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## Estimating the population size of the endangered Cantabrian brown bear through genetic sampling

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The Cantabrian brown bear *Ursus arctos* population can be seen as a paradigm in conservation biology due to its endangerment status and genetic uniqueness. Therefore, the need to obtain basic demographic data to inform management actions for conservation is imperative. Despite this, empirical data on the size and trends of the Cantabrian bear population are scarce. Here we present the first estimates of population size ( $N_c$ ) and effective population size ( $N_e$ ) of the whole Cantabrian brown bear population. We genotyped 270 non-invasive samples collected during 2006 throughout the entire range of the population and subsequently identified 130 individuals. Different model estimators of  $N_e$  based on capture–mark–recapture (CMR) procedures were compared. The average for the best three models (Mh Chao, Mh Darroch and CAPWIRE TIRM) yielded a total estimate of  $N_e = 223$  individuals ( $CI_{95\%} = 183–278$ ) and  $N_e = 50$  ( $CI_{95\%} = 36–75$ ) providing an  $N_e / N_c$  ratio of 0.22. Estimates for the two subpopulations commonly recognized in the Cantabrian range were  $N_c = 203$  ( $CI_{95\%} = 168–260$ ) and  $N_c = 47$  ( $CI_{95\%} = 36–70$ ) for the western subpopulation and  $N_c = 19$  ( $CI_{95\%} = 12–40$ ) and  $N_e = 9$  ( $CI_{95\%} = 8–12$ ) for the eastern subpopulation. These data suggest that the Cantabrian brown bear population has increased recently, mainly in the western subpopulation, after a long period of decline and isolation which lead to the split of the population at the beginning of the 20th century. Population sizes in the early 1990s were thought to be only 60 individuals for the western subpopulation and 14 individuals in the eastern one. The efforts to improve conservation policies made since then have probably contributed, to some extent, to the population increase during the last couple of decades.

The brown bear *Ursus arctos* population in the Cantabrian Mountains (northwest Spain) is one of the most endangered in the world (Zedrosser et al. 2001), mainly because of low numbers and limited habitat availability (Wiegand et al. 1998, Naves et al. 2003, Martin et al. 2012). This brown bear population is the most southwestern in Europe and it has been completely isolated from other bear areas for centuries (Sorensen 1990, Zedrosser et al. 2001). From a phylogeographic point of view it represents one of the three mitochondrial clades described in European brown bears, currently limited to the Cantabrian Mountains, the Pyrenees and south Scandinavia (Taberlet and Bouvet 1994, Davison et al. 2011). Cantabrian bears are the only remaining bears of this clade whose genetic diversity has not been altered during the last few centuries by introgression from other lineages (genetic mixing with the northern subpopulations in Scandinavia, Swenson et al. 2000) and human-assisted in the Pyrenees (Zedrosser et al. 2001). With such a context of endangerment and genetic uniqueness, the Cantabrian population is a paradigm in conservation biology. The need to obtain basic demographic data such as population size ( $N_c$ )

and effective size ( $N_e$ ) to inform management actions for conservation is imperative, as management decisions would otherwise be uncertain and potentially controversial.

Brown bears, once distributed throughout the entire Iberian Peninsula, suffered a sharp range contraction that began more than 300 years ago which lead to the eventual split of the Cantabrian and Pyrenean populations (Naves and Nores 1997). At the beginning of the 19th century the Cantabrian population occupied an area of 14 000 km<sup>2</sup> which continued to decrease throughout the century (Naves and Nores 1997). Since the first half of the 20th century, the Cantabrian bear population has been divided into two subpopulations (western and eastern), both occupying roughly the same amount of area (~ 3700 km<sup>2</sup>). Estimates of population size in the early 1990s yielded figures of only 55–60 individuals for the western subpopulation (Wiegand et al. 1998) and about 14 individuals in the eastern one (Clevenger and Purroy 1991). This reduction has also been noted at the level of genetic diversity (Valdiosera et al. 2008, Pérez et al. 2009). The high genetic differentiation observed between the two Cantabrian subpopulations is due to their

complete isolation for several generations in conjunction with an extremely low population size in the eastern subpopulation for decades (Pérez et al. 2009). The western subpopulation exhibits a level of genetic diversity which is among the lowest observed in the species ( $H_e = 0.48$ ; Skrbinek et al. 2012), while diversity in the eastern subpopulation is even lower ( $H_e = 0.28$ ; Skrbinek et al. 2012) and is only comparable to the values reported for the isolated population of the Kodiak Islands in Alaska (Paetkau et al. 1998a) and the Gobi brown bears in Mongolia (McCarthy et al. 2009). However, movement of individuals between the two subpopulations has been detected quite recently, and there is evidence of recent genetic exchange (Pérez et al. 2010).

To avoid extinction, a temporary ban on hunting was introduced in Spain in 1967. This ban became permanent when brown bears were declared a protected species in 1973 and subsequently considered in serious danger of extinction on the National list of threatened species. Since 1989 the Spanish government and four regional governments have developed several recovery plans for brown bear populations. Such initiatives were among the first legally approved in Spain for any endangered population of wild vertebrate with the aim of its recovery and conservation.

The implementation of regional recovery plans involved considerable budgetary and conservation efforts (e.g. establishing reserves and protected areas, conducting long-term field research, mapping and monitoring bear distribution, habitat analysis, damage compensation schemes, increasing the number of wildlife rangers and NGO personnel, etc.). It is important to note that until today these conservation efforts have not considered population reinforcement measures (translocation, captive breeding, supplementary feeding, etc.) that are standard actions in conservation projects of small European brown bear populations.

