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Frédéric Jiguet[a]*, Kevin J. Kardynal[b], Keith A. Hobson[b,c]

*corresponding author: frederic.jiguet@mnhn.fr, tel +33(0)140793423

[a] CESCO, UMR7204 MNHN-CNRS-Sorbonne Université, CP135, 43 Rue Buffon, 75005 Paris, France

[b] Environment and Climate Change Canada, 11 Innovation Boulevard, Saskatoon, Saskatchewan, S7N 3H5, Canada

[c] Dept. Biology and Environment and Climate Change Canada, University of Western Ontario, Room 2025 BGS Building, 1151 Richmond St. N., London, Ontario, N6A 5B7, Canada

Abstract

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

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

Keywords

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

Highlights

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

1. Introduction

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

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

Inferring the origin of migratory animals in terrestrial systems can be based on various stable isotopes, especially nitrogen, hydrogen and carbon. Nitrogen ($^{15}N/^{14}N$) stable isotopes are mainly used to determine the trophic level of a species. Hydrogen (^1H) and deuterium (^2H) has revolutionized stable isotope analysis in the study of animal migration. Deuterium ratios vary strongly with weather conditions, resulting in highly predictable spatial variation across continents (Hobson & Wassenaar 2008). Constructing continental maps that predict deuterium levels is relatively straightforward, given the large amount of existing data on continental weather patterns, and biologists further use such maps to determine probabilistic migratory origins of species. Deuterium ratios in feathers are effective indicators of breeding latitude in European birds (Hobson & Wassenaar 2008, Bearhop et al. 2003). Carbon isotopic signatures ($^{13}C/^{12}C$) are used to distinguish plants that use C3, C4 and CAM modes of 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 Palaearctic 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 δ^2H values (δ^2H_f) proportional to local
109 amount-weighted mean δ^2H_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 δ^2H values than local precipitation or wild grown seeds. This
112 discrepancy in δ^2H 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 δ^2H_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 δ^2H_f

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

2. Materials and methods

2.1 Sample collection and hydrogen isotopic measurements

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

Feathers were cleaned in 2:1 chloroform:methanol solvent rinse and prepared for δ^2H analysis at the Stable Isotope Laboratory of Environment Canada, Saskatoon, Canada. The δ^2H of the non-exchangeable hydrogen of feathers was determined using the method described by Wassenaar & Hobson [10] based on two calibrated keratin hydrogen-isotope reference materials (CBS: -197 ‰, KHS: -54.1 ‰). We performed hydrogen isotopic measurements on H_2 gas derived from high-temperature (1350 °C) flash pyrolysis (Eurovector 3000; Milan, Italy) of 350 ± 10 ug feather subsamples and keratin standards loaded into silver capsules. Resultant separated H_2 was analysed on an interfaced Isoprime (Crewe, UK) continuous-flow isotope-ratio mass spectrometer. Measurement of the two keratin laboratory reference materials corrected for linear instrumental drift were both accurate and precise with typical within-run measurement error < 2 ‰. All results are reported for non-exchangeable H expressed in the typical delta notation, in units of per mil (‰), and normalized on the Vienna Standard Mean Ocean Water–Standard Light Antarctic Precipitation (VSMOW-SLAP) standard scale.

2.2 Statistical analyses

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

predicting δ^2H_f values by year of capture for birds captured and ringed in the wild by forensic ringers ($F_{2,37}$ = 28.3, P < 0.001).

3. Results

3.1 Normality

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

3.2 Mixing proportions

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

4. Discussion

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

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

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

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

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

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

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

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

Year	Proportion 1 (in %)	$\delta^2H_f\ \bar{x}\ 1$	SD 1	Proportion 2 (in %)	$\delta^2H_f\ \bar{x}\ 2$	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

330 Figure 1. Boxplot of deuterium concentration values in Ortolan Bunting feathers (δ^2H_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, 1st and 3rd quartiles, min and

333 max values.

