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Subordinate male cichlids retain reproductive competence during social suppression

Jacqueline M. Kustan, Karen P. Maruska* and Russell D. Fernald

Department of Biology, Stanford University, 371 Serra Mall, Stanford, CA 94305-5020, USA

Subordinate males, which are excluded from reproduction often save energy by reducing their investment in sperm production. However, if their position in a dominance hierarchy changes suddenly they should also rapidly attain fertilization capability. Here, we asked how social suppression and ascension to dominance influences sperm quality, spermatogenesis and reproductive competence in the cichlid *Astatotilapia burtoni*, where reproduction is tightly coupled to social status. Dominant territorial (T) males are reproductively active while subordinate non-territorial (NT) males are suppressed, but given the opportunity, NT males will perform dominance behaviours within minutes and attain T male testes size within days. Using the thymidine analogue 5-bromo-2-deoxyuridine (BrdU) to label germ cell proliferation, we found that the spermatogenic cycle takes approximately 11–12 days, and social status had no effect on proliferation, suggesting that spermatogenesis continues during reproductive suppression. Although sperm velocity did not differ among social states, NT males had reduced sperm motility. Remarkably, males ascending in status showed sperm motility equivalent to T males within 24 h. Males also successfully reproduced within hours of social opportunity, despite four to five weeks of suppression and reduced testis size. Our data suggest that NT males maintain reproductive potential during suppression possibly as a strategy to rapidly improve reproductive fitness upon social opportunity.

Keywords: *Astatotilapia burtoni*; social status; reproduction; sperm; spermatogenesis; teleost

1. INTRODUCTION

The ability to reproduce determines the fitness of all organisms, yet this ability can be significantly influenced by the social environment. In social animals, inequity in mating success can arise when subordinates are behaviourally and physiologically suppressed by the aggression of dominant conspecifics (i.e. mice [1]; baboons [2]; Atlantic salmon [3]). In the most extreme cases, social suppression can result in reduction of reproductive structures (i.e. accessory sex glands in white rhinoceros [4]; testes, seminal vesicles and coagulating glands in bank voles [5]), as well as physical exclusion from mating opportunities. However, while such 'social contraception' and its consequences are well documented among vertebrates [6,7], relatively little is known about the cellular changes that occur within the testes during a shift in status from an extreme socially and reproductively suppressed state, to a dominant reproductively active phenotype. Further, little is known about what consequences these testicular changes may have for gamete quality and reproductive success in the context of highly dynamic social environments [8].

For promiscuous males, a critical measure of reproductive potential is the production and quality of sperm [9]. The costly nature of ejaculate production encourages sperm economy [10,11], and many species possess the ability to adjust sperm characteristics and sperm allocation in response to social cues, such as female presence and mating opportunity [12–14], female attractiveness [15–18] and perceived levels of sperm competition and female promiscuity [5,19–22]. While dominant males often father the majority of offspring in hierarchical species [23,24], subordinate males can compensate for decreased mating opportunities through the adoption of alternative reproductive strategies and increased investment in ejaculate quality [25–30]. Previous research has primarily focused on comparing sperm characteristics in the context of sperm competition theory, which postulates that males should increase their ejaculate investment when sperm competition is high [31]. However, it has been suggested that reproductive suppression through social stress excludes the adoption of these alternative strategies in some species [1,8,32,33]. Understanding how sperm characteristics and sperm production compare between dominant and subordinate individuals, and how they might change during a shift in social status, can provide insight into how subordinate males may resist suppression to invest in future reproductive success [7], or adapt their sperm allocation in response to reduced mating opportunities [12,13].

The African cichlid fish *Astatotilapia burtoni* is an ideal model to study how the social environment influences reproduction because male reproductive physiology and behaviour are tightly coupled to social status [34]. Dominant, territorial (T) males (approx. 10–30% of population) aggressively defend a territory, display reproductive behaviours, and have large testes in relation to body size, while subordinate, non-territorial (NT) males are submissive, do not actively court females or hold a territory, and have

* Author for correspondence (maruska@stanford.edu).

smaller testes [35,36]. Importantly, these social states are highly plastic and reversible, such that when given the opportunity, an NT male will ascend in social rank, display reproductive and territorial behaviours within minutes, and gain T male testes size within a few days [37–40]. While previous research in *A. burtoni* has examined how social inputs modulate the brain [34,41,42], little is known about the functional consequences of social suppression on sperm quality, spermatogenesis and reproductive competence in *A. burtoni*, or the implications this has for resource allocation and life-history strategies of reproductively suppressed individuals.

The goal of this study was to quantify the differences in reproductive competence among NT, T and ascending (individuals rising in social status from NT to T) male *A. burtoni* based on the production and the quality of sperm and the latency of spawning as indicators of reproductive potential. Specifically, we asked (i) how does sperm production and sperm quality differ among social states? and (ii) how quickly after a rise in social rank can previously suppressed NT males successfully reproduce?

2. MATERIAL AND METHODS

(a) *Study population and social manipulation*

Adult *A. burtoni* were derived from wild-caught stock in Lake Tanganyika, Africa, and kept in aquaria under conditions similar to their native habitat (pH 8.0, 24°C–26°C and 12 L : 12 D cycle with full spectrum illumination).

