



Extra-pair paternity explains cooperation in a bird species

Indrikis A. Krams^{a,b,c,1}, Adèle Mennerat^{d,e}, Tatjana Krama^{c,f}, Ronalds Krams^{c,f}, Priit Jõers^g, Didzis Elferts^h, Severi Luoto^{i,j}, Markus J. Rantala^k, and Sigrunn Eliassen^d

^aDepartment of Zoology and Animal Ecology, Faculty of Biology, University of Latvia, Riga 1004, Latvia; ^bInstitute of Ecology and Earth Sciences, University of Tartu, Tartu 50409, Estonia; ^cDepartment of Biotechnology, Daugavpils University, Daugavpils 5401, Latvia; ^dDepartment of Biological Sciences, University of Bergen, Bergen 5020, Norway; ^eEDYSAN, CNRS, Université de Picardie Jules Verne, Amiens 80039, France; ^fChair of Plant Health, Estonian University of Life Sciences, Tartu 51006, Estonia; ^gDepartment of General and Microbial Biochemistry, University of Tartu, Tartu 51010, Estonia; ^hDepartment of Botany and Ecology, Faculty of Biology, University of Latvia, Riga 1004, Latvia; ⁱSchool of Population Health, University of Auckland, Auckland 1023, New Zealand; ^jSchool of Psychology, University of Auckland, Auckland 1010, New Zealand; and ^kDepartment of Biology, Section of Ecology, University of Turku, Turku 20014, Finland

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In many social animals, females mate with multiple males, but the adaptive value of female extra-pair mating is not fully understood. Here, we tested whether male pied flycatchers (*Ficedula hypoleuca*) engaging in extra-pair copulations with neighboring females were more likely to assist their neighbors in antipredator defense. We found that extra-pair sires joined predator-mobbing more often, approached predators more closely, and attacked predators more aggressively than males without extra-pair offspring in the neighboring nest. Extra-pair mating may incentivize males to assist in nest defense because of the benefits that this cooperative behavior has on their total offspring production. For females, this mating strategy may help recruit more males to join in antipredator defense, offering better protection and ultimately improving reproductive success. Our results suggest a simple mechanism by which extra-pair mating can improve reproductive success in breeding birds. In summary, males siring extra-pair offspring in neighboring nests assist neighbors in antipredator defense more often than males without extra-pair offspring.

cooperation | promiscuity | extra-pair offspring | birds | antipredator defense

Prey react to predators in different ways: by moving away, staying motionless waiting for the predator to leave, or summoning others to cooperatively attack or harass the predator (1, 2). Prey mob predators by emitting repeated, loud, and easily localizable calls and performing stereotyped movements that recruit more prey individuals to join the mobbing (3). Such behavioral patterns are considered adaptive antipredator strategies, and they occur in a variety of species (4, 5). Mobbing benefits prey by reducing hunting efficiency of the predator, either through distracting it or by driving it away (6, 7). At an individual level, mobbing also carries costs (8) as cooperative individuals spend time and energy and risk injury or death. Cooperative antipredator behaviors may hence be prone to freeloaders that benefit from collective defense but refrain from joining forces with others.

Cooperation in mobbing may be understood as byproduct mutualism when mobbing predators at nests of close neighbors directly affects protection of own offspring (9). Cooperation among neighbors breeding further apart may be explained as direct reciprocity (10–12) in which only cooperating individuals are supported by their neighbors in a tit-for-tat-like strategy (4, 13–15). Predator harassment entails a risk of injury and death (14, 16), while driving predators away is the main function of mobbing, which significantly decreases future risk of predation (6, 17). Cooperative actions are often more efficient in larger groups (1). Larger aggregations of breeders may also increase opportunities for male and female neighbors to interact, with extra-pair mating being more prevalent in dense aggregations (18) and among close neighbors (19, 20).

