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Assessing Contamination Profiles in Livers from Road-Killed Owls

Maria Dulsat-Masvidal,[a] Rui Lourenço,[b] Rafael Mateo,[c] and Silvia Lacorte[a,*]

[a]Department of Environmental Chemistry, Institute of Environmental Assessment and Water Research (IDAEA-CSIC), Barcelona, Spain
[b]Mediterranean Institute for Agriculture, Environment and Development, Universidade de Évora (MED), Laboratory of Ornithology, and Global Change and Sustainability Institute (CHANGE), University of Évora, Évora, Portugal
[c]Institute for Game and Wildlife Research (IREC), Consejo Superior de Investigaciones Científicas, Universidad de Castilla-La Mancha, Junta de Comunidades de Castilla-La Mancha (CSIC-UCLM-JCCM), Ciudad Real, Spain

Abstract: Raptors are recognized as valuable sentinel species for monitoring environmental contaminants owing to their foraging behavior across terrestrial and aquatic food webs and their high trophic position. The present study monitored environmental contaminants in livers from road-killed owls to evaluate differences in the exposure patterns due to factors such as species, age, and sex of individuals. Carcasses of road-killed individuals of eagle owl (*Bubo bubo*), long-eared owl (*Asio otus*), little owl (*Athene noctua*), tawny owl (*Strix aluco*), and barn owl (*Tyto alba*) were collected in Alentejo (Portugal). Eighty-one organic contaminants were analyzed, including organochlorine pesticides (OCPs), per- and polyfluoroalkyl substances (PFAS), polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), pharmaceuticals, in-use pesticides, and organophosphate esters (OPEs). Overall, 21 contaminants were detected. In all species ∑OCPs were prevalent at concentrations from 3.24 to 4480 ng/g wet weight, followed by perfluorooctane sulfonic acid (PFOS), the only PFASs detected (from 2.88 to 848 ng/g wet wt) and ∑PCBs (1.98–2010 ng/g wet wt); ∑PAHs were ubiquitous but detected at the lowest concentrations (7.35–123 ng/g wet wt). Differences among species were observed according to principal component analysis. Eagle owl and long-eared owl presented the highest levels of ∑OCPs, ∑PCBs, and PFOS, consistent with its higher trophic position, while ∑PAHs prevailed in tawny owl, barn owl, and little owl, related to their frequent use of urban areas for nesting and roadsides for hunting. Adults presented higher concentrations of ∑OCPs and ∑PCBs than juveniles, while no differences were observed for PFOS and ∑PAHs. Pharmaceuticals, in-use pesticides, and OPEs were not detected. Overall, the present study shows specific contamination patterns in five species with similar diet but with differences in habitat preferences. *Environ Toxicol Chem* 2024;00:1–12. © 2023 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

Keywords: Biomonitoring; Contaminants; Wildlife toxicology; Birds; Organic contaminants

INTRODUCTION

Birds are exposed to a myriad of organic contaminants, affecting the well-being of many species and posing a serious threat to biodiversity and ecosystem services. Most studies focus on persistent organic pollutants such as organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs), and per- and polyfluoroalkyl substances (PFAS), which are global environmental contaminants despite efforts to regulate them (Stockholm Convention, 2019). Other contaminants affecting birds are polycyclic aromatic hydrocarbons (PAHs), which, despite being metabolized, are recurrently detected in bird species as a result of exposure to petroleum and combustion (Custer et al., 2001). Finally, the presence and impact of emerging contaminants such as pharmaceuticals, in-use pesticides, or plasticizers in birds are still under discussion, with only a few studies reporting their presence (González-Rubio et al., 2021). Contaminants have been detected in eggs, feathers, blood, livers, and other internal tissues (Espín et al., 2016), providing evidence on their bioaccumulation through the food web, affecting particularly top predators (Rodríguez-Jorquera et al., 2017).

Biomonitoring programs that use birds as sentinel species have been proven to be a good approach for the early

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* Address correspondence to silvia.lacorte@idaea.csic.es
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detection of adverse effects of contaminants in ecosystems and to assess the effectiveness of legislation (Smits & Fernie, 2013). Raptors have been described as good sentinel species for monitoring legacy and emerging contaminants (Gómez-Ramírez et al., 2014; Movalli et al., 2018), mostly because of their foraging habits through both terrestrial and aquatic food webs and high trophic position (Badry et al., 2020). Also, raptors are a widely studied group of birds with a great number of monitoring schemes that provide data about their population, reproduction, and potential adverse effects caused by contaminants (Derlink et al., 2018). However, working with raptors can also be challenging because they are protected species and sensitive to human disturbance. For this reason, the use of noninvasive and nonintrusive sampling methods to perform monitoring studies with raptors is strongly recommended (Espín et al., 2016). The collection of individuals found dead is a sampling approach that allows the analysis of internal tissues without animal disturbance. Internal tissues such as livers or muscles are excellent matrices to analyze a wide number of pollutants, especially the most persistent ones (Espín et al., 2016).

Roads can represent feeding opportunities for some diurnal raptor species because road-killed animals or prey are abundant along road verges (Hanmer & Robinson, 2021; Meunier et al., 2000). However, for many owls (Strigiformes), roads represent a major threat, with frequent mortality caused by collision with vehicles (Gomes et al., 2009; Santos et al., 2013; van der Horst et al., 2019). Because of their large size, raptor carcasses are often easily detected along roadsides (berms and verges) and reported by citizens to authorities or wildlife rehabilitation centers. Owls are the most frequent victims among raptors (Hanmer & Robinson, 2021). Therefore, road-killed owls can be a valuable source of samples and contextual information to assess the exposure of contaminants in a noninvasive approach.

The present study monitored 81 organic micropollutants, including emerging and legacy compounds, in 47 livers from road-killed owls collected in rural areas of Alentejo (Portugal). Differences in exposure patterns were evaluated in five species: eagle owl (*Bubo bubo*), long-eared owl (*Asio otus*), little owl (*Athene noctua*), tawny owl (*Strix aluco*), and barn owl (*Tyto alba*). These species have similar biology but different habitat preferences, and we discuss the potential causes of exposure according to species and diet.

