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Research

Heterozygosity, genetic similarity and extra-pair paternity variation in two populations of rock sparrow *Petronia petronia*: a within and between populations comparison

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Extra-pair paternity (EPP) has been broadly reported in socially monogamous bird species and it has been hypothesized that females engage in extra-pair copulations to increase the genetic variability of the offspring and to reduce the risk of inbreeding and genetic incompatibilities. This hypothesis makes two predictions: within populations, females should engage with more dissimilar/heterozygous males and, among populations, females should pursue more frequently EPP in populations characterized by a lower genetic variability and a higher homozygosity. However, support is still unclear throughout literature, and usually involves the study of a single population. We compared a peripheral population of rock sparrow *Petronia petronia* living at the marginal distribution of the species and characterized by a high EPP level (> 50%) (Italian Alps) with a population located in the centre of the species' distribution (central Spain), to understand if variations in EPP could be linked to differences in mean heterozygosity and genetic similarity both between and within populations. EPP in the Spanish population was 18.1%, three times lower than that observed in the Alpine one (51.2%), and this difference remained fairly constant across different years. Supporting the between populations prediction, we found lower heterozygosity and reduced allelic richness in the Alpine population compared to the Spanish one. In contrast, social and extra-pair males, as well as within and extra-pair offspring, did not significantly differ in terms of genetic similarity and heterozygosity within brood in either population. Social and extra-pair males did not differ in tarsus size, body weight or yellow badge size, suggesting that females were not choosing extra-pair partners based on heterozygosity, genetic similarity or phenotypic quality. Although based on a limited sample in the within population analysis, our results indicate that EPP may evolve in response to a low level of genetic variability in the population.

Keywords: extra-pair paternity, genetic similarity, heterozygosity, *Petronia petronia*, rock sparrow



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Introduction

Extra-pair paternity (EPP) is a widespread phenomenon that has been reported in 75% of the 342 socially monogamous bird species whose parentage has been genetically determined (Brouwer and Griffith 2019). Whereas this mating strategy provides obvious fecundity benefits to the males, it is debated to which extent it is beneficial for the females (Petrie and Kempenaers 1998, Forstmeier et al. 2014, Arct et al. 2015), as engaging in this activity is expected to be associated with direct costs for the female due to, for example, the reduction of the social partner's parental investment (Matysiokova and Remes 2013), the costs of searching for an extra-pair partner (Dunn and Whittingham 2007) and the risk of sexually transmitted diseases (Poiani and Wilks 2000).

Several hypotheses have been proposed to explain occurrence of EPP in birds (reviewed by Brouwer and Griffith 2019), such as fertility insurance (as a guard against infertility; Sheldon 1994), direct benefits (in terms of accessibility to resources held by males; Burke et al. 1989), breeding synchrony (which facilitates the comparison between different males; Westneat et al. 1990) and population density (higher encounter rates favour occurrence of EPP; Westneat et al. 1990). In terms of potential genetic benefits, among others, it has been hypothesized that females may seek EPP to increase the genetic diversity of the offspring (Williams 1975, Westneat et al. 1990) and to reduce the risk of inbreeding and genetic incompatibility (Kempenaers et al. 1999, Tregenza and Wedell 2000). These hypotheses predict that there should be an association between the genetic similarity of social partners and the incidence of EPP, and a difference in genetic heterozygosity between extra-pair and within-pair offspring. Although empirical data support these predictions in some species (Arct et al. 2015), it does not seem to be the case for the majority of the species investigated (reviewed by Hsu et al. 2015). Non-significant patterns may indicate that genetic similarity/incompatibility between social partners is not a general evolutionary explanation for EPP in birds, but may also reflect the fact that females are limited in their capability to assess the extent of genetic similarity between themselves and males (Williams 1975, Westneat et al. 1990). EPP, however, may also evolve from a bet-hedging polyandry, in which EPP may be favoured when the risk of genetic incompatibility between partners is high, and females may be selected to engage in extra-pair copulations irrespective of their capability to assess the actual genetic similarity with interacting males (Yasui and Yoshimura 2018). This is expected, for example, in small isolated populations such those living on small islands (Reid et al. 2015) or in stable group sizes with limited dispersal (Lichtenauer et al. 2019).

The genetic diversity/compatibility hypotheses therefore lead to two distinct, not mutually exclusive predictions regarding the variation of EPP within a species: 1) within populations, females are expected to engage with extra-pair partners that are genetically less similar and/or more heterozygous than the social partner (Brooked et al. 1990); 2) among populations, the mean rate of EPP is expected to be higher in

those populations in which the risk of genetic incompatibility and/or inbreeding is higher. Although these two predictions are based on the same genetic benefit of EPP for the female, the first prediction may depend on the ability of females to assess their genetic similarity with social and extra-pair partners, though similar patterns may be observed in the absence of partner recognition if embryo survival is influenced by genetic compatibility or heterozygosity. Either way, the second prediction does not require this partner recognition capability, and females within a population may engage in extra-pair copulations irrespective of the genetic similarity with the social partner. Therefore, an association between genetic similarity and the occurrence of EPP might be observed among, but not necessarily, within populations.

