



Neto, J. M., de Oliveira Gordinho, L., Vollot, B., Marín, M., Monrós, J. S., and Newton, J. (2017) Stable isotopes reveal differences in diet among reed bunting subspecies that vary in bill size. *Journal of Avian Biology*, 48(2), pp. 284-294. (doi:[10.1111/jav.01069](https://doi.org/10.1111/jav.01069))

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Deposited on: 7 November 2016

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## 22 Abstract

23 Reed bunting (*Emberiza schoeniclus*) subspecies vary considerably in bill size and shape and  
24 seem to be at an early stage of speciation, in which bill might be indirectly causing  
25 reproductive isolation. Hence, we evaluated whether bill size, as well as age and sex, are  
26 associated with foraging niche in three West European subspecies of reed bunting: the thin-  
27 billed *schoeniclus*, the intermediate-billed *lusitanica* and the thick-billed *witherbyi*. Blood  
28 sampling was undertaken at three sites in southwest Europe during the winter (when these  
29 subspecies co-occur), and stable isotope analyses (carbon and nitrogen) were performed to  
30 assess their foraging niches. Stable isotope analyses of potential food items confirmed  
31 uniform baseline isotopic composition among sites. *E. s. schoeniclus* showed a significantly  
32 broader isotopic niche than *lusitanica* and *witherbyi*, which seemed otherwise similar despite  
33 the fact that *witherbyi* is more divergent in bill traits. Stable isotope ratios were consistent  
34 with the latter two subspecies feeding on C3-plant-feeding insects, whereas *schoeniclus* diet  
35 also included C4 plant material. Despite its lower sexual dimorphism, sex and age differences  
36 were found only in *schoeniclus*, but these differences vary between locations in a complex  
37 manner. Our results suggest that bill size and shape differentiated between northern,  
38 migratory and southern, resident subspecies as a consequence of natural selection through  
39 competition during the winter, which is now reflected in isotopic niche divergence between  
40 subspecies. The potential roles of sexual selection, reed thickness and summer temperature on  
41 the difference in bill size (and greater sexual dimorphism) between *lusitanica* and *witherbyi*  
42 are discussed.

## 43 Introduction

44 Ecological speciation is a process through which new species arise as a consequence of  
45 disruptive or of divergent natural selection that directly or indirectly causes the evolution of  
46 reproductive isolation (Rundle and Nosil 2005). This process may be particularly fast when  
47 sexually selected traits are the subject of local adaptation (often called “magic traits”), as  
48 there is a direct link between natural selection and reproductive isolation (Servedio et al.  
49 2011). One such trait is bill size in birds, as bills may be locally adapted for particular food  
50 types and simultaneously be associated with the divergence of sexual signals (song traits) that  
51 are constrained by bill size (Podos et al. 2001, but see Grant and Grant 1997); or be under  
52 direct sexual selection (Olsen et al. 2013). In addition, when individuals are adapted to  
53 particular environmental conditions and have a “good genes” sexual selection system, hybrids  
54 will be maladapted to any of the parental environments and so will be selected against by  
55 females due to their low condition (van Doorn et al. 2009).

56 Determining the ecological pressures that cause divergent selection is a crucial step for  
57 understanding the speciation process. In this study, we aim to achieve this in one of the most  
58 variable species of the large bunting family (Emberizidae): the reed bunting (*Emberiza*  
59 *schoeniclus*). Over 30 subspecies have been described in this non-model species, of which 20  
60 are currently recognized (del Hoyo et al. 2011). They are often divided into thick-billed  
61 subspecies, which reside in the southern part of the Palearctic, and thin-billed subspecies,  
62 which occur further north and tend to be migratory (co-occurring with the southern, resident  
63 ones during winter); but also vary in several other traits including body size, plumage colour  
64 and song (Byers et al. 1995, Matessi et al. 2000a, b, Neto et al. 2013, Gordinho et al. 2015).  
65 This study system is particularly interesting because the processes that are causing divergence  
66 among populations of reed bunting can potentially be generalized to at least some of the other  
67 40 species of *Emberiza* (and over 320 species of Emberizidae), as well as to species of other

68 large seed-eating bird families such as the Fringillidae, all of which have largely continental  
69 distributions.

70 Population genetic studies have shown that West European subspecies of reed bunting are  
71 slight but significantly different at neutral markers (Grapputo et al. 1998, Kvist et al. 2011),  
72 and probably diverged only since the last glaciation; whereas in Asia there are four partially  
73 overlapping closely related lineages (Zink 2008). There is asymmetric song discrimination  
74 between different subspecies in Western Europe (Matessi et al. 2000, Gordinho et al. in  
75 prep.), and no evidence for hybridization at a contact zone between the thick-billed *E. s.*  
76 *intermedia* and the thin-billed *E. s. schoeniclus* (Grapputo et al. 1998), which differ in diet  
77 (Matessi et al. 2002, see below). Therefore, this species seems to be at an early stage of  
78 speciation, with populations/subspecies still showing incomplete mtDNA lineage sorting, but  
79 significant genetic and behavioural divergence. It is particularly important to study organisms  
80 at this critical stage of evolution, when the actual ecological and genetic mechanisms of  
81 speciation can be witnessed.

82 Here, we propose to determine the ecological pressures that drove the evolution of bill size  
83 differences among the three subspecies of reed bunting that occur in southwest Europe: *E. s.*  
84 *schoeniclus* (hereafter *schoeniclus*), which breeds in central and northern Europe and winters  
85 in the Mediterranean area; *E. s. witherbyi* (hereafter *witherbyi*), which is resident in northern  
86 Morocco, central and eastern Spain and southern France; and *E. s. lusitanica* (hereafter  
87 *lusitanica*), which is resident in Portugal and northwest Spain (Byers et al. 1995). *Emberiza s.*  
88 *witherbyi* has a much thicker bill, whereas the bill of *lusitanica* is larger on average than, but  
89 overlaps extensively with, that of *schoeniclus* (Neto et al. 2013). The increasing bill thickness  
90 towards the east among southern, thick-billed subspecies, and the existence of an  
91 intermediate-billed subspecies (*lusitanica*) suggests that these three subspecies may form the

92 closest link between thin-billed and thick-billed populations, and therefore are especially  
93 interesting to study the current level of ecological and reproductive isolation.

