



Paternity Loss in Relation to Male Age, Territorial Behaviour and Stress in the Pied Flycatcher

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Abstract

For sexual selection to operate in monogamous species, males of poor quality in some factor like age, ornamentation, condition or aggressiveness, should lose paternity compared with higher quality males. We tested this idea in an Iberian population of pied flycatchers (*Ficedula hypoleuca*). Microsatellite analysis of 67 broods revealed moderate levels of extra-pair paternity (22.4% of broods, 7.5% of young). In a sample of 58 broods for which the caring male was identified, a higher paternity loss was associated with younger males, males that were less aggressive during territorial intrusion tests performed before the commencement of laying, and with males that showed higher levels of corticosterone metabolites in faecal samples collected at the end of the nestling period. Plumage darkness, forehead patch size and condition were not related to paternity loss. Paternity loss is more related to behavioural or physiological traits than to morphological ones in this population.

Introduction

Paternity loss in socially monogamous birds has been intensely studied over the last two decades given the ability to do so with increasingly accurate molecular tools (Møller 1998). This work has shown that extra-pair paternity in avian nests is a widespread phenomenon, raising the issue of its differential impact on individual male fitness (Westneat et al. 1990). Males should try to minimise the risk of cuckoldry through aggressive mate and nest defence, while females could be more or less prone to accept extra-pair copulations depending on social mate attractiveness. Thus both morphological and behavioural traits of males could be involved in cuckoldry risk. In this scenario the crucial question may be the identification of the traits distinguishing the cuckolded social mates. Cuckolded males could

be different from non-cuckolded males in certain traits related to ornamentation (Lifjeld et al. 1997a; Johnsen et al. 1998; but see Lifjeld et al. 1997b), polygyny status (Pinxten et al. 1993; Cordero et al. 2003; Pearson et al. 2006; but see Westneat & Mays 2005), age (Bouwman & Komdeur 2005; Johannessen et al. 2005; Lubjuhn et al. 2007), condition (Kempnaers et al. 2001) or size (Green et al. 2004; Hutchinson & Griffith 2008), with less ornamented, polygamous, young, debilitated and smaller mates losing more paternity.

An extra-pair fertilisation (EPF) requires that an extra-pair male encounters a female and achieves a copulation (EPC). Although females of some species seek out such encounters (Gray 1996; Currie et al. 1998; Stutchbury et al. 2005; Dolan et al. 2007; Dunn & Whittingham 2007), in many others males initiate them. Because the social mate gains by

preventing extra-pair males from gaining access, mate guarding has benefits in terms of ensuring paternity (Chuang-Dobbs et al. 2001; Komdeur et al. 2007) but is costly in terms of energy balance (Komdeur 2001). Cuckolded males could be those not able to guard their mates effectively when confronted with harassing male intruders (Low 2005). Territorial behaviour could also be involved as less effective territory defence would lead to intruders more frequently approaching females. This hypothesis predicts that cuckolded males should show more signs of subordinate social status or lowered aggressiveness towards intruders. Subdominance in territorial interactions could also be expressed in higher levels of physiological stress both during territorial encounters and chronically.

We investigated these ideas in the Pied Flycatcher *Ficedula hypoleuca*, a small (12–13 g) hole-nesting migratory passerine of European woodlands (Lundberg & Alatalo 1992), which is also a well-studied model organism given its propensity for breeding in artificial nest-boxes (Lundberg & Alatalo 1992). Clutch size in our population ranges from 4 to 7 with a mode of six eggs. There is information in the literature showing low to moderate levels of cuckoldry in this species (Lifjeld et al. 1991, 1997b; Gelter & Tegelström 1992; Rätti et al. 1995; Brun et al. 1996; Lubjuhn et al. 2000; Lehtonen et al. 2009a), especially after the development of microsatellite typing (Ellegren 1992; Primmer et al. 1996). The pied flycatcher is sexually dimorphic during the breeding season, with males especially in Iberian populations showing a highly contrasting black and white plumage (Lehtonen et al. 2009b) and a white forehead patch, in contrast with duller brown and white females. Males with more black feathers on the head, wings and back are more attractive to females in some populations (Slagsvold & Saetre 1991; Saetre et al. 1994; Galván & Moreno 2009), while larger forehead patches also seem to be preferred by females (Potti & Montalvo 1991a; Dale et al. 1999; Osorno et al. 2006). However, the evidence concerning traits affecting paternity loss in this species is confusing (Rätti et al. 1995; Lifjeld et al. 1997b; Lehtonen et al. 2009), and no study concerning behavioural and physiological correlates of paternity loss has been published.

