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RESEARCH ARTICLE

Toxic metals and associated sporulated bacteria on Andean hummingbird feathers

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Abstract Human activities in the Sabana de Bogotá, Colombia, release toxic metals such as lead (Pb) and chromium (Cr) into the environment polluting the air, water, and soil. Because birds are in contact with these pollutants and their sources, they may serve as bioindicator organisms. We evaluated the use of hummingbird feathers obtained from individuals captured in three sites of the Sabana de Bogotá as bioindicators of toxic metal pollution using spectrophotometric and spectroscopic methods based on single-feather samples. We also characterized the bacterial microbiota associated with hummingbird feathers by molecular identification using the 16S rRNA with a special focus on sporulated bacteria. Finally, we described the interactions which naturally occur among the feathers, their associated bacteria, and pollutants. We found differences in Pb and Cr concentrations between sampling sites, which ranged from 2.11 to 4.69 ppm and 0.38 to 3.00 ppm, respectively. This may reflect the impact of the activities held in those sites which release pollutants to the environment. Bacterial assemblages mainly consisted of sporulated bacilli in the *Bacillaceae* family (65.7 % of the identified morphotypes). We conclude that the feathers of wild tropical birds, including hummingbirds, can be used as lead and chromium bioindicators and that bacteria growing on feathers may in fact interact with these two toxic metals.

Keywords Feathers · Trochilidae · Bioindicator · Lead · Chromium · Spore-forming bacteria · Sabana de Bogotá

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Introduction

Toxic metal pollution is a pressing issue worldwide mainly due to the exploitation and usage of resources in processes involving the release of various compounds containing impurities, the intentional extraction of heavy metals, and waste incineration (He et al. 2013). Lead (Pb) and chromium (Cr) are two of the most important toxic metal pollutants at many sites (Flora, Gupta & Tiwari, 2012; Harrison & Laxen 1984; Lay & Levina 2012; Patrick, 2006; Song et al. 2012; Zhitkovich 2005). Pb is a metal with no known biological activity that cannot be biodegraded (Flora et al. 2012), and for this reason, it can be easily bioaccumulated by many organisms. Pb appears to be toxic at any particular dosage because it is present in products such as batteries, pipelines, paint, and gasoline and is even frequently found in water sources; it is an ubiquitous toxic metal that may represent an environmental hazard (Flora et al. 2012; Harrison & Laxen 1984, Patrick 2006). Chromium is a metal with three main stable oxidation states: Cr(0), the inert metallic state; Cr(III), a low-toxicity ionic form with some biological activity; and Cr(VI), the most toxic state, whose toxicity appears to be dosage-dependent (Lay & Levina 2012; Song et al. 2012; Zhitkovich 2005). Cr environmental pollution mainly comes as a major by-product of the tannery, electroplating, cement, photography, and metal finishing

industries (Baral et al. 2006; Carrero et al. 2011, Kathiravan et al. 2010; Song et al. 2012).

The Sabana de Bogotá, a high elevation plateau in the Eastern Andes of Colombia, has been significantly impacted by various industries throughout the years. Inadequate disposal of industrial residues into water sources, mostly attributed to tanneries (Cuberos et al. 2009; Suárez et al. 2012), has increased the levels of toxic metals in the Bogotá River and surrounding areas (Corporación Autónoma Regional de Cundinamarca 2009). Various studies have evaluated the presence of toxic metals in water sources, soils, and plants associated with agricultural processes in the region (Acherman 2007; Corporación Autónoma Regional de Cundinamarca 2009; Miranda et al. 2008a, b; Reyes & Avendaño 2012; Soto et al. 2010; Suárez et al. 2012), and in the urine of workers of the tanning industry (Cuberos et al. 2009). These studies revealed that the levels of toxic metals, including Pb and Cr, are often above nationally or internationally safety thresholds. However, studies are limited to pollution sources that affect human activities and do not consider the pollutant levels on native flora and fauna. Studies concerning the presence of toxic metals and compounds in birds have been either suggested or performed in other regions of Colombia (Argumedo et al. 2013; Estrada-Guerrero & Soler-Tovar 2014; Gischler 2005; Olivero-Verbel et al. 2013; Olivero-Verbel et al. 2006) but not in the Sabana de Bogotá or nearby areas in the Eastern Colombian Andes.

As animals take part in food chains, they are prone to suffer from bioaccumulation processes; such is the case with birds (Malik & Zeb 2009). Because birds can be exposed to toxic metals both through their diets and via environmental pollution (Markowski et al. 2013) and because their feathers can be easily sampled and manipulated non-invasively, they can be useful metal bioindicators (Bond et al., 2015; Hahn et al., 1993; Malik & Zeb 2009; Markowski et al. 2013, Smith et al. 2003). Previous studies have used feathers obtained from birds such as egrets (Malik & Zeb 2009), tits (Dauwe et al. 2000; Markowski et al. 2013, Markowski et al. 2014), oceanic and coastal birds (Bond et al. 2015; Burger & Gochfeld 1995, Burger & Gochfeld 1997; Pérez-López et al. 2005) as well as birds of prey (Hahn et al. 1993) as bioindicators, but no studies on the topic have been conducted using species from tropical mountain environments. Hummingbirds (Trochilidae) are a diverse and abundant group occurring in several habitats affected by different levels of human intervention (Hilty & Brown 1986; Remsen et al., 2015). They show a broad range of feeding behaviors including nectarivorous and insectivorous diets (Hilty & Brown 1986; Montgomerie & Redsell 1980; Yanega & Rubega 2004). Additionally, hummingbirds are distributed throughout the Andes, especially on the high mountain ecosystems, in which they are characteristic. Here, we explore the use of hummingbird feathers as potential bioindicators of toxic metals.

Bird feathers host a wide variety of bacterial assemblages including feather-degrading bacteria (FDB) which possess enzymes that can degrade the main constituent of feathers: β-keratin (Czirják et al., 2013; Jacob et al., 2014). Some spore-forming FDB are especially important for their biotechnological potential. For example, *Bacillus licheniformis* is important for its enzymatic production (Ramnani et al. 2005), whereas *Lysinibacillus sphaericus* plays a role in various environmental processes including bioremediation of toxic metals and hydrocarbon compounds such as crude oil (Carrero et al. 2011; Córdoba et al. 2008; Manchola & Dussán, 2014; Shaw & Dussan 2015; Velásquez & Dussán 2009) and environmental control (Lozano & Dussán 2013; Peña-Montenegro et al. 2015).