To compare spermatogenesis between different social states, NT and T males were established using a paradigm similar to that described previously [43]. Two size-matched T males were taken from community tanks and placed into an experimental tank that contained three to four females and a single half terracotta pot to serve as a territory. After the first day, one male remained T while the other displayed behaviours typical of NT males (i.e. performed only submissive behaviours such as fleeing). Focal observations were made daily to verify that both males maintained their social status for four to five weeks to ensure full suppression of the hypothalamic-pituitary-gonadal (HPG) axis [37]. To establish ascending animals (males who had recently transitioned from subordinate NT to dominant T status), the T male was removed while the NT was injected (see below) and placed back into the tank alone, providing an opportunity for ascent. In this situation, the NT displays dominance behaviours typical of T males within approximately 15 min and is considered ascended [39].

(b) *Comparison of germ cell proliferation by social status*

To determine whether social status influenced spermatogenesis, we examined cell proliferation within the testis among Ts, NTs and males who had recently ascended from NT to T status (now referred to as 'ascending (A) males') by injection with and immunohistochemical detection of the thymidine analogue 5-bromo-2-deoxyuridine (BrdU). BrdU is commonly used across vertebrates to examine spermatogenesis because it is incorporated into DNA during the S-phase of mitosis, and thus is an indicator of cell proliferation [44–46].

(i) *BrdU injection and preparation of testes*

After the four to five weeks suppression period, males were briefly anaesthetized in ice-cold tank water, weighed to determine injection volume, given an intraperitoneal BrdU injection (100 $\mu g\,g^{-1}$ body mass; 16–30 μl total volume), and immediately placed back into their tanks for recovery. T and NT males were sacrificed 24 h after injection, while A males were sacrificed 24 h after the first display of dominance behaviours as described previously [39]. All fish were injected and sacrificed at the same time of day (10:00 $\pm$ 2 h). For animals used in all experiments, body mass (range 5.2–5.8 g), standard length (range 54–59 mm) and testes mass (range 0.014–0.055 g) were measured, and gonadosomatic index (GSI) was calculated [GSI = (gonad mass/body mass) $\times$ 100].

To estimate the length of the spermatogenic cycle, individual T males were housed with three to four females, injected with BrdU as above, and sacrificed at 3, 24, 195, 267 and 288 h post-injection. Testes were fixed in 4 per cent buffered-formalin at 4°C overnight, rinsed in phosphate-buffered saline (PBS, pH 7.4), cryoprotected in 30 per cent sucrose overnight, embedded in mounting media (Neg 50, Thermo Scientific), sectioned in the transverse plane at 10 μm with a cryostat, and collected onto charged slides (Superfrost Plus, VWR).

(ii) *BrdU immunohistochemistry*

To visualize germ cell proliferation, BrdU staining was detected using standard immunohistochemistry: 30 min 1$\times$ PBS (Gibco) wash; 30 min quenching of endogenous peroxidases with 1.5 per cent hydrogen peroxide (H_20_2); 3 $\times$ 5 min PBS wash; 5 min incubation in DNAse I buffer (40 mM Tris-HCL, 10 mM NaCl, 6 mM $MgCl_2$, 10 mM $CaCl_2$, pH 7.9); 10 min denaturing of DNA with 500 U ml^{-1} DNAse I in DNAse buffer; 3 $\times$ 5 min PBS wash; 1 h blocking of non-specific staining (5% normal goat serum, 0.2% bovine serum albumin (BSA) and 0.3% Triton-X 100 in 1$\times$ PBS); overnight incubation with rat anti-BrdU (Abcam, no. ab6326) at 1 : 1000 in blocking solution at 4°C; 3 $\times$ 10 min PBS wash; 1 h incubation with biotinylated goat anti-rat secondary (Vector Laboratories) in PBS with 5 per cent normal goat serum; 3 $\times$ 10 min PBS wash; 10 min quenching with 1.5 per cent H_20_2; 3 $\times$ 10 min PBS wash; 2 h incubation with Vectastain avidin–biotin complex (Vector Laboratories); 3 $\times$ 10 min PBS wash; and reaction with 3′3′-diaminobenzidine substrate. Slides were then counterstained with 0.5 per cent cresyl violet, dehydrated in an ethanol series (50–100%), cleared in xylene, and coverslipped with cytoseal 60 mounting media (Richard Allen Scientific).

(iii) *Quantification of germ cells and estimation of spermatogenic cycle length*

In fishes, the testes are organized into membrane-bound spermatocysts (now referred to as 'cysts'), with each cyst containing a group of synchronously developing germ cells derived from the same spermatogonial stem cell [47]. To determine the frequencies of germ cell types and the duration of the spermatogenic cycle, cell stages were identified based on cell morphology, size and number (as reviewed in Schulz *et al.* [48]). Owing to the difficulty of distinguishing between different spermatogonial generations and spermatid stages, and the relative infrequency of secondary spermatocytes [48], only three spermatogenic stages were quantified: B type spermatogonia, primary spermatocytes and all spermatids. For each animal, all cysts contained within each of five randomly chosen cross sections were counted, and

stage and staining frequencies were determined by the following formulae:

$$\text{stage frequency} = \frac{\text{number of cysts of given stage}}{\text{total number of cysts}}$$

and

$$\text{staining frequency} = \frac{\text{number of stained cysts of a given stage}}{\text{total number of cysts of a given stage}}.$$

To estimate the length of the spermatogenic cycle, staining in the most advanced cell type at 3, 24, 195, 267 and 288 h after the BrdU injection was noted in the testes of T males, and sections were examined for the presence of BrdU staining in the spermatozoa at all time points.

