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Stable isotopes reveal differences in diet among reed bunting subspecies that vary in bill size

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Running Title: Bill size and diet in reed buntings

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Abstract

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

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

Determining the ecological pressures that cause divergent selection is a crucial step for understanding the speciation process. In this study, we aim to achieve this in one of the most variable species of the large bunting family (Emberizidae): the reed bunting (Emberiza schoeniclus). Over 30 subspecies have been described in this non-model species, of which 20 are currently recognized (del Hoyo et al. 2011). They are often divided into thick-billed subspecies, which reside in the southern part of the Palearctic, and thin-billed subspecies, which occur further north and tend to be migratory (co-occurring with the southern, resident ones during winter); but also vary in several other traits including body size, plumage colour and song (Byers et al. 1995, Matessi et al. 2000a, b, Neto et al. 2013, Gordinho et al. 2015).

This study system is particularly interesting because the processes that are causing divergence among populations of reed bunting can potentially be generalized to at least some of the other 40 species of Emberiza (and over 320 species of Emberizidae), as well as to species of other
large seed-eating bird families such as the Fringillidae, all of which have largely continental
distributions.

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

Here, we propose to determine the ecological pressures that drove the evolution of bill size
differences among the three subspecies of reed bunting that occur in southwest Europe: E. s.
schoeniclus (hereafter schoeniclus), which breeds in central and northern Europe and winters
in the Mediterranean area; E. s. witherbyi (hereafter witherbyi), which is resident in northern
Morocco, central and eastern Spain and southern France; and E. s. lusitanica (hereafter
lusitanica), which is resident in Portugal and northwest Spain (Byers et al. 1995). Emberiza s.
witherbyi has a much thicker bill, whereas the bill of lusitanica is larger on average than, but
overlaps extensively with, that of schoeniclus (Neto et al. 2013). The increasing bill thickness
towards the east among southern, thick-billed subspecies, and the existence of an
intermediate-billed subspecies (lusitanica) suggests that these three subspecies may form the
closest link between thin-billed and thick-billed populations, and therefore are especially interesting to study the current level of ecological and reproductive isolation.

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

Traditional studies of diet are hampered by the fact that the digestibility of the food items is variable, and the subspecies also vary in the conspicuousness of foraging birds, as they use
different feeding techniques and microhabitats. Therefore, in order to compare the diets among subspecies, ages and sexes, we used an indirect method: stable isotope analysis. This approach relies on the variation of stable carbon and nitrogen isotope ratios with the diet: the former being mostly dependent on the C3 v C4 photosynthesis at the base of the food chain and on the water-use efficiency within C3 plants; whereas nitrogen varies mostly with the trophic level in the food chain (Bearhop et al. 2005, Inger and Bearhop 2008). We test the hypothesis (H1) that the nitrogen isotope ratio in the blood, which correlates positively with trophic level (DeNiro et al. 1981, Inger and Bearhop 2008), is higher in the thick-billed subspecies (presumed to eat mostly insects) than in the thin-billed subspecies (whose diet seems to include a large component of seeds). As in at least some areas schoeniclus seems to prefer seeds of Chenopodiaceae, Cyperaceae and Poaceae (which include many C4 plants; Cramp and Perrins 1994, Holland et al. 2006, Pyankov et al. 2010, Kandereit et al. 2012), we also hypothesize (H2) that this subspecies has the most ¹³C-rich values. In addition, if the diet closely follows the morphological differentiation, we hypothesize (H3) that the isotopic niche of lusitanica is intermediate between the other two subspecies (and closer to schoeniclus), and the difference in isotopic niche between the sexes is greater in lusitanica and witherbyi than in schoeniclus.

Materials and methods

Fieldwork

Fieldwork was carried out during December-February 2010/11 and 2012/13 at Salreu marshlands (40°43′41.10″N, 08°35′06.51″W), Portugal, as well as December-February 2011/12 and 2012/2013 at Lagunas de Villafranca (39°27′22.52″N, 03°20′09.03″W), Ciudad Real, Spain. In order to increase the sample size of witherbyi and the geographic representativeness, additional fieldwork was undertaken from the end of November to
December 2014 in southern France, mostly in the Camargue (43°36'24.62"N, 04°31'58.58"E), Arles, but a few samples (four in each location, including two subspecies) were also collected in Saint-Laurent D’Aigouze (43°35'43.61"N, 04°12'47.64"E) and Courthézon (44°04'21.68"N, 04°52'01.53"E).

