

# 1 **Stable isotopes reveal captive vs wild origin of illegally captured songbirds in France**

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12

## 13 **Abstract**

14 Naturally occurring stable isotopes have become important tracers in the study the wildlife ecology  
15 including the identification of origins for migration research, investigations of trophic relationships,  
16 and in the forensic determination of illegally harvested and captive fauna. Extensive illegal trapping for  
17 use as decoys and in cuisine has contributed to drastic declines of Ortolan Bunting *Emberiza hortulana*  
18 populations breeding and migrating through France. We contrasted feather hydrogen isotope ( $\delta^2\text{H}_f$ )  
19 values in illegally captured and subsequently confiscated Ortolan Buntings ( $n = 234$ ), including feathers  
20 known to have grown in captivity ( $n = 34$ ) or of unknown growing environment (wild or cage), with  
21  $\delta^2\text{H}_f$  values in birds legally caught (ringed and released) in the wild ( $n = 40$ ). We sought to determine if  
22 these bird groups could be differentiated based on this single isotope. Feathers grown in captivity had  
23 considerably lower  $\delta^2\text{H}_f$  relative to feathers of wild birds, which is potentially indicative of tap water  
24 consumption and the use of different diets in captive birds. Further, applying mixing models to  $\delta^2\text{H}_f$

25 values revealed similar proportions of captive vs. wild origins for birds illegally captured in 2012, 2014  
26 and 2015, and a larger proportion of individuals with feathers grown in the wild in 2013. This  
27 potentially mirrors the confiscation of birds at poaching sites only in the former years, but also of  
28 recently caught wild buntings kept captive in 2013. Our results show that even a single stable isotope  
29 ( $\delta^2\text{H}$ ) with good association with origins where feather keratins are produced may be useful in  
30 understanding origins of captive birds and may advance the monitoring of illegally captured birds.

31

## 32 **Keywords**

33 Bird; *Emberiza hortulana*; Illegal hunting; Ortolan Bunting; Poaching; seized birds

34

## 35 **Highlights**

- 36 - The ortolan bunting is a protected migratory passerine poached in south-west France for  
37 gastronomic purposes
- 38 - Captive-grown feathers of ortolan buntings have very low deuterium concentrations  
39 compared to wild-grown feathers
- 40 - This difference is used to estimate the relative proportion of recently captured vs. long caged  
41 ortolan buntings seized by the police from poachers
- 42 - Stable isotopes can be useful tools to infer the wild vs. captive origin of seized birds and further  
43 adapt the care time before being released in the wild

44

## 45 **1. Introduction**

46 Naturally occurring patterns of stable isotopes across large geographic areas enable the tracking of  
47 bird migration. This is based on the principle that stable isotope ratios in food and water consumed by  
48 birds can be correlated with those values in growing tissues (e.g. feathers, blood) following well-  
49 described processes. Protium ( $^1\text{H}$ ) and its heavier isotope, deuterium ( $^2\text{H}$ ), occur naturally in  
50 environmental waters, and the ratio of the heavier to lighter isotope ( $^2\text{H}/^1\text{H}$  measured as  $\delta^2\text{H}$ ) in

51 precipitation has been shown to change predictably at continental scales (i.e. “isoscapes”) [1]. Indeed,  
52  $\delta^2\text{H}$  values in precipitation ( $\delta^2\text{H}_p$ ) generally have a latitudinal structure across continents [2] and these  
53 patterns form the basis of tracing animal movements because such patterns are transferred up  
54 foodwebs [3]. Foodweb isotopic signatures are reflected in the tissues of organisms, and such  
55 signatures can vary spatially based on a variety of biogeochemical processes. Organisms moving  
56 between isotopically distinct foodwebs carry with them information on the location of previous  
57 feeding (Hobson 1999). Migration connectivity can thus be studied by tracking chemicals contained in  
58 tissue, notably stable isotopes in keratins (Hobson & Wassenaar 2008). As feathers grow during a short  
59 time period during a precise phase of the annual cycle, they are preferred to the continuously growing  
60 claws for inferring geographical origins. Determination of migratory origins using isotopes does not  
61 require sacrificing individuals, just sampling a feather of known growth timing. Migrants wearing  
62 feathers grown on breeding grounds can be attributed to probabilistic breeding areas by comparing  
63 the concentrations of some stable isotopes in their feathers and the geographical variations of the  
64 same isotopes in the natural environments where the feathers could have grown. The same holds true  
65 if analyzing feathers of breeding individuals that have molted on their wintering grounds.

