

Estrategias reproductivas alternativas en la carraca europea (*Coracias garrulus*): cuando la genética se convierte en herramienta indispensable



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UNIVERSIDAD DE CÓRDOBA



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Alternative reproductive strategies in the European roller (*Coracias garrulus*): when genetics become an indispensable tool

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Resumen

No hace mucho tiempo que la mayoría de paseriformes eran considerados monógamos. Sin embargo, el uso de herramientas moleculares ha revolucionado nuestra visión de la biología reproductiva de las aves hasta el punto de que la paternidad fuera de la pareja (EPP) y el parasitismo intraespecífico (CBP) son ahora considerados como estrategias reproductivas alternativas comunes. La carraca europea es una ave casi amenazada que anida de manera secundaria en oquedades y que se considera socialmente monógama. Sin embargo, hasta ahora, su biología reproductiva nunca ha sido explorada mediante el uso de herramientas moleculares. En el presente trabajo hemos usado 10 marcadores microsatélites divididos en 4 reacciones de PCR multiplex diferentes para analizar muestras correspondientes a 5 años de estudio. En total, 325 descendientes y 113 adultos fueron incluidos. La ocurrencia de EPP así como de CBP varía a lo largo de los 5 años estudiados, estando la media entre el 3.1 y el 4.9 % de los nidos para el caso de EPP y entre el 12.4 y el 14.9 % para el caso de CBP. Se discuten varias posibles explicaciones para la existencia/prevalencia de esas estrategias reproductivas alternativas. Concretamente, se discuten las posibles relaciones entre esas estrategias y variables tales como la fecha de puesta, la densidad de nidos y la asincronía de puesta. A pesar de nuestro limitado tamaño muestral, esas discusiones abren nuevas y excitantes cuestiones en el estudio de la biología reproductiva de la carraca.

Abstract

It was not long ago when the majority of passerine species were considered monogamous. However, the use of molecular tools has revolutionized our view of the avian breeding biology to the point that extra-pair paternity (EPP) and conspecific brood parasitism (CBP) are considered widespread alternative reproductive strategies. The European roller is a near-threatened secondary hole-nesting bird that is considered to be socially monogamous. However, so far, its breeding biology has never been explored by means of genetic tools. Here, we used 10 microsatellite markers divided into 4 different multiplex PCR reactions for that purpose using samples from a 5-years study. In all, 325 offspring and 113 adults were analyzed. The occurrence of EPP as well as CBP varies throughout the 5 years studied, being the average 3.1 – 4.9 % of the nests in the case of EPP and 12.4 – 14.9 % in the case of CBP. Several possible explanations for the existence/prevalence of those alternative reproductive strategies are discussed. Specifically, the relation between those alternative reproductive strategies and variables such as laying date, nest density and hatching asynchrony are addressed. Despite our limited sample size, those discussions open new and exciting avenues in the study of the breeding biology of the roller.

1. Introduction

It was not long ago when the majority of passerine species were considered monogamous (Lack 1968). However, with the onset of molecular tools for the study of paternity, our view of avian mating systems has been revolutionized to the point that the discovery of extra-pair paternity (EPP) via those tools has been proposed to be the most important empirical discovery in avian mating systems over the last 30 to 40 years (Bennett & Owens 2002).

In fact, nowadays, EPP has been found to be very common in birds (Westneat et al. 1990; Birkhead & Møller 1992; Griffith et al. 2002; Westneat & Stewart 2003), so that many species are socially but not genetically monogamous. In spite of the plethora of articles published in the last 30 years on this topic and that it seems clear that extra-pair copulation (EPC) is one of the most widespread reproductive behaviour by which males can increase their fitness (Trivers 1972; Birkhead & Møller 1992), there is no consensus on the adaptive nature of extra-pair mating for females (Griffith et al. 2002; Westneat & Stewart 2003; Arnqvist & Kirkpatrick 2005; Griffith 2007; Akçay & Roughgarden 2007; Eliassen & Kokko 2008; Slatyer et al. 2011).

Several hypotheses have been put forward for explaining the benefits that females may obtain by copulating with extra-pair males. Those benefits are usually divided into (1) direct benefits, which comprise either nongenetic resources (e.g. courtship feeding, nest defence or parental care by the extra-pair male) or a female's guarding strategy against infertility in its social mate; and, (2) indirect (genetic) benefits which may as well be separated into three different hypotheses: (a) the genetic diversity hypothesis which posits that females are trying to maximize genetic diversity among their offspring; (b) the genetic compatibility hypothesis which says that females seek EPP to maximize genetic compatibility between themselves and the father of the offspring; and, (c) the good genes hypothesis which posits that is precisely good genes what females are looking for (see more in Griffith et al. 2002). Most attention has been paid to the last two hypotheses and some studies have supported the compatibility hypothesis (e.g. Johnsen et al. 2000; Veen et al. 2001; Garvin et al. 2006), some others the good genes hypothesis (e.g. Kempenaers et al. 1992;

Hasselquist et al. 1995; Saino et al. 1997; Sheldon et al. 1997) and even both strategies have been found to be used by females in the same population (Foerster et al. 2003). However, many other studies did not find support for none of them (e.g. Edler & Friedl 2008; Schmoll et al. 2009; Sardell et al. 2011, 2012; Moreno et al. 2013). That inconsistency of results across EPP studies has been argued to be caused by either context-dependent genetic effects (Schmoll 2011) or even maternal effects such as laying order (Magrath et al. 2009).

On the other hand, another widespread avian reproductive strategy that has received much less attention than EPP is conspecific brood parasitism (CBP; Yom-Tov 2001; Arnold & Owens 2002). This phenomenon appears when a female lays one or more eggs in nest(s) belonging to other conspecifics, being, therefore, her offspring raised by the foster parents. This way the parasitic female avoids most of the parental costs (Yom-Tov 1980). CBP offers a novel opportunity to explore conflicts of interest within species (Lyon & Eadie 2008) and it can be divided into two different forms: (1) both foster parents are unrelated to the offspring; and, (2) only the tending male is related to the offspring, but not the female, also called extra-pair maternity (EPM) or quasi-parasitism (QP, Grønstøl et al. 2006). Likewise, from the point of view of the parasitic female, another important division that can be done is whether or not the parasite owns a nest (Lyon & Eadie 2008), that is whether parasitic females lay eggs in their own nest as well as in other conspecific nests, or they only lay eggs in conspecific nests.