Extra-pair mating behaviors are widespread in nature, but the adaptive reasons of this reproductive strategy in females are far from being understood (18, 21). Extra-pair mating causes paternity uncertainty, which reduces the risk of infanticide in multimale groups (22, 23). Extra-pair mating may also shift male incentives from a sole focus on care at the nest to investing in activities that benefit close neighbors (18, 20). Since males potentially sire offspring in neighboring broods, they may benefit from providing antipredator protection and assist neighbors in mobbing activities. Field studies suggest a link between sex-related cooperative behavior, especially in the context of antipredator protection (7, 20, 24), clumped distribution of breeding individuals (25), and extra-pair mating (26–28).

Several recent studies have linked predation risk to the occurrence of extra-pair paternity: higher frequencies of extra-pair offspring have been reported in populations of Japanese great tit (*Parus minor*) that were naturally exposed to stronger nest predation pressures (29). In blue tits (*Cyanistes caeruleus*), whole-brood mortality due to predation is negatively associated with levels of extra-pair paternity (20). Studies manipulating the level of perceived predation risk found that repeated playbacks of predator calls delayed dawn singing in songbird communities (30) but had no effect on extra-pair paternity in blue

Significance

In many monogamous species, a substantial proportion of offspring is sired by other males than the one providing care at the nest. Although females often solicit extra-pair mating, the benefits of extra-pair copulations to females are not fully understood. In this study on pied flycatchers, we tested whether extra-pair paternity in neighboring nests may have affected the likelihood that males helped defend extra-pair offspring against predators. We found that extra-pair sires were more often engaged and invested more heavily in cooperative predator defense than males without extra-pair offspring. For female flycatchers, extra-pair mating may thus be an adaptive strategy for enhancing offspring survival and breeding success.

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¹To whom correspondence may be addressed. Email: indrikis.krams@ut.ee.

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tits (31) or great tits (*Parus major*) (32). In contrast, blue tit females that were exposed to predator models during the period of peak fertility intensified their promiscuous behavior along with or as a consequence of the disruption of morning copulatory routines within the pair (33). In some cases, cooperative investment in increased safety of the whole neighborhood comes at the expense of paternity loss in own brood for some males, while concomitantly increasing the reproductive output of other males (34). However, the specific link between extra-pair paternity and investment in neighborhood defense has never been explicitly addressed.

In this study, we tested whether the propensity of male pied flycatchers (*Ficedula hypoleuca*) to engage in antipredator defense at neighboring nests (9) is associated with the presence of extra-pair offspring in that nest. The pied flycatcher often breeds semicolonally, and to provide an ecologically valid breeding environment for the birds, we created experimental neighborhoods in the form of triplets of nest boxes in close proximity. Males in focal nests (A) were allowed to be near fertile females and thus potentially sire extra-pair offspring in neighboring nests (B and C) but not the other way around. Using stuffed predators at nests B and C, we monitored whether focal males (nest A) joined antipredator mobbing at these neighboring nests. Genotype-based paternity assignment enabled us to test whether participation in antipredator defense varied according to extra-pair paternity within neighborhoods.

Results

A total of 44 experimental triplet nest boxes were included in the study. Only nest box A males assisted their neighbors at B and C nest boxes; nest box A females, although present and defending their own nest boxes, were never observed being involved in mobbing events at neighboring nest boxes. Within each 10-min trial, nest box A males were observed attending one neighboring nest box at most, mobbing predators at either nest box B or C. Nevertheless, nest box A males with offspring in both nest boxes B and C did defend both nest boxes B and C across trials.

There were marked differences across groups in the frequency with which A nest box males joined mobbing at neighboring nest boxes (Fig. 1A). When there were no extra-pair offspring at neighboring nest boxes ($n = 17$), three nest box A males assisted their neighbors during both trials, and two males assisted only once. The other 12 males remained at nest box A. When there were extra-pair offspring in one neighboring nest box ($n = 14$), all nest box A males assisted their neighbors during both trials, and all but one attended the nest box containing their extra-pair offspring. When there were extra-pair offspring in both neighboring nest boxes ($n = 13$), not only did all nest box A males assist in mobbing during both trials, but they all switched nest boxes between trials, attending both nest boxes B and C over the course of the two trials.