66         Inferring the origin of migratory animals in terrestrial systems can be based on various stable  
67 isotopes, especially nitrogen, hydrogen and carbon. Nitrogen ( $^{15}\text{N}/^{14}\text{N}$ ) stable isotopes are mainly used  
68 to determine the trophic level of a species. Hydrogen ( $^1\text{H}$ ) and deuterium ( $^2\text{H}$ ) has revolutionized stable  
69 isotope analysis in the study of animal migration. Deuterium ratios vary strongly with weather  
70 conditions, resulting in highly predictable spatial variation across continents (Hobson & Wassenaar  
71 2008). Constructing continental maps that predict deuterium levels is relatively straightforward, given  
72 the large amount of existing data on continental weather patterns, and biologists further use such  
73 maps to determine probabilistic migratory origins of species. Deuterium ratios in feathers are effective  
74 indicators of breeding latitude in European birds (Hobson & Wassenaar 2008, Bearhop et al. 2003).  
75 Carbon isotopic signatures ( $^{13}\text{C}/^{12}\text{C}$ ) are used to distinguish plants that use C3, C4 and CAM modes of  
76 photosynthesis, as the C4 and CAM pathways lead to lower carbon fractionation than C3

77 photosynthesis (Karasov & Martínez del Rio 2007). Carbon isotopes can thus be used to reconstruct  
78 migratory routes, if the geographical distribution of C3, C4 and CAM plants and the diet preferences  
79 of the study species are known (Hobson 1999). As C3 and C4 plants have not the same occurrence rate  
80 north and south of the Sahara, carbon isotopic signatures in winter-grown feathers can discriminate  
81 individual birds that have overwintered south and north of the Sahara. Clearly, these isotopes provide  
82 rougher estimates of origins than deuterium, while C and N should be avoided when studying farmland  
83 species growing their feathers or hair in environments receiving agricultural fertilizers, disturbing  
84 natural concentrations of their isotopes.

85         The Ortolan Bunting *Emberiza hortulana* is a long-distance migrant Palearctic songbird, that  
86 breeds across Eurasia and winters south of the Sahara. It breeds mainly in farmed habitats, while its  
87 migratory strategy has been confirmed by isotopic investigations of deuterium concentrations in  
88 winter-grown feathers [4]. The species is protected in the European Union and listed in Appendix I of  
89 the Birds Directive (1979/409/EEC), and formally protected in France since 1999. However, this species  
90 was illegally harvested in southwest France for culinary interest, as migrants have the outstanding  
91 capacity to double their mass, primarily as fat, within a short period prior to their fall migration. While  
92 bird conservation organizations have repeatedly denounced this practice, hunters, local politicians and  
93 leading French Michelin-starred chefs [5] have defended the persistence of this hunting and  
94 gastronomic practice as part of traditional cultural heritage. The European Commission referred France  
95 to the Court of Justice of the European Union (EU) in December 2016 on the charge of illegal hunting  
96 of Ortolan Buntings, as France could not prove it was satisfactorily taking action to stop the poaching  
97 activities. In this context, the French nature police intervened in the field by interrogating some alleged  
98 offenders and seizing captive Ortolan Buntings. These illegally captured birds were taken into care and  
99 later released in the wild before the end of the migration season.

100         In all birds, the renewal of feathers through moult is necessary to maintain the functions of  
101 these keratinous appendages. Songbirds typically renew flight feathers once each year and body  
102 feathers twice each year. Adult Ortolan Buntings moult the body feathers twice each year [6], on the

103 breeding grounds in June to August, before the autumn migration to Africa, and on the wintering  
104 grounds before the spring migration. Their flight feathers are moulted only once, during the post-  
105 breeding moult in Europe. First-year individuals have feathers grown in the nest on the breeding  
106 grounds. Hence, during the fall migration, all individuals have a complete set of feathers recently grown  
107 on their breeding grounds regardless of their age.

