

Group composition affects male reproductive partitioning in a cooperatively breeding cichlid

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Abstract

Individuals within groups of cooperatively breeding species may partition reproduction, with the dominant pair often taking the largest share. The dominant's ability to reproductively control subordinates may depend on differences in competitive ability, due to, e.g. body size differences, but may also depend on the number of same-sex competitors inside the group. We tested experimentally whether subordinates reproduce more when these subordinates are large or when a second subordinate of the same sex need to be controlled by the dominants, using the cooperatively breeding cichlid *Neolamprologus pulcher*. Dominant pairs were assisted by a large and a small unrelated subordinate; sexes of these fish were varied in a full-factorial design (giving four treatments). Dominant males lost significantly more parentage to the large subordinate male when a small subordinate male was also present, compared to when a small subordinate female was present. However, subordinate paternity was generally low and did not significantly curb total dominant male reproductive output, which was more affected by the sizes and numbers of reproductive females present inside his group. Dominant female maternity, clutch sizes and total output did not depend on the treatments. Subordinate–subordinate reproduction was virtually absent (one out of 874 offspring). Female subordinates were more likely to provide care for their own broods. In contrast, male subordinates did not adjust their level of care to their parentage. Variability in female subordinate alloparental brood care was particularly high, with females showing more care than males in general. We also detected effects of growth rate and food ration on parentage independent of the treatments, most notably: (i) a trade-off between dominant male growth rate and paternity; (ii) a decrease in dominant male paternity with increasing food ration; (iii) a positive effect of growth rate on paternity in small males. We conclude that dominant males should be sensitive to the number and sizes of subordinate males present in their group, particularly when these subordinates are not helpful or grow fast, and food is plentiful. Dominant females should be less sensitive, because female subordinates do not appear to impose reproductive costs and can be helpful through alloparental brood care.

Keywords: brood care, Cichlidae, cooperative breeding, helping behaviour, maternity, microsatellite DNA, paternity, reproductive skew

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Introduction

Dominants and subordinates within cooperatively breeding groups may partition reproduction, but the amount of partitioning, 'reproductive skew', varies widely between and within species (e.g. Haydock *et al.* 1996; Richardson *et al.* 2001; Williams 2004; Bradley *et al.* 2005; Heg *et al.*

2006b). In some vertebrates, dominants claim virtually all reproduction inside their group (high skew), whereas in others, reproduction is partitioned more or less evenly between the group members of the same sex (low skew). Dominants are expected to concede some reproduction to the subordinates, if this increases the likelihood of these subordinates staying in the group, and provided subordinates are in some way helpful (Reeve *et al.* 1998; Reeve & Jeanne 2003, e.g. alloparental brood care increasing the quantity or quality of dominant offspring produced). On

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the other hand, subordinates and dominants may also engage in a tug-of-war over reproduction, where both actively compete for reproduction at a cost to total group productivity (Reeve *et al.* 1998; Nonacs 2007). In these cases, the ability of dominants to control subordinate reproduction may depend on internal (e.g. body size differences, Heg *et al.* 2006b; Heg & Hamilton 2008) and external factors (e.g. distribution of breeding resources, Heg & Hamilton 2008). Under tug-of-war, dominants are expected to compete over reproduction with same-sex subordinates, but interference by group members of the other sex may influence this conflict (Heg *et al.* 2006b; Hamilton & Heg 2007; see also Fitzpatrick *et al.* 2008).

Effects of within-group relatedness and ecological constraints (e.g. the availability of breeding resources) on reproductive skew have been studied (Langer *et al.* 2004; Heg *et al.* 2006b), but experimental studies of the effects of group composition, i.e. number, sex and types of subordinates, are lacking (see for correlative studies, e.g. Webster *et al.* 2004; Bradley *et al.* 2005). This is unfortunate, because a group's composition may determine how much reproduction dominants share with the subordinates (e.g. when more same-sex group members are present, more dominant reproduction is lost to those subordinates). In turn, this may determine whether a dominant should accept same-sex immigrant subordinates as additional group members, or whether a dominant should expel a certain type of subordinate from the group (e.g. expulsion of a male subordinate by the dominant male after the subordinate has engaged in reproduction, Dierkes *et al.* 1999; Skubic *et al.* 2004). Actual group sizes found in nature may thus, in part, be based upon this interplay between subordinate reproduction, subordinate helpfulness and dominant acceptance of same-sex subordinates. Our purpose is to bridge the gap between standard cooperation theory, which usually only addresses interactions between two individuals (see review Bergmüller *et al.* 2007), and more tailored theories, which take the dynamics of multimember groups into account (e.g. Reeve & Emlen 2000; Reeve & Jeanne 2003).

In group-living fish, dominance hierarchies are usually based on the size differences between the group members (Heg *et al.* 2004b, 2005a; Hamilton *et al.* 2005a; Mitchell 2005; Heg & Bachar 2006; Wong *et al.* 2007; Hamilton & Heg 2008). For instance, in anemonefish (Ross 1978; Fricke 1979; Ochi 1989), and *Amphiprion percula* in particular, growth rates are adjusted to maintain certain size differences between adjacent-ranked group members (Buston 2003b; Buston & Cant 2006). If the smallest group member reaches a certain size, it may accept a new very small recruit into the group, and this process is sufficient to explain the differences in group sizes founded (Buston 2003a; Buston & Cant 2006). However, in anemonefish, subordinates neither help nor reproduce (Mitchell 2003; Buston 2004). In cooperatively breeding cichlid fish, conflicts appear more severe, because

subordinate fish of both sexes are perfectly capable of reproducing (Dierkes *et al.* 1999; Awata *et al.* 2005, 2006; Heg *et al.* 2006; Heg 2008). In *Neolamprologus pulcher*, subordinates increase the reproductive success of dominants (Brouwer *et al.* 2005), but some subordinates are more helpful than others and may also impose costs on the dominants (Bergmüller & Taborsky 2007; Heg & Hamilton 2008).