94 It is well known that during spring/summer reed buntings feed on a large variety of insects  
95 and spiders (Cramp and Perrins 1994, Holland et al. 2006, JMN pers. obs.). However,  
96 previous work indicates that, during winter, some thick-billed subspecies (*intermedia* and  
97 *pyrrhuloides*) feed on insect larvae and pupae that are dormant inside the reed (*Phragmites*  
98 *australis*) stems (Shtegman, 1948 cited by Prys-Jones 1984, Matessi et al. 2002), whereas  
99 thin-billed subspecies eat mostly seeds and often occur in other habitats such as farmland  
100 (Cramp and Perrins 1994, Holland et al. 2006, Orłowski and Czarnecka 2007), but can eat  
101 insects in some areas opportunistically (Orłowski et al. 2013, JMN pers. obs.). The diets of  
102 *witherbyi* and of the intermediate-size *lusitanica* have never been studied before, but given  
103 their distribution and relatively convex, thick bill, they might feed on insects in a manner  
104 similar to the other thick-billed subspecies described above (using their bills to crush and  
105 open up the reed stems to get access to the larvae); although the large overlap in morphology  
106 between *lusitanica* and *schoeniclus* make predictions difficult (Neto et al. 2013). In addition,  
107 Neto et al. (2013) showed that sexual dimorphism in bill size and shape varies, with thick-  
108 billed subspecies having a greater dimorphism than *schoeniclus* and males showing greater  
109 divergence between subspecies than the females. However, the ecological implications of the  
110 sexual differences in bill size have never been tested, and thus it is not known which forces  
111 were involved in the evolution of this dimorphism. The comparison of diet between the sexes  
112 allows the evaluation of whether ecology may have played a role in this differential evolution  
113 of bill size, or whether alternative explanations like sexual selection might have been  
114 involved.

115 Traditional studies of diet are hampered by the fact that the digestibility of the food items is  
116 variable, and the subspecies also vary in the conspicuousness of foraging birds, as they use

117 different feeding techniques and microhabitats. Therefore, in order to compare the diets  
118 among subspecies, ages and sexes, we used an indirect method: stable isotope analysis. This  
119 approach relies on the variation of stable carbon and nitrogen isotope ratios with the diet: the  
120 former being mostly dependent on the C3 v C4 photosynthesis at the base of the food chain  
121 and on the water-use efficiency within C3 plants; whereas nitrogen varies mostly with the  
122 trophic level in the food chain (Bearhop et al. 2005, Inger and Bearhop 2008). We test the  
123 hypothesis (H1) that the nitrogen isotope ratio in the blood, which correlates positively with  
124 trophic level (DeNiro et al. 1981, Inger and Bearhop 2008), is higher in the thick-billed  
125 subspecies (presumed to eat mostly insects) than in the thin-billed subspecies (whose diet  
126 seems to include a large component of seeds). As in at least some areas *schoeniclus* seems to  
127 prefer seeds of Chenopodiaceae, Cyperaceae and Poaceae (which include many C4 plants;  
128 Cramp and Perrins 1994, Holland et al. 2006, Pyankov et al. 2010, Kandereit et al. 2012), we  
129 also hypothesize (H2) that this subspecies has the most <sup>13</sup>C-rich values. In addition, if the diet  
130 closely follows the morphological differentiation, we hypothesize (H3) that the isotopic niche  
131 of *lusitanica* is intermediate between the other two subspecies (and closer to *schoeniclus*), and  
132 the difference in isotopic niche between the sexes is greater in *lusitanica* and *witherbyi* than in  
133 *schoeniclus*.

134

## 135 Materials and methods

### 136 **Fieldwork**

137 Fieldwork was carried out during December-February 2010/11 and 2012/13 at Salreu  
138 marshlands (40°43'41.10"N, 08°35'06.51"W), Portugal, as well as December-February  
139 2011/12 and 2012/2013 at Lagunas de Villafranca (39°27'22.52"N, 03°20'09.03"W), Ciudad  
140 Real, Spain. In order to increase the sample size of *witherbyi* and the geographic  
141 representativeness, additional fieldwork was undertaken from the end of November to

142 December 2014 in southern France, mostly in the Camargue (43°36'24.62"N, 04°31'58.58"E),  
143 Arles, but a few samples (four in each location, including two subspecies) were also collected  
144 in Saint-Laurent D'Aigouze (43°35'43.61"N, 04°12'47.64"E) and Courthézon  
145 (44°04'21.68"N, 04°52'01.53"E).

146 Intensive mist netting was undertaken in order to capture and sample (whole blood) reed  
147 buntings of the nominate subspecies, which winters at all study sites, as well as the local  
148 resident subspecies *witherbyi* at the Spanish and French sites and *lusitanica* at the Portuguese  
149 site. Some potential food items (n = 52), particularly, C3-plant material (inflorescences of  
150 reeds, sedges and rushes; from all sites), insect larvae and pupae from inside the reed stems  
151 (from Portugal and Spain), spiders (Portugal and France) and a beetle (common red soldier  
152 beetle *Rhagonycha fulva*; Portugal) were collected along the mist nets to assess differences in  
153 baseline isotopic composition between the sites. As more than 50% of *lusitanica* occur at the  
154 Portuguese sampling site (showing very high gene flow with the other much smaller  
155 populations where they occur; Kvist et al. 2011), and the Spanish and French sites are located  
156 close to the latitudinal limits of *witherbyi* distribution, the isotopic divergence found at these  
157 three sites is considered to be representative of the whole subspecies. Also, as the blood tissue  
158 is renewed at a high rate (half-lives of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  in the blood of a similar-sized species  
159 are 5.4 and 11 days, respectively; Hobson and Bairlein 2003), its stable isotopes reflect the  
160 diet that the birds had during the previous weeks (i.e. the winter diet, as the sampling took  
161 place c.2 months after reed buntings arrived into the winter quarters).