108 Bird feathers grown at a given location generally have  $\delta^2\text{H}$  values ( $\delta^2\text{H}_f$ ) proportional to local  
109 amount-weighted mean  $\delta^2\text{H}_p$  [7]. However, this is not necessarily true for feathers grown in captivity.  
110 Captive birds are assumed to be provided tap water and commercial food of unknown and varied  
111 origins, which can have different  $\delta^2\text{H}$  values than local precipitation or wild grown seeds. This  
112 discrepancy in  $\delta^2\text{H}$  is especially true if tap water does not correspond well to amount-weighted growing  
113 season precipitation where feathers are grown and if food provided to the captive birds is not issued  
114 from local production [2],[8]. Under a global research program dedicated to unravel the migration  
115 strategy of the Ortolan Bunting [4], we had access to numerous individuals captured illegally during  
116 four consecutive years. These birds included recently captured wild individuals and caged decoys  
117 placed on hunting sites. Caged decoys could be either individuals kept year-long in captivity (i.e. with  
118 captive-grown feathers), or recently captured wild individuals (i.e. wild-grown feathers) placed in cages  
119 to complement/renew the stock of long-captive decoy birds. All seized first-calendar individuals (i.e.  
120 hatch year) were of conclusively wild origin, thus having wild-grown feathers, since captive breeding  
121 of the species is not possible. Most other individuals could have feathers of wild or captive origin, but  
122 the plumage of a few adults displayed color aberration with either a lack of or an excess of dark  
123 pigmentation (leucism or melanism), signaling captive-grown feathers [9].

124 Within the poached and seized birds, our main aim was to study  $\delta^2\text{H}_f$  values in order to  
125 estimate the proportion of individuals that were recently captured compared to those individuals  
126 captured in earlier years and further kept in captivity. All seized birds hatched in the wild, as there is  
127 no captive breeding of the species, so our aim was to address the question of the temporality of their  
128 capture, either recent or older. By first considering obvious caged buntings, we first assessed  $\delta^2\text{H}_f$

129 values for cage-grown feathers to determine if  $\delta^2\text{H}_f$  could be used to separate recently captured and  
130 caged birds. We expected birds with suspected cage-grown feathers to have potentially different  $\delta^2\text{H}_f$ ,  
131 either lower or higher. As most Ortolan Buntings migrating by southwest France come from Poland  
132 and Scandinavia [4] where deuterium concentrations in the environment are lower than in France, we  
133 expected higher  $\delta^2\text{H}_f$  in caged birds. We then analyzed the distributions of  $\delta^2\text{H}_f$  in feathers collected  
134 from Ortolan Buntings illegally captured during four consecutive hunting seasons to estimate the  
135 proportions of individuals of wild vs captive origins. We finally propose possible interpretations for the  
136 observed inter-annual variations in these proportions, in terms of confiscation efforts by the police.

137

## 138 **2. Materials and methods**

### 139 *2.1 Sample collection and hydrogen isotopic measurements*

140 We collected feathers from Ortolan Buntings seized by the nature police in Landes department,  
141 southwestern France, when interrogating owners of illegal trapping sites. Seized birds were placed in  
142 wildlife care centers where licensed forensic ringers processed them before release. Seized birds could  
143 include caged and recently trapped birds found at capture sites, and potentially birds kept in captivity  
144 to be fattened for consumption. All surviving confiscated individuals were ringed and feather-sampled  
145 prior to release in the wild before the end of the autumn migration season. We organized the collection  
146 of one tail feather from each bird cared for in 2012 (n=50), 2013 (n=18), 2014 (n=53) and 2015 (n=113).  
147 Individual birds were classified into three groups: 1) decoys (n=34) that were illegally captured birds  
148 with plumage abnormalities or highly developed feet scabies, 2) illegally captured birds from each year  
149 (2012, 2013, 2014 and 2015; these groups include the previously cited obvious decoys, but also illegally  
150 captured first-calendar year individuals), and 3) wild birds legally captured and ringed in autumn  
151 (n=40). For birds with tail feathers that were too heavily damaged from being in captivity, we instead  
152 sampled one tertial feather. We stored feathers in individual envelopes at ambient temperature until  
153 isotopic analysis.