In this study, we have conducted a paternity analysis of a moderately large sample of nests in a southern European population of pied flycatchers to test the following predictions concerning paternity loss: (1) cuckolded males are younger than non-cuckolded males; (2) cuckolded males are less ornamented

than non-cuckolded males; (3) cuckolded males are less aggressive in territorial contests than non-cuckolded males; and (4) cuckolded males show higher levels of stress than non-cuckolded males. To test the effects of ornamentation on paternity loss, a manipulation of the white forehead patch of males on arrival at the breeding area was performed (experiment fully described in Osorno et al. 2006). The blackness of male dorsal plumage was also scored as a measure of ornamentation (Galván & Moreno 2009). We have also included two measures related to territorial aggressiveness obtained in territorial intrusion tests, namely the minimum distance of approach to a recorder playing pied flycatcher song and the song frequency during these tests. Finally, we have measured corticosterone (CORT) metabolites in faecal samples to estimate physiological stress.

Methods

Capture and Handling

The study was conducted during the 2003 breeding season in a deciduous forest of *Quercus pyrenaica* in central Spain (40°48'N, 4°01'W), where 300 nest-boxes were installed in 1991. Between 60 and 100 nest-boxes are occupied by pied flycatchers every year. All flycatcher broods in the study area were included in the present study.

From mid-Apr. the study area was intensively searched by a team of six experienced ornithologists for newly arrived males. In this species, males start to advertise their territorial claims at nest-boxes as soon as they arrive (Lundberg & Alatalo 1992). Forty-five males were captured with nestbox traps on their first day of observation in the study area to manipulate forehead patch size (Osorno et al. 2006). Males were classified as first-year birds or older from the colour of primaries and primary coverts (brown in 1-yr birds). Age of males older than 1 yr were established as exact age if ringed as nestlings in the study area, or estimated assuming that males were 2 yrs when captured for the first time as breeders in full adult plumage (Lundberg & Alatalo 1992). Mass was recorded with a Pesola spring balance (accuracy of 0.25 g) and tarsus length was measured with a digital calliper to the nearest 0.01 mm. We used the cubic root of mass divided by tarsus length as a measure of body condition. The largest height and width of the white forehead patch was measured to the nearest 0.1 mm with a calliper. Width and height are positively correlated (Osorno et al. 2006), so we

have used their product as an estimate of patch size. Patch size was enlarged, reduced or kept constant in an experiment on differential allocation by females with respect to patch size (see Osorno et al. 2006 for details). The modified patch size was measured for all birds. The experimental manipulation of forehead patch size lasted at least until cessation of laying (Osorno et al. 2006). The percentage of black feathers in the head and mantle was scored as 'blackness', which is the exact inverse of the brownness score used by Potti & Montalvo (1991b). This score is highly correlated (Potti & Montalvo 1991b) with Drost's (1936) scale from I (darkest) to VII (lightest) used by other researchers such as Dale et al. (1999) for Scandinavian populations. Blackness scores were converted to 10 point intervals, with values ranging from 0 (0–10%) to 9 (90–100%). Males were individually marked with colour rings to allow subsequent observations during territorial intrusion tests (see below). Many initially captured males ($n = 25$) defended (see territorial intrusion tests) and bred (see final captures) in a different nest-box from that in which they were captured, suggesting that capture altered nest-box selection.