162 Birds were measured for wing (maximum chord), tail, tarsus and bill (to skull) lengths, bill  
163 width and bill depth (at the distal side of the nostrils), as well as muscle, fat and weight (for  
164 details see Neto et al. 2013). Portuguese and Spanish birds were measured by JMN, whereas  
165 French birds were measured by BV, thus being analysed separately. All individuals were  
166 identified to subspecies in the field: *lusitanica* (n = 26) from Salreu and *witherbyi* (n = 16)



167 from southern France were positively identified by their darker plumage and small size,  
168 whereas the few ( $n = 4$ ) *witherbyi* individuals caught at the Spanish site were readily  
169 identified by their much thicker bill, which does not overlap with *schoeniclus* ( $n = 44$  at  
170 Salreu,  $n = 36$  at Villafranca,  $n = 17$  in France) (see Neto et al. 2013). Age (first-year or adult)  
171 and sex were determined from the plumage wear and pattern using standard methods  
172 (Svensson 1992, de la Puente and Seoane 2001), with only one bird being of indeterminate  
173 age. Part of these morphological data were included in Neto et al. (2013) namely: the  
174 *lusitanica* and *schoeniclus* captured during the first winter at Salreu, as well as the three  
175 *witherbyi* captured during the first winter at Villafranca, but not the *schoeniclus* from  
176 Villafranca, the individuals captured during the second year of sampling at both sites, neither  
177 the French birds. Blood samples were collected for stable isotope analysis by puncturing the  
178 brachial vein and were stored in centrifuge tubes.

179

#### 180 **Geometric morphometrics of the bill**

181 A photograph of the bill in profile was taken from a subset of individuals ( $n = 4$  *witherbyi*, 21  
182 *lusitanica* and 27 *schoeniclus* from Portugal and Spain), and subjected to geometric  
183 morphometric analysis in software of the tps series (Rohlf 2010). A tps file was built from  
184 images using tpsUtil and used in tpsDig, where seven landmarks and eight semi-landmarks  
185 were digitized (Supplementary material Appendix, Fig. A1, Foster et al. 2007, Neto et al.  
186 2013). We then applied a Generalized orthogonal least-squares Procrustes Analysis (GPA)  
187 (Rohlf 1999) in order to obtain a consensus configuration, computed partial and relative  
188 warps, and extracted relative warp scores with a  $\alpha = 0$ , all using the tpsRelw software.

189

#### 190 **Stable isotope analysis**

191 The potential food items and the blood samples were dried and analysed for stable carbon and

192 nitrogen isotope ratios in order to determine the differences in isotopic niche between the  
193 subspecies at the wintering quarters (Inger and Bearhop 2008). Carbon and nitrogen isotope  
194 ratios were analyzed simultaneously on the same sample, using approximately 0.5–0.7 mg of  
195 dry blood, which was put into a clean tin capsule and analyzed by continuous-flow isotope  
196 ratio mass spectrometry (CF-IRMS): tin capsules were combusted on an Elementar Pyrocube,  
197 the analytes N<sub>2</sub> and CO<sub>2</sub> separated by purge-and-trap, and the stable isotope ratios measured  
198 on a Thermo Delta XP stable isotope ratio mass spectrometer. All stable isotope ratios are  
199 reported in permil (‰) using the  $\delta$  notation:

$$200 \quad \delta_{\text{sample}} = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000$$

201 where  $\delta_{\text{sample}}$  is the isotope ratio of the sample relative to a standard,  $R_{\text{sample}}$  and  $R_{\text{standard}}$  are  
202 the fractions of heavy to light isotopes (i.e. <sup>13</sup>C/<sup>12</sup>C or <sup>15</sup>N/<sup>14</sup>N) in the sample and standard  
203 respectively.  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  are reported relative to their respective international standards,  
204 i.e. V-PDB and AIR. Isotope ratios are normalized against internal laboratory standards  
205 gelatine, alanine and glycine. Replicate analyses of laboratory gelatin standard implied a  
206 precision lower than 0.20‰ for  $\delta^{15}\text{N}$  and 0.12‰  $\delta^{13}\text{C}$ , and all internal standards are routinely  
207 checked against international reference materials:  $\delta^{13}\text{C}$  was calibrated against the glutamic  
208 acid reference materials USGS40 and USGS41 (Coplen et al. 2006),  $\delta^{15}\text{N}$  was calibrated  
209 against the glutamic acid reference materials as well as the ammonium sulphate standards  
210 IAEA-N1, IAEA-N2 and USGS25.

211

## 212 **Statistical analyses**

213 Differences in bill traits between subspecies and sexes of the sampled birds were determined  
214 in order to provide the background for evaluating the association between form (bill  
215 morphology) and function (diet/isotopic niche). A measurement of bill size for Iberian birds  
216 was obtained through Principal Component Analysis (PCA), based on the correlation matrix,

217 of bill length, depth and width, resulting in one component (PCbill) with eigenvalue greater  
218 than one that explained 66% of the variance (KMO = 0.653, Bartlett's test of sphericity:  $\chi^2_3 =$   
219 78.2,  $P < 0.001$ ). PCA was also used to obtain a measure of body size from the variables  
220 wing, tail and tarsus lengths, resulting in one component (PCsize) with eigenvalue greater  
221 than one that explained 70.5% of the variance (KMO = 0.586, Bartlett's test of sphericity:  $\chi^2_3$   
222 = 150.7,  $P < 0.001$ ). Then, in order to evaluate the differences in bill size relative to body size  
223 between the ages, sexes and subspecies, a General Linear Model (GLM) was used with these  
224 three categorical variables and PCsize as covariate. The same procedure was used to evaluate  
225 the differences in bill shape between ages, sexes and subspecies, for which the first axis of  
226 variation derived from the geometric morphometric analysis (RW1), which represents the  
227 curvature of the culmen, was used (Neto et al. 2013). The interactions between subspecies and  
228 sex, and subspecies and age were also included initially, but the latter interaction and the  
229 variable age were removed from the final models, as they were not significant. French birds  
230 (measured by a different ringer) were analyzed separately, for which PCsize and PCbill  
231 explained 68.4% (KMO = 0.569, Bartlett's test of sphericity:  $\chi^2_3 = 35.7$ ,  $P < 0.001$ ) and 43.6%  
232 (KMO = 0.5, Bartlett's test of sphericity:  $\chi^2_3 = 2.77$ ,  $P = 0.43$ ) of the variance, respectively.  
233 As the latter PCA does not adequately depict the variation in bill size amongst French birds,  
234 we also describe bill depth for this population.