Nest box A males without extra-pair offspring at nest boxes B or C stayed significantly further away from predators (98.32 ± 17.04 m, mean \pm 95% CI) than nest box A males with extra-pair offspring in one (14.31 ± 12.13 m) or two (6.09 ± 0.74 m) neighboring nest boxes (*glmer*, $F_{2,41} = 36.922$, $P < 10^{-4}$; Table 1). The two latter groups did not differ significantly in their distance of approach to predator (Tukey post hoc test, $t_{41} = 0.839$, $P = 0.68$; Fig. 1B and Table 1). This result holds also when excluding those A males that did not mob (*SI Appendix*, Fig. S1; *glmer*, $F_{2,29} = 13.698$, $P = 0.05$; *SI Appendix*, Table S1).

Nest box A males with no extra-pair offspring in neighboring nest boxes mobbed at a significantly lower intensity (0.21 ± 0.17 , mean \pm 95% CI; mobbing score) than males with extra-pair offspring in either one (1.0 ± 0.15) or two (1.31 ± 0.18) nest boxes (*lmer*, $F_{2,41} = 35.772$, $P < 10^{-4}$; Table 1). The two

latter groups did not differ significantly in their mobbing intensity (Tukey post hoc test, $t_{41} = -2.147$, $P = 0.080$; Fig. 1C and Table 1, b). The same trend remains when excluding those A males that did not mob, although weaker and marginally significant (*SI Appendix*, Fig. S1; *glmer*, $F_{2,29} = 3.0102$, $P = 0.065$; *SI Appendix*, Table S1).

Mobbing intensity of males B and C did not differ significantly (*lmer*, $F_{2,41} = 0.7234$, $P = 0.49$; Fig. 1D).

Discussion

Our study suggests that engagement in antipredator defense at a neighboring nest depends on whether the male sires extra-pair offspring in that nest. Males with extra-pair offspring in neighboring nest boxes were more likely to assist their neighbors, mobbed predators more intensely, and approached predators closer than males without extra-pair offspring in neighboring nest boxes. We found that extra-pair sires attacked predators more frequently and more aggressively at the neighboring nest boxes with their extra-pair offspring than at nest boxes without extra-pair young. These findings support the idea that female extra-pair mating may enhance safety by recruiting more males to join forces in alarm-calling and mobbing of predators (18, 20).

While not directly manipulating male behavior, our study did manipulate the opportunity for males to attain extra-pair mating in the neighborhood: males in nest box A were present during the fertile period of nest B and C females. It is unlikely that males B and C might have been around during the fertile period of nest A females, as they were neither seen nor heard singing until a few days after B and C nest boxes were opened. Because extra-pair copulation was not manipulated directly, it remains possible that the association between extra-pair paternity and cooperative antipredator defense might be due, at least partly, to confounding factors. Nest A males that sired extra-pair offspring in neighboring nests did not differ in body size, body mass, antipredator defense at own nest, or clutch size at own nest (*SI Appendix*, Figs. S3–S5) from those not siring extra-pair offspring, but they were on average older (*SI Appendix*, Fig. S2). Intriguingly, similar links between male age and propensity to sire extra-pair offspring have been found in other small passerine birds (20, 35). Older and more-experienced males may be better at attaining extra-pair copulations, and/or they may be preferred by females (20, 35). Our results suggest that one reason why females may preferentially seek extra-pair copulations with older male neighbors may be that these males are better at providing defense for their extra-pair offspring.

For prey individuals, group size is important when mobbing predators, because larger groups repel predators more efficiently and thus provide better protection (6, 36). Here, extra-pair paternity seems to facilitate the recruitment of neighbors in antipredator defense. Noticeably, the amount of mobbing performed by resident males (B and C) at their own nests did not vary according to the extra-pair status of those broods (*SI Appendix*, Fig. S6). This implies that the assistance that extra-pair (A) males provided resulted in overall higher levels of collective protection at B and C nests, thus enhancing safety for females and their broods.

