Non-receding hare lines: genetic continuity since the Late Pleistocene in European mountain hares (*Lepus timidus*)

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1   **ABSTRACT**

2   Throughout time, climate changes have caused substantial rearrangements of habitats which
3   have alternately promoted and disfavoured different types of taxa. At first glance, the
4   mountain hare (*Lepus timidus*) shows the typical hallmarks of a cold-adapted species that has
5   retreated to refugia since the onset of the current Holocene interglacial. In contrary to
6   expectations, however, the species has a high contemporary genetic diversity with no clear
7   differentiation between geographically isolated populations. In order to clarify the
8   phylogeographic history of European mountain hares, we here analysed ancient DNA from
9   the glacial populations that inhabited the previous midlatitude European tundra region. Our
10 results reveal that the Ice Age hares had similar levels of genetic variation and lack of
11 geographic structure as observed today, and the ancient samples were intermingled with
12 modern individuals throughout the reconstructed evolutionary tree. This suggest a temporal
13 genetic continuity in Europe, where the mountain hares were able to keep pace with the rapid
14 changes at the last glacial/interglacial transition, and successfully track their shifting habitat to
15 northern and alpine regions. Further, the temporal demographic analyses showed that the
16 species' population size in Europe appear to have been tightly linked with palaeoclimatic
17 fluctuations, with increases and declines occurring during periods of global cooling and
18 warming, respectively. Taken together, our results suggest that neither habitat shifts nor
19 demographic fluctuations have had any substantial impact on the genetic diversity of
20 European mountain hares. This remarkable resilience, which contrasts to a majority of
21 previously investigated cold-adapted species, is likely due to its generalist nature which
22 makes it less vulnerable to environmental changes.

23

24 **Keywords**: Ancient DNA; approximate Bayesian computation; coalescent simulations;
25 glacial cycles; climate change; phylogeography; range shift

26

INTRODUCTION

Background

Many species currently inhabiting temperate as well as arctic environments are likely to have a demographic history that includes periods of stability, followed by contraction into suitable habitat during unfavourable environmental conditions. The timing of such retreats into refugia and the subsequent mode of expansion has important implications for species' evolutionary trajectories. These trajectories depend a great deal on the life histories of the various taxa and particularly to what extent they are adapted to cold or temperate environments (Stewart *et al.*, 2010).

Cold-adapted species have generally been more abundant and widespread during glaciations, and experienced population bottlenecks and range contractions during interglacials (Muster *et al.*, 2009) such as the current Holocene period. These contractions occurred into polar refugia, as well as to the so-called cryptic southern refugia like the European Alps and Pyrenees (Stewart *et al.*, 2010), and therefore many cold-adapted taxa today show disjunct distributional patterns. Further, past local population extinctions and re-colonisations are expected to have left corresponding signatures of founder events in previously glaciated areas, which show genetic associations with the source populations. These source populations usually inhabited the surrounding lowlands, the tundra of periglacial Europe, or further to the east, as the European high latitude and altitude habitats were largely covered by ice caps during the Late Pleistocene glacial period (Fig. 1). One important parameter that has affected the genetic diversity in cold-adapted species is to what extent the glacial populations that inhabited non-refugial areas were able to track the decreases in habitat that took place at the Pleistocene/Holocene transition. A lack of such ability has been seen for true lemmings (*Lemmus* spp.) and arctic foxes (*Vulpes lagopus*), whose southern populations were unable to follow their shifting habitat during postglacial warming and became locally extinct (Dalén *et al.*, 2007; Lagerholm *et al.*, 2014). Similarly, extant collared lemmings (*Dicrostonyx torquatus*) only retain a small fraction of the genetic diversity observed in Late Pleistocene samples, due to a series of local extinctions associated with past periods of climate fluctuations (Palkopoulou *et al.*, 2016; Prost *et al.*, 2010).

Taxa adapted to other environments show different patterns and responses to global climate fluctuations. Temperate species tend to expand their ranges and population sizes in the warmer interglacial periods (Hewitt, 1999), and during glacials go through range contractions

into southern as well as cryptic northern refugia. The latter are restricted areas with favourable climate that allow the survival of temperate species at higher latitudes than would be anticipated (Stewart *et al.*, 2010). Modern temperate species are thus expected to show a distinct and separate population genetic pattern compared to their cold-adapted neighbours, with strong signals of postglacial expansion from different southern refugia and decreasing diversity towards the north (Hewitt, 1996). Examples of temperate species with phylogeographic signals reflecting traditional expansions from southern refugia are hedgehogs (*Erinaceus* spp.), water voles (*Arvicola terrestris*) and crested newts (*Triturus cristatus*) (Hewitt, 1999).

Although species with similar climatic adaptations are generally expected to have similar demographic histories, it has become increasingly clear that species within each category (*i.e.* cold-adapted and temperate) also have responded in an individual manner to changes in habitat availability associated with past climatic fluctuations (Stewart, 2008). Indeed, the ecological characteristics of each species likely determine the pace of population expansion, endurance in refugia, and the degree of any associated genetic differentiation (Stewart *et al.*, 2010). For example, moose (*Alces alces*), grey wolf (*Canis lupus*) and brown bear (*Ursus arctos*) all display relatively large distributions today as well as during the Late Pleistocene. Consequently, these species do not conform to the typical pattern of range expansions and contractions in concert with the glacial cycles (Niedziałkowska *et al.*, 2014). Moreover, due to their wide dispersal capabilities, they have experienced significant levels of gene flow and population admixture and show relatively constant effective population sizes (N_e's) through time.

The mountain hare (*Lepus timidus*) is an interesting case amongst the Late Pleistocene fauna. The glacial distribution of the species (Fig. 1) seems broadly consistent with the Mammoth steppe biome (Kahlke, 1999), and mountain hare fossil finds are frequently reported from non-cultural and Palaeolithic sites across Europe (e.g. Koby, 1960; López-Martínez, 1980; Napierala, 2008; Wojtal *et al.*, 2012). Today, the European range is restricted to the Alps, Ireland and Scotland, as well as being more continuous throughout Fennoscandia and north-eastern Europe (Fig. 1). Mountain hares from the Irish population have also in recent times been introduced to some Scottish islands (Angerbjörn & Flux, 1995). From the fossil remains and its modern distribution, the mountain hare consequently shows the hallmarks of a cold-adapted species that retreats to northern and alpine refugia during interglacials. This disjunct Holocene distribution might be expected to create genetic differentiation between recently

isolated populations, as has been observed in other cold-adapted species that inhabit similar regions in Europe today, such as the rock ptarmigan, *Lagopus muta* (Sahlman, Segelbacher & Höglund, 2009). However, the phylogeographic relationships between modern-day mountain hare populations are very confusing. There is little geographical structuring among genetic lineages, and the overall and regional sequence diversity is high (Melo-Ferreira *et al.*, 2007). It is difficult to deduce whether this pattern is shaped by contemporary gene flow or is a consequence of shared ancestral polymorphisms among postglacial colonisers. Hamill *et al.* (2006) and Melo-Ferreira *et al.* (2007) favour the latter scenario, in which the diversity in modern arctic and alpine populations is explained by recent colonisation from a large and continuous glacial mountain hare population. Based on Fu's F statistics and mismatch distributions, Melo-Ferreira *et al.* (2007) also estimate that this ancestral population increased substantially in size during the cold climate of an earlier glacial period (ca 164,000 years BP). On the other hand, Walteri & Cook (2005) and Pierpaoli *et al.* (1999) hypothesise that the lack of modern phylogeographic structure may reflect ongoing high levels of gene flow throughout Eurasia, but also acknowledge that it may be a result of incomplete lineage sorting. Further, Pierpaoli *et al.* (1999) dates the ancient population expansion of mountain hares to the late Middle Pleistocene, just before start of the last warm interglacial period, some 135,000 years BP. The time of the first fossil appearance of *L. timidus* is much earlier, at least before 0.4 Myrs (Fostowicz-Frelik & Gasparik, 2006). It is thus plausible that the species evolved from an Early Pleistocene ancestor, which was adapted to the spreading of steppe biota triggered by the onset of the Ice age. The competing views by molecular biologists on the extant species consequently fuel an ongoing debate over the preferred ecological niche of the mountain hare (Hamill *et al.*, 2006). The division of species into categories that conform to the traditional expansion/contraction model would suggest that *L. timidus* is a cold-adapted species that is now confined to polar and cryptic southern refugia. Populations thus remain fragmented with limited dispersal across temperate zones (Hamill *et al.*, 2006). The question remains, however, why the phylogeographic pattern does not indicate a recent population decline if the species has recently shifted its range into isolated refugia which were previously glaciated during the Pleistocene. One possible explanation for this pattern could be that the species managed to quickly and efficiently track suitable habitat as the climate warmed, thus preserving much of the genetic diversity despite some reduction in population size. On the other hand, the argument of high contemporary gene flow supposes that mountain hares have the general phylogeographic history of temperate species, and thus have expanded into artic and alpine regions following postglacial climate warming, while also