Traditionally, several hypotheses have been used to explain CBP: (1) the best-of-a-bad-job hypothesis posits that females lay parasitically when environmental or phenotypic factors act as a constraint by limiting breeding or when environment conditions are unfavourable such that nesting implies low prospects of successful reproduction; (2) the nest lost hypothesis proposes that females become parasites when their nest get destroyed; (3) the lifelong specialist parasites hypothesis put forward the idea that some individuals show this alternative strategy during their entire life, being therefore a frequency-dependent strategy; and, finally, (4) the fecundity enhancement hypothesis posits that this strategy acts as a way of increasing fitness beyond that possible through nestling alone (see more in Lyon & Eadie 2008). Nevertheless, Lyon and Eadie (2008) have convincingly argued that,

instead of that four-hypotheses division, CBP needs rather to be considered in the context of a flexible life-history strategy, which implies both considering the developmental basis of parasitism (Sorenson 1991) and studying the benefits of CBP in different ecological and social contexts.

Although traditionally CBP has been identified by non-genetic clutch characteristics (e.g. Jackson 1992; McRae 1997, Pöysä et al. 2001), to study why CBP appears and what is the relative success of each reproductive strategy within the population, telling apart the different forms of CBP is crucial and, for doing so, molecular evidences are required (Grønstøl et al. 2006). In fact, as for the case of extra-pair paternity, the use of genetic tools has also revolutionized the study of CBP, raising the number of species described showing this behaviour from 53 in the first review about the matter (Yom-Tov 1980) to more than 200 species described in the last one (Yom-Tov 2001). CBP is particularly common in Anseriformes (74 sp.), Passeriformes (66), Galliformes (32) and Charadriiformes (20), but it also appears in other orders such as Columbiformes (9), Coraciiformes (1) and Falconiformes (1) (reviewed by Yom-Tov 2001). In fact, CBP has been found to be more common in species with precocial young (Rohwer & Freeman 1989; Yom-Tov 2001), that breed in either cavities or colonies (Eadie et al. 1988; Eadie 1991; Beauchamp 1997; Yom-Tov 2001; but see Geffen & Yom-Tov 2001) and that show large clutch sizes and fast life histories, i.e. high fecundity, high reproductive effort, early age of sexual maturity (Eadie 1991; Beauchamp 1997; Geffen & Yom-Tov 2001; Arnold & Owens 2002).

Regarding the order Coraciiformes, the only family where CBP has been described is the family Meropidae, specifically in the white-fronted bee-eater (*Merops bullockoides*, Emlen & Wrege 1986; Wrege & Emlen 1987). Within that order, the only member of the Coraciidae family that breeds in Europe is the European roller (*Coracias garrulus*; Snow et al. 1998), a near-threatened bird species that has apparently undergone rapid declines across its range (*sensu* BirdLife International 2012) and in which alternative reproductive strategies such as CBP or EPP have not been described yet. Since CBP (and also EPP) may have significant consequences at the population level (Lyon & Eadie 2008), the study of those phenomena becomes an important task in avian conservation biology.

The goal of the present study is to find out by means of genetic tools whether or not European rollers present alternative reproductive strategies to the social monogamy described so far for the species. Finally, we will discuss several possible causes that might explain the existence/prevalence of those alternative reproductive strategies in our population such as laying dates of nests, nest density, and, in the case of CBP, brood hatching asynchrony.

2. Materials and Methods

2.1. Study system

The European roller (*Coracias garrulus*; roller hereafter) is a near-threatened (*sensu* BirdLife International 2012) secondary hole-nesting bird, i.e. that breeds in natural cavities or holes excavated by other species. It is considered as a socially monogamous species that shows a very small sexual dimorphism, being males slightly bigger and showing slightly brighter plumage coloration than females (Avilés 2006). Furthermore, both sexes incubate the eggs, brood and feed the young (Cramp & Simmons 1988), and aggressively defend their territory towards intruders (Avilés 2006). Nevertheless, it has been also found breeding in small or loose colonies in some particular cases (Noval 1975; Glutz & Bauer 1980; Cramp & Simmons 1988; Sosnowski & Chmielewski 1996), and even three adult individuals have been seen feeding the nestlings of a single nest (Avilés & Sánchez 1995). Finally, damaged eggs at the nest and rare laying sequences (i.e. with a between-egg time greater than the common 48 hours found in this species [Noval 1975; Sosnowski & Chmielewski 1996]) are occasionally found in this species (D. Parejo & J.M. Avilés, unpublished data).

The field study was carried out in May-July 2007-2011 at the surroundings of Guadix (37°18' N, 3°11' W), southeast Spain, where rollers breed in nest-boxes that are mainly attached to trees (Rodríguez et al. 2011). The vegetation is sparse in the area, including cultivated cereals, some remains of holm oaks forests, pines, groves of almond trees and olive trees, and other tree crops in irrigated areas surrounding villages (more details in Avilés et al. 2008).

2.2. Data collection

Nest-box positions were GPS-logged in order to allow us to estimate distances between different nests each year. Those nest-boxes were monitored every 10 days from early May to fledging to determine laying dates, clutch sizes, and fledging success. When the older chick in each brood was 19 days old, chicks were weighted with a Pesola spring balance (± 1 g) and their wing lengths and tarsus lengths measured with a rule (± 1 mm) and a caliper (± 1 mm), respectively. Breeding males and females were captured at the nests by means of nest-traps either at clutch completion or at the beginning of the nestling period, and body size measurements (wing length, tarsus length and body mass) were also taken in the same way as for the nestlings. Finally, small (≈ 25 μ l) blood samples were collected via the brachial vein from all adults and nestlings and stored in 1 ml of 95% ethanol at room temperature for the molecular analyses. All individuals were ringed for further identification. This work was carried out by D. Parejo and J.M. Avilés as part of a long-term study of the species.

2.3. Molecular analyses

DNA was extracted from red blood cells by boiling them in 100 μ l of 50 mM NaOH for 20 min in a thermocycler. After that, each individual was molecularly sexed using the primers 2550F and 2718R described by Fridolfsson and Ellegren (1999). Polymerase chain reaction (PCR) was performed in 20 μ l volumes on an Applied Biosystems GeneAmp PCR System 9700. Final concentrations were: 5 mM MgCl₂, 0.2 mM dNTPs (each; Bioron), 0.25 mM (each) primers, 0.098 mg/ml BSA (Amersham Biosciences), 0.5 U Taq DNA polymerase (Bioron) and 1 μ l raw extract. The sexing thermal profile used was: 94°C for 2 min, 55°C for 30 s, 72°C for 1 min, followed by 36 cycles (92°C for 30 s, 52°C for 30s, 72°C for 30 s), and a final 72°C for 5 min step. PCR products were separated in 3% agarose gel run in standard TBE buffer and visualized by SyBRSafe (Invitrogen) staining.