154           Feathers were cleaned in 2:1 chloroform:methanol solvent rinse and prepared for  $\delta^2\text{H}$  analysis  
155 at the Stable Isotope Laboratory of Environment Canada, Saskatoon, Canada. The  $\delta^2\text{H}$  of the non-  
156 exchangeable hydrogen of feathers was determined using the method described by Wassenaar &  
157 Hobson [10] based on two calibrated keratin hydrogen-isotope reference materials (CBS: -197 ‰, KHS:  
158 -54.1 ‰). We performed hydrogen isotopic measurements on  $\text{H}_2$  gas derived from high-temperature  
159 (1350 °C) flash pyrolysis (Eurovector 3000; Milan, Italy) of  $350 \pm 10$  ug feather subsamples and keratin  
160 standards loaded into silver capsules. Resultant separated  $\text{H}_2$  was analysed on an interfaced Isoprime  
161 (Crewe, UK) continuous-flow isotope-ratio mass spectrometer. Measurement of the two keratin  
162 laboratory reference materials corrected for linear instrumental drift were both accurate and precise  
163 with typical within-run measurement error  $< 2$  ‰. All results are reported for non-exchangeable H  
164 expressed in the typical delta notation, in units of per mil (‰), and normalized on the Vienna Standard  
165 Mean Ocean Water–Standard Light Antarctic Precipitation (VSMOW-SLAP) standard scale.

166

## 167 *2.2 Statistical analyses*

168 We first tested if  $\delta^2\text{H}_f$  values measured from each bird group followed normal distributions by  
169 performing Shapiro-Wilk tests. We also performed a general linear model to compare  $\delta^2\text{H}_f$  values  
170 among five groups (wild birds and the four annual datasets). We then applied the normalmixEM  
171 function of the ‘mixtools’ package in R v3.5.2 [11] to the datasets for each year (2012, 2013, 2014 and  
172 2015) to obtain the mixing proportions, means and standard deviations of both estimated mixed  
173 normal distributions. We could have pre-defined the means and standard deviations of the two mixed  
174 normal distributions using values estimated from the obvious wild and decoy birds. However, we did  
175 not do so as we expected that inter-annual variations in meteorological conditions during migration  
176 (e.g. predominance of eastern vs. western winds) and relative breeding productivity across the range  
177 (i.e. origins) should create variation in the annual distribution of  $\delta^2\text{H}_f$  in wild birds migrating through  
178 southwestern France. This expectation was supported by a year effect in an analysis of variance

179 predicting  $\delta^2\text{H}_f$  values by year of capture for birds captured and ringed in the wild by forensic ringers  
180 ( $F_{2,37} = 28.3$ ,  $P < 0.001$ ).

181

## 182 **3. Results**

### 183 *3.1 Normality*

184 We report the mean  $\pm$  s.d. values of  $\delta^2\text{H}_f$  for the wild individuals and obvious decoys in Table 1, while  
185 Figure 1 shows the boxplot of the different groups studied here (including obvious decoys). The boxplot  
186 illustrates that illegally captured birds include individuals of wild and captive origins in unknown  
187 proportions. Shapiro-Wilk's tests confirmed the normal distribution of the deuterium values for the  
188 following datasets: wild birds ( $W=0.967$ ,  $P=0.051$ ), obvious decoys ( $W=0.961$ ,  $P=0.268$ ), and 2013 birds  
189 ( $W=0.925$ ,  $P=0.161$ ), but not for birds confiscated in 2012 ( $W=0.900$ ,  $P<0.001$ ), 2014 ( $W=0.878$ ,  $P <$   
190  $0.001$ ) and 2015 ( $W=0.899$ ,  $P < 0.001$ ). A general linear model performed on data from wild birds and  
191 all years revealed that all annual groups differed from the wild sample (year effects predicting  $\delta^2\text{H}_f$   
192 compared to the wild sample defined as the intercept; intercept estimate  $\pm$  s.d. =  $-88.18\pm 2.69$  ‰):  
193 2012 ( $-11.18\pm 3.46$  ‰,  $t=-3.10$ ,  $P=0.002$ ); 2013 ( $-13.06\pm 4.83$  ‰,  $t=-2.71$ ,  $P=0.007$ ); 2014 ( $-11.30\pm 3.41$   
194 ‰,  $t=-3.56$ ,  $P=0.002$ ), 2015 ( $-12.16\pm 3.13$  ‰,  $t=-3.89$ ,  $P<0.001$ ). Fig. 1 illustrates the differences  
195 between these groups.