Here, we test experimentally whether the level of reproductive sharing with male and female subordinates depends on a group's composition, using the *N. pulcher* model system. Parentage assignment was conducted with microsatellite DNA markers. In this cichlid fish, artificial groups can be created in various compositions (Heg *et al.* 2004b; Bergmüller *et al.* 2005; Heg & Hamilton 2008). We created groups of four individuals, each group containing a dominant breeder pair, a large subordinate and a small subordinate. The sexes of these subordinates varied in a full-factorial design, giving four treatments (Mm, large male, small male; Ff, large female, small female; Mf, large male, small female; Fm, large female, small male). The null hypothesis is that dominants can control reproduction irrespective of the number and sizes of same-sex competitors inside their group (e.g. dominant paternity $Mm = Mf = Fm$). The first alternative hypothesis is that if dominants have more difficulty in controlling multiple subordinates of the same-sex, particularly if they are large, they should lose parentage accordingly (e.g. dominant paternity $Fm > Mf > Mm$, see Heg *et al.* 2006 for subordinate size-effect). The second alternative hypothesis is that if subordinates not only compete with the dominant for reproduction, but also with other same-sex subordinates, such interference may lead to subordinates siring fewer offspring compared to similar subordinates living in groups with no such additional competitors (e.g. dominant paternity $Mm > Fm = Mf$).

Males may trade-off growth with investment in their gonads (Fitzpatrick *et al.* 2008) and female growth may influence her reproductive capacity through changes in body size (Heg & Hamilton 2008); both maybe mediated by differences in food abundance. Therefore, we also assessed whether male and female reproduction depended on their growth rate and daily food ration. Finally, to assess whether dominants may share reproduction to ensure help by the subordinates, we test whether subordinate brood care depends on the subordinate's sex and their level of parentage in the brood.

Materials and methods

Experimental set-up

We created artificial groups of four unrelated mature individuals. All fish were laboratory-reared descendants of fish caught near Mpulungu, Zambia, at the southern end of Lake Tanganyika, and had been housed in large aggregation

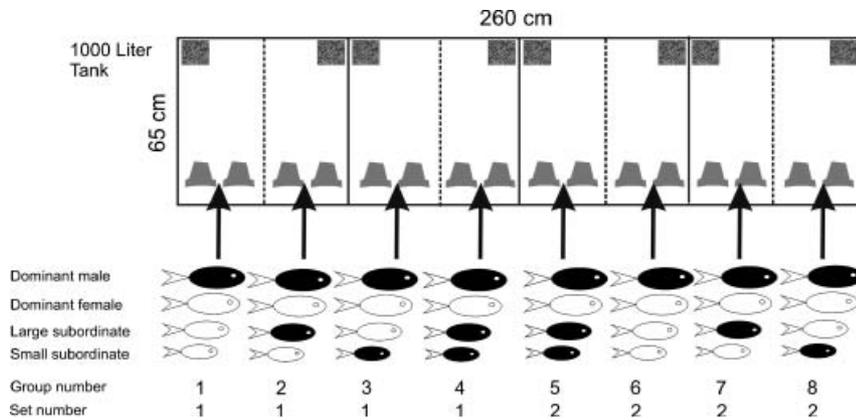


Fig. 1 Setup of the experiment exemplified for one tank. Each 1000-L tank contained eight groups from two sets, where two adjacent groups had visual contact (dashed partitions), but could not see other groups (continuous partitions). Each group had two pot halves for breeding, a filter (grey square) and an air stone.

aquaria without access to breeding substrate; thus, were not breeding before the experiment started. We created 12 sets of four groups ($n = 48$ groups). In each set, the four breeder males were approximately size-matched, as were the four breeder females, the four large subordinates and the four small subordinates (Fig. 1). We measured the size of the fish at the start of the experiment (body mass in mg; standard length SL in mm measured by two to three observers and averaged), sexed by examining the genital papilla, and individually marked by taking a small fin-clip from the dorsal and/or anal fin. Compartments were separated by alternating clear and opaque partitions, such that each group could see one adjacent group from the same set of four (Fig. 1). Eight groups (two sets) were maintained in adjacent 125-L compartments within one 1000-L aquarium (Fig. 1). We used three aquariums sequentially (3×2 sets, and again 3×2 sets after completion of the first six sets, gives 12 sets in total). Compartments measured 65 cm length \times 32.5 cm breadth \times 65 cm height (Fig. 1). The floor of the aquarium was covered with c. 6 cm of sand. Each compartment contained: two clay flower pot halves (used as shelters and for breeding, Fig. 1), a translucent tube (suspended near the surface, used as a refuge from aggression), and a suspended filter (also used as a refuge). The availability of refugia ensured that subordinates could always avoid interactions with dominants, which usually stayed near the pot halves. The treatments of neighbouring groups were randomized (e.g. Fig. 1).

After the body measurements, all subordinates were released directly into their respective compartments. Dominants were kept overnight in single isolation nets within their compartments before being released. Water temperature was maintained at an average of 28 °C (± 0.7 SD). The light regime was a 13:11 h light:dark cycle. Groups were fed daily with commercial TetraMin flake food. The amount of food was tailored to the sizes of the fish in each group per sequence (2.5% of the group's combined mass per day; group's combined mass was determined at the start of each sequence). Food ration was defined as the total amount of

food provided per day (mg), determined for each group \times sequence separately. After 30 and 60 days, body measurements were taken of all individuals.

Group compositions and experimental procedure

Subordinates' sexes were varied with the intention that all four possible subordinate gender combinations be represented within each set. Re-sexing of subordinates at the conclusion of the experiment revealed that some individuals had been initially mis-sexed, such that the final group compositions were as follows (mean SL \pm SD of the subordinates): (i) 11 groups with large subordinate male (50.2 ± 2.3 mm), small subordinate male (40.8 ± 1.7 mm), treatment 'Mm'; (ii) 12 groups with large subordinate male (50.4 ± 2.0 mm), small subordinate female (41.4 ± 2.4 mm), treatment 'Mf'; (iii) 11 groups with large subordinate female (50.6 ± 2.3 mm), small subordinate male (41.6 ± 2.4 mm), treatment 'Fm'; (iv) 14 groups with large subordinate female (49.8 ± 1.9 mm), small subordinate female (41.3 ± 1.7 mm), treatment 'Ff'. Breeder males measured 67.0 ± 5.9 mm and breeder females 61.7 ± 4.0 mm (mean SL \pm SD, both $n = 48$). Body condition was defined as ($K = \text{body mass}/\text{SL}^3 \times 100$, Taborsky *et al.* 2007). Large subordinate, small subordinate, dominant male and dominant female SL, body mass and body condition did not differ between the treatments ($4 \times 3 = 12$ separate ANOVA tests, all $P > 0.14$).