Despite being an important issue in the conservation of endangered species, empirical data on the size and trends of the Cantabrian bear population are scarce. Opportunistic data of females with cubs-of-the-year (Fcub), based on direct observations and track signs collected by rangers and researchers, have been used to perform a non-spatial demographic population viability analysis of the western subpopulation with data for the period 1982–1995 (Wiegand et al. 1998). More recently, Fcub data from 1994 to 2004 were analyzed and a trend was interpreted as an increase in population size (Palomero et al. 2007a). In 2004, Palomero et al. (2007b) estimated 105–130 bears for the entire population. Nevertheless, the use of opportunistic Fcub data to estimate population size and trends (Eberhardt and Knight 1996, Wiegand et al. 1998, Palomero et al. 2007a, b) has been cautioned for small bear populations (Brodie and Gibeau 2007, Harris et al. 2007, Fernández-Gil et al. 2010).

Estimating census population size ( $N_c$ ) in large carnivores usually involves considerable logistical concerns and constraints, due to their elusive behavior and low densities (Miller et al. 1997, Karanth and Nichols 2002). When the concerned populations are small and endangered, robust estimates of census (including variance estimates) and monitoring procedures are called for because they are needed to implement conservation-oriented actions. In addition, the required sample sizes are even more difficult to obtain in small populations, such as the one studied here. Besides

the actual size, it is important the effective population size ( $N_e$ ), “the number of individuals that would result in the same loss of genetic diversity, inbreeding or genetic drift if they behaved in the manner of an idealized population” (Frankham et al. 2010). Thus,  $N_e$  can be used to predict the effects of finite population size on inbreeding, loss of genetic variation and random drift. Its importance becomes evident after considering that  $N_e$  is generally only about 10% of  $N_c$  in wildlife, due to unequal sex-ratio, variance in family size and fluctuations in abundance over generations (Frankham 1995). The need to obtain basic demographic data such as  $N_c$  and  $N_e$  is imperative, as management decisions would otherwise be uncertain and potentially controversial.

To date, estimates of bear population size in other areas have been provided by capture–mark–recapture (CMR) of radio-tagged animals (Miller et al. 1997) and more recently by adapting CMR procedures to the use of individual data from DNA analysis (Bellemain et al. 2005, Kendall et al. 2009). Also, methods have recently been developed to estimate  $N_e$  from the genetic constitution of individuals in one generation (Tallmon et al. 2008). An unpublished technical report using non-invasive genotyping provided an estimate of 107 individuals for the western subpopulation ( $CI_{95\%} = 85–143$  bears) in 2002 (García-Garitaigoitia et al. 2004). This report showed an astounding male sex bias, which was also previously reported for the eastern subpopulation (Rey et al. 2000).

Thus, given the virtual absence of demographic estimates for the Cantabrian brown bear population, the main goals of this study are: 1) to estimate the size of the bear population ( $N_c$ ) through DNA analysis of non-invasive samples obtained from multiple sources and gathered throughout the entire home range of the population during the year 2006; 2) to estimate the effective population size ( $N_e$ ) and 3) to discuss whether the current population status may be derived from previous conservation policies.

Through the use of non-invasive genetic sampling we can estimate population size using CMR procedures modified to fit genetic implementation (Baillargeon and Rivest 2007, Miller et al. 2005). In addition to the minimum number of individual genotypes identified, we will compare the results obtained from different estimators to determine the size of the population. Also, the effective population size ( $N_e$ ) will be calculated using the method ONEsAMP proposed by Tallmon et al. (2008).

## Material and methods

### Study area

The Cantabrian brown bear currently inhabits a total range of approximately 5000–7000 km<sup>2</sup> along the whole Cantabrian mountain chain. The two subpopulations are separated by some 30–50 km of mountainous terrain, a gap which has been interpreted as the result of habitat encroachment due to the development of heavy transport infrastructures during the 20th century and, in general, due to high human-related mortality (Wiegand et al. 1998, Naves et al. 1999). The Cantabrian Mountains (northwestern Spain) are the least forested landscape currently occupied by brown

bears in Europe (ca 30% of remaining forest cover, Naves et al. 2003). The range runs east–west along the Atlantic coast of northwestern Spain, with a maximum elevation of 2648 m a. s. l. Beech *Fagus sylvatica*, oak *Quercus* spp., birch *Betula alba* and chestnut *Castanea sativa* forests are interspersed with pasturelands and shrubs of broom *Cytisus* spp., *Genista* spp., heather *Erica* spp., *Calluna vulgaris* and bilberry *Vaccinium myrtillus*, the latter usually dominating montane and subalpine levels (above ca 1000–1700 m a. s. l.; see more details in Naves et al. 2003, Martin et al. 2012).

### Collection of field samples

The study is based on 270 samples collected in 2006 throughout the study area. Figure 1b illustrates the distribution area of the species and the sampling locations. Samples used for the analyses were obtained from several sources.

1) Systematic surveys conducted on foot looking for bear signs (scat and hair) were carried out twice during the year (summer season: mid-August–mid-September, and autumn: mid-October–mid-November) by wildlife biologists and rangers over a grid (cell of 6.25 km<sup>2</sup>) covering an area of 750 km<sup>2</sup> (total of 120 cells), with roughly 680 km surveyed each season (average = 5.61 km, SD = 1.39 km for each cell in summer; average = 5.39, SD = 1.35 km in autumn). Systematic foot surveys were performed in the western subpopulation (Fig. 1a).

