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Direct fitness benefits of group living in a complex cooperative society of carrion crows *Corvus corone corone*

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The social behaviour of carrion crows varies between populations. In northern Spain cooperatively breeding groups form through delayed natal dispersal and/or immigration of individuals (usually males) into the territory. In this population, carrion crows therefore breed as either unassisted pairs, pairs with nondispersing 1–2-year-old helpers (nondispersers), pairs with immigrant helpers or mixed groups (pairs with both immigrants and nondispersers). We used a microsatellite-based genotyping system to determine the parentage of 57 nestlings (19 broods). Polygamous mating was involved in 26% of the broods and reproduction was shared among group members of both sexes in at least three groups. Immigrants of both sexes can therefore gain access to mates by living in a group, while reproduction is unlikely to involve nondispersers. This implies that nondispersers and immigrants gain different sorts of benefits from group living and helping at the nest. Our genetic data confirmed that nondispersers associated with their parents on the natal territory and therefore that delayed natal dispersal leads to family formation in the carrion crow. Polygamous mating was not found in groups without immigrants, suggesting that, in this population, breeders lose parentage in their brood when sociality is extended beyond the limit of the nuclear family.

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Cooperative breeding in birds is a reproductive system where more than two individuals provide care to the young. In cooperative species, social groups usually involve either delayed offspring dispersal (family-based societies) or formation of alliances among adult individuals (Brown 1987). However, more complex societies are not uncommon. In some species both retained offspring and adult birds recruited from outside the territory are found in a social group (Whittingham et al. 1997; Li & Brown 2000; Baglione et al. 2002a). Among the many possible explanations of helping behaviour (reviewed by Cockburn 1998) enhanced production of nondescendant kin and direct access to parentage have proved to be important in the evolution of cooperative kin and nonkin societies, respectively. Little evidence is available to assess whether these factors co-occur in complex social groups. Such evidence would expand our understanding of cooperative breeding in birds by showing that, even within the same group, different members gain different sorts of benefits from group living and helping at the nest. Molecular techniques of assigning parentage have opened new perspectives to the study of helping behaviour (Smith 1990; Queller et al. 1993), and allow us to address this question.

In the carrion crow social behaviour is variable. In most European populations, carrion crows breed as unassisted pairs, but cooperative breeding is frequent in a population in northern Spain (Baglione et al. 2002a). In this population, cohesive groups of up to nine birds (average group size 3.2) hold all-purpose territories year-round and unassisted pairs are found in about 25% of the territories. The simplest social groups consist of a pair with one or more individuals, mainly yearlings, which have delayed natal dispersal and remain on their natal territory (nondispersers hereafter). Other groups contain one or more immigrants to the territory. The majority of these birds are sexually mature (2 years at least, but typically older) and 'mixed' groups containing both nondispersers and immigrants are common. Group members typically move and forage together in the territory and cooperate to evict conspecific intruders (Baglione et al. 2002a). The sex ratio in social groups is male biased, and groups with more than one adult female are rare. In social groups, nestling

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care always involves more than two birds. Both nondispersers and immigrants can help at the nest. Since carrion crows are long lived and territory turnover is usually low (Cramp & Perrins 1994), delayed natal dispersal is likely to lead to the association between offspring and parents. However, conclusive genealogical and genetic evidence of such association in the carrion crow is lacking. The presence of sexually mature immigrants in social groups raises the question of whether group members share in reproduction. Our aim in this study was to describe the mating system of this cooperative population and in particular to examine whether breeding is shared among group members. We addressed this question by applying a microsatellite-based genotyping system to determine parentage (Ellegren 1992; Primmer et al. 1995).

METHODS

Field Data Collection

We have been studying a cooperatively breeding population of carrion crows at La Sobarriba, northern Spain (42°N, 5°W) since 1995. All nestlings reared in a 45 km^2 study area were banded with individual codes of colour rings. In this area, carrion crows are territorial year round. Territory boundaries are stable and, since nesting sites are not abundant, typically only a few traditional sites are used in each territory. This makes territories easy to survey and recognize through the years. We surveyed 55–64 territories each year from 1998 to 2001 (N=236 territory-years). As juveniles can delay dispersal up to 2 years and they never inherit the natal territory (N=334; see also Baglione et al. 2002a), since 1997 we have been able to recognize, using binoculars or spotting scopes, all crows living in their natal territory. In 1999 and 2000 we captured free-flying crows (N=61) to collect blood samples, by using two-compartment walk-in traps and a 'snap trap' specifically developed for this work (for details on trapping methods see Baglione et al. 2002a). The carrion crows were aged in three categories as 1, 2 or older than 2 years according to the internal colour of the upper mandible (Svensson 1992).

