



A link between historical population decline in the threatened great bustard and human expansion in Iberia: evidence from genetic and demographic data

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Early anthropogenic impacts on the abundance and distribution of wild species are difficult to document, but can help us to understand the causes and relative importance of current declines. Genetic data can be of use in inferring historical demographic events, but the accuracy of these inferences depends on the availability and precision of demographic parameters that are difficult to obtain in the field. Here, we use demographic data on Iberian populations of the threatened great bustard (Aves: *Otis tarda*), obtained from an intensive population monitoring programme over the last 20 years, to estimate critical population parameters (population size and generation time), which are then used in a Bayesian Skyline Plot (BSP) analysis of mitochondrial DNA sequence data to assess changes in population size over the last several thousand years. BSP showed a sudden and sharp great bustard population decline coinciding with human expansion in Iberia, and the associated agricultural and urban development and increased hunting pressure. These results illustrate the importance of human population size as a possible ultimate cause of an environmental impact that occurred in the historical past, a fact that has often been neglected. Our results also suggest the role of human activities in driving historical population declines in great bustards, and underscore the importance of precise, long-term field data to infer past demographic trends from parameters of extant populations. © 2013 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2013, **110**, 518–527.

ADDITIONAL KEYWORDS: BSP – conservation – control region – demography – generation time – growth rate – Middle Ages – *Otis tarda* – Romans.

INTRODUCTION

Humans have been a major factor determining distribution changes and population declines of wild species since prehistoric times, often driving them to extinction (Prescott *et al.*, 2012; Rule *et al.*, 2012). Recently, it has been acknowledged that both human population size and resource use may be the cause of many environmental problems (Allendorf & Allendorf, 2012). However, the factors connecting cause and effect in this relationship are complex and sometimes difficult to understand (Axinn & Ghimire, 2011). Anthropogenic impacts have been both direct

(e.g. exploitation through hunting and collecting) and indirect (e.g. through habitat disturbance and introduction of domestic species), and have sometimes acted in combination with climatic change, as proposed for the extinction of the woolly mammoth (Nogues-Bravo *et al.*, 2008). Inferring past demographic events that took place thousands of years ago is a challenging endeavour that has greatly benefited from advances in the analysis of genetic data. Information inferred from genetic data is crucial to the understanding of past events directly related to the present conditions and distributions of the species, from humans (Gutenkunst *et al.*, 2009) to flowers (Ross-Ibarra *et al.*, 2008), including crustaceans (Babbucci *et al.*, 2010), fishes (Turrero, Horreo & Garcia-Vazquez, 2012), birds (Mila, Smith & Wayne,

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2006), amphibians (Canestrelli, Sacco & Nascetti, 2012) and mammals (Gallardo *et al.*, 2013).

The great bustard (*Otis tarda*) is a steppe bird that has coexisted with humans in the Iberian Peninsula for thousands of years, as suggested by the fossils dated 350 000–118 000 years ago (Sánchez, 1995). Great bustards were persecuted by prehistoric humans, probably as prey, and later from Medieval times to the 20th century as a game species (Chapman & Buck, 1910; Hernández, 1993; Bernis, 1995). Hunting led to minimum population levels of this species in the late 1970s, with a later recovery following the hunting ban established in 1980 (Alonso *et al.*, 2003a). At present, great bustards are a globally endangered bird, classified as vulnerable in the *Red List of Threatened Species* (IUCN, 2012). Their distribution covers the Palaearctic region from northern Morocco to eastern China, with the main stronghold in Spain, where 60–70% of the world's population is found today (Alonso & Palacin, 2010). In addition to hunting, the species decreased markedly in the last century as a result of habitat loss and fragmentation, agricultural intensification and collision with power lines, and nowadays survives in highly fragmented populations, mainly in cereal pseudo-steppes (Palacin & Alonso, 2008). Threats have forced birds to aggregate in the most suitable areas (Alonso, Palacin & Martin, 2003b; Alonso *et al.*, 2004), where human activities are still compatible with the habitat requirements of the species (Sastre *et al.*, 2009; Torres *et al.*, 2011). The great bustard metapopulation located north of the city of Madrid is composed of eight reproductive aggregations connected through gene flow, which form an independent management unit (Martin *et al.*, 2002; Alonso *et al.*, 2009b). Intensive demographic monitoring and long-term radio tracking over the last 20 years have generated a robust record of census figures, movement records and longevity data that are essential to estimate population sizes and trends with a level of precision that is rarely achieved for a threatened species (Alonso *et al.*, 2004; Martin *et al.*, 2007, 2008; Palacin *et al.*, 2011).