(c) Spawning latency by social status

To determine the time to first spawning after ascension, dyads were established as described above. At the end of the fourth week, an opportunity to rise in social status was created for the suppressed NT by removing the T male and females 1 h before light onset with the help of night-vision goggles (Bushnell night vision, Model 26-1020). At light onset, five to six gravid (ready-to-spawn) females were added to the tank and the fish were fed. Observations were then made every hour to check for mouthbrooding females (females that have spawned will carry the eggs in their mouth, which can be easily observed). Latency to spawn was defined as the time between light onset and observation of a brooding female. Since females will carry eggs for several days even if they have not been fertilized (J. M. Kustan, K. P. Maruska & R. D. Fernald 2010, unpublished observation), brooding females were moved to an isolation tank after spawning and whether or not the eggs were developing was checked 4–6 days later (total brooding time in this species is approximately two weeks). As a comparison, the time to spawn for T males was also assessed. A T male was allowed to establish a territory with three to four females and no other males were present for four to five weeks. Prior to light onset, females were removed, and at light onset, five to six gravid females were added, fish were fed and behavioural observations recorded as described above.

(d) Ejaculate collection and sperm quality analysis by social status

To quantify sperm characteristics among T, NT and A males, a small drop of milt (seminal fluid containing sperm) was collected by touching a pipette tip to the testes within 15 min of dissection. Sperm was activated by mixing in 1 ml of tank water (21°C–23°C) with 0.02 per cent BSA for 2–3 s. Roughly, 5 µl of this sperm mixture was transferred to a glass slide, and sperm were filmed for 60 s post-activation at 101 frames s^{-1} (Basler A600f; UNIBRAIN FIRE-I software) under an inverted microscope (32× objective). An average of 46 sperm were analysed per video (range 7–189) and sperm parameters were measured using a computer assisted sperm analysis (CASA) plugin developed and tested for NIH IMAGEJ [49]. One second (101–102 frames) was analysed for each animal at three different time points (15, 30 and 55 s post-activation). Curvilinear velocity (VCL; total point-to-point distance travelled over time, averaged to a per-second value) and fraction of motile sperm (number of sperm that showed positive velocity over the time analysed/total number of sperm detected in the field) were analysed for each animal.

To determine sperm longevity, sperm samples were filmed at 30 fps. The fraction of motile sperm was analysed at 30 s intervals beginning at 15 s post-activation, and sperm longevity was defined as the time it took to reach a fraction motility of less than or equal to 0.05 as measured by the CASA plugin.

(e) Statistical analyses

Datasets that were normally distributed (Shapiro–Wilk test) with equal variances (Levene median test) were analysed with either Student's t-tests, one-way ANOVA or repeated measures two-way ANOVA with post hoc Holm–Sidak tests for multiple comparisons, while data that did not meet the assumptions of parametric statistics and could not be normalized by transformation (e.g. log, ln or arcsine) were compared with Kruskal–Wallis tests (KW) and post hoc Dunn's tests. Correlations were assessed with Pearson product moment tests. For consistency, however, all data are plotted as mean ± standard errors (s.e.) with appropriate statistical test values reported in the text. Statistical comparisons were performed with SIGMAPLOT v. 11.0 (Systat Software, Inc., San Jose, CA, USA.).

3. RESULTS

(a) Timing of spermatogenesis

To estimate spermatogenic cycle length, testes were examined at 3 ($n = 3$), 24 ($n = 8$), 195 ($n = 3$), 267 ($n = 3$) and 288 h ($n = 2$) after BrdU injection (table 1). Average GSI of all males was 0.930 ± 0.225 s.d., with no relationship between GSI and hours post-injection (linear regression, $r^2 = 0.10$, d.f. $= 18$, $p = 0.283$). The most advanced cysts containing BrdU stain at 3 and 24 h were spermatocytes, while at 195, 267 and 288 h, the most advanced stained cysts were spermatids (table 1). BrdU-labelled spermatozoa were also observed within the sperm ducts at 267 and 288 h, but no earlier (see figure 1g, inset). Thus, the spermatogenic cycle was estimated at 267–288 h or 11–12 days.

(b) Spermatogenesis and cell proliferation across social status

To test for differences in spermatogenesis among animals of different social status, we administered BrdU to T ($n = 8$), NT ($n = 8$) and A males ($n = 7$), and collected testes 24 h later (figure 1a). GSI of T males was higher than both NT and A males (ANOVA, $F_{2,20} = 26.443$, $p < 0.001$; Holm–Sidak, $p < 0.001$), but GSIs of A and NT males did not differ (Holm–Sidak, $p > 0.05$). T males had a greater total number of cysts per testes area than NT males (KW, d.f. $= 2$, $p = 0.004$; Dunn's, $p < 0.05$), with the remaining area in NTs devoted to interstitial tissue and luminal space containing spermatozoa. There was no difference in the total number of cysts between A males and NT or T males (Dunn's, $p > 0.05$); however, there was an overall positive correlation between the number of cysts per testes area and GSI (Pearson's, $r = 0.584$, $p = 0.003$).