In most of the species, the hypotheses that females seek extra-pair copulations to increase the genetic diversity/heterozygosity of their offspring has been tested within a population, by comparing the genetic similarity of the social and the extra-pair partner (meta-analysis by Arct et al. 2015), with contrasting results (Kleven and Lifeld 2005, Smith et al. 2005, Ferretti et al. 2011). A relatively less explored approach is the one comparing, within species, different populations characterized by different levels of genetic variability, such as isolated or marginal populations (with increased rates of homozygosity and reduced genetic diversity) versus populations in the core of a species' distribution, where genetic variability is expected to be higher and the risk of genetic incompatibility and inbreeding consequently lower (Eckert et al. 2008, Cheptou and Donohue 2011). This approach, although logistically more complex, may reveal those cases in which females have been selected for a higher level of polyandry in response to the risk of inbreeding or genetic incompatibility (Michalczyk et al. 2011), even when they cannot assess their genetic similarity with the males of the population (Williams 1975, Westneat et al. 1990).

In contrast, among populations studies have the disadvantage that populations may differ for a number of characteristics, which may influence the level of EPP (and may covary with the within population genetic similarity), yet are difficult to control.

Bearing in mind these intrinsic limitations, we explored the pattern of EPP and genetic similarity between two geographically separated populations of a cavity-nesting passerine species, the rock sparrow *Petronia petronia*. One small, peripheral population of this species, located in the Alps, at the boundary between France and Italy (Fig. 1, population A), is characterized by a level of EPP (around 50% of the broods contain extra-pair offspring; Pilastro et al. 2002, Nemeth et al. 2012), which is among the highest reported for passerines (Brouwer and Griffith 2019). The location of this population in the range limit of the species distribution (Mingozzi et al. 2021) may support the hypothesis that the high level of EPP may occur to compensate reductions in the population's genetic diversity expected in marginal populations (Eckert et al. 2008). To answer this question, we estimated the rate of EPP in a rock sparrow population located in the core of its distribution range (central Spain, Fig. 1,

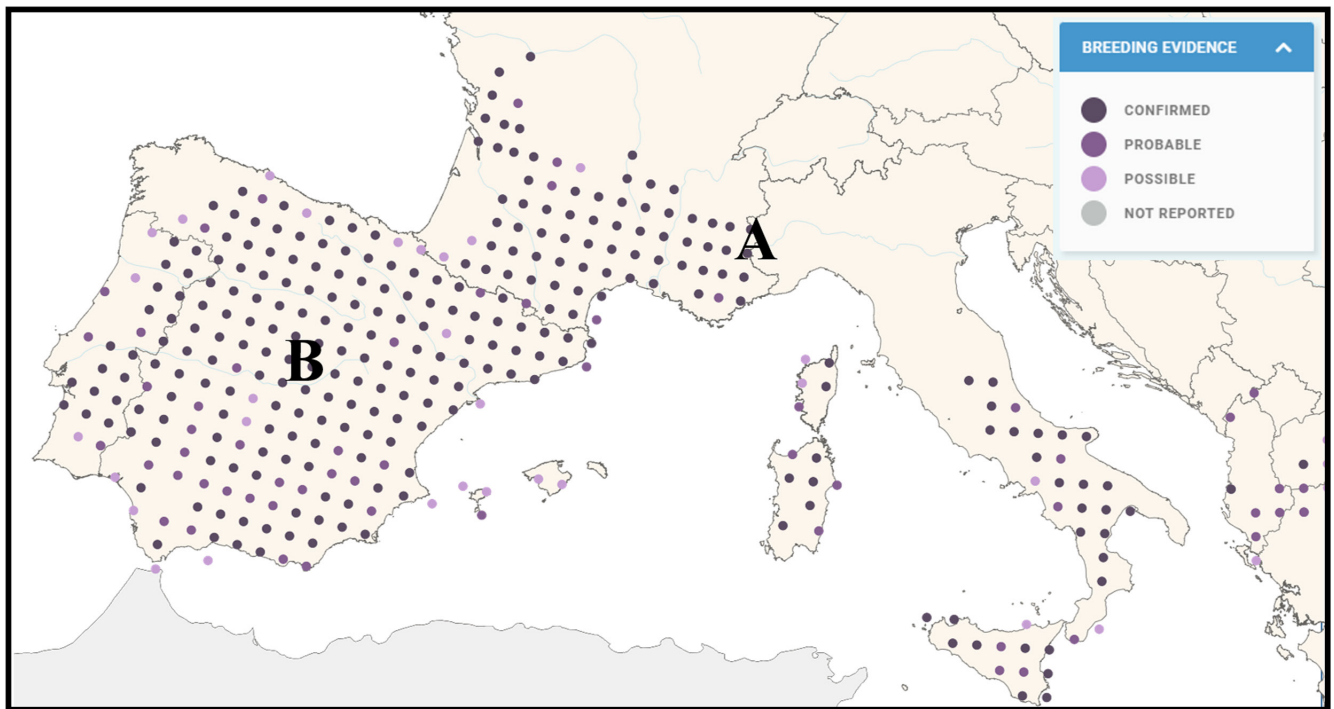


Figure 1. Distribution of the rock sparrow population according to the first Atlas of European breeding birds (2022) and location of the study areas: (A) Alpine population at the edge of the species' geographic range; (B) Spanish population in the central range of the species distribution.

population B); we subsequently compared the genetic variability of the two populations by estimating the allele richness of the adults and the heterozygosity of adults and offspring; finally we compared, for broods with EPP, the heterozygosity of the social and extra-pair males and their genetic similarity with the female, and, within brood, the heterozygosity of the within and extra-pair offspring and their genetic similarity with the mother.

