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Intra-specific brood parasitism revealed by DNA r analyses in a sub-oscine bird, the vermilion fl

Parasitismo intraespecífico revelado mediante análisis de micr en un ave suboscina, el cardenalito o saca tu rea

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ABSTRACT

Extra-pair reproduction is known to occur in many avian species. However, among studies on extra-pair reproduction have been carried out in oscine birds from temperate sub-oscines species, and particularly, species that inhabit tropical regions, have been limited in extent. Given that a majority of avian species live in the tropics, it is important to study extra-pair reproduction in sub-oscine species to have a more accurate picture of the rates of extra-pair reproduction and a better understanding of the adaptive function of extra-pair reproduction in relation to different modes of extra-pair reproduction and their prevalence. In this study we

reproduction occur in a sexually dimorphic and socially monogamous sub-oscine, (Pyrocephalus rubinus) We report cases of extra-pair paternity, extra-pair maternity and brood parasitism, and discuss our results in the view of other studies with passerines.

Key words: extra-pair reproduction, vermilion flycatcher *Pyrocephalus rubinus* sub-brood parasitism.

RESUMEN

Se sabe que la reproducción extrapareja ocurre en muchas especies de aves. Sin embargo, la mayoría de los estudios se han llevado a cabo en aves oscinas de regiones templadas y especies suboscinas, y en particular las especies que habitan regiones tropicales y subtropicales. Por lo tanto, es importante estudiar más especies tropicales y suboscinas para determinar las tasas de reproducción extrapareja en paserinos y un mejor entendimiento de la adaptativa de la reproducción extrapareja en aves. Las especies tropicales difieren en las tasas de ocurrencia de diferentes modos de reproducción extrapareja. En este estudio se reportamos casos de reproducción extrapareja en el cardenalito o saca tu real (*Pyrocephalus rubinus* suboscino sexualmente dimórfico y socialmente monógamo). Reportamos casos de maternidad extrapareja y parasitismo intraespecífico, y discutimos nuestros resultados en paserinos.

Palabras clave: reproducción extrapareja, Saca tu real *Pyrocephalus rubinus* suboscino intraespecífico.

INTRODUCTION

Although it is accepted that a majority of passerine birds are socially monogamous, there is increasing evidence that individuals seek

extra-pair matings in the majority of passerine species (reviewed in Griffith et al. 2002). Understanding the significance of inter-specific variation in

extra-pair paternity (EPP; 0- > 70 % between species, Møller & Birkhead 1994) has become a paramount challenge for behavioural ecologists. Yet, after 20 years of studies and accumulating information on the topic, a general and convincing explanation of the observed inter-specific variation in rates of EPP remains elusive.

Among passerines, most studies on extra-pair reproduction (EPR) have been carried out

in the Neotropics (Chesser 2004) because differences in ecology between tropical and temperate seasonality, Stutchbury & Morton (2001) relate to different rates of EPP in species.

Because oscine species in temperate regions of the world have been studied and tropical species have been studied (Stutchbury & Morton (2001)

in oscine birds from temperate regions, while there is a “temperate zone” knowledge of avian mating ecology in sub-oscines and tropical species have been studied to a much lesser extent (Stutchbury & Morton 2001, Griffith et al. 2002). Sub-oscine birds” can be seen as a “cal

species differ from their "sister group" (i.e., oscine or songbirds) in some life history and ecological traits that may influence the mechanism and occurrence of extra-pair reproduction: (1) Ontogeny of song production. Evidence indicates that while songbirds learn to sing, sub-oscines do not (i.e., song production in sub-oscines seem to be more determined by an "endogenous" mechanism; review in Kroodsma 1982). The process of learning to sing has been related to the evolution of complex and elaborate songs in oscines (Kroodsma 1982), and therefore relatively less variable and less complex songs could be expected in sub-oscines. Regarding oscines, different aspects of song structure (e.g., the presence of particular phrases in canaries Serinu canaries Vallet & Kreuzer 1995, Vallet et al. 1998) have been related to mate preferences, and song repertoire size has been shown to play a key role in determining extra-pair reproductive success (Hasselquist et al. 1996); however, it is not known how, or even whether, any aspects of song structure in sub-oscines may influence the occurrence of EPR. (2) Geographic distribution. While sub-oscines occur mostly in their inferred geographical areas of origin, oscines have undergone extensive geographical dispersal from Australasia (Barker et al. 2004), resulting in a current worldwide distribution. This makes oscines, in terms of radiation, one of the most successful groups of birds. On the other hand, sub-oscines do not occur in Europe and are more widely distributed in tropical regions of the New World, Africa and Asia (Ericson et al. 2003, Moyle et al. 2006), accounting for more than 30 % of the world's richest avifauna, which occurs in the