A small sample of blood from the brachial vein was collected into Eppendorf tubes with a lysis buffer (100 mM TRIS, 100 mM ethylenediaminetetraacetic acid, 2% sodium dodecyl sulphate, pH 8) to conduct paternity analyses (see below). Males were introduced for 2 min in a dark wooden box to collect faeces from aluminium foil placed on the bottom of the box.

Frequent (every 2–3 d) checks of nestboxes provided data on laying date, clutch size, hatching date, number of chicks alive at 12 d of age and number of fledged young. On day 12 of nestling age (hatching day = day 0), provisioning adults were captured at the nest with nest-box traps and their identity checked or they were ringed if necessary. Birds were measured as above if not captured previously (see above). A blood sample was taken if birds had not been captured earlier in the season (see above). Faecal samples were obtained as at first capture (see above). These faecal samples were analysed for metabolites of CORT (see below). Some males ($n = 10$) tending nests could not be captured at this stage. Observations at nests during nestling care did not detect any case of secondary females without male help, which could be evidence of secondary status and the existence of polyterritorial polygyny (Lundberg & Alatalo 1992).

A small blood sample (10–20 μ l) from each nestling alive at 5 d of age was obtained from the

brachial vein for paternity analyses. Hatching failure affected 33 (8.5%) of 387 eggs in less than half of the nests. However, some of them did not show any trace of embryonic development so they may have been infertile. Five nestlings (1.3% of the sample) died before sampling.

Territorial Intrusion Tests

Two sets of territorial intrusion tests were conducted 5.5 ± 0.8 and 13 ± 1.0 d after initial capture and before nest-building commenced (Osorno et al. 2006). We will only analyse the first set to avoid including effects of habituation. During experiments, colour-ringed males who had previously been observed singing at a certain nest-boxes were submitted to recordings of the song of a territorial intruder (a single strange male) during 10 min to estimate aggressiveness. We used sound stimuli instead of visual presentations of models or captive males as response may be much more rapid and easily estimated. We used song from a single bird in all cases, so the responses to it have to be interpreted cautiously (Kroodsma et al. 2001). We assume that responses to this specific song reflect responses of males to circumstances when paternity could be influenced by territorial intrusions.

We used the commercial digital recorder Mundi Sound PQ10 produced in Spain (Cantos de Pájaros Digitales S.L., c/Rey Jaime I 22, 12589 Cáliz; <http://www.capadi.com>), capable of playing different bird songs, depending on the chip acquired. The recorder included an EPROM chip with songs performed by eight different species, including pied flycatcher song of one individual from an unspecified pied flycatcher population. It is highly unlikely that the song recorded would be individually recognizable. The volume of the loudspeaker was adjusted so that it could only be heard at a short distance from the nest-box of the subject male (at 50 m, the average distance between nest-boxes in the study area, the playback song could not be heard by us). The song rate (number of songs per minute) of the digital recorder (40 strophes per 5 min) was in the natural range of song rate typically produced by an adult pied flycatcher (average of 37.9 strophes per 5 min; Gottlander 1987). The recorder was placed on the ground below the nest-box defended by a focal colour-banded male, and his response to the emitted pied flycatcher song estimated by an observer (always the same) hidden at a distance of at least 15 m. A single song bout was used only once on each subject (see above) and gave rise to a unique

data point. It is not likely that neighbours of subjects could hear the recording as the digital recorder was played at a volume that could presumably only be heard at a short distance from the subject.

The minimum distance at which the male owner approached the nest-box above the recorder was registered as well as the number of song bouts emitted (Osorno et al. 2006). Both variables are assumed to indicate the propensity of males to repel territorial intruders. Only 35 males could be assigned to a certain nest-box at this stage and tested.

Paternity Analyses

We obtained samples corresponding to 67 families (466 individuals) and 10 males captured initially and not assigned to any nest, giving a total of 476 individuals. Of the sampled families (348 nestlings and 118 adults), we obtained blood samples from all the chicks, mother and social father in 54 nests, only chicks and mother in six nests, only chicks and putative father in four nests and only the chicks in three nests. Thus, only 58 nests were available for establishing extra-pair paternity.