On the other hand, genotyping was carried out using 10 microsatellite loci that were previously suggested as appropriate for paternity analysis in this species (D. Martín-Galvéz et al., unpublished data). Using the software Multiplex Manager 1.2. (Holleley

& Geerts 2009), we designed four new multiplex PCR reactions in which we amplified loci SAP47-ZEST, Pte24-CEST and TGG15 in the first reaction (set I); HvoB1-TTG, TGG17 and Be48 in the second reaction (set II); TG13-017 and Bb111-TG in the third reaction (set III); and TG03-098 and TGG18 in the last reaction (set IV). Further information about the microsatellite markers used can be found in Table 1. Each 2 μ l PCR contained approximately 50 ng of genomic DNA, 1 μ l of QIAGEN® Multiplex PCR Kit, 0.017 μ l of each 5 μ M primer and, finally, milli-Q water was added up to the 2 μ l total reaction volume was reached. The same PCR profile was used for all the reactions and it consisted of 94°C for 3 min, then 10 cycles of 94°C for 30 s, 65°C 30 s (and decreasing 1°C in each new cycle), 72°C for 1 min; afterwards 28 cycles of 94°C for 30 s, 50°C for 30 s and 72 min for 30 s; followed by one cycle of 5 min at 72°C. PCRs were performed on an Px2 Thermal Cycler (Thermo Electron Corporation) and PCR products were sent to the genetic unit of the SCAI in Cordoba (Spain) for genotyping.

2.4 Paternity analyses

The scoring of genotypes was carried out with the software GeneMapper v. 3.7 (Applied Biosystems). The software Cervus v. 3.0.3 was used for calculating both observed and expected heterozygosities, and predicted null alleles frequencies (Table 1; Kalinowski et al. 2007). Furthermore, the EXCELL macro IRmacroN3 (see Amos et al. 2001) was used for taking into account a common source of scoring error, specifically scoring heterozygotes as homozygotes and vice versa when the alleles are adjacent, being the results nonsignificant for all of our markers (p-value > 0.270 in all cases). For all those analyses, only adults were included in order to use only unrelated individuals in the calculations (N=113 adults from the 5 years together).

The combined non-exclusion probabilities as calculated by Cervus (Marshall et al. 1998) were 0.256 for the first parent and 0.008 for the parent pair. Those are the probabilities of not excluding a single unrelated candidate parent or parent pair from parentage of a given offspring, respectively. Genetic parentage was assessed by comparing the genotypes of the nestlings with those of their putative parents and also by running paternity analyses in Cervus. Since we had already found some ca-

Table 1. Microsatellite markers information

Locus	Primer sequence (5'-3')	N	Observed allele size range (bp)	Number of alleles	H _o	H _E	Estimated null allele frequency
Bb111-TG	F: CTTTGTGTCAGTTTTCCCTGTAGC R: ATCTAAGCATTAAAAATGCARAYCTT	103	182 - 190	4	0.146	0.139	-0.029
Be48	F: CATCAACCCACAGCTTCCTC R: GCGTTACTTCCCCTTTAAGC	112	151 - 161	4	0.286	0.254	-0.0701
HvoB1-TTG	F: AAGCAAGGACTTTCCTTCCAG R: TCTCAAATTGGAACAGAGAAAGG	113	91 - 113	5	0.142	0.152	+0.020
Pte24-CEST	F: AACAAAGGACGCCGAGTAG R: TCATTTAATGGCTYTACTTCATACAT	105	215 - 235	9	0.743	0.766	+0.0147
SAP47-ZEST	F: GGAAGTTTTTGGTACTGCT R: GGGAGAATGACCTCATCA	107	159 - 167	5	0.673	0.693	+0.0104
TG03-098	F: TTTGCCTTAATTCTTACCTCATTTG R: TTGCAACCTCTGTGGAAGC	45	246 - 252	7	0.622	0.737	+0.0886
TG13-017	F: GCTTTGCATCTTGCCTTAAA R: GGTAAC TACAACATTCCA ACTCCT	99	214 - 220	3	0.414	0.357	-0.0802
TGG15	F: SGACGACTCCTTTATTTCCC R: TTCTGACTTCCYCAGGTAACAC	106	268 - 280	6	0.330	0.337	+0.0040
TGG17	F: CGGGTTGTAATCAAGAAGATGC R: CTGCGGAGCAATTAACGC	112	183 - 185	2	0.063	0.077	+0.1037
TGG18	F: TTAAGAAGTTTACACCCAGCG R: GCTAAATAACAGAGCCAGGAAG	98	328 - 330	2	0.051	0.050	-0.0052

N: Number of adults individuals genotyped

H_o: Observed heterozygosity

H_E: Expected heterozygosity

bp: base pair

ses of CBP by comparing the genotypes of nestlings and putative parents, we did not specify neither the putative mother nor the putative father of each nestling for those paternity analyses. A level of confidence of 95 %, a proportion of candidate parents sampled of 75 % (D. Parejo & J.M. Avilés, unpublished data), a proportion of loci mistyped of 1 % and a minimum number of six loci typed for calculations, were established for the analyses.

Each year was analyzed separately, therefore only adults captured in a specific year were available for the paternity analyses of that particular year. The criterion used for assigning a nestling as either a case of EPP or a case of CBP consisted of finding mismatches between the parents and the nestling genotypes in at least one typed locus. However, those cases where only one mismatch was found were assumed to be due to mutation if the difference between the parental and the offspring alleles was of only a mutation step (a total of 2 nestlings from 2 different nests), that is so because the markers used are dinucleotide repeats and the mutation of microsatellites mainly follows a stepwise model (Primmer & Ellegren 1998), so such a discrepancy may be easily the result of a single mutation.

2.5. Statistical analyses

In order to combine the 5 years laying date values altogether, we calculated a standardized laying date consisting of the laying date of each nest minus the average laying date of that particular year. Furthermore, an estimate of the nest density of each year was calculated as the average value of the number of occupied nests within a 500 metres radius from each nest. Since some adults avoided nest-traps, the number of nests where both tending parents were captured differs from the total number of nests monitored each year. Therefore, when calculating EPP-related parameters (frequency, nest density, standardized laying date), the nests included were those where both tending parents were captured and those where only the male was captured. In the same way, when calculating CBP-related parameters (same as before), the nests included were those where both tending parents were captured and those where only the female was captured.

Hatching asynchrony was quantified following the method proposed by Kontiainen et al. (2010) that allows comparison across different clutch sizes and also takes into account egg hatchability, which are two variables likely affecting measures of hatching asynchrony. In brief, hatching asynchrony (HA) was obtained from the residuals from a linear regression of brood size at hatching on hatching span, treating brood size as a continuous variable. Therefore, it takes more positive values when the eggs hatch more asynchronously once brood size at hatching is taken into account. If females parasites are very synchronized with their host, HA is expected to be low, and vice versa.