MATERIALS AND METHODS

Sample collection

Seventy-three road-killed owls found in Alentejo (Portugal) were collected from 2010 to 2019 during regular monitoring of road killings (Santos et al., 2013; van der Horst et al., 2019). Biometric data for all individuals were measured and included wing, tarsus, bill, mouth, weight, wingspan, body length, and ulna (data detailed in Supporting Information, Table S1). Individuals were aged through the molting pattern of feathers and sexed by biometric differences and gonad inspection (Martínez et al., 2002). Necropsies were conducted in all owls to obtain liver samples for chemical analysis. However, nonfresh livers or those with severe damage due to collision were discarded. From the initial 73 carcasses collected, 47 liver samples suitable for chemical analysis were obtained for five owl species breeding in the area: eagle owl ($n = 7$), long-eared owl ($n = 5$), little owl ($n = 12$), tawny owl ($n = 12$), and barn owl ($n = 11$). Samples were frozen at –21 °C and freeze-dried in a lyophilizer (FreeZone; LabConco, Kansas, MO), and the tissue was homogenized with a mortar and a pestle. Liver samples were weighed before and after freeze-drying to determine the water content. The average content of water in livers was 74% for eagle owl, 73% for long-eared owl, 72% for tawny owl, 69% for barn owl, and 71% for little owl.

Chemical standards

Compounds studied are indicated in Supporting Information, Table S2. A total of 81 organic compounds were analyzed including 14 OCPs, seven marker PCB congeners, 16 PAHs, 14 PFASs, 15 pharmaceuticals, 12 in-use pesticides, and three organophosphate esters (OPEs). Certified standards of 98% to 99% purity were purchased from Wellington Laboratories, AccuStandard, Sigma-Aldrich, and Dr. Ehrenstorfer. The surrogate standards used were triphenyl phosphate-d15 (TPhP-d15), acetaminophen-methyl-d3, carbamazepine-d2, lidocaine-diethyl-d10, sulfamethoxazole-d4, isoproturon-d6, estrone-d2, ^{13}C-perfluorooctanoic acid (PFOA; M-PFOA), ^{13}C-perfluorooctane sulfonic acid (PFOS; M-PFOS), and a PAH solution mix (naphthalene d-8, acenaphthene d-10, phenanthrene d-10, chrysene d-12, and perylene d-12; Supporting Information, Table S2). Solvents used were acetonitrile (ACN) purchased from Fisher Scientific Chemical (Bridgewater, NJ), methanol (MeOH) and high-performance liquid chromatography (HPLC)–grade water from Merck, ammonium formate, ammonium acetate, and formic acid from Sigma-Aldrich.

Sample extraction and analysis

The analytical methodology developed to analyze contaminants in blood (Dulsat-Masvidal et al., 2023) was adapted for the analysis of liver samples. Three different extraction and analytical methods were used: Method A for the determination of OCPs, PCBs, and PAHs; Method B for PFAS; and Method C for the determination of pharmaceuticals, in-use pesticides, and OPEs. Results are expressed as nanograms per gram wet weight, taking into account the percentage of water in livers from each species, to be consistent with previous studies and compare our results according to the open bibliography.

Analysis of OCPs, PCBs, and PAHs (Method A). Each freeze-dried liver sample (50 mg) was weighed in a 2-mL polypropylene vial (Eppendorf) and spiked with 50 ng of mixture of internal standards (ISs; naphthalene d-8, acenaphthylene d-10, phenanthrene d-10, chrysene d-12, and perylene d-12). A generic solid–liquid extraction was performed by adding 1.5 mL of hexane:dichloromethane (1:1) into the vials. Samples were vortexed (1 min) and ultrasonicated (10 min), and this procedure was repeated three times without changing solvent.

Samples were centrifuged for 10 min at 2370 rcf (Eppendorf 5415C Centrifuge), and the supernatants were collected. Cleanup was performed with 5-g florisil Bond Elut cartridges (Agilent Technologies) using 30 mL of hexane:dichloromethane (1:1) as the conditioning and elution mixture solvent. The extracts were evaporated to near dryness with ReactiVap® and reconstituted with 250 µL of hexane. Samples were analyzed by gas chromatography (GC) coupled to a triple quadrupole mass spectrometer (Agilent 7890A chromatograph and 7000A MS analyzer; Agilent Technologies) with electron ionization at 70 eV. An HP-5MS Agilent column of 30 m length × 0.25 mm inner diameter × 0.25 µm film thickness (Agilent Technologies) was used for the separation of compounds according to a previous method (Dulsat-Masvidal et al., 2023). The initial temperature was set at 70 °C and kept for 1 min, then increased to 175 °C for 4 min, from 175 °C to 235 °C for 20 min, and to 305 °C for 8 min. The data were processed through MassHunter quantitative software.

Analysis of PFAS (Method B). Each freeze-dried liver sample (50 mg) was weighed in a 2-mL polypropylene vial (Eppendorf) and spiked with 50 ng of the ISs M-PFOA and M-PFOS. Samples were solid–liquid-extracted with 1.5 mL of ACN. Samples were vortexed (1 min) and ultrasonicated (10 min), and this procedure was repeated three times without changing the solvent. After centrifugation (10 min, 2370 rcf), the supernatant was collected in a new vial, and 25 mg of active carbon and 50 µL of glacial acid were added. The extract was vortexed (1 min) and centrifuged (10 min) and extracts filtered through 0.2 µm × 13 mm nylon syringe (Clarify; Phenomenex). Samples were evaporated to dryness with ReactiVap and reconstituted with 100 µL of MeOH and 100 µL HPLC water with 10 mM ammonium acetate. The analysis was performed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) with an electrospray ion source (Waters). An Acquity BEH C18 analytical column (100 mm length × 2.1 mm inner diameter, 1.7 µm particle size) with a VanGuard precolumn was deployed (Acquity). An Acquity BEH C18 trap column (30 mm length × 2.1 mm inner diameter, 1.7 µm particle size) was placed before the injector to avoid PFAS contamination from the chromatographic system. The mobile phase consisted of (A) a mixture of methanol and acetonitrile (80:20) with 10 mM of ammonium acetate and (B) water with 10 mM of ammonium acetate. Gradient elution started at 50% A and 50% B (condition kept for 3 min), increased to 100% B for 7 min, and returned to initial conditions for 5 min. All compounds were measured under negative electrospray ionization (ESI–). The data were processed through MassLynx software.

Analysis of pharmaceuticals, pesticides, and OPEs (Method C). Each freeze-dried liver sample (50 mg) was weighed in a 2-mL polypropylene vial (Eppendorf) and spiked with 50 ng of ISs (acetaminophen-d-13, lidocaine-d-10, isoproturon-d6, TPhP-d-15, and sulfamethoxazole-d-2). Then, 1.5 mL of ACN was added, samples were vortexed and ultrasonicated for 10 min (procedure repeated three times without changing the solvent) and then centrifuged (10 min, 2370 rcf), and the supernatant collected. Samples were directly purified with 30 mg Oasis PRIME HLB cartridges (1 cm^3 cartridge tube; Waters) without prior conditioning, and elution was performed with 1.5 mL of ACN and water (80:20) solution. The samples were evaporated until dryness with ReactiVap and reconstituted with 200 µL of ACN and water (1:1). The analysis was performed by LC-MS/MS with an electrospray ion source (Waters). An Acquity BEH C18 analytical column (100 mm × 2.1 mm internal diameter, 1.7 µm particle size) with a VanGuard C18 precolumn was deployed. The mobile phase consisted of (A) acetonitrile with 0.1% formic acid and (B) water with 0.1% formic acid. Gradient elution started at 5% A and 95% B (3 min hold time) and increased to 100% of A in 21 min (1 min hold time). All compounds were measured under ESI+ except diclofop and furosemide, which were measured with ESI– in the same run. The data were processed through MassLynx software.