As stage frequency can be indicative of the relative duration of each stage of the spermatogenic cycle [44], we next compared stage frequencies of different cyst types among social states (figure 1b). The most common cyst types in all testes were spermatogonia (type B), which accounted for more than half of the cysts in all animals

Table 1. Germ cell stage and staining frequency of BrdU-injected territorial male *A. burtoni* at different time points (Data are expressed as mean ($\pm$s.d.). GSI, gonadosomatic index; SPG, spermatogonia type B; SPC, spermatocytes; SPT, spermatids.)

hours post-injection	3 ($n = 3$)	24 ($n = 8$)	195 ($n = 3$)	267 ($n = 3$)
total cysts counted	241.50 ($\pm$6.36)	244.33 ($\pm$15.99)	274.67 ($\pm$37.07)	240.67 ($\pm$9.29)
total staining frequency	0.39 ($\pm$0.02)	0.41 ($\pm$0.03)	0.31 ($\pm$0.31)	0.13 ($\pm$0.06)
GSI	0.83 ($\pm$0.06)	0.84 ($\pm$0.20)	0.88 ($\pm$0.26)	0.99 ($\pm$0.18)
SPG: stage frequency	0.67 ($\pm$0.02)	0.63 ($\pm$0.03)	0.55 ($\pm$0.03)	0.61 ($\pm$0.02)
staining frequency	0.59 ($\pm$0.05)	0.68 ($\pm$0.16)	0.05 ($\pm$0.08)	0.06 ($\pm$0.06)
SPC: stage frequency	0.05 ($\pm$0.002)	0.05 ($\pm$0.01)	0.04 ($\pm$0.006)	0.06 ($\pm$0.007)
staining frequency	0.08 ($\pm$0.19)	0.38 ($\pm$0.15)	0.39 ($\pm$0.24)	0.21 ($\pm$0.17)
SPT: stage frequency	0.29 ($\pm$0.01)	0.32 ($\pm$0.03)	0.40 ($\pm$0.03)	0.33 ($\pm$0.02)
staining frequency	0	0	0.61 ($\pm$0.05)	0.25 ($\pm$0.08)

(figure 1*b*). Stage frequency of type B spermatogonia was significantly greater for A males compared with T males (KW, d.f. = 2, $p = 0.016$, Dunn's, $p < 0.05$) with no difference between A or T versus NT males (Dunn's, $p > 0.05$). Across social states, GSI was negatively correlated with spermatogonial stage frequency (Pearson's, $r = -0.607$, $p = 0.002$). There was no difference in stage frequency of spermatocytes across social status (KW, d.f. = 2, $p = 0.528$) and no correlation between GSI and spermatocyte stage frequency (Pearson's, $r = -0.019$, $p = 0.931$). Spermatids appeared most often in T males, which showed a significantly greater frequency compared with both A and NT males (KW, $p = 0.003$; Dunn's, $p < 0.05$; figure 1*b*), with a resultant positive correlation between GSI and spermatid stage frequency (Pearson's, $r = 0.683$, $p < 0.001$).

To test whether cell proliferation and spermatogenesis were altered in suppressed and ascending individuals, we next compared the BrdU staining frequencies of different cyst types among social states (figure 1*c*). Within social groups, there was no correlation between GSI and any stage or staining frequency for A or T males (Pearson's, all $r \leq 0.40$; all $p > 0.05$). For NT males, however, there was a negative correlation between GSI and stage frequency of B type spermatogonia (Pearson's, $r = -0.776$, $p = 0.024$), but no relationships to any other staining or stage frequencies (Pearson's, $p > 0.05$). At 24 h after injection, spermatocytes were the most advanced stained cysts in all animals. Across social status, there was no significant difference in staining frequency of any cyst stage (ANOVA$_{SPG}$, $F_{2,20} = 0.568$, $p = 0.575$; KW$_{SPC}$, $p = 0.219$), or of all cyst types together (ANOVA, $F_{2,20} = 1.905$, $p = 0.175$), and there was no correlation between GSI and any staining frequency overall (Pearson's, $p > 0.05$). Thus, although testes of T males are larger and contained more overall cysts, the relative percentage of germ cells actively proliferating within the testes was similar both during suppression and when males were given an opportunity to become dominant.

(c) *Influence of social status on sperm quality*
To quantify sperm quality, the VCL and fraction of motile sperm were compared among fish of different social status ($n = 22$NT, 16T and 12A males at 24 h after ascent; figure 2). As expected, T males had greater GSIs than both NT and A males (KW, d.f. = 2, $p < 0.001$; Dunn's, $p < 0.05$), but GSI did not differ between A and NT males (Dunn's, $p > 0.05$).

VCL decreased significantly over time between 15, 30 and 55 s post-activation for all animals (RM two-way ANOVA, $F_{time} = 255.779$, d.f. = 2,49,80, $p < 0.001$; Holm–Sidak $p < 0.001$), while the fraction of motile sperm did not significantly decrease until 55 s post-activation (RM two-way ANOVA, $F_{time} = 9.133$, d.f. = 2,49,80, $p < 0.001$; Holm–Sidak, $p_{15versus30} = 0.102$, $p_{15versus55} = 0.010$, $p_{30versus55} < 0.001$).

