

LETTER

Genetic relatedness in groups is sex-specific and declines with age of helpers in a cooperatively breeding cichlid

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Abstract

Kin selection can explain the evolution of cooperative breeding and the distribution of relatives within a population may influence the benefits of cooperative behaviour. We provide genetic data on relatedness in the cooperatively breeding cichlid *Neolamprologus pulcher*. Helper to breeder relatedness decreased steeply with increasing helper age, particularly to the breeding males. Helper to helper relatedness was age-assortative and also declined with age. These patterns of relatedness could be attributed to territory take-overs by outsiders when breeders had disappeared (more in breeding males), between-group dispersal of helpers and reproductive parasitism. In six of 31 groups females inherited the breeding position of their mother or sister. These matrilineal inheritance were more likely to occur in large groups. We conclude that the relative fitness benefits of helping gained through kin selection vs. those gained through direct selection depend on helper age and sex.

Keywords

Age distribution, cichlids, cooperative breeding, genetic relatedness, matrilineal inheritance, size distribution, spatial distribution.

Ecology Letters (2005) 8: 968–975

INTRODUCTION

Cooperative breeding is a widespread phenomenon in insects, birds and mammals (Emlen 1997), but has only rarely been reported in fish (Taborsky 1994). Kin selection plays an important role in the evolution of many of these societies, e.g. subordinates gain indirect fitness benefits by helping relatives raising offspring (Choe & Crespi 1997; Emlen 1997; Solomon & French 1997; Cockburn 1998; Hatchwell & Komdeur 2000; Clutton-Brock 2002). However, molecular genetic relatedness studies show more complex kin-structuring within vertebrate cooperatively breeding groups than previously thought, i.e. because of multiple breeder females, multiple paternity and emigration/immigration of subordinates and dominants (recent studies include, e.g. Creel & Creel 2002; Richardson *et al.* 2002; Griffin *et al.* 2003; Baker *et al.* 2004; Van Horn *et al.* 2004). Therefore, evidence suggests that many subordinates are unrelated to the breeders and cannot derive kin-selected benefits from remaining in the group. Instead, these subordinates are expected to gain direct benefits from

living in a group, i.e. increased survival (e.g. Heg *et al.* 2004a) or participation in reproduction (e.g. reproductive skew theory, see for review Johnstone 2000; Reeve & Keller 2001).

Extended families have been less intensively studied in fish species, except in the Lake Tanganyika cichlid *Neolamprologus pulcher/Neolamprologus brichardi* Poll (Taborsky & Limberger 1981; Taborsky 1984, 1985; Balshine-Earn *et al.* 1998; Dierkes *et al.* 1999; Balshine *et al.* 2001; Stiver *et al.* 2004). *Neolamprologus pulcher* lives in the southern part of the lake, *N. brichardi* in the northern part, but the lake-wide distribution of interconnected populations showing clinal morphological variation suggests they are the same species (S. Balshine, E. Skubic, P. Dierkes, A. Grantner, M. Taborsky unpublished data). Taborsky & Limberger (1981) predicted that because of a high turn-over of breeders, helper age should be negatively correlated with helper relatedness to the breeders. Further studies have shown that either extra-group members may take up vacant breeding positions and may accept unrelated helpers of all sizes, or that large helpers may take the breeding position after a breeder

has disappeared (Taborsky 1984; Balshine-Earn *et al.* 1998; Balshine *et al.* 2001). Hence, the relative fitness benefits of group living gained through kin selection vs. those gained through direct selection should be helper age-dependent. This might be a general phenomenon in all the cooperatively breeding fish species, but until now genetic data on within-group (WG) relatedness are missing to test this idea.

