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Supplementary Information accompanies the paper on **www.nature.com/nature**.

Acknowledgements We thank R. Rinaldo, E. Prince, A. Seitz, T. Sippel, R. Schallert, N. Tulloch, G. Rosenwaks, S. Beemer, G. Shillinger, C. Perle, S. Vermillion, J. Bonaventura, D. Barber, M. Orbach, J. Jenkins, G. Stuve, P. Wright, D. Britt, B. Eakes, C. Perry, D. Brower, W. Whippen, R. Whorley, R. Jansenius, G. Sharp, R. Hill, T. Lindstrom, P. Ekstrom, P. Manuel, R. Ruais and S. Loga. We are indebted to the late Richard Novak for his contributions and sacrifice on behalf of the Tag-A-Giant programme. We thank the National Marine Fisheries Service (NMFS) for providing access to the GOM scientific observer data and US pelagic longline logbook data. The Tag-A-Giant programme was supported by grants and donations from the Packard, Pew, MacArthur, Disney, Marine Ventures, Gordon and Betty Moore, and Monterey Bay Aquarium Foundations. This research was supported in part by the NOAA NMFS, the NSF and the National Fish and Wildlife Federation. We acknowledge the extensive cooperation of the commercial and recreational captains and crews of fishing vessels in North Carolina, New England, Nova Scotia, Louisiana and Texas. We thank N. Miyabe of the National Research Institute of Far Seas Fisheries, ICCAT, A. Dinatale, G. DeMetrio, M. de la Serna and the EU COPEMED programme for return of electronic tags.

Competing interests statement The authors declare that they have no competing financial interests.

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Learned kin recognition cues in a social bird

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In many cooperatively breeding birds, kin selection has an important role in the evolution and maintenance of social behaviour, and 'helpers' can maximize indirect fitness gains by preferentially allocating care to close relatives[1–3]. Although there is evidence for kin-biased helping behaviour in several species[1,4,5], the mechanism of kin recognition underlying this behaviour is poorly understood[2]. Vocalizations are the most commonly used cues in avian recognition systems[6,7], but the effectiveness of vocal signals as reliable recognition cues must depend on how they are acquired[6–9]. However, there have been no experimental studies of the development of vocal recognition cues in cooperative birds; indeed, the ontogeny of all bird vocalizations other than song is poorly known in any species[10–12]. Here, we show that cooperatively breeding long-tailed tits (*Aegithalos caudatus*) can discriminate between kin and non-kin according to the individual-specific characteristics of contact calls, and show experimentally that individuals learn these calls from provisioning adults during the nestling period. Finally, we show that the pattern of cooperative behaviour in this species is consistent with the use of recognition cues learned through association.

In long-tailed tits, all adults attempt to breed independently in pairs each year, but most nests fail due to depredation[13,14]. Failed breeders often re-nest, but later in the season may instead become helpers[14]; this switch from re-nesting to helping corresponds with a seasonal change in the potential fitness benefits of each strategy[15]. No significant direct fitness benefits of helping have been found, but helpers preferentially care for close relatives[16] and accrue indirect fitness benefits by increasing brood productivity[14,15]; this kin-selected benefit represents a substantial component of inclusive fitness and is the sole source of fitness for many individuals[17]. Thus, helping is beneficial to both helpers and recipients, and selection should favour kin recognition[6,8]. Kin-biased helping occurs in the absence of reliable spatial cues to kinship[16], and a previous study suggested that long-tailed tits can discriminate between the vocalizations of close relatives and non-relatives[18]. Here, we describe an experiment that determines the characteristics of contact calls used in discrimination, and a second experiment that investigates the acquisition of these recognition cues.