Material and methods

Datasets used for genetic analyses

For this study, we used both already published and original unpublished data for two rock sparrow populations. We obtained data regarding EPP (expressed as the proportion of broods with at least one extra-pair offspring over the total broods analysed) and polygyny (expressed as proportion of males that were socially mated with two females within the same reproductive season over the total number of males) for the Alpine population from Pilastro et al. (2002), Tavecchia et al. (2002), Griggio et al. (2003a) and Nemeth et al. (2012), with the addition of unpublished data from 26 broods collected in 2001 (detailed lists of the broods sampled in the different years and study sites are given in Table 1 and 2). All data used for heterozygosity, allele richness and genetic similarity analyses were unpublished and based on the blood samples collected during the years 1997–2001

for the Alpine population, and 2016–2018 for the Spanish population.

Study sites

This study is based on the comparison of two populations of rock sparrow breeding in two well-studied nestbox systems, one located in the core of the species distribution (referred to as Spanish population) and one located in the range limit of the species distribution (referred to as Alpine population). The Spanish population breeds in a deciduous forest of Pyrenean oak *Quercus pyrenaica* located in central Spain (40°54'N, 4°01'W; Cantarero et al. 2017, 2019; Fig. 1, population B), and was monitored during the years 2016–2018. The Alpine population was sampled in two nearby study sites: one located in the upper Susa Valley in the Italian Cottian Alps (45°01'N, 6°66'E) (Pilastro et al. 2001, Griggio et al. 2003a), and another in the Clarée Valley in the French Alps (44°57'N, 6°53'E) (Matessi et al. 2005) (Fig. 1, population A), monitored during the years 1997–2001 and considered as a single loose colony (Matessi et al. 2009). Both populations (Spanish and Alpine) were monitored regularly to obtain information on first day of laying and hatching, and bred almost exclusively in the provided nestboxes. For genetic and biometric analyses, adult and chicks were trapped at the nest during the chick-rearing period. In total, we trapped 115 adults (63 males and 52 females) and 199 chicks in the Spanish population, and 193 adults (108 males and 85 females) and 292 chicks in the Alpine population.

Table 1. Detailed list of data origin for the present study regarding EPP variation in two populations of rock sparrow. The percentage of broods that contain extra-pair paternity (%EPB) was calculated by dividing the number of broods that contained at least one extra-pair offspring (EPB) by the total number of broods sampled in the population for said year. Similarly, the percentage of extra-pair offspring (%EPO) was calculated by dividing the number of offspring that belonged to an extra-pair male (EPO) by the total number of offspring in the population for said year. Data origin corresponds to the published papers from where this information has been collected, where 'unpublished' refers to original data.

Population	Year	%EPB	%EPO	Brood total	Offspring total	EPB	EPO	Data origin
Alpine	1997–1999	57.1	32.0	42	181	24	58	Pilastro et al. 2002
	2001	58.3	43.1	26	111	16	48	unpublished
	2008	41.2	21.9	29	137	12	30	Nemeth et al. 2012
	2009	48.1	27.1	27	140	13	38	Nemeth et al. 2012
Spanish	2016	11.8	4.5	17	66	2	3	unpublished
	2017	26.7	25.9	15	58	4	15	unpublished
	2018	15.8	6.7	19	75	3	5	unpublished

All trapped birds, including the chicks of an age of about 14 days, were ringed using aluminium rings and a unique combination of plastic colour rings.

Since females may be able to indirectly recognize more heterozygous partners if heterozygosity covaries with phenotypic quality (Ryder et al. 2010, Griggio et al. 2011), we measured tarsus length, body weight and yellow badge size, an indicator of male quality in the rock sparrow (Pilastro et al. 2002), for the adults. We used the same data on yellow badge size for the Alpine population in the years 1997–1999 as in Pilastro et al. (2002). Tarsus length was measured with a digital calliper to the nearest 0.01 mm and weight obtained with a Pesola spring balance (0.1 g accuracy). The size of the yellow badge size was measured by placing a transparent acetate strip over the breast, while holding the bird on its back. The contour was drawn and measured as the length of the major axis (i.e. the horizontal) with a digital calliper (± 0.01 mm). For parentage and genetic analyses 50 μ l of blood were collected from the brachial vein using disposable heparinized capillaries and stored them either at -20°C or dried on Flinders Technology Associates reagent loaded cards (Whatman Bioscience, Florham Park, NJ, USA) until needed for the paternity analyses.