To minimize the loss of data from early deaths of nestlings and to collect sufficient blood without harming the chicks, we sampled the nestlings 7–10 days after hatching. Every bird received a unique combination of colour rings. All procedures of bird ringing and blood sampling were authorized by the Junta de Castilla y León. During the breeding seasons of 2000 and 2001 we surveyed all groups banded in the previous years, collecting information on juvenile retention on the natal territory and adult turnover. The sample for parentage analysis (Table 1) included 17 breeding groups with different composition: three unassisted pairs, four pairs with one or two nondispersers, six pairs with immigrants and four mixed groups. Two groups were sampled in both years. All nondispersers were yearlings. Six groups were incompletely sampled: we failed to obtain samples from three incubating females and three unknown group members that were not involved in nestling care (Table 1). For all groups, videorecorded observations at the nest provided information on the identity of the incubating female and on the participation of group members in nestling care.

We visited all nests daily during laying to record any joint laying or egg dumping (McRae & Burke 1996). Each egg in the brood was marked with a tiny piece of tape of a different colour. This method allowed us to minimize disturbance at the nest. The majority of crows' nests were placed at less than 8 m from the ground, so that most nest visits were carried out within a few minutes.

Molecular Methods

We collected between 50 and 200 μl of blood from each individual from the alar vein. The blood was transferred into 200 μl of SSC buffer (0.15 m NaCl, 0.15 mM trisodium citrate, 0.5 mM EDTA, pH 7.0) and stored at −20 °C. To extract DNA from blood samples we used the standard proteinase K/phenol:chloroform procedure (Sambrook et al. 1989) on the samples collected in 1999 and the Qiagen 'DNeasy' extraction kit on samples of 2000. We tested 18 published primers were tested for amplification and polymorphism on 5–10 individual carrion crows. Nine of them were originally cloned for the mariana crow, *Corvus kubaryi* (Tarr & Fleisher 1998), and are thought to have a wide application within the genus *Corvus*. The rest were isolated in other passerine species. Eight primers gave polymorphic products. The PCR reactions (10 μl) were performed on Perkin Elmer 2400 and 9600 PCR machines and contained 0.33 units of Taq DNA polymerase, 1 μl of Jeffreys' buffer (final concentration 20 mM $(NH_4)_2SO_4$, 75 mM tris-HCl pH 8.8, 0.15 mM DNAse free BSA, 10 mM β-mercaptoethanol, 2.5 mM $MgCl_2$), 20 μM dNTPs, 10 μM of each primer and ca. 100 ng of genomic DNA. We used a 'touchdown' PCR profile for the first attempt at amplification. The profile consisted of 10 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, with the annealing temperature decreasing 1 °C at each cycle, followed by 20 cycles of 94 °C for 1 min, 50 °C for 30 s and 72 °C for 30 s. Some of the loci that showed polymorphic products were optimized by varying the annealing temperature (Table 2). For these primers the final thermal profile was, after 10 min of incubation at 92 °C, 92 °C for 40 s, 1 min annealing, 75 °C for 1 min (35 cycles). PCR products were electrophoresed on 6% denaturing polyacrylamide gels and visualized with silver staining. Samples from individuals of the same group were run together on the same gel to ensure reliable detection of mismatches.