196

### 197 *3.2 Mixing proportions*

198 Fitting normal mixture densities to the annual datasets, we identified for each year two mixed normal  
199 distributions closely matching those of obvious decoys and of wild individuals (Table 1 and Fig. 2). The  
200 estimated proportion of birds with captive- vs. wild-grown feathers were relatively balanced in 2013  
201 (45% vs 55%) but were skewed in 2012 (61% vs 39%), 2014 (75% vs 25%) and 2015 (72% vs 28%), with  
202 a larger proportion of caged birds.



203

#### 204 **4. Discussion**

205 Ortolan Buntings illegally captured in France in 2012-2015 included some individuals that were obvious  
206 decoys with plumage abnormalities, leucism or melanism, which are likely linked to nutrient  
207 deficiencies [9],[12]. All of these individuals were captive for long periods prior to sampling and so they  
208 grew their feathers in captivity. The feathers sampled on these birds provided a good opportunity to  
209 obtain estimates of the  $\delta^2\text{H}_f$  concentrations in captive-grown feathers, and further compare them to  
210 similar measures obtained from Ortolan feathers grown in the wild. The latter came from active  
211 migrants captured in nature by licensed forensic ringers. The decoys had notably lower  $\delta^2\text{H}_f$   
212 concentrations than wild birds which is consistent with the expectation that captive birds, presumably  
213 drinking tap water and eating commercial food, can have different  $\delta^2\text{H}_f$  than those of wild birds derived  
214 from diets and drinking water driven by local precipitation at the site of feather development. Here,  
215 by analyzing  $\delta^2\text{H}_f$  concentrations of seized Ortolans, we were able to predict their wild or captive origin,  
216 as long as the captive birds consumed only minimal rainwater and wild-grown seeds while growing  
217 their feathers. For birds that have not molted feathers while in captivity, analysis of  $\delta^2\text{H}$  in other  
218 chemically active tissues (e.g. toenails, blood) with differing turnover periods may provide further  
219 insights into their capture history and requires further study [14]. However, the expectation of a  
220 difference of isotopic concentrations in feathers of wild vs. captive individuals will be entirely case  
221 specific and depend very much on the molt origin of wild birds compared to captive birds and their  
222 artificial diets [2],[8],[13].

223         Within the datasets obtained for the different years, 2013 appears distinct, with balanced  
224 proportions of wild and captive birds, while the other three years included more captive than wild  
225 birds. The difference could arise from variations in confiscation efforts and procedures, concerning  
226 mainly long-lasting or recently captured decoys present on field poaching stations, possibly  
227 complemented by additional wild and recently captured individuals moved indoors for fattening. If the

228 police operated mainly at field capture sites, confiscated birds would concern only caged decoys, with  
229 a majority of long-caged individuals with feathers grown in captivity, and fewer recently captured  
230 individuals kept in the field to complement the available decoys surviving from the precedent year. If  
231 the police further confiscated the wild individuals captured recently but then moved indoor for  
232 fattening, we would expect a higher proportion of individuals wearing wild-grown feathers. The first  
233 scenario would produce a larger proportion of birds of captive origin (see 2012, 2014, 2015), while the  
234 second scenario would produce a comparatively larger proportion of wild individuals (as in 2013).

