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Divergent selection and reproductive isolation: an empirical study on reed buntings

Luís de Oliveira Rijo Gordinho

Tese de Doutoramento apresentada à
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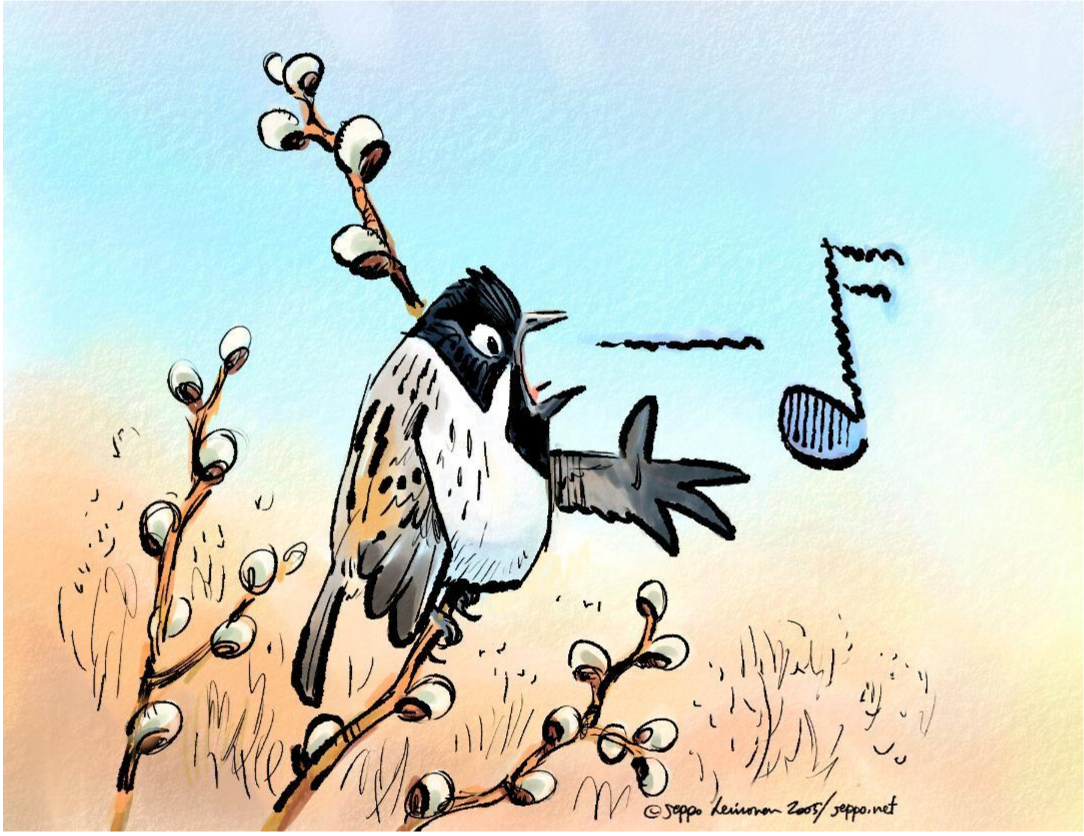

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Departamento de Biologia
2020

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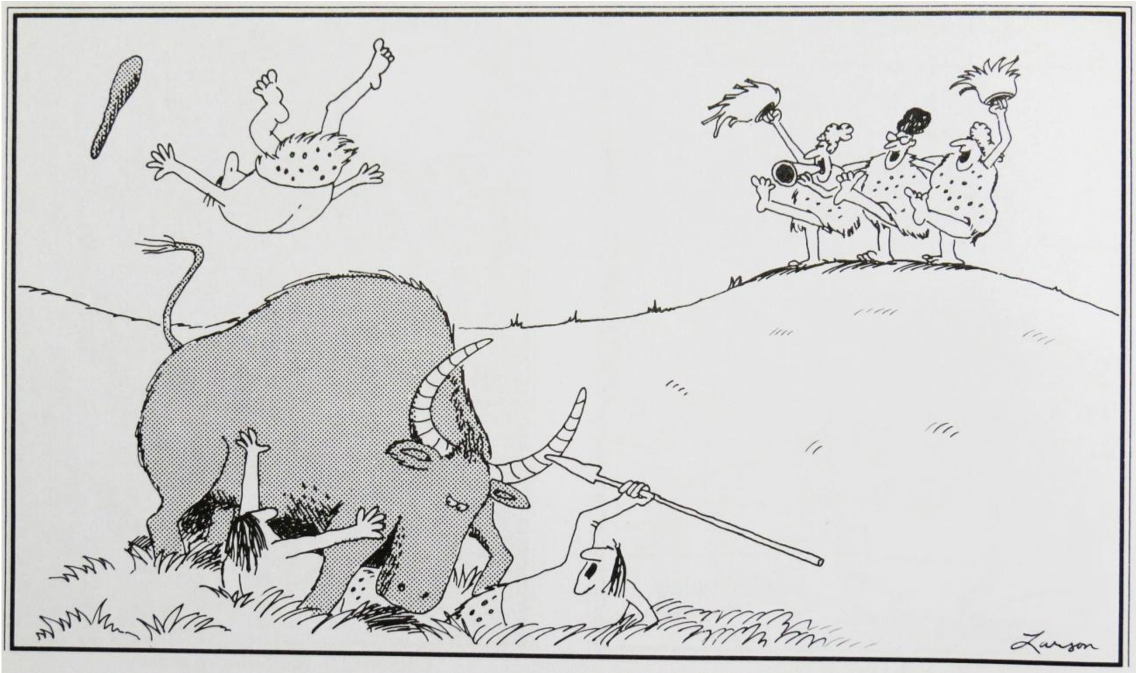
"*Yo soy yo y mi circunstancia y si no la salvo a ella no me salvo yo*" (aka context matters)
José Ortega y Gasset in Meditaciones del Quijote (1914)

"*Je n'ai fait celle-ci plus longue que parce que je n'ai pas eu le loisir de la faire plus courte*" (aka be synthetic, shorter text is much harder to write but easier to read)
Blaise Pascal in Lettres Provinciales (1657)

"*The hurrier I go, the behinder I get*" (aka pace yourself)
Lewis Carroll in Alice in Wonderland (1865)

"*You will never always be motivated, so you must learn to be disciplined*" (aka the two ways to perseverance)
Gym "Quote of the Day" (author and date unknown)



Cartoon 1 - Context matters (from Larson 1986).

Preliminary remark

In the elaboration of this dissertation, and in compliance with number 2 of Article 4 of the General Regulation of the Third Cycles of Studies of the University of Porto and Article 31 of D.L. 74/2006, of March 24, with the new wording introduced by D.L. 63/2016, of 13 September, it was made the full use of a coherent set of research articles already published in peer-reviewed journals with selection committees of recognized international merit, which are part of some of the chapters of this thesis. Taking into account that these works were carried out with the collaboration of other authors, the candidate elucidates that in all of them he participated actively in its conception, in obtaining, analyzing and discussing the results, as well as in the preparation of its published form. In addition, for this thesis, the four articles were adapted, updated and fully formatted to match the other two chapters.

During the PhD years (including those with suspended registration, 2015-18), the candidate co-authored six other papers published in international journals but not directly related to the thesis subject. Those are, of course, not included here. Also, the PhD programme and some preliminary results were presented in four scientific meetings. Finally, the candidate has peer-reviewed three manuscripts by other authors for two journals with recognized international merit. More details about these outputs are presented in the final Appendix.

List of articles

Chapter 2 - Neto JM, **Gordinho L**, Belda EJ, Marín M, Monrós JS, et al. (2013) Phenotypic Divergence among West European Populations of Reed Bunting *Emberiza schoeniclus*: The Effects of Migratory and Foraging Behaviours. *PLoS ONE* 8(5): e63248. doi.org/10.1371/journal.pone.0063248 (Impact Factor [2017] = 2.766)

Chapter 3 - Gordinho LO, Matheu E, Hasselquist D, Neto JM (2015) Song divergence between subspecies of Reed Bunting is more pronounced in singing styles under sexual selection. *Animal Behaviour* 107: 221-231. doi.org/10.1016/j.anbehav.2015.06.024 (Impact Factor [2017] = 3.067)

Chapter 4 - Gordinho LO, Hasselquist D, Neto JM (2016) Asymmetric song recognition between recently diverged subspecies of reed bunting. *Behavioral Ecology* 27(5): 1413-1423. doi.org/10.1093/beheco/arw062 (Impact Factor [2017] = 3.347)

Chapter 5 - Neto JM, **Gordinho LO**, Vollot B, Marín M, Monrós JS, Newton J (2017) Stable isotopes reveal differences in diet among reed bunting subspecies that vary in bill size. *Journal of Avian Biology* 48(2): 284–294. doi.org/10.1111/jav.01069 (Impact Factor [2017] = 2.488)

Nota prévia

Na elaboração desta dissertação, e nos termos do número 2 do Artigo 4º do Regulamento Geral dos Terceiros Ciclos de Estudos da Universidade do Porto e do Artigo 31º do D.L. 74/2006, de 24 de Março, com a nova redação introduzida pelo D.L. 63/2016, de 13 de Setembro, foi efectuado o aproveitamento total de um conjunto coerente de trabalhos de investigação objecto de publicação em revistas com comissões de selecção de reconhecido mérito internacional, os quais integram alguns dos capítulos da presente tese. Tendo em conta que os referidos trabalhos foram realizados com a colaboração de outros autores, o candidato esclarece que, em todos eles, participou ativamente na sua concepção, na obtenção, análise e discussão de resultados, bem como na elaboração da sua forma publicada. Adicionalmente, para a presente tese, os quatro artigos foram adaptados, atualizados e formatados de modo a ficarem coerentes com os outros dois capítulos.

Durante os anos de doutoramento (incluindo aqueles em que esteve matriculado mas não inscrito, 2015-18), o candidato foi co-autor de outros seis artigos publicados em revistas internacionais mas não diretamente relacionados com o tema da tese. Naturalmente, esses artigos não foram aqui incluídos. Adicionalmente, o programa de trabalho e alguns resultados preliminares foram apresentados em quatro reuniões científicas. Por fim, o candidato reviu três manuscritos de outros autores para duas revistas científicas internacionais de mérito reconhecido. Mais detalhes sobre estas tarefas são apresentados no Anexo final.

Lista de artigos

Capítulo 2 - Neto JM, **Gordinho L**, Belda EJ, Marín M, Monrós JS, et al. (2013) Phenotypic Divergence among West European Populations of Reed Bunting *Emberiza schoeniclus*: The Effects of Migratory and Foraging Behaviours. *PLoS ONE* 8(5): e63248. doi.org/10.1371/journal.pone.0063248 (Fator de impacto [2017] = 2,766)

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It took me four and a half years to complete this PhD. However, that period was spread over a nine year time-frame, making it a very long and exhausting journey. It started in February 2010, with a grant and an unpaid leave from my full-time job in a company experiencing financial problems. In February 2014, I went back to my old job and suspended the PhD registration for four years. Nevertheless, by April 2018, I terminated the 16.5 year-old contract, due to 6 months of unpaid salaries, and went on unemployment benefits. In July, I got my compensation through Labor Court, and in October I registered in the academic year of 2018-19, to submit this thesis in June 2019. Therefore, first of all, I'd like to thank the Portuguese Science and Technology Foundation for financing the first four years of my PhD, through grant no. SFRH/BD/64645/2009, and the Portuguese Social Security for financing me (beneficiary no. 11339398802) during the last six months. I also express my gratitude to the Portuguese Institute of Employment and Professional Training (IEFP), for not demanding too much from user no. 6714211 during this stage. In addition, some fieldwork for the thesis was supported financially by ICETA, University of Porto, thanks to the mediation of Pedro Beja.

I never seriously considered doing a PhD before July 2009. Fortunately, I got a grant the first time I run for it, in August 2009. The idea was triggered by resentment towards colleagues resigning from the company, to move up on their careers, leaving me behind. I now thank them; the resentment is gone. But to register in a PhD, a minimum level of self-confidence is required. Key events that built up my self-confidence were the article published in the American Birding Association (ABA) technical journal *Birding* (18,000 copy circulation) in January 2009 (Gordinho 2009a), and the fieldwork done for HOS (Hellenic Ornithological Society), in Greece, during June 2009 (Gordinho 2009b). I therefore thank Ted Floyd, José Pedro Tavares and Tasos Dimalexis for these opportunities. The good old formative years were probably important too, for those I'm grateful to António Teixeira, Rui Rufino, António Araújo, Renato Neves, Jorge Saraiva and Luís Lopes Silva. Before getting the PhD grant, in September 2009, I went to the XXII International Bioacoustics Council (IBAC) conference for free with Magnus Robb, which was a good introduction to scientific bioacoustics, so I thank Magnus and Mark Constantine for the opportunity.

Only one person closely accompanied most of this nine year long journey, so my deepest gratitude goes to my Mother, Graça Maria de Oliveira Martins. Maybe she never realized what this was all about, maybe she was not always in the best mood, or able to set the best example. But she was always there, and I know her love for me is

unconditional. That should be enough. My Girlfriend, Ana Filipa de Melo Alves, only come on board this rocky boat on the sunny day of April the 15th, 2017. My motivation regarding the PhD was very low at the time, so reconnecting with Filipa and building this relationship with her was probably a turning point for my thesis. I remember the first time I saw her, in Grândola, on April the 12th, 2006. I remember the SMS she sent me the day I got the PhD grant ("Congratulations! Show them your worth"). I remember so many things, that my heart is about to burst just writing these lines.

Júlio Neto, friend and supervisor, was the only person who accompanied most of my work. I was his first PhD student, and we both have strong personalities, so the stakes were high. However, we decided that, no matter how bad thing got, this venture would never jeopardize our friendship. In the end, I think our relationship got even stronger. We became brothers in arms (but, like *The Man Who Stare at Goats*, we don't fight with guns, we fight with our minds). Almost to the end of the four years, Júlio seemed sure I could pull this through; much surer than I was. That was important. I also understand his view of the PhD as a proof of absolute and utter autonomy (that many others share). His PhD was like that, and he lived to show it can be done. In addition, I thank him for loaning me his sound recording equipment. I also thank Júlio's family, Ulrika Björkman, Bruno and Markus, for their hospitality and kindness in my many visits, both to Veberöd and to Verdemilho. Dennis Hasselquist made it clear from the start that his availability would be limited, so Júlio would act as the hands-on supervisor. This was indeed the case, but Dennis gave us his opinion about the best direction to follow in several occasions and reviewed all the papers giving valid input. I thank him for that. Dennis is very kind and an excellent communicator, and also he has that restrain than Júlio and me sometimes lack. Paulo Célio only became my 2nd co-supervisor in December 2018, when I registered in the academic year of 2018-19, because Júlio was no longer affiliated to Porto University. However, from my first visit to CIBIO, back in March 2010, Paulo was one of the friendliest and most welcoming professors there. I thank him for his availability, help with administrative procedures, and thesis review.

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In 2009, I had this dream that may be about to come true. It has been a long way, but now I can see light in the end of the tunnel. I hope it’s not the Alfa train coming from Oporto.

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Abstract

Divergent selection and local adaptation are responsible for many phenotypic differences between populations, potentially leading to speciation through the evolution of reproductive barriers. Acoustic divergence, in particular, is often the basis of assortative mating and behavioral isolation in birds. However, some species have different singing styles used in distinct social contexts, and songs of each style may change at different rates over time and space. Mate recognition largely depends on learning, generally resulting in discrimination against nonlocal stimuli. Nevertheless, there may also be geographical variation in discrimination, allowing inference on the mechanisms behind recognition. Morphologic divergence can cause reproductive isolation too, and a strong association between bill traits and foraging niche, at a given place and time, may indicate ecological speciation is at play and how.

This thesis focused on reed buntings (*Emberiza schoeniclus*), a small passerine that breeds in marshlands throughout the Palearctic. Populations of three subspecies were studied: migratory *E. s. schoeniclus*, breeding in northern and central Europe; and the residents *E. s. lusitanica* from northwest Iberia and *E. s. witherbyi* from southeast Iberia and southern France (hereafter *schoeniclus*, *lusitanica* and *witherbyi*, respectively).

Four main objectives were addressed using different approaches: (1) phenotypic divergence was evaluated to determine the extent of local adaptation to migration and diet, using linear measurements and geometric morphometrics; (2) song divergence between subspecies and between the three singing styles of each subspecies were tested searching for effects of sexual selection, using spectral traits and derived synthetic variables; (3) song discrimination level was determined to infer premating reproductive isolation, using playback of fast song to test territorial males; (4) association between bill morphology and foraging niche was evaluated searching for evidence of natural selection, by sampling blood in southwest Europe during the winter (when target subspecies co-occur) and performing stable isotope analyses (carbon and nitrogen).

As predicted, migratory subspecies were smaller and had longer and more pointed wings. Unexpectedly, their tail was longer, probably due to correlation with wing length. *witherbyi*, which feed on insects hiding inside reed stems during the winter, had a very thick bill; whereas *schoeniclus*, which feed on seeds, had thinner bills. Although smaller, *lusitanica* had thicker and longer bill than *schoeniclus*. Geometric morphometrics revealed that southern subspecies have a more convex culmen than *schoeniclus*, and that *lusitanica* and *schoeniclus* bills differ more in shape than in linear measurements. The two singing styles under sexual selection (dawn and fast songs, related to obtaining extrapair and social mates, respectively) diverged more than the slow songs (an all-clear signal to nest attending females). Multiple song traits differed between subspecies in all styles, with intermediate values for *lusitanica*. Cluster analyses of populations indicate that sexually selected styles better discriminate subspecies, describing a major split in song features between *schoeniclus* and the resident subspecies. It was found that *witherbyi*, and to some extent *lusitanica*, males largely ignored *schoeniclus* songs. However, *witherbyi* reacted less strongly to *lusitanica* than the converse. In contrast, *schoeniclus* males reacted equally strongly to all subspecies. *schoeniclus* showed a broader isotopic niche than southern subspecies, which seemed similar despite *witherbyi* more divergent bill. Stable isotope ratios were consistent with resident subspecies feeding on C3-plant-feeding insects, whereas *schoeniclus* diet also included C4 plant material.

Subspecies differing in several traits as theoretically predicted indicate local adaptation through natural selection. Greater song divergence in fast and dawn styles implies that sexual selection is important in this evolutionary process. Differential territorial defense provides evidence of premating reproductive isolation, and discrimination by southern subspecies seems associated with local adaptation. Recognition pattern agrees with ecological rather than neutral divergence, as predicted in ecological speciation processes. Isotopic niche divergence between northern and southern subspecies suggests that bill differentiated by natural selection through competition during winter.

Keywords: morphologic divergence, *Emberiza schoeniclus*, local adaptation, acoustic divergence, subspecies, singing style, birdsong, speciation, playback, asymmetric recognition, song discrimination, isotopic niche, bill, geometric morphometrics.

Resumo

A seleção divergente e a adaptação local são responsáveis por muitas diferenças fenotípicas entre populações que, por sua vez, poderão resultar em especiação através da evolução de barreiras reprodutivas. A divergência acústica, em particular, é frequentemente a base do acasalamento seletivo e do isolamento comportamental nas aves. No entanto, algumas espécies têm diferentes estilos de cantar que são usados em distintos contextos sociais, e as canções de cada estilo podem ter diferentes taxas de alteração no tempo e no espaço. O reconhecimento de potenciais parceiros depende sobretudo de aprendizagem, geralmente resultando na discriminação negativa de estímulos não locais. Não obstante, também pode existir variação geográfica na discriminação, permitindo inferências sobre os mecanismos subjacentes ao reconhecimento. A divergência morfológica pode igualmente causar isolamento reprodutivo, e uma forte associação entre carateres do bico e o nicho alimentar, num dado momento e local, poderá indicar que está a ocorrer especiação ecológica e como.

Esta tese focou-se na escrevedeira-dos-caniços[1] (*Emberiza schoeniclus*), um pequeno passeriforme que nidifica em zonas húmidas do Paleárctico. Foram estudadas populações de três subespécies: a migratória *E. s. schoeniclus*, que nidifica na Europa central e do norte; e as residentes *E. s. lusitanica* do noroeste da Península Ibérica e *E. s. witherbyi* do sudeste dessa península e sul de França (doravante *schoeniclus*, *lusitanica* e *witherbyi*, respetivamente).

Foram abordados quarto objetivos principais, utilizando metodologias distintas: (1) a divergência fenotípica foi avaliada para determinar a extensão da adaptação local à migração e à dieta, utilizando biometria linear e morfometria geométrica; (2) a divergência no canto entre as subespécies e entre os três estilos de cantar de cada subespécie foram comparadas em busca de efeitos da seleção sexual, utilizando caracteres espectrais e variáveis sintéticas derivadas destes; (3) o nível de discriminação do canto foi determinado para inferir isolamento reprodutivo pré copulatório, reproduzindo artificialmente canto rápido para testar machos territoriais; (4) a associação entre a morfologia do bico e o nicho alimentar foi avaliada procurando evidências de seleção natural, através da amostragem de sangue no sudoeste da Europa durante o inverno (altura em que as subespécies alvo aí coocorrem) e realizando análises de isótopos estáveis (carbono e azoto).

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[1] O candidato segue a lista de nomes portugueses para as aves da Europa de Sacarrão & Soares (1979, Arquivos do Museu Bocage 2.ª Série. Vol. VI, n.º 23) e a respectiva Adenda e Errata (Soares 1986).

Como previsto, as aves de populações migratórias eram mais pequenas e tinham asas mais longas e pontiagudas. Inesperadamente, a sua cauda era mais comprida, provavelmente devido a correlação com o comprimento das asas. *witherbyi*, que se alimenta de insetos ocultos no interior dos caules de caniço durante o inverno, tinha um bico muito grosso; ao passo que *schoeniclus*, que se alimenta de sementes, tinha bico mais fino. Apesar de mais pequena, *lusitanica* tinha um bico mais grosso e comprido que *schoeniclus*. A morfometria geométrica revelou que as subespécies do sul têm um cúlmen mais convexo que *schoeniclus*, e que os bicos de *lusitanica* e *schoeniclus* diferem mais na forma do que na biometria linear. Os dois estilos de cantar sob seleção sexual (canto da madrugada e canto rápido, relacionados com a obtenção de fêmeas extra par e de fêmea social, respetivamente) divergiram mais do que o canto lento (um sinal tranquilizador para a fêmea no ninho). As subespécies diferiram em múltiplos caracteres do canto em todos os estilos, com valores intermédios para *lusitanica*. A análise de agrupamento das populações com base em caracteres do canto indica que os estilos sob seleção sexual discriminam melhor as subespécies, identificando uma grande dicotomia entre *schoeniclus* e as subespécies residentes. Verificou-se que os machos de *witherbyi*, e até certo ponto de *lusitanica*, ignoraram amplamente o canto de *schoeniclus*. No entanto, *witherbyi* reagiu menos a *lusitanica* que o inverso. Pelo contrário, os machos de *schoeniclus* reagiram de forma igualmente forte ao canto de todas as subespécies. *schoeniclus* exibiu um nicho isotópico mais largo que o das subespécies meridionais, cujos nichos parecem muito semelhantes, apesar do bico mais divergente de *witherbyi*. As proporções de isótopos estáveis foram consistentes com as subespécies residentes a predarem insetos que se alimentam de plantas C3, e com a dieta de *schoeniclus* a incluir também plantas C4.

Subespécies que diferem em múltiplos caracteres de acordo com as previsões teóricas, indica adaptação local através de seleção natural. Maior divergência do canto nos estilos rápido e da madrugada sugere que a seleção sexual é importante neste processo evolutivo. Defesa territorial diferencial constitui uma evidência de isolamento reprodutivo pré copulatório, e a discriminação pelas subespécies meridionais parece associada à adaptação local. O padrão de reconhecimento está de acordo com divergência ecológica e não com divergência neutral, como previsto nos processos de especiação ecológica. A divergência no nicho isotópico entre subespécies setentrionais e meridionais sugere que o bico se diferenciou por seleção natural, devido a competição durante o inverno.

Palavras-chave: divergência morfológica, *Emberiza schoeniclus*, adaptação local, divergência acústica, subespécies, estilo de cantar, canto, especiação, playback, reconhecimento assimétrico, discriminação acústica, nicho isotópico, morfometria geométrica.

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Abbreviations

AIR	Air N2 (δ^{15}N international standard)
ANOVA	Analysis of Variance
AR	Aspect Ratio of a wing (AR=b^2/S, where b is wing span and S is wing area)
ca.	*Circa* (about or approximately)
cf.	*Confer* (compare)
CF-IRMS	Continuous-Flow Isotope Ratio Mass Spectrometry
csv	Comma-separated values (file format)
d	Day/s
D.L.	Decreto-Lei (Decree-Law)

dB	Decibel, one-tenth of a bel (B), a measure of comparative power or loudness
df (df1, df2)	Degrees of freedom (numerator & denominator, respectively)
DFA	Discriminant Function Analysis
DMIs	Dobzhansky-Muller genetic Incompatibilities
e.g.	*Exempli gratia* (for example)
EMM	Estimated Marginal Means
et al.	*Et alii* (and others)
etc.	*Et cetera* (and so forth)
FCT	Fundação para a Ciência e a Tecnologia (Portuguese Foundation of Science and Technology)
g	gram
GLM	General Linear Model
GLMM	General Linear Mixed Model
GPA	Generalized orthogonal least-squares Procrustes Analysis
h	Hour
Hz	Hertz
i.e.	*Id est* (that is)
IQR	Interquartile ranges
kHz	kilo Hertz
km	kilometer
KMO	Kaiser-Meyer-Olkin test (for sampling adequacy)
ku, u	kilounits (kU) and units (in the waveform vertical amplitude axis)
LWS	Long-wavelength-sensitive opsin gene
m	Meter
min	Minutes
mm	millimeter
ms	Milliseconds
mtDNA	mitochondrial DNA
n	Number of sampling units or observations (sample size)
N, S, W, E	North, South, West and East (in geographic coordinates)
ns	non-significant
P & p	Probability level (probability that H_0 is true)
P1-9	Primary feathers number one to nine, counted from the innermost primary (P1) outwards
PC & PCA	Principal Component and Principal Component Analysis

pers. obs.	Personal observations
PhD	*Philosophiae Doctor* (Doctor of Philosophy)
Photoshop CS	Photoshop integrated in Creative Suite package
POPH	Programa Operacional Potencial Humano (Human Potential Operational Program)
QREN	Quadro de Referência Estratégico Nacional (Portuguese Strategic Reference Table)
QTL	Quantitative Trait Loci
REML	Restricted Maximum Likelihood parameter estimate
RW	Relative Warp (axis derived from geometric morphometric analysis)
S	Supporting information, in table and figure codes
s	second
SD	Standard Deviation
SE & Std. Error	Standard Error
SEA_B	Bayesian Standard Ellipse Area
SEA_C	Standard Ellipse Area Corrected for small sample size
Sig.	Statistical Significance
SPSS	“Statistical Package for the Social Sciences” (statistical analysis software)
ssp.	Subspecies
stricto sensu	In a narrow sense or with a narrow interpretation
tps	thin-plate spline function based family of geometric morphometrics software programs
t-test	Student's t-test, a parametric statistic test to compare means
UK	United Kingdom
unpubl.	Unpublished
Viz.	*Videlicet* (that is or namely)
V-PDB	Vienna Pee Dee Belemnite ($\delta^{13}C$ international standard)
WAV	Waveform audio (file format)

Abbreviations of author and subspecies names

BV	Benjamin Vollot
EJB	Eduardo J. Belda
JMN	Júlio Manuel Neto
JSM	Juan S. Monrós

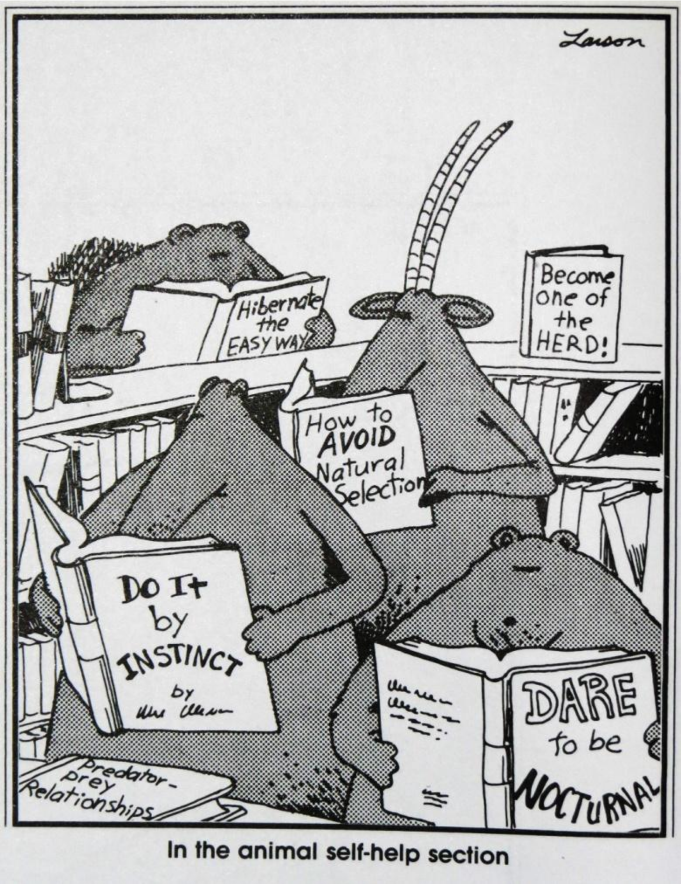
LG & LOG	Luís Gordinho and Luís de Oliveira Gordinho
MM	Marcial Marín
PF	Peter Fearon Jr.
RC	Ross Crates
lus	*Emberiza schoeniclus lusitanica*
sch	*Emberiza schoeniclus schoeniclus*
wit	*Emberiza schoeniclus witherbyi*

Symbols

δ	stable isotope ratio [$\delta_{sample} = (R_{sample}/R_{standard} - 1) \times 1000$]
±	Plus or minus
‰	Permil
$\delta^{13}C$	stable carbon isotope ratio, dependent on C_3 vs C_4 photosynthesis and water-use within C_3 plants
$\delta^{15}N$	stable nitrogen isotope ratio, varies with the trophic level
χ^2	Chi square, a test statistic
Φ_{ST}	Genetic divergence estimated from mtDNA
F	Test statistic of the Snedecor F test used to evaluate H_0 e.g. in GLMs
Fst	Genetic divergence
G_{ST}	Genetic divergence estimated from microsatellites
H	test statistic of Kruskal–Wallis nonparametric test, used to compare averages of more than two samples
º, ‘	Degrees and minutes (in geographic coordinates)
Pst	Phenotypic divergence
Q	Cochran’s Q test statistic
Qst	Quantitative trait divergence
r^2	coefficient of determination, the square of the correlation
R_{sample} & $R_{standard}$	The fractions of heavy to light isotopes (i.e. $^{13}C/^{12}C$ or $^{15}N/^{14}N$) in the sample and standard, respectively
Z	Test statistic of the Z-test, any statistical test for which the distribution of Z under H_0 can be approximately normal
H_0	Null hypothesis
H_n	Alternative hypotheses
Wilk's λ	Wilk's lambda, F test used in DFA

Chapter 1

General introduction



Cartoon 2 - 4 books useful for reed buntings: song is partly inate, dawn song is nocturnal, natural selection is behind ssp. divergence, and they are gregarious in winter (from Larson 1986)

1 General introduction

1.1 Ecological speciation

Speciation

Speciation, i.e. the formation of new species, is usually seen as the evolution of reproductive barriers, particularly in sexually reproducing organisms, for which the biological species concept is more frequently applied (Coyne & Orr 2004; Futuyma 2013; Kirkpatrick & Ravigné 2002). The reproductive or isolating barriers are those biological features of organisms that impede de exchange of genes with members of other populations of a species (Coyne & Orr 2004). These barriers are usually based on genetic differences between populations, though learning and cultural evolution may also play a role. The diverse forms of isolating barriers are classified in three major groups: premating, postmating prezygotic, and postzygotic (see Box 1).

To understand the speciation process, it is important to identify which reproductive barriers were involved and which evolutionary forces produced them. That is difficult because: (1) barriers continue to accumulate long after gene flow is reduced to nearly zero, (2) the relative importance of isolating barriers may vary during the speciation process, and (3) barriers act sequentially, reducing only the gene flow that escaped previous barriers. The latter difficulty highlights the importance of studying premating isolating barriers, which are the main focus of the present thesis.

Speciation can occur in either geographically isolated populations (allopatry) or in populations with no physical barriers to gene flow (sympatry or parapatry). When gene exchange is physically impossible, the conditions under which reproductive isolation can evolve are nonrestrictive: allopatric speciation can be driven by strong or weak divergent selection, sexual selection, uniform selection, or even stabilizing selection (Coyne & Orr 1989, 1997; Via 2009). It may occur quickly under divergent selection or extremely slowly under uniform or balancing selection. In contrast, the conditions under which sympatric or parapatric speciation with gene flow can occur are more demanding: genetically based phenotypic divergence requires much stronger selection to occur and be maintained when gene flow is possible than when geography makes it an impossibility. In the presence of migration, the establishment of genomic regions that resist gene flow sufficiently to maintain phenotypic differentiation is only likely if divergent (or possibly sexual) selection is strong, and so the initial barriers to gene flow in sympatry are likely to evolve quickly (Schluter 2001, Via 2001, Rice & Hostert 1993, Hendry et al. 2007). Speciation with gene flow is thus unlikely to occur under weak divergent selection, and it is certainly not expected under uniform or balancing selection (except perhaps by polyploidy). A variety of conditions that facilitate speciation with gene flow are now well described. They include strong divergent selection on multiple traits associated with resource or habitat use and ecologically based selection against migrants and/or hybrids. Recent work suggests that assortative mating can evolve rather easily if habitat choice determines the choice of mates, if mate choice is a correlate of the traits under divergent selection, or if recombination is reduced by physical linkage, pleiotropy (Via 2009) or genomic (mechanic) effects such as inversions (Dagilis & Kirkpatrick 2016).

BOX 1. Reproductive barriers.

1. Premating isolating barriers
    a. Behavioral isolation
    b. Ecological isolation
        i. Habitat isolation
        ii. Temporal (allochronic) isolation
        iii. Pollinator isolation
    c. Mechanical isolation
    d. Mating system “isolation”

2. Postmating, prezygotic isolating barriers
    a. Copulatory behavioral isolation
    b. Gametic isolation
        i. Noncompetitive gametic isolation
        ii. Competitive gametic isolation

3. Postzygotic isolating barriers (hybrid sterility and inviability)
    a. Extrinsic
        i. Ecological inviability
        ii. Behavioral sterility
    b. Intrinsic
        i. Hybrid inviability
        ii. Hybrid sterility
            1. Physiological sterility
            2. Behavioral sterility

Up to a decade ago, genetic studies of speciation in natural populations focused almost exclusively on retrospective analyses of reproductive isolation between species or subspecies and on hybrid sterility or inviability (through Dobzhansky-Muller genetic incompatibilities). Via (2009) called this approach “the spyglass” and claimed that, if we were to fully understand the origin of species, we should analyze the process from additional points of view. By studying the genetic causes of partial reproductive isolation between specialized ecological races, early barriers to gene flow could be identified before they become confounded with other species differences. This population-level approach, named “the magnifying glass”, can reveal patterns that become invisible over time, such as the mosaic nature of the genome early in speciation. Under divergent selection in sympatry, the genomes of incipient species become temporary genetic mosaics in which ecologically important genomic regions resist gene exchange, even as gene flow continues over most of the genome. Analysis of such mosaic genomes suggests that surprisingly large genomic regions around divergently selected quantitative trait loci (QTL) can be protected from interrace recombination by "divergence hitchhiking".

In allopatric populations, where there is no possibility for gene exchange, virtually any type or strength of selection will eventually lead to reproductive isolation, and barriers to gene flow may be of virtually any kind. In contrast, for speciation to occur without physical barriers to gene flow, divergent selection must be strong and affect several different traits, which causes ecologically based isolation to evolve relatively rapidly. The primacy of ecologically based isolation in speciation with gene flow is supported by empirical analyses of taxa in which divergent selection is thought to have been involved in speciation. They reveal extensive prezygotic ecologically based isolation, with little or no isolation attributable to postzygotic genetic incompatibilities.

The path for purely sympatric speciation can be divided into two stages: In stage one, there is rapid divergence at genomic regions harboring QTLs for traits under divergent selection, leading to significant ecologically based reduction of successful interbreeding between incipient species and ecological allopatry. In stage two, genetic incompatibilities can then accumulate to reinforce the ecologically based isolation and make it permanent (Via 2009).

In contrast, allopatric speciation cannot be divided into the same kind of distinct stages, because the accumulation of Dobzhansky-Muller genetic incompatibilities by independent responses to uniform or balancing selection can occur at the same time as the evolution of ecologically based isolation driven by divergent selection. In allopatry, any combination of divergent selection, uniform selection and genetic drift could produce speciation. Because the rapid divergence through selection that characterizes ecological speciation with gene flow is not required when populations are geographically isolated (although it can happen), allopatric speciation will often take much longer than speciation with gene flow (Hendry et al. 2007).

Ecological speciation

Ecological speciation is a process through which new species arise as a consequence of disruptive or divergent natural selection that directly or indirectly causes the evolution of reproductive isolation (Schluter 2001; Rundle & Nosil 2005; Nosil 2012). Selection is divergent when it acts in contrasting directions in two populations, often leading to local adaptation (when resident individuals have, on average, a higher fitness in their local habitat than those originating from other habitats; Williams 1966; Kawecki & Ebert 2004); whereas disruptive selection favours opposite, usually extreme, phenotypes within a single population, as occurs during sympatric speciation. This contrasts with other processes of speciation such as those in which genetic drift (in allopatry) and "pure", non-ecological sexual selection are the main drivers of divergence (Rundell & Price 2009; Nosil 2012). The alternatives to ecological speciation tend to involve stochastic events, such as random changes in gene frequencies and stochastic differences among populations in which mutations arise. Such alternatives can be classified into two main categories. The first considers mechanisms of speciation that do not involve selection. The second considers mechanisms that do involve selection, but in which selection is not divergent between ecological environments. All models that do not involve divergent selection are explicit alternatives to ecological speciation and generate different predictions. However, the different models are not mutually exclusive, and more than one may be operating simultaneously. Table 1.1 presents a classification of these different mechanisms of speciation (from Nosil 2012).

Table 1.1 - List of alternative mechanisms of speciation and examples of their predictions (from Nosil 2012)

Mechanism of speciation	Description	Example process causing divergence	Example prediction
1. "Ecological speciation"	Divergent selection between ecological environments drives the evolution of reproductive isolation	Divergent selection	Reproductive isolation is correlated with adaptive and ecological divergence
2. "Speciation without selection"	The evolution of reproductive isolation without a key role for selection	Genetic drift in stable populations	Reproductive isolation is correlated with time and not ecological divergence
		Genetic drift in small populations ("founder effect" speciation)	Reproductive isolation is correlated with the occurrence of population bottlenecks, perhaps also time
		Hybridization and polyploidy	Postzygotic isolation due to genetic incompatibilities and rapid speciation
3. "Mutation-order speciation"	Separate populations adapting to similar selection pressures fix different advantageous mutations (alleles) that are incompatible with one another	Selection arising from sexual or genetic conflict	Reproductive isolation is uncorrelated with ecological divergence and correlated with the intensity of conflict

Ecological speciation 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 ‘magic 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 2001; but see Grant and Grant 1997); or be under direct sexual selection (Grant and Grant 1997; Olsen et al. 2013). In addition, when individuals are adapted to particular environmental conditions and have a ‘good genes’ sexual selection system (i.e. viability benefits accruing to choosy females, cf. e.g. Møller & Alatalo 1999), hybrids will be maladapted to any of the parental environments and so will be selected against by locally-adapted individuals due to their low condition (van Doorn et al. 2009).

According to Nosil (2012), there are three main components in a process of ecological speciation: (1) a source of divergent selection, (2) a form of reproductive isolation, and (3) a link between selection and reproductive isolation. In this thesis, the focus was on the first two aspects of this process, which will be further described below. But, briefly, there are two ways by which divergent selection on ecological traits can be transmitted to the traits causing reproductive isolation: (1) pleiotropy, when the genes under divergent selection and those causing reproductive isolation are the same (i.e. a single gene has effects on two phenotypic traits: an ecological trait and reproductive isolation); and (2) linkage disequilibrium, when genes under divergent selection are physically different from those causing reproductive isolation (which may be neutral) but are associated (for instance by being closely positioned on a chromosome). It is important to determine how ecology and genetics interact to cause the evolution of the first reproductive barriers, before they are confounded by further barriers and differences evolving subsequently among populations/species (Via 2009). However, it is important to note that when studying divergence from the “magnifying glass perspective”, it is not possible to know whether speciation will ever be completed (Nosil et al. 2009).

1.1.1 Sources of divergent selection

1.1.1.1. Differences between environments

Divergent selection and local adaptation are responsible for many phenotypic differences found across populations, and may lead to the evolution of reproductive barriers and speciation (van Dorn et al. 2009; Winker 2010). The characterization of diverging phenotypes and the identification of relevant evolutionary forces acting on those phenotypes are crucial first steps to study the causes of speciation (Shaw & Mullen 2011). Even in widespread habitat generalists, natural selection alone may favor local specialization (e.g. Taylor et al. 2018). Alternatively, divergence in some traits may be entirely due to plastic changes derived from seasonal or environmental effects, without any genetic encoding (e.g. increased minimum song frequency in response to anthropogenic noise; Gross et al. 2010). One way of determining to which extent phenotypic divergence is genetic or plastic is using common garden experiments (Martin II et al. 2004; Bears et al. 2008; Nuismer & Gandon 2008; Ballentine & Greenberg 2010). However, this is difficult or often impossible with birds and especially threatened species. Alternative approaches must be used, such as comparisons between genetic and phenotypic divergence (Fst-Qst, Whitlock 2008, or Fst-Pst, Gay et al. 2009); and focusing on traits known to have high heritabilities (like bill traits, cf. Grant and Grant 2002; Eroukhmanoff et al. 2013) and thus can respond to sexual and natural selection. Additionally, the observation that the morphology fit well with the environment also strongly suggests local adaptation (but, because of its correlational character, this approach has limitations – see Kawecki & Ebert 2004). Morphological characters such as those analyzed in this thesis are generally highly heritable (Keller et al. 2001; Tarka et al. 2010), and given that the genetic divergence is very small (Zink et al. 2008; see also 2.3. below), the morphological differences among populations are likely to be meaningful (adaptive), especially if the predictions are confirmed, showing that the individuals ‘‘fit’’ their environments.