our efforts on studying many species. Because a larger number of species occur in the tropics zones (Stutchbury & Morton 2001), it may think as the "rule" that is emerging in many passerines that is, based on temperate systems), may be the "exception" in tropical species (studies in tropical species (sub-oscines) are needed to Another form of EPR that different ecological conditions brood parasitism (ISBP, Re This mode of EPR occurs with eggs in the nest of a conspecific and the host female incubates young (Andersson & Åhlund several forms of extra-pair has been studied to a lesser extent (Reyer et al. 1990), and has been cons the rate of ISBP can greatly among avian species. For proportion of nest parasitism estimated from 5-46 % for starlings (*Sturnus vulgaris*) 50 % for some species of doves (Davis 1988). It is not known if rates of ISBP are related to seasonality, but if ISBP is related to the degree on the probability of finding a mate then we could expect higher rates in temperate regions, where the shorter period of time in the breeding period of tropical species, and therefore a higher probability of finding a mate during the breeding period.

Here, we studied the reproductive system of the vermilion flycatcher, *Pyrocephalus rubinus* (Tyrannidae, Boddaert, 1783), in a population of central Mexico. The vermilion flycatcher is a socially monogamous and sexually dichromatic sub-oscine. Sexual dimorphism is also related to the singing behaviour since females do not usually sing. Females build the nests and incubate the clutch of one to three eggs (usually three) and both parents feed the young (Díaz Ríos 2002, A. Ríos-Chelén personal observations). We investigated whether or not the reproductive system of the vermilion flycatcher includes the occurrences of EPR or, conversely, whether this species can be considered genetically monogamous.

MATERIAL AND METHODS

Study area

We studied a population of vermilion flycatchers ($n =$ approximately 24 pairs) located in the forest of San Diego Metepec ($19^{\circ}17.97$ N, $98^{\circ}14.60$ W), Tlaxcala, Mexico, where blood samples were collected in 2001 and 2003. The study site is a mixture of native *Pinus* spp. and introduced *Eucalyptus* spp. forest and open areas. Most vermilion flycatchers in this population are migrants, with the exception of two males that were year round residents. The reproductive season for this population starts around February, when most migrant males begin to establish territories, and finishes around late July or early August, by which time most males have departed for the non-breeding grounds.

DNA extraction and PCR products

Adults were captured with mist nets and baited spring traps and colour ringed. Chicks were

collected at their nests when 8 days old and returned to their nest once their blood was obtained. Blood samples (70-240 μ L) were

et al. 1989). We used three markers (micro-satellites Py390) developed for this species for method). Approximately 100 ng of genomic DNA were amplified in PCR reactions containing 1x buffer (for micro-satellites Py274) and 1.5 mM $MgCl_2$ (for micro-satellites Py274) of each primer, 0.5 U of Taq polymerase in a total volume with sterile distilled water. PCR reactions were denatured at $95^{\circ}C$ followed by 30 cycles of 95 $^{\circ}C$ for 30 seconds, primer-specific annealing at $56^{\circ}C$ for 30 seconds or $56^{\circ}C$ for micro-satellite Py274 respectively) for 45 seconds and final elongation at $72^{\circ}C$ for 10 minutes. PCR products were separated on 8% polyacrylamide gels, visualized by silver staining, and allele length determined by inspection using a 10 base pair ladder (Invitrogen) as a reference.

Development of micro-satellite markers

A microsatellite-enriched genomic library was developed using modification of Hamilton et al. (1999). Genomic DNA was extracted from whole blood of the species and digested with *NotI* (Promega) and hybridized with a $(CA)_{24}$ oligonucleotide (Coryell et al. 1993) to single tandem repeats (STIR). The STIR fragments were cloned into pUC18 plasmid and used for transformation by heat shock of *Escherichia coli* XL-1 (Stratagen). One hundred colonies were grown on a YEA medium (Electran+®, BDH) and then cross-linked. The membrane was probed with a P^{32} 5' end-labeled oligonucleotide and used for autoradiography. From a total of 99 colonies, 10 positive colonies were identified. The positive colonies were sequenced with universal primers using the Perfectprep kit (Eppendorf).