Long-tailed tits have a limited vocal repertoire, with five call types and a very rarely used song[13,19,20]. The 'churr' call is a contact call given frequently by both sexes that is important for short-range communication; for example, during nest-building or aggressive interactions[13,18–20]. This call develops in the nest before fledging[20] and is highly stereotyped within individuals[21], remaining unchanged throughout adulthood (S.P.S., unpublished data); multivariate analysis showed that maximum and minimum frequency are the two most individual-specific call parameters[21]. Using a playback experiment, we tested the ability of long-tailed tits to discriminate between the churr calls of kin and non-kin according to variation in these two parameters. We conducted playback trials with four treatments at the nests of focal birds using the following

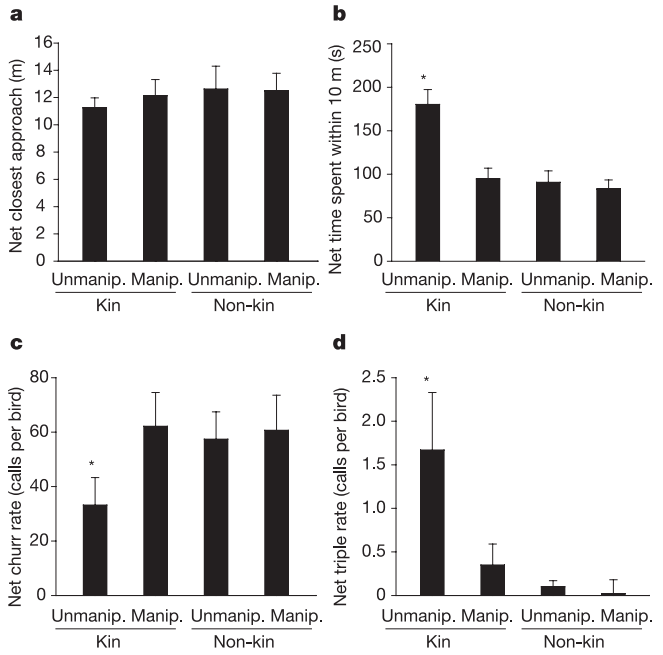

Figure 1 Responses to playback trials ($n = 8$) using the churr calls of kin and non-kin with maximum and minimum frequency unmanipulated (Unmanip.) and manipulated (Manip.). Net responses (error bars indicate mean ± standard error) were calculated as the difference in response during playback and quiet periods. **a**, Closest approach to the speakers (Friedman test, $s = 0.63$, $P = 0.889$). **b**, Time spent within 10 m of the speakers ($s = 14.85$, $P = 0.002$). **c**, Churr rate ($s = 12.15$, $P = 0.007$). **d**, Triple rate ($s = 15.93$, $P = 0.001$). Tests remained significant after sequential Bonferroni correction. Asterisks indicate significant differences after treatment comparison tests[28].

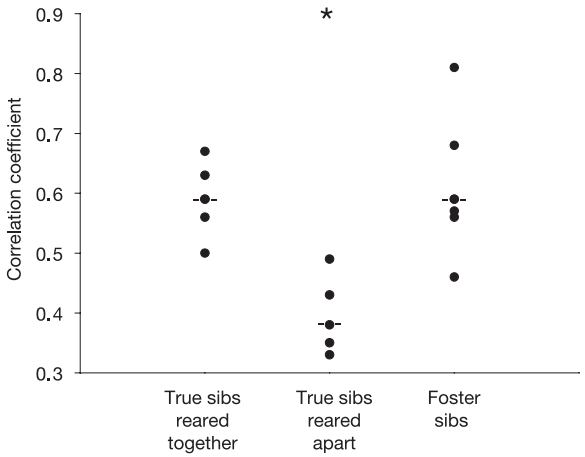

Figure 2 Call similarity between different groups of siblings. Correlation coefficients (dashed horizontal bars indicate means) for pairwise comparisons of churr calls in each group were obtained using SPCC (Kruskal–Wallis test, $\chi^2 = 9.752$, $P = 0.008$). Asterisks indicate significant differences after treatment comparison tests[28].