Intensive mist netting was undertaken in order to capture and sample (whole blood) reed buntings of the nominate subspecies, which winters at all study sites, as well as the local resident subspecies witherbyi at the Spanish and French sites and lusitanica at the Portuguese site. Some potential food items (n = 52), particularly, C3-plant material (inflorescences of reeds, sedges and rushes; from all sites), insect larvae and pupae from inside the reed stems (from Portugal and Spain), spiders (Portugal and France) and a beetle (common red soldier beetle Rhagonycha fulva; Portugal) were collected along the mist nets to assess differences in baseline isotopic composition between the sites. As more that 50% of lusitanica occur at the Portuguese sampling site (showing very high gene flow with the other much smaller populations where they occur; Kvist et al. 2011), and the Spanish and French sites are located close to the latitudinal limits of witherbyi distribution, the isotopic divergence found at these three sites is considered to be representative of the whole subspecies. Also, as the blood tissue is renewed at a high rate (half-lives of δ¹³C and δ¹⁵N in the blood of a similar-sized species are 5.4 and 11 days, respectively; Hobson and Bairlein 2003), its stable isotopes reflect the diet that the birds had during the previous weeks (i.e. the winter diet, as the sampling took place c.2 months after reed buntings arrived into the winter quarters).

Birds were measured for wing (maximum chord), tail, tarsus and bill (to skull) lengths, bill width and bill depth (at the distal side of the nostrils), as well as muscle, fat and weight (for details see Neto et al. 2013). Portuguese and Spanish birds were measured by JMN, whereas French birds were measured by BV, thus being analysed separately. All individuals were identified to subspecies in the field: lusitanica (n = 26) from Salreu and witherbyi (n = 16)
from southern France were positively identified by their darker plumage and small size, whereas the few (n = 4) *witherbyi* individuals caught at the Spanish site were readily identified by their much thicker bill, which does not overlap with *schoeniclus* (n = 44 at Salreu, n = 36 at Villafranca, n = 17 in France) (see Neto et al. 2013). Age (first-year or adult) and sex were determined from the plumage wear and pattern using standard methods (Svensson 1992, de la Puente and Seoane 2001), with only one bird being of indeterminate age. Part of these morphological data were included in Neto et al. (2013) namely: the *lusitanica* and *schoeniclus* captured during the first winter at Salreu, as well as the three *witherbyi* captured during the first winter at Villafranca, but not the *schoeniclus* from Villafranca, the individuals captured during the second year of sampling at both sites, neither the French birds. Blood samples were collected for stable isotope analysis by puncturing the brachial vein and were stored in centrifuge tubes.

**Geometric morphometrics of the bill**

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

**Stable isotope analysis**

The potential food items and the blood samples were dried and analysed for stable carbon and
nitrogen isotope ratios in order to determine the differences in isotopic niche between the subspecies at the wintering quarters (Inger and Bearhop 2008). Carbon and nitrogen isotope ratios were analyzed simultaneously on the same sample, using approximately 0.5–0.7 mg of dry blood, which was put into a clean tin capsule and analyzed by continuous-flow isotope ratio mass spectrometry (CF-IRMS): tin capsules were combusted on an Elementar Pyrocube, the analytes N2 and CO2 separated by purge-and-trap, and the stable isotope ratios measured on a Thermo Delta XP stable isotope ratio mass spectrometer. All stable isotope ratios are reported in permil (‰) using the δ notation:

\[ \delta_{\text{sample}} = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000 \]