There was no difference in VCL among A, T and NT males at any time point (RM ANOVA, $F_{status} = 1.555$, d.f. = 2,49,80, $p = 0.221$; $F_{interaction} = 0.504$, $p = 0.733$), nor was there any correlation between GSI and VCL at any time point (Pearson's, $r < 0.06$, $p > 0.05$). However, T males had a significantly greater fraction of motile sperm than did NT males at all time points (figure 2*b*). This is reflected in a weak correlation between GSI and fraction motility at all time points (Pearson's, $r = 0.401$–0.428, $p < 0.05$). Surprisingly, only 24 h after ascent, sperm motility in A males was significantly higher than that of NT males and not different from T males (RM two-way ANOVA, $F_{status} = 20.087$, d.f. = 2,49,80, $p < 0.001$; $F_{time} = 0.106$, $p = 0.466$; $F_{interaction} = 0.183$, $p = 0.947$, Holm–Sidak, $p_{AversusNT} < 0.001$, $p_{AversusT} = 0.959$, $p_{TversusNT} < 0.001$).

Sperm longevity, defined as the time when sperm motility decreased to 5 per cent or less, was measured for NT, T and A males ($n = 3$ each). While sperm motility was consistently higher for A males at each time point, there was no detectable difference among different social states (ANOVA, $F_{2,7} = 2.467$, $p = 0.165$), probably because of limited sample size (figure 2*c*). When data from all nine males were pooled, the average longevity was 3.97 min (range 2.25–4.75 min).

(d) *Spawning latency by social status*
While *A. burtoni* males ascending in social rank display reproductive behaviours within minutes of social opportunity [38,39], it was not known how quickly males can successfully reproduce (i.e. fertilize eggs). To test this, we compared the time it took for A ($n = 12$) and T ($n = 12$) males to spawn with introduced gravid females.

Ascending males that were previously suppressed for four to five weeks had lower GSI than T males (Student's *t*-test, $t_{22} = -4.90$, $p < 0.001$), but there was no relationship between either body mass or GSI and latency to spawn (Pearson's, $p > 0.05$). Males from both groups successfully spawned within 48 h of exposure to gravid females (A: 12.7 ± 4.1 h, range 2–48 h; T: 14.6 ± 3.4
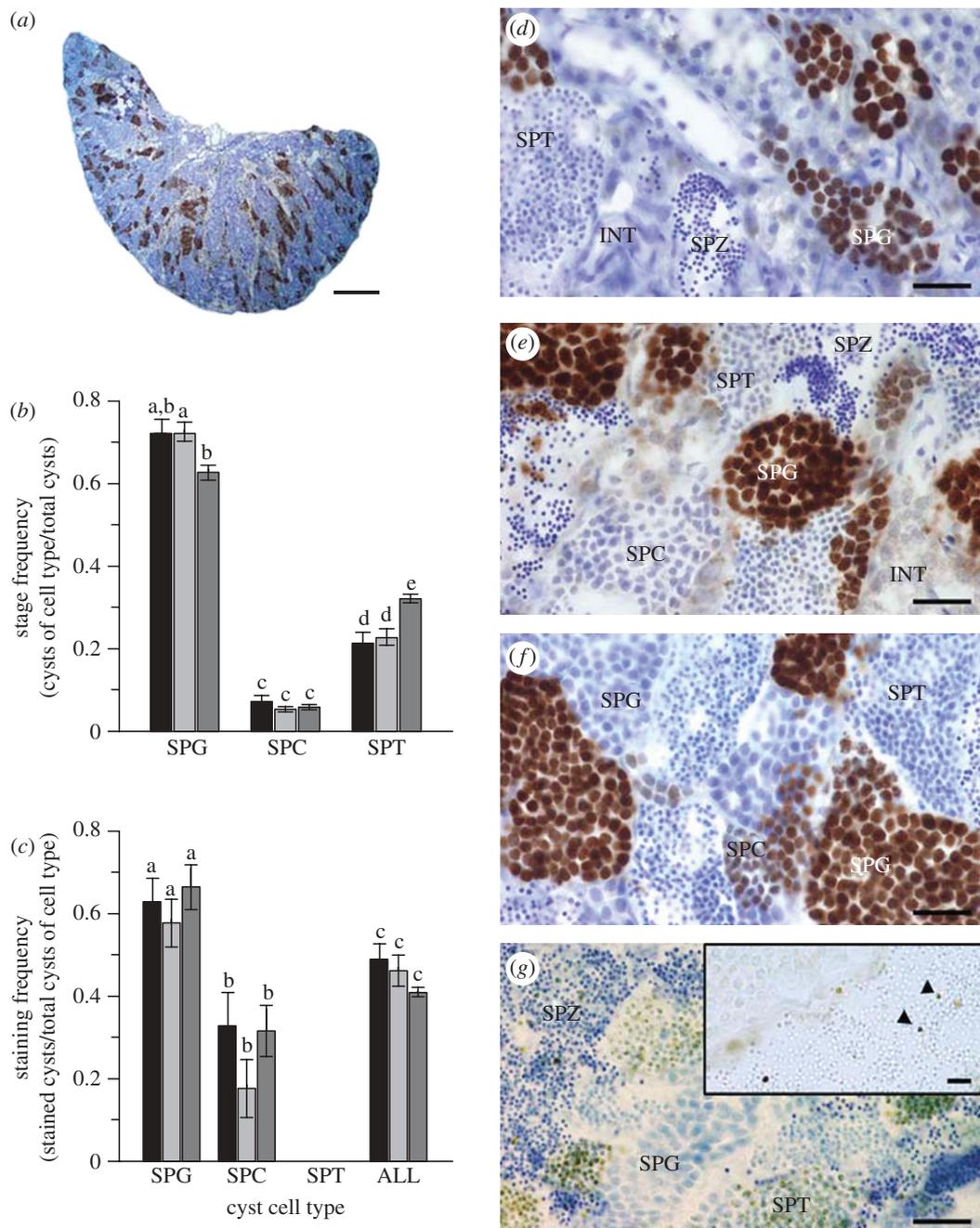
Figure 1. Germ cell types and BrdU staining in testes of different social states in the African cichlid fish *Astatotilapia burtoni*. (*a*) Representative cross section of a territorial (T) male testis 24 h after BrdU injection. Brown is BrdU staining, violet is cresyl violet counterstain. Scale bar, 100 μm. (*b*) T males have a lower fraction of B type spermatogonia (SPG) compared with non-territorial (NT) males and a higher fraction of spermatids (SPT) than NT and ascending (A) males. (*c*) There was no difference in staining frequency of any cell type, or of all cell types together, among T, NT and A testes at 24 h after injection. No staining appears in the spermatids of any animal at 24 h. Letters indicate statistical differences at $p < 0.05$; bars represent means $\pm$ s.e. (*d–f*) Representative cross sections of testes from (*d*) NT, (*e*) A and (*f*) T males 24 h after injection show BrdU staining (brown) in SPG and SPC. INT, interstitial tissue. Scale bars, 50 μm. (*g*) Testis of T male at 195 h shows BrdU staining in SPT. Inset: mature spermatozoa (SPZ) containing BrdU staining (arrowheads) are evident in the sperm ducts at 12 days after injection. Scale bar, 10 μm. (*b*) Black bars, NT ($n = 8$); light grey bars, A ($n = 7$); dark grey bars, T ($n = 8$).