stimuli: (1) the churr calls of a close relative (coefficient of relatedness, $r = 0.5$); (2) the churr calls from treatment 1 but with maximum and minimum frequency manipulated; (3) the churr calls of a non-relative ($r < 0.125$); and (4) the churr calls from treatment 3 but with maximum and minimum frequency manipulated. The frequency parameters of manipulated calls remained within the range of natural variation observed in this species. The difference in each of four behavioural responses of focal birds during periods of playback and periods of quiet (that is, with no playback) was calculated to give four 'net' responses for each treatment. For three of the four responses measured there was a significant difference in net response during the four treatments (Fig. 1). In each case, the net response during playback of the unmanipulated churr calls of a close relative was significantly different from that during the other three treatments, between which there were no significant differences (Fig. 1). Long-tailed tits therefore responded differently to the manipulated and unmanipulated churr calls of kin, yet manipulation had no significant effect on the birds' responses to calls of non-kin. Thus, individuals were able to discriminate between the vocalizations of kin and non-kin based at least in part on variation in maximum and minimum frequency. This result does not imply that the churr call is the only kin recognition cue used by long-tailed tits: several different cues may be used, either in combination or separately according to context[7,9]. However, the results do show that the churr call alone is sufficient for successful discrimination.

The churr call may function as a vocal cue for kin recognition, but the reliability of such cues will depend in part on the nature of their development. The use of genetically determined cues may lead to recognition errors due to the effects of recombination, whereas cues derived from the environment are only reliable if acquired at a time when there is good evidence of kinship[6–9]. We conducted a cross-fostering experiment to investigate the relative contribution of genetic and environmental influences on the development of the churr call. In a previous observational study, spectrographic cross-correlation (SPCC) revealed that the churr calls of siblings were more similar than those of non-siblings (mean ± s.d. correlation coefficient for the calls of siblings = 0.54 ± 0.10, $n = 46$ pairs of siblings; for the calls of non-siblings = 0.47 ± 0.08, $n = 500$ pairs of non-siblings; S.P.S., unpublished data). The aims of the cross-fostering experiment were to compare the churr calls of foster siblings and true siblings, and the churr calls of fostered birds with those of their foster and biological parents. Nestlings from 24 partial broods were marked and swapped between synchronous nests of unrelated birds ($r < 0.125$). The churr calls of recruits from cross-fostered broods were recorded in the following year when they had reached reproductive maturity and commenced breeding; these calls were then compared using SPCC. The churr calls of foster siblings were just as similar as those of true siblings reared together, whereas those of true siblings reared apart were significantly less similar (Fig. 2). Correlation coefficients for foster siblings and true siblings reared apart were comparable with those in the observational study for siblings and non-siblings, respectively. Furthermore, the churr calls of fostered individuals were significantly more similar to those of their foster parents than to those of their biological parents, whether comparisons were made with female (Fig. 3a) or male (Fig. 3b) parents. There must therefore be a significant learned component in the development of these calls.

Avian calls were traditionally thought to be genetically determined[11,12], but our results support the more recent idea that learning can have an important role in call development[10,12,22,23], just as it does in song development. Reliance on kin recognition cues that develop through learning may result in recognition errors (that is, the acceptance of non-kin as kin) if interactions with non-kin occur during cue development, or if social relationships are not effective predictors of genetic relatedness[6,8]. However, in long-tailed tits, extra-pair paternity and brood parasitism are rare[24] and, as in most cooperatively breeding birds, the association between offspring and their relatives is extended over a relatively long period[25]—the risk of making recognition errors is therefore reduced.

Long-tailed tit helpers exhibit a kin preference when controlling for spatial cues[16], so if recognition is achieved through learning we would predict that the pattern of helping reflects associations during periods of call development. We tested this prediction by examining whether those helpers whose entire life history was documented became helpers at nests belonging to an individual with whom they had been associated during the nestling phase,

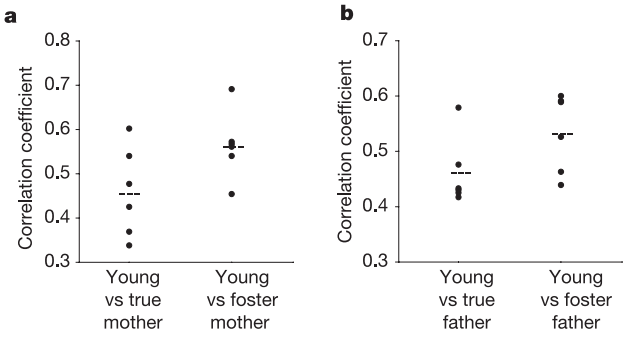

Figure 3 Call similarity between recruits from cross-fostered broods and their true and foster parents. Correlation coefficients (dashed horizontal bars indicate means) for pairwise comparisons of churr calls were obtained using SPCC. **a**, Comparisons between the calls of recruits and their true and foster mothers (Wilcoxon's signed rank test, $z = -2.201$, $P = 0.028$). **b**, Comparisons between the calls of recruits and their true and foster fathers (Wilcoxon's signed rank test, $z = -2.201$, $P = 0.028$).