2) Hair traps (20 × 20 cm metallic-trellis, placed in a tree) in 75 sites systematically distributed over a 2.5 × 2.5 km grid, with 10 traps per site, baited with scent lure. Sites with hair traps were distributed in both subpopulations (Fig. 1a) and placed in previous years. Hair traps were activated (baited) in March and checked three times (June, August, October).

3) Samples gathered opportunistically by rangers and wildlife biologists (in bear damage inspections, trophic ecology studies, among others) in both subpopulations.

Fecal samples were dry stored with silica after ethanol soaking upon collection, following Nsubuga et al. (2004) and Roeder et al. (2004). Hairs were placed in individual envelopes without further manipulation until DNA extraction.

### DNA extraction and typing

DNA was extracted from feces using the ‘Qiamp DNA stool kit’ specially developed for this type of material, as described in Pérez et al. (2009). For hair DNA extraction, 5–10 hair roots were used; these were incubated overnight with a PCR buffer and proteinase K (Allen et al. 1998, Vigilant 1999). All hair and feces extractions were carried out in a separate room used exclusively for processing samples of this type. DNA extraction was performed at the population genetics laboratory at the Functional Biology Dept, Oviedo Univ. To test for DNA quality, a preliminary round of PCR was conducted. Three microsatellite loci and the sex marker were amplified. If the genotype for at least two out of the four markers was not retrieved for a given sample, the sample was discarded and no further analysis was made.

Remaining samples were genotyped for the other loci. A total of 18 microsatellite markers (G1A, G1D, G10B, G10C, G10J, G10L, G10O, G10P, G10X, MU05, MU09,

MU10, MU23, MU50, MU51, MU59, MU61, MU64), as well as the sex marker (Pérez et al. 2009) were therefore analyzed. Two methods were used indiscriminately for sex determination: the SRY method (Bellemain and Taberlet 2004), and the SRY / ZF method (Pagès et al. 2009). All PCRs included positive and negative controls to detect contamination issues.

### Reliability of the genotyping

In order to increase the reliability of the genotyping from fecal samples we performed DNA amplifications in a two-step PCR, following Taberlet et al. (1997); but we used only one PCR for hair samples. The amplification was carried out following the multiplex preamplification method (Bellemain and Taberlet 2004, Piggot et al. 2004). Three positive PCRs were first analyzed, a consensus genotype was assigned using the GIMLET ver. 1.3.2 software (Valière 2002), and its reliability was tested using the RELIOTYPE software (Miller et al. 2002). If we found a multilocus genotype with reliability lower than 95%, more repetitions were carried out until that level of reliability was attained. We discarded samples that could not be reliably typed for at least 13 of the 18 loci after the entire process was completed. We determined genotypic mismatches between all scores to identify different samples from the same individual (Pérez et al. 2009, 2010).

### $N_c$ and $N_e$ estimates

Population size ( $N_c$ ) estimates were performed using several capture–mark–recapture (CMR) estimators. Calculations were made for the total sample for the whole population and for the two subpopulations independently.

Different estimates of population size produced by the CMR-based approaches were compared. We started by grouping identical multilocus genotypes and then generated a matrix with two columns in which the first column contained the number of captures and the second column contained the observed frequency. We estimated the population size employing several models (Mh Chao, Mh Poisson2, Mh Gamma3.5 and Mh Darroch) available in the Rcapture software package (Baillargeon and Rivest 2007). The latest version of this R software package (1.3-1; 2012-05-31) allows working with capture occasions that are not well-defined, so captures occur in continuous time.

We also calculated the population size estimate produced by a CMR-based program, CAPWIRE, specially designed to work with non-invasive genetic sampling (Miller et al. 2005). This method accommodates data with multiple observations of an individual within a single session (continuous time), and also accounts for capture heterogeneity (the two innate rates model; TIRM). This method appears to work particularly well for small populations (< 100 individuals; Miller et al. 2005).

Effective population size ( $N_e$ ) estimates were performed using the Bayesian method implemented in ONeSAMP (Tallmon et al. 2008), which is based on linkage disequilibrium (LD) together with seven additional summary statistics. Calculations were also made for the total sample for the whole population and for the two subpopulations independently. Priors on lower and upper bounds were 2