Sequences were determined in an automated ABI Prism 3100XL DNA sequencer. For each sequence, primer pairs were identified using Primer 3 program (Rosenberg et al. 2001).

taken by puncturing the brachial vein, and collecting the blood with capillary tubes. Samples were stored in lysis buffer at 4 °C until DNA was extracted.

DNA was obtained by phenol-chloroform extraction and ethanol precipitation (Sambrook

2000). For each primer set concentration and $T_{annealing}$ optimise amplification yielding no extra-bands and Table 1 for characteriza

TABLE 1

Characterization of *Pyrocephalus rubinus* micro-satellite loci. For locus Py448 no clear motif (sequence added in GenBank). See “DNA extraction” for primer temperatures and primer specific $MgCl_2$ concentration for PCR. Accession numbers and sequences, which have been deposited in GenBank

Caracterización de microsátélites de *Pyrocephalus rubinus*. Para el locus Py448 no hubo un motivo claro (secuencia agregada en GenBank). Ver “DNA extraction” para información sobre las temperaturas y $MgCl_2$ específicas a cada primer, para realizar el PCR. Los números de acceso son para las secuencias, las cuales han sido depositadas en GenBank

Locus	Accession number	Repeat motif	Primer sequence (5'-3')
Py274	DQ834920	(TG) ₁₀ N ₆₀ (CT) ₂ (GT) ₇	AGGCATGATGAGGAACTCCA CTCCCAAGGGAGGATGTCTA
Py390	DQ834919	(CA) ₃ N ₈ (CA) ₄ GA (CA) ₄ N ₁₂ (AC) ₈	CACACTCACACTCACGCTCA GTGTGTGCACGAACACCTG
Py448	DQ834918		CACTGTACACAAAATCACACG GTCCCCTCTGTGCCTTGAG

DNA analysis

In 2001 six families were genotyped. From these families we obtained blood samples of all six males, three of the females and all but two of the 20 chicks. For this year we also obtained blood sample from five more adults in the population (two males, three females). In 2003,

along with positive and negative controls we ran each gel with individual samples from formerly typed and untyped individuals. The samples served as a reference for new samples.

TABLE 2

Data on micro-satellite alle

we sampled eight families, obtaining DNA from all eight males, five of the females and all 21 chicks. For this year we also obtained blood samples from eight more adults in the

from 14 and 21 adult individuals in the years 2001 and 2003 respectively. In the year 2003 our sample lack

samples from eight more adults in the population (five males, three females). All adult individuals (14 in 2001 and 21 in 2003) were used to obtain allele frequencies (Table 2). We ran the PCR products using the three microsatellite markers from the putative father, mother and offspring in adjacent columns to facilitate the comparison of allele bands between family members. We considered a chick to be the result of EPR if at least one allele (band) was not shared between the nestling and either of the putative parents. The micro-satellites had relatively low numbers of alleles in this population (Table 2). This relatively low variability, plus the fact that not all adult birds were sampled, made it impossible for us to assign genetic parentage. However, we were able to identify chicks that had a different genetic father or mother from the adult on the nest. To lower the risk of typing errors and misreading some alleles,

locus Py448		
Datos sobre frecuencias alélicas de 14 y 21 individuos adultos m 2001 y 2003 respectivamente. El al no se encontró en nuestra m		
Locus	Allele (bp)	Year
Py448	250	0.2
	260	0.3
	298	0.0
	318	0.1
	322	0.1
	330	0.0
	332	0.0
Py274	245	0.3
	255	0.3
	312	0.2
	450	0.0
Py390	202	0.8
	205	0.1

INTRA-SPECIFIC BROOD PARASITISM IN A SUB-OSCINE BIRD

To obtain basic statistics such as observed and expected heterozygosity, and to verify if our loci were under Hardy-Weinberg equilibrium, we used Cervus software (Marshall et al. 1998) and Genepop software (Raymund & Rousset 1995), respectively.