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

**Statistical analyses**

Differences in bill traits between subspecies and sexes of the sampled birds were determined in order to provide the background for evaluating the association between form (bill morphology) and function (diet/isotopic niche). A measurement of bill size for Iberian birds was obtained through Principal Component Analysis (PCA), based on the correlation matrix,
of bill length, depth and width, resulting in one component (PCbill) with eigenvalue greater
than one that explained 66% of the variance (KMO = 0.653, Bartlett's test of sphericity: \( \chi^2_3 = 78.2, P < 0.001 \)). PCA was also used to obtain a measure of body size from the variables
wing, tail and tarsus lengths, resulting in one component (PCsize) with eigenvalue greater
than one that explained 70.5% of the variance (KMO = 0.586, Bartlett's test of sphericity: \( \chi^2_3 = 150.7, P < 0.001 \)). Then, in order to evaluate the differences in bill size relative to body size
between the ages, sexes and subspecies, a General Linear Model (GLM) was used with these
three categorical variables and PCsize as covariate. The same procedure was used to evaluate
the differences in bill shape between ages, sexes and subspecies, for which the first axis of
variation derived from the geometric morphometric analysis (RW1), which represents the
curvature of the culmen, was used (Neto et al. 2013). The interactions between subspecies and
sex, and subspecies and age were also included initially, but the latter interaction and the
variable age were removed from the final models, as they were not significant. French birds
(measured by a different ringer) were analyzed separately, for which PCsize and PCbill
explained 68.4% (KMO = 0.569, Bartlett's test of sphericity: \( \chi^2_3 = 35.7, P < 0.001 \)) and 43.6%
(KMO = 0.5, Bartlett's test of sphericity: \( \chi^2_3 = 2.77, P = 0.43 \)) of the variance, respectively.
As the latter PCA does not adequately depict the variation in bill size amongst French birds,
we also describe bill depth for this population.

In order to determine whether there were differences in baseline isotope ratios between sites,
general linear models (GLM) were used with \( \delta^{15}N \) or \( \delta^{13}C \) as dependent variables and site
(Portugal, Spain, France), type of food item (plant, insect larvae, spider) and their interaction
and predictors. For these analyses, the few beetles collected at the Portuguese site were
excluded (because no similar samples were available from other sites), as was one outlier (an
insect larva from Spain with a \( \delta^{15}N \) of +30.76‰; even though its inclusion produced virtually
similar results), resulting in a total sample size of 47 food items.
GLMs were used to compare the stable isotope ratios between sites, subspecies (*schoeniclus, lusitanica, witherbyi*), sexes (male, female) and ages (first-year, adult). Initially, year was also included, but since it was not significant, neither in the global model (i.e. including all samples and sites) nor in site-specific models (F-test: $P > 0.5$), this variable was excluded from further analyses. GLMs were also used to determine the influence of bill size (PCbill) on the stable isotope ratios within each subspecies, for which site, age and sex were included in the models. Levene tests indicated the existence of significantly different variances between the subspecies for $\delta^{13}C$ in France and Portugal, but as non-parametric tests produced qualitatively similar results (all comparisons resulting in $P < 0.001$), we present only the GLM results. Uncorrected pairwise comparisons of estimated marginal means were performed with least significant difference used for confidence interval adjustment in SPSS 22.0 (IBM Corp. 2013). Results are presented as mean ± SE.

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

Results

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

The first axis of bill shape variation (RW1), which represents the curvature of the culmen (see Neto et al. 2013), was also highly significantly different between subspecies ($F_{2,48} = 17.2; P < 0.001$), with the thick-billed subspecies showing more curved culmens (Supplementary material Appendix, Fig. A2b). Bill shape differed to a great extent between the sexes of *lusitanica*, which had greater sexual dimorphism than the remaining subspecies, in which
sexes were similar (Sex: $F_{1,50} = 0.1; P = 0.764$; Interaction Sex x ssp: $F_{2,50} = 5.8; P = 0.005$; see Supplementary material Appendix, Fig. A2b).

