Molecular Ecology (2007) **16**, 3679–3688 doi: 10.1111/j.1365-294X.2007.03420.x

Explicit experimental evidence for the effectiveness of proximity as mate-guarding behaviour in reducing extra-pair fertilization in the Seychelles warbler

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Abstract

Extra-pair copulations (EPCs; copulations outside the pair bond) are widespread in birds and may result in extra-pair fertilizations (EPFs). To increase reproductive success, males should not only seek to gain EPFs, but also prevent their own females from gaining EPFs. Although males could reduce the number of EPCs by their mates, this does not necessarily mean that they reduce the number of EPFs; indeed several studies have found no association between EPCs and EPFs. Male Seychelles warblers (*Acrocephalus sechellensis*) follow their partner closely during the period when the pair female is most receptive (fertile period). We show that males that guarded their mates more closely were less likely to have extra-pair young in their nest. This study on the Seychelles warbler is the first to provide explicit experimental evidence that mate guarding is effective in reducing EPFs. First, in territories where free-living males were induced to stop mate guarding during the pair female's fertile period, extra-pair parentage was higher than in the control group. Second, in the experimental group, the probability of having an extra-pair nestling in the nest was positively associated with the number of days during the fertile period for which mate guarding was artificially stopped. Thus, male mate guarding was effective in reducing the risk of cuckoldry.

Keywords: experimentally ceased mate guarding, extra-pair paternity, mate guarding, microsatellites, Seychelles warbler

Received 25 January 2007; revision accepted 10 May 2007

Introduction

In socially monogamous species, females often copulate with males other than their social mates (extra-pair copulations, EPCs). In birds, EPCs are widespread and often result in extra-pair fertilizations (EPFs) (e.g. Birkhead *et al*. 1990; Gibbs *et al*. 1990; Birkhead & Møller 1992; Kempenaers *et al*. 1992; Dixon *et al*. 1994; Wetton *et al*. 1995), which reduce the reproductive success of the cuckolded male. Males are therefore expected to adopt behaviours that prevent their mates from engaging in EPCs (Birkhead *et al*. 1987; Birkhead & Møller 1992; Kempenaers *et al*. 1995; Stockley 1997). If, for instance, a male can remain near his mate during her fertile period and physically prevent other males from copulating with her, then such mate guarding would be strongly favoured by selection (Birkhead 1998). However, the idea of mate guarding as a method of paternity guarding is controversial. Even if males do attempt to remain near their mates to prevent EPCs, the effectiveness of this strategy has been called into question (Stutchbury & Neudorf 1998). Females may often adopt behaviours that allow them to escape from male-guarding efforts and seek EPCs, and males may be prevented from effectively guarding their mates over the entire fertile period because of mate-guarding energy costs and time constraints. Empirical studies indicate that females can circumvent the guarding efforts of their mate (Kempenaers *et al*. 1995; Johnsen *et al*. 1998; Stutchbury & Neudorf 1998; Komdeur *et al*. 1999), that males cannot guard their mate continuously during the fertile period

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(Westneat 1994; Gowaty & Bridges 1991; Currie *et al*. 1998; Komdeur 2001), and that the intensity of male mate guarding is often not associated with the level of EPF (e.g. Kempenaers *et al*. 1995; Riley *et al*. 1995; Wagner *et al*. 1996; Schleicher *et al*. 1997; Currie *et al*. 1998; Johnsen *et al*. 1998). Such results have led to discussion about sexual conflict over fertilization and over which sex is in control of fertilization (Wagner 1991; Björklund *et al*. 1992; Lifjeld & Robertson 1992; Blakey & Norris 1994; Osorio-Beristain & Drummond 1998; Stutchbury & Neudorf 1998; Komdeur *et al*. 1999).