235 While isotope profiling or fingerprinting are often used in food quality controls or criminal  
236 cases [15], there is a growing literature showing that stable isotope analyses are a powerful tool in  
237 forensic tracing of the origins of legally or illegally traded animals and plants [16],[17]. Studying the  
238 African Grey Parrot *Psittacus erithacus*, Alexander et al. [18] found a systematic difference in feather  
239 carbon ( $\delta^{13}\text{C}_f$ ) and deuterium ( $\delta^2\text{H}_f$ ) values for known wild and captive birds, and a match between  
240 isotopic ratios obtained for imported birds that deceased in captivity and for wild birds, attesting that  
241 these imported birds had been captured illegally in the wild. Castelli and Reed [19] identified unique  
242 patterns of three isotopic ratios ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$  and  $\delta^{34}\text{S}$ ) to distinguish wild and pen-raised Northern  
243 Bobwhite *Colinus virginianus*. Dittrich et al. [20] investigated the isotopic composition ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$  and  
244  $\delta^{18}\text{O}$ ) of legally traded frog legs from suppliers in Vietnam and Indonesia, and segregated naturally  
245 born from intensively farmed frogs. Indeed, the use of stable isotope analyses can definitely improve  
246 the monitoring of legally and illegally traded wildlife species across the globe.

247 Analysis of stable isotopes in recently grown feathers of illegally captured birds in Europe is a  
248 promising forensic tool to help authorities identify wild poached individuals of protected species that  
249 are otherwise bred legally in captivity. This could be highly efficient to segregate legally from illegally  
250 traded Goldfinches *Carduelis carduelis*, which suffers from a long-term poaching pressure that has  
251 resulted in a drastic range retraction in North Africa [21]. International illegal trade of poached  
252 Goldfinches has also recently increased across southern Europe, following the decline in North African

253 breeding numbers [21]. Indeed, French authorities have recurrently seized Goldfinches in recent years,  
254 and analyzing the stable isotope concentrations (notably  $\delta^2\text{H}$ ) in their feathers could help to determine  
255 the captive vs wild origin of seized birds, and the geographic origin of wild seized individuals [22]. As  
256 the principal strategy used to deal with animals recovered from illegal traffic is to release them back  
257 into nature [23], this would allow rapid release of individuals with recently wild-grown feathers, and  
258 give longer recovery time to individuals having been captive for longer durations.

259 As a conclusion, we acknowledge that differences in isotopic concentrations in bird feathers  
260 grown in captivity and in the wild are generally case specific, though if attested as was the case for  
261 ortolan buntings in southwest France, they can provide a useful tool to determine the duration of  
262 previous captivity for seized birds, and therefore adapt their rehabilitation period before an optimal  
263 release back in the wild. The information on the relative proportion of wild and recently captured  
264 individuals, vs. individuals captured since at least one year, can further inform on the efficiency of the  
265 confiscation efforts to target wild birds whose captures impeded directly the fate of wild populations [4].

266

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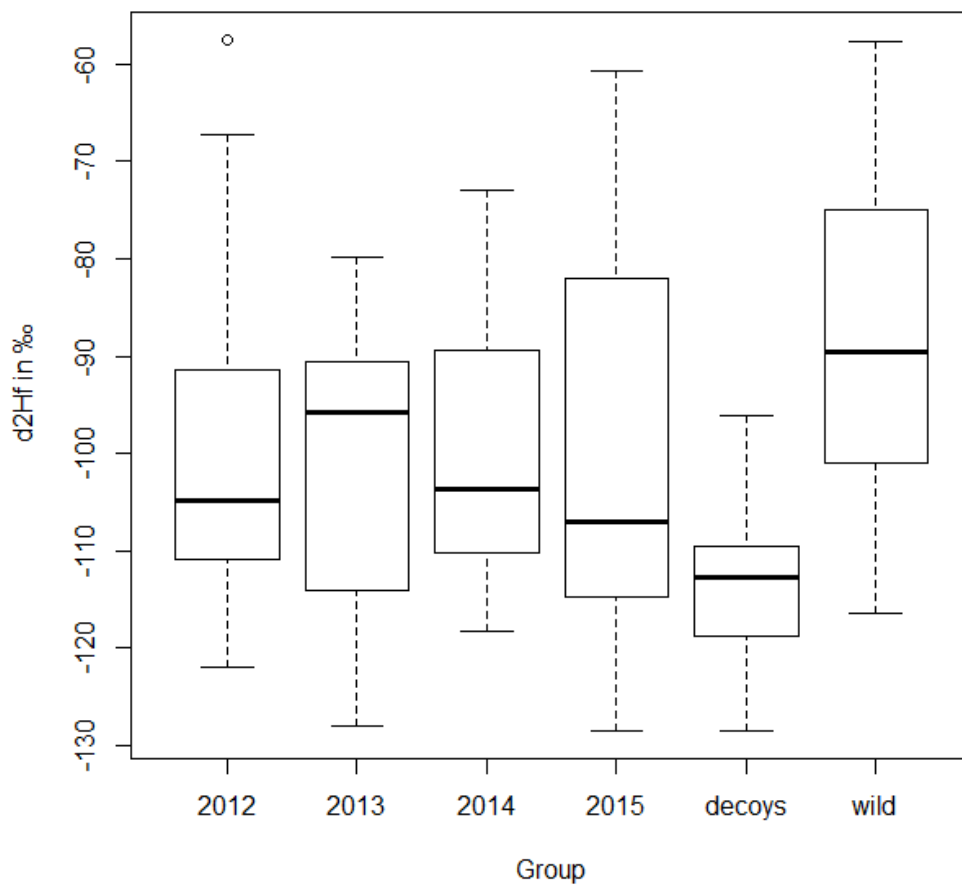
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326 Table 1. Mixing proportions, mean ( $\bar{x}$ ) and standard deviation (SD) of deuterium concentrations in  
 327 Ortolan Bunting feathers ( $\delta^2\text{H}_f$ ) for each of the mixed normal distribution. The last line provides the  
 328 mean and standard deviation for obvious dummies and wild birds also reported in Fig. 1.