Because the social mating status (monogamous or polygynous) of the male is associated with the rate of EPP in the Alpine rock sparrow population (monogamous males suffer a lower cuckoldry rate than polygynous males, Pilastro et al. 2002), we obtained the mating status of the social males from the Spanish population, and compared it to that observed in the Alpine population (Table 2). We defined a male as socially monogamous if chicks from its nest fledged before it

attracted a second mate (Griggio et al. 2003b), and socially polygynous when we observed the same male caring for the broods of two females. In addition, we used the difference between the first and last laying date as a proxy of breeding synchrony for the different populations, and measured the minimum distance between neighbour occupied nestboxes (one nest and its closet neighbour) to account for population density effects.

Microsatellite analysis

We used the blood collected from the trapped individuals to determine paternity (Table 1), heterozygosity and genetic similarity of individuals. Total genomic DNA was extracted using Qiagen tissue kit following the included protocol for the DNA stored at -20°C , whereas for the dry samples we used the salting out protocol (Miller et al. 1988). For the paternity analysis, we used ten of the eleven microsatellite primers developed for this species by Grapputo et al. (2006) with the exclusion of the sex specific locus. Microsatellite loci of Alpine population samples were amplified according to Grapputo et al. (2006). We labelled the product of amplification for loci PP01, PP11, PP15, PP14 and PP38 with IDR dyes and run in a LI-COR autosequencer. We scored the alleles by eye using the sequence of the plasmid pUC18 as size standard. We labelled loci PP18111, PP18112, PP18113, PP18114 and PP18117 with ABI dyes and run in an ABI3100 autosequencer using the GS500-LIZ size standard. We scored the alleles using Genemapper ver. 3.7 (Applied Biosystems). Loci amplification of Spanish samples were multiplexed as follow: PP01 + PP15, PP11 + PP18113 + PP18117,

Table 2. Detailed list of data origin for the study regarding polygyny variation in two populations of rock sparrow. The percentage of polygyny was calculated as the number of polygynous males divided by the total number of males in the population. Data origin corresponds to the published papers from where this information has been collected, where 'unpublished' refers to original data.

Year	Population	%Polygyny	Males total	Polygynous males	Data origin
1990–1998	Alpine – S. Sicario	37.3	67	25	Tavecchia et al. 2002
1990–1998	Alpine – C. Janvier	14.0	57	8	Tavecchia et al. 2002
1997–1999	Alpine	23.1	39	9	Pilastro et al. 2002
1999–2000	Alpine	25.0	28	7	Griggio et al. 2003a
2016	Spanish	13.3	15	2	unpublished
2017	Spanish	15.4	13	2	unpublished
2018	Spanish	26.7	15	4	unpublished

PP14 + PP18111 and PP38 + PP18114. PP18112 was amplified alone. The amplification process was carried out in a total volume of 10 μ l containing 1 \times of Wonder buffer (Euroclone containing dNTPs and MgCl_2), 1.4 μ M of each primer (with the forward labelled), 0.5 U of Wonder Taq (Euroclone) and 1.5 μ l of extracted DNA at an approximate concentration of 12.5 ng μ l⁻¹ (measured with Nanodrop 2000c). PCR consisted of 4 min at 94°C followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, followed by one cycle at 72°C for 5 min. Spanish samples were genotyped at BMR Genomics (<www.bmr-genomics.it>) with ROS 400 as size standard and alleles were scored in Geneious ver. 8.1.9 (<www.geneious.com>).

Identification of the genetic father in EPP broods

In order to identify the genetic fathers in EPP broods, we assigned paternity separately within each population using Cervus 3.0.7 (<www.fieldgenetics.com>) (Kalinowski et al. 2007) with a 95% strict confidence and allowing for up to one mismatch between chicks and parents. All loci were in Hardy–Weinberg equilibrium except for PP01 in the Spanish population. The probability of non-exclusion of the second parent was 1.4×10^{-4} in the Spanish population, and 3.9×10^{-4} in the Alpine one. We determined paternity for 100% (199/199 offspring from 51 broods) and 80.8% (236/292 offspring from 68 broods) of the chicks in our sample of the Spanish (2016–2018) and Alpine population (1997–2001), respectively. A male was assigned the category ‘social male’ (SM) when it was considered as the bonded pair of the nesting female, and ‘extra-pair male’ (EPM) when the male that sired a chick was not the social male. We considered as within-pair offspring (WPO) all chicks genetically related to the SM, and extra-pair offspring (EPO) all that were related to a EPM. The proportion of broods containing EPO and the proportion of EPO for each year and population are given in the Table 1. For the Alpine population, we used the published data to determine the frequency of EPP in the population. The above genetic analyses were conducted on a subset of DNA available samples to identify the EPM and to determine the genetic variability of the adult population and the WPO and EPO.

Genetic variability: heterozygosity and allele richness

To measure genetic variability, we used two measures of heterozygosity and calculated allele richness. In particular, to estimate heterozygosity we used the multi locus heterozygosity (MLH) (Hansson et al. 2004) and the mean-squared distance between alleles (mean d^2 , Coulson et al. 1998). MLH was calculated as in Hansson et al. (2004), assigning a value of ‘1’ to each heterozygous locus and ‘0’ to each homozygous locus, and then divided by the total number of loci. To obtain mean d^2 , we calculated the mean of the squared difference between sizes among alleles per locus (Neff 2004). As recommended by Coltman et al. (1998) we log-transformed the values of mean d^2 to improve the statistical analysis. Individuals lacking

information on three or more loci were excluded ($n=90$) from these heterozygosity measurements. We calculated the allele richness as the number of allele shared between each pair of adults within populations. The allele sharing was calculated as twice the number of alleles shared between the two individuals over the total number of alleles present in the two individuals (Wetton et al. 1987) using the software MSA (Dieringer and Schlotterer 2003).

