=2.10, P=0.006	$F_{1,41}$=5.13, P<0.001

Fig. 1

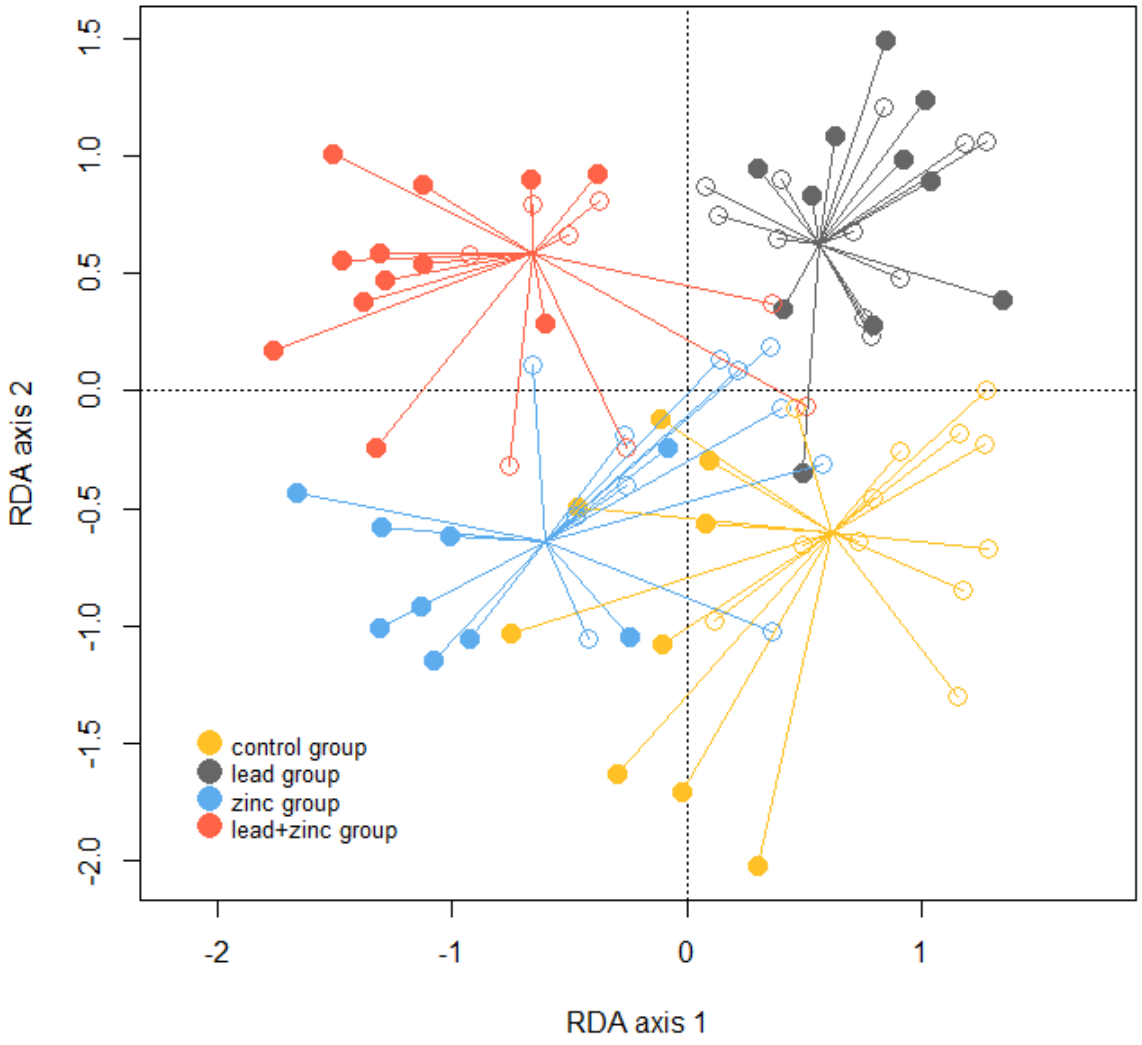

RDA axis 2
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2
-1
0
1
RDA axis 1
control group
lead group
zinc group
lead+zinc group

