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Male European robins (*Erithacus rubecula*) and mercury transference in the Tatra Mountains of Slovakia

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Abstract. Pollutants such as heavy metals are increasingly prevalent in the environment. For example, Organic methylmercury is known for its persistence and bio-accumulative capacity, and thus for its high toxicity in various organisms. Birds are often used as bioindicators for measuring and monitoring the concentrations of contaminants in the environment. In this report, the mercury content in different tissues of 32 male robins (*Erithacus rubecula*) was investigated. The level of mercury contamination in 29 of 32 birds was within tolerable limits. Significantly more mercury was found in feathers than in muscle tissues ($\alpha = 0.05$). Migratory robins showed slightly higher Hg levels in soft tissues, while residential birds showed higher Hg levels in their plumage. The latter could be an effect of increased Hg deposition in mountain ecosystems, while the higher amounts of Hg ingested by robins in wintering areas may be excreted near the northern montane nesting areas, contributing to increased Hg exposure.

Key words: Erithacus rubecula, Hg exposure, migratory robins, residential robins, Slovakia

Introduction

Since the Industrial Revolution, pollutants such as the heavy metal mercury (Hg), are entering the environment to a higher degree (Sonke *et al.* 2023). Their emission as recurrent waste products of agricultural and industrial activities results in contamination of surrounding areas (Costa *et al.* 2011; Grúz *et al.* 2018). Although elemental Hg is considered a mutagen, teratogen, and carcinogen, potentially affecting behaviour, physiology, and reproductive success, it has no metabolic function with relatively low toxicity (Eisler 1987; Tsipoura *et al.* 2008). Its methylated organic form MeHg, on the other hand, has the potential to accumulate and magnify in organisms and along trophic levels throughout the food web.

To monitor mercury concentrations and contamination in the environment, bio indicative species are studied. Birds have been used in numerous cases as bioindicators for the assessment of heavy metal contamination (e.g., Ackerman *et al.* 2016; Condon and Cristol 2009; Frederick *et al.* 2002; Low *et al.* 2020). Exposure levels differ between species according to direct contact, food and foraging type, habitat area, and moulting (Bianchi *et al.* 2008). Higher levels of contamination are expected for species that feed on soil- or water-borne food and live in wet, open habitats near industrial sites and along contaminated water bodies. Due to the bio accumulative nature of Hg, the highest levels are expected at the end of the food chain, where it biomagnifies, for example, in fish- and bird-eating raptors (Ackerman *et al.* 2016; Grúz *et al.* 2019; Solonen and Lodenius 1990; Shrum 2009). An acute dietary intake of 4-40 mg/kg total Hg is considered lethal for birds (Eisler 1987). The threshold in feathers, which has been shown to have adverse effects such as reproductive performance, is 5.0-5.43 mg/kg (Burger and Gochfeld 1997; Eisler 1987; Grúz *et al.* 2019). A minimum of 70% of a bird's Hg burden is accumulated in the feathers. This is due to the high affinity of Hg to the thiol group in the keratin of bird feathers (Tsipoura *et al.* 2008). During feather growth, the feather is supplied with blood through which Hg is transported. The Hg in feathers corresponds to the level circulating in the blood during the period of feather growth, reflecting the acute dietary intake and the amount already stored in other tissues (Solonen and Lodenius 1990). Once feather growth is complete, the Hg in the feather remains physically and chemically stable, even after death (Condon and Cristol 2009; Low *et al.* 2020). The moult allows birds to excrete the stored mercury and to partially detoxify their bodies on a regular basis (Grúz *et al.* 2018; Poláček and Haas 2018). This could be the reason why Hg tolerance in birds is relatively high compared to mammals (Shrum 2009). Despite the presence of Hg in many tissues, it is usually highest in concentration in feathers, followed by the liver, as a storage organ and a major site for selenium-induced demethylation of Hg (Eisler 1987; Low *et al.* 2020). Muscle tissue usually contains the smallest amount of Hg in examined tissues (Solonen and Lodenius 1990; Zaman *et al.* 2022). Feathers can be obtained non-invasively from the ground, from nests or even from deceased specimens (Bianchi *et al.* 2008; Parmar *et al.* 2016; Solonen and Lodenius 1990). The Hg levels in rectrices (tail feathers) tends to be less than in primaries (long flight feathers) but more than in secondary flight feathers (Furness *et al.* 1986).

