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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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1 **Mercury stable isotopes in seabirds in the Ebro Delta (NE Iberian Peninsula):**
2 **inter-specific and temporal differences**

3
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26
27 **Keywords:** feathers, Laridae, anthropogenic mercury, Mediterranean, Audouin's
28 gull

31 **Abstract**

32 Mercury (Hg) is a global pollutant, which specially affects aquatic ecosystems, both marine
33 and freshwater. Top-predators depending on these environments, such as seabirds, are regarded
34 as suitable bioindicators of Hg pollution. In the Ebro Delta (NE Iberian Peninsula), legacy Hg
35 pollution from a chlor-alkali industry operating in Flix and located ca. 100 km upstream of the
36 Ebro River mouth has been impacting the delta environment and the neighboring coastal area.
37 Furthermore, natural occurring levels of Hg in the Mediterranean Sea are known to be high
38 compared to other marine areas. In this work we used a Hg stable isotopes approach in feathers
39 to disentangle the Hg sources and processes leading to different Hg concentrations in three
40 *Laridae* species breeding in sympatry in the area (Audouin's gull *Ichtyaethus audouinii*, black-
41 headed gull *Chroicocephalus ridibundus*, common tern *Sterna hirundo*). These species have
42 distinct trophic ecologies and make a different use of marine resources and rice paddies prey.
43 Moreover, for Audouin's gull in which in the Ebro Delta colony temporal differences in Hg
44 levels were documented previously, we investigated the change in the contribution of different
45 Hg sources using the same approach. Hg stable isotopes differentiated the three different
46 species according to their trophic ecologies. Furthermore, for Audouin's gull we observed
47 temporal variations in Hg isotopic signatures possibly owing to anthropogenic-derived
48 pollution in the Ebro Delta. To the best of our knowledge this is the first time Hg stable isotopes
49 have been reported in seabirds from the NW Mediterranean.

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65 1. Introduction

66

67 Mercury (Hg) is considered a global pollutant, which is present in all Earth compartments, and
68 exerts detrimental effects on both humans and wildlife (Clarkson and Magos 2006; Discroll et
69 al. 2013; Evers 2018). Although Hg naturally occurs in the environment, it can also be found
70 as result of human activities (Discroll et al. 2013). Among anthropogenic activities introducing
71 mercury into the environment are artisanal gold mining or industrial processes (e.g., coal
72 combustion, chlor-alkali plants) (UNEP 2019). Anthropogenic activities have significantly
73 increased mercury levels in the environment since the pre-industrial era (Amos et al. 2013;
74 UNEP 2019), which poses an urge to monitor environmental loads of this pollutant and be able
75 to distinguish between anthropogenic and natural sources in order to help environmental-
76 related decisions (Wiener et al. 2003).

77

78 Particularities of this element include a complex biogeochemical cycle resulting in chemical
79 speciation (Selin 2009; Gustin et al. 2020), which depends on both the biotic and abiotic factors
80 in the environment where mercury is found (Ullrich et al. 2001; Lyman et al. 2020; Bowman
81 et al. 2020). For instance, in aquatic environments, inorganic mercury (Hg^{2+}) is known to be
82 methylated by mainly sulfate- and iron-reducing bacteria (Podar et al. 2015), but also by abiotic
83 processes (Celo et al. 2006), transforming Hg^{2+} into methylmercury (MeHg). MeHg is a readily
84 bioavailable and highly toxic form of mercury which can bioaccumulate in organisms and get
85 biomagnified along food webs (Morel et al. 1998; Lavoie et al. 2013; Evers 2018).

86

87 In this context, top-predator and long-lived species, such as seabirds, are more exposed to Hg
88 and its negative effects including physiological, reproductive, neurological and teratogenic
89 effects (Whitney and Cristol 2017). In fact, seabirds have been extensively used as bioindicator
90 species of environmental pollution for an array of contaminants, including mercury (Furness
91 and Camphuysen 1997; Becker et al. 2002; Burger and Gochfeld 2004; Sanpera et al. 2007;
92 Blévin et al. 2013; Albert et al. 2019; Bond and Lavers 2020).

93

94 The Ebro Delta (NE Iberian Peninsula) comprises 30 000 ha of wetlands, with 75% of its area
95 used for rice-farming, 20% protected as a natural reserve, and 5% is urbanized (Mañosa et al.
96 2001). Since the late 19th century, a chlor-alkali plant has been operating ca. 100 km upstream
97 the Ebro River mouth. During the active period of the plant, 550 000 – 700 000 m³ of
98 contaminated sediments, mainly with mercury and organochlorine compounds, were

99 accumulated on the bottom of the river dam, close to the factory (Grimalt et al. 2003; ACA
100 2013). Although the production of chlorine using mercury ceased in 2017, legacy mercury
101 pollution persists and has been impacting all the environments in the Ebro River delta, because
102 of the transport of contaminated particles along the river (Carrasco et al. 2010, 2011; Cotín et
103 al. 2012; Campillo et al. 2019; Palanques et al. 2020). Moreover, during 2013-2014, a sediment
104 remediation plan, including the dredging and disposal of polluted sediments, was implemented
105 to remove toxicants from the riverbed in the vicinity of the factory. In a previous study,
106 Sánchez-Fortún et al. (2020) reported the possible influence of the remediation tasks which
107 took place in 2013-2014 on the increase of mercury levels observed in the Audouin's gull
108 breeding colony in the Ebro Delta area. Furthermore, due to its biogeochemical characteristics
109 and local Hg reservoirs, methylmercury levels in the Mediterranean Sea are high compared to
110 other oceanic regions (Cossa and Coquery 2005). Thus, seabird species depending on food
111 webs from the Ebro Delta area might be also exposed to naturally high levels of mercury (Arcos
112 et al. 2002; Sanpera et al. 2007; Cotín et al. 2011; Sánchez-Fortún et al. 2020). However, the
113 relative importance of mercury sources impacting aquatic habitats from the area (freshwater or
114 coastal areas) has never been analyzed in depth.

115

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

121

122 Mercury has seven stable isotopes with atomic masses 196, 198, 199, 200, 201, 202, and 204
123 amu, which can undergo both mass-dependent fractionation (MDF) and mass-independent
124 fractionation (MIF). MDF occurs as consequence of physical, chemical and biological
125 processes including mercury biological methylation and demethylation (Kritee et al. 2009;
126 Rodríguez-González et al. 2009; Janssen et al. 2016), photoreduction (Bergquist and Blum
127 2007), trophic transfer (Laffont et al. 2009), metabolic reactions (Laffont et al. 2009; Feng et
128 al. 2015; Renedo et al. 2020; 2021). MIF occurs mainly as consequence of photochemical
129 reactions in the water column prior to bioaccumulation of Hg (Bergquist and Blum 2007).
130 Although MIF processes have been described for isotopes ^{200}Hg and ^{204}Hg (i.e., even-MIF),
131 and for ^{199}Hg and ^{201}Hg (i.e., odd-MIF; Kwon et al. 2020), odd-MIF has been observed
132 commonly in biological samples (Tsui et al. 2020). Thus, MDF and MIF signatures offer a

133 framework to trace Hg pollution and may provide information on Hg sources in organisms.
134 This information coupled with carbon and nitrogen stable isotopes values, which have been
135 used extensively as proxies of the trophic ecology of organisms (Post 2002; Boecklen et al.
136 2011), might provide valuable information on the causes leading to high Hg levels in wildlife.

137
138 The main aim of this study is to relate the trophic ecology of different *Laridae* species
139 inhabiting in the Ebro Delta area to the total-Hg (THg) burden found in the organism and
140 potential Hg sources at local scale. We have analyzed Hg stable isotopes in feathers of fledgling
141 individuals of three different species breeding in the Ebro Delta, that present contrasted dietary
142 characteristics: the common tern (*Sterna hirundo*; SH), an epipelagic marine forager (Cotín et
143 al. 2011), the black-headed gull (*Chroicocephalus ridibundus*; CR), which mostly forages in
144 rice-fields (Antón-Tello et al. 2021), and the Audouin's gull (*Ichthyaetus audouinii*; IA), which
145 forages in both marine environments (fisheries discards) and in rice-fields (Sanpera et al. 2007;
146 García-Tarrasón et al. 2015). We then analyzed the stable isotopes of Hg to unravel the
147 differential contribution of various Hg sources over years on the Audouin's gull breeding
148 colony in the Ebro Delta, to comprehend if differences of this metal observed from before and
149 after 2013, when the remediation plan was applied (Sánchez-Fortún et al. 2020), are related to
150 changes in Hg sources.