126   maintaining connectivity with continental populations at lower latitudes and to the east. This
127   view, however, cannot explain the high diversity observed at the northern extent of the
128   species' range, which for temperate species should be lower than that at the core of the range
129   due to genetic founder effects (e.g. Hewitt, 1996). A final alternative may be that a decline in
130   genetic variation and N_e did occur but has not been detected, because extinct Pleistocene
131   lineages cannot be directly observed in modern sequence datasets (Brace *et al.*, 2012).

132   These conflicting interpretations of the mountain hare's phylogeographic history are unlikely
133   to be reconciled with modern genetic data alone, due to the similar patterns produced by
134   contemporary gene flow and incomplete lineage sorting. Further, the complex climatic history
135   of the Pleistocene period gives good reasons to expect that modern arctic/alpine species'
136   distributions are the result of multiple and varied phases of population expansions and
137   contractions. Empirical data to resolve biogeographical histories of these taxa, however,
138   remain very scarce (Muster & Berendonk, 2006; Muster *et al.*, 2009). One solution is to
139   investigate genetic variation in ancient samples from the final stages of the Late Pleistocene
140   glaciation. A comparison of past and present diversity makes it possible to detect series of
141   historical local extinctions and recolonization events (Brace *et al.*, 2012) that possibly have
142   been associated with the Pleistocene/Holocene transition. This approach is thus of great value
143   for understanding how habitat and climate changes have differentially affected the
144   evolutionary history of temperate and cold-adapted species (Hofreiter & Barnes, 2010). More
145   specifically, ancient sequence data can help resolve whether mountain hare haplotypes have
146   disappeared in certain areas and been replaced by those carried by recent colonisers, or if the
147   weak phylogeographic structure observed in modern hares was a feature also of ancient
148   populations. Further, temporally sampled data makes it possible to infer the demographic
149   history and to resolve the trajectory of post-glacial expansions or contractions.

150   The aim of this study is to use subfossil remains of mountain hares dating back to the Late
151   Pleistocene from several palaeontological sites in mainland Europe, to clarify the
152   phylogeographic history of the species. We use mitochondrial DNA sequencing of the control
153   region (CR) and Cytochrome B gene (CytB) together with Bayesian phylogenetic analyses
154   and approximate Bayesian computation (ABC) to examine the historical distribution of
155   genetic diversity and to determine whether, and if so in what direction, the effective
156   population size has changed since the Pleistocene/Holocene transition.

157

MATERIAL AND METHODS

Sampling

We collected ancient mountain hare bones from 45 individuals sampled from 20 palaeontological sites across Europe (Table S1), ranging in age from approximately 44 thousand (k) calendar years before present (BP) to 10 k BP. Samples were selected from different natural history collections to cover the extent of the modern-day European range of the mountain hare and also to provide a reasonable representation of the range during the Late Pleistocene period (see Fig. 1). Additionally, we compiled sequence data from Melo-Ferreira *et al.* (2007) of portions of the mtDNA control region and Cytochrome B gene from 124 modern mountain hares, originating from 10 different Eurasian countries.

DNA extraction and sequencing

DNA extractions from the ancient samples were conducted in the ancient DNA laboratory at the Swedish Museum of Natural History, Stockholm, according to Brace *et al.* (2012). Briefly, 50 mg of bone powder was incubated in an EDTA buffer with urea and proteinase K for 24 hours at 55 °C. The supernatants were then concentrated using Vivaspin filters before mixing with PB buffer (Qiagen, Germany) and application to Qiaquick spin columns. Spin columns were washed with PE buffer and then eluted with EB buffer to a final volume of 100 μl.

In order to cover the equivalent length fragments of Cytochrome B and control region as in Melo-Ferreira *et al.* (2007), we designed primers for five overlapping segments of each mtDNA region (see Table S2). PCRs were performed in 25 μl volumes containing $1 \times$ PCR buffer, 0.2 μM of each primer, 200 μM dNTPs, 2.5 mM $MgCl_2$, 0.1 mg/mL BSA, 0.4 Units HotStar Taq DNA polymerase (Qiagen, Germany), purified water, and 2 μL of DNA extract. PCR conditions were 10 min at 95 °C, followed by 55 cycles of 30 s at 94 °C, 30 s at 50 °C or 51 °C (dependent on primer pair specifications), 30 s at 72 °C, and a final extension of 7 min at 72 °C.

Amplicons were purified using Exonuclease I and Shrimp Alkaline Phosphatase and sequenced on an ABI3130XL at the Konrad Lorenz Institute for Ethology, Vienna. Sequencing chromatograms were assembled and analysed using Geneious 5.5.4. Throughout our procedures, protocols to prevent contamination and ensure accurately coded (undamaged)

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189 mtDNA were followed, including isolation of work areas, negative controls, reduced fragment
190 length amplification, and repeated PCRs and sequencing of all samples and sub-amplicons.

191

192 *Sequence analysis and network construction*

193 Ancient DNA sequence data were aligned with modern sequences from Melo-Ferreira *et al.*
194 (2007), and grouped into 5 geographic regions (see Table S3). Three datasets were
195 constructed; one for cytochrome B (CytB), one for control region (CR), and one consisting of
196 concatenated CytB and CR sequences. Arlequin v.3.5.2.2 (Excoffier & Lischer, 2010) was
197 used to calculate standard diversity estimates for the two gene regions, during both the present
198 and the Late Pleistocene period. Further, temporal statistical parsimony networks were
199 created with the R-script TempNet v1.4 (Prost & Anderson, 2011) to display the relationships
200 between all haplotypes found in the two time periods. This was done for the concatenated
201 CytB+CR dataset, as well as for each mitochondrial region separately.

202

203 *Phylogenetic and demographic analyses*

204 We used BEAST v1.8.0 (Drummond *et al.*, 2012) to create a dated phylogeny, based on the
205 concatenated dataset. The mean of the approximate ages of the Late Pleistocene samples were
206 used as tip dates, and a strict molecular clock of 7.7 % substitutions per million years (Myr^{-1})
207 was employed, following the rates used in (Cheng *et al.*, 2014) and the proportion of the two
208 mitochondrial regions in our dataset. The mutation rate is a sensitive parameter that could bias
209 demographic analyses if accurate estimates are not employed. In addition, the so called
210 temporal dependency of the mutation rate, a phenomenon whereby substitution rates are faster
211 on shorter time scales, can be a source of uncertainty (Ho, 2014). For that reason, we assessed
212 the robustness of our conclusions by replicating the entire set of analyses with mutation rates
213 of 15.8 % (following Melo-Ferreira *et al.*, 2007) and 50 % Myr^{-1}. We also performed parallel
214 analyses with both the complete Eurasian dataset, as well as using European sequences only
215 (*i.e.* the geographic extent of the ancient sampling). The substitution model was set to
216 HKY+I+G, as recommended by the AIC and hLRT criterion in MrModeltest v2.3 (Nylander,
217 2004), and a Coalescent Constant size tree prior was used together with a randomly generated
218 starting tree. The length of the Markov chain Monte Carlo (MCMC) was set to 50 million
219 generations, with parameters logged to file every 1,000 generations. Two independent runs

220 were made on the same input file and the results were checked in Tracer v1.4.0 (Rambaut &
221 Drummond, 2007) to ensure effective sampling and convergence on the same posterior
222 distributions. The trees from two runs were then combined in LogCombiner v1.8.0
223 (Drummond *et al.*, 2012) and annotated to a maximum clade credibility tree in TreeAnnotator
224 v1.8.0 (Drummond *et al.*, 2012), using a burnin of 10% and a posterior probability limit of
225 0.5. Finally, FigTree v1.4.0 (Rambaut, 2012) was used to graphically edit the output.

