Sperm-limited males save ejaculates for future matings when competing with superior rivals

Dolores Schütz¹, Linda Tschirren¹, Gudrun Pachler¹, Pia Grubbauer¹ & Michael Taborsky^{1,*}

1 **Abstract**

2

3 Ejaculate adjustments to sperm competition can lead to sperm limitation. Particularly in
4 polygynous species, males may face a trade-off between investing sperm in current or future mating
5 opportunities. The optimal sperm allocation decision should depend on the relative intensity of
6 sperm competition experienced in a mating sequence. Here we ask how males respond to this trade-
7 off in polygynous fish with alternative male mating tactics, intense sperm competition and sperm
8 limitation. Large bourgeois males of the shell brooding cichlid *Lamprologus callipterus* build nests
9 consisting of empty snail shells, in which females spawn and raise offspring. During spawning, nest
10 males release ejaculates into the shell opening. Genetically distinct, parasitic dwarf males enter
11 shells during spawning to fertilize the eggs from inside the shell. These dwarf males were
12 previously shown to be superior sperm competitors to nest males. Here we show that when
13 spawning with several females simultaneously, nest males reduce the spawning duration for each
14 clutch and the number of ejaculations per female tends to decrease, reflecting sperm limitation.
15 Experimental exposure of nest males to sperm competition with dwarf males caused a reduction of
16 the number and duration of ejaculations roughly by half. Hence, when exposed to competition with
17 a superior rival, nest males do not increase their sperm expenditure as predicted by sperm
18 competition risk models, but in fact save sperm for future mating opportunities as predicted by
19 sperm competition intensity theory. This seems to be adaptive because of the considerable sperm
20 demands in this species, which is partly due to their high degree of polygyny.

21

22 **Keywords:** alternative mating tactics, bourgeois and parasitic males, ejaculate allocation, sperm
23 competition, sperm depletion

24

25 **Word count** (including everything): 9533 words

26

27 **Introduction**

28

29 Sperm competition, where sperm of two or more males compete for the fertilisation of eggs (Parker,
30 1970), can cause behavioural and morphological adaptations in males. Adaptive adjustment to
31 sperm competition includes prolonged copulations (Schöfl & Taborsky, 2002), mate guarding and
32 an increase in copulation frequency (Birkhead, 1998), sperm displacement (Parker & Simmons,
33 1994), the development of copulatory plugs (Dunham & Rudolf, 2009), and breakage of copulatory
34 organs (Snow, Abdel-Mesih, & Andrade, 2006). Males may monopolise either limited resources for
35 breeding or females directly to prevent rival males from gaining access to females (bourgeois tactic;
36 Taborsky, 1997; Taborsky & Brockmann, 2010). As a consequence of such mating monopolisation,
37 male reproductive success is typically strongly skewed, and alternative reproductive tactics (ARTs;
38 Oliveira, Taborsky, & Brockmann, 2008) may evolve, where parasitic males invest relatively more
39 in testes than bourgeois males due to their higher risk of sperm competition (Byrne, Roberts, &
40 Simmons, 2002; Stockley, Gage, Parker, & Møller, 1997; Taborsky, 1998).

41

42 Adaptations to sperm competition have been modelled extensively, with two types of game
43 theoretical approaches considering either how males should respond to sperm competition risk
44 (SCR; whether it occurs or not, Kelly & Jennions, 2011, Parker, 1998) or to sperm competition
45 intensity (SCI; number of ejaculates competing for a set of ova, Parker, Ball, Stockley, & Gage,
46 1996; 1997). SCR models predict that ejaculate expenditure increases when males are exposed to a
47 single competitor, which has been confirmed by empirical data (for review see Wedell, Gage, &
48 Parker, 2002). SCI models predict that ejaculate expenditure decreases with an increasing number
49 of competitors because males save sperm for better future spawning opportunities, which has
50 received less empirical support (for reviews see Engqvist & Reinhold, 2005; Kelly & Jennions,
51 2011; Wedell et al., 2002). These models imply that generally speaking, if males can succeed in

52 sperm competition they should increase ejaculate expenditure, whereas if they have little chances to
53 succeed against superior competitors, they should reduce ejaculate expenditure to save sperm for
54 future matings. This may be particularly important in polygynous species, where both mating
55 frequency and sperm competition can lead to sperm limitation (Wedell et al., 2002). Additional
56 causes of sperm limitation (Shapiro & Giraldeau, 1996) include low fertilization efficiency, large
57 clutch size, and high reproductive costs for males (Wedell et al., 2002).