The objectives of the study were to test for the presence of two toxic metals (Pb and Cr) on hummingbird feathers in three different locations of the Colombian Andes. As they differ in their degree of pollution, we also evaluated the use of the feathers as potential bioindicators of environmental toxic metal contamination which allow to differentiate the pollutant levels among sites. Another objective of our study was to characterize the bacterial assemblages present on such feathers with a particular focus in sporulated bacteria. Since this study is the first that has been performed on the Sabana de Bogotá using hummingbird feathers, we believe our results are important for the measurement of toxic metal levels naturally present in animals of the area and also give an insight to the description of the microbiota of Andean hummingbirds.

Materials and methods

Sampling sites

We captured 12 species of hummingbirds using mist nets at sites located in three different municipalities of the Sabana de Bogotá between January and May, 2015: Reserva Biológica El Encenillo (henceforth, Encenillo) is a biological reserve consisting of patches of cloud forest habitat interspersed with pastures and second growth areas, located between 2800 and 3200 m in elevation in the municipality of Guasca (4° 47′ N–73° 54′ W) at approximately 5.5 km from a major road. Hacienda San Carlos in the municipality of Zipacón (henceforth, Zipacón) is a cattle farm with various cloud forest patches, located between 2000 and 2600 m in elevation (4° 46′ N–74° 25′ W) at approximately 12 km from a major road. Finally, we worked at a small forest patch in a highly urbanized area on the campus of the Universidad de los Andes (henceforth, Uniandes) located at an approximate elevation of 2700 m in downtown Bogotá (4° 35′ N–73° 3′ W).

Capture and sample collection

Birds were identified using field guides (Hilty & Brown 1986; Asociación Bogotana de Ornitología 2000). Before manipulating the birds, we sterilized our hands with antibacterial hand sanitizer to avoid bacterial contamination (Bisson et al. 2007). We then swabbed feathers from all over their bodies using a sterile cotton swab previously submerged in a sterile Luria-Bertani (LB) broth. Swabs were kept in Falcon tubes with LB broth at room temperature until they were taken to the laboratory where they were stored at 4 °C until processed. Additionally, a minimum of five feathers were plucked from the breast region of each bird and stored in sealed plastic bags for further analysis. Although it has been suggested that both tail and breast feathers should be sampled (Smith et al. 2003), we focused on the breast feathers because they are more abundant, can provide more replicates for our analyses, removing them is less likely to negatively affect the animals (i.e., hummingbird tail feathers play important roles in flight), and they have shown to be ideal for the use as metal bioindicators (Burger and Gochfeld, 1992; Burger and Gochfeld, 1996; Burger and Gochfeld, 1997; Burger et al., 1993; Gochfeld et al., 1991; Hofer, Gallagher & Holzapfel, 2010; Hogstad, Nygård, Gätzschmann, Lierhagen & Thingstad, 2003; Mansouri, Babaei & Hoshyari, 2012; Morrissey et al., 2005). Feathers that fell off the animals due to manipulation were also collected. After collecting the swab and feather samples, the birds were released as close to the capture site as possible. All applicable international, national, and institutional guidelines for the care and use of animals were followed during the capture and sampling of the hummingbirds used in this study. A total of 22 hummingbirds were captured: 9 at Encenillo, 9 at Zipacón, and 4 at Uniandes. These 22 individuals belonged to 12 different species (Table 1).

Table 1 Hummingbirds captured in the three sampling sites. Encenillo: Reserva Biológica El Encenillo; Zipacón: Hacienda San Carlos; Uniandes: Universidad de los Andes

Species	Encenillo	Zipacón	Uniandes
Eriocnemis vestitus	4		
Eriocnemis cupreoventris	2		
Chaetocercus mulsant	1		
Metallura tyrianthina	1		2
Ramphomicron microrhynchum	1		
Ocreatus underwoodii		3	
Adelomyia melanogenys		2	
Coeligena bonapartei		2	
Coeligena prunellei		1	
Thalurania colombica		1	
Lesbia nuna			1
Ensifera ensifera			1

Metal detection

We employed two detection methods, each using a single feather to detect and quantify Pb and Cr on hummingbird feathers.

Spectrophotometric method

To quantify the total metal concentration on feathers, organic material in the sample has to first be digested so that all metal particles are available for detection. This was accomplished by placing individual feathers in a test tube with a cap and covering it completely with ca. 1–2 mL of HNO_3 65 % which was then processed for 30 min at 111 °C with the Spectroquant® Crack set 10 (Merck) according to the manufacturer's instructions. Pb and Cr in the samples were subsequently quantified with the Spectroquant® NOVA 60A photometer using the Spectroquant® Lead and Chromate test (Merck) following the manufacturer's instructions. This procedure was performed by triplicate for each individual bird.

Energy dispersive X-ray (EDX) analysis

Pb and Cr, among other elements were also detected by X-ray energy disperse spectroscopy (EDS) using a JEOL JSM-6490LV scanning electron microscope (SEM) coupled with an OXFORD Inca Energy 250 EDS System LKIE250. Feathers were first mounted on clean copper coins with double-sided adhesive carbon tape and were then coated with gold-plate on the Desk®IV cold sputtering platform (Denton Vacuum). Feathers were then observed and analyzed by EDX on the SEM at 20 kV in the characterization laboratory (MEB) at Universidad de los Andes (Shaw & Dussan 2015).

Bacterial assemblage characterization

To simulate the micro-environment where the obtained bacteria were swabbed from and to minimize the amount of feathers needed for cultivation, we developed liquid and solid feather media by modifying a feather meal agar (Tork, Aly & Nawar, 2010). The composition of the liquid feather medium was: NaCl (0.5 g/L), K_2HPO_4 (1.4 g/L), KH_2PO_4 (0.7 g/L), $MgSO_4$ (0.1 g/L) and one ground feather (1.5×10^{-2} g/L) obtained from a hummingbird as a carbon and nitrogen source. The solid feather medium had the same composition as the liquid medium with the addition of agar (15 g/L). Because each medium was prepared using feathers from the bird the bacteria were swabbed from, it was specific to each bacterial assemblage.