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

**Baseline isotopic differences between sites**

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

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

The global GLMs (i.e. including all samples and sites) revealed highly significant differences in both stable isotope ratios between subspecies and sites: the age classes differed significantly in $\delta^{13}C$ and the sexes in $\delta^{15}N$ (Table 1, Fig. 1). Interactions between these variables were not significant, thus being excluded from the final models. The parameters of the models (Table 1) indicate that the migratory *schoeniclus* differs in both isotope ratios from the two resident subspecies, which otherwise seem similar (Fig. 1). This is supported by
statistical comparisons of the estimated marginal means, which revealed significant
differences between *schoeniclus* and *lusitanica* ($\delta^{15}$N: mean difference = -1.3 ± 0.6‰; P =
0.029; $\delta^{13}$C: mean difference = 4.2 ± 0.8‰; P < 0.001), *schoeniclus* and *witherbyi* ($\delta^{15}$N:
mean difference = -1.6 ± 0.7‰; P = 0.023; $\delta^{13}$C: mean difference = 4.1 ± 0.9‰; P < 0.001),
but not between *lusitanica* and *witherbyi* ($\delta^{15}$N: mean difference = -0.3 ± 0.9‰; P = 0.781;
$\delta^{13}$C: mean difference = -0.1 ± 1.2‰; P = 0.930). There were significant differences between
the sites for both isotope ratios with Portugal having the highest and France the lowest $\delta^{15}$N,
and the Spanish site having a significantly lower $\delta^{13}$C than the other two locations (Table 1,
Fig. 1).

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

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

**Relationship between bill size/shape and stable isotopes**
No significant relationships were found between bill size and either of the stable isotope ratios for *lusitanica* and *witherbyi* independently of whether age and sex were taken into account in the models (all P > 0.4; see Fig. 5). For *schoeniclus*, the relationship between δ¹⁵N and bill size was not significant (F₁;₈₈ = 2.0; P = 0.16), although the negative relationship at Salreu was significant (B = 1.04 ± 0.513, t₁;₃₈ = 2.0, P = 0.049; Fig. 5a). But, there was a significant negative relationship between δ¹³C and bill size (F₁;₈₈ = 5.6; P = 0.02) with large-billed birds having lower δ¹³C values in all populations (Fig. 5b). All the relationship between bill shape (RW1) and stable isotope ratios within *lusitanica* and within *schoeniclus* were not significant (all P > 0.26).

**Discussion**

**Subspecies differentiation in isotopic niche**

Our approach revealed clear differences in isotopic niche among subspecies, but these differences do not strictly follow the divergence in bill size and shape. The stable isotope signatures of the two resident subspecies (*lusitanica* and *witherbyi*) have higher δ¹⁵N and lower δ¹³C than *schoeniclus* at each site (which supports the first two hypotheses described in the Introduction). This indicates that, on average, *schoeniclus* feeds on a lower trophic level than the resident subspecies, and that C₄-plant material (δ¹³C < -20) is included in the diet of many individuals. In addition, both resident subspecies showed a much narrower isotopic niche than *schoeniclus* at each site, but with *schoeniclus* including some isotopic values similar to those of the resident subspecies (Fig. 1, Fig. 2). Therefore, our results show that: (1) there are consistent ecological differences between thin- and thick-billed subspecies of reed bunting across its distribution, expanding from what was known from a traditional diet study between *schoeniclus* and *intermedia* (Matessi et al. 2002); (2) the intermediate-billed subspecies (*lusitanica*) is also ecologically distinct from the thin-billed (*schoeniclus*) in the
same direction and magnitude as the thick-billed form; and (3) the intermediate- and thick-billed subspecies are ecological specialists whereas the thin-billed is a generalist. These results imply that natural selection could have had an important role in the phenotypic divergence of reed bunting subspecies; and indicate that the southern subspecies, being more specialized, may be more locally-adapted than the generalist *schoeniclus*, which agrees with the asymmetric response to song playback (and presumed asymmetric reproductive isolation) that we found among these subspecies (Gordinho et al. in review). These results also imply that conservation measures that have taken place for the benefit of *schoeniclus* (e.g. winter feeding in Britain) are not adequate for the more highly-threatened *lusitanica* and *witherbyi*.