Eight species of fish with 'helpers at the nest' have been described as yet, six of which are cichlids from Lake Tanganyika (Taborsky 1994; Cichlidae, tribe Lamprologini). Lamprologini are substrate-breeding cichlids, showing extensive parental care (Taborsky 1994). In the cooperatively breeding species both mature and immature helpers assist the breeders in brood care, maintenance and territory defence against predators and space competitors (Taborsky & Limberger 1981; Taborsky 1984, 1994). In *N. pulcher*/*N. brichardi*, both male and female offspring remain in the territory and become helpers. Experimental manipulations of group composition in the laboratory and in the field showed that helpers may increase the fitness of breeders (Taborsky 1984; Brouwer *et al.* 2005). Small helpers assist in brood care, digging behaviour and chasing away of small conspecific and heterospecific intruders, while large helpers assist in territory defence against all intruders including large piscivores (Taborsky 1984). Helpers and offspring show submissive behaviour towards all larger sized individuals within the group, leading to a size-dominance hierarchy with (usually a single) breeding female and breeding male in the top-dominant positions. Group membership and social cohesion are maintained by individual recognition (Balshine-Earn & Lotem 1998).

Here we present data on genetic relatedness in the cooperatively breeding cichlid *N. pulcher*. We test whether (i) there is a decline in helper to breeder relatedness with helper age as predicted, and explore the causes for changes in relatedness with helper age. Additionally, we test whether (ii) helpers are less related to the breeding male than to the female, for example because of extra-pair paternity (Dierkes *et al.* 1999, in press) and whether this may lead to (iii) helpers of either sex inheriting the dominant breeding positions of related individuals, as the largest helpers of both sexes may occupy breeding vacancies in their group (Balshine-Earn *et al.* 1998). Finally, we test (iv) how these combined effects influence helper-to-helper relatedness.

MATERIALS AND METHODS

Study site and DNA sampling

We studied four colonies of *N. pulcher* near Mpulungu, Zambia by scuba diving in 1995–1998 (8°46.849' S, 31°04.882' W). The four colonies were located at 7–11 m depth. We sampled 20, 9, 1 and 1 breeding groups per

colony (total of $n = 31$ groups). Groups were defined according to the criteria of Balshine *et al.* (2001). All fish were caught, body size measured [standard length (SL) in cm, i.e. measured from the tip of the snout to the base of the tail fin] and DNA sampled ($n = 330$; 30 female breeders, 21 male breeders, 279 helpers and offspring). Two groups did not have a clear female or male breeder at the time of sampling, and in one group the male breeder went unsampled. The sampled groups also included harem breeding males defending extended territories encompassing several groups (polygyny, see Limberger 1983). Four of these males defended two groups, and one male each defended three and four groups (and each group had a breeder female). All breeders and 35% of all helpers could be sexed by outer inspection of the genital papilla (sexed helpers had a SL of 2.7–5.9 cm). We refer to 'helpers' to indicate all the group members, except the breeding male and breeding female, because offspring start to defend the territory against same-sized intruders at a very early age (at this age within the seclusion of the shelters, but visible to observers from *c.* 1 cm SL onwards, D. Heg personal observations). We used the Blumberg growth curve of Skubic *et al.* (2004) to estimate the age (days) of each individual in the population.

Microsatellite analyses

Genomic DNA was extracted from tissue samples using proteinase K and phenol/chloroform/isoamylalcohol. Microsatellite DNA genotypes of all individuals were characterized for five loci developed for cichlid species (Parker & Kornfield 1996; Brandtmann *et al.* 1999; Schlieven *et al.* 2001). We used the loci 773, 780, 007, 101 and UME003. DNA was amplified in two 15- μ L multiplex reactions, using 40 ng of genomic DNA, 0.2 mM dNTPs, 0.5 μ M of each primer (all forward primers labelled with fluorescent dyes), 1.5 μ L 10X polymerase chain reaction (PCR) buffer, 2.5 mM MgCl and 0.75 units of *Taq* polymerase. PCR was started with an initial denaturation at 94 °C for 10 min, followed by 30 cycles of 94 °C for 30 s, 56 °C for 40 s and 72 °C for 70 s followed by a final extension step at 72 °C for 60 min. PCR products were analysed using an ABI Prism 310 automatic capillary sequencer and GENESCAN and GENOTYPER software (Applied Biosystems, Foster City, California, USA). Allele frequencies, observed and expected heterozygosities and tests for deviations from the Hardy–Weinberg equilibrium (all non-significant) were generated with GENEPop software (Raymond & Rousset 1995, see for details Dierkes *et al.* in press).