226 BEAST v1.8.0 (Drummond *et al.*, 2012) was also employed to analyse demographic histories
227 using the Coalescent Bayesian Skyline approach (Drummond *et al.*, 2005). The analysis was
228 made both for Eurasia and Europe (again with the concatenated dataset), with a piecewise-
229 constant Skyline model, 10 groups and a random starting tree, and run for 70 million
230 generations. The tip dates, clock rates and evolutionary model were the same as in the
231 previous phylogenetic reconstruction. Log and tree files from 2 replicate runs were combined
232 in LogCombiner v1.8.0 (Drummond *et al.*, 2012) and then used as input to Tracer v1.4.0
233 (Rambaut & Drummond, 2007) in which Skyline plots were created.

234

235 *Approximate Bayesian computation*

236 We employed approximate Bayesian computation (ABC) to infer the model of demographic
237 history that best fitted the temporal pattern of genetic diversity in Eurasian mountain hares.
238 Inferring a demographic history by ABC is usually performed following a model choice
239 approach, in which a collection of feasible models are simulated and compared via Bayes
240 factors, which are ratios of their estimated Bayesian likelihoods. Although such an approach
241 has been successfully applied in numerous studies, concerns have been raised that it is highly
242 subjective when applied to complex models (Templeton, 2010). Moreover, the hypotheses
243 regarding the demographic history of *L. timidus* are diverse and debated. For those reasons,
244 we conducted an analysis that bypassed the problem of defining relevant models. It consisted
245 of sequentially performing ABC with simulations that were constrained according to the
246 posterior sample of the previous run, until a demographic history with a recognizable shape
247 was obtained. This procedure was operated in a "let the data speak" fashion that reduced
248 subjective choices because the posteriors obtained by ABC are conditioned exclusively to the
249 observed data, and the initial run itself started from completely random demographic
250 histories. Afterwards, additional model choice analyses were performed to test simple models
251 pre-defined on both the "random histories" and on the results obtained with the Coalescent
252 Bayesian Skyline approach.

8

253 Our ABC procedure employed coalescent simulations with the concatenated CytB+CR
254 dataset as the observed data. Following a dedicated analysis, we selected a working set of 15
255 summary statistics that typically are sensitive to demographic change, including three
256 multivariate statistics (mismatch distributions, Table S4). The simulation of "random
257 histories" was carried out by randomly sampling: the number of demographic changes
258 (exponential prior, Λ=1.0); the time to those changes (uniform prior in 0-200 k BP); and the
259 N_e values from each time period (exponential prior, Λ=100,000). Exponential priors were
260 employed in the initial round because they sample uniformly in logarithmic scales, which is
261 optimal for screening large parametric spaces, but these were later substituted by uniform
262 priors or fixed values. Changes in population size were sudden, and the populations remained
263 constant between changes. The sequential process included five ABC analyses with 20
264 million simulations each, retaining 0.005% of the simulations for further analysis. The
265 performance of our sequential procedure was assessed by posterior predictive checks, in
266 which the observed summary statistics were compared with their posterior predictive
267 distributions (Csillery *et al.*, 2010).

268 We then performed three separate model-choice analyses henceforth called "old-change test",
269 "growth test", and "all-models test"; the first two were intended to obtain simple tendencies
270 while the last one aimed to infer the historical demography with improved resolution. They
271 included models that were suggested by the "random histories" and the Bayesian Skyline
272 analyses, as well as alternative models that were based on expectations from previous
273 research or associations with climatic records (Fig. 2). The "old-change test" compared three
274 models of a single, sudden demographic change (increase, decrease and constant) occurring
275 70-140 k BP. The "growth test" compared recent scenarios of positive versus negative
276 exponential growth (uniformly sampled in -0.005 to 0.005) around the last glacial/interglacial
277 transition, starting at 9-15 k BP and continuing until the present. The "all-models test"
278 included five models differing in two recent demographic changes, one occurring at 9-15 k
279 BP and the other occurring between the first one and 40 k BP. Also, the "all-models test"
280 included a palaeoclimate records model (PCRM) and its inverse version (PCRM^{-1}) in which
281 N_e had a perfect correlation (positive for PCRM and negative for PCRM^{-1}) with past
282 temperature records, averaged at stadial/interstadial level. Palaeoclimate records were taken
283 from the Eemian interglacial, ca 130 k BP, to the present following Blockley *et al.* (2014). N_e
284 values were sampled uniformly in the interval 10,000-2,000,000. The mean ages of the
285 ancient samples were simulated from normal distributions to account for uncertainties in
286 stratigraphic inferences. We employed a generation time of two years and mutation rates of

287 7.7, 15.8 and 50% Myr^{-1}, as in the phylogenetic analysis, but focused on the first two after

288 obtaining poor fitting with 50% Myr^{-1}. Model likelihoods were estimated with three methods:

289 *i*) a direct approach; *ii*) a weighted method in which the model likelihoods were adjusted by

290 the Epanechnikov kernel evaluated on the distances to the observed data, as implemented in

291 the software BaySICS (Sandoval-Castellanos, Palkopoulou & Dalén, 2014); and *iii*) a logistic

292 regression (Fagundes *et al.*, 2007). One million simulations were run for each model and 20

293 different acceptance proportions were employed to assess consistency of the estimates

294 (0.0025% to 0.05%).

295 We performed additional analyses to adjust the methodology as well as to test relevant

296 hypotheses. They included: *i*) pilot essays for adjusting simulation scenarios, priors, and

297 summary statistics; *ii*) an analysis for assessing the effect of contemporary genetic

298 differentiation (initiated during the Holocene) following the geographic structure reported by

299 Hamill *et al*. (2006); and *iii*) a procedure for assessing the "temporal signal" of our sample,

300 following Firth *et al*. (2010). In the last analysis, 10,000 temporally random datasets were

301 used to build null distributions that were employed for testing the significance of our

302 parameter estimates.

303 Finally, we validated our model choice methodology using pseudo observed datasets (PODs),

304 which are simulated datasets that are used as real data to obtain rates of correct and incorrect

305 model choices. One complete PODs analysis included the iteration of 500 model choice

306 procedures, treating one POD at the time as the real data. As we needed to know the rates of

307 right/wrong choices for every model, it was necessary to carry out a complete PODs analysis

308 (with its 500 replicates) with every model that was considered in a specific analysis. Then we

309 replicated that procedure for the three different model choice analyses ("old-change test",

310 "growth test", and "all-models test"), and again for the entire set of analyses of both the

311 Eurasian and European datasets, as well as for mutation rates of 7.7 % and 15.8 % Myr^{-1}. Due

312 to the massive amount of computational time required for all those analyses, it was necessary

313 to reduce the amount of simulations analysed in each run (from 1 million in the main model

314 choice analyses to 250,000) and to include only 500 PODs per analysis, and to skip the

315 analyses of the 50% mutation rate since it was a poor fit with the data and was also left out of

316 the model choice analyses anyway.

317 Simulations were performed in the software BaySICS (Sandoval-Castellanos *et al.*, 2014).

318 The "random histories" analyses as well as the "temporal signal test" were programed in a

319 custom software (available on request) that employed the source code of BaySICS, using the

320 platform provided by Microsoft Visual Studio Professional 2011 and Intel Visual Fortran
321 Composer XE 2013. Minor estimations such as transition/transversion bias and the gamma
322 parameter were made with the software MEGA 6 (Tamura *et al.*, 2013).