DNA was obtained from blood samples using a standard salt-extraction protocol (Millar et al. 1988). Genotyping was carried out for five microsatellite loci: FhU1, FhU2, FhU3, FhU4 and FhU5, using the primers and conditions previously specified in Ellegren et al. (1995). In our study population these loci were all polymorphic, with 7, 6, 11, 26 and 35 alleles respectively each, and a combined exclusion probability of 0.99 as calculated by CERVUS (Marshall et al. 1998). We assessed genetic parentage by comparing the genotypes of chicks with those of female and male nest owners. We considered that chicks were the offspring of the adults attending the nests when their genotypes were all compatible with those of male and female in the five loci typed. Those cases in which nestlings mismatched the paternal alleles in two or more loci were considered to be cases of extrapair paternity (EPP). A mismatch between the genotypes of the male and any chick in only one allele was considered to be as a result of mutation and not to be evidence of EPP. Despite the fact that the probability of exclusion of 0.99 relates to the probability of excluding an offspring at one or more loci, we have not considered as an extra-pair chick the only case of mismatch at a single locus with the male. This is because we also observed a single case of mismatch at a single locus between a chick and the female, and it was the only case of mismatch in that brood (whereas in 10 of 13 cases

of broods with EPP there were more than one chick identified as extra-pair), and in both cases the mismatch was because of a difference of only one pair of bases between the parental allele and the offspring allele. As the markers used are dinucleotide repeats, and the mutation of microsatellites mainly follows a stepwise model (Primmer & Ellegren 1998), this discrepancy may easily be as a result of a single mutation step. Two of the markers (FhU3 and FhU5) deviated from Hardy–Weinberg equilibrium indicating null alleles, but none of the extra-pair paternity cases assigned was because of mismatches in only one or both markers and the frequency of null alleles estimated by CERVUS was low (<0.01).

Corticosterone Analyses

We could only use samples collected at the end of the nestling period, as we only obtained faecal samples from four cuckolded males out of 13 at initial capture (there were seven samples at final capture). Faecal samples ($n = 30$) were analysed for metabolites of corticosterone (CORT) following the protocol by Rettenbacher et al. (2004) and Lobato et al. (2008). Lobato et al. (2008) validated the measurement of excreted CORT metabolites as a measure of CORT levels in pied flycatchers from the study population by adrenocorticotrophic hormone administration and subsequent analysis of collected excreta. All faecal samples were analysed in duplicates and if the coefficient of variation between results was higher than 8%, the samples were reanalysed. The inter-assay coefficient of variation was 14% (Lobato et al. 2008). Final values were not affected by the number of captures (1 or 2) to which males had been subjected ($F_{1,28} = 1.23$, $p = 0.28$).

Statistical Analyses

The proportion of EPY (number of EPY/brood size) was subjected as dependent variable to generalized linear models (GENMOD procedure in SAS; SAS Institute 2001) with binomial distributions to test for effects of different factors. Three analyses were performed: a model including only the 35 males which were captured at the beginning and tested for territorial aggression, a model including the 58 males captured during the nestling stage, and a model including only the 30 males for which we had final CORT values. In the first model we included as independent variables male age, condition at first capture, experimentally modified forehead patch size, blackness of plumage, number of song bouts during

intrusion tests and minimum distance to the nest-box during intrusion tests. In the second model only variables estimated during the nestling stage were included, namely age, condition at final capture, forehead patch size at final capture (because of abrasion it was not identical to modified patch size, see Osorno et al. 2006) and male blackness at final capture. In the analysis including CORT, the same variables as in the second model were included adding final CORT values. Full models will be presented plus final models obtained by a backward deletion procedure; variables were removed from the full model when the variance explained did not significantly improve the model ($\alpha = 0.05$). There were missing values for some variables for some males.