Although mate guarding has been described in many bird species, there are only seven experimental tests of the mate-guarding hypothesis and all of these have involved removing males. These have shown that, in the absence of their mates, females obtained more EPCs (Björklund & Westman 1983; Westneat 1994; Kempenaers *et al*. 1995; Dickinson 1997; Rodrigues 1998; Currie *et al*. 1999; Chuang-Dobbs *et al*. 2001). However, the results of these experiments are difficult to interpret. First, only three of these experiments examined the paternity of nestlings, and these revealed mixed results. Although the temporary detention of males during their mates' fertile period increased EPCs in all three studies, it only translated into increased EPFs in the broods of black-throated blue warblers (*Dendroica caerulescens*; Chuang-Dobbs *et al*. 2001). In wheatears (*Oenanthe oenanthe*; Currie *et al*. 1999), there was a slight but nonsignificant increase in EPFs, while there was no increase in EPFs in the broods of red-winged blackbirds (*Agelaius phoeniceus*; Westneat 1994). Furthermore, an increase in EPCs does not always translate into an increase in EPFs in other species. For example, in the collared flycatcher (*Ficedula albicollis*; Michl *et al*. 2002), nearly all females whose social males were experimentally prevented from fertilizing them engaged in a high level of EPC, but the rate of EPFs still remained low. More importantly, male-removal experiments inadequately tease apart the effects of mate guarding on the one hand, and the costs and benefits to females of accepting EPCs and the role of female choice in EPCs on the other hand, as they artificially create a situation in which the male disappears, rather than one in which only mate guarding is reduced (Currie *et al*. 1999; Komdeur *et al*. 1999). In such a situation, the complete absence of the male may dramatically alter the behaviour of the female, for instance leading the female to initiate re-pairing strategies, which may include copulating with encountered males. Even if the effect of removal is not this extreme, previous work has shown that when females are outside the view of their mate, they are better able to deceive him about his paternity, and thus avoid the costs of obtaining EPCs (e.g. less paternal care from male partner; e.g. Lifjeld & Robertson 1992; Westneat 1994; Johnsen *et al*. 1998; Komdeur *et al*. 1999). In the present study on the Seychelles warbler (*Acrocephalus sechellensis*), we have devised an experiment that by manipulating the mate- guarding activities of the male without removing him from the territory avoids the problems listed above and allows us to directly, and unequivocally, assess the consequences of mate-guarding effort on levels of EPF. Although the present study uses the same focal pairs, it is not a repeat of the earlier study by Komdeur *et al*. (1999) which showed a negative association between mate-guarding intensity and the number of EPCs. The only difference is that in this study, we use only 20 controls, the same controls as in Komdeur (2001), and not the 21 controls in Komdeur *et al*. (1999). This is because the female of one control pair had been replaced by another female during the incubation period, resulting in nest failure. As critics of the earlier study correctly pointed out, a lower level of EPCs does not necessarily translate into lower rates of EPFs for the reasons outlined above. The current study, which experimentally reduces mate guarding and then uses molecular parentage analysis to accurately assess levels of EPFs, was designed specifically to determine the relationship between mate guarding and paternity.

The Seychelles warbler is a rare island endemic which, until 1988, was entirely restricted to Cousin Island (29 ha) in the Seychelles. Genetic analysis has demonstrated that the frequency of EPFs is high (40%; Richardson *et al*. 2001) in this species. Furthermore, the fitness costs of cuckoldry are considerable for males because: (i) breeding opportunities are restricted, as warblers usually have a single egg clutch (91.0%), are single-brooded within a breeding season and have no time for a successful replacement clutch (Komdeur 1996a); (ii) males make the same number of provisioning visits to the nestlings as do females (Richardson *et al*. 2003) and; (iii) fledglings have a 4-month period of dependence (Komdeur 1991). Consequently, male Seychelles warblers are expected to be under strong selection to develop efficient paternity guards. Previous work on the Seychelles warbler has shown that all successful within-pair copulations and EPCs occurred during the period beginning 5 days before and up until the first — and normally only — egg was laid (the fertile period). To minimize EPCs, males guard their females intensively during this fertile period; males maintain close proximity with their female by following her around the territory (Komdeur *et al*. 1999), and calling her for courtship feeding (D.S. Richardson, personal observation). The male then abruptly ceases mate guarding from the moment he spots the single-egg clutch in the nest. However, even after the male stops intense mate guarding, the male and female are often within sight of each other, perhaps due to the restricted size of the territory or because both the male and female remain close to the nest (Komdeur *et al*. 1999). Consequently, by introducing a model egg in the nest of pairs during the fertile period of the female before egg laying, we are able to advance this sudden switch in male behaviour, thus reducing the intensity and duration of his mate guarding without otherwise disturbing the pair bond. The use of this protocol has previously been shown to increase the number of EPCs gained by a female, and strongly supported the idea that mate guarding was acting as a paternity insurance (Komdeur *et al*. 1999).

In the present study, we combine field observations and experimental manipulations with the molecular analysis of paternity to examine the effectiveness of mate guarding in reducing the risk of EPF. We are specifically interested in whether mate guarding had a direct and significant effect on the probability of being cuckolded but have not devised the experiment to distinguish between the subtle mechanisms at work. Although Seychelles warblers often breed cooperatively, we concentrate in this study on pairs without helpers. We predicted that the time allocated to mate guarding during the fertile period will be positively associated with the probability that a male retains paternity of the single-egg clutch. We therefore expected females whose partners were induced to stop mate guarding during the fertile period to have EPFs more often than the guarded control females. This is to our knowledge the first time such an experiment, in which the effect on paternity of inducing free-living males to stop mate guarding, has been performed.