Genetic relatedness

We used all samples from the four different colonies, plus an independent sample of mature breeders and helpers ($n = 80$,

subsample from Stiver *et al.* 2004) to estimate population allele frequencies. Subsequently, we calculated global measures of genetic relatedness for colonies 1–4 to obtain within-group (WG, $n = 338$), between-group (BG, $n = 312$), within-colony (WC, $n = 338$), and between-colony (BC, $n = 338$) estimates of relatedness, using the software Relatedness 5.08 (Queller & Goodnight 1989). The calculations were based on WG: comparing individuals within the same group; BG: comparing individuals from different groups within the same colony (excludes colonies 3 and 4 with only a single group each); WC: comparing individuals from the same colony; and BC: comparing individuals from different colonies. We included the bias correction of the population background allele frequencies in terms of groups and weighted the estimates per group. Standard errors were obtained by Jackknife resampling over loci. Statistical significance of the differences between the measures of global relatedness were obtained from Relatedness 5.08 for WG vs. BG and WC vs. BC (P -values of the relatedness differences by jackknifing over loci).

Likewise, we calculated pairwise relatedness measures for WG helper to breeder and helper to helper comparisons, again weighing per group (Queller & Goodnight 1989). Genetic relatedness was normally distributed ($n = 4392$). Two major analyses were conducted using general linear mixed modelling (GLMM) in SPSS 11.0 (SPSS Inc., Chicago, Illinois, USA), both corrected for groups nested within harems as random effects (SPSS uses the 'restricted maximum likelihood method' to decompose variances).

First, helper to breeder genetic relatedness was related to $\ln[SL]$ of the helper as a continuous fixed effect, mating status (monogamy or polygyny) and breeder sex (male or female) as fixed effects (the natural logarithm of SL gave a significantly better fit than SL). The same analysis was performed on a subset of sexed helpers, adding sex of the helper as a fixed effect.

Second, helper to helper genetic relatedness was related to helper body size, $\ln[SL]$, and the difference in helper size, $\ln[SL \text{ larger helper} - SL \text{ smaller helper}]$, which includes same-sized helpers, as fixed continuous effects. In all analyses mating status (monogamy or polygyny) and all interactions between the independent variables or squared effects were not significant and hence omitted from the results.

Genotype reconstruction

First, genotypes of the former breeders in each group were reconstructed by manual comparison of the genotypes of all group members for each locus. The precondition for this analysis is one known breeder sex, i.e. a breeder still alive, sexed and genotyped, and the availability of an adequate large sample of same-sized helper cohorts within the group.

This pre-condition was met in 17 groups. We concluded that no breeder exchange has occurred when nearby cohorts were full-siblings. A single breeder exchange occurred, when a cohort were half-siblings of the previous cohort. A double breeder exchange occurred when a cohort was unrelated to the previous cohort.