Results

In 13 of 58 nests (22.4%) where we had sampled the male attending the nest, we found at least one offspring that mismatched his genotype (only one case of intraspecific brood parasitism was detected). These nests contained 26 EPY of 348 nestlings sampled (7.5%). There were 2.0 ± 0.2 EPY per nest with EPY (range = 1–3, $n = 13$). These values are very similar to other previously reported for the species.

Both the full and minimal models for males that were captured on arrival to the breeding site showed that age and minimum approach distance had significant effects on paternity loss (Table 1). Younger males (Fig. 1) and males that kept farther away from the nest-box (Fig. 2) suffered a higher paternity loss. The full model for late captures including all males showed condition and male age as significant effects, but the minimal model retained only age as significant (Table 2). The full model including final CORT measurements showed significant effects of age and CORT levels, but the minimal model only retained CORT as significant (Table 3). Males with higher CORT levels lost more paternity (Fig. 3).

Discussion

We found that behavioural and physiological attributes of male pied flycatchers were better predictors of paternity loss than ornamental traits. Younger males and males that responded less intensely to a territorial intrusion test suffered a higher paternity loss. Also, males with higher levels of CORT during the nestling stage lost more paternity. The effects of territorial behaviour and physiological stress were not solely because of age as effects were detected in analyses including age as independent variable.

Ornaments like the white forehead patch and blackness of dorsal plumage did not affect paternity loss, even though forehead patch size was manipulated to increase variation in this trait.

Age has been frequently related to cuckoldry (Bouwman & Komdeur 2005; Johannessen et al. 2005; Lubjuhn et al. 2007; but see Stutchbury et al. 1997; Westneat & Mays 2005). Although 1-yr males can be easily identified by their plumage, there were only five 1-yr males in the sample and only two of them were cuckolded. This indicates that the age effect is more subtle than just separating 1-yr from older males. Although male age affects forehead patch size and plumage blackness (Galván & Moreno 2009), these plumage traits were not involved in the effect of age. Younger males may be less capable of

Table 1: Generalized linear models (GENMOD procedure in SAS) with binomial distributions to test for effects of different factors on proportion of EPY in the brood for the sample of initially captured and tested males

| | df | χ^2 -Value | p-Value | β -Value |
|------------------|------|-----------------|---------|----------------|
| Full model | | | | |
| Age | 1,26 | 3.92 | 0.048 | -0.807 |
| Condition | 1,26 | 0.33 | 0.566 | -0.208 |
| Forehead patch | 1,26 | 0.00 | 0.963 | -0.017 |
| Blackness | 1,26 | 1.12 | 0.289 | -0.325 |
| Minimum distance | 1,26 | 3.90 | 0.048 | 0.395 |
| Song rate | 1,26 | 0.10 | 0.753 | -0.150 |
| Minimal model | | | | |
| Age | 1,32 | 5.27 | 0.022 | -0.839 |
| Minimum distance | 1,32 | 6.01 | 0.014 | 0.450 |

Minimal models are obtained from full models by successive backward deletion of variables when the variance explained do not significantly improve the model ($\alpha = 0.05$).

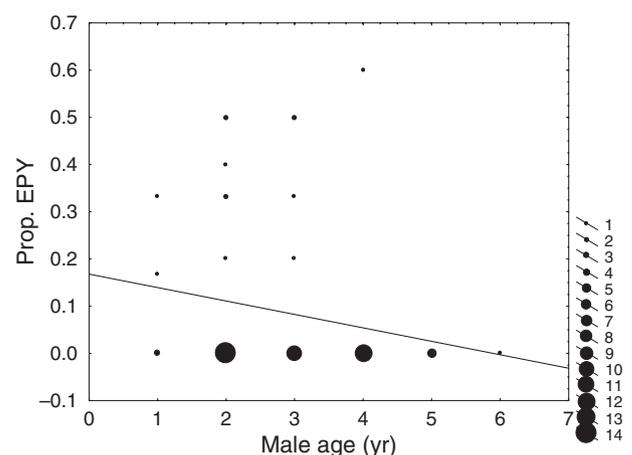


Fig. 1: Proportion of extra-pair young in relation to male age. Lines are illustrative.

