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Mercury stable isotopes in seabirds in the Ebro Delta (NE Iberian Peninsula): Inter-specific and temporal differences[☆]

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ABSTRACT

Mercury (Hg) is a global pollutant, which particularly affects aquatic ecosystems, both marine and freshwater. Top-predators depending on these environments, such as seabirds, are regarded as suitable bioindicators of Hg pollution. In the Ebro Delta (NE Iberian Peninsula), legacy Hg pollution from a chlor-alkali industry operating in Flix and located ca. 100 km upstream of the Ebro River mouth has been impacting the delta environment and the neighboring coastal area. Furthermore, levels of Hg in the biota of the Mediterranean Sea are known to be high compared to other marine areas. In this work we used a Hg stable isotopes approach in feathers to understand the processes leading to different Hg concentrations in three *Laridae* species breeding in sympatry in the area (Audouin's gull *Ichthyetus audouinii*, black-headed gull *Chroicocephalus ridibundus*, common tern *Sterna hirundo*). These species have distinct trophic ecologies, exhibiting a differential use of marine resources and freshwater resources (i.e., rice paddies prey). Moreover, for Audouin's gull, in which in the Ebro Delta colony temporal differences in Hg levels were documented previously, we used Hg stable isotopes to understand the impact of anthropogenic activities on Hg levels in the colony over time. Hg stable isotopes differentiated the three *Laridae* species according to their trophic ecologies. Furthermore, for Audouin's gull we observed temporal variations in Hg isotopic signatures possibly owing to anthropogenic-derived pollution in the Ebro Delta. To the best of our knowledge this is the first time Hg stable isotopes have been reported in seabirds from the NW Mediterranean.

1. Introduction

Mercury (Hg) is a global pollutant, which is present in all Earth's compartments, and exerts detrimental effects on both humans and wildlife (Clarkson and Magos, 2006; Driscoll et al., 2013; Evers, 2018). Although Hg naturally occurs in the environment, it can also be found as a result of human activities (Driscoll et al., 2013). Among anthropogenic activities introducing mercury into the environment are artisanal gold mining or industrial processes (e.g., coal combustion, chlor-alkali plants) (UNEP, 2019). Anthropogenic activities have significantly increased mercury levels in the environment since the pre-industrial era

(Amos et al., 2013; UNEP, 2019), which poses an urge to monitor environmental loads of this pollutant. Moreover, being able to estimate the relative contribution of anthropogenic vs. natural sources of mercury in the environment would help environmental-related decisions (Wiener et al., 2003).

Particularities of this element include a complex biogeochemical cycle resulting in chemical speciation (Selin, 2009; Gustin et al., 2020), which depends on both the biotic and abiotic factors in the environment where mercury is found (Ullrich et al., 2001; Lyman et al., 2020; Bowman et al., 2020). For instance, in aquatic environments, inorganic mercury (Hg²⁺) is known to be methylated by mainly sulfate- and

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iron-reducing bacteria (Podar et al., 2015), but also by abiotic processes (Celo et al., 2006), transforming Hg^{2+} into methylmercury (MeHg). MeHg is a readily bioavailable and highly toxic form of mercury which can bioaccumulate in organisms and get biomagnified along food webs (Morel et al., 1998; Lavoie et al., 2013; Evers, 2018).

In this context, top-predator and long-lived species, such as seabirds, are more exposed to Hg and its negative effects including physiological, reproductive, neurological and teratogenic effects (Whitney and Cristol, 2017). In fact, seabirds have been extensively used as bioindicator species of environmental pollution for an array of contaminants, including mercury (Furness and Camphuysen, 1997; Becker et al., 2002; Burger and Gochfeld, 2004; Sanpera et al., 2007; Blévin et al., 2013; Albert et al., 2019; Bond and Lavers, 2020). Feathers are one of the main Hg excretion routes in birds, reflecting blood mercury concentrations at the time of growth (Furness et al., 1986; Thompson et al., 1998; Bearhop et al., 2000). In seabirds, Hg in feathers is mainly in MeHg form (Bond and Diamond, 2009; Renedo et al., 2017). In this regard, juveniles from many species of colonial seabirds are fed with prey items from the surroundings of the breeding colonies, and consequently, their feathers grown in breeding grounds will reflect Hg levels from the surrounding environments (Sanpera et al., 2007; Blévin et al., 2013).

The Ebro Delta (NE Iberian Peninsula) comprises 30 000 ha of wetlands, with 75 % of its area used for rice-farming, 20 % protected as a natural reserve, and 5 % is urbanized (Mañosa et al., 2001). Since the late 19th century, a chlor-alkali plant has been operating ca. 100 km upstream the Ebro River mouth. During the active period of the plant over the past century, 550 000–700 000 m^3 of contaminated sediments, mainly with mercury and organochlorine compounds, were accumulated on the bottom of the river dam, close to the factory (Grimalt et al., 2003; ACA, 2013). Although the production of chlorine using mercury ceased in 2017, legacy mercury pollution persists and has been impacting all the environments in the Ebro River delta, due to the transport of contaminated particles along the river (Carrasco et al., 2010, 2011; Cotín et al., 2012; Campillo et al., 2019; Palanques et al., 2020). Moreover, during 2013–2014, a sediment remediation plan, including the dredging and disposal of polluted sediments, was implemented to remove toxicants from the riverbed in the vicinity of the factory. In a previous study, Sánchez-Fortún et al. (2020) reported the possible influence of the remediation tasks which took place in 2013–2014 on the increase of mercury levels observed in the Audouin's gull breeding colony in the Ebro Delta area, due to a possible remobilization and resuspension of polluted sediments and transport downstream. Furthermore, due to its biogeochemical characteristics and local Hg reservoirs, methylmercury levels in the Mediterranean Sea are high compared to other oceanic regions (Cossa and Coquery, 2005). Thus, seabird species depending on food webs from the Ebro Delta area might be also exposed to naturally high levels of mercury (Arcos et al., 2002; Sanpera et al., 2007; Cotín et al., 2011; Sánchez-Fortún et al., 2020). However, the relative importance of mercury sources impacting aquatic habitats from the area (freshwater or coastal areas) has never been analyzed in depth.

In systems with acknowledged anthropogenic sources of Hg pollution, Hg stable isotopes have proven as a useful tool to unravel origins of Hg burdens in an array of environmental compartments (e.g., sediments [Gehrke et al., 2011; Wiederhold et al., 2015; Reinfelder and Janssen, 2019], atmosphere [Estrade et al., 2010]), and in humans (Laffont et al., 2009, 2011) and wildlife (Day et al., 2012; Kwon et al., 2014; Bonignore et al., 2015).

Mercury has seven stable isotopes with atomic masses 196, 198, 199, 200, 201, 202, and 204 amu, which can undergo both mass-dependent fractionation (MDF) and mass-independent fractionation (MIF). MDF occurs as consequence of physical, chemical and biological processes including mercury biological methylation and demethylation (Kritee et al., 2009; Rodríguez-González et al., 2009; Janssen et al., 2016), photoreduction (Bergquist and Blum, 2007), trophic transfer (Laffont et al., 2009), metabolic reactions (Laffont et al., 2009; Feng et al., 2015;

Renedo et al., 2020, 2021). MIF occurs mainly as consequence of photochemical reduction reactions in the water column prior to bioaccumulation of Hg (Bergquist and Blum, 2007). Although MIF processes have been described for isotopes ^{200}Hg and ^{204}Hg (i.e., even-MIF), and for ^{199}Hg and ^{201}Hg (i.e., odd-MIF; Kwon et al., 2020), odd-MIF has been observed commonly in biological samples (Tsui et al., 2020). Thus, MDF and MIF signatures offer a framework to trace Hg pollution and may provide information on Hg sources in organisms. This information coupled with carbon and nitrogen stable isotopes values, which have been used extensively as proxies of the trophic ecology of organisms (Post, 2002; Boecklen et al., 2011), might provide valuable information on the causes leading to high Hg levels in wildlife.

The aims of this study were, firstly, to relate the trophic ecology of different *Laridae* species inhabiting in the Ebro Delta area to the total-Hg (THg) burden found in the organisms and potential Hg sources at local scale using Hg stable isotopes. We have analyzed Hg, C and N stable isotopes in feathers of fledgling individuals of three different species breeding in the Ebro Delta (Fig. S1), with contrasted dietary characteristics: the common tern (*Sterna hirundo*), the black-headed gull (*Chroicocephalus ridibundus*), and the Audouin's gull (*Ichthyaeus audouinii*). And secondly, explore the temporal dynamics of Hg levels in the Audouin's gull breeding colony in the Ebro Delta using Hg stable isotopes, to further understand the differences of this metal observed before and after 2013, when the remediation plan was applied (Sánchez-Fortún et al., 2020).