235 In order to determine whether there were differences in baseline isotope ratios between sites,  
236 general linear models (GLM) were used with  $\delta^{15}\text{N}$  or  $\delta^{13}\text{C}$  as dependent variables and site  
237 (Portugal, Spain, France), type of food item (plant, insect larvae, spider) and their interaction  
238 and predictors. For these analyses, the few beetles collected at the Portuguese site were  
239 excluded (because no similar samples were available from other sites), as was one outlier (an  
240 insect larva from Spain with a  $\delta^{15}\text{N}$  of +30.76‰; even though its inclusion produced virtually  
241 similar results), resulting in a total sample size of 47 food items.

242 GLMs were used to compare the stable isotope ratios between sites, subspecies (*schoeniclus*,  
243 *lusitanica*, *witherbyi*), sexes (male, female) and ages (first-year, adult). Initially, year was also  
244 included, but since it was not significant, neither in the global model (i.e. including all  
245 samples and sites) nor in site-specific models (F-test:  $P > 0.5$ ), this variable was excluded  
246 from further analyses. GLMs were also used to determine the influence of bill size (PCbill) on  
247 the stable isotope ratios within each subspecies, for which site, age and sex were included in  
248 the models. Levene tests indicated the existence of significantly different variances between  
249 the subspecies for  $\delta^{13}\text{C}$  in France and Portugal, but as non-parametric tests produced  
250 qualitatively similar results (all comparisons resulting in  $P < 0.001$ ), we present only the  
251 GLM results. Uncorrected pairwise comparisons of estimated marginal means were  
252 performed with least significant difference used for confidence interval adjustment in SPSS  
253 22.0 (IBM Corp. 2013). Results are presented as mean  $\pm$  SE.

254 We also analyzed  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  simultaneously to compare the isotopic niche space among  
255 groups. We calculated bivariate standard ellipse areas corrected for small sample size ( $\text{SEA}_C$ )  
256 and Bayesian standard ellipse areas ( $\text{SEA}_B$ ) using the SIBER routine in “siar”, a package in  
257 the R programming environment (Jackson et al. 2011, R Development Core Team 2011).  
258 Standard area ellipses estimated by Bayesian inference take into account uncertainties such as  
259 sampling biases and small sample sizes into niche metrics and, unlike the convex hulls, can be  
260 readily used for comparisons between groups, especially the posterior distribution of  $\text{SEA}_B$ ,  
261 which was estimated based on 10000 posterior draws of the  $\text{SEA}_B$  parameters (Jackson et al.  
262 2011).

263

## 264 Results

### 265 Morphological differences

266 There was no significant difference in bill size (PCbill) of *schoeniclus* between the  
267 Portuguese and Spanish sites (GLM:  $F_{1,77} = 0.09$ ;  $P > 0.7$ ), and so they were grouped in the  
268 remaining biometric comparisons. There were highly significant differences in bill size  
269 between the subspecies ( $F_{2,102} = 42.6$ ;  $P < 0.001$ ), but also significant differences between the  
270 sexes ( $F_{1,102} = 4.3$ ;  $P = 0.04$ ) and interaction between subspecies and sex ( $F_{2,102} = 3.2$ ;  $P =$   
271  $0.019$ ). These resulted from *schoeniclus* having the smallest and *witherbyi* the largest bills (all  
272 subspecies being highly significant different from each other), males having larger bills than  
273 females, and the sexual dimorphism in bill size increasing from *schoeniclus* to *lusitanica* and  
274 especially to *witherbyi* (Supplementary material Appendix, Fig. A2a). Birds caught in France  
275 also showed significant differences in bill size between the subspecies ( $F_{1,29} = 4.5$ ,  $P = 0.044$ ),  
276 but with a *schoeniclus* sample including only two females, no significant differences were  
277 detected between the sexes ( $F_{1,29} = 1.6$ ,  $P = 0.221$ ) and the interaction between sex and  
278 subspecies (also not significant  $P = 0.8$ ) was excluded from the model (Supplementary  
279 material Appendix, Fig. A3). Bill depth was highly significantly different between subspecies  
280 at the French site ( $F_{1,29} = 42.4$ ,  $P < 0.001$ ), and significantly different between the sexes ( $F_{1,29}$   
281  $= 6.5$ ,  $P = 0.017$ ), whereas the interaction between subspecies and sex was not significant  
282 ( $F_{1,29} = 0.09$ ,  $P = 0.767$ ). Interestingly, we found overlap in bill measurements between  
283 *schoeniclus* and *witherbyi* at this location, where *witherbyi* seems to have smaller bills than  
284 those from Spain, thus being somewhat closer in morphology to *lusitanica*.

285 The first axis of bill shape variation (RW1), which represents the curvature of the culmen (see  
286 Neto et al. 2013), was also highly significantly different between subspecies ( $F_{2,48} = 17.2$ ;  $P <$   
287  $0.001$ ), with the thick-billed subspecies showing more curved culmens (Supplementary  
288 material Appendix, Fig. A2b). Bill shape differed to a great extent between the sexes of  
289 *lusitanica*, which had greater sexual dimorphism than the remaining subspecies, in which

290 sexes were similar (Sex:  $F_{1;50} = 0.1$ ;  $P = 0.764$ ; Interaction Sex x ssp:  $F_{2;50} = 5.8$ ;  $P = 0.005$ ;  
291 see Supplementary material Appendix, Fig. A2b).

292 These morphological differences are similar to the ones found by Neto et al. (2013) for a  
293 larger sample size (but excluding the French *witherbyi*), thus establishing that the sampled  
294 birds are representative and suitable for determining the relationship between morphology and  
295 diet/isotopic niche.

296

### 297 **Baseline isotopic differences between sites**

298 There were no significant differences between sites in  $\delta^{15}\text{N}$  of the potential food items, but the  
299 there was a highly significant difference between the types of food items, reflecting the  
300 expected increase with the trophic level (GLM: Type of item:  $F_{2;40} = 18.93$ ;  $P < 0.001$ ; Site:  
301  $F_{2;40} = 1.45$ ;  $P_{2;41} = 0.25$ ; TypeXSite:  $F_{2;40} = 0.31$ ;  $P = 0.74$ ; see Supplementary material  
302 Appendix, Fig. A4). There were also no significant differences between sites in  $\delta^{13}\text{C}$  of food  
303 items (GLM: Type of item:  $F_{2;40} = 1.17$ ;  $P = 0.32$ ; Site:  $F_{2;40} = 2.19$ ;  $P_{2;40} = 0.13$ ; TypeXSite:  
304  $F_{2;40} = 5.57$ ;  $P = 0.007$ ), but there was a significant interaction between site and type of food  
305 item because in France spiders had lower  $\delta^{13}\text{C}$  values than plants (which were similar in  
306 Portugal; see Supplementary material Appendix, Fig. A4).