Both dominant and subordinate females potentially produce clutches, at least in groups where all members are unrelated (Heg 2008; Heg & Hamilton 2008). To ensure that mothers were correctly identified, groups were checked daily for courtship, spawning and egg laying behaviour. *Neolamprologus pulcher* females clean the substrate of the breeding shelter starting 1 to 2 days before spawning and dig away excessive sand. Prelaying females also intensively court the dominant male and may engage in 'pseudo-spawning' (behaviourally identical to spawning, but without eggs being laid). Spawning takes several hours. Some spawning events were observed directly or through

video-recording of the compartments. During spawning by dominant females, subordinates are usually not allowed inside the breeding shelter by the dominants, but some exceptions occur. During spawning by subordinate females, dominant females often try to enter the shelter, but may be prevented from doing so by the dominant male (chasing) or subordinate female (blocking of the entrance while tail-quivering). DNA microsatellite analyses of a subset of broods confirmed that mothers were assigned correctly (see below).

After spawning was completed, parental and alloparental brood care (frequency of cleaning and fanning eggs combined) was determined for all four group members simultaneously during three 5-min observations by E.J. and J.M. (data were summed over these 15 min) and the clutch size was determined. Frequency of brood care was determined for 58 broods from 31 groups, where also paternity and maternity assignment by microsatellite DNA was successful (see below). In the evening, clutches were removed. Note that cichlids may eat eggs (Heg & Hamilton 2008), but the proportion of dominant female eggs eaten (6.5%) and subordinate female eggs eaten (7.2%) did not depend on the treatments (probit GEEs on eaten eggs/clutch size: dominant females, $P = 0.43$, d.f. = 3; subordinate females, $P = 0.53$, d.f. = 3). About half of the clutch was removed, eggs counted and weighed as part of other data analyses presented elsewhere. The other half of the clutch remained attached to the pots and was transferred to separate isolation nets, where the eggs were incubated artificially. After 4 to 12 days, hatched offspring were stored in 99% ethanol inside 1.5 mL DNA storage tubes for future microsatellite DNA analyses. Note, therefore, that we measured reproductive skew on the day that the eggs were laid.

Microsatellite DNA analyses

Eleven polymorphic microsatellite loci developed for different cichlid species (see Table S1, Supporting Information): loci NP007 (= UME002), NP773 (= US-758/773), ULI2 (= US-780/783): Schliewen *et al.* 2001; Pzeb4: Van Oppen *et al.* 1997; TmoM11, TmoM13, TmoM25 and TmoM27: Zardoya *et al.* 1996; UME003: Parker & Kornfield 1996; UNH106 (GenBank Accession no. G12259) and UNH154 (GenBank Accession no. G12306): Lee & Kocher 1996; were used to determine the parentage of the broods (see Table 1 for sample sizes). All the loci had at least two to maximally 18 alleles per 152 unrelated individuals (combined breeders and helpers in the experiment, see Table S1).

Genomic DNA was extracted from ethanol-preserved fin clip samples of breeders and subordinates (*c.* 1–2 mm² in size each) or from whole ethanol-preserved offspring using Magnetic Beads (MagneSil Blue, Promega, White *et al.* 1998) and a MagnaBot 96 Magnetic Separation Device

Table 1 Assigned paternity and maternity of offspring per treatment

| Father | Mother | No. of broods | No. of offspring |
|---|----------|---------------|------------------|
| Treatment: male, male ($n = 28$ broods, 9 groups) | | | |
| Dominant | Dominant | 28* | 460 |
| Large | Dominant | 8 | 19 |
| Small | Dominant | 3 | 12 |
| Treatment: male, female ($n = 15$ broods, 7 groups) | | | |
| Dominant | Dominant | 14 | 308 |
| Large | Dominant | 0 | 0 |
| Dominant | Small | 1‡ | 14 |
| Large | Small | 1‡ | 1 |
| Treatment: female, male ($n = 27$ broods, 10 groups) | | | |
| Dominant | Dominant | 25† | 528 |
| Dominant | Large | 2 | 18 |
| Small | Dominant | 3 | 5 |
| Small | Large | 0 | 0 |
| Treatment: female, female ($n = 28$ broods, 12 groups) | | | |
| Dominant | Dominant | 24§ | 403 |
| Dominant | Large | 3 | 58 |
| Dominant | Small | 2§ | 17 |

Treatment: sex of large subordinate, sex of small subordinate.

Note that in all broods, the dominant male at least had some paternity; and the subordinate males never gained simultaneously paternity within the same brood.

*includes 17 broods with 187 offspring where the dominant male had 100% paternity.

†includes 22 broods with 446 offspring where the dominant male had 100% paternity.

‡mixed paternity in same single brood by small subordinate female.

§The only case of mixed maternity was detected in a brood where 15 offspring were assigned to the dominant female and 16 offspring to the small subordinate female.

(Promega). Tissue lysis was carried out in 96-well deep-well plates with a Lysis-Buffer containing Nuclei Lysis Solution (Promega), 0.5 M EDTA and Proteinase K according to the Wizard Genomic DNA Purification Protocol (Technical Manual no. TM050, Promega). DNA was captured by adding MagneSi Blue to the lysate and washed three times with 80% EtOH. Finally, DNA was eluted by adding 50–100:1 of H₂O.