58
59 It is generally little understood to which extent variation in sperm and ejaculation characteristics
60 result from either sperm competition or sperm limitation. In bitterling fish, for example, differences
61 in the mating system (Pateman-Jones et al., 2011) or breeding resource distribution (Reichard,
62 Ondrackova, Bryjova, Smith, & Bryja, 2009) significantly affect ejaculate characteristics under
63 high levels of both sperm competition and sperm limitation. An interspecific comparison showed
64 that the species with the shortest spawning season (*Rhodeus amarus*), which corresponds to a high
65 probability of sperm limitation, showed the greatest level of investment in sperm production, the
66 highest ejaculation rate, the smallest clutch size, and spermatozoa apparently adapted to fast
67 swimming (Pateman-Jones et al., 2011). With a clumped breeding resource distribution in
68 comparison to an even resource distribution, stronger selection on traits that evolve due to sperm
69 competition was detected (Reichard et al., 2009). In sea urchins, high population densities result in
70 selection on sperm traits associated with sperm competition, whereas low population densities result
71 in selection on sperm traits associated with sperm limitation (Levitan, 2002).

72
73 A possibility to disentangle the influence of these two challenges on sperm and ejaculate
74 characteristics is to manipulate the degrees of sperm competition and polygyny independently of
75 each other in species that combine polygyny with the existence of ARTs. To test for an influence of
76 sperm limitation, it can be determined how males adjust ejaculation characteristics to the number of

77 females they mate with at a time. To test for an influence of sperm competition, ejaculation
78 characteristics can be compared between bourgeois males mating alone or together with parasitic
79 males. However, in externally fertilising species releasing their gametes into the water, it is difficult
80 to determine ejaculation characteristics under different experimental conditions (Shapiro,
81 Marconato, & Yoshikawa, 1994).

82
83 In the cichlid fish *Lamprologus callipterus*, large bourgeois males construct and defend nests
84 consisting of empty snail shells, in which the much smaller females breed (further on called ‘nest
85 males’; Schütz & Taborsky, 2005). Females lay clutches containing on average 95 eggs, which are
86 deposited one by one inside a shell with intervals of more than two minutes between subsequent
87 eggs (Schütz, Heg-Bachar, Taborsky, & Heg, 2012). Each egg needs to be fertilized by a separate
88 ejaculate, leading to a total spawning duration of nearly seven h on average (Schütz et al., 2012) and
89 to severe sperm limitation of nest males (Schütz, Pachler, Ripmeester, Goffinet, & Taborsky, 2010).
90 The latter reduce the number of sperm released per ejaculate drastically after 5 h of continuous
91 spawning, even when spawning with only one female (Schütz et al., 2010), and even though eggs
92 are deposited at a similar rate over the whole duration of a spawning (Schütz et al., 2012).

93
94 In *L. callipterus*, two distinct parasitic male types exist: Sneaker males opportunistically try to
95 enter territories where nest males are spawning to fertilize eggs by releasing ejaculates into the shell
96 opening when the nest male is inattentive. This tactic is transitional and performed by males of the
97 nest male type (Schütz, Parker, Taborsky, & Sato, 2006). Parasitic dwarf males constitute a
98 genetically distinct male morph (Wirtz Ocana, Meidl, Bonfils, & Taborsky, 2014) remaining small
99 throughout life (Taborsky, 2001). They try to enter shells in which a female is spawning to fertilize
100 the eggs from inside the shell. Once succeeded, dwarf males stay in the shell during the whole
101 spawning event, and therefore they are in much closer vicinity to the female and eggs during laying