Method validation/quality control

Commercial chicken liver was used as a matrix for method validation to evaluate the extraction efficiency. Chicken liver (50 mg freeze-dried, $n = 5$) was spiked with 50 ng of the mixture of target analytes and extracted using the three methods described above. Unspiked chicken liver was also analyzed to ensure the lack of initial contaminant contribution. Also, three procedural blanks (no matrix) were analyzed to determine background contamination. All samples and quality controls were spiked with 50 ng of ISs. The calibration curve was built in hexane from 0.001 to 0.8 ng/µL with IS at 0.05 ng/µL for GC-MS/MS analysis and in methanol and water from 0.001 to 0.3 ng/µL with IS at 0.05 ng/µL for LC-MS/MS methods. Concentrations were calculated using IS quantification. Instrumental limits of detection were calculated as the amount of analyte that gave a signal-to-noise (S/N) ratio of 3 using the standard solution at 0.001 ng/µL. Method detection limits (MDL) were calculated as the concentration that gave an S/N ratio of 3 using spiked liver. Values below the MDL were given a value of zero when the compound was detected in <20% of the samples above the MDL; for compounds detected in >20% of the samples above the MDL, the MDL/2 for each compound was used as a replacement value to handle zero data (Bustnes et al., 2010; Hammond et al., 2023). The frequency of detection of the compounds was calculated considering only those values detected above the MDL. All of the quality parameters of the methods used are detailed in Supporting Information, Table S3, for PAHs, OCPs, and PCBs; in Supporting Information, Table S4, for PFAS; and in Supporting Information, Table S5, for pharmaceuticals, in-use pesticides, and OPEs.

Data analysis

The data distribution was tested through normality plots (Supporting Information, Figure S1); logarithmic transformation ($\log x + 1$) was used to obtain a normal distribution of the variables. Considering that ∑PCBs still presented some skewing in distribution (Supporting Information, Figure S1), we used the nonparametric Kruskal-Wallis test followed by post hoc Dunn test to determine differences between mean chemical groups. When

all tested variables exhibited a normal distribution, two-way analysis of variance followed by the Tukey test were used to assess differences between mean groups. Principal component analysis (PCA) was used to assess the different profiles of contamination among species. The Kaiser-Meyer-Olkin test was used to assess the usefulness of the PCA, where Kaiser-Meyer-Olkin >0.5 indicates variables interdependent enough for using PCA (Dziuban et al., 1979). All statistical analyses were performed in RStudio (R, Ver. 4.0.3), and the figures were elaborated using the ggplot2 and factoextra package.

RESULTS AND DISCUSSION

Occurrence of contaminants in owl livers

All individuals analyzed contained residues of contaminants in livers, indicating exposure to multiple contaminants of different chemical families. A total of 21 out of 81 target compounds were detected in liver samples (Table 1). Supporting Information, Figure S2, lists all compounds according to the detection frequency considering the 5 species. The ∑contaminants ranged from 29.4 to 4661 ng/g wet weight and according to the median values decreased following the order eagle owl > long-eared owl > tawny owl > barn owl > little owl (Figure 1A). Different patterns of contaminants were observed among species, with ∑OCPs being the most prevalent in all species, except for barn owl, followed by ∑PFASs, ∑PCBs, and ∑PAHs (Figure 1B). Barn owl had a profile dominated by ∑PCBs, followed by ∑PFASs and ∑OCPs. Pharmaceuticals, in-use pesticides, and OPEs were not detected in any sample, despite the method being effective to determine those emerging compounds (Supporting Information, Table S5).

Table 1 shows the detection frequency and the mean, minimum, and maximum concentrations of each detected contaminant in the five species. Among chemical families, ∑OCPs were detected at concentrations from 3.34 to 4480 ng/g wet weight, with higher mean values in eagle owl (1197 ± 738) compared to the other species (424 ± 235 ng/g wet wt in long-eared owl to 117 ± 57.2 ng/g wet wt in barn owl). The most ubiquitous OCP was 4,4′-dichlorodiphenyldichloroethylene (DDE; the most stable and toxic metabolite of 4,4′-dichlorodiphenyltrichloroethane [DDT]), which was detected in all individuals at concentrations from 2.19 to 4475 ng/g wet weight (Table 1); 4,4′-DDE is a recognized legacy contaminant detected in biota (Turusov et al., 2002). 2,4′-dichlorodiphenyldichlorethane was detected in 8% to 29% of the analyzed birds at concentrations from 0.06 to 2.58 ng/g wet weight, and other DDT isomers were seldom detected (Table 1), with 4,4′-DDT never being detected, indicating that it has not been used over the last decades, coinciding with the Stockholm Convention (2019) phase out of this compound. Our results are consistent with those of Roque et al. (2022), who found 4,4′-DDE as the most prevalent OCP in livers from road-killed barn owls in Portugal at mean concentrations from 1.93 to 162 ng/g wet weight, lower that the ones found in barn owls in the present study, which were from 2.19 to 636 ng/g wet weight. Also, 4,4′-DDE was the main OCP detected in livers from 18 diurnal birds of prey in Spain (van Drooge et al., 2008) and in white-tailed eagles (*Haliaeetus albicilla*) in Germany (Badry et al., 2022). The minimum concentration associated with lethality in birds is 2000 ng/g (Beyer & Meador, 2011); in our study two adult eagle owls presented concentrations above these limits at 4475 ng/g wet weight (female) and 3577 ng/g wet weight (male). These concentrations are considered high enough to be the underlying cause of mortality; however, concentrations 10 to 100 times lower than the lethal concentrations can also cause behavioral effects in birds including decreased aggression, impaired avoidance, as well as reduced defense and attentiveness in the nest (Hellou et al., 2013). Also, 4,4′-DDE is known as an endocrine disruptor in birds. High levels of 4,4′-DDE in eggs from eagle owls in Spain have been related with eggshell thinning, potentially affecting the reproduction of the species (Gómez-Ramírez et al., 2012). Another OCP detected in owl liver was lindane, with a higher detection frequency and mean concentration in eagle owl (57%, 1.82 ± 0.66 ng/g wet wt) than the rest of the species (20%–27%, from 0.27 ± 0.20 to 0.67 ± 0.32 ng/g wet wt). Hexachlorobenzene was detected in all species (17%–71% of detection frequency depending on the species), with a special prevalence in eagle owl (mean 2.11 ± 0.62 ng/g wet wt) but with the highest concentrations in two little owl individuals (5.12 and 8.61 ng/g wet wt). Finally, α-endosulfan was only detected in tawny owl, barn own, and little owl at concentrations from 0.10 to 0.23 ng/g wet weight. Our results evidence a high exposure of nocturnal raptors to OCPs, which illustrates that, despite the existing restrictions, persistent legacy pesticides are still a potential threat to wildlife. The persistence of the target OCPs poses a particular concern to top predators, such as owls. The eagle owl is one of the species proposed as a sentinel species for OCPs (Gómez-Ramírez et al., 2019). In the present study, eagle owl was the species with the highest levels of OCPs confirming its suitability to monitor these persistent compounds.