To recover total bacteria found in the samples, an aliquot of 1 mL of the stored LB broth that contained the cotton swabs was added to its respective liquid feather medium and incubated at 30 °C and 150 RPM for 3–4 days (until the medium

became turbid). To recover sporulated bacteria, before adding the aliquot to the liquid medium, we exposed the samples to heat shock by adding 1 mL of the LB broth to a sterile Eppendorf tube which was heated for 20 min at 80 °C and then quickly placed at 4 °C until the samples were cooled. The heat-shocked samples were then added to the liquid feather medium and incubated under the same conditions as for the total bacteria. After this initial incubation period, the media were subcultured to their respective solid liquid medium by triplicate using the replica plating technique. The solid media were incubated at 30 °C for 4–5 days. Finally, the bacterial colonies obtained from the solid feather medium were streaked on standard plate count (SPC) agar to obtain pure isolates.

The microscopic morphology of the pure isolates was observed by Gram staining, which also allowed us to confirm that a single morphology was present in each isolate. These isolates were then identified by sequencing the 16S ribosomal RNA (rRNA) gene using primers 27F and 1492R (Villegas-Torres et al., 2011). The cycling parameters and the PCR amplification conditions followed Villegas-Torres et al. (2011). The obtained sequences were identified via comparison to sequences available in databases using the basic local alignment search tool (BLAST) algorithm (Zhang et al., 2000) and were deposited on GenBank under the accession numbers KX611431 - KX611456.

Feather-bacteria-metal interaction

To determine whether the metals present on feathers could interact with the bacteria there present, feathers from the different hummingbirds were fixed overnight in an Eppendorf tube with 2.5 % glutaraldehyde at 4 °C in Millonig's phosphate buffer, pH 7.2, and were then dehydrated consecutively with 70, 95, and 100 % ethanol (Shaw & Dussán 2015). Fixed feathers were mounted on copper coins, plated with gold plate, and observed on the SEM to detect bacteria present (see the "Energy Dispersive X-ray (EDX) Analysis" section for details on the mounting and plating processes). When bacteria were found, an EDX analysis was performed to detect the presence of Pb and Cr on the surface of both the bacteria and the feathers.

To obtain a reference sample for bacteria-feather-metal interaction observation in the SEM and EDX, we used *Lysinibacillus sphaericus* strain OT4b.31, which can absorb/adsorb Pb (Data not shown). A pure culture of this strain obtained from the CIMIC bacteria collection was incubated for 5 h in an LB broth supplemented with Pb at a concentration of 5 mM. Feathers were briefly submerged in this medium, dried for 1 h at 30 °C and then fixed, mounted, and observed in the SEM as described above.

Statistical analysis

Triplicate samples used to quantify Pb and Cr concentrations were averaged for each bird sampled. Mean values from individuals of the same species from the same sampling location were taken to indicate the metal concentrations per species per site which we then used to compare the Pb and Cr levels among sampling sites. For species of which only one individual was captured, we used its respective mean concentration value for analyses. A Shapiro-Wilk test was used to evaluate whether the data were normally distributed. We transformed the Cr data using a Log_{10} transformation to normalize them. ANOVA or Kruskal-Wallis rank sum test were used to compare metal concentrations among sites depending on whether the data were normally distributed (Cr) or not (Pb). Tukey's honest significant difference method was used to determine which groups were significantly different from each other. We also compared the Pb and Cr concentrations among *Eriocnemis vestitus* and *Eriocnemis cupreoventris* and *Ocreatus underwoodii*, *Adelomyia melanogenys*, and *Coeligena bonapartei* at Encenillo and Zipacón, respectively, in order to observe whether there are differences among certain species captured on a given site (these species were chosen as they were the ones for which more than one individual was captured). For this, we used *t* test for the two species from Encenillo and ANOVA and Tukey's honest significant difference method for the three species from Zipacón. A significance value of $p < 0.05$ was established for all tests. All statistical analyses were conducted in R (R Core Team, 2015).

Results

Metal detection

We detected Pb using both detection methods in all the feathers we sampled. Cr was also detected in all of the analyzed feathers by the spectrophotometric method although in 27.3 % of the samples, the concentration obtained was under the detection limit of the chromate test. Cr was only detected in a fraction (36.3 %) of the feathers using the EDX analysis, in comparison to the spectrophotometric analysis.

Spectrophotometric method

Mean Pb concentration per species was 3.25 ± 3.31 (ppm ± SD) for Encenillo, 4.05 ± 0.65 (ppm ± SD) for Zipacón and 4.69 ± 1.3 (ppm ± SD) for Uniandes (Supplemental Fig. 1a) but differences among sites were not significant (Kruskal-Wallis, $p = 0.15$, $df = 2$, $n = 13$). Mean Cr concentration also did not differ significantly among sites, $F(2,10) = 3.68$, $p = 0.06$, $n = 13$; values were 0.89 ± 1.31

(ppm ± SD) at Encenillo, 0.49 ± 0.18 (ppm ± SD) at Zipacón, and 3.00 ± 1.22 (ppm ± SD) at Uniandes (Supplemental Fig. 1b).

Although no significant differences in metal concentration among sites were found, results appear to be influenced by an outlier individual of *Chaetocercus mulsant* captured at Encenillo (Supplemental Fig. 1). Therefore, we repeated analysis discarding data from this individual in order to give further insight to the overall pollution level from this site (Fig. 1). The mean Pb at Encenillo after removing the outlier was 2.11 ± 0.57 (ppm ± SD) and, with this individual excluded, we found statistically significant differences in Pb concentration among groups (Kruskal-Wallis, $p = 0.02$, $df = 2$, $n = 12$). Although the data were not normally distributed, Tukey's test indicated that Pb concentrations were significantly higher at Zipacón and Uniandes than at Encenillo ($p = 0.01$ and $p = 2.7 \times 10^{-3}$, respectively), but did not differ between Zipacón and Uniandes. Mean Cr concentration for Encenillo excluding the outlier was 0.38 ± 0.35 (ppm ± SD), we found statistically significant differences in Cr concentrations among groups, $F(2,9) = 11.06$, $p = 3.8 \times 10^{-3}$, $n = 12$. Tukey's test indicated that Cr concentration at Uniandes was significantly higher than at Encenillo and Zipacón ($p = 4.0 \times 10^{-3}$ and $p = 9.7 \times 10^{-3}$, respectively), but did not differ between Encenillo and Zipacón.