Divergent natural selection may lead to local adaptation and speciation (Nosil 2012). Local adaptations are expected to constrain gene flow among populations, as hybrids would be maladapted relative to their parents (van Dorn et al. 2009). That is, local adaptation often directly leads to some level of reproductive isolation (post-zygotic barriers). Determining the ecological pressures that cause divergent selection is a crucial step for understanding the speciation process. Any ecological differences between populations can lead to local adaptation and many have been studied in birds: e.g. altitude, climate, habitat, temperature, acoustic environment etc. Here, we focus on responses to ecological selection pressures that have also been associated with the evolution of reproductive isolation (and not just local adaptation) in birds, namely: migratory and foraging behaviours.

Migratory Behaviour

Divergence in migratory behaviour has been suggested to be an important factor promoting speciation, by causing selection against hybrids (Ruegg & Smith 2002; Pérez-Tris et al. 2003; Irwin & Irwin 2005; Bensch et al. 2009; Rohwer & Irwin 2011). Migratory movements are predominantly genetically determined in passerines, while in large soaring birds it is presumed that social factors play the largest role (Väli et al.

2018). Successful seasonal migration requires a specific set of behavioral, morphological and physiological traits. The fact that many of these traits have a genetic basis, indicates there is the potential for two closely related taxa to differ in their "migratory program". Hybrids between these groups might therefore have a suboptimal combination of genes. For instance, reproductive isolation seems to be evolving as a consequence of new migratory strategies in Blackcaps (*Sylvia atricapilla*; Bearhop et al. 2005) and Red-Eyed Vireos (*Vireo olivaceus*; Battey & Klicka 2017). However, some study systems that differ in migratory behaviour (Swainson's thrushes *Catharus ustulatus*, bluethroats *Luscinia svecica*, wheatears of *Oenanthe hispanica–pleschanka–cypriaca* complex) also differ in many other traits that may have evolved as the two migratory groups/subspecies were in (and expanded from) different glacial refugia (Kakhki et al. 2018). Overall, although migratory species represent a minority (18.5%) of all extant birds, they have a higher net diversification rate than sedentary species (Rolland et al. 2014).

As a general rule, migratory birds have longer and more pointed wings, shorter tails and lower body mass than residents (Hedenström 2008). Since flight is costly, there should be adaptations in the flight apparatus, i.e. wings and musculoskeletal systems, that reduce these costs. Aerodynamic theory provides predictions regarding wing and tail morphology (Rayner 1988; Thomas 1993). Migrants generally have wings of higher aspect ratio ($AR=b^2/S$, where b is wing span and S is wing area; a high value of AR means a long and slender wing) and more pointed wing tips than residents (Mönkkönen 1995; Lockwood et al. 1998; Voelker 2001), and the tails tend to be short and square rather than long and graduated (Leisler & Winkler 2003). A more general prediction is that migrants using flapping flight should be favoured by small overall body size (Hedenström & Alerstam 1998), which has gained some empirical support (Sol et al. 2005; Milá et al. 2008). In other animal groups, the locomotor behaviour may also lead to the evolution of different body shapes. One such group are the three-spined sticklebacks (*Gasterosteus aculeatus*), in which both a deep profile and small size enhance maneuverability in littoral environments, while both a streamlined profile and large size increase velocity and locomotor efficiency in pelagic environments (Walker 1997).

Foraging Behaviour

Niche divergence has been shown to promote reproductive isolation in a large variety of taxa (Funk et al. 2006). Primarily since 1990, explicit empirical studies of ecology's role in speciation have been conducted on natural populations, providing new insights into the mechanisms by which ecological divergence causes reproductive isolation. In the first 15 years, these studies covered subjects like phytophagous insects (Berlocher & Feder 2002) including apple maggot *Rhagoletis pomonella* (Filchak et al. 2000), leaf beetles *Neochlamisus bebbianae* (Funk 1998; Funk et al. 2002), *Heliconius* butterflies (Jiggins et al. 2001), three-spined sticklebacks (Nagel & Schluter 1998), walking-stick insect *Timema cristinae* (Nosil et al. 2002), Darwin's finches (Podos 2001), and monkeyflowers *Mimulus* (Ramsey et al. 2003). In benthic and limnetic threespine sticklebacks, cross-fostered females prefer mates of their foster father's species. That happens because daughters imprint on father odour and colour during a critical period early in development, and such traits have diverged between the species owing to differences in ecology (Kozak et al. 2011).

Avian foraging ecology has been associated with divergent selection and speciation, particularly in seed-eating species. In a 30-year study of two populations of Darwin's finches on the Galapagos island of Daphne Major, eight events with strong directional natural selection on beak and body size traits have been detected in *Geospiza fortis* (medium ground finch) (body size, four; beak size, three; beak shape, one) and in *Geospiza scandens* (cactus finch) seven times (body size, two; beak size, five). Most selection events have been when a scarcity of rain caused a change in the composition of the seed supply that forms their dry season diets (Grant & Grant 2002). *Nesospiza* buntings are another classic example of a simple adaptive radiation, with two species on each island in the Tristan da Cunha archipelago: an abundant small-billed dietary generalist and a scarce large-billed specialist. Their morphological diversity closely matches the available spectrum of seed sizes. Speciation is complete on the smaller island, where there is a single habitat with strongly bimodal seed size abundance, but is incomplete on the larger island, where a greater diversity of habitats has resulted in three lineages (Ryan et al. 2007). In red crossbills (*Loxia curvirostra* complex) bill depth is the target of stabilizing selection and, on an estimated fitness surface based on foraging data, each of five species belonging to the adaptive radiation resides on a summit corresponding to a different conifer species (Benkman 2003; Smith & Benkman 2007).

1.1.1.2. Competition

Competition with other species, in different communities, can result in accelerated divergence (Bolnick 2004). Competition occurs during periods of sympatry, but could also be the direct cause of parapatry (Bournez et al. 2015; McEntee et al. 2016). Competition may also be important for the full evolution of reproductive isolation, when populations with incomplete reproductive isolation meet by secondary contact potentially leading to character displacement and reinforcement of reproductive barriers

(Schluter 1988; Schluter & McPhail 1992). If populations under study are currently allopatric or parapatric as breeders, then contemporary intra-specific competition is probably not a source of divergent selection. However, historically, those populations may have been part of a larger meta-population with more continuous range, at which time intraspecific competition (for food, mates, etc) could have acted as an important agent of divergent selection.

1.1.1.3. Sexual selection

Environmentally dependent sexual selection can act as a source of divergent selection in the ecological speciation process. In such cases, sexual selection interacts with natural selection to accelerate speciation. Ecology may interact with sexual selection if it influences the signals or the perception differently in different environments. Sexual selection may also become associated with ecological selection, through magic traits, in reinforcement, etc. Vocalizations, in particular songs, are important for sexual selection and species recognition among birds (Newton 2003; Price 2008). Consequently, geographical differences in such vocalizations among populations may allow individuals to distinguish local birds from immigrants, for example to avoid inbreeding or mating with maladapted individuals (Bensch et al. 1994; Edmands 2007; Hansson et al. 2004; Keller & Waller 2002; Marr et al. 2002; Wilkins et al. 2018). Thus bird vocalizations are highly relevant in the context of incipient speciation. In general, evolutionary changes in the quantity of syllables are thought to be due to changes in pressure from sexual selection (Baker 1996; Lynch 1996; Read & Weary 1992). Several studies suggest that sexual selection promotes the evolution of reproductive isolation and two meta-analyses found small but significant overall trends (Kraaijeveld et al. 2011; Seddon et al. 2013). For instance in antbirds (Thamnophilidae), a positive relationship was found between species diversity and the intensity of sexual selection, measured by the production of lower pitched and more complex songs (Seddon et al. 2008).

In many birds and some marine mammals, there can be differences in the patterns of within-species spatial variation from one type of vocalization to another (Baker 2011). Among passerines, some species have several singing styles: functionally nonequivalent song types used in specific contexts (Bradbury & Vehrencamp 2011; Brunner & Pasinelli 2010; Ewin 1976; Hasselquist & Bensch 1991; Nemeth 1996). The songs used in each singing style may show distinct geographical patterns (Byers 1996; Kroodsma 1981) and change over time at different rates (Byers et al. 2010), suggesting that divergence between populations may be more pronounced in certain singing styles. Thus, as some social contexts are more relevant to reproductive isolation, certain singing styles could be of greater importance for speciation.

The environmentally dependent sexual selection that may lead to speciation is not just about acoustic signals: visual signals can be important too. The following sensory drive speciation scenario has been proposed for cichlid fish. First, divergent natural selection between light regimes at different water depths acts on long-wavelength-sensitive opsin gene (LWS). Second, sexual selection for conspicuous colouration is also divergent because perceptual biases differ between light regimes. Third, their interaction generates initial deviation from linkage equilibrium between LWS and nuptial colour alleles on all but the steepest gradients. Fourth, subsequent disruptive selection due to reduced fitness of genotypes with a mismatch between LWS and colour alleles causes speciation, perhaps involving reinforcement-like selection for mating preferences, whereby male nuptial colour may serve as a marker trait for opsin genotype (Seehausen et al. 2008).

In birds, sexual selection also acts on morphology, both on plumage and structure, including bill size (Grant & Grant 1997; Olsen et al. 2013) and plumage pattern, which may simultaneously evolve for ecological reasons as described above (see 1.1.1). There are several examples of this, for instance, in the swamp sparrow (*Melospiza georgiana*), bill sexual dimorphism as been shown to be driven by sexual selection and not by ecology/ natural selection (Olsen et al. 2013). In Darwin's finches, birds that hybridize tend to be morphologically similar to the species with which they breed (in bill, wing and tarsus length), and this suggests that they may pair assortatively according to morphology (Grant & Grant 1997). Morphology (including bill size) should therefore be under sexual selection in Darwin's finches. In the four bellbird species (genus *Procnias*), which appear to be ecologically quite similar and all have similar looking females, males show very different morphological traits (plumage and bare parts) thought to be the sole result of sexual selection in arbitrary directions (Snow 1976 in Price 2008). In trogons, sexual selection on male (carotenoid-derived) plumage coloration may be stronger than on acoustic traits (Ornelas et al. 2009).

Behaviour and Communication

Few anatomical or biochemical adaptations in animals are effective without some coupled behavior that invokes their use. This behavior typically takes the form of movements or the emission of signals, or both. Integrated models of animal communication address the question of if and when a receiver should incorporate signals into a decision about subsequent actions and invoke the value of information as the relevant criterion subject to selection. The value of information measure compares the average Darwinian fitness of a receiver when it incorporates a given set of signals in its decisions against when it does not incorporate them. Signal usage will only be favored if the value of information is positive (Bradbury & Vehrencamp 2011). Information sharing is recognized as one of the key adaptations that has led to major evolutionary changes throughout organismal history (Maynard Smith & Szathmàry 1995; Maynard Smith 1999, 2000; Lachmann et al. 2000; Jablonka 2002).

Reproductive isolation may arise when male mating signals and female preferences differ among populations. Habitat differences are important in generating diversity in mating signals and preferences (e.g. Laverde & Cadena 2018). Such differences in ecology are at the basis of the Sensory Drive Hypothesis for divergence in sexual signaling. That hypothesis focuses on how communication systems adapt to local environments and predicts that divergence in communication systems will occur when environments differ. Reproductive isolation can arise as a byproduct of this adaptive divergence in behavior (Boughman 2002).

Signal divergence

Song is an important premating isolation barrier between passerine species (Kroodsma & Miller 1996; Marler & Slabbekoorn 2004; Kroodsma 2005; Catchpole & Slater 2008), and song divergence between populations of a species can lead to reproductive isolation and speciation (Martens 1996; Price 2008). Song divergence often follows morphological divergence (e.g. in bill or body size) resulting from a shift in ecology (e.g. in diet or feeding actions), or adaptation to the local acoustic environment. However, it could also result from random cultural evolution and/or drift, especially in small, isolated populations. In some cases, acoustic adaptation and cultural isolation together influence song, as shown in the rufous-and-white wren *Thryophilus rufalbus* (Graham et al. 2018).

Shifts in ecology may include feeding on larger and harder seeds requiring strong bill musculature for crushing, but which slows the bill movements and hence constrains song production, as shown for the large ground finch *Geospiza magnirostris* (Podos 2001). Within the population of *Geospiza fortis* on Santa Cruz Island (Galápagos), known to possess a bimodal distribution in beak size, it was shown that that birds with longer, deeper and wider beaks produce songs with significantly lower frequencies; but beak morphology correlated positively with ‘vocal deviation’, a composite index of vocal performance (Huber & Podos 2006). A study covering the six different species of Darwin's ground finches (Geospiza) on the Galápagos Islands suggested that, while species' songs are sufficiently distinct at the local level to permit recognition, further divergence has been slow because birds ultimately rely on visual cues to recognize conspecifics (Ratcliffe & Grant 1985). Darwin's small tree finch *Camarhynchus parvulus* has a bill shape and musculature adapted to forage on trees, where they consume mostly insects, but still song is a reliable signal of bill morphology (Christensen et al. 2006). Relative to their body mass, tidal marsh sparrows have longer and thinner bills than their non-tidal marsh counterparts, which is likely an adaptation for consuming more invertebrates and fewer seeds, as well as for probing in sediment crevices to capture prey (Grenier & Greenberg 2005). Variation in bill morphology has subsequently been shown to influence divergence in song between swamp sparrow subspecies (Ballentine 2006; Liu et al. 2008).

Adaptation to the local acoustic environment potentially also plays an important role in ecological speciation, through its impact on species recognition and mate choice. For instance, the green hylia (*Hylia prasina*) sings at lower frequencies at higher elevations and under reduced canopy cover, but also to avoid masking by lower frequency insect sounds (Kirschel et al. 2009). Also, a study comparing the songs of European and North American Mediterranean climate passerine communities in open and closed habitats, found that frequency varied across different habitats but not continents, indicating community convergence due to acoustic adaptation (Cardoso & Price 2010).

Random cultural evolution and/or drift are also well known causes of song divergence. Cultural evolution can be defined as the change in the frequency of cultural traits through differential transmission from one generation to the next. The evolution of cultural traits is driven by processes analogous to those involved in biological (or genetic) evolution. The population memetic approach to birdsong aims to explain cultural evolution quantitatively, by identifying cultural analogues of the evolutionary forces responsible for changes in gene frequency (mutation, migration, drift and selection) and applying population genetics mathematical theory (Lynch 1996). For instance, between 1980 and 2011, changes within different segments of the songs of male Savannah sparrows *Passerculus sandwichiensis* were examined in an island population and both trill duration and the number of clicks increased in variance, suggesting directional cultural selection (Williams et al. 2013). Another example is the northward expansion of the Light-vented Bulbul *Pycnonotus sinensis* into north China since the 1980s, showing that song evolution and vocal trait shifts can arise rapidly and that 'founder effects', geographical isolation, and recent rapid expansions can play an important roles in the evolution of song dialects (Xing et al. 2013).

Perception divergence

Most studies on the perception of sexual signals across populations have found that individuals discriminate against nonlocal stimuli (e.g. Uy et al. 2009; Brumm et al. 2010; Parker et al. 2018), but there are a few exceptions (e.g. Baker 1982; Balaban 1988). In species with distinct singing styles, song discrimination by males has been shown to occur even for the singing style directed mostly to females (e.g. Regelski & Moldenhauer 1996; Matessi et al. 2000b, 2001b). In a few bird species, it has been shown that song is a reliable signal of bill morphology and that individuals display stronger response to songs of males with similar bill size (Christensen et al. 2010). In the latter case, positive assortative pairing based on bill size has been found (Christensen & Kleindorfer 2007).

Most studies have focused on single populations, but when the levels of discrimination between own and foreign songs are compared between populations, one of 4 main patterns of geographical variation will emerge (Colbeck et al. 2010): 1) symmetric discrimination, in which nonlocal signals elicit a uniform response across populations; 2) asymmetric self-assessment, in which individuals in some populations respond more strongly to all stimuli than individuals in other populations; 3) asymmetric opponent assessment, in which individuals from some populations are perceived to be of higher quality and are responded to more strongly (or weakly) across populations; and 4) asymmetric nonlocal recognition, in which the difference in how local and nonlocal signals are perceived (and responded to) is reduced in some populations due to recognition errors. Self-assessment and opponent assessment are the main causes of different responsiveness within a dialect (assessments based on the quality of the respondent and of the quality of the challenger, respectively), but across dialects responsiveness varies with assessment strategy and decreases with increasing dissimilarity to the local signal (Colbeck et al. 2010).

Three main proximate causes of asymmetric responses have been described (Dingle et al. 2010): 1) relaxation of female choice, 2) intrasexual interactions, and 3) skewed perceptual sensitivity. Kaneshiro hypothesized that asymmetric responses were due to relaxation of female choice in derived populations, driven by a full or partial loss of a male's sexual signal during a founding event (Kaneshiro 1976; Kaneshiro and Boake 1987). However, this explanation, which is based on intersexual interactions, has gained little empirical or theoretical support (Arnold et al. 1996).

A second possibility is that asymmetric responses to heterospecific signals are a consequence of intrasexual interactions and due to asymmetric competitive ability or aggressiveness between two taxa. Such differences in aggressiveness are thought to explain the asymmetric response to heterospecific playback in Townsend's warblers

(*Setophaga townsendi*) and hermit warblers (*S. occidentalis*, Pearson & Rohwer 2000). Townsend’s warblers respond strongly to mounts of both species, whereas hermit warblers respond more strongly to conspecific mounts. Townsend’s warblers also respond more strongly to hermit warbler mounts than do hermit warblers themselves, suggesting that Townsend’s warblers are more aggressive overall. Pearson (2000) argues that this asymmetry in aggression results in Townsend’s warbler males more easily establishing territories and attracting mates, thereby outcompeting and replacing hermit warblers across a moving hybrid zone.

A third, mechanistic, explanation for the asymmetric response pattern is that the subspecies have a skewed perceptual sensitivity due to distinct overlap differences with respect to the frequency ranges used by each subspecies. This seems to explain the asymmetric response to playback in gray-breasted wood-wren (*Henicorhina leucophrys*) subspecies, *H. l. hilaris* and *H.l. leucophrys* (Dingle et al. 2010). *Leucophrys* songs overlap almost completely in frequency range with hilaris songs, although *hilaris* songs only cover part of the frequency range of *leucophrys* and do not contain the particularly high-frequency end of the distinctive wide bandwidth notes typical of leucophrys songs. It is well known that spectral features can play a critical role in triggering behavioral responses (Nelson 1988; Slabbekoorn and ten Cate 1998) and in gray-breasted wood-wrens the amount of frequency overlap between the songs used for playback and the songs of the local population correlated well with response strength.

These behavioral mechanisms, together with the ultimate mechanisms described above, are all probably important to speciation.

1.1.1.4. Interactions between the three sources

The combination of natural and sexual selection can promote rapid evolution of local adaptation and reproductive isolation, even in the presence of gene flow (van Doorn et al. 2009; Wilkins et al. 2013), although in birds the importance of this process is debated (Price 2008). Sexual preferences for traits such as birdsong may evolve simultaneously with divergent ecological selection and cause assortative mating between ecotypes, further strengthening divergence (Nosil 2012). In some circumstances, birdsong can be genetically associated (by pleiotropy) with morphological (“magic”) traits such as bill size that diverged due to natural selection, potentially leading to assortative mating, reproductive isolation, and speciation (Podos 2001; Servedio et al. 2011). Also, there are a few studies where song traits and bill traits have been shown to be correlated. In Darwin's finches of the Galápagos Islands, birds with large beaks and body sizes have evolved songs with comparatively low rates of syllable repetition and narrow frequency bandwidths, whereas the converse is true for small birds (Podos 2001; Christensen et al. 2006; Huber & Podos 2006). However, also in Darwin's finches, there is evidence that thick-billed birds can learn and use the songs of thin-billed birds, sometimes leading to hybridization (Grant & Grant 1997).

1.1.2 Forms of reproductive isolation

According to Nosil (2012), in ecological speciation there are seven possible forms of reproductive isolation to consider, some of which are common in birds and may apply to this study system. These forms of reproductive isolation are part of the isolating barriers listed in Box 1, the barriers specific to ecological speciation and connected to particular ecological traits.

(1) **Habitat and temporal isolation.** The former occurs when populations exhibit genetically based preferences for separate habitats, reducing the likelihood of interbreeding. The latter occurs when populations exhibit divergent developmental schedules, such that mating occurs at different times in different populations. In allopatric and parapatric taxa/populations, the roles of habitat divergence and phenology divergence on reproductive isolation are hard to determine. Simulating sympatry through common garden experiences is one way to deal with this (more details above, under 1.1.1.).

(2) **Immigrant inviability** occurs when immigrants into a population suffer reduced survival because they are poorly adapted to the foreign habitat (e.g., Hansson et al. 2004). Gene flow between populations can be reduced by the lower rate of heterospecific mating encounters.

(3) **Divergent mating and pollinator preferences.** The former occurs when individuals from different populations, adapted to different environments, are less attracted to, or do not recognize, one another as potential mates. In the latter, populations in different environments can be exposed to selection to adapt to different pollinators, or habitat-specific selection might incidentally act on traits that affect pollinator preferences. As we have seen above, prezygotic isolation due to divergent mating preferences can arise because of deficient recognition. Recognition can be measured by the response toward the individuals of one group (as compared with individuals of another group), with species and subspecies recognition being an extension or form of mate recognition (Ryan & Rand 1993; Mendelson & Shaw 2012

2013; but see Padian & Horner 2013). In at least some bird species, the recognition of suitable mates depends to a large extent on learning (Hultsch & Todt 2004; Verzijden et al. 2012; Grant & Grant 2018) in which, among other processes, juveniles may imprint on species-specific traits during a sensitive period, leading to a sexual preference for members of their own species when reaching adulthood (Balakrishnan et al. 2009). In birds and other animals, the identification of suitable mates is based on traits such as morphology and song (Searcy 1992; Collins 2004; Grant & Grant 2009), as shown by cross-fostering experiments that result in complete assortative mating (Clayton 1990; Slagsvold et al. 2002).

Speciation by sexual selection is thought to proceed most commonly via the divergent coevolution of male sexual signals and female preferences, leading to reproductive isolation between populations (Safran et al. 2013). Male territorial behavior responses are often correlated with female preferences (e.g. Searcy et al. 1997). Therefore, in many cases, song playback experiments with territorial males allow inferring the level of pre-mating reproductive isolation among populations/taxa. However, mating signals sometimes involve a suite of display traits of different sensory modalities, like vision, audition or olfaction. In these situations, it is important to test the consequences of multimodal signal divergence. That can be accomplished by using additional test methods, like taxidermic mount presentation (Uy et al. 2009) or odour treatment trials (Mihailova et al. 2014).

(4) **Post-mating, pre-zygotic incompatibility**: it exists when there is a reduction in the fertilization success of between-population matings, or a reduction in female fitness following between-population copulation.

(5) **Intrinsic hybrid incompatibilities**: refers to intrinsic genetic incompatibilities between loci of ecologically divergent populations that arises because different alleles are favoured by divergent selection in each environment. Alleles within each environment are selected to work well together, whereas alleles from different environments are not.

(6) **Ecologically dependent selection against hybrids** arises when hybrid fitness is reduced because of an ecological mismatch between hybrid phenotype and the environment.

(7) **Sexual selection against hybrids** occurs if hybrids, despite surviving to sexual maturity, are less likely to secure a mate (at least partly) for ecological reasons (e.g., Hansson et al. 2004).

1.2 Reed bunting: the study system

1.2.1 Geographic distribution

The reed bunting (*Emberiza schoeniclus*) is a Palearctic species, widespread over much of Europe, and extending east across northern Asia to Lena river (with isolated population in Kamchatka), Sakhalin island and northern Japan, and south to Iran, Tien Shan mountains and northern China (Byers et al. 1995; Cramp & Perrins 1994).

This study focused on three subspecies of reed bunting breeding in northern and western Europe: *E. s. schoeniclus* (hereafter *schoeniclus*), widespread breeder from France and the U.K. north and eastwards to beyond the Western Palearctic, wintering in the Mediterranean area; *E. s. lusitanica* (hereafter *lusitanica*), resident and endemic to northwestern Iberia; and *E. s. witherbyi* (hereafter *witherbyi*), resident from eastern Iberia, Balearics, southern France and, at least formerly, North Africa and Sardinia (Atienza 2006; Cramp & Perrins 1994; Figure S 2.1). Historically, there were probably contact zones between *lusitanica* and *witherbyi* in the upper Ebro river valley, and between *lusitanica* and *schoeniclus* in the coastal western Pyrenees, but in the late 1990s, *lusitanica* disappeared from these areas (Atienza 2006). Currently, there is also no known contact zone between subspecies *witherbyi* [*stricto sensu*, *i.e.* excluding *E. s. intermedia* (hereafter *intermedia*)] and subspecies *schoeniclus* (Issa and Muller 2015).

1.2.2 Phenotypic variation

1.2.2.1. Morphologic variation

The reed bunting is a small (12-22g) passerine of the large Emberizidae family, with clear sexual dimorphism in both plumage and size (males are much brighter and slightly larger). It is one of the most variable species within that family, having numerous subspecies described on the basis of bill size, body size and plumage colour (Vaurie 1956, 1958; Cramp & Perrins 1994; Byers et al. 1995).

The variation in phenotype is complex and to a large extent clinal (Cramp & Perrins 1994; Byers et al. 1995). Birds with a thick bill occur in the southern part of the distribution, where the thickness of the bill (as well as body size) increases towards the east, whereas thin-billed birds occur further north. In addition, western individuals are the darkest in plumage, becoming increasingly light in colour towards the east (Vaurie 1956; Cramp & Perrins 1994; Byers et al. 1995). Southern populations are resident, but further north partial, short- and medium-distance migration occurs in various directions, with thin-billed subspecies often co-occurring with thick-billed birds during the winter (Prŷs-Jones 1984).

Individual variation and the existence of intermediate populations led to some instability in reed bunting's taxonomy, with the number of recognized subspecies varying from 15 to over 20 (Vaurie 1956; Cramp & Perrins 1994; Byers et al. 1995; Clements et al. 2018; del Hoyo et al. 2011). One of the subspecies for which little data exist and has not been recognized by most authors before Byers et al. (1995) is *lusitanica* (first described by Steinbacher 1930), which resides in the northwest part of the Iberian Peninsula (see Figure S 2.1; Atienza 2006). It was lumped with *witherbyi* pending further study by Vaurie (1956) and by Cramp & Perrins (1994), though they admitted that it should probably belong to the thin-billed group, close to *schoeniclus*, as was later described by Byers et al. (1995). Previous studies addressing phenotypic divergence amongst reed bunting subspecies generally analysed very few individuals of each population and no statistical comparisons were made (but see Copete et al. 1999; Belda et al. 2009).

1.2.2.2. Acoustic variation

Signal

The reed bunting has three singing styles, two of which are used for female attraction: fast song, the only style used by unpaired males, has been related to the attraction of a social mate (Ewin 1976; Nemeth 1996), and dawn song, which is sung by paired males and is associated with obtaining extrapair paternity (Suter et al. 2009). In contrast, slow songs are used by paired males apparently as an all-clear signal to their nest-attending females (Wingelmaier et al. 2007) and do not influence extrapair paternity or the number of fertilizations (Bouwman et al. 2007). In addition, all three singing styles are probably important in the context of territory defense (Brunner & Pasinelli 2010; Ghiot 1976), which is relevant because speciation is facilitated by intrasexual male competition (Tinghitella et al. 2018). Individual males have repertoires of 10-30 different syllables that are used to build the songs of the three singing styles (Cramp & Perrins 1994; Ewin 1978; Suter et al. 2009). Dawn and fast singing are stereotyped styles because of the stricter rules of syllable and song arrangement (Brunner & Pasinelli 2010; Suter et al. 2009). Concomitantly, compared to the slow singing style, the fast style has greater syllable sequence predictability and greater syllable repetition (Ewin 1978). Song sharing, matched counter-singing and local dialects have been recorded at the syllable level, but not at the song level (Ehrengruber et al. 2006; Ewin 1976, 1978). Previous work has shown that the fast songs of non migratory, thick-billed central and western Mediterranean birds (*intermedia* and *witherbyi*) differ from those of the largely migratory, thin-billed central European *schoeniclus* (Matessi et al. 2000a, 2001a). However, the divergence in other singing styles and differential divergence across styles have never been studied. For *lusitanica* there was no previous quantitative study of song (for qualitative notes see Martínez & Romay 2012).

Perception

Song discrimination between different subspecies is slight (Matessi et al. 2000b, 2001b). However, there seems to be no interbreeding between thick-billed and thin-billed subspecies in contact zones (Graputo et al 1998). The differences in fast song seem to be recognized by the birds, as males react differently to the playback of their own and foreign fast songs (Matessi et al. 2000b, 2001b).

Previous studies of song recognition in reed buntings focused only on one subspecies, either *schoeniclus* (Ewin 1978; Matessi et al. 2000b) or *intermedia* (including *witherbyi*, Matessi et al. 2001b). Hence, song recognition studies between these subspecies have so far not been conducted. Thus, there is still little information about the patterns of song discrimination across populations and subspecies.

1.2.3 Ecological variation

The reed bunting breeds mostly in marshland, thriving on stands of common reed (*Phragmites australis*), great fen-sedge (*Cladium mariscus*), bulrush (*Typha latifolia*) etc (Gordinho 2012), though some populations also nest in agricultural land (Ewin 1978). Outside the breeding season, birds from more populations forage on cultivated fields (Orłowski & Czarnecka 2007). It is well known that during spring/summer reed buntings feed on a large variety of insects and spiders (Cramp & Perrins 1994; Holland et al. 2006). However, previous work indicates that, during winter, some thick-billed subspecies (*intermedia* and *E. s. pyrrhuloides*) feed on insect larvae and pupae that are dormant inside the reed (*Phragmites australis*) stems (Shtegman 1948 cited by Prŷs-Jones 1984; Matessi et al. 2002), whereas thin-billed subspecies eat mostly seeds and often occur in other habitats such as farmland (Cramp & Perrins 1994; Holland et al. 2006; Orłowski & Czarnecka 2007), but can eat insects in some areas opportunistically (Orłowski et al. 2013). The diets of *witherbyi* and of the intermediate-size *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 *lusitanica* and *schoeniclus* make predictions difficult (Chapter 2).

1.2.4 Genetic variation

Genetic studies have shown that the neutral genetic divergence between the Italian subspecies *intermedia* (thick-billed) and the central-European *schoeniclus* (thin-billed) is slight but significant (Graputo et al. 1998). This was confirmed by an analysis of mitochondrial DNA (ND2 gene) describing three partially overlapping closely-related lineages in Asia (Zink et al. 2008), and by our own analysis of mtDNA (control region) and microsatellites of Iberian and central European subspecies (Kvist et al. 2011).

1.2.5 Why study speciation in reed buntings?

This species seems to be at an early stage of speciation, with populations/subspecies still retaining ancient polymorphisms (see 2.3. above), but showing significant genetic, morphological and behavioural divergence. Bill and body size are interesting, as these traits are likely to influence song characteristics involved in mate choice (Podos 2001; Chapter 3), potentially acting as magic traits of (ecological) speciation (Servedio et al. 2011). It is important to study organisms at this stage of evolution, when the actual ecological and genetic mechanisms of speciation can be witnessed.

Studying local adaptation in the west European populations of reed bunting is useful because *schoeniclus* includes both resident and migratory populations, and Iberia is inhabited by two resident populations/subspecies that differ markedly in size and bill characters thereby allowing to separate the effects of migration and foraging. With its intermediate characteristics, *lusitanica* is exciting because it allows us to evaluate the level of reproductive isolation between the two bill-size groups.

This study system is promising 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.

1.3 Objectives

This thesis aims to describe the causes and consequences of phenotypic divergence in a continental bird, for which the following eight specific objectives were addressed:

1. To characterize morphological divergence comparing with predictions derived from the migratory behaviour and diet/feeding technique, thereby inferring adaptive divergence [Chapter 2];
2. To compare the foraging niche between subspecies and its relation to bill morphology [Chapter 5];
3. To compare quantitative properties of songs from each of the three singing styles between three subspecies [Chapter 3];
4. To quantify the extent to which males can be assigned to the correct subspecies on the basis of song properties in each singing style [Chapter 3];
5. To evaluate whether the geographical pattern of song differentiation conforms to the subspecies distributions [Chapter 3];
6. To test how territorial males react to songs of their own subspecies versus to songs of two distinct foreign subspecies (i.e., investigate to what extent there is discrimination within and between subspecies), i.e., signs of incipient pre-mating isolation [Chapter 4];
7. To determine whether discrimination is symmetric or asymmetric across the three subspecies (inferring the possible mechanisms involved from the pattern of asymmetry) [Chapter 4];
8. To assess whether the variation in responses across subspecies (i.e., proxies for premating reproductive isolation) is best explained by morphological/ecological factors (e.g., beak size/food niche) or by genetic distance between the three subspecies [Chapter 4];

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Chapter 2

Phenotypic divergence among west European populations of reed bunting

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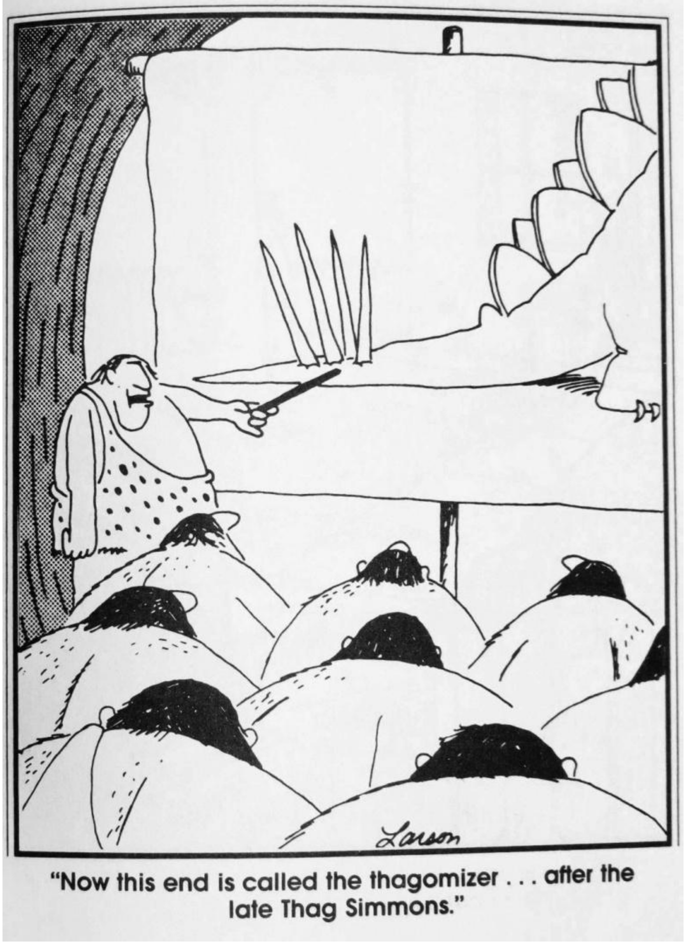

Cartoon 3 - Mankind interest in morphologic detail is ancient (from Larson 1984).

2 Phenotypic divergence among west European populations of reed bunting *Emberiza schoeniclus*: the effects of migratory and foraging behaviours

2.1 Abstract

Divergent selection and local adaptation are responsible for many phenotypic differences between populations, potentially leading to speciation through the evolution of reproductive barriers. Here we evaluated the morphometric divergence among west European populations of reed bunting in order to determine the extent of local adaptation relative to two important selection pressures often associated with speciation in birds: migration and diet. We show that, as expected by theory, migratory *E. s. schoeniclus* had longer and more pointed wings and a slightly smaller body mass than the resident subspecies, with the exception of *E. s. lusitanica*, which despite having rounder wings was the smallest of all subspecies. Tail length, however, did not vary according to the expectation (shorter tails in migrants) probably because it is strongly correlated with wing length and might take longer to evolve. *E. s. witherbyi*, which feed on insects hiding inside reed stems during the winter, had a very thick, stubby bill. In contrast, northern populations, which feed on seeds, had thinner bills.

Despite being much smaller, the southern *E. s. lusitanica* had a significantly thicker, longer bill than migratory *E. s. schoeniclus*, whereas birds from the UK population had significantly shorter, thinner bills. Geometric morphometric analyses revealed that the southern subspecies have a more convex culmen than *E. s. schoeniclus*, and *E. s. lusitanica* differs from the nominate subspecies in bill shape to a greater extent than in linear bill measurements, especially in males. Birds with a more convex culmen are thought to exert a greater strength at the bill tip, which is in agreement with their feeding technique. Overall, the three subspecies occurring in Western Europe differ in a variety of traits following the patterns predicted from their migratory and foraging behaviours, strongly suggesting that these birds have became locally adapted through natural selection.

2.2 Introduction

Divergent selection and local adaptation are responsible for many phenotypic differences found across populations, and may lead to the evolution of reproductive barriers and speciation (Van Dorn et al. 2009; Winker 2010). Local adaptations are expected to constrain gene flow among populations, as hybrids would be maladapted relative to their parents (Van Dorn et al. 2009). In addition, the speciation process is greatly facilitated, even in the presence of gene flow, when the traits subject to divergent selection are also involved in mate choice (often called ‘magic traits’; Servedio et al. 2011). In order to understand the speciation process, it is important to determine how ecology and genetics interact to cause the evolution of the first reproductive barriers, before they are confounded by further barriers and differences evolving subsequently among populations/species (Via 2009). The characterization of diverging phenotypes and the identification of relevant evolutionary forces acting on those phenotypes are crucial first steps to study the causes of speciation (Shaw & Mullen 2011).

In birds, two of the most significant selection pressures associated with the evolution of reproductive barriers are migratory and foraging behaviours. For instance, reproductive isolation seems to be evolving as a consequence of a new migratory direction in Blackcaps (*Sylvia atricapilla*; Bearhop et al. 2005), and migratory behaviour has been suggested to be an important factor promoting speciation (Ruegg & Smith 2002; Pérez-Tris et al. 2003; Irwin & Irwin 2005; Bensch et al. 2009; Rohwer & Irwin 2011). On the other hand, foraging ecology has been associated with divergent selection and speciation, particularly in seed-eating species such as Darwin’s finches, *Nesospiza* buntings and crossbills (Grant & Grant 2002; Ryan et al. 2007; Benkman 2003). Other organisms have also evolved in foraging behaviour leading to speciation, such as the benthic and limnetic threespine sticklebacks (Kozak et al. 2011); and niche divergence has been shown to promote reproductive isolation in a large variety of taxa (Funk et al. 2006).

The reed bunting (*Emberiza schoeniclus*) is the most variable species within the large Emberizidae family, having numerous subspecies described on the basis of bill size, body size and plumage colour (Vaurie 1956, 1958; Cramp & Perrins 1994; Byers et al. 1995). The variation in phenotype is complex and to a large extent clinal (Cramp & Perrins 1994; Byers et al. 1995). Birds with a thick bill occur in the southern part of the distribution, where the thickness of the bill (as well as body size) increases towards the east, whereas thin-billed birds occur further north. In addition, western individuals are the darkest in plumage, becoming increasingly light in colour towards the east

(Vaurie 1956; Cramp & Perrins 1994; Byers et al. 1995). Southern populations are resident, but further north partial, short- and medium-distance migration occurs in various directions, with thin-billed subspecies often co-occurring with thick-billed birds during the winter (Prŷs-Jones 1984). During spring and summer, reed buntings feed mostly on insects, but during the winter, thick-billed birds seem to feed on insects lying dormant inside the reed (*Phragmites australis*) stems; whereas the thin-billed birds feed almost exclusively on small seeds (Shtegman 1948 cited by Prŷs-Jones 1984; personal observations; Matessi et al. 2002; Orłowski & Czarnecka 2007; Orłowski et al. 2013).

Individual variation and the existence of intermediate populations led to some instability in reed bunting's taxonomy, with the number of recognized subspecies varying from 15 to over 20 (Vaurie 1956, 1958; Cramp & Perrins 1994; Byers et al. 1995; Clements et al. 2018; del Hoyo et al. 2011). One of the subspecies for which little data exist and has not been recognized by most authors before Byers et al. (1995) is *E. s. lusitanica* (hereafter *lusitanica*; first described by Steinbacher 1930), which resides in the northwest part of the Iberian Peninsula (see Figure S 2.1, Atienza 2006). It was lumped with *E. s. witherbyi* (hereafter *witherbyi*) pending further study by Vaurie (1956) and by Cramp & Perrins (1994), though they admitted that it should probably belong to the thin-billed group, close to *E. s. schoeniclus* (hereafter *schoeniclus*), as was later described by Byers et al. (1995).

Previous studies addressing phenotypic divergence amongst reed bunting subspecies generally analysed very few individuals of each population and no statistical comparisons were made (but see Copete et al. 1999; Belda et al. 2009). Genetic studies, however, have shown that the neutral genetic divergence between the Italian subspecies *E. s. intermedia* (thick-billed) and the central-European *schoeniclus* (thin-billed) is slight but significant (Graputo et al. 1998). This was confirmed by the analysis of mitochondrial DNA (ND2 gene) describing three partially overlapping closely-related lineages in Asia (Zink et al. 2008), and by our own analysis of mtDNA (control region) and microsatellites of Iberian and central European subspecies (Kvist et al. 2011). Song discrimination between different subspecies is also slight (Matessi et al. 2000), but the bill size differences between *E. s. intermedia* and *schoeniclus* are correlated with diet suggesting local adaptation (Matessi et al. 2002). Furthermore, there seems to be no interbreeding between thick-billed and thin-billed subspecies in contact zones (Graputo et al. 1998). Therefore, this species seems to be at an early stage of speciation, with populations/subspecies still retaining ancient polymorphisms, but showing significant genetic, morphological and behavioural divergence. Bill and body size are especially interesting, as these traits are likely to influence song characteristics involved in mate choice (Podos 2001; Chapter 3), potentially acting as magic traits of (ecological) speciation (Servedio et al. 2011). It is particularly interesting to study organisms at this stage of evolution, when the actual ecological and genetic mechanisms of speciation can be witnessed.