2. Materials and methods

2.1. Fieldwork and sample collection

As part of a continued monitoring program of the Audouin's gull (*Ichthyaeus audouinii*) breeding colony in the Ebro Delta (NE Iberian Peninsula: 40°33' N, 00°39' E), 10–15 mantle feathers of juvenile individuals (approx. 3 weeks old) were sampled on years 2010, 2013, 2014, 2017 (N = 10 individuals each year; see Sánchez-Fortún et al., 2020 for further details on sampling). Additionally, during the breeding season of 2017, 10–15 mantle feathers of juvenile individuals from the following species breeding in the Ebro Delta area (Fig. S1) were sampled (N = 10 individuals for each species): common tern (*Sterna hirundo*; approx. 3 weeks old), black-headed gull (*Chroicocephalus ridibundus*; approx. 3 weeks old). We sampled one individual per brood to avoid pseudoreplication. Feathers were stored in zip-lock bags until analysis.

2.2. Trophic ecologies of Audouin's gull, common tern and black-headed gull

The three *Laridae* species sampled in this study differ in their trophic ecologies: Audouin's gulls and common terns depend mainly on marine resources, whereas black-headed gulls forage mainly on rice-fields (Antón-Tello et al., 2021), with potential prey items including the American crayfish (*Procambarus clarkii*). Moreover, Audouin's gulls and common terns exploit different marine resources. Essentially, common terns are epipelagic foragers (Cotín et al., 2011), with preferred food items being clupeiform fish (> 90 % of their prey; Arcos et al., 2002) while Audouin's gulls are opportunistic foragers on fisheries discards, comprised mainly of mesopelagic and demersal fish species (e.g., *Boops boops*, Arcos et al., 2002; Pedrocchi et al., 2002; Sanpera et al., 2007; García-Tarrasón et al., 2015). Besides, to a lesser extent Audouin's gulls also forage in terrestrial and freshwater environments (e.g., rice-fields), with potential preys including American crayfish (Oro et al., 1996; Ruiz et al., 1996; Pedrocchi et al., 2002; Sanpera et al., 2007). Likewise, common terns are known to a lesser extent to rely on other prey (e.g., terrestrial prey, or demersal/mesopelagic prey from fisheries discards; Oro and Ruiz, 1997; Cotín et al., 2011).

2.3. Total mercury determination

Total mercury (THg) determination in feather samples was performed at the Scientific and Technological Centers of the University of Barcelona (Spain). Feather samples were washed using 0.25 M NaOH and rinsed with distilled water to remove external Hg contamination (Bearhop et al., 2000). After cleaning, samples were oven dried to constant mass at 50 °C and then ground to a homogenous powder with an impactor mill (Freezer Mill 6850, SpexCertiPrepH Inc., Metuchen, NJ, USA) operating at liquid nitrogen temperature. THg in feathers (ng/g dry weight) was determined by combustion using a Direct Mercury Analyzer (Milestone® DMA-80) for all the species samples from 2017, or by Induction Coupled Plasma-Mass Spectrometer (ICP-MS) for Audouin's gull samples from 2010, 2013 and 2014. DMA-80 analyses were directly analyzed by combustion using 20–30 mg of feather sample. For the ICP-MS mercury determinations, ~30 mg of feather sample were acid digested in a microwave oven using 1–2 mL of nitric acid (HNO₃ 70 %) and 0.5–1 mL of hydrogen peroxide (H₂O₂ 30 %) in Teflon containers. To check for the accuracy of measurements for both methods, we analyzed a certified reference material in duplicate (NIES-13 Human Hair CRM) every 10 to 15 samples with recoveries ranging from 91 % to 94 % for DMA-80 analyses and 97–103 % in ICP-MS analyses. Thus, no corrections were applied to the THg values of the samples prior to statistical analyses. Detection limits for both methods were 0.001 µg/L for ICP-MS and 0.005 ng Hg for DMA-80.

2.4. Analysis of mercury stable isotopes

Stable isotopes of mercury were analyzed at the Institute of Analytical Sciences and Physico-Chemistry for Environment and Materials (IPREM) facilities (Pau, France) following method and protocol reported by Renedo et al. (2018). Aprox. 0.2 g of feather powder was digested with 5 mL of HNO₃ (65 %, INSTRA quality) and 1/3 of the total volume of H₂O₂ (30 %, ULTREX quality), and extracted in Hotblock at 75 °C during 8h (6h in HNO₃, plus 2h after the addition of H₂O₂). Hg isotopic composition for each of the 6 most abundant stable isotopes of Hg (¹⁹⁸Hg, ¹⁹⁹Hg, ²⁰⁰Hg, ²⁰¹Hg, ²⁰²Hg and ²⁰⁴Hg) was determined by means of a cold-vapor generator (CVG)-multicollector (MC)-ICPMS (Nu Instruments). Hg isotopic values were reported using the delta notation relative to the bracketing standard NIST-3133 certified reference material. Instrumental mass-bias was corrected using the internal Tl standard, NIST 997 CRM. A secondary standard NIST RM-8160 (UM-Almadén standard) was used for validation of the analytical session. Furthermore, NIES-13 Human Hair CRM and an internal reference material composed of king penguin feathers (F-KP) were used for the Hg stable isotopes analyses. Results on mercury stable isotopes analyses are reported in detail in Table S1, Table S2 and Table S3 from the Supplementary Materials.

Mass-dependent fractionation (MDF) is reported as $\delta^{202}\text{Hg}$ values (‰), odd-MIF is reported as $\Delta^{199}\text{Hg}$ (‰) and $\Delta^{201}\text{Hg}$ (‰) and even-MIF as $\Delta^{200}\text{Hg}$ (‰) and $\Delta^{204}\text{Hg}$ (‰). Detailed calculations of mercury stable isotopes signatures are reported in the Supplementary Materials.

Additionally, we calculated the ratio $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$, which has proven to be indicative of different photochemical transformation processes and speciation of Hg (i.e., photoreduction of Hg(II) vs. photodemethylation of MeHg) in experimental settings (Bergquist and Blum, 2007). $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg} > 1$ has been determined on samples containing predominantly MeHg, for instance, biota samples, whereas $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg} \approx 1$ has been observed in environmental samples (e.g., sediments, water) containing predominantly Hg(II) (Tsui et al., 2020).

2.5. Stable isotopes of carbon and nitrogen analysis

Stable isotopic analyses for carbon and nitrogen were carried out at the Scientific and Technological Centers of the University of Barcelona (Spain). 0.30–0.35 mg of feather powder were placed into tin capsules

and analyzed using a Thermo-Finnigan Flash 1112 elemental analyzer (CE Elantech, Lakewood, NJ, USA) coupled to a Delta-C isotope ratio mass spectrometer via a CONFLO III interface (Thermo Finnigan MAT, Bremen, Germany). Stable isotope signatures are expressed using the standard delta notation relative to Vienna Pee Dee Belemnite ($\delta^{13}\text{C}$) and atmospheric nitrogen ($\delta^{15}\text{N}$). IAEA standards (IAEA CH₇, IAEA CH₆, USGS 24 for ¹³C, and IAEA N₁, IAEA N₂, IAEA NO₃ for ¹⁵N) were applied every 12 samples to calibrate the system. Replicate assays of standards indicated analytical measurement errors of ±0.1 ‰ and ±0.2 ‰ for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively.

2.6. Statistical analyses

THg values and stable isotopes signatures ($\delta^{202}\text{Hg}$, $\Delta^{199}\text{Hg}$, $\Delta^{201}\text{Hg}$, $\Delta^{200}\text{Hg}$, $\Delta^{204}\text{Hg}$, $\delta^{13}\text{C}$, $\delta^{15}\text{N}$) were tested using the Shapiro-Wilk test to assess normality. THg values were log-transformed to approximate residual normality. Homogeneity of variances was tested using the Breusch-Pagan test.

2.6.1. Total mercury and stable isotopes signatures in Laridae species

ANOVA tests were performed to test differences among the three species studied (IA, CR and SH) in THg, $\delta^{202}\text{Hg}$, $\Delta^{199}\text{Hg}$, $\Delta^{201}\text{Hg}$, $\Delta^{200}\text{Hg}$ and $\Delta^{204}\text{Hg}$, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ observed in feathers. Pairwise comparisons were evaluated using Tukey tests. A linear model was fitted to evaluate the association between logTHg (response variable) and $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and Species and the interactions $\delta^{13}\text{C} \times \text{Species}$ and $\delta^{15}\text{N} \times \text{Species}$ as covariates.

Intraspecific correlation tests were performed for each species for each of the mercury isotopic tracers and THg in feathers, each of the mercury isotopic tracers and $\delta^{13}\text{C}$, each of the mercury isotopic tracers and $\delta^{15}\text{N}$. For the correlations analyses we omitted a data point from Audouin's gull that was an outlier in terms of $\delta^{13}\text{C}$.

Linear models were fitted to estimate the slopes of $\Delta^{199}\text{Hg}/\delta^{202}\text{Hg}$, $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ and $\Delta^{200}\text{Hg}/\Delta^{204}\text{Hg}$ for all the species studied.