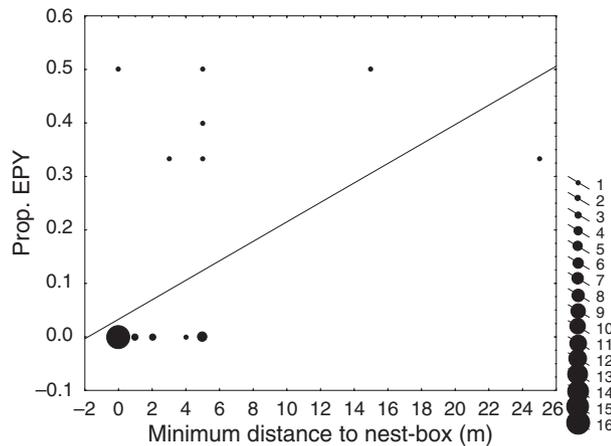


Fig. 2: Proportion of extra-pair young in relation to minimum distance of approach by males to own nest-box during territorial intrusion tests conducted prior to laying. Lines are illustrative.

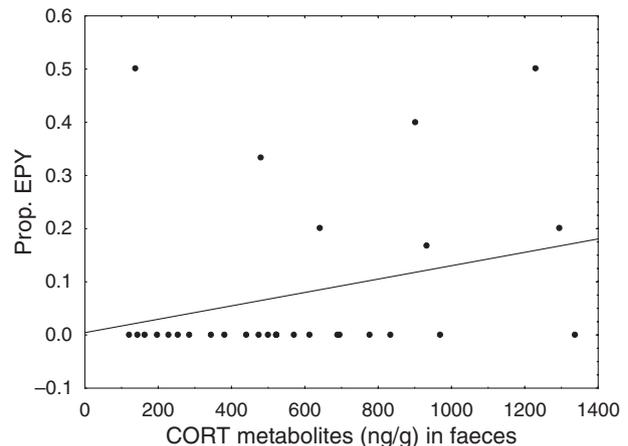


Fig. 3: Proportion of extra-pair young in relation to concentration of immunoreactive corticosterone (CORT) metabolites in faecal samples collected at the end of the nestling period. Lines are illustrative.

Table 2: Generalized linear models (GENMOD procedure in SAS) with binomial distributions to test for effects of different factors on proportion of EPY in the brood for the sample of late captured males

| | df | χ^2 -Value | p-Value | β -Value |
|----------------------|------|-----------------|---------|----------------|
| Full model | | | | |
| Age | 1,50 | 4.28 | 0.039 | -0.501 |
| Condition | 1,50 | 5.97 | 0.015 | -0.617 |
| Forehead patch | 1,50 | 1.91 | 0.167 | 0.297 |
| Blackness | 1,50 | 0.01 | 0.925 | -0.020 |
| Minimal model | | | | |
| Age | 1,55 | 5.77 | 0.016 | -0.538 |

Minimal models are obtained from full models by successive backward deletion of variables when the variance explained do not significantly improve the model ($\alpha = 0.05$).

Table 3: Generalized linear models (GENMOD procedure in SAS) with binomial distributions to test for effects of different factors on proportion of EPY in the brood for the sample of late captured males for which we had measures of corticosterone (CORT) metabolite levels in faecal samples

| | df | χ^2 -Value | p-Value | β -Value |
|----------------------|------|-----------------|---------|----------------|
| Full model | | | | |
| Age | 1,24 | 3.97 | 0.046 | -0.874 |
| Condition | 1,24 | 1.75 | 0.186 | -0.477 |
| Forehead patch | 1,24 | 3.25 | 0.071 | 0.708 |
| Blackness | 1,24 | 0.46 | 0.499 | 0.224 |
| CORT | 1,24 | 4.53 | 0.033 | 0.728 |
| Minimal model | | | | |
| CORT | 1,28 | 5.21 | 0.022 | 0.657 |