All 11 microsatellite primer pairs were multiplexed in one polymerase chain reaction (PCR) using the QIAGEN Multiplex PCR Kit (QIAGEN). PCRs were carried out in a 10 µL volume containing 1 µL of the genomic DNA, 1H QIAGEN Multiplex PCR Master Mix (consisting of QIAGEN Multiplex PCR Buffer with a final concentration of 3 mM MgCl₂, dNTP mix and HotStar *Taq* DNA Polymerase), 2 µM of locus-specific fluorescent-labelled forward primer and nonlabelled reverse primer. Fluorescent dyes were 6-FAM, HEX (Microsynth), NED and PET (Applied Biosystems). In order to improve allele-calling efficiency, eight microsatellite

primers (NP007, NP773, ULI2, UME003, TmoM11, TmoM13, TmoM25, TmoM27) were modulated by adding the sequence GTTCTT to the 5' end of the reverse primer sequence. This reverse-primer tailing improves allele calling by driving the nontemplate directed nucleotide addition ('plus A addition') of the 3' end of the forward strand to completion, facilitating accurate genotyping as a result of consistent allele calls (Brownstein *et al.* 1996).

Amplification was achieved in a 96-well GeneAmp 9700 PCR System (Applied Biosystems) by using the following cycling parameters: 15 min at 95 °C; 35 cycles consisting of 30 s at 94 °C, 3 min at 57 °C and 1 min at 72 °C, followed by a final 15-min extension at 72 °C. Fluorescent PCR fragments were visualized by capillary electrophoresis on an ABI PRISM 3100 Genetic Analyzer and analyzed by the GeneMapper Analysis Software version 3.7 (Applied Biosystems).

Parentage assignment

Potential parents for each offspring were the group member breeder male, breeder female and the two subordinates. Note that we used unrelated individuals to create breeding groups in all cases. Based on a previous publication suggesting low levels of extra-pair maternity (Heg *et al.* 2006b), maternity was assigned first and paternity was assigned after mothers had been assigned, using the Cervus version 3.0 software and the resulting number of mismatching alleles for the different parent combinations (Kalinowski *et al.* 2007). All offspring had zero mismatching alleles with their putative parents and could be unambiguously assigned. The average number of offspring per brood per treatment successfully genotyped and assigned were (\pm SD, range, n broods): Mm: 17.5 ± 14.8 (2–69, $n = 28$); Mf: 21.5 ± 9.2 (2–31, $n = 15$); Fm: 20.4 ± 14.9 (2–66, $n = 27$); Ff: 17.1 ± 12.1 (1–31, $n = 28$).

Statistical analyses

Allele frequencies, observed and expected heterozygosities, and exclusion probabilities were determined using the Cervus version 3.0 software package (Kalinowski *et al.* 2007, see Table S1). DNA microsatellite analysis from a subsample of broods confirmed that we correctly identified the female who had spawned the eggs in 91 out of 91 dominant female broods and seven out of eight subordinate female broods (see Results). In the one case where we failed to identify subordinate female spawning, DNA maternity analysis showed she had spawned simultaneously with the dominant female inside the same breeding shelter during the weekend, when observations were less intensively conducted (see also Heg & Hamilton 2008). Therefore, for the final analyses of total female reproduction (number of eggs produced/60 days), we assumed all 260 broods were correctly assigned

to their mothers based on the behavioural observations of prespawning courtship and actual spawning. For the final analyses of total male reproduction (number of eggs sired/60 days), we summed the siring rate \times clutch size for each clutch and male, implementing average values of siring rate for each male \times female combination for the missing values. Treatment effects were analysed using generalized estimating equations (GEE) in *SPSS* 15.0 (Norusis 2007), corrected for group effects (entered as 'subjects'), as we had multiple broods from the same group in most cases. The GEE procedure adjusts the standard errors for multiple observations on the same unit. Scaling parameters were adjusted using the deviance method (Norusis 2007). Coefficients for the effects are reported as $B \pm SE$ throughout.

Growth of dominant males and females, subordinate large males and females, and subordinate small males and females did not correlate with food ration (Pearson's correlations r : $P = 0.58$ and 0.29 , 0.47 and 0.08 , 0.83 and 0.48 , respectively). In subordinate large females, the relationship was almost significant, but negative ($r = -0.25$). Therefore, both growth and food ration were entered as independent covariates into the GEEs.

Results

The dominant males (98%) and dominant females (94.1%) sired the majority of offspring ($n = 1843$ offspring, Table 1). Nevertheless, subordinate reproduction occurred in all treatments (Table 1). In total, 17 subordinates were involved: five large subordinate males, five small subordinate males, four large subordinate females and three small subordinate females. In the treatment Ff, all offspring were successfully assigned to the dominant male (Table 1) and in the treatment Mm, all offspring were successfully assigned to the dominant female (Table 1). Only one case of a mixed maternity brood was detected (Table 1): this brood was produced during the weekend, when unfortunately less-intensive spawning observations were conducted.

Paternity

Dominant male paternity depended significantly on the treatments (Fig. 2a, Table 2). This was due to dominant males losing parentage to large and small subordinate males, and to both males when both were present (Mm treatment). Moreover, the loss in parentage was nonadditive in this Mm treatment: large male subordinates significantly increased their paternity when an additional small male subordinate was present, compared to when a small female subordinate was present (compare Mm vs. Mf in Fig. 2a, Table 2). Small male subordinates did not sire more offspring when a large male subordinate was present, compared to when a large female subordinate was present (compare Mm vs. Fm in Fig. 2a, Table 2).