In this study, we evaluated the morphometric divergence among west European populations including two resident southern subspecies from the Iberian Peninsula, *witherbyi* and *lusitanica*, as well as migratory and resident populations of *schoeniclus*. Our purpose was to determine the extent of local adaptation, evaluating the effects of migratory behaviour and diet/feeding technique, and to describe for the first time the morphometrics of *lusitanica*. In particular, we tested the expectations that migratory birds should have longer and more pointed wings, shorter tails and lower body mass than residents (Hedenström 2008; Milá et al. 2008). In addition, we evaluated to which extent *lusitanica* differed from *witherbyi* and *schoeniclus* in terms of bill size and shape. As a southern resident subspecies, *lusitanica* is expected to feed on insects lying inside reed stems during winter (Chapter 5), thus being close to *witherbyi* in foraging-related traits, even though recent authors include it in the small-billed group (Byers et al. 1995). Morphological characters such as the ones analysed here are generally highly heritable (Keller et al. 2001; Tarka et al. 2010) and, given that the genetic divergence is very small (Kvist et al. 2011), the morphological differences among populations are likely to be meaningful (adaptive), especially if the predictions are confirmed, showing that the individuals ‘‘fit’’ their environments. It is especially useful to study local adaptation in the west European populations of reed bunting because *schoeniclus* includes both resident and migratory populations, and Iberia is inhabited by two resident populations/subspecies that differ markedly in size and bill characters thereby allowing to separate the effects of migration and foraging. With its intermediate characteristics, *lusitanica* is of considerable interest because it allows us to evaluate the level of reproductive isolation between the two bill-size groups.

2.3 Materials and Methods

Fieldwork

Biometric data of reed buntings were obtained from several populations (Figure S 2.1): (1) the resident *lusitanica* was measured at Salreu marshlands, Portugal, from 2008 to 2011 (n =201); (2) the resident *witherbyi*, measured at several sites in Spain from 2002 to 2012 (n= 76); (3) wintering *schoeniclus* measured at Salreu marshlands from 2008 to 2011 (n= 94); (4) the resident *schoeniclus* from the United Kingdom, sampled in the Liverpool and Oxford regions in autumn 2011 (n= 47); and (5) Scandinavian migrants (*schoeniclus*) sampled at lake Krankesjön, Skåne, Sweden, just prior to autumn migration in 2011 (n= 22). The two subspecies that occur in Salreu were distinguished on the basis of date and plumage traits, with *lusitanica* being obviously darker in the head, upper parts and flanks, and having also darker and more intensely coloured wing coverts than the wintering *schoeniclus* (Figure 2.1, see also Byers et al. 1995; del Hoyo et al. 2011). Judging from the many local and foreign retraps, the experience gathered during the last few years allowed us to classify each bird to subspecies with 100% certainty, although there are no quantitative data on plumage traits. Spanish birds of the subspecies *witherbyi* were distinguished from the wintering *shoeniclus* on the basis of date, plumage and genetics (Belda et al. 2009).

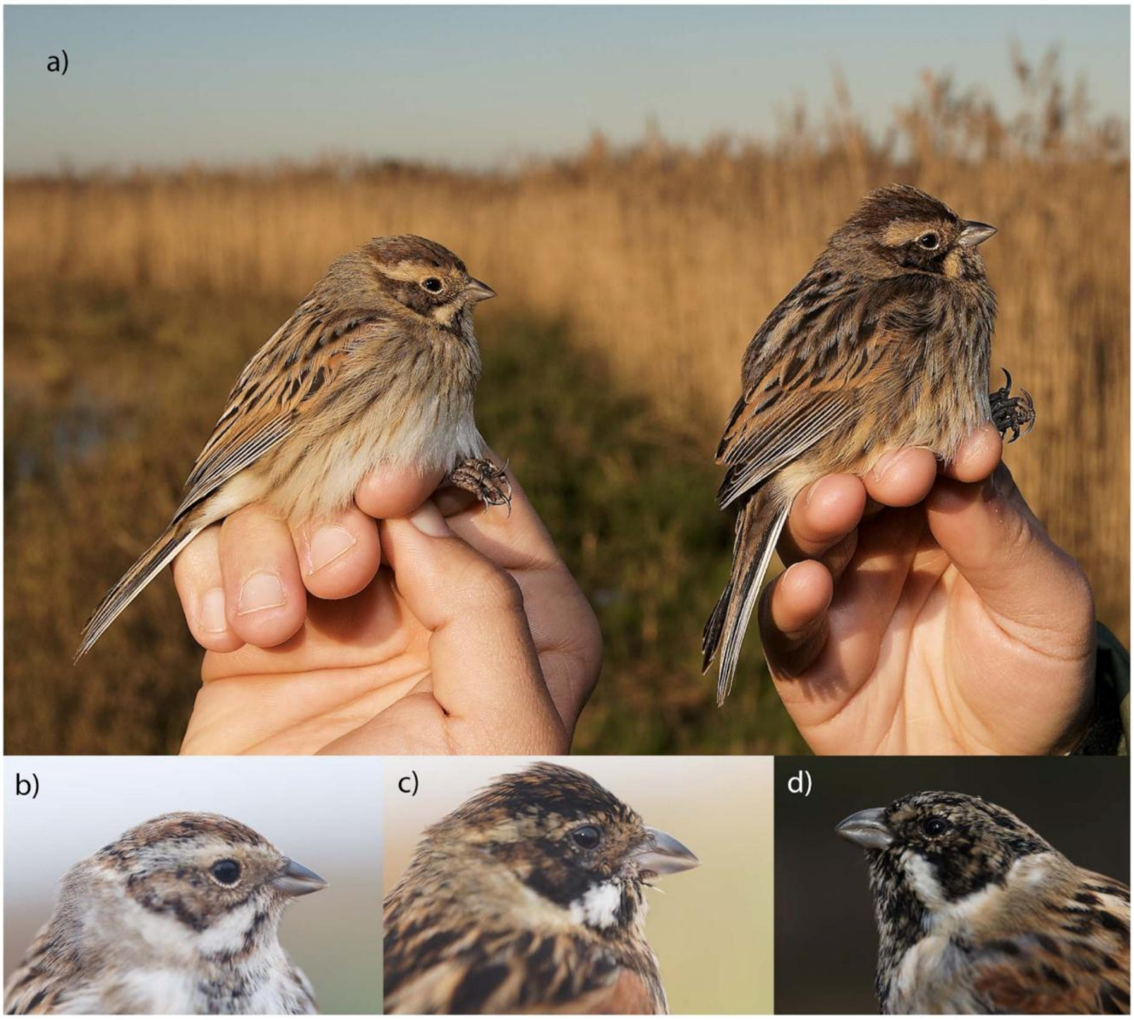

Figure 2.1 - Examples depicting plumage and bill shape differences among reed bunting subspecies. a) first-year females *E. s. schoeniclus* (left) and *E. s. lusitanica* (right); b) first-year male *E. s. schoeniclus*; c) first-year male *E. s. lusitanica* and d) first-year male *E. s. witherbyi*, captured at Salreu, Estarreja, Portugal, except the latter, which was captured at Lagunas de Villafranca, Toledo, Spain. All pictures were taken by JMN.

Birds were captured with mist-nets, marked with a metal ring issued by the ringing centre of the country where ringing took place, and the age and sex were determined using published criteria (Svensson 1992; de la Puente & Seoane 2001). The wing (maximum chord) and tail lengths were measured with a ruler to the nearest 0.5 mm, tarsus and bill (to skull) lengths, bill depth and bill width (at the nostrils) were measured with callipers to the nearest 0.1 mm, weight was measured either with a Pesola spring balance or a digital balance to the nearest 0.1 g and the subcutaneous fat reserves were recorded following Kaiser (1993). In addition, the length of each primary was measured as described by Jenni & Winkler (1989) in birds with fresh feathers in autumn and winter. The sample size for each individual measurement is variable, as it was not possible to measure all traits in all birds.

The Portuguese (*lusitanica* and wintering *schoeniclus*) and Swedish reed buntings were measured by JMN, whereas Spanish birds were measured by JMN, MM, JSM, EJB and others, and the birds from the U.K. were measured by PF and RC. Differences in measuring technique between the ringers (especially wing and bill lengths, which are more difficult to measure) could potentially be a problem for population comparisons because they will result in significant differences given enough sample size. However, JMN and PF have been ringing together for many years and their measurements were calibrated and are comparable (the same was done at a later stage between JMN, MM and JSM). In addition, although preliminary analysis showed that many statistical comparisons between *schoeniclus* wintering in Portugal (measured by JMN) and in Spain (by several Spanish ringers) are significant (despite these birds probably having the same origin and biometrics), the actual differences in the means are very small relative to the differences that we found among the populations/ subspecies. For instance, the difference in wing length (when controlling for age and sex) between Portuguese and Spanish schoeniclus was only 1.37 mm ($F_{[1;744]}$=29.8; P<0.001), whereas the difference in tail length was 0.84 mm ($F_{[1;737]}$=2.76; P=0.097), tarsus 0.36 mm ($F_{[1;741]}$=20.0; P<0.001) and bill depth 0.18 mm ($F_{[1;683]}$=34.1; P<0.001; see also Gosler et al. 1998 for a general inter-observer comparisons of measurements of the same individual birds). Hence, the phenotypic divergence found between populations (see Table 2.1) is real and not caused by inter-observer differences. Furthermore, analyses restricted to birds measured by JMN produced qualitatively similar results (although the UK population was not included), and so we provide the results obtained from the full dataset.

Table 2.1 - Unstardardized parameters and t-tests of the General Linear Models evaluating the effects of age, sex and subspecies/ population on the various biometrics.

	Age	Sex	Population
Wing	**-1.454±0.211*****	**-5.652±0.210*****	**(lus) -1.545±0.370***** **(sch) 3.543±0.377***** **(UK) 2.351±0.436*****
Tail	**-0.898±0.252****	**-3.619±0.251*****	**(lus) -3.861±0.464***** (sch) -0.524±0.471ns **(UK) -2.087±0.534*****
Tarsus	0.017±0.093ns	**-0.650±0.091*****	**(lus) -0.959±0.153***** **(sch) -0.603±0.157**** (UK) 0.242±0.186ns
Bill length	-0.009±0.054ns	**-0.390±0.053*****	**(lus) 0.511±0.089***** **(sch) 0.278±0.091**** **(UK) -0.275±0.117****
Bill depth	-0.052±0.027#	**-0.254±0.027*****	**(lus) -0.911±0.045***** **(sch) -1.094±0.047***** **(UK) -1.411±0.055*****
Bill width	-0.039±0.040ns	**-0.135±0.039*****	**(lus) -1.305±0.066***** **(sch) -1.314±0.068***** **(UK) -1.728±0.086*****
Bill shape index	-0.027±0.014#	**0.035±0.014***	**(lus) 0.437±0.023***** **(sch) 0.479±0.023***** **(UK) 0.530±0.030*****
Body mass	-0.155±0.128ns	**-2.238±0.127*****	**(lus) -1.917±0.232***** **(sch) -0.625±0.239**** (UK) 0.056±0.276ns
Tail/Wing	-0.004±0.003ns	**0.015±0.003*****	**(lus) -0.033±0.005***** **(sch) -0.045±0.005***** **(UK) -0.051±0.006*****
$PC1_{WING}$	0.205±0.179ns	0.282±0.180ns	**(lus) -1.707±0.416**** **(sch) -2.126±0.396***** **(UK) -1.379±0.394*****
$PC2_{WING}$	**0.598±0.176*****	**0.466±0.176****	**(lus) -1.049±0.408**** (sch) -0.117±0.38ns (UK) 0.259±0.386ns
PC_{BILL}	0.008±0.057ns	**-0.469±0.056*****	**(lus) -1.936±0.093***** **(sch) -2.207±0.095***** **(UK) -3.047±0.132*****
PC_{SIZE}	**0.293±0.067*****	**-1.394±0.067*****	**(lus) -1.061±0.124**** (sch) 0.058±0.126ns (UK) -0.058±0.151ns
RW1	-0.004±0.005ns	**-0.026±0.005*****	**(lus) -0.112±0.013***** **(sch) -0.151±0.013***** **(UK) -0.163±0.014*****

– P = 0.059; *** – P<0.001; ** – P<0.01; * – P<0.05; ns – non-significant. Fat and muscle scores were included as covariates in the model analysing body mass. The parameters represent the difference relative to adults, males and *E. s. witherbyi*. Models with significant interactions are presented in the main text.

Geometric Morphometrics of the Bill

A photograph of the bill in profile was taken from 208 individuals of all populations/subspecies, and subjected to geometric morphometric analysis, a powerful method with few a priori assumptions to explicitly define shape (Rohlf & Marcus 1993;

Marcus et al. 1996; Klingenberg 1996; Zelditch et al. 2004). This method has recently been applied to a growing number of animal groups, including in a few bird studies that compare bill shapes (Foster et al. 2007; Navarro et al. 2009; Berns & Adams 2010). Prior to analysis, photographs were edited in Adobe Photoshop CS4 (for details see Protocol S 2.1), and then all geometric morphometric analyses were conducted in software of the tps series (Rohlf 2010). A tps file was built from images using tpsUtil (Rohlf 2010a; Kaliontzopoulou et al. 2007) and used in tpsDig (Rohlf 2010b), where seven landmarks and eight semilandmarks were digitized following Foster et al. (2007). The semilandmarks were placed by reference to a standardized grid superimposed onto each image (cf. Figure S 2.2 and Protocol S 2.2). Files containing links (between landmarks) and sliders (for each semi-landmark) were built in tpsUtil and an image list was obtained. Using the tpsSmall software (Rohlf 2003), we confirmed that shape variation between the specimens was sufficiently small and therefore the distribution of points in the shape space can be represented satisfactorily by their distribution in the tangent space. We then applied a Generalized orthogonal least-squares Procrustes Analysis (GPA; Rohlf & Slice 1990; Rohlf 1999) using tpsRelw (Rohlf 2010c), in order to standardize the size and to translate and rotate the configurations of landmark coordinates, therefore obtaining a consensus configuration. We computed partial and relative warps and extracted relative warp scores with a $\alpha=0$, using the tpsRelw software (Rohlf 2010c). tpsRelw output files were saved in NTS format, converted to csv using tpsUtil, and merged with the image list in Microsoft Excel. Because of logistical constraints that prevented inclusion of a standardized scale in each image, allometry was evaluated by reference to a Principal Component based on univariate measurements (Foster et al. 2007; see below).

Statistical Analysis

As the variables were approximately normally distributed and there were no obvious deviations from model assumptions judging from the variance comparisons, covariance structure and residuals, General Linear Models (GLM) were used to determine and evaluate the effects of age, sex and population/subspecies on each trait. Two-way interactions were also tested and kept in the final model if significant. The basic biometrics (wing, P8, tail, tarsus, bill length, bill depth and bill width) were included in stepwise (forward) discriminant analyses (using default parameters, i.e., a variable was entered in the model if it improved significantly the significance of Wald’s test, having an $F>3.84$, and dropped if $F<2.71$) in order to determine to which extent birds of different subspecies and populations were correctly classified and by which variables.

The size of the feeding apparatus (bill length, depth and width) was reduced to one variable using principal component analysis (PC_{BIIL}, Table S 2.1), which represents overall bill size and explains 60.1% of the variance. A bill shape index was calculated by dividing bill length by bill depth. Tail to wing ratio was also calculated for each bird by dividing these variables. The primary lengths were first corrected for body size isometrically following Lleonart et al. (2000) and using a standard wing length of 78 mm. Subsequently, adjusted primary lengths were reduced to two variables (representing wing shape) using principal component analysis ($PC1_{WING}$ and $PC2_{WING}$), which explained 46.6% and 21.0% of the variance, respectively (Table S 2.2). $PC1_{WING}$ represents (the inverse of) wing convexity, as it is strongly correlated with the length of the inner primaries, but not with the outer primaries (Table S 2.2); whereas $PC2_{WING}$ reflects wing pointedness because it is strongly correlated with the longest primaries (Table S 2.2; see also Pérez-Tris et al. 2003; Copete et al. 1999). Overall body size, estimated as the first principal component of an analysis including wing, tail, tarsus and bill lengths (PC_{SIZE}, 51.7% of variance explained, Table S 2.3), was included as a covariate in some analyses in order to control for allometric differences. Whenever one of the four variables contributing to PC_{SIZE} was the dependent variable in the statistical model, body mass (and fat score) were used as covariates to control for allometry. Statistical analyses were undertaken in SPSS 20.0 [61], and results are presented as mean ± SE (n).

Ethical Treatment of Animals

The capture and ringing of birds was conducted under the licenses required by the corresponding national authorities, following standard protocols and releasing the birds unharmed on site. Permits were given by the following institutions: Daimiel National Park, Marjal Pego-Oliva Natural Park, S'Albufera de Mallorca Natural Park, Conselleria de Medi Ambient, Aigua, Urbanisme i Habitatge, Generalitat Valenciana (440066); Consejería de Medio Ambiente y Desarrollo Rural de Castilla La Mancha; Direcció General de Medi Natural, Educació Ambiental i Canvi Climàtic, Conselleria d'Agricultura, Medi Ambient i Territori, Govern de les Illes Balears (13123/2012); Consejería de Medio Ambiente de la Junta de Andalucía (6305); Ringmärkningscentralen, Naturhistoriska Riskmuseet; CEMPA, Instituto de Conservação da Natureza e Florestas (99/2011, 112/2012); British Trust for Ornithology (RC =5435, AF5394).

2.4 Results

General Morphological Differences

Swedish birds were statistically indistinguishable in all traits (GLM, all $P>0.1$) to the *schoeniclus* wintering at Salreu, Portugal, which, according to ringing controls, originate from northern France, Sweden, Germany, Poland and Czech Republic (Neto et al., in preparation). Therefore, these two populations were lumped in all subsequent analyses. Otherwise, biometrics differed markedly among the studied populations/subspecies (Table 2.1, Table S 2.4). Age significantly influenced the length of feathers (wing and tail) and consequently body size (PC_{SIZE}), with adults being larger than first-year birds. Also, with the exception of wing shape (PC_{WING}), all measurements differed between the sexes, with females being significantly smaller than males, but having higher values of bill shape index (bill length/bill depth) and tail/wing ratio. Hence, these factors had to be taken into account for population comparisons.

Stepwise discriminant analysis indicated that 100% of male (Wilk's lambda=0.142, $\chi^2_{[4]}=161.83$, $P<0.001$) and 97.9% of female (Wilk's lambda=0.192, $\chi^2_{[4]}=108.89$, $P<0.001$) *witherbyi* can be correctly distinguished from *lusitanica* (and from the other populations studied here) on the basis of bill depth, bill width, bill length and tarsus length (but note that bill depth alone was enough to correctly classify 100% of male and 98% of female *witherbyi* from *lusitanica*; see also Belda et al. 2009). Wing length, bill depth and bill width allowed the correct classification of 94.8% of male (Wilk's lambda=0.328, $\chi^2_{[3]}=109.70$, $P<0.001$) and bill and wing lengths 92.6% of female (Wilk's lambda=0.321, $\chi^2_{[2]}=142.09$, $P<0.001$) *lusitanica* and *schoeniclus* (see Figure 2.2). On the other hand, discriminant functions of the two populations of *schoeniclus* (migratory and UK residents) were able to correctly classify 88.3% of male (Wilk's lambda=0.542, $\chi^2_{[3]}=30.91$, $P<0.001$) and 71.4% of female (Wilk's lambda=0.943, $\chi^2_{[1]}=4.043$, $P=0.044$) on the basis of bill width (both sexes), bill depth and tarsus length (the latter two for males only).

Adaptations to Migration

Body mass (with fat and muscle scores as covariates) was similar between *witherbyi* and *schoeniclus* resident in the UK, but was slightly, but significantly, smaller in migrant *schoeniclus* and even smaller in *lusitanica*. Body size (PC_{SIZE}), however, was similar across populations except for *lusitanica*, which was significantly smaller than the other subspecies (Table 2.1). The discrepancy in the comparisons of body mass and body size across populations can be explained by migrant *schoeniclus* having the longest wings (Table 2.1, Table S 2.4), which was the most important factor loading for PC_{SIZE} (Table S 2.3). Although *lusitanica* appeared equally small in mass and size (PC_{SIZE}) relative to the other subspecies, it actually had the longest bill, but was smaller in all other body measurements (wing, tail, tarsus; Table 2.1).

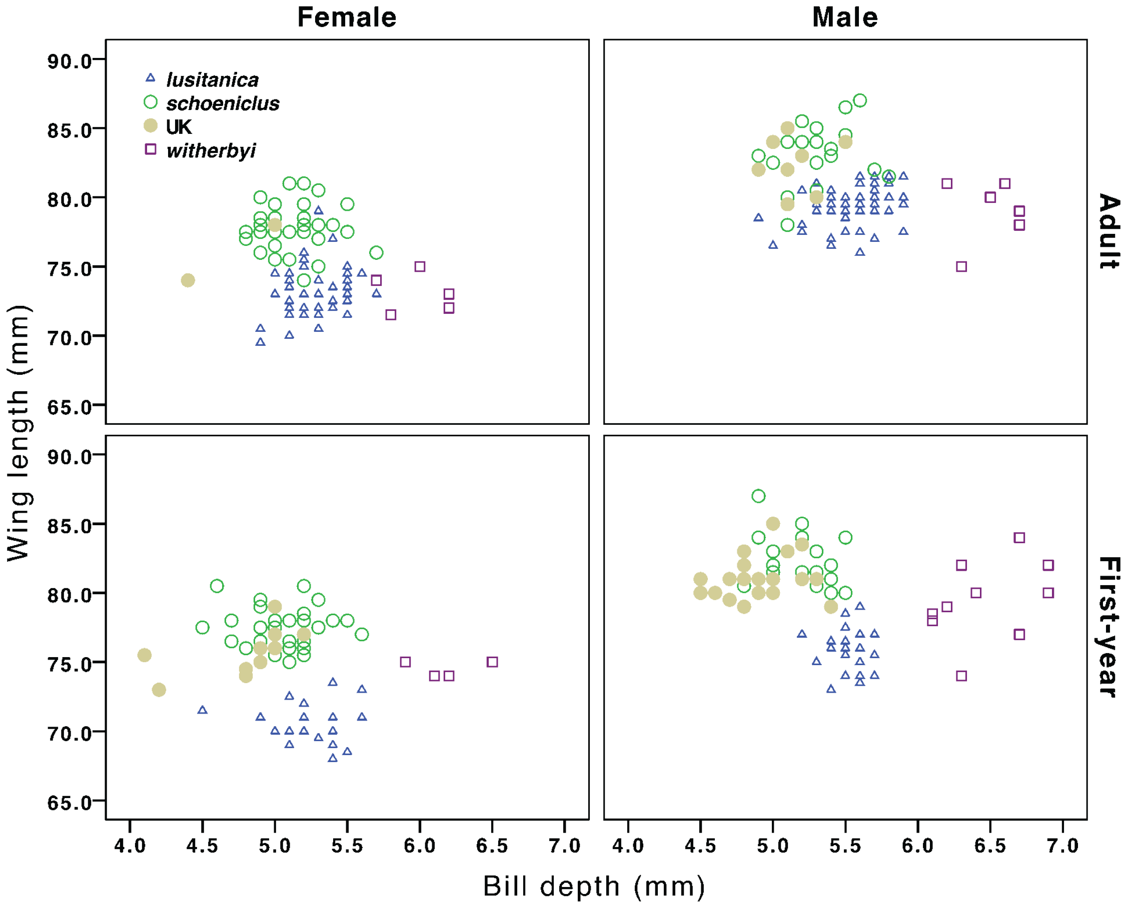

Figure 2.2 - Scatterplot of bill depth and wing length for each age, sex and subspecies/population. *E. s. schoeniclus* includes birds trapped in Portugal during winter as well as those measured in Sweden.

As predicted by theory, migratory populations of *schoeniclus* had the longest wings, followed by resident *schoeniclus* from the UK, *witherbyi* and *lusitanica*, which had almost no overlap in wing length with the other populations (Table 2.1, Figure 2.2, Table S 2.4). Wing convexity ($PC1_{WING}$) also varied significantly across populations, with migratory *schoeniclus* having the most negative values (i.e. more convex wings), followed by *lusitanica*, resident *schoeniclus* from the UK and *witherbyi* (Table 2.1; see also Copete et al. 1999). On the other hand, *lusitanica* had significantly less pointed ($PC2_{WING}$) wings than the remaining populations, which were otherwise similar (Table 2.1). Differences in wing shape are better illustrated between *lusitanica* and the migratory *schoeniclus*, as both have a large sample size and were measured by the same person (JMN), allowing for detailed comparisons between the primaries (Figure

2.3). As predicted by theory, migratory birds had significantly longer outer primaries and shorter inner primaries, and a tendency to have P6 longer than P5, whereas in *lusitanica* P5 seems slightly longer on average than P6 (Figure 2.3). The inclusion of body size (PC_{SIZE}) as a covariate in the statistical model does not affect the comparison of wing shape ($PC1_{WING}$ and $PC2_{WING}$) between populations (GLM: $PC1_{WING}$: PC_{SIZE}: $F_{[1;96]}$ =0.49, P= 0.486; Age: $F_{[1;96]}$ = 0.91, P =0.342; Sex: $F_{[1;96]}$ = 1.56, P =0.215; Population: $F_{[3;96]}$ = 9.92, P,0.001; $PC2_{WING}$: PC_{SIZE}: $F_{[1;96]}$ = 0.49, P= 0.486; Age: $F_{[1;96]}$ =9.77, P= 0.002; Sex: $F_{[1;96]}$ =0.01, P= 0.919; Population: $F_{[3;96]}$ =6.38, P= 0.001), and so the difference is not caused by allometry.

Although the tail of the migratory populations of *schoeniclus* was significantly longer than that of the resident *schoeniclus* from the UK and of *lusitanica* (but not significantly different from *witherbyi*), the tail/wing ratio was significantly smaller in migratory *schoeniclus* than that of other subspecies except the resident UK population (GLM with *schoeniclus* and males as reference and B representing the unstandardized coefficients/parameters of the fitted model: Sex: B=0.01460.03, P<0.001; Population: (lus) B=0.010±0.003, P=0.001, (UK) B=-0.008±0.004, P=0.066, (wit) B=0.041±0.005 P<0.001). However, if body mass (rather than PC_{SIZE}, which depends on tail length) is used as a covariate to adjust for differences in body size, the tail length of migratory *schoeniclus* and *witherbyi* are not significantly different (B=0.255±0.494mm, P=0.605), whereas the tail of *schoeniclus* from the UK are significantly shorter (B=-1.966±0.443mm, P<0.001) and even shorter in *lusitanica* (B=-2.705±0.317mm, P<0.001).

Adaptations to Foraging

Although there is a large overlap in measurements, all bill traits differed significantly between *lusitanica* and *schoeniclus*, particularly bill depth and width, the former being 2.6–3.9% (females–males) larger in *lusitanica* (Table 2.1, Figure 2.2). This is particularly remarkable given that *schoeniclus* is 7.4–8.3% heavier and have 6.1–4.9% longer wings than *lusitanica* (Table S 2.4). As described above, there was virtually no overlap in bill depth between the thick-billed *witherbyi* and the remaining subspecies, with *witherbyi* having a bill 14.3–17.3% deeper than *lusitanica*, but being only 8.0–7.8% heavier (Figure 2.2, see also Belda et al. 2009). On the other hand, resident *schoeniclus* from the UK had significantly shorter (3.2–5.3%) and less deep (7.3–8.6%) bills than the migratory *schoeniclus* (Table 2.1, Figure 2.2). In contrast to the measurements of the flight apparatus, there were significant interactions (not shown in Table 2.1) between population and sex in bill length ($F_{[3;317]}$=2.97, P=0.032) and bill depth ($F_{[3;323]}$=3.98, P=0.008), which result from the fact that males differ more between populations than females in these traits (see Figure 2.2 and above). The inclusion of PC_{SIZE} as a covariate in the model comparing bill depth between populations still resulted in highly significant differences (GLM: PC_{SIZE}: $F_{[1;281]}$=23.2, P<0.001; Sex: $F_{[1;281]}$=2.3, P=0.130; Population: $F_{[3;281]}$=190.2, P<0.001; Sex vs. Population: $F_{[3;281]}$=3.9, P=0.009). Hence, allometry does not explain the patterns found, particularly between *witherbyi* and *lusitanica*, which vary in size and bill depth in the same direction. Bill width largely follows the variation described for bill depth, as does the overall bill size (PC_{BILL}), whereas the bill shape index varied in the opposite direction with *witherbyi* having the deepest bill in relation to its length, followed by *lusitanica*, migratory *schoeniclus* and the resident *schoeniclus* from the UK (Table 2.1).

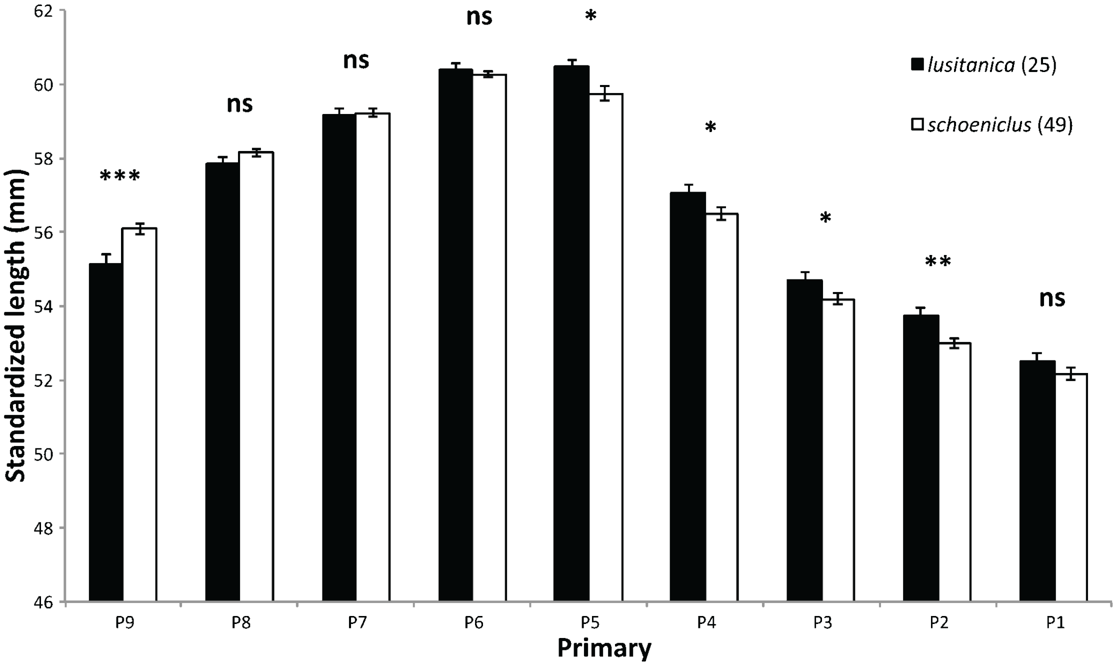

Figure 2.3 - Isometrically-adjusted primary lengths of the resident *E. s. lusitanica* and the migratory *E. s. schoeniclus* wintering in Portugal and from Sweden. Sample size is indicated between parentheses. T-tests indicate that primaries 9, 5, 4, 3, and 2 are significantly different between the subspecies (ns – non-significant; * – P<0.05; ** – P<0.01; *** – P<0.001).

Geometric morphometrics of the bill in profile revealed significant differences for the first nine axis (RW1-9) of variation between the populations/subspecies (for RW1 see Table 2.1). The first axis (RW1), which is the most important for population discrimination, represents variation in the curvature of the culmen, with *witherbyi* having the highest values, followed by *lusitanica*, migratory *schoeniclus* and then by resident *schoeniclus* from the UK (Table 2.1, Figure 2.4). As with the linear measurements, the interaction between sex and population is highly significant

($F_{[3;190]}$=5.78, P=0.001) because females do not differ as much between populations as males (see Figure 2.4). When body size (and birds of unknown age, since age is not significant, see Table 2.1) is included in the statistical model, the comparisons among populations and the interaction with sex, remain highly significant (GLM: PC_{SIZE}: $F_{[1;174]}$=0.33, P=0.569; Sex: $F_{[1;174]}$=1.86, P=0.174; Population: $F_{[3;174]}$=32.11, P<0.001; Sex vs. Population: $F_{[3;174]}$=4.754, P=0.003), and so differences in bill shape cannot be explained by allometry. RW3, the second most important bill shape variable to discriminate the populations (representing variation from short, stubby to long, shallow bills, see Figure 2.4), produces similar results to RW1 (GLM: PC_{SIZE}: $F_{[1;174]}$=0.23, P=0.629; Sex: $F_{[1;174]}$=1.82, P=0.179; Population: $F_{[3;174]}$=5.93, P=0.001; Sex vs. Population: $F_{[3;174]}$=3.03, P=0.031). The difference in RW1 between *lusitanica* and migratory *schoeniclus* is greater than for any linear measurement of the bill, especially in males (Figure 2.4). Indeed, discriminant analyses (using RW1-5) between these two populations resulted in 95.1% of the males and 75.5% of females being correctly classified to their original population; whereas linear measurements of the bill (length, depth, width and bill shape index) resulted in 80.2% of the males and 74.7% of the females being correctly classified.

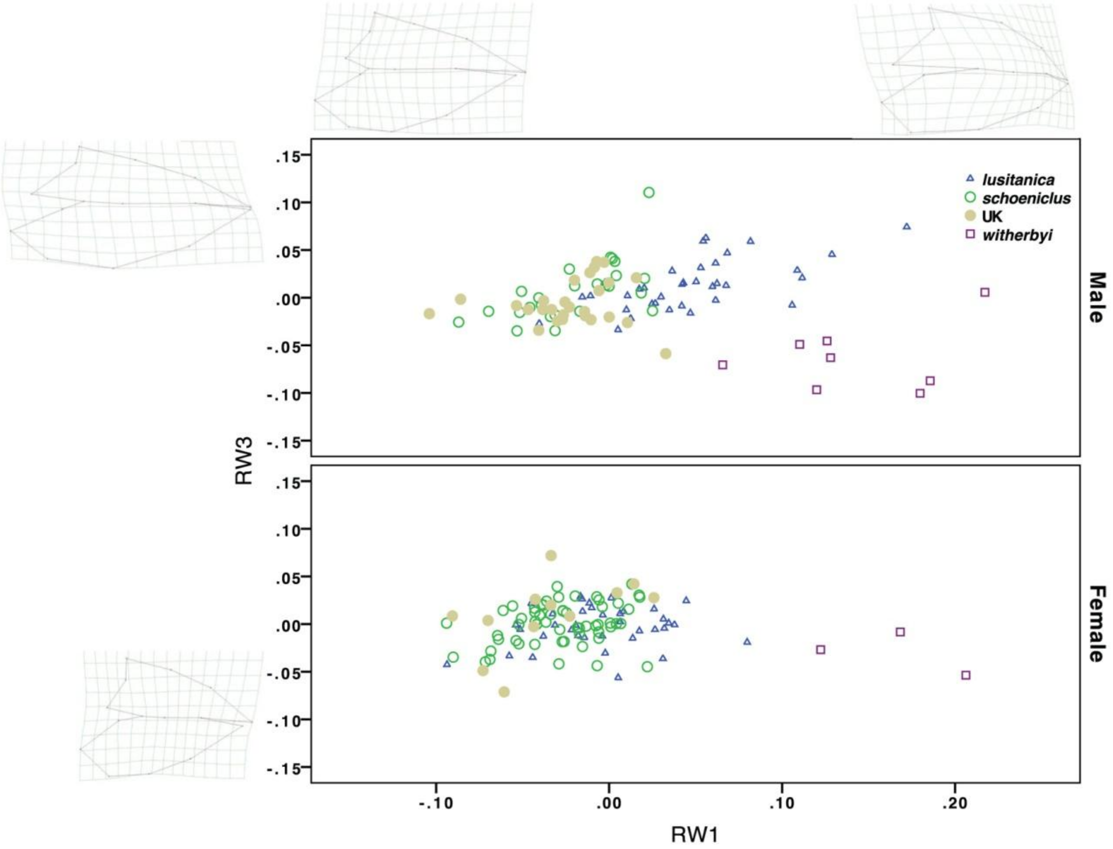

Figure 2.4 - Bill shape in relation to population and sex, as measured by the two most important axis of variation for population discrimination (RW1 and RW3) derived from geometric morphometric analysis.

2.5 Discussion

In this study, we described the phenotypic divergence amongst reed bunting populations likely to be relevant for the seemingly on-going speciation process in this species. We chose to analyse traits for which clear predictions of the direction of evolution could be made relative to two selection pressures that are known to influence speciation in birds: migration and diet. In particular, we showed that, according to predictions, migratory *schoeniclus* had longer and more convex wings than the resident Iberian subspecies (see also Copete et al. 1999), and similar patterns have been found in other bird species (Milá et al. 2008; Tarka et al. 2010; Baldwin et al. 2010; Förschler & Bairlein 2010). The migratory *schoeniclus* also had slightly smaller body mass than the other populations, as predicted by theory, except for *lusitanica*, which is much smaller than the other subspecies. These variations/adaptations seemed to have occurred despite northern reed buntings being short to medium distance migrants, rather than long-distance migrants (Prŷs-Jones 1984), and so the selection pressure for high aspect-ratio wings might not be as high as in other species that have been studied (e.g. Milá et al. 2008). Comparisons of migratory and resident populations of the nominate subspecies reveal slight differences in wing shape, which is rounder (less convex) in the resident population (UK) than in migratory *schoeniclus*. Also, birds from the UK were heavier than the migrants, but in contrast to expectations had relatively short tails. A recent common ancestry, large gene flow and the occurrence of short-distance seasonal movements in UK birds (particularly in some years when snow cover might prevent them to have access to seeds; Prŷs-Jones 1984; Wernham et al. 2002) might explain the small differences found. Tail length, however, did not vary according to the expectation of shorter tail in migratory birds, and tail/wing ratio seemed to reflect mostly the longer wings of migrants (see also Milá et al. 2008). This may be a consequence of tail and wing lengths being strongly correlated both phenotypically and genetically in birds, and for this reason it is possible that tail length takes longer to evolve and may even act as a morphological constrain to adaptation in wing lengths (Tarka 2012).

We also show that the southern subspecies, which have been observed feeding on dormant insects lying inside reed stems during winter, have thicker bills (which they use to open the reed stems; pers. observations, Prŷs-Jones 1984). In contrast, northern populations, which switch their diets to seeds during the winter (Orłowski & Czarnecka 2007; although they can also feed on insects opportunistically by gleaning Orłowski et al. 2013; pers. observations), have much shorter and especially thinner bills (see also Belda et al. 2009). Particularly interesting is the small, resident, Iberian subspecies *lusitanica*, for which we present for the first time statistical comparisons with other populations. This subspecies has a disproportionally long and thick bill for its small body size, having a significantly larger bill than the large-bodied nominate subspecies, but smaller/thinner than all *witherbyi*. In contrast, birds from the UK have smaller and thinner bills than those of migratory *schoeniclus*, which could be associated with a diet composed of smaller seeds (although this has so far not been studied in any detail).

In addition to the linear measurements, our geometric morphometric analyses revealed important differences in bill shape, particularly in culmen curvature. The resident *witherbyi* remains especially distinct regarding bill shape from the remaining populations; but in contrast to the linear measurements, there is some overlap in culmen curvature (RW1) between *lusitanica* and *witherbyi* males (Figure 2.4). On the other hand, *lusitanica* differs from the nominate subspecies in bill shape to a greater extent than when only the linear bill measurements are used, especially in males, although there is still overlap between these subspecies (Figure 2.4). This is most likely associated with differences in diet (Chapter 5) because birds with a more convex culmen are able to exert a greater strength at the bill tip, which is probably very useful to open the reed stems, whereas seed-eating birds tend to crack the seeds at the base of the bill (Foster et al. 2007). Given these results, and despite the overlap in bill traits with *schoeniclus* (especially in females), *lusitanica* appears to share morphological traits with the thick-billed subspecies (as expected by their ecology and distribution), but it is still quite distinctive from both groups due to its much smaller size and dark plumage (in addition to the feeding apparatus).

One interesting morphological difference clearly shown by our analyses is the sexual dimorphism in bill size and shape, which is consistent across subspecies. Sexual differences in bill size and shape do not result from the overall small body size of females, as sex remains significant when body size is taken into account in the statistical models. Females have shorter, thinner bills and a less convex culmen than males and, independently of its origin (sexual selection or intra-specific competition), these differences are probably associated with ecological differences that have hitherto not been studied. It is possible that females prefer smaller seeds in northern populations or search for insects in thinner reeds in southern populations, but more radical foraging niche differences may occur between the sexes. Interestingly, bill size and shape diverged more between populations in males than in females, which could suggest that in addition to ecology, sexual selection could have also played a role in population divergence. Our results are comparable to those described for tidal-marsh (North American) sparrows, for which intraspecific competition for food (and/or possibly male-male competition for territories/females) was considered the most likely cause for the greater increase in male than female bill size associated with the colonization of marshes by a variety of emberizid species (Greenberg & Olsen 2010). As shown theoretically and empirically (in threespine stickebacks), both sexual dimorphism and speciation can co-occur as long as the effects of loci underlying sexually dimorphic traits are orthogonal to those underlying sexually selected traits (Cooper et al. 2011). The role of sexual selection and competition in producing the sexual differences found in reed buntings deserve further research.