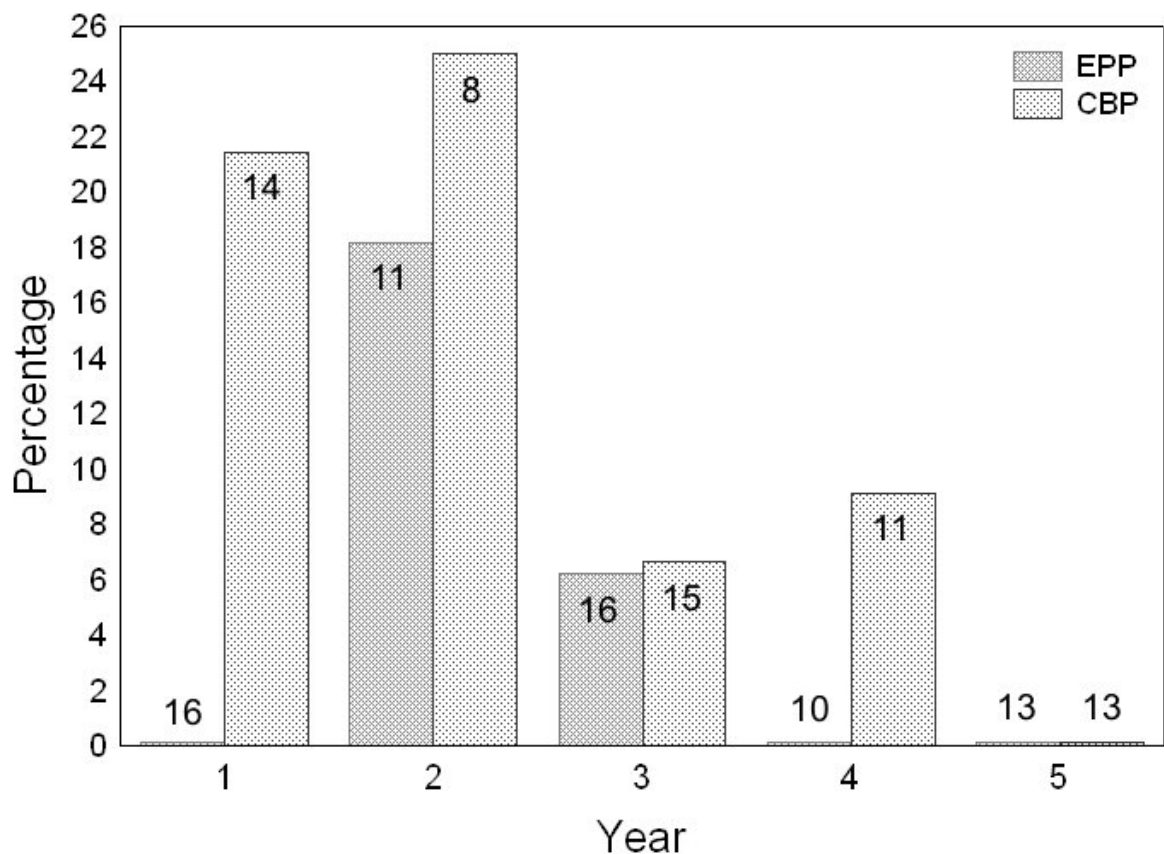


Figure 1. Percentage of nests showing EPP and CBP throughout the 5-years study in the European roller. Years 2007 to 2011 correspond to the numbers 1 to 5 presented in the X-axis. Numbers on bars represent the amount of nests considered in each case for the calculation of those percentages.

The Spearman correlation was used for analyzing the percentage of either EPP or CBP in relation to nest density. Finally, the Mann-Whitney U test was used for comparing nests with and without CBP in terms of HA. The software Statistica v. 7.1 (Statsoft) was used for statistical analyses and plotting of the results.

3. Results

In all, 325 nestlings were included in the study, 84 from 2007, 71 from 2008, 69 from 2009, 30 from 2010 and 72 from 2011. Regarding adults, a total of 113 individuals were included, where 57 were females and 56 were males. The number of adults included each year consisted of 33 in 2007 (14 females and 19 males), 21 in 2008 (8 females and 13 males), 38 in 2009 (19 females and 19 males), 43 in 2010 (23 females and 20 males) and 34 in 2011 (18 females and 16 males). Since some adults bred in several years, the overall sum of adults is lower than expected by adding up each year separately.

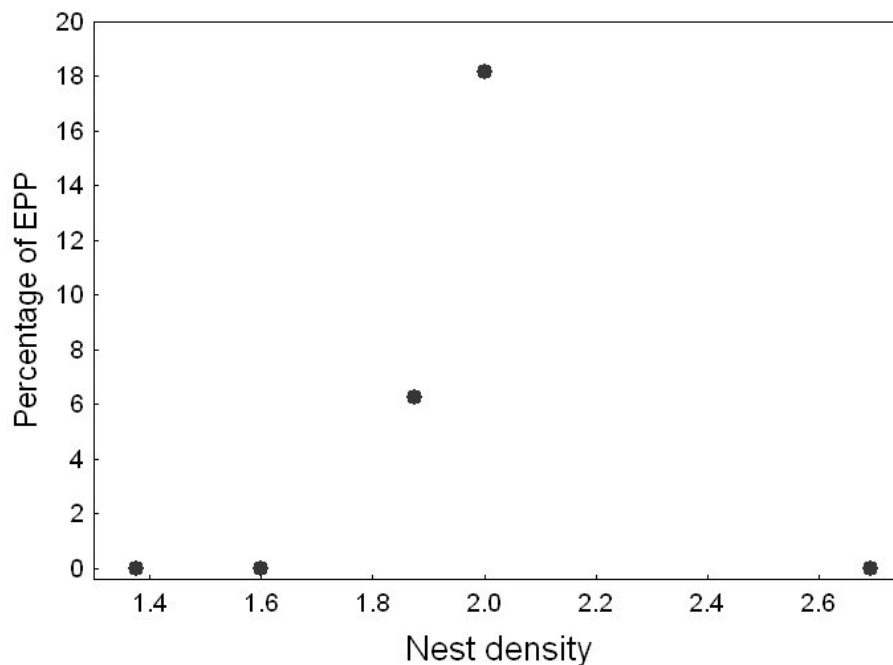


Figure 2. Correlation between nest density and percentage of EPP. Each point represents the average nest density value for each of the 5 years studied.