307

### 308 **Subspecies, sex and age differences in isotopic niche**

309 The global GLMs (i.e. including all samples and sites) revealed highly significant differences  
310 in both stable isotope ratios between subspecies and sites: the age classes differed  
311 significantly in  $\delta^{13}\text{C}$  and the sexes in  $\delta^{15}\text{N}$  (Table 1, Fig. 1). Interactions between these  
312 variables were not significant, thus being excluded from the final models. The parameters of  
313 the models (Table 1) indicate that the migratory *schoeniclus* differs in both isotope ratios  
314 from the two resident subspecies, which otherwise seem similar (Fig. 1). This is supported by

315 statistical comparisons of the estimated marginal means, which revealed significant  
316 differences between *schoeniclus* and *lusitanica* ( $\delta^{15}\text{N}$ : mean difference =  $-1.3 \pm 0.6$  ‰;  $P =$   
317  $0.029$ ;  $\delta^{13}\text{C}$ : mean difference =  $4.2 \pm 0.8$  ‰;  $P < 0.001$ ), *schoeniclus* and *witherbyi* ( $\delta^{15}\text{N}$ :  
318 mean difference =  $-1.6 \pm 0.7$  ‰;  $P = 0.023$ ;  $\delta^{13}\text{C}$ : mean difference =  $4.1 \pm 0.9$  ‰;  $P < 0.001$ ),  
319 but not between *lusitanica* and *witherbyi* ( $\delta^{15}\text{N}$ : mean difference =  $-0.3 \pm 0.9$  ‰;  $P = 0.781$ ;  
320  $\delta^{13}\text{C}$ : mean difference =  $-0.1 \pm 1.2$  ‰;  $P = 0.930$ ). There were significant differences between  
321 the sites for both isotope ratios with Portugal having the highest and France the lowest  $\delta^{15}\text{N}$ ,  
322 and the Spanish site having a significantly lower  $\delta^{13}\text{C}$  than the other two locations (Table 1,  
323 Fig. 1).

324 Bivariate  $\text{SEA}_C$  show clear differences in isotopic niche between subspecies within each site  
325 as well as between *schoeniclus* populations wintering at each site (Fig. 2). The Bayesian  
326 standard ellipse areas ( $\text{SEA}_B$ ) show highly significant differences in isotopic niche breadth  
327 between *schoeniclus* and the remaining subspecies, which are otherwise similar, whereas the  
328 three populations of *schoeniclus* seem equally generalist (Fig. 3).

329 The comparisons of stable isotope ratios between the age and sex classes of *lusitanica* and  
330 *witherbyi* did not produce any significant differences (all  $P > 0.3$ ). However, in Iberian  
331 *schoeniclus* (French birds were excluded as there were only two females sampled), both age  
332 and sex influenced the stable isotope ratios, but their effect was not similar at both sites. There  
333 was a significant three-way interaction for  $\delta^{15}\text{N}$  (Sex\*Age\*Site:  $F_{1;71} = 7.2$ ;  $P = 0.009$ ; all  
334 other variables with  $P > 0.2$ ), whereas for  $\delta^{13}\text{C}$  there was a highly significant effect of age and  
335 a significant interaction between sex and site (Site:  $F_{1;1} = 2.4$ ;  $P = 0.361$ ; Age:  $F_{1;73} = 16.1$ ;  $P$   
336  $< 0.001$ ; Sex:  $F_{1;73} = 0.04$ ;  $P = 0.831$ ; Sex\*Site:  $F_{1;73} = 8.3$ ;  $P = 0.005$ ) producing the complex  
337 pattern of variation depicted in Fig. 4.

338

339 **Relationship between bill size/shape and stable isotopes**

340 No significant relationships were found between bill size and either of the stable isotope  
341 ratios for *lusitanica* and *witherbyi* independently of whether age and sex were taken into  
342 account in the models (all  $P > 0.4$ ; see Fig. 5). For *schoeniclus*, the relationship between  $\delta^{15}\text{N}$   
343 and bill size was not significant ( $F_{1,88} = 2.0$ ;  $P = 0.16$ ), although the negative relationship at  
344 Salreu was significant ( $B = 1.04 \pm 0.513$ ,  $t_{1,38} = 2.0$ ,  $P = 0.049$ ; Fig. 5a). But, there was a  
345 significant negative relationship between  $\delta^{13}\text{C}$  and bill size ( $F_{1,88} = 5.6$ ;  $P = 0.02$ ) with large-  
346 billed birds having lower  $\delta^{13}\text{C}$  values in all populations (Fig. 5b). All the relationship between  
347 bill shape (RW1) and stable isotope ratios within *lusitanica* and within *schoeniclus* were not  
348 significant (all  $P > 0.26$ ).

349

## 350 Discussion

### 351 **Subspecies differentiation in isotopic niche**

352 Our approach revealed clear differences in isotopic niche among subspecies, but these  
353 differences do not strictly follow the divergence in bill size and shape. The stable isotope  
354 signatures of the two resident subspecies (*lusitanica* and *witherbyi*) have higher  $\delta^{15}\text{N}$  and  
355 lower  $\delta^{13}\text{C}$  than *schoeniclus* at each site (which supports the first two hypotheses described in  
356 the Introduction). This indicates that, on average, *schoeniclus* feeds on a lower trophic level  
357 than the resident subspecies, and that C4-plant material ( $\delta^{13}\text{C} < -20$ ) is included in the diet of  
358 many individuals. In addition, both resident subspecies showed a much narrower isotopic  
359 niche than *schoeniclus* at each site, but with *schoeniclus* including some isotopic values  
360 similar to those of the resident subspecies (Fig. 1, Fig. 2). Therefore, our results show that: (1)  
361 there are consistent ecological differences between thin- and thick-billed subspecies of reed  
362 bunting across its distribution, expanding from what was known from a traditional diet study  
363 between *schoeniclus* and *intermedia* (Matessi et al. 2002); (2) the intermediate-billed  
364 subspecies (*lusitanica*) is also ecologically distinct from the thin-billed (*schoeniclus*) in the