RESULTS

Information on allele frequencies and individual genotypes are given in Table 2 and 4 respectively. Table 3 shows that locus Py274 was not under Hardy-Weinberg equilibrium, presenting a statistically significant heterozygote deficit both in 2001 and 2003.

these putative parents are putative offspring. For these 1000 simulations and, in a P value of < 0.05 as a criterion, putative relationship fits the better than the alternative analyses showed that in 20 contained extra-pair offspring in the sample had extra-pair 87 % families had extra-pair 4 % chicks resulted of extra-pair is very likely that given the data, the frequencies of EP 0.05 were overestimated. If re-analysed our data, but t

Heterozygote deficit could be the result of several factors, for instance endogamy or presence of null alleles; this latter possibility is

conservative approach, the < 0.2. In other words, this v least an 80 % probability t

a potential source of bias in paternity analyses (Dakin & Avise 2004). Therefore, to take into account this possibility, we re-analysed our data using ML-Relate software (Kalinowski et al. 2006), for this software can take into account the presence of null alleles when estimating maximum-likelihood relationships between individuals. To this end, we tested in ML-Relate the hypothesis that putative parents had a Parent-Offspring relationship with their putative offspring (putative relationship) against the alternative relationship, that is, that

TABLE 3

Descriptive statistics of micro-satellite loci, H_o observed heterozygosity, H_e heterozygosity, H heterocigocidad, H_e heterocigocidad, los valores de P en HW (para probar equilibrio de Hardy Weinberg) y Hdef (para probar heterocigotos) por medio del método de cadena de Markov en el programa Genepop. Val significant P values are in bold. See “DNA extraction and PCR products annealing temperatures and primer specific MgCl₂ concentration f

Estadística descriptiva de loci de microsatélites, H_o heterogocidad observada, H_e heterogocidad, los valores de P en HW (para probar equilibrio de Hardy Weinberg) y Hdef (para probar heterocigotos) por medio del método de cadena de Markov en el programa Genepop. Val significativos se muestran en negrilla. Ver “DNA extraction and PCR products” para informac concentraciones de MgCl₂ específicas a cada primer, para realizar el PC

Locus	2001 (n = 14)				2002 (n = 2)	
	H_o	H_e	HW	Hdef	H_o	H_e
Py274	0.429	0.696	0.004	0.007	0.429	0.682
Py390	0.357	0.304	1.000	1.000	0.095	0.093
Py448	1.000	0.828	0.764	1.000	0.762	0.664

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ML-Relate results (taking into account the possibility of null alleles)

In 2001, six out of eight broods (75.0 %) contained extra-pair offspring. Among these eight broods, EPP was found in four (50.0 %), and extra-pair maternity (EPM) in two (25.0 %). In terms of all chicks in the sample (n = 18), nine (50.0 %) were probably the result of EPP, and two (11.1 %) the result of EPM. Overall, 11 chicks (61.1 %) were extra-pair offspring. In 2003, four out of eight broods (50.0 %) contained extra-pair offspring. From these eight broods, three (37.5 %) contained EPP chicks, one (12.5 %) contained an EPM chick, and two (25.0 %) contained a chick that probably resulted from ISBP. In terms of chicks in the whole sample (n = 21), seven (33.3 %) were the result of EPP, one (4.7 %)

was the result of EPM, and two (9.5 %) the result of ISBP. Hence, 10 chicks (47.6 %) had extra-pair parentage.

from the father could not be male on the nest). In the other broods there was EPM. In this brood, the offspring of the female could probably be the offspring of the male, but there is no evidence to state that. In terms of genotype of the nestling shared the allele with its social father and mother. In terms of all nestlings in the sample, three chicks (16.6 %) resulted from EPP, one (5.5 %) from EPM. Overall, 11 chicks (22.2 %) probably resulted from extra-pair reproduction (Table 5). In terms of eight broods (37.5 %) were the result of extra-pair offspring. Among these eight broods, EPP was found in two of them (25.0 %), in one of them (12.5 %), and in three of them (37.5 %). In terms of chicks in the whole sample (n = 21), 10 chicks (47.6 %) were the result of extra-pair reproduction.

not the offspring of the male (11.1 %), and for the result of extra-pair paternity), and for the result of ISBP. As in 2001, 11 chicks (52.4 %) were the result of extra-pair reproduction.