Genetic similarity

To calculate genetic similarity among individuals we used the Microsoft Excel (Microsoft Corporation 2018) add-in GENALEX ver. 6.51 (Peakall and Smouse 2012). This software allowed the analysis of pairwise relatedness parameters, providing with different relatedness estimators, such as RI (Ritland 1996), LRM (Lynch and Ritland 1999) and QGM (Queller and Goodnight 1989), between each pair of individuals. Values of genetic similarity refer to values between SM/EPM and the female, and WPO/EPO and the mother.

Statistical analysis

Between populations, we tested for differences in the proportion of broods with EPP and polygyny level using χ^2 tests, and breeding synchrony and population density using t-tests. The analyses of genetic variability, genetic similarity and biometric differences both at between and within populations were conducted using paired t-tests among broods that contained simultaneously SM and EPM, or WPO and EPO. Thus, we used ‘brood’ as the statistical unit (values of sample size represent the number of broods considered for the analysis), and obtained values of heterozygosity and genetic similarity as mean values for the similar ‘roles’ within the brood (e.g. average WPO and EPO value in brood 1). In the Spanish population, we detected 9 broods with both WPO and EPO, whereas our available sample of broods with multiple paternity in the Alpine population was 24. Because of the low sample size in the number of broods available for the within population analyses, we conducted a post hoc power analysis to understand the achievable statistical power when comparing MLH, RI and the different biometric variables, using the R package pwr (Champely 2020). We set the significance level of the power test at 0.05. We tested the data for normality using the Shapiro–Wilk test (Shapiro 1985), and used parametric and non-parametric tests accordingly: t-test for normally distributed data and Wilcoxon rank-sum test for non-normally distributed. All the statistical analyses were conducted using the statistical software R (<www.r-project.org>).

Results

Extra-pair paternity

We observed significant differences in the total number of broods that contained EPO between the Spanish and the

Alpine population ($\chi^2=59.35$, $p < 0.001$). In the Spanish population, 18.1% (yearly averages ranging from 11.8 to 26.7%) of the broods contained at least one EPO, compared to a 51.2% (yearly averages ranging from 41.2 to 58.3%) in the Alpine one (Table 1). We also found differences in the number of total EPO in both populations ($\chi^2=48.56$, $p < 0.001$), where 12.4% of the chicks in Spanish population were product of EPP (yearly averages ranging from 4.5 to 25.9%), compared to 33.4% (yearly averages ranging from 21.9 to 43.1%) in the Alpine population (Table 1).

Genetic variability between populations

As an indicator of genetic variability between populations, we used the mean allele richness of the adults, and the mean MLH of the adults and the offspring. We found that mean allelic richness of the Alpine population ($n=93$) was significantly lower (9.1 ± 5.2) than the Spanish population ($n=55$; 10.3 ± 6.2) ($t_9=-2.42$, $p=0.038$). Similarly, we found differences in the mean MLH of the adults between both populations ($V=7532$, $p=0.03$), with lower values in the Alpine population ($n=192$; 0.71 ± 0.16) compared to the Spanish one ($n=115$; 0.75 ± 0.14). We observed a similar trend when comparing the mean MLH of WPO and EPO between populations. Mean within-pair offspring MLH of the Alpine population (0.70 ± 0.08) was significantly lower ($t_{31}=-2.85$, $p=0.008$) than for the WPO in the Spanish population (0.84 ± 0.08). Similarly, the mean value of MLH for the EPO from the Alpine population (0.64 ± 0.14) was

significantly lower ($t_{28}=-2.62$, $p=0.014$) than for the EPO in the Spanish population (0.77 ± 0.17).

Heterozygosity and genetic similarity within populations

Social and extra-pair males

The analysis of MLH for the siring males provided non-significant differences between SM (0.73 ± 0.18) and EPM (0.81 ± 0.14) in the Spanish population ($n=8$; $t_7=1.28$, $p=0.30$; power=0.29; Fig. 2). In a similar way, we did not find significant differences in MLH between SM (0.68 ± 0.12) and EPM (0.79 ± 0.17) in the Alpine population ($n=18$; $V=125.5$, $p=0.08$; power=0.73; Fig. 2). No significant differences were observed with the complementary measure of heterozygosity, the mean d^2 : males in the Spanish population ($n=8$) were not different among each other ($V=4$, $p=0.055$; power=0.53), same as males in the Alpine population ($n=18$; $V=109$, $p=0.32$; power=0.09).

Similarly, we did not find any differences regarding genetic similarity between SM and EPM with the female when using neither the RI parameter (Spanish population: $n=4$, $t_3=0.48$, $p=0.66$, power=0.09; Alpine population: $n=11$, $V=35$, $p=0.90$, power=0.10; Fig. 3), nor the LRM or the QGM (Spanish population: $n=4$; LRM: $t_3=0.14$, $p=0.90$, power=0.05; QGM: $t_3=0.11$, $p=0.92$, power=0.08; Alpine population: $n=11$; LRM: $V=38$, $p=0.70$, power=0.05; QGM: $t_{10}=0.38$, $p=0.71$, power=0.07).