The aim of this study is to use demographic parameters obtained from long-term monitoring to inform population genetics models designed to estimate major changes in the past population size of Iberian great bustards employing sequence data and Bayesian inference analysis. Although detailed demographic parameter estimates are mostly available for the Madrid population, we also extrapolate these values to sequences obtained from other Iberian populations in order to test whether historical trends are consistent at larger geographical scales. Information on historical demographic trends over the last several thousand years, available only through the analysis of molecular data, can be very useful to gain insight into

Table 1. Number of great bustard samples (*N*) from different regions of the Iberian Peninsula

Region	<i>N</i>
Madrid N	114
Albacete	8
Aragón	4
Burgos	3
Cáceres	13
Castilla	28
Córdoba	16
Huelva	11
Madrid SW	27
Madrid SE	16
Navarra	11
Sevilla	22
Toledo E	10
Toledo W	6
Villafáfila	13
Total Iberian Peninsula	302

both past events and the present distribution of the species.

MATERIAL AND METHODS

STUDIED POPULATION

In total, 114 blood samples of great bustards (Table 1) were obtained between 1995 and 1999 from the eight leks found in the management unit identified in the northern part of Madrid province (hereafter Madrid) (Martin *et al.*, 2002; Alonso *et al.*, 2009a; Pitra *et al.*, 2011). Sampling was quite representative of this population, which has been carefully monitored and censused annually since 1998 (Alonso *et al.*, 2003a; J. C. Alonso *et al.*, unpubl. data). The average number of breeding females in this population was estimated at 709 (95% confidence interval, 670–748) in the period 1999–2012. Because female age cannot be determined in the field, this figure was obtained by assuming the age of first successful breeding to be the third calendar year or later in females (Morales, Alonso & Alonso, 2002; Magaña, 2007), and thus subtracting from the census carried out each year in March the estimated number of females hatched 1 and 2 years previously, based on the numbers of chicks counted in autumn (when the age and sex of the chicks can be determined), and applying mortality rates obtained from a sample of 132 females marked and radio-tracked between 1995 and 2006 (Martín, 2008; J. C. Alonso *et al.*, unpubl. data). To ensure that the patterns observed were not caused by local dynamics, we also analysed 188 blood samples from

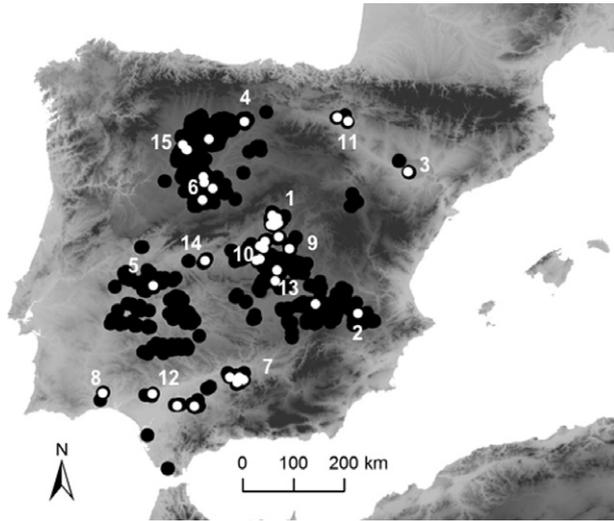


Figure 1. Map of the Iberian Peninsula showing the great bustard distribution in Spain (black dots indicate the location of the 350 currently identified leks; see Alonso, Alvarez-Martinez & Palacin, 2012) and the locations in which blood samples were collected (white dots). Numbers 1–15 are the corresponding region names: Madrid N, Albacete, Aragón, Burgos, Cáceres, Castilla, Córdoba, Huelva, Madrid SW, Madrid SE, Navarra, Sevilla, Toledo E, Toledo W and Villafáfila, respectively (see Pitra *et al.*, 2011).

across Spain (Table 1), encompassing most of the great bustard's Iberian distribution (Fig. 1).