Materials and methods

General field methods and behavioural observations

Seychelles warblers were studied between June and September (the main breeding period) on the island of Cousin in 1996 and 1997. During this time, most birds (adults and nestlings) were already individually colour-ringed, blood-sampled, and of known age and sex. Behavioural data were collected from 40 different breeding pairs (26 in 1996, 14 in 1997). To control for potential effects of age (Welling *et al*. 1995) and breeding experience (Kempenaers *et al*. 1995) on mate-guarding behaviour, all focal pairs consisted of warblers between 3 and 6 years of age (during which there are no age effects on mate guarding; Komdeur 1996b) that had successfully produced a fledgling before the onset of this study. All territories were checked weekly for initiation of nest building by following females for 30 min (Komdeur 1991). During the nest-building period, which lasts up to 19 days (Komdeur 1991), nests were checked daily for the presence of an egg. We used only pairs without helpers, and the eventual clutch size of all pairs was one egg. Focal pair observations were conducted daily during the female's fertile period (starting five days before egg laying and lasting until the day of egg laying). This period was based on the evidence that in the Seychelles warbler successful within- and extra-pair copulations — defined as when the female allows the male to mount for a few seconds (see also Arvidsson 1992) — only occurred during this narrow time-window (Komdeur *et al*. 1999). This defined fertile period concurs with studies on several other bird species showing that most EPFs arise from copulations occurring during this period (see Birkhead & Møller 1992; Colegrave *et al*. 1995; Lifjeld *et al*. 1997; Birkhead 1998). The warblers are remarkably tame and easily observed. Every day, between 30 min after sunrise (approximately 06.30 h local time) and 11.30 h the male and female of each pair were observed simultaneously and continuously for 30 min; one observer watched the female while the other kept track of the male. Which observer would follow each sex was decided by the toss of a coin. The 30-min observation interval of each pair was randomized each day during the morning observations. When both members of the pair were located, the following four measures were recorded. (i) Mate-guarding behaviour, which is the distance between pair members, was recorded as less than or greater than 5 m (the maximum distance at which both warblers can be kept in view), at 30-s intervals. Proximity was analysed as the percentage of observations where the male and female were within 5 m of each other when the female was off the nest. This measure has previously been shown to be positively correlated with the percentage of mate following by the male (Komdeur *et al*. 1999). In the remainder of this study, we refer to proximity rather than mate guarding to avoid ascribing an inferred purpose to an objective measure, as the purpose of our study is to show whether close proximity acts as an efficient mate-guarding technique. (ii) During the mate-guarding observation period, which coincides with the focal female's fertile period starting 5 days before egg laying and lasting until the day of egg laying (Komdeur *et al*. 1999), male intrusions were measured as territorial or aggressive incidents between pair male and intruding males. (iii) Successful copulations were recorded, as was the identity of the copulating male, where possible. (iv) For each pair, we accurately determined the number of reproductive males (i.e. over 8 months of age; Komdeur 1996b) present in adjacent territories during the observation period, because this was positively correlated with the average time a male spent in proximity (mate guarding) to his mate' before egg laying (Komdeur *et al*. 1999; Komdeur 2001).

After hatching, a blood sample was taken from the nestling for parentage analyses (approximately on day 2). Blood samples (*c*. 15 μL) were collected by brachial venipuncture, diluted in 800 μL of 100% ethanol in a screw-cap microfuge tube and stored at room temperature. The blood sampling of parents took place outside the females' fertile period so as not to interfere with the observations.