Among PFAS, PFOS was the only one detected, present in 83% to 100% of the analyzed birds at concentrations from 0.24 to 848 ng/g wet weight (Table 1). It was identified in all samples from long-eared owl, tawny owl, and barn owl at mean concentrations between 133 ± 38.9 and 187 ± 64.5 ng/g wet weight. In eagle owl, the concentrations were much higher (86% detection frequency, mean 285 ± 124 ng/g wet wt), and little owl was the least impacted species (83% detection frequency, mean 36.2 ± 16 ng/g wet wt). Because of its potential for bioaccumulation and biomagnification through the food web, PFOS is commonly reported in birds of prey (Eriksson et al., 2016; Monclús et al., 2022). Also, PFOS was the main PFAS detected in livers from the top predator common buzzard (*Buteo buteo*) in Belgium, with mean concentrations between 41.8 and 67.1 ng/g wet weight (Groffen et al., 2023). High PFOS concentrations are reported in road-killed barn owls close to a PFAS chemical plant in Belgium, with median concentrations of 304 ng/g wet weight (42–992 ng/g wet wt; Jaspers et al., 2013). The toxic reference value for PFOS in liver of avian top predators is estimated to be 600 ng/g wet weight (Newsted et al., 2005); in the present study, one individual of eagle owl and one of tawny owl surpassed this limit.

Total PCBs were detected in all individuals of eagle owl and long-eared owl at mean concentrations of 111 ± 47.3 and

TABLE 1: Compounds detected out of 81 studied and ∑chemical families in liver samples from five species of nocturnal raptors, indicating frequency of detection (percentage) and number of individuals with positive findings for each contaminant, mean with standard error, minimum and maximum concentrations, expressed in nanograms per gram wet weight

	Eagle owl ($n = 7$)				Long-eared owl ($n = 5$)				Tawny owl ($n = 12$)				Barn owl ($n = 10$)				Little owl ($n = 12$)			
Compounds	f.d. (n)	Mean ± SE	Min.	Max.	f.d. (n)	Mean ± SE	Min.	Max.	f.d. (n)	Mean ± SE	Min.	Max.	f.d. (n)	Mean ± SE	Min.	Max.	f.d. (n)	Mean ± SE	Min.	Max.
4,4′-DDE	100 (7)	1193 ± 738	19	4475	100 (5)	423 ± 235	43	1341	100 (12)	182 ± 94.4	14.9	1179	100 (11)	116 ± 56.9	2.19	636	100 (12)	144 ± 109	3.24	1342
Naphthalene	86 (6)	10.6 ± 2.39	0.32	17.5	100 (5)	13.8 ± 2.22	7.35	19.7	100 (12)	13.2 ± 1.53	8.21	27.7	91 (10)	18.2 ± 4.34	0.315	55.1	100 (12)	20.7 ± 1.99	13.4	38.6
Acenaphthene	71 (5)	4.81 ± 2.37	0.13	18	80 (4)	2.02 ± 0.80	0.13	4.83	100 (12)	47 ± 5.99	24.3	92.8	100 (11)	17.8 ± 2.62	3.45	34.9	100 (12)	12.8 ± 2.70	3.49	33.5
PFOS	86 (6)	285 ± 124	0.24	848	100 (5)	133 ± 38.9	8.89	245	100 (12)	187 ± 64.5	15.1	774	100 (11)	135 ± 31	37.2	306	83 (10)	36.2 ± 16.0	0.24	200
PCB-138	100 (7)	50.8 ± 20.7	1.98	131	100 (5)	17.3 ± 5.51	3.98	34.2	67 (8)	19.5 ± 14.3	2.64	176	82 (9)	11.8 ± 4.13	0.09	46.9	17 (2)	4.45 ± 3.85	0.09	46.5
Phenanthrene	29 (2)	0.81 ± 0.20	0.55	1.92	20 (1)	0.83 ± 0.29	0.55	1.98	58 (7)	1.4 ± 0.26	–	3.12	91 (10)	3.41 ± 0.72	0.54	8.59	75 (9)	3.01 ± 0.55	0.55	6.1
PCB-180	71 (5)	24.8 ± 9.90	0.17	63.6	80 (4)	9.23 ± 3.63	0.17	21.2	42 (5)	11.2 ± 8.56	1.96	104	82 (9)	6.07 ± 1.86	0.165	21.3	8 (1)	1.97 ± 1.81	0.17	21.8
PCB-153	57 (2)	26.9 ± 13.1	0.09	79.8	60 (3)	6.36 ± 3.02	0.09	13.9	17 (2)	1.79 ± 1.15	9.77	10.9	82 (9)	6.70 ± 2.82	0.08	32.2	17 (2)	4.45 ± 3.85	0.09	25.6
Lindane	57 (4)	1.82 ± 0.66	0.07	4.04	20 (1)	0.27 ± 0.20	0.07	1.07	25 (3)	0.37 ± 0.15	0.07	1.4	27 (3)	0.65 ± 0.35	0.07	3.69	25 (3)	0.67 ± 0.32	0.07	2.74
HCB	71 (5)	2.11 ± 0.62	0.03	4.65	20 (1)	0.88 ± 0.76	0.03	3.42	17 (2)	0.58 ± 0.37	0.03	3.7	18 (2)	0.72 ± 0.46	0.03	3.91	17 (2)	1.17 ± 0.80	0.03	8.61
α-Endosulfan	–	–	–	–	–	–	–	–	33 (4)	0.05 ± 0.02	0.1	0.2	18 (2)	0.03 ± 0.02	0.12	0.23	17 (2)	0.03 ± 0.02	0.1	0.22
PCB-118	57 (4)	8.41 ± 3.89	2.12	23.6	40 (2)	0.82 ± 0.52	1.55	2.54	–	–	–	–	9 (1)	–	797	797	8 (1)	–	2.53	2.53
2,4′-DDD	29 (2)	0.07 ± 0.06	0.06	0.43	20 (1)	–	0.11	0.11	8 (1)	–	0.1	0.1	9 (1)	–	0.16	0.16	17 (2)	0.24 ± 0.21	0.32	2.58
Pyrene	–	–	–	–	20 (1)	–	3.73	3.73	8 (1)	–	2.15	2.15	–	–	–	–	25 (3)	0.91 ± 0.49	3.12	4.46
Acenaphthylene	57 (4)	2.69 ± 1.12	2.84	7.27	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2,4′-DDE	–	–	–	–	–	–	–	–	17 (2)	0.01 ± 0.01	0.07	0.1	–	–	–	–	8 (1)	–	0.09	0.09
4,4′-DDD	–	–	–	–	–	–	–	–	–	–	–	–	9 (1)	–	1.46	1.46	8 (1)	–	6.6	6.6
Fluoranthene	–	–	–	–	20 (1)	–	2.47	2.47	–	–	–	–	–	–	–	–	8 (1)	–	2.81	2.81
2,4′-DDT	–	–	–	–	–	–	–	–	–	–	–	–	9 (1)	–	2.67	2.67	–	–	–	–
Fluorene	14 (1)	–	6.43	6.43	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Benzo[a] anthracene	–	–	–	–	–	–	–	–	8 (1)	–	0.59	0.59	–	–	–	–	–	–	–	–
∑OCPs	100 (7)	1197 ± 738	25.7	4480	100 (5)	424 ± 235	43.2	1341	100 (12)	183 ± 94.4	15.0	1179	100 (11)	117 ± 57.2	3.84	640	100 (12)	147 ± 110	3.34	1353
∑PFASs	86 (6)	285 ± 124	0.24	848	100 (5)	133 ± 38.9	8.89	245	100 (12)	187 ± 64.5	15.1	774	100 (11)	135 ± 31.0	37.2	306	83 (10)	36.2 ± 16.0	0.24	200
∑PCBs	100 (7)	111 ± 47.3	2.23	298	100 (5)	33.7 ± 12.1	4.23	69.2	67 (8)	32.5 ± 22.9	0.34	280	82 (9)	24.5 ± 8.77	0.34	100	17 (2)	9.22 ± 7.80	0.34	94
∑PAHs	100 (7)	19.4 ± 3.21	8.57	30.5	100 (5)	17.4 ± 3.66	7.35	26.5	100 (12)	61.6 ± 7.42	33.0	123	100 (11)	39.4 ± 5.36	20.1	81.6	100 (12)	37.6 ± 4.32	21.4	68.5