2.6.2. Temporal changes in total mercury and stable isotope signatures in Audouin's gull fledglings

ANOVA tests were performed to test the differences in THg, $\delta^{202}\text{Hg}$, $\Delta^{199}\text{Hg}$, $\Delta^{201}\text{Hg}$ and $\delta^{15}\text{N}$ observed in feathers among the years studied (2010, 2013, 2014, 2017). Pairwise comparisons were evaluated using Tukey tests. Since assumptions of normality and homogeneity of variances were not met for $\delta^{13}\text{C}$ residuals, Kruskal Wallis test was used to examine the among years differences for $\delta^{13}\text{C}$ values and Wilcoxon Rank Sum Test for pairwise comparisons.

A linear mixed effects model was fitted to evaluate the association between log THg (response variable) and $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ (covariates), with year as a random effect. We assessed correlations between each of the mercury isotopic tracers and THg in feathers, $\delta^{13}\text{C}$, and $\delta^{15}\text{N}$ for each of the years separately and for all the years pooled together.

Besides, a linear model was fitted to test the association between $\Delta^{199}\text{Hg}$ and $\delta^{202}\text{Hg}$. Finally, a linear model of $\Delta^{199}\text{Hg}$ as a response variable and $\Delta^{201}\text{Hg}$ as covariate was fitted to estimate the slope $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ for the years considered in the study (data for all the years pooled in a single dataset; for the regressions for each year see Supplementary Materials). In this linear model one extreme value was omitted from the dataset.

All statistical analyses used a significance level of $\alpha = 0.05$. Summary statistics are reported as mean ± SD, unless otherwise stated. All statistical analyses were performed using R v.4.0.3 (R Core Team, 2020).

3. Results

3.1. Species differences in total mercury concentrations and isotopic composition

3.1.1. Total mercury concentrations, carbon and nitrogen stable isotopes

Table 1 shows the mean ± SD for the variables studied for each of the species under study. THg observed ranged from 1128.31 to 9205.55 ng/g. Significant differences in THg concentrations in fledglings' feathers were observed among species ($F_{2,27} = 167.64, p < 0.0001$; Fig. 1). Post-hoc tests showed significant differences among the three species considered (Fig. 1). Mean THg concentrations observed in feathers was greater for Audouin's gull, followed by common tern, and black-headed gull fledglings (Table 1).

Significant differences in both $\delta^{13}C$ and $\delta^{15}N$ were observed among species ($\delta^{13}C: F_{2,27} = 68.64, p < 0.0001$ and $\delta^{15}N: F_{2,27} = 29.02, p < 0.0001$). Post-hoc pairwise comparisons showed significant differences in $\delta^{13}C$ between Audouin's gull and black-headed gull ($p < 0.0001$), and black-headed gull and common tern ($p < 0.0001$), but not between Audouin's gull and common tern ($p = 0.29$) with $\delta^{13}C$ values being (Audouin's gull \approx common tern) $>$ black-headed gull (Fig. 2). Regarding $\delta^{15}N$ values, we detected differences between Audouin's gull and common tern ($p < 0.0001$), and black-headed gull and common tern ($p < 0.0001$), but not between Audouin's gull and black-headed gull ($p = 0.48$). $\delta^{15}N$ values were higher in Audouin's gull and black-headed gull than common tern fledglings (Fig. 2; Table 1).

The linear model did not show a significant association between $\delta^{15}N$ or $\delta^{13}C$ and the observed THg levels in feathers ($\delta^{15}N: F_{1,21} = 0.025, p = 0.88$; $\delta^{13}C: F_{1,21} = 0.004, p = 0.95$). The interaction terms in the model had no effect on THg levels ($\delta^{15}N \times \text{Species}: F_{2,21} = 0.52, p = 0.60$; $\delta^{13}C \times \text{Species}: F_{2,21} = 1.22, p = 0.32$). However, the results from this model might be a consequence of a low statistical power.

3.1.2. Mercury stable isotopes composition

Regarding mercury stable isotopes (Table 1) we found significant interspecific differences for $\delta^{202}Hg$ values ($F_{2,27} = 101.50, p < 0.0001$), $\Delta^{199}Hg$ ($F_{2,27} = 198.41, p < 0.0001$) and $\Delta^{201}Hg$ ($F_{2,27} = 181.49, p < 0.0001$). Post-hoc pairwise comparisons showed significant differences among the three species for $\delta^{202}Hg$ (all comparisons: $p < 0.0001$; Fig. 3) and between common tern and black-headed gull, and black-headed gull and Audouin's gull, but not between Audouin's gull and black-headed gull for both $\Delta^{199}Hg$ (Audouin's gull-common tern: $p < 0.0001$; black-headed gull-common tern: $p < 0.0001$; Audouin's gull-black-headed gull: $p = 0.53$; Fig. 3) and $\Delta^{201}Hg$ (Audouin's gull-common tern: $p < 0.0001$; black-headed gull-common tern: $p < 0.0001$; Audouin's gull-black-headed gull: $p = 0.50$). Finally, no significant differences were found among species for $\Delta^{200}Hg$ ($F_{2,27} = 1.99, p = 0.15$) or $\Delta^{204}Hg$ ($F_{2,26} = 1.12, p = 0.34$).

Regression of $\Delta^{199}Hg$ against $\delta^{202}Hg$ showed a significant positive relationship ($F_{1,24} = 144.37, p < 0.0001$; Fig. 3), with a regression slope of 2.21. The ratio obtained from the regression between odd-MIF values $\Delta^{199}Hg/\Delta^{201}Hg$ for the three species studied was 1.16 (Fig. S1). The ratio between $\Delta^{200}Hg/\Delta^{204}Hg$ was not significantly different from 0 (Fig. S3), possibly due to the low variability encountered in both $\Delta^{200}Hg$ and $\Delta^{204}Hg$ signatures in our dataset.

Correlations among Hg stable isotopes and THg, $\delta^{15}N$ and $\delta^{13}C$, for each of the species are shown in Table 2. We found significant positive correlations ($p < 0.05$) between $\delta^{13}C$ and odd-MIF signatures ($\Delta^{199}Hg$ and/or $\Delta^{201}Hg$) values for Audouin's gull and black-headed gull, and significant negative correlations between THg and odd-MIF ($\Delta^{199}Hg$ and $\Delta^{201}Hg$) for common tern.

Table 1

Summary statistics (mean, SD) for each of the parameters ($\delta^{15}N, \delta^{13}C, THg, \Delta^{199}Hg, \Delta^{201}Hg, \delta^{202}Hg$ and $\Delta^{200}Hg$) in fledglings feathers for each of the three species studied during the 2017 breeding season in the Ebro Delta: *I. audouinii* (Audouin's gull; N = 10 individuals sampled), *C. ridibundus* (black-headed gull; N = 10 individuals sampled), *S. hirundo* (common tern; N = 10 individuals sampled).

Species	$\delta^{15}N$ (‰)			$\delta^{13}C$ (‰)			THg (ng/g dw)			$\Delta^{199}Hg$ (‰)			$\Delta^{201}Hg$ (‰)			$\delta^{202}Hg$ (‰)			$\Delta^{200}Hg$ (‰)									
	Mean	SD	Min	Mean	SD	Min	Mean	SD	Min	Mean	SD	Min	Mean	SD	Min	Mean	SD	Min	Mean	SD	Min							
<i>I. audouinii</i>	13.77	0.38	14.52	13.28	-17.65	0.82	-16.83	-19.69	7982.97	951.94	9205.55	6439.79	0.89	0.16	1.11	0.60	0.68	0.14	0.85	0.38	0.26	0.10	0.43	0.11	0.009	0.05	0.1	-0.05
<i>C. ridibundus</i>	13.50	0.66	14.80	12.82	-21.33	1.15	-19.32	-22.50	1555.94	704.45	3494.11	1128.31	1.00	0.20	1.34	0.77	0.78	0.19	1.13	0.54	0.57	0.17	0.84	0.37	0.02	0.03	0.06	-0.03
<i>S. hirundo</i>	12.16	0.43	13.15	11.64	-17.05	0.59	-16.57	-18.13	1916.14	284.04	2379.45	1381.47	2.70	0.30	3.13	1.98	2.23	0.26	2.61	1.62	1.05	0.09	1.20	0.94	0.04	0.03	0.00	0.1