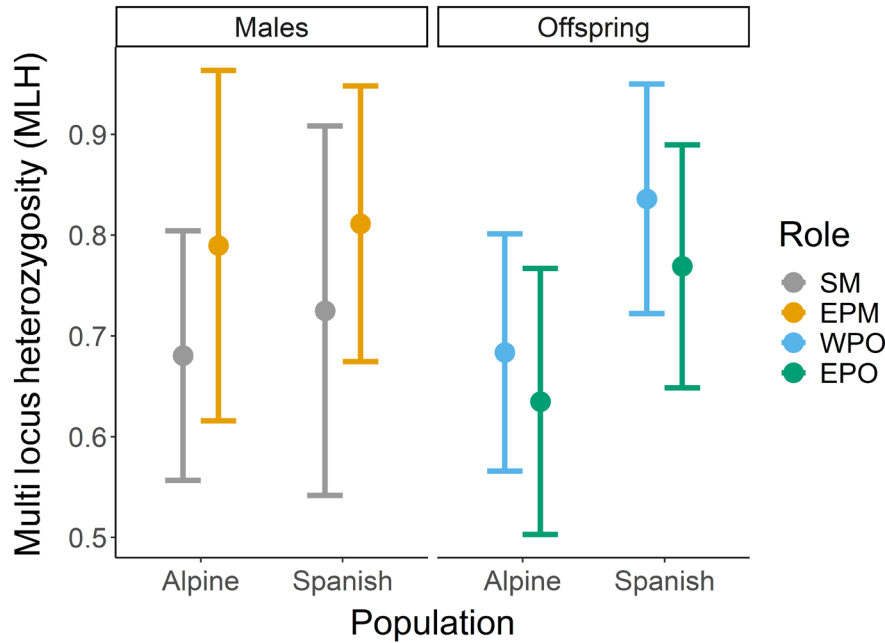


Figure 2. Mean values (and standard deviation bars) of multi-locus heterozygosity (MLH) per role for each of the broods: 1) SM (social male, in grey); 2) EPM (extra-pair male, in orange); 3) WPO (within-pair offspring, in light blue); 4) EPO (extra-pair offspring, in dark green). The values are given for the two different populations (Alpine and Spanish), where values closer to 1 indicate a high heterozygosity level in the different alleles marked, and 0 shows complete homozygosity. Sample sizes (i.e. number of broods compared) for each of the pair comparisons: Alpine males=18 broods; Spanish males=8 broods; Alpine offspring=21 broods; Spanish offspring=9 broods.

Within and extra-pair offspring

We did not find differences in the mean MLH between WPO (0.84 ± 0.11) and EPO (0.77 ± 0.12) neither in the Spanish population ($n=9$; $t_8=2.10$, $p=0.07$, power=0.31), nor in the Alpine population (WPO= 0.68 ± 0.12 ; EPO= 0.64 ± 0.13 ; $n=21$; $t_{20}=1.52$, $p=0.14$, power=0.37; Fig. 2). Similar results were obtained when testing for differences in the mean d^2 : in the Spanish population ($n=9$), WPO and EPO did not present significant differences ($V=11$, $p=0.20$, power=0.20), same as in the Alpine population ($n=21$; $V=85$, $p=0.30$, power=0.15).

Regarding the genetic similarity of WPO and EPO with the female, none of the parameters provided statistically significant differences (Spanish population: $n=6$; RI: $t_5=0.72$, $p=0.50$, power=0.08; LRM: $t_5=0.43$, $p=0.69$, power=0.07; QGM: $t_5=0.00$, $p=0.99$, power=0.05; Alpine population: $n=12$; RI: $t_{11}=-1.76$, $p=0.11$, power=0.53; LRM: $t_{11}=-1.72$, $p=0.11$, power=0.23; QGM: $t_{11}=-1.08$, $p=0.30$, power=0.13; Fig. 3).

Polygyny

The proportion of socially polygynous males observed in the different years was significantly lower in the Spanish population (18.5%; yearly averages ranging from 13.3 to 26.7%) than in the Alpine population (24.8%; range 14.0–37.3%) ($\chi^2=20.33$ $p=0.002$) (Table 2). However, and as opposed to the values found in the Alpine population by Pilastro et al. (2002), we did not find evidence of

higher EPP for polygynous males in the Spanish population: 12.5% of the polygynous males suffered from EPP (1/8), compared to a 22.9% in monogamous broods (8/35) ($t_{1,43}=-0.61$, $p=0.54$).

Biometric measurements

We did not observe any significant differences in the yellow badge size between SM and EPM neither in the Spanish population ($n=7$; $t_6=0.20$, $p=0.85$; power=0.07) nor in the Alpine population ($n=12$; $t_{11}=0.76$, $p=0.46$; power=0.06). In addition, we did not find any differences in the tarsus size or weight between SM and EPM neither in the Spanish population (tarsus: $n=7$; $V=18$, $p=0.58$, power=0.09; weight: $n=7$; $V=16.5$, $p=0.73$, power=0.09) nor in the Alpine population (tarsus: $n=9$; $t_8=1.06$, $p=0.32$, power=0.05; weight: $n=11$; $t_{10}=0.39$, $p=0.70$, power=0.05).