3.1. Percentages of EPP and CBP

On the one hand, the occurrence of EPP varies throughout the 5 years studied, ranging from 0 to 18.2 % of nests. Specifically, the percentage of EPP were 0.0 % in 2007 (0 out of 16 nests), 18.2 % in 2008 (2 out of 11 nests), 6.25 % in 2009 (1 out of 16 nests), 0.0 % in 2010 (0 out of 10 nests) and 0.0 % in 2011 (0 out of 13 nests;

Figure 1). On the other hand, the percentage of CBP also changed over time from 0 to 25.0 % of the nests, being 21.4 % in 2007 (3 out of 14 nests), 25.0 % in 2008 (2 out of 8 nests), 6.7 % in 2009 (1 out of 15 nests), 9.1 % in 2010 (1 out of 11 nests) and 0.0 % in 2011 (0 out of 13 nests; Figure 1). Overall, the percentage of EPP combining the 5 years was 4.9 % (3.1 % when a nest where only the tending male was captured was not accounted for), whereas the percentage of CBP was higher, specifically 12.4 % (14.9 % when that nest was included).

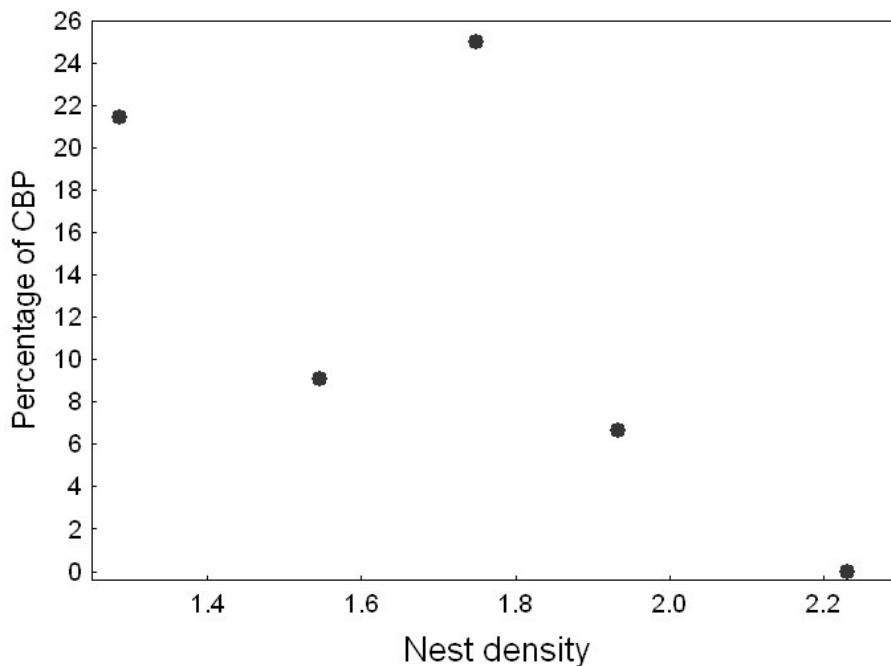


Figure 3. Correlation between nest density and percentage of CBP. Each point represents the average nest density value for each of the 5 years studied.

3.2. Paternity assignments

The total number of assignments reached by Cervus was 61 (18.7 % of the offspring, all years combined), 44 corresponded to mothers and 17 to fathers. Furthermore, 9 pairs were also assigned. Unfortunately, none of those assignments coincide with cases of EPP nor CBP. On the other hand, when analysing the comparisons between the genotypes of the nestlings and their putative parents in those nests where CBP was detected, we found that some of those cases could be interpreted as cases of QP, i.e. the tending mother did not match with the offspring but the tending male did

so. In all, 4 possible cases of QP were found throughout the five years studied, 1 nest in 2007 (7.7 %), 1 in 2008 (25 %), 1 in 2009 (9.1 %) and 1 in 2010 (10.0 %). Only nests with both tending parents captured were used for calculating those frequencies.

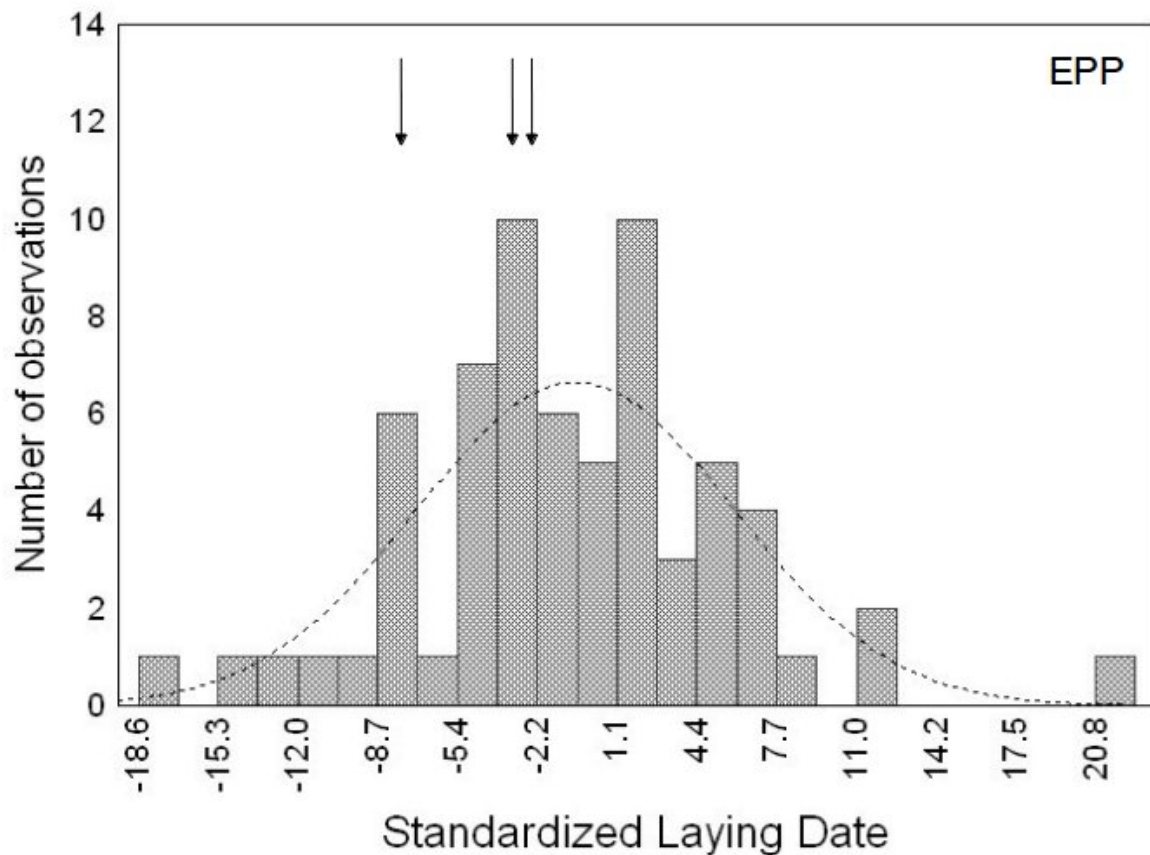


Figure 4. Histogram of the standardized laying date throughout the 5 years studied. Rows represent nests showing EPP and point out their standardized laying date value.