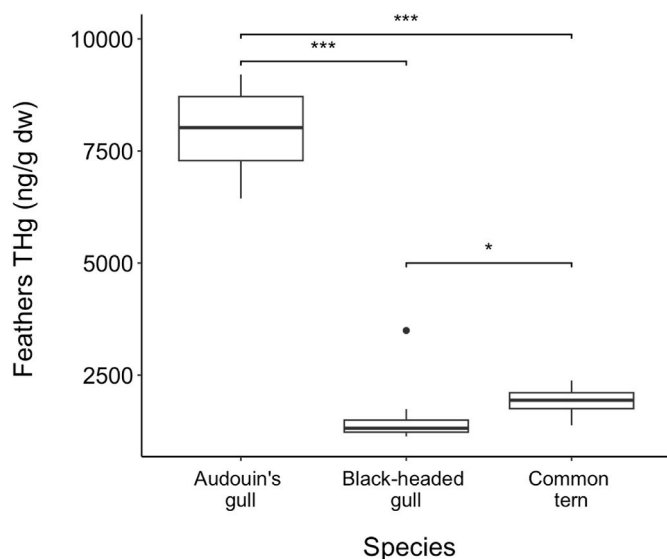


Fig. 1. Boxplots for THg (ng/g dw) in feathers of Audouin's gull (*I. audouinii*), black-headed gull (*C. ridibundus*) and common tern (*S. hirundo*) fledglings from the Ebro Delta in 2017. Pairwise significant differences between species are represented above the boxplots (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

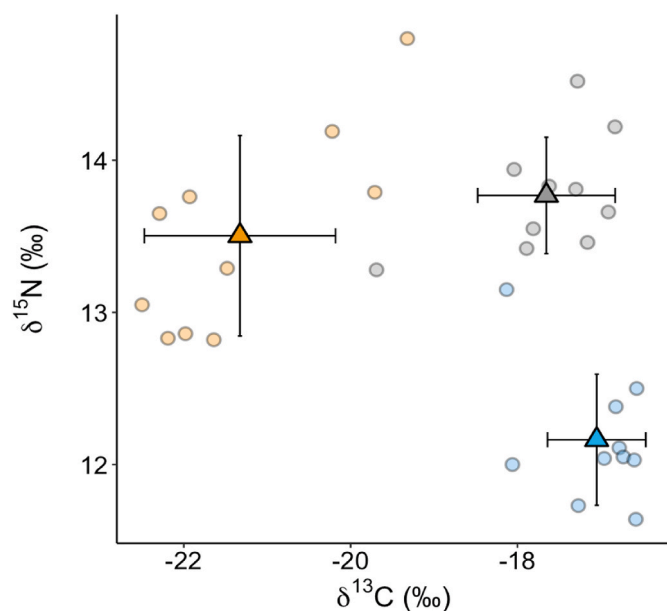


Fig. 2. Biplot of $\delta^{15}\text{N}$ (‰) and $\delta^{13}\text{C}$ (‰) in feathers of Audouin's gull (in gray), black-headed gulls (in orange) and common tern (in blue) fledglings from the Ebro Delta in 2017. Individual data are plotted as dots. Triangles and error bars represent mean \pm SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2. Temporal variation in stable isotopic composition in Audouin's gull fledglings

3.2.1. Total mercury concentrations, carbon and nitrogen stable isotopes

Along with the Audouin's gull feather samples from $N = 10$ fledglings analyzed in 2017, we also determined the THg in feathers from 10 Audouin's gull fledglings in 2010, 2013, 2014, for a total of 40 feather samples (Table 3), ranging from 1588.99 to 15009.73 ng/g. We found significant differences among years ($F_{3,36} = 55.24$, $p < 0.0001$). Post-hoc pairwise comparisons detected significant differences among all years' pairs except 2014–2017 (Fig. 4). Mean THg concentrations in

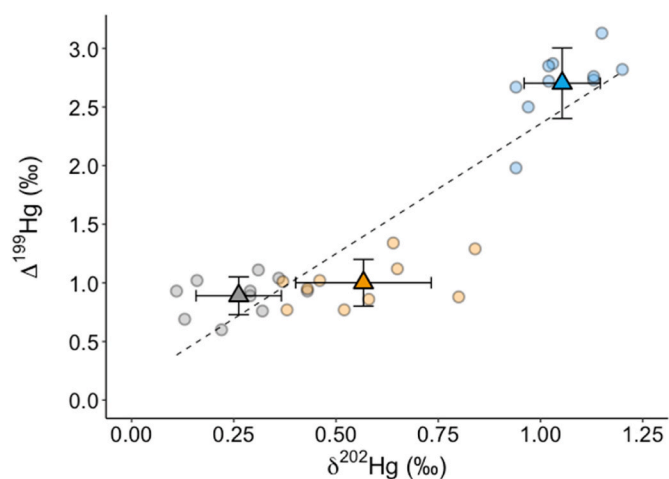


Fig. 3. Biplot of MDF $\delta^{202}\text{Hg}$ (‰) and odd-MIF $\Delta^{199}\text{Hg}$ (‰) in feathers of Audouin's gull (in gray), black-headed gull (in orange) and common tern (in blue) fledglings from the Ebro Delta in 2017. Individual data are plotted as dots. Triangles and error bars represent mean \pm SD. Dashed line represents linear regression between these variables for the three species: $y = 0.1407 + 2.2167x$; $R^2 = 0.7963$; $p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Audouin's gull fledgling feathers were higher in 2017 \approx 2014 > 2010 > 2013 (Table 3).

For the Audouin's gull samples, significant differences were observed for $\delta^{13}\text{C}$ values ($H = 10.16$, $p = 0.017$) and $\delta^{15}\text{N}$ values ($F_{3,36} = 32.90$, $p < 0.0001$) among years. Multiple pairwise comparisons for $\delta^{13}\text{C}$ values in feathers showed significant differences between 2013 and 2014 ($p = 0.011$) and 2013–2017 ($p = 0.019$), but not for the rest of pairwise comparisons (2010–2013: $p = 0.22$; 2010–2014: $p = 0.12$; 2010–2017: $p = 0.052$; 2014–2017: $p = 0.50$; Fig. 5). Pairwise comparisons for $\delta^{15}\text{N}$ signatures showed significant differences among all years' pairs ($p < 0.0001$ in all cases), except 2010–2013 ($p = 1.00$) and 2014–2017 ($p = 0.96$; Fig. 5), with $\delta^{15}\text{N}$ values higher in 2014 and 2017 than in 2010 and 2013 (Table 3).

The linear mixed effects model did show a significant positive association between $\delta^{13}\text{C}$ and the observed THg levels in feathers ($F_{1,34} = 5.20$, $p = 0.029$). Nonetheless, no significant association was observed between $\delta^{15}\text{N}$ values and THg levels in feathers ($F_{1,34} = 1.29$, $p = 0.26$).

3.2.2. Mercury stable isotopes composition

We found significant differences among years for $\delta^{202}\text{Hg}$ values ($F_{3,36} = 25.30$, $p < 0.0001$), $\Delta^{199}\text{Hg}$ ($F_{3,36} = 11.68$, $p < 0.0001$) and $\Delta^{201}\text{Hg}$ ($F_{3,36} = 8.25$, $p = 0.0002$) in gulls' feathers (Table 3). Post-hoc pairwise comparisons showed significant differences for $\delta^{202}\text{Hg}$ signatures among all years' pairs ($p < 0.0001$) except the pairs 2013–2017 ($p = 0.30$) and 2010–2014 ($p = 1.00$). $\Delta^{199}\text{Hg}$ pairwise comparisons showed significant differences between 2010–2013 ($p = 0.02$), 2010–2017 ($p < 0.0001$) and 2014–2017 ($p = 0.001$), although no significant differences were detected among the other years' pairs (in all cases, $p > 0.05$). For $\Delta^{201}\text{Hg}$ values pairwise comparisons showed significant differences between years 2010–2017 ($p = 0.0003$) and 2014–2017 ($p = 0.003$), but not among the other years' pairs (in all cases, $p > 0.05$).

For $\Delta^{200}\text{Hg}$ and $\Delta^{204}\text{Hg}$, values ranged from -0.05 to 0.1 (Table 3). We found significant differences among years for $\Delta^{200}\text{Hg}$ ($F_{3,36} = 3.45$, $p = 0.026$) but not for $\Delta^{204}\text{Hg}$ ($F_{3,36} = 0.86$, $p = 0.47$). Post-hoc comparisons showed significant differences in $\Delta^{200}\text{Hg}$ values for 2010–2013 ($p = 0.037$) but not among the other years' pairs (in all cases, $p > 0.05$).

Regression of $\Delta^{199}\text{Hg}$ against $\delta^{202}\text{Hg}$ values showed a significant positive relationship ($F_{1,38} = 23.05$, $p < 0.0001$; Fig. 6). The ratio between odd-MIF values $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ for all the years was 1.09

Table 2

Pearson's correlation tests between each of the mercury isotopic tracers ($\delta^{202}\text{Hg}$, $\Delta^{199}\text{Hg}$ and $\Delta^{201}\text{Hg}$) and THg, $\delta^{13}\text{C}$, and $\delta^{15}\text{N}$ in fledglings feathers for each of the three species studied during the 2017 breeding season in the Ebro Delta: *I. audouinii* (Audouin's gull), *C. ridibundus* (black-headed gull), *S. hirundo* (common tern). Significant correlations are highlighted in bold.