Table 2 Treatment, growth rate (SL mm/30 days) and food ration (mg/day) effects on dominant and subordinate paternity and maternity

| Group member | Treatments compared | <i>n</i> Groups, Broods | Treatment | | | Growth rate | | | Food ration | | | |
|-------------------|---------------------|-------------------------|-----------|---------------|----------|-------------|---------------|----------|-------------|---------------|----------|--|
| | | | d.f. | Wald χ^2 | <i>P</i> | d.f. | Wald χ^2 | <i>P</i> | d.f. | Wald χ^2 | <i>P</i> | |
| Paternity | | | | | | | | | | | | |
| Dominant male | Mm, Mf, Fm | 26,70 | 2 | 25.8 | < 0.001 | 1 | 14.0 | < 0.001 | 1 | 6.2 | 0.013 | |
| Large subordinate | Mm, Mf | 16,43 | 1 | 8.7 | 0.003 | 1 | 0.2 | 0.63 | 1 | 13.2 | < 0.001 | |
| Small subordinate | Mm, Fm | 19,55 | 1 | 1.0 | 0.32 | 1 | 6.9 | 0.008 | 1 | 1.4 | 0.23 | |
| Maternity | | | | | | | | | | | | |
| Dominant female | Ff, Fm, Mf | 29,70 | 2 | 1.7 | 0.44 | 1 | 0.1 | 0.77 | 1 | 4.0 | 0.045 | |
| Large subordinate | Ff, Fm | 22,55 | 1 | 1.6 | 0.21 | 1 | 1.9 | 0.17 | 1 | 3.1 | 0.078 | |
| Small subordinate | Ff, Mf | 19,43 | 1 | 0.004 | 0.95 | 1 | 2.0 | 0.16 | 1 | 0.01 | 0.92 | |

Results from separate generalized estimating equations, with group identifier as subjects, scale adjusted using the deviance method. The response variable is the number of offspring assigned/number of offspring genotyped as weighted binomial distribution with a negative log-log link (subordinates) or probit-link (dominants). Treatments compared: sex of large subordinate, sex of small subordinate, where M and m, large and small male; F and f, large and small female.

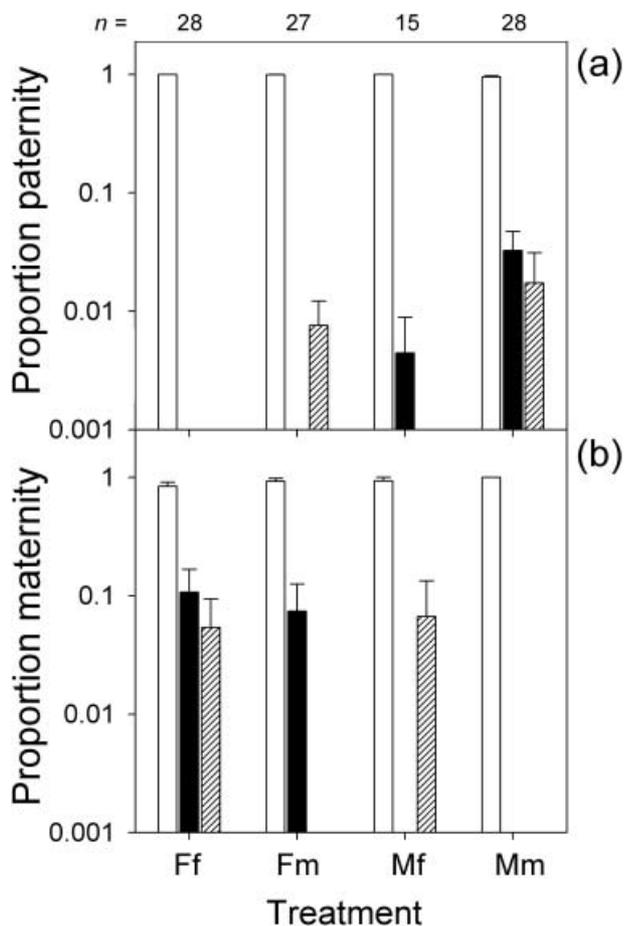


Fig. 2 The average proportion of offspring assigned per brood, (a) paternity and (b) maternity for the different treatments (\pm SE, log-scale). Dominants, white bars; large subordinates, black bars; small subordinates, hatched bars. Sample sizes, number of broods, are indicated on top of the graph.

Additionally, fast-growing dominant males lost significantly more paternity (Fig. 3a, Table 2, $B \pm$ SE: 0.43 ± 0.11) as did dominant males receiving relatively high rations (Fig. 3b, Table 2, $B \pm$ SE: 0.0017 ± 0.0007). The latter effect was consistent with large subordinate males siring significantly more offspring when food ration was high (Tables 2, $B \pm$ SE: 0.0021 ± 0.0006). Moreover, small subordinate males sired significantly more offspring when they were growing fast (Tables 2, $B \pm$ SE: 0.23 ± 0.09).

Maternity

Dominant female maternity was not affected by the treatments (Fig. 2b, Table 2). Likewise, large female subordinates were not more engaged in reproduction when a small male subordinate was present, compared to when a small female subordinate was present (Fig. 2b, Table 2). Also, small female subordinates shared on average an equal amount of maternity across the treatments (Fig. 2b, Table 2).

Female growth rate did not significantly affect maternity (Table 2). In contrast, food ration significantly increased dominant female maternity (Table 2, $B \pm$ SE: 0.0034 ± 0.0017) and tended to decrease large subordinate female maternity (Table 2, $B \pm$ SE: 0.006 ± 0.0036).

Total female reproduction

Since mixed-maternity broods were so rare (one out of 98 DNA analysed broods), we can safely assume that our spawning observations were sufficient to determine the mother of the brood in the vast majority of cases. In total, 246 dominant female broods were detected (per treatment: Ff: 66, Fm: 65, Mf: 48, Mm: 67 broods) with a clutch size of 100.0 ± 51.4 eggs (\pm SD, range 1 to 322 eggs). We also detected 10 large subordinate female broods (Ff: 5, Fm: 5

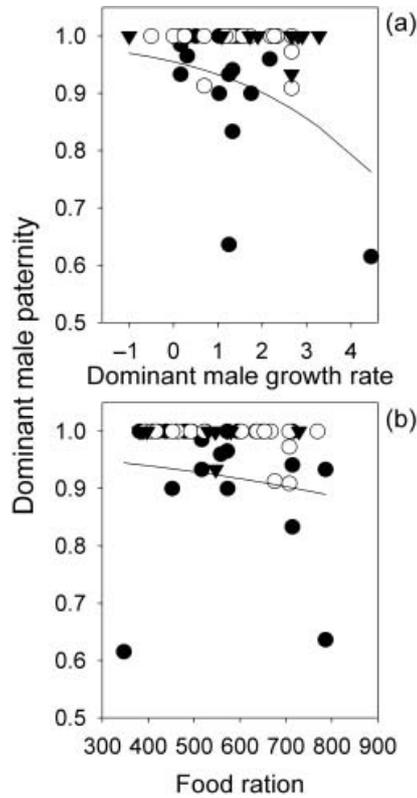


Fig. 3 Dominant male parentage depending on his (a) growth rate (SL mm/30 days) and (b) food ration (mg/day). Treatments: Mm (black dots, $n = 28$), Mf (black triangles, $n = 15$), Fm (white dots, $n = 27$). Also depicted are probit regression lines from the models presented in Table 2, corrected for the other effects [in (a) corrected for average treatment and average food ration; in (b) corrected for average treatment and growth rate effects].