3.3. Alternative reproductive strategies and nest density

Neither the percentage of EPP ($N = 5$ years; $R_{\text{Spearman}} = 0.34$; $t_3 = 0.62$; $p\text{-value} = 0.581$; Figure 2) nor the percentage of CBP ($N = 5$ years; $R_{\text{Spearman}} = -0.70$; $t_3 = -1.70$; $p\text{-value} = 0.188$; Figure 3) were significantly correlated with the average density of occupied nests within the 500 m radius.

3.4. Alternative reproductive strategies and laying date

The distribution of both the EPP and the CBP cases throughout the 5 years in relation to the standardized laying date is represented in figures 4 and 5.

3.5. CBP and HA

There were not significant differences between nests with CBP ($N = 6$; $\text{mean} \pm \text{SD} = -0.75 \pm 1.36$) and nests without it in terms of HA ($N = 38$; $\text{mean} \pm \text{SD} = 0.18 \pm 1.28$; $Z_{\text{adjusted}} = 1.61$; $p\text{-value} = 0.108$; Figure 6). Year 2011 was discarded from this analysis due to the absence of CBP during that year.

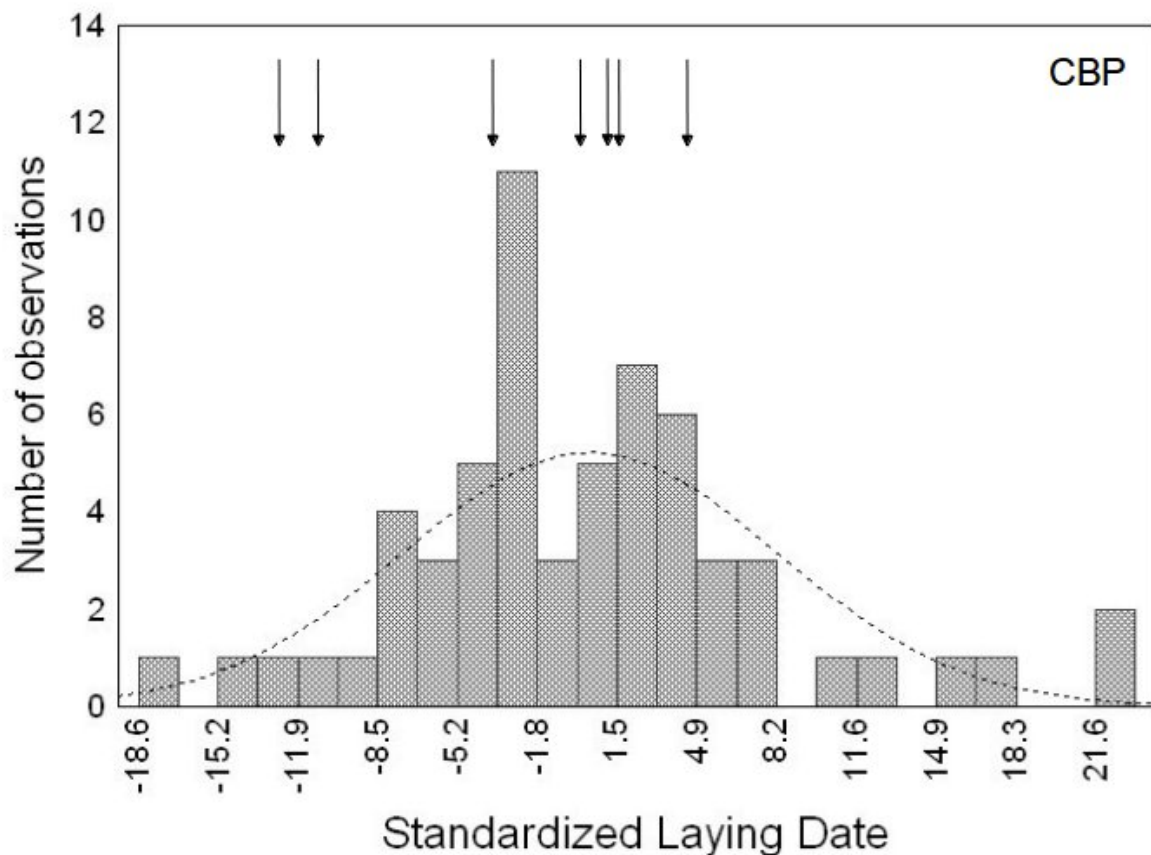


Figure 5. Histogram of the standardized laying date throughout the 5 years studied. Rows represent nests showing CBP and point out their standardized laying date value.

4. Discussion

4.1. Microsatellites performance

The 10 microsatellites used in the present study were chosen from an unpublished work carried out by D. Martín-Gálvez et al., where only 28 polymorphic microsatellites were found after testing 147 markers that were previously published for birds. Among those 28 polymorphic microsatellites, only 15 were suggested as appropriate for parentage analyses due to their low null allele frequencies, being 6 of them very difficult to work with because of their 1 base pair allele size increments (D. Martín-Gálvez et al., unpublished data). In this study, after running 113 adult individuals, both the average number of alleles (4.7 alleles / microsatellite) and the a-