Six polymorphic markers were used for parentage analysis (Table 2). Two other markers, CK.1B5G and CK.1B6G, gave a product that was difficult to score consistently. These were used as supplementary markers in cases when more than one putative father matched the genotype of an offspring at the other six loci (N=4) or when polygamous mating was revealed by a mismatch at one locus only (N=2). No loci were found to deviate from Hardy–Weinberg expectation. The combined paternity exclusion probability of the markers assuming random mating (Chakravarti & Li 1983) was 0.950 and increased

Table 1. Composition of the groups included in the parentage analysis and mating system assigned according to the results

Territory	Year	Group size	Immigrant males	Immigrant females	Nonsampled unknown individuals	Male nondispersing yearlings	Female nondispersing yearlings	Number of sampled nestlings	Group type	Mating
56	2000	2	—	—	—	—	—	3	UP	Monogamy
66	2000	2	—	—	—	—	—	3	UP	Monogamy
75	2000	2	—	—	—	—	—	4	UP	Monogamy
65	2000	4	—	—	—	1	1	2	PN	Monogamy
52	1999–2000	3–3	—	—	—	1*	—	6	PN	Monogamy
84	2000	3	—	—	—	1	—	4	PN	Monogamy
38	2000	3	—	—	—	1	—	3	PN	Monogamy
85	2000	4	1	—	—	1	—	5	MG	Undetermined
14	1999	5	—	—	1	—	2	3	MG	Polyandry§
3	2000	6	1	1†	—	1	1	5	MG	Within group polygamy
70	1999	4	1	—	—	1	—	3	MG	Within group polyandry
82	2000	4‡	2	—	—	—	—	3	PI	Monogamy
68	2000	3	1	—	—	—	—	3	PI	Monogamy
87	2000	3‡	1	—	—	—	—	3	PI	Monogamy
27	1999	3	—	—	1	—	—	3	PI	Polyandry§
67	1999–2000	4–3	1–1	—	1–0	—	—	3	PI	Within-group polyandry
69	1999	4	—	2	—	—	—	1	PI	Not applicable

Group type: UP=unassisted pair, PN=pair with nondispersers, MG=mixed group, PI=pair with immigrants. For sex determination see molecular methods.
*Two different individuals in the 2 years.
†Not sampled.
‡Breeding female not sampled.
§Suspected within-group polyandry.

Table 2. Polymorphic microsatellite loci used in the parentage analysis

Locus	Annealing temperature (°C)	Number of alleles	Observed heterozygosity (%)	Reference source
Ck.5A5F	54	8	77	Tarr & Fleisher 1998
Ck.5A4B	60	7	76.3	Tarr & Fleisher 1998
Ck.5A4D	62–52	6	72.5	Tarr & Fleisher 1998
Ck.B6D	61	4	62.8	Tarr & Fleisher 1998
Ck.5A5G	57	3	39.8	Tarr & Fleisher 1998
Ase18	62–52	2	47.8	Richardson et al. 2000
Ck.1B5D	62–52	6	77.6	Tarr & Fleisher 1998
Ck.1B6G	62–52	5	71.4	Tarr & Fleisher 1998

to 0.984 when the supplementary markers were considered. The assumption of random mating probably holds for unassisted pairs and pairs with nondispersers. When dispersing, crows do not tend to settle close to the natal territory (V. Baglione & J. M. Marcos, unpublished data). Such a lack of philopatry implies that close relatives are not overrepresented among the potential extrapair mates of breeders living in groups without immigrants (Double et al. 1997). Groups with immigrants represent a different case. We did not take into account the degree of relatedness between breeders and immigrants (currently under investigation), so the exclusionary power of the genotyping system might have been overestimated. This could explain the high proportion of ambiguous paternity in groups with immigrants (see Results), but it does not affect our conclusions.

P2/P8 DNA-based identification provided the sex of the banded carrion crows (Griffiths et al. 1998 for details). The molecular method for sexing proved to be reliable, classifying correctly 22 carrion crows (seven males, 15 females), where the sex was simultaneously assessed by behavioural observations (egg incubation for females, mating behaviour for males).

Parentage Analysis

Nondispersing yearlings were initially not considered as putative parents, but this assumption was checked a posteriori (see below). Only two sampled groups contained more than one sexually mature female. The genotype of all nestlings was always fully consistent with the genotype of the incubating female. When indications of multiple laying or egg dumping were not found, we assumed that the incubating female was the true genetic mother. All males within the cooperative group were regarded as putative fathers of the nestlings reared in their own territory. We excluded paternity by a putative father when the paternal alleles of the nestling mismatched the genotype of that male at one or more loci. If the mother was not sampled we excluded paternity if neither of the nestling's alleles matched the genotype of the putative father. We assigned a putative father as the true genetic father when all paternal alleles of the nestling matched only that male. If two males within a group matched the genotype of the nestling, paternity was considered undetermined. For all nestlings we calculated the probability that a random male could have shown the same combination of paternal alleles as the assigned father did ('probability of chance inclusion', Jeffreys et al. 1992) in order to validate our paternity assignment.