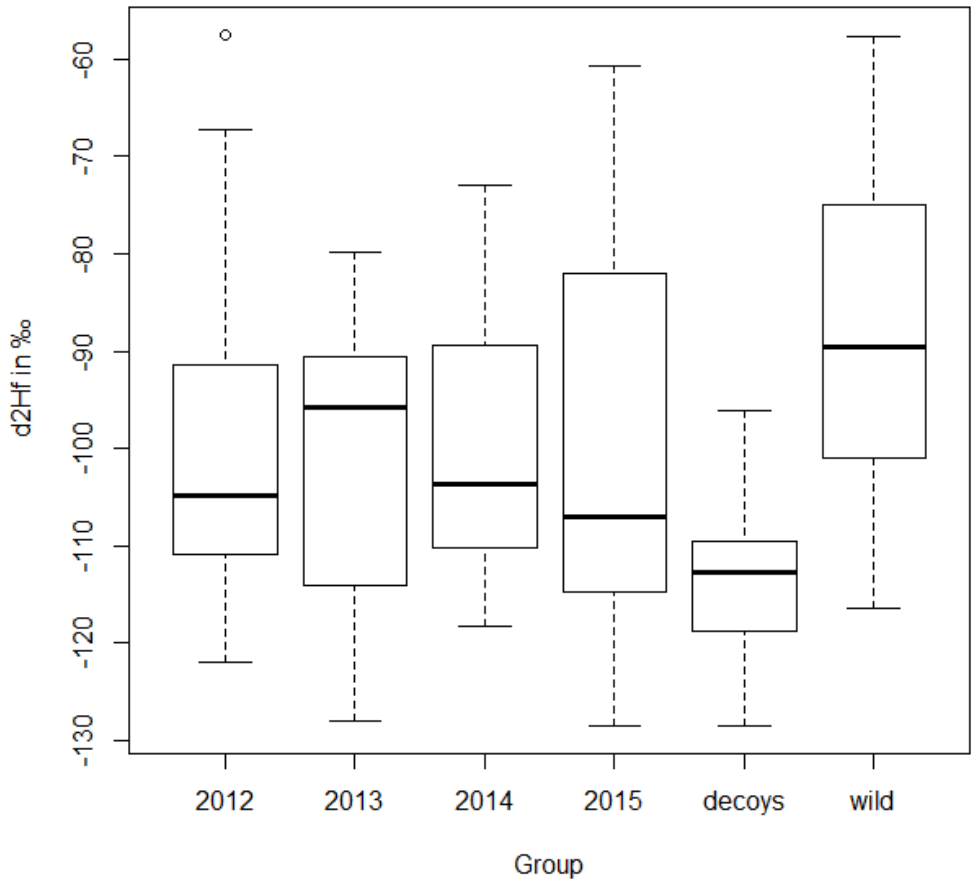

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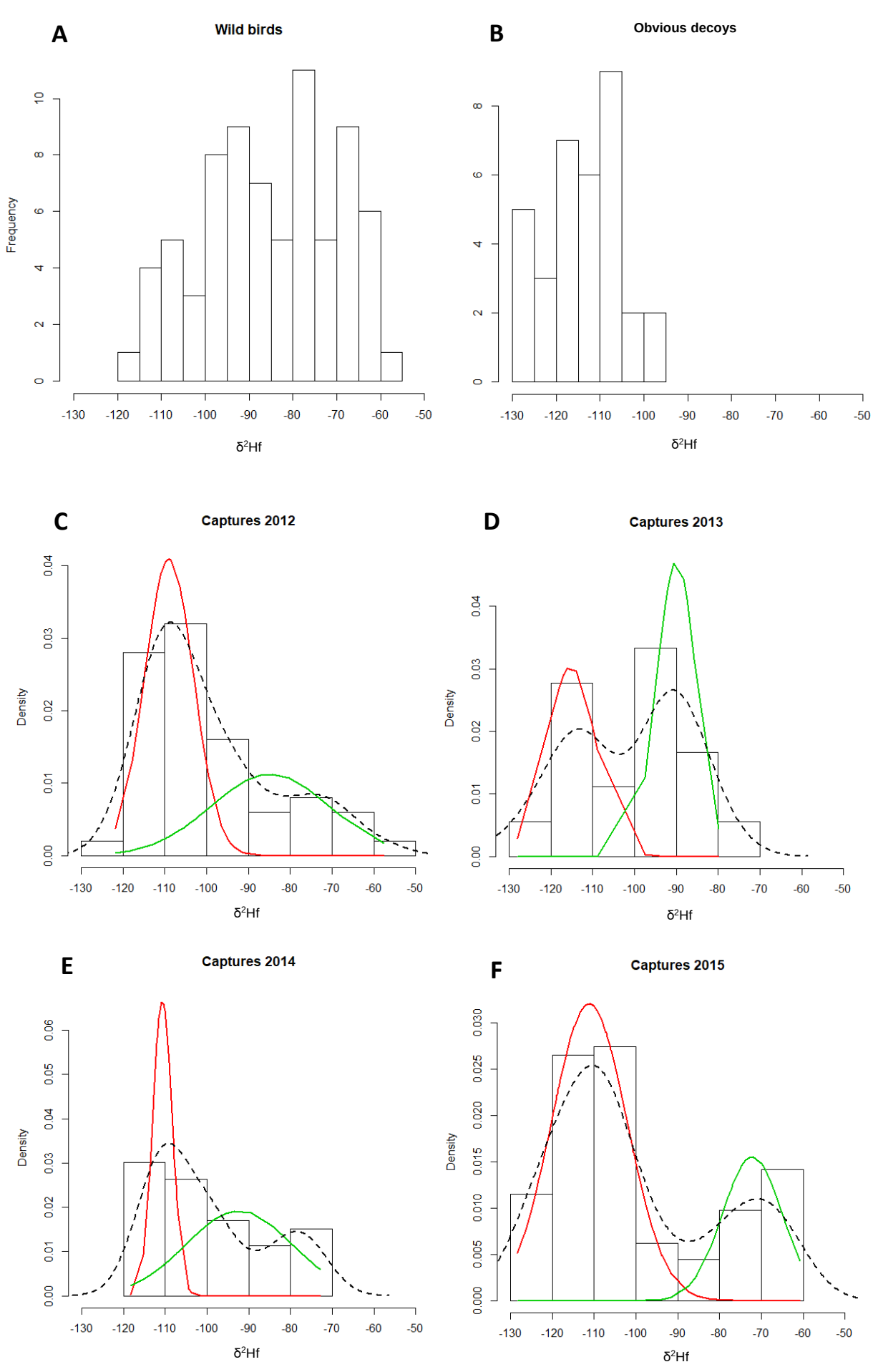
Figure 2. Distributions of deuterium concentrations in Ortolan Bunting feathers (δ^2H_f, in ‰) for (A) wild birds (upper left, n=40) and (B) obvious decoys (upper right, n=34) (in frequency), then (C-D-E-F) as obtained from fitting normal mixture densities to the annual datasets (with global and mixed density curves).


A
Wild birds
Frequency
δ²Hf
B
Obvious decoys
δ²Hf
C
Captures 2012
Density
δ²Hf
D
Captures 2013
Density
δ²Hf
E
Captures 2014
Density
δ²Hf
F
Captures 2015
Density
δ²Hf