Another interesting morphological difference that we described is the much smaller size of *lusitanica* relative to the remaining subspecies, for which we have no obvious adaptive explanation. This subspecies lives in close proximity to the large and thick-billed *witherbyi*, but uses mostly coastal reedbeds located in the Atlantic influenced (wet, mild) geographical region, whereas the latter occurs mostly in inland (occasionally coastal) reedbeds in the Mediterranean influenced (dry, hot or continental) region. Both the small size and dark plumage of *lusitanica* could perhaps be explained by adaptations to the mild, wet climate where they occur (following Gloger's rule); whereas its thinner bill (in comparison with *witherbyi*) could be related to their occurrence in brackish sites, where the reeds tend to be shorter and thinner, although this is not sufficiently studied. As *witherbyi* have a thicker bill than lusitanica, even when controlling for body size, and the foraging ecology seems to be similar (Chapter 5), it is possible that bill size between these subspecies has evolved to dissipate heat in the warmer eastern Iberian sites. In fact, summer temperatures might be responsible for the clinal variation of increasing bill size towards the east among thick-billed subspecies of reed buntings. This has recently been shown to occur in several North American emberizids (Greenberg et al. 2012; Greenberg et al. 2012; Greenberg & Danner 2012). The relative roles of diet and temperature on the evolution of bill size should be further studied in reed buntings, especially among subspecies with similar diets.

In previous studies, we have shown that the genetic divergence among the reed bunting subspecies is very small, but significant, with G_{ST} (microsatellites) ranging from 0.03 to 0.04 and Φ_{ST} (mtDNA) from 0.04–0.05 between *schoeniclus* and each Iberian subspecies; and 0.04 (microsatellites) and 0.14 (mtDNA) between the two Iberian subspecies (Kvist et al. 2011). In addition, the shallow mtDNA phylogeny indicates that these subspecies diverged very recently, after the last glacial maxima (Kvist et al. 2011). Therefore, and given that the morphological traits studied here generally have high heritabilities (Keller et al. 2001; Tarka et al. 2010) and showed limited plasticity in a common garden experiment with a North American emberizid (Ballentine & Greenberg 2010), differences among populations found in this study probably evolved very rapidly through natural selection. However, genetic drift, especially in the threatened Iberian subspecies, cannot be excluded as a potential explanation for some of the morphological differences that were found, nor does (adaptive) plasticity. Detailed comparisons between genetic and phenotypic divergence are clearly needed (for which additional genetic markers need to be used relative to those already available for this system; Kvist et al. 2011), as well as common garden experiments, in order to confirm whether these traits are indeed under selection or locally adapted (Ballentine & Greenberg 2010; Whitlock 2008).

In conclusion, our morphometric analyses clearly show that the three subspecies of reed buntings occurring in Western Europe differ in a variety of traits in the direction predicted by their migratory and foraging behaviours, strongly suggesting that these birds became locally adapted through natural selection. Whether these traits contribute to reproductive isolation is currently being investigated in this interesting study system (Chapter 4). This study contributes to improve upon the limited knowledge on speciation phenotypes that is available for a variety of organisms (Shaw & Mullen 2011).

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2.7 Author Contributions

Conceived and designed the experiments: JMN LG. Performed the experiments: JMN LG EJB MM JSM PF RC. Analyzed the data: JMN LG. Contributed reagents/materials/analysis tools: JMN LG EJB MM JSM PF RC. Wrote the paper: JMN LG.

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2.9 Supporting Information

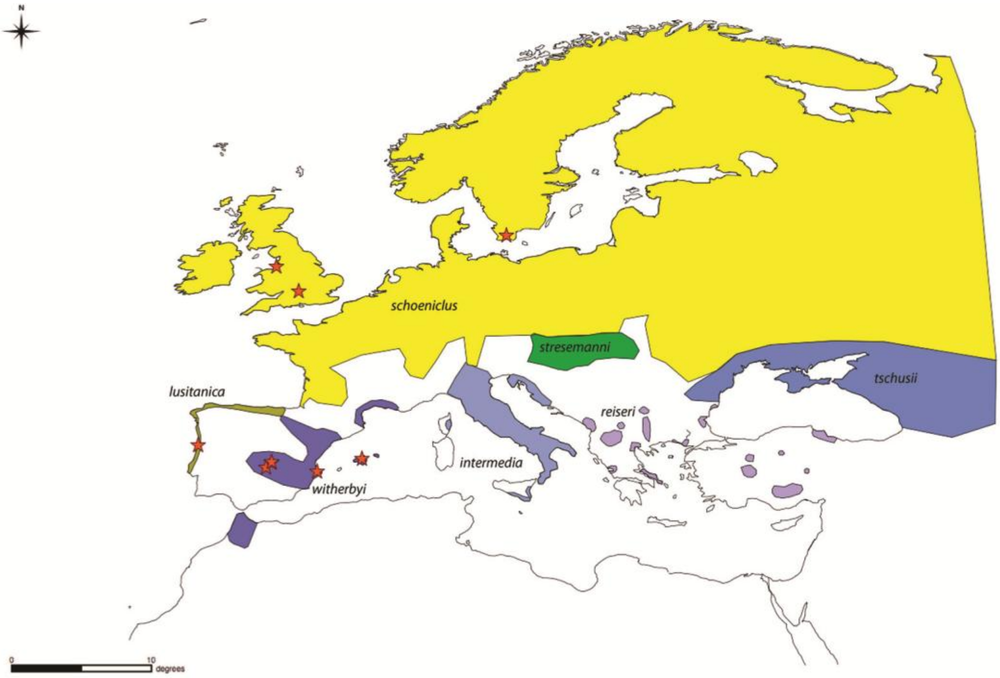

Figure S 2.1 - Approximate breeding distributions of reed bunting subspecies occurring in Europe. (based on Vaurie 1956, 1958; Cramp & Perrins 1994; Byers et al. 1995). Sampling sites are indicated with a red star.

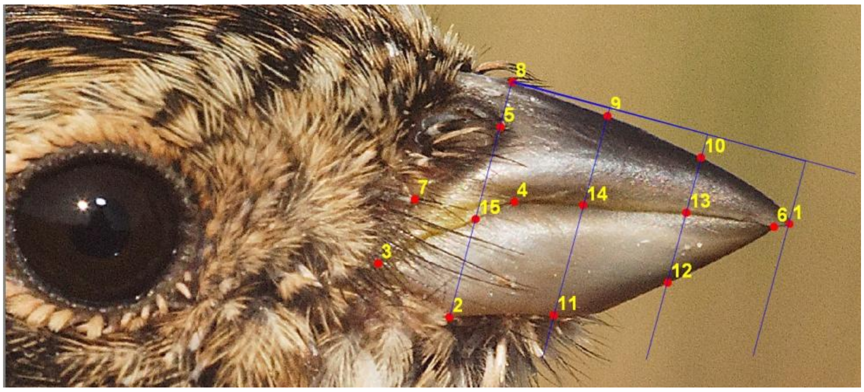

Figure S 2.2 - Location of the seven landmarks and eight semi-landmarks (calculated from the landmarks) used in geometric morphometric analyses.

Table S 2.1 - Principal component analysis of bill size measurements, used to extract PCBILL.

Total Variance Explained

Component	Initial Eigenvalues			Extraction Sums of Squared Loadings		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	1.802	60.071	60.071	1.802	60.071	60.071
2	0.994	33.142	93.213			
3	0.204	6.787	100.000			

Component Matrix

	Component 1
Bill length	0.231
Bill depth	0.947
Bill width	0.923

Table S 2.2 - Principal component analysis of the isometrically-adjusted primary lengths, used to extract PC1WING and PC2WING, which represent wing convexity and wing pointedness, respectively.

Total Variance Explained

Component	Initial Eigenvalues			Extraction Sums of Squared Loadings		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	4.194	46.597	46.597	4.194	46.597	46.597
2	1.885	20.948	67.545	1.885	20.948	67.545
3	1.058	11.759	79.304			
4	0.607	6.745	86.049			
5	0.415	4.614	90.663			
6	0.316	3.512	94.175			
7	0.263	2.921	97.096			
8	0.143	1.585	98.681			
9	0.119	1.319	100.000			

Component Matrix

	Component	
	1	2
P9	0.297	0.675
P8	0.433	0.777
P7	0.576	0.564
P6	0.595	0.308
P5	0.698	0.005
P4	0.831	-0.240
P3	0.846	-0.351
P2	0.853	-0.314
P1	0.782	-0.365

Table S 2.3 - Principal component analysis of body size measurements, used to extract PCSIZE.

Total Variance Explained

Component	Initial Eigenvalues			Extraction Sums of Squared Loadings		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	2.067	51.679	51.679	2.067	51.679	51.679
2	0.988	24.691	76.370			
3	0.716	17.894	94.264			
4	0.229	5.736	100.000			

Component Matrix

	Component 1
Wing	0.901
Tail	0.891
Tarsus	0.657
Bill	0.172

Table S 2.4 - Descriptive statistics of morphological traits for each population, sex and age class. Individuals captured in Sweden did not differ from individuals of the nominate subspecies wintering in Portugal, and so they were lumped.

a) *E. s. schoeniclus*, adult males

	N	Minimum	Maximum	Mean	Std. Error
Wing	20	78.00	87.00	83.2000	0.48177
P8	20	58.00	65.50	62.3500	0.40572
Tail	20	64.5	73.0	67.950	0.4321
Tarsus	20	18.5	20.5	19.540	0.1572
Bill length	20	12.5	14.2	13.310	0.0951
Weight	20	15.9	23.1	19.365	0.4590
Bill depth	19	4.9	5.8	5.326	0.0545
Bill width	19	4.2	5.4	4.632	0.0693
Bill shape	19	2.4	2.7	2.505	0.0223
Tail/Wing	20	0.7857	0.8598	0.816858	0.0041543
PC1$_{WING}$	10	-3.00935	0.39745	-0.6806189	0.30247526
PC$_{BILL}$	19	-1.00679	0.96456	-0.1757131	0.11546877
PC$_{SIZE}$	20	-0.08508	2.50380	1.1919476	0.14110635
RW1	12	-0.08693661	0.02510300	-0.0165500528	0.00972898694

b) *E. s. schoeniclus*, first-year males

	N	Minimum	Maximum	Mean	Std. Error
Wing	18	80.00	87.00	82.3611	0.44245
P8	17	58.50	65.50	61.5000	0.40674
Tail	18	66.5	71.0	68.583	0.3688
Tarsus	18	18.5	21.1	19.967	0.1799
Bill length	18	12.4	13.9	13.156	0.1017
Weight	18	17.4	22.0	19.372	0.2723
Bill depth	18	4.8	5.5	5.206	0.0521
Bill width	18	3.9	5.5	4.700	0.1010
Bill shape	18	2.4	2.7	2.533	0.0181
Tail/Wing	18	0.8036	0.8650	0.832865	0.0038480
PC1$_{WING}$	8	-0.87762	0.24838	-0.2442220	0.14420557
PC$_{BILL}$	18	-1.36798	0.68003	-0.2850498	0.14492142
PC$_{SIZE}$	18	0.69927	2.19804	1.3089171	0.09510728
RW1	13	-0.06963698	0.02051702	-0.0235799350	0.00762650852

c) *E. s. schoeniclus*, adult females

	N	Minimum	Maximum	Mean	Std. Error
Wing	36	74.00	81.00	77.6250	0.28339
P8	33	53.50	61.00	57.6364	0.26046
Tail	36	59.0	69.0	64.694	0.3329
Tarsus	36	17.2	20.2	19.117	0.1204
Bill length	36	11.7	13.5	12.861	0.0786
Weight	36	15.6	19.5	17.078	0.1532
Bill depth	35	4.8	5.7	5.134	0.0357
Bill width	35	3.2	4.9	4.466	0.0528
Bill shape	35	2.3	2.8	2.503	0.0194
Tail/Wing	36	0.7815	0.8846	0.833442	0.0031959
PC1$_{WING}$	16	-1.65101	1.44060	-0.2622198	0.19756004
PC$_{BILL}$	35	-1.76389	0.38166	-0.6208201	0.07793469
PC$_{SIZE}$	36	-1.84005	1.06817	-0.0950370	0.09467121
RW1	28	-0.06126072	0.02208257	-0.0232523349	0.00414189258

d) *E. s. schoeniclus*, first-year females

	N	Minimum	Maximum	Mean	Std. Error
Wing	42	73.50	80.50	77.1190	0.22849
P8	40	53.00	60.50	57.1250	0.20782
Tail	41	60.0	67.5	64.354	0.2597
Tarsus	42	16.8	20.4	19.098	0.1193
Bill length	42	11.8	13.9	12.869	0.0756
Weight	39	13.7	22.2	17.156	0.2190
Bill depth	38	4.5	5.6	5.068	0.0413
Bill width	38	3.9	5.0	4.447	0.0444
Bill shape	38	2.3	2.9	2.539	0.0201
Tail/Wing	41	0.8000	0.8710	0.834758	0.0027068
$PC1_{WING}$	13	-1.67402	0.67211	-0.4255270	0.18777186
PC_{BILL}	38	-1.87810	0.27450	-0.7054309	0.08560620
PC_{SIZE}	41	-1.20273	0.92260	-0.2120669	0.06896011
RW1	29	-0.09397113	0.01772159	-0.0321491578	0.00579737456

e) *E. s. lusitanica*, adult males

	N	Minimum	Maximum	Mean	Std. Error
Wing	50	76.00	81.50	79.2800	0.20412
P8	43	55.50	61.00	58.9884	0.21789
Tail	47	61.0	69.5	65.436	0.2570
Tarsus	57	18.1	20.8	19.423	0.0743
Bill length	57	12.9	14.5	13.514	0.0445
Weight	58	15.5	19.4	17.886	0.1019
Bill depth	57	4.9	5.9	5.533	0.0294
Bill width	57	4.1	5.2	4.595	0.0345
Bill shape	56	2.3	2.7	2.441	0.0144
Tail/Wing	47	0.7673	0.8608	0.825018	0.0026897
$PC1_{WING}$	10	-1.09944	1.07280	-0.1378834	0.20969688
PC_{BILL}	56	-1.16143	0.89492	0.0677461	0.05885045
PC_{SIZE}	47	-0.68210	1.55336	0.3982752	0.07001222
RW1	24	-0.01552546	0.11164010	0.0413689387	0.00704651597

f) *E. s. lusitanica*, first-year males

	N	Minimum	Maximum	Mean	Std. Error
Wing	21	73.00	79.00	75.8810	0.35001
P8	20	54.00	59.00	55.9500	0.28308
Tail	20	59.0	66.5	63.375	0.4244
Tarsus	21	18.4	20.5	19.310	0.1165
Bill length	21	12.5	14.6	13.310	0.1125
Weight	21	14.4	20.0	17.529	0.3147
Bill Depth	21	5.2	5.7	5.524	0.0284
Bill width	21	4.1	5.3	4.671	0.0614
Bill shape	21	2.2	2.8	2.419	0.0281
Tail/Wing	20	0.7922	0.8836	0.834273	0.0051950
$PC1_{WING}$	3	-2.04398	0.15733	-0.6640759	0.69411514
PC_{BILL}	21	-0.57204	0.51597	0.0728374	0.06462813
PC_{SIZE}	20	-1.42868	0.64174	-0.3291862	0.09985520
RW1	11	-0.04037748	0.17203692	0.0546367634	0.01845410362

g) *E. s. lusitanica*, adult females

	N	Minimum	Maximum	Mean	Std. Error
Wing	44	69.50	79.00	73.1932	0.27261
P8	37	51.00	59.00	54.2027	0.26242
Tail	42	57.5	68.0	62.060	0.3815
Tarsus	48	17.4	20.6	18.838	0.1022
Bill length	48	12.6	14.0	13.113	0.0506
Weight	49	13.9	19.1	15.904	0.1703
Bill depth	48	4.9	5.7	5.269	0.0273
Bill width	48	3.9	4.9	4.460	0.0351
Bill shape	48	2.3	2.7	2.488	0.0148
Tail/Wing	41	0.8014	0.9178	0.848270	0.0038054
$PC1_{WING}$	8	-0.84989	1.68372	0.5058748	0.30880820
PC_{BILL}	48	-1.27930	0.43358	-0.4200244	0.05455421
PC_{SIZE}	41	-1.90889	0.67883	-1.0202798	0.09048879
RW1	25	-0.09368186	0.07977888	-0.0061319140	0.00738256743

h) *E. s. lusitanica*, first-year females

	N	Minimum	Maximum	Mean	Std. Error
Wing	21	68.00	73.50	70.6429	0.31837
P8	21	49.50	55.00	52.0238	0.32085
Tail	21	57.5	64.0	60.667	0.4116
Tarsus	21	17.9	19.3	18.519	0.0758
Bill length	21	12.5	13.4	13.148	0.0533
Weight	21	13.8	17.4	15.514	0.2190
Bill depth	21	4.5	5.6	5.214	0.0570
Bill width	20	4.2	4.9	4.530	0.0411
Bill shape	21	2.4	2.9	2.529	0.0260
Tail/Wing	21	0.8099	0.9197	0.858819	0.0047392
$PC1_{WING}$	3	-0.90078	1.05026	-0.0528652	0.57749062
PC_{BILL}	20	-1.05999	0.33917	-0.3757133	0.08255773
PC_{SIZE}	21	-2.29326	-0.84524	-1.6011798	0.09515144
RW1	12	-0.05773664	0.03137872	-0.0037080004	0.00856799138

i) *E. s. witherbyi*, adult males

	N	Minimum	Maximum	Mean	Std. Error
Wing	8	75.00	81.00	79.0000	0.70711
P8	8	56.00	63.50	60.3750	0.91977
Tail	6	61.0	77.0	69.833	2.1042
Tarsus	9	17.4	21.3	20.089	0.3942
Bill length	7	12.1	13.5	12.900	0.1864
Weight	9	16.5	22.0	19.278	0.5570
Bill depth	8	6.2	6.7	6.488	0.0789
Bill width	7	5.0	7.2	5.829	0.3168
Bill shape	7	1.9	2.2	1.971	0.0474
Tail/Wing	6	0.8133	0.9872	0.883696	0.0232501
$PC1_{WING}$	1	3.05784	3.05784	3.0578388	0.0
PC_{BILL}	7	1.32281	2.99059	2.0158336	0.25200454
PC_{SIZE}	5	-0.59576	1.99815	1.1106233	0.44938200
RW1	1	0.18559707	0.18559707	0.1855970650	0.0

j) *E. s. witherbyi*, first-year males

	N	Minimum	Maximum	Mean	Std. Error
Wing	11	74.00	84.00	79.6818	0.84819
P8	10	57.00	64.50	61.3500	0.71899
Tail	11	65.0	73.0	68.955	0.7150
Tarsus	12	19.8	21.3	20.617	0.1364
Bill length	12	12.1	15.3	13.300	0.2705
Weight	10	17.8	21.4	19.900	0.3445
Bill depth	12	6.1	6.9	6.517	0.0911
Bill width	12	4.7	6.8	5.925	0.2132
Bil shape	12	1.8	2.3	2.042	0.0452
Tail/Wing	11	0.8228	0.9189	0.865792	0.0082412
PC1$_{\mathbf{WING}}$	4	-0.53504	3.57906	1.4094423	1.08514850
PC$_{\mathbf{BILL}}$	12	0.78482	3.41628	2.1697482	0.22987731
PC$_{\mathbf{SIZE}}$	11	0.14432	2.64153	1.3204985	0.24332499
RW1	5	0.06568295	0.17981081	0.1238896468	0.01809956408

k) *E. s. witherbyi*, adult females

	N	Minimum	Maximum	Mean	Std. Error
Wing	6	71.50	75.00	73.4167	0.61124
P8	5	53.50	56.50	55.3000	0.51478
Tail	5	60.5	66.0	63.500	0.8944
Tarsus	9	18.8	20.3	19.367	0.1803
Bill legth	9	11.2	13.3	12.289	0.2003
Weight	6	15.3	19.0	17.183	0.6901
Bill depth	9	5.7	6.4	6.022	0.0722
Bill width	9	5.0	6.3	5.889	0.1522
Bill shape	9	1.9	2.3	2.056	0.0475
Tail/Wing	4	0.8533	0.8951	0.872863	0.0092379
PC1$_{\mathbf{WING}}$	1	0.67119	0.67119	0.6711887	0.0
PC$_{\mathbf{BILL}}$	9	0.50526	1.97739	1.3773026	0.17538541
PC$_{\mathbf{SIZE}}$	4	-0.80161	-0.14795	-0.5610769	0.15151417
RW1	2	0.12229824	0.16834301	0.1453206255	0.02302238150

l) *E. s. witherbyi*, first-year females

	N	Minimum	Maximum	Mean	Std. Error
Wing	5	72.00	75.00	74.0000	0.54772
P8	5	51.00	58.00	55.8000	1.25100
Tail	4	61.5	67.0	64.250	1.4506
Tarsus	6	18.7	20.8	20.083	0.3429
Bill length	5	11.7	13.2	12.420	0.2634
Weight	6	16.5	17.9	17.200	0.2380
Bill depth	5	5.7	6.5	6.080	0.1356
Bill width	5	5.0	6.7	5.780	0.3277
Bill shape	5	1.9	2.2	2.060	0.0510
Tail/Wing	4	0.8200	0.9054	0.862680	0.0227994
PC1$_{\mathbf{WING}}$	0				
PC$_{\mathbf{BILL}}$	5	0.61059	2.42598	1.3810382	0.31861776
PC$_{\mathbf{SIZE}}$	4	-0.80487	0.12202	-0.3493920	0.19143066
RW1	0				

m) *E. s. schoeniclus*, adult males from the U. K.

	N	Minimum	Maximum	Mean	Std. Error
Wing	10	79.50	85.00	82.2500	0.60208
P8	10	59.50	65.50	62.3000	0.55377
Tail	10	65.0	71.0	68.000	0.5323
Tarsus	10	18.9	24.5	20.760	0.5860
Bill length	8	12.1	13.3	12.688	0.1481
Weight	10	18.4	22.0	19.490	0.3446
Bill depth	9	4.9	5.5	5.156	0.0580
Bill width	6	3.8	4.4	4.083	0.0910
Bill shape	7	2.3	2.6	2.500	0.0378
Tail/Wing	10	0.8095	0.8625	0.826845	0.0048451
PC1$_{\text{WING}}$	8	-0.00831	1.26194	0.4743480	0.19978963
PC$_{\text{BILL}}$	5	-1.18673	-0.93583	-1.0518969	0.04897743
PC$_{\text{SIZE}}$	8	0.22593	2.12748	1.3126658	0.19502805
RW1	9	-0.08583785	0.03274251	-0.0127423861	0.01185048262

n) *E. s. schoeniclus*, first-year males from the U. K.

	N	Minimum	Maximum	Mean	Std. Error
Wing	20	79.00	85.00	81.1000	0.34527
P8	20	59.00	65.00	61.1000	0.32967
Tail	20	62.0	69.0	65.725	0.4537
Tarsus	20	19.3	23.3	20.495	0.2864
Bill length	16	11.8	13.1	12.638	0.1060
Weight	20	18.0	21.8	19.470	0.2394
Bill depth	20	4.5	5.4	4.905	0.0559
Bill width	15	3.5	4.6	4.193	0.0836
Bill shape	16	2.4	2.9	2.600	0.0387
Tail/Wing	20	0.7590	0.8500	0.810612	0.0059847
PC1$_{\text{WING}}$	18	-2.04722	1.61758	-0.0602602	0.22212294
PC$_{\text{BILL}}$	13	-1.71273	-0.42887	-1.1671276	0.12731486
PC$_{\text{SIZE}}$	16	0.23691	1.61369	0.8638221	0.11584239
RW1	16	-0.10391997	0.00005960	-0.0270659251	0.00606408108

o) *E. s. schoeniclus*, adult females from the U. K.

	N	Minimum	Maximum	Mean	Std. Error
Wing	2	74.00	78.00	76.0000	2.00000
P8	2	54.50	58.50	56.5000	2.00000
Tail	2	60.0	66.0	63.000	3.0000
Tarsus	2	19.5	20.2	19.850	0.3500
Bill length	0				
Weight	2	16.0	17.8	16.900	0.9000
Bill depth	2	4.4	5.0	4.700	0.3000
Bill width	2	3.9	4.5	4.200	0.3000
Bill shape	0				
Tail/Wing	2	0.8108	0.8462	0.828482	0.0176715
PC1$_{\text{WING}}$	2	0.52028	0.54866	0.5344698	0.01418934
PC$_{\text{BILL}}$	0				
PC$_{\text{SIZE}}$	0				
RW1	2	-0.06999003	-0.06065344	-0.0653217345	0.00466829950

p) *E. s. schoeniclus*, first-year females from the U. K.

	N	Minimum	Maximum	Mean	Std. Error
Wing	10	73.00	79.00	75.7000	0.54365
P8	10	52.00	59.00	56.4500	0.63004
Tail	10	58.0	65.0	62.600	0.7446
Tarsus	10	18.1	22.2	20.020	0.4912
Bill length	6	11.8	13.4	12.467	0.2124
Weight	10	15.6	19.2	17.390	0.3391
Bill depth	10	4.1	5.2	4.790	0.1130
Bill width	7	4.0	4.3	4.186	0.0404
Bill shape	6	2.4	2.7	2.533	0.0422
Tail/Wing	10	0.7945	0.8553	0.826838	0.0065494
$PC1_{WING}$	7	-0.79604	1.75062	0.4512214	0.39547803
PC_{BILL}	4	-1.27801	-0.88836	-1.1287710	0.09184407
PC_{SIZE}	6	-0.70443	0.91425	-0.1466503	0.24567195
RW1	9	-0.09061147	0.02586803	-0.0343655421	0.01183131005

Protocol S 2.1 - Photograph editing in Photoshop CS4.

1. When necessary, the photograph was flipped horizontally so that all bills would face right;
2. Zoom Level was set at 100%;
3. When necessary, the image was rotated so that pupil center and bill tip would lay exactly on the same imaginary horizontal line;
4. The picture was cropped so that the output would have a fixed width to height ratio of 5/3, the same distance left of the bill gape and right of the bill tip, and the same distance above the culmen and below the gonys (to make the best use of tpsDig window shape);
5. Each photograph was saved in jpg format using the ring number of the depicted bird as file name.

Protocol S 2.2 - Grid drawing in tpsDig.

1. Click the “Make angle measurements” button and next the “Draw background curves” button. Starting on landmark 2 draw a straight line going through landmark 5 and ending in the culmen ridge and then another straight line at a 90 degree angle from the first extending beyond bill tip;
2. Click the “Make linear measurements button” and measure the minimum distance between landmark 1 and the line going from landmark 2 to landmark 5 and to the culmen and divide the value by three;
3. Measure this last value from the 90 degree angle along the line going towards bill tip;
4. Use the “Make angle measurements” and the “Draw background curves” buttons again to draw a new straight line from the 90 degree angle to the measured point (over part of the preexisting line) and then another straight line at a 90 degree angle from the first extending down beyond the gonys;
5. Repeat the above procedure three times (the last one is for accuracy checking only);
6. Digitize semi-landmarks 8 to 15 in the order illustrated in Figure S 2.2.

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Chapter 3

Song divergence between subspecies of reed bunting is more pronounced in singing styles under sexual selection

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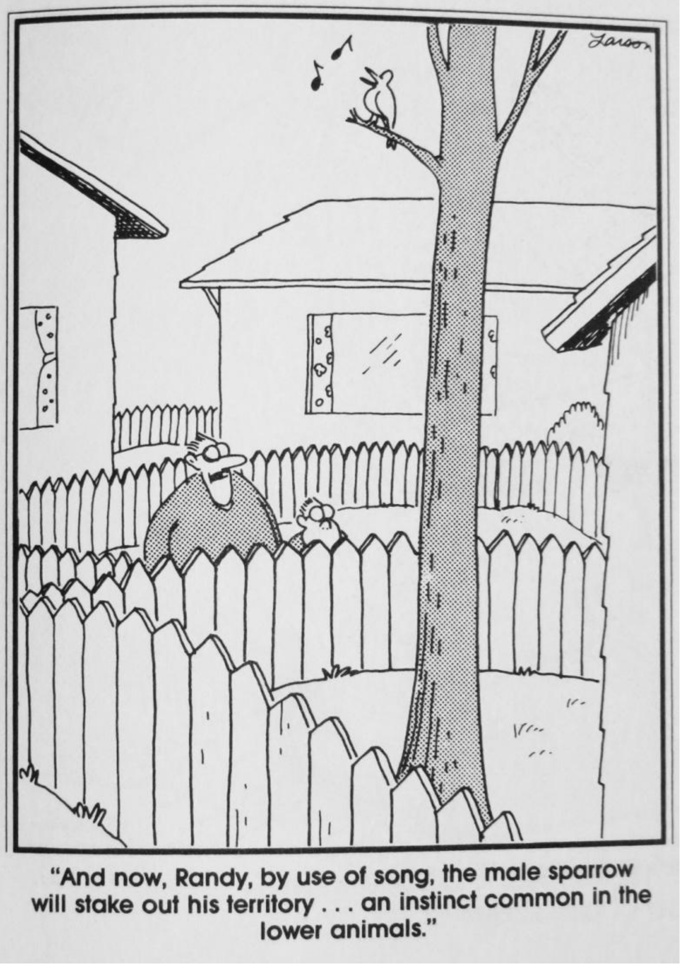

Cartoon 4 - All three reed bunting singing styles are probably important in the context of territory defence (from Larson 1984)

3 Song divergence between subspecies of reed bunting is more pronounced in singing styles under sexual selection

3.1 Abstract

Song divergence between populations of a species can lead to reproductive isolation and speciation. However, birds may have different singing styles used in distinct social contexts, and songs of each style may change at different rates over time and space. Here, we tested whether song divergence between subspecies of reed bunting, *Emberiza schoeniclus*, differs with singing style, by comparing song traits of its three singing styles among three subspecies breeding in northern and western Europe. We show that the two singing styles under sexual selection (dawn and fast songs, related to obtaining extrapair and social mates, respectively) diverged significantly more than the slow songs (used as an all-clear signal to nest-attending social females). Multiple song traits differed significantly between the subspecies in all singing styles, with *E. s. lusitanica* generally being intermediate between *E. s. schoeniclus* and *E. s. witherbyi*, and the pattern of song complexity opposing the expected latitudinal gradient (of increasing complexity with increasing latitude). Cluster analyses of populations indicate that sexually selected singing styles are better for discriminating subspecies, describing a scenario of a major split in song features between the migratory, northern *E. s. schoeniclus* and the two resident, southern subspecies, rather than a clinal variation. The greater song divergence in fast and dawn singing styles suggests that sexual selection may be playing an important role in the incipient speciation of reed buntings.

3.2 Introduction

Song is an important premating isolation barrier between passerine species (Catchpole & Slater 2008; Marler & Slabbekoorn 2004), and song divergence between populations of a species can lead to reproductive isolation and speciation (Martens 1996; Price 2008). Song divergence often follows morphological divergence (e.g. in bill or body size) resulting from a shift in ecology (e.g. in diet or feeding actions; Christensen et al. 2006; Grenier & Greenberg 2005; Huber & Podos 2006; Ratcliffe & Grant 1985), or adaptation to the local acoustic environment (Cardoso & Price 2010; Kirschel et al. 2009). However, it could also result from random cultural evolution and/or drift, especially in small, isolated populations (Lynch 1996; Williams et al. 2013; Xing et al. 2013).

In many birds and some marine mammals, there can be differences in the patterns of within-species spatial variation from one type of vocalization to another (Baker 2011). Among passerines, some species have several singing styles: functionally nonequivalent song types used in specific contexts (Bradbury & Vehrencamp 1998; Brunner & Pasinelli 2010; Ewin 1976; Hasselquist & Bensch 1991; Nemeth 1996). The songs used in each singing style may show distinct geographical patterns (Byers 1996; Kroodsma 1981) and change over time at different rates (Byers et al. 2010), suggesting that divergence between populations may be more pronounced in certain singing styles. Thus, as some social contexts are more relevant to reproductive isolation, certain singing styles could be of greater importance for speciation.

The reed bunting, *Emberiza schoeniclus*, is a Palearctic passerine with several subspecies described on the basis of morphology (Byers et al. 1995; Cramp & Perrins 1994). It has three singing styles, two of which are used for female attraction: fast song, the only style used by unpaired males, has been related to the attraction of a social mate (Ewin 1976; Nemeth 1996), and dawn song, which is sung by paired males and is associated with obtaining extrapair paternity (Suter et al. 2009). In contrast, slow songs are used by paired males apparently as an all-clear signal to their nest-attending females (Wingelmaier et al. 2007) and do not influence extrapair paternity or the number of fertilizations (Bouwman et al. 2007). In addition, all three singing styles are probably important in the context of territory defence (Brunner & Pasinelli 2010; Ghiot 1976). Individual males have repertoires of 10-30 different syllables that are used to build the songs of the three singing styles (Cramp & Perrins 1994; Ewin 1978; Suter et al. 2009). Dawn and fast singing are stereotyped styles because of the stricter rules of syllable and song arrangement (Brunner & Pasinelli 2010; Suter et al. 2009). Concomitantly, compared to the slow singing style, the fast style has greater syllable sequence predictability and greater syllable repetition (Ewin 1978). Song sharing, matched countersinging and local dialects have been recorded at the syllable level, but not at the song level (Ehrengruber et al. 2006; Ewin 1976, 1978).

Previous work has shown that the fast songs of non-migratory, thick-billed central and western Mediterranean birds (*E. s. intermedia* and *E. s. witherbyi*) differ from those of the largely migratory, thin-billed central European *E. s. schoeniclus* (Matessi et al. 2000b, 2001b). However, the divergence in other singing styles and differential divergence across styles have never been studied. The differences in fast song seem to be recognized by the birds, as males react differently to the playback of their own and foreign fast songs (Chapter 4; Matessi et al. 2000a, 2001a).

In this study, we analysed quantitative properties of songs of the three singing styles in three subspecies of reed bunting breeding in northern and western Europe: *E. s. schoeniclus* (hereafter *schoeniclus*), widespread from France and the U.K. north and eastwards to beyond the Western Palearctic; *E. s. lusitanica* (hereafter *lusitanica*), endemic to northwestern Iberia, for which there was no previous quantitative study of song (for qualitative notes see Martínez & Romay 2012); and *E. s. witherbyi* (hereafter *witherbyi*), from eastern Iberia, Balearics, southern France and, at least formerly, North Africa and Sardinia (Atienza 2006; Cramp & Perrins 1994; Figure 3.1). These three subspecies probably constitute the closest link between thin- and thick-billed groups of subspecies, as *lusitanica* is intermediate in bill traits and bill thickness increases towards the east among the thick-billed subspecies; it is thus particularly interesting to study speciation in this system (Chapter 2).

Our aims were (1) to compare quantitative properties of songs from each of the three singing styles between the three subspecies, (2) to quantify the extent to which males can be assigned to the correct subspecies on the basis of song properties in each singing style and (3) to evaluate whether the geographical pattern of song differentiation conforms to the subspecies distributions. Considering that (1) vocal signals with different functions can exhibit different geographical patterns (Baker 2011), (2) singing styles used for female attraction can act as a behavioural isolating mechanism (Kroodsma 1981) and (3) stronger sexual selection results in faster evolution/divergence (Kraaijeveld et al. 2011; Seddon et al. 2013), we hypothesized that fast and dawn singing styles (those used for female attraction) diverged faster and more extensively between reed bunting subspecies than the slow style.

3.3 Methods

Ethical Note

Field procedures used to obtain the sound recordings in which this study was based comply with the current laws of the countries where they were obtained (Portugal, Spain, France, Switzerland and Sweden).

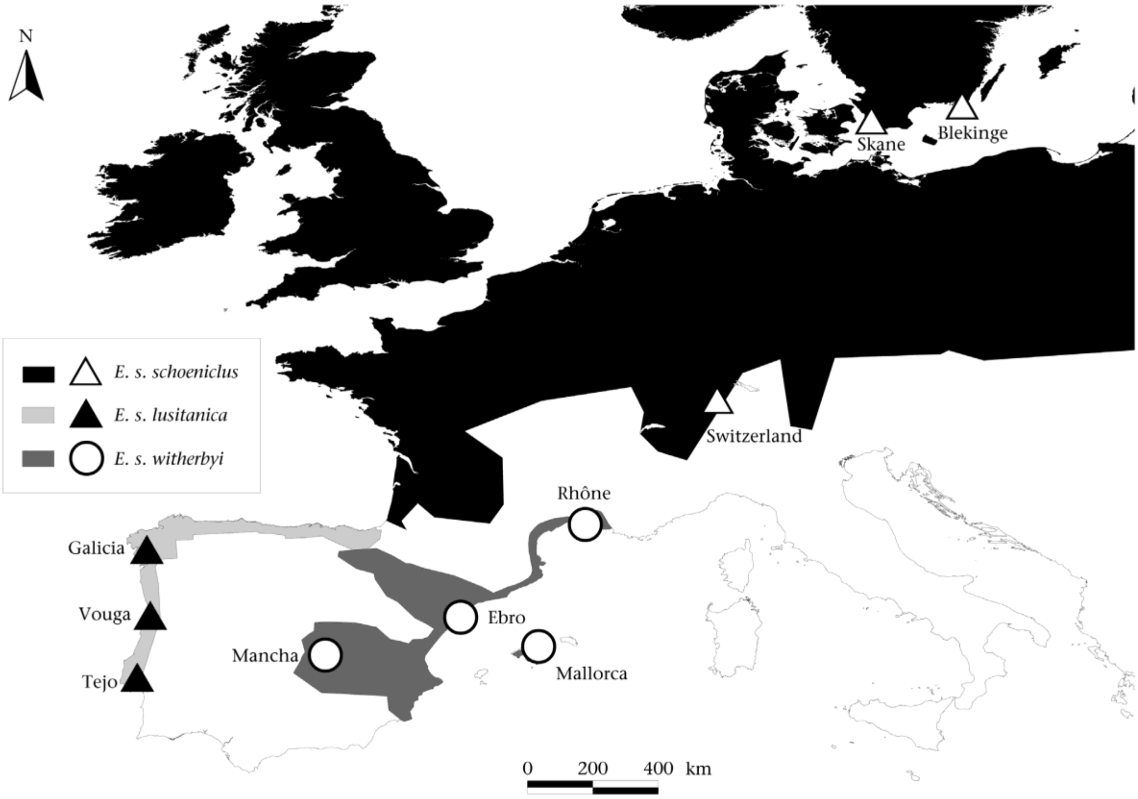

Figure 3.1 - European breeding range of the studied subspecies of reed bunting (*Emberiza schoeniclus schoeniclus*, *E. s. witherbyi* and *E. s. lusitanica*) and positions of recording sites. Iberian range according to Atienza (2006), distribution in France from Byers et al. (1995).

Fieldwork

Most of the recordings were obtained using a 570 mm Telinga parabola with a Twin Science microphone and a Marantz PMD660 solid-state recorder (settings: 48 kHz sampling frequency, WAV 16-bit format) by L.G. (Figure 3.1, Table 3.1). Additional recordings were obtained by E.M. using a similar Telinga parabola with a Stereo mic and a DA-P1 Tascam recorder (in 2004 and earlier), by the ‘Sound Approach to Birding’ team, and by Jean Roché (Figure 3.1, Table 3.1). Further recordings were obtained at three Swiss lakes in 2006 by Patrick Brunner (part of which were published in Brunner & Pasinelli 2010) and in 2009-2010 by Gilberto Pasinelli (Figure 3.1, Table 3.1).

Given the rate of cultural evolution in birdsong (Byers et al. 2010), large temporal gaps in data may be a potential source of bias. However, in our data set, the overall distribution of recording years (Mann-Whitney U test: P>0.05) and the median year of recording (median test: P>0.05) did not differ between subspecies.

Table 3.1 - Location, year and sample size of recordings of reed bunting song analysed in this study.

Area	Coordinates	Subspecies	Dawn	Fast	Slow	All styles	Years	Recorders
Galicia	42°22'N, 8°44'W	*lusitanica*	5	1	4	10	2004	EM
Vouga	40°50'N, 8°37'W	*lusitanica*	10	12	12	34	2010, 2011	LG
Tejo	38°51'N, 8°58'W	*lusitanica*	-	5	3	8	2010, 2011	LG
Total		*lusitanica*	15	18	19	52		
Rhône	43°26'N, 4°31'E	*witherbyi*	-	2	2	4	1983	JR
Ebro	40°39'N, 0°45'E	*witherbyi*	5	18	6	29	88,94,02,09,12	LG,EM,SA
Mallorca	39°47'N, 3°06'E	*witherbyi*	-	3	1	4	01,02,03	EM,SA
Mancha	39°31'N, 3°19'W	*witherbyi*	10	4	3	17	2004, 2011	EM,LG
Total		*witherbyi*	15	27	12	54		
Switzerland	47°15'N, 8°25'E	*schoeniclus*		13		13	2006,09,10	PB,GP
Skåne	55°39'N, 13°11'E	*schoeniclus*	5	2	14	21	2011,13	LG,JN
Blekinge	56°05'N, 15°51'E	*schoeniclus*		1	2	3	2013	LG,JN
Total		*schoeniclus*	5	16	16	37		
All range		**All ssp.**	35	61	47	143		

Samples are given as number of males of each subspecies (ssp.) for each singing style (fast, slow or dawn). Only one singing style was analysed for each male (i.e. there is no repetition of individuals across singing styles). Coordinates are in latitude/longitude, WGS84 datum. Recordings by the authors, Patrick Brunner (PB), Gilberto Pasinelli (GP), Jean Roché (JR) and the Sound Approach to Birding (SA).

Sound Processing

We screened an initial pool of 239 recordings (116 *witherbyi*, 76 *lusitanica*, 47 *schoeniclus*) and visually attributed each of them to one of the three singing styles (114 fast, 80 slow, 45 dawn). Dawn song is highly distinctive because it is sung 'continuously', with intervals between songs of similar magnitude to intervals between syllables within songs (Brunner & Pasinelli 2010; Suter et al. 2009). Fast and slow songs were distinguished based on the length of the intervals between the first two syllables (>0.3 s suggesting slow song) and the length of the interval between songs (if shorter than the length of songs, being suggestive of slow song; Nemeth 1996; Brunner & Pasinelli 2010). We only analysed songs from individual males that could be unambiguously identified based on the location of song posts and, especially, on the introductory syllables used by each bird, which are individual specific (Nemeth 1996). We therefore excluded recordings that could possibly be of the same individuals, resulting in a sample size of 143 different males (Table 3.1). From each male, we analysed songs of just one singing style, digitized 100 consecutive syllables and classified them into syllable types, using syllable type catalogues for each area created for this work (following Suter et al. 2009). For dawn songs, the frequency of occurrence of syllables and short pauses between song bouts were used to identify introductory syllables (Brunner & Pasinelli 2010). Those introductory syllables were then used to define songs within the continuous song bouts. We digitized up to 20 songs per individual male (following Brunner & Pasinelli 2010), but in some cases this was not possible due to recording length. Overall, on average 18 ± 4 SD (range 6-20) songs per male were used.