Population parameters

We found differences in both the mean breeding synchrony ($t_3=4.26$; $p=0.02$) between the Alpine (49 ± 7.91 days) and the Spanish population (18 ± 11 days), and the population density ($t_5=-4.23$; $p=0.01$), measured as the minimum distance between occupied nestboxes: Alpine population (54 ± 21 m; range: 16–205 m); Spanish population (119 ± 25 m; range: 11–368 m).

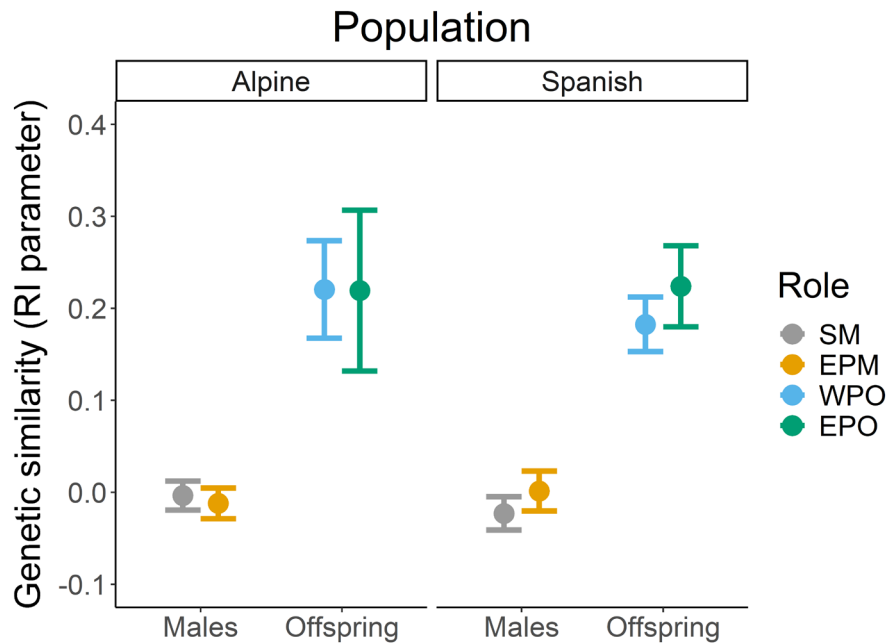


Figure 3. Mean values (and standard deviation bars) of genetic similarity according to the Ritland (1996) estimator per role: 1) SM (social male, in grey); 2) EPM (extra-pair male, in orange); 3) WPO (within-pair offspring, in light blue); 4) EPO (extra-pair offspring, in dark green) in the different populations. The values are represented as mean values per brood. Sample sizes (i.e. number of broods compared) for each of the pair comparisons: Alpine males=11 broods; Alpine offspring=12 broods; Spanish males=4 broods; Spanish offspring=6 broods.

Discussion

We found that the level of EPP for the Spanish population, in the centre of the rock sparrow distribution, was three times lower (18.1%) than the EPP level reported for the peripheral Alpine population (51.2%), as reported in Pilastro et al. (2002) and Nemeth et al. (2012). In addition, within each of the two populations, the levels of EPP were fairly constant across the years (%EPB, Table 1), suggesting that this difference was not contingent to annual variations that may exist in environmental conditions possibly influencing the level of EPP (e.g. habitat degradation due to climate change) but rather seems to be an intrinsic feature of the population.

At a between population level, we found that: 1) the allelic richness from the adults in the Alpine population was significantly lower than in Spain (Spanish population: 10.3; Alpine population: 9.1), 2) the heterozygosity of adults from the Alpine population was significantly lower than in Spain (Spanish population: 0.75; Alpine population: 0.71), 3) the heterozygosity of the offspring was lower in the Alpine population (WPO: 0.70; EPO: 0.64) than in the Spanish population (WPO: 0.84; EPO: 0.77). These results are in line with our expectations, as the Alpine population is a marginal one, and thus is more likely to suffer from inbreeding depression (Eckert et al. 2008, Cheptou and Donohue 2011). Thus, at a between population level, the higher level of polyandry in the Alpine population could be explained by the overall lower allelic richness and reduced heterozygosity of the population, as a mechanism to increase the genetic variability of the offspring (Foerster et al. 2003, Stapleton et al. 2007) and to reduce the risk of genetic incompatibility (Yasui and Yoshimura 2018).

When comparing at a within population level, we did not find differences in the mean heterozygosity between the different actors (SM/EPM and WPO/EPO; Fig. 2). This contrasts with our first prediction that females engage with extra-pair partners that are genetically less similar and/or more heterozygous than their social partner in order to produce more heterozygous offspring (Griffith 2010). Our results, from this point of view, are therefore similar to those obtained in previous studies where neither SM and EPM (Aparicio et al. 2001, Kleven and Lifjeld 2005), nor WPO and EPO (Smith et al. 2005, Ferretti et al. 2011) differed in heterozygosity levels. Similarly, we did not find differences in the partner's genetic similarity to the mother when comparing SM and EPM (Kempnaers et al. 1996), nor WPO and EPO (Fig. 3).