The mean Pb and Cr concentrations for *E. cupreoventris* were 2.01 ± 0.74 (ppm ± SD) and 0.49 ± 0.44 (ppm ± SD), respectively. For *E. vestitus*, the respective concentrations were 1.89 ± 0.04 (ppm ± SD) and 0.21 ± 0.04 (ppm ± SD). There were no statistically significant differences on the Pb (*t* test, $p = 0.80$, $df = 2.02$, $n = 5$; Supplemental Fig. 2a) and Cr (*t* test, $p = 0.36$, $df = 2.3$, $n = 5$; Supplemental Fig. 2b) concentrations between these two species captured in Encenillo. Mean Pb concentrations for the species captured in Zipacón were 4.80 ± 0.34 (ppm ± SD) for *O. underwoodii*, 4.00 ± 0.36 (ppm ± SD) for *A. melanogenys*, and 3.48 ± 0.41 (ppm ± SD) for *C. bonapartei*. The respective Cr for the same species were 0.31 ± 0.14 (ppm ± SD), 0.50 ± 0.17 (ppm ± SD), and 0.64 ± 0.08 (ppm ± SD). There were statistically significant differences for the Pb concentrations among these species, $F(2,4) = 8.06$, $p = 0.04$, $n = 7$ (Supplemental Fig. 2c) but not for the Cr concentrations, $F(2,4) = 2.58$, $p = 0.19$, $n = 7$ (Supplemental Fig. 2d). Tukey's test allowed to observe that the differences found among the Pb concentrations were between *O. underwoodii* and *C. bonapartei* ($p = 0.04$), but not between *O. underwoodii* and *A. melanogenys* or *A. melanogenys* and *C. bonapartei*.

EDX analysis

Metal detection using the SEM allowed us to observe microscopic particulate material entrapped in feathers (Fig. 2a) and the EDX allowed us to determine whether such particles contained Pb, Cr, or other elements (Fig. 2). Furthermore, the EDX analysis served as a validation method to confirm the presence of the two metals detected by spectrophotometry and enabled us to estimate a percentage of the sampled area containing particular elements (Fig. 2b). However, because we analyzed microscopic material, we could not determine or estimate the weight particles to obtain a concentration based on the percentage results.

The elemental composition of the particles present in the feathers was obtained based on the results of the EDX analysis (Fig. 3). Lead was found in 100 % of the examined feathers, whereas chromium was found in only 36.3 %. Other elements which can be considered toxic pollutants such as arsenic or mercury were found in 4.5 % of the sampled feathers.

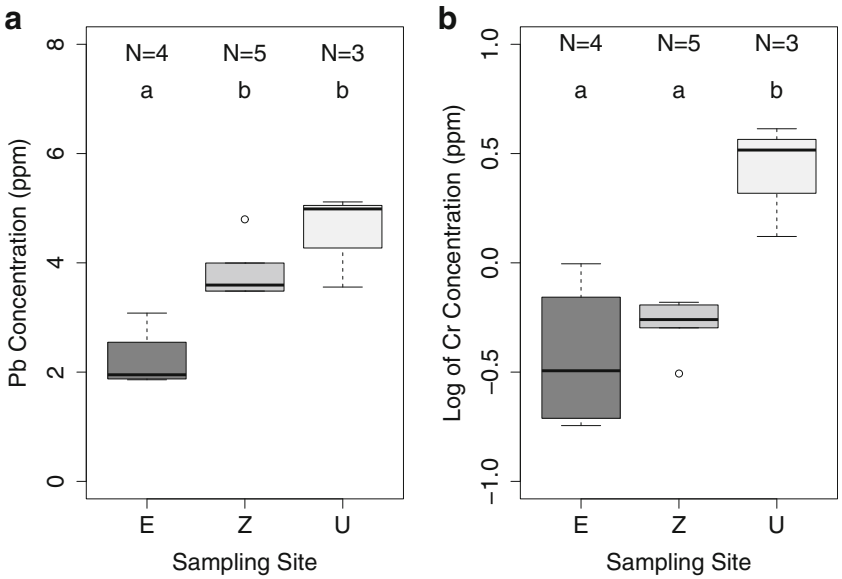

Fig. 1 Variation in lead (**a**) and chromium (**b**) concentrations (in ppm) by site after removing an outlier datum in the Encenillo dataset. *E*: Encenillo; *Z*: Zipacón; *U*: Uniandes. The number of samples for each site is shown above each boxplot. *Letters* above boxplots indicate statistical differences between groups