102 than nest males, i.e. they are in a privileged position to fertilise the eggs (Sato, Hirose, Taborsky, &
103 Kimura, 2004). This is in contrast to most other species, where bourgeois males are usually much
104 closer to eggs than parasitic males (Taborsky, 2008; Taborsky, Oliveira, & Brockmann, 2008).
105 Thus, spawning of a nest male with a parasitic dwarf male resembles a loaded raffle (Parker, 1990a)
106 where dwarf males have a fertilization advantage, which is revealed also by the much greater
107 fertilization success of dwarf males in comparison to nest males in nature (Wirtz Ocana et al.,
108 2014). Theory predicts that the unprivileged male type (here the nest male) should compensate his
109 disadvantage by investing more in the present ejaculate than the privileged male type (here the
110 dwarf male; Parker, 1990a). Hence, in accordance with sperm competition risk models nest males
111 should increase their reproductive effort when spawning with a parasitic dwarf male (Parker, 1998).
112 Alternatively, nest males might decrease ejaculate expenditure in such competitive situations to
113 save sperm for future spawning opportunities without participating dwarf males, as predicted by
114 sperm competition intensity models especially if they involve highly loaded raffles (Parker et al.,
115 1996, Parker et al., 1997).

116

117 In species with external fertilisation, ejaculate sizes can hardly be determined exactly because
118 sperm diffuse into water right after release. This is different in *L. callipterus*, since males ejaculate
119 into a snail shell, which allows collection of their sperm and determination of ejaculation
120 characteristics. Additionally, the perceived risk of sperm competition for nest males can be
121 manipulated directly by adding a dwarf male into a shell where the nest male is spawning. Thus,
122 physiological responses of males to the perceived risk of sperm competition before a test spawning
123 are prevented, avoiding this pitfall in testing predictions from sperm competition models (Engqvist
124 & Reinhold, 2005).

125

126 Here we aim to clarify the relative roles of sperm limitation and sperm competition for shaping

127 ejaculation characteristics of *L. callipterus* nest males. Specifically, we ask how much they invest in
128 ejaculates in dependence of increasing sperm limitation and sperm competition risks. We compared
129 nest male sperm and ejaculation characteristics when spawning (i) with one or more females
130 simultaneously to test for the influence of sperm limitation, and (ii) with or without a parasitic
131 dwarf male present to test for the influence of sperm competition.

132

133 We investigated how nest males deal with the apparent trade-off regarding sperm allocation in
134 dependence of the current sperm competition risk and future mating opportunities (Wedell et al.,
135 2002). When facing sperm competition with a superior dwarf male, nest males may either increase
136 ejaculate expenditure to compensate for their `devalued` fertilisation opportunity (Parker, 1990b;
137 Parker, 1998) or decrease it to save sperm for future matings without dwarf males (cf. Parker et al.,
138 1996, Parker et al., 1997, Wedell et al., 2002). In the laboratory, we experimentally added a dwarf
139 male into a shell where a female was spawning with a nest male and analysed nest male behaviour
140 and ejaculate characteristics before and after this manipulation.

141

142 To estimate the risk of sperm competition and sperm limitation in the natural situation, we
143 determined the number of females, intruders of the nest male type (mainly territorial neighbours),
144 sneakers, and dwarf males entering a nest per day from long-term video recordings obtained in the
145 field. Sperm limitation of nest males should be even higher when nest males spawn with more than
146 one female at a time. Thus, from these long-term video recordings we determined the total
147 spawning duration for each clutch and the number of ejaculations per female for nest males that
148 were spawning simultaneously with different numbers of females.