We also found that males always arrived alone at mobbing events, whereas in previous studies, pairs of birds came to assist their neighbors together (14, 37). In earlier studies, nest boxes were placed closer together than in the current study, and this proximity may have facilitated cooperation as a form of byproduct mutualism, whereby the presence of a predator at a neighboring nest box represented a direct threat to the home nest. This may also explain why, in those studies, both males and females joined the mob (14, 37). For the current experiment, good relationships may be based more clearly on previous mating encounters, giving stronger incentives for males to join their

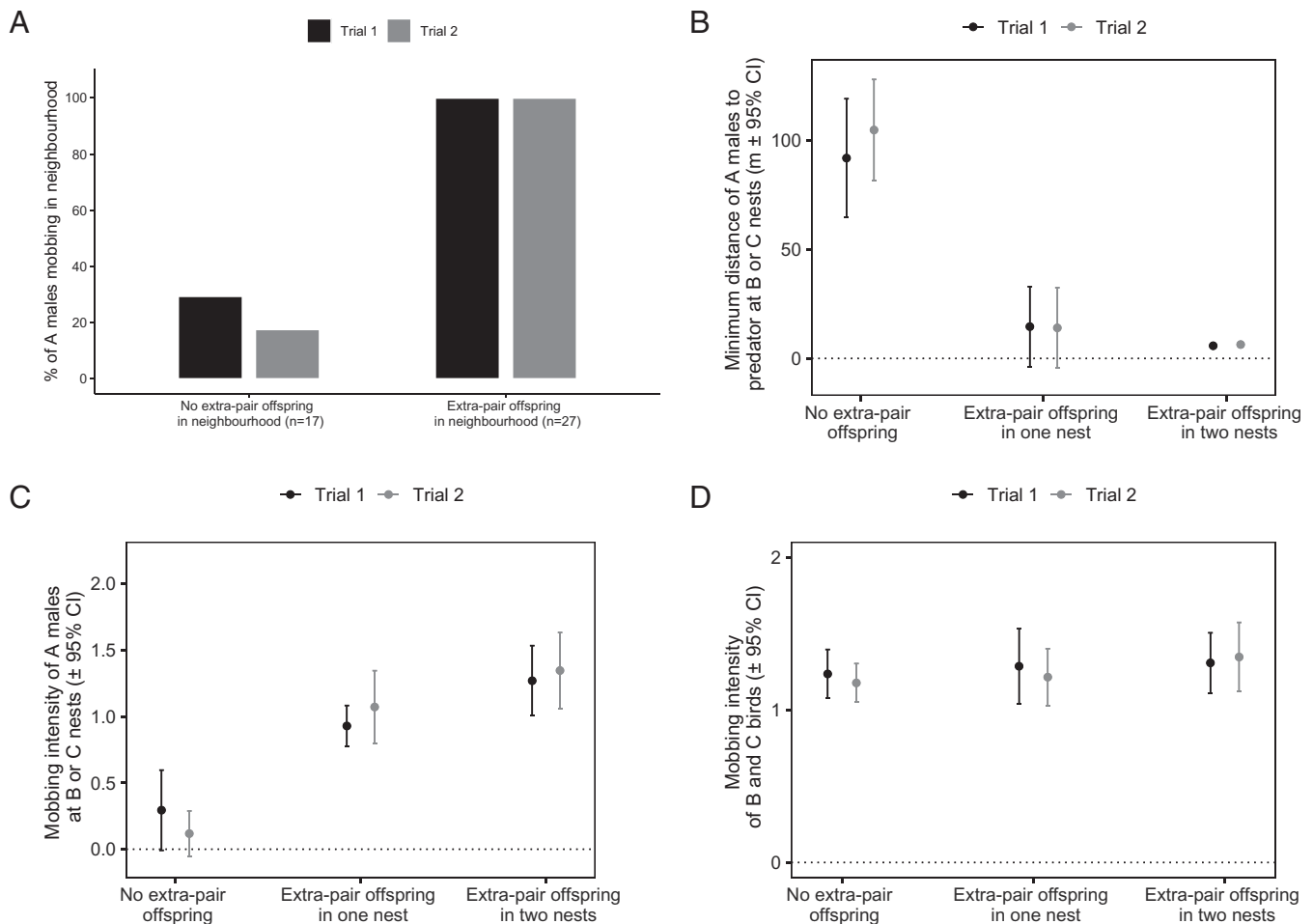


Fig. 1. (A) Proportion of nest box A males assisting in mobbing at neighboring nest boxes (B or C), in relation to whether they sired extra-pair offspring in at least one of those nest boxes. (B) Minimum distance of approach (meters) by A nest box males to the predators placed near neighboring nest boxes (B or C), in relation to whether they sired extra-pair offspring in these nest boxes. (C) Intensity of mobbing behavior by nest box A males to the predators placed near neighboring nest boxes (B or C), in relation to whether they sired extra-pair offspring in these neighboring nest boxes. (D) Average intensity of mobbing behavior by nest box B and C birds to the predator placed near their own nest boxes in relation to whether nest box A males sired extra-pair offspring in the neighborhood. B, C, and D represent all males, including those that stayed at their own nests and did not mob. Refer to *SI Appendix, Fig. S1* for a representation of only those males that participated in mobbing.

neighbors if they potentially sire offspring in these nests (18), while the benefit–cost ratio for females of leaving their own nest unattended to mob at a nest further away might be too low.

In pied flycatchers (9, 14, 37) and red-winged blackbirds (*Agelaius phoeniceus*) (13), mobbing behavior has been shown to depend on previous actions of others in a reciprocal manner. In the current study, we induced shifts in timing of nest building in nest box triplets by preventing males B and C from establishing themselves in the neighborhood during the fertile period of nest box A females. The early-arriving focal males (A) had a relatively high extra-pair paternity rate, and the likelihood of their assisting at neighboring nests was linked to presence of extra-pair offspring. Our results, however, do not provide information on whether extra-pair paternity and mobbing behaviors are based on previous social encounters with neighbors (9, 13, 14).