151

152 **2. Materials and methods**

153

154 *2.1. Fieldwork and sample collection*

155

156 As part of a continued monitoring program of the Audouin's gull (*Ichthyaetus audouinii*, IA)
157 breeding colony in the Ebro Delta (NE Iberian Peninsula: 40°33' N, 00°39' E), 10-15 mantle
158 feathers of juvenile individuals (approx. 3 weeks old) were sampled on years 2010, 2013, 2014,
159 2017 (N = 10 individuals each year; see Sánchez-Fortún et al. 2020 for further details on
160 sampling). Additionally, during the breeding season of 2017, 10-15 mantle feathers of juvenile
161 individuals from the following species breeding in the Ebro Delta area were sampled (N = 10
162 individuals for each species): common tern (*Sterna hirundo*, SH; approx. 3 weeks old), black-
163 headed gull (*Chroicocephalus ridibundus*, CR; approx. 3 weeks old). Feathers were stored in
164 zip-lock bags until analysis.

165

166 *2.2. Total mercury determination*

167

168 Total mercury (THg) determination in our samples was performed at the Scientific and
169 Technological Centers of the University of Barcelona (Spain). Feather samples were washed
170 using 0.25 M NaOH and rinsed with distilled water to remove external Hg contamination
171 (Bearhop et al. 2000). After cleaning, samples were oven dried to constant mass at 50°C and
172 then ground to a homogenous powder with an impactor mill (Freezer Mill 6850,
173 SpexCertiPrepH Inc., Metuchen, NJ, USA) operating at liquid nitrogen temperature. THg in
174 feathers (ng/g dry weight) was determined by combustion using a Direct Mercury Analyzer
175 (Milestone® DMA-80) for all the species samples from 2017, or by Induction Coupled Plasma-
176 Mass Spectrometer (ICP-MS) for Audouin's gull samples from 2010, 2013 and 2014. DMA-
177 80 analyses were directly analyzed by combustion using 0.20-0.30 mg of feather sample. For
178 the ICP-MS mercury determinations, ~30 mg of feather sample were acid digested in a
179 microwave oven using 1-2 mL of nitric acid (HNO₃ 70%) and 0.5-1 mL of hydrogen peroxide
180 (H₂O₂ 30%) in Teflon containers. To check for the accuracy of measurements for both methods,
181 we analyzed a certified reference material in duplicate (NIES-13 Human Hair CRM) every 10
182 to 15 samples with recoveries ranging from 91% to 94% for DMA-80 analyses and 97 to 103%
183 in ICP-MS analyses. Thus, no corrections were applied to the THg values of the samples prior
184 to statistical analyses.

185

186 *2.3. Analysis of mercury stable isotopes*

187

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

200 stable isotopes analyses are reported in detail in Table S1, Table S2 and Table S3 from the
201 Supplementary Materials.

202

203 Mass-dependent fractionation (MDF) is reported as $\delta^{202}\text{Hg}$ values (‰) calculated as follows
204 (Blum and Bergquist 2007):

205

$$206 \quad \delta^{202}\text{Hg} = [({}^{202}\text{Hg}/{}^{198}\text{Hg})_{\text{sample}}/({}^{202}\text{Hg}/{}^{198}\text{Hg})_{\text{NIST3133}} - 1] \times 1000$$

207

208 Mass-independent Hg isotopic fractionation (MIF) is reported as $\Delta^{199}\text{Hg}$, $\Delta^{200}\text{Hg}$, $\Delta^{201}\text{Hg}$,
209 $\Delta^{204}\text{Hg}$ (‰), calculated using the difference by the $\delta^{\text{xxx}}\text{Hg}$ value and a value predicted based
210 on MDF (Bergquist and Blum 2007):

211

$$212 \quad \Delta^{204}\text{Hg} \approx \delta^{204}\text{Hg} - (\delta^{202}\text{Hg} \times 1.493)$$

$$213 \quad \Delta^{201}\text{Hg} \approx \delta^{201}\text{Hg} - (\delta^{202}\text{Hg} \times 0.752)$$

$$214 \quad \Delta^{200}\text{Hg} \approx \delta^{200}\text{Hg} - (\delta^{202}\text{Hg} \times 0.502)$$

$$215 \quad \Delta^{199}\text{Hg} \approx \delta^{199}\text{Hg} - (\delta^{202}\text{Hg} \times 0.252)$$

216

217 Where $\Delta^{199}\text{Hg}$ and $\Delta^{201}\text{Hg}$ are known as odd-MIF, and $\Delta^{200}\text{Hg}$ and $\Delta^{204}\text{Hg}$ as even-MIF.
218 Additionally, we calculated the ratio $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$, which has proven to be indicative of
219 different photochemical transformation processes and speciation of Hg (i.e., photoreduction of
220 Hg(II) vs. photodemethylation of MeHg) in experimental settings (Bergquist and Blum 2007).
221 $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg} > 1$ has been determined on samples containing predominantly MeHg, for
222 instance, biota samples, whereas $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg} \approx 1$ has been observed in environmental
223 samples (e.g., sediments, water) containing predominantly Hg(II) (Tsui et al. 2020).

224

225 *2.4. Stable isotopes of carbon and nitrogen analysis*

226

227 Stable isotopic analyses for carbon and nitrogen were carried out at the Scientific and
228 Technological Centers of the University of Barcelona (Spain). 0.30-0.35 mg of feather powder
229 were placed into tin capsules and analyzed using a Thermo-Finnigan Flash 1112 elemental
230 analyser (CE Elantech, Lakewood, NJ, USA) coupled to a Delta-C isotope ratio mass
231 spectrometer via a CONFLO III interface (Thermo Finnigan MAT, Bremen, Germany). Stable
232 isotope signatures are expressed using the standard delta notation relative to Vienna Pee Dee

233 Belemnite ($\delta^{13}\text{C}$) and atmospheric nitrogen ($\delta^{15}\text{N}$). IAEA standards (IAEA CH₇, IAEA CH₆,
234 USGS 24 for ^{13}C , and IAEA N₁, IAEA N₂, IAEA NO₃ for ^{15}N) were applied every 12 samples
235 to calibrate the system. Replicate assays of standards indicated analytical measurement errors
236 of $\pm 0.1\%$ and $\pm 0.2\%$ for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively.

237

238 *2.5. Statistical analyses*

239

240 THg values and stable isotopes signatures ($\delta^{202}\text{Hg}$, $\Delta^{199}\text{Hg}$, $\Delta^{201}\text{Hg}$, $\Delta^{200}\text{Hg}$, $\Delta^{204}\text{Hg}$, $\delta^{13}\text{C}$,
241 $\delta^{15}\text{N}$) were visually inspected to assess normality. THg values were log-transformed to
242 approximate residual normality. Homogeneity of variances was tested using the Breusch-Pagan
243 test.

244

245 *2.5.1. Total mercury and stable isotopes signatures in Laridae species*

246

247 ANOVA tests were performed to test differences among the three species studied (IA, CR and
248 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.
249 Further pairwise comparisons were evaluated using Tukey tests. A linear model was fitted in
250 order to evaluate the association between logTHg (response variable) and $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and
251 Species and the interactions $\delta^{13}\text{C} \times \text{Species}$ and $\delta^{15}\text{N} \times \text{Species}$ as covariates.

252

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

257

258 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
259 $\Delta^{200}\text{Hg}/\Delta^{204}\text{Hg}$ for all the species studied.

260

261

262 *2.5.2. Temporal changes in total mercury and stable isotope signatures in Audouin's gull* 263 *fledglings*

264

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

271

272 A linear mixed effects model was fitted to evaluate the association between log THg (response
273 variable) and $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ (covariates), with year as a random effect. Correlation tests were used
274 to assess correlations between each of the mercury isotopic tracers and THg in feathers, $\delta^{13}\text{C}$,
275 and $\delta^{15}\text{N}$ for each of the years studied and for all the years studied.

276

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

282

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

286

287 **3. Results**

288

289 *3.1. Species differences in total mercury concentrations and isotopic composition*

290

291 3.1.1. Total mercury concentrations

292

293 We analyzed feather samples from 10 individuals sampled in 2017 breeding season for each of
294 the species of study (Audouin's gull (IA), black-headed gull (CR), common tern (SH)). Table
295 1 shows the mean \pm SD for each of the variables studied. Significant differences in THg
296 concentrations in fledglings' feathers were observed among species ($F_{2,27} = 167.64$, $p < 0.0001$;
297 Fig. 1). Post-hoc tests showed significant differences among the three species considered (Fig.

298 1). Mean THg concentrations in feathers observed was greater for IA, followed by SH, and CR
299 fledglings (Table 1).