either as siblings or as a recipient or donor of care. In 57 out of 64 (89%) cases the helper assisted at least one breeder with whom it had been associated during the nestling phase, either as a sibling (38 out of 57, 67%), an offspring (13 out of 57, 23%), a parent (2 out of 57, 3%), a helper (1 out of 57, 1%), or as a recipient of helper care (3 out of 57, 5%). In 3 out of 64 (5%) cases, helpers assisted at nests belonging to a sibling of either a parent or a helper who fed it as a nestling. These instances suggest that kin recognition might occasionally be achieved indirectly through shared call characteristics, but we cannot exclude the possibility that there was some direct prior association that we had not observed. Finally, in just 4 out of 64 (6%) cases the helper and recipients were unrelated and we had no record of prior association, either direct or indirect, between the helper and the assisted breeders. In these few instances, it is possible that the helpers made recognition errors, as might be expected to occur in any recognition system[8].

The cooperative associations of long-tailed tits are broadly consistent with a recognition mechanism of learning through direct association, as expected among cooperatively breeding birds[2,16,18,23,26]. Development of the churr call in the nest provides the opportunity to learn kin recognition cues from provisioning adults at a time when the presence of non-kin is unlikely. However, such cues would not function effectively as a kinship label for individuals who were not associated during the appropriate period of development because of the rapid diluting effect of learning from parents in an outbred population. Thus, this learning mechanism limits the pool of potential beneficiaries of kin-directed cooperation to the subset of kin within the population with whom the helper has had direct association. □

Methods

Field recordings and acoustic analysis

We studied a colour-ringed population of 63–90 pairs of long-tailed tits in Melton Wood, Doncaster, UK (53° 20′ N, 1° 30′ W) in 2001–03; relatedness was determined from pedigrees. Calls were recorded between February and June at a distance of <15 m using a Sennheiser MKH 416P48U(during 2001–02) or MKH 60P48 (2003) microphone. Recordings were made on one side of TDK type II SA cassettes using a Sony WM-D6C Walkman and digitized with 16-bit accuracy at a sampling rate of 22,050 Hz. Spectrograms were produced in Avisoft SASLab Pro (version 4.23b, 2003) using a 256-point fast Fourier transform length with a Hamming window function, 100% frame size and 75% window overlap.

Playback experiment

In 2003, we identified focal nests ($n = 8$) at which one parent, the 'target bird', had a close relative ($r = 0.5$) alive whose churr call had been recorded; target birds were also randomly allocated a living non-relative ($r < 0.125$). Each target bird was subjected to four playback treatments using churr calls: (1) kin; (2) manipulated kin; (3) non-kin; and (4) manipulated non-kin. For treatments 1 and 3, we randomly selected one churr call from recordings of the appropriate bird, then created a 1-min sequence of 36 randomly spaced copies of the call (mean natural calling frequency in conspecific interactions at the nest = 35.7 ± 6.4 calls per min, $n = 50$). For treatments 2 and 4, sequences from treatments 1 and 3, respectively, underwent a frequency domain transformation in Avisoft, moving the entire spectrogram along the frequency axis by shifting a Fourier-transformed signal by a specified amount, then performing an inverse Fourier transformation. This procedure introduced no significant artefacts into the signal. The maximum frequency of churr calls ranges from 8.48 kHz to 10.47 kHz ($n = 169$ individuals), the mid-point being 9.475 kHz. For calls with a maximum frequency above or below this mid-point, the spectrogram was decreased or increased by 1 kHz respectively; thus all calls remained within the normal frequency range. Sequences were then transferred to TDK endless cassettes.