GENERATION TIME IN GREAT BUSTARDS

The generation time (T) has been described by Lande, Engen & Saether (2003) as:

$$T = \alpha + s/(\lambda - s)$$

where α is the age in years at first breeding, λ is the annual geometric growth rate of the population and s is the probability of adult annual survival. Based on long-term field data from the studied population, as well as from other Iberian populations, we know that $\alpha = 2$ (Morales *et al.*, 2002; Magaña, 2007), $s = 0.87$ (Martín, 2008) and $\lambda = 1$, as this population has been in relative demographic stability between 1995 and the present (Alonso *et al.*, 2003b; Martín, 2008). The generation time of the great bustard for the Madrid population was therefore estimated at 8.69 years.

DNA ANALYSES

Methods for DNA extraction, polymerase chain reaction (PCR) amplification and DNA sequencing of the mitochondrial control region (CR) have been described in previous studies (Martin *et al.*, 2002).

DNA sequences were aligned with MUSCLE software (Edgar, 2004) implemented in MEGA5 (Tamura *et al.*, 2011). The length of the CR sequences was 657 base pairs (bp), and GenBank accession numbers and sequence details have been provided elsewhere (Pitra *et al.*, 2011).

Different indicators of genetic diversity, including the number of haplotypes, number of variable sites, and haplotype and nucleotide diversity indices, were calculated with DNAsp v.5.10.01 (Librado & Rozas, 2009). Median-joining networks (Bandelt, Forster & Rohl, 1999) were constructed to represent haplotype relationships in the Madrid and Iberian populations with NETWORK 4.6.1.0 (<http://www.fluxus-technology.com>).

DEMOGRAPHIC INFERENCE

Signals of recent population expansion were searched with Tajima's D (Tajima, 1989) and Fu's F_s (Fu, 1997) neutrality tests implemented in ARLEQUIN v.3.5 (Excoffier & Lischer, 2010). Both detect departures from neutrality (Kimura, 1983), but the first is based on the fact that, under the neutral model, estimates of the number of segregating sites and of the average number of nucleotide differences are correlated, and the second proposes a different statistic based on the infinite sites model of mutation. To infer long-term demographic evolution, Bayesian Skyline Plots (BSPs) were generated with BEAST v.1.7.2 (Drummond *et al.*, 2012) after determining the model of DNA sequence evolution with jModelTest v.0.1.1 (Posada, 2008). BSP is a powerful method for estimating past population dynamics through time from a sample of molecular sequences without dependence on a pre-specified parametric model of demographic history; it allows us to discover novel demographic signatures that are not readily described by simple demographic models (Drummond, Pybus & Rambaut, 2003).

In order to date the BSP, a mutation rate for the bustard CR is needed. The CR is known to vary considerably across bird species, and values ranging from 0.02 to 0.15 substitutions/site/million years have been reported (Mila, Smith & Wayne, 2007). We thus carried out analyses using four different values spanning those observed in other bird studies: 0.02, 0.04, 0.10 and 0.15 substitutions/site/million years (Lambert *et al.*, 2002; Mila *et al.*, 2007). Once BSP results were obtained, we selected the mutation rate most consistent with the available field estimates of population size and generation time for the great bustard in order to calibrate the time axis of these BSPs (see Results section for details). Twenty-five million steps were sufficient to obtain reliable likelihood values (effective sample size, ESS, higher than 200). We assumed a strict molecular clock for the

Table 2. Genetic diversity indices and tests of population expansion (Tajima's D and Fu's F_s) for the Madrid and Iberian Peninsula great bustard datasets. π , nucleotide diversity

	Variable sites	Haplotypes	π	Hd	F_s	D
Northern Madrid	10	7	0.0045	0.585	3.31; n.s.	1.38; n.s.
Iberian Peninsula	14	19	0.0049	0.828	-1.79; n.s.	0.99; n.s.