Fig. 2

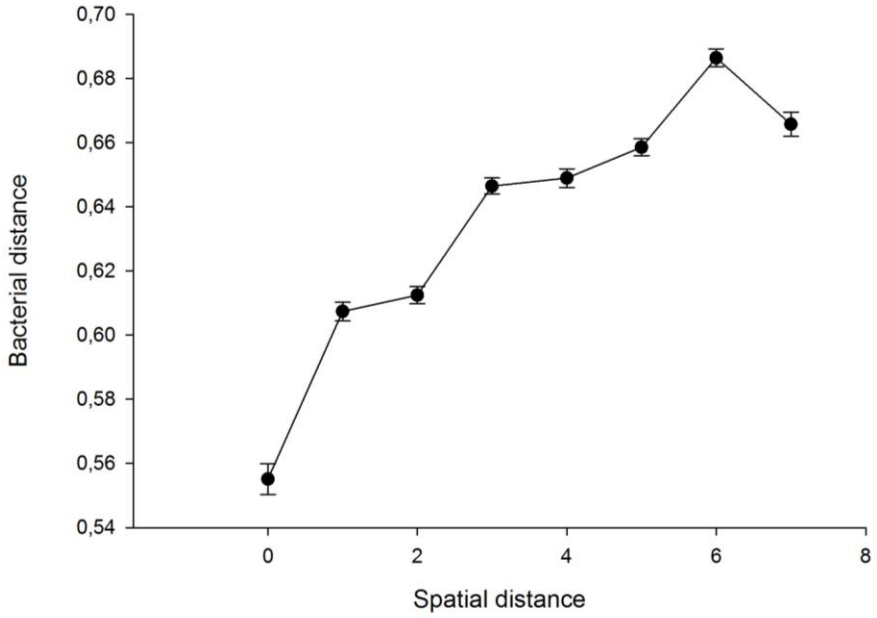

0,70
0,68
0,66
0,64
0,62
0,60
0,58
0,56
0,54
Bacterial distance
0
2
4
6
8
Spatial distance

Fig. 3

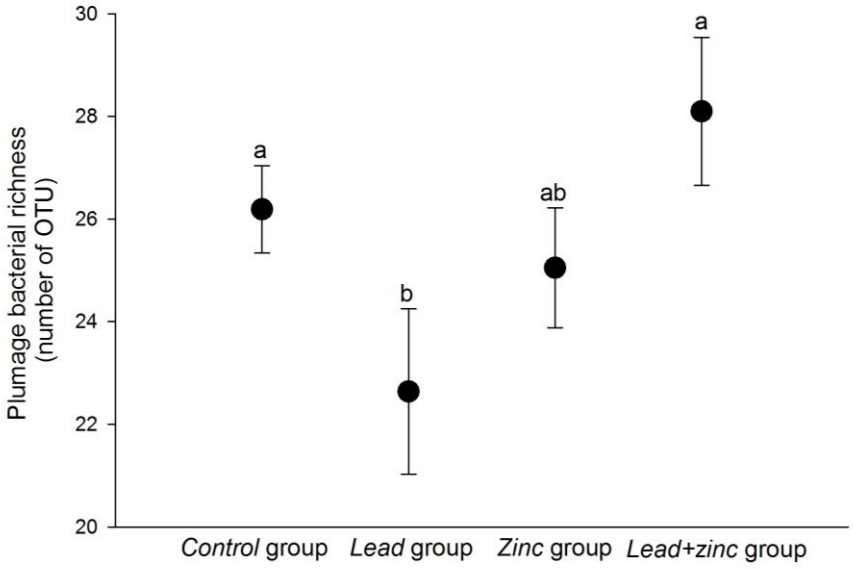
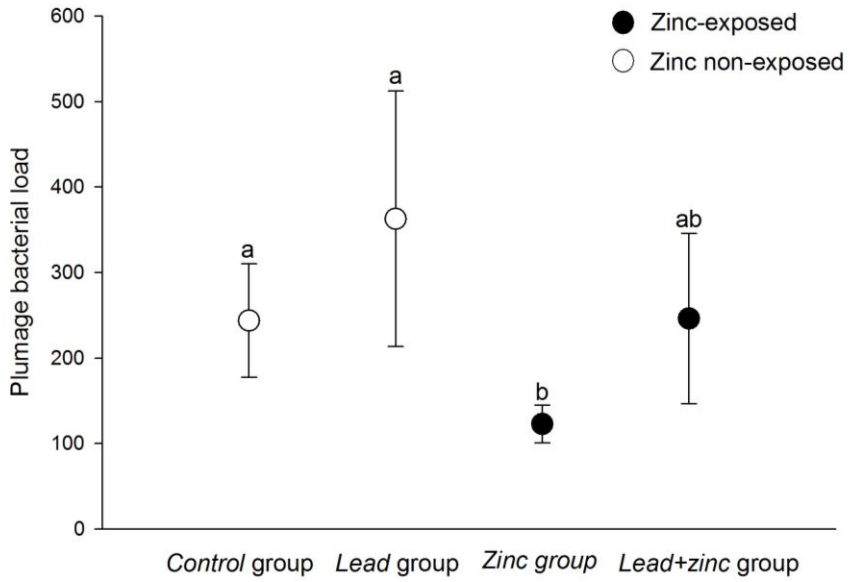

Plumage bacterial richness
(number of OTU)
30
28
26
24
22
20
a
b
ab
a
Control group
Lead group
Zinc group
Lead+zinc group

Fig. 4


Zinc-exposed
Zinc non-exposed
Plumage bacterial load
600
500
400
300
200
100
0
a
a
b
ab
Control group
Lead group
Zinc group
Lead+zinc group