	Chick1	298/260	245/245	21
	Chick2	298/260	245/245	21
	Chick3	298/330	245/245	21
3 Kind	Male	260/250	312/312	21
	Female	332/322	312/312	21
	Chick1	332/260	312/312	21
	Chick2	250/250	245/312	21
4 Esq2	Male	260/250	255/255	21
	Chick1	318/250	450/255	21
	Chick2	318/250	450/255	21
5 Lic	Male	322/260	245/245	21
	Chick1	322/250	450/245	21
	Chick3	260/260	245/245	21
	Chick5	322/260	245/245	21
6 Mor	Male	260/250	245/255	21
	Chick1	260/322	255/255	21
	Chick2	260/322	255/255	21
	Chick3	260/260	255/255	21

Visual inspection results

In 2001 two out of eight broods (25.0 %) contained at least one chick that resulted from extra-pair reproduction. In one (12.5 %) of those broods, all chicks (n = 3) presumably resulted from EPP (in all of them, one allele

all 21 chicks in the sample, (4.7 %) whose mother was in the nest, but whose father was the male at the nest (i.e., EPM (Yezerinac 1995). Hence, eight (38.0 %) had extra-pair paternity. Table 5 summarizes both “visual inspection” and the “ML-Relate

TABLE 5

Percentage of broods and chicks that were the result of different modes of reproduction in 2001 and 2003. EPP = extra-pair paternity, EPM = extra-pair maternity, ISBP = intra-specific brood parasitism, EPR = extra-pair reproduction. Results from visual inspection and ML-Relate are shown for comparison

Porcentaje de nidadas y pollos que fueron resultado de diferentes modos de reproducción en 2001 y 2003. EPP = paternidad extrapareja, EPM = maternidad extrapareja, ISBP = parasitismo intra-específico, EPR = reproducción extrapareja. Se muestran resultados de inspección visual y de EM-Relate para comparación

Variable	Mode	Year		
		2001 Visual inspection	2001 ML-Relate	2003 Visual inspection
Broods (%)	EPP	12.5	50.0	25.0
	EPM	12.5	25.0	12.5
	ISBP	0.0	0.0	37.5
	EPR	25.0	75.0	37.5
Chicks (%)	EPP	16.6	50.0	14.2
	EPM	5.5	11.1	4.7
	ISBP	0.0	0.0	19.0
	EPR	22.2	61.1	38.0

INTRA-SPECIFIC BROOD PARASITISM IN A SUB-OSCINE BIRD

DISCUSSION

Despite our small sample size, we found evidence that the mating system of the vermilion flycatcher is far more complicated

than simple monogamy. Our results point to cases of extra-pair paternity, extra-pair maternity (quasi-parasitism) and intra-specific

Depending on the method used (visual inspection or ML-Relate), extra-pair paternity ranged from 16.6 % to 50.0 % in 2001 and from 14.2 % to 33.3 % in 2003. Other studies in socially monogamous birds

have found that on average 11.1 % of broods result from extra-pair paternity (Griffiths et al. 2004)

brood parasitism.

The most striking possible effect of null alleles in our data might have been an underestimation of EPP and EPM in 2001, and an overestimation of ISBP combined with an underestimation of EPP in 2003 (see Table 5). However, since null alleles are not the only possible source of heterozygote deficit, it may be better to take those results as an upper boundary of extra-pair reproduction in this species, rather than accepting that the possible effects of null alleles provide an accurate picture of extra pair parentage (see below). Besides null alleles, other potential sources of bias in our results would be a high mutation rate, and having misread some alleles in our gels. Based on studies made with *Drosophila melanogaster* and humans, mutation rates in micro-satellites have been considered to be relatively low (i.e., 10^{-2} - 10^{-4} ; DeWoody & Avise 2000, Bailey et al. 2007). This, with the relatively low numbers of alleles reported for each micro-satellite in this study combined with the relatively large number of chicks presenting an extra band does not point to mutation as a likely source of bias in our results. It is also unlikely that we might have misread some alleles. This is because the use of a positive control (i.e., from a plasmid) aided us in determining the gel area where we should expect to find our micro-satellite bands. The use of other individuals as positive controls (i.e., alleles coming from individuals that had already been run in previous gels) also provided a reference for new individual's alleles. Moreover, mismatched bands were very different in size (3-72 bp, and 5-200 bp for years 2001 and 2003 respectively; Table 2) which made identification of mismatched bands a relatively easy task. Nevertheless, the rates of EPR found in this study should be viewed with caution since, in our visual inspection we found that only two out of four (50.0 % for 2001) and one out of eight (12.5 % for 2003) extra-pair chicks were found to have a mismatch at more than one locus