Experimentally terminated mate guarding and paternity

In total, 40 nests of different breeding pairs were followed from before egg laying. Of these, 20 were used as experimental nests, in which the male was induced to stop mate guarding, and 20 as control nests. We induced the male to stop mate guarding by placing a model egg in the empty nest during the period when the female was observed lining the nest (between 1 to 4 days before the first egg was laid; Komdeur *et al*. 1999). Model eggs were made of wax and painted with a waterproof marker to match the pattern of Seychelles warbler eggs. The models were placed in the nest during the early morning (approximately 06.30 h local time) to mimic the laying time of real eggs (when males would be expected to inspect their nests for a newly laid egg; Komdeur 1991). In a previous study, it was found that warbler females laid between 06.00 h and 07.00 h local time (Komdeur 1991). The presence or absence of the model egg and real egg was checked daily during the observation period. The model-egg experiments were conducted on 20 pairs, and these were all successful, i.e. model eggs were 'laid' within the female's fertile period and the females then laid an egg in the nest containing the model egg. Pair birds were observed on the days prior to adding the model egg, and on the days between adding the model egg and the laying of the real egg, using the protocol described above. As a control, we performed the same intensity of actions (disturbance) around the nests of an additional 20 pairs, but without adding a model egg. To minimize interference with experimental pairs, these control pairs were at randomly chosen synchronized nests (i.e. nests that were active at the same time) located at least two territories away from the experimental pairs. Immediately (*c*. 1 h) after placing the model egg, males stopped mate guarding (Komdeur *et al*. 1999, see Results below) and copulating with their mates, despite the fact that the females were still soliciting within-pair copulations (Komdeur *et al*. 1999). None of the experimental and control nests were deserted after performing these actions, and in all nests, a natural egg was laid. Six to 14 days before the addition of the model egg, seven control males and five control females, and nine experimental males and six experimental females, were trapped to take measurements of mass and tarsus length. Body mass was measured to the nearest 0.1 g, and tarsus length was measured to the nearest 0.1 mm. In the experimental group, eight eggs hatched, 10 were depredated and two failed to hatch. Of the experimental nests where the egg did hatch, a real egg was either laid 4 days ($N = 2$), 3 days ($N = 3$), 2 days ($N = 2$), or 1 day ($N = 1$) after the addition of the model egg. On average, the model egg was laid 2.75 (22/8) days before egg laying. In the control group, nine eggs hatched while 10 were depredated and one failed to hatch. The fraction of eggs that hatched in the nonguarding experimental group was similar to that in the guarding control group (8/20 vs. 9/20); therefore, these experimental nests seem to represent an unbiased sample.

Parentage analyses

DNA was extracted and genotypes were identified for all individuals using 14 polymorphic microsatellite markers previously isolated in the Seychelles warbler (Richardson *et al*. 2001). Parentage was determined for all nestlings (for methodology see Richardson *et al*. 2001) and was conducted without knowledge of the origin and the manipulation status of the warbler pairs. The extra-pair young identified this way have been further confirmed using a full Bayesian probability model that incorporated demographic data and the estimation of genotype scoring error rates (Hadfield *et al*. 2006; in contrast, a high proportion of the earlier assignments of the paternity of extra-pair young were not supported, but they are not used here). We compared the frequency of extra-pair young in the nine control nests to that in the eight experimental nests.

Data analyses

The focal pairs observed in 1996 and 1997 were all different, thus avoiding the potential problems of pseudoreplication between years. Observations were related to the laying of the first real egg (day 0). For each variable, we calculated a mean daily value across all pairs from day –5 to day 0. Furthermore, we calculated the change in proximity as proximity observed on the day the model egg was 'laid' minus proximity observed on the previous day. For the control pairs, we calculated the change in proximity as the proximity measured on days –5 and –3 (before day –2, the median 'laying' day of the model egg) minus the proximity measured on days –2 and –1. The average number of males in neighbouring territories was similar for control and experimental groups (mean 2.7 ± 0.7 and 3.3 ± 0.7, respectively; $t = 0.68$, d.f. = 15, $P = 0.55$). Most variables deviated from normality; therefore, nonparametric statistics were used for most of the analyses. In parametric tests, the proximity intensity values, which were not normally distributed, were angular transformed. Binary logistic regression analysis was used to test the effects of multiple independent terms on the probability of extra-pair fertilization. The number of neighbouring reproductive males was included, as this has previously been shown to increase the number of successful EPCs gained by a female ($r^2 = 0.50$, $N = 20$, $P < 0.001$; Komdeur *et al*. 1999). The year of nesting and the average proximity intensity across all the days during the female's fertile period (which differs significantly between control and experimental males) were also included in the analysis. Year of breeding was entered as a fixed factor; all other terms were entered as variables. The minimum adequate model is presented following parsimonious streamlining (the stepwise backwards removal of nonsignificant terms) of the original model. The *Wald* test statistic is given. The influence of the onset of experimentation (the number of days during the fertile period for which mate guarding was artificially stopped) on paternity was analysed separately because studies of sperm usage in birds indicate that most EPFs arise from copulations occurring close to egg laying (reviewed in Birkhead & Møller 1992). Statistical analyses were performed using SPSS version 12.0.1 (SPSS 2001). Mean values are expressed with standard errors; probability values are two-tailed, and the level of significance was set at $P < 0.05$.

