Ecotoxicology (2016) 25:469–480
DOI 10.1007/s10646-015-1604-8

# Barn owl feathers as biomonitors of mercury: sources of variation in sampling procedures

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Accepted: 22 December 2015 / Published online: 30 December 2015


**Abstract** Given their central role in mercury (Hg) excretion and suitability as reservoirs, bird feathers are useful Hg biomonitors. Nevertheless, the interpretation of Hg concentrations is still questioned as a result of a poor knowledge of feather physiology and mechanisms affecting Hg deposition. Given the constraints of feather availability to ecotoxicological studies, we tested the effect of intra-individual differences in Hg concentrations according to feather type (body vs. flight feathers), position in the wing and size (mass and length) in order to understand how these factors could affect Hg estimates. We measured Hg concentration of 154 feathers from 28 un-moulted barn owls (*Tyto alba*), collected dead on roadsides. Median Hg concentration was 0.45 (0.076–4.5) mg $kg^{-1}$ in body feathers, 0.44 (0.040–4.9) mg $kg^{-1}$ in primary and 0.60 (0.042–4.7) mg $kg^{-1}$ in secondary feathers, and we found a poor effect of feather type on intra-individual Hg levels. We also found a negative effect of wing feather mass on Hg concentration but not of feather length and of its position in the wing. We hypothesize that differences in feather growth rate may be the main driver of between-feather differences in Hg concentrations, which can have implications in the interpretation of Hg concentrations in feathers. Finally, we recommend that, whenever possible, several feathers from the same individual should be analysed. The five innermost primaries have lowest mean deviations to both between-feather and intra-individual mean Hg concentration and thus should be selected under restrictive sampling scenarios.

**Keywords** Biomonitor · Barn Owl · Mercury · Feathers · Intra-individual variations

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## Introduction

Mercury (Hg) is a metal naturally present in the environment (prolific in coal and metal-rich geologic deposits) and also an introduced contaminant—its main anthropogenic sources are mining and fossil fuel combustion (Krabbenhoft and Sunderland 2013). Hg is mostly available to living organisms after conversion in its toxic organic form of methylmercury (MeHg), which is reported to be harmful both to humans and wildlife, mainly due to neurological and immunological effects, and reproductive impairment (Evans et al. 1982; Burger and Gochfeld 1997; Scheuhammer et al. 2007). Methylation of the element can occur in aquatic environments, and so Hg ecotoxicological studies have been focused mainly in aquatic organisms (Seewagen 2010). Nevertheless, toxicity thresholds have also been reported in terrestrial organisms in agricultural wetlands (Ackerman and Eagles-Smith 2010; Ackerman et al. 2010). Despite Hg compounds were banned as plant protection products in Europe since 1991 (Commission Directive 91/188/EEC), Hg availability appears to be increasing globally through atmospheric deposition

(Windham-Myers et al. 2014), highlighting the urgent need for Hg contamination biomonitors in farmlands for ecological and food safety concerns (Jiang et al. 2014; Chen et al. 2015).

Given the pronounced bioaccumulation and biomagnification of Hg in food webs, the highest concentrations are often attained in top predator species (Lindberg and Odsjö 1983; Dietz et al. 2000; Lourenço et al. 2002). Both owls (Strigiformes) and diurnal raptors (Accipitriformes, Falconiformes) have been used as sentinels of environmental contamination in Europe since the late 1950s (Gómez-Ramírez et al. 2014), and most monitoring schemes used feathers as a non-invasive sampling method for several contaminants, including metals (García-Férnandez et al. 2008; Castro et al. 2011; Bustnes et al. 2013). Since feathers can be collected from both live and dead individuals, they are extremely versatile as reservoirs of contaminants, allowing for monitoring direct effects in contemporary populations, as well as for studying long time trends, using for instance specimens stored in museum collections (Bustnes et al. 2013; Gómez-Ramírez et al. 2014).

Feathers are the key excretory pathway for Hg in birds because they hold from 50 % to more than 90 % of the body Hg burden (Honda et al. 1986; Braune 1987; Lewis and Furness 1991; Agusa et al. 2005). Mercury concentrations in feathers result mostly from endogenous deposition of blood-circulating Hg and are not or slightly affected by external deposition (Burger and Gochfeld 1997; Dauwe et al. 2003). Since the transfer of blood-circulating substances in feathers is interrupted after total feather growth, Hg is trapped and remains stable, bonded to keratin fibbers, mainly in the form of MeHg (Furness et al. 1986).

However, the interpretation of Hg concentrations in feathers for biomonitoring purposes is not straightforward. There is no general agreement on which factors influence Hg deposition, and the biological meaning of the observed between-feathers variation is still unclear. While some authors recommend the use of smaller body feathers for Hg quantification (Furness et al. 1986; Solonen and Lodenius 1990), others state that feathers cannot be indiscriminately selected and therefore flight feathers (remiges) should be used, given they can be consistently located (Bortolotti 2010). The correlations found in many bird species between Hg concentration in primary feathers and species-specific moulting sequence (i.e. feathers replaced earlier have higher Hg concentrations) are generally interpreted as a cause-effect pattern linked to Hg deposition: (1) circulating Hg levels drop as this metal is retained in growing feathers (Lindberg and Odsjö 1983; Furness et al. 1986; Dauwe et al. 2003); or (2) individuals select less contaminated prey during the moult than before (Lindberg and Odsjö 1983). However, it is also hypothesized that this pattern is an artefact of variation in feather mass for elements whose incorporation is time dependent, such as Hg. Thus heavier (and often longer) feathers show a more diluted concentration since they have a wider growth period (Bortolotti 2010). Moreover, there is evidence that the decrease in Hg concentrations along with the moult sequence is not generalized to all species. For instance, a study with barn owl (*Tyto alba*) primaries did not show any relationship between the two (Dauwe et al. 2003).