The mercury content in various tissues of 32 male European robins (*Erithacus rubecula*), found deceased in more than 10 montane areas in Slovakia was examined in this research. The robin is a typical forest passerine species belonging to the family Muscicapidae, with a wide distribution in the western Palearctic (Bianchi *et al.* 2008; Cramp 1988). It is mainly territorial and only forages over a small area, leading to a firm association with an area and the level of contamination present within it (Costa *et al.* 2011). Robins mainly feed on worms and insects on the ground, but may also consume berries when these other food sources are unavailable (Miller and Harley 1996). In addition to a relatively high metabolic rate, robins have a higher potential to accumulate Hg than other passerines (Costa *et al.* 2011). Examination of their feathers is thus considered to be a reliable method to evaluate Hg contamination during periods of feather growth and between moults. Moult is indicated by the length of day and/or exposure to daylight (Payne 1972). It may thus vary between migratory species or individuals. Robins are considered partly migratory, with males spending winters close to their nesting site, while females and young migrate further away, (e.g., to southern Slovakia and Hungary) (Janiga 2021). In general, the moulting period is between May or June, and September (Bianchi *et al.* 2008). Due to the physiology of moulting and Hg storage, the first moulted primaries should have the highest Hg content in the plumage of passerine birds (Furness *et al.* 1986).

The robins studied in this report were collected in alpine and mountainous areas of the Tatra Mountains in Slovakia. Mountain ecosystems are exposed to higher atmospheric Hg concentrations, which varies among biotopes and may depend on the type of forest cover (Poláček and Haas 2018). MeHg availability in birds is linked to atmospheric Hg deposition in montane areas (Rimmer *et al.* 2005). There is a higher concentration of pollutants in the High Tatra Mountains (Martinková *et al.* 2019). The nearest coal power plants, (i.e., Hg emission sources) are in south Poland. Poland is one of Europe's largest Hg emitters, and southern Poland is considered an air pollution "hot-spot" (Jędruch *et al.* 2021). The elevated Hg levels in the mountains of south-eastern Poland, bordering the High Tatras in Slovakia, are also due to the naturally elevated Hg content in their bedrock. Much of the Hg deposited on the land accumulates in moist, organically enriched soils, is washed out and eventually enters water bodies as runoff. The Hg transported via air and deposited among mountain ranges is exacerbated by increased precipitation and humidity (Martinková *et al.* 2019).

The main objective of this study was to compare Hg concentrations in the feathers of individuals to determine mercury concentrations. The impact of migrating behavior on the accumulation of Hg in different tissues was also investigated. The results were compared with Hg concentrations from other studies and with toxic reference values (TRV) to determine trends in the current contamination risk in the samples area within the Tatra Mountains of Slovakia.

Material and Methods

Sampling sites and sample collection

Between the 2000 and 2021, 32 deceased specimens of *E. rubecula* were collected. 14 samples were found in 7 different areas of the High Tatras and 18 were collected in 3 different areas of the Low Tatras. 16 of the latter were found deceased on the same day in southern Chopok. Two additional specimens were collected from an unknown locality. The birds were packed in individual sterile plastic bags and stored in a freezer until analysis was performed.

Morphological measurements were taken, including weight in a half-frozen state and length of tarsus, bill, wings, and tail. Heart and liver, as well as breast and thigh muscle tissues were dissected. Internal organs were examined for the presence of intestinal parasites. All tissues were dried in a laboratory incubator (IF 160 Plus) (Memmert, Germany) at 40 °C, with 30% air circulation, for 48 hours. After drying, samples were homogenized in a cryomill (Retsch, Germany).

Laboratory analyses

Mercury: the 1st and 3rd primary feather and the 4th and 5th tail feather (rectrices) from the left side were used. A piece weighing approximately 0.0010-0.0016 g was cut with the use of sterile steel scissors and scalpels. Exact weight determination of each sample (tissues and feathers) needed for mercury analysis was performed using an accuracy of 0.001 g on a Kern 770 balance scale (Kern and Sohn, Germany). Mercury concentrations in different sample types were measured with the direct mercury analyzer DMA-80 (DMA-80 Dual Cell, Milestone srl., Italy), according to the manufacturer's instructions.