Extra-pair mating may affect decisions to assist or desert a neighbor, but it does not preclude a role for reciprocal altruism. On the contrary, these mechanisms may be linked. Several studies have reported that long-term relationships among territorial neighbors benefit breeders (25). Great tits were more likely to join forces to defend the nest of neighbors if those individuals were breeding close by in previous years. A recent

study showed that associations during the winter months predicted future social and extra-pair relations in great tits (38). Individuals that were strongly associated during winter tended to establish nests close together at the breeding ground (38). Neighbors are known to dominate as extra-pair sires (20, 39), but correcting for this, familiar individuals were still more likely to have extra-pair young than unfamiliar neighbors (38). Our findings on pied flycatchers suggest a simple mechanism by which extra-pair mating can improve reproductive success in breeding birds and more generally that paternity distributions are important for explaining sociality and the evolution of cooperative behaviors.

Materials and Methods

Study Site and Birds. The pied flycatcher is a small passerine bird, which often exhibits semicolonial breeding behavior (37), builds its nests in tree holes and artificial nest boxes, and breeds in most of Europe. It is a migratory bird that winters mainly in tropical Africa. The study was carried out in a population of pied flycatchers breeding in nest boxes near Krāslava, south-eastern Latvia (55°90'N, 27°19'E), where pied flycatchers have been studied since 1980 (40). The study was conducted in May and June 2006 through 2011 and 2013 through 2016, but none of the birds were included for more than a single breeding season.

Table 1. Relationship between mobbing behavior and the presence of extra-pair young (EPY) at neighboring nests, for all A males included ($n = 44$)

(a) Distance to predator

| Output from <i>glmer</i> | Estimate | SE | <i>t</i> | <i>P</i> |
|--------------------------------|----------|--------|----------|------------|
| Intercept | 0.0330 | 0.0140 | 2.360 | 0.018 |
| 1 neighbor nest with EPY | 0.1166 | 0.0213 | 5.478 | $<10^{-4}$ |
| 2 neighbor nests with EPY | 0.1360 | 0.0216 | 6.293 | $<10^{-4}$ |
| Tukey's pairwise contrasts | Estimate | SE | <i>z</i> | <i>P</i> |
| 0 nest versus 1 nest with EPY | 0.1166 | 0.0213 | 5.478 | $<10^{-4}$ |
| 0 nest versus 2 nests with EPY | 0.1360 | 0.0216 | 6.293 | $<10^{-4}$ |
| 1 nest versus 2 nests with EPY | 0.0195 | 0.0232 | 0.839 | 0.678 |