Owing to its ecological requirements and its closeness to humans, the barn owl is potentially a good sentinel of environmental Hg contamination, particularly in farmland habitats. This owl is a generalist and opportunistic predator that hunts in open farmland, feeding mostly on small mammals, and in many regions using man-made structures (e.g. barns, sheds, old houses) as nesting sites (Bunn et al. 1982; Roulin 2002). The same nests may be continuously monitored for long time periods: at nest sites, feathers can be collected from nestlings and sometimes shed flight feathers (from adults' moult) are also available (adults can also be captured to take feather samples). Another straightforward source of barn owl feathers for ecotoxicological analysis is collecting carcasses on roadsides. Owls are frequent victims of collision with vehicles, as for example the road-killing estimates of 0.35–0.49 owls/km/year for Southern Portugal (Silva et al. 2008; Gomes et al. 2009; Grilo et al. 2014). Literature reporting Hg levels measured in owl feathers is still modest (see review in Espín et al. 2014), and to the best of our knowledge only a few studies have analysed Hg in barn owl feathers (Westermark et al. 1975; Denneman and Douben 1993; Dauwe et al. 2003; Lourenço et al. 2002). None of these studies examined the implications of feather sampling methods.

For ethical and legal reasons sampling live birds requires the use of non-invasive methods. Body feathers from the breast are therefore frequently used, since they are easy to pluck and it is possible to collect a few without causing harmful effects to the bird. Also, since body feathers can be collected from both live and dead individuals (while not possible for blood samples) these tissues are good candidates for a standard assessment of environmental contamination levels. Therefore, considering that many ecotoxicological studies rely mostly on opportunistic sampling, i.e. with access to a limited number and/or type of tissue samples, it is important to understand how the characteristics of the available samples affect the accuracy of the results and thus the quality of the conclusions.

Our main goal in this study is to verify if feathers of different types and also flight feathers (remiges) varying in size and position in the wing show considerable variation in Hg levels, independently of feather age, with

implications in the use of barn owl feathers as biomonitors and in sampling procedures. We focused on feathers collected from road killed un-moulted barn owls (moult starts in the 2nd calendar year; Martínez et al. 2002), thus restricting the analysis to feathers from the same generation, which were simultaneously developed while the birds were nestlings (i.e. in each individual the available Hg in blood during growth is identical for all feathers). We tackled the following issues: (1) is the variation in Hg concentration between body and flight feathers small, so that these feather types can be interchangeably used to compare contamination levels in different sites? and (2) is the Hg concentration in flight feathers similar despite feather length, mass and position in the wing, so that remiges (primary and secondary feathers) can be indiscriminately used to assess environmental Hg contamination?

## Methods

### Study area

Samples were collected along roads in central Portugal, between Vila Franca de Xira and Évora (7°53′–8°59′W; 38°32–38°59′N). The climate in the study area is Mediterranean, with mild winters and hot dry summers, and the rain period mainly concentrated in winter. Landscape is mostly plain or undulating and is dominated by cork oak *Quercus suber* and holm oak *Quercus rotundifolia* traditional woodland systems named 'montados', with varying tree density. 'Montados' are managed for different uses (e.g. cork extraction, grazing, cereals), resulting in a multifunctional landscape. Agricultural areas occupy 10–30 % of the study area and consist mainly of irrigated annual cultures, rice fields, rainfed cereal crops, vineyards and olive groves.

### Sampling procedures

A total of 154 feathers were plucked from 28 barn owl carcasses collected on roadsides from 2009 to 2012: 29 samples of body feathers, 62 primary feathers and 64 secondary feathers. Whenever possible, five feather samples were collected from each individual: (1) at least three body feathers from the breast, and (2) one primary feather from the outermost group (P10–P6), (3) one primary feather from the innermost group (P5–P1), (4) one secondary feather from the outermost group (S1–S6) and (5) another secondary feather from the innermost group (S7–S12), in order to represent all the wing length. Feathers were stored in transparent plastic bags until analysis. We followed the feather numbering system of Martínez et al. (2002). Regarding position in the wing, flight feathers were numbered from 1 to 24 from the outermost primary (P10) to the innermost secondary (S14). Feather mean mass (dry weight) and length were obtained by weighing and measuring all flight feathers from the right wing of two barn owls in the range of extreme wing lengths for the species (277 and 296 mm; range in our data: 269–295 mm ($n = 12$ individuals); range in Martínez et al. 2002: 270–300 mm). Prior to weighing, feathers were dried in an oven for 2 h at 35 °C in order to remove excess moisture resulting from freeze storing. Feathers were weighed on a precision scale (0.1 mg) and measured with a wing ruler (1 mm).