h, range 3–30 h), and all mouthbrooding females contained developing embryos 4–6 days later. Importantly, there was no significant difference in spawn latency between A and T males (Student's *t*-test, $t_{22} = -0.359$, $p = 0.723$), which indicates that reproductively suppressed males are capable of successful fertilization within hours of an opportunity.

4. DISCUSSION
Our results show that despite suppression of the reproductive axis, NT males maintain reproductive potential with active spermatogenesis and viable sperm, and that males ascending from NT to T status increase their sperm motility and successfully mate with females within hours of social opportunity.
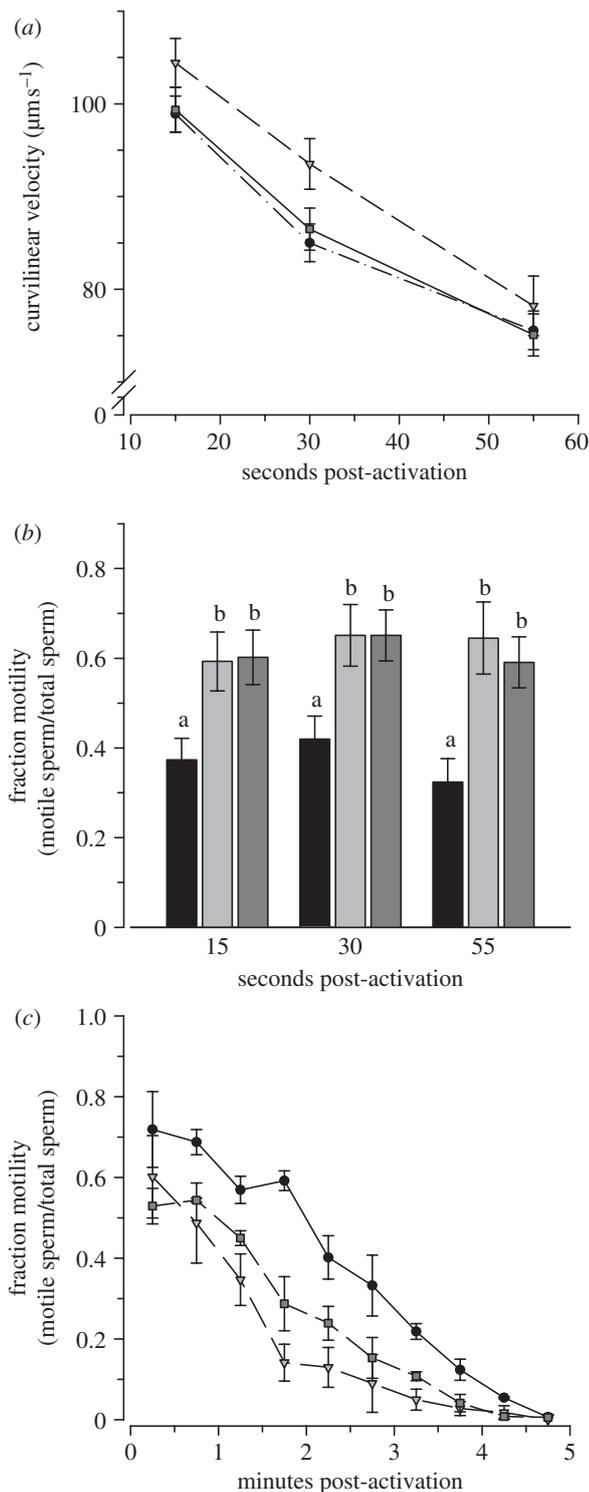
Figure 2. Curvilinear velocity (VCL) and fraction of motile sperm in *A. burtoni* of different social status. (*a*) There was no statistical difference in VCL across social status at any time point. (*b*) Fraction of motile sperm was greater for both T and A males than for NT males, but there was no difference among time points. Letters indicate statistical differences at $p < 0.05$; data are plotted as means $\pm$ s.e. (*c*) Fraction of motile sperm declines to near zero by 4.75 min after activation for all social states ($n = 3$ fish per status per time). (*a*) Circles with dashed line, NT ($n = 22$); inverted triangles with dashed line, A ($n = 12$); squares with solid line, T ($n = 16$). (*b*) Black bars, NT ($n = 22$); light grey bars, A ($n = 12$); dark grey bars, T ($n = 16$). (*c*) Circles with solid line, A ($n = 3$); inverted triangles with dashed line, NT ($n = 3$); squares with solid line, T ($n = 3$).