		Audouin's gull (<i>I. audouinii</i>)			Black-headed gull (<i>C. ridibundus</i>)			Common tern (<i>S. hirundo</i>)		
		$\delta^{202}\text{Hg}$	$\Delta^{199}\text{Hg}$	$\Delta^{201}\text{Hg}$	$\delta^{202}\text{Hg}$	$\Delta^{199}\text{Hg}$	$\Delta^{201}\text{Hg}$	$\delta^{202}\text{Hg}$	$\Delta^{199}\text{Hg}$	$\Delta^{201}\text{Hg}$
THg	r	-0.21	-0.41	-0.34	0.05	0.19	0.17	-0.51	-0.85	-0.83
	p-value	0.56	0.23	0.34	0.88	0.60	0.63	0.13	0.002	0.002
$\delta^{13}\text{C}$	r	-0.14	0.66	0.72	-0.03	0.56	0.65	0.07	0.44	0.44
	p-value	0.70	0.04	0.02	0.94	0.09	0.04	0.86	0.21	0.20
$\delta^{15}\text{N}$	r	-0.50	-0.09	-0.03	0.03	0.48	0.52	0.18	-0.09	-0.16
	p-value	0.14	0.80	0.92	0.93	0.16	0.12	0.63	0.80	0.65

(Fig. S4). The ratio between $\Delta^{200}\text{Hg}/\Delta^{204}\text{Hg}$ was not significantly different from 0 (Fig. S5), possibly due to the small variabilities for $\Delta^{200}\text{Hg}$ and $\Delta^{204}\text{Hg}$ in our samples (Table S2).

Correlations among mercury stable isotopes and THg, $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$, for each of the years and all the years pooled are shown in Table 4. We found significant positive correlations ($p < 0.05$) in 2017 between $\delta^{13}\text{C}$ and odd-MIF signatures, and significant negative correlations ($p < 0.05$) between $\delta^{15}\text{N}$ and odd-MIF values when pooling data from all the years together.

4. Discussion

4.1. Species differences in total mercury concentrations and isotopic composition

4.1.1. Total mercury concentrations

Fledglings from the three species studied (Audouin's gull, black-headed gull and common tern) presented differences in THg levels in feathers. Audouin's gull fledglings' feathers had higher mercury concentration than those from common terns or black-headed gulls. This would be related to the different trophic resources exploited by each species, as mercury is mainly acquired through diet. Although the three species coexist in the same area and their breeding colonies are few km apart, they have distinct trophic ecologies (see Materials and Methods section). Audouin's gull fledglings are fed with a greater proportion of mesopelagic and demersal fish compared to common terns (Pedrocchi et al., 2002). Mesopelagic and demersal fish species present higher trophic levels than epipelagic fish (Arcos et al., 2002) while inhabiting environments in the water column with enhanced mercury methylation (Chouvelon et al., 2012). Therefore, those fish species accumulate higher Hg levels compared to epipelagic fish species (Arcos et al., 2002), which constitute main prey items in the case of common terns. Conversely, black-headed gulls rely on rice-field prey, which are known to accumulate Hg to a lesser extent than marine species (e.g., Suárez-Serrano et al., 2010).

4.1.2. Mercury, carbon and nitrogen stable isotopes

Differences among the three species were found for $\delta^{202}\text{Hg}$, whereas $\Delta^{199}\text{Hg}$ and $\Delta^{201}\text{Hg}$ only differed between common tern and Audouin's gull, and between common tern and black-headed gull. The differences observed for the odd-MIF and MDF signatures might reflect differences in sources of mercury, the distinct trophic ecologies among species, and differences among processes linked to Hg speciation and bioavailability among foraging environments. Differences in $\Delta^{200}\text{Hg}$ and $\Delta^{204}\text{Hg}$ signatures were not observed among the three species.

$\Delta^{199}\text{Hg}$ values reflect differences in MeHg sources owing to photo-demethylation in the water column (Bergquist and Blum, 2007). Since odd-MIF signatures are maintained along the food webs (Kwon et al., 2012), they reflect the foraging habitats from which fledglings of the different species are fed. Common terns consumed epipelagic resources (mainly clupeiforms), where the incidence of sunlight and photo-demethylation processes predominate, leading to larger odd-MIF values

(Blum et al., 2013; Bonsignore et al., 2015; Renedo et al., 2018). Conversely, Audouin's gull fledglings are fed mainly on benthic and mesopelagic species from fisheries discards (e.g., Sanpera et al., 2007; García-Tarrasón et al., 2013). In the mesopelagic and benthic zones of the water column the incidence of sunlight and thus photo-demethylation rates are not as high as in the epipelagic zone (Blum et al., 2013). As such, odd-MIF signatures observed in Audouin's gull fledglings' feathers are lower than in common tern individuals. Analogously, black-headed gulls fed juveniles with rice-field prey associated to the paddy soils (e.g., crayfish). In this case, possibly due to rice canopy and the turbidity of waters in rice paddies, photochemical reactions are not enhanced in these environments (Gantner et al., 2009; Senn et al., 2010; Sherman and Blum, 2013), resulting in low odd-MIF values in black-headed gull fledglings' feathers.

The information obtained from the Hg stable isotopic signatures regarding the trophic ecology for each species is consistent with the information obtained from the trophic markers ($\delta^{15}\text{N}$ and $\delta^{13}\text{C}$). Audouin's gull fledglings are fed mainly on benthic and mesopelagic discarded fish which have higher trophic levels (e.g., Pedrocchi et al., 2002) resulting in higher $\delta^{15}\text{N}$ than SH, which are fed mainly with epipelagic fish species of lower trophic levels (Cotín et al., 2011). This is consistent with $\delta^{15}\text{N}$ reported in the literature for potential prey for these seabird species, where small pelagic fish present lower nitrogen isotopic signatures than demersal fish (small pelagic fish: $\delta^{15}\text{N}$ [mean \pm SD]: 7.95 ± 1.08 ; demersal fish: $\delta^{15}\text{N}$ [mean \pm SD]: 9.54 ± 1.39 ; Navarro et al., 2009). $\delta^{15}\text{N}$ values in black-headed gull fledglings are similar to those observed in Audouin's gull fledglings. Nevertheless, it is important to consider that the application of fertilizers in agricultural fields might be increasing $\delta^{15}\text{N}$ in these environments' food webs (Hebert and Wassenaar, 2001; Cole et al., 2004). Therefore, black-headed gull nitrogen signatures need to be interpreted with caution, since they might not reflect accurately their trophic position, when comparing species across habitats. In addition, Audouin's gull $\delta^{15}\text{N}$ signatures could also be influenced by the baseline signatures of oceanic food webs where the fishing activity takes place (Senn et al., 2010). The $\delta^{13}\text{C}$ signatures observed in each of the three species are consistent with the foraging habitats for each of the species, with Audouin's gull and common tern fledglings presenting higher values than black-headed gull fledglings, associated to trophic sources from marine environments, as reported in Navarro et al. (2009): small pelagic fish: $\delta^{13}\text{C}$ (mean \pm SD): -18.99 ± 0.71 and demersal fish: $\delta^{13}\text{C}$ (mean \pm SD): -18.19 ± 0.76 . Conversely, the lower $\delta^{13}\text{C}$ signatures observed in black-headed gull fledglings, are similar to those found in other avian species in the area foraging freshwater habitats, as reported in Cotín et al. (2011): *Chlidonias hybridus* (whiskered tern): $\delta^{13}\text{C}$ (mean \pm SD): -24.6 ± 0.7 and *Sterna nilotica* (gull-billed tern): $\delta^{13}\text{C}$ (mean \pm SD): -24.8 ± 0.5 . This lower $\delta^{13}\text{C}$ values are also consistent with those from *Procambarus clarkii* individuals found in the literature: $\delta^{13}\text{C}$ [mean \pm SD]: -27.73 ± 1.56 (Navarro et al., 2010) and -28.5 ± 2.8 (authors' unpublished data).

Rice-fields are flooded with water coming from the Ebro River, that transports anthropogenic Hg downstream from the chlor-alkali site

Table 3

Summary statistics (mean, SD, max and min) for each of the parameters ($\delta^{15}\text{N}$, $\delta^{13}\text{C}$, THg, $\Delta^{199}\text{Hg}$, $\Delta^{201}\text{Hg}$, $\delta^{202}\text{Hg}$ and $\Delta^{200}\text{Hg}$) in fledglings' feathers for each of the years studied (2010, 2013, 2014 and 2017; N = 10 individuals sampled for each year) on the Audouin's gull (*I. audouinii*) breeding colony in the Ebro Delta.