323

324 **RESULTS**

325 *Sequencing success rate*

326 We successfully obtained consensus sequences from 18 ancient samples (between 10 k and 23
327 k BP in age) originating from 10 palaeontological sites, for a 266 bp part of the mitochondrial
328 cytochrome B (CytB) region. For the control region (CR), 226 bp was obtained from 17
329 ancient samples (covering the same temporal span as above), originating from 9
330 palaeontological sites. Further, we built a concatenated 542 bp dataset for the 17 individuals
331 that produced sequences from both gene regions, containing 316 bp CytB (*i.e.* an additional
332 49 bp to the strictly CytB dataset) and 226 bp CR (see Table S3 for an overview). All datasets
333 were aligned with modern Eurasian sequences from Melo-Ferreira *et al.* (2007).

334 The most southern ancient sample from Loutra Almopias Cave in northern Greece did not
335 provide DNA for all sub-amplicons, and could therefore not be included in the phylogenetic
336 analysis. It did however produce enough sequence data (424 bp in total) to be confirmed as *L.
337 timidus* from GenBank Blast searches, indicating that the mountain hare may have had a more
338 southern Late Pleistocene range limit than previously thought. Conversely, one bone sample
339 from the Belgian site Caverne Marie-Jeanne (ca 44 k BP in age) was genetically determined
340 as *L. europaeus*. While such a northern glacial distribution may seem surprising at first
341 glance, we propose that this could be explained by a northwards expansion of brown hares
342 during one of the warm interstadials during Marine Isotope Stage 3.

343

344 *mtDNA diversity and population structure*

345 Within the entire mountain hare dataset (ancient and modern combined), there were 93
346 control region and 27 CytB haplotypes. In the control region for modern samples, gene (H)
347 and nucleotide (π) diversities were 0.988 ($\pm$ 0.003) and 0.075 ($\pm$ 0.037) respectively. For the
348 ancient samples, gene and nucleotide diversities were slightly lower ($H = 0.956 \pm 0.044$; $\pi = $
349 0.052 ± 0.028). For CytB, the diversity in the modern samples ($H = 0.786 \pm 0.033$; $\pi = 0.0067$

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350 ± 0.004) was slightly lower than in the ancient samples ($H = 0.889 \pm 0.053$; $\pi = 0.010 \pm$
351 0.006). It could be noted that within the ancient data set, there were no general tendencies for
352 changed diversity over time, with ~ 20 k BP old samples having slightly higher CR diversity
353 but lower CytB diversity than samples with an age of around 10 k BP.

354 The temporal haplotype network for the modern and ancient CytB sequences is shown in Fig.
355 3. Three haplotypes are shared among the time periods, and the network pattern is similar in
356 the modern and ancient datasets: We find a common main clade with star-shaped topology
357 and a secondary clade connected to the main one only three base pairs distant, but there is no
358 clear geographic structure. There are a number of missing haplotypes in the ancient network
359 that could be interpreted either as historically absent or as a result of reduced sample numbers
360 and sampling sites for this dataset. The overall pattern for this gene region, however, has not
361 changed markedly since the final stages of the Late Pleistocene. In the control region network
362 (Fig. 3), there are no shared haplotypes between the two time periods and the pattern reflects
363 high levels of diversity and historic admixture. The ancient haplotypes, although different
364 from the modern ones, still appear within and in proximity to the contemporary types.

365

366 *Phylogenetic and demographic analyses*

367 The Bayesian phylogeny constructed from the concatenated CytB and CR dataset (Fig. 4)
368 further illustrates the lack of clear geographic structure among the analysed Eurasian
369 mountain hares. Also, the Late Pleistocene individuals are generally not positioned in close
370 proximity to the modern hares from the same region. A Bayesian skyline plot (BSP) was
371 initially made on European individuals only, in line with the software assumption of a single
372 population over time (Drummond *et al.*, 2005). However, since the phylogeny indicated a
373 general lack of phylogeographic structure across Eurasia (see Fig. 4), we also performed the
374 BSP on the complete Eurasian dataset. The results for the two geographic regions were very
375 similar, revealing a fairly constant population size up until ca 150-100 k BP when the
376 numbers increased (ca 4,5 and 2,5 fold, respectively, in Eurasia and Europe). Then, following
377 a longer period of stability, the populations experience a rapid decline after ca 25 k BP of less
378 than 8 fold (Fig. 5, Eurasia and Fig S1, Europe).

379

380 *Approximate Bayesian computation*

381 *i)* Random demographic histories

382 Five sequential rounds of ABC analyses were necessary to obtain a final posterior sample of

383 random histories with a recognizable shape for all mutation rates and both geographic datasets

384 (Figs. S2-S6). Attempts to further refine the obtained shape failed as disparate histories

385 obtained similar support. The demographic histories in the final posterior sample displayed a

386 population increase occurring at some time between 70 and 140 k BP (supported by >95% of

387 the posterior simulations), and a set of demographic changes occurring after 40 k BP, where a

388 bottleneck was the most frequent pattern (supported by >50% of posterior simulations). The

389 posterior distributions of the parameters from the final analysis yielded estimations of N_e

390 broadly agreeing with those obtained by the BSP, while the time to the demographic changes

391 were informative only for the oldest change (Figs. S2-S6). Interestingly, the mode of that

392 parameter occurred around 110-125 k BP for most combinations of datasets and mutation

393 rates, which roughly corresponds with the end of the last interglacial (the distribution has a

394 large variance however). The fit of the simulations to the observed data was very good with

395 the intervals of 95 % of HPD capturing 100% of the observed values, despite some being well

396 outside the intervals in the initial rounds. Furthermore, the 95% HPD intervals were up to one

397 order of magnitude narrower in the final analysis than in the initial run, indicating that our

398 procedure indeed generated random histories that matched the observed data (Figs. S2-S11).

399

400 *ii)* Model choice analyses

401 The "old change test" was a model choice aimed to assess the support for the demographic

402 increase observed in the random histories and BSP at 70-140 k BP, when tested against a

403 model with constant population size and a model with a reduction. The increase received

404 ample and consistent support across methods, mutation rates, and datasets (Table S5).

405 The "growth test" was a model choice comparing a continuous exponential decline in N_e with

406 an exponential growth starting around the onset of the Holocene (9-15 k BP), to support or

407 contradict the pattern of reduction observed in the BSP. In agreement with the BSP, the

408 results supported a demographic decline in Europe. However, only the logistic regression

409 supported this scenario in the Eurasian dataset (Table S5).

410 As the "random histories" failed to provide a clear picture of the demographic history of *L.*

411 *timidus* after the LGM, and the most common pattern (a bottleneck) contrasted with the pure

13

412 decline observed in the BSP, we performed a third model choice analysis, "all-models test",
413 aimed to infer the post-LGM demographic history at higher resolution. For the European
414 dataset, this analysis indeed improved our reconstruction of demographic history as it
415 supported the inverse palaeoclimate records model (PCRM^{-1}), consistently across methods
416 and mutation rates (Table S5). However, with the Eurasian dataset the logistic regression
417 supported the PCRM^{-1} as the best model (for all mutation rates) whiles the direct and the
418 weighted methods supported the bottleneck model. This result is probably not surprising since
419 the employed palaeoclimate records were modelled only for Europe, and Eurasia has a very
420 large area with a more heterogeneous climate record.

421 Since the presence of population genetic structure could mimic demographic change (for
422 instance by promoting deep genealogical branching which also occurs with demographic
423 growth), we performed a model choice analysis between a model with structure (growth being
424 flexible) and a model with a panmictic population since the start of Holocene. However, our
425 model choice with population structure failed to obtain more support than a model without
426 population structure (Bayes factors = 0.6-1.2), thus rejecting the genetic structure as a factor
427 affecting our inference of demographic history.