300

301 3.1.2. Carbon and nitrogen stable isotopes and total mercury concentrations

302

303 Significant differences in both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ were observed among species ($\delta^{13}\text{C}$: $F_{2,27} = 68.64$,
304 $p < 0.0001$ and $\delta^{15}\text{N}$: $F_{2,27} = 29.02$, $p < 0.0001$). Post-hoc pairwise comparisons showed
305 significant differences in $\delta^{13}\text{C}$ between IA-CR ($p < 0.0001$) and CR-SH ($p < 0.0001$), but not
306 between IA-SH ($p = 0.29$) with $\delta^{13}\text{C}$ values being (IA \approx SH) $>$ CR (Fig. 2). Regarding $\delta^{15}\text{N}$
307 values, we detected differences between IA-SH ($p < 0.0001$) and CR-SH ($p < 0.0001$), but not
308 between IA-CR ($p = 0.48$). $\delta^{15}\text{N}$ values were higher in IA and CR than SH (Fig. 2).

309

310 The linear model did not show a significant association between $\delta^{15}\text{N}$ or $\delta^{13}\text{C}$ and the observed
311 THg levels in feathers ($\delta^{15}\text{N}$: $F_{1,21} = 0.025$, $p = 0.88$; $\delta^{13}\text{C}$: $F_{1,21} = 0.004$, $p = 0.95$). The
312 interaction terms in the model had no effect on THg levels ($\delta^{15}\text{N} \times \text{Species}$: $F_{2,21} = 0.52$, $p =$
313 0.60 ; $\delta^{13}\text{C} \times \text{Species}$: $F_{2,21} = 1.22$, $p = 0.32$).

314

315 3.1.3. Mercury stable isotopes composition

316

317 Regarding mercury stable isotopes we found significant interspecific differences for $\delta^{202}\text{Hg}$
318 values ($F_{2,27} = 101.50$, $p < 0.0001$), $\Delta^{199}\text{Hg}$ ($F_{2,27} = 198.41$, $p < 0.0001$) and $\Delta^{201}\text{Hg}$ ($F_{2,27} =$
319 181.49 , $p < 0.0001$). Post-hoc pairwise comparisons showed significant differences among the
320 three species for $\delta^{202}\text{Hg}$ (all comparisons: $p < 0.0001$; Fig. 3) and between SH and CR, and CR
321 and IA, but not between IA and CR for both $\Delta^{199}\text{Hg}$ (IA-SH: $p < 0.0001$; CR-SH: $p < 0.0001$;
322 IA-CR: $p = 0.53$; Fig. 3) and $\Delta^{201}\text{Hg}$ (IA-SH: $p < 0.0001$; CR-SH: $p < 0.0001$; IA-CR: $p =$
323 0.50). Finally, no significant differences were found among species for $\Delta^{200}\text{Hg}$ ($F_{2,27} = 1.99$, p
324 $= 0.15$) or $\Delta^{204}\text{Hg}$ ($F_{2,26} = 1.12$, $p = 0.34$).

325

326 Regression of $\Delta^{199}\text{Hg}$ against $\delta^{202}\text{Hg}$ showed a significant positive relationship ($F_{1,24} = 144.37$,
327 $p < 0.0001$; Fig. 3), with a regression slope of 2.21. The ratio obtained from the regression
328 between odd-MIF values $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ for the three species studied was 1.16 (Fig.S1). The
329 ratio between $\Delta^{200}\text{Hg}/\Delta^{204}\text{Hg}$ was not significantly different from 0 (Fig. S2).

330

331 Correlations among Hg stable isotopes and THg, $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$, for each of the species are
332 shown in Table 2.

333

334 *3.2. Temporal variation in stable isotopic composition in Audouin's gull fledglings*

335

336 3.2.1. Total mercury concentrations

337

338 Along the IA samples (N = 10 individuals) analyzed in 2017, we also determined the THg in
339 feathers from 10 IA fledglings in 2010, 2013, 2014, for a total of 40 feather samples (Table 3).
340 We found significant differences among years ($F_{3,36} = 55.24$, $p < 0.0001$). Post-hoc pairwise
341 comparisons detected significant differences among all year's pairs except 2014-2017 (Fig. 4).
342 Mean THg concentrations in Audouin's gull fledgling feathers were higher in
343 2017 \approx 2014 $>$ 2010 $>$ 2013 (Table 3).

344

345 3.2.2. Carbon and nitrogen stable isotopes and total mercury concentrations

346

347 Significant differences were observed for $\delta^{13}\text{C}$ values ($H = 10.16$, $p = 0.017$) and $\delta^{15}\text{N}$ values
348 ($F_{3,36} = 32.90$, $p < 0.0001$) among years. Multiple pairwise comparisons for $\delta^{13}\text{C}$ values in
349 feathers showed significant differences between 2013-2014 ($p = 0.011$) and 2013-2017 ($p =$
350 0.019), but not for the rest of pairwise comparisons (2010-2013: $p = 0.22$; 2010-2014: $p = 0.12$;
351 2010-2017: $p = 0.052$; 2014-2017: $p = 0.50$; Fig. 5). Pairwise comparisons for $\delta^{15}\text{N}$ signatures
352 showed significant differences among all years' pairs ($p < 0.0001$ in all cases), except 2010-
353 2013 ($p = 1.00$) and 2014-2017 ($p = 0.96$; Fig. 5), with $\delta^{15}\text{N}$ values higher in 2014 and 2017
354 than in 2010 and 2013 (Table 3).

355

356 The linear mixed effects model did show a significant association between $\delta^{13}\text{C}$ and the
357 observed THg levels in feathers ($F_{1,34} = 5.20$, $p = 0.029$). Nonetheless, no significant
358 association was observed between $\delta^{15}\text{N}$ values and THg levels in feathers ($F_{1,34} = 1.29$, $p =$
359 0.26).

360

361 3.2.3. Mercury stable isotopes composition

362

363 We found significant differences among years for $\delta^{202}\text{Hg}$ values ($F_{3,36} = 25.30$, $p < 0.0001$),
364 $\Delta^{199}\text{Hg}$ ($F_{3,36} = 11.68$, $p < 0.0001$) and $\Delta^{201}\text{Hg}$ ($F_{3,36} = 8.25$, $p = 0.0002$) in gulls' feathers. Post-

365 hoc pairwise comparisons showed significant differences for $\delta^{202}\text{Hg}$ signatures among all
366 years' pairs ($p < 0.0001$) except the pairs 2013-2017 ($p = 0.30$) and 2010-2014 ($p = 1.00$).
367 Regarding $\Delta^{199}\text{Hg}$ pairwise comparisons showed significant differences between 2010-2013 (p
368 $= 0.02$), 2010-2017 ($p < 0.0001$) and 2014-2017 ($p = 0.001$), although no significant differences
369 were detected among the other years' pairs (in all cases, $p > 0.05$). For $\Delta^{201}\text{Hg}$ values pairwise
370 comparisons showed significant differences between years 2010-2017 ($p = 0.0003$) and 2014-
371 2017 ($p = 0.003$), but not among the other years' pairs (in all cases, $p > 0.05$).

372

373 Regarding $\Delta^{200}\text{Hg}$ and $\Delta^{204}\text{Hg}$, we found significant differences among species for $\Delta^{200}\text{Hg}$
374 ($F_{3,36} = 3.45$, $p = 0.026$) but not for $\Delta^{204}\text{Hg}$ ($F_{3,36} = 0.86$, $p = 0.47$). Post-hoc comparisons
375 showed significant differences in $\Delta^{200}\text{Hg}$ values for 2010-2013 ($p = 0.037$) but not among the
376 other years' pairs (in all cases, $p > 0.05$).

377

378 Regression of $\Delta^{199}\text{Hg}$ against $\delta^{202}\text{Hg}$ values showed a significant positive relationship ($F_{1,38} =$
379 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
380 was 1.09 (Fig. S3). The ratio between $\Delta^{200}\text{Hg}/\Delta^{204}\text{Hg}$ was not significantly different from 0
381 (Fig. S4).

382

383 Correlations among mercury stable isotopes and THg, $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$, for each of the years and
384 all the years pooled are shown in Table 4.

385

386 4. Discussion

387

388 4.1. Species differences in total mercury concentrations and isotopic composition

389

390 4.1.1. Total mercury concentrations

391

392 Fledglings from the three species studied (IA, CR and SH) presented differences in THg levels
393 in feathers. IA fledglings feathers had higher mercury concentration than those from SH or CR.
394 This would be related to the different trophic resources exploited by each species, as mercury
395 is mainly acquired through diet. Although the three species coexist in the same area and their
396 breeding colonies are few km apart, they have distinct trophic ecologies: IA and SH depend
397 mainly on marine resources, whereas CR forage mainly on rice-fields. Moreover, IA and SH
398 exploit different marine resources. Essentially, SH are epipelagic foragers (Cotín et al. 2011),

399 while IA are opportunistic foragers on fisheries discards (Sanpera et al. 2007; García-Tarrasón
400 et al. 2015). Besides, IA also forage in terrestrial and freshwater environments (e.g., rice-fields;
401 Oro et al. 1996; Ruiz et al. 1996; Pedrocchi et al. 2002; Sanpera et al. 2007).