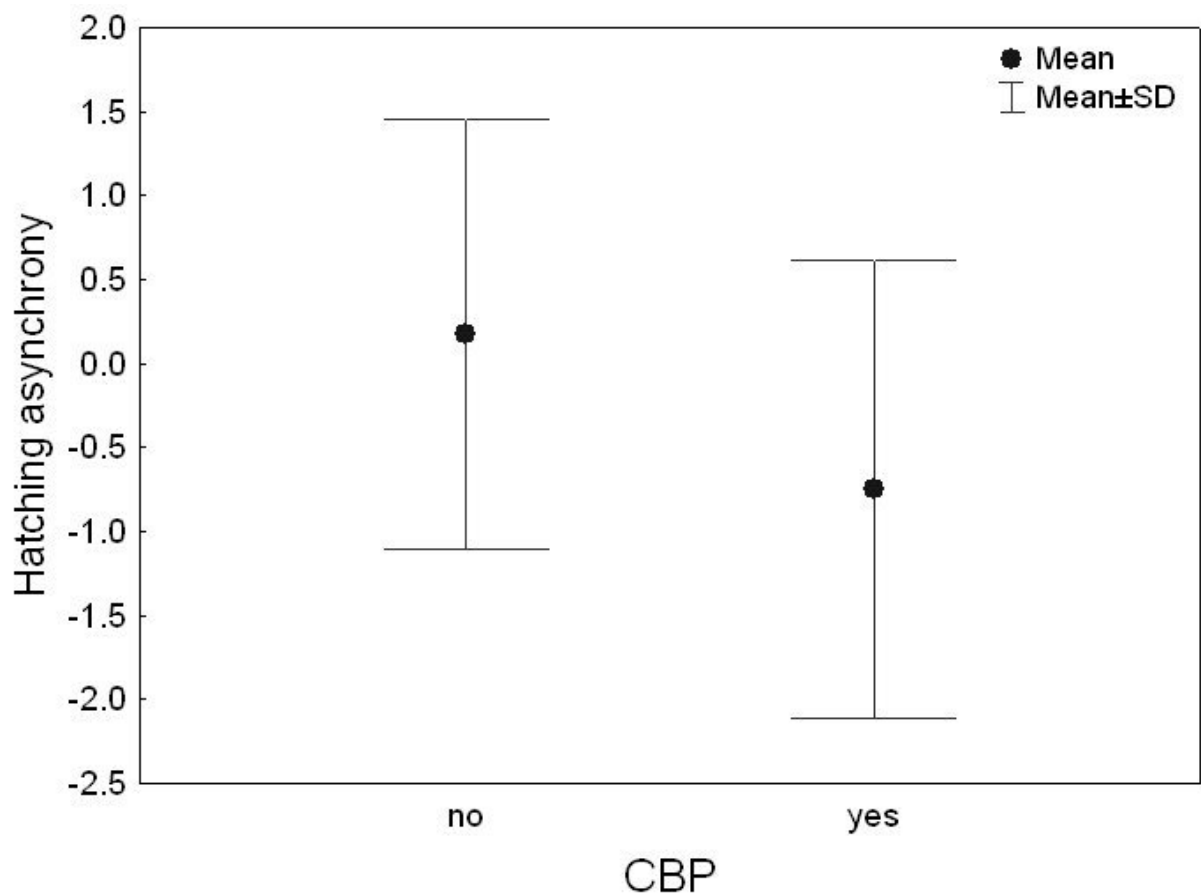


Figure 6. Differences between nests with (N = 6) and without CBP (N = 38) in terms of HA.

verage observed heterozygosity (0.347 / microsatellite) for those 10 microsatellites were quite low. Furthermore, after all, 2 of them presented null allele frequencies greater than 0.05 (see Table 1). Altogether, this has led us to a low resolution power during the parentage analyses carried out by Cervus. This fact has prevented us for getting more insight about the exciting breeding biology of the roller. However, that also could be suggesting some interesting points to dig into in future investigations. The low polymorphism of the markers together with the relatively low observed heterozygosity could be due to a rather small population size and/or some degree of population isolation which, in a near-threatened species like this one, is something to find out as soon as possible.

4.2. *Extra-pair paternity*

The overall percentage of EPP in the roller (3.1 - 4.9 %, but see results) is similar to that of other members of the order Coraciiformes, e.g. the white-fronted bee-eater (<9 -12 %; Wrege & Emlen 1987) and the European bee-eater (*Merops apiaster*; 0.7 %; Jones et al. 1991). In general, it can be considered as a low EPP percentage compared to that usually found in socially monogamous bird species (18.7 %, Griffith et al. 2002). In fact, levels of EPP below 5 % (as ours) are considered something worthy to dig into (Petrie & Kempenaers 1998; Griffith et al. 1999; Griffith 2000; Robertson et al. 2001) which, together with the fact that EPP was found only in 2 of the 5 years studied here, adds more interests to the study of this phenomenon in rollers.

Since the number of nests showing EPP is very limited in our study and the paternity assignments achieved by Cervus are also scarce, we cannot discuss hypotheses such as the direct benefits, the genetic compatibility or the good genes hypothesis (see introduction). However, some comments about possible ecological factors influencing EPP can be put forward. First of all, the average density of occupied nests each year did not correlate with the percentage of EPP. Breeding density has been proposed as an ecological factor that could possibly affect the appearance and prevalence of EPP (e.g. Birkhead 1979; Stutchbury & Morton 1995). Some authors have found a correlation between nest density and percentage of EPP (e.g. Augustin et al. 2007; Canal et al. 2012) and others have experimentally found that nest density

and EPP percentage are indeed positively related (e.g. Stewart et al. 2010). Conversely, some correlational studies have found no relation between those two variables (e.g. Tarof et al. 1998; Chuang et al. 1999), several reviews have diminished the role of breeding density in EPP (Westneat & Sherman 1997; Bennett & Owens 2002; Griffith et al. 2002) and others have found that, indeed, cuckolders are not necessarily the closest neighbours (e.g. Westneat & Mays 2005; Albrecht et al. 2007; Canal et al. 2012; Moreno et al. 2013). Our results seem to be in accordance with the latter studies but a greater sample size would be necessary to confirm that trend.

Secondly, another ecological factor that has also been proposed to play a role in EPP is breeding synchrony (Stutchbury & Moront 1995; Stutchbury 1998a, b). As for the case of breeding density, though with less conviction, the importance of synchrony has also been diminished (Westneat & Sherman 1997; Bennett & Owens 2002; Griffith et al. 2002). Our only 3 cases of EPP appear in the first half of the season and they coincide with peaks of high breeding synchrony within the population, i.e. with a high availability of fertile females (Figure 4). A recent study in pied flycatchers (*Ficedula hypoleuca*) have found that not only the breeding synchrony at the population level but the social context of a particular male (i.e. whether his social female has already started to lay or not) affects the probability of engaging in EPP, being therefore those males that arrived earlier to the breeding areas in advantage to sire extra-pair young (Canal et al. 2012). Unfortunately, we could not identify the extra-pair fathers in those 3 cases but, because rollers are migratory birds that arrive to the Iberian Peninsula between late March and early April (Noval 1975), the study of those aspects would be of great interest in determining, for example, whether those males that arrive earlier (supposedly males of better quality) achieve a greater fitness by means of more extra-pair young sired elsewhere.

4.3. Conspecific brood parasitism

The overall percentage of CBP in the roller (12.4 - 14.9 %, but see results) is very similar to that of the only member of the order where CBP has been described, the white-fronted bee-eater (16% over a 4-years study; Emlen & Wrege 1986). CBP is a widespread phenomenon that appears in several bird species and in different

frequencies. For instance, cases of CBP showed up in 9.6 % of the nests in the greater sage-grouse (*Centrocercus urophasianus*; Bird et al. 2013), ranged between 17.5 and 21 % in both wild and domesticated zebra finch (*Taeniopygia guttata*) populations (Griffith et al. 2010; Schielzeth & Bolund 2010) and went up to 36 % in the barnacle goose (*Branta leucopsis*; Anderholm et al. 2009). In general, the percentage of nests showing CBP ranges from 0 % to 50 % (Arnold & Owens 2002). Although widespread, CBP is a less well described phenomenon than EPP, in a smaller amount when taking into account only studies carried out with genetic tools (Griffith et al. 2004). Therefore, the discovery of such percentages of CBP in rollers opens a lot of new opportunities to work on.