Hd , haplotype diversity; n.s., not significant.

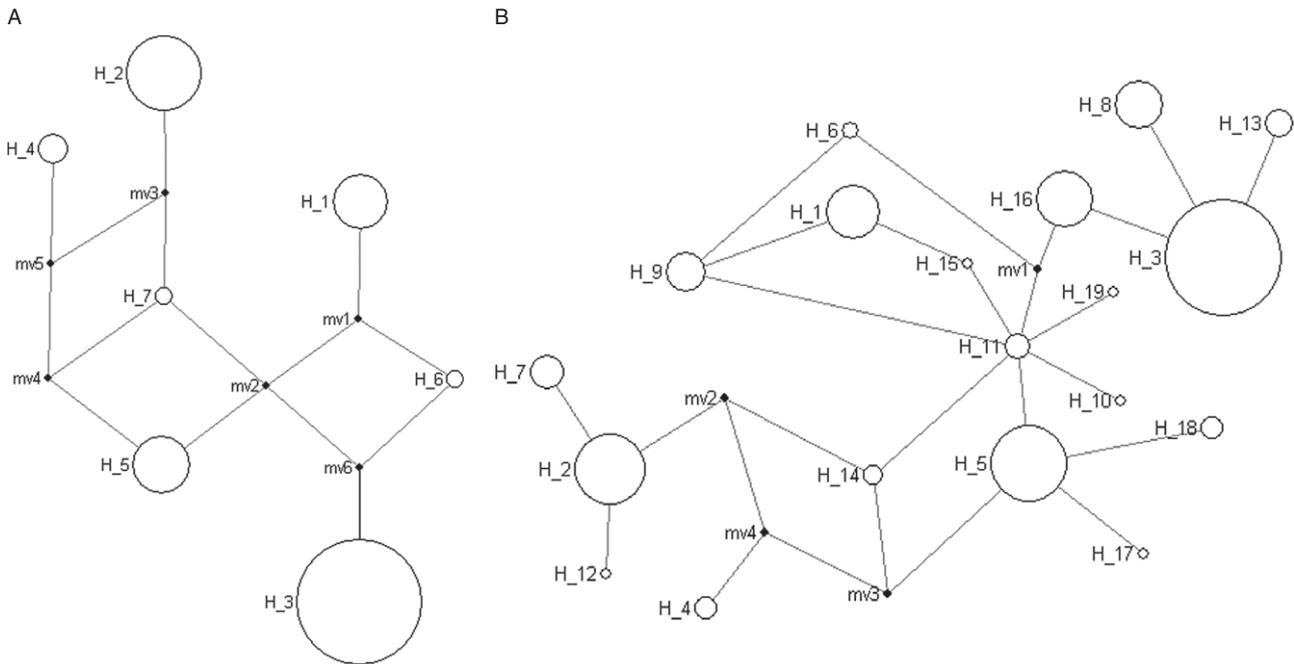


Figure 2. Median-joining network of great bustard mitochondrial DNA (mtDNA) haplotypes identified in Spain. Each circle represents one haplotype, its area being proportional to its frequency in the population. Each branch represents one nucleotide change and black dots are unsampled or extinct haplotypes. A, Madrid. B, Iberian Peninsula.

range of mutation rates mentioned above, a log-normal distribution for the prior, a Bayesian Skyline tree prior and a piecewise-constant skyline model (with a random starting tree). Results from BEAST were visualized and analysed with Tracer v.1.5 (Rambaut & Drummond, 2007).

To apply the BSP method, the data must fulfil some preconditions (Ho & Shapiro, 2011). In this work, these issues have been carefully addressed: individuals have been randomly sampled from across the range of the populations of interest and are sufficient (in number) to capture the diversity of the populations. In addition, subpopulations (Madrid in this case) have been analysed separately to satisfy the assumption of panmixia.