The assumption that nondispersing yearlings did not sire any nestlings was based on the general consensus that sexual maturity is delayed in the carrion crow until the second year (Madge & Burn 1999). The biology of this species is a well known in several European populations and reproduction in the first year has never been observed (Goodwin 1986; Cramp & Perrins 1994; J. M. Marcos & V. Baglione, unpublished data), even when breeding vacancies were experimentally provided (Charles 1972). Furthermore, we have never observed any attempt at copulation involving a first-year carrion crow. However, to check the assumption that male nondispersing yearlings do not sire any nestlings, we compared their genotypes with those of the nestlings they helped to rear.

Parent–Offspring Association

Where possible, we checked whether delayed natal dispersal led to parent–offspring association in the studied population. Fifteen nestlings (12 territories) with at least one parent ringed delayed natal dispersal and we could verify their association with their parents from the group composition in the following breeding seasons. The pedigree information was lacking for another 13 nondispersers (nine territories) since the group that reared them was not ringed the year when they hatched. We compared their genotype with those of the adults in their group classified as breeders in our parentage analysis, considering lack of mismatches as an indication of parent–offspring relationship.

RESULTS

Group Composition in the Population

Unassisted pairs represented 25% of breeding groups, pairs with nondispersers 17%, pairs with immigrants 40% and mixed groups 18% (N=236 group-years).

Parentage Analysis

We analysed 19 broods from 17 groups. Overall, we found evidence of shared reproduction in three groups (groups 3, 70 and 67; 17.6% of sampled groups). Polyandrous mating was detected in another two groups (groups 27 and 14) but, owing to incomplete sampling, we could not disentangle within-group shared reproduction from extragroup paternity. There were no confirmed cases of polygamy involving extragroup mates in our sample. Evidence of polygamous mating was found only in pairs with immigrants and mixed groups (five of nine groups) but never in unassisted pairs or families (N=8; Table 1). The presence of immigrants in the group was significantly associated with the occurrence of polygamous mating (Fisher's exact test: P=0.026; groups sampled in 2 years were considered only once to avoid pseudoreplication).

We assigned a father to 53 of 57 nestlings (93%). Paternity could not be assigned to a specific male for two nestlings that matched both of the males of their group (groups 3 and 85; Table 1). Another two nestlings, both from groups that had been incompletely sampled (groups 27 and 14, both sampled in 1999), mismatched their putative fathers. Three different paternal alleles were found at two loci among the genotypes of the three nestlings of brood 27, indicating multiple paternity. One nestling mismatched the genotype of the putative father at two loci. At the beginning of the breeding season in 1999, one bird disappeared after egg laying from the trio that had held territory 27 through 1998, and it could not be sampled. Conceivably, that bird might have fertilized the female before disappearing from the territory. In the brood of group 14 one nestling mismatched the putative father at one locus only. This was the only case where polygamous mating was revealed by a single mismatch (about the interpretation of this case, see Discussion).

Groups 67 and 70 contained two adult males, which shared reproduction. In group 67 one male sired the two nestlings reared in 1999 while the other male sired the single nestling of 2000. Multiple paternity was detected within a single brood in group 70. In both groups (67 and 70), nestlings assigned to one male mismatched the other male at two loci at least. The probability of chance paternity inclusion for these nestlings was low ($\overline{X} \pm \text{SE}$=0.014 ± 0.008; mean for the whole sample 0.034 ± 0.006). We therefore regarded these data as evidence of male-shared reproduction in the two groups ('within-group polyandry' in Table 1).