402

403 4.1.2. Mercury, carbon and nitrogen stable isotopes

404

405 Differences among the three species were found for $\delta^{202}\text{Hg}$, whereas $\Delta^{199}\text{Hg}$ and $\Delta^{201}\text{Hg}$ only
406 differed between SH and IA, and between SH and CR. The differences observed for the odd-
407 MIF and MDF signatures might reflect differences in sources of mercury, the distinct trophic
408 ecologies among species, and differences among processes linked to Hg speciation among
409 foraging environments. Differences in $\Delta^{200}\text{Hg}$ and $\Delta^{204}\text{Hg}$ signatures were not observed among
410 the three species.

411

412 $\Delta^{199}\text{Hg}$ values reflect differences in MeHg sources owing to photodemethylation in the water
413 column (Bergquist and Blum 2007). Since odd-MIF signatures are maintained along the food
414 webs (Kwon et al. 2012), they reflect the foraging habitats from which fledglings of the
415 different species are fed. SH consumed epipelagic resources, where the incidence of sunlight
416 and photodemethylation processes predominate, leading to larger odd-MIF values (Blum et al.
417 2013; Bonsignore et al. 2015; Renedo et al. 2018). Conversely, IA are fed mainly on benthic
418 and mesopelagic species from fisheries discards (Sanpera et al. 2007; García-Tarrasón et al.
419 2013), for which the incidence of sunlight and thus photodemethylation rates are not as high
420 (Blum et al. 2013). As such, odd-MIF signatures observed in AI fledglings' feathers will be
421 lower. Analogously, CR feed juveniles with rice-field preys associated to the paddy soils (e.g.,
422 crayfish). In this case, possibly due to rice canopy and the turbidity of waters in rice paddies,
423 photochemical reactions are not enhanced in these environments (Gantner et al. 2009; Senn et
424 al. 2010; Sherman and Blum 2013), resulting in low odd-MIF values in CR fledglings' feathers.

425

426 The information obtained from the Hg stable isotopic signatures regarding the trophic ecology
427 for each species is consistent with the information obtained from the trophic markers. $\delta^{13}\text{C}$
428 values are regarded as a proxy of carbon sources in diet and $\delta^{15}\text{N}$ is a proxy of trophic position
429 (Post et al. 2002). IA fledglings are fed mainly on benthic and mesopelagic discarded fish
430 which have higher trophic levels (e.g., Pedrocchi et al. 2002) resulting in higher $\delta^{15}\text{N}$ than SH,
431 which are fed mainly with epipelagic fish species of lower trophic levels (Cotín et al. 2011).
432 $\delta^{15}\text{N}$ values in CR fledglings are similar to those observed in IA. Nevertheless, it is important

433 to consider that the application of fertilizers in agricultural fields might be increasing $\delta^{15}\text{N}$ in
434 these environments' food webs (Herbert and Wasseenar 2001; Cole et al. 2004). Therefore, CR
435 nitrogen signatures need to be interpreted with caution, since they might not reflect accurately
436 their trophic position, when comparing species across habitats. The $\delta^{13}\text{C}$ signatures observed
437 in each of the three species are consistent with the foraging habitats for each of the species,
438 with IA and SH fledglings presenting higher values than CR fledglings, associated to trophic
439 sources from marine environments (García-Tarrasón et al. 2013). Conversely, the lower $\delta^{13}\text{C}$
440 signatures observed in CR fledglings, are similar to those found in other avian species in the
441 area foraging freshwater habitats (Cotín et al. 2011).

442

443 Rice-fields are flooded with water coming from the Ebro river, that transports anthropogenic
444 Hg downstream from the chlor-alkali site (Palanques et al. 2020). Generally, significant MIF
445 has not been observed during river transport of Hg downstream (e.g., Washburn et al. 2017;
446 Schudel et al. 2018; Reinfelder and Janssen 2019). Thus, we would expect CR fledglings will
447 present odd-MIF signatures closer to those from the polluted site. And since IA fledglings
448 present odd-MIF signatures similar to those from CR, this could point out that part of its
449 mercury has a continental origin from the sediments transported by the river as influenced by
450 the upstream polluted site. Indeed, although marine sedimentary Hg is often dominated by Hg
451 of geogenic origin (e.g., volcanic activity, cinnabar deposits), surface layers of marine
452 sediments have been shown to retain anthropogenic contributions (Ogrinc et al. 2019).
453 Therefore, it is possible that anthropogenic mercury transported by the river has been deposited
454 over decades in the sea sediment (Gustin et al. 2020), and it has accessed the
455 benthic/mesopelagic food web (Perrot et al. 2010; Bonsignore et al. 2015), from which IA
456 fledglings are fed. This observation is consistent with several studies, that have reported in
457 sediments and biota from polluted sites, MIF signatures are close to 0‰ (eKwon et al. 2014;
458 Wiederhold et al. 2015; Feng et al. 2019).

459

460 The species studied presented a $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ slope of 1.17 (Fig. S1). $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ ratios
461 are used to identify mechanisms involving MIF variations. Also, they are assumed to be
462 preserved along food webs biomagnification after MeHg assimilation by primary producers
463 (Bergquist and Blum 2007). Experimentally theoretical $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ slopes have been
464 determined for aquatic systems, being for MeHg photodemethylation 1.36 (± 0.02 , 2 SE) and
465 1.00 (± 0.02 , 2 SE) for inorganic Hg photoreduction (Bergquist and Blum 2007). Several studies
466 reported in marine and freshwater biota $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ slopes higher than 1.00 and slightly

467 lower than 1.30 (e.g., Senn et al. 2010; Gehrke et al. 2011; Point et al. 2011; Day et al. 2012;
468 Renedo et al. 2018). This deviation from the experimentally determined slopes is probably due
469 to the organic ligands binding of Hg in the freshwater or marine environments (Bergquist and
470 Blum 2007; Zheng and Hintelmann 2009).

471

472 In the case of MDF ($\delta^{202}\text{Hg}$) values, both CR and IA were significantly lower (IA: 0.26 ± 0.10 ;
473 CR: 0.57 ± 0.17) than those from SH (1.05 ± 0.09) and are closer to 0‰. Although odd-MIF is
474 much more sensible to photochemical reactions, the higher positive MDF values observed in
475 SH feathers might reflect photochemical demethylation of MeHg in the photic zone of the
476 marine water column, leaving a residual MeHg enriched in $\delta^{202}\text{Hg}$ (Blum et al. 2013;
477 Bonsignore et al. 2015; Renedo et al. 2018) compared to CR and IA. Also, MDF signatures in
478 fish from estuarine freshwater ecosystems compared to marine environments are shown to be
479 more negative (Li et al. 2016). Thus, this reflects the trophic ecologies of CR and IA, and the
480 influence of Hg derived from terrestrial/freshwater environments in the diet of IA fledglings.
481 We also detected significant differences in MDF signatures between CR and IA, with IA
482 $\delta^{202}\text{Hg}$ values being lower than CR values (Fig. 3). These differences possible arise from the
483 impact of Hg legacy pollution from the chlor-alkali industry. Indeed, Cransveld et al. (2017)
484 compared sea bass (*Dicentrarchus labrax*) populations among coastal polluted areas, and those
485 subject to local chlor-alkali derived Hg pollution, presented higher $\delta^{202}\text{Hg}$ signatures than those
486 in which background atmospherically Hg-contamination was assumed to constitute the main
487 driver of $\delta^{202}\text{Hg}$ values. Chlor-alkali processes induce MDF, which help to trace Hg coming
488 from industrial waste waters (Wiederhold et al. 2015). Although this possibly explains the
489 higher MDF values of CR fledglings compared to those from IA fledglings we cannot rule out
490 other factors that could be modifying MDF values and that would need further studying (e.g.,
491 trophic transfer (Laffont et al. 2009; Perrot et al. 2010, but see Kwon et al. 2012), metabolic
492 reactions or physiological processes from the individual (Renedo et al. 2021).

