Male reproductive suppression in the cooperatively breeding fish *Neolamprologus pulcher*

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In most cooperative breeders, dominants suppress the reproduction of subordinates. However, two previous studies of *Neolamprologus pulcher*, a cooperatively breeding cichlid fish, have suggested that socially subordinate helper males sneak fertilizations from dominant breeding males. If such sneaking does occur, both theoretical work and empirical studies of other fish species suggest that sperm competition will select for increased reproductive investment by sneaker males, relative to more dominant males. To address these issues, we quantified gonadal investment and sperm characteristics of 41 *N. pulcher* male breeders and 62 male helpers from 55 groups in Lake Tanganyika. Gonadal investment followed patterns consistent with reproductive suppression, with breeders having considerably larger testes masses than helpers. Breeders also had faster and longer swimming sperm and a higher percentage of motile sperm compared to helpers. However, sperm characteristics of large helpers were similar to those of breeders, but these same helpers had lower testes masses. Thus, large helpers had sperm that were physiologically equivalent to that of breeders, but their relatively small gonads imply that they were reproductively suppressed.

**Key words:** Cichlidae, dominance hierarchies, Lake Tanganyika, reproductive physiology, social status, sperm competition.  
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regularly compete to fertilize eggs (Parker, 1970). For example, larger testes or faster swimming sperm may provide males with a competitive advantage (Ball and Parker, 1996; Parker, 1996a,b, 1998). In a laboratory study, Dierkes et al. (1999) demonstrated that large, subordinate Neolamprologus pulcher males engaged in sneak fertilizations and successfully fathered between 12.5% and 35.8% of the young. In a complementary field study, mixed paternity was found, but sampled subordinate male helpers from those groups were not the fathers; however, the researchers argued that the subordinate males did successfully sneak fertilizations and father young and were subsequently expelled from the group (Dierkes P, Taborisky M, and Achmann R, personal communication). Hence, it remains unclear whether helper males engage in sneak fertilizations in the wild.

Reproductive suppression and sperm competition should select for different, and opposing, levels of gonadal/gametic investment in breeders and helpers, providing testable predictions regarding an individual’s reproductive investment. Reproductive suppression typically results in males in the disadvantaged role (physically subordinate or sneaking males) investing less in gonads and gametes (Johnstone and Cant, 1999). The three studies performed to date describing reproductive investment in cooperatively breeding species provide empirical support for these predictions. In the naked mole-rat, sperm from nonbreeding males was less concentrated in the ejaculate and exhibited impaired motility (Faulkes et al., 1994), and adult-sized, nonbreeding females had reproductive tracts in a prepubescent state of development (Faulkes and Abbott, 1997). Similarly, nonbreeding male Damaraeland mole-rats had a greater proportion of immature sperm in their ejaculate relative to breeding males in the same social group (Maswanganye et al., 1999), but in this system helpers do not invest in gonads and gametes due to the costs associated with inbreeding rather than active reproductive suppression by dominant breeders (Bennett et al., 1996). Finally, in the dwarf mongoose, subordinate males had smaller gonadal sizes relative to dominant male breeders (Maswanganye et al., 1999).

In contrast, with sperm competition, disadvantaged males (physically subordinate or sneaking males) are expected to invest relatively more energy in gonads and gametes than do dominant males (Parker, 1999b). Both theoretical and empirical studies demonstrate that sperm competition can select for different, but adaptive, levels of gonadal investment by males of the same species (Taborsky, 1994, 1998, 2001). In several fish species where some males are known to engage in sneak fertilizations, sneaker males invest relatively more resources in their gonads than do dominant individuals, including salmonids (Gage et al., 1995; Vladic and Järvi, 2001), bluegill (Lepomis macrochirus; Gross and Charnov, 1980; Leach and Montgomerie, 2000), bluehead (Thalassoma bifasciatus) and saddleback wrasses (Thalassoma duperrey; Henson and Warner, 1997; Ross et al., 1983; Warner, 1982), plainfin midshipman (Porichthys notatus; Brantley and Bass, 1994; Foran and Bass, 1998; Grober et al., 1994), and platyfish (Xiphophorus maculatus; Halpern-Scholz et al., 1986), to mention only a few of the well-studied species.