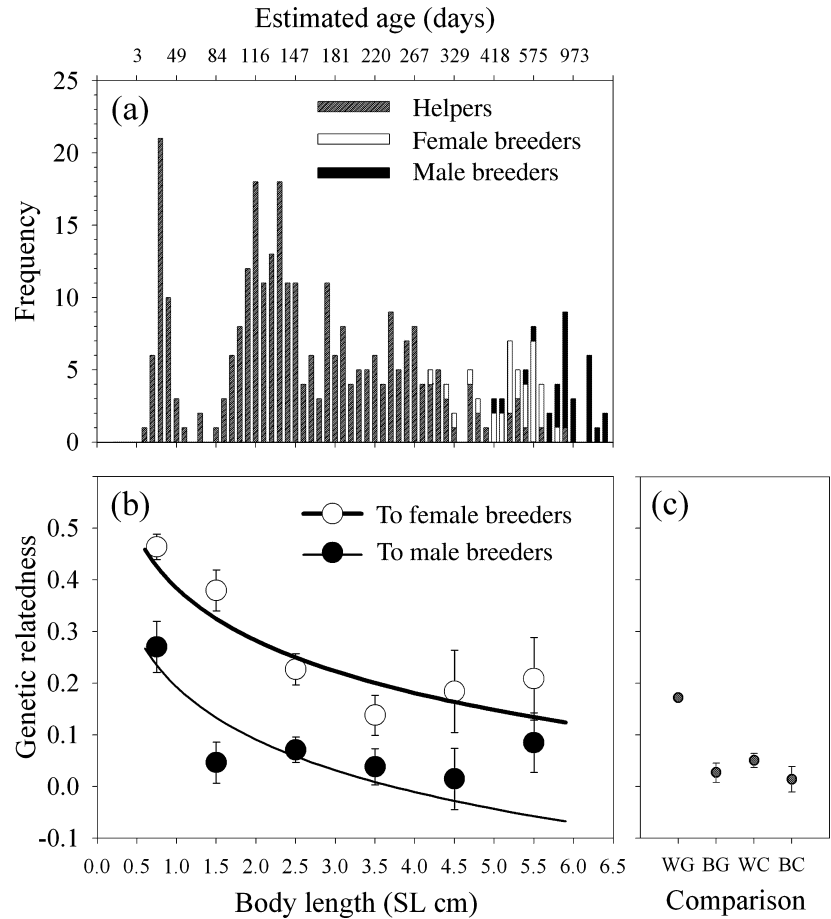
Second, the manual assignments were checked and confirmed by reconstructing kin-groups using the software KINGROUP version 050513 (Kononov *et al.* 2004, available from <http://www.kingroup.org>). As only five microsatellite markers were available, complex hypotheses testing was not attempted. Instead, we tested for each pair of individuals within each group whether they were significantly more likely to be: (i) full-siblings vs. unrelated, (ii) half-siblings vs. unrelated, (iii) full-siblings vs. half-siblings. We then compared the significance values and the kingroup assignments across these three analyses, and the manual assignments were confirmed in each case. Note that the correct sex of the replaced breeder could only be assigned in cases where both breeders were not replaced simultaneously. This situation occurred in one group, and therefore also one earlier replacement in this same group could not be assigned to breeder sex, leading to three missing values for breeder sex. Finally, the same genotype reconstruction could also be used to detect immigrant helpers, i.e. helpers neither related to both the (reconstructed) breeders nor to same-sized helpers within the group.

The Blumberg growth curve of Skubic *et al.* (2004) was used to estimate the age (days) of each individual in the population, and we calculated the tenure of current and former breeders in each group as follows. The minimum tenure was estimated as the difference in age between the oldest and youngest group member sired, the maximum tenure was calculated as the difference in age between the nearest oldest and the nearest youngest group members not sired by the breeder (Life Table Analysis, $n = 73$; 51 censored and 22 uncensored cases, including three cases where the sex of the breeder could not be reconstructed with certainty). We concluded that focal breeders had inherited the territory from a close relative (parent or full-sibling) when large siblings (sharing the same mother or father with the focal breeder based on the genotype reconstruction) were present in the group. Comparing the age estimates with the minimum size of breeders (from Balshine *et al.* 2001) confirmed that these focal breeders could not have produced these large siblings themselves.