Results

Age and biometrics of Seychelles warblers of treatment groups

There was no difference between the average age, tarsus length, or body mass of control and experimental males, and of control and experimental females ($P > 0.5$ in all cases; data not shown). This suggests that individuals of each treatment group were of similar quality and that the results of the experiment were unlikely to be influenced by quality differences of individuals present in the treatment groups.

Male mate guarding and paternity in the control group

Of the nine nestlings from the control nests, four (44%) were not sired by the female's social mate. This frequency of extra-pair young was similar to that observed for this population in other years (40%). In all nine cases, the pair female present on the territory was the genetic mother of the nestling produced in that territory. Males and females stayed in close proximity (< 5 m) to each other for 51.9 ± 6.2% of the time during the fertile period. Males who spent more time in proximity to their mate had a lower probability of having an extra-pair nestling in their nest (Fig. 1). In total, nine male intrusions were recorded, and the probability of having an extra-pair nestling in that territory was positively associated with the male intrusion rate per territory when tested in a univariate regression (*Wald* = 4.56, d.f. = 1, $P = 0.033$).

Experimentally terminated mate guarding and paternity

The average time that experimental males spent in close proximity to their mates in the days before the introduction of the model egg was similar to that of control males in the corresponding period (mean time in close proximity: 51.1 ± 2.1% vs. 50.4 ± 2.7%, Wilcoxon signed-rank test $Z = 0.482$, $N = 17$, $P = 0.63$). The amount of time an experimental male spent in close proximity to his mate immediately after adding the model eggs was dramatically reduced compared to the control males (8.1 ± 0.6% vs. 53.3 ± 2.5%, $Z = 3.470$, $N = 17$, $P = 0.001$). The change in proximity on the day the model egg was introduced compared with that on the day before was significantly higher for experimental males than for control males (–43.0 ± 2.1% vs. 2.9 ± 3.4%, $Z = 3.488$, $N = 17$, $P < 0.001$). Moreover, the difference in the change in proximity between experimental and control males after addition of the model and real eggs, respectively, was similar (–43.0 ± 2.1% vs. –44.4 ± 2.4%, $Z = 0.483$, $N = 17$, $P = 0.629$), indicating that experimental males were not reacting differently, in terms of mate guarding behaviour, to the appearance of the model egg than they would to a natural one. On average, across all the days during the female's fertile period, the experimental males spent almost half the amount of time in close proximity to their female compared to the control males (27.5 ± 3.0% vs. 51.9 ± 2.1%, $Z = 3.466$, $N = 17$, $P = 0.001$). Furthermore, the rates of male intrusions and EPCs during the female's fertile period were significantly higher for the experimental males than for the control males (mean intrusions per hour 0.95 ± 0.13 ($N = 8$) vs. 0.40 ± 0.13 ($N = 9$), $Z = 2.49$, $P = 0.013$; mean EPCs per hour 1.25 ± 0.25 ($N = 8$) vs. 0.22 ± 0.12 ($N = 9$), $Z = 2.84$, $P = 0.005$). The placement of a fake egg in the nest did therefore appear to reduce mate guarding significantly in the Seychelles warbler. However, the rates of within-pair copulations during the female's fertile period were similar for the experimental and the control males (mean per hour 0.36 ± 0.16 ($N = 8$) vs. 0.50 ± 0.19 ($N = 9$, $Z = 0.41$, $P = 0.68$). The reason that the rates of within-pair copulations do not differ between experimental and control males is because both groups of

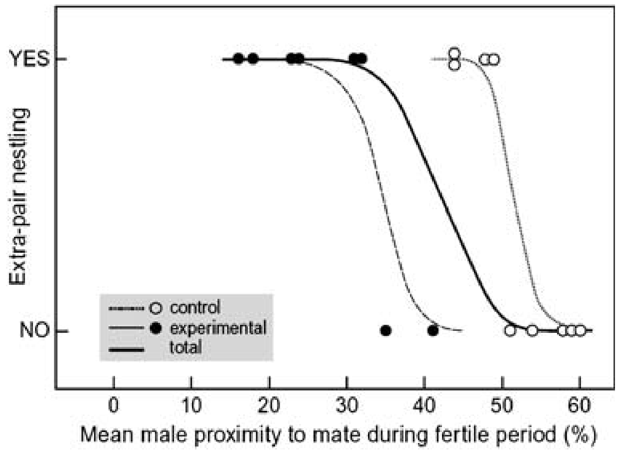

Fig. 1 The influence of proximity of the male to his mate, as measured by the average percentage of time the male spent within 5 m of his mate during her fertile period, on the probability that a nest would contain an extra-pair nestling in the Seychelles warbler (control nests: *Wald* = 6.71, d.f. = 1, $P = 0.010$; probability extra-pair nestling = $1/(1 + e^{-z})$; $z = -17.09 * \text{proximity} + 854.28$; experimental nests: *Wald* = 9.00, d.f. = 1, $P = 0.003$; $z = -11.40 * \text{proximity} + 381.71$; control and experimental nests combined: *Wald* = 8.58, d.f. = 1, $P = 0.003$, $z = -0.148 * \text{proximity} + 6.76$).

males were compared across the entire fertile period of the female (thus for experimental males this means some days with mate guarding and within pair copulations and some days without mate guarding and no within-pair copulations.