broods) with a clutch size of 40.9 ± 26.9 eggs (\pm SD, range 1 to 81 eggs); and 4 small subordinate female broods (Ff: 2, Mf: 2 broods) with a clutch size of 37.0 ± 35.3 eggs (\pm SD, range 2 to 86 eggs). Clutch sizes were averaged per individual female before further analyses.

Dominant female clutch size and total number of eggs produced did not depend on the treatments (Fig. 4a, b, Table 3). Large and small subordinate females produced on average smaller clutches than dominants did, but this was due to body size effects (Fig. 4a, ANOVA on average clutch size per individual, $n = 45$ dominants, seven large and four small subordinates; effect of covariate body size SL mm: $F_{1,56} = 21.2$, $P < 0.001$), not due to their rank (effect of rank: dominant, large or small subordinate: $F_{2,56} = 1.3$, $P = 0.27$). Both large and small subordinate female reproduction did not depend on the treatments (Fig. 4a, b, Table 3). However, body size had a significant positive effect on large subordinate female reproduction ($B \pm$ SE: 7.51 ± 3.22) and these females were significantly less likely to produce eggs when food ration was high (Fig. 4b, Table 3, $B \pm$ SE: 0.183 ± 0.056).

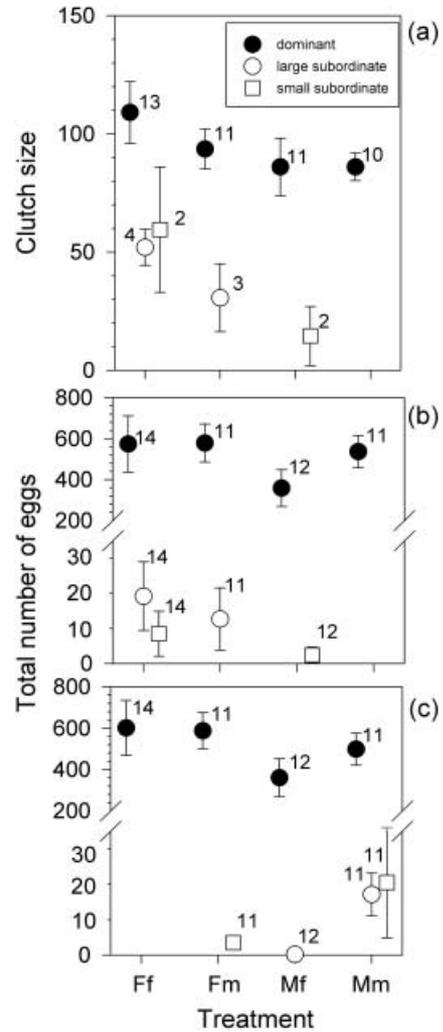


Fig. 4 (a) Average female clutch size (SE), (b) total number of eggs spawned over 60 days of the females, and (c) total number of eggs sired by the males depending on the treatments. Dominants: black circles; large subordinates: white circles; small subordinates: white squares. Sample sizes, which are the number of individuals, are indicated in the graph.

Total male reproduction

The previous results have shown that dominant males incur parentage loss to both large and small subordinate males. Assuming average paternity for all unsampled broods (see Methods), we also calculated the total number of eggs sired by each male depending on the treatments, male body size, male growth rate and food ration (Table 3). Apparently, the dominant parentage loss was so slight that dominant male reproduction did not depend on the treatments (c. 350 to 600 eggs sired, Fig. 4c, Table 3), which is understandable given the very low number of eggs sired by the subordinate males (c. 0.2 to 20 eggs sired, Fig. 4c). Nevertheless, large subordinate males sired significantly

Table 3 Treatment, body size (SL mm), growth rate (SL mm/30 days) and food ration (mg/day) effects on female clutch size, and female and male reproduction

| Group member | Treatments compared | <i>n</i> | Treatment | | | | Body size | | | Growth rate | | | Food ration | | |
|-----------------------------------|---------------------|----------|-----------|----------|----------|---|-----------|----------|----------|-------------|----------|----------|-------------|----------|----------|
| | | | d.f. | <i>F</i> | <i>P</i> | | d.f. | <i>F</i> | <i>P</i> | d.f. | <i>F</i> | <i>P</i> | d.f. | <i>F</i> | <i>P</i> |
| Average clutch size females | Ff, Fm, Mf, Mm | 45 | 3 | 1.3 | 0.29 | 1 | 2.5 | 0.12 | 1 | 0.06 | 0.81 | 1 | 0.03 | 0.87 | |
| Dominant female | | | | | | | | | | | | | | | |
| Large subordinate | Ff, Fm | 7 | 1 | 7.9 | 0.11 | 1 | 0.09 | 0.80 | 1 | 1.1 | 0.41 | 1 | 6.8 | 0.12 | |
| Small subordinate | Ff, Mf | 4 | — | — | — | — | — | — | — | — | — | — | — | — | |
| Total no. of eggs/60 days females | | | | | | | | | | | | | | | |
| Dominant female | Ff, Fm, Mf, Mm | 48 | 3 | 0.7 | 0.56 | 1 | 1.9 | 0.17 | 1 | 0.2 | 0.69 | 1 | 0.27 | 0.61 | |
| Large subordinate | Ff, Fm | 25 | 1 | 1.4 | 0.26 | 1 | 5.4 | 0.03 | 1 | 3.0 | 0.10 | 1 | 10.6 | 0.004 | |
| Small subordinate | Ff, Mf | 26 | 1 | 0.5 | 0.47 | 1 | 0.0 | 0.99 | 1 | 0.9 | 0.36 | 1 | 0.2 | 0.65 | |
| Total no. of eggs/60 days males* | | | | | | | | | | | | | | | |
| Dominant male | Ff, Fm, Mf, Mm | 48 | 3 | 0.6 | 0.60 | 1 | 0.5 | 0.48 | 1 | 2.6 | 0.11 | 1 | 1.9 | 0.18 | |
| Large subordinate | Mm, Mf | 23 | 1 | 7.3 | 0.014 | 1 | 0.02 | 0.90 | 1 | 0.3 | 0.59 | 1 | 0.6 | 0.45 | |
| Small subordinate | Mm, Fm | 22 | 1 | 1.2 | 0.29 | 1 | 0.02 | 0.89 | 1 | 0.1 | 0.76 | 1 | 1.3 | 0.27 | |