We conducted trials by broadcasting calls through Sony SRS-58 speakers placed 10 m from focal nests containing nestlings. Treatments were run in different sequences at each nest, with two conducted on each of two consecutive days at the same times. Trials comprised 5 min of no playback followed by 5 min of playback. Target birds >20 m from the speakers were considered absent; if birds were absent throughout the quiet period the trial was restarted. An observer, who was unaware of which treatment was being conducted, stood 25–30 m from the nest and recorded the closest approach to the speaker and time spent <10 m from the speaker by the target bird. Calls could not always be assigned to individuals so the total numbers of churr calls and 'triple' calls (a long-range contact call[13,18–20]) were recorded and then divided by the number of birds present (two in most cases, but three for nests with a helper) to give the 'churr rate' and 'triple rate'.

Cross-fostering experiment

In 2002, we marked partial broods (mean = 4.50 ± 0.66 nestlings, $n = 24$ broods; mean brood at hatching = 9.1 nestlings[14]) of 4–5-day-old nestlings and switched them between synchronous nests of unrelated birds. In 2003, the churr calls of philopatric recruits from cross-fostered nests were recorded and spectrograms produced of one randomly selected call per individual. Three categories were identified: (1) true siblings reared together; (2) true siblings reared apart; and (3) foster siblings. For dyads in each category, we compared their spectrograms by SPCC using Avisoft Correlator with a tolerated frequency deviation of 50 Hz and a high-pass filter of 1 kHz. Recordings of the churr calls of foster and biological parents were available for six recruits. We randomly selected one call from each foster parent and biological parent and compared their spectrograms with those of the corresponding fostered recruit using SPCC.

Helper–breeder associations

Data were available from a long-term study (1994–2004) of a colour-ringed population of 18–68 pairs of long-tailed tits in the Rivelin Valley, Sheffield, UK (53° 12′ N, 1° 34′ W); relatedness was determined from pedigrees. On alternate days during the 16-day nestling period, provisioning behaviour was observed (usually for 1 h) and the identities and provisioning rates of all birds that fed nestlings recorded[27]. A complete history of associations as nestling and provisioning adult was determined for a total of 64 helpers who were first ringed as nestlings. A further five 'helpers' at four nests were excluded as they were observed to feed a brood on just one occasion despite extensive observations (mean = 9.5 ± 3.1 h, $n = 4$ nests; range 6–13 h); typical helpers have a provisioning rate of 5.0 feeds per hour to day-8 nestlings[27].

Received 11 January; accepted 9 March 2005; doi:10.1038/nature03522.

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Acknowledgements We thank T.R. Birkhead, N.B. Davies, J. Slate and A.P. Beckerman for their advice; A.D.C. MacColl, D.J. Ross, M.K. Fowlie and A.F. Russell for their assistance with fieldwork; and Doncaster and Sheffield City Councils, Yorkshire Water and Hallam Golf Club for allowing us to watch birds on their land. This research was supported by NERC and the University of Sheffield.

Competing interests statement The authors declare that they have no competing financial interests.

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A synthetic multicellular system for programmed pattern formation

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Pattern formation is a hallmark of coordinated cell behaviour in both single and multicellular organisms[1–3]. It typically involves cell–cell communication and intracellular signal processing. Here we show a synthetic multicellular system in which genetically engineered 'receiver' cells are programmed to form ring-like patterns of differentiation based on chemical gradients of an acyl-homoserine lactone (AHL) signal that is synthesized by 'sender' cells. In receiver cells, 'band-detect' gene networks respond to user-defined ranges of AHL concentrations. By fusing different fluorescent proteins as outputs of network variants, an initially undifferentiated 'lawn' of receivers is engineered to form a bullseye pattern around a sender colony. Other patterns, such as ellipses and clovers, are achieved by placing senders in different configurations. Experimental and theoretical analyses reveal which kinetic parameters most significantly affect ring development over time. Construction and study of such synthetic multicellular systems can improve our quantitative understanding of naturally occurring developmental processes and may foster applications in tissue engineering, biomaterial fabrication and biosensing.