All of the eight nestlings from the experimental nests were produced by the focal female on the territory, but six (75%) were not sired by the female's social mate. The experimental termination of mate guarding did appear to increase the probability that the nest would contain extra-pair young; the percentage of extra-pair young in experimental nests was almost twice as high as that in control nests, but due to the small sample size, there was no statistical power to detect a real difference of that magnitude (75% vs. 44%, respectively; Fisher's exact one-tailed test, $P = 0.218$). However, within the experimental group, the fact that we were unable to exactly predict the egg-laying day of each female meant that the timing of the fake egg being placed in the nest differed among experimental nests (from day –1 to –4). Consequently, the number of days for which the experimental nest males guarded during their females' fertile period also differed between nests, and this could be predicted to affect their chance of being cuckolded. For experimental males, the male intrusion rate ($r^2 = 0.65$, $N = 9$, $P = 0.016$) and the probability of having an extra-pair nestling in their nest (Fig. 2) were each positively associated with the number of days during the fertile period for which mate guarding was artificially stopped.

The experimental and control groups can be regarded as a continuum with the control birds having a full 5-day period of mate guarding and the experimental birds having a 4- to 1-day mate-guarding period. By combining both groups in an analysis, we can investigate more accurately the effect that the number of mate guarding has on the probability of having an EPF. When both control and experimental males were combined, the average time a male spent mate guarding during the female's fertile period was negatively associated with male intrusion rate ($r^2 = 0.59$, $N = 17$, $P = 0.001$) but not with number of neighbouring males ($r^2 = 0.03$, $N = 17$, $P = 0.51$). There was a significant negative relationship between average time spent mate guarding and the probability of having an extra-pair young (Table 1, Fig. 1). The year of study and the number of neighbouring males were not included in the final model (Table 1). Given that mate guarding time and intrusion rate were so tightly correlated, it was no surprise that the probability of having extra-pair young was positively correlated with the male intrusion rate (*Wald* = 10.70, d.f. = 1, $P = 0.001$). For the experimental males, and for control and experimental males combined, the probability of having an extra-pair nestling in the nest was negatively associated with the number of days spent mate guarding during the female's fertile period (Fig. 2).

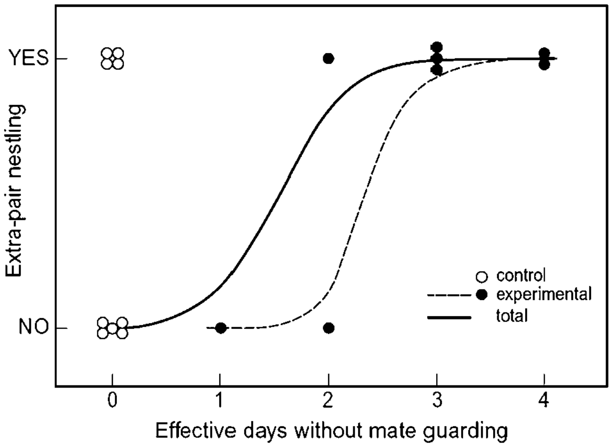

Fig. 2 The influence of the number of effective days without mate guarding days on the probability that a nest would contain an extra-pair nestling in the Seychelles warbler (experimental nests: *Wald* = 6.23, d.f. = 1, $P = 0.013$; probability extra-pair nestling $= 1/(1 + e^{-z})$; $z = -20.27 * \text{days} - 40.54$; control and experimental nests combined: *Wald* = 4.35, d.f. = 1, $P = 0.037$; $z = -0.81 * \text{days} - 0.50$).