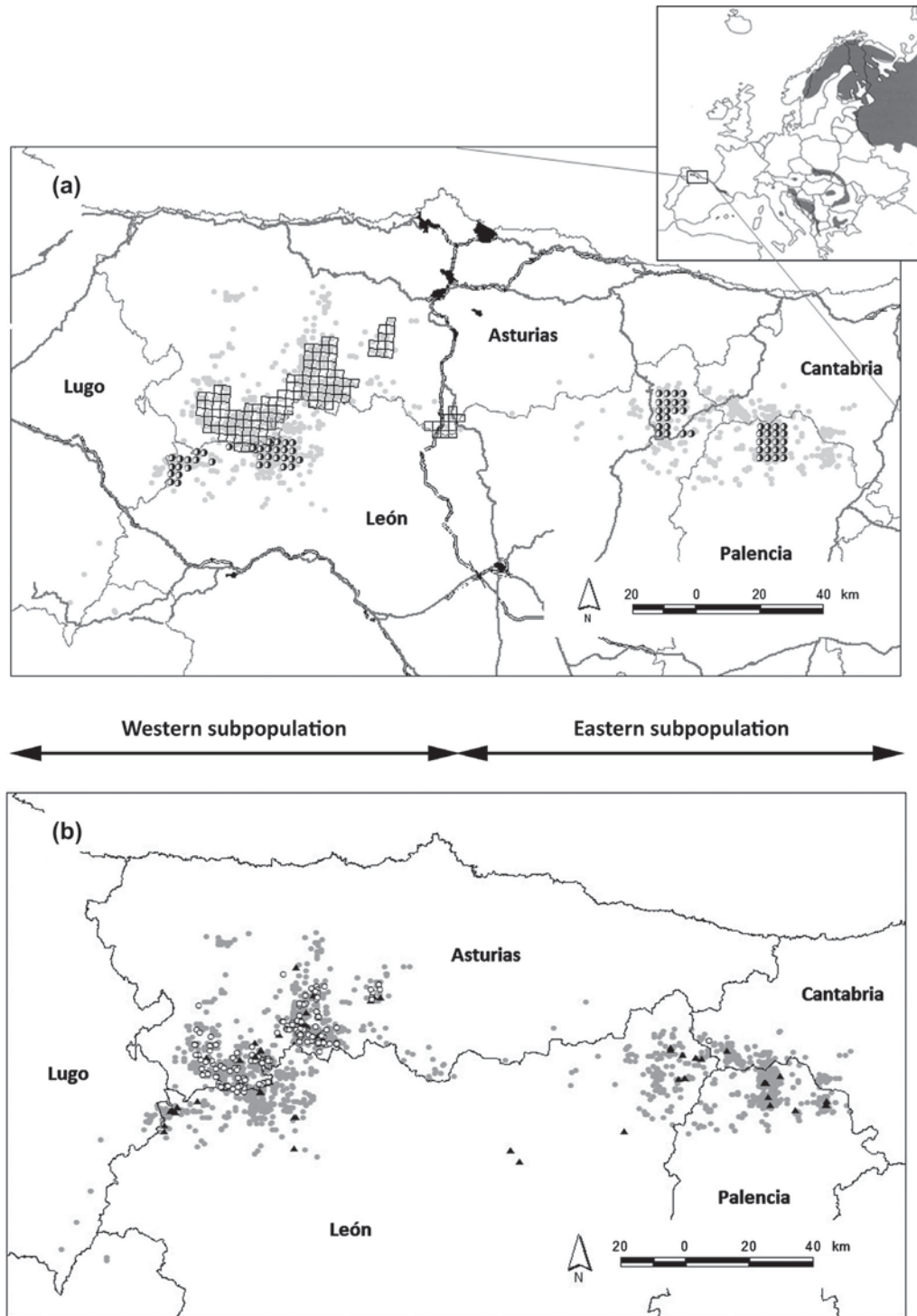


Figure 1. Distribution of brown bears in the Cantabrian Mountains, sampling areas and locations of samples used in the study. (a) Distribution of the Cantabrian brown bear depicting motorways, railways and major cities. Light grey dots show areas where brown bear presence has been detected (based on 3093 observations of brown bears during the period 1996–2001). Transects for systematic surveys (squares) and hair traps (circles) are also presented. (b) Location of the 207 samples genotyped in this study (open circles represent feces samples and black triangles represent hair samples; light grey dots same as in (a)).

and 300, respectively, for both the total population and the western subpopulation, and 2 and 50 for the eastern subpopulation.

We tested for different capture probability between males and females fitting generalized linear models (hereafter GLM) with capture frequency as the explanatory

variable and sex (male, female) as the response variable (GLM, binomial error distribution). We considered the model adequately supported if the explanatory variable (capture frequency) was significant ( $p < 0.05$ ). Analyses were performed in R (ver. 2.15.0). We also used GML to test for differences in the efficacy of sex determination depending on

the sex of the individual, in order to assess methodological bias of the sex determination.

## Results

### DNA extraction and reliability of the genotyping for both sexes

In 2006 a total of 605 samples (443 from feces and 162 from hair) were collected throughout the study area. Only 270 of the samples yielded reliable genotypes and thus could be used in this study, of which 203 were from feces (45.82% success) and 67 were from hair (73.63% success of the 91 rooted samples; 71 samples had no roots and were therefore discarded). Most of the samples (87.41%;  $n = 236$ ) were collected between August and November. Of the 270 samples that gave reliable genotypes, 178 samples came from systematic foot surveys (mainly scat), 20 samples from sites with hair traps and 72 samples were gathered opportunistically. Most samples were collected in the western subpopulation ( $n = 242$ ).

Out of the 270 samples successfully genotyped, a total of 130 unique genotypes were obtained, of which 47 were males, 42 females and 41 of unknown sex (we were unable to amplify the sex marker). Each multilocus genotype was observed from 1 to 13 times (Fig. 2), with a mean of  $2.08 \pm 1.96$  (SE) times ( $3.02 \pm 2.69$  for males,  $1.74 \pm 1.24$  for females and  $1.34 \pm 0.81$  for individuals of unknown sex). The difference in the success rate of the sexing procedure in relation to the sex of the sample was not statistically significant (GML sex ~ failure\_rate:  $z = -0.103$ ;  $p = 0.918$ ; GLM binomial function). To increase the number of samples analyzed and therefore the statistical power, we used not only the samples from 2006, but also samples collected over several years (2004–2009) for this test. Data are based on a total number of 314 samples belonging to 112 individuals

for whom sex was determined at least once: 85 out of the 314 samples could not be sexed.

### Population and subpopulation $N_c$ and $N_e$

As mentioned above, population sizes were calculated using five different models: Mh Chao, Mh Poisson2, Mh Gamma3.5, Mh Darroch and the TIRM models. All of these models assume individual heterogeneity (IH) of sampling probabilities.