### Mercury analysis

Total Hg concentration in feather samples was determined by thermal atomization followed by atomic absorption spectroscopy, using an AMA-254 spectrophotometer (LECO, Czech Republic). This methodology is simple, and requires minimum sample handling prior to analysis, since no digestion procedure or sample pre-treatment is necessary. Homogenized, dried samples are placed into a pre-cleaned combustion boat and inserted in a quartz combustion catalytic tube. The sample is firstly dried at 120 °C prior to combustion at 680–700 °C in an oxygen atmosphere. The mercury vapour is collected in a gold amalgamator and after a delay period heated at 900 °C. The released mercury is transported to a heated (120 °C) cuvette and then quantified by atomic absorption spectroscopy using a silicon UV diode detector (for more details please see Costley et al. 2000). Given the reduced mass of a single body feather, for analytical reasons mean Hg concentration was calculated analysing a pool of body feathers per individual. Concerning single flight feathers, Hg concentration was determined using the mean of the measurements in successive cuts starting from the distal part of the feather. All Hg concentrations are presented in mg $kg^{-1}$ on a dry weight basis.

### Quality assurance

Precision, accuracy and analytical detection limits were continuously monitored as means of assessing analytical performance, and hence the validity of results. Sample treatment and analyses were performed using ultra-clean protocols. All glassware used was previously soaked for at least 24 h in a bath containing 5 % Decon, then 24 h in 25 % $HNO_3$ and finally thoroughly rinsed with ultra-pure water obtained from a Millipore Milli-Q Integral System.

Precision was assessed through the analysis of dispersion between replicate analyses. Acceptance criteria were established (three replicate results with relative standard deviation below 10 %) above which samples were re-analysed.

The main tool employed to measure analytical accuracy was parallel analyses of certified reference materials (CRM), using reference material (TORT-2) throughout the day to assure correct response of the equipment. No significant differences ($p < 0.05$) were found between the certified concentration (±confidence interval) and the laboratory concentration (±confidence interval) for all CRM replicate analyses.

The analytical limit of detection (LOD) of the methodology was mass dependent, given the overall 0.01 ng absolute Hg LOD. Therefore, for a 100 mg sample, the detection limit was considered as 0.1 ng $g^{-1}$.

### Statistical analysis

The data were screened to detect outliers and check distribution normality of the variables (Quinn and Keough 2002), and a logarithmic transformation was applied to the variable Hg concentration. Linear mixed-effects models (Pinheiro and Bates 2000) were used in order to evaluate the variation of the mean Hg concentration (1) between body and flight feathers (sample size = 154 feathers, from 28 individuals) and (2) according to position on the wing and mass of flight feathers (sample size = 125 feathers, from 28 individuals). We included the individual as a random effect in all models, since for each individual we had several feather samples. In a first analysis, feather type (body (B), primary (P) and secondary (S)) was used as the fixed factor; and in a second analysis feather position in the wing, feather mean length and feather mean mass were used as fixed effects. Since the three variables used in the second analysis were highly correlated (Pearson $r > 0.7$), competing models with one fixed effect only were built. Information-theoretic methods were used for model inference based in AICc values–second-order Akaike's information criterion (Burnham and Anderson 2002; Burnham et al. 2011). This criterion measures the contribution of each candidate model to explain the variation in Hg concentration, with a lower AICc scoring a best fitting model (Burnham and Anderson 2002). For each model it was calculated the number of parameters (degrees of freedom), log-likelihood value, AICc difference (ΔAICc), Akaike weight ($w_i$; i.e. the probability of each model given the data and the models considered), and evidence ratio. The random effects model (i.e. a model with intercept and random effects, but without fixed effects) was included in model selection to provide inferential information (Burnham et al. 2011). Model diagnostic plots were used to validate model results (Pinheiro and Bates 2000). All statistical analyses were conducted using R software 3.1.1 (R Core Team 2014) with packages gplots (Warnes et al. 2015), MuMIn (Barton 2015), nlme (Pinheiro et al. 2015).

## Results

### Inter and intra-individual mercury variation

Median Hg concentration measured in 154 feather samples from 28 un-moulted barn owls, was 0.47 mg $kg^{-1}$ (range: 0.040–4.9 mg $kg^{-1}$). Corresponding mean (±standard deviation; SD) Hg concentration was 0.62 ± 0.76 mg $kg^{-1}$ and geometric mean was 0.41 mg $kg^{-1}$. Mean Hg per individual ranged between 0.054 and 3.7 mg $kg^{-1}$, with a corresponding inter-individual SD of 0.70 mg $kg^{-1}$. Intra-individual SD in Hg concentration (i.e. Hg measurements in different feathers from a same individual) ranged between 0.013 and 1.7 mg $kg^{-1}$, with a mean intra-individual SD of 0.21 mg $kg^{-1}$. These results indicate that inter-individual variation in mean Hg concentration is in general higher than intra-individual variation in Hg measurements (Fig. 1).

### Effect of feather type on mercury concentration

Median Hg concentration was 0.45 mg $kg^{-1}$ in body feathers (range: 0.076–4.5; $n = 29$), 0.44 mg $kg^{-1}$ in primary feathers (range: 0.040–4.9; $n = 62$) and 0.60 mg $kg^{-1}$ in secondary feathers (range: 0.042–4.7; $n = 63$). Corresponding mean Hg concentration was 0.72 ± 0.94 mg $kg^{-1}$ in body feathers, 0.59 ± 0.77 mg $kg^{-1}$ in primary feathers and 0.60 ± 0.66 mg $kg^{-1}$ in secondary feathers. Geometric mean Hg concentration was, respectively, 0.43, 0.39 and 0.42.

Our data supported best the random effects model ($w_i = 0.95$) compared to the model testing the effect of feather type on Hg concentration ($w_i = 0.05$; Table 1). The evidence ratio for the two models indicated that the empirical support for the random effects model was 2.6 times that of the model including the variable feather type. These results suggest that the feather type did not have a strong effect on Hg concentration.