149

150 In addition, we determined the relationship between ejaculation characteristics and sperm numbers
151 in laboratory experiments in which nest males spawned with a female alone, since it is impossible

152 to determine sperm numbers of nest males reliably when dwarf males participate in spawning. We
153 video-taped nest male ejaculations, collected and counted their sperm, and tested the relationship
154 between different ejaculation characteristics. We determined the number of sperm per ejaculation,
155 the duration of each ejaculation bout, and the sperm release rate (sperm number per ejaculate /
156 duration of ejaculation bout), and tested how these parameters relate to the total number of sperm
157 released during the entire spawning.

158

159 **Methods**

160

161 Study species

162

163 *L. callipterus* is a cichlid fish endemic to Lake Tanganyika, which shows the greatest sexual size
164 dimorphism among animals with males being bigger than females (Schütz & Taborsky, 2000). Two
165 genetically determined alternative reproductive tactics exist: (i) Nest males: after passing a size
166 threshold of 9 cm standard length (Schütz & Taborsky, 2005), these males build and defend nests
167 consisting of clumps of empty snail shells, in which females lay eggs and care for the brood for 10
168 to 14 days (Sato, 1994; Sato et al., 2004; Schütz & Taborsky, 2000). Nest males collect empty snail
169 shells from the vicinity of their nests or steal them from neighbouring males (Mitchell, Wirtz
170 Ocana, & Taborsky 2014; Sato, 1994). Since nest males hardly feed when defending a territory,
171 their condition decreases during territory maintenance until their territories are taken over by larger
172 or heavier males (Sato, 1994; Schütz et al., 2010). Males pursuing this life history pathway may
173 adopt also a parasitic mating tactic, particularly when they are still too small to successfully defend
174 a nest. Such ‘sneaker’ males try to enter a nest owner’s territory during spawning to fertilize eggs
175 by releasing ejaculates into the shell opening (Sato et al., 2004). (ii) Dwarf males: These males
176 remain even smaller than females throughout life (Taborsky, 2001; Wirtz Ocana et al., 2014); in the

177 field, they weigh on average only 2.5% of nest males (Sato et al., 2004). Dwarf males try to enter a
178 shell with a spawning female inside by wriggling past the female in order to enter the inner whorl of
179 the shell, from where they can fertilize the eggs (Sato et al., 2004). If successful in their attempt to
180 enter the shell, they fertilize the majority of the eggs from this privileged position (Wirtz Ocana et
181 al., 2014).

182

183 Holding conditions for fish in the laboratory

184

185 All fish used in laboratory experiments were offspring of wild-caught fish originating from the
186 southern end of Lake Tanganyika, Zambia. They were fed twice per day on 6 days of the week,
187 with protein-rich food (frozen zooplankton) in the morning and with flake food in the evening.
188 Water parameters were kept close to the values known from the Southern end of Lake Tanganyika.
189 Light was kept at a 13:11 light:dark cycle with 10-min dimmed light phases in between. All tanks
190 were equipped with a biological filter.

191

192 Female visits and sperm competition in nature

193

194 To check for the potential risk of sperm competition and sperm limitation, we determined how
195 many females and males of different tactics are found around territories at Lake Tanganyika. As the
196 spawning activity in this species varies significantly with the lunar cycle (Nakai, Yanagisawa, Sato,
197 Niimura, & Gashagaza, 1990), we attempted to monitor nests throughout the different phases of the
198 moon cycle. During two field seasons, seven nests at Wonzye Point (1995) and four nests at
199 Kasakalawe Point (1997) were continuously recorded via underwater video-cameras from new
200 moon to one week after full moon. In ten of these nests, spawning was recorded. All videos were
201 analysed for the entire daylight period (6:00h-18:00h). From these video recordings, we determined

202 the number of females, nest male intruders (mainly neighbouring males that try to steal shells or to
203 take over the nest), sneaker males and dwarf males (males trying to steal fertilizations) entering a
204 nest per day, and we calculated averages per moon day.