Population and subpopulation size estimates, confidence interval ( $\pm 95\%$  CI) and other parameters resulting from the five models are provided in the Supplementary material Appendix 1 Table A1–A3.

For the whole population, the Gamma 3.5 model yielded the highest estimate, 419 individuals, and also displayed the widest CI ( $CI_{95\%} = 291–634$ ). The Mh Poisson exhibited the narrowest CI but the lowest estimate: 164 individuals ( $CI_{95\%} = 149–184$ ). However, the Mh Chao, Mh Darroch and CAPWIRE TIRM models yielded nearly the same estimates and the CIs were also very similar, although they were slightly wider for Mh Chao model. In addition the  $CI_{95\%}$  of Gamma and Poisson estimates barely overlap with those of Mh Chao, Mh Darroch and CAPWIRE models (Supplementary material Appendix 1 Table A1).

Estimates for the Mh Chao model, Mh Darroch model and CARWPIRE TIRM model were 222 individuals ( $CI_{95\%} = 177–294$ ), 225 individuals ( $CI_{95\%} = 188–274$ ) and 223 individuals ( $CI_{95\%} = 185–270$ ), respectively. The average of these three models provides an estimate of 223 individuals ( $CI_{95\%} = 183–279$ ; Fig. 3) for the whole population. When we repeated the analysis for each subpopulation the utilized estimators displayed the same pattern of estimates and CI values, as Mh Chao, Mh Darroch and CAPWIRE TIRM models yielded nearly the same estimated value within each subpopulation and the CIs were also very similar. The average of the three models provided an

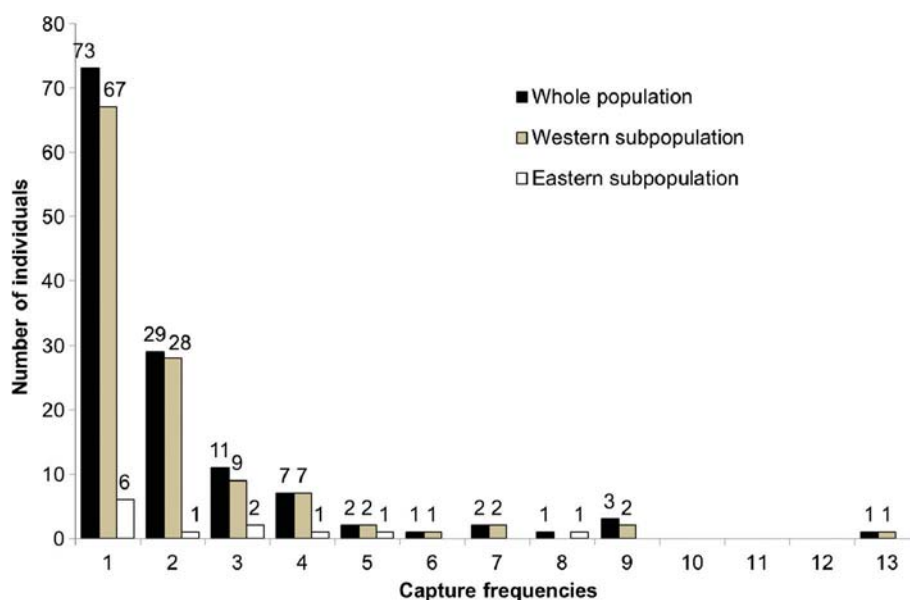


Figure 2. Capture frequencies of the 130 individual brown bears genotyped in 2006 via field samples collected in the Cantabrian population (119 in the western subpopulation and 12 in the eastern subpopulation; one individual captured in both subpopulations).