### Effect of flight feather mass, length, and position in the wing on mercury concentration

Feathers with highest and lower median Hg concentration were P5 (0.78 mg $kg^{-1}$; range: 0.63–0.92) and P9 (0.19 mg $kg^{-1}$; range: 0.097–0.59), respectively. Considering mean Hg concentrations, feathers with highest and lower values were P6 (1.4 ± 1.5 mg $kg^{-1}$) and P9 (0.22 ± 0.14 mg $kg^{-1}$), respectively. Hg concentrations apparently followed no order from inner to outer position in the wing and did not reflect a consistent between-feather pattern, i.e. the difference in Hg concentration between each feather and the previous one was not systematically

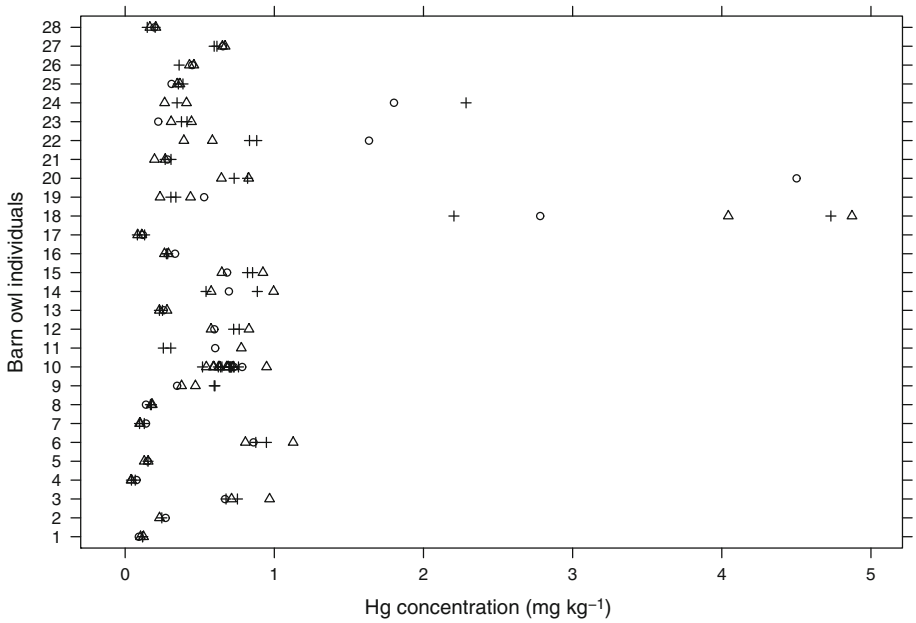


**Fig. 1** Mercury concentration (mg $kg^{-1}$) measurements in all barn owl individuals by feather type: body feathers (*circles*); primary feathers (*triangles*); secondary feathers (*crosses*)

**Table 1** Information-theoretic model selection results for the analysis of the effect of feather type on Hg concentration in un-moulted barn owls

| Model | df | Log-likelihood | AICc | ΔAICc | Akaike weight ($w_i$) |
|---|---|---|---|---|---|
| Random effects model (intercept + random effect) | 3 | −90.96 | 188.07 | 0.00 | 0.95 |
| Feather type + random effect | 5 | −91.77 | 193.95 | 5.88 | 0.05 |

positive or negative regarding position in the wing (Table 2, Fig. 2). The information-theoretic analysis of the effects of feather mass, length and position in the wing on Hg concentration, showed that our data supported best the model with feather mass ($w_i = 0.69$; evidence ratio to second best model = 2.2; Table 3). However, the random effects model also received some support, with a probability ($w_i$) of 0.31 of being the best model ($\Delta AICc = 1.62$). The models with feather length and position in the wing were little supported by our data. These results suggest that when analysing flight feathers from the same barn owl individual, feathers with lower mass may often show higher Hg concentration, however mass does not seem to have a very strong and clear effect (Table 4). On the other hand, both feather length and its position in the wing have no strong linear relationship with Hg concentration in barn owls.

## Discussion

### Mercury contamination in barn owl feathers

In general, the Hg concentrations we measured in barn owl feathers ($0.62 \pm 0.76$ mg $kg^{-1}$) were below the concentrations previously detected for this species in the Iberian Peninsula ($1.2 \pm 1.1$ mg $kg^{-1}$ in body and flight feathers, $n = 13$; Lourenço et al. 2002), in Belgium ($0.77 \pm 0.44$ to $0.90 \pm 0.53$ mg $kg^{-1}$ in primary feathers, $n = 5$; Dauwe et al. 2003), in the Netherlands ($1.8 \pm 0.93$ mg $kg^{-1}$ in primary feather P4, $n = 3$; Denneman and Douben 1993) and in Sweden ($15 \pm 32$ mg $kg^{-1}$ in indiscriminate feathers, range 0.4–6.0 mg $kg^{-1}$ during alkyl Hg ban and 0.19–126 mg $kg^{-1}$ during alkyl Hg use in agriculture, $n = 16$; Westermark et al. 1975). The toxicity threshold for Hg is highly variable among bird species and reported sub-lethal effects are mainly associated with reproductive impairment (Scheuhammer et al. 2007). Concentrations from 2.4 mg $kg^{-1}$ in body feathers have been reported to cause a reduction in nest success by 10 % in songbirds (Jackson et al. 2011), whereas concentrations over 40 mg $kg^{-1}$ are associated with sterility in the white-tailed eagle *Haliaaetus albicilla* (Berg et al. 1966). In our data set, 3 % of samples (five samples from two individuals) showed Hg concentrations in the range of the values reported to produce negative effects on terrestrial birds (between 2.8 and 4.9 mg $kg^{-1}$). Therefore, despite in our study area barn owls are in general not exposed to very high Hg contamination, we should consider some of our values as sufficiently high to potentially impair