Sex determination: For the determination of gender, 1 mm of the very tip and top of the calamus (quill) were cut off each feather of each individual with use of a sterile scalpel, and stored in an Eppendorf tube. DNA was then extracted from the cut-offs using commercially available DNeasy Blood & Tissue Kits (QIAGEN, Germany), according to the manufacturer's instructions. Amplification of a fragment of the CHD gene by PCR with use of P2 and P8 primers and GoTaq® Hot Start Polymerase (Promega, USA) was done (Griffiths *et al.* 1998). PCR was performed using a C1000 Touch ThermalCycler (Bio-Rad, USA). The PCR cycling conditions were as follows: 95 °C for 3 min, followed by 34 cycles of 95 °C for 30 s, an annealing temperature of 48 °C for 45 s and 72 °C for 45 s, then one final cycle of 72 °C for 5 min. Amplified products were visualized using electrophoresis for 40 mins at 7-10 V/cm in 2% agarose gel stained with EliDNA PS Green (Elisabeth Pharmacon).

Statistical analysis

All measured values were recorded in a data sheet in Microsoft© Excel 2016. Mean values (MV), as well as median and standard deviation (SD) were calculated using the commands "Stabw" (SD), "Median", and "Mittelwert" (MV) in the German ver-

sion of Excel. Diagrams and statistical tests (analysis of variance, one- sided ANOVA, Tukey's test, Mann-Whitney U test) were generated using IBM© SPSS (version 29.0.0.0) with a significance level of $\alpha = 0.05$. Multivariate principal component analysis (PCA) for more easily accessible and comprehensive visualisation of complex data was performed using Past software (version 4.13).

Results

Sex determination revealed that all sampled *E. rubecula* specimens (n = 32) were indeed male. The concentration of mercury (Hg) was measured in the sampled rectrices (T) and primaries (W) of all 32 robins (Fig. 1). The median concentration in rectrices was 1.26 mg/kg, with 29 values under 4.0 mg/kg. The median concentration in primaries was 1.16 mg/kg, with all but three values under 3.0 mg/kg. The samples ER7 and ER23 were statistical extremes with Hg levels above the toxic reference values (TRV) of 5.0 mg/kg in both feather tissues. There was no statistical difference in the concentration levels between feather tissues.

The Hg concentrations in heart and lung tissues, as well as femoral and pectoral muscle tissue were obtained for 28 of the birds (Fig. 2). ER2, ER6, ER19, and ER24 were excluded from further analysis because Hg concentrations could not be measured in all tissues. No parasites were found in the organs or muscle tissues. Only one specimen had a measured concentration of higher than 5.0 mg/kg. Median concentration in liver tissue was 0.425 mg/kg, 0.326 mg/kg in heart tissue, and 0.198 mg/kg and 0.185 mg/kg in femoral and pectoral muscle tissues, respectively. Samples ER12, ER16, and ER32 were statistical extremes and had the highest levels of Hg in all four soft tissues. No differences in Hg concentration between tissue types were significantly different from on another.

A one-sided analysis of variance in all tissues examined suggested a significant difference in the Hg concentration values. Using the Tukey post-hoc test at a significance level of $\alpha = 0.05$, it was found that both feather tissues differed significantly from both muscle tissues in Hg concentrations. Furthermore, Hg levels in rectrices also differed significantly from those in heart tissues. High conformities ($\alpha > 0.9$) of Hg contents were found between muscle and heart tissues, as well as between heart and liver tissues.

To investigate whether the concentration of Hg in the sampled *E. rubecula* differed according to their spatial wintering behaviour, Mann-Whitney U tests were conducted for each tissue type (Fig. 4). Migratory individuals had higher mercury levels in all examined soft tissues than residential individuals. The biggest difference between the two groups was observed in heart tissues, with a significance of 0.06, followed by liver tissues and pectoral muscles, each with a significance of 0.09. Conversely, residential birds showed higher levels of Hg in both feather tissues. Both clear trends were not significant, but can be visualized using multivariate principal component analysis (PCA). It was, for example, evident that migrating birds had accumulated significantly more Hg in their livers (as in all soft tissues), while resident birds had accumulated a comparatively higher proportion of Hg in or on their primary feathers (as well as in/on their rectrices) (Fig. 3).