Year	$\delta^{15}\text{N}$ (‰)			$\delta^{13}\text{C}$ (‰)			THg (ng/g dw)			$\Delta^{199}\text{Hg}$ (‰)			$\Delta^{201}\text{Hg}$ (‰)			$\delta^{202}\text{Hg}$ (‰)			$\Delta^{200}\text{Hg}$ (‰)										
	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max					
2010	12.67	0.30	12.37	13.27	12.38	-17.97	0.36	-17.46	-18.73	4288.55	1260.41	6441.93	2438.68	1.27	0.12	1.45	1.12	1.02	1.11	1.16	0.89	0.59	0.23	0.88	0.27	0.04	0.03	0.1	-0.02
2013	12.65	0.28	13.14	12.35	-19.82	-19.82	2.57	-17.26	-24.10	2227.59	443.56	2710.85	1588.99	1.05	0.16	1.25	0.85	0.85	0.12	1.00	0.67	0.15	0.10	0.32	0.00	-0.005	0.03	0.05	-0.04
2014	13.85	0.47	14.83	13.21	-17.45	-17.45	1.10	-16.20	-19.60	8032.03	3119.42	15009.73	5084.04	1.17	0.16	1.49	0.92	0.96	0.25	1.57	0.63	0.60	0.10	0.77	0.46	0.032	0.02	0.06	0.01
2017	13.77	0.38	14.52	13.28	-17.65	-17.65	0.82	-16.83	-19.69	7982.97	951.94	9205.55	6439.79	0.89	0.16	1.11	0.60	0.68	0.14	0.85	0.38	0.26	0.10	0.43	0.11	0.009	0.05	0.1	-0.05

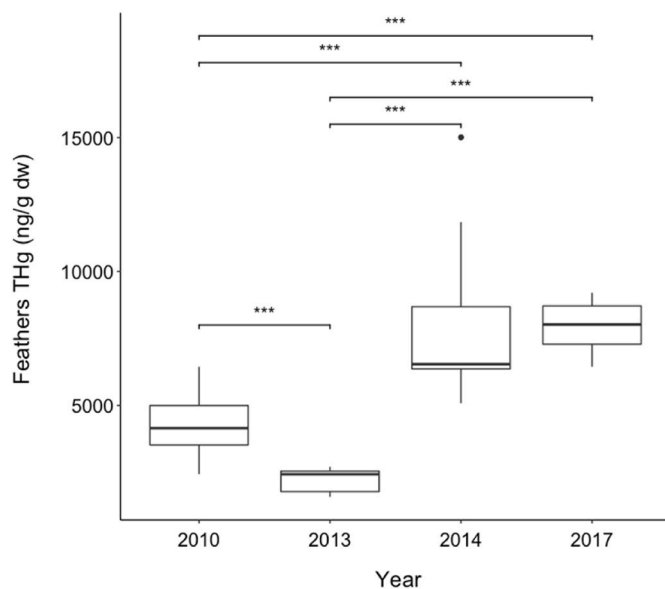


Fig. 4. Boxplots for THg (ng/g dw) in feathers of Audouin's gull (*I. audouinii*) fledglings from breeding seasons of 2010, 2013, 2014, 2017 (N = 10 individuals for each year) in the Ebro Delta breeding colony. Pairwise significant differences between years are represented above the boxplots (*p < 0.05, **p < 0.01, ***p < 0.001).

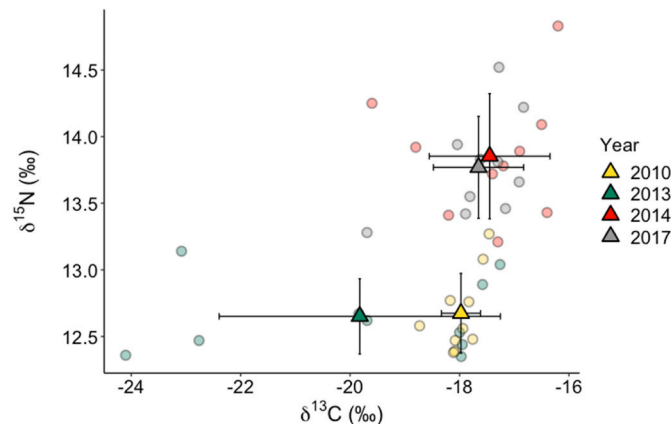


Fig. 5. Biplot of $\delta^{15}\text{N}$ (‰) and $\delta^{13}\text{C}$ (‰) in feathers of Audouin's gull (*I. audouinii*) fledglings from the Ebro Delta breeding colony in 2010, 2013, 2014 and 2017 (N = 10 individuals for each year). Individual data are plotted as dots. Triangles and error bars represent mean \pm SD.

(Palanques et al., 2020). Generally, significant MIF has not been observed during river transport of Hg downstream (e.g., Washburn et al., 2017; Schudel et al., 2018; Reinfelder and Janssen, 2019). Thus, we would expect black-headed gull fledglings will present odd-MIF signatures closer to those from the polluted site. Several studies have reported in sediments and biota from polluted sites, odd-MIF signatures are close to 0 ‰ (Kwon et al., 2014; Wiederhold et al., 2015; Feng et al., 2019). This is consistent with our data, as black-headed gulls present relatively low odd-MIF values ($\Delta^{199}\text{Hg}$: 1.00 ± 0.20). And since Audouin's gull fledglings present the lowest odd-MIF signatures ($\Delta^{199}\text{Hg}$: 0.89 ± 0.16) similar to those from black-headed gulls, this could point out that part of its mercury has a continental origin from the sediments transported by the river as influenced by the upstream polluted site. Indeed, although marine sedimentary Hg is often dominated by Hg of geogenic origin (e.g., volcanic activity, cinnabar deposits), surface layers of marine sediments have been shown to retain anthropogenic contributions (Ogrinc et al., 2019). Therefore, it is

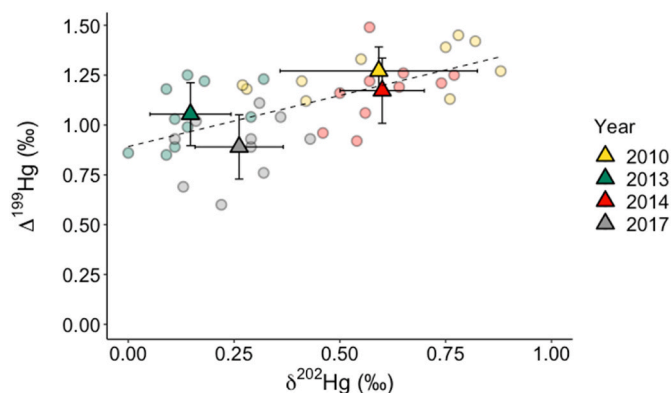


Fig. 6. Biplot of MDF $\delta^{202}\text{Hg}$ (‰) and odd-MIF $\Delta^{199}\text{Hg}$ (‰) in feathers of Audouin's gull fledglings from the Ebro Delta breeding colony in 2010, 2013, 2014 and 2017 (N = 10 individuals for each year). Individual data are plotted as dots. Triangles and error bars represent mean \pm SD. Dashed line represents linear regression between these variables for all the years studied: $y = 0.8920 + 0.5115x$; $R^2 = 0.3611$; $p < 0.0001$.

possible that anthropogenic mercury transported by the river has been deposited over decades in the sea sediment (Gustin et al., 2020), and it has accessed the benthic/mesopelagic food web (Perrot et al., 2010; Bonsignore et al., 2015), from which Audouin's gull fledglings are fed.

The species studied presented a $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ slope of 1.17 (Fig. S2). $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ ratios are used to identify mechanisms involving MIF variations. Also, they are assumed to be preserved along food webs biomagnification after MeHg assimilation by primary producers (Bergquist and Blum, 2007). Experimentally theoretical $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ slopes have been determined for aquatic systems, being $1.36 (\pm 0.02, 2 \text{ SE})$ for MeHg photodemethylation and $1.00 (\pm 0.02, 2 \text{ SE})$ for inorganic Hg photoreduction (Bergquist and Blum, 2007). Several studies reported in marine and freshwater biota $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ slopes higher than 1.00 and slightly lower than 1.30 (e.g., Senn et al., 2010; Gehrke et al., 2011; Point et al., 2011; Day et al., 2012; Renedo et al., 2018). This deviation from the experimentally determined slopes is probably due to the fact that in natural conditions, $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ slopes signatures depend on a variety of factors such as DOC levels relative to Hg concentrations in the environment, type of DOC present, organic ligands binding to Hg, irradiance, and the proportion of MeHg and inorganic mercury in biota (Bergquist and Blum, 2007; Zheng and Hintelmann, 2009; Li et al., 2022).