Songs were screened and digitized in Raven Pro 1.3 (www.birds.cornell.edu/brp/raven) using default spectrogram settings, resulting in a time resolution of 2.67 ms and a frequency resolution of 187.5 Hz. In each song, seven traits were measured from the spectrogram (following Suter et al. 2009): maximum frequency (MaxF); minimum frequency (MinF); song length (SL), the duration of each song; first interval (FI), the duration of the interval between the first two syllables; song interval (SI), the interval between two consecutive songs; number of syllables (NS); and number of different syllables (NDS). Although extracting frequency measures from spectrograms can be problematic (Zollinger et al. 2012), it nevertheless produces good results (Cardoso & Atwell 2012) and, importantly, does not introduce biases to the subspecies and style comparisons.

Statistical Analysis

The quantitative properties of song were compared between the three subspecies with general linear mixed models (GLMMs), in which the male identity was included as a random (subject) effect and subspecies and singing style were fixed factors (Grafen & Hails 2002). The song variables were approximately normally distributed within each subspecies and singing style, with the exception of SI, which was log-transformed for this analysis. In addition to the seven individual variables, we reduced the dimensionality of the data with a principal component analysis (PCA) from which two principal components (PC) with eigenvalues greater than one were extracted (explaining 67% of the variance; KMO=0.63, Bartlett's test of sphericity: χ^2_{21}=8034, P<0.001; Table S 3.1). The two PCs are easily interpretable (Table S 3.1): PC1 (which explains 37% of the variance) mostly concerns differences in frequency and syllable number whereas PC2 (30% of the variance) mainly represents the differences in components of cadence (intervals between syllables and intervals between songs). Parameters were estimated using restricted maximum likelihood (REML; Type III) in SPSS 21.0 (IBM Corporation, Armonk, NY, U.S.A.) using default parameters. The magnitude of subspecies divergence for each singing style was quantified using estimated marginal means (EMM) for the interaction between style and subspecies, and compared with log-likelihood tests.

To quantify the extent to which males could be assigned to the correct subspecies on the basis of song properties in each singing style, we applied the discriminant function analysis (DFA) on the average values of the song variables for each of the 143 males. A tolerance test was performed to determine whether there were any highly correlated variables (Tabachnick & Fidell 2007). As none of the seven variables were highly correlated (all tolerance values >0.10), we subsequently performed a DFA entering all raw song variables together as independent and subspecies as grouping variable. Post hoc probabilities of correct assignment in DFA were cross-validated by the jackknife method, in which each observation was omitted one at a time, the classification function recalculated with the remaining data, and the omitted observation reclassified (Baker 2011). In all DFAs, we used F tests (Wilk's lambda) to examine whether the overall discriminant models were significant (e.g. Tobias & Seddon 2009).

Finally, we built hierarchical cluster analysis dendrograms to evaluate the extent to which song traits group the sampling areas into subspecies within each singing style. For that, we used all raw variables in a database with average values for each area, selecting the between-groups linkage cluster method and squared Euclidean distance interval measure.

3.4 Results

Acoustic Divergence

According to the GLMMs, all variables were significantly different between subspecies and between singing styles, and significant interactions between subspecies and style were detected for FI, SL and NDS, whereas PC1 (representing variation in frequency and syllable numbers) and NS closely approached the significance threshold (Table 3.2, Figure 3.2). Estimated marginal means and their 95% confidence limits indicate that the divergence between subspecies across singing styles was generally greater for dawn and fast songs than for slow songs (Table S 3.2, Figure 3.2). For instance, the EMM of PC1 was 1.342, 1.175 and 0.520 for dawn, fast and slow songs, respectively. This is further supported by subspecies comparisons within each singing style, which resulted in greater overall significances and subspecies divergence in fast and dawn songs, relative to slow songs (Table S 3.2, Table 3.3). Indeed, when we looked at the overall pattern of the nine investigated song parameters (see Table 3.3), parameters for which at least two of the three subspecies pairwise comparisons differed significantly were much more frequent in the fast (seven of nine) and the dawn (five of nine) singing styles than in the slow (one of nine) singing style. This supports the conclusion that the singing styles under stronger sexual selection (fast and dawn song) have diverged more than the singing style used in another context (slow song).

Table 3.2 - General linear mixed models comparing subspecies and singing styles for each song trait.

Dependent Variable	Effect	*df*1	*df*2	*F* test	*P*
PC1	Intercept	1	132.812	2.937	0.089
	Style	2	133.047	**22.376**	**<0.001**
	ssp	2	132.814	**24.130**	**<0.001**
	Style * ssp	4	133.086	2.324	0.060
PC2	Intercept	1	132.517	**16.616**	**<0.001**
	Style	2	132.738	**340.381**	**<0.001**
	ssp	2	132.519	**3.979**	**0.021**
	Style * ssp	4	132.774	1.939	0.108
Minimum Frequency	Intercept	1	132.657	**11666.604**	**<0.001**
	Style	2	132.856	**3.344**	**0.038**
	ssp	2	132.637	**13.784**	**<0.001**
	Style * ssp	4	132.881	1.001	0.410
Maximum Frequency	Intercept	1	134.471	**31526.241**	**<0.001**
	Style	2	134.649	**7.219**	**0.001**
	ssp	2	134.452	**24.206**	**<0.001**
	Style * ssp	4	134.670	1.942	0.107
Song Length	Intercept	1	132.015	**1908.479**	**<0.001**
	Style	2	132.374	**63.539**	**<0.001**
	ssp	2	131.980	**5.406**	**0.006**
	Style * ssp	4	132.421	**2.750**	**0.031**
First Interval	Intercept	1	132.467	**1354.372**	**<0.001**
	Style	2	132.729	**140.373**	**<0.001**
	ssp	2	132.440	**9.656**	**<0.001**
	Style * ssp	4	132.762	**4.225**	**0.003**
Log$_{10}$(Song Interval)	Intercept	1	132.456	**480.511**	**<0.001**
	Style	2	132.652	**348.310**	**<0.001**
	ssp	2	132.458	2.115	0.125
	Style * ssp	4	132.683	.351	0.843
Number of Syllables	Intercept	1	133.468	**1328.939**	**<0.001**
	Style	2	133.610	**38.683**	**<0.001**
	ssp	2	133.454	**4.241**	**0.016**
	Style * ssp	4	133.627	2.381	0.055
Number of Different Syllables	Intercept	1	132.149	**1715.307**	**<0.001**
	Style	2	132.324	**18.294**	**<0.001**
	ssp	2	132.131	**20.758**	**<0.001**
	Style * ssp	4	132.345	**3.335**	**0.012**

Male was included as a random effect, dependent variables were subspecies (ssp.) singing style (style) and their interaction, and the dependent variables were the two principal components (PC1 and PC2) and the seven variables measured from the songs. *df*1 and *df*2 refer to the numerator and denominator degrees of freedom, respectively. Significant *P* values are shown in bold.

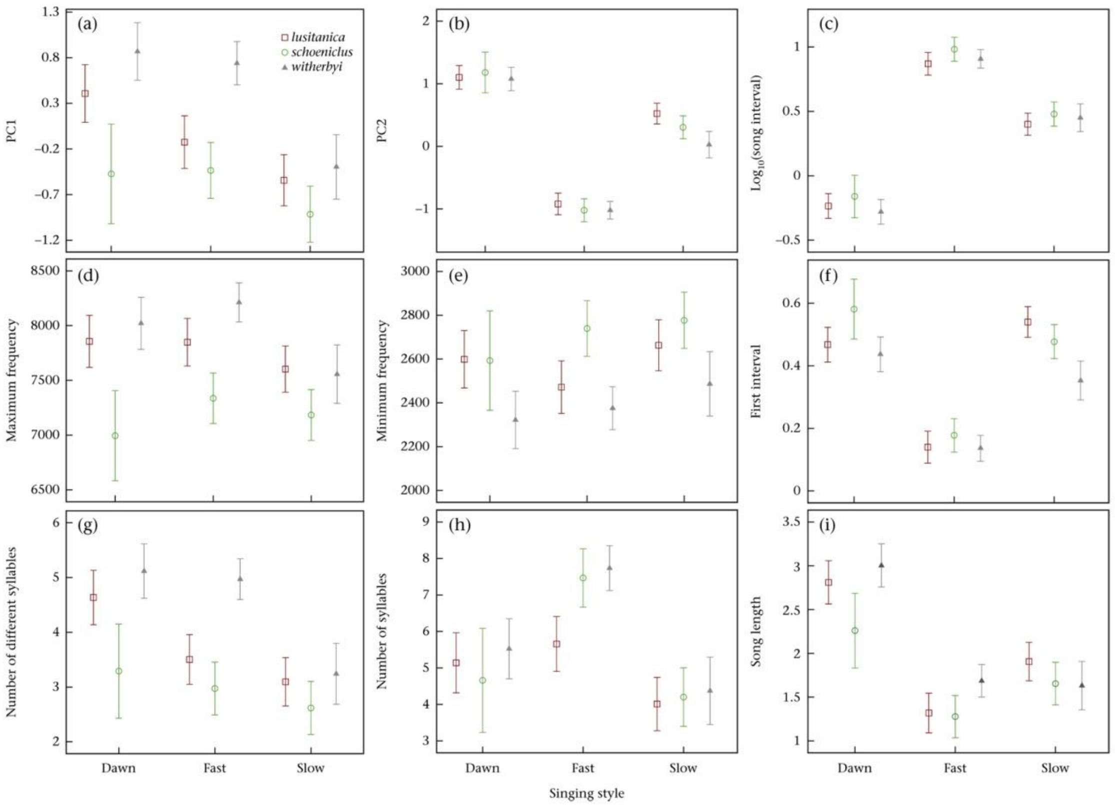

Figure 3.2 - Estimated marginal means and 95% confidence intervals of (a) PC1 (describing differences in frequency and syllable numbers), (b) PC2 (describing differences in intervals between syllables and intervals between songs), (c) $\log_{10}$ (song interval), (d) maximum frequency, (e) minimum frequency, (f) first interval, (g) number of different syllables, (h) number of syllables and (i) song length per subspecies and singing style, derived from general linear mixed models. For statistics see Table 3.2.

Table 3.3 - General linear mixed models comparing subspecies within each singing style.

	Dawn		Fast		Slow	
	F test	*P*	*F* test	*P*	*F* test	*P*
PC1	**12.42** sch≠wit;lus≠wit;sch≠lus	**<0.001**	**15.92** sch≠wit;lus≠wit	**<0.001**	**3.56** sch≠wit	**0.037**
PC2	0.65 none	0.527	**5.35** sch≠wit;sch≠lus	**0.007**	**3.42** lus≠wit	**0.042**
First interval	**6.47** sch≠wit;sch≠lus	**0.004**	2.01 none	0.144	**5.11** sch≠wit	**0.01**
Log10 (song interval)	1.21 none	0.313	2.86 sch≠lus	0.066	0.42 none	0.657
Maximum frequency	**13.70** sch≠wit;sch≠lus	**<0.001**	**18.73** sch≠wit;lus≠wit;sch≠lus	**<0.001**	3.02 sch≠lus	0.059
Minimum frequency	**4.54** lus≠wit	**0.018**	**8.51** sch≠wit;sch≠lus	**0.001**	**6.34** sch≠wit;lus≠wit	**0.004**
Song length	**4.25** sch≠wit;sch≠lus	**0.024**	**9.99** sch≠wit;lus≠wit	**<0.001**	1.07 none	0.351
Number of syllables	**2.98** sch≠wit	**0.066**	**5.71** sch≠lus;lus≠wit	**0.005**	0.28 none	0.755
Number of different syllables	**11.56** sch≠wit;sch≠lus	**<0.001**	**16.01** sch≠wit;lus≠wit	**<0.001**	2.78 none	0.073

The table shows the *F* tests with associated probability (*P*), as well as the subspecies pairs that differed significantly in their estimated marginal means according to log-likelihood tests. lus = *lusitanica*, sch = *schoeniclus*, wit = *witherbyi*. Significant results are shown in bold.

Subspecies Assignment

The DFAs were significant for dawn (Wilk's $\lambda=0.132$, $\chi^2_{14}=58.702$, $P<0.001$), fast (Wilk's $\lambda=0.319$, $\chi^2_{14}=62.915$, $P<0.001$) and slow singing styles (Wilk's $\lambda=0.433$, $\chi^2_{14}=34.326$, $P=0.002$). The three functions showed slightly different abilities to correctly classify cases, with 77.1% for dawn style (80.0% lusitanica, 100.0% schoeniclus, 66.7% witherbyi), 75.4% for fast style (61.1% lusitanica, 87.5% schoeniclus, 77.8% witherbyi) and 74.5% for slow style (78.9% lusitanica, 75.0% schoeniclus, 66.7% witherbyi). Subspecies diagnosis using song traits is presented in Table S 3.3.

Geographical Structure

The hierarchical cluster dendrograms for dawn and fast songs clearly segregated the populations of *schoeniclus* from those of the other two subspecies (Figure 3.3a, b). In addition, the dendrogram for dawn style agreed with the separation of Iberian populations into two subspecies (Figure 3.3a; Atienza 2006). For fast style, the separation between *lusitanica* and *witherbyi* was less clear, as *witherbyi* populations from Mallorca and Ebro clustered within the *lusitanica* clade (Figure 3.3b). As expected from the weaker subspecies discrimination based on slow songs (Table 3.3 and DFAs results above), the dendrogram for slow songs did not segregate the populations of the three subspecies (Figure 3.3c).

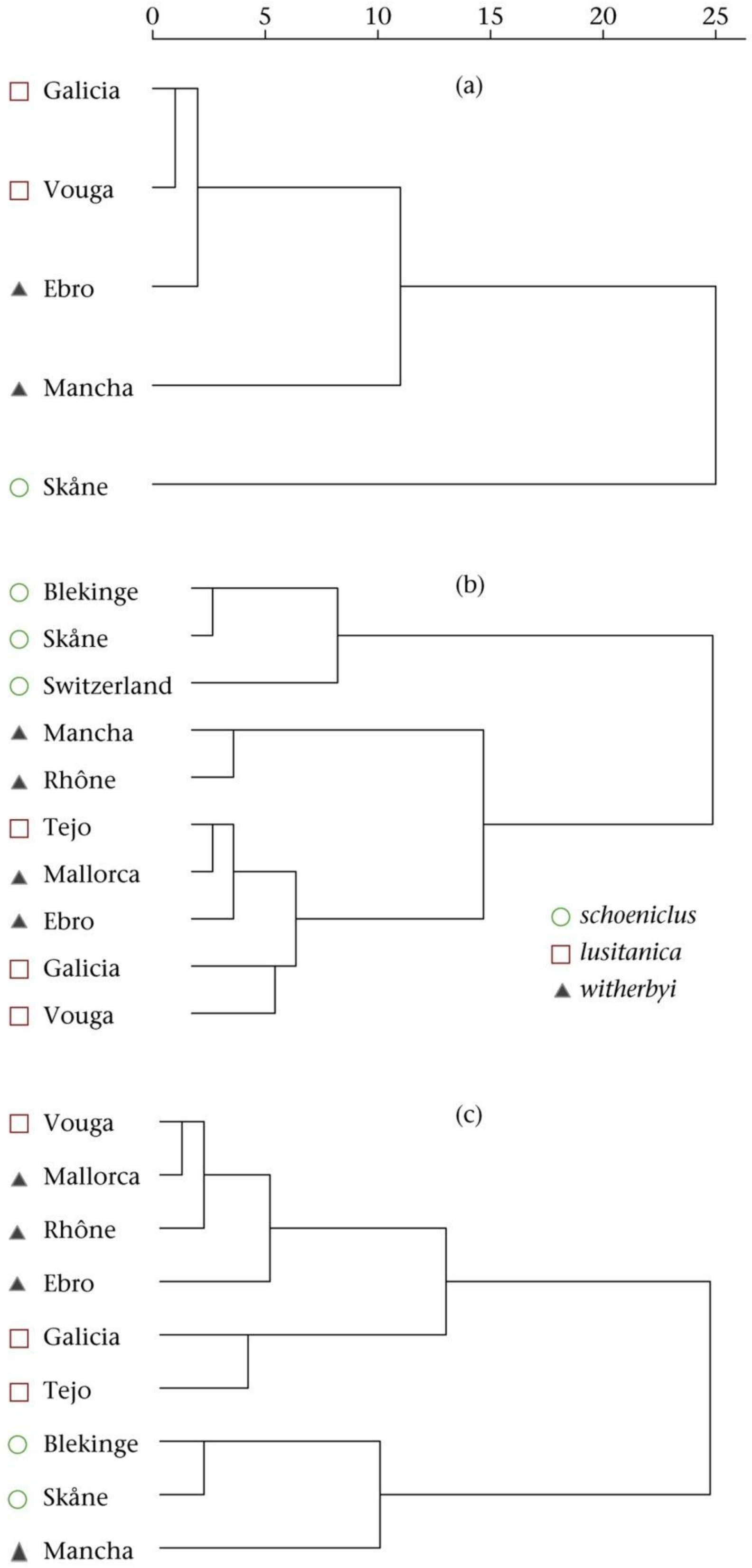

Figure 3.3 - Dendrograms resulting from hierarchical cluster analysis using the between-groups linkage cluster method and squared Euclidean distances to group the sites where (a) dawn, (b) fast and (c) slow songs were recorded, according to the seven raw song variables. White circles = *Emberiza schoeniclus witherbyi*, black triangles = *E. s. lusitanica*, white triangles = *E. s. schoeniclus*. Dawn songs came from only five sites because this singing style was not described until 2009 and we used only fast songs from Switzerland to even the sample size across styles.

3.5 Discussion

Geographical variation in bird vocalizations, such as the large divergence we describe in reed buntings, can arise through several mechanisms, and may have profound consequences for the segregation of populations within a species. In passerines, vocalizations are, to a large extent, learnt from parents and neighbours, but the learning process is imperfect and copying errors are common. In such cases, the appearance of geographical variation in vocalizations depends on the timing of the learning period and on the dispersal of the juveniles (Podos &Warren 2007). However, part of the vocalizations, in particular their general structure (including many of the traits studied here), do not depend on social experience and are subject to evolutionary forces such as selection and drift (Podos et al. 2004). In addition, most bird species often have several kinds of vocalizations that are used in different contexts, and hence may be subject to different evolutionary forces. Thus, the greater divergence of structural traits in dawn and fast singing styles that we describe in reed buntings strongly suggests that sexual selection was an important cause of acoustic divergence in this species. Vocalizations, in particular songs, are important for sexual selection and species recognition among birds (Price 2008). Consequently, geographical differences in such vocalizations among populations may allow individuals to distinguish local birds from immigrants, for example to avoid inbreeding or mating with maladapted individuals (Chapter 4; Bensch et al. 1994; Edmands 2007; Hansson et al. 2004; Keller & Waller 2002; Marr et al. 2002), being highly relevant in the context of the incipient speciation occurring in reed buntings.

Song divergence in reed buntings

In this study, we compared quantitative properties of three singing styles with known functions among populations of three subspecies of reed bunting, which differ morphologically in traits that seem to confer local adaptation (e.g. bill size and shape; Byers et al.1995; chapters 2 & 5). Although subspecies divergence in relation to the singing style varied depending on the particular trait that was measured, it is clear that, overall, dawn and fast songs diverged more extensively than slow songs, as is reflected by the first principal component (PC1) of variation (see Figure 3.2). The number of different syllables contributed most to this pattern, whereas song length and the total number of syllables were particularly divergent in dawn and fast songs, respectively. On the other hand, the first interval, which also showed a significant interaction between subspecies and singing style, seems to have contributed little to the overall pattern, having only a slightly greater divergence in dawn and slow songs, and produced a different relative positioning of the subspecies (see Figure 3.2). Slow songs are less differentiated, but significant differences between the subspecies were nevertheless detected in three of the seven original variables (contrasting with six of seven variables with significant differences for both dawn and fast songs; see Table 3.3). The subspecies *witherbyi* stands out as being particularly divergent from the other two subspecies in most variables, especially in fast songs, as they produce the longest and more varied songs; whereas *lusitanica* is intermediate in most acoustic variables relative to the other two subspecies.

Reed buntings can increase their repertoire, and thus song diversity, between successive years (Suter et al. 2009), but it is unlikely that variation in the proportion of older males affected our results. Plastic changes in some song traits due to seasonal or environmental effects (e.g. increased minimum frequency in response to anthropogenic noise; Gross et al. 2010) are also unlikely to have affected our results because birds were sampled only during the breeding season and all study sites were in natural habitats, distant from loud sources of anthropogenic noise. Additionally, the rather small differences in timing of sampling of each population should not have affected our results because the breeding season is very long, and the singing style potentially showing a greater seasonal variation (fast; for which we have the largest sample size) is actually used later on by mated males in secondary territories (where they try to attract additional social females; LOG & JMN personal observations).

The substantial song divergence found among subspecies (as well as the morphological differences) must have evolved rapidly, as indicated by the star shape of the haplotype network presented by Kvist et al. (2011) supporting a recent expansion from a single glacial refugium of the reed buntings occurring in Europe. Interestingly, our findings oppose the expected latitudinal gradient resulting from higher rates of evolution of syllable diversity and song length in north European passerines than in Mediterranean passerines (Cardoso et al. 2012; Weir & Wheatcroft 2011). Bill morphology has also been shown to affect song output (Christensen et al. 2006; Huber & Podos 2006; Podos 1997; but see Grant & Grant 2002a, b; Slabbekoorn & Smith 2000), so this trait could potentially explain the differences found between subspecies, especially the generally higher song divergence of *witherbyi*, which has the most differentiated bill (Chapter 2). In our study, we found that the thick-billed subspecies *witherbyi* sang songs with the lowest minimum frequency, thin-billed *schoeniclus* sang with the highest minimum frequency, and the intermediate-billed subspecies *lusitanica* used intermediate frequencies. Similar results have been described for Darwin's finches (Huber & Podos 2006; Podos 2001). However, *witherbyi* songs also have a higher maximum frequency than those of *schoeniclus* and, consequently, for dawn and fast styles, a wider frequency bandwidth that does not fit with the expectations from the differences in bill morphology (although this expectation is specific to trilled vocalizations, which are common in this species but were not studied here in detail). In swamp sparrows, *Melospiza georgiana*, it has been suggested that males with larger bills and lower 'song performance' (ability to produce rapid, broadband trills) may compensate for that by increasing song complexity (Ballentine 2006; Cardoso & Hu 2011). Because higher song complexity (larger number of different syllables) was also found in the 'thick-billed' *witherbyi* and was positively correlated with frequency bandwidth (Pearson correlation: r=0.470), it is possible that this subspecies has been selected for higher song complexity in a similar way. A detailed study of song performance, comparing trill rates and frequency bandwidth in the three subspecies, is needed to clarify this issue. In general, evolutionary changes in the quantity of syllables are thought to be due to changes in pressure from sexual selection (Baker 1996; Lynch 1996; Read & Weary 1992). Perhaps the larger number of syllables found in *witherbyi* is the result of a shift in female preferences from vocal performance to song complexity, as suggested for coastal swamp sparrows (Ballentine 2006). In a study restricted to the fast singing style, Matessi et al. (2000b) also found higher syllabic complexity in thick-billed subspecies (*intermedia* and *witherbyi*) and a strong tendency for higher minimum frequency in the thin-billed *schoeniclus*. The fact that dawn and fast songs are relatively complex agrees with these styles being under sexual selection, because the production of complex songs has been associated with female mate choice in many previous studies (e.g. Brunner & Pasinelli 2010; Catchpole & Leisler 1996; Forstmeier & Balsby 2002; Hasselquist et al. 1996; Neubauer 1999).

Nevertheless, the causes of the differential song divergence relative to singing style in reed buntings are unclear. The relative importance of imitation and improvisation could vary with singing style, and this cultural evolution may be dependent on whether the particular singing style is constrained by selection. Indeed, the song of two species of *Cistothorus* wrens, which differ in the relative importance of imitation and improvisation at the syllable level, generated different geographical patterns of song variation (Kroodsma & Verner 1978). In addition, a population of chestnut-sided warblers, *Dendroica pensylvanica*, studied across time showed a high rate of cultural evolution in the singing style unconstrained by sexual selection, and a low rate in a sexually selected style (Byers et al. 2010). Other Nearctic warblers (e.g. Bay 1999; Janes & Ryker 2006, 2011; Moldenhauer 1992), however, fit our prediction of greater divergence in singing styles under sexual selection. Overall, this prediction does not seem to hold for species in which such singing styles have a highly variable syntax (Martens 1996; Salomon & Hemim 1992) or contain syllables not used for intrasexual countersinging (Byers 1996; Byers et al. 2010; Kroodsma 1981; Kroodsma 1996; Lein 2008; Nelson 1992; Nelson & Croner 1991; Soha et al. 2009). Different types of sound analysis can generate different patterns (e.g. Baker & Logue 2003) and distinct methods have been used in the abovementioned studies, so comparisons with our study may have limited value.

Geographical Congruence Between Morphology and Song

In the hierarchical cluster dendrogram for dawn songs (Figure 3.3a) the grouping can be explained by either geographical or subspecies divergence. However, the dendrogram for fast singing style shows that songs from birds in central Iberia are more similar to songs of geographically distant birds from southern France than to songs of birds from the nearby western Iberia (Figure 3.3b). This suggests an abrupt acoustic change, rather than clinal variation, although the location of sampling sites is not ideal to distinguish these patterns. The island population of *witherbyi* clustered within *lusitanica*, possibly due to undersampling or to the effects of insularity (Baker et al. 2001; Griffith 2000; Marler & Slabbekoorn 2004). The fact that the well-sampled Ebro population of *witherbyi* also clustered within *lusitanica* is harder to explain, but may be due to side-effects of the small and undersampled *lusitanica* population of Tejo on clustering. Like geographical distance, habitat connectivity is probably not a confounding parameter, because reed beds are a rare and highly fragmented habitat in southwestern Europe, so connectivity between all 10 populations sampled is low to nonexistent (see also Gammon et al. 2005; Laiolo et al. 2008). However, many *schoeniclus* migrate to spend the winter in the Mediterranean area, using many reed bed patches during their annual cycle and co-occurring with *lusitanica*, *witherbyi* and other subspecies at their wintering quarters (Byers et al. 1995; Cramp & Perrins 1994; Chapter 2). Hence, *schoeniclus* males could potentially learn the songs of *lusitanica* and *witherbyi* among others, especially the fast songs, which start to be used by local residents before *schoeniclus* departs to the breeding areas (LOG & JMN, personal observations). Nevertheless, large differences between the subspecies were found, especially between *schoeniclus* and the two resident subspecies (Figure 3.3b).

Evolutionary Implications

Given that reed bunting subspecies seem to be at an incipient stage of speciation (Matessi et al. 2000a; chapters 2 & 5), the finding of greater divergence in sexually selected singing styles is interesting, suggesting that sexual selection may be playing an important role in this process (probably in conjunction with natural selection). Moreover, some of the song traits measured in this study do not seem to rely on social experience for development (Ewin 1978; Glutz von Blotzheim & Bauer 1997; Stewart 1955; Thorpe 1964), and are likely to be targets of selection potentially leading to an evolutionary response (i.e. of song traits within populations/subspecies). Several studies suggest that sexual selection promotes the evolution of reproductive isolation and two recent meta-analyses found small but significant overall trends (Kraaijeveld et al. 2011; Seddon et al. 2013). For instance in antbirds (Thamnophilidae), a positive relationship was found between species diversity and the intensity of sexual selection, measured by the production of lower pitched and more complex songs (Seddon et al. 2008). When divergence between populations in singing styles related to female attraction is greater than in other styles, as shown in our study and for some Nearctic warblers (Bay 1999; Janes & Ryker 2006, 2011; Moldenhauer 1992), there seems to be an enhanced potential for premating reproductive isolation to evolve. Indeed, population differences in sexually selected singing styles seem to be associated with different signal perceptions in reed buntings, and may be causing some level of reproductive isolation between the subspecies (Chapter 4).

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3.8 Supporting Information

Table S 3.1 - Factor loadings on principal components (PC) for acoustic measurements.

	PC1	**PC2**
Eigenvalue	2.601	2.112
% Variance	37.159	30.166
Minimum Frequency	-0.577	0.080
Maximum Frequency	0.678	-0.182
Song Length	0.678	0.632
First Interval	-0.188	0.862
Number of Syllables	0.705	-0.397
Number of Different Syllables	0.895	0.083
Log_{10}(Song Interval)	-0.124	-0.874

Acoustic traits were taken from song spectrograms of *Emberiza schoeniclus lusitanica*, *E. s. schoeniclus*, and *E. s. witherbyi* (143 males, up to 20 songs per male).

Table S 3.2 - Estimated marginal means (EMM) of the GLMMs comparing subspecies and singing styles for each song trait.

Variable	**Style**	**subspecies**	**EMM**	**SE**	***df***	**95% Confidence Interval**	
						Lower Bound	**Upper Bound**
PC1	Dawn	*lusitanica*	0.407	0.159	132.279	0.092	0.723
		schoeniclus	-0.474	0.276	131.019	-1.019	0.071
		witherbyi	0.868	0.159	131.597	0.553	1.183
	Fast	*lusitanica*	-0.126	0.146	133.287	-0.414	0.163
		schoeniclus	-0.436	0.155	133.943	-0.743	-0.13
		witherbyi	0.739	0.119	134.569	0.503	0.976
	Slow	*lusitanica*	-0.544	0.142	131.851	-0.824	-0.264
		schoeniclus	-0.916	0.156	137.318	-1.224	-0.608
		witherbyi	-0.396	0.179	133.844	-0.75	-0.043
PC2	Dawn	*lusitanica*	1.102	0.095	132.014	0.914	1.291
		schoeniclus	1.18	0.164	130.835	0.855	1.505
		witherbyi	1.076	0.095	131.376	0.888	1.264
	Fast	*lusitanica*	-0.922	0.087	132.956	-1.094	-0.749
		schoeniclus	-1.022	0.092	133.574	-1.205	-0.839
		witherbyi	-1.022	0.071	134.161	-1.162	-0.881
	Slow	*lusitanica*	0.523	0.084	131.613	0.356	0.69
		schoeniclus	0.304	0.093	136.742	0.12	0.488
		witherbyi	0.026	0.107	133.497	-0.185	0.237
MinF	Dawn	*lusitanica*	2598.736	66.365	132.299	2467.463	2730.01
		schoeniclus	2592.802	114.68	131.11	2365.94	2819.664
		witherbyi	2321.613	66.268	131.559	2190.523	2452.703
	Fast	*lusitanica*	2471.605	60.643	132.82	2351.655	2591.555
		schoeniclus	2739.541	64.457	133.872	2612.055	2867.028
		witherbyi	2375.585	49.596	133.655	2277.49	2473.681
	Slow	*lusitanica*	2662.945	58.91	131.817	2546.413	2779.477
		schoeniclus	2776.751	64.862	137.066	2648.491	2905.011
		witherbyi	2486.556	74.36	133.337	2339.479	2633.633
MaxF	Dawn	*lusitanica*	7856.514	120.308	134.146	7618.569	8094.46
		schoeniclus	6994.513	207.956	133.089	6583.187	7405.839
		witherbyi	8021.11	120.155	133.488	7783.456	8258.765
	Fast	*lusitanica*	7848.267	109.921	134.608	7630.872	8065.661
		schoeniclus	7336.409	116.805	135.552	7105.413	7567.406
		witherbyi	8212.446	89.88	135.354	8034.695	8390.197
	Slow	*lusitanica*	7603.011	106.807	133.717	7391.762	7814.26
		schoeniclus	7183.558	117.45	138.41	6951.33	7415.786
		witherbyi	7556.62	134.767	135.088	7290.094	7823.145
SL	Dawn	*lusitanica*	2.81	0.125	131.411	2.563	3.057
		schoeniclus	2.26	0.215	129.166	1.833	2.686
		witherbyi	3.003	0.125	130.023	2.757	3.25
	Fast	*lusitanica*	1.319	0.114	132.437	1.093	1.545
		schoeniclus	1.278	0.122	134.281	1.037	1.518
		witherbyi	1.686	0.094	133.949	1.501	1.872
	Slow	*lusitanica*	1.906	0.111	130.53	1.686	2.125
		schoeniclus	1.654	0.123	140.053	1.411	1.898
		witherbyi	1.631	0.14	133.105	1.353	1.908
FI	Dawn	*lusitanica*	0.468	0.028	132.005	0.412	0.523
		schoeniclus	0.581	0.048	130.41	0.486	0.677
		witherbyi	0.437	0.028	131.015	0.381	0.492
	Fast	*lusitanica*	0.14	0.026	132.717	0.089	0.191
		schoeniclus	0.178	0.027	134.09	0.124	0.231
		witherbyi	0.137	0.021	133.82	0.095	0.178

Variable	Style	subspecies	EMM	SE	*df*	95% Confidence Interval	
						Lower Bound	Upper Bound
	Slow	*lusitanica*	0.54	0.025	131.367	0.491	0.589
		schoeniclus	0.477	0.027	138.307	0.423	0.532
		witherbyi	0.353	0.031	133.325	0.291	0.415
LogSI	Dawn	*lusitanica*	-0.235	0.048	132.007	-0.331	-0.139
		schoeniclus	-0.161	0.084	130.97	-0.327	0.004
		witherbyi	-0.28	0.048	131.446	-0.376	-0.184
	Fast	*lusitanica*	0.869	0.044	132.834	0.782	0.957
		schoeniclus	0.982	0.047	133.385	0.889	1.075
		witherbyi	0.907	0.036	133.902	0.836	0.979
	Slow	*lusitanica*	0.4	0.043	131.652	0.315	0.486
		schoeniclus	0.479	0.047	136.186	0.385	0.573
		witherbyi	0.45	0.054	133.341	0.343	0.558
NS	Dawn	*lusitanica*	5.14	0.417	133.207	4.317	5.964
		schoeniclus	4.66	0.72	132.37	3.235	6.085
		witherbyi	5.525	0.416	132.685	4.701	6.348
	Fast	*lusitanica*	5.656	0.381	133.569	4.904	6.409
		schoeniclus	7.466	0.404	134.325	6.666	8.265
		witherbyi	7.735	0.311	134.163	7.12	8.35
	Slow	*lusitanica*	4.012	0.37	132.865	3.28	4.744
		schoeniclus	4.201	0.406	136.603	3.398	5.004
		witherbyi	4.373	0.466	133.971	3.45	5.295
NDS	Dawn	*lusitanica*	4.636	0.251	131.83	4.138	5.133
		schoeniclus	3.29	0.435	130.79	2.43	4.15
		witherbyi	5.117	0.251	131.182	4.621	5.614
	Fast	*lusitanica*	3.503	0.23	132.284	3.049	3.957
		schoeniclus	2.972	0.244	133.213	2.489	3.454
		witherbyi	4.97	0.188	133.019	4.598	5.341
	Slow	*lusitanica*	3.095	0.223	131.407	2.654	3.537
		schoeniclus	2.617	0.245	136.025	2.132	3.102
		witherbyi	3.241	0.282	132.756	2.684	3.798

Results are shown for each acoustic variable, subspecies and singing style resulting from the general linear mixed models, where male identity was included as a random effect (see also Table 3.2). MinF = minimum frequency, MaxF = maximum frequency, SL = song length, FI = first interval, SI = song interval, NS = number of syllables; NDS = number of different syllables.

Table S 3.3 - General rules for separating typical songs of the three subspecies (ssp.) for each singing style.

Style/ Ssp. ≠	lus ≠ sch	lus ≠ wit	sch ≠ wit
Dawn	NS < 4 = sch, FI < 0.5 = lus	NDS & NS < 5 = lus	MaxF < 7500 = sch, NDS < 4.5 = sch
Fast	MinF > 2600 Hz = sch	NDS < 4 & NS < 7 = lus	MaxF < 7600 = sch, NDS < 4 = sch
Slow	FI < 0.5 = sch	FI > 0.5 = lus	MaxF < 7300 = sch, NDS > 3 = wit

The table shows rules based on the GLMM results summary (Table 3.2) and on 95% confidence intervals built for our sample (Table S 3.2). lus = *lusitanica*, sch = *schoeniclus*, wit = *witherbyi*. For dependent variable abbreviations see Table S 3.2. In all styles, *schoeniclus* songs can be distinguished from those of *witherbyi* by a combination of MaxF lower than 7.3 kHz and NDS lower than 3. For slow songs, FI above 0.5 s is typical of *lusitanica*, whereas FI under 0.4 s is exclusive of *witherbyi*. Finally, dawn and fast songs of *lusitanica* can be separated from those of *witherbyi* by the lower NS and NDS, and from those of *schoeniclus* by the FI (<0.5 = *lusitanica* for dawn) and MinF (>2.6 kHz = *schoeniclus* for fast). Accurate identification of less typical songs using multivariate discriminant functions is possible for fast and, especially, for dawn songs, but not for slow songs.

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Chapter 4

Asymmetric song recognition between recently diverged subspecies of reed bunting

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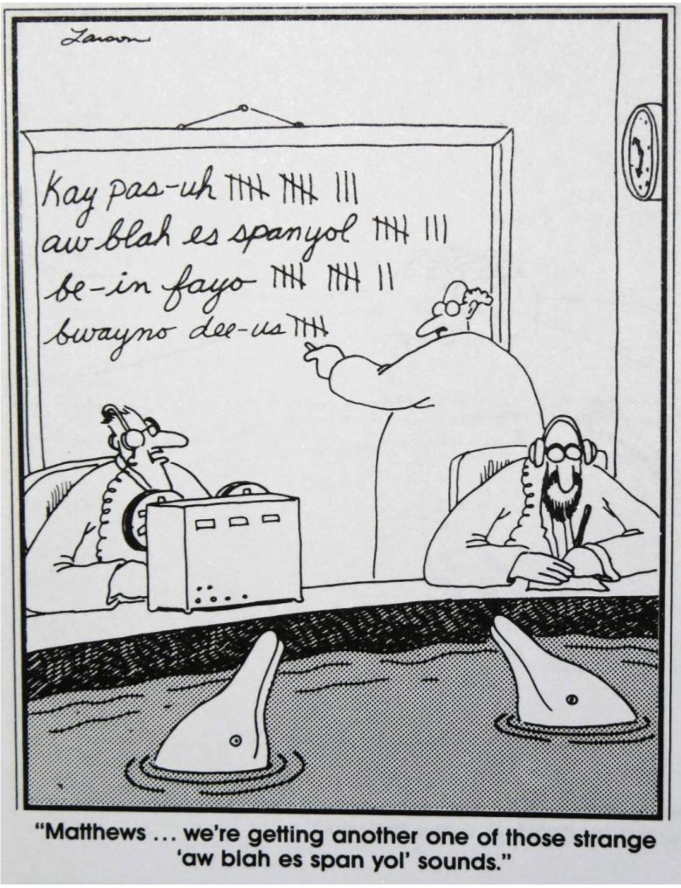

Cartoon 5 - Song recognition studies in dolphins (from Larson 1986)

4 Asymmetric song recognition between recently diverged subspecies of reed bunting

4.1 Abstract

Acoustic divergence among populations may result in assortative mating, behavioral isolation, and speciation. In birds, the recognition of suitable mates depends to a large extent on learning, generally resulting in a tendency to discriminate against nonlocal stimuli. However, there may be geographical variation in the discrimination against nonlocal stimuli, and this may allow inferring the mechanisms behind the evolution of vocal recognition. We tested territorial males of 3 west European subspecies of reed bunting (*Emberiza schoeniclus schoeniclus*, *Emberiza schoeniclus lusitanica*, and *Emberiza schoeniclus witherbyi*) using song playback to determine the level of song discrimination. We found that *witherbyi* and, to some extent *lusitanica*, males largely ignored *schoeniclus* songs. However, *witherbyi* reacted less strongly to the songs of *lusitanica* than *lusitanica* did to songs of *witherbyi*. In contrast, *schoeniclus* males did not discriminate the songs of the different subspecies, reacting strongly to all. Differential territorial defense behavior suggest that intruding males with different songs do not represent the same competitive threat, and provide evidence of premating reproductive isolation among these recently evolved subspecies. The high discrimination exhibited by *witherbyi* and *lusitanica* seems associated with the high level of local adaptation. Overall, the pattern of premating reproductive isolation appears to agree more with the ecological than with the neutral genetic divergences between subspecies, suggesting that there is an ongoing process of ecological speciation in this study system.

4.2 Introduction

The combination of natural and sexual selection can promote rapid evolution of local adaptation and reproductive isolation, even in the presence of gene flow (van Doorn et al. 2009; Wilkins et al. 2013), although in birds the importance of this process is debated (Price 2008). Sexual preferences for traits such as birdsong may evolve simultaneously with divergent ecological selection and cause assortative mating between ecotypes, further strengthening divergence (Nosil 2012). In some circumstances, birdsong can be genetically associated (by pleiotropy) with morphological (“magic”) traits such as bill size that diverged due to natural selection, potentially leading to assortative mating, reproductive isolation, and speciation (Podos 2001; Servedio et al. 2011). However, behavioral isolation depends on the evolution of recognition, which can be measured by the response toward the individuals of one group (as compared with individuals of another group), with species and subspecies recognition being an extension or form of mate recognition (Ryan & Rand 1993; Mendelson and Shaw 2012; 2013 but see Padian & Horner 2013).