RESULTS

As reported previously (Taborsky 1984; Balshine *et al.* 2001), breeding males were on average larger than breeding females and both were larger than the largest helper in the group (Fig. 1a, mean \pm SEM; SL = 5.9 ± 0.1 , 5.2 ± 0.1 and

Figure 1 Genetic relatedness in *Neolamprologus pulcher*. (a) Size distribution of the genotyped fish with estimated age in days. (b) Relatedness of helpers to the breeders in the group declined significantly with helper body size and age. Helpers were significantly less related to the male ($n = 264$) than to the female breeder ($n = 260$ pairwise comparisons). Depicted are mean values \pm SEM per 1 cm class, with the two regression lines from a GLMM (see text) and estimated age in days. (c) Within-group genetic relatedness (WG, 0.1722) was significantly higher than between-group relatedness from groups within the same colony (BG, 0.0270), and within-colony relatedness (WC, 0.0506) was significantly higher than between-colony relatedness (BC, 0.0143). Depicted are mean values of the estimates \pm SE by jackkniving over loci. Note the very small standard errors of the estimates (0.0029, 0.0187, 0.0138 and 0.0247 for the WG, BG, BC and WC estimates respectively).



4.4 ± 0.1 cm, $n = 18$, 30 and 31 respectively; GLM $F_{2,79} = 45.2$, $P < 0.001$, Tukey-B *post hoc* test: all three groups significantly different). Helpers were on average 3.1 ± 0.1 cm SL long (range 2.0–5.9 cm, excluding fish smaller than 2.0 cm SL for comparison with Balshine *et al.* 2001).

As predicted, helper to breeder genetic relatedness decreased with the body size and thus age of the helper (Fig. 1b, GLMM with correction for random group within harem effects: $F_{1,499.4} = 39.1$, $P < 0.001$; estimate \pm SE: -0.1464 ± 0.0232). Also, helpers were significantly more related to the female than to the male breeder ($F_{1,509.6} = 72.8$, $P < 0.001$, estimate to the female breeders: 0.1916 ± 0.0225 , estimate to the male breeders was set to zero; with intercept \pm SE: 0.1924 ± 0.0331). Sex of the helpers did not significantly alter these relationships when entered into the model and was in itself non-significant (GLMM $F_{1,145.0} = 2.5$, $P = 0.11$). The global measures of relatedness showed that the study population was spatially structured. First, WG relatedness was significantly higher than BG relatedness (Fig. 1c, difference \pm SE = 0.145 ± 0.017 , $P < 0.0001$). Second, WC relatedness was

significantly higher than BC relatedness (Fig. 1c, difference \pm SE = 0.036 ± 0.028 , $P = 0.027$, confirming results obtained by Stiver *et al.* 2004). Helper relatedness towards the female was significantly higher than all three non-WG global relatedness values for all helper sizes, whereas towards the male it was only significantly higher for the smallest 0–1-cm cohort (Fig. 1b vs. 1c, as indicated by non-overlapping standard errors).

The genetic relatedness between helpers and the current breeding pair in the group might be influenced by three major factors: (i) breeder replacement (Taborsky & Limberger 1981), (ii) helper immigration and (iii) extra-pair paternity (Dierkes *et al.* 1999). We used genotype reconstruction and daily growth rate estimates (see Materials and methods) to derive the genotypes of the shared parents of helpers and offspring through time. Thereby, we determined the rate of breeder exchange and helper immigration. A breeder was replaced every 198 days (median minimum estimate; median maximum estimate was 274 days from the Life Table Analysis, $n = 73$ breeders). This corresponds to helpers growing up to a size of *c.* 3.2 cm (median minimum or 4.1 cm maximum), and hence can explain the rapid

reduction in helper to breeder relatedness with helper size and age. Breeder males were more often replaced than breeder females (31 vs. 16 cases, Binomial test, $P = 0.04$), which is in agreement with the more rapid decline in helper to breeder male relatedness. Seven immigrants were detected of 205 helpers (3.5%, in seven of 31 groups, range SL immigrants was 2.4–3.8 cm, excluding all 74 helpers smaller than 2.0 cm, which are too small to disperse). These replacement and immigration rate values were minimum estimates. Particularly in small, less productive groups and groups where fast events of replacement or immigration occurred, no unequivocal assignment of the cause and timing of these changes could be inferred (large groups are more stable and productive than small groups, Heg *et al.* in press). Finally, the low relatedness of offspring to the breeding males (0.25 instead of 0.5, see 0–1-cm cohort in Fig. 1b) is because of the high level of extra-pair paternity, which is treated in more detail elsewhere (Dierkes *et al.* in press).