Year	Proportion 1 (in %)	$\delta^2\text{H}_f \bar{x} 1$	SD 1	Proportion 2 (in %)	$\delta^2\text{H}_f \bar{x} 2$	SD 2
2012	60.1	-109.01	5.87	39.9	-84.82	14.16
2013	44.6	-115.24	5.87	55.4	-89.98	4.67
2014	74.9	-106.42	6.63	25.1	-78.75	4.15
2015	72.2	-111.21	8.98	27.8	-72.14	7.14
References	Decoys (n=34)	-114.1	8.50	Wild (n=40)	-85.73	15.32

329

330 Figure 1. Boxplot of deuterium concentration values in Ortolan Bunting feathers ( $\delta^2\text{H}_f$ , in ‰) for  
331 annual datasets of confiscated birds, for confiscated obvious decoys and for individuals captured  
332 legally captured in the wild by ringers. Each boxplot reports median, 1<sup>st</sup> and 3<sup>rd</sup> quartiles, min and  
333 max values.



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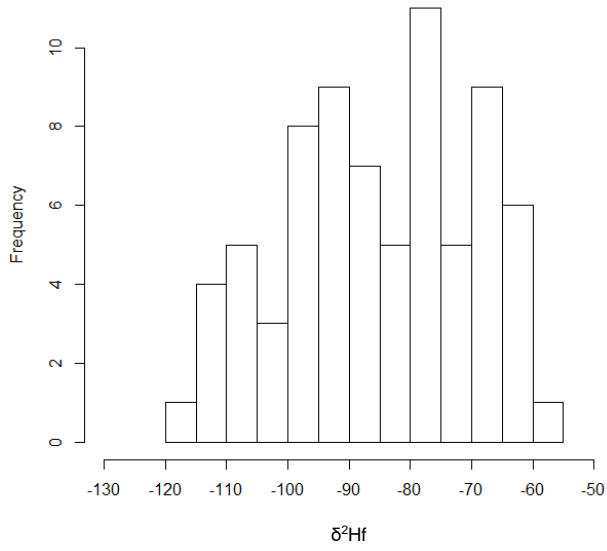
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336 Figure 2. Distributions of deuterium concentrations in Ortolan Bunting feathers ( $\delta^2\text{H}_f$ , in ‰) for (A)  
337 wild birds (upper left, n=40) and (B) obvious decoys (upper right, n=34) (in frequency), then (C-D-E-F)  
338 as obtained from fitting normal mixture densities to the annual datasets (with global and mixed  
339 density curves).