Compounds are ordered from the highest to lowest concentration and frequency of detection considering eagle owl.
f.d. = frequency of detection; SE = standard error; DDE = dichlorodiphenyldichloroethylene; PFOS = perfluorooctane sulfonic acid; PCB = polychlorinated biphenyl; HCB = hexachlorobenzene; DDD = dichlorodiphenyldichlorethane; DDT = dichlorodiphenyltrichloroethane; OCP = organochlorine pesticide; PFAS = per- and polyfluoroalkyl substances; PAH = polycyclic aromatic hydrocarbon.

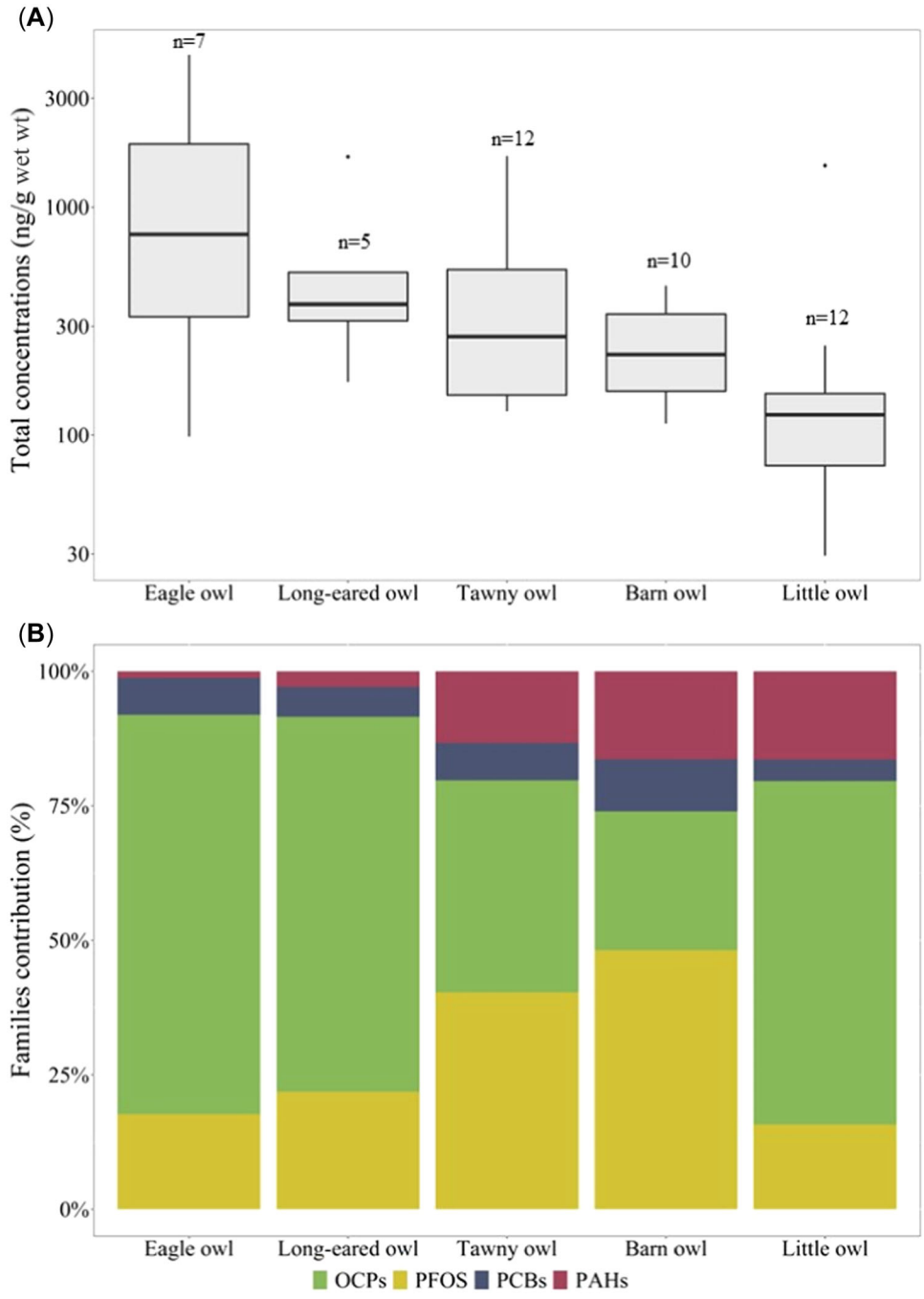

FIGURE 1: (**A**) Concentration of total contaminants in the 47 analyzed owl livers from the different species. (**B**) Contamination profile in each species according to the chemical family. OCP = organochlorine pesticide; PFOS = perfluorooctane sulfonic acid; PCB = polychlorinated biphenyl; PAH = polycyclic aromatic hydrocarbon.

33.7 ± 12.1 ng/g wet weight, respectively; in 67% of tawny owls and 82% of barn owls at mean concentrations of 32.5 ± 22.9 ng/g wet weight and 24.5 ± 8.77 ng/g wet weight, respectively; and in 17% of little owls at mean concentrations of 9.22 ± 7.80 ng/g wet weight. Tawny owl and little owl were the species with the lowest detection frequency and concentrations. The highest levels were found in a juvenile female of barn owl that presented ∑PCBs of 2010 ng/g because of high concentrations of PCB-138 (511 ng/g wet wt), PCB-153 (615 ng/g wet wt), and PCB-118 (797 ng/g wet wt). Because of the extreme values, this individual was identified as an outlier and excluded from both descriptive statistics and subsequent data analyses. The levels found in barn owls are below those found in barn owls collected in Italy, with mean concentration of 651 ng/g (range 55–2688 ng/g; Naso et al., 2003). In all species, the PCB profile was dominated by PCB-138, found in 17% to 100% of the birds at levels from 1.98 to 511 ng/g, followed by PCB-180 and PCB-153 present in 8% to 82% of the individuals depending on the species at concentrations ranging from 1.79 to 104 ng/g wet weight and from 2.52 to 615 ng/g

wet weight, respectively. The PCB profile is similar to those reported in livers from birds where high chlorinated PCBs (118, 138, 153) were more abundant than low chlorinated PCBs (28, 52, and 101; Buck et al., 2020). Due to the lack of unsubstituted adjacent meta- and para-positions on the biphenyl rings, high chlorinated PCBs are more resistant to cytochrome P450–mediated metabolism and are more persistent and bioacumulative in soft tissues than low chlorinated PCBs, which are more rapidly metabolized and excreted by organisms (Tomza-Marciniak et al., 2019). The exposure of PCBs in birds has been associated with decreased nest attentiveness in glaucous gulls (*Larus hyperboreus*; Bustnes et al., 2001) and disruption of feather coloration in American kestrels (*Falco sparverius*; Bortolotti et al., 2003).