Experimental breeder removals have shown that large helpers might inherit the breeding position of disappeared breeders in *N. pulcher* (Balshine-Earn *et al.* 1998). The relatively high average relatedness of the large helpers to the female breeders (*c.* 0.2) suggests that large helpers might take over the breeding position of relatives. Using genotype reconstruction we detected six groups ($n = 31$, 19%) where a female helper had taken over the breeding position of her mother or sister. Females were more likely to take over their natal territory in large groups (Fig. 2). Likewise, we detected one probable case where the male helper took over the breeding position of the father or brother. Related males inherited the territory significantly less often than related

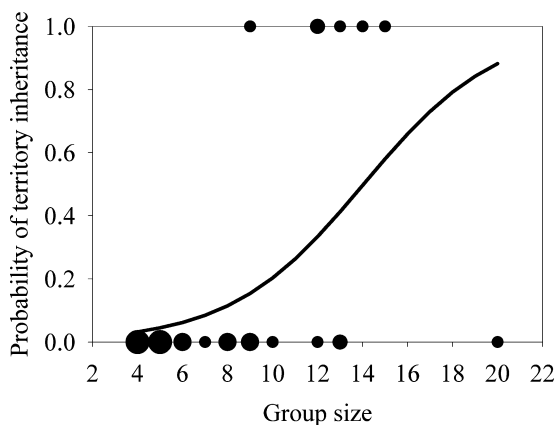


Figure 2 Females are more likely to inherit the breeding position of their mother (or sister) in large groups. Multiple cases are indicated by increasing symbol sizes (1–5 cases). Depicted is the increase in probability with group size from a logistic regression ($-2 \log$ likelihood = 23.1, parameter estimates \pm SE of intercept: -4.75 ± 1.71 , d.f. = 1, $P = 0.005$; of group size 0.34 ± 0.15 , d.f. = 1, $P = 0.025$).