It is noteworthy that *schoeniclus* showed obvious differences in stable isotope ratios between sites, particularly a lower $\delta^{13}$C in Spain and a lower $\delta^{15}$N in France, but *lusitanica* and *witherbyi* are remarkable similar (Fig. 1, Table 1). As the stable isotope ratios of food items do not vary significantly between sites, this observation indicates that there are differences in the diet (or food availability) of *schoeniclus* between sites. Indeed, although the food preferences of wintering *schoeniclus* should be similar across sites (as these birds originate from the same breeding locations and ringing recoveries even show that individuals occasionally move between Spain and Portugal in subsequent winters; JMN unpublished data), the actual diet might differ depending on food availability, which can then be explored by this generalist subspecies. In contrast, the similar isotopic values across sites showed by the specialized subspecies may reflect similar diets. Overall, the isotopic data is consistent with previous observations of thick-billed birds feeding almost exclusively on insects lying dormant inside reed stems and with *schoeniclus* eating seeds of various plants, including many C4 plants, as well as (flying) insects and spiders whenever available (Cramp and Perrins 1994, Matessi et al. 2002, Holland et al. 2006, Orłowski and Czarnecka 2007, Orłowski et al. 2013, pers. obs.), although the stable isotope technique cannot distinguish the
type of insects/spiders consumed by these morphological groups, nor whether the foraging
technique of *schoeniclus* when eating insects is indeed different from the resident subspecies,
not requiring strong, convex bills to access food (Neto et al. 2013, JMN pers. obs., Emilio
Martínez pers. comm.). However, dietary interpretations of the stable isotopes are difficult
and necessarily speculative. This is because there may be spatial heterogeneity in isotope
ratios within sites, for which a more extensive sampling of food items would have been
useful; and different food items (involving different feeding techniques and bill sizes/shapes)
may have similar isotope signatures, in which case systematic observations of feeding birds
and analyses of stomach contents or faeces would be required (this is probably the case
between insects lying dormant inside reed stems vs. flying insects and spiders [see
Supplementary material Appendix, Fig. A4], for which isotope mixing models would be
useless). Future studies should sample additional food items, include isotope mixing
modelling and combine traditional and isotope methods, as the various insects/spiders
dependent on C3 plants probably have similar isotope signatures.

As mentioned above, the isotopic data indicate that *witherbyi* has a similar isotopic niche to
*lusitanica* (Fig. 1, Fig. 2). However, if bill size and diet were strictly associated (third
hypothesis), *witherbyi* should differ to a greater extent from the other two subspecies than the
latter among each other, so this expectation (H3) is not supported by the results. Hence,
ecologically (and to some extent on the basis of bill shape; Supplementary material Appendix,
Fig. A2b, Neto et al. 2013), *lusitanica* should belong to the thick-billed group of subspecies of
reed bunting rather than to the thin-billed group with which it may appear more similar from
linear measurements (Byers et al. 1995, Neto et al. 2013). It is not possible to completely
exclude the possibility that *lusitanica* and *witherbyi* have different diets that happen to
coincide in their isotope ratios, but assuming that the foraging niches are indeed similar, we
suggest three potential, non-exclusive explanations for their large difference in bill size: (1)
they could be adapted to feed on insects lying inside reeds of different thicknesses (or at different heights of the reeds); (2) bill size could have diverged due to differences in climate at the locations where *lusitanica* and *witherbyi* occur. For instance, bill has been shown to have a role in dissipating heat, varying with summer air temperature among populations of emberizids that have similar, generalist diets (Greenberg et al. 2012a,b); and other climate variables are also known to affect bill size in a variety of bird species (Grant and Grant 2002, Symonds and Tattersall 2010, Eroukhmanoff et al. 2013). (3) Sexual selection could be involved in the evolution of bill size and shape differences among the subspecies, and this could also explain the differences in sexual dimorphism among subspecies in these traits. Male *witherbyi* with thicker, curved bills could be preferred by the females (see Olsen et al. 2013) which, due to genetic correlations, would also increase in these traits relative to other subspecies. Unfortunately, no studies on sexual selection or quantitative genetics were undertaken on any thick-billed subspecies of reed bunting that could explain these patterns, but the possibility that the phenotypic variation is entirely plastic is unlikely due to the high heritabilities of bill traits (e.g. Grant and Grant 2002, Eroukhmanoff et al. 2013). The interplay between natural and sexual selection relative to bill size and shape in reed buntings is a promising research avenue.