Results from nine separate ANOVA tests. Treatments compared: sex of large subordinate, sex of small subordinate, where M and m, large and small male; F and f, large and small female.

—, not computed due to the low sample size.

*implementing average paternity per male status × female status broods for missing values.

Table 4 Subordinate brood care depending on subordinate sex, subordinate parentage and their interaction with the covariate clutch size cared for ($n = 116$ focal brood care observations, including 56 female and 60 male subordinates)

| Parameter | d.f. | Wald χ^2 | <i>P</i> | Coefficient SE |
|-----------------|------|---------------|----------|----------------|
| Intercept | | | | -2.019 ± 0.407 |
| Sex | 1 | 7.70 | 0.006 | 0.983 ± 0.355* |
| Parentage | 1 | 3.66 | 0.056 | -1.334 ± 1.372 |
| Sex × parentage | 1 | 9.62 | 0.002 | 5.145 ± 1.660* |
| Clutch size | 1 | 5.77 | 0.016 | 0.006 ± 0.003 |

Results from a generalized estimating equation, with group identifier as subjects, scale adjusted using the deviance method. The response variable is focal subordinate brood care/total brood care as weighted binomial distribution with a negative log-log link to account for the many values near zero.

*Coefficients for females, males are the reference category.

more eggs in the Mm treatment ($B \pm SE$: 16.3 ± 6.0 eggs) than in the Mf treatment (Fig. 4c, Table 3). Small subordinate male reproductive success did not depend on the treatments (Fig. 4c, Table 3). Male size, male growth rate and food ration did not affect reproductive success in any of the males (Table 3).

Subordinate parentage and brood care

In general, brood care significantly increased with clutch size, and further results were corrected for this effect (Table 4). Female subordinates showed significantly more

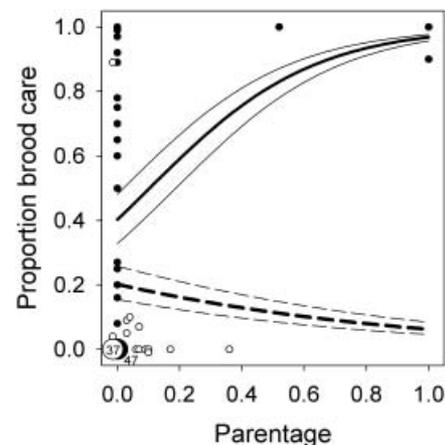


Fig. 5 Subordinate brood care depending on their parentage in the brood (proportion sired offspring). Females: black circles with continuous lines, $n = 56$. Males: white circles with dotted lines, $n = 60$. Depicted are also regression lines from the model presented in Table 4, lower lines: clutch size 50; middle bold lines: clutch size 100; upper lines: clutch size 150. Note the many overlapping cases with no brood care and no parentage (47 females, 37 males).

brood care than male subordinates, even when they gained no parentage inside the brood (Fig. 5, Table 4). Females also increased their level of care with increasing maternity (i.e. cared more for own broods with parentage of 100% vs. broods with 0% parentage), but no such effect was apparent in the males (Fig. 5), as indicated by the significant interaction between subordinate sex and parentage (Table 4). Note that within the subordinate males, the five cases where

these males shared the highest proportion of paternity, they did not show any brood care, and vice versa, the male showing the highest level of care of all subordinate males, actually did not gain parentage at all (Fig. 5). Also note the high variability in true alloparental brood care by the female subordinates, i.e. when their parentage is zero, ranging between zero care and 100% care (Fig. 5).

Discussion

Our results show that dominant males incur a cost of having multiple subordinate males inside their group. To our knowledge, this is the first experimental study showing this effect. When only a large subordinate male was present, 99.7% of all offspring were sired by the dominant male. With only a small subordinate male present, 99.1% were sired by the dominant male. In contrast, when both a large and small subordinate male were present, dominant males sired only 93.6% of the offspring. This effect appears entirely due to the large subordinate males siring on average more offspring in the latter situation, compared to the first situation. We did not find any evidence that dominant males may gain from conceding some reproduction to the subordinate males: subordinate males were usually not helpful and did not show an increase in brood care with the level of paternity they gained. We note that subordinate males may have benefited dominant males in other ways, for instance through territory defence. It appears that dominant males have difficulty in controlling paternity of multiple subordinate males, and adding subordinate males to a group has nonadditive effects on the loss of dominant paternity. This corroborates previous results (Heg *et al.* 2006b) suggesting male reproductive partitioning in *Neolamprologus pulcher* is governed by a tug-of-war (*sensu* Reeve 2000; Nonacs 2007). However, since subordinate male parentage was very low, the overall reproductive output of dominant males was hardly curbed and will more depend on the number and sizes of reproductive females available inside his group.

Subordinate males never gained simultaneous parentage in the same brood, which suggests that both males may also compete among each other for parentage, not only with the dominant male. Nevertheless, small subordinate males do not appear to gain *less* paternity when a large male subordinate is around. This suggests that although male subordinates may compete for paternity among each other, this cost is offset with a greater access to broods for both subordinate males. Our study complements the study by Heg *et al.* (2006b), who showed that large subordinate males reproduced more than small subordinate males (Heg *et al.* 2006b had data mainly from Mf and Fm treatments).