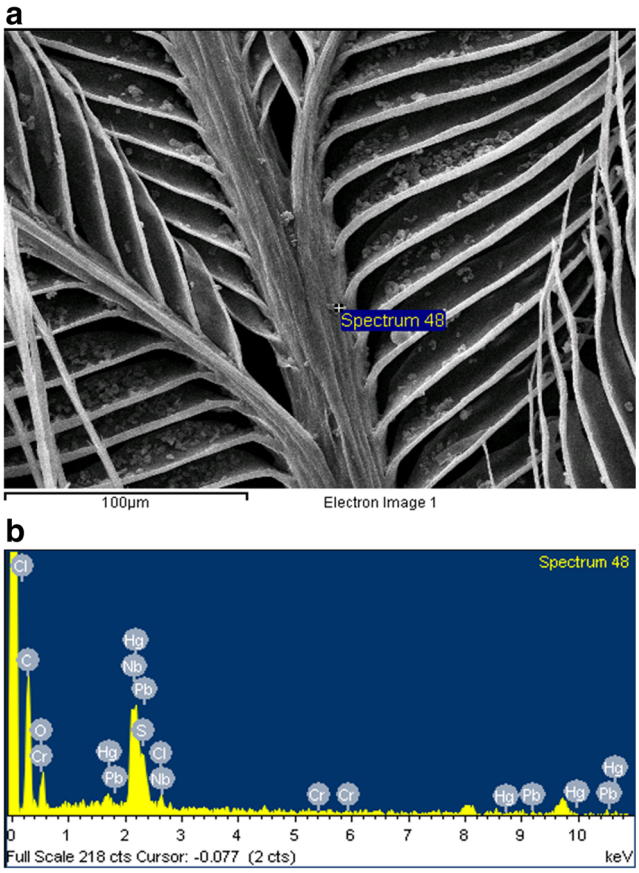

Fig. 2 EDX analysis of particulate material on the surface of a hummingbird feather. **a** SEM image (at 20 kV) of a feather. Particulate material can be observed on various points of the feather. The image was used to select the point where the EDS spectrum is measured. **b** EDS spectrum of the selected point. *Peaks* represent elements that are present in the sampled point

Bacterial assemblage characterization

We recovered over 50 bacterial morphotypes using the two methods; we chose 35 of them based on their colony morphology and microscopic characteristics to be identified by sequencing the 16S rRNA gene (Fig. 4; Supplemental Table 1). Out of the 35 selected morphotypes, 65.7 % of the isolates belonged to the *Bacillaceae* family and 8.6 % belonged to the *Pseudomonadaceae* family. The rest of the morphotypes could not be identified either because no sequences were obtained for the isolates as they did not amplify during the PCR or sequencing processes (22.9 % of the 35 morphotypes, "Unidentified" in Fig. 4) or because the microscopic characteristics and the recovery method did not match the molecular identification for the bacteria (2.9 %, "Misidentified" in Fig. 4).

We could only identify the species of four of the isolates using the 16S rRNA (Supplemental Table 1): *Lysinibacillus sphaericus* (isolate 3), *Bacillus anthracis* (isolate 23), *Bacillus cereus* (isolate 30), and *Bacillus thuringiensis* (isolate 25). We were able to identify the rest of the bacterial isolates classified in this study only up to genus. For the *Bacillaceae* family, the genera *Bacillus* (14 isolates), *Cohnella* (3 isolates), and *Paenibacillus* (2 isolates) were present on the plumage of the sampled hummingbirds. For *Pseudomonadaceae*, the only genus present was *Pseudomonas* with three isolates.

Feather-bacteria- metal interaction

Images of bacteria found on hummingbird feathers along with their respective EDS spectra suggest that the bacteria can

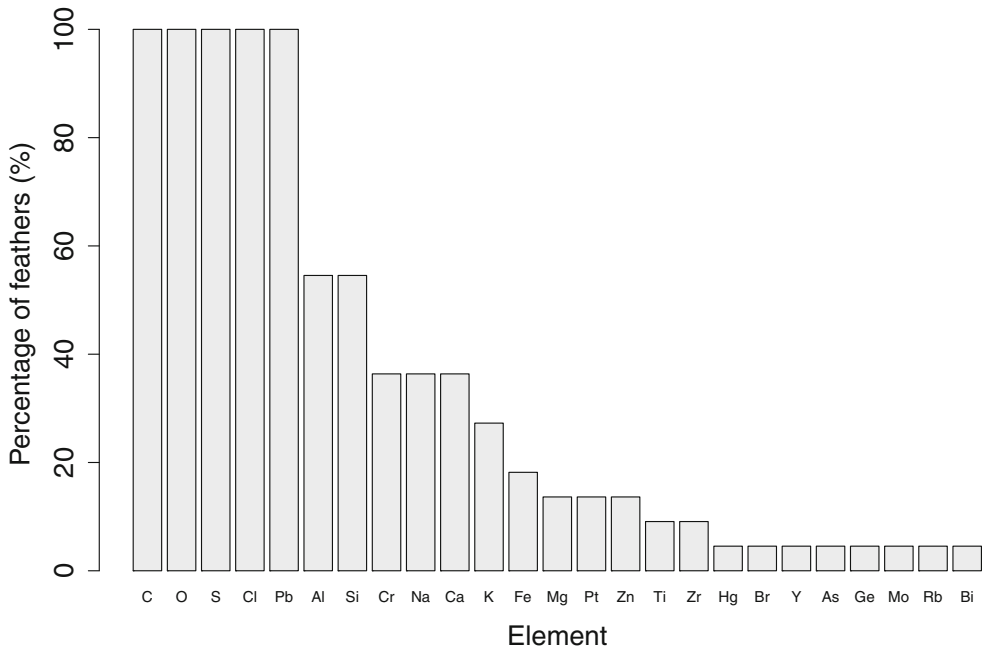

Fig. 3 Percentage of the total number of feathers ($N = 22$) that contained a given element. Elemental compositions were obtained by EDS