Two females laid eggs in nest 3. In this nest the brood was exceptionally large (nine eggs, whereas the maximum brood size in this population is usually six, N=490, J. M. Marcos, unpublished data) and after the first egg, two eggs of two clearly different patterns were laid per day (carrion crows lay one egg per day; Cramps & Perrins 1994; J. M. Marcos, unpublished data). Furthermore, two females incubated the eggs, which we have never observed in normal-sized broods. Only one of the two females was captured and genotyped. We marked every egg and followed hatching to identify the chicks hatched from each egg. We eventually sampled three nestlings hatched from the eggs of one female and two from the other. One male (individual 14) was excluded as father of four of the five nestlings since this male had none of the alleles of these nestlings at one, two, three and three loci, respectively. For these nestlings the other male (individual 13) was assigned as father, which implies that he fertilized both females (Table 3) and simultaneously excludes the possibility that two monogamous pairs had shared the nest. Since both males matched the genotype of one nestling (Table 3), we could not exclude polyandrous mating for one female. We therefore cannot distinguish between polygyny (male 13 mating with two females) and promiscuity (male 13 mating with two females, and one female mating with both males) as the mating system in this group.

Table 3. Paternity exclusion and assignment in group 3, where two females laid

Female	Nestling	Excluded father*	Assigned father
1	a	14 (1)	13
1	b	14 (2)	13
1	c	14 (3)	13
2	d	None	—
2	e	14 (3)	13

The two putative fathers are identified with their individual code (male 13 and 14).
* The number of mismatching loci is shown in parentheses.

We checked whether genetic data confirmed the assumption that nondispersing yearlings did not reproduce in their group. Eighteen of 25 nestlings (72%) mismatched the genotype of the male nondispersing yearlings of their group at one or more loci and 50% of male nondispersing yearlings (N=8) showed at least one mismatch with all nestlings they helped to rear. There were no cases where a nondisperser was the only male in the group that matched the genotype of a nestling.

Parent–Offspring Association

Delayed natal dispersal invariably led to the association of offspring with their parents. At least one of the parents was always present through the period of retention on the natal territory of the 15 nestlings of known or partially known pedigree (in five groups, seven nestlings associated with both parents). Twelve of 13 nondispersers whose pedigree information was lacking showed no mismatches with the genotype of the current breeders of their group, suggesting a parent–offspring relationship. The remaining nondisperser mismatched the genotype of the breeding female at three loci.

DISCUSSION

Our microsatellite-based parentage analysis showed that immigrant carrion crows gain direct fitness by living in a cooperative group, through shared reproduction with the breeders. In our sample (19 broods), polygamous mating occurred only in groups with immigrants, while

unassisted pairs and pairs with nondispersers were invariably monogamous.

Microsatellite mutations represent a potential confounding factor in parentage analysis, but cannot explain our results. In all but one case the mismatches between the genotype of a nestling and that of its putative father occurred at two or more loci, strongly indicating lack of paternity rather than mutations. The mixed group of territory 14 was the only case where we found a mismatch at one locus only (locus Ck.5A4D). Since mutations are more likely to occur at very polymorphic loci (Queller et al. 1993) and locus Ck.5A4D showed only six alleles in the present sample, this mismatch was likely to indicate extrapair fertilization. However, the interpretation of this single case did not affect our main conclusions.

In the population studied it is unlikely that nondispersers reproduce within their social group. The majority of nondispersers are yearlings (only 11.7% of young banded from 1995 to 1998 delayed dispersal until the second year; N=188) so that, according to available data, they are probably not sexually mature when they help at the nest. Despite the difficulty of resolving parentage when first-order relatives (i.e. father–son) compete for mating, the genetic data confirmed that nondispersing yearlings did not sire offspring. Furthermore, as our data showed, delayed natal dispersal in crows results in a parent–offspring association. Therefore, in most cases, participation of nondispersers in reproduction within the group would lead to incest, which is usually strongly avoided in vertebrates (Pusey & Wolf 1996; Cockburn 1998). We conclude that offspring do not seek direct fitness benefits when they stay at home and help. Conversely, our data showed that immigrants can reproduce within their social group. The genetic data are supported by two direct observations of polyandrous copulation. In both cases a female copulated with two group members in sequence (no aggression was observed between the two males). The dichotomy between cooperative polygamy and kin-based cooperation is widely accepted as a criterion to classify cooperative societies, but our data show that both processes can occur within a population and even within the same social group.