205

206 We divided the moon cycle into 3 day intervals (7 intervals for 3 weeks of recording) and checked
207 whether the frequency of dwarf plus sneaker males, nest male intruders and visiting females
208 differed from equal distributions with Chi^2 -tests. Additionally, we used Chi^2 -tests to check whether
209 the frequency of dwarf plus sneaker males and of nest male intruders differed from the frequency of
210 females visiting a nest per day. For statistical analyses, we used the software package IBM SPSS
211 statistics 22 and tested for two-tailed probabilities. For the LMM of sperm number per ejaculation
212 in dependence of time of sperm release we used the package “lme4” of the software R 3.2.2. (R
213 Core Team 2015).

214

215 Adjustment of ejaculations to spawning with multiple females

216

217 To find out how *L. callipterus* nest males adjust ejaculation characteristics when spawning with
218 more than one female at a time, we determined the total spawning duration of nest males per clutch
219 and the number of ejaculations per female when they were spawning with up to four females
220 simultaneously. The number of ejaculations is representative for the total number of sperm if 1)
221 these two variables correlate positively with each other, and 2) the number of sperm per ejaculation
222 does not change significantly with time. We checked and verified these two assumptions with data
223 from the first laboratory experiment (see below).

224

225 From continuous video recordings in the field (see above), we could identify the exact start and end
226 of laying a clutch for 28 individual females spawning in different shells (each female spawns in a

227 separate shell in this species; see Schütz et al., 2012 for details and methods). To check whether the
228 two variables differ between males spawning with 1, 2, 3, or 4 females at a time, we used general
229 linear models (GLM) with the number of simultaneously spawning females as fixed factor, and
230 male ID as random factor. Additionally, with Spearman rank correlation analyses we tested whether
231 the two variables relate to the number of females with which a nest male was concurrently
232 spawning.

233

234 Sperm release rate versus the duration of ejaculations

235

236 We aimed to check whether ejaculation characteristics are a reliable measure for the numbers of
237 sperm released during a whole spawning event by counting sperm numbers of nest males when
238 spawning alone with a female and relating these to ejaculation characteristics. These experiments
239 were carried out in 400 litre tanks divided into two differently-sized (1:2) compartments with a
240 clear Plexiglas partition. The bigger compartment was set up as a territory for a nest male. Five
241 manipulated shells were secured in a position that enabled us to videotape any activity in front of
242 them. Eight males between 8.3 and 9.9cm standard length were investigated with groups of four
243 females each. Before starting the experiment we put the four females in the large compartment
244 (containing the shells) and the territorial male in the small compartment, so that he could see the
245 females but could not physically interact with them until they were ready to spawn.

246

247 When a female was ready to spawn (showing an extended whitish belly), the experiment started at
248 9:00 a.m. on the following morning. The other 3 females were taken out of the compartment, the
249 male was transferred from the small into the large compartment, and continuous video recording
250 was started. To collect the sperm released by the male, we prepared shells by attaching a silicone
251 tube (inside diameter = 2mm) to a hole drilled into the first spiral of the shell, where the eggs are

usually placed during spawning. After each ejaculation the sperm was sucked out with a motor pump and all samples were then analysed for the presence of sperm using the methods of Leong (1989) and Shapiro et al. (1994) modified to our needs (see below).

Water samples of 30 ml each were sucked out from the shell via the tube when the male positioned his genital papilla over the opening of the shell and supposedly released sperm (cf. Schütz et al., 2012). The amount of water contained in the tube itself was 5 ml, and the average shell-volume was 15.4 ± 0.84 ml (mean $\pm$ standard deviation). We took 30 ml for each water-sample to ensure that the tube plus shell volumes were collected completely for each extraction. The water sample was immediately mixed with the same amount of 0.1M phosphate buffer to eliminate the effect of osmotic pressure on sperm heads. To analyse our samples for the presence of sperm, we modified the methods of Leong (1989) and Shapiro et al. (1994) as follows. After removal, two drops of Rose Bengal were added for staining the head of the spermatozoa before passing the sample through a Millipore filter (0.22 μ m pore size) under vacuum. The filter paper was dried and cleared with immersion oil. The number of sperm was counted under a light microscope at a magnification of 400x in an area of 0.185x0.185 mm, and the count was repeated 20 times at randomly selected portions of the filter. The mean value of these counts was used to estimate the total number of sperm present in the sample (total filter area = 160.61mm², therefore the sperm count was multiplied by 4692.65).