428

429 *iii)* PODs analyses and temporal signal

430 The statistical power for the models of the "old-change test" was moderate but rose to high
431 levels when a Bayes factor of 2.0 was used as a threshold criterion (Table S6). Such high
432 power is interesting because in those specific models the demographic change is very old and
433 a reduced power was expected. However, the increased power could be due to two factors: the
434 large population sizes of mountain hares which stretch the coalescent genealogies and widen
435 the time window of detectable change; and (2) the ancient samples that gave additional depth
436 to the genealogies. The statistical power for differentiating a continuous demographic growth
437 from a decline during the Holocene ("growth test"; Table S6) was in general high for the
438 growth, but a little lower for the decline, which could explain our lack of consistent support
439 for the decline in the Eurasian dataset. Finally, the statistical power for detecting changes in
440 the "all-models test" (Table S6) was very disparate and frequently low, also for the best
441 supported model (PCRM^{-1}). The reason seems to be some overlap between the models that
442 can produce similar scenarios despite being nominally different. The moderate power of the
443 model choice could therefore be attributed to the limitations of the technique and sampling.

14

444   However, we believe that our power was substantially improved by assessing the consistency

445   of the results among different types of tests, mutations rates, datasets, acceptance thresholds,

446   and methods.

447   In addition, the temporal signal test showed that our parameter estimations were highly

448   significant in respect to their null distributions, indicating that our temporal sampling

449   significantly contributed to the inference (p <0.001).

450

DISCUSSION

451

Contrast between hares and other arctic/alpine species

452

453   Our analyses of mtDNA sequence data derived from subfossil mountain hare remains, and

454   comparisons with modern data, can provide answers for many of the questions relating to the

455   species' phylogeographic history. Despite the very complicated pattern in the historical

456   haplotype network, we can start to resolve the ambiguity about the driving forces with some

457   certainty. The sharing of haplotypes between modern regions (at least for CytB) is not a

458   recent phenomenon, and extensive phases of dispersal from the east appear to have occurred

459   well before the most recent glaciation. This is a scenario already suggested by Melo-Ferreira

460   *et al*. (2007) based on modern mtDNA phylogenies, but also by others with the inclusion of

461   nuclear DNA (Hamill *et al.*, 2006; Suchentrunk *et al.*, 1999). However, the historically high

462   levels of diversity revealed in our analyses suggest that the mountain hare has maintained

463   diversity over time unlike many classic arctic/alpine specialists.

464   Both the Bayesian Skyline Plot (BSP) and Approximate Bayesian Computation (ABC)

465   analyses show clear signals of an expansion that started approximately at the onset of the last

466   glaciation, which may have continued throughout much of the Late Pleistocene, although the

467   actual starting time of the population growth has a degree of uncertainty. The BSP then shows

468   a decline in N_e after the LGM which could coincide with the start of the Holocene warming

469   (Fig. 5; Suppl. Fig. S1). The ABC model choice of "growth test" agrees with the post-LGM

470   decline identified in the BSP, but its support is consistent only with the European dataset.

471   Further, the results from the "all-models test" and the "random histories" analysis weakly

472   suggested a "true" bottleneck (reduction and recovery) and the inverse palaeoclimate records

473   model (PCRM[-1]) received the highest support, at least in Europe (note that the PCRM[-1] has a

474   bottleneck around the Pleistocene-Holocene transition during the warm Bølling-Allerød

475 interstadial immediately prior to the Younger Dryas cold stadial). It seems reasonable that the

476 varying levels of support in these analyses is mostly due to a limited statistical power, and

477 does not rule out demographic dynamics between Europe and Asia as well as the presence of

478 more complex models than the tested ones. However, we consider it unlikely that the

479 consistent support for the PCRM^{-1} is due to a random fit to a fluctuating climate pattern

480 (which would also support the PCRM), or to a long term noisy pattern (which would also

481 support the constant size model). Further, the support for the PCRM^{-1} is not only provided by

482 the most recent demographic changes, since both the bottleneck and the anti-bottleneck

483 models failed to obtain much support in the analyses of the European dataset. Combined,

484 these results support the notion that the PCRM^{-1} is the most parsimonious explanation for our

485 data and that a negative correlation between palaeoclimate temperature records and the

486 demographic history of *L. timidus* is likely real rather than a methodological artefact. This

487 implies that *L. timidus* populations not only grew and decreased following the glacial-

488 interglacial cycles (expand during glacials, decline during interglacials), but also followed the

489 finer scale temperature changes at the stadial-interstadial level.

490 One important question to address is why the BSP only identified demographic changes

491 associated with the glacial-interglacial cycles, whereas the ABC analyses also revealed much

492 finer millennial-scale fluctuations. The answer could be that BSP inference includes the use of

493 a smoothing function that tends to buffer sudden changes provided by short coalescent

494 intervals, in order to prevent the noisy pattern observed in early versions of skyline plots (Ho

495 & Shapiro, 2011). But in doing so, it may absorb some of the finer scale recent demographic

496 changes and therefore fail to identify complicated patterns of N_e shifts. Thus, sudden

497 demographic peaks or short bottlenecks, as those predicted by a demographic history linked to

498 stadial-interstadial cycling, are usually absent from BSPs.

499 Similarly, violation of the assumption of panmixia in BSP estimation combined with an

500 unbalanced sampling strategy can lead to a false signal of population decline (Heller, Chikhi

501 & Siegismund, 2013). Our sampling design was by its very nature unbalanced, as we were

502 reliant upon those few locations where bone fragments were discovered and adequately

503 preserved to allow retrieval of DNA. Further, despite the complicated pattern of the haplotype

504 network, it is clear from the current species distribution that gene flow is restricted across the

505 contemporary range, and has likely been restricted at many points historically for the species.

506 However, the incorporation of population structure failed to improve support in the model

507 choice analyses across the Eurasian dataset. Future studies would thus benefit from larger

508   sample sizes as well as analysis of older samples, in order to allow further testing of the
509   conclusions and hypotheses presented in this paper.

510   In summary, our analyses lend some support for signatures of population contraction and
511   expansion in and out of interglacial refugia, but also intriguing signals of more complex
512   changes likely correlated with millennial-scale events of interstadial warming as well as
513   stadial cooling. However, as apparent in the comparisons of glacial and modern genetic
514   variation, as well as in the haplotype network, there seems to have been no major losses of
515   genetic diversity across the Pleistocene/Holocene transition. Consequently, the pattern of
516   temporal genetic change in the mountain hare does not resemble that seen in other cold-
517   adapted species such as collared lemmings (Palkopoulou *et al.*, 2016; Prost *et al.*, 2010), true
518   lemmings (Lagerholm *et al.*, 2014) and arctic foxes (Dalén *et al.*, 2007), which experienced
519   local extinctions and large declines in genetic diversity following Holocene warming. The
520   mountain hare instead seems to have been able to keep pace with the rapid changes at the
521   Pleistocene/Holocene transition, and successfully track their contracting habitat to northern
522   and alpine regions as their former areas became unsuitable. The classification of the mountain
523   hare as a typical cold-adapted species under the expansion/contraction model framework is
524   thus complicated as its generalist nature appears to offer certain resilience to climate effects,
525   which may allow the mountain hare to buffer sudden demographic impacts and maintain
526   genetic diversity.

527   There are aspects of the mountain hare's ecology and evolutionary history that have
528   similarities with those of temperate species. For example, its current distribution covers large
529   temperate areas in Europe and north-western Asia, similar in some respects to red squirrels
530   and water voles. It also displays high levels of diversity in the core of its range similar to
531   these and other rodents but, unlike those species, the mountain hare shows no reduction in
532   diversity towards the northern margins of its range (Alves *et al.*, 2008; Waltari & Cook,
533   2005). This feature of its phylogeography seems to place the mountain hare in a separate
534   category compared to many modern Eurasian mammals in that it can fulfil the roles of both
535   temperate and cold-adapted species. Over long time scales, the expansion/contraction model
536   predicts that both cold-adapted and temperate species will have a reduced level of genetic
537   diversity due to bottlenecks, either during interglacials or glaciations (*i.e.* the effect of
538   refugia). The mountain hare appears to be an exception to this pattern, and could thus be one
539   of the few species whose mtDNA diversity is more or less in mutation/drift equilibrium. The
540   high diversity and lack of structure in the ancient concatenated networks and phylogeny (Fig.