Discussion

In the Seychelles warbler, as in many other passerine species, males maintain close proximity to their social partner during the female's fertility period (see above and Komdeur *et al.* 1999). Most observational studies and a few experimental studies of this behaviour support the hypothesis that this proximity functions to prevent EPCs (reviewed in Birkhead 1998). By remaining near his mate, a male is able to physically prevent other males from approaching and copulating with the subject female. However, the effectiveness of such mate guarding in preventing EPFs has been unclear. First, in many species unguarded

Table 1 Logistic regression analyses of the effects of average proximity of males to their mates (as measured by the average percentage of time the male spent within 5 m of his mate during her fertile period), year of study, and number of neighbouring males on the probability that a nest would contain an extra-pair young in Seychelles warblers (data set combined for control ($N = 9$) and experimental ($N = 8$) pairs). Explanatory variables that remained in the model after the stepwise backwards removal of nonsignificant terms are highlighted in bold

Explanatory terms	*Wald*	d.f.	*P*
Average proximity	**–6.95**	**1**	**0.008**
Year of study	2.66	1	0.103
Number of neighbouring males	0.67	1	0.412

d.f. = 1, 16; $r^2 = 0.53$, *Wald* = –6.95.

females are able to reject unwanted EPCs (e.g. Björklund *et al*. 1992; Komdeur *et al*. 1999). This suggests that not all unguarded females will engage in EPCs. Second, it may be relatively easy for females to evade the mate-guarding efforts of their mates, such that male mate-guarding behaviour has little effect on the female's ability to gain EPCs (Gowaty & Bridges 1991; Chek & Robertson 1994; Kempenaers *et al*. 1995; Gray 1996) or EPFs (Wagner *et al*. 1996; Currie *et al*. 1998; Johnsen *et al*. 1998). Several studies found no relationship between mate-guarding behaviour and EPFs (Gowaty & Bridges 1991; Kempenaers *et al*. 1995; Riley *et al*. 1995; Krokene *et al*. 1996; Schleicher *et al*. 1997), and two studies showed that mate guarding may even be positively correlated with levels of EPF (Wagner *et al*. 1996; Johnsen *et al*. 1998). These last, apparently counterintuitive, results may be explained if certain confident males do not have to mate-guard, because their females are unlikely to seek EPFs, while other, unconfident males mate-guard intensively, because their females are seeking EPFs. These studies also suggest that mate guarding by males may be ineffective at deterring EPF in, at least, some species. This may not be surprising, given that a single well-timed copulation can potentially fertilize many young (Birkhead & Møller 1992; Colegrave *et al*. 1995), and there is evidence to suggest that males deliver unusually large numbers of sperm during EPC (Birkhead *et al*. 1994; Birkhead & Fletcher 1995; Birkhead & Petrie 1995). Furthermore, the ability to guard the mate can be confounded by other factors, for example individual quality, if it affects a male's ability to guard, or a female's ability to evade her mate (Gowaty 1996; Lifjeld *et al*. 1994; Bouwman & Komdeur 2005). Therefore, experimental tests are essential to properly investigate the function and effectiveness of mate guarding (Björklund & Westman 1983; Krokene *et al*. 1996; Komdeur *et al*. 1999; Chuang-Dobbs *et al*. 2001).

The present study on the Seychelles warbler is the first to provide explicit experimental evidence that mate guarding by males functions as a paternity guard. First, mate guarding did reduce the frequency of extra-pair fertilizations. In our study, we found a negative relationship between the amount of time the male spent in close proximity to his female during her fertile period and the probability that a nest would contain an extra-pair nestling. This result supports the hypothesis that proximity can be used as an accurate index of mate guarding and that this mate guarding is effective in reducing extra-pair paternity. In addition, in the experimental group, the probability of having an extra-pair nestling in the nest was positively associated with the number of days (and hence the proportion of overall time) during the fertile period for which mate guarding was artificially stopped. Such experiments — in which the consequences of inducing free-living males to stop mate guarding are measured — have not been performed before.

The results of this experiment are unlikely to have been influenced by male or female quality, because pairs were assigned to treatment groups at random (see Materials and methods), or by age or breeding experience, because all pairs fell within the same age categories and were all experienced breeders. Furthermore, we realize that it is hard to distinguish between the direct mechanisms that reduce or increase EPP, and that there are various alternative pathways which may have resulted in our study's results. The increase in extra-pair paternity with reduced mate guarding by the resident male might be because these experimental males do not physically prevent intruding males from interacting with the female. Alternatively, it may be the effect of females showing adaptive responses to changes in payoffs arising from reduced male proximity. A third alternative is that, following the manipulations, the female may be more likely to solicit EPCs or accept EPCs because her social male is not mate guarding or responding to her solicitations for within-pair copulations (Komdeur *et al*. 1999). The latter explanation may have been the case because level of within-pair copulations for experimental males tended to be lower (0.36) than for control males (0.50). However, due to the small statistical power (small sample sizes), this was not significant. Future studies are needed to elucidate between the alternative mechanisms.