Total PAHs were detected in all samples although at low concentrations compared with other contaminants (7.35–123 ng/g wet wt). Naphthalene and acenaphthene were the most frequently found PAHs in the different species in 86% to 100% and 71% to 100% of the samples, respectively, followed by phenanthrene with a detection frequency of 20% to 91% (Table 1). The mean concentrations ranged from 17.4 ± 3.66 to 61.6 ± 7.42 ng/g wet weight, and in contrast to OCPs, eagle owl and long-eared owl had the lowest concentrations among the studied species. Other PAHs sporadically detected are indicated in Table 1. Low–molecular weight (LMW) PAHs such as naphthalene and acenaphthene presented a higher contribution than high-molecular-weight PAHs, which is indicative of petrogenic PAH sources including crude oil and petroleum products such as kerosene, gasoline, diesel fuel, lubricating oil, and asphalt (Saha et al., 2009). High concentrations of naphthalene, acenaphthene, and phenanthrene have been related to exposure to coal tar (McCormick et al., 2022). Luzardo et al. (2014) also reported a higher prevalence of LMW PAHs in livers from six species of birds of prey from the Canary Islands (Spain), including barn owl and long-eared owl, with naphthalene being the main compound detected. Similar to our results, barn owls presented higher levels of PAHs than long-eared owls; however, the levels detected in the present study are lower than those reported by Luzardo et al. (2014).

Differences of exposure among owl species

The PCA grouped owl species according to the exposure profile to the different chemical families (Figure 2). Principal Component 1 explained 46% of the variance and is positively related to ∑PCBs, ∑OCPs, and PFOS and negatively related to ∑PAHs. Component 2 explained 24.6% of the variance, and it is explained by individuals with high contribution of ∑PAHs and PFOS and negatively related to ∑PCBs and ∑OCPs. Eagle owl and long-eared owl samples are distributed in the right Principal Component 1 axis, indicating a similar profile of contaminants. These two species are characterized by the prevalence of the most persistent compounds such as ∑OCPs, ∑PCBs, and PFOS