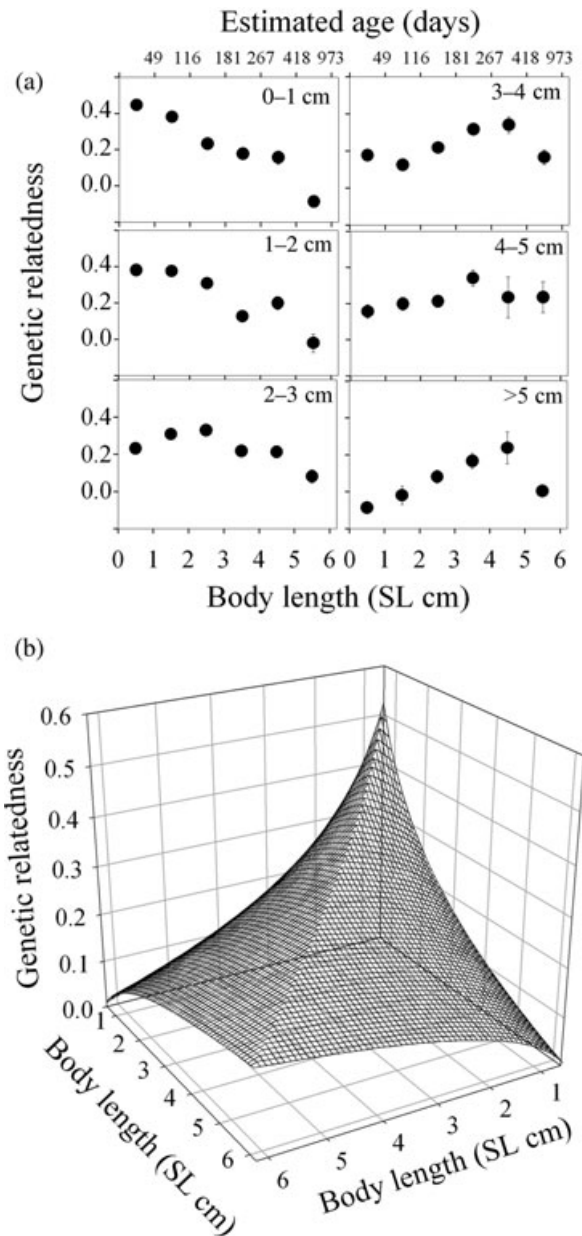


Figure 3 Helper to helper genetic relatedness is size- and age-assortative and declines with helper size and age in *Neolamprologus pulcher*. (a) Each panel shows a 1 cm SL cohort, and their mean \pm SEM relatedness to each cohort within the group ($n = 581, 680, 1109, 634, 238$ and 46 respectively) with estimated age in days. (b) Helper to helper relatedness landscape, showing the higher relatedness to nearby cohorts (the 'slopes') and the general decline in relatedness to same-sized cohorts with size (the 'ridge', GLMM correction for random group within harem effects, $n = 3288$, estimates \pm SE of the fixed effects: intercept: 0.3652 ± 0.0302 , $F_{1,50.7} = 146.5$, $P < 0.001$, $\ln[\text{SL}]$: -0.0801 ± 0.0178 , $F_{1,1594.5} = 20.3$, $P < 0.001$, $\ln[\text{difference in SL}]$: -0.1404 ± 0.0162 , $F_{1,1709.6} = 74.9$, $P < 0.001$). For estimated age see (a).

females ($G_1 = 4.4$, $P = 0.04$). No other cases of inheritance, nor evidence of offspring breeding with parents were detected. Also these values are minimum estimates, because of the difficulty to detect territory inheritance when all large sibling helpers have either died or dispersed.

The three major factors affecting the helper to breeder relatedness translated into size- and thus age-dependent helper to helper relatedness. As expected, *N. pulcher* helpers showed significant age-assortative genetic relatedness (Fig. 3a). First, relatedness between approximately same-aged helpers declined significantly with helper age (represented by the ridge in Fig. 3b). Second, relatedness between any two helpers decreased with their difference in helper age (represented by the slopes in Fig. 3b).

DISCUSSION

We conclude that the combination of breeder exchange, helper immigration and extra-pair paternity quickly reduces the genetic relatedness of helpers to their beneficiaries through time in our study species. In effect, many large, old helpers live in groups with unrelated breeders, nevertheless, they show risky and expensive helping behaviours like predator defence (Taborsky 1984; Grantner & Taborsky 1998). To our knowledge, the only species where similar results have been found are dwarf mongooses (Creel & Waser 1997) and wild dogs (Creel & Creel 2002), where relatedness to the dominants declines with subordinates' age. On the contrary, relatedness to breeding females in *N. pulcher* remains relatively high, because of the lower female breeder exchange rates, the low level of extra-pair maternity and the high level of extra-pair paternity (Dierkes *et al.* in press). Hence, group members in this study were on average still significantly higher related to each other than to members of other groups in the same colony (WG vs. BG estimates). This suggests a potential for multilevel selection operating on cooperative kin groups (*sensu* Keller 1999). The existence of unrelated helpers in our study species may explain why helpers apparently pay-to-stay for being tolerated in the group (Taborsky 1984, 1985; Balshine-Earn *et al.* 1998; Bergmüller *et al.* 2005; Bergmüller & Taborsky 2005). Moreover, strategic adjustments in helper growth may decrease the likelihood of eviction (Heg *et al.* 2004b), as after breeder replacement, helpers similar in size to the new breeders stand a high chance of being evicted (Balshine-Earn *et al.* 1998).