**Table 2** Mercury concentration (mean ± standard deviation, geometric mean, median and range), mean mass and mean length for primary (P10–P1) and secondary (S1–S13) feathers of un-moulted barn owls, ordered from the outermost to the innermost feather

| Feather | Hg (mg $kg^{-1}$) | Mean mass (g) | Mean length (mm) | Sample size |
|---|---|---|---|---|
| P10 | 0.52 ± 0.27<br>0.46<br>0.51 (0.20–0.97) | 0.4600 | 226 | 6 |
| P9 | 0.24 ± 0.18<br>0.20<br>0.19 (0.097–0.59) | 0.4518 | 238 | 6 |
| P8 | 0.37 ± 0.27<br>0.26<br>0.37 (0.040–0.78) | 0.4261 | 237 | 9 |
| P7 | 0.44 ± 0.22<br>0.40<br>0.38 (0.23–0.81) | 0.3627 | 221 | 7 |
| P6 | 1.4 ± 1.5<br>0.97<br>0.72 (0.43–4.0) | 0.3091 | 209 | 5 |
| P5 | 0.78 ± 0.21<br>0.77<br>0.78 (0.63–0.92) | 0.2709 | 194 | 2 |
| P4 | 0.43 ± 0.22<br>0.36<br>0.45 (0.11–0.68) | 0.2251 | 180 | 7 |
| P3 | 1.1 ± 1.6<br>0.59<br>0.56 (0.10–4.8) | 0.2007 | 171 | 8 |
| P2 | 0.42 ± 0.32<br>0.29<br>0.34 (0.042–0.83) | 0.1861 | 165 | 8 |
| P1 | 0.45 ± 0.30<br>0.36<br>0.48 (0.12–0.71) | 0.1772 | 157 | 4 |
| S1 | 0.52 ± 0.34<br>0.35<br>0.68 (0.042–0.88) | 0.1493 | 152 | 5 |
| S2 | 0.26 ± 0.15<br>0.22<br>0.25 (0.096–0.52) | 0.1542 | 153 | 7 |
| S3 | 1.1 ± 1.6<br>0.56<br>0.71 (0.13–4.7) | 0.1502 | 153 | 7 |
| S4 | 0.58 ± 0.21<br>0.53<br>0.61 (0.20–0.83) | 0.1396 | 146 | 6 |
| S5 | 0.42 ± 0.19<br>0.40<br>0.32 (0.26–0.62) | 0.1321 | 144 | 3 |

**Table 2** continued

| Feather | Hg (mg $kg^{-1}$) | Mean mass (g) | Mean length (mm) | Sample size |
|---|---|---|---|---|
| S6 | 0.55 ± 0.29 | 0.1222 | 141 | 2 |
| | 0.51 | | | |
| | 0.55 (0.35–0.76) | | | |
| S7 | 0.73 ± 0.086 | 0.1150 | 137 | 3 |
| | 0.73 | | | |
| | 0.73 (0.65–0.82) | | | |
| S8 | 0.66 ± 0.79 | 0.1118 | 135 | 6 |
| | 0.41 | | | |
| | 0.39 (0.13–2.2) | | | |
| S9 | 0.41 ± 0.33 | 0.1047 | 137 | 9 |
| | 0.30 | | | |
| | 0.28 (0.067–0.89) | | | |
| S10 | 0.67 ± 0.078 | 0.0979 | 127 | 4 |
| | 0.66 | | | |
| | 0.66 (0.60–0.75) | | | |
| S11 | 0.85 ± 0.88 | 0.0881 | 124 | 5 |
| | 0.49 | | | |
| | 0.70 (0.082–2.3) | | | |
| S12 | 0.57 ± 0.32 | 0.0733 | 117 | 5 |
| | 0.47 | | | |
| | 0.73 (0.15–0.85) | | | |
| S13 | 0.639 | 0.0493 | 102 | 1 |

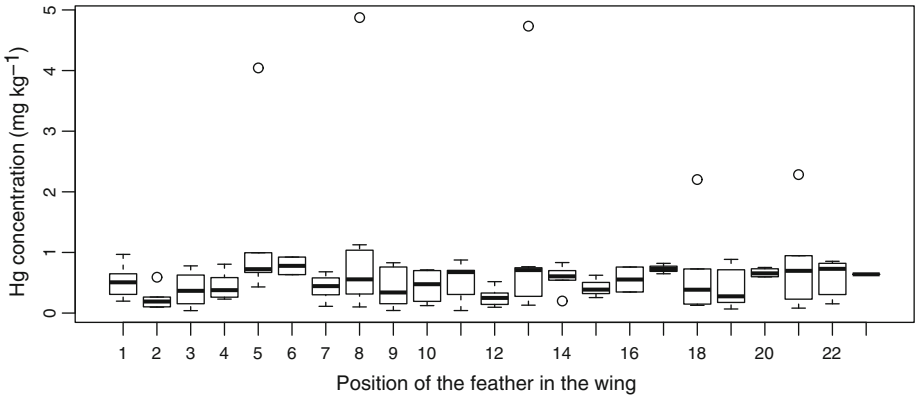


**Fig. 2** Mercury (Hg) concentration (mg $kg^{-1}$) in barn owl feathers with different position in the wing, from outermost primary feather—P10 (1) to innermost secondary feather—S13 (23). *Box* and *whisker plots* show the median, 25 % quartiles and range