(Table 4).

the small sample sizes in our study may not be representative of the whole, and thus should serve as a guide to the occurrence of EPR in the vermilion flycatcher, rather than to other avian populations. Some studies have shown a relation between extra-pair paternity (i.e., song repertoire size) and extra-pair paternity in females may seek genetic diversity in offspring (Hasselquist et al. 2000). Paternity has been shown to be high levels in a few subspecies of the vermilion flycatcher (Empidonax hammondi; Woolfenden et al. (2005) found that 58 % of nests contained extra-pair offspring, and 40 % of nestlings were extra-pair offspring. Tarof (2001) reported up to 100 % of extra-pair offspring in the vermilion flycatcher (Empidonax minimus). This incidence of extra-pair offspring in the vermilion flycatcher may be explained by a "polygamous mating system" characterized by clusters of individuals whereby clusters of individuals from other sites may be present during the breeding season (Kasumović et al. 2005). On the other hand, Dolan et al. (2005) found that those males singing in the chorus were more successful in reproductive success via extra-pair reproduction which reached a level of 61 % of offspring. It is still not known if a particular male phenotype might predict extra-paternity in the vermilion flycatcher.

Since we could not assign paternity, we cannot discard the possibility that the extra-pair offspring we found EPP or EPM are in fact ISBP. Because of this, we focus the remainder of our discussion on the occurrence of extra-pair reproduction in the vermilion flycatcher.

This is the first time that extra-pair reproduction has been reported in the Vermilion Flycatcher. It has been reported in other species including the blue jay (1990, Petrie & Møller 1991), the blue tit (1996, McRae & Burke 1996, Lyons

McRae & Burke 1996, Lyons

relatively low frequencies of occurrence in some (e.g., zebra finch, *Taeniopygia guttata* 10.9 % of offspring, Birkhead et al. 1990; the sand martin, *Riparia riparia*: 1.8 % of offspring, Alves & Bryant 1998) and relatively high in others (e.g., nests parasitized: up to 46 % in starlings *Sturnus vulgaris* up to 24 % in cliff swallows *Hirundo pyrrhonota* up to 31 % in swallows *H. rustica* review in Davies 1988, and perhaps up to 39 % of offspring in the eastern kingbird, McKittrick 1990). However, in this latter case, it could not be discerned whether these extra-pair reproduction events were result of ISBP, quasi-parasitism or both. Several hypotheses have been brought forward to explain the occurrence of ISBP. One possibility is that neither the male nor the female are aware of other female laying egg(s) on their nest. In this hypothesis the social partner gain no benefit from ISBP. In the case of the vermilion flycatcher, this hypothesis seems likely because only females incubate eggs and thus the nest may occasionally be left unattended for relative long periods of time. On the other hand, it is also possible that one or both pair members may obtain some benefits by allowing the parasitic female to lay eggs in the host nest (McRae & Burke 1996). For example, males may cooperate with parasitic females by letting her lay eggs in their nests in exchange for copulations. If this is the case, we should expect this male to sire some offspring in the parasitic female's own nest or in his own nest (i.e., quasi-parasitism or extra-pair maternity).

We found that in 2003 between 9.5 % and 19.0 % of chicks in our sample was probably the result of ISBP. Regardless of the method used (visual inspection or ML-Relate), we

found that in one of the nests where ISBP presumably occurred also occurred a case of extra-pair maternity. While we could not

on the mating system of a group of passerines that de The observed frequency of underlines the need to con to assess which individual either morphological (e.g., plumage colouration) or be and flight display: Smith 1 Chelén & Macías 2004, Río 2005), promote extra-pair r sub-oscine species.

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determine whether the mother of this quasi-parasitic offspring was the same female that laid the ISBP egg in this nest, this result points to the “in exchange of copulations” hypothesis. However, for the time being this interpretation is speculative and a more detailed study, where paternity and maternity can be assigned, is needed to address this hypothesis.

To conclude, we found evidence that the vermilion flycatcher, although socially monogamous, incur in different modes of extra-pair reproduction. This expands our knowledge

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