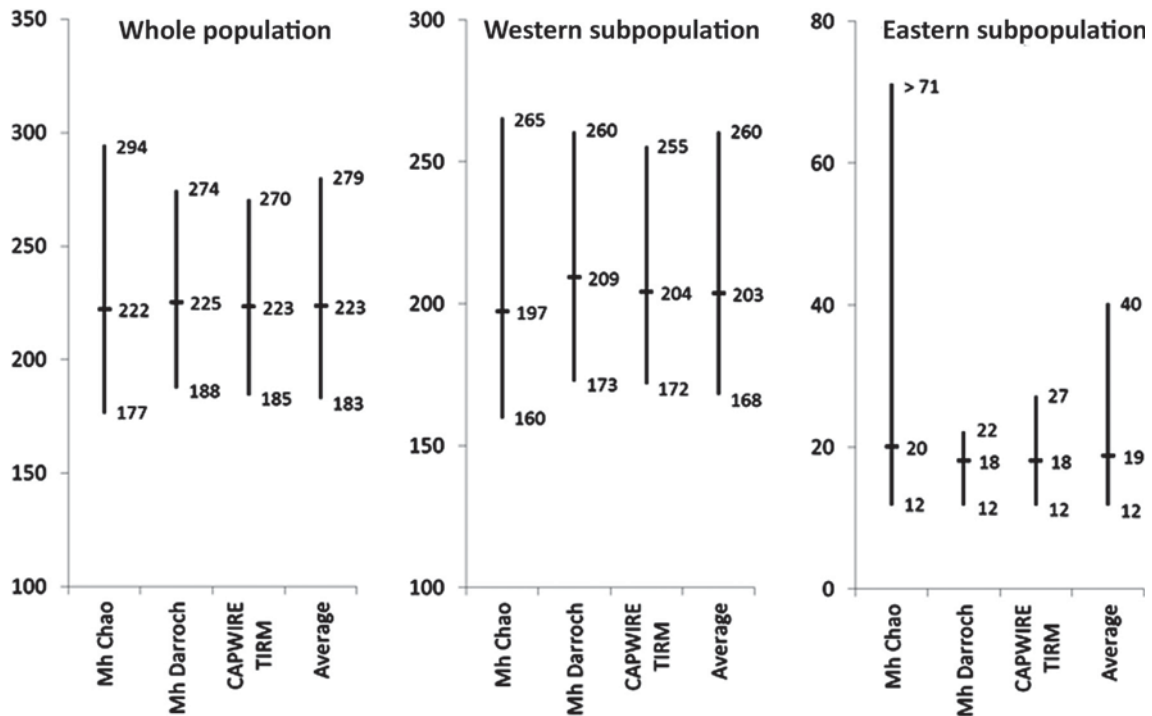


Figure 3. Cantabrian brown bear population estimates (estimate  $\pm$  95% CI) based on the selected estimators (Mh Chao, Mh Darroch and CAPWIRE TRIM; see text) and average of the models. (From left to right: whole population, western subpopulation, eastern subpopulation).

estimate of 203 individuals ( $CI_{95\%} = 168\text{--}260$ ) for the western subpopulation and 19 individuals ( $CI_{95\%} = 12\text{--}40$ ) for the eastern subpopulation.

Estimates of  $N_e$  for the whole population obtained from the ONeSAMP software yielded a value of 50 ( $CI_{95\%} = 36\text{--}75$ ). The value for western subpopulation was  $N_e = 47$  ( $CI_{95\%} = 36\text{--}70$ ) and for the eastern subpopulation  $N_e = 9$  ( $CI_{95\%} = 8\text{--}12$ ).

We were able to determine the sex of 89 of the 130 identified individual genotypes (47 males and 42 females) and we observed a clear difference between sexes in the mean number of captures per individual, with a larger proportion of the sampled males captured multiple times (mean number of captures per male  $3.02 \pm 2.69$ ) compared to females (mean number of captures per female  $1.74 \pm 1.24$ ). This difference between sexes was statistically significant (GML sex~total: estimate/parameter = 0.375; intercept = -0.7151; SE = 0.156;  $z = 2.406$ ;  $p = 0.0161$ ; GLM binomial function).

## Discussion

### Some cautions concerning the methods

The use of non-invasive sampling methods (including DNA based procedures) can be an efficient way to estimate population size and effective population size, mainly when individuals are difficult to capture or observe. However, we must be careful when interpreting the estimates obtained in the current study. By definition, molecular tags are marks that cannot be lost or duplicated, but genotyping errors could still

occur (Pompanon et al. 2005). A previous pilot study carried out on this population followed a strict protocol in order to determine the number of markers necessary for individualization thus minimizing genotyping errors. The protocol included the use of multiple amplifications, the application of the consensus rule and testing for genotyping reliability (see Pérez et al. 2009 for details). Measures to avoid contamination were taken and we consider that problems related with low-quantity DNA samples are not an issue in our data set. In addition, we did not find a statistical difference in the probability of determining the sex of an analyzed sample depending on the sex.

The capture estimators used in this study assume population closure. Population changes over the time period of interest must be sufficiently small so that the assumption of closure is a reasonable approximation (Amstrup et al. 2005). In our case, although samples were collected throughout the year, 87.40% of the 270 samples (122 of the 130 individuals) were collected within a reasonably short period of time, i.e. less than four months (during the hyperphagia period of the bears). Besides, brown bear mortality rates are usually low (McLellan et al. 1999). With regard to geographic closure, we do not believe that this is a large source of bias in our estimate since bear presence outside the study area is only sporadically reported (Fig. 1b).

Another drawback of the method is the individual heterogeneity (IH) in the probability of capture. IH occurs as a result of biological characteristics of the study species, individual attributes such as sex, age, seasonal changes of habitat use due to individual or habitat features, territoriality or even sampling procedures, e.g. seasons or sessions (Ebert et al. 2010). As we have already shown, sex is an

important source of IH in our data set, similar to that reported in many bear studies (Mace et al. 1994, Boulanger et al. 2002, Bellemain et al. 2005). We minimized these problematic issues by combining different sampling strategies (systematic and opportunistic) and sample types (feces and hair) (Pollock et al. 1982, Williams et al. 2002, Ebert et al. 2010). Hair sampling using traps requires an active approach by the animal that could affect the sampling probability. Therefore, passive sampling, i.e. feces, along transects appears to be a good approach as it is less affected by individual behavior. However, passive sampling can be affected by other sources of IH such as differences in deposition rate and pattern (Ebert et al. 2010). The fact that the combination of different sampling strategies is a good approach to reduce IH in the field has already been reported in the literature (Boulanger et al. 2008). When multiple approaches are used simultaneously, the impact of IH caused by any single method can be minimized (Pollock et al. 1982, Williams et al. 2002, Boulanger et al. 2008, Ebert et al. 2010). Furthermore, we adapted the estimates of population size from CMR-estimators to IH.

A serious issue for capture heterogeneity is that different estimates can be obtained from the same capture history (Buckland et al. 2001, Huggins 2001, Link 2003, 2004). One approach to deal with this problem is to try to model all possible (and presumed) sources of variation for capture heterogeneity. However, when few samples are available a simpler and superior method is to use several estimators (models) and then select and average those that best fit the data, make more biological sense and perform robustly and consistently.