**Table 3** Information-theoretic model selection results for the analysis of the effect of feather mass, length and position in the wing on Hg concentration in un-moulted barn owls

| Model | Df | log-likelihood | AICc | ΔAICc | Akaike weight ($w_i$) |
|---|---|---|---|---|---|
| Feather mass + random effect | 4 | −60.29 | 128.91 | 0.00 | 0.69 |
| Random effects model (intercept + random effect) | 3 | −62.17 | 130.53 | 1.62 | 0.31 |
| Feather position + random effect | 4 | −64.80 | 137.93 | 9.02 | 0.01 |
| Feather length + random effect | 4 | −66.43 | 141.20 | 12.28 | 0.00 |

**Table 4** Model results for the analysis on the effect of feather mass on Hg concentration in un-moulted barn owls

| Fixed effect | Estimate | SE | df | *t* | *p* |
|---|---|---|---|---|---|
| Intercept | −0.92 | 0.17 | 96 | −5.29 | <0.001 |
| Feather mass | −0.43 | 0.18 | 96 | −2.32 | 0.022 |
| Random effect | SD—intercept (between-individual variation) | | SD—residuals (within-individual variation) | | |
| Individual | 0.882 | | 0.252 | | |

reproduction. Nevertheless, we cannot completely exclude the possibility that the highest values reported in this study could have resulted from sporadic external contamination, such as small particles retained in feathers.

### Mercury contamination in body feathers versus flight feathers

Our results suggest that either body feathers from the breast, primaries and secondaries are adequate to evaluate Hg levels in first-year barn owls, since no consistent differences between these three feather types were observed. Thus, opportunistic sampling should be applicable provided mean concentrations are calculated from several feathers: given the considerable variation in Hg levels between different feathers, irrespectively of feather type, it is advisable to use more than one feather to estimate an Hg value per individual. If this procedure is adopted, it is expected that Hg concentration measured in a juvenile could be considered a reliable indicator of local contamination (i.e. the area surrounding the nest site).

### Mercury concentration in flight feathers: variations with position in the wing, feather mass and length

The effect of feather position in the wing seems to be small as it showed no linear relationship with Hg variation in our data set. However, the widest range in Hg concentration was found among the outermost primaries (between P9 and P5–P6), and hence we recommend caution when using these particular feathers to estimate and compare Hg contamination in barn owls, mainly in studies with small sample sizes. Greater variation between primaries than other parts of plumage was also reported for other species (Furness et al. 1986). Given the negative effect of feather mass on Hg concentration (due to dilution), the largest outermost primaries, being the heaviest feathers in the barn owl wing, might contain lower Hg concentrations when compared to smaller feathers. However, while feather length and mass in general decreases inwards (P10–S13; with exception of an increase in length in P10–P9 and in both mass and length in S1–S2), our results did not show a comparable trend inwards-outwards in Hg concentration in barn owl remiges, contradicting the general pattern described in the literature (see Bortolotti 2010 and references therein).

Bortolotti (2010) has demonstrated that the relationship between the position of a primary and its relative mass is the inverse of the relationship between the position of a primary and its relative Hg concentration. Based on this finding he proposed that Hg concentration in primaries is confounded by a variation in feather mass. In his data ($n = 5$ individuals; adapted from Furness et al. 1986), Hg concentrations followed the general pattern of contaminant concentrations found in several studies: a decrease from P1 to P8 or P9 and then an increase in P10. In our study, although the relationship between the position of barn owl primaries and their relative mass and length followed a pattern similar to that found by Bortolotti (2010), the relative Hg concentrations in primaries showed a very different pattern (Fig. 3a), thus supporting independence from relative feather mass and length. Despite the current poor understanding of feather physiology, Bortolotti (2010) also hypothesized that Hg passively accumulates in the feather in a time-dependent manner, i.e. the length of time growing cells are exposed while Hg passes from the circulation to the growing feather is critical. Hence, we suggest that differences in the growth rate, i.e. in daily increase in feather mass and/or length, should be determinant to differences in Hg concentrations between feathers. Therefore, the pattern found by Bortolotti may not illustrate a cause-effect relationship between feather mass and Hg concentration but is eventually a consequence of the position of the primaries in his data set being correlated with the growth rates of individual feathers.

The post-moult growth of the outermost primaries was described for barn owl by Lenton (1984), and their daily increase in length and mass can be calculated from his data. The pattern of mean Hg concentration we found among the outermost primary feathers is concurrent with Hg deposition being influenced by differences in daily increase in mass and length during feather growth: both increase from P10 to P8, then decrease to P6 and rise again in P5 (Fig. 3b). Mean Hg concentration showed an opposite