In the case of MDF ($\delta^{202}\text{Hg}$), both black-headed gull and Audouin's gull values were significantly lower (Audouin's gull: 0.26 ± 0.10 ; black-headed gull: 0.57 ± 0.17) than those from common tern (1.05 ± 0.09), and are closer to 0‰. Although odd-MIF is much more sensitive to photochemical reactions, the higher positive MDF values observed in common tern feathers might reflect photochemical demethylation of MeHg in the photic zone of the marine water column, leaving a residual MeHg enriched in $\delta^{202}\text{Hg}$ (Blum et al., 2013; Bonsignore et al., 2015; Renedo et al., 2018) compared to black-headed gulls and Audouin's gulls. Also, MDF signatures in fish from estuarine freshwater ecosystems compared to marine environments are shown to be lower (Li et al., 2016). Thus, this reflects the trophic ecologies of black-headed gulls and Audouin's gulls, and the influence of Hg derived from terrestrial/freshwater environments in the diet of Audouin's gull fledglings. We also detected significant differences in MDF signatures between black-headed gull and Audouin's gull, with Audouin's gull $\delta^{202}\text{Hg}$ values being lower than black-headed gull values (Fig. 3). These differences possibly arise from the impact of Hg legacy pollution from the chlor-alkali industry. Indeed, Cransveld et al. (2017) compared sea bass (*Dicentrarchus labrax*) populations among coastal polluted areas, and those subject to local chlor-alkali derived Hg pollution, presented higher $\delta^{202}\text{Hg}$ signatures than those in which background atmospherically

Table 4 Pearson's correlation tests between each of the mercury isotopic tracers ($\delta^{202}\text{Hg}$, $\Delta^{199}\text{Hg}$ and $\Delta^{201}\text{Hg}$) and THg, $\delta^{13}\text{C}$, and $\delta^{15}\text{N}$ in fledglings' feathers for each of the years studied (2010, 2013, 2014 and 2017) and for all the years together on the Audouin's gull (*I. audouinii*) breeding colony in the Ebro Delta. Significant correlations are highlighted in bold.

	2010		2013		2014		2017		All Years	
	$\delta^{202}\text{Hg}$	$\Delta^{199}\text{Hg}$	$\delta^{202}\text{Hg}$	$\Delta^{201}\text{Hg}$	$\delta^{202}\text{Hg}$	$\Delta^{199}\text{Hg}$	$\delta^{202}\text{Hg}$	$\Delta^{201}\text{Hg}$	$\delta^{202}\text{Hg}$	$\Delta^{199}\text{Hg}$
THg	r	0.49	-0.32	-0.47	-0.07	-0.41	-0.18	-0.33	0.29	-0.09
	p-value	0.15	0.37	0.17	0.84	0.24	0.61	0.33	0.07	0.57
$\delta^{13}\text{C}$	r	-0.07	-0.35	-0.37	0.46	0.66	-0.14	0.72	0.29	0.04
	p-value	0.84	0.32	0.29	0.18	0.04	0.70	0.02	0.07	0.83
$\delta^{15}\text{N}$	r	0.11	0.17	-0.37	0.45	-0.09	-0.50	-0.03	0.15	-0.32
	p-value	0.77	0.63	0.29	0.20	0.80	0.14	0.92	0.33	0.04

Hg-contamination was assumed to constitute the main driver of $\delta^{202}\text{Hg}$ values. Chlor-alkali processes induce MDF, which help to trace Hg coming from industrial waste waters (Wiederhold et al., 2015). Although this possibly explains the higher MDF values of black-headed gull fledglings compared to those from Audouin's gull fledglings we cannot rule out other factors that could be modifying MDF values and that would need further studying (e.g., trophic transfer [Laffont et al., 2009; Perrot et al., 2010, but see Kwon et al., 2012], metabolic reactions or physiological processes from the individual [Renado et al., 2021]).

The regression between $\Delta^{199}\text{Hg}$ and $\delta^{202}\text{Hg}$ values showed a significant positive relationship with a slope of 2.21. This value is similar to those reported in previous studies, for lake and marine organisms (Bergquist and Blum, 2007; Motta et al., 2019; Kwon et al., 2020), and consistent with experimental studies, reporting increasing $\delta^{202}\text{Hg}$ and $\Delta^{199}\text{Hg}$ in remaining MeHg during photodegradation (Bergquist and Blum, 2007), thus, implying a benthic-epipelagic gradient.

Finally, $\Delta^{200}\text{Hg}$ values are consistent with those reported in previous studies using seabirds as model organisms (Point et al., 2011; Day et al., 2012) and in Mediterranean biota (Jiskra et al., 2021). Our results show $\Delta^{200}\text{Hg}$ values close to 0 ‰ or slightly positive, which other studies have pointed out to be values related to some extent to anthropogenic sources (Obrist et al., 2018; Kwon et al., 2020).

Correlation analyses did not show significant correlations for any of the species studied between MDF and THg, $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ in feathers. This reflects the fact that MDF mercury isotopic signatures are not only affected by the trophic ecology of the individuals and biomagnification processes along food webs (Laffont et al., 2009; Perrot et al., 2010) but also intrinsic metabolism and physiological processes, especially in high trophic level organisms (Laffont et al., 2009; Jackson, 2018; Rua-Ibarz et al., 2019; Tsui et al., 2020; Renado et al., 2020, 2021) and other environmental factors, including differences in Hg dynamics and fractionation on rice-paddies (e.g., Yin et al., 2013; Qin et al., 2020) vs. marine environments (e.g., Archer and Blum, 2018), which need further study and are beyond the scope of the present work. However, THg levels in feathers of common terns did correlate negatively with odd-MIF values ($\Delta^{199}\text{Hg}$ and $\Delta^{201}\text{Hg}$), but not for Audouin's gull or black-headed gull fledglings. The observed negative correlation might appear since, even if major diet items foraged by common terns in the colony studied to feed their chicks are epipelagic fish species (mainly clupeids; Arcos et al., 2002), some of the individuals might also rely on a wider range of food items, including terrestrial prey or mesopelagic/demersal resources obtained from fisheries discards (Oro and Ruiz, 1997; Guitart et al., 2003; Cotín et al., 2011). This is consistent with a higher variability in odd-MIF signatures in this species. Conversely, both gulls' species present low odd-MIF variability (Fig. 3). Finally, we found positive correlations between $\delta^{13}\text{C}$ and odd-MIF values in black-headed gulls and Audouin's gulls (Table 2). These positive correlations are in accordance to those reported in guillemots (*Uria aalge* and *U. lomvia*) breeding in the Alaskan Arctic, which was attributed to a $\delta^{13}\text{C}$ gradient from terrestrial to oceanic systems' sources and a lower photochemical demethylation in coastal systems due to higher turbidity (Day et al., 2012). In our study, these positive correlations might reflect individual variability within species in dietary sources with different proportions of continental and coastal marine resources, especially in Audouin's gull (Sanpera et al., 2007; García-Tarrasón et al., 2015).

Thus, mercury stable isotopes confirm the trophic ecologies for the species under study and point out a possible influence of Hg from anthropogenic origin in Audouin's gull fledglings, as signatures are similar to those in black-headed gull fledglings. Furthermore, they distinguish the foraging ecologies in a vertical gradient of Audouin's gull and common tern, with Audouin's gull consuming mainly benthic/mesopelagic species and common tern mainly consuming epipelagic prey.

4.2. Temporal variation in stable isotopic signatures in Audouin's gull fledglings

4.2.1. Total mercury concentrations

Total mercury concentrations in the Audouin's gull colony varied among years (Fig. 4). Previous studies in this colony (Sánchez-Fortún et al., 2020), have suggested this could be possibly related to the sediment dredging that took place in 2013–2014 in the site next to the chlor-alkali plant, which remobilized legacy mercury. In the present study, Hg levels were 2017 \approx 2014 > 2010 > 2013. This is consistent with the results obtained by Sánchez-Fortún et al. (2020) for a longer time series from the same breeding colony using larger sample sizes. Accordingly, we would expect that the mercury found in 2014 is predominantly mercury sourced from the chlor-alkali plant surroundings that has been transported through the river. The peak in THg in feathers of Audouin's gull fledglings in 2017 could be attributed to the deposition over time of mercury transported by the river on the coastal seabed (Palanques et al., 2020).

4.2.2. Mercury, carbon and nitrogen stable isotopes

We found differences in both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ stable isotopes signatures in feathers among years. Nonetheless, the differences observed need to be interpreted with caution due to low sample sizes. The $\delta^{13}\text{C}$ signatures from 2010, 2014 and 2017 are consistent with carbon stable isotopes signatures from marine environments (Fig. 5; García-Tarrasón et al., 2013; Sánchez-Fortún et al., 2020). Individuals from 2013 presented the largest range of $\delta^{13}\text{C}$ values, indicating significant differences in carbon sources among the individuals sampled. According to the $\delta^{13}\text{C}$ observed in 2013 at least 3 individuals were being fed predominantly with rice-field sources (Fig. 5), with their $\delta^{13}\text{C}$ ranging from -19.7 to -24.1 ‰. This is consistent with this and other studies in the area using aquatic birds feeding from freshwater resources (e.g., black-headed gull [Table 1, this study], Cotín et al., 2011). $\delta^{13}\text{C}$ values were positively associated with THg values in feathers, accounting for the different dietary proportions from rice-fields or marine environments, with individuals fed with higher proportions of rice-field preys (lower $\delta^{13}\text{C}$), having lower THg levels in feathers (García-Tarrasón et al., 2013).