In this study, the estimators Mh Chao, Mh Darroch and CAPWIRE TIRM yielded similar estimates of population size and CIs, although the CIs for the Mh Chao model are slightly wider (see Fig. 3 and Supplementary material Appendix 1 Table A1–A4). Moreover, the estimates obtained from the Mh Chao and CAPWIRE TIRM models are barely affected (for both average and CI) by the presence or absence of a ‘outlier’ individual that has

been caught on a large number of occasions (13 times; Supplementary material Appendix 1 Table A4), thus showing a high capture probability.

The Mh Poisson model typically yields a smaller correction for heterogeneity than other models that implement IH (Baillargeon and Rivest 2007), which occurred in our study sample. On the other hand, the Mh Gamma 3.5 model can lead to very large estimates of abundance. Indeed, in the case of the brown bear, such large estimates seem to have little biological consistency, i.e. leading to unreported densities of these bears in southern Europe (Zedrosser et al. 2001). Baillargeon and Rivest (2007) suggest considering the Mh Gamma estimator only when very small capture probabilities are likely. Furthermore, estimates using Mh Gamma models are seriously affected by the inclusion or exclusion of the ‘outlier’ individual with high capture probability in the analyses (Supplementary material Appendix 1 Table A4).

We therefore consider that the average of these three estimators (Mh Chao, Mh Darroch and CAPWIRE TIRM) provides a reliable population estimate (Fig. 3).

### Population and subpopulation $N_c$ and $N_e$ : conservation implications

The results of this study are quite important from a conservation standpoint as we established the first direct size estimate of the whole endangered Cantabrian brown bear population, providing the largest census recorded from any documentary source (technical reports, scientific papers, etc.) in the last 60 years (Fig. 4). This census demonstrates a positive trend in Cantabrian brown bear population size, mainly for the western subpopulation during the last few decades. Population size estimates from the early 1990s gave figures of 55–60 individuals for the western subpopulation (using a demographic model based on Fcub data; Wiegand et al. 1998) and about 14 individuals in the eastern one (Clevenger and Purroy 1991). Palomero et al. (2007b)

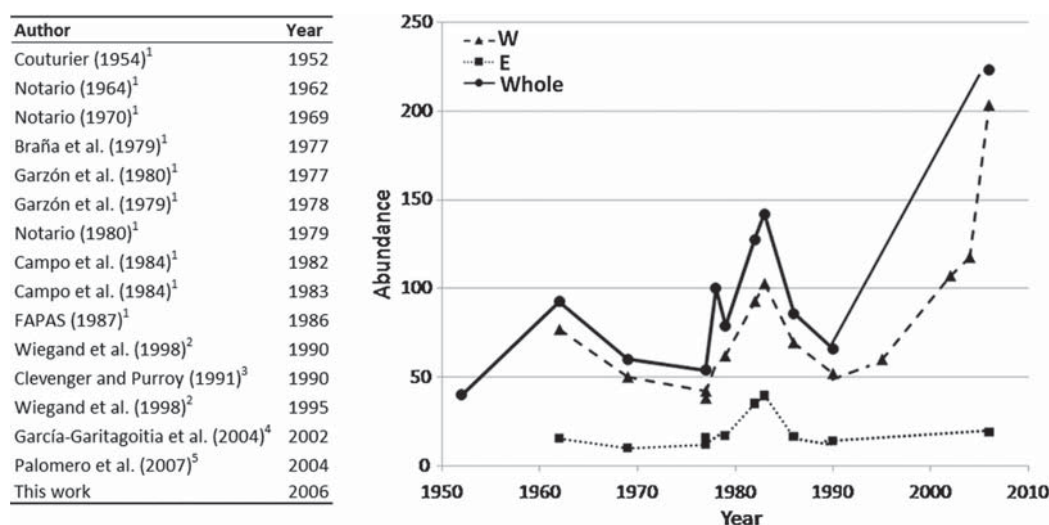


Figure 4. Cantabrian brown bear population size estimates during the last 60 years. Population sizes for the whole population (Whole) western subpopulation (W) and eastern subpopulation (E) are depicted. Size estimates in different works are based either on surveys, direct observations, females with cubs (Fcub) or non-invasive genotyping. References: <sup>1</sup>Palomero et al. (1993), <sup>2</sup>Wiegand et al. (1998), <sup>3</sup>Clevenger and Purroy (1991), <sup>4</sup>García-Garitagoitia et al. (2004), <sup>5</sup>Palomero et al. (2007b) and this work.



proposed population sizes of 105–130 individuals in 2004 from the observation of the number of Fcub and other data. This estimate is much lower than the one obtained in the present study for 2006, namely 223 individuals for the whole population ( $CI_{95\%} = 183\text{--}279$ ). Marucco et al. (2011) reported that estimates obtained from non-invasive sampling tend to be 30–50% larger than traditional ones. This discrepancy has both been attributed to residual genotyping errors, which would cause overestimation, and a higher probability of detecting individuals (Marucco et al. 2011). Even taking this into consideration, our estimates show a much larger census than previous ones.

Our figures for the western subpopulation are more than three times larger than the size estimate obtained in the early 1990s (Wiegand et al. 1998), which indicates a growth rate of approximately 5%, a rate that has been reported in other European populations (Sæther et al. 1998). The trend changes detected for the Cantabrian population have been accompanied by an inter-population connection that has been reported recently, for the first time in decades (Pérez et al. 2009, 2010). In addition, other observations, such as the presence of individuals in areas where they had not been seen for decades, seem to suggest an actual increase in the number of individuals, and therefore the estimates we present here are quite consistent with the information available. Interestingly, Zedrosser et al. (2011) highlighted the fact that European brown bears appear to respond faster, via conservation policies or less persecution, than North American ones, and suggested this is the result of population differences in adaptations to persecution through changes in life-history traits.