RESULTS

In the sequences from Madrid ($N = 114$), we found 10 variable sites and seven haplotypes, and a total of 14

variable sites and 19 different haplotypes were found in the Iberian Peninsula sample ($N = 302$; Table 2). Nucleotide diversity in the Madrid population was similar to the nucleotide diversity reported for all Iberian populations (0.0045 and 0.0049, respectively), whereas the haplotype diversity was somewhat lower in Madrid than in Iberia (0.585 vs. 0.828). Indices of population expansion (Fu's and Tajima's) were not significant for either dataset (Table 2).

The median-joining haplotype network of Madrid (Fig. 2A) showed a most common haplotype (H3), followed by one less common haplotype (H2) and five low-frequency haplotypes separated by more than one substitution. In the network for the larger Iberian Peninsula sample (Fig. 2B), haplotype H3 remained the highest frequency haplotype, accompanied by two less common haplotypes (H2 and H5) and 16 low-frequency haplotypes.

The HKY model of sequence evolution (Hasegawa, Kishino & Yano, 1985) with invariant sites (HKY + I)

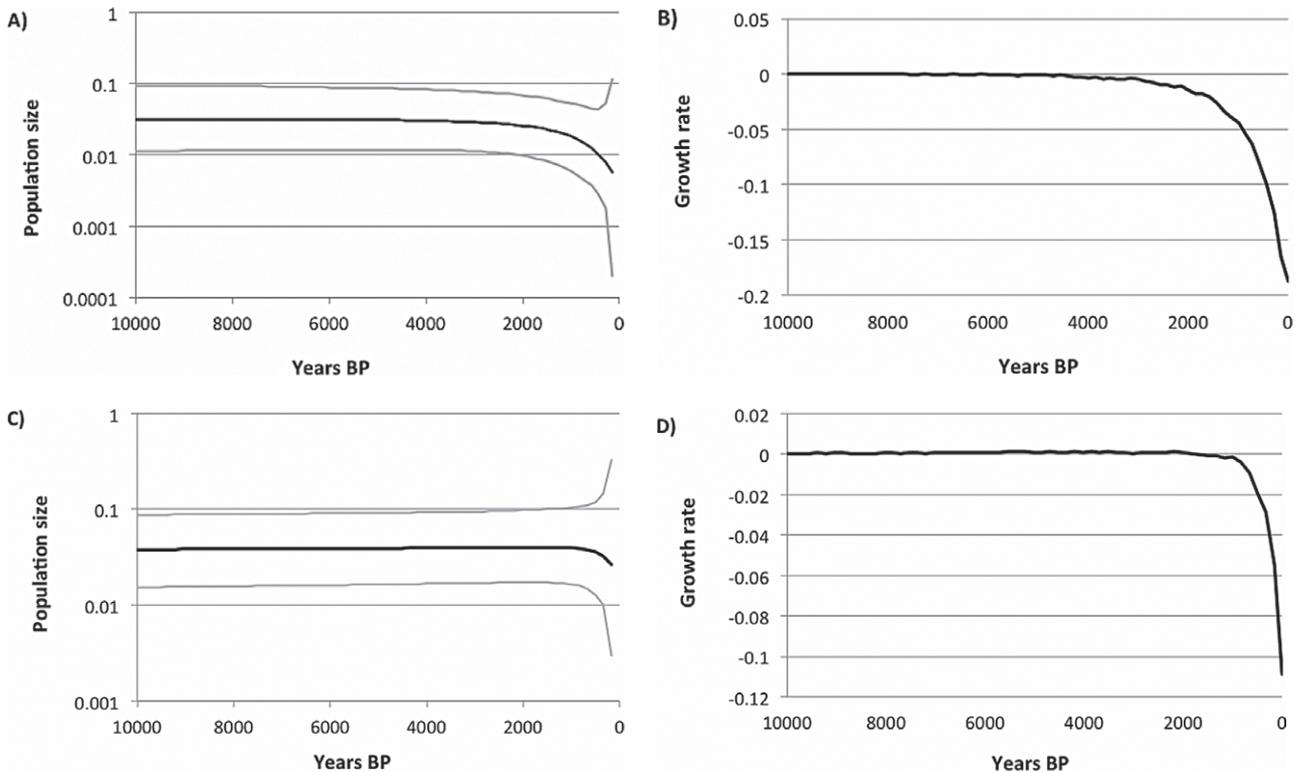


Figure 3. Bayesian Skyline Plot (A, Madrid population; C, Iberian Peninsula) and rates of population growth (B, Madrid population; D, Iberian Peninsula) for Spanish great bustards, assuming a mutation rate of 0.10 substitutions per site per million years. The x axis represents the time in years before present (BP). The population size is the product of the effective size and generation time. The full black line represents the median population size values and the grey lines indicate the 95% highest posterior density interval.

was identified as the most appropriate for our dataset, and was consequently employed for the BSP analyses. Plots for both geographical scales (Madrid and Iberian Peninsula) showed a sudden, steep decline in both population size (Fig. 3A, C) and growth rate (Fig. 3B, D). In order to estimate the time since the sudden decline, we first determined a mutation rate for the CR. BSP results using four different mutation rates (0.02, 0.04, 0.10 and 0.15 substitutions/site/million years) resulted in corresponding effective female population sizes of 2607, 1043, 521 and 304, respectively. As the y axis in the BSP plots is the product of effective population size and generation time, we used available field data for the great bustard to infer the mutation rate most consistent with the demographic parameter values. Given that the generation time is estimated at 8.69 years (see Material and Methods), and the female effective population size (N_{ef}) of the Madrid population during the study period is approximately 709 females, the mutation rate most consistent with great bustard demography is 0.10 substitutions/site/million years. This rate was therefore used to calibrate the x axis on BSPs and to estimate the timing of historical