The development of assortative mating, a tendency to mate with own kind, is a critical step in speciation (Price 2008). In most bird species, the recognition of suitable mates depends to a large extent on learning (Hultsch & Todt 2004; Verzijden et al. 2012) in which, among other processes, juveniles may imprint on species-specific traits during a sensitive period, leading to a sexual preference for members of their own species when reaching adulthood (Balakrishnan et al. 2009). In birds and other animals, the identification of suitable mates is based on traits such as morphology and song (Searcy 1992; Collins 2004), as shown by cross-fostering experiments that result in complete assortative mating (Clayton 1990; Slagsvold et al. 2002). As male territorial behavior responses are often correlated with female preferences (e.g., Searcy et al. 1997), song playback experiments allow inferring the level of pre- mating reproductive isolation among populations/taxa.

Most studies on the perception of sexual signals across populations have found that individuals discriminate against nonlocal stimuli (e.g., Uy et al. 2009; Brumm et al. 2010), but there are a few exceptions (e.g., Baker 1982; Balaban 1988). In species with distinct singing styles, song discrimination by males has been shown to occur even for the singing style directed mostly to females (e.g., Regelski & Moldenhauer 1996; Matessi et al. 2000b, 2001). In a few bird species, it has been shown that song is a reliable signal of bill morphology and that individuals display stronger response to songs of males with similar bill size (Christensen et al. 2010). In the latter case, positive assortative pairing based on bill size has been found (Christensen & Kleindorfer 2007).

The majority of research has focused on single populations, but when the levels of discrimination between own and foreign songs are compared between populations, one of 4 main patterns of geographical variation will emerge (Colbeck et al. 2010): 1) symmetric discrimination, in which nonlocal signals elicit a uniform response across populations; 2) asymmetric self-assessment, in which individuals in some populations respond more strongly to all stimuli than individuals in other populations; 3) asymmetric opponent assessment, in which individuals from some populations are perceived to be of higher quality and are responded to more strongly (or weakly) across populations; and 4) asymmetric nonlocal recognition, in which the difference in how local and nonlocal signals are perceived (and responded to) is reduced in some populations due to recognition errors. Self-assessment and opponent assessment are the main causes of different responsiveness within a dialect (assessments based on the quality of the respondent and of the quality of the challenger, respectively), but across dialects responsiveness varies with assessment strategy and decreases with increasing dissimilarity to the local signal (Colbeck et al. 2010).

Three main proximate causes of asymmetric responses have been described (Dingle et al. 2010): 1) relaxation of female choice, 2) intrasexual interactions, and 3) skewed perceptual sensitivity. These behavioral mechanisms, together with the ultimate mechanisms described above, are all probably important to speciation.

We have studied song recognition and its geographical variation in the reed bunting (*Emberiza schoeniclus*), a Palearctic passerine with circa 20 subspecies described on the basis of differences in bill size, body size, and plumage color (Cramp and Perrins 1994; Byers et al. 1995; Chapter 2). Song has also been shown to differ at least among some subspecies (Matessi et al. 2000a; Chapter 3), especially in singing styles under sexual selection (Chapter 3), and local song dialects may exist (e.g., Ehrengruber et al. 2006). This is a particularly interesting species for studies of reproductive isolation, as its subspecies differentiated morphologically very rapidly still sharing many genetic polymorphisms, but do not seem to interbreed in a contact zone in the Alps (Grapputo et al. 1998; Kvist et al. 2011), thus being at an incipient stage of speciation. Moreover, bill size and shape are partly associated with diet, which differs between northern (thin-billed) and southern (thick-billed) subspecies (Chapter 5). Therefore, bill size could be acting as a “magic trait” of speciation (viz. Podos 2001). However, previous studies of song recognition in reed buntings focused only on 1 subspecies, either *schoeniclus* (Ewin 1978; Matessi et al. 2000b) or *intermedia* (including *witherbyi*, Matessi et al. 2001). Hence, song recognition studies between these subspecies have so far not been conducted. Thus, there is still little information about the patterns of song discrimination across populations and subspecies.

In this study, we aimed to find the possible role of song and song discrimination in the premating reproductive isolation mechanisms among 3 West European subspecies of reed bunting: the intermediate-billed *Emberiza schoeniclus lusitanica* (hereafter *lusitanica*), the thin-billed *Emberiza schoeniclus schoeniclus* (hereafter *schoeniclus*), and the thick-billed *Emberiza schoeniclus witherbyi* (hereafter *witherbyi*), which probably constitute the closest link between the southern, resident, thick-billed forms, and the northern, migratory, thin-billed forms (chapters 2 and 5). Genetic divergence (Φ_{ST} from mtDNA) is small between *schoeniclus* and the 2 other subspecies (0.05 to *witherbyi*, 0.04 to *lusitanica*), but is relatively large between *witherbyi* and *lusitanica* (0.14) (Kvist et al. 2011). Subspecies *lusitanica* breeds in costal northwestern Iberia; *witherbyi* across eastern Iberia, Balearics, southern France and, at least formerly, North Africa and Sardinia; and *schoeniclus* is widespread from France and the UK north and eastwards to beyond the Western Palearctic (Figure 4.1). Historically, there were probably contact zones between *lusitanica* and *witherbyi* in the upper Ebro river valley, and between *lusitanica* and *schoeniclus* in the coastal western Pyrenees, but in the late 1990s, *lusitanica* disappeared from these areas (Atienza 2006). Currently, there is no known contact zone between subspecies *witherbyi* (stricto sensu, i.e., excluding *intermedia*) and *schoeniclus* either (Issa & Muller 2015). We used song playback experiments to 1) test how territorial males react to songs of their own subspecies versus to songs of 2 distinct foreign subspecies (i.e., investigate to what extent there is discrimination within and between subspecies); 2) determine whether discrimination is symmetric or asymmetric across the 3 subspecies (and discuss the possible mechanisms involved from the pattern of asymmetry); and 3) assess whether the variation in responses across subspecies (i.e., proxies for premating reproductive isolation) is best explained by morphological/ecological factors (e.g., beak size/food niche) or by phylogenetic distance between the 3 subspecies.

4.3 Methods

Experimental design and test song files

The experiments with *lusitanica* males were conducted in 2010, 2011, and 2012, between 18 March and 21 June, in *Phragmites australis* reed beds at Salreu (40º44'N, 8º35'W), Aveiro Lagoon, Portugal. The experiments with *witherbyi* males were conducted in 2012, between 25 May and 3 June, in reed beds of Illa de Buda (40º42'N, 0º51'E) and in great fen-sedge (*Cladium mariscus*) stands of Vilacoto (40º38'N, 0º38'E), Ebro Delta, Spain. The experiments with *schoeniclus* males were conducted in 2013, between 27 May and 11 June, in reed beds of 3 wetlands located in southwestern Skåne, Sweden: Krankesjön (55º42'N, 13º29'E), Löddesnäs (55º43'N, 12º59'E), and Falsterbo (55º23'N, 12º52'E). The different lengths of sampling periods at the 3 European areas should not influence the results, as the magnitude of the reactions of territorial reed bunting males to playback songs does not change through the season (Ewin 1978).

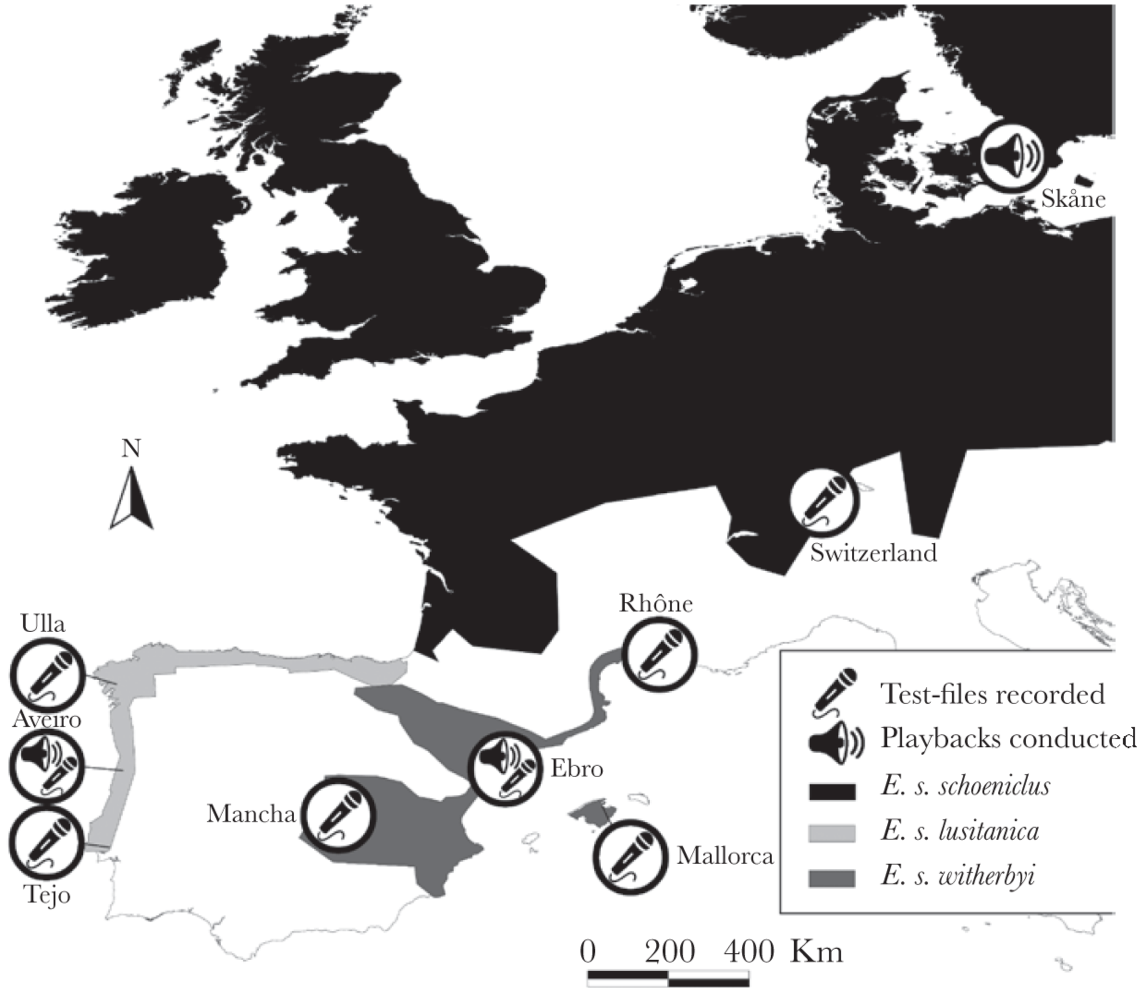

Figure 4.1 - European breeding range of the studied subspecies of reed bunting (*Emberiza schoeniclus schoeniclus*, *Emberiza schoeniclus witherbyi*, and *Emberiza schoeniclus lusitanica*), locations where sound recordings used in test files were obtained, and areas where playback experiments were conducted. Iberian range according to Atienza (2006), rest of European distribution from Byers et al. (1995).

We compared the reactions of territorial males to the playback of songs from 3 subspecies: *lusitanica*, *witherbyi*, and *schoeniclus*. Playback experiments simulate secondary contact (Seddon & Tobias 2007) and have previously been used to evaluate song discrimination in a wide variety of animals, ranging from invertebrates (e.g., Fonseca & Revez 2002) to mammals (e.g., Waser 1975). To avoid pseudoreplication (see Kroodsma 1989, 1990; Kroodsma et al. 2001) and response-intensity bias due to variation in signal performance (de Kort et al. 2009), test song files were built from songs of 12 different males of each subspecies. To avoid bias from any local dialects within subspecies, we used male songs from several populations across the subspecies distributions (Dingle et al. 2010). Because we were not interested in neighbor–stranger differences within populations (inc. dear–enemy relationships), males were exposed to songs of other males from their own population, but never to songs of familiar individuals (cf. Searcy et al. 2014). Most songs used in test files were less than 10 years old, so the fact that some species respond more strongly to current than to historical (>20 year old) songs (Derryberry 2007) probably did not skew our results. In some species that respond strongly to local call variants but not to foreign variants, the call variants in a 5-year interval appear to be stable (Nicholls 2008). Files were prepared as described by Matessi et al. (2000b), but directly from digital recordings using the Audacity 1.3 software. Songs were filtered using a 2000–9050 Hz bandpass and peak amplitude was normalized to 30 000 u in all test files (Amrhein & Lerch 2010). Only songs of the fast (or type I) singing style, used by males to attract a social female, were used in the song files (the other 2 song types are used by males to obtain extrapair copulations [type II] and as an all clear signal to nest-attending females [type III] and are not relevant for territory defense and social mate attraction; see Chapter 3). Presentation order of the 3 files to each male was randomized to counterbalance any order effects (McGregor et al. 1992; McGregor 2000). This was done by sorting all files in a random order and, in the first trial, consecutively presenting the first files of the first, second, and third subspecies; the second trial started with the second file of the second subspecies and so forth. Details of the recordings used to build the 36 test files are given in Table 4.1. We used *lusitanica* recordings from northwestern Portugal and Spain, *witherbyi* recordings from eastern Spain and southern France, and *schoeniclus* recordings from northern Switzerland (part from Brunner & Pasinelli 2010). The songs were selected for high recording quality, that is, low background noise and no masking by vocalizations of other birds.

Previous studies indicate that fast songs of the 3 subspecies differ in frequency and structure, and so discrimination is possible. Briefly, Matessi et al. (2000a) showed that *schoeniclus* songs have a lower total number of syllables and a lower number of different syllables than the songs of *witherbyi* (grouped together with the subspecies *intermedia* by these authors). We found similar differences between the fast songs of *lusitanica* and *witherbyi*, plus a higher maximum frequency in the songs of the latter (Chapter 3). It should be noted, however, that within the range of *schoeniclus*, the total number of syllables in each song seems variable, with lower values in northern Switzerland (Brunner & Pasinelli 2010) than in any of the 7 areas sampled by Matessi et al. (2000a). In our study, the populations of *lusitanica* and *witherbyi* were tested with songs from the same population and from other populations of the same subspecies, whereas the population of *schoeniclus* was tested only with songs from other populations. This happened because, when the experiments with *schoeniclus* were conducted, no high-quality local fast songs were available. Later, we were able to record 3 *schoeniclus* males from the population tested (see Table 3.1) and to compare them with 13 nonlocal males. We concluded that there were no significant differences in songs between them (general linear mixed model [GLMM] on PC1, explaining 42% of variance in 7 song parameters, setting country as fixed factor and male as random factor. Type III test of the fixed effect: $Z_{1,14.113} = 2.118$; $P = 0.167$).

Table 4.1 - Details of recordings used to build test files, including recording locations, number of recordings of different males from each location used (number of males), recording years, and recordists (source)[a]

Subspecies	Area	Coordinates	No. males	Years	Source
lusitanica	Ulla	42º42'N, 8º41'W	1	2004	EM
	Aveiro	40º50'N, 8º37'W	6	2010	LG
	Tejo	38º51'N, 8º58'W	5	2010-11	LG
witherbyi	Rhône	43º26'N, 4º31'E	2	1983	JR
	Ebro	40º39'N, 0º45'E	3	88,94,02	EM,SA
	Mallorca	39º47'N, 3º06'E	3	2001-03	EM,SA
	Mancha	39º31'N, 3º19'W	4	2004, 2011	EM,LG
schoeniclus	Greifensee	47º21'N, 8º41'E	2	2006	PB
	Pfäffikersee	47º21'N, 8º47'E	6	2006	PB
	Sempachersee	47º07'N, 8º11'E	4	2009-10	GP

[a]Coordinates are in latitude/longitude, WGS84 datum. Recordings by Eloisa Matheu (EM), Luís Gordinho (LG), Jean Roché (JR), Sound Approach to birding team (SA), Patrick Brunner (PB), and Gilberto Pasinelli (GP).

Field experiments and response measures

The 16-bit WAV files were played using an Edirol R09 digital recorder connected to a Logitech Pure-Fi Anywhere II iPod dock (speaker) via a double 3.5 jack cable. For playback in the field, the speaker was set on a hand-cut wood board screwed to the top of a 1-m-high pole. Stability was provided by elastic cables and camouflaged by an olive-green hood covering the setup. In Sweden, a more portable setup was used: Sunstech Dedalo player and Conceptronic CLLSPKTRV speaker. Sound pressure levels of both setups were measured using a Brüel & Kjaer 2238 Mediator. We used an output level of 65-dB sound pressure level at 10 m, lower than that of natural song (Matessi et al. 2000b, 2007). Because a 10-fold change in pressure is equal to 20 dB (Simmons et al. 2003), that corresponds to about 85 dB at 1 m. Both setups had a very similar acoustic performance (directionality and fidelity, including signal to noise ratio and frequency range).

During the experiments, the speaker was placed near a song post regularly used by a male reed bunting (that we had located previously). Two minutes of silence were included in the beginning of all test files, to allow the observer to get away from the speaker after setup and before the playback started. An experiment was started when 1) the test male was within hearing range of the speaker and 2) the male did not sing himself. A single observer (LOG) performed all playback experiments, standing approximately 25 m away from the speaker. An experiment was considered successful when the tested male approached the speaker (to within 30 m or less, see below) at least during one of the subexperiments; unsuccessful experiments were excluded.

At Aveiro Lagoon, 12 successful unmatched playback experiments were conducted with different males in May 2010 (following Matessi et al. 2000b, 2001 to ensure comparability). A single *lusitanica* or *witherbyi* song was used in each experiment with presentation order randomized. Because unmatched experiments would not allow us to control for male personality (Amy et al. 2010), territory habitat structure (Barker et al. 2009) or early parasite exposure (Bischoff et al. 2009), in late May and June 2010, we conducted 13 successful paired experiments testing each male (different males from previous unmatched experiments) with both *lusitanica* and *witherbyi* recordings. To increase the scope and replication of our study, during springs of 2011 and 2012, we conducted 23 successful triple playback experiments testing each male with recordings of *lusitanica*, *witherbyi*, and *schoeniclus*. In Ebro Delta, 29 different males were tested with 3 recordings, one of each of the subspecies under study, but 2 experiments were considered invalid (because some of the males' movements were toward nests with young) and 16 males were completely unresponsive. Thus, 11 successful experiments carried out at the Ebro Delta remained, corresponding to 33 subexperiments. Finally, in Skåne, 25 triple experiments with different males were conducted, of which 14 were successful.

All unmatched experiments (and subexperiments in the matched designs) included 2 min of song playback followed by 2 min of silence and, during this period, we collected 9 variables. These included the following 6 continuous variables (Matessi et al. 2001): latency of approach (the time it took for a male to first approach the speaker and to get within 30 m of it, in seconds), minimum distance from the speaker during and after playback (both in meters), time spent within 10 m of the speaker during and after playback (both in seconds), and latency of song (the time it took for a male to start singing after the playback started, in seconds). The following 3 categorical (binary) variables were also collected: approach, song during playback, and song after playback (all presence/absence). Songbird studies show that the distance to the speaker is often a significant predictor of attack, whereas most measures of singing behavior (including song-type matching, type-switching frequency, and song rate) are not (Searcy et al. 2006).

In the raw data, several values are missing in the following 4 response variables: latency of approach, latency of song and minimum distance to the speaker, both during and after playback. If a male did not approach the speaker (down to 30 m) or if it did not sing during the experiment (240 s), no value was recorded for the first 2 variables. Also, if a male did not respond to the playback and was not heard or seen, no minimum distance to the speaker was estimated. Because the nonparametric tests do not allow missing values, semiarbitrary values were used in those 4 situations: 241 s in the first 2 cases, and 100 m in the last two. Regarding the distances, a second and less conservative approach was also used: In the matched experiments and for subexperiments with no response, a minimum distance equal to the largest minimum distance recorded in a successful subexperiment of the same group (n) plus 1 m (n + 1) was assumed, ensuring that n + 1 > 30 m (if n + 1 < 30 m, the value 31 m was used, keeping the consistency with the variable latency of approach). Both approaches produced similar results. The use of semiarbitrary numbers to deal with missing values is a common practice in studies using nonparametric statistics (e.g., Hirsch & Slack 1984; Broadhurst & Kell 2007), including avian song recognition studies (e.g., Colbeck et al. 2010; Kirschel et al. 2011).

Potential limitations of the experimental design

Different positions within the territory (Briefer et al. 2009), movements (Amrhein & Lerch 2010), and visual cues (Uy et al. 2009) of the virtual territorial intruder were not simulated in any of our experiments, and so overall responses may have been underestimated. Also, long-term singing reactions (e.g., after 24 h) were not measured, and these may reveal additional differences (Amrhein & Lerch 2010). We focused on the classic sender–receiver dyad, but animals communicate in networks, often including male–female signaling interactions (Matessi et al. 2007). Hence, our approach should be viewed as a necessary oversimplification.

Data analysis

With the data from the 48 successful experiments conducted at Aveiro, 3 matrices were built: 1) one from the successful triple experiments (n = 23 males); 2) another from the 13 paired experiments plus the triple experiments, but excluding the subexperiments with *schoeniclus* recordings (n = 35 males); and 3) a third matrix including the 12 successful unmatched experiments, 7 experiments from the paired design, and 22 from the triple design, retaining only the first subexperiment and excluding repeated males to obtain a similar number of experiments with songs of the 3 subspecies (n = 41 males). The total number of successful unmatched experiments and subexperiments of successful matched tests was 182.

Data from the 3 matrices were analyzed using different methods for continuous variables and for categorical variables: With triple experiments, we used Friedman's analyses of variance (Anovas) for continuous variables and Cochran's Q test for categorical variables; with paired experiments, Wilcoxon's tests for continuous variables and McNemar's tests for categorical variables; and with unmatched experiments, Kruskal–Wallis tests for continuous variables and chi-square (χ^2) tests for categorical variables. Only nonparametric tests were used because most of the original variables were not normally distributed, even after transformation. The overall patterns of song discrimination were assessed by creating 2 synthetic variables from the triple experiment data set using principal component analysis. PC1 explained 53% of the total variance and was positively correlated with the approach to the speaker and the time spent around it, and PC2 explained 26% of the variance and reflected the vocal responses (Table S 4.1). All analyses were computed in SPSS 21 (IBM Corporation, Armonk, NY).

In order to evaluate which pattern of geographical variation in nonlocal discrimination (Colbeck et al. 2010) fitted our system better, a matrix with all triple experiments from the 3 areas was built (n = 48): *lusitanica* was coded as own for Aveiro subexperiments, *schoeniclus* was coded as own for Skåne, and *witherbyi* was coded as own for Ebro. Using this matrix, 2 orthogonal descriptors of the 9 response variables were extracted by principal component analyses, and these proved to be normally distributed. For each component, a GLMM was built using a normal probability distribution and an identity link function. Subspecies recordings were nested within subjects (males), area, and subspecies were set as fixed factors and male as random effect. The 3 areas considered were Aveiro (where subspecies *lusitanica* was tested), Ebro (where *witherbyi* was tested), and Skåne (where *schoeniclus* was tested). In addition, in order to determine whether the data fitted the specific pattern of asymmetric nonlocal recognition, one of its distinctive features was tested: that local recognition is symmetric, that is, that responses to own subspecies song are equal across the populations. For that, a Kruskal–Wallis test for independent samples was used to compare the responses to own song obtained in the 3 areas. Complementarily, to determine if the data fitted the pattern of opponent assessment, we tested whether the response to a more complex foreign song differed across the populations, considering that song complexity increases from *schoeniclus* to *lusitanica* and then to *witherbyi* (Matessi et al. 2000a; Chapter 3). Again, a Kruskal–Wallis test for independent samples was used to compare the responses to the most complex foreign song obtained in the 3 areas.

4.4 Results

The experiments conducted at Aveiro showed that *lusitanica* males produced the strongest response to *lusitanica* songs, followed by *witherbyi* songs, whereas *schoeniclus* songs generated the weakest response (Figure 4.2). However, in a few cases (4%), some males from Aveiro responded to the foreign subspecies songs, but did not respond to the songs of their own subspecies (a situation not recorded at Ebro or Skåne). Among the 2 Iberian subspecies, responses to foreign song were much stronger in *lusitanica* (at Aveiro) than in *witherbyi* (at Ebro; Figure 4.3, see also Geographical variation in song discrimination for details). The triple playback experiments revealed an overall difference in the responses of *lusitanica* males to the song of the 3 subspecies ($n = 23$ males, $\chi^2 = 6.689$, $P = 0.035$; Friedman's Anova on PC2), but no significant pairwise differences. Regarding the original variables (Table 4.2) and in comparison with *schoeniclus* songs, *lusitanica* songs elicited the males to approach the speaker more often (Figure 4.4d; $Q = 0.391$, $P = 0.002$), get closer (both during [Figure 4.4a; $\chi^2 = -0.717$, $P = 0.15$] and after playback [Figure 4.4b; $\chi^2 = -0.913$, $P = 0.002$]), and spend more time within 10 m of it after playback (Figure 4.4c; $\chi^2 = 0.826$, $P = 0.005$). However, no significant differences were found between the responses of *lusitanica* males to *lusitanica* and to *witherbyi* songs. The same was true for the paired playback experiments ($n = 35$ males), as they revealed no significant differences between the responses of *lusitanica* males to *lusitanica* and *witherbyi* songs. Unmatched experiments ($n = 41$ males) resulted in male *lusitanica* approaching the speaker faster during playback of their own song than during playback of either *schoeniclus* (Figure 4.4a; $H = -17.495$, $P < 0.001$, Kruskal–Wallis test) or *witherbyi* songs ($H = -13.250$, $P = 0.003$, Kruskal–Wallis test) and in approaching less during playback of *schoeniclus* song than during playback of song from their own subspecies (Figure 4.4b; $H = -11.813$, $P = 0.028$, Kruskal–Wallis test).

In the Ebro Delta, we found that *witherbyi* males showed an overall stronger response to songs of *witherbyi* than to songs of either *lusitanica* ($\chi^2 = 1.045$, $P = 0.014$) or *schoeniclus* ($\chi^2 = -1.409$, $P = 0.001$) (Friedman 2-way Anova on PC1, n = 11; Figure 4.3a). Their overall response to *lusitanica* and *schoeniclus* recordings was similar ($\chi^2 = -0.364$, $P = 0.394$). From the analysis of the 9 original variables (Table 4.2), we found that *witherbyi* males approached the speaker faster and came closer to it in response to *witherbyi* songs than to *lusitanica* songs ($\chi^2 = 1.136$, $P = 0.008$ for latency; $\chi^2 = 1.182$, $P = 0.006$ for distance during, Figure 4.4a; $\chi^2 = 1.045$, $P = 0.014$ for distance after, Figure 4.4b) or *schoeniclus* songs ($\chi^2 = -1.455$, $P = 0.001$ for latency; $\chi^2 = -1.545$, $P < 0.001$ for distance during, Figure 4.4a; $\chi^2 = -1.273$, $P = 0.003$ for distance after, Figure 4.4b). Also, *witherbyi* males approached more often in response to *witherbyi* song playback than to *schoeniclus* song playback (Q = 0.909, P < 0.001; Figure 4.4d), and sang less in response to *witherbyi* song (Q = −0.455, P = 0.006). Finally, an overall difference in the response of *witherbyi* to songs of the 3 subspecies was found for the time spent in a 10-m radius around the speaker (Figure 4.4c), and for the latency of song (Table 4.2), but without significant pairwise differences between subspecies.

In Skåne, *schoeniclus* males responded strongly to the songs of all 3 subspecies (Figure 4.3), and we found a similar global response to the different song playbacks (Friedman 2-way Anova on PC1: χ^2 = 0.429, P = 0.807, n = 14; Figure 4.2a). The analysis of the 9 original variables (Table 4.2) did not reveal any differences either (Figure 4.4).

Hence, both *witherbyi* and *lusitanica* strongly discriminate *schoeniclus* song. However, *witherbyi* shows a more consistent discrimination of *lusitanica* song than *lusitanica* does of *witherbyi* song. Discrimination of *lusitanica* song by *witherbyi* was found in 11 successful triple playback experiments, but discrimination of *witherbyi* song by *lusitanica* was not clear in 23 similar experiments. To uncover some degree of *witherbyi* song discrimination by *lusitanica*, a matrix of 41 successful unmatched experiments was needed. On the other hand, male schoeniclus did not discriminate foreign songs from different subspecies, or even own songs from foreign songs. This contrasting pattern was found despite successfully testing more males of *schoeniclus* than of *witherbyi*.

Geographical variation in song discrimination

The GLMM resulted in significant effects on PC1 response score for area ($F_{2,135}$=27.290, P<0.001), for subspecies ($F_{2,135}$=23.389, P<0.001), and for the interaction area × subspecies ($F_{4,135}$=13.173, P<0.001; Figure 4.5; Table S 4.2). This was mainly because the responses to *schoeniclus* song playback differed between the 3 areas. In contrast, the responses to *witherbyi* playback were very similar in all 3 areas, as were the responses to lusitanica in Skåne and Aveiro (Figure 4.5). Overall, we found asymmetric nonlocal discrimination between the subspecies and variable patterns of discrimination within subspecies. Those include almost identical responses (very low discrimination) in *schoeniclus*, very variable responses (much higher discrimination) in *witherbyi*, and an intermediate pattern in *lusitanica*.

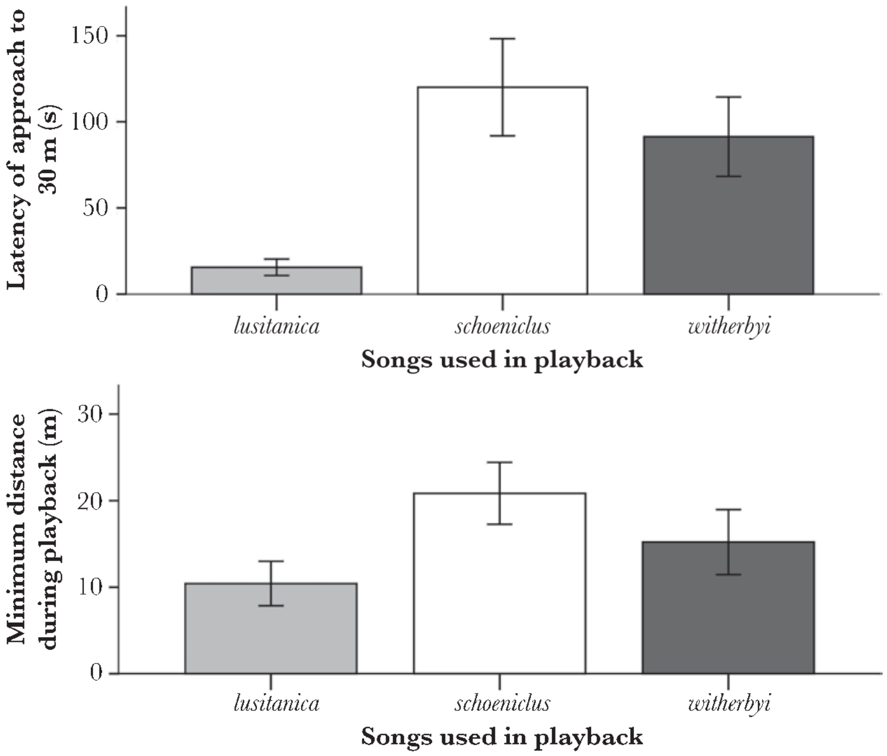

Figure 4.2 - Results of the unmatched playback experiments performed with subspecies *lusitanica* at Aveiro Lagoon for 2 of the 9 original response variables collected in the field. In the unmatched experiments, each male (n = 41) was tested only once, with either 1 *lusitanica*, 1 *schoeniclus*, or 1 *witherbyi* test file. Error bars represent the standard error of the mean (1 × SE).

Table 4.2 - Statistical analyses of the 9 original variables from the 48 triple playback experiments using nonparametric tests for k-related samples[a]. Significant P values (<0.05) are shown in bold typeface.

Area/ ssp tested	**Song used in playback**	**Approach latency (s)**	**Minimum distance (m)**		**Time within 10 m (s)**		**Song latency (s)**	**Presence of song**		**Presence of approach**
			During	After	During	After		During	After	
Aveiro	*lusitanica*	1.74	1.65	1.57	2.13	2.41	1.98	73%	47%	95%
lusitanica	*schoeniclus*	2.39	2.37	2.48	1.78	1.59	1.96	73%	47%	56%
(n = 23)	*witherbyi*	1.87	1.98	1.96	2.09	2.00	2.07	78%	52%	82%
	Test statistic	5.793	7.583	10.571	2.000	10.314	0.215	0.222	0.133	9.692
	P	0.055	**0.023**	**0.005**	0.368	**0.006**	0.898	0.895	0.936	**0.008**
Ebro	*lusitanica*	2.27	2.27	2.27	1.77	1.77	1.95	45%	54%	45%
witherbyi	*schoeniclus*	2.59	2.64	2.50	1.73	1.73	1.64	63%	63%	9%
(n = 11)	*witherbyi*	1.14	1.09	1.23	2.50	2.50	2.41	18%	45%	100%
	Test statistic	15.297	16.632	11.737	8.273	8.273	7.684	7.600	3.000	15.200
	P	**< 0.001**	**< 0.001**	**0.003**	**0.016**	**0.016**	**0.021**	**0.022**	0.223	**0.001**
Skåne	*lusitanica*	2.29	2.11	1.93	1.64	2.18	1.82	79%	8%	100%
schoeniclus	*schoeniclus*	1.75	1.96	2.00	2.18	1.79	2.11	86%	22%	100%
(n = 14)	*witherbyi*	1.96	1.93	2.07	2.18	2.04	2.07	79%	8%	100%
	Test statistic	2.111	0.467	0.222	2.778	2.067	0.717	0.250	2.000	-
	P	0.348	0.792	0.895	0.249	0.356	0.699	0.882	0.368	-

[a]For continuous variables, Friedman's Anovas were computed, mean ranks are provided for each subspecies, and the test statistic is χ^2. For categorical variables, Cochran's tests were used, frequency of successes is shown for each subspecies, and the test statistic is *Q*. df equals 2 for all tests.

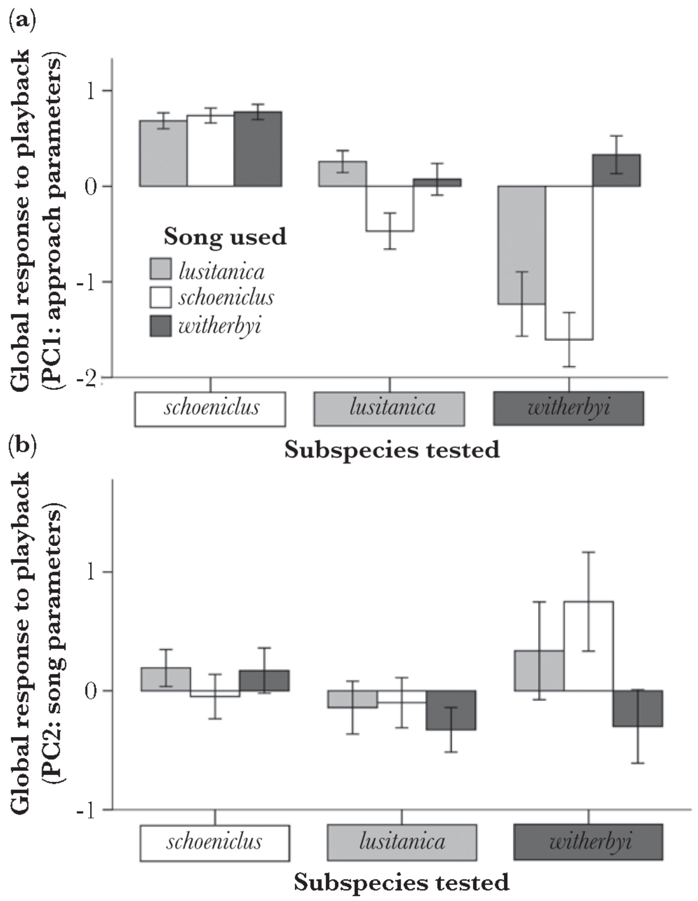

Figure 4.3 - Mean and SE of the global responses to song playback of 48 males from the 3 western subspecies of reed bunting (*schoeniclus*, n = 14; *lusitanica*, n = 23; *witherbyi*, n = 11). Global response was calculated by Principal component (PC) analysis of 9 variables collected during the experiments: (a) PC1 (53% of the total variance) represents approach to the speaker and the time spent around it and (b) PC2 (26% of the variance) represents song responses. Each of the 3 clustered bar graphs represents a different study area (Skåne, Aveiro, and Ebro) and, therefore, a different subspecies tested (*schoeniclus*, *lusitanica*, and *witherbyi*, respectively, as indicated in the x axis).

The responses to own song obtained in the 3 areas differed (Kruskal-Wallis test for independent samples: *H’*=9.618, degrees of freedom [df]=2, *P*=0.008), mostly due to differences between Aveiro and Skåne (pairwise comparison: *H’*=3.101, *P*=0.006, cf. Figure 4.5). This means that responses to the own subspecies song are not of the same intensity (symmetric) across the populations. Because symmetric local recognition is one of the features of the pattern of asymmetric nonlocal recognition, we conclude that our data do not fit this pattern.

The responses to the most complex foreign song also differed in the 3 areas (Kruskal–Wallis test for independent samples, *H’*=23.104, df=2, *P*<0.001), mostly due to differences between Skåne and the other 2 areas (cf. pairwise comparisons). Such different response to a more complex foreign song across the populations does not fit the pattern of opponent assessment either.

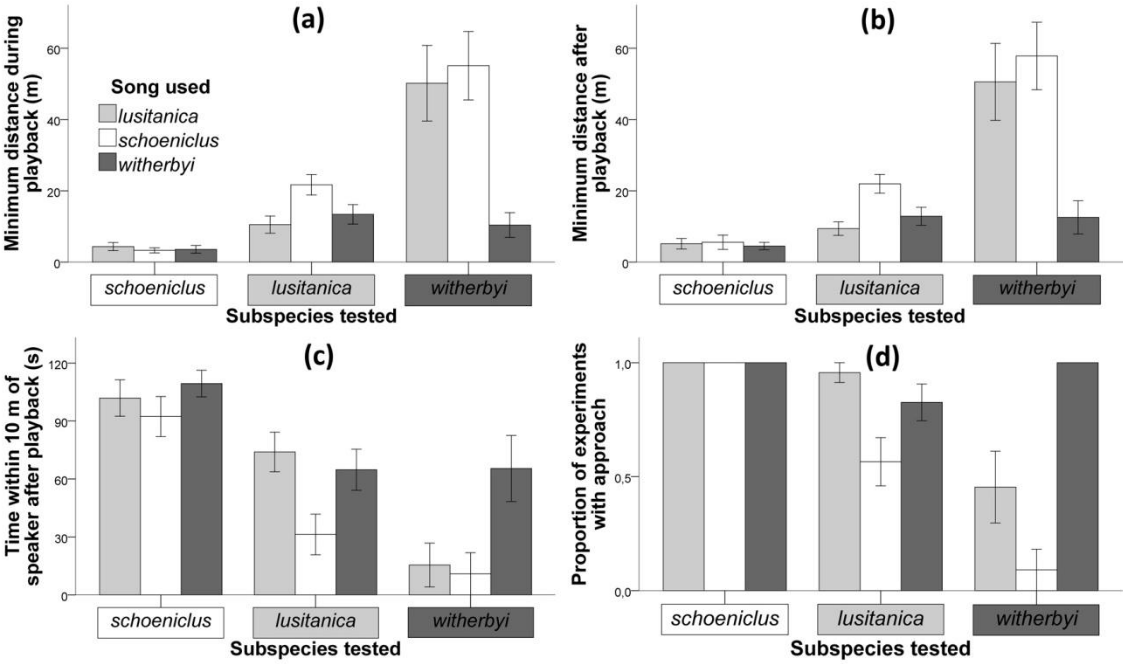

Figure 4.4 - Response of 48 males from the 3 western subspecies of reed bunting (*schoeniclus*, n = 14; *lusitanica*, n = 23; *witherbyi*, n = 11) to playback of their own song and of songs from the other 2 subspecies as measured from 4 of the 9 variables used: minimum distance to the speaker (in meters) during (a) and after playback (b), time spent within 10 m of the speaker (in seconds) after playback (c), and proportion of experiments with approach (d) to at least 30 m of the speaker. Error bars represent the standard error of the mean (1 × SE).

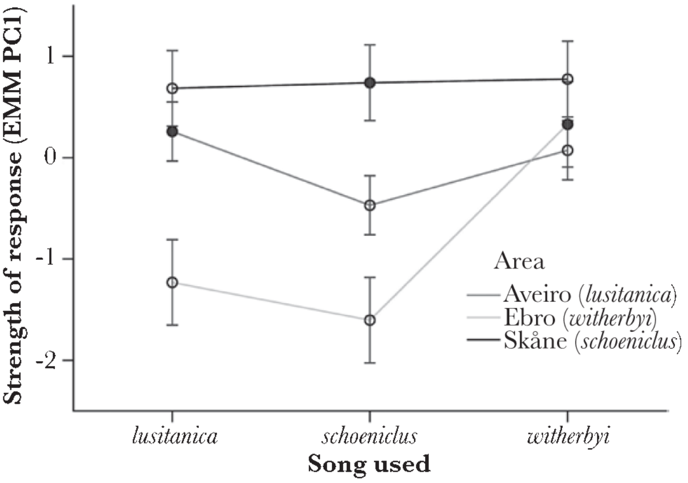

Figure 4.5 - Results from the GLMM for geographical variation in nonlocal discrimination. Effects on PC1 playback response score for area (Ebro, Skåne, and Aveiro, corresponding to the 3 subspecies tested: *witherbyi*, *schoeniclus*, and *lusitanica*, respectively), subspecies (song used in playback), and interaction between area and subspecies. Estimated means chart for significant effects (*P*<0.05). Error bars represent 95% confidence intervals. Filled circles stand for responses to own subspecies song.

4.5 Discussion

Our experiments show that the song differences between subspecies are perceived by most territorial males and lead to different behaviors of aggressiveness toward the simulated intruder. However, the responses were not similar across populations/subspecies tested nor across the types of intruder simulated within each population. Two out of 3 populations showed stronger responses to its own subspecies song than to the songs of foreign subspecies. This differential territorial defense in relation to own versus foreign subspecies suggests that an intruding male with foreign songs does not represent a large threat, a pattern that is expected when there is some degree of premating isolation based on song (Balakrishnan & Sorenson 2006). Also, male territorial responses are often correlated with female preferences (e.g., Searcy et al. 1997). Thus, our results indicate the existence of premating reproductive isolation among these recently evolved reed bunting subspecies and support the view that sexual signals, in this case male song, can have an important role during speciation, but it would be interesting to further test this by studying female choice in the future.