The data presented in this study demonstrate a population increase in the past 30 years accompanying movement between the two subpopulations, probably resulting from conservation policies, lower persecution or habitat improvement. Thus, more drastic and controversial measures such as translocations, captive breeding or supplementary feeding appear to be unnecessary at the moment. This present situation may be a result of a reduction in bear mortality, a reflection of the efforts made during the last 20 years concerning the conservation of the species, such as implemented protected areas, hunting policies, etc. For instance, protected areas (including National and Natural Parks, European Natura 2000 areas and Hunting Regional Reserves) currently cover about 88% of the reproductive range of the species, compared to only 55% in 1980. Likewise, the number of wildlife rangers in these areas has increased during this period from 70 to 130 (including NGO personnel). Nevertheless, population size is still so low that prior endangerment diagnoses are probably justified (Servheen et al. 1999, Zedrosser et al. 2001), as prospects of range habitat availability and thus potentials for recovery are limited (Naves et al. 2003, Martin et al. 2012). Indeed, given the present conditions regarding current size estimates, habitat availability, and the apparently unique nature of the Cantabrian brown bear population, management and conservation efforts should focus on preventing habitat loss and controlling human-caused mortality. In fact, habitat management (e.g. paved and unpaved road use) is the most important factor in bear management (Sorensen 1990, Ordiz et al. 2014). The translocation of bears has

been commonly used in recovery programs of small bear populations in Europe (see e.g. Mustoni et al. 2003 for the Italian Alps, Quenette et al. 2001 for the Pyrenees, Rauer 1997 for the Austrian Alps). Supplementary feeding has also been implemented in Europe, directed specifically to the species (for conservation or hunting purposes) or to other game species but used by bears as well (Swenson et al. 2000). Captive-reared bears have also been used to reinforce some European populations (Huber 2010). Although none of these actions have been carried out in the Cantabrian population, the number of individuals in this population has increased in the last couple of decades.

The male to female ratio of the sexed individuals was 1.14:1. Given that females have a lower probability of capture than males, the observed sex ratio is probably skewed towards females. This result contrasts with the male-biased sex ratio previously reported (Rey et al. 2000, García-Garitaigoitia et al. 2004). The number of females, which is the limiting factor for population growth, should therefore be larger than the number of males in the Cantabrian population, as is usual in most brown bear populations (Schwartz et al. 2003).

In addition to population size, the estimation of  $N_e$  is essential for evaluating the conservation risk of wild populations, because it determines the rate of inbreeding and the strength of genetic drift (Crow and Kimura 1970). The extremely low  $N_e$  obtained for the eastern subpopulation ( $N_e = 9$ ) is consistent with its very low genetic diversity and strong differentiation from the main nucleus (Pérez et al. 2009). The ratios of effective to actual population size for the whole population,  $N_e/N_c = 0.22$ , and for the western subpopulation,  $N_e/N_c = 0.23$ , are within the values reported for brown bears in other studies (Paetkau et al. 1998b, Miller and Waits 2003). The value of 0.47 found for the eastern subpopulation may be related to the relatively higher ratio in small populations previously reported (Palstra and Ruzzante 2008). These authors hypothesized that this could be due to changes in biological interactions at low abundance levels which would reduce individual reproductive variance (Frankham 1995). Variance in reproductive success is a factor reducing  $N_e$  with respect to  $N_c$ . We also surmise that this high  $N_e/N_c$  ratio may be derived from an actual underestimation of population size due to the small sample size in this subpopulation and the fact that samples were mainly collected through active hair trap sampling which could lead to downward-biased census estimates.

It has been suggested that the minimum effective size for a population to be viable in the short term is 50 and between 500 and 5000 when considering the long term protection of adaptive potential (Frankham et al. 2010). Thus, the western Cantabrian subpopulation is above the limit of the inbreeding avoidance criterion of  $N_e > 50$ , but far below the long-term minimum viable population size of  $N_e = 500$  (Franklin and Frankham 1998). The effective population size of the eastern subpopulation is even farther from the threshold of short-term viability. In addition, the subpopulation is so small that the risk derived from demographic stochasticity is even higher. Therefore, the eastern nucleus is at a high risk of extinction, unless the connection between the two subpopulations becomes effective and strengthened in the near future.

In the light of our results we suggest that population size estimates based on similar sampling procedures should be carried out every 10–12 years (one generation) in order to obtain robust and comparable estimates of population size. Such procedures should aim at collecting 2.5–3 times the number of non-invasive samples as the ‘assumed’ number of bears (considering that a number of the samples will not be genotyped) (Solberg et al. 2006). Special attention should be paid to the eastern subpopulation as well as to increasing the efficiency of the sexing method in order to obtain a better estimate of the sex ratio.

In summary, we consider that our approach for estimating the population size of the endangered Cantabrian brown bear population has important conservation applications and implications. We also consider that the approach presented in this study can be applied to other small populations of brown bear or other large carnivores. Our approach provided the most robust estimate of the sex ratio and size of the whole population, and may also be used as a metric reference for the monitoring of other field approaches of this population.

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Supplementary material (available online as Appendix wlb.00069 at <[www.wildlifebiology.org/readers/appendix](http://www.wildlifebiology.org/readers/appendix)>). Appendix 1