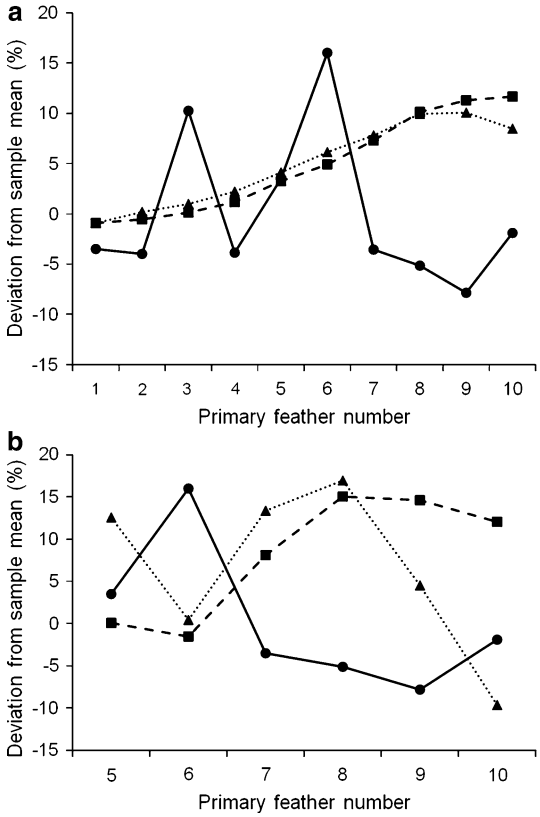


**Fig. 3** Patterns of variation in Hg concentration (*circles* and *solid line*) in barn owl primary feathers versus feather size (**a**) and growth rate (**b**). *Squares* and *dashed lines* represent feather mass (**a**) and daily increase in feather mass (**b**); *triangles* and *dotted lines* represent feather length (**a**) and daily increase in feather length (**b**). All values are expressed in % deviation from the sample mean. Daily increase in mass and length (**b**) was adapted from Lenton (1984) and was only available for the six outermost primaries (P5–P10). *Lines* joining *points* are for visual emphasis

trend, with its highest value in P6, which is the primary with the lowest daily increase in mass and length. Our data is in agreement with this rationale, since feather mass and not length showed a stronger effect on Hg concentration, and accordingly differences between feathers are more pronounced in the daily increase in mass than in length (Fig. 3b). Moreover, feather mass and length do not seem to fully explain the total Hg excreted in the feather (mean Hg concentration multiplied by feather mass), since relative excreted Hg follows the pattern of Hg concentration irrespectively of feather size (Fig. 4). Differences in Hg concentrations can be so accentuated that a smaller and lighter feather could excrete more Hg (see for example P6 and P9). Therefore, the contribution of a single feather to Hg elimination may be more dependent on its susceptibility to incorporate Hg due to its growth rate than on its size, suggesting that besides mass and length other factors, possibly related to feather physiology, can be determinant to the process of Hg deposition in feathers.

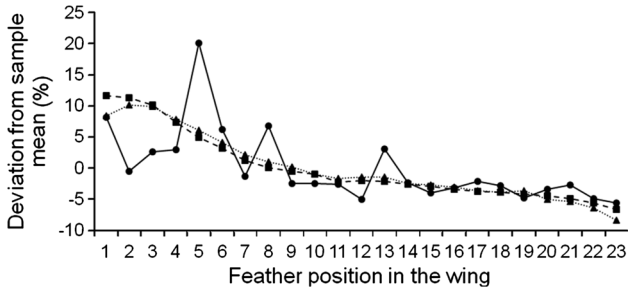


**Fig. 4** Variation in the relative estimated amount of Hg excreted (*circles* and *solid line*) in barn owl flight feathers versus relative feather mass (*squares* and *dashed lines*) and length (*triangles* and *dotted lines*). All values are expressed in % deviation from the sample mean. *Lines* joining points are for visual emphasis. Feathers are ordered from outermost primary feather—P10 (1) to innermost secondary feather—S13 (23)

Although in our study the feather with the lowest Hg concentration is simultaneously the longest feather in the barn owl wing (P9), in Lenton (1984) P8 was the longest feather and had the highest daily increase both in mass and length (most likely this reflects the deviation to the pattern in Fig. 3b). Since feather morphogenesis is genetically determined (Yu et al. 2002), we assume that feather growth pattern is equivalent in juveniles and adults. Nevertheless, further detailed studies on feather development in barn owl nestlings are needed.

Our study seems to support the hypothesis that Hg deposition is time dependent as stated by Bortolotti (2010). However, our results suggest that feather growth rate is possibly the main determinant of differences in Hg concentration found in flight feathers. Future studies with detailed data on growth rate of all flight feathers in barn owl nestlings are needed to confirm this hypothesis. As a consequence of this conclusion, the correction method suggested by Bortolotti (2010) of using length as a proxy of time for quantifying Hg in feathers (instead of concentration calculated as Hg mass divided by sample mass) may not be valid for the barn owl (and possibly for other bird species as well), since differences in feather length do not fully represent differences in feather growth rate.

## Implications to sampling procedures

The use of barn owl feathers to biomonitor Hg contamination, as in raptors in general, is often subjected to sampling and analytical constraints. If researchers are sampling live birds, the most ethical option is to collect a few body feathers. On the other hand, in studies relying on bird carcasses or shed feathers found in nest sites and perches, the limitations are related to feather availability and the sample size that can be analysed. Such opportunistic sampling implies that for some individuals or sites only a