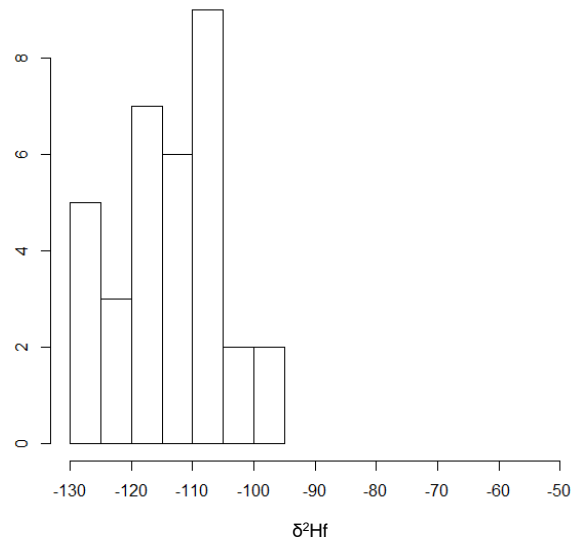


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**A** Wild birds



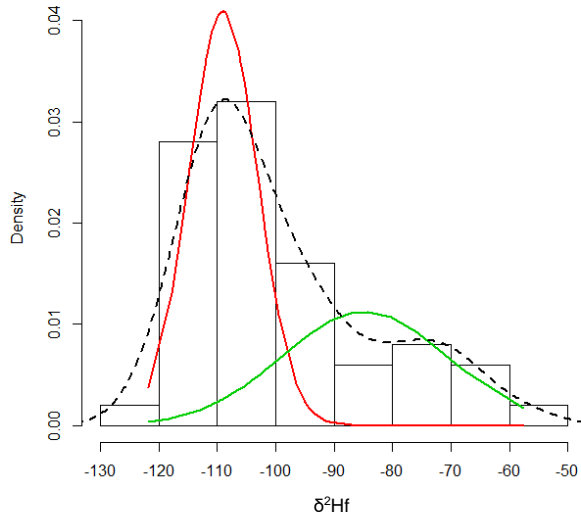
**B** Obvious decoys



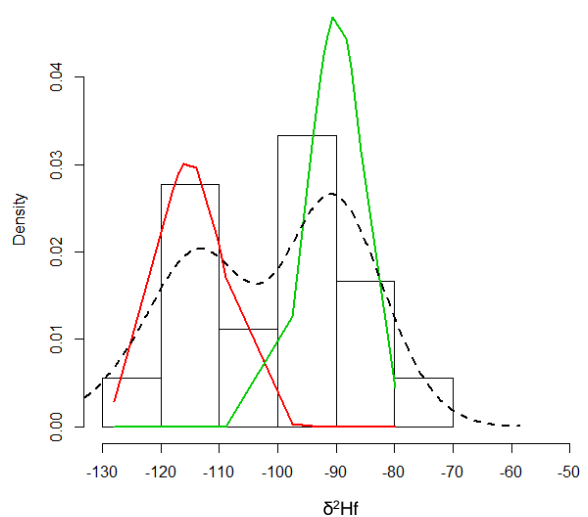
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**C** Captures 2012



**D** Captures 2013



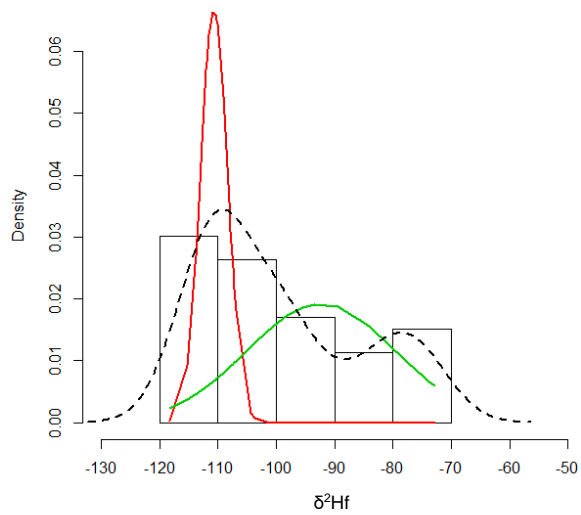
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**E** Captures 2014



**F** Captures 2015