Sperm swimming velocity and duration have often been used as indices of sperm quality. Recently, studies examining sperm characteristics in a variety of species have demonstrated a positive relationship between sperm swimming velocity and fertilization success (Geresko and Dabrowski, 1994; Gage et al., 2004; Lahnsteiner et al., 1998; Levitan, 2000; Malo et al., 2005; Moccia and Munkittrick, 1987) and a negative relation between the duration of sperm motility and fertilization success (Ginsburg, 1963; Hoyasak and Liley, 2001). To date, sperm characteristics of sneaker males in relation to territorial (or parental) male fishes have only been examined in Atlantic salmon (Gage et al., 1995; Vladic and Järvi, 2001; Vladic et al., 2002) and bluegill (Burness et al., 2004; Leach and Montgomerie, 2000). In salmon, sperm from sneaker males had a greater percentage of motile spermatozoa and greater energy stores (Gage et al., 1995; Vladic and Järvi, 2001) but no differences in sperm velocity, duration of motility, or morphology compared to dominant males (Vladic and Järvi, 2001; Vladic et al., 2002). In bluegill, sperm from sneaker males initially swim faster, had greater energy stores (Burness et al., 2004), and was 50% more concentrated in ejaculates (Leach and Montgomerie, 2000) than the sperm of parental males.

In this study, we examine the gonadal investment and gametic characteristics of Neolamprologus pulcher, comparing our results to predictions from reproductive suppression and sperm competition theory. This is the first study to offer a detailed analysis of sperm characteristics in a nonmammalian cooperative breeder and only the third examination of sperm characteristics in a cooperatively breeding animal (the other two were on mole-rats: the naked mole-rat, Faulkes and Abbott, 1991; Faulkies et al., 1994; Jarvis, 1991, and Damaraland mole-rat, Maswanganye et al., 1999).

**METHODS**

We studied 55 Neolamprologus pulcher groups between 5 February and 15 April 2004 in Kasakalawe Bay, Lake Tanganyika (8° 46′ S; 31° 46′ E). Groups were studied at depths of 10–13 m using SCUBA. After an initial habituation period (approximately 3–5 min) for each group, group compositions and dominance hierarchies were ascertained in two to four separate observational sessions. Groups were visited three to eight times throughout the course of this study. In each group, 10-min focal watches were also conducted on the breeding pair and the two largest helpers, and the number of aggressive (ramming, chasing, biting, mouth fighting) and submissive (tail quivers, submissive postures) acts performed by each focal fish was recorded. Two watches were conducted on each individual. On each visit to a territory, group size was recorded. Social dominance was assigned using an index that combined behavioral observations, relative body size, and body color markings (see Buchner et al., 2004). Helpers were categorized by their position in the dominance hierarchy, as shown in Table 1. Between groups, an individual’s position within this dominance hierarchy did not necessarily correlate with its absolute size because the largest helpers (helper 1) in any one group may have actually been quite small. Conversely, helpers further down in the dominance hierarchy could be relatively large if there were several large helpers in the group. Fish were individually identified based on territory affiliation, estimates of body length, natural body markings, and experimental markings with a nontoxic latex paint (see Balshine et al., 2001 for further details). All data were recorded on PVC slates.

**Table 1**

<table>
<thead>
<tr>
<th>Social status</th>
<th>n</th>
<th>Size range (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breeding males</td>
<td>41</td>
<td>66–49</td>
</tr>
<tr>
<td>All helper males</td>
<td>62</td>
<td>57–24</td>
</tr>
<tr>
<td>Helper 1</td>
<td>25</td>
<td>57–29</td>
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<td>Helper 2</td>
<td>12</td>
<td>50–22</td>
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<td>Helper 3</td>
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<td>Helper 5</td>
<td>10</td>
<td>42–24</td>
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<td>Helper 6</td>
<td>4</td>
<td>34–27</td>
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</table>
After the final observation period, we attempted to collect all the individuals in each group by placing a conical tent net, equipped with weights at the bottom and a float at the top, directly over the territory and brood chamber area and securing the net perimeter to the substrate with rocks (see Morley and Balshine, 2002 for further details). A small volume (3–7 ml) of quinaldine [2-methylquinoline; C₆H₄N:C(CH₃)CH:CH] was then released inside the brood chamber, temporarily sedating the fish. Fish were placed in mesh cloth collection bags and brought to the surface.