Water samples of ejaculates were taken at intervals of about 10 minutes, and one sample from every half-hour was analysed as described above. We determined the numbers of sperm per ejaculation and the total numbers of sperm released for each test male. For each ejaculate of the analysed water-samples, from the video recordings we measured the exact duration of the ejaculation bout, i.e. how long the male placed his genital papilla above the shell entrance and calculated the ejaculation rate

277 (ejaculations/min) for each male. The number of sperm per ejaculate divided by the duration of
 278 ejaculation bouts gave the mean sperm release rate for each ejaculation (number of sperm/s).
 279
 280 With Spearman's rank correlation analyses we tested whether the ejaculation bout duration
 281 correlated with the number of sperm released per ejaculate, and whether the total number of sperm a
 282 male released during spawning correlated with the mean number of sperm released per ejaculation,
 283 the sperm release rate, and the mean ejaculation bout duration. With Spearman's rank correlation
 284 analyses we also checked if the total number of sperm correlates with the number of ejaculations.
 285 Furthermore, we tested whether the number of sperm released per ejaculation is constant throughout
 286 a spawning during the first 5 hours of spawning. For this purpose, we standardized the data from the
 287 eight involved males as follows: [time] = time of ejaculation sample minus time of first sperm
 288 release; [sperm per ejaculation] = sperm of ejaculation sample / average sperm per ejaculation. We
 289 calculated a linear mixed-effect model (LMM, Bates et al 2014) with time as fixed factor, male ID
 290 as random factor and sperm number per ejaculate as dependent variable. The dependent variable
 291 was reciprocally transformed as $\frac{1}{\sqrt{\text{sperm per ejaculation} + 0.5}}$. The residuals of the model were checked
 292 for normal distribution by visual inspection of the Q-Q plot, and the Kolmogorow Smirnov and
 293 Shapiro normality tests. The significance of time was tested using likelihood ratio test between the
 294 full model that included time as fixed effect and a null model in which the fixed effect was
 295 excluded.

297 Response of nest males to sperm competition with dwarf males

298
 299 We checked whether nest males increase their ejaculate investment when faced with a dwarf male
 300 competitor or whether instead they conserve sperm for future spawning opportunities. Before the
 301 experiment, 24 large nest males (>9 cm; to ensure that they were able to transport shells; Schütz &

302 Taborsky, 2005) were placed individually into 200 litre experimental tanks and given one week of
303 acclimatization time. At the same time, dwarf males were held in a 200 litre storage tank and
304 stimulated with unusable shells and 2 females. Females that had not reproduced for at least 8 weeks
305 were transferred to a 400 litre stimulation tank, in which a nest male was present to stimulate them.
306 When they showed signs of being ready to spawn, two such gravid females were placed in a
307 separate compartment of the experimental tanks (approximately $\frac{1}{4}$ of the tank, separated from the
308 nest male by a clear partition) for two days to allow for acclimatisation. A video camera was set in
309 front of the experimental tank to record the nest and surroundings (approximately $\frac{1}{3}$ of the
310 experimental tank). Experimental tanks were protected with black curtains from visual disturbance
311 by the observer.

312

313 On the day of the experiment, the partition was removed and 3 manipulated shells were put into the
314 tank at 9.00 a.m. The experimental snail shells had a hole (ca. 0.5x1 cm) covered with Plexiglas,
315 where normally the dwarf male's body is located during spawning (approximately in the second
316 whorl). This Plexiglas 'window' was fixed to the shell with a rubber band. Tanks were checked
317 every 30 min for courtship activity.