(a) Spawning latency and spermatogenesis

We found that the spermatogenic cycle in *A. burtoni* is roughly 11–12 days. This is comparable to another cichlid, the Nile tilapia *Oreochromis niloticus*, for which the combined spermatocyte and spermiogenic phases were estimated to be six and 10–11 days at 30°C and 25°C, respectively [50]. Most importantly, we found that the time to produce new sperm far exceeds the time to successful spawning after social ascent, as all A males spawned within 48 h of social opportunity, and BrdU-stained spermatozoa were only observed 11 days after injection. Furthermore, there was no difference in the latency to spawn for A versus T males, suggesting that reproductively suppressed NTs retain viable sperm during suppression rather than producing it upon perception of social opportunity. BrdU injections also revealed similar active proliferation across all social states, indicating that NTs continue some sperm production, at least during the four to five week suppression used in this study. This strategy may be an adaptation for suppressed NT males to facilitate the transition to dominance and rapidly improve their reproductive fitness upon social opportunity, or alternatively, may be an adaptation to reduce investment in sperm production when mating opportunities are limited.

The total number of all testicular cysts was greater in Ts than in NT and A males, but there was no difference in BrdU staining frequency of any cyst type among social states. This is consistent with recent data showing that while the testes of NTs do contain all germ cell stages, they have a smaller percentage of each cell type compared with Ts [40]. In A males, the relative percentages of spermatogonia and spermatocytes are not elevated above NT levels until 3 days after social ascent [40]. This spermatocyte increase is also coincident with an elevation of the fish androgen 11-ketotesterone (11-KT), which is initially low in suppressed males but rises to T male levels by 3 days after ascent [39]. 11-KT is important for the induction of spermatogonial proliferation in fishes [51]; thus we might expect to see an increase in staining frequency of spermatogonia following this 3 day time point, as the volume of sperm production is increased.

The proportion of both spermatogonia and spermatids differed across social status. As the different stages of spermatogenesis (i.e. spermatogonial proliferation, initiation of meiosis and spermiogenesis) are controlled by different genetic and endocrine mechanisms [48], there is potential for plasticity in the initiation of each stage to occur separately. The similar germ cell proliferation among social states provides evidence against the hypothesis that social suppression limits the speed of the spermatogenic process, despite lower androgen levels in subordinates [39,52]. This is consistent with studies in social mole rats (*Cryptomys hottentotus natalensis*), which found that spermatogenesis occurs in non-reproductive males, despite low testosterone and sperm motility [53], and studies in mice show that the maintenance of spermatogenesis requires lower androgen levels than does the initiation of spermatogenesis [54]. Nonetheless, an increase in B-type spermatogonial stage frequency in A males, and an overall correlation between GSI and B type spermatogonia stage frequency, may reflect an increased investment in the initiation of spermatogenesis in A males, even as relative

proliferation remains constant across social status. Future studies are needed to understand the timing and hormonal mechanisms by which *A. burtoni* males vary testicular cell composition in response to social cues, and whether apoptotic regulatory pathways of spermatogenesis might also play a role during this phenotypic plasticity [55].

(b) Sperm quality and social status

We used VCL and fraction of motile sperm as indicators of relative sperm quality in *A. burtoni*. Surprisingly, we found that while sperm from NTs had lower fraction motility than Ts, A males achieved greater sperm motility compared with NTs by only 24 h after a rise in social rank. To our knowledge, this is the first study to show such a rapid change in per cent sperm motility in relation to a shift in social status from a suppressed state, but adjustments of other ejaculate quality measures in response to various social cues can occur within days in guppy fish (sperm velocity) [13], minutes-hours in humans (per cent motile sperm) [19], and within minutes in stickleback fish (ejaculate size) [56], reef fishes (no. of sperm per spawn) [15,16] and fowl (sperm velocity) [18]. Interestingly, the higher percentage of motile sperm at 24 h after ascent also corresponds to a shift in the behavioural repertoire of A males from primarily territorial behaviours to more reproductive behaviours [39]. This suggests that within 24 h of an opportunity, or possibly sooner, ascended males have asserted their dominance to signify territory ownership and improved their chances of successfully attracting and spawning with a female. It is important to note, however, that mating opportunity also differed among T, NT and A males in our study because even with equal exposure to females, NT males are behaviourally prevented from spawning by aggressive attacks from the T male. Several studies in fishes show changes in sperm production and/or sperm quality when males are in the presence of females (high mating opportunity) compared with isolation (low mating opportunity) [12,13,57,58], but it is not known whether altered mating chances owing to behavioural strategies, as in our study, have similar effects on testicular function.