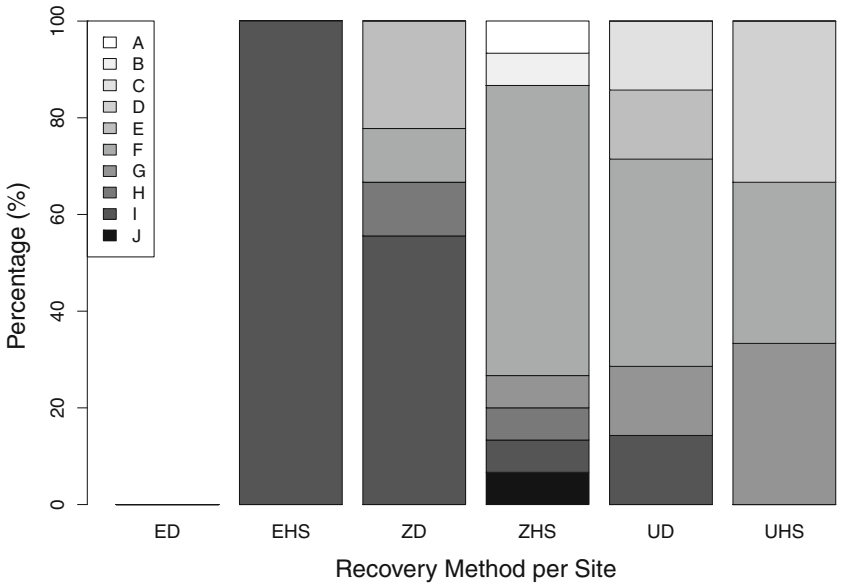

Fig. 4 Molecular identification of the 35 bacterial isolates obtained from feathers of the three sites and both recovery methods. *ED*: Bacteria isolated from Encenillo by direct method (0 isolates); *EHS*: bacteria isolated from Encenillo by heat shock method (1 isolate); *ZD*: bacteria isolated from Zipacón by direct method (9 isolates); *ZHS*: bacteria isolated from Zipacón by heat shock method (15 isolates); *UD*: bacteria isolated from Uniandes by direct method (7 isolates); UHS: bacteria isolated from Uniandes by heat shock method (3 isolates); *A*: *Lysinibacillus sphaericus*; *B*: *Bacillus anthracis*; *C*: *Bacillus cereus*; *D*: *Bacillus thuringiensis*; *E*: *Pseudomonas* sp.; *F*: *Bacillus* sp.; *G*: *Cohnella* sp.; *H*: *Paenibacillus* sp.; *I*: unidentified (Isolates for which no sequences were obtained); *J*: misidentified (isolates for which the molecular identification did not match the microscopic morphology and/or the recovery method). Isolates identified up to the same genus do not belong to the same species; see Supplemental Table 1 for more information on each particular isolate

interact with the metals, as both are naturally present on the sampled feathers (Fig. 5). Because patterns seen in those images are similar to those observed for the *L. sphaericus* OT4b.31 cells which were artificially placed on the feathers (Fig. 6), we can suggest that the isolated bacteria in this study may also be able to absorb/adsorb toxic metals.

Discussion

Metal detection and quantification

The presence of toxic metals at the three study sites can be explained by the activities performed in them. Reserva Biológica El Encenillo is a biological reserve, where formerly degraded areas are currently being reforested; however, the reserve is located in an area which used to be a clay pit. The mining activity may have released toxic metals into the environment and this might explain the presence of lead and chromium, and the other metals detected, on the bird feathers. Hacienda San Carlos in Zipacón is a cattle farm located at approximately 10 km from a mining site for a cement company and approximately 15 km from a main road. Mining and fuel combustion from motor vehicles might explain the pollution levels we observed. Finally, Universidad de los Andes is located in downtown Bogotá where air pollution is high and various construction sites are present. The forest patch studied is surrounded on its eastern and southern sides by streets, heavily and frequently used by motor vehicles. Volatile particles originating from the construction sites, the combustion of gasoline and other petroleum derivatives nearby, and the general pollution of the city may explain why the lead and chromium levels found at Uniandes where the highest in this study.

A variety of previous studies used feathers as bioindicators of Pb and Cr pollution (Table 2). The concentrations measured in the present study are similar to those found by other researchers. These results cover diverse geographic regions with varying characteristics and focus on different bird species. Due to the limited number of existing studies and the variation in multiple factors among study species and sites, we cannot readily compare our results with those of other studies. Also, generalizations about patterns of variation among species, regions, or environments cannot yet be made. To gain better insight into the pollution levels present in our study region and focal sites with respect to other systems, additional data are necessary. We note, however, that the highest metal concentrations reported to date are from a study conducted in the Punjab province of Pakistan (Table 2) at sites where agricultural, industrial, or urban residues were disposed into water sources. Because waste disposal is a major issue causing contamination in the Sabana de Bogotá, its effect on the accumulation of pollutants in animals should be considered in future studies by sampling sites located closer to the Bogotá River, where much of the city's residues are disposed via drainage systems.