CBP has been found to be more common in species breeding in cavities or in colonies (Eadie et al. 1988; Eadie 1991; Beauchamp 1997; Yom-Tov 2001; but see Geffen & Yom-Tov 2001) but experimental manipulations of nest site availability (usually by increasing/decreasing nest-box availability) have reached controversial results. While some authors have found that an increase in nest site availability lead to an increase in CBP (e.g. Semel et al. 1988), others have found the opposite trend (e.g. Gowaty & Bridges 1991; Jacot et al. 2009). On the one hand, since rollers are secondary hole-nesting birds and they have been found breeding in small or loose colonies in some particular cases (Noval 1975; Glutz & Bauer 1980; Cramp & Simmons 1988; Sosnowski & Chmielewski 1996), they could, a priori, be considered as a good target to look for CBP. On the other hand, though with a small sample size (only 7 cases of CBP), our results do not show any significant correlation between nest density in one particular year and the percentage of nests showing CBP. However, that correlation shows a nonsignificant negative tendency (see results) that would not agree with Semel et al. (1988). Anyway, a much greater sample size is needed to draw powerful conclusions about the effect of nest density in CBP in rollers. Since nest-boxes are currently used in different populations of rollers and some authors have suggested that large numbers of visible nest-boxes may lead to extremely high levels of CBP in other species (Semel & Sherman 1986, 2001), the understanding of how nest density affects CBP percentages in rollers could help us in the conservation and management of this near threatened species.

In relation to how those cases of CBP are distributed throughout the breeding season. Our results show that cases of CBP are preferentially distributed at the beginning and mid-part of the breeding season, being the nests with CBP quite spread during that range (Figure 5). CBP is currently treated as a flexible life-history trait (Lyon & Eadie 2008), however, the four-hypotheses division previously used for explaining this phenomenon can still be partially used as a continuum of possible states of a single female. The best-of-a bad-job hypothesis posits that several constraints or restrictions can push some females to become parasites (see introduction). Therefore, CBP would be expected to be in a greater extent when environmental conditions are worse, i.e. at the end of the breeding season. Since our cases of CBP preferentially occur within the first half and mid-part of the breeding season, i.e. when environmental conditions are supposed to be more suitable, our results do not seem to support such a hypothesis. Another hypothesis, the nest lost hypothesis, proposes that females become parasites when their nest get destroyed. Two evidences could support this hypothesis in rollers. First, rollers never present replacement nests (J. Rodríguez, personal observations) so, when nest lost occurs, CBP could be the only alternative to get any fitness in that particular year. Second, although nest lost could, a priori, occur throughout the breeding season, the probabilities of finding a conspecific nest to lay the eggs are higher during the mid-part of the season, when many others are laying (see results). The distribution of the majority of our cases of CBP seems to be in accordance to that idea.

A different perspective is presented by the fecundity enhancement hypothesis which proposes that CBP is a strategy to increase fitness beyond that possible through nesting alone. In principle, that strategy could be carried out by all kind of females (low and high quality females), however, it seems logical to think that females that are less constrained in terms of resources, i.e. high quality females, could spend additional resources in both looking for conspecific nests and forming additional eggs which future is, indeed, not clear at all. Therefore, if we assume that high quality females mainly breed at the beginning of the season, CBP would be chiefly found at that time and that is in accordance with our results. Finally, the fourth hypothesis to consider falls out of the flexible life-history trait hypothesis suggested by Lyon and Eadie (2008) and it poses the existence of lifelong specialist parasites. One can expect that those specialists will choose the best parents for their offspring, so, it

could be expected that they preferentially lay eggs at the beginning and mid-part of the season, when the best quality pairs are supposed to breed. Therefore, our results would also agree with that hypothesis. The identification of the parasite females is essential for disentangling all those possibilities, therefore, the lack of that point in this study together with the small sample size achieved makes impossible to rule out any of those potential explanations for the existence/prevalence of CBP in our population of rollers. Further studies in rollers should focus on the use of a wider set of microsatellites together with a deeper recording of behavioural observations.

Another variable that could help us in understanding CBP is HA. If parasites are laying their parasitic eggs as a response to either a nest loss or the existence of constraints/restrictions, one would not expect a very high synchronization between the host's and the parasite's laying date, therefore, a greater HA would be expected in nests with CBP than in nests without it. Our results show no significant differences between nests with and without CBP in terms of HA. In fact, there is a nonsignificant trend for HA to be lower in nests with CBP than in nests without it but that could be due to HA is usually lower at the beginning of the breeding season (D. Parejo, unpublished data). Anyhow, these results show a high synchrony between the parasite and its host which points out directly to the other two possibilities, the lifelong specialist parasites hypothesis and the fecundity enhancement hypothesis.

Finally, regarding CBP, a caution note must be done. Since we have some evidences that some of the cases of CBP could indeed be cases of QP, the percentage of CBP presented here could be lower. Specifically, it would change to an overall of 5.4 – 7.9 %, with only 2 of the 5 years studied showing cases of CBP, whereas the percentage of QP would reach 10.4 % in total, appearing in 4 of the 5 years studied. The number of species where QP has been confirmed is rather small (e.g. Küpper et al. 2004; Li et al. 2009; but see Griffith et al. 2004), therefore, further investigation in that rare reproductive alternative strategy in the roller would be of great interest. Since trios have been observed breeding in a single nest (Avilés & Sánchez 1995), the next step if those cases of QP were genetically confirmed, would be to assure that they are really QP cases instead of cooperative breeding where a female helper gets the opportunity of laying some eggs within that nest (Baglione et al. 2002).

5. Conclusions

Our work is the first one using genetic tools (microsatellites markers) for studying the breeding biology of the roller and it describes for the first time a set of 4 multiplex PCRs for doing so. In fact, this study shows for the first time that rollers are not exclusively monogamous birds but that they exhibit a wide range of alternative reproductive strategies from EPP to CBP and even possibly QP. However, the low resolution power of our set of markers should warn us that the percentages of EPP and CBP (as well as QP) could be greater than observed here, i.e. we could have underestimated those percentages due to that low both polymorphism and heterozygosity. Finally, regarding the possible causes for the existence/prevalence of those alternative reproductive strategies, we have discussed some possible explanations. However, and despite our limited sample size, those discussions open new and exciting avenues in the study of the breeding biology of the roller.

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