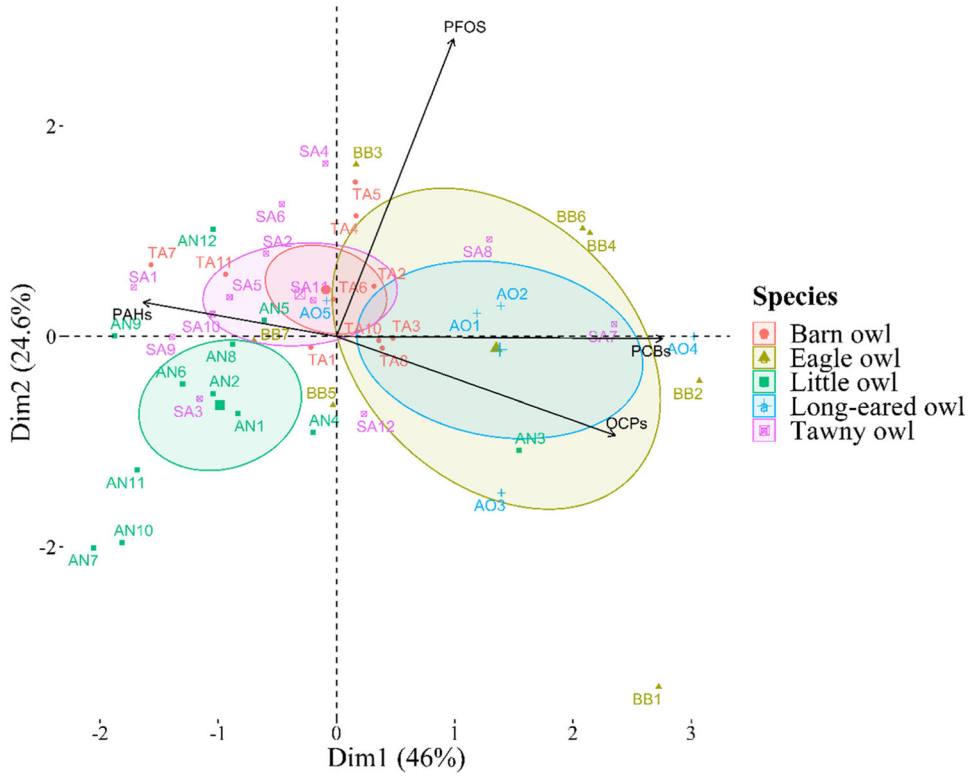

FIGURE 2: Principal component analysis of concentrations (logarithmic transformation log x + 1) of compounds detected in owl livers. Plot showing components 1 and 2, which explain 70.6% of the total variance. Kaiser-Meyer-Olkin score = 0.50. Dim1/2 = dimensions 1/2; PFOS = perfluorooctane sulfonic acid; PAH = polycyclic aromatic hydrocarbon; PCB = polychlorinated biphenyl; OCP = organochlorine pesticide; SA = *Strix aluco* (Tawny owl); BB = *Bubo bubo* (Eagle owl); TA = *Tyto alba* (Barn owl); AN = *Athene noctua* (Little owl); AO = *Asio otus* (Long-eared owl).

and low ∑PAH concentrations. Tawny owl and barn owl samples are distributed in the center of the plot, showing intermediate levels of ∑OCPs, ∑PCBs, and PFOS but also a high contribution of ∑PAHs. Little owl samples are grouped in the PCA bottom left quadrant, indicating a lower contribution of ∑OCPs, ∑PCBs, and PFOS and a high contribution of PAHs. The Dunn test was used to assess the pairwise comparisons among species and chemical families; the statistical measures are detailed in Supporting Information, Table S6.

Despite all species being nocturnal raptors and being collected in the same Alentejo region in Portugal, they present differences in their diet, nesting site characteristics, life span, and habitat preferences, as summarized in Table 2. These differences represent a key factor explaining the specific contamination profiles among species. Eagle owl and long-eared owl are distinguished by feeding in higher trophic levels and hunting and nesting in woodland areas (Table 2). Eagle owl is a long-lived superpredator, and its diet is composed of medium-sized mammals such as rabbits, hares, hedgehogs, rats, and birds such as partridges, pigeons, and jays (Lourenço, 2006). Eagle owl was the species with the highest total concentration levels (Figure 1 and Table 1), mainly due to the high levels of ∑OCPs (25.7–4480 ng/g wet wt), PFOS (0.24–848 ng/g wet wt), and ∑PCBs (2.23–298 ng/g wet wt). The high exposure of persistent compounds in eagle owls is due to its superpredator diet, which makes this species prone to a higher bioaccumulation of persistent compounds through the food web, compared with other species feeding on lower trophic levels (Lourenço et al., 2011). It must be considered that the eagle owl and long-eared owl were the less frequently road-killed carcasses found compared with the three other species. In the Alentejo region, eagle owl and long-eared owl inhabit and forage in areas farther away from roads and urban areas (Lourenço et al., 2015), which could contribute to lower ∑PAH levels.

Tawny owl, barn owl, and little owl showed a contaminant profile characterized by the presence of higher concentrations of ∑PAHs than the other species (Supporting Information, Figure S3). In birds, exposure to PAHs usually occurs by breathing air contaminated by coal tar or wild fires or by eating polluted foods (González et al., 2002). Therefore, the foraging behavior on small mammals or invertebrates at roadsides or closer to urban areas could be a source of PAHs for nocturnal raptors which hunt or nest in these areas, explaining the higher exposure of ∑PAHs found in tawny owl, barn owl, and little owl in our study.

Individual factors of exposure to contaminants

Individuals were classified as juveniles when the molt pattern indicated they were in their first calendar year (year of birth) and as adults when the date and molt of flight feathers were indicative of the second or more calendar year (Supporting Information, Table S1). From the 47 individuals analyzed, 16 were juveniles and 26 were adults. Five individuals were not aged because of unclear plumage pattern. We obtained representatives of both age classes in all species except

TABLE 2: Species studied, hunting habitat, nesting sites, life span, and diet of the sampled bird species in the study area (Alentejo, Portugal)

Species	Hunting habitat	Nesting sites	Life span[a]	Diet	References
Eagle owl (*Bubo bubo*)	Woodlands, shrublands	Old quarries, riverbanks	27	Rabbits, hares, partridges, rats, hedgehogs, pigeons; sometimes other mammals and birds	Lourenço et al. (2015), 2021; Lourenço (2002)
Long-eared owl (*Asio otus*)	Woodlands, cereal fields	Nests from other birds (raptors, crows, etc.)	13–17	Mainly small mammals; sometimes passerine birds	de Magalhaes (1974); Lourenço et al. (2015), 2021; Lourenço (2002)
Tawny owl (*Strix aluco*)	Woodlands with varying densities	Old trees; sometimes buildings	22	Invertebrates, small mammals, and birds	Lourenço et al. (2015); Lourenço (2002); Roque et al. (2021); Santos (1998); Silva et al. (2012); van der Horst et al. (2019)
Barn owl (*Tyto alba*)	Pastures, cereal fields, open woodlands	Mostly buildings (barns, old houses)	15–17	Mainly small mammals (mice, voles, shrew); sometimes passerine birds	Lourenço et al. (2015); Lourenço (2002); Roque et al. (2021); Santos (1998)
Little owl (*Athene noctua*)	Pastures, cereal fields, olive groves	Buildings, old trees	10–11	Mainly invertebrates; sometimes small mammals	Lourenço et al. (2015), 2021; Lourenço (2002); Santos (1998); Tomé et al. (2008)

[a]Fransson et al. (2010).

for long-eared owl, for which only adults were collected. In all species, adults presented higher mean concentrations of ∑OCPs, PFOS, and ∑PCBs than juveniles (Figure 3). The Kruskal-Wallis test followed by Dunn's test indicated a significant difference among adults and juveniles for ∑OCP concentrations ($\chi^2 = 8.17$, $df = 1$, Dunn's test $p = 0.0021$) and ∑PCBs ($\chi^2 = 18.6$, $df = 1$, Dunn's test $p < 0.001$). Adults also presented higher PFOS concentrations than juveniles, although this difference was nearly significant ($\chi^2 = 3.36$, $df = 1$, Dunn's test $p = 0.07$). This is expected because these compounds tend to bioaccumulate along the life of the birds, usually leading to higher concentrations in adult individuals. Concentrations of ∑PAHs were not different among age classes ($\chi^2 = 0.65$, $df = 1$, Dunn's test $p = 0.42$; Figure 3), probably because although PAHs are lyophilic and therefore have the potential to be bioaccumulated, they are also easily metabolized and excreted by vertebrates (Malcolm & Shore, 2003), thus limiting their bioaccumulation and explaining the similar concentrations between adults and juveniles.