Regarding $\delta^{15}\text{N}$ signatures, there were clear differences between 2010 vs. 2013, and 2014 vs. 2017, although we did not detect any association with THg levels in feathers. In this colony, it has been observed, that besides a possible pollution episode derived from the cleansing of the chlor-alkali plant site in 2013–2014, a parallel eutrophication process could have been occurred (Sánchez-Fortún et al., 2020), varying the baseline $\delta^{15}\text{N}$ over time (Cole et al., 2004), thus influencing the consumer's $\delta^{15}\text{N}$, but not the trophic level of the individuals.

Mercury stable isotopes showed differences among years for $\delta^{202}\text{Hg}$, $\Delta^{199}\text{Hg}$ and $\Delta^{201}\text{Hg}$ values. Regarding mean $\Delta^{199}\text{Hg}$ values among years, we observed the following: year 2017 < 2013 \approx 2014 < 2017 (Table 3), although statistically significant differences were not always detected due to intra-annual variability in individual signatures. $\Delta^{199}\text{Hg}$ in 2017 ranged from 0.60 to 1.11‰, whereas in 2010 from 1.12 to 1.45‰, in 2013 from 0.85 to 1.25‰ and in 2014 from 0.92 to 1.49‰. $\Delta^{199}\text{Hg}$ (and $\Delta^{201}\text{Hg}$) showed a significant negative correlation with THg (when pooling the data for all the years (Table 4). Since individuals being fed with more proportion of fisheries discards (mesopelagic and benthic species) rather than epipelagic or freshwater/terrestrial sources will likely present higher levels of Hg in their bodies (García-Tarrasón et al., 2013) and lower odd-MIF isotopic composition (Blum et al., 2013) this might explain the negative correlation encountered. The $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ slope obtained from the regression on this dataset was 1.09, which lays in between the experimentally derived slopes for Hg(II) photoreduction (~ 1.00) and MeHg photodemethylation (~ 1.30). The odd-MIF slope found for all the years in this study is consistent with the one found in common murre's eggs by Day et al. (2012), that was 1.09. However, with the existing knowledge on Hg fractionation in the

environment, 1.09 is inconclusive in terms of the process leading to $\Delta^{199}\text{Hg}$ and $\Delta^{201}\text{Hg}$ signatures prior to the incorporation in the food web (Day et al., 2012). As previously stated, factors including type and relative abundance of DOC to Hg in the environment or irradiance, might develop an important role in determining $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ slope (Bergquist and Blum, 2007; Zheng and Hintelmann, 2009), and for our samples, we assume they will vary widely among years and due to the complexity of the ecosystems under study.

No significant correlation was found between odd-MIF signatures and $\delta^{13}\text{C}$, possibly due to the low ranges observed in $\delta^{13}\text{C}$ values in the years studied, except for $N = 3$ individuals in 2013 which presented lower $\delta^{13}\text{C}$ values. We found significant negative correlation between MIF signatures and $\delta^{15}\text{N}$ values when considering the data for all the years (Table 4). However, due to $\delta^{15}\text{N}$ variations in food webs baselines over years, interpretations on this correlation in terms of trophic levels might be misleading (Sánchez-Fortún et al., 2020). Nonetheless, even if data on Hg stable isotopes in sediments from the polluted site at Flix is not available, for all the years studied, odd-MIF values ranged from 0.60 to 1.49 ‰. These values seem to indicate that overall, there might be an influence of Hg proceeding from the polluted site in the Audouin's gull colony in the Ebro Delta over the years in a varying degree.

Regarding even-MIF values, for the years considered in this study, both $\Delta^{200}\text{Hg}$ and $\Delta^{204}\text{Hg}$ signatures obtained were close to 0 ‰ or slightly positive, thus indicating that possibly Hg input in the colony is influenced to some extent by anthropogenic sources (Obriest et al., 2018; Kwon et al., 2020).

Interestingly, MDF ($\delta^{202}\text{Hg}$) in Audouin's gull fledgling feathers presented similar values between 2013 and 2017 (mean \pm SD: 0.20 \pm 0.11) and between 2010 and 2014 (mean \pm SD: 0.60 \pm 0.18), being MDF values in 2010 and 2014 higher than those from 2013 to 2017. Since $\delta^{202}\text{Hg}$ values were not correlated with $\delta^{15}\text{N}$ nor $\delta^{13}\text{C}$, this $\delta^{202}\text{Hg}$ difference among years was not due to an altered trophic ecology (i.e., as a result of trophic level exploited [Point et al., 2011; Tsui et al., 2020]). Furthermore, MDF values for 2010 and 2014 are closer to those obtained for black-headed gull in this study (mean \pm SD: 0.57 \pm 0.17), a species considered to be more impacted by anthropogenic derived mercury.

Several studies in the Ebro River and the Ebro Delta area pointed out the possible influence of the presence of the Flix chlor-alkali plant on Hg levels in biota (Sanpera et al., 2007; Faria et al., 2010; Cotín et al., 2011; Carrasco et al., 2011; Sánchez-Fortún et al., 2020). The phase-down of activities using Hg in the plant started in 2004 with a consequent decrease in Hg emissions (Pujadas, 2016), until the complete cessation of them in 2017. In 2013 a management plan to remediate the contaminated soils nearby the chlor-alkali site was performed, which could have had an impact on biota downstream the river, due to mobilization of legacy mercury. Sánchez-Fortún et al. (2020) analyzed the temporal trends on THg in feathers from juveniles of the Audouin's gull colony in the Ebro Delta from 2004 to 2019: from 2004 to 2013 a progressive decrease was observed in THg, possibly due to ecosystem recovery (Kopeck et al., 2019), followed by a tipping point with increased THg levels in 2014, and a slight increase in THg concentrations from 2014 onwards, although with fluctuations among years. Using compound-specific stable isotopes analyses of amino acids (CSIA-AA), the study concluded that a trophic shift of the individuals in the colony was not responsible for the increased THg levels observed from 2014 onwards (Sánchez-Fortún et al., 2020). Thus, we expected the Audouin's gull colony in the years 2010 and 2014 to be affected by a major extent with Hg transported from the chlor-alkali plant site, while in 2013 and 2017 would be less impacted by anthropogenic pollution due to ecosystem recovery (Kopeck et al., 2019). The results of the present study regarding MDF values on these years seem to be consistent with this idea, with individuals presenting higher MDF values in 2010 and 2014, associated to higher influence of chlor-alkali derived Hg (Cransveld et al., 2017; Feng et al., 2019).

Nonetheless, although we did find a weak positive correlation between $\delta^{202}\text{Hg}$ and THg levels in feathers, which would reinforce the idea

of an increase of $\delta^{202}\text{Hg}$ coupled to the additional pollution from the chlor-alkali site, it was not significant ($r = 0.29$; $p = 0.07$). Unfortunately, with the available data we are not able to discern among the possible factors and sources leading to the different concentrations in total mercury in feathers from the different years. Possibly, river dynamics over decades has had an influence in transporting Hg from the polluted site towards the coastal seabed sediments surrounding the colony (Palanques et al., 2020), which would lead to an increase in Hg on the marine food web (e.g., Sánchez-Fortún et al., 2020), although other factors not taken into account in this study (e.g., physiology of species; Renedo et al., 2020, 2021; fractions of inorganic mercury and MeHg accumulated in organisms; Pinzone et al., 2021) and the complexity of estuarine environments could be influencing THg levels or MDF signatures, and thus, modifying the correlation between MDF and THg values.

5. Conclusions

Stable isotopes of mercury have been shown to be a promising tool in investigating processes and sources leading to Hg body burdens in biota. In this study, we found a relation between Hg stable isotopes signatures and the trophic ecologies of three species of seabirds breeding in the Ebro Delta area (NE Iberian Peninsula), which has been long impacted by the legacy mercury pollution from a chlor-alkali plant. Furthermore, we have also investigated how temporal changes in anthropogenic activities leading to Hg release or remobilization in the environment could be traced by Hg stable isotopes measured in a bioindicator species such as the Audouin's gull. Nonetheless, due to complexity of ecosystems, trophic ecology, physiology of species and other processes affecting mercury isotopic signatures, particularly MDF, more studies in this direction should be performed in order to be able to discern processes related to Hg accumulation in top-predators foraging in complex ecosystems such as wetlands and marine environments.

CRedit authorship contribution statement

Moisés Sánchez-Fortún: Conceptualization, Formal analysis, Investigation, Project administration, Writing – original draft, Writing – review & editing. **David Amouroux:** Conceptualization, Formal analysis, Investigation, Writing – review & editing. **Emmanuel Tessier:** Investigation, Writing – review & editing. **Josep Lluís Carrasco:** Conceptualization, Formal analysis, Supervision, Writing – review & editing. **Carola Sanpera:** Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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