Only three other experimental studies have investigated the effectiveness of male mate guarding on EPFs; however, these studies used procedures very different from our own. Overall, the temporary detention of males from their territories during their mates' fertile period did increase the probability that a brood would contain extra-pair young in black-throated blue warblers (Chuang-Dobbs *et al*. 2001) but not in red-winged blackbirds (Westneat 1994) and wheatears (Currie *et al*. 1999). However, the complete experimental removal (albeit temporary) of males from the territory can lead to difficulties in interpreting the results of such studies. First, female copulatory behaviour may differ under the experimental conditions used because in some studies, males were removed from their territories for far longer periods (e.g. 24 h removals in Currie *et al*. 1999) than would be normal during the fertile period and this may simulate mate predation (Blakey & Norris 1994). Detention may disturb the pair bond and makes it difficult to distinguish extra-pair fertilizations from mate replacement (Blakey & Norris 1994). However, it is argued that the 1-h removal period used in the black-throated blue warblers makes mate replacement an unlikely explanation for the increase in EPFs (Chuang-Dobbs *et al*. 2001). Second, even though the short period of time for which males were detained should make mate replacement an unlikely explanation for the observed results, it could well be that male detention changes the cost-benefit trade-off involved in the gaining of EPFs by females. Females (or intruding males) may adjust their behaviour in response to the presence of a male rather than close mate guarding *per se*; for example, when a female is outside the view of her own mate, she can potentially cuckold her mate without paying any costs (e.g. reduced paternal care; Lifjeld & Robertson 1992; Westneat 1994; Sheldon 2002).

In an earlier study, we showed that mate guarding in the Seychelles warbler is energy-costly (Komdeur 2001). Although our results show that males can reduce the probability of EPF by guarding their mates, this does not indicate that males are in 'control' of fertilization in these species. Indeed, the high frequency of EPFs observed in this species, despite intense mate guarding, would suggest otherwise. Females may adopt behaviours to partially circumvent male mate guarding, and may also be able to reject copulation attempts by undesirable males. On a couple of occasions, Seychelles warbler females were observed to escape being in close proximity to their mate and then proceed to engage in a copulation with an extra-pair mate (J. Komdeur, personal observation). Eventually, the probability that a brood will contain extra-pair young is likely to be influenced by the behaviours and strategies adopted by both sexes (Gowaty 1996; Komdeur *et al*. 1999). This requires further testing. A recent study has shown that, in the Seychelles warbler, males of low major histo-compatibility complex (MHC) diversity are more likely to lose paternity in their nest than males with an average or high level of MHC diversity (Richardson *et al*. 2005). Whether this result is due to female choice or male competition or pre- or post-copulatory mechanisms is, as yet, unknown; however, one possibility worth investigating would be whether the MHC diversity of a male is correlated with his mate-guarding ability. Low MHC-diversity males may be in poorer condition and may therefore be less able to spend time and effort mate guarding.

In most bird species, fertilization occurs about 24 h before an egg is laid, and there is only a short period, about 15–30 min after ovulation (termed as the fertilization or insemination window, Cheng *et al*. 1983), in which the egg can be fertilized (Birkhead & Møller 1992). This means that extra-pair copulations during the period 24 h before egg laying have no chance of fertilizing the egg. Consequently, it could be argued that experimental males that were induced to stop mate guarding only 1 day before egg laying were irrelevant to the experiment (as the egg would already have been fertilized) and should be excluded from the analyses. The data from our study, although minimal, does support this view, as the one individual which was only prevented from mate guarding on the last day before egg laying did not lose his paternity. If this male is removed from the analysis, then six of the seven social males who were prevented from mate guarding (85.7%) lost their paternity. Although the exclusion of this male from the data set resulted in a higher percentage of extra-pair young in experimental nests (86% vs. 75%), this was not significantly different to that in control nests (44%; Fisher's exact test, $P = 0.145$).

Acknowledgements

We are grateful to Nature Seychelles for allowing us to work on Cousin Island and for providing accommodation and facilities during our stay. The Department of Environment and the Seychelles Bureau of Standards gave permission for fieldwork and sampling. We thank Ken Kraaijeveld, Femmie Kraaijeveld-Smit, Pim Edelaar and Karen Blaakmeer for help in the field, Marco van der Velde for help with genotyping in the laboratory, and two anonymous reviewers for their constructive comments on the manuscript. This work was supported by grants from the UK Natural Environment Research Council, from the Australian Research Council, by a NERC postdoctoral fellowship to DSR, and the Netherlands Organization for Scientific Research.

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