The sex of the individuals is another important factor to consider when explaining contaminant exposure because breeding females may have lower concentrations of lipophilic compounds compared with males, due to the contaminant deposition in eggs (Bustnes et al., 2017). However, sex could not be assessed as a factor of exposure to contaminants owing to the opportunistic sampling in our study. Of the 47 individuals, 19 were identified as females and eight as males, but for a large number of individuals it was not possible to determine the sex ($n = 20$) because of unclear morphological differences in juvenile individuals and in the most deteriorated cadavers, with gonads severely damaged as a result of collision. Individual contextual data are of interest to improve the interpretation of variations in exposure pattern between individuals. In the present study, this comparison was limited by the uneven dating and sexing of individuals between species as a result of opportunistic sampling based on carcass collection.

CONCLUSIONS

The use of road-killed birds is a useful approach to monitor the exposure of organic micropollutants without animal disturbance, which is preferred in sensitive species such as nocturnal raptors. Twenty-one contaminants were detected in livers from five nocturnal raptor species. Owls were found to be exposed to a variety of persistent compounds such as OCPs, PFOS, PCBs, and PAHs, while pharmaceuticals, OPEs, and in-use pesticides were not detected. In all individuals 4,4′-DDE was detected at high concentrations (2.19–4475 ng/g wet wt), exceeding the minimum lethal concentrations in two adult eagle owls. The only perfluorinated compound detected was PFOS, but it was ubiquitous in all species and at concentrations

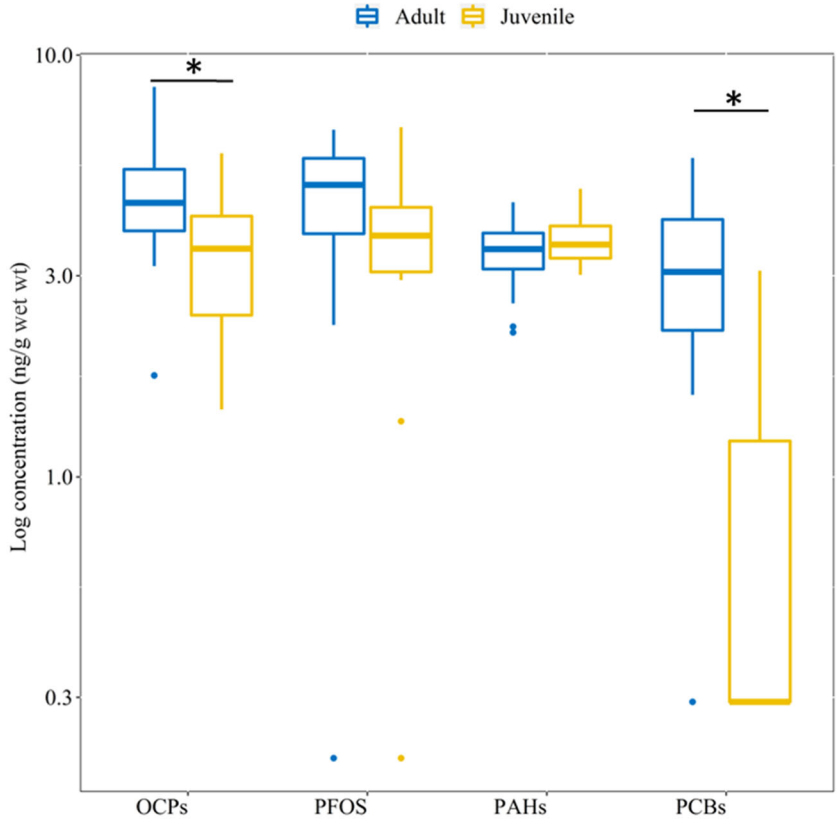

FIGURE 3: Total concentrations (logarithmic transformation log $x + 1$) of each chemical group in adults (second calendar year or more) and juveniles (first calendar year). *Significant differences between age classes, $p < 0.05$. ∑OCPs: $\chi^2 = 8.17$, $df = 1$, Dunn test $p = 0.0021$; PCBs: $\chi^2 = 18.6$, $df = 1$, Dunn test $p < 0.001$. OCP = organochlorine pesticide; PFOS = perfluorooctane sulfonic acid; PAH = polycyclic aromatic hydrocarbon; PCB = polychlorinated biphenyl.

up to 848 ng/g wet weight. Total PCBs were detected in all species at concentrations ranging from 1.98 to 298 ng/g wet weight, and ∑PAHs were also found in all individuals, with the LMW PAHs naphthalene and acenaphthene being the most frequently detected. These results provide evidence of widespread exposure of owls collected in Portugal to environmental contaminants, highlighting the importance of ongoing monitoring and conservation efforts to address the potential adverse effects of chemicals on wildlife. Individual factors such as age are important to consider when assessing contaminant exposure in long-lived species such as raptors. In the present study, adults presented a higher concentration of the persistent compounds ∑OCPs, ∑PCBs, and PFOS than juveniles; but no statistical differences were found in ∑PAHs between age classes. The lack of difference in PAH levels has been related to their rapid metabolization and low accumulation rate in vertebrates. In addition, high levels of ∑OCPs, ∑PCBs, and PFOS were observed in woodland species feeding at higher trophic levels, particularly in the eagle owl but also in the long-eared owl. However, these species presented a significantly lower concentration of ∑PAHs than tawny owl, barn owl, and little owl. The differences observed can be attributed to more regular foraging in roadsides of the latter species.

Supporting Information—The Supporting Information is available on the Wiley Online Library at https://doi.org/10.1002/etc.5816.

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Author Contributions Statement—**Maria Dulsat-Masvidal**: Conceptualization; Formal analysis; Investigation; Methodology; Software; Validation; Writing—original draft; Writing—review & editing. **Rui Lourenço**: Conceptualization; Data curation; Funding acquisition; Investigation; Methodology; Resources; Supervision; Writing—review & editing. **Rafael Mateo**: Investigation; Writing—review & editing. **Silvia Lacorte**: Conceptualization; Investigation; Methodology; Supervision; Validation; Writing—review & editing.

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