great bustard declines. According to this rate, the steep decline in great bustard population size and growth rate started around 2000 BP (Fig. 3B). Alternative calibrations using mutation rates of 0.15 or 0.04 substitutions/site/million years would yield time estimates of 1500 and 3000 BP, respectively.

DISCUSSION

Our analyses of great bustard genetic data, informed by critical demographic parameters obtained from long-term field studies, indicate that the Iberian population of the species underwent a sudden population decline within historical times. Steep population declines reflected in the BSPs are corroborated by positive (except F_s in the Iberian Peninsula) and non-significant values of Tajima's and Fu's tests, consistent with situations in which rare alleles are eliminated from a population through past population decline and genetic bottlenecks (Tajima, 1989). The lack of intermediate haplotypes connected by only one mutation step in the Madrid population (Fig. 2) supports this hypothesis. Bottlenecks in natural populations can be produced by inadequate management

and can lead to local and even global species extinctions (Luikart *et al.*, 1998), as genetic variability is the basis of the evolutionary potential of populations (Frankel & Soulé, 1981; Frankham, 1996). In great bustard populations, hunting and habitat transformations are known to have led to local extinctions in some areas (Alonso *et al.*, 2003a, 2009a, 2003b; Palacín *et al.*, 2004) and could be the origin of the population decline documented here.

During the Holocene and upper Pleistocene (10 000–30 000 BP), great bustards were probably distributed throughout the Iberian Peninsula (Sánchez, 2004). Long-term demographic reconstructions based on BSPs suggest that Iberian great bustard populations have been in demographic stability until relatively recent times. According to our results, the population was stable for at least 8000 years, until about 2000 BP, when the growth rate became negative (below -1.5% ; Fig. 3B) and the population started to decline until the present. This decline was particularly marked during the last 1000 years, when the population was reduced by 70% as a result of negative growth rates of -20% . With other possible mutation rates (0.02, 0.04 and 0.15 substitutions/site/million years, see Material and methods), the decline (growth rate below -1.5%) would have started around 3100, 3000 and 1500 BP, respectively.