(b) Mobbing intensity

| Output from <i>lmer</i> | Estimate | SE | <i>t</i> | <i>P</i> |
|--------------------------------|----------|--------|----------|------------|
| Intercept | 0.2059 | 0.0902 | 2.282 | 0.075 |
| 1 neighbor nest with EPY | 0.7941 | 0.1343 | 5.915 | $<10^{-4}$ |
| 2 neighbor nests with EPY | 1.1018 | 0.1371 | 8.039 | $<10^{-4}$ |
| Tukey's pairwise contrasts | Estimate | SE | <i>z</i> | <i>P</i> |
| 0 nest versus 1 nest with EPY | 0.7941 | 0.1343 | 5.915 | $<10^{-4}$ |
| 0 nest versus 2 nests with EPY | 1.1018 | 0.1371 | 8.039 | $<10^{-4}$ |
| 1 nest versus 2 nests with EPY | 0.3077 | 0.1433 | 2.147 | 0.080 |

(a) Minimal distance of approach to predator; (b) mobbing intensity (*Materials and Methods*).

Nest boxes were arranged in triplets mounted in a triangular spatial pattern and were placed in young pine forests (45 to 65 y) with sparse understorey. Each nest box contained one male and one female pied flycatcher and their offspring (5.72 ± 1.56 fledglings, mean \pm SD; 6.14 ± 1.39 eggs, mean \pm SD). The distance between nest boxes in triplets was ca. 125 m (mean \pm SD = 125.41 ± 3.22 m). This is far enough to exclude the possibility that antipredator responses of the focal males at neighboring nest boxes are driven by byproduct mutualism, which occurs when the distance between neighboring nest boxes is ca. 25 m (9). The triplets of nest boxes were separated by at least 550 m ($1,360 \pm 762.05$ m, mean \pm SD).

Adult flycatchers were not banded with metal or plastic rings before behavioral trials. It was easy to identify every bird because they marked themselves by touching a piece of nonwaterproof, ink-saturated, foam rubber attached to the entrance of their nest boxes 2 to 5 d before behavioral trials (14, 25).

We are not aware that any of the male pied flycatchers in the study were socially polygynous. We are, however, confident that all females in nest boxes A, B, and C were primary ones and not widows, because their male partners were constantly observed in the vicinity of their nest boxes during nestling period while providing parental care and antipredator protection. Secondary females can only partially compensate for the absence of a male, which leads to secondary females raising fewer offspring.

Experimental Design. In early April, we opened the entrance of one nest box (A) in each triplet. Upon arrival of the first females, we checked the A nest boxes daily to assess the start of nest building and first egg laying. The rest of the nest boxes (nest boxes B and C) were closed until female A laid her fourth egg. Thus, we had an experimental setup with one older and two younger nests in each nest box triplet. The experimental design made it possible for males in nest box A to sire extra-pair offspring in nest boxes B and C. Males in nest boxes B and C did not have this opportunity as males B and C arrived and established their territories when the eggs in nest box A had been laid. The offspring of males in nest box A could hence be distributed in three ways: extra-pair offspring in both nest box B and C, extra-pair offspring in only one nest box (either B or C), or no extra-pair offspring in neighboring nest boxes.

Behavioral Trials. We started behavioral trials when nestlings in nest boxes B and C were at least 5 d-old and nestlings in nest boxes A were ca. 12 to 14 d-old. Two stuffed tawny owls (*Strix aluco*), a common predator of pied flycatchers in Europe (41), were simultaneously presented near nest boxes B and C. The presence of tawny owls significantly affects the behavior of small passerine birds, including the cavity-nesting birds (9, 14, 37), because the owls readily depredate nestlings if the entrance of the cavity or a nest box is large enough to reach them. The owls were mounted on a pole on a small platform 1.3 m above ground. They were installed when no pied flycatcher was

detected nearby and were positioned c. 1.0 m from the nest box. The owls were looking toward the nest. We presented the owls at nest boxes B and C and monitored the behavior of males and females in nest boxes A, B, and C. Nest box A birds had the opportunity to assist their neighbors but had to make a choice between joining nest box B or C birds. We were specifically interested in whether nest box A males would assist their neighbors based on the presence of extra-pair nestlings in those nest boxes. Importantly, the presence and location of extra-pair offspring was not known at the time behavior was assessed, thus reducing the risk of observational bias.