On shore, we sexed the fish and measured standard length (SL) to the nearest millimeter and body mass to the nearest 0.001 g. In total, we collected 41 dominant breeding males and 62 subordinate helper males from the 55 groups we studied, and every group had a breeding pair and at least one helper. In some cases, the whole group was collected, whereas in other cases only the breeding pair or only the helpers were collected from a group. Fish were anesthetized in benzocaine, quickly sacrificed via cervical severance, and their testes were removed.

Sperm analysis

Testes were placed on a dry glass microscope slide and weighed, taking care to avoid contact with water or mucus. Relative gonad investment was measured using the gonadosomatic index ($\text{GSI} = [\text{gonad mass/body mass}] \times 100\%$). Testes were then split open with a scalpel, allowing access to milt. A drop of milt was placed in a 2-ml microcentrifuge tube and activated by quickly adding 0.25 ml of lake water (previously boiled to exclude microorganisms and allowed to cool to ambient temperature). The video recordings (see below) began as soon as lake water was added to the sample. The water/sperm mixture was agitated for 1–2 s, and a 60-μl subsample was immediately placed in a 1-mm deep well on a slide, with a cover slip covering half of the depression (see Liley et al., 2002 for similar methods). Videos of sperm motility were captured at 60 frames/s using a PixeLINK Megapixel PL-A662 digital video camera (PixeLINK, Ottawa, Ontario, Canada) mounted on a Leica DME light microscope (Leica Microsystems Inc., Buffalo, New York, USA) at 200× magnification. Images were recorded using PixeLINK PL-A600 Series Camera Software (v. 3.1, PixelINK).

Videos taken in the field were brought back to the lab where sperm velocity was measured using a CEROS (v.12) video sperm analysis system (Hamilton-Thorne Research, Beverly, Maine, USA). For each male, sperm velocity was quantified for 1 s at 2, 5, and 7 min postactivation. We analyzed only those spermatozoa whose forward movement was recorded for at least 0.33 s (≥20 frames; see Burness et al., 2004, and Lahnsteiner et al., 1998, for a similar criterion). The median sperm velocity (median smooth path velocity [VAP]) for all spermatozoa recorded at each time period after activation (mean ± SE: 40 ± 4 cells per time period; range: 6–279).

The duration of sperm movement was measured as the time since activation at which 95% of the sperm no longer exhibited forward movement (see Gage et al., 1998, 2002; Hoytak and Liley, 2001). Two, five, and seven minutes after sperm activation, sperm motility was evaluated by eye on a 0–6 scale, similar to that described by DeFrapont and Sorensen (1993) and Hoytak and Liley (2001): 0 = no motility; 1 = 1–10% of sperm showing forward movement (very low); 2 = 11–29% (low); 3 = 30–49% (moderately low); 4 = 50–74% (moderately high); 5 = 75–94% (high); and 6 = 95–100% (very high). Two independent observers scored both the duration and rank of sperm motility (2, 5, and 7 min postactivation), and mean estimates were used in further analyses. Repeatability between observers, using the intraclass correlation coefficient ($\eta$), was high (in all cases $\eta \geq 0.80, p < .0001$).

Statistical analysis

Statistical analyses were performed with JMP (version 5.1, SAS Institute Inc., 2004) and Resampling Stats for Excel (version 3, Resampling Stats Inc., 2004). Nonparametric statistics were used when the data were not normally distributed and could not be transformed to correct this; all rank data were corrected for ties. To distinguish between the effects of helper status and body size on gonadal investment, we performed multiple regression analyses using body length (log-transformed SL) as a measure of body size and coded helper status as an ordinal variable (1–6) in the model. We reached the same conclusions using soma mass (body mass–testes mass) as a measure of body size. To avoid pseudoreplication, all statistical analyses of sperm characteristics were performed using a single (median) value from each male. All other measures are presented as mean ± SE. To compare the slopes of reduced major axis (RMA) regressions, we used a randomization test (Manly, 1991) with 1000 iterations to generate 95% confidence limits (CL) for each slope, as well as a sampling distribution of differences between slopes, which was compared to the expected difference of zero that would be obtained if there was no difference between the slopes.