However, these results should be considered carefully, as our post hoc power analyses showed that these within population analyses had low statistical power, and thus should be our confidence to trust them. Given that the differences seemed to be larger (e.g. EPM and SM in Spain differed in 0.08 MLH units) when compared to the relative differences in the between population analysis (0.04 MLH units), lack of significant differences could have been because of a lack of statistical power due to a reduced sample size, rather than a lack of effect. Provided we could have accessed a bigger sample

size, differences between actors in the within population analysis may have been found, similar to previously reported studies (Varian-Ramos and Webster 2012). Nonetheless, these effects should not be big, as they would have been detected even with small sample sizes.

Thus, the pattern of EPP and genetic variability/similarity between and within populations is therefore consistent with the hypothesis that the females of the Alpine population engage more frequently in extra-pair copulations to maximize the genetic diversity of their offspring (Williams 1975, Westneat et al. 1990) and/or to reduce the risk of genetic incompatibility with their partner (Yasui and Yoshimura 2018), but may not be able to assess the extent of genetic similarity between themselves and males. Of course this is only one possible interpretation of our results, and should be taken with caution, as we compared only two populations that could differ for many other ecological and social characteristics that may determine the observed EPP and genetic variability pattern. For example, we found differences in population density between the populations that could explain differences in the frequency of EPP, as higher encounter rates between individuals may increment the frequency of EPP (Westneat et al. 1990). However, these differences alone (around 65 m between populations) may not explain EPP differences in our study species due to its high mobility, as we found cases of EPP between individuals in boxes separated up to 3.5 km. In addition, median and minimum distances among occupied nestboxes between populations were similar throughout the period (Supporting information) when considering this scale of mobility.

The main factor that has been shown to be associated with the occurrence of EPP in the rock sparrow (so far) is the polygynous state of the male in relation to the breeding synchrony: males mated with two females whose fertile period is overlapping have a reduced capability to guard them, and the higher is the degree of overlapping the higher is the EPP (Pilastro et al. 2002). Although the rate of polygyny and differences in the breeding synchrony in the Alpine population (polygyny rate: 24.8%; breeding synchrony: 49 days) were higher than in the Spanish population (p.r: 18.5%, b.s: 18 days), polygynous males in the Spanish population showed a lower EPP rate (12.5%) than that of monogamous males (22.9%), thus excluding this explanation as a possible source of EPP variation between these two populations. It is also interesting to note that in other territorial passerine species in which EPP has been compared among populations at a large geographical scale, a negative association between EPP and latitude has been reported (Brouwer and Griffith 2019, Valcu et al. 2021), in contrast with the pattern that we found in the rock sparrow, suggesting that the difference in latitude between the Spanish and the Alpine population may not explain the difference in EPP.

In addition, while previous studies have evidenced mating preferences for phenotypic quality in the rock sparrow (Griggio et al. 2007, 2011), we did not find any differences in the tarsus size, body weight or yellow badge size of SM and EPM. Thus, with our limited data available, we cannot

support the prediction that, within population, EPP pattern is driven by a low phenotypic quality of the social partner (Nemeth et al. 2012).

In conclusion, although our analysis was based on the comparison between only two populations, our results are in agreement with the prediction that in small populations with high risk of inbreeding, a higher level of EPP may be favoured. Other ecological factors, such as habitat fragmentation (Evans et al. 2009), that have been shown to be associated with the variation of EPP among populations of other species, may therefore account for our results.

For all the above reasons, there is an obvious limitation in the conclusions we can draw from comparing just two populations. However, we think that our results are important because they suggest that empirical evidence of the genetic diversity/compatibility hypotheses should not be looked solely at the within population level, by comparing the genetic diversity between the female and the extra-pair and social partners, but should also consider the variation in EPP among populations and their overall genetic diversity. In conclusion, even if alternative explanations of the observed EPP variation between populations cannot be discounted, our results suggest that EPP may evolve in response to a low genetic variability and a higher risk of inbreeding/incompatibility (Reid et al. 2015, Lichtenauer et al. 2019) more frequently than the results of within population analyses may suggest.

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Author contributions

Alejandro Corregidor-Castro: Data curation (equal); Formal analysis (equal); Writing – original draft (lead). **Matteo Griggio:** Conceptualization (equal); Funding acquisition (equal); Methodology (equal). **Alessandro Grapputo:** Formal analysis (equal). **Toni Mingozzi:** Methodology (equal). **Andrea Pilastro:** Data curation (equal); Formal analysis (equal); Funding acquisition (equal); Writing – original draft (supporting). **Mireia Plaza:** Methodology (equal). **Alejandro Cantarero:** Conceptualization (equal); Funding acquisition (equal); Methodology (equal); Writing – original draft (supporting).

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Data availability statement

The data that support the findings of this study are openly available in Digital CSIC at <<https://doi.org/10.20350/digitalCSIC/14499>>.

Supporting information

The supporting information associated with this article is available from the online version.

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