In fishes, the motility of sperm is dependent on the composition of the seminal fluid [59,60] as well as the composition of external factors such as sea water and ovarian fluid [61]. For example, sperm of salmonids, although morphologically mature, does not acquire motility until activated by the elevated pH of the sperm duct [62]. Lahnsteiner *et al.* [63] also found that differences in motility between males of the cyprinid fish *Alburnus alburnus* correlated with factors of seminal fluid composition, including ion, protein and lactate concentrations, and a recent study in *A. burtoni* showed sperm can be rapidly adjusted by changes in osmolality and ionic environment [64]. Recently, ascended males might, therefore, quickly increase sperm motility by regulating the composition of the seminal fluid, or possibly the number of sperm that come in contact with activating factors.

(c) The possibility of alternative reproductive strategies

The ability of NTs to spawn within hours of an opportunity raises the question of alternative reproductive strategies. While sneak fertilization attempts have been
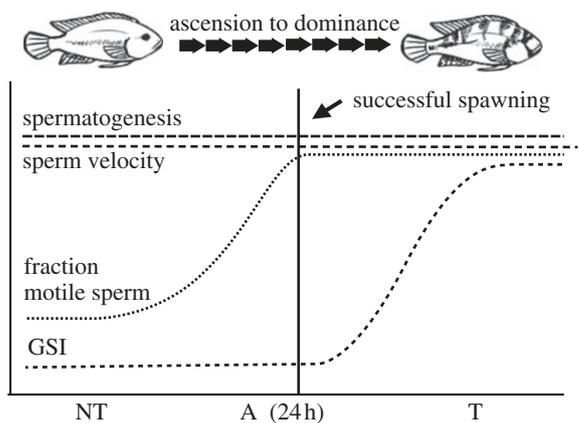

Figure 3. Diagrammatic summary of testicular condition and reproductive competence during social ascent in male *A. burtoni*. While gonadosomatic index (GSI) does not reach T male levels until several days after perception of social opportunity, suppressed NT males still show relative spermatogenic proliferation and sperm velocity comparable to T males. Sperm motility in A males is also elevated to T male levels at 24 h after social opportunity, and successful spawning generally occurs within the first 24 h of ascent (arrow). Data are expressed as arbitrary values to illustrate overall patterns of temporal changes among measures, and are extrapolated between the quantified NT, A (24 h after opportunity) and T states.

observed on multiple occasions in aquaria (see the electronic supplementary material), it is currently unknown to what extent this tactic plays a role in the reproductive biology of *A. burtoni*. NTs exhibit both appearance and behaviour typical of sneakers in other fishes, including coloration similar to females and submissive behaviours, so as to remain inconspicuous to dominant males (see [65]). Sperm competition theory predicts that, should subordinates sneak and always spawn in the presence of dominants, their investment in ejaculate quality should exceed that of dominants [31]. In fishes with alternative mating tactics, sneakers often have larger testes [26], faster sperm [25,27], greater sperm motility [28,29] and/or greater sperm concentration or number [30] than dominants. In *A. burtoni*, however, there is no evidence that NTs invest more in testes growth or ejaculate quality than Ts. Nonetheless, while a lower GSI, lower testicular sperm density [40] and lower per cent motility are consistent with social suppression [1,33], NTs do not exhibit reduced sperm velocity as seen in some other socially suppressed species [32]. In the light of the dynamic social environment and relatively short territory tenure in *A. burtoni* [66], the propensity to engage in sneaking behaviour becomes a trade-off and may vary depending on how long an individual has been suppressed, his internal physiological state and relative body size, the structure of the community and other factors.

(d) Conclusions

Overall, our data show that despite suppression of the entire HPG axis, including reduction in testes size, GnRH1 neuron size and circulating gonadotrophin and androgen levels [34,37,39,40,42,52,67], suppressed NT males maintain sperm production and reproductive capability for at least four to five weeks (figure 3). In highly

dynamic physical and social environments, reminiscent of natural conditions, *A. burtoni* males hold territorial status for no more than three to four weeks on average [66]. In such an environment, where both gain and loss of opportunity are common, there should be a clear advantage to adaptations that allow rapid transition to the more favoured dominant role. NTs have higher somatic growth rates than Ts, and it was suggested that this is at the cost of maintaining reproductive capacity [66]. Our data suggest, however, that while such increased investment in growth may occur at the expense of testes size, percentage of motile sperm and total volume of sperm produced, it has little effect on relative cell proliferation, quality of motile sperm or the ability of a male to successfully spawn with females (figure 3). These data also highlight the importance of using physiological measures other than mere testes size (or GSI) to determine reproductive competence, which can provide insight towards understanding the selective pressures that drive the evolution of reproductive systems in all socially dynamic species.

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