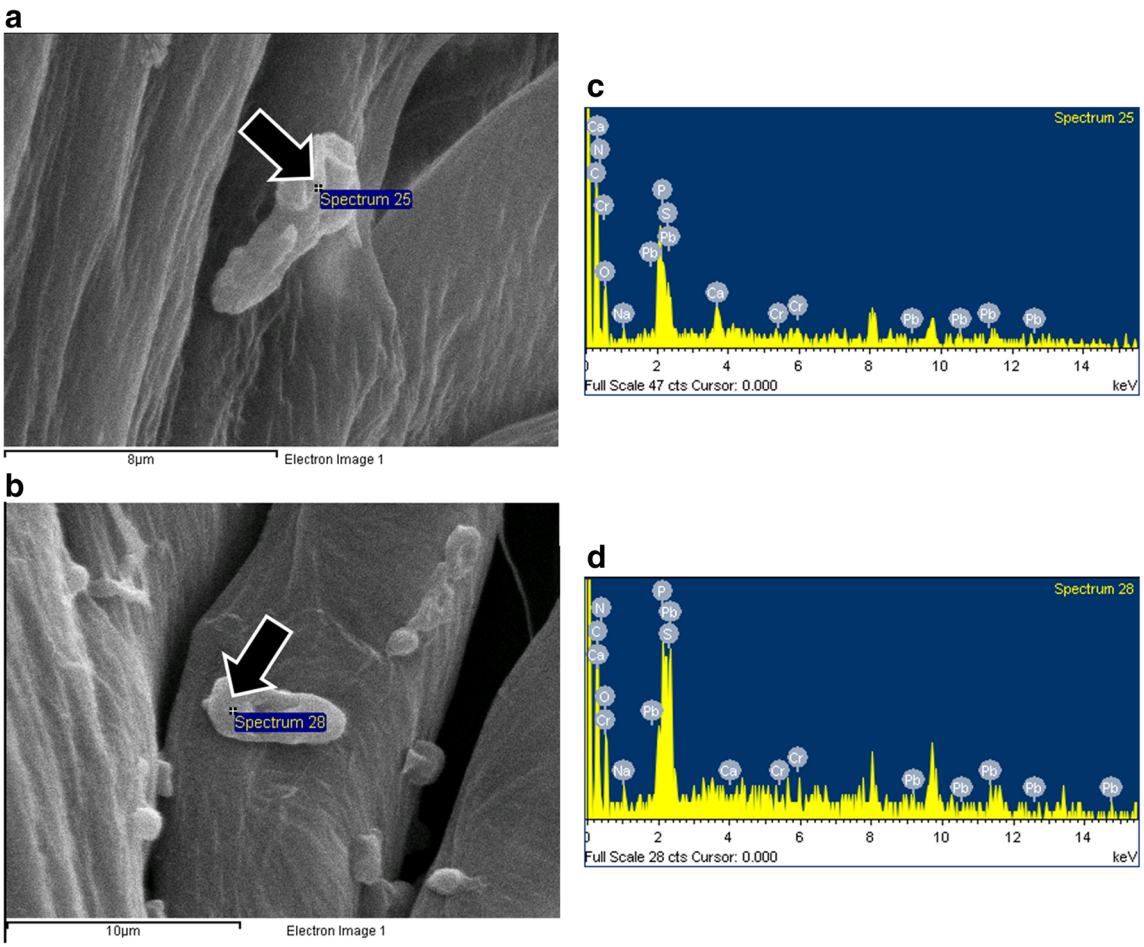

Fig. 5 EDX analysis of native bacteria present on the surface of a hummingbird feather. **a, b** SEM image (at 20 kV) of bacteria on feathers. *Black arrows* indicate the location of analyzed bacteria. *Black squares* indicate points selected to perform the EDX analysis. **c, d** EDS spectra of the selected points. *Peaks* represent elements present in the sampled point

As was previously mentioned, the *C. mulsant* individual we sampled is believed to be an outlier that does not correctly represent the general state of the sampling area where it was captured. However, we recognize the need to further sample this species at the Encenillo site as well as the other two sites used in this study in order to detect whether the increase in Pb and Cr is caused by specific contact of this individual with pollutants or a general phenomenon for this species. The statistical analysis we performed in order to compare the metal concentrations among different species captured from the same location showed that the Pb levels differed significantly only between *O. underwoodii* and *C. bonapartei* at Zipacón while the levels among the rest of species remained statistically alike. On the other hand, we found no significant difference among species for the Cr levels. This may allow us to further suggest the use of hummingbirds as a pollution bioindicator regardless of the species because, statistically speaking, most species provided us with the same information. However, in order to further validate and assure the previous statement, we would have to increase the sampling size in future studies so that we include, both, a higher number of individuals captured per species and a higher number of species. This would allow us to confirm our assessment of the different environments regarding the presence of metal pollutants therein using this family of birds.

Our results suggest that both spectrophotometry and EDS are valid methods for detecting the presence of lead and chromium in hummingbird feathers. An advantage of these methods relative to other approaches is that they are based on a single feather and thus do not require a specific feather weight (Bond et al. 2015; Hahn et al. 1993; Pérez-López et al. 2005; Malik & Zeb 2009), implying a reduction of sampling efforts. Additionally, our strategy is less invasive and largely reduces stress for the birds, as only feathers from the breast region, not primary and secondary flight feathers or tail feathers, are used (Harvey et al., 2006; Smith et al., 2003).

Bacterial assemblage characterization

The two feather media which were developed for this study, along with the initial enrichment in LB broth, allowed us to recover a large amount of bacterial morphotypes associated to

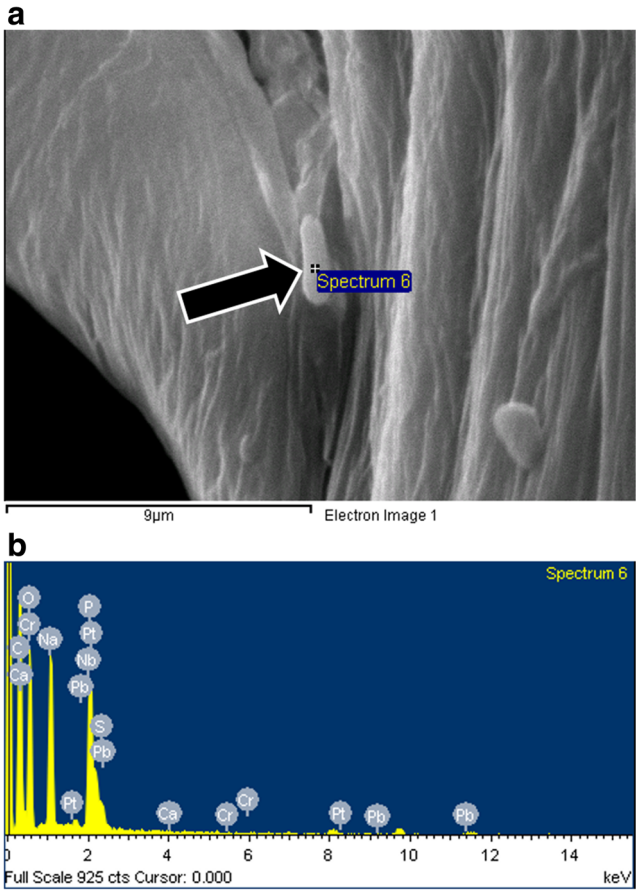

Fig. 6 EDX analysis of *L. sphaericus* OT4b.31 bacteria present on the surface of a hummingbird feather. **a** SEM imaging at 20 kV of bacteria on the feather. The *black arrow* indicates where the analyzed bacterium is located. The *square* represents the selected point to perform the EDX analysis. **b** EDS spectrum of the selected point. Peaks represent elements present in the sampled point

the hummingbirds' plumage. However, the molecular identification was not very effective because over 25 % of the isolates chosen for sequencing could not be identified. Other studies have had similar difficulties when using molecular identification for bacterial morphotypes recovered from feathers (Shawkey et al., 2005; Shawkey et al., 2003). The majority of the morphotypes which were correctly identified belonged to the *Bacillaceae* family and included the genera *Lysinibacillus*, *Bacillus*, *Paenibacillus* and *Cohnella*. This was expected as we focused on the recovery of sporulated bacteria which mainly belong to this family. Additionally, both the *Bacillaceae* and *Pseudomonadaceae* families include environmental bacteria previously found in other studies describing the feather microbiome and feather-associated bacteria (Table 3). It is noteworthy that, even without performing the heat shock, most of the isolates were spore-forming bacteria. This was also expected because most sporulated bacilli are environmental bacteria and they use their spores as resistance and dispersal structures. As spores are highly volatile (Hill et al., 1999), this could allow them to easily come in contact with birds. However, the ability of these spores to germinate and grow in vivo on the feathers after the initial contact should be studied in the future.