The complexity of carrion crow society indicates that a single general process is unlikely to explain the behaviour of all extrabirds in a social group (Cockburn 1998). Since access to mating is restricted to immigrant birds, the kinds of benefits that immigrants and nondispersers can gain from group living and helping at the nest are not identical. We suggest that the two routes that lead to group living in this species (delayed dispersal and immigration) reflect different selective forces involved in the evolution of cooperative breeding within the population. The traditional approach to the study of cooperative bird societies consists of seeking separate adaptive explanations of delayed offspring dispersal and associations not based on parent–offspring relations. However, the carrion crow cooperative system cannot be completely understood unless both processes are addressed in parallel. By sharing reproduction with resident birds, immigrants dilute the relatedness between nestlings and nondispersers, decreasing the indirect fitness benefits of helping of the latter. How this conflict has been overcome over evolutionary time is currently under investigation.

To what extent the carrion crow represents an exception among cooperative bird societies is difficult to assess. A few cooperative species are known to show a combination of helping by unrelated and related individuals. In the white-browed scrubwren, *Sericornis frontalis* (Whittingham et al. 1997), the white-winged trumpeter, *Psophia leucoptera* (Sherman 1995a,b) and the Mexican jay, *Aphelocoma ultramarina* (Li & Brown 2000), unrelated helpers have access to mates within the group, while in the red-cockaded woodpecker, *Picoides borealis* (Haig et al. 1994), the pied kingfisher, *Ceryle rudis* (Reyer 1980), and the rifleman, *Acanthisitta chloris* (Sherley 1989, 1990) unrelated helpers do not sire offspring. The application of molecular methods has dramatically changed our understanding of some well-studied cooperative societies (i.e. Li & Brown 2000). Therefore, only the use of such techniques on a wide range of species will clarify whether the complexity of cooperative bird societies has been overlooked. If so, a reassessment of current theories on the evolution of cooperative behaviour will be needed.

Within carrion crow social groups, shared reproduction seems to arise mostly as polyandry rather than as polygyny or promiscuity. Exceptionally large broods indicate multiple laying in the carrion crow. We have detected only four enlarged broods (including the one considered in this study) in the population studied in 490 nests surveyed since 1995 (J. M. Marcos, unpublished data). Based on circumstantial information on laying sequence, colour pattern and shape of the eggs, which were available for almost all broods, we did not suspect any other case of egg dumping. Within-group polyandry has occurred instead in at least 22% of groups with immigrants sampled in this study. Immigrant males might therefore have a better chance of mating than immigrant females, and should be more likely to settle permanently in an already established social group. This hypothesis is consistent with the observation that immigration is male biased (eight of 11 immigrants were males in our sample).

The parentage analysis did not reveal any polygamous mating in unassisted pairs or nuclear families. Conversely, polygamous mating was likely in groups that contained immigrants (55%). The correlation between the presence of immigrants and the occurrence of polygamous mating was close to statistical significance (Fisher's exact test: P=0.07) even if we conservatively exclude group 14 from the analysis. Although extragroup mating could not be ruled out in two groups, owing to incomplete sampling, our data suggest that, in crows, immigrants are the cause of loss of parentage. This scenario raises the question of why breeders (especially males) tolerate immigrants of the same sex in their territories. It is unlikely that immigrants stay just because they cannot be evicted. Carrion crows are efficient at chasing conspecific intruders away from the territory. Furthermore, field observations showed that groups that contain immigrants are as cohesive as family groups. Breeders and immigrants typically cooperate in defending the territory and peacefully share foraging grounds. Preliminary data

suggest that birds involved in reproduction feed the nestlings at a higher rate than nonbreeders and that the presence of immigrants enhances the reproductive success of a pair (V. Baglione, J. M. Marcos & D. Canestrari, unpublished data). By accepting an immigrant and sharing reproduction, a breeder might obtain high-quality help at the nest and therefore might increase its fitness despite the loss of parentage in the brood. Further study will assess the potential of this trade-off in explaining breeders–immigrant association in Spanish carrion crows.

Another intriguing question is why such associations do not form in carrion crows elsewhere in Europe. In some noncooperative European populations the proportion of birds that cannot find a breeding vacancy (up to 75% of the total population, Picozzi 1975; Loman 1985) is much higher than in Spain (34%; Baglione et al. 2002a), yet these crows breed as unassisted pairs. Little is known about the selective advantages of alliences and the mechanisms that lead to them in animals. In the carrion crow, experimental evidence shows the role of the rearing environment on the dispersal behaviour of the juveniles and the tendency to form families (Baglione et al. 2002b). We suggest that environmental factors are also important in determining the formation of nonfamily groups.

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