541   3 and 4) suggests large populations that were widely connected throughout the Pleistocene,
542   including earlier warm interglacials. Indeed, the present-day diversity shows that the
543   mountain hare maintains similar levels of variation and haplotype sharing across a wide
544   geographic range during the current interglacial period.

545   To find similar phylogeographic arrangements, one has to look beyond the classic
546   arctic/alpine and temperate species. A resemblance to the pattern we observe in mountain
547   hares can be seen in modern habitat generalists such as the red fox and grey wolf. Despite
548   recent population crashes due to persecution (Jansson *et al.*, 2012) and the proliferation since
549   the Pleistocene of a previously rare haplogroup (Pilot *et al.*, 2010), grey wolves show a
550   relatively stable phylogeographic history throughout Europe (Vilà *et al.*, 1999). Similar to the
551   mountain hare haplotype network, a network constructed from ancient and modern Eurasian
552   wolf samples shows weak geographic structuring and high levels of diversity (Germonpré *et*
553   *al.*, 2009). Hunting of wolves has acted to reduce diversity somewhat in modern times, but
554   their high dispersal ability and generalist habitat requirements have acted to maintain a large
555   effective population size (Flagstad *et al.*, 2003; Pilot *et al.*, 2010). Red foxes also show little
556   genetic structuring in both ancient and modern populations and a relatively stable effective
557   population size since the Pleistocene (Teacher, Thomas & Barnes, 2011). The reason for this
558   pattern is also hypothesised to relate to the high dispersal ability and adaptability of red foxes
559   which has allowed them to thrive throughout climate oscillations and in diverse habitat zones.
560   These ancient DNA studies have indicated that dispersal and the ability of species to adapt
561   quickly are important features in shaping evolutionary histories, which together with
562   interspecific interactions create the observed phylogeographic patterns.

563   A further aspect to consider when studying phylogeography is the dynamics of co-existing
564   species. Predation and inter-specific competition are likely to have played significant roles in
565   shaping the realised niche of the mountain hare. In particular, interactions with the European
566   brown hare (*Lepus europaeus*) have strongly influenced the distribution of modern mountain
567   hare populations in Europe (Alves *et al.*, 2006; Kasapidis *et al.*, 2005). The brown hare is
568   common throughout western and central Europe whereas the mountain hare is found in
569   northern and north-eastern Europe, as well as in the Alps and British Isles (see Fig. 1). There
570   is a range overlap in Finland, Sweden and Eastern Europe, where recent expansions of the
571   brown hare are replacing resident populations of mountain hares (Thulin & Tegelstrom,
572   2001). Historically, *L. timidus* populations in the Iberian Peninsula were also displaced by
573   brown hares and their close relatives *L. granatensis* and *L. castroviejoi* (Melo-Ferreira *et al.*,

574   2007). Our data showing a historical continuity of high gene flow and genetic diversity,
575   combined with the modern distribution of the mountain hare relative to the brown hare,
576   indicates that climate is not the only factor influencing the more recent phylogeographic
577   pattern of *L. timidus*. Instead, an additional, yet related impact is likely to be competitive
578   exclusion by brown hares during the Holocene.

579   Although the close historical contact between *Lepus* species in the Iberian Peninsula is still
580   evident from the modern-day persistence of introgressed *L. timidus* mtDNA in their relatives
581   (Alves *et al.*, 2008; Melo-Ferreira *et al.*, 2005), results from northern Europe show that
582   acquired mountain hare mtDNA subsequently disappears from brown hare populations when
583   there is no continuous interspecific gene flow (Thulin & Tegelström, 2002). The bones
584   collected for this study were morphologically identified as *L. timidus*, and since the mountain
585   hare and the brown hare mainly had allopatric European distributions during the Late
586   Pleistocene period (Averianov, 2001) we find it highly unlikely that some of our analysed
587   ancient sequences are derived from hybrids with introgressed mountain hare mtDNA. Also,
588   the high number of haplotypes detected in our dataset means that a potential inclusion of an
589   admixed individual would have little influence on the overall results of our analyses.

590   Despite some recent population declines (Jennings *et al.*, 2006; Marboutin *et al.*, 2003),
591   brown hares have thrived in landscapes modified for agriculture (Tapper & Barnes, 1986).
592   They have better body condition and reduced mortality in high intensity arable landscape
593   types, compared to marginal upland and pastoral areas (Smith, Vaughan Jennings & Harris,
594   2005). Farmland is the primary habitat for brown hares, and as the extent of farmlands has
595   increased with human expansion, so too has the range and abundance of the brown hare. The
596   corresponding retreat of the mountain hare from much of central and western Europe is thus
597   likely affected by decreases in forest cover and competitive exclusion, rather than being solely
598   a temperature mediated process. Based on that assumption, we would expect that *L. timidus*
599   had a wider distribution in Europe and connections with Asia during previous interglacials,
600   when forest cover was more extensive than today (Birks & Birks, 2004). This hypothesis is
601   supported by our finding that the mountain hare has a less dramatic demographic history than
602   other cold-adapted species that conform to the classic expansion/contraction model.

603   Our results indicate that the mountain hare has shown a remarkable resilience throughout the
604   Quaternary climate changes, likely due to its capacity for occupying habitats that range from
605   temperate steppe to Alpine landscapes. Its effective population size in Europe seems to have
606   been tightly coupled with palaeoclimatic fluctuations, with growth and declines occurring in

607   line with temperature cooling and warming, respectively. However, although the range has

608   shifted substantially in space there is no apparent decrease in genetic variation following the

609   last glacial-interglacial transition. This is probably due to the high dispersal capability in the

610   species, which may have enabled it to track shifts in habitat availability as well as led to long

611   term gene flow among regions. The latter would have resulted in weak population structure,

612   which may have acted as an additional buffer against major declines in diversity during range

613   shifts, since local extinctions would not have led to any losses of locally distributed clades or

614   haplogroups. However, the mountain hare's modern day distribution may have been

615   additionally influenced by competitive interactions with the brown hare, which has expanded

616   due to anthropogenic landscape changes during the Holocene.

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References

Alves PC, Harris DJ, Melo-Ferreira J, Branco M, Ferrand N, Suchentrunk F, Melo-Ferreira J, and Boursot P. 2006. Hares on thin ice: introgression of mitochondrial DNA in hares and its implications for recent phylogenetic analyses. *Molecular Phylogenetics and Evolution* **40:** 640-1.

Alves PC, Melo-Ferreira J, Freitas H, and Boursot P. 2008. The ubiquitous mountain hare mitochondria: multiple introgressive hybridization in hares, genus Lepus. *Philosophical Transactions of the Royal Society B: Biological Sciences* **363:** 2831-2839.

Angerbjörn A, and Flux JEC. 1995. Lepus timidus. *Mammalian Species:* 1-11.

Averianov AO. 2001. Lagomorphs (Mammalia) from the Pleistocene of Eurasia. *Paleontological Journal* **35:** 191-199.

Birks HH, and Birks HJB. 2004. The Rise and Fall of Forests. *Science* **305:** 484-485.

Blockley SPE, Bourne AJ, Brauer A, Davies SM, Hardiman M, Harding PR, Lane CS, MacLeod A, Matthews IP, Pyne-O'Donnell SDF, Rasmussen SO, Wulf S, and Zanchetta G. 2014. Tephrochronology and the extended intimate (integration of ice-core, marine and terrestrial records) event stratigraphy 8–128 ka b2k. *Quaternary Science Reviews* **106:** 88-100.

Brace S, Palkopoulou E, Dalén L, Lister AM, Miller R, Otte M, Germonpré M, Blockley SPE, Stewart JR, and Barnes I. 2012. Serial population extinctions in a small mammal indicate Late Pleistocene ecosystem instability. *Proceedings of the National Academy of Sciences* **109:** 20532-20536.

Cheng E, Hodges KE, Melo-Ferreira J, Alves PC, and Mills LS. 2014. Conservation implications of the evolutionary history and genetic diversity hotspots of the snowshoe hare. *Molecular Ecology* **23:** 2929-2942.