As the winter isotopic niche (and presumably diet) differs between the resident and wintering populations of reed buntings, but both feed mostly on insects during spring/summer (Cramp and Perrins 1994, Holland et al. 2006, JMN pers. obs.), we suggest that intraspecific competition at the winter quarters (when food is scarce, temperatures low and natural selection presumably strong) might have been the main driver of ecological differentiation in reed buntings (see also Smith 1990 and Benkman 1993). An alternative hypothesis is that the smaller, straighter bill of migratory birds evolved as the populations of reed buntings expanded north after the last glaciation and locations with different available food and climate
were colonized. These hypotheses are not mutually exclusive, as the concomitant evolution of migration and smaller bills in northern, expanded populations (currently represented by *schoeniclus*), might have driven the evolution of larger, convex bills in southern, resident populations. Testing these hypotheses will be challenging, but diet studies reed bunting populations that do not co-occur (compete) with northern migrants might prove useful, as well as detailed phylogenetic and character-evolution studies.

**Age and sex differences in isotopic niche**

The comparisons of stable isotopes among the age and sex classes at the two Iberian sites revealed an interesting pattern. Despite its relatively lower sexual dimorphism, differences among sexes (and ages) were only found in *schoeniclus*. These differences are rather complex and depend on the site, which could be associated with variation in food availability. In Portugal, the stable isotopes of reed buntings indicate the existence of two major food types with few intermediates (Fig. 2a): one with high δ¹⁵N and low δ¹³C (presumably C3-plant-eating insects) and another with high δ¹³C and low δ¹⁵N (C4-plant material). This apparent bimodality is not adequately described by the standard ellipses. In Spain, however, there are almost no C4-plant isotopic signatures, but there are many individuals showing low δ¹³C and δ¹⁵N (presumably C3-plant material), which are largely absent at the Portuguese site. Although first-year birds had higher δ¹³C values than adults at both locations, their food preferences seem to differ between locations and sexes (Fig. 4). Males, especially first-years, seem to show a preference for C4-plant material at Salreu (high δ¹³C and low δ¹⁵N) and C3-plant material at Villafranca (low δ¹³C and low δ¹⁵N), but adult females at Salreu and first-year females at Villafranca deviated from most other birds presumably by including insects in their diet (high δ¹⁵N in both cases). However, as pointed out above, these dietary interpretations are tentative. It is possible that dominance is involved in the sex and age
differences within *schoeniclus*, with adults and males conditioning the access to the best available food types of the subdominant females and first-year birds (Radford and du Plessis 2003), but behavioural development (learning) might also effect age differences in stable isotope ratios. These results imply that age and sex differences in isotopic niche cannot be fully ascertained by single-site studies.

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

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

The relationship between bill size and stable isotope ratios was only significant for *schoeniclus*, and it was independent of age and sex classes, as these factors were also included in the statistical models. The relationship between bill size and δ¹⁵N varied with location (Fig. 5), which could be explained by the combination of large-billed *schoeniclus* eating more insects/spiders and possibly a lower abundance of insects at Villafranca, where δ¹⁵N is lower than at Salreu (Fig. 1, Fig. 2); but this variation was no longer significant after including age and sex in the statistical model. However, there was a effect of bill size on diet as measured by δ¹³C, with smaller-billed birds presumably tending to eat seeds of C4 plants. The birds
wintering in the Iberian Peninsula belong to various populations of the subspecies *schoeniclus* (according to ringing recaptures, from northern France, Sweden, Czech Republic, Poland, Germany, Holand etc; Neto et al. in prep.), and are quite variable in bill size (Neto et al. 2013). It is possible that the relationship between bill size and stable isotope ratios results at least in part from different (breeding) populations with slightly different bill sizes also having different food preferences, but competition could also play a role in this relationship (Radford and du Plessis 2003). Although there was no evidence for individual differences in isotopic niche relative to bill size/shape within *lusitanica*, stable isotopes do not provide any information on the size of the reeds where they feed nor on prey size.