365 same direction and magnitude as the thick-billed form; and (3) the intermediate- and thick-  
366 billed subspecies are ecological specialists whereas the thin-billed is a generalist. These  
367 results imply that natural selection could have had an important role in the phenotypic  
368 divergence of reed bunting subspecies; and indicate that the southern subspecies, being more  
369 specialized, may be more locally-adapted than the generalist *schoeniclus*, which agrees with  
370 the asymmetric response to song playback (and presumed asymmetric reproductive isolation)  
371 that we found among these subspecies (Gordinho et al. in review). These results also imply  
372 that conservation measures that have taken place for the benefit of *schoeniclus* (e.g. winter  
373 feeding in Britain) are not adequate for the more highly-threatened *lusitanica* and *witherbyi*.  
374 It is noteworthy that *schoeniclus* showed obvious differences in stable isotope ratios between  
375 sites, particularly a lower  $\delta^{13}\text{C}$  in Spain and a lower  $\delta^{15}\text{N}$  in France, but *lusitanica* and  
376 *witherbyi* are remarkable similar (Fig. 1, Table 1). As the stable isotope ratios of food items  
377 do not vary significantly between sites, this observation indicates that there are differences in  
378 the diet (or food availability) of *schoeniclus* between sites. Indeed, although the food  
379 preferences of wintering *schoeniclus* should be similar across sites (as these birds originate  
380 from the same breeding locations and ringing recoveries even show that individuals  
381 occasionally move between Spain and Portugal in subsequent winters; JMN unpublished  
382 data), the actual diet might differ depending on food availability, which can then be explored  
383 by this generalist subspecies. In contrast, the similar isotopic values across sites showed by  
384 the specialized subspecies may reflect similar diets. Overall, the isotopic data is consistent  
385 with previous observations of thick-billed birds feeding almost exclusively on insects lying  
386 dormant inside reed stems and with *schoeniclus* eating seeds of various plants, including  
387 many C4 plants, as well as (flying) insects and spiders whenever available (Cramp and  
388 Perrins 1994, Matessi et al. 2002, Holland et al. 2006, Orłowski and Czarnecka 2007,  
389 Orłowski et al. 2013, pers. obs.), although the stable isotope technique cannot distinguish the

390 type of insects/spiders consumed by these morphological groups, nor whether the foraging  
391 technique of *schoeniclus* when eating insects is indeed different from the resident subspecies,  
392 not requiring strong, convex bills to access food (Neto et al. 2013, JMN pers. obs., Emilio  
393 Martínez pers. comm.). However, dietary interpretations of the stable isotopes are difficult  
394 and necessarily speculative. This is because there may be spatial heterogeneity in isotope  
395 ratios within sites, for which a more extensive sampling of food items would have been  
396 useful; and different food items (involving different feeding techniques and bill sizes/shapes)  
397 may have similar isotope signatures, in which case systematic observations of feeding birds  
398 and analyses of stomach contents or faeces would be required (this is probably the case  
399 between insects lying dormant inside reed stems vs. flying insects and spiders [see  
400 Supplementary material Appendix, Fig. A4], for which isotope mixing models would be  
401 useless). Future studies should sample additional food items, include isotope mixing  
402 modelling and combine traditional and isotope methods, as the various insects/spiders  
403 dependent on C3 plants probably have similar isotope signatures.

404 As mentioned above, the isotopic data indicate that *witherbyi* has a similar isotopic niche to  
405 *lusitanica* (Fig. 1, Fig. 2). However, if bill size and diet were strictly associated (third  
406 hypothesis), *witherbyi* should differ to a greater extent from the other two subspecies than the  
407 latter among each other, so this expectation (H3) is not supported by the results. Hence,  
408 ecologically (and to some extent on the basis of bill shape; Supplementary material Appendix,  
409 Fig. A2b, Neto et al. 2013), *lusitanica* should belong to the thick-billed group of subspecies of  
410 reed bunting rather than to the thin-billed group with which it may appear more similar from  
411 linear measurements (Byers et al. 1995, Neto et al. 2013). It is not possible to completely  
412 exclude the possibility that *lusitanica* and *witherbyi* have different diets that happen to  
413 coincide in their isotope ratios, but assuming that the foraging niches are indeed similar, we  
414 suggest three potential, non-exclusive explanations for their large difference in bill size: (1)

415 they could be adapted to feed on insects lying inside reeds of different thicknesses (or at  
416 different heights of the reeds); (2) bill size could have diverged due to differences in climate  
417 at the locations where *lusitanica* and *witherbyi* occur. For instance, bill has been shown to  
418 have a role in dissipating heat, varying with summer air temperature among populations of  
419 emberizids that have similar, generalist diets (Greenberg et al. 2012a,b); and other climate  
420 variables are also known to affect bill size in a variety of bird species (Grant and Grant 2002,  
421 Symonds and Tattersall 2010, Eroukhmanoff et al. 2013). (3) Sexual selection could be  
422 involved in the evolution of bill size and shape differences among the subspecies, and this  
423 could also explain the differences in sexual dimorphism among subspecies in these traits.  
424 Male *witherbyi* with thicker, curved bills could be preferred by the females (see Olsen et al.  
425 2013) which, due to genetic correlations, would also increase in these traits relative to other  
426 subspecies. Unfortunately, no studies on sexual selection or quantitative genetics were  
427 undertaken on any thick-billed subspecies of reed bunting that could explain these patterns,  
428 but the possibility that the phenotypic variation is entirely plastic is unlikely due to the high  
429 heritabilities of bill traits (e.g. Grant and Grant 2002, Eroukhmanoff et al. 2013). The  
430 interplay between natural and sexual selection relative to bill size and shape in reed buntings  
431 is a promising research avenue.