Feather-bacteria- metal interaction

We observed that the bacteria which naturally occur on feathers appear to be in contact with metals and other elements found on the feathers. Because some of these bacteria are FDB, this suggests that they may in fact interact with the lead and chromium present. It is then possible that these bacteria may be able to tolerate toxic metals (Lozano & Dussán, 2013), reduce them (Córdoba et al., 2008), and even absorb/adsorb them by different mechanisms such as efflux pumps (Shaw & Dussan 2015), using the S-layer protein (Carrero et al. 2011; Velásquez & Dussan 2009) or accumulating the metals in the outer structure of the spores, which we have shown to be present on the feathers (Francis, Casciotti & Tebo 2002; Soltmann et al. 2003; Dick et al. 2008; Allievi et al. 2011). Because FDB use the keratin in the feathers as a source of carbon and nitrogen, the presence of toxic metals reduces their ability to degrade keratin (Kainoor & Naik 2010; Vigneshwaran et al. 2010). Future studies should focus on evaluating the metal tolerance and accumulation ability of the isolated strains and the change in their keratinase activity under pressure from toxic metals. This will allow us to have a better understanding of the feather-bacteria-metal interactions that naturally occur on the avian plumage. Another area of study that should be evaluated later on is the ability of the spores of the isolated strains to absorb metals.

Table 2 Lead and chromium concentrations obtained from feathers as bioindicators for various studies

Pb (ppm)	Cr (ppm)	Study site	Study
4.06 ± 1.78	1.26 ± 1.59	Sabana de Bogotá, Colombia	This study
37.5 ± 10.7	5.38 ± 1.0	Punjab province, Pakistan	Malik & Zeb 2009
2.72 ± 0.70	0.60 ± 0.08	Bali, Indonesia	Burger & Gochfeld 1997
1.04	7.02	Papua New Guinea	Burger & Gochfeld, 1995
4.83 ± 1.08	N/A	Antwerp, Belgium	Dauwe et al., 2000
~16	N/A	Central Poland	Markowski et al., 2013
4.28 ± 0.59	N/A	Central Poland	Markowski et al., 2014

Table 3 Bacterial taxa associated with feathers of different birds as described in various studies

Bacterial taxa	Study
Lysinibacillus, *Bacillus*, *Paenibacillus*, *Cohnella*, *Pseudomonas*	This study
Bacillus, *Pseudomonas*, *Xanthomonas*, *Rhizobium*	Bisson et al. 2007
Bacillus, *Lysinibacillus*, *Paenibacillus*, *Staphylococcus*, *Clavibacter*, *Curtobacterium*, *Microbacterium*, *Rathayibacter*, *Frigoribacterium*, *Kitasatospora*, *Cellulomonas*, *Promicromonospora*, *Rhodococcus*, *Blastococcus*, *Humicoccus*, *Nocardioides*, *Agrobacterium*, *Aurantimonas*, *Brevundimonas*, *Methylobacterium*, *Rhizobium*, *Rhodobacter*, *Sphingomonas*, *Pantoea*, *Stenotrophomonas*, *Pseudomonas*, *Variovorax*, *Pedobacter*, *Spirosoma*	Dille, Rogers & Schneegurt 2016
Paenibacillus, *Bacillus*, *Rhodococcus*, *Sphingobacterium*, *Pseudomonas*, *Aeromonas*, *Azotobacter*	Ghosh, Maity, Chakrabarti & Chattopadhyay 2007
Aeromonas, *Bacillus*, *Enterobacter*, *Escherichia*, *Paenibacillus*, *Pseudomonas*, *Roseomonas*, *Staphylococcus*	Goodenough & Stallwood 2010
Bacillus, *Staphylococcus*, *Enterococcus*, *Kocuria*, *Micrococcus*, *Steptomyces*, *Nesterenkonia*, *Pseudomonas*, *Stenotrophomonas*, *Vibrio*, *Chryseobacterium*, *Flavobacterium*	Gunderson 2008
Bacillus, *Stenotrophomonas*, *Fervidobacterium*, *Streptomyces*, *Thermoactinomyces*	Korniłłowicz-Kowalska & Bohacz 2011
Bacillus, *Streptomyces*, *Fervidobacterium*, *Vibrio*	Lucas et al., 2003
Bacillus, *Microbacterium*, *Pantoea*, *Serratia*	Shawkey et al. 2005
Bacillus, *Pseudomonas*, *Staphylococcus*	Shawkey et al. 2003
Arthrobacter, *Bacillus*, *Bervibacterium*, *Dermacoccus*, *Flavimonas*, *Kocuria*, *Micrococcus*, *Paenibacillus*, *Pseudomonas*, *Staphylococcus*, *Streptomyces*	Shawkey, Pillai & Hill 2009
Bacillus, *Klebsiella*, *Enterobacter*, *Hafnia*, *Pseudomonas*, *Brevundimonas*, *Stenotrophomonas*, *Micrococcus*, *Dermacoccus*, *Acinetobacter*, *Pantoea*, *Escherichia*, *Corynebacterium*	Verea et al. 2014

Conclusions

Although our sample size is relatively low, this study showed that hummingbirds from various habitat types in the Sabana de Bogotá can be used as bioindicators of environmental pollution by toxic metals such as lead and chromium through samples consisting of a single feather-based method. Our approach enabled us to characterize the feather's bacterial microbiome which, as expected, was mainly composed of members of the *Bacillaceae* family and a few members of the *Pseudomonadaceae* family, given our focus on sporulated bacteria. Our work represents a baseline for future analyses of the interactions occurring among birds, their associated bacteria, and the environments they inhabit.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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