RESULTS

Gonadal investment: testes size and GSI

Breeder males ($n = 41$) had both larger testes mass (Figure 1a) and higher GSI (Figure 1b) than helper males ($n = 62$) (t tests: testes mass, $t = 13.9, p < .0001$; GSI, $t = 2.8, p = .006$). The average breeder testes mass was 3.6 times that of helpers at the top of the dominance hierarchy (H1; Figure 1a). Testes mass did not differ among helpers in any position in the dominance hierarchy (ANOVA, $F_{3,56} = 1.64, p = .17$; Figure 1a), though the testes of male helpers at the top of the hierarchy (H1) were about twice the mass of the other helpers’ testes.

 Helpers at the bottom of the dominance hierarchy had significantly larger GSI than those at the top of the hierarchy (log GSI data, $F_{3,56} = 2.65, p = .03$; Figure 1b), but this effect disappeared when the effects of body size were controlled statistically (analysis of covariance, $F_{3,56} = 1.52, p = .20$), suggesting that size and not helper dominance status determined GSI. Thus, within helpers, GSI was negatively correlated with body length (SL) ($r = -.42, n = 62, p = .001$; Figure 2a). Within breeders, however, GSI and body length (SL) were not correlated ($r = .13, n = 41, p = .42$; Figure 2a).

The relation between testes mass and soma mass (both log transformed) differed significantly between breeders and helpers (randomization test comparing RMA slopes, $p < .001$). In breeders, the slope ($v$) of the RMA regression ($v = 2.21, 95\% \text{ CL} = 1.72–2.93, n = 41$) was significantly >1.0, whereas the slope of the RMA regression in helpers was not ($v = 1.04, 95\% \text{ CL} = 0.85–1.29, n = 62$; Figure 2b). Thus, testes mass was larger relative to body size in breeders than in helpers.

Male breeders invested more in testes (i.e., had higher GSI values) when they were in larger groups ($r = .42, n = 34, p = .02$; Figure 3a). This was not simply a result of breeders being larger in larger groups as breeder size and group size were not correlated in our sample (body mass, $r = .20, n = 34, p = .25$; SL, $r = .30, n = 34, p = .09$). Further, controlling for body mass had little effect on the relation between group size and breeder GSI (partial $r = .44, n = 34, p = .03$). Breeder GSI was also significantly correlated with the number of male ($r = .35, n = 32, p = .05$; Figure 3b) but not female ($r = .32, n = 32, p = .08$) helpers in the group.
Sperm swimming speed

In both breeders and helpers, sperm velocity (VAP) declined significantly at the three time periods measured after activation (Breeders: Kruskal-Wallis sign rank test, $H = 36.25, n = 41, p < .001$; Helpers: $H = 42.6, n = 62, p < .001$; Figure 4a). In both breeders and helpers, mean VAP at 5 min after activation was less than 75% of the VAP at 2 min.

The mean sperm velocity (VAP) of breeders was higher than that of helper males (Figure 4a) by between 2 and 6 $\text{lm/s}$ at all times measured after activation. This difference between breeder and helper sperm velocities was not significant at 2 min postactivation ($U = 1.82, n_b = 32; n_h = 48, p = .18$) but was significant at both 5 ($U = 5.21, n_b = 32; n_h = 44, p = .02$) and 7 min ($U = 4.77, n_b = 27; n_h = 33, p = .03$) after sperm activation (Figure 4a).

Sperm velocity at each time postactivation was not correlated with breeder size (e.g., Figure 4b). In helpers (controlling for status), sperm swimming speed did not vary with body size (SL) at any of the times that speed was measured (multiple regressions: 2 min, $F_{1,41} = 1.15, p = .37$; 5 min, $F_{1,39} = 0.67, p = .67$; 7 min, $F_{1,28} = 0.69, p = .46$).