432 As the winter isotopic niche (and presumably diet) differs between the resident and wintering  
433 populations of reed buntings, but both feed mostly on insects during spring/summer (Cramp  
434 and Perrins 1994, Holland et al. 2006, JMN pers. obs.), we suggest that intraspecific  
435 competition at the winter quarters (when food is scarce, temperatures low and natural  
436 selection presumably strong) might have been the main driver of ecological differentiation in  
437 reed buntings (see also Smith 1990 and Benkman 1993). An alternative hypothesis is that the  
438 smaller, straighter bill of migratory birds evolved as the populations of reed buntings  
439 expanded north after the last glaciation and locations with different available food and climate

440 were colonized. These hypotheses are not mutually exclusive, as the concomitant evolution of  
441 migration and smaller bills in northern, expanded populations (currently represented by  
442 *schoeniclus*), might have driven the evolution of larger, convex bills in southern, resident  
443 populations. Testing these hypotheses will be challenging, but diet studies reed bunting  
444 populations that do not co-occur (compete) with northern migrants might prove useful, as  
445 well as detailed phylogenetic and character-evolution studies.

446

#### 447 **Age and sex differences in isotopic niche**

448 The comparisons of stable isotopes among the age and sex classes at the two Iberian sites  
449 revealed an interesting pattern. Despite its relatively lower sexual dimorphism, differences  
450 among sexes (and ages) were only found in *schoeniclus*. These differences are rather complex  
451 and depend on the site, which could be associated with variation in food availability. In  
452 Portugal, the stable isotopes of reed buntings indicate the existence of two major food types  
453 with few intermediates (Fig. 2a): one with high  $\delta^{15}\text{N}$  and low  $\delta^{13}\text{C}$  (presumably C3-plant-  
454 eating insects) and another with high  $\delta^{13}\text{C}$  and low  $\delta^{15}\text{N}$  (C4-plant material). This apparent  
455 bimodality is not adequately described by the standard ellipses. In Spain, however, there are  
456 almost no C4-plant isotopic signatures, but there are many individuals showing low  $\delta^{13}\text{C}$  and  
457  $\delta^{15}\text{N}$  (presumably C3-plant material), which are largely absent at the Portuguese site.  
458 Although first-year birds had higher  $\delta^{13}\text{C}$  values than adults at both locations, their food  
459 preferences seem to differ between locations and sexes (Fig. 4). Males, especially first-years,  
460 seem to show a preference for C4-plant material at Salreu (high  $\delta^{13}\text{C}$  and low  $\delta^{15}\text{N}$ ) and C3-  
461 plant material at Villafranca (low  $\delta^{13}\text{C}$  and low  $\delta^{15}\text{N}$ ), but adult females at Salreu and first-  
462 year females at Villafranca deviated from most other birds presumably by including insects in  
463 their diet (high  $\delta^{15}\text{N}$  in both cases). However, as pointed out above, these dietary  
464 interpretations are tentative. It is possible that dominance is involved in the sex and age

465 differences within *schoeniclus*, with adults and males conditioning the access to the best  
466 available food types of the subdominant females and first-year birds (Radford and du Plessis  
467 2003), but behavioural development (learning) might also effect age differences in stable  
468 isotope ratios. These results imply that age and sex differences in isotopic niche cannot be  
469 fully ascertained by single-site studies.

470 The similarity in isotopic niche between the ages and sexes of *lusitanica* and *witherbyi*,  
471 despite their greater sexual dimorphism in bill size and especially shape, probably results  
472 from their specialization, having a much lower range of stable isotope ratios than *schoeniclus*  
473 (Fig. 3). The sexual dimorphism in the feeding apparatus of *lusitanica* (and perhaps  
474 *witherbyi*) could have been driven by sexual selection, as seems to have happened in Swamp  
475 Sparrows (*Melospiza georgiana*) (Olsen et al. 2013). But, as for the difference in bill traits  
476 between *lusitanica* and *witherbyi*, the sexes could differ in the choice of reed thickness when  
477 searching for dormant insects (which could be tested experimentally in captivity), feed on  
478 different prey items with similar isotopic signatures, or the females could be constrained in  
479 their evolutionary potential thus being more similar between subspecies than males.

480

#### 481 **Bill size/shape and isotopic niche within subspecies**

482 The relationship between bill size and stable isotope ratios was only significant for  
483 *schoeniclus*, and it was independent of age and sex classes, as these factors were also included  
484 in the statistical models. The relationship between bill size and  $\delta^{15}\text{N}$  varied with location (Fig.  
485 5), which could be explained by the combination of large-billed *schoeniclus* eating more  
486 insects/spiders and possibly a lower abundance of insects at Villafranca, where  $\delta^{15}\text{N}$  is lower  
487 than at Salreu (Fig. 1, Fig. 2); but this variation was no longer significant after including age  
488 and sex in the statistical model. However, there was a effect of bill size on diet as measured  
489 by  $\delta^{13}\text{C}$ , with smaller-billed birds presumably tending to eat seeds of C4 plants. The birds

490 wintering in the Iberian Peninsula belong to various populations of the subspecies *schoeniclus*  
491 (according to ringing recaptures, from northern France, Sweden, Czech Republic, Poland,  
492 Germany, Holand etc; Neto et al. in prep.), and are quite variable in bill size (Neto et al.  
493 2013). It is possible that the relationship between bill size and stable isotope ratios results at  
494 least in part from different (breeding) populations with slightly different bill sizes also having  
495 different food preferences, but competition could also play a role in this relationship (Radford  
496 and du Plessis 2003). Although there was no evidence for individual differences in isotopic  
497 niche relative to bill size/shape within *lusitanica*, stable isotopes do not provide any  
498 information on the size of the reeds where they feed nor on prey size.

499 In summary, phenotypic divergence in bill size/shape is partially associated with differences  
500 in isotopic niche, suggesting that reed bunting subspecies evolved through natural selection  
501 and became locally adapted. The southern, resident subspecies are more specialized than the  
502 migratory populations, which have a broad isotopic niche and probably use different feeding  
503 techniques as described in the literature (Shtegman 1948 cited by Prys-Jones 1984, Matessi et  
504 al. 2002). The foraging (isotopic) niche, however, is not the only factor explaining the  
505 divergence in bill size, neither between subspecies nor between sexes, since the two resident  
506 subspecies and their sexes seem to have similar foraging ecologies. Further work is required  
507 to determine the selection pressures associated with the phenotypic evolution in reed  
508 buntings, in particular to test the effects of sexual selection, climate and reed thickness, as  
509 well as evaluate the amount of plasticity in bill traits. Furthermore, age and sex differences in  
510 isotopic niche were found in *schoeniclus*, but not in the other subspecies (which have a  
511 greater sexual dimorphism), suggesting that the greater population divergence in bill size in  
512 males than in females could have been caused by sexual selection rather than intra-specific  
513 competition, or there could be constraints in the evolutionary potential of the females.