Discussion

Given that all sampled individuals were male, differences in Hg accumulation and excretion based on gender could be eliminated for this study. However, the literature is not clear on whether sex has an influence on Hg levels of bird tissues (Low *et al.* 2020), whether the observation of differences in Hg levels of different sexes is tissue-dependent, (Vizuete *et al.* 2022), or whether sex is related to Hg accumulation at all (Grúz *et al.* 2019). Our sampling of only male specimens may be attributed to

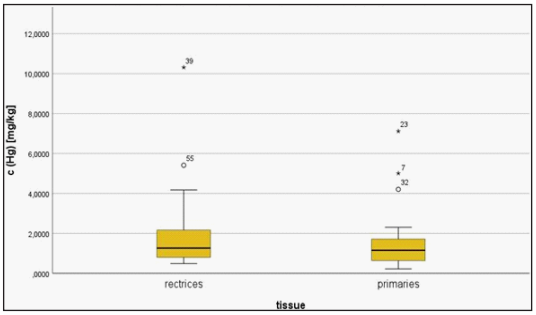

Fig. 1. The concentration of mercury (Hg) was measured with the DMA-80 in the barbs of the 1st and 3rd primary feather and 4th and 5th rectrices of n = 32 European robins (*E. rubecula*). The dotted line marks the threshold at 5.0 mg/kg, above which adverse effects of Hg have been reported in birds (Eisler 1987). Most concentrations were within this limit. Samples ER7 (7, 39), ER23 (23, 55), and. ER32 (32) are statistical extremes in this diagram. The mercury concentration in rectrices and primaries was not statistically different but was slightly higher in rectrices than primaries.

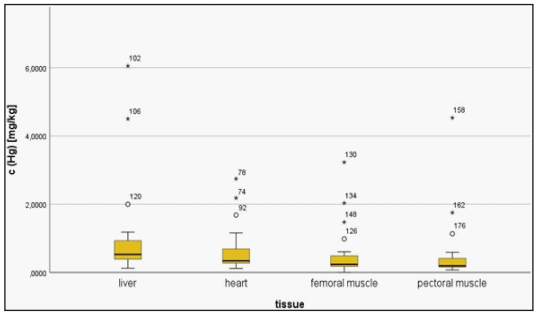

Fig. 2. The concentration of mercury (Hg) was measured with the DMA-80 in the tissues of liver, heart, femoral, and pectoral muscle of n = 28 European robins (*E. rubecula*). The dotted line marks the threshold at 5.0 mg/kg, above which adverse effects of Hg have been reported in birds (Eisler, 1987). All but one of the measured concentrations lay within this limit. The samples ER12 (74, 102, 130, 158), ER16 (78, 106, 134, 162), and ER32 (92, 120, 148, 176) were statistical extremes displaying the highest levels in all 4 tissues. None of the differences in mercury concentration per tissue were significantly different.

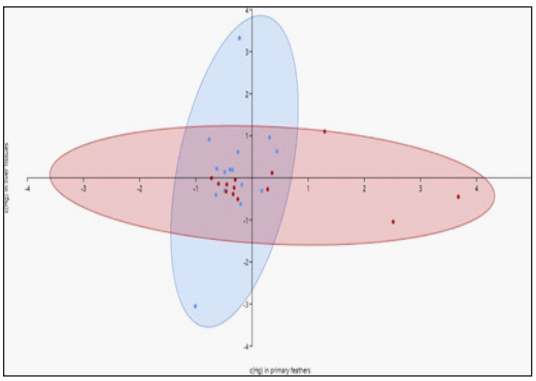

Fig. 3. Multivariate principal component analysis (PCA) of the mercury concentration in primaries and liver tissue of migratory (blue) and residential (red) male *E. rubecula*. The distribution clearly shows opposing trends of the two groups. Migrating birds show higher accumulation of Hg in the liver as resident specimens. The latter show higher accumulation of Hg in the primaries. Created with the software Past (version 4.03).

the territorial nature of the species, with females remaining near males during breeding season (Adriaensen and Dhondt 1990).