493

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

500

501 Finally, $\Delta^{200}\text{Hg}$ values are consistent with those reported in previous studies using seabirds as
502 model organisms (Point et al. 2011; Day et al. 2012) and in Mediterranean biota (Jiskra et al.
503 2021). These signatures have been used to trace atmospheric inputs of Hg via precipitation. In
504 precipitation samples, $\Delta^{200}\text{Hg}/\Delta^{204}\text{Hg}$ ratio has been observed to be of -0.2 (Kwon et al. 2020).
505 In our study, although it was negative, it was not significantly different from 0. Possibly, even-
506 MIF values might be influenced by other sources of contamination, trophic transfers or other
507 environmental factors that might be modifying the slope between these two isotopic tracers
508 observed in precipitation samples (Kwon et al. 2020).

509

510 Correlation analyses did not show significant correlations for any of the species studied
511 between MDF and THg, $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ in feathers. This reflects the fact that MDF mercury
512 isotopic signatures are not only affected by the trophic ecology of the individuals and
513 biomagnification processes along food webs (Laffont et al. 2009; Perrot et al. 2010) but also
514 intrinsic metabolism and physiological processes, especially in high trophic level organisms
515 (Laffont et al. 2009; Jackson 2018; Rua-Ibarz et al. 2019; Tsui et al. 2020; Renedo et al. 2020;
516 2021) and other environmental factors, including differences in Hg dynamics and fractionation
517 on rice-paddies (e.g., Yin et al. 2013; Qin et al. 2020) vs. marine environments (e.g., Archer
518 and Blum 2018), which need further study and are beyond the scope of the present work.
519 However, Hg levels in feathers of SH did correlate negatively with odd-MIF values ($\Delta^{199}\text{Hg}$
520 and $\Delta^{201}\text{Hg}$), but not for IA or CR fledglings. The negative correlation observed might appear
521 since, even if major diet items foraged by common terns in the colony studied to feed their
522 chicks are epipelagic fish species (Arcos et al. 2002), some of the individuals might also rely
523 on mesopelagic/demersal resources obtained from fisheries discards (Guitart et al. 2003; Cotín
524 et al. 2011). This is consistent with a higher variability in odd-MIF signatures in this species.
525 Conversely, both gulls' species present low odd-MIF variability (Fig. 3). Finally, we found
526 positive correlations between $\delta^{13}\text{C}$ and odd-MIF values in CR and IA (Table 2). These positive
527 correlations are in accordance to those reported in guillemots (*Uria aalge* and *U. lomvia*)
528 breeding in the Alaskan Arctic, which was attributed to a $\delta^{13}\text{C}$ gradient from terrestrial to
529 oceanic systems' sources and a lower photochemical demethylation in coastal systems due to
530 higher turbidity (Day et al. 2012). In our study, these positive correlations might reflect
531 individual variability within species in dietary sources with different proportions of continental
532 and coastal marine resources, especially in IA (Sanpera et al. 2007; García-Tarrasón et al.
533 2015).

534

535 Thus, mercury stable isotopes confirm the trophic ecologies for the species under study and
536 point out a possible influence of Hg from anthropogenic origin in IA fledglings, as signatures
537 are similar to those in CR fledglings. Furthermore, they distinguish the foraging ecologies in a
538 vertical gradient of IA and SH, with IA consuming mainly benthic/mesopelagic species and
539 SH consuming epipelagic fish.

540

541 *4.2. Temporal variation in stable isotopic signatures in Audouin's gull fledglings*

542

543 *4.2.1. Total mercury concentrations*

544

545 Total mercury concentrations in the Audouin's gull colony varied among years (Fig. 4).
546 Previous studies in this colony (Sánchez-Fortún et al. 2020), have suggested this could be
547 related to the sediment dragging that took place in 2013-2014 in the site next to the chlor-alkali
548 plant, which remobilized legacy mercury. In the present study, Hg levels were
549 2017~2014>2010>2013. This is consistent with the results obtained by Sánchez-Fortún et al.
550 (2020) for a longer time series from the same breeding colony using larger sample sizes.
551 Accordingly, we would expect that the mercury found in 2014 is predominantly mercury
552 sourced from the chlor-alkali plant surroundings that has been transported through the river.
553 The peak in THg in feathers of Audouin's gull fledglings in 2017 could be attributed to the
554 deposition over time of mercury transported by the river on the coastal seabed (Palanques et
555 al. 2020).

556

557 *4.2.2. Mercury, carbon and nitrogen stable isotopes*

558

559 We found differences in both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ stable isotopes signatures in feathers among years.
560 The $\delta^{13}\text{C}$ signatures from 2010, 2014 and 2017 are consistent with carbon stable isotopes
561 signatures from marine environments (Fig. 5; García-Tarrasón et al. 2013; Sánchez-Fortún et
562 al. 2020). Individuals from 2013 presented the largest range of $\delta^{13}\text{C}$ values, indicating
563 significant differences in carbon sources among the individuals sampled. According to the $\delta^{13}\text{C}$
564 observed in 2013 at least 3 individuals were being fed predominantly with rice-field sources
565 (Fig. 5), with their $\delta^{13}\text{C}$ ranging from -19.7 to -24.1‰. This is consistent with this and other
566 studies in the area using aquatic birds feeding from freshwater resources (e.g., black-headed
567 gull [Table 1, this study], Cotín et al. 2011). $\delta^{13}\text{C}$ values were positively associated with THg

568 values in feathers, accounting for the different dietary proportions from rice-fields or marine
569 environments, with individuals fed with higher proportions of rice-field preys (lower $\delta^{13}\text{C}$),
570 having lower THg levels in feathers (García-Tarrasón et al. 2013).

571

572 Regarding $\delta^{15}\text{N}$ signatures, there were clear differences between 2010 vs. 2013, and 2014 vs.
573 2017, although we did not detect any association with THg levels in feathers. In this colony, it
574 has been observed, that besides a possible pollution episode derived from the cleansing of the
575 chlor-alkali plant site in 2013-2014, a parallel eutrophication process could have been occurred,
576 as suggested by Sánchez-Fortún et al. (2020) in a longitudinal study using compound specific
577 stable isotopes of nitrogen, which might variate baseline $\delta^{15}\text{N}$ over time influencing the
578 consumer's $\delta^{15}\text{N}$ (Cole et al. 2004), thus not reflecting the actual trophic position of the
579 individuals if comparing the values among years.

580

581 Mercury stable isotopes showed differences among years for $\delta^{202}\text{Hg}$, $\Delta^{199}\text{Hg}$ and $\Delta^{201}\text{Hg}$
582 values. Regarding $\Delta^{199}\text{Hg}$, the year 2017 presented the lowest values among the years studied,
583 followed by 2013, 2014 which presented similar values, and 2010 with the highest values,
584 although statistically significant differences were not always detected due to intra-annual
585 variability in individual signatures. $\Delta^{199}\text{Hg}$ in 2017 ranged from 0.60–1.11‰, whereas in 2010
586 from 1.12–1.45‰, in 2013 from 0.85–1.25‰ and in 2014 from 0.92–1.49‰. $\Delta^{199}\text{Hg}$ (and
587 $\Delta^{201}\text{Hg}$) showed a significant negative correlation with THg when pooling the data for all the
588 years (Table 4). Since individuals being fed with more proportion of fisheries discards
589 (mesopelagic and benthic species) rather than epipelagic or freshwater/terrestrial sources will
590 likely present higher levels of Hg in their bodies (García-Tarrasón et al. 2013) and lower odd-
591 MIF isotopic composition (Blum et al. 2013) this might explain the negative correlation
592 encountered. The $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ slope obtained from the regression on this dataset was 1.09,
593 which lays in between the experimentally derived slopes for Hg(II) photoreduction (~ 1.00)
594 and MeHg photodemethylation (~ 1.30). The odd-MIF slope found for all the years in this study
595 is consistent with the one found in common murre's eggs by Day et al. (2012), that was 1.09.
596 However, with the existing knowledge on Hg fractionation in the environment, 1.09 is
597 inconclusive in terms of the process leading to $\Delta^{199}\text{Hg}$ and $\Delta^{201}\text{Hg}$ signatures prior to the
598 incorporation in the food web (Day et al. 2012). $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ slopes signatures depend on a
599 variety of factors such as DOC levels relative to Hg concentrations in the environment, type of
600 DOC present and irradiance (Bergquist and Blum 2007; Zheng and Hintelmann 2009). In

601 natural conditions, these factors might develop an important role in determining $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$
602 slope, and for our samples, we assume they will vary widely among years and due to the
603 complexity of the ecosystems under study.