Minimal models are obtained from full models by successive backward deletion of variables when the variance explained do not significantly improve the model ($\alpha = 0.05$).

repelling male intruders during the fertile period of their mates, or females may detect male age through behavioural traits and reduce paternity with younger males. Old age of a mate can be used as an indicator of genetic quality as viability has been proven (Kokko 1998). The effect of age on paternity loss has not been detected in another study of pied flycatchers (Lehtonen et al. 2009).

Males that avoid confronting territorial intruders near their nest-box may be unable to guard their mates efficiently. Intruding males would thus have more easy access to females in territories owned by less aggressive males. The literature on mate guarding in pied flycatchers is ambiguous concerning its importance in different populations (Alatalo et al. 1987; Chek et al. 1996), but just poor territory defence as evidenced during territorial intrusion tests could be sufficient to allow EPC to take place. Our results agree with this possibility as young and prudent males could be more easily frightened away from intruding males trying to force copulations with their mates. Low (2005) has suggested that despite the current trend focussing on female fitness benefits associated with EPF, males may gain EPC through forced copulation without females attaining any benefit. Rapid mate switching as observed near the time of nest initiation (Lifjeld et al. 1997b) cannot explain EPP as copulations leading to fertilization take place no earlier than 2 d before the first egg is laid (Lifjeld et al. 1997a). It is also possible that females may prefer aggressive males to timid ones in this species. This preference may be expressed in females through paternity reduction when paired to mates easily frightened by intruding males.

Unfortunately the sample of males for which it was possible to test simultaneously for the effects of CORT level and territorial aggressiveness was too small to allow analyses, so we cannot totally separate the effects of stress and behaviour on paternity loss. Both effects may be related through less aggressive males being more easily stressed. However, given that CORT level was measured long after paternity loss was consummated, it is possible that the conditions relating to paternity loss itself could have increased stress levels in cuckolded males. Easily stressed males could also be less attractive to females inducing them to seek extra-pair copulations. If the propensity for high CORT levels is heritable (Evans et al. 2006), females could be avoiding bad genes (Velando et al. 2008) for their offspring through paternity reduction when paired with easily stressed males.

Given the association of paternity loss with age, stress and behaviour, it is striking that male ornaments did not differ between cuckolded and non-cuckolded males. Even the manipulation of the supposedly attractive white forehead patch (Potti & Montalvo 1991a; Sanz 2001; Osorno et al. 2006) did not affect paternity loss. Although females in our population adjusted their investment in eggs according to the manipulated forehead patch of their mates (Osorno et al. 2006), there was no evidence that they discriminated against social mates according to this trait in terms of extra-pair matings. The forehead patch may in fact be more related to male-male competition for territories (Pärt & Qvarnström 1997) than to mate choice in our population. However, paternity loss was more affected by territorial behaviour than by plumage traits presumably involved in social interactions. Male plumage blackness was positively associated with higher rates of cuckoldry in a Norwegian pied flycatcher population where many males are similar to females in colour (Lifjeld et al. 1997b), but in our population most males are black (mean blackness score $70 \pm 3\%$) so the possibility of discriminating mates as a function of this trait is presumably reduced. Rätti et al. (1995) did not find that plumage darkness in males explained paternity loss. Lifjeld et al. (1997b) were unable to explain why more attractive males according to Saetre et al. (1994) suffered more from cuckoldry. Lehtonen et al. (2009) have also shown that plumage is not involved in paternity gain through extra-pair matings either. Why plumage traits apparently used in social mate choice in some populations do not affect extra-pair mating decisions remains a paradox.

To conclude, our results indicate that younger males, males less responsive to our territorial intrusion tests, and more easily stressed males, suffer a higher risk of cuckoldry. Behavioural traits appear more predictive of paternity loss than morphological ones in our study population.

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