The relative amount of Hg was highest in feathers, followed by liver tissue. Concentrations in heart tissue were higher than in muscles, where levels were lowest. This order of accumulated Hg concentrations per tissue is consistent with the literature (e.g., Low *et al.* 2020; Tsipoura *et al.* 2008; Solonen and Lodenius 1990), though the highest concentration were found in rectrices instead of primaries (Furness *et al.* 1986). The levels of Hg contamination in 29 of 32 birds were < 2.40 mg/kg, and thus within tolerable limits (Grúz *et al.* 2019). Three birds had elevated levels, two of them above the threshold of 5.43 mg/kg in wing and tail feathers, which has been shown to impair reproductive performance (Eisler 1987; Grúz *et al.* 2019). These samples were all found in relatively urbanized areas in montane northern Slovakia than specimens with mercury levels well below the threshold. The amount of Hg measured in plumage may be locally impacted by external factors. As mentioned above, aquatic and mountain habitats are particularly vulnerable to pollution from Hg deposition (Rimmer *et al.* 2005). Ground-feeding insectivorous passerines such as the robin are particularly vulnerable to elevated Hg levels. The increased amount of Hg deposition in the mountains could also be reflected in increased external Hg levels on the feathers of individuals that exhibit year- round site fidelity. External and internal Hg content was not distinguished in the analytical method used. This may be accounted for based on differences in acute dietary intake, delayed accumulation in the feathers during and shorty after moult, and on the feathers due to atmospheric deposition.

Hg concentrations were usually highest in the rectrices, which usually moult first, and thus start to accumulate Hg before other feathers and may contain more Hg overall (Jenni and Winkler 2020). According to Bianchi *et al.* (2008), the moulting period in robins takes place in September. It is not clear whether feathers accumulate Hg from other body tissues during growth (Furness *et al.* 1986, Solonen and Lodenius 1990), although the opposite has been shown to occur (Whitney and Cristol 2017). This could reduce the Hg concentration in the body during and shortly after feather growth in autumn. The same phenomena apply to lower concentrations observed in feathers shortly after the typical moulting period ends in September, when feathers have not completely regrown, and have thus not accumulated their full load of Hg yet.

In organ tissue, the highest Hg concentrations were measured in ER12 and ER16, exceeding the values of comparable samples. Both individuals were found in April in southern Chopok. This area is in the Low Tatras. A total of 16 robins were found frozen to death on the same day in mid-April 2013. It is likely that this group was on its migration route. They likely wintered in more southern locales and were returning to their breeding grounds, in northern Slovakia. Male robins tend to overwinter near nesting sites and these birds are rare finds of scientific interest (Janiga 2021). Significantly higher levels of Hg were detected in soft tissues of the migratory group. Orally accumulated Hg takes about 2-3 months to degrade and be excreted (Rimmer *et al.* 2005). It can, therefore, be assumed that they ingested an increased amount of Hg in their diet in their wintering habitat.

Mercury may be excreted into the environment by birds through excrement, glandular secretions, eggshells, and moulting (Bianchi *et al.* 2008; Costa *et al.* 2011; Grúz 2019). While Hg remains stable in feathers even after moult, Hg can enter the environment via these other pathways (Low *et al.* 2020). Although these amounts are small in contrast to the amounts released by human activity, the excretions of migratory birds represent an additional Hg load in nesting areas.

Statistically significant differences in Hg concentration in the sampled male robins were most clearly observed between those in feathers and those in muscle tissues. This distribution is consistent with migratory behavior and what is known about deposition patterns in mountainous regions to date. Individuals that migrate to areas with elevated Hg content (e.g., in food) during winter, transport additional Hg to the nesting site when they return during breeding season. This increases exposure and risk of poisoning for adults, hatchlings, and higher trophic levels of the food web. Furthermore, mercury concentrations increased rather than decreased during the sampling period from 2000 until 2021. Certainly, climate change will have further implications on the distribution of heavy metals such as mercury, and additional, more detailed studies on avifauna as bioindicators could begin to show us the extent of its influence. With this additional information, appropriate responses could be identified and implemented at an early stage.

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