604

605 No significant correlation was found between odd-MIF signatures and $\delta^{13}\text{C}$, possibly due to
606 the low ranges observed in $\delta^{13}\text{C}$ values in the years studied, except for $N = 3$ individuals in
607 2013 which presented lower $\delta^{13}\text{C}$ values (Fig. 5). We found significant negative correlation
608 between MIF signatures and $\delta^{15}\text{N}$ values when considering the data for all the years (Table 4).
609 However, due to $\delta^{15}\text{N}$ variations in food webs baselines over years, interpretations on this
610 correlation in terms of trophic levels might be misleading (Sánchez-Fortún et al. 2020).
611 Nonetheless, even if data on Hg stable isotopes from the polluted site at Flix is not available,
612 for all the years studied, odd-MIF values range from 0.60–1.49‰. These values seem to
613 indicate that overall, there might be an influence of Hg proceeding from the polluted site in the
614 Audouin's gull colony in the Ebro Delta over the years in a varying degree.

615

616 Regarding even-MIF values, for the years considered in this study, the $\Delta^{200}\text{Hg}/\Delta^{204}\text{Hg}$ ratio in
617 IA fledglings feathers was not significantly different from 0. This might be indicative of other
618 sources of contamination, trophic transfers or other environmental factors that might be
619 modifying the slope between these two isotopic tracers found in global precipitation samples
620 (-0.2; Kwon et al. 2020).

621

622 Interestingly, MDF ($\delta^{202}\text{Hg}$) in Audouin's gull fledgling feathers presented similar values
623 between 2013 and 2017 (mean \pm SD: 0.20 ± 0.11) and between 2010 and 2014 (mean \pm SD:
624 0.60 ± 0.18), being MDF values in 2010 and 2014 higher than those from 2013 and 2017. Since
625 $\delta^{202}\text{Hg}$ values were not correlated with $\delta^{15}\text{N}$ nor $\delta^{13}\text{C}$, this $\delta^{202}\text{Hg}$ difference among years was
626 not due to an altered trophic ecology (i.e., as a result of trophic level exploited [Point et al.
627 2011; Tsui et al. 2020]). Furthermore, MDF values for 2010 and 2014 are closer to those
628 obtained for CR in this study (mean \pm SD: 0.57 ± 0.17), a species more impacted by
629 anthropogenic derived mercury.

630

631 Several studies in the Ebro river and the Ebro Delta area pointed out the possible influence of
632 the presence of the Flix chlor-alkali plant on Hg levels in biota (Sanpera et al. 2007; Faria et
633 al. 2010; Cotín et al. 2011; Carrasco et al. 2011; Sánchez-Fortún et al. 2020). The phase-down
634 of activities using Hg in the plant started in 2004 with a consequent decrease in Hg emissions

635 (Pujadas 2016), until the complete cessation of them in 2017. In 2013 a management plan to
636 remediate the contaminated soils nearby the chlor-alkali site was performed, which could have
637 had an impact on biota downstream the river, due to mobilization of legacy mercury. From
638 2004 to 2013, in the Audouin's gull colony in the Ebro Delta a progressive decrease in Hg
639 levels in feathers of fledglings has been described, followed by a slightly increase from 2014
640 onwards (Sánchez-Fortún et al. 2020). Thus, we expected the Audouin's gull colony in the
641 years 2010 and 2014 to be affected by a major extent with Hg transported from the chlor-alkali
642 plant site, while in 2013 and 2017 would be less impacted by anthropogenic pollution due to
643 ecosystem recovery (Kopec et al. 2019). The results of the present study regarding MDF values
644 on these years seem to be consistent with this idea, with individuals presenting higher MDF
645 values in 2010 and 2014, associated to higher influence of chlor-alkali derived Hg (Cransveld
646 et al. 2017; Feng et al. 2019).

647
648 Nonetheless, although we did find a weak positive correlation between $\delta^{202}\text{Hg}$ and THg levels
649 in feathers, which would reinforce the idea of an increase of $\delta^{202}\text{Hg}$ coupled to the additional
650 pollution from the chlor-alkali site, it was not significant ($r = 0.29$; $p = 0.07$). Unfortunately,
651 with the available data we are not able to discern among the possible factors and sources
652 leading to the different concentrations in total mercury in feathers from the different years.
653 Possibly, river dynamics over decades has had an influence in transporting Hg from the
654 polluted site towards the coastal seabed sediments surrounding the colony (Palanques et al.
655 2020), which would lead to an increase in Hg on the marine food web (e.g., Sánchez-Fortún et
656 al. 2020), although other factors not taken into account in this study (e.g., physiology of
657 species; Renedo et al. 2020, 2021; fractions of inorganic mercury and MeHg accumulated in
658 organisms; Pinzone et al. 2021) and the complexity of estuarine environments could be
659 influencing THg levels or MDF signatures, and thus, modifying the correlation between MDF
660 and THg values.

661

662 **5. Conclusions**

663

664 Stable isotopes of mercury have been shown to be a promising tool in investigating processes
665 and sources leading to Hg body burdens in biota. In this study we applied this methodology to
666 understand differences in Hg levels observed in three species of seabirds breeding in the Ebro
667 Delta area (NE Iberian Peninsula), which has been long impacted by the legacy mercury
668 pollution from a chlor-alkali plant. Furthermore, we have also investigated how temporal

669 changes in anthropogenic activities leading to Hg release or remobilization in the environment
670 can be traced by Hg stable isotopes measured in a bioindicator species such as the Audouin's
671 gull. Nonetheless, due to complexity of ecosystems, trophic ecology, physiology of species and
672 other processes affecting mercury isotopic signatures, particularly MDF, more studies in this
673 direction should be performed in order to be able to discern processes related to Hg
674 accumulation in top-predators foraging in complex ecosystems such as wetlands and marine
675 environments.

676

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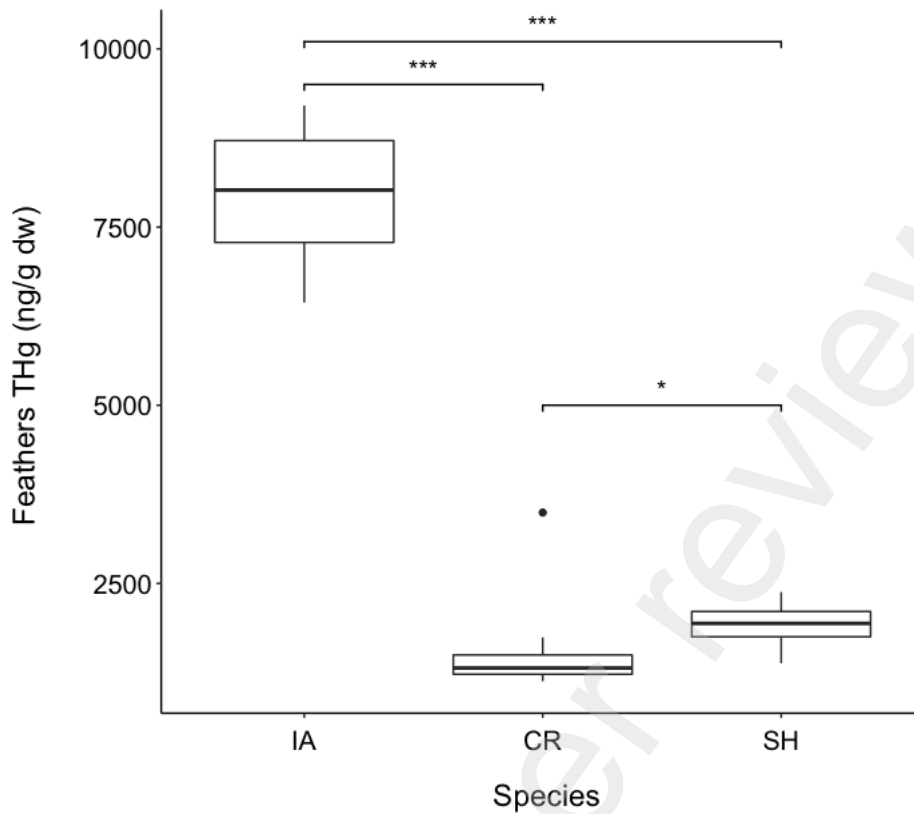
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1036 **Figures and Tables**