Figure 1a depicts the design of the synthetic bacterial multicellular system, showing how only receivers at intermediate distances from senders express the output protein. Cell–cell communication from the senders is initiated by expression of the LuxI enzyme[4,5] (Fig. 1b). LuxI catalyses the synthesis of AHL, which diffuses through the cell membrane and forms a chemical gradient around the senders. AHL diffuses into nearby receiver cells and is bound by LuxR, an AHL-dependent transcriptional regulator, which activates the expression of lambda repressor (CI) and Lac repressor ($LacI_{M1}$, a product of a codon-modified *lacI*). Receiver cells in close proximity to the senders receive high concentrations of AHL, resulting in high cytoplasmic levels of CI and $LacI_{M1}$ and repression of the green fluorescent protein (GFP). Receivers that are far from the senders have low AHL concentrations, and accordingly $LacI_{M1}$ and CI are expressed only at basal levels. This enables the expression of a wild-type LacI, again resulting in GFP repression. At intermediate distances from the senders, intermediate AHL concentrations result in moderate levels of CI and $LacI_{M1}$. However, because the repression efficiency of CI is significantly higher than that of $LacI_{M1}$, CI effectively shuts off LacI expression while the $LacI_{M1}$ concentration is below the threshold required to repress GFP production. This difference between the CI and $LacI_{M1}$ repression efficiencies, in combination with a feed-forward loop[6] that begins with LuxR and culminates in GFP, affords the circuit the desired non-monotonic response to AHL dosages.

Guided by a mathematical model, the band-detect behaviour was engineered by combining a high-detect component (pHD plasmids; Fig. 1c) with a low-detect component (pLD plasmid; Fig. 1d) as described below. The high-detect component determines the AHL threshold above which GFP expression is muted. We engineered three high-detect strains (HD1, HD2 and HD3), each harbouring a variant of the high-detect plasmid (pHD{*x*}; Fig. 1c). The HD1 strain contains a hypersensitive LuxR mutant[7], HD2 incorporates the wild-type LuxR, and HD3 cells express LuxR from a reduced-copy-number plasmid. In agreement with model predictions (Fig. 2a), the liquid-phase dosage responses of these three HD strains showed inverse correlations to AHL concentrations with different sensitivities (Fig. 2b). The low-detect component determines the lowest concentration of AHL that elicits GFP response. By combining the low-detect plasmid with each of the high-detect plasmid variants, we obtained three different band-detect strains named BD1, BD2 and BD3 accordingly. The BD strains showed a non-monotonic response to AHL with different thresholds (Fig. 2d), which correlated well with model predictions (Fig. 2c). Taken together, the responses of the three variants cover a wide range of biologically relevant AHL concentrations. Further analysis showing the effects of LacI and CI repression efficiencies on band-detect behaviour is included in the Supplementary Information.

Spatiotemporal simulations of a band-detect system predicted that by placing sender cells capable of AHL synthesis next to receiver cells, the above network could direct pattern formation on solid media. The model showed that given the appropriate kinetics for circuit elements, a distinct ring pattern would form in an initially undifferentiated 'lawn' of receiver cells around a group of sender cells (see Methods). Furthermore, a bullseye pattern could be achieved by mixing band-detect network variants such as BD1, BD2 and BD3. We tested these model predictions by plating on a Petri dish a mixture of BD3 cells and BD2-Red cells (similar to BD2 with *dsRed-Express* replacing *gfp*). A disk containing sender cells was placed in the middle of the dish (Fig. 3a), and the dish was incubated overnight. Microscope fluorescence images were subsequently captured. As seen in Fig. 3b, BD3 cells formed a green fluorescent ring near the senders, whereas BD2-Red cells formed a red fluorescent ring located further from the senders, creating a bullseye pattern. Similarly, when BD1 and BD2-Red cells were mixed and plated with a sender disk, an outer green fluorescent ring appeared around the red fluorescent ring (Fig. 3c). Although the relative positions of BD1, BD2 and BD3 cells were consistent in the two experiments, the diameters of the two BD2-Red rings were somewhat different (30 mm versus 22 mm). This can be attributed to variations in the AHL gradients due to differences in the growth rates and population