Csillery K, Blum MGB, Gaggiotti O, and Francois O. 2010. Approximate Bayesian Computation (ABC) in practice. *Trends in Ecology & Evolution* **25:** 410 - 418.

Dalén L, Nyström V, Valdiosera C, Germonpré M, Sablin M, Turner E, Angerbjörn A, Arsuaga JL, and Götherström A. 2007. Ancient DNA reveals lack of postglacial habitat tracking in the arctic fox. *Proceedings of the National Academy of Sciences* **104:** 6726-6729.

Drummond AJ, Rambaut A, Shapiro B, and Pybus OG. 2005. Bayesian Coalescent Inference of Past Population Dynamics from Molecular Sequences. *Molecular Biology and Evolution* **22:** 1185-1192.

Drummond AJ, Suchard MA, Xie D, and Rambaut A. 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution*.

Excoffier L, and Lischer HEL. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* **10:** 564-567.

Fagundes NJR, Ray N, Beaumont M, Neuenschwander S, Salzano FM, Bonatto SL, and Excoffier L. 2007. Statistical evaluation of alternative models of human evolution. *Proceedings of the National Academy of Sciences* **104:** 17614-17619.

Firth C, Kitchen A, Shapiro B, Suchard MA, Holmes EC, and Rambaut A. 2010. Using time-structured data to estimate evolutionary rates of double-stranded DNA viruses. *Molecular Biology and Evolution* **27:** 2038-2051.

Flagstad O, Walker CW, Vila C, Sundqvist AK, Fernholm B, Hufthammer AK, Wiig O, Koyola I, and Ellegren H. 2003. Two centuries of the Scandinavian wolf population: patterns of genetic variability and migration during an era of dramatic decline. *Molecular Ecology* **12:** 869-680.

Fostowicz-Frelik U, and Gasparik M. 2006. The taxonomic status of leporid remains from Ördöglyuk Cave, Solymár (Hungary). *Acta Zoologica Cracoviensia - Series A: Vertebrata* **49:** 151-161.

Germonpré M, Sablin MV, Stevens RE, Hedges RE, Hofreiter M, Stiller M, and Després VR. 2009. Fossil dogs and wolves from Palaeolithic sites in Belgium, the Ukraine and Russia: osteometry, ancient DNA and stable isotopes. *Journal of Archaeological Science* **36:** 473-490.

Hamill RM, Doyle D, and Duke EJ. 2006. Spatial patterns of genetic diversity across European subspecies of the mountain hare, Lepus timidus L. *Heredity* **97:** 355-365.

Heller R, Chikhi L, and Siegismund HR. 2013. The confounding effect of population structure on Bayesian skyline plot inferences of demographic history. *PLoS One* **8:** e62992.

Hewitt GM. 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. *Biological Journal of the Linnean Society* **58:** 247-276.

Hewitt GM. 1999. Post-glacial re-colonization of European biota. *Biological Journal of the Linnean Society* **68:** 87-112.

Ho SYW. 2014. The changing face of the molecular evolutionary clock. *Trends in Ecology & Evolution* **29:** 496-503.

Ho SYW, and Shapiro B. 2011. Skyline-plot methods for estimating demographic history from nucleotide sequences. *Molecular Ecology Resources* **11:** 423-434.

Hofreiter M, and Barnes I. 2010. Diversity lost: are all Holarctic large mammal species just relict populations? *BMC biology* **8:** 46.

Hughes PD, and Woodward JC. 2008. Timing of glaciation in the Mediterranean mountains during the last cold stage. *Journal of Quaternary Science* **23:** 575-588.

Jansson E, Ruokonen M, Kojola I, and Aspi J. 2012. Rise and fall of a wolf population: genetic diversity and structure during recovery, rapid expansion and drastic decline. *Molecular Ecology* **21:** 5178-5193.

Jennings N, Smith RK, Hacklander K, Harris S, and White PCL. 2006. Variation in demography, condition and dietary quality of hares Lepus europaeus from high-density and low-density populations. *Wildlife Biology* **12:** 179-189.

Kahlke R-D. 1999. *The History of the Origin, Evolution and Dispersal of the Late Pleistocene Mammuthus–Coelodonta Faunal Complex in Eurasia (Large Mammals)*. Mammoth Site of Hot Springs, SD.

Kasapidis P, Suchentrunk F, Magoulas A, and Kotoulas G. 2005. The shaping of mitochondrial DNA phylogeographic patterns of the brown hare (Lepus europaeus) under the combined influence of Late Pleistocene climatic fluctuations and anthropogenic translocations. *Molecular Phylogenetics and Evolution* **34:** 55-66.

Koby F. 1960. Sur l'extension maxima vers le sud-ouest de quelques représentants de la faune froide Wurmienne. *Anthropos 1, Supplement***:** 101-107.

Lagerholm VK, Sandoval-Castellanos E, Ehrich D, Abramson NI, Nadachowski A, Kalthoff DC, Germonpré M, Angerbjörn A, Stewart JR, and Dalén L. 2014. On the origin of the Norwegian lemming. *Molecular Ecology* **23:** 2060-2071.

López-Martínez N. 1980. Les lagomorphs (Mammalia) du pléistocène supérieur de Jaurens. *Nouvelles Archives du Museum d'Histoire Naturelle de Lyon***:** 5-16.

Marboutin E, Bray Y, Péroux R, Mauvy B, and Lartiges A. 2003. Population dynamics in European hare: breeding parameters and sustainable harvest rates. *Journal of Applied Ecology* **40:** 580-591.

Melo-Ferreira J, Boursot P, Randi E, Kryukov A, Suchentrunk F, Ferrand N, and Alves PC. 2007. The rise and fall of the mountain hare (Lepus timidus) during Pleistocene glaciations: expansion and retreat with hybridization in the Iberian Peninsula. *Molecular Ecology* **16:** 605-618.

Melo-Ferreira J, Boursot P, Suchentrunk F, Ferrand N, and Alves PC. 2005. Invasion from the cold past: extensive introgression of mountain hare (Lepus timidus) mitochondrial DNA into three other hare species in northern Iberia. *Molecular Ecology* **14:** 2459-2464.

Muster C, and Berendonk TU. 2006. Divergence and diversity: Lessons from an arctic-alpine distribution (Pardosa saltuaria group, Lycosidae). *Molecular Ecology* **15:** 2921-2933.

Muster C, Maddison WP, Uhlmann S, Berendonk TU, and Vogler AP. 2009. Arctic-alpine distributions - Metapopulations on a continental scale? *American Naturalist* **173:** 313-326.

Napierala H. 2008. Viele Jäger sind des Hasen Tod? Magdalénienzeitliche Hasenjagd am Kesslerloch. *Mitteilungen der Gesellschaft für Urgeschichte***:** 55-67.

Niedziałkowska M, Hundertmark KJ, Jędrzejewska B, Niedziałkowski K, Sidorovich VE, Górny M, Veeroja R, Solberg EJ, Laaksonen S, Sand H, Solovyev VA, Shkvyria M, Tiainen J, Okhlopkov IM, Juškaitis R, Done G, Borodulin VA, Tulandin EA, and Jędrzejewski W. 2014. Spatial structure in European moose (Alces alces): genetic data reveal a complex population history. *Journal of Biogeography* **41:** 2173-2184.

Nylander JAA. 2004. MrModeltest v2. http://www.abc.se/~nylander/mrmodeltest2/mrmodeltest2.html.

Palkopoulou E, Baca M, Abramson NI, Sablin M, Socha P, Nadachowski A, Prost S, Germonpré M, Kosintsev P, Smirnov NG, Vartanyan S, Ponomarev D, Nyström J, Nikolskiy P, Jass CN, Litvinov YN, Kalthoff DC, Grigoriev S, Fadeeva T, Douka A, Higham TFG, Ersmark E, Pitulko V, Pavlova E, Stewart JR, Węgleński P, Stankovic A, and Dalén L. 2016. Synchronous genetic turnovers across Western Eurasia in Late Pleistocene collared lemmings. *Global Change Biology* **22:** 1710-1721.