Sperm longevity

The mean duration of sperm motility for all fish (breeders and helpers) was 7.03 ± 0.23 min ($n = 75$, range 124–766 s). Breeder sperm swam significantly longer than sperm from helpers ($t = 3.14, n_b = 32; n_h = 46, p = .003$; Figure 5a). Within breeders, there was no relation between body length (SL) and sperm longevity ($r = .05, n = 32, p = .78$), but within helpers, sperm swimming duration was positively correlated with SL ($r = .51, n = 46, p = .04$; Figure 5b). Similarly, sperm longevity increased up the dominance hierarchy ($F_{5,45} = 2.84, n = 43, p = .03$), with sperm from H1 males swimming significantly longer than sperm from H6 (Tukey-Kramer test, $p < .05$; Figure 5a). Controlling for social status, helper sperm longevity was
not related to body size (multiple regression, standardized beta = 0.05, $F_{1,36} = 0.08$, $p = .78$) but was positively correlated with testes mass (standardized beta = 0.31, $F_{1,36} = 4.71$, $p = .04$).

Percentage of motile sperm

The proportion of sperm exhibiting forward movement decreased significantly over time since activation in both breeders ($H = 41.1$, $n = 33$, $p < .0001$) and helpers ($H = 67.8$, $n = 48$, $p < .0001$; Figure 6). Moreover, at each time period analyzed, a significantly greater proportion of breeder sperm was motile compared to helper sperm (2 min after activation; $U = 5.35$, $n_b = 33$; $n_h = 48$, $p < .02$; 5 min: $U = 7.63$, $n_b = 33$; $n_h = 44$, $p < .005$; 7 min: $U = 11.44$, $n_b = 27$; $n_h = 35$, $p < .0007$; Figure 6).

Group size and aggression

All aggression was directed down the dominance hierarchy by breeders toward helpers, or from more dominant helpers (e.g., helper 1s) toward more subordinate helpers (e.g., helper 2s). While breeders ($1.84 \pm 0.45$ acts/10 min) and helpers ($2.19 \pm 0.52$) performed aggression toward members of their own social groups at roughy equal frequencies ($U = 0.14$, $n = 32$, $p = .71$), helpers performed submissive behavior at a much higher rate ($2.70 \pm 0.67$) than breeders ($0.44 \pm 0.13$; $U = 12.0$, $p = .0005$).

None of the sperm characteristics measured were correlated with group size or helper sex ratio within each group ($p > .05$ in all cases).

DISCUSSION

Our investigation of gonadal investment patterns in male breeders and helpers provides support for the idea that reproductive suppression occurs in *N. pulcher*. First, helpers had smaller absolute testes mass (Figure 1a) and less gonadal investment (Figure 1b) than breeders, suggesting that they would have less sperm available per ejaculate (Gage et al.,...
Second, helpers had slower swimming sperm (Figure 4), shorter lived sperm after activation (Figure 5), and a lower percentage of motile sperm (Figure 6) than breeders, suggesting that their sperm would be less competitive (Hoysak and Liley, 2001). Finally, breeding males were aggressive toward helpers, and helpers were much more submissive than breeders, suggesting that breeder males may behaviorally control reproduction in the helpers.

Among helpers, testes mass increased isometrically with body size (Figure 2b), whereas among breeders this relation was allometric, suggesting that their sperm would be less competitive (Hoysak and Liley, 2001). Finally, breeding males were aggressive toward helpers, and helpers were much more submissive than breeders, suggesting that breeder males may behaviorally control reproduction in the helpers.

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sneak fertilizations. Furthermore, the higher GSI values observed in smaller helpers (Figure 1b) is driven by the fact that testes mass does not change significantly as helper size increases (Heg et al., 2004b), using stored energy only to rapidly enhance gonad development as breeding opportunities arise. Future work could provide a critical test of this idea by examination of subordinate physiology before and after the assumption of breeder status, focusing in particular on gonad development and use of stored energy reserves. We found little evidence from male physiology to support the idea that sneak fertilizations by helpers are responsible for mixed paternity in wild *N. pulcher* populations (Dierkes P, Taborsky M, and Achmann R, personal communication). Instead, this mixed paternity may be the result of extragroup fertilizations by neighboring males, as has been seen in other cooperatively breeding species (Cant et al., 2002; Double and Cockburn, 2000; Hatchwell et al., 2002).