Sex-specific differences in parentage appear to be the main causes of the helper to breeder sex-biased genetic relatedness (i.e. the intercepts in Fig. 1b). Moreover, our data showed that breeder exchange was the main reason for the decline in helper to breeder relatedness with age (i.e. the slopes in Fig. 1b). These exchanges create cohorts of siblings and half-siblings in the family groups. As

helper immigration was very rare (3.5% of the helpers) and none of the immigrant helpers sired offspring (see also Dierkes *et al.* in press), helper immigration cannot explain the rapid decline in helper to breeder relatedness with helper age.

The turnover of breeders, and the relatively higher turnover rate of breeder males compared with breeder females results in a complex within-group genetic structuring. Explicitly, size cohorts within groups can be characterized by 'sib-ships', i.e. cohorts of sibs, which are half sibs of other cohorts or unrelated to them. At the same time, the pattern of breeder turnover results in a high within-group variation of relatedness. The actual turnover of breeders may be even larger, because parental genotype reconstruction was only possible if a number of same-sized helper cohorts were present in the group during DNA sampling. Similar sub-structuring has been found in cooperatively breeding primates (Altmann *et al.* 1996; Nievergelt *et al.* 2000). Colony takeovers have been suggested to result in low levels of relatedness also in a primitively eusocial sweat bee (Paxton *et al.* 2002).

Some female helpers directly inherited the breeding territory of their mother (or sister), and polygyny was common. To our knowledge, this is the first proof of the existence of matriline in fish, which is otherwise common in group-living primates (predominantly matrilineal, e.g. de Ruiter & Geffen 1998; Pope 2000; Kappeler *et al.* 2002; but see Gerloff *et al.* 1999) and sometimes found in birds (predominantly patrilineal, see Cockburn 1998). The higher incidence of matriline compared with patriline in our study species is most likely caused by the sex-bias in the breeder turnover rate combined with a high level of extra-pair paternity, since Stiver *et al.* (2004) found no evidence for sex-biased dispersal.

Helpers of both sexes assisting unrelated group members are uncommon in vertebrates (Creel & Macdonald 1995), where usually either male or female offspring help parents raising sibs (Solomon & French 1997; Cockburn 1998). Our results in *N. pulcher* corroborate recent insights (Clutton-Brock 2002; Griffin & West 2003; Koenig & Dickinson 2004) that direct benefits might be more important in cooperative breeding systems than previously thought. In fact, the exponential decline in helper to breeder relatedness with helper age suggests that the importance of direct benefits should increase with helper age (see Taborsky 1985). These benefits might be an increase in survival because of group enhancement (Heg *et al.* 2004a) or reproductive parasitism (Dierkes *et al.* 1999; Skubic *et al.* 2004). Young helpers are likely to live in groups with their parents still present and hence may accrue indirect benefits by assisting their parents in raising offspring (Taborsky 1984; Brouwer *et al.* 2005).

We conclude that the ratio of kin selected vs. directly selected fitness benefits should decline with helper age. The

high variation in within-group genetic relatedness in *N. pulcher* and the experimental possibilities to alter the group composition in the field and laboratory offer excellent opportunities to test general models about how direct and indirect fitness benefits should affect cooperative breeding and helping behaviour.

ACKNOWLEDGEMENTS

We are grateful to the Zambia Ministry of Agriculture, Food and Fisheries for their continuous support of our work, in particular our colleagues at the Fisheries Department in Mpulungu, especially C. Kapasa, H. Phiri, R. Shapola, L. Makasa, D. Sinyinza and C. Lukwesa. We thank M. Müller and G. Brem for providing laboratory facilities, A. Kunz for field assistance and K. Goodnight and U. Dieckmann for helpful discussions and comments. We thank D. Schütz, R. Bergmüller, I. Hamilton, J. Strassmann and anonymous referees for comments on the manuscript. This work was supported through a grant from the Austrian Science Fund (FWF: project P10916-BIO) and a grant of the Swiss National Science Foundation (SNSF: project 3100-064396), both to M.T.

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Editor, Joan Strassmann

Manuscript received 7 February 2005

First decision made 17 March 2005

Manuscript accepted 3 June 2005