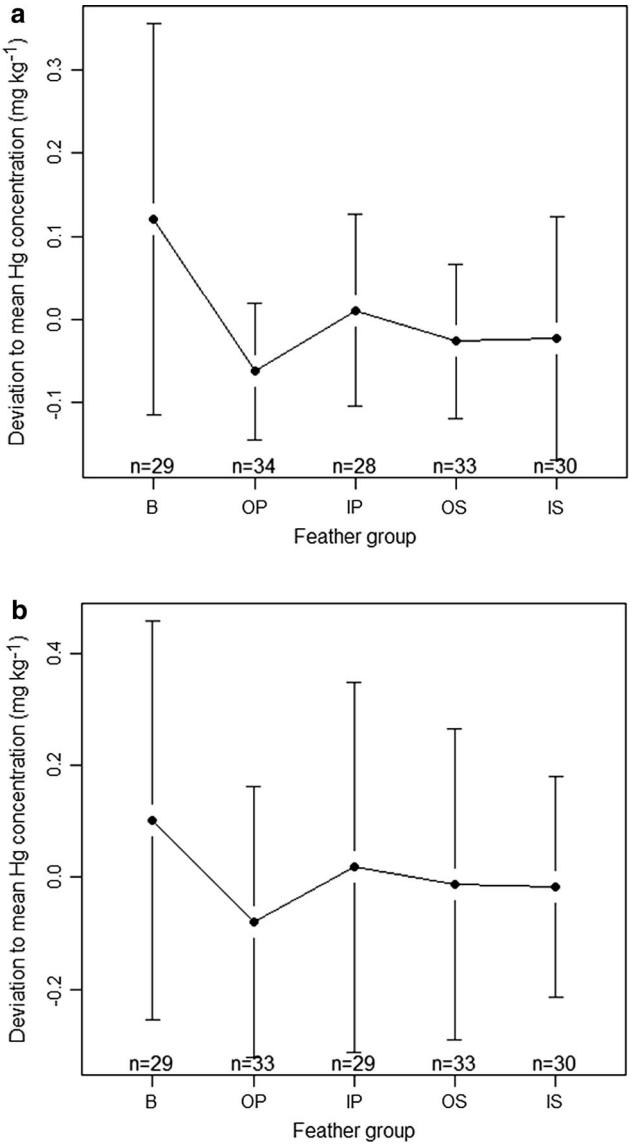


**Fig. 5** Inter-feather **a** and intra-individual **b** deviations to mean Hg concentration. *B* body feathers. Remiges are grouped in mass classes: *OP* mean mass 0.402 g (5 outermost primaries); *IP* mean mass 0.212 g (5 innermost primaries); *OS* mean mass 0.138 g (7 outermost secondaries); *IS* mean mass 0.088 g (6 innermost secondaries). *Dots* represent mean and *error lines* represent 95 % confidence intervals. The *line* adjoining *dots* is for visual emphasis

certain type or number of feathers can be used. Sampling constraints are particularly restrictive when relying on shed feathers, because the barn owl has a complex moult and can shed a small number of feathers in some years (1–2 feathers, Martínez et al. 2002). Moreover, the exact position in the wing is seldom identifiable in shed feathers (exception to P10, owing to its particular structure) and also age is undetermined, meaning additional variability is introduced by possible differences in bioaccumulation when using moulted feathers.

Our results suggest that it is not crucial to discriminate between feather type, position in the wing and length, since these characteristics seem to have little importance on the feather ability to accumulate Hg. The similarity of Hg concentrations between feather types and among wing feathers with different lengths and positions was also reported for other species (Lindberg and Odsjö 1983; Martínez et al. 2002; Calle et al. 2015). Considering that simultaneously-grown remiges (i.e. with equal Hg concentration available in the blood) differ in their ability to incorporate Hg because they have different growth rates, then the best estimate of individual Hg level should overcome between-feather variation. To accomplish this, a mean value should be obtained by using several feathers from the same individual. Moreover, in obtaining the best estimate possible of the individual mean Hg concentration, intra-individual variation should also be considered.

Since mass has a dilution effect in Hg concentration in remiges, we grouped these feathers in four classes by decreasing mean mass, in order to examine the effect of mass in the deviations to mean Hg concentrations: the five outermost primaries (OP–0.40 g), the five innermost primaries (IP–0.21 g), the seven outermost secondaries (OS—0.14 g) and the six innermost secondaries (IS—0.088 g). The group that contributes to minimise both inter-feather and intra-individual variations includes the five innermost primaries (Fig. 5). Accordingly, under a restrictive scenario, i.e. when choices must be done on which feathers to analyse, feathers from this group seem the best possible option (i.e. primaries 5–1). Its average deviations from sample and individual mean Hg concentration are low (respectively $-0.019$ and $-0.009$ mg $kg^{-1}$). This roughly corresponds to remiges in the range of length of 157–194 cm. Although this criterion is by principle applicable to adult birds, the increasing trend in Hg with age and the complex stepwise moult of the barn owl are probably more relevant to explain differences in Hg concentrations between feathers in adults than feather growth rate.

**Acknowledgments** IR was supported by a doctoral Grant (SFRH/BD/72163/2010), and RL and JPC by post-doctoral degree Grants (BPD/78241/2011 and BPD/102870/2014, respectively) from Fundação para a Ciência e a Tecnologia—Portugal. Fieldwork was partly supported by QREN/INALENTEJO 2007–2013 under the project ECOMEDBIRDS (ALENT-04-0331-FEDER000205) and by Companhia das Lezírias, S.A. through a Business & Biodiversity Protocol with the University of Évora, under the project TYTOTAGUS.

**Compliance with ethical standards**

**Conflict of interest** The authors have no known conflicts of interest associated with this publication and there has been no financial support for this work that could have influenced its outcome.

**Human and animal rights** All samples were obtained under the permits of Instituto da Conservação da Natureza e das Florestas (Portugal) numbers: 40, 204-205, 265/2009/CAPT; 165-166/2010/CAPT and 258-260/2012/CAPT (IR, RL and AM). All applicable international, national, and/or institutional guidelines for care and use of animals were followed.

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