Behavioral mechanisms that may operate within and between subspecies song dialects

Across populations, we found that foreign subspecies song elicited different responses in males of the 3 western subspecies of reed buntings, indicating that the pattern of discrimination against foreign subspecies song is asymmetric. However, we also found that responses to song of the own subspecies differed between populations. Although the first result is compatible with the asymmetric nonlocal recognition hypothesis, the second result does not comply with the predictions of this hypothesis. The asymmetric self-assessment hypothesis predicts that males from one/some subspecies should have the strongest reactions to all song stimuli than males of other subspecies. This was true for individuals from the Skåne population (*schoeniclus*) where males responded more strongly to all song stimuli. However, the asymmetric self-assessment hypothesis cannot explain the differences found between *witherbyi* and *lusitanica* reed buntings. The much smaller *lusitanica* males (Byers et al. 1995; Chapter 2), likely candidates for being the less aggressive subspecies (e.g., Robinson & Terborgh 1995; Martin & Martin 2001), reacted stronger to the song of *witherbyi* than *witherbyi* to songs of *lusitanica*. Moreover, both subspecies showed a similarly weak reaction to *schoeniclus* song. A possible explanation for this pattern could be that males from Ebro (*witherbyi*) may perceive songs from males of the other 2 subspecies (that on average have simpler songs) as being sung by inferior males and therefore respond more weakly to them. This would then be in line with the asymmetric opponent assessment hypothesis. Nevertheless, the latter hypothesis cannot alone explain all the differences found. We therefore suggest that a combination of the self-assessment (in *schoeniclus*) and the opponent assessment (in *witherbyi*) hypothesis could explain the general pattern we found in western reed buntings.

Two main behavioral mechanisms could help to explain the asymmetric patterns of response to foreign subspecies songs found in our study: 1) intrasexual interactions due to asymmetric competitive ability or intersubspecies aggressiveness (with *schoeniclus* being the more aggressive subspecies) and 2) skewed perceptual sensitivity due to distinct overlap differences with respect to the frequency ranges or number of syllables used by each taxon (mechanistic explanation, see below).

Comparison with previous studies and overall patterns and processes

Previous studies have shown a difference in singing response to playback of own and foreign subspecies song in *schoeniclus* males (Matessi et al. 2000b), but not in *intermedia* males (including *witherbyi*, Matessi et al. 2001). For *schoeniclus*, that difference was found assuming the frequency of singing during playbacks of yellowhammer (*Emberiza citrinella*) song (used as a control in the experiments) to be the baseline singing frequency, and by comparing frequencies of singing during *schoeniclus* playback and during intermedia playback with the baseline. However, the comparison of the 5 original variables resulted in smaller *P* values for *intermedia* (3 under 0.07) than for *schoeniclus* (none under 0.09), and given the small sample size used in these studies (n = 20), a larger number of experiments with *intermedia* might have given somewhat different results.

Our results, together with those from the original variables in Matessi et al. (2000b, 2001), suggest that Mediterranean thicker-billed birds (*witherbyi* and *intermedia*) show stronger discrimination against the songs of Atlantic and Northern thinner-billed birds (*lusitanica* and *schoeniclus*) than the latter 2 subspecies discriminate the songs of the Mediterranean birds. Two factors might contribute to such pattern. First, the variation in song complexity (number of syllables and number of different syllables) and frequency ranges shown by northern birds completely overlaps with part of the variation shown by southern birds, but the remaining variation in the southern birds (the most complex and broadband songs) is exclusive and distinctive (Matessi et al. 2000a; Chapter 3). This could make the discrimination task of southern birds more feasible. Second, the southern areas are important for wintering northern birds, while southern birds are not known to visit areas far north at all (Villarán Adánez 1999). Hence, during the southern breeding season (March to June), local birds often come in contact with northern birds (singing during their late wintering period - October to April) and, as the northern subspecies does not compete with the southern subspecies for mates, selection might favor stronger song discrimination to avoid investing energy by reacting to a nonthreat. This is consistent with the idea that song recognition allows territory owners to modulate their response according to the threat posed by each intruder and thus to reduce the costs associated with territorial defense (Briefer et al. 2009). Alternatively, even if northern males sometimes compete with southern males for mates in southern areas, it would be selectively advantageous for southern females to discriminate against northern males because the potential costs of outbreeding for a locally adapted population may be high. For the black-throated blue warbler (*Setophaga caerulescens*), it has also been suggested that individuals in the north have limited opportunity to perceive and/or learn the southern song, but that the same is not true in the south, possibly due to asymmetrical exposure during migration (Colbeck et al. 2010).

Evolutionary mechanisms and implications

Given that male and female response patterns to local and foreign songs are often similar (e.g., Searcy et al. 1997), so that the former has been examined as a proxy for the latter in several studies (e.g., Christensen et al. 2010), our results also imply that females could discriminate between males on the basis of song. However, the assumption that females prefer the same signals that elicit strong responses from the males is not true for every case (e.g., Nelson & Soha 2004; Anderson et al. 2007). It would therefore be very important to study song recognition by females, but this needs to be conducted in captivity (e.g., Ceugniet & Aubin 2001; Nelson & Soha 2004) raising considerable experimental problems and should probably not be conducted in the threatened southwestern subspecies (*lusitanica* and *witherbyi*).

The 3 subspecies of reed bunting have differentiated in a variety of morphological traits (Chapter 2), with the 2 southern subspecies (*lusitanica* and *witherbyi*) having a specialized foraging behavior, which is associated with thicker, convex bills and a much narrower foraging niche than *schoeniclus* (chapters 2 & 5; LOG & JMN personal observations). For these 2 resident subspecies, the cost of mating with a migratory thin-billed subspecies (*schoeniclus*) should be high, given the presumably lower fitness of descendents with intermediate bill sizes. Accordingly, these subspecies responded weakly (or not at all) to *schoeniclus* songs. In contrast, the ecological generalist *schoeniclus* responded strongly to all subspecies songs, perhaps indicating that the potential costs of subspecies mixing (“hybridization”) are not so high for this subspecies. The fact that *lusitanica* showed a stronger response to *witherbyi* songs than *witherbyi* did to *lusitanica* songs could potentially be related to the low genetic variation of *lusitanica* (Kvist et al. 2011), a possibility that deserves further research.

The patterns of foreign subspecies song discrimination described in this study indicate a strong premating reproductive isolation between each of the 2 southern, resident subspecies (*lusitanica* and *witherbyi*) and the northern, migratory *schoeniclus*, and a slightly less strong isolation between *lusitanica* and *witherbyi* (see Figure 4.5). As *lusitanica* is genetically closer to *schoeniclus* than to *witherbyi* based on mtDNA and microsatellites (Kvist et al. 2011) as well as nuclear intron sequences (Neto JM, unpublished data), premating reproductive isolation is in line with ecological rather than genetic differentiation, indicating early stages of ongoing ecological speciation (i.e., isolation-by-ecology) in reed buntings (see Shafer & Wolf 2013). Further work testing the association between reproductive isolation, ecological, and genetic divergences is needed and should point to the mechanisms involved in differentiation in this interesting study system.

4.6 Acknowledgments

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4.8 Supporting Information

Table S 4.1 - Factor loadings on principal components (PC) for response measures[a]

	PC1	PC2
Eigenvalue	4.786	2.350
% Variance	53.180	26.115
Latency of approach to 30 m	-0.926	0.001
Minimum distance from speaker during playback	-0.918	0.009
Minimum distance from speaker after playback	-0.906	0.018
Time spent within 10 meters of speaker during playback	0.817	-0.058
Time spent within 10 meters of speaker after playback	0.826	-0.060
Song during playback	-0.137	0.880
Song after playback	0.340	0.798
Latency of song	0.034	-0.966
Approach to 30 m	0.884	0.005

[a]Response measures were obtained from triple playback experiments with *Emberiza schoeniclus lusitanica* (23 males), *E. s. schoeniclus* (14 males), and *E. s. witherbyi* (11 males).

Table S 4.2 - Full GLMM results for PC1[a]

Source	F	df1	df2	Sig.
Corrected Model	18.072	8	135	< 0.001
Ssp	23.389	2	135	< 0.001
Area	27.290	2	135	< 0.001
Ssp*Area	13.173	4	135	< 0.001

[a]Subspecies recordings were nested within subjects (males), Area and subspecies (Ssp) were set as fixed factors and male as random effect. Positive covariance for the single random effect tested (Intercept = 0.037)

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Chapter 5

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

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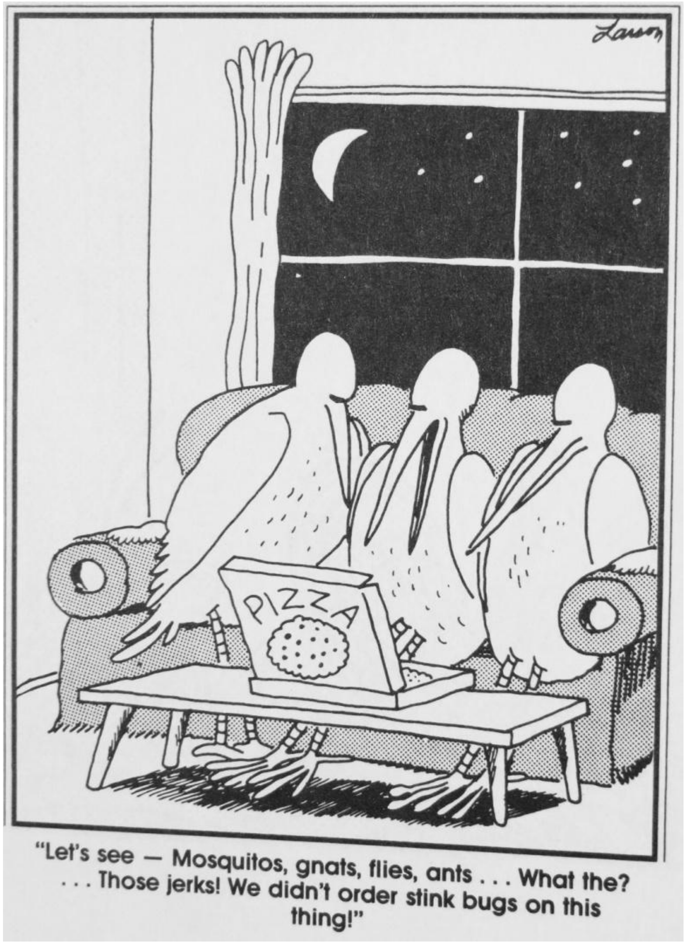

Cartoon 6 - The variety of C3-plant-feeding insects in avian diet (from Larson 1984)

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

5.1 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. *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.

5.2 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 & 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 2001, but see Grant & Grant 1997); or be under direct sexual selection (Grant & Grant 1997; 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 locally-adapted individuals 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 (chapters 2 and 3; Byers et al. 1995; Matessi et al. 2000a, b). 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 et al. 2008). There is asymmetric song discrimination between different subspecies in western Europe (Chapter 4; Matessi et al. 2000a, b), 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). 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 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* (Chapter 2). The increasing bill thickness towards the east among all the 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 & Perrins 1994; Holland et al. 2006). However, previous work indicates that, during winter, some thick-billed subspecies (*intermedia* and *pyrrhuloides*) feed on insect larvae and pupae that are dormant inside the reed (*Phragmites australis*) stems (Shtegman 1948 cited by Prŷs-Jones 1984; Matessi et al. 2002), whereas thin-billed subspecies eat mostly seeds and often occur in other habitats such as farmland (Cramp & Perrins 1994; Holland et al. 2006; Orłowski & Czarnecka 2007), but can eat insects in some areas opportunistically (Orłowski et al. 2013; JMN unpubl.). The diets of *witherbyi* and of the intermediate-size *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 *lusitanica* and *schoeniclus* make predictions difficult (Chapter 2). In addition, in Chapter 2, it was shown that sexual dimorphism in bill size and shape varies, with thick-billed subspecies having a greater dimorphism than *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 C_3 vs C_4 photosynthesis at the base of the food chain and on the water-use efficiency within C_3 plants; whereas nitrogen varies mostly with the trophic level in the food chain (Bearhop et al. 2005; Inger & Bearhop 2008). We test the hypothesis (H_1) that the nitrogen isotope ratio in the blood, which correlates positively with trophic level (DeNiro & Epstein 1981; Inger & 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 C_4 plants; Cramp & Perrins 1994; Holland et al. 2006; Pyankov et al. 2010; Kandereit et al. 2012), we also hypothesize (H_2) that this subspecies has the most ^{13}C-rich values. In addition, if the diet closely follows the morphological differentiation, we hypothesize (H_3) 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*.

5.3 Material and methods

Fieldwork

Fieldwork was carried out during December–February 2010/2011 and 2012/2013 at Salreu marshlands (40º43’41.10’’N, 08º35’06.51’’W), Portugal, as well as December–February 2011/2012 and 2012/2013 at Lagunas de Villafranca (39º27’22.52’’N, 03º20’09.03’’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 (43º36’24.62’’N, 04º31’58.58’’E), Arles, but a few samples (four in each location, including two subspecies) were also collected in Saint-Laurent D’Aigouze (43º35’43.61’’N, 04º12’47.64’’E) and Courthézon (44º04’21.68’’N, 04º52’01.53’’E).

Intensive mist netting was undertaken in order to capture 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. Blood samples (whole blood) were collected for stable isotope analysis by puncturing the brachial vein and were stored in centrifuge tubes. Some potential food items (n=52), in particular C_3-plant material (inflorescences of reeds, sedges and rushes; from all sites), insect larvae and pupae lying 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 (previous estimates of half-lives of $\delta^{13}C$ and $\delta^{15}N$ in the blood of a similar-sized species are 5.4 and 11 d, respectively [Hobson & Bairlein 2003]; and 12.0 and 17.6 d, respectively [Pearson et al. 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 ca. 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 Chapter 2). 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) (Chapter 2). Age (first-year or adult) and sex were determined from the plumage wear and pattern using standard methods (Svensson 1992; de la Puente & Seoane 2001), with only one bird being of indeterminate age. Part of these morphological data were included in Chapter 2, 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.

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 (see Supporting Information below, Figure S 5.1; Foster et al. 2007; Chapter 2). 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 α=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 & 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:

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

where δ_{sample} is the isotope ratio of the sample relative to a standard, R_{sample} and $R_{standard}$ are the fractions of heavy to light isotopes (i.e. $^{13}C/^{12}C$ or $^{15}N/^{14}N$) in the sample and standard respectively. $\delta^{13}C$ and $\delta^{15}N$ 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 $\delta^{15}N$ and 0.12‰ $\delta^{13}C$, and all internal standards are routinely checked against international reference materials: $\delta^{13}C$ was calibrated against the glutamic acid reference materials USGS40 and USGS41 (Coplen et al. 2006), $\delta^{15}N$ 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: χ^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: χ^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 (Chapter 2). 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: χ^2_3=35.7, p<0.001) and 43.6% (KMO=0.5, Bartlett's test of sphericity: χ^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.

Differences in baseline isotope ratios between sites were evaluated using GLMs with δ^{15}N or δ^{13}C as dependent variables and site (Portugal, Spain, France), type of food item (plant, insect larvae, spider) and their interaction as predictors. For these analyses, the few beetles collected in Portugal were excluded (because no similar samples were available from other sites), as was one outlier (an insect larva from Spain with a δ^{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 also used to compare the stable isotope ratios of the birds' blood 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 sitespecific models (F-test: p>0.5), this variable was excluded from further analyses. GLMs used to determine the influence of bill size (PCbill) on the stable isotope ratios within each subspecies included site, age and sex as predictors. Levene tests indicated the existence of significantly different variances between the subspecies for δ^{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 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). 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 10 000 posterior draws of the SEA_B parameters (Jackson et al. 2011).

Data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.p3nt7 (Neto et al. 2016).

5.4 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* (see Supporting Information below, Figure S 5.2a). 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 (see Supporting Information below, Figure S 5.3). 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 (Chapter 2), was also highly significantly different between subspecies ($F_{2;48}$=17.2; p<0.001), with the thick-billed subspecies showing more curved culmens (see Supporting Information below, Figure S 5.2b). 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 × ssp: $F_{2;50}$=5.8; p=0.005; see Supporting Information below, Figure S 5.2b).

These morphological differences are similar to the ones found in Chapter 2 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 δ^{15}N of the potential food items, but 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=0.25; type × site: $F_{2;40}$=0.31; p=0.74; see Supporting Information below, Figure S 5.4). There were also no significant differences between sites in δ^{13}C of food items (GLM: type of item: $F_{2;40}$=1.17; p=0.32; site: $F_{2:40}$=2.19; p=0.13; type × site: $F_{2;40}$=5.57; p=0.007), but there was a significant interaction between site and type of food item because in France spiders had lower δ^{13}C values than plants (which were similar in Portugal; Supporting Information, Figure S 5.4).

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 δ^{13}C and the sexes in δ^{15}N (Table 5.1, Figure 5.1). Interactions between these variables were not significant, thus being excluded from the final models. The parameters of the models (Table 5.1) indicate that the migratory *schoeniclus* differs in both isotope ratios from the two resident subspecies, which otherwise seem similar (Figure 5.1). This is supported by statistical comparisons of the estimated marginal means, which revealed significant differences between *schoeniclus* and *lusitanica* (δ^{15}N: mean difference =-1.3±0.6‰; p=0.029; δ^{13}C: mean difference =4.2±0.8‰; p<0.001), *schoeniclus* and *witherbyi* (δ^{15}N: mean difference =-1.6±0.7‰; p=0.023; δ^{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 5.1, Figure 5.1).

Bivariate SEA_C show clear differences in isotopic niche between subspecies within each site as well as between *schoeniclus* populations wintering at each site (Figure 5.2). 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 (Figure 5.3).

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 δ15N (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 Figure 5.4.

Table 5.1 - Unstandardized coefficients (B±SE) resulting from the general linear models comparing $\delta^{13}C$ ($R^2=0.36$) and $\delta^{15}N$ ($R^2=0.33$) between subspecies (*lusitanica*/ *schoeniclus*/ *witherbyi*), sexes (male/ female), ages (first-year/ adult) and sites (Portugal/ Spain/ France).

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

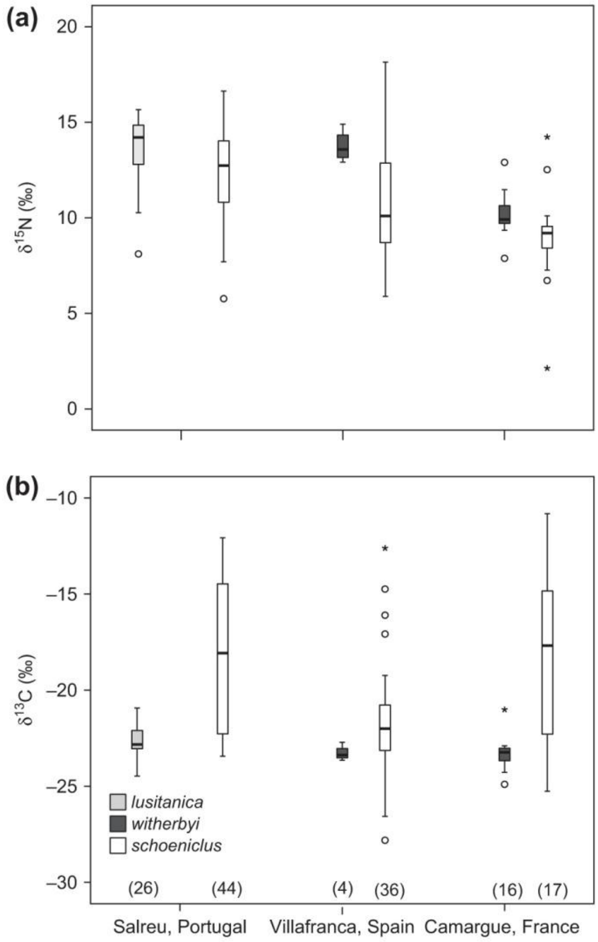

Figure 5.1 - Boxplots of isotopic composition ((a) $\delta^{15}N$ and (b) $\delta^{13}C$) of the blood of reed bunting subspecies collected during winter at three sites in southwest Europe. Medians (lines) and interquartile ranges (IQR; boxes) are shown, together with outliers greater than 1.5×IQR (circles) and greater than 3×IQR (asterisks). Sample size is indicated between parentheses.

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; Figure 5.5). For *schoeniclus*, the relationship between $\delta^{15}N$ and bill size was not significant ($F_{1;88}$=2.0; p=0.16), although the positive relationship at Salreu was significant (B=1.04±0.513, $t_{1;38}$=2.0, p=0.049; Figure 5.5a). But, there was a significant negative relationship between $\delta^{13}C$ and bill size ($F_{1;88}$=5.6; p=0.02) with large-billed birds having lower $\delta^{13}C$ values in all populations (Figure 5.5b). All the relationship between bill shape (RW1) and stable isotope ratios within *lusitanica* and within *schoeniclus* were not significant (all p>0.26).

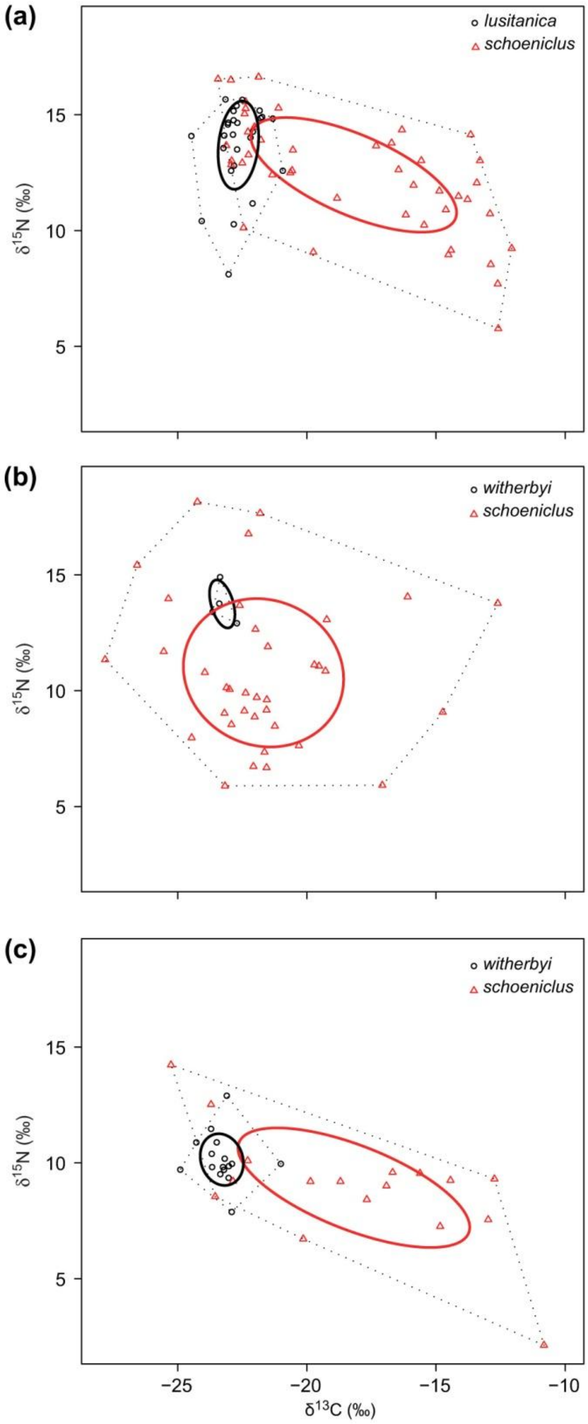

Figure 5.2 - Convex hull ellipses (dashed lines) and standard ellipse areas corrected for small sample size (SEA_C; red/full lines) of each subspecies and location ((a) – Salreu, Portugal; (b) – Villafranca, Spain; (c) – Camargue, France).

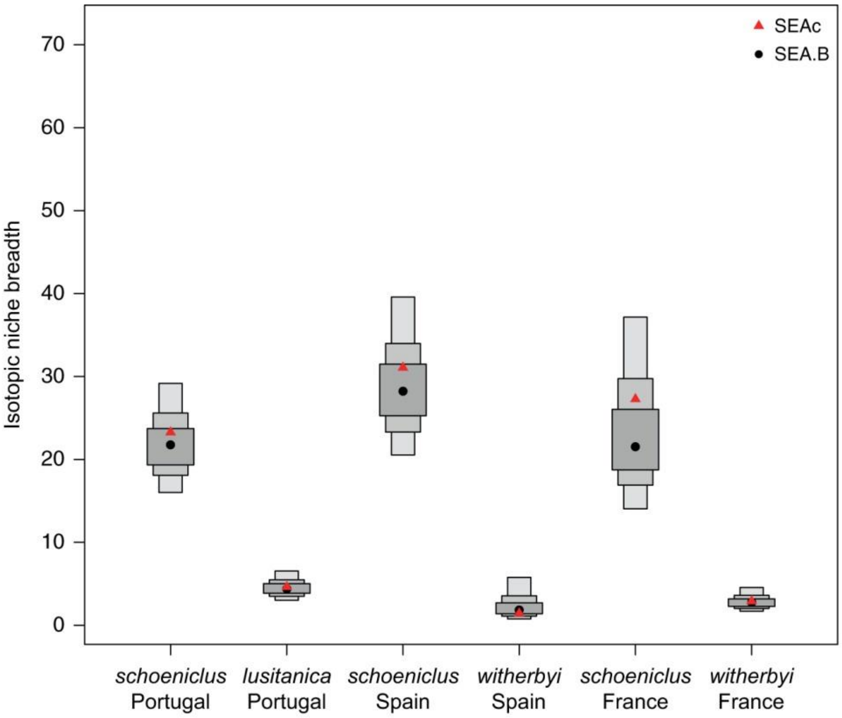

Figure 5.3 - Posterior distribution of the isotope niche widths, as measured by the Bayesian standard ellipses (SEA_B), showing mean, 50, 75 and 95% confidence limits, as well as mean SEA_C.

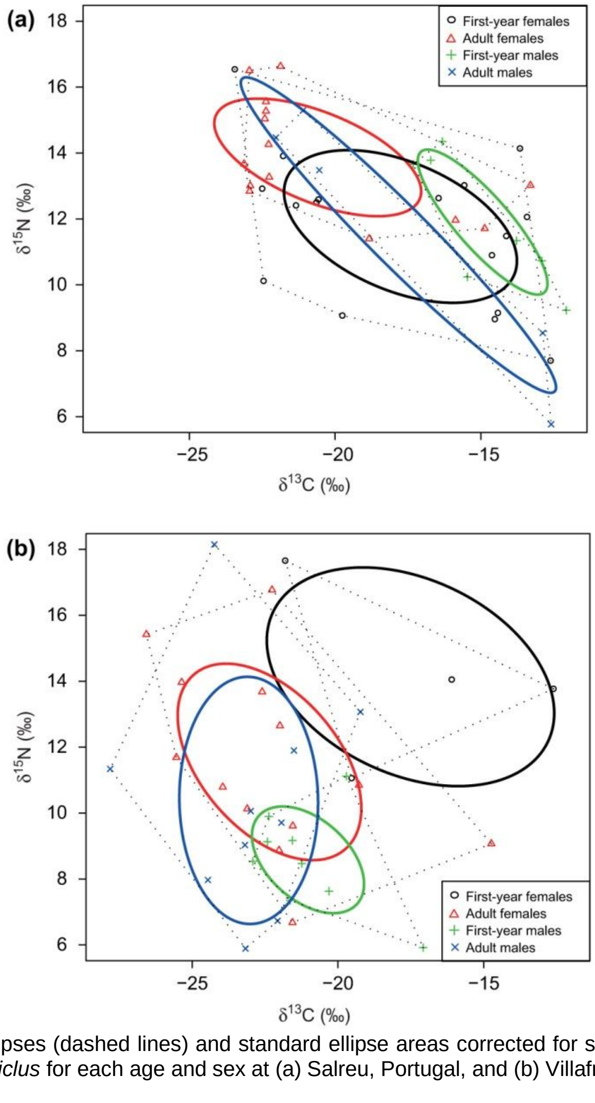

Figure 5.4 - Convex hull ellipses (dashed lines) and standard ellipse areas corrected for small sample size (SEA_C; full lines) of *Emberiza s. schoeniclus* for each age and sex at (a) Salreu, Portugal, and (b) Villafranca, Spain.

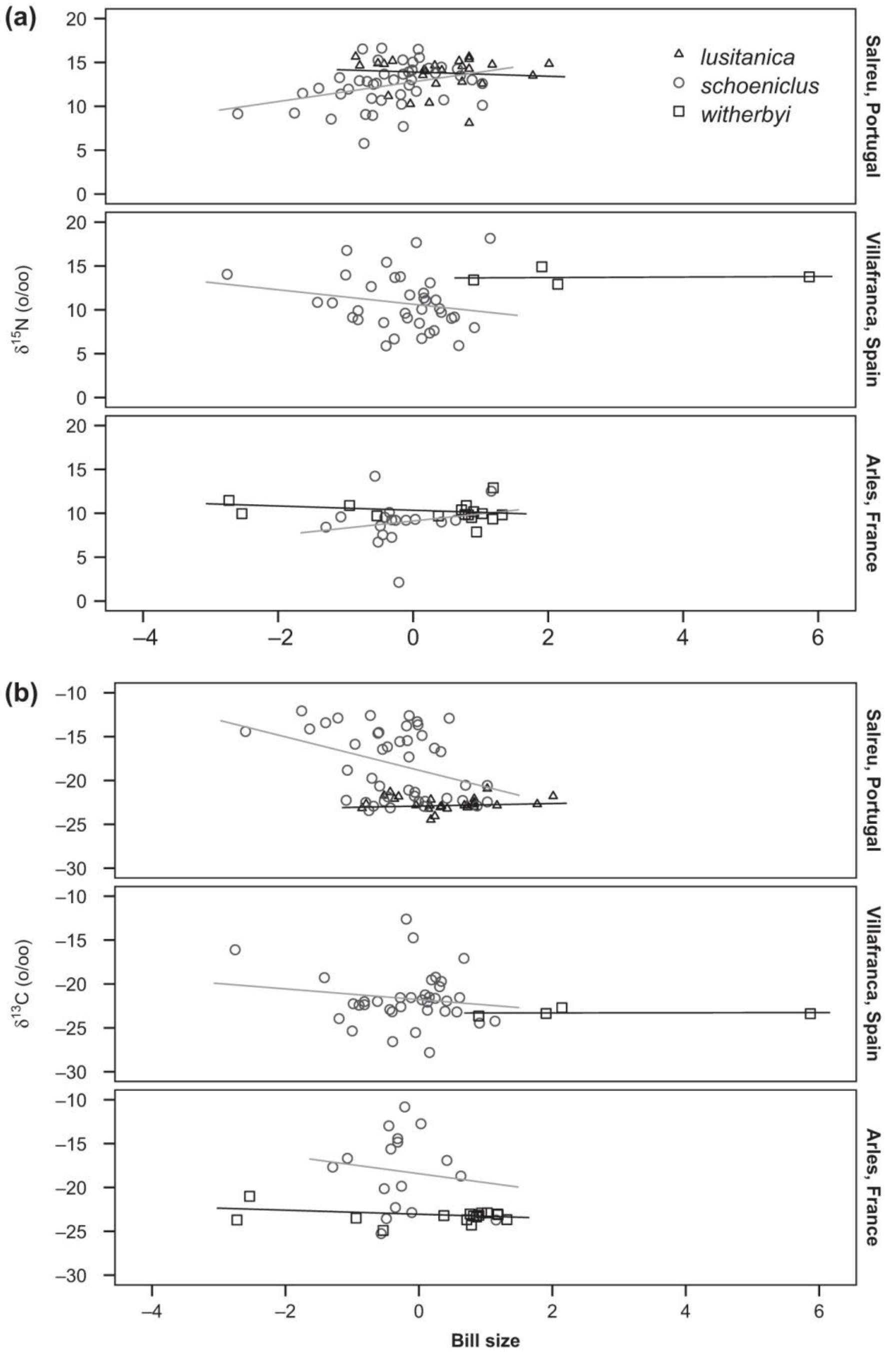

Figure 5.5 - Linear Regressions between bill size (PCbill) and the stable isotope ratios ((a) δ^{15}N and (b) δ^{13}C) for each location and subspecies (for statistics see the main text).

5.5 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 δ^{15}N and lower δ^{13}C 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 (or its consumers; $\delta^{13}C<-20$) 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 (Figure 5.1, Figure 5.2). 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 (Chapter 4). 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 (Figure 5.1, Table 5.1). 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 unpubl.]), 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 & Perrins 1994; Matessi et al. 2002; Holland et al. 2006; Orłowski & Czarnecka 2007; Orłowski et al. 2013), 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 (Chapter 2, JMN unpubl., E. 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 [Supporting Information, Figure S 5.4], 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* (Figure 5.1, Figure 5.2). 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 (H_3) is not supported by the results. Hence, ecologically (and to some extent on the basis of bill shape; Supporting Information, Figure S 5.2b, Chapter 2), *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; Chapter 2). 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 & Grant 2002; Symonds & 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 (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 (Grant & 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 & Perrins 1994; Holland et al. 2006), 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; 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 (as the food items have similar isotope ratios between the sites). In Portugal, the stable isotopes of reed buntings indicate the existence of two major food types with few intermediates (Figure 5.2a): one with high $\delta^{15}N$ and low $\delta^{13}C$ (presumably C3-plant-eating insects) and another with high $\delta^{13}C$ and low $\delta^{15}N$ (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 δ^{13}C and δ^{15}N (presumably C3-plant material), which are largely absent at the Portuguese site. Although first-year birds had higher δ^{13}C values than adults at both locations, their food preferences seem to differ between locations and sexes (Figure 5.4). Males, especially first-years, seem to show a preference for C4-plant material at Salreu (high δ^{13}C and low δ^{15}N) and C3-plant material at Villafranca (low δ^{13}C and low δ^{15}N), but adult females at Salreu and first-year females at Villafranca deviated from most other birds presumably by including insects in their diet (high δ^{15}N 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 & 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* (Figure 5.3). 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 δ^{15}N varied with location (Figure 5.5), 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 δ^{15}N is lower than at Salreu (Figure 5.1, Figure 5.2); but this variation was no longer significant after including age and sex in the statistical model. However, there was an effect of bill size on diet as measured by δ^{13}C, 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. unpubl.), and are quite variable in bill size (Chapter 2). 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 & 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 Prŷs-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 chapter shows that, in addition to phenotypic (Chapter 2), acoustic (Chapter 3) 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 (but see Chapter 4).

5.6 Acknowledgements

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5.8 Supporting Information

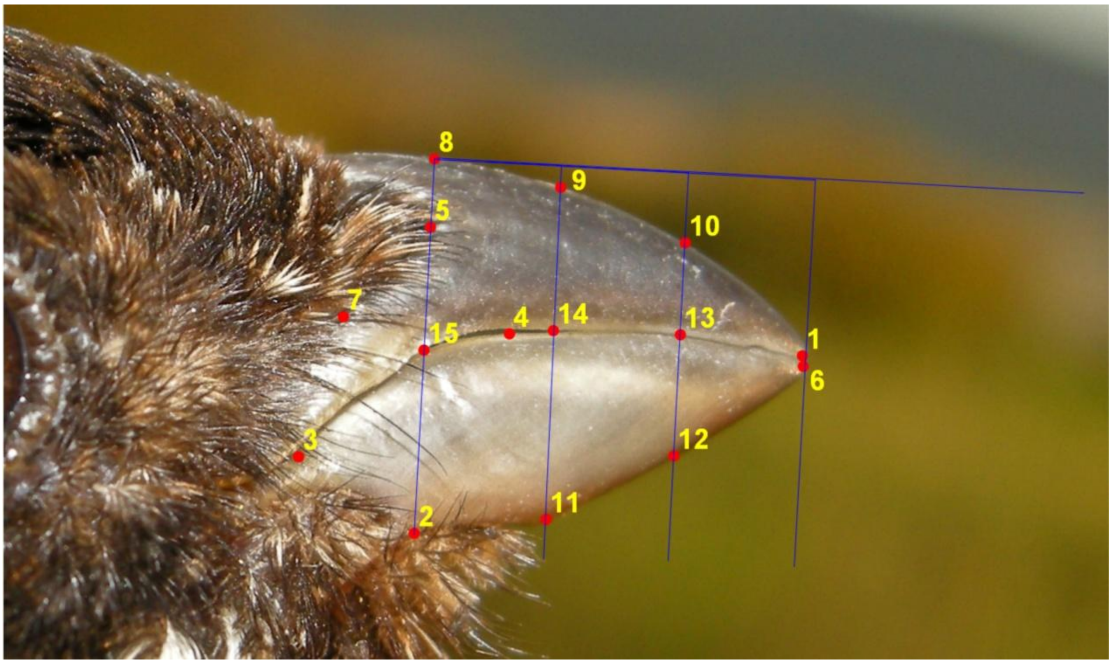


Figure S 5.1 - Positioning of landmarks and semi-landmarks used in geometric morphometric analysis on a male *Emberiza schoeniclus witherbyi*.

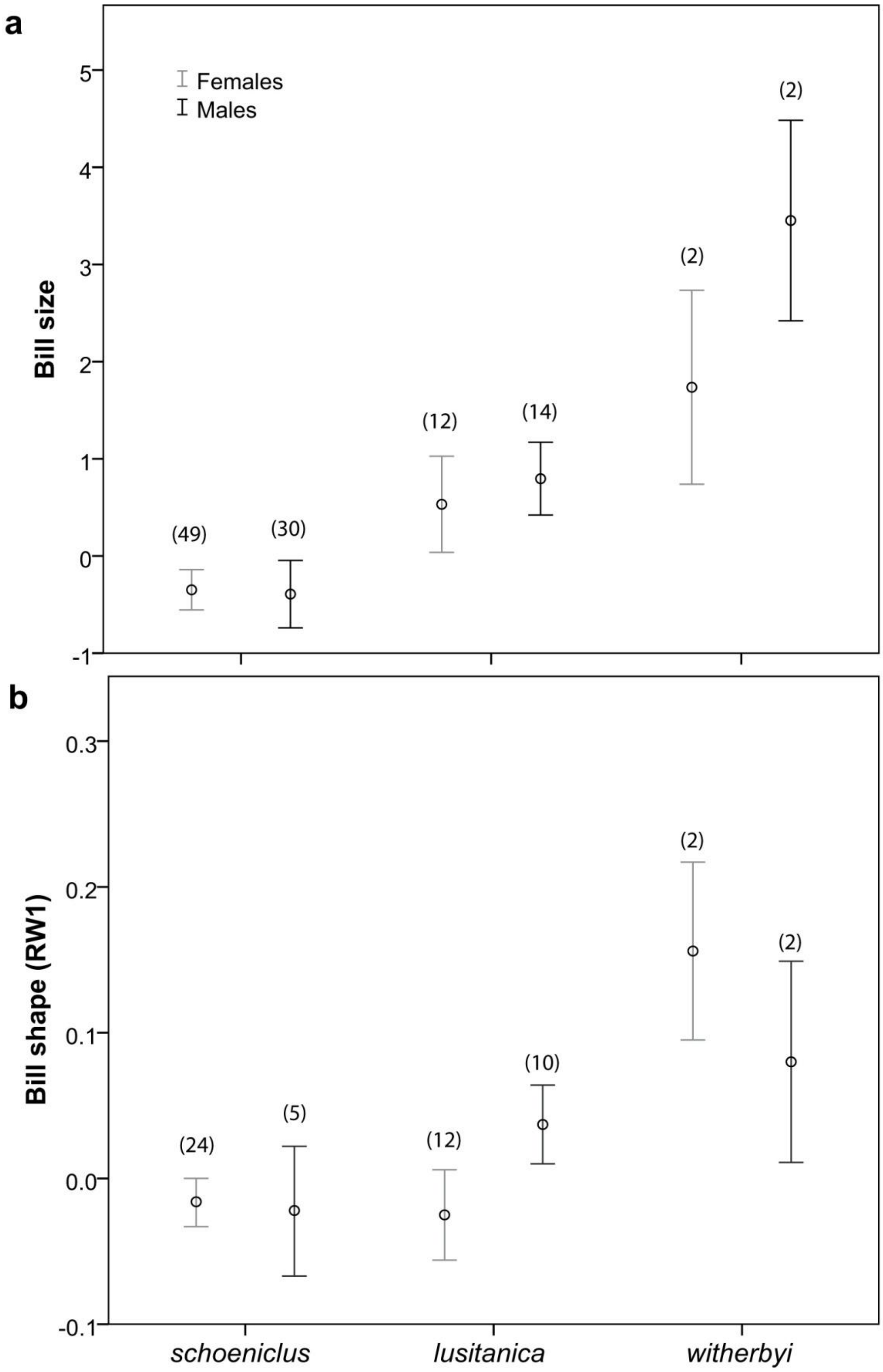


Figure S 5.2 - Estimated marginal means (EMM) and 95% confidence interval of a) bill size (PCbill) and b) bill shape (RW1) for each subspecies and sex of Iberian reed buntings. EMMs were derived from a general linear model that included body size (PCsize) as a covariate. As there was no significant effect 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 Figure S 5.3.