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REFERENCES


Borowsky R, 1973; Buston, 2003; Hofmann et al., 1999). So, helpers may delay final sexual maturation until a favorable situation is presented (Wickings and Dixson, 1992), at which time rapid sexual maturation may be possible, where gonads grow at a faster rate than the soma (Figure 3b). Indeed, dramatic sexual maturation and gonadal development is observed in many teleosts, over a period of days to weeks, after their acquisition of socially dominant positions in their social group (Hofmann et al., 1999; Munday et al., 1998; Warner and Swearer, 1991).

In contrast to the sperm characteristics reported in other cooperatively breeding species (naked mole-rat, Faulkes and Abbott [1991], Faulkes et al. [1994], Jarvis [1991]; and Damaraland mole-rat, Maswanganye et al., 1999), large subordinate male *N. pulcher* did not have impaired sperm physiology, though they did have much smaller testes size than breeders (Figure 1). Large subordinate males had physiologically equivalent sperm characteristics to breeders, but their relatively small gonads suggest that they are limited in reproductive capacity. Rather than investing in gonadal development, subordinate helpers may invest in strategic somatic growth (Heg et al., 2004b), using stored energy only to enhance gonad development as breeding opportunities arise. Therefore, unlike species where sneak fertilizations are common, in *N. pulcher* none of the sperm characteristics of males utilizing different reproductive tactics in fish. Males engaging in sneak fertilizations in both the Atlantic salmon and bluegill have different sperm characteristics than dominant males (Burness et al., 2004; Gage et al., 1995; Leach and Montgomery, 2006; Vladic and Järvi, 2001; Vladic et al., 2002; but see Liley et al., 2002). Sneaker males attained high levels of reproductive success in both species, fertilizing 5–40% of eggs in salmon (Hutchings and Myers, 1988; Jordan and Youngson, 1992; Thomaz et al., 1997) and up to 92% of eggs in bluegill (Fu et al., 2001). Therefore, unlike species where sneek fertilizations are common, in *N. pulcher* none of the sperm characteristics of males in the disadvantaged role (helpers) surpassed those of breeders—in fact, we found just the opposite. Therefore, if *N. pulcher* helps engage in sneak fertilizations, as has been suggested (Dierkes et al., 1999), sperm characteristics have not been influenced. Previous theoretical and empirical research suggests that competition will increase ejaculate quality of the potential sneaks (Burness et al., 2004; Gage et al., 1995; Vladic and Järvi, 2001), but we found the opposite pattern in that helpers had sperm that was less motile and slower than that of breeders. Thus, it seems highly unlikely that helpers were responsible for the mixed paternity (Dierkes P, Taborsky M, and Achmann R, personal communication) observed in wild populations.

The disparity in testes size and GSI between large helpers and breeders may represent an adaptive trade-off between immediate and future reproductive success (Williams, 1966). Helpers may put energy into growth or energy stores, rather than gonad development, subsequently using energy stores to overcome the observed large difference in gonad, and presumably ejaculate, size. Several teleost species exhibit socially mediated gonadal growth (Berglund, 1991; Schultz et al., 1991) and many, including *N. pulcher* (Heg et al., 2004b), are known to exhibit socially modulated growth rates (Borowsky, 1973; Buston, 2003; Hofmann et al., 1999). So, helpers may delay final sexual maturation until a favorable situation is presented (Wickings and Dixson, 1992), at which time rapid sexual maturation may be possible, where gonads grow at a faster rate than the soma (Figure 3b). Indeed, dramatic sexual maturation and gonadal development is observed in many teleosts, over a period of days to weeks, after their acquisition of socially dominant positions in their social group (Hofmann et al., 1999; Munday et al., 1998; Warner and Swearer, 1991).

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