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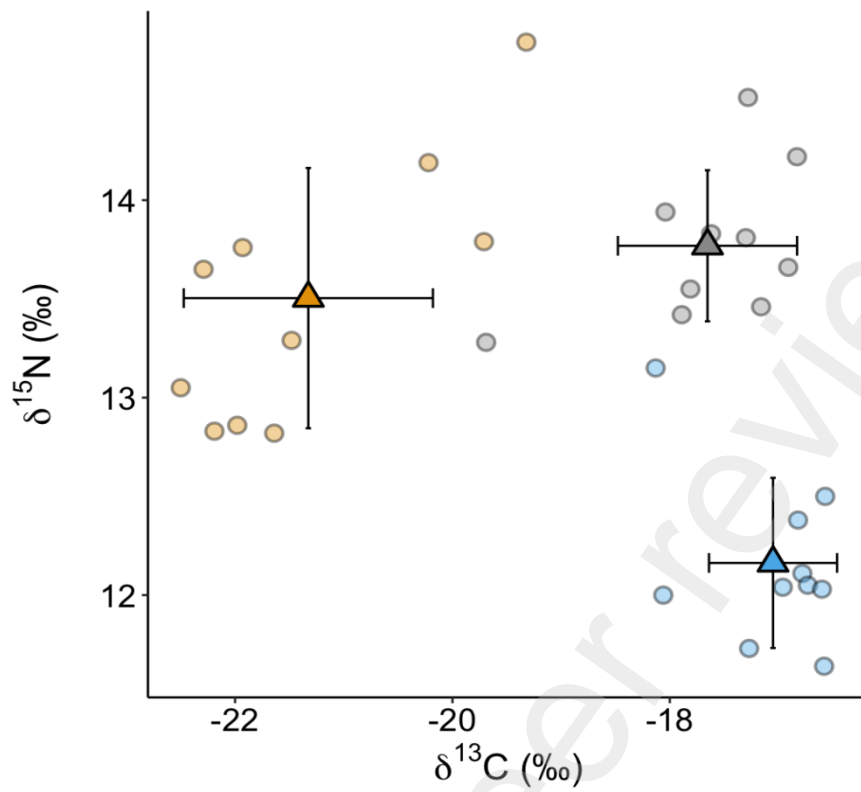
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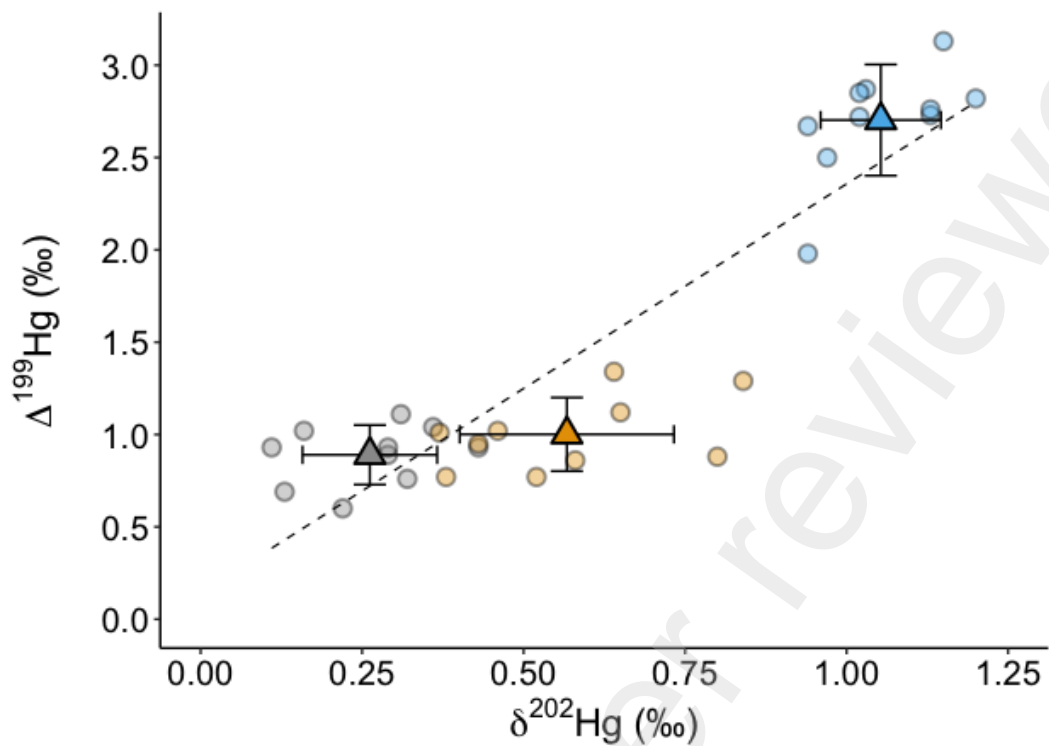
Figure 1. Boxplots for THg (ng/g dw) in feathers of Audouin's gulls (IA), black-headed gull (CR) and common tern (SH) fledglings from the Ebro Delta in 2017. Pairwise significant differences between species are represented above the boxplots (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

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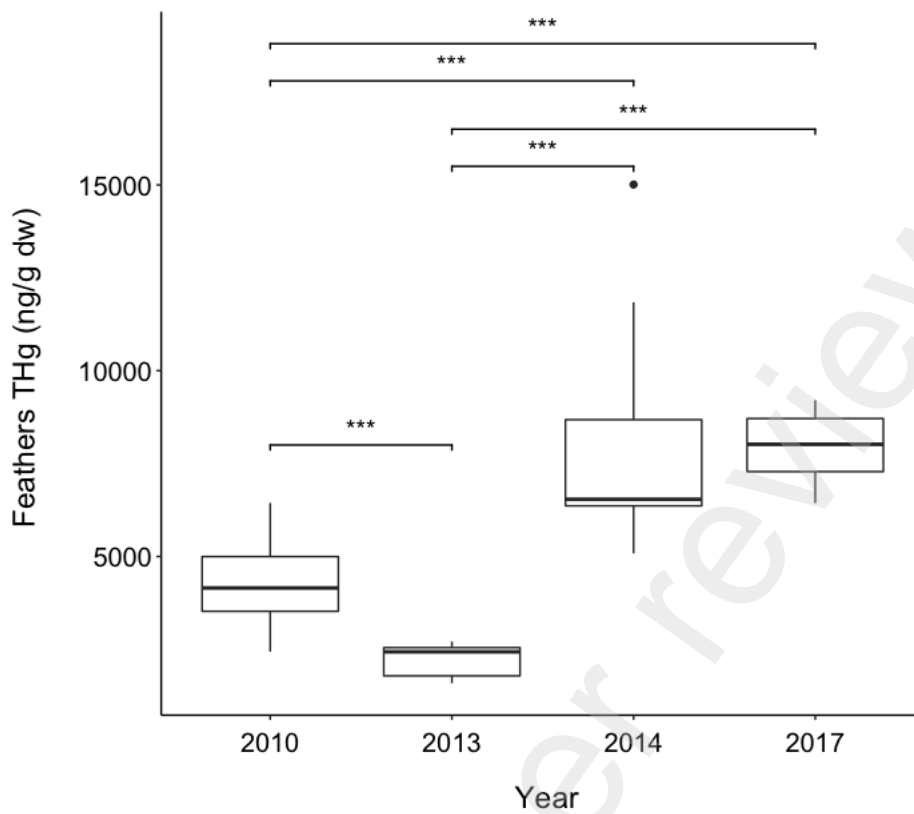
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Figure 2. Biplot of $\delta^{15}\text{N}$ (‰) and $\delta^{13}\text{C}$ (‰) in feathers of Audouin's gulls (IA, in gray), black-headed gull (CR, in orange) and common tern (SH, in blue) fledglings from the Ebro Delta in 2017. Individual data are plotted as dots. Triangles and error bars represent mean \pm SD.



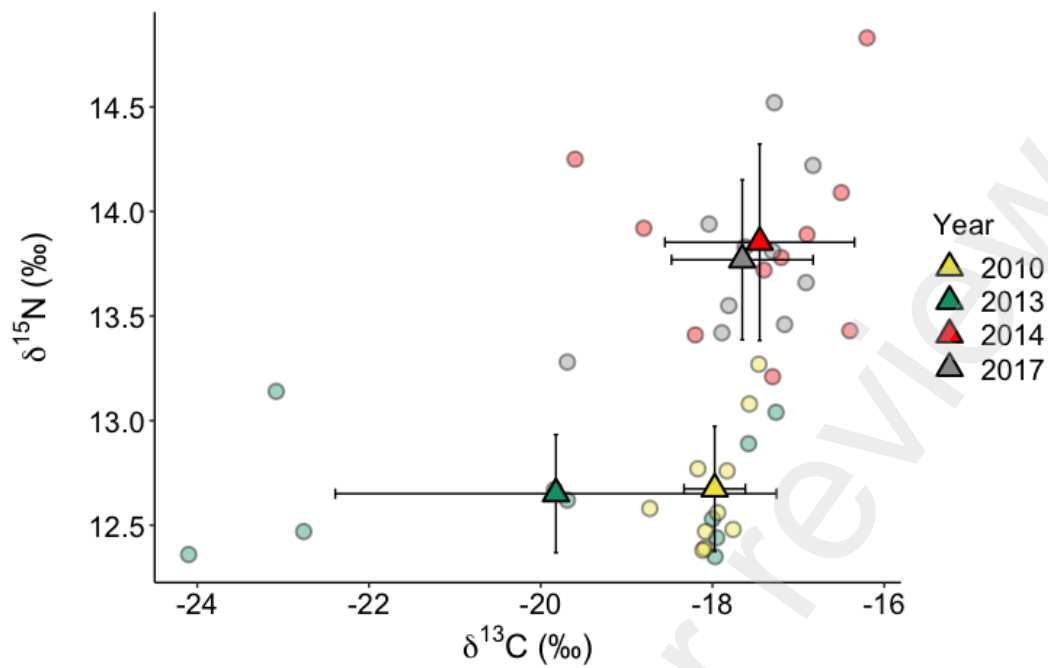
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 1056 **Figure 3.** Biplot of MDF $\delta^{202}\text{Hg}$ (‰) and odd-MIF $\Delta^{199}\text{Hg}$ (‰) in feathers of Audouin's gulls (IA, in gray), black-
 1057 headed gull (CR, in orange) and common tern (SH, in blue) fledglings from the Ebro Delta in 2017. Individual
 1058 data are plotted as dots. Triangles and error bars represent mean \pm SD. Dashed line represents linear regression
 1059 between these variables for the three species: $y = 0.1407 + 2.2167x$; $R^2 = 0.7963$; $p < 0.0001$.