Using the most probable mutation rate (0.10 substitutions/site/million years), we obtain similar BSPs for both the Iberian and Madrid datasets (Fig. 3), with the only difference being that the final decline is more recent and slightly less marked in Iberia than in the Madrid population (growth rate reaches -11% , whereas, in Madrid, it reaches -19%). The BSP starts to decline around 1000 BP (Fig. 3D), coinciding with the start of the marked decline of the Madrid population. This suggests that the signal of population decline found in Madrid is representative of the Iberian Peninsula.

What could be the reason for the decline in these great bustard populations? The effect of climate is unlikely, as alternating warm and cool periods have occurred during the last 10 000 years (Ruddiman, Shackleton & McIntyre, 1986; Rimbu *et al.*, 2004; Frigola *et al.*, 2007; Morellon *et al.*, 2008), a period during which BSPs show demographic stability (Fig. 3). The great bustard is highly sensitive to changes in agricultural regimes and patterns of habitat fragmentation (Palacín *et al.*, 2004). Since the Neolithic (from 5000 BC onwards), agricultural development has changed the Iberian landscape and the adoption of farming has resulted in significant ecological, economic, dietary and social changes (Zapata *et al.*, 2004; Palomo *et al.*, 2011). Around 2200 BP, the Romans invaded the Iberian Peninsula, modifying agricultural methods and dramatically

increasing production by promoting large-scale cultivation, irrigation development and crop rotation (Curchin, 2003). The Romans also introduced grape and olive cultivation to the Iberian Peninsula, which represented profound changes in the agricultural landscape, previously composed of cereals and legumes (Brun, 2003; Buxo, 2008). The Roman invasion also marked the beginning of urban development in Iberia, with associated infrastructure, such as major roads (Curchin, 2003). Hughes (1994) indicated that, in Roman times, habitat alteration and excessive hunting pressures contributed to gradual population declines of wildlife in the Mediterranean Basin. After the fall of the Roman Empire, in the Middle Ages, triennial rotation was implanted in Iberia (see Mazoyer & Roudart, 2006 for complete information). This agricultural practice is based on the succession in a given field of winter cereal in the first year, spring cereal in the second year and fallow in the third year, allowing cereals to be grown on the same piece of land in winter and spring and reducing the time of fallow to one in every 3 years. The combination of this improvement, together with others, such as the use of ploughs with wheels and horsepower, contributed to an increase in production accompanied by an extensive agricultural expansion that destroyed wide areas of forest (Bartlett, 1994). As a consequence, human nutrition and diet improved and the Iberian population grew (Mazoyer & Roudart, 2006; Epstein, 2009) (Fig. 4). Our data on historical great bustard demography indicate that the sudden and steep decline in numbers coincided in time with these dramatic transformations of the Iberian landscape and the onset of human population growth (Fig. 4). The latter indeed shows a significant correlation with the bustard's decline ($r = -0.982$, $P < 0.01$; based on Teran & Sabaris, 1978; Nadal, 1988; INE, 2012). The temporal congruence between human (infrastructural and agricultural) expansion and bustard decline suggests a possible causal relationship between the two processes. However, other factors, such as disease or local climate changes, cannot be ruled out.

An additional effect of human expansion could have been active persecution. Great bustards seem to have often been hunted by humans in the western Mediterranean since the Mesolithic (8000 BC; Cuisin & Vigne, 1998). The first evidence of bustard hunting in the Iberian Peninsula dates back to the Neolithic, and consists of *c.* 4000-year-old paintings discovered in prehistoric caves in eastern and southern Spain (Brodick, 1965; Acosta, 1968). However, it was probably during the Middle Ages when bustard hunting started to become a widespread activity, as suggested by the presence of bustard bones in deposits from the