318

319 Approximately 3 h after the first ejaculation of the nest male (i.e., approximately 2 h after the first
320 egg had been laid; cf. Schütz et al., 2012), we opened the shell's Plexiglas window and added a
321 dwarf male head first into the shell (N = 12). The window was closed again with the rubber band,
322 and the shell was put back into the nest at the same position it had been before handling. If
323 spawning did not start until 12 a.m., the trial was aborted (i.e., the manipulated shells were taken
324 out), and the procedure was started again on the next day to ensure that 9 h of light were available
325 for the spawning. In the control treatment (N = 8), the procedure was exactly the same (tank
326 equipment, timing, shell handling, camera installation, etc.), except that no dwarf male was added

327 into the shell during shell handling.

328

329 After spawning was finished, the shell with the female was isolated. Shells of the experimental
330 treatment were briefly opened to enable the dwarf male to leave the shell without disturbing the
331 female. The dwarf male was returned to the storage tank. Three days after spawning, the larvae
332 (hatched embryos) were collected by releasing them together with the female with help of light
333 anaesthesia (MS222). Larvae were stored in 95% ethanol, as were fin clips from nest males,
334 females, and dwarf males.

335

336 15 successfully manipulated spawnings ($N = 7$ of the experimental treatment, $N = 8$ of the control
337 treatment) could be completely video recorded and analysed using Observer 5.0 (Noldus
338 Information Technology). We could not analyse the videos of five spawnings of the experimental
339 treatment because of video system malfunction. We continuously noted the rate of three behaviours
340 known to be of key importance during spawning (Schütz et al., 2012): ‘mouthing’, when the nest
341 male opens and closes his mouth within the entrance of a shell containing a female; ‘ejaculation’,
342 when the male stays motionless with his genial papilla over the shell entrance; and ‘sniffing’, which
343 corresponds to mouthing into an empty shell. Additionally, the duration of each ejaculation bout (as
344 an estimate of sperm release duration), the number of ejaculations, and the time the nest male stayed
345 in his nest were determined over the whole spawning event. The latency from handling until the
346 first ejaculation by the nest male was determined for the experimental and control group to check
347 whether handling affected the two groups differently. A different latency in the experimental
348 treatment compared to the control treatment would indicate different disturbance levels of nest
349 males and females between the two groups. All broods from the experimental treatments (i.e. with a
350 dwarf male contributing to spawning; $N = 12$) were tested for paternity using microsatellite analysis
351 (details of DNA sampling and analysis see Appendix). The aim was to analyse at least 20

352 individuals per brood, if the clutch was large enough.

353

354 To analyse the role of the dwarf male presence (treatment: present, control: absent), we subdivided
355 the behavioural recordings into 10-min intervals and calculated means (of rates and durations) per
356 interval for each behavioural variable, starting one h after the first ejaculation. As with increasing
357 spawning time more and more spawnings end, only the first thirty 10-min intervals were analysed to
358 keep sample size constant (12 intervals before and 18 intervals after handling the shell). From these
359 intervals, we calculated means of behaviours shown before and after the experimental manipulation.
360 With repeated measures ANOVAs, we tested whether the number of ejaculations per female, the
361 ejaculation duration, the time the nest male was in the nest, and the sniffing and mouthing rates
362 differed between before and after the manipulation (within-subjects variables), and whether a
363 difference was due to the treatment effect (between-subjects factor; treatment versus control). We
364 used a Mann-Whitney *U*-test to compare the latency until the first ejaculation by the nest male after
365 handling, between treatment and control conditions. Paternity results were compared with the
366 percentage of total spawning time for which the dwarf male was inside the shell, and with total
367 clutch size.

368

369 Ethical note

370

371 Prior to the laboratory experiments, fish were held in mixed groups of various sizes in tanks
372 between 200 and 500 litres. They were fed twice per day on 6 days of the week as in the
373 experiments. Each individual was used only once for an experiment. After the experiments, fish
374 were placed back into the tanks from which they had been taken. In accordance with the Veterinary
375 office of the Kanton Bern granting permission for our experiments, fish were not anaesthetized for
376 fin clipping in order to reduce stress and potential negative effects of anaesthetization. Fin clips

377 were taken from the dorsal and anal fins by removing one fin ray