In summary, phenotypic divergence in bill size/shape is partially associated with differences in isotopic niche, suggesting that reed bunting subspecies evolved through natural selection and became locally adapted. The southern, resident subspecies are more specialized than the migratory populations, which have a broad isotopic niche and probably use different feeding techniques as described in the literature (Shtegman 1948 cited by Prys-Jones 1984, Matessi et al. 2002). The foraging (isotopic) niche, however, is not the only factor explaining the divergence in bill size, neither between subspecies nor between sexes, since the two resident subspecies and their sexes seem to have similar foraging ecologies. Further work is required to determine the selection pressures associated with the phenotypic evolution in reed buntings, in particular to test the effects of sexual selection, climate and reed thickness, as well as evaluate the amount of plasticity in bill traits. Furthermore, age and sex differences in isotopic niche were found in *schoeniclus*, but not in the other subspecies (which have a greater sexual dimorphism), suggesting that the greater population divergence in bill size in males than in females could have been caused by sexual selection rather than intra-specific competition, or there could be constraints in the evolutionary potential of the females.
This study shows that in addition to phenotypic (Neto et al. 2013), acoustic (Gordinho et al. 2015) and genetic (Kvist et al. 2011) differences, reed bunting subspecies differ in (foraging) ecology. It remains to be determined whether local adaptation is (in)directly causing reproductive isolation among the subspecies, providing evidence for ecological speciation in this study system.

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**References**


Figure legends

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

Figure 2. Convex hull ellipses (dashed lines) and standard ellipse areas corrected for small sample size (SEA$_C$; red/full lines) of each subspecies and location (a – Salreu, Portugal; b – Villafranca, Spain; c – Arles, France).

Figure 3. Posterior distribution of the isotope niche widths, as measured by the Bayesian standard ellipses (SEA$_B$), showing mean, 50, 75 and 95% confidence limits, as well as mean SEA$_C$.

Figure 4. Convex hull ellipses (dashed lines) and standard ellipse areas corrected for small sample size (SEA$_C$; full lines) of Emberiza s. schoeniclus for each age and sex at a Salreu, Portugal, and b Villafranca, Spain.

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

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

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

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

Figure A4. Boxplot showing variation in stable isotope ratios of the various food items for each site.
Table 1. Unstandardized coefficients (B ± SE) resulting from the General Linear Models comparing δ¹³C and δ¹⁵N between subspecies (*lusitanica*/schoeniclus/witherbyi), sexes (male/female), ages (first-year/adult) and sites (Portugal/Spain/France).

<table>
<thead>
<tr>
<th>Model</th>
<th>Variable</th>
<th>B ± SE</th>
<th>F test</th>
<th>Significance</th>
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<tr>
<td>δ¹³C</td>
<td>Subspecies</td>
<td>(lus) -0.105 ± 1.200</td>
<td>26.5</td>
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<td></td>
<td></td>
<td>(sch) 4.119 ± 0.892</td>
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<tr>
<td>R² = 0.36</td>
<td>Age</td>
<td>(first-year) 1.690 ± 0.518</td>
<td>10.7</td>
<td>P = 0.001</td>
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<tr>
<td></td>
<td>Sex</td>
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<td>0.5</td>
<td>P = 0.500</td>
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<tr>
<td></td>
<td>Site</td>
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<td>11.4</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(France) 2.597 ± 1.035</td>
<td></td>
<td></td>
</tr>
<tr>
<td>δ¹⁵N</td>
<td>Subspecies</td>
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<td>5.3</td>
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<td></td>
<td></td>
<td>(sch) -1.609 ± 0.722</td>
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<td></td>
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<tr>
<td>R² = 0.33</td>
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<td>P = 0.037</td>
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<tr>
<td></td>
<td>Site</td>
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<td>P &lt; 0.001</td>
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<td>(France) -1.947 ± 0.815</td>
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