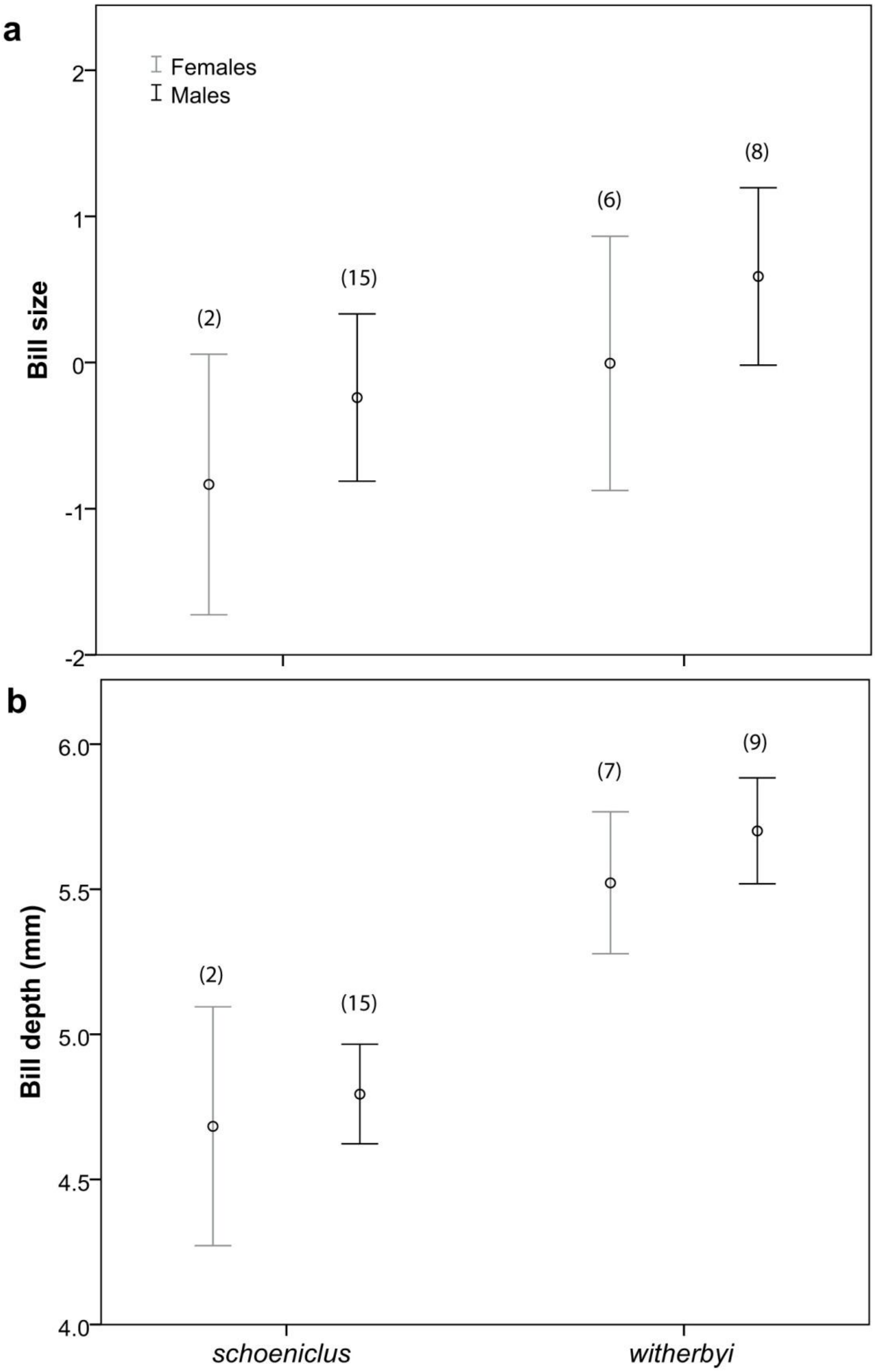


Figure S 5.3 - Estimated marginal means (EMM) and 95% confidence interval of a) bill size and b) bill depth of French birds, for each subspecies and sex. Sample size is indicated between parenthesis and statistics are described in the main text.

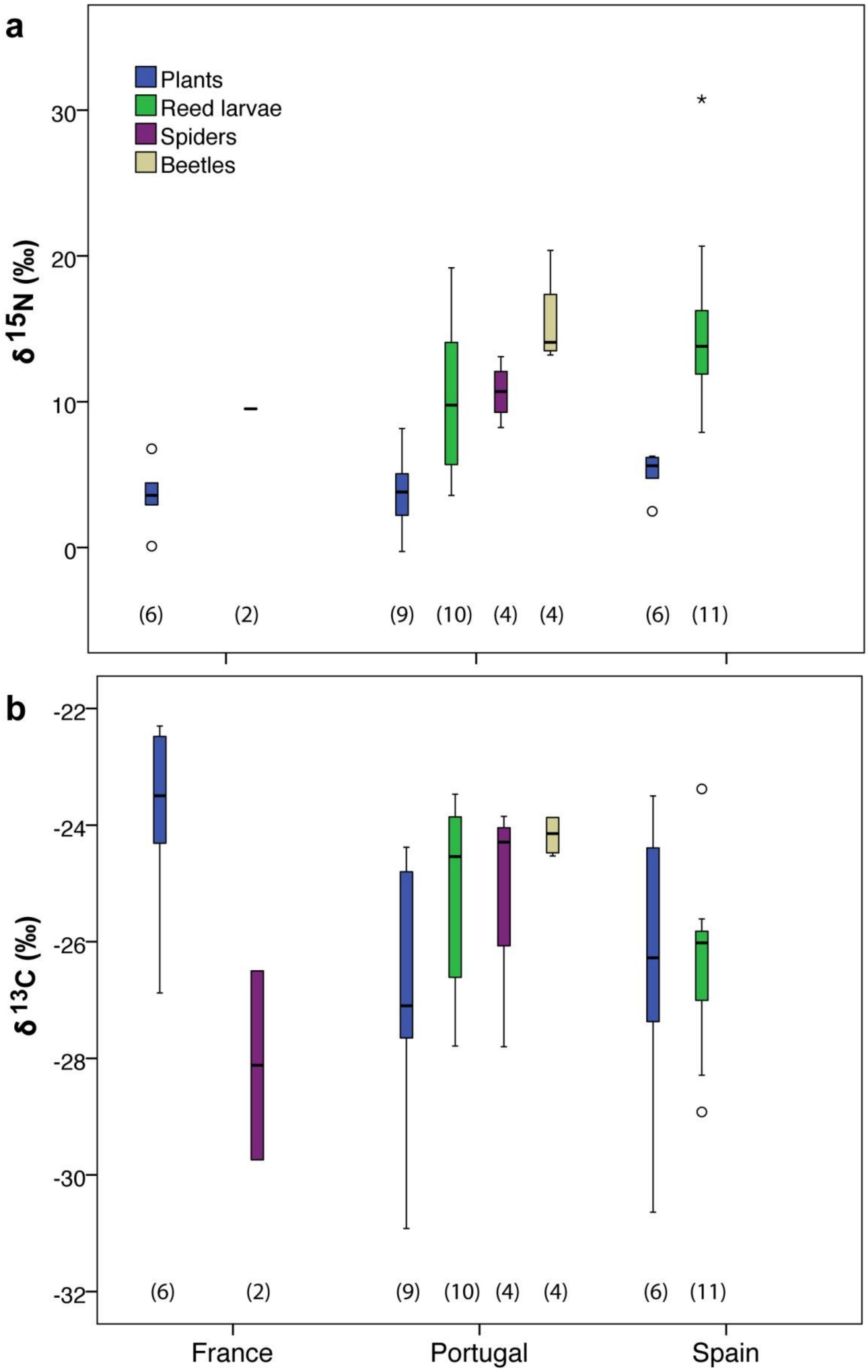
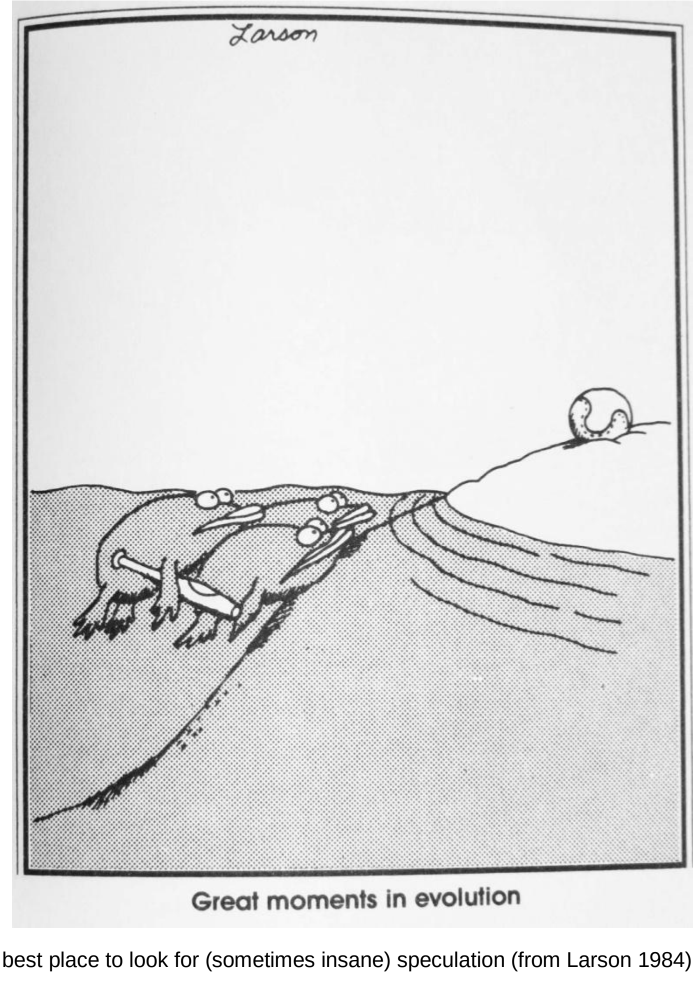


Figure S 5.4 - Boxplot showing variation in a) and b) of the various food items for each site. Medians (lines) and interquartile ranges (IQR; boxes) are shown, together with outliers greater than 1.5xIQR (circles) and greater than 3xIQR (asterisks). Sample size is indicated between parentheses.

Chapter 6

General discussion



Cartoon 7 - The discussion is the best place to look for (sometimes insane) speculation (from Larson 1984)

6 General discussion

This study describes the morphological and ecological divergence amongst reed bunting populations likely to be relevant for the seemingly on-going speciation process in this species. In addition, it reports the acoustic divergence of the various singing styles used by this species, and evaluates the extent to which these differences are perceived by territorial males of the different subspecies by measuring their behavioural responses relevant for reproductive isolation.

This study adds to previous work on genetic divergence among reed bunting subspecies thus contributing to an integrated perspective of speciation, for which there is still limited knowledge except for a few model organisms (Shaw & Mullen 2011).

6.1 Sources of divergent selection

6.1.1 Differences between environments

6.1.1.1. Morphologic divergence

The morphologic analysis focused on traits for which the direction of evolution could be predicted from two selection pressures that are known to influence speciation in birds: diet and migration.

Foraging niche adaptation

Data showed that the southern subspecies, which have has been observed feeding on dormant insects inside reed stems during winter, have thicker bills (which they use to open the reed stems; pers. observations, Prys-Jones 1984). In contrast, northern populations, which switch their diets to seeds during the winter (Orłowski & Czarnecka 2007), have shorter and thinner bills (see also Belda et al. 2009). In addition to the linear measurements, geometric morphometric analyses revealed important differences in bill shape, particularly in culmen curvature. This is most likely associated with differences in diet (Chapter 5) because birds with a more convex culmen are able to exert a greater strength at the bill tip, which is probably very useful to open the reed stems, whereas seed-eating birds tend to crack the seeds at the base of the bill (Foster et al. 2007). Given these results, and despite the overlap in bill traits with *schoeniclus* (especially in females), *lusitanica* appears to share morphological traits with the thick-billed subspecies (as expected by their ecology and distribution), but it is still quite distinctive from both groups due to its much smaller size and dark plumage (in addition to the feeding apparatus). No obvious adaptive explanation could be proposed for the much smaller body size of *lusitanica* relative to the remaining subspecies. This subspecies lives in close proximity to the large and thick-billed *witherbyi*, but uses mostly coastal reedbeds located in the Atlantic influenced (wet, mild) geographical region, whereas the latter occurs mostly in inland (occasionally coastal) reedbeds in the Mediterranean influenced (dry, hot or continental) region. Both the small size and dark plumage of *lusitanica* could perhaps be explained by adaptations to the mild, wet climate where they occur (following Gloger's rule).

Adaptation to migration

In agreement with predictions, migratory *schoeniclus* had longer and more convex[2] wings than the resident Iberian subspecies (see also Copete et al. 1999), and similar patterns have been found in other bird species (Milá et al. 2008; Tarka et al. 2010; Baldwin et al. 2010; Förschler & Bairlein 2010). As predicted by theory, the migratory *schoeniclus* also had slightly smaller body mass than the other populations; except for *lusitanica*. These variations/adaptations seemed to have occurred despite northern reed buntings being short to medium distance migrants, rather than long-distance migrants (Prys-Jones 1984), and so the selection pressure for high aspect-ratio wings might not be as strong as in other species that have been studied (e.g. Milá et al. 2008). Tail length, however, did not vary according to the expectation of shorter tail in migratory birds, and tail/wing ratio seemed to reflect mostly the longer wings of migrants (see also Milá et al. 2008). This may be a consequence of tail and wing lengths being strongly correlated both phenotypically and genetically in birds, and for this reason it is possible that tail length takes longer to evolve and may even act as a morphological constrain to adaptation in wing lengths (Tarka et al. 2010).

6.1.1.2. Behavioural divergence

Bill morphology has been shown to affect song output (Christensen et al. 2006; Huber & Podos 2006; Podos 1997; but see Grant & Grant 2002a, b; Slabbekoorn & Smith 2000), so this trait could potentially explain the differences found between subspecies, especially the generally higher song divergence of *witherbyi*, which has the most differentiated bill (Chapter 2). In this study, it was found that the thick-billed subspecies *witherbyi* sang songs with the lowest minimum frequency, thin-billed *schoeniclus* sang with the highest minimum frequency, and the intermediate-billed subspecies *lusitanica* used intermediate frequencies. Similar results have been described for Darwin's finches (Huber & Podos 2006; Podos 2001). However, *witherbyi* songs also have a

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[2] A more convex wing has longer outer primary feathers and shorter inner primaries and secondaries (see Chapter 2).

higher maximum frequency than those of *schoeniclus* and, consequently, for dawn and fast styles, a wider frequency bandwidth that does not fit with the expectations from the differences in bill morphology (although this expectation is specific to trilled vocalizations, which are common in reed bunting but were not studied separately here).

Some of the song traits measured in this study are directly genetically inherited, without much influence from tutors (Ewin 1978; Glutz von Blotzheim & Bauer 1997; Stewart 1955; Thorpe 1964), and are likely to be targets of selection potentially leading to an evolutionary response (i.e. of song traits within populations/subspecies).

6.1.1.3. Ecological divergence

The results show that: 1) there are consistent ecological differences in food choice 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.

Overall, the isotopic data are consistent with previous observations of thick-billed birds that feed almost exclusively on dormant insects inside reed stems, whereas *schoeniclus* eats seeds of various plants, including many C4 plants, as well as (flying) insects and spiders whenever available (Cramp & Perrins 1994; Matessi et al. 2002; Holland et al. 2006; Orłowski & Czarnecka 2007; Orłowski et al. 2013; JMN unpubl.). However, 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, in a way that does not require strong, convex bills to get access to this food (Chapter 2).

If bill size and diet were strictly associated, *witherbyi* should differ to a greater extent from the other two subspecies than the latter among each other, but this expectation is not supported by the results. Assuming that the foraging niches are indeed similar, three potential, non-exclusive explanations for the large difference in bill size are suggested: 1) they could be adapted to feed on insects inside reeds of different thickness or at different heights of the reeds (e.g., at brackish sites reeds tend to be shorter and thinner); 2) bill size could have diverged due to differences in climate at the locations where *lusitanica* and *witherbyi* occur (warmer eastern Iberian sites). 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). In addition, other climate variables are also known to affect bill size in a variety of bird species (Grant & Grant 2002c; Symonds & Tattersall 2010, Eroukhmanoff et al. 2013; Tattersall et al. 2016; Luther & Danner 2016; Danner et al. 2017). In fact, summer temperatures might be responsible for the clinal variation of increasing bill size towards the east among all thick-billed subspecies of reed buntings. 3) divergence due to sexual selection (see below under 6.1.3.).

Isotopic measurements indicated that intermediate- and thick-billed subspecies are ecological specialists whereas the thin-billed is a generalist. Although this could have resulted from competition between the co-occurring subspecies at the winter quarters, the association between bill traits and the isotopic niche suggests that natural selection could have had an important role in the phenotypic divergence of reed bunting subspecies. It further suggests that intermediate- and thick-billed subspecies (*lusitanica* and *witherbyi*) are more locally adapted than the more generalist *schoeniclus* because their higher degree of specialisation should result in a greater decrease in fitness if they are moved (or disperse) to different environments.

The finding that the intermediate- and thick-billed subspecies are ecological specialists may also have conservation implications, as the two threatened subspecies, being more specialized, seem to require more strict environmental conditions (Stockwell et al. 2003).

6.1.2 Competition

As the winter niche (and presumably diet) suggested by the isotope analyses differs between the resident and wintering populations of reed buntings, but both feed mostly on insects during spring/summer (Cramp & Perrins 1994; Holland et al. 2006; JMN unpubl.), 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; 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 food types and climate conditions 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, to avoid overlap in diet during the wintering periods when the three sub-species partly coincide in distribution range.

6.1.3 Sexual selection

Morphological divergence

Isotopic data from this study indicate that *witherbyi* has a similar isotopic niche to *lusitanica*. Three potential, non-exclusive explanations for their large difference in bill size are suggested, two of which were presented above, under ecological divergence (6.1.1.3.). The third is that sexual selection could be involved in the evolution of different bill size and shape among the subspecies. Male *witherbyi* with thicker, curved bills could be preferred by the females (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 (Grant & Grant 2002c; Eroukhmanoff et al. 2013).

Behavioural divergence: Signal

Most songbird species have several kinds of vocalizations that are used in different contexts, and hence may be subject to different evolutionary forces (Byers 1996; Kroodsma 1981; Byers et al. 2010). The greater divergence of structural traits in dawn and fast singing styles that was described for reed buntings in this study, strongly suggests that sexual selection was an important cause for acoustic divergence in this species.

Interestingly, findings from this work oppose the expected latitudinal gradient resulting from higher rates of evolution of syllable diversity and song length in north European passerines than in Mediterranean passerines (Cardoso et al. 2012; Weir & Wheatcroft 2011). In general, evolutionary changes in the quantity of song syllables are thought to be due to changes in pressure from sexual selection (Baker 1996; Lynch 1996; Read & Weary 1992). Perhaps the larger number of syllables found in *witherbyi* is the result of a shift in female preferences from vocal performance to song complexity, as suggested for coastal swamp sparrows (Ballentine 2006). In a study restricted to the fast singing style, Matessi et al. (2000b) also found higher syllabic complexity in thick-billed subspecies (*intermedia* and *witherbyi*) and a strong tendency for higher minimum frequency in the thin-billed *schoeniclus*. The fact that dawn and fast songs are relatively complex agrees with these styles being under sexual selection, because the production of more complex songs has been associated with female mate choice in many previous studies (e.g. Brunner & Pasinelli 2010; Catchpole & Leisler 1996; Forstmeier & Balsby 2002; Hasselquist et al. 1996; Neubauer 1999).

Given that reed bunting subspecies seem to be at an incipient stage of speciation (Matessi et al. 2000a; Chapters 2 to 5), the finding of greater divergence in sexually selected singing styles is interesting, suggesting that sexual selection may be playing an important role in this process (probably in conjunction with natural selection). Several studies indicate that sexual selection promotes the evolution of reproductive isolation and two meta-analyses found small but significant overall trends (Kraaijeveld et al. 2011; Seddon et al. 2013). When divergence between populations in singing styles related to female attraction is greater than in other styles, as shown in the current study and for some Nearctic warblers (Bay 1999; Janes & Ryker 2006, 2011; Moldenhauer 1992), there seems to be an enhanced potential for premating reproductive isolation to evolve.

Behavioural divergence: Perception

The results from this study, together with the (raw) data described in Matessi et al. (2000b, 2001b), suggest that Mediterranean thicker-billed birds (*witherbyi* and *intermedia*) show stronger discrimination against the songs of Atlantic and Northern thinner-billed birds (*lusitanica* and *schoeniclus*) than the latter two subspecies discriminate the songs of the Mediterranean birds. Two factors might contribute to such pattern. First, the variation in song complexity (number of syllables and number of different syllables) and frequency ranges shown by northern birds completely overlaps with part of the variation shown by southern birds, but the remaining variation in the southern birds (the most complex and broadband songs) is exclusive and distinctive (Matessi et al. 2000a; Chapter 3). This could make the discrimination task of southern birds more feasible (Dingle et al. 2010). Second, the southern areas are important for wintering northern birds, while southern birds are not known to visit areas far north at all (Villarán Adánez 1999). Hence, during the southern breeding season (March to June), local birds often come in contact with northern birds (singing during their late wintering period—October to April) and, as the northern subspecies does not compete with the southern subspecies for mates, selection might favor stronger song discrimination to avoid investing energy by reacting to a non-threat. This is consistent with the idea that song recognition allows territory owners to modulate their response according to the threat posed by each intruder and thus to reduce the costs associated with territorial defense (Briefer et al. 2009). Alternatively, even if northern males sometimes compete with southern males for mates in southern areas, it would be selectively advantageous for southern females to discriminate against northern males because the potential costs of out-breeding for a locally adapted population may be high (Bensch et al. 1994; Edmands 2007; Hansson et al. 2004; Keller & Waller 2002;

Marr et al. 2002). For the black-throated blue warbler (*Setophaga caerulescens*), it has also been suggested that individuals in the north have limited opportunity to perceive and/or learn the southern song, but that the same is not true in the south, possibly due to asymmetrical exposure during migration (Colbeck et al. 2010). Other explanations, like stronger response to high-performance songs (Phillips & Derryberry 2017) or to less divergent song stimuli (Sosa-López et al. 2016; Lipshutz et al. 2017), alone cannot explain the pattern found in this study. Population differences in sexually selected singing styles seem to be associated with different signal perceptions in reed buntings, and may be causing some level of reproductive isolation between the subspecies (Chapter 4).

6.1.4 Interactions between the three sources

The interpretation that interactions between several sources of divergent selection are the cause of the differences found between reed bunting subspecies is in line with the latest research. For instance, in hermit thrush (*Catharus guttatus*), Eastern and Western song structure differ dramatically, notably introductory note frequencies, and song is probably shaped by a variety of forces, ranging from large-scale geological events to factors such as habitat differences and cultural drift (Roach & Phillmore 2017). Even in the very well studied Darwin’s finches, song structure differences have been linked both to changes in beak morphology via natural selection (Podos & Nowicki 2004; Huber & Podos 2006) and to cultural drift (Grant & Grant 1997).

Congruence between morphology and song

In this thesis, morphological divergence was studied as an adaptation to diet and migration, which is due to differences between environments (and possibly competition). On the other hand, song divergence was studied mostly in relation to sexual selection (but also to some extent in relation to differences between environments). Therefore, looking at the congruence between morphology and song is in a way similar to discussing interactions between two of the main sources of divergent selection during speciation: differences between environments and sexual selection.

As mentioned above, reed bunting subspecies were described mostly on the basis of morphology (Byers et al. 1995; Cramp & Perrins 1994). Song, however, could potentially produce different patterns of intra-specific variation. The hierarchical cluster dendrogram for fast singing style (Figure 3.3b) shows that songs from birds in central Iberia are more similar to songs of geographically distant birds from southern France than to songs of birds from the nearby western Iberia (Figure 3.3b). This suggests an abrupt acoustic change, rather than clinal variation. Like geographical distance, habitat connectivity is probably not a confounding parameter, because reedbeds are a rare and highly fragmented habitat in southwestern Europe, so connectivity between all 10 populations sampled is low to nonexistent (see also Gammon et al. 2005; Laiolo et al. 2008). However, many *schoeniclus* migrate to spend the winter in the Mediterranean area, using many reedbed patches during their annual cycle and co-occurring with *lusitanica*, *witherbyi* and other subspecies at their wintering quarters (Byers et al. 1995; Cramp & Perrins 1994; Chapter 2). Hence, *schoeniclus* males could potentially learn the songs of *lusitanica* and *witherbyi* among others, especially the fast songs, which start to be used by local residents before *schoeniclus* departs to their northern breeding areas (personal observation). Nevertheless, large differences between the subspecies were found, especially between *schoeniclus* and the two resident subspecies (Figure 3.3b). Geographical congruence between morphology and song is a pattern often found in birds, even in recent studies (e.g. Kryukov et al. 2017). According to Wilkins et al. (2018), species with post-dispersal learning (i.e. in which immigrants can learn nonlocal songs and breed successfully after natal dispersal) will show an association between acoustic and geographic distance, but not acoustic and genetic distance (Podos & Warren 2007; Sun et al. 2013; González & Ornelas 2014).

6.2 Forms of reproductive isolation

6.2.1 Divergent habitat & phenology

The main general habitat of all populations studied is the same (reedbed). However, *schoeniclus* has been shown to be more eclectic, using several types of farmland habitats. Even between the Iberian subspecies, some habitat differences can be found, with *lusitanica* showing preference for costal Atlantic reedbeds and *witherbyi* for inland Mediterranean reedbeds. Regarding phenology, both subspecies breeding in Iberia seem to be mostly resident and breed at the same time, so phenology is probably not an important cause of reproductive isolation (less so if compared with divergent mating preferences). *Schoeniclus* is migratory and breeds later in the season so phenology may play a role in isolating it from the Iberian subspecies, but, again, this probably plays a smaller role compared to the divergent mating preferences found.

The three subspecies of reed bunting have differentiated in a variety of morphological traits (Chapter 2), with the two southern subspecies (*lusitanica* and *witherbyi*) having a specialized foraging behavior, which is associated with thicker, convex bills and a much narrower foraging niche than *schoeniclus* (Chapters 2 and 5;

personal observation). For these two resident subspecies, the cost of mating with a migratory thin-billed subspecies (*schoeniclus*) is expected to be high, given the presumably lower fitness of descendants with intermediate bill sizes. Accordingly, males of these subspecies responded weakly (or not at all) to *schoeniclus* songs. In contrast, males of the ecological generalist *schoeniclus* responded strongly to all subspecies songs, perhaps indicating that the potential costs of subspecies mixing ("hybridization") are not so high for this subspecies.

6.2.2 Divergent mating preferences

The differential territorial defense in relation to own versus foreign subspecies discussed above (6.1.3. Behavioural divergence: perception) suggests that an intruding male with foreign songs does not represent a large threat, a pattern that is expected when there is some degree of premating isolation based on song (Balakrishnan & Sorenson 2006). Also, male territorial responses are often correlated with female preferences (e.g., Searcy et al. 1997). Thus, the results obtained here indicate the existence of premating reproductive isolation among these recently evolved reed bunting subspecies and support the view that sexual signals, in this case male song, can have a role during speciation, but it would be interesting to further test this by studying female choice in the future.

6.3 Conclusions

The morphometric analyses from this study (Chapter 2) clearly show that the three subspecies of reed bunting occurring in Western Europe differ in a variety of traits in the direction predicted by their migratory and foraging behaviours, strongly suggesting that these birds became locally adapted through natural selection. An increase in the rate of phenotypic divergence has been associated with speciation events (Cardoso & Mota 2008; Arbuthnott et al. 2010; Maia et al. 2013; Gomes et al. 2016). Furthermore, early pulses of trait divergence have been suggested to promote subsequent transitions to sympatry, rather than such pulses occurring after sympatry has been established, for example via character displacement (McEntee et al. 2018).

The finding that the intermediate- and thick-billed subspecies are ecological specialists whereas the thin-billed is a generalist (Chapter 5) 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) found among these subspecies (Chapter 4).

Phenotypic divergence in bill size/shape was partially associated with differences in isotopic niche, also suggesting that reed bunting subspecies evolved through natural selection and became locally adapted. The southern, resident subspecies have a narrow isotopic niche and probably use different feeding techniques as described in the literature (Shtegman 1948 cited by Prŷs-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 (Chapter 5).

The patterns of foreign subspecies song discrimination indicate a strong premating reproductive isolation between each of the two southern, resident subspecies (*lusitanica* and *witherbyi*) and the northern, migratory *schoeniclus*, and a slightly less strong isolation between *lusitanica* and *witherbyi* (see Figure 4.5). As *lusitanica* is genetically closer to *schoeniclus* than to *witherbyi* based on mtDNA and microsatellites (Kvist et al. 2011) as well as nuclear intron sequences (Neto JM, unpublished data), premating reproductive isolation is in line with ecological rather than genetic differentiation (Figure 6.1), indicating early stages of ongoing ecological speciation (i.e., isolation-by-ecology) in reed buntings (see Shafer & Wolf 2013). Song, the basis for acoustic recognition (an isolating barrier), and genotype are not tightly coupled in several other species either (e.g. Kenyon et al. 2017).

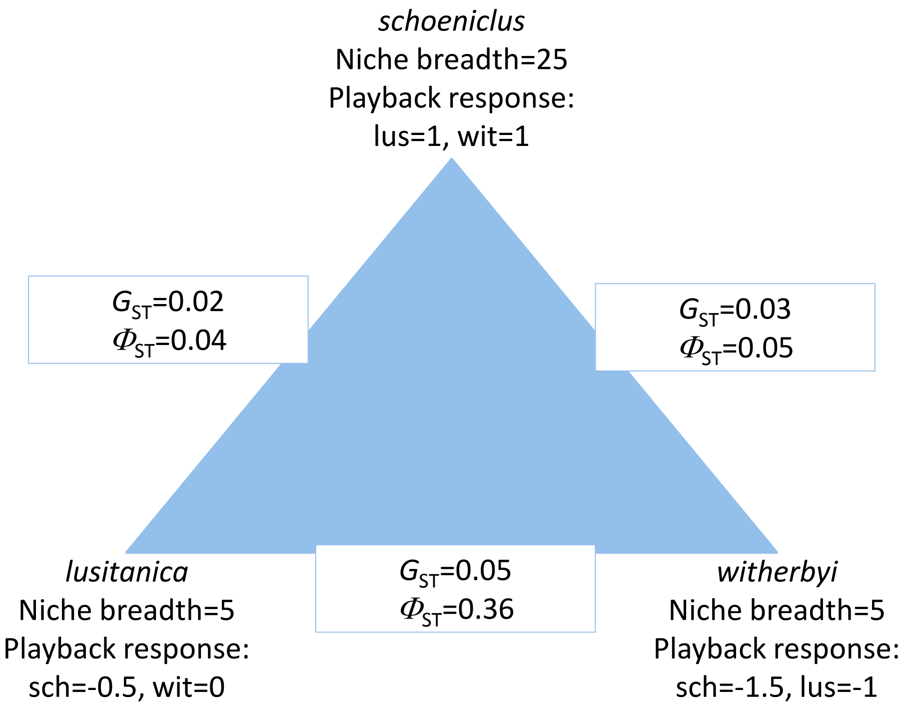

Figure 6.1 - Relative isolation between each subspecies pair (from the strength of response to playback, estimated as EMM PC1 in Chapter 4) versus ecological divergence (isotopic niche breadth, estimated as SEA in Chapter 5) and genetic divergence (from Kvist et al. 2001, for *witherbyi* regarding Ebro birds only).

The phenotypic differences among populations found in this study probably evolved very rapidly through natural selection. This is because there is a small but significant genetic divergence among the reed bunting subspecies, and a shallow mtDNA phylogeny, indicating that they diverged very recently, after the last glacial maxima (Kvist et al. 2011). The star shape of the haplotype network presented by Kvist et al. (2011) also indicates an expansion from a single glacial refugium. In addition, the morphological traits studied here generally have high heritabilities (Keller et al. 2001; Tarka et al. 2010) and showed limited plasticity in a common garden experiment with a North American emberizid (Ballentine & Greenberg 2010). However, genetic drift, especially in the threatened Iberian subspecies, cannot be excluded as a potential explanation for some of the morphological differences that were found, nor does (adaptive) plasticity. Several theoretical models have shown that ecological differences can drive the evolution of partial reproductive barriers in dozens to hundreds of generations. Barriers likely to evolve fast include dispersal rate, habitat preference and selection against migrants/hybrids (Hendry et al. 2007). Birds evolving different migratory routes can mate assortatively within at least 10–20 generations (Bearhop et al. 2005).

This study shows that in addition to phenotypic (Chapter 2), acoustic (Chapter 3) and genetic (Kvist et al. 2011) differences, reed bunting subspecies differ in (foraging) ecology (Chapter 5). Also, Chapter 4 suggests that local adaptation is (in)directly causing reproductive isolation among the subspecies, providing evidence for ecological speciation in this study system. Although Wilkins et al. (2018) highlight a role for stochastic processes for song evolution in barn swallows, they also mention that deterministic ecological processes clearly dominate the evolution of some song parameters for some species (Boncoraglio & Saino 2007; Ey & Fischer 2009; Mason & Burns 2015).

6.4 Limitations and future directions

One important limitation of this study is that reproductive isolation was not actually measured; a more quantitative approach should be used in the future. Despite that, the relative isolation between each subspecies pair is discussed above, based on their responses to each others song. This is important to compare with ecological and genetic divergence and thereby directly test the importance of ecology vs. time of divergence (genetic) in the evolution of reproductive isolation.

6.4.1 Song divergence

Plastic changes in some song traits due to seasonal or environmental effects (e.g. increased minimum frequency in response to anthropogenic noise; Gross et al. 2010) are unlikely to have affected results significantly because birds were sampled only during the breeding season and all study sites were in natural habitats, distant from loud sources of anthropogenic noise. In addition, recent research suggests that either noise is not the causal force driving the divergence of song frequency between urban and forest populations, or that noise induces population-wide changes over a time scale of several generations (Zollinger et al. 2017).

A detailed study of song performance, comparing trill rates and frequency bandwidth in the three subspecies, is needed to clarify if, like in swamp sparrows, 'thick-billed' *witherbyi* males with larger bills and lower 'song performance' may compensate for that by increasing song complexity. Call divergence should be studied as well, since this kind of vocal output is known to include a larger innate component (Marler 2004), and may potentially affect communication/recognition among subspecies.

6.4.2 Divergence in song recognition

Male and female response patterns to local and foreign songs are often similar (e.g., Searcy et al. 1997), so that the former has been examined as a proxy for the latter in several studies (e.g., Christensen et al. 2010). Therefore, the results from this study imply that females can discriminate between males on the basis of song. However, the assumption that females prefer the same signals that elicit strong responses from the males is not true for every case (e.g., Nelson & Soha 2004; Anderson et al. 2007). Therefore, it would be important to study song recognition by females. However, this would be better conducted in captivity (e.g., Ceugniet & Aubin 2001; Nelson & Soha 2004) raising considerable experimental problems and should probably not be conducted in the threatened southwestern subspecies (*lusitanica* and *witherbyi*). Condition- and context-dependent factors related to courtship behavior of paired and unpaired males might also have affected playback results in this study. Recently, it has been shown that paired male dark-eyed Juncos (*Junco hyemalis*) approached the female more rapidly, spent more time in close proximity, were more active and spent more time with body feathers erect, but sang fewer songs (Reichard et al. 2017). However, in the present study there was no evidence that the ratio of paired to unpaired males in playback areas differed significantly, so this potential source of bias was probably not important. Studies of signal recognition among reed bunting subspecies should not be restricted to acoustic signals. Visual recognition should be tested as well, namely using models of singing males. Some recent studies highlight the importance of visual signal recognition (Rek & Magrath 2017). Since only song was used, but recognition is likely to involve also plumage, body size and bill size, all of which differ to some extent between subspecies, reproductive isolation may have actually been underestimated. Future studies of acoustic signal recognition should also cover Dawn song, which has been shown to be under strong sexual selection (Suter et al. 2009; Chapter 3). With Dawn song, testing female preferences in the field might be possible. However, it would require performing playback experiments in the dark, for instance using an infra-red camera, which would considerably complicate the procedures (Bolton 2007, Lourenço et al. 2008, Santos et al. 2008).

6.4.3 Ecological divergence

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. Moreover, 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 Figure S 5.4), for which isotope mixing models would be useless. It is not possible to completely exclude the possibility that *lusitanica* and *witherbyi* have different diets that happen to coincide in their isotope ratios.

Future studies should therefore 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. It will be challenging to test the hypotheses that; (1) intraspecific competition at the winter quarters may have been the main driver of ecological differentiation in reed buntings, and (2) the smaller, straighter bill of migratory birds evolved as the populations of reed buntings expanded north after the last glaciations. However, diet studies of reed bunting populations that do not co-occur (compete) with northern migrants might prove useful, as well as detailed phylogenetic and character evolution studies. The relative roles of diet and temperature on the evolution of bill size should be further studied in reed buntings, especially among subspecies with similar diets.

6.5 References

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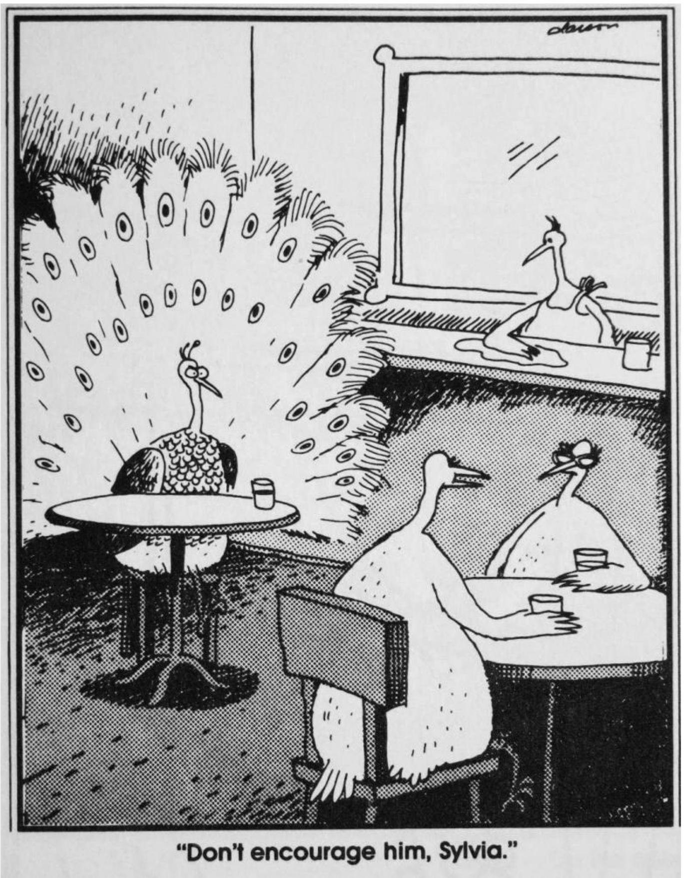
Appendix

Thesis outreach, other papers published during the PhD & peer-reviewed manuscripts



Cartoon 8 - As seen in Chapter 3, sexual selection is important in reed bunting divergence (from Larson 1984)

Appendix

Thesis outreach

Gordinho L & Neto JM (2013) Signal design and perception in Iberian reed buntings. Poster D23SY02PS0404. Symposium 2: Selection and Evolution in Natural Populations. p. 80 in *Abstract book of the 14th Congress of the European Society for Evolutionary Biology (ESEB), Lisbon, Portugal, 19 - 24 August 2013*.

Neto JM, **Gordinho L** & Newton J (2013) Phenotypic divergence among west european populations of reed bunting *Emberiza schoeniclus* agrees with migratory and foraging behaviours indicating local adaptation. Poster D23SY02PS0366. Symposium 2: Selection and Evolution in Natural Populations. p. 75 in *Abstract book of the 14th Congress of the European Society for Evolutionary Biology (ESEB), Lisbon, Portugal, 19 - 24 August 2013*.

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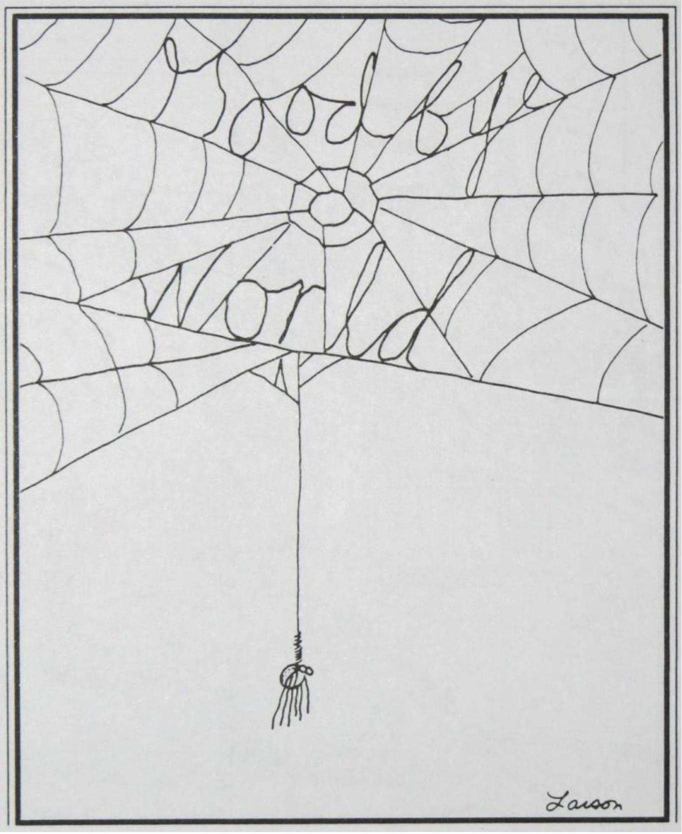

Cartoon 9 - The end of this web (the thesis), the spider lives on… (from Larson 1986)

Maybe I flatter myself that people might think I can help with basic bird identification. Such as when a woman fumbles through Birds of Europe [Jonsson 1992] *trying to put a name to the female Reed Bunting perched just a few meters away. I ask, 'Would you like to know what bird that is?' She replies, 'Oh, it's all right. I've found it on page 498. It's only a Rock Sparrow* [no records in Great Britain or Ireland]*.' Now what? There was a time when I would have gently explained that her misidentification was a plausible and understandable slip. However, this approach can go down like a lead balloon when indignant novices feel patronized and stick to their guns. These days I tend to smile sweetly and say, 'You may well be right.'* (McGeehan 2002)

Very few people I knew [at Oxford] *from the science side made jokes, they just went on splitting the atom instead of splitting their sides.* (Palin *in* Python Pictures 2003)

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