Dominant males appear to benefit from the presence of subordinate females, because these females also reproduce (see also Heg & Hamilton 2008), and virtually all of these

offspring are sired by the dominant male (107 out of 108 offspring, i.e. 99.1%). Moreover, subordinate females showed more alloparental brood care than subordinate males (i.e. for broods with 0% parentage), despite the subordinate females' certainty about their parentage, whereas subordinate males can be uncertain as their sperm have to compete for fertilization with those of dominant males. Subordinate males are likely to be less successful in sperm competition, due to subordinate males having smaller gonads with less viable sperm compared to dominant males (Fitzpatrick *et al.* 2006).

Field studies indicate that dominant males may incur an even larger loss of siring success due to the presence of subordinate males (Dierkes *et al.* 2008, but unfortunately the identity of extra-pair males could not be determined), possibly augmented by extra-group paternity (Heg *et al.* 2006b). This is probably because territories are highly substructured in the field, were labyrinths of shelters are excavated under the stones, and subordinates and intruders have more opportunity to sneak spawn without being detected. Either way, our results show that dominant males should be sensitive to the sizes and numbers of subordinate males present inside their group, although they may only represent a risk for paternity loss. This may explain why dominant males are likely to evict subordinate males from their group (Balshine-Earn *et al.* 1998), particularly if these subordinates have engaged in competitive spawning (Dierkes *et al.* 1999, but see Heg *et al.* 2006b). Although subordinate males hardly provided (alloparental) brood care, it must be conceded that subordinate males may have other beneficial effects on the fitness of dominant males under natural conditions, for instance, due to positive effects on dominant survival rate (Heg *et al.* 2004a, 2005b), e.g. due to territory defence (Bergmüller & Taborsky 2005; Bergmüller *et al.* 2005).

Female subordinates cannot directly reduce the reproductive output of a dominant female, except by eating her eggs after spawning (von Siemens 1990), which rarely occurs (Heg & Hamilton 2008). Nevertheless, indirect effects might be more subtle. For instance, the presence of subordinate females may reduce dominant female growth rate (Heg & Hamilton 2008), and thereby reduce her future reproductive output (due to positive effects of female body size on clutch size). The presence of subordinates may affect clutch size and egg mass of dominant females (Taborsky 1984; Heg *et al.* 2006b; Taborsky *et al.* 2007). We have shown that subordinate females also produce clutches, and this may draw away resources from the dominant female's broods (e.g. food for egg-laying, subordinate help). Whether effects are direct or indirect, we have found no evidence to suggest that total dominant female reproductive output is reduced due to the presence of female subordinates, at least in the short term. However, in our experiment, dominant females do appear to produce fewer clutches with heavier

eggs when the small subordinate is a female, and female subordinates show more alloparental care than male subordinates (D. Heg *et al.*, unpublished data).

Interestingly, we found only 1 out of 874 offspring produced by a mating between a male and female subordinate, although both sexes readily spawned with the dominants. The mechanisms and functional aspects of this finding remain unclear, but should receive more attention. If this result holds true for the natural situation, it might imply that subordinates have not much to gain from their opposite-sex group members in terms of current reproduction, although they might provide future partners. It is also a feature which is highly variable, comparing patterns of paternity and maternity across the different cooperatively breeding vertebrates. In the red fox *Vulpes vulpes*, for instance, dominant females did not produce cubs with the subordinate males, whereas dominant males sired offspring with all group member females (Baker *et al.* 2004).

We also detected a trade-off between dominant male growth rate and his paternity, suggesting that the trade-off between male investment in soma and gonads (Fitzpatrick *et al.* 2008) may have direct reproductive repercussions. Small subordinate males sired more offspring when growing fast, suggesting they may improve success by growing bigger, in line with previous findings of a subordinate males size effect on reproduction (this study and Heg *et al.* 2006), maybe mediated by an increased investment in gonads (Fitzpatrick *et al.* 2006). Food ration decreased parentage of the dominant males, whereas it increased parentage of the large subordinate males. This suggests that the latter males profit more from a rich food supply and maybe shunt food towards sperm production to increase siring success. Growth rate did not affect maternity and the total number of eggs produced in females. Food ration increased maternity of the dominant females, but did not have effects on clutch size and total egg production. A rich food supply may have been mainly used by the dominant females at a cost to the subordinate females, since large subordinate females tended to have less maternity and produced significantly fewer eggs when food was plentiful. However, tailored supporting experiments are needed to measure the effects of food ration and food competition on reproduction and reproductive partitioning in these cichlids, e.g. by giving certain group members access to supplemental food (Wong *et al.* 2008). This is important, because (i) food ration was not experimentally altered in our study; and (ii) food ration was fixed at 2.5% of the summed body masses of all group members, so by default correlated with the sizes of the fish in our experiment.

Taken together, our results suggest that dominant females should be less sensitive to the presence of female subordinates, than dominant males should be to the presence of male subordinates, because dominant females incur lower direct costs from the presence of these same-sex group

members, where females are also likely to show alloparental care. We have also shown mediating effects of growth rate and food ration on parentage, deserving experimental verification. How and whether these effects change predictions from the various reproductive skew models (e.g. Reeve & Emlen 2000; Hamilton & Heg 2007) remains to be tested. We have shown subordinates can gain direct reproductive benefits from group-living and might adjust their help accordingly (Magrath & Whittingham 1997). The relative importance of subordinate reproduction and other reasons why subordinates might show cooperative behaviour should receive more attention in the future (e.g. pay-to-stay hypothesis, Bergmüller & Taborsky 2005; Bergmüller *et al.* 2005; Hamilton & Taborsky 2005b; Stiver *et al.* 2005).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Number of adults successfully typed (n), number of different alleles (Alleles), number of heterozygotic (Het) and homozygotic individuals (Hom), observed (H_O) and expected heterozygosities (H_E), the polymorphic information content (PIC), exclusionary power of the first (Excl₁) and second parent (Excl₂), and estimated null allele frequencies (Null), respectively, per locus. Based on the Cervus 3.0 analysis of 152 unrelated, adult individuals (breeders and subordinates in the experiment)

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