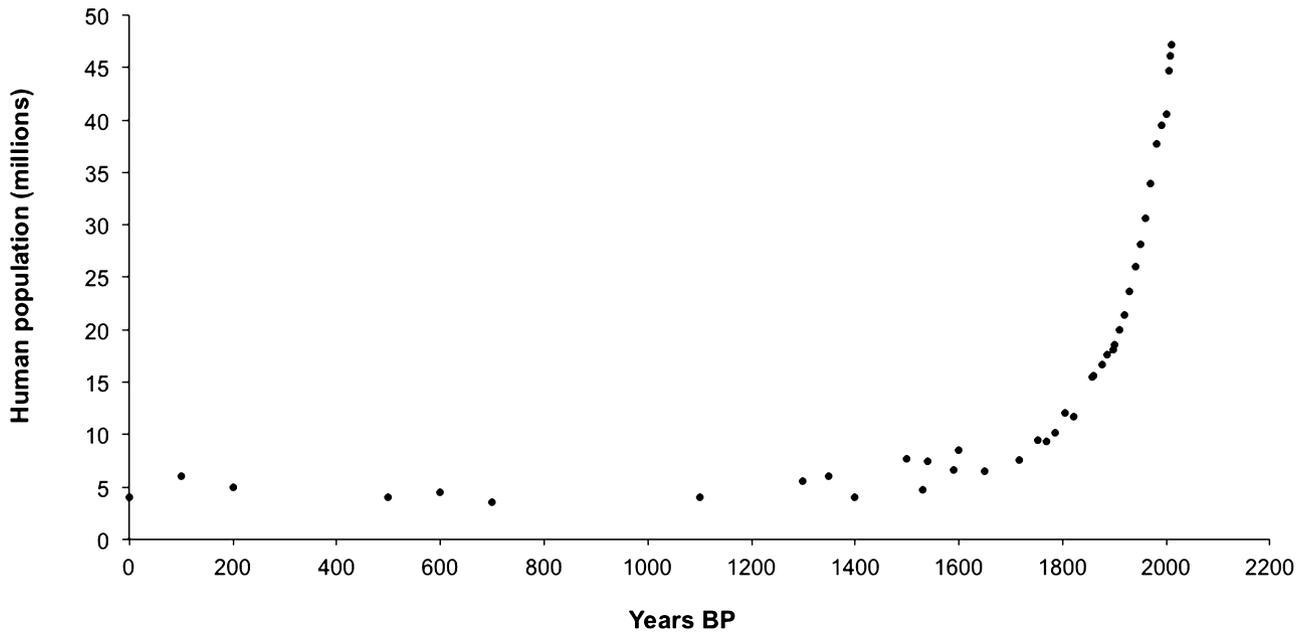


Figure 4. Human population growth in Spain over the last 20 centuries (based on Teran & Sabaris, 1978; Nadal, 1988; INE, 2012).

11th to the 13th centuries (Aguilar, 1990; Hernández, 1993). Hunting pressure probably increased with the advent of firearms, and continued to increase in proportion to human expansion up to the present time. In 1970, up to 2057 great bustards were shot officially in Spain (Trigo de Yarto, 1971). There are strong reasons to believe that, together with agricultural intensification, hunting has been one of the main causes of local bustard extinctions in Spain, at least in the last century (Alonso *et al.*, 2003b).

Finally, the decline of the Iberian bustard population is coincident with the accelerated decline in Madrid in the last 1000 years (Fig. 3B), a city founded by the year AC 932.

In conclusion, our study presents, for the first time, evidence suggestive of a direct impact of human population growth and expansion on a bird species with which humans have coexisted in the same agrosteppe habitat for thousands of years. Human expansion, and the associated agricultural and urban development and hunting pressure, have run parallel to the rapid decline of bustard populations through the last thousands of years, and this process has probably resulted in the currently fragmented distribution of this species in Iberia. Similar processes may have taken place in other parts of the distribution range of the species.

Our study also highlights the importance of using high-quality field data to infer reliable conclusions from genetic analyses. Without accurate field data to estimate population parameters (in our case, genera-

tion time and population size), the choice of the correct mutation rate for the mitochondrial CR would not have been possible, and the results and interpretation might have changed dramatically. Finally, Iberian great bustards have probably suffered a recent genetic bottleneck, with a consequent loss of genetic variation (genetic variability is related to the effective population size) (Frankham, 1996). As genetic diversity provides populations with the capacity to adapt and resist adverse environmental conditions (Frankel & Soulé, 1981), under the current global change scenario, it is especially important to conserve as much genetic variability as possible in Iberian great bustards, which represent the main stronghold of this species in the world.

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