Pierpaoli M, Riga F, Trocchi V, and Randi E. 1999. Species distinction and evolutionary relationships of the Italian hare (Lepus corsicanus) as described by mitochondrial DNA sequencing. *Molecular Ecology* **8:** 1805-1817.

Pilot M, Branicki W, Jedrzejewski W, Goszczynski J, Jedrzejewska B, Dykyy I, Shkvyrya M, and Tsingarska E. 2010. Phylogeographic history of grey wolves in Europe. *BMC Evolutionary Biology* **10:** 104.

Prost S, and Anderson CNK. 2011. TempNet: a method to display statistical parsimony networks for heterochronous DNA sequence data. *Methods in Ecology and Evolution* **2:** 663-667.

Prost S, Smirnov N, Fedorov VB, Sommer RS, Stiller M, Nagel D, Knapp M, and Hofreiter M. 2010. Influence of Climate Warming on Arctic Mammals? New Insights from Ancient DNA Studies of the Collared Lemming *Dicrostonyx torquatus*. *PLoS ONE* **5:** e10447.

Rambaut A. 2012. FigTree v1.4.0: Tree Figure Drawing Tool. http://tree.bio.ed.ac.uk/software/figtree/.

Rambaut A, and Drummond AJ. 2007. Tracer v1.4: MCMC trace analyses tool. http://tree.bio.ed.ac.uk/software/tracer/.

Sahlman T, Segelbacher G, and Höglund J. 2009. Islands in the ice: colonisation routes for rock ptarmigan to the Svalbard archipelago. *Ecography* **32:** 840-848.

Sandoval-Castellanos E, Palkopoulou E, and Dalén L. 2014. Back to BaySICS: A User-Friendly Program for Bayesian Statistical Inference from Coalescent Simulations. *PLoS ONE* **9:** e98011.

Smith RK, Vaughan Jennings N, and Harris S. 2005. A quantitative analysis of the abundance and demography of European hares Lepus europaeus in relation to habitat type, intensity of agriculture and climate. *Mammal Review* **35:** 1-24.

Stewart JR. 2008. The progressive effect of the individualistic response of species to Quaternary climate change: an analysis of British mammalian faunas. *Quaternary Science Reviews* **27:** 2499-2508.

Stewart JR, Lister AM, Barnes I, and Dalén L. 2010. Refugia revisited: individualistic responses of species in space and time. *Proceedings of the Royal Society B: Biological Sciences* **277:** 661-671.

Suchentrunk F, Polster K, Giacometti M, Ratti P, Thulin CG, Ruhle C, Vasil'ev AG, and Slotta-Bachmayr L. 1999. Spatial partitioning of allozyme variability in European mountain hares (Lepus timidus): gene pool divergence across a disjunct distributional range? *Zeitschrift Fur Saugetierkunde-International Journal of Mammalian Biology* **64:** 308-318.

Tamura K, Stecher G, Peterson D, Filipski A, and Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution* **30:** 2725-2729.

Tapper SC, and Barnes RFW. 1986. Influence of Farming Practice on the Ecology of the Brown Hare (Lepus europaeus). *Journal of Applied Ecology* **23:** 39-52.

Teacher AG, Thomas JA, and Barnes I. 2011. Modern and ancient red fox (Vulpes vulpes) in Europe show an unusual lack of geographical and temporal structuring, and differing responses within the carnivores to historical climatic change. *BMC Evolutionary Biology* **11:** 214.

Templeton AR. 2010. Coherent and incoherent inference in phylogeography and human evolution. *Proceedings of the National Academy of Sciences* **107:** 6376-6381.

Thulin C-G, and Tegelström H. 2002. Biased geographical distribution of mitochondrial DNA that passed the species barrier from mountain hares to brown hares (genus Lepus): an effect of genetic incompatibility and mating behaviour? *Journal of Zoology* **258:** 299-306.

Thulin CG, and Tegelstrom H. 2001. High mtDNA haplotype diversity among introduced Swedish brown hares Lepus europaeus. *Acta Theriologica* **46:** 375-384.

Waltari E, and Cook JA. 2005. Hares on ice: phylogeography and historical demographics of Lepus arcticus, L. othus, and L. timidus (Mammalia: Lagomorpha). *Molecular Ecology* **14:** 3005-3016.

Vilà C, Amorim IR, Leonard JA, Posada D, Castroviejo J, Petrucci-Fonseca F, Crandall KA, Ellegren H, and Wayne RK. 1999. Mitochondrial DNA phylogeography and population history of the grey wolf Canis lupus. *Molecular Ecology* **8:** 2089-2103.

Wojtal P, Wilczyński J, Bocheński ZM, and Svoboda JA. 2012. The scene of spectacular feasts: Animal remains from Pavlov I south-east, the Czech Republic. *Quaternary International* **252:** 122-141.

Figure 1. Extant (A) and Late Pleistocene (B) distribution of the mountain hare (*Lepus timidus*) in Europe, with locations indicated for the palaeontological sites that yielded ancient *L. timidus* DNA. Also shown are the extended coastlines resulting from lowered sea levels during the last glacial maximum (LGM), as well as the extent of the LGM ice sheets. Further isolated peaks and regions in the Mediterranean area, the Carpathians and Anatolia were glaciated at different times throughout the Pleistocene (Hughes & Woodward, 2008), but are not shown due to restrictions of scale and the fact that they are diachronous and not necessarily dated to the LGM. Modern European localities with available sequence data are taken from Melo-Ferreira *et al.* (2007). Palaeontological sites are 1: Trou Al'Wesse; 2: Kesslerloch; 3: Luegloch; 4: Nixloch and Gamssulzenhöhle; 5: Smrcka Lorenz-Abris; 6: Krucza Skala; 7: Deszczowa Cave; 8: Oblazowa Cave; 9: Kostenki 1; 10: Loutra Almopias Cave. See Table S1 for a complete list of all Late Pleistocene sites from which bone samples were retrieved.

Figure 2. Schematic view of the models compared in the three ABC model choice analyses. The vertical axis shows time and to the bottom left the ancient hare samples are represented by filled red circles, with the vertical position indicating their ages. The black shapes represent populations and the width represents their relative size over time. The curve to the bottom right shows the variations in δ^{18}O levels over the last 130 thousand years (Blockley *et al.*, 2014), which is the temperature proxy that our two palaeoclimate models are directly based upon. The PCRM (palaeoclimate records model) predicts population growth during warm periods, while in the PCRM^{-1} (the inverse of PCRM) populations increase in size during periods of cold climate.

Figure 3. Temporal networks of cytochome B (A) and control region (B) haplotypes found in modern and Late Pleistocene mountain hares (upper and lower layer, respectively). Shared haplotypes among the two temporal layers are connected by vertical lines, while empty circles illustrate haplotypes that are missing during one time period but present in the other. The number of individuals sharing a haplotype is reflected by its size, and more than one mutational difference between these is shown as a black dot.

Figure 4. Bayesian phylogeny of modern and Late Pleistocene mountain hares, based on the Eurasian concatenated CytB and CR dataset and a mutation rate of 7.7 % Myr^{-1}. The time scale is in calendar years before present, and asterix (*) denotes internal nodes with posterior probabilities above 0.8. Sequences obtained from ancient samples are indicated with filled ovals.

Figure 5. Effective population size (N_e) over time, obtained from the Bayesian Skyline Plot (upper part) and the best performing model in the the "random histories" ABC analysis (middle part). The N_e scale is shown to the right and left of each graph, respectively, while time (in thousands of calendar years before the present) is found below. Both analyses are based on the European dataset and a mutation rate of 7.7% Myr^{-1}. The small stepwise graphs at the bottom show the shape of N_e in the two palaeoclimate records models (PCRM and PCRM^{-1}) employed in the ABC model choice analyses, which are based on the average $\delta^{18}O$ records (black curve) (Blockley *et al.*, 2014). The light blue and orange bands show the placement of the cold Younger Dryas (YD) and Last Glacial Maximum (LGM), and the warm Eemian interglacial, as a time reference.