As soon as the tawny owls were discovered by nest owners, we began documenting the mobbing response of the nest owners and their neighbors for 10 min. Pied flycatchers collect the food near their nest boxes; hence, males and females arrived at the predator decoy within 1 to 2 min at the latest. We recorded the occurrence of nest box A birds joining birds from nest boxes B and C, their minimum distance of approach (in meters), and the intensity of mobbing by nest box A birds as well as nest owners (B and C birds). While measuring the minimum distance from the predator, we evaluated the shortest distance of the focal bird to the predator during each trial. Before conducting the trials, we marked nearby tree trunks and branches, which allowed us to more accurately estimate the birds' approach distances. We considered neighbors to have assisted the nest owners if they appeared in the vicinity of the nest (<30 m). We scored the intensity of mobbing responses as follows: 0 points, no response: flycatchers investigate the predator from a distance usually without any alarm calls while continuing activities such as foraging or singing; 1 point, weak response: involves frequent approaching and retreating to/from a predator—alarm calls are given often but not continuously; 2 points, intermediate response: where the birds tend to be close to the predator, and they restlessly move around the object of alarm by bowing, pivoting, tail-flicking, and hovering in the air in front of the predator and giving non-stop alarm calls; and 3 points, strong response: involves intense movements and displays including dive-attacks on the predator, and alarm calls are given continuously. The trials were repeated the next day for 10 min so that antipredator reactions were assessed twice for each experimental triplet. Whenever nest box A birds did not join mobbing at B or C nest boxes, the distance between nest boxes was used as the minimum distance of approach and the mobbing intensity was scored as 0.

Paternity Analysis. As soon as the behavioral trials were done, we caught all males and females and took blood samples from the adults ($n = 264$) and their offspring ($n = 755$) for paternity analysis. Blood samples (50 mg) were taken from the tarsal vein and were stored in SET buffer (0.015 M NaCl, 0.05 M Tris, and 0.001 M EDTA; pH 8) at -86°C (42). We also collected dead nestlings ($n = 55$) and unhatched eggs ($n = 3$), stored at -86°C , and used those tissue samples for paternity analysis.

Applied Biosystems MagMAX DNA Isolation Kit (Thermo Fisher Scientific, Waltham) was used to isolate the DNA from tissues and blood, following the manufacturer's protocol. PCR was done using five microsatellite DNA loci: Fhyu336, Fhyu234, Fhyu304, Fhyu453, and Fhyu448 (28, 43, 44). Applied Biosystems 3130xl Genetic Analyzer (Thermo Fisher Scientific, Waltham) was used to separate the PCR product fragments. The results of capillary electrophoresis were analyzed, and the sizes of the amplification products were done using GeneMapper 4.0 software (Thermo Fisher Scientific, Waltham).

Paternity was analyzed using Cervus version 3.0.7 software (Field Genetics, London, United Kingdom) (45). This software estimates a probability of paternity for each father-offspring pair. It assigns paternity according to the difference in the likelihood ratio scores between the most-likely and second-most-likely fathers (28). Five microsatellite loci were used to determine genotype and assign parentage with one mismatch allowed. Confidence was set at 95% level.

Statistical Analyses. To study the minimum approach distance of nest box A birds in all three experimental groups, generalized, linear mixed-effects model with gamma error structure as implemented in software R 3.5.0 (46) package *lme4* (47) was used. Nest ID was used as a random effect, as there were two repeated measurements for each nest box. Mobbing intensity of birds A, B, and C between the groups was analyzed using linear mixed-effects models. R package *emmeans* (48) was used to perform post hoc tests to compare estimated marginal means of experiment groups.

Data Availability. All data have been deposited to Zenodo repository, a publicly accessible database (DOI: [10.5281/zenodo.5590020](https://doi.org/10.5281/zenodo.5590020)).

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