514 This study shows that in addition to phenotypic (Neto et al. 2013), acoustic (Gordinho et al.  
515 2015) and genetic (Kvist et al. 2011) differences, reed bunting subspecies differ in (foraging)  
516 ecology. It remains to be determined whether local adaptation is (in)directly causing  
517 reproductive isolation among the subspecies, providing evidence for ecological speciation in  
518 this study system.

519

## 520 **Acknowledgements**

521 All applicable institutional and/or national guidelines for the care and use of animals were  
522 followed. The capture, ringing and sampling of birds was conducted under the licenses  
523 required by the corresponding national authorities, following standard protocols and releasing  
524 the birds unharmed on site. Permits were given by the following institutions: Consejería de  
525 Agricultura, Dirección General de Montes y Espacios Naturales de Castilla-La Mancha  
526 (DGMEN/SEN/avp\_13\_011\_aut), CEMPA, Instituto de Conservação da Natureza e Florestas  
527 (99/2011, 112/2012) and Centre de Recherches sur la Biologie des Populations d'Oiseaux,  
528 Museum National D'Histoire Naturelle (1764, 783). We are indebted to Santiago Moraleda,  
529 José Luis Hernández de Santos, Edna Correia and Camilo Carneiro for help with the  
530 fieldwork, and to Dennis Hasselquist for critically reviewing the manuscript. Some fieldwork  
531 in Portugal was supported financially by ICETA, University of Porto. J.S.M. was partly  
532 financed by Projects CGL2005-02041 and CGL2010-21933/CO2-02 of the Spanish Ministry  
533 of Science and Innovation. L.O.G. and J.M.N. were financed by the Portuguese Foundation  
534 for Science and Technology through grants SFRH/BD/64645/2009 and  
535 SFRH/BPD/40667/2007, respectively.

536

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651

652 Figure legends

653 Figure 1. Boxplots of isotopic composition (**a**  $\delta^{15}\text{N}$  and **b**  $\delta^{13}\text{C}$ ) of the blood of reed bunting  
654 subspecies collected during winter at three sites in southwest Europe. Medians (lines) and  
655 interquartile ranges (IQR; boxes) are shown, together with outliers greater than 1.5xIQR  
656 (circles) and greater than 3xIQR (asterisks).

657

658 Figure 2. Convex hull ellipses (dashed lines) and standard ellipse areas corrected for small  
659 sample size ( $\text{SEAC}$ ; red/full lines) of each subspecies and location (**a** –Salreu, Portugal; **b** –  
660 Villafranca, Spain; **c** – Arles, France).

661

662 Figure 3. Posterior distribution of the isotope niche widths, as measured by the Bayesian  
663 standard ellipses (SEA.B), showing mean, 50, 75 and 95% confidence limits, as well as mean  
664  $\text{SEAC}$ .

665

666 Figure 4. Convex hull ellipses (dashed lines) and standard ellipse areas corrected for small  
667 sample size ( $\text{SEAC}$ ; full lines) of *Emberiza s. schoeniclus* for each age and sex at **a** Salreu,  
668 Portugal, and **b** Villafranca, Spain.

669

670 Figure 5. Linear Regressions between bill size (PCbill) and the stable isotope ratios (**a**  $\delta^{15}\text{N}$   
671 and **b**  $\delta^{13}\text{C}$ ) for each location and subspecies (for statistics see the main text).

672

673 Supplementary material Appendices:

674 Figure A1. Positioning of landmarks and semi-landmarks used in geometric morphometric  
675 analysis on a male *Emberiza schoeniclus witherbyi*.

676

677 Figure A2. Estimated marginal means (EMM) and 95% confidence interval of a) bill size  
678 (PCbill) and b) bill shape (RW1) for each subspecies and sex of Iberian Reed buntings.  
679 EMMs were derived from a general linear model that included body size (PCsize) as a  
680 covariate. As there was no significant effect of site, *E. s. schoeniclus* trapped in Spain and  
681 Portugal were grouped. Sample size is indicated between parenthesis and statistics are  
682 described in the main text. Birds trapped in France were analysed separately and are described  
683 in Fig. A3.

684

685 Figure A3. Estimated marginal means (EMM) and 95% confidence interval of a) bill size and  
686 b) bill depth of French birds, for each subspecies and sex. Sample size is indicated between  
687 parenthesis and statistics are described in the main text.

688

689 Figure A4. Boxplot showing variation in stable isotope ratios of the various food items for  
690 each site.

Table 1. Unstandardized coefficients ( $B \pm SE$ ) resulting from the General Linear Models comparing  $\delta^{13}C$  and  $\delta^{15}N$  between subspecies (*lusitanica/schoeniclus/witherbyi*), sexes (male/female), ages (first-year/adult) and sites (Portugal/Spain/France).

<b>Model</b>	<b>Variable</b>	<b>B <math>\pm</math> SE</b>	<b>F test</b>	<b>Significance</b>	
$\delta^{13}C$	<b>Subspecies</b>	(lus) $-0.105 \pm 1.200$	26.5	<b>P &lt; 0.001</b>	
		(sch) $4.119 \pm 0.892$			
	$R^2 = 0.36$	<b>Age</b>	(first-year) $1.690 \pm 0.518$	10.7	<b>P = 0.001</b>
		<b>Sex</b>	(female) $-0.369 \pm 0.545$	0.5	P = 0.500
		<b>Site</b>	(Portugal) $3.020 \pm 0.694$	11.4	<b>P &lt; 0.001</b>
(France) $2.597 \pm 1.035$					
$\delta^{15}N$	<b>Subspecies</b>	(lus) $-0.263 \pm 0.944$	5.3	<b>P = 0.006</b>	
		(sch) $-1.609 \pm 0.722$			
	$R^2 = 0.33$	<b>Age</b>	(first-year) $-0.749 \pm 0.408$	3.4	P = 0.068
		<b>Sex</b>	(female) $0.906 \pm 0.429$	4.5	<b>P = 0.037</b>
		<b>Site</b>	(Portugal) $1.432 \pm 0.547$	11.9	<b>P &lt; 0.001</b>
(France) $-1.947 \pm 0.815$					