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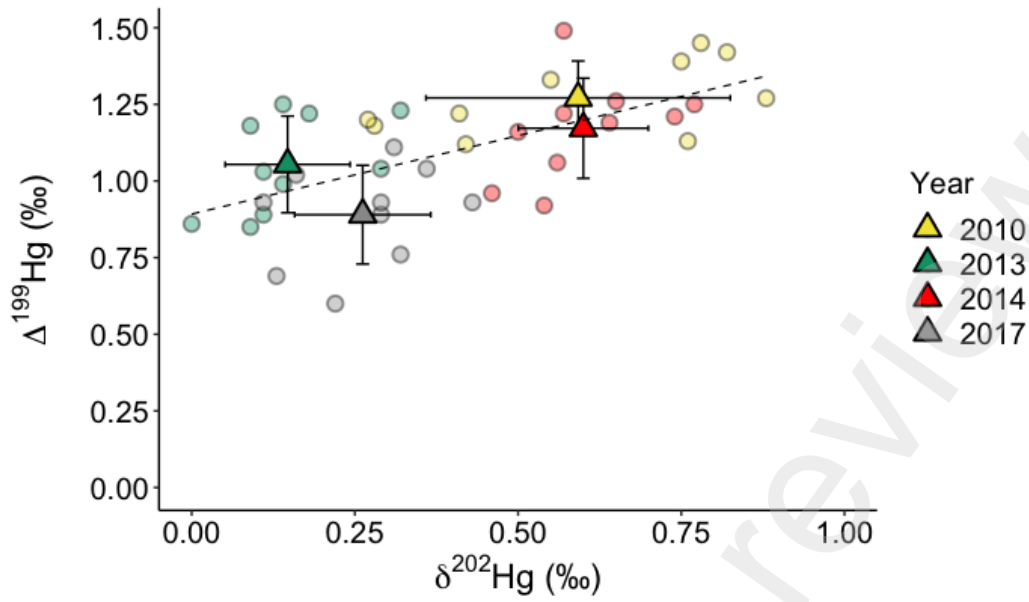
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Figure 4. Boxplots for THg (ng/g dw) in feathers of fledglings Audouin's gulls (*I. audouinii*) from breeding seasons of 2010, 2013, 2014, 2017 (N = 10 individuals for each year) in the Ebro Delta breeding colony. Pairwise significant differences between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



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Figure 5. Biplot of $\delta^{15}\text{N}$ (‰) and $\delta^{13}\text{C}$ (‰) in feathers of Audouin's gulls (*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.



1117 **Figure 6.** Biplot of MDF $\delta^{202}\text{Hg}$ (‰) and odd-MIF $\Delta^{199}\text{Hg}$ (‰) in feathers of Audouin's gulls (IA) fledglings
 1118 from the Ebro Delta breeding colony in 2010, 2013, 2014 and 2017 (N = 10 individuals for each year). Individual
 1119 data are plotted as dots. Triangles and error bars represent mean \pm SD. Dashed line represents linear regression
 1120 between these variables for all the years studied: $y = 0.8920 + 0.5115x$; $R^2 = 0.3611$; $p < 0.0001$.
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Table 1. Summary statistics (mean, SD) for each of the parameters ($\delta^{15}\text{N}$, $\delta^{13}\text{C}$, THg, $\Delta^{199}\text{Hg}$, $\Delta^{201}\text{Hg}$) in fledglings feathers for each of the three species studied during the 2017 breeding season in the Ebro Delta. IA: *I. audouinii* (Audouin's gull), CR: *C. ridibundus* (black-headed gull), SH: *S. hirundo* (common tern).

Species	$\delta^{15}\text{N}$ (‰)				$\delta^{13}\text{C}$ (‰)				THg (ng/g dw)				$\Delta^{199}\text{Hg}$ (‰)				$\Delta^{201}\text{Hg}$ (‰)				$\delta^{202}\text{Hg}$ (‰)			
	Mean	SD	Max	Min	Mean	SD	Max	Min	Mean	SD	Max	Min	Mean	SD	Max	Min	Mean	SD	Max	Min	Mean	SD	Max	Min
IA	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
CR	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
SH	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

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. IA: *I. audouinii* (Audouin's gull), CR: *C. ridibundus* (black-headed gull), SH: *S. hirundo* (common tern). Significant correlations are highlighted in bold.

		Audouin's gull (IA)			Black-headed gull (CR)			Common tern (SH)		
		$\delta^{202}\text{Hg}$	$\Delta^{199}\text{Hg}$	$\Delta^{201}\text{Hg}$	$\delta^{202}\text{Hg}$	$\Delta^{199}\text{Hg}$	$\Delta^{201}\text{Hg}$	$\delta^{202}\text{Hg}$	$\Delta^{199}\text{Hg}$	$\Delta^{201}\text{Hg}$
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

Table 3. Summary statistics (mean, SD, max and min) for each of the parameters ($\delta^{15}\text{N}$, $\delta^{13}\text{C}$, THg, $\Delta^{199}\text{Hg}$, $\Delta^{201}\text{Hg}$ and $\delta^{202}\text{Hg}$) in fledglings' feathers for each of the years studied (2010, 2013, 2014 and 2017) 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)				$\Delta^{199}\text{Hg}$ (‰)				$\Delta^{201}\text{Hg}$ (‰)				$\delta^{202}\text{Hg}$ (‰)			
	Mean	SD	Max	Min	Mean	SD	Max	Min	Mean	SD	Max	Min	Mean	SD	Max	Min	Mean	SD	Max	Min	Mean	SD	Max	Min
2010	12.67	0.30	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	0.11	1.16	0.89	0.59	0.23	0.88	0.27
2013	12.65	0.28	13.14	12.35	-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
2014	13.85	0.47	14.83	13.21	-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
2017	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

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		
		$\delta^{202}\text{Hg}$	$\Delta^{199}\text{Hg}$	$\Delta^{201}\text{Hg}$	$\delta^{202}\text{Hg}$	$\Delta^{199}\text{Hg}$	$\Delta^{201}\text{Hg}$	$\delta^{202}\text{Hg}$	$\Delta^{199}\text{Hg}$	$\Delta^{201}\text{Hg}$	$\delta^{202}\text{Hg}$	$\Delta^{199}\text{Hg}$	$\Delta^{201}\text{Hg}$	$\delta^{202}\text{Hg}$	$\Delta^{199}\text{Hg}$	$\Delta^{201}\text{Hg}$
THg	r	0.49	0.38	0.28	-0.32	-0.52	-0.47	-0.07	0.46	0.49	-0.18	-0.41	-0.33	0.29	-0.09	-0.02
	p-value	0.15	0.28	0.43	0.37	0.13	0.17	0.84	0.19	0.15	0.61	0.24	0.33	0.07	0.57	0.91
$\delta^{13}\text{C}$	r	-0.07	-0.38	-0.44	-0.35	-0.39	-0.37	0.46	0.47	0.22	-0.14	0.66	0.72	0.29	0.04	0.03
	p-value	0.84	0.28	0.20	0.32	0.26	0.29	0.18	0.17	0.54	0.70	0.04	0.02	0.07	0.83	0.85
$\delta^{15}\text{N}$	r	0.11	-0.29	-0.49	0.17	-0.41	-0.37	0.45	-0.14	-0.38	-0.50	-0.09	-0.03	0.15	-0.32	-0.34
	p-value	0.77	0.42	0.15	0.63	0.24	0.29	0.20	0.71	0.28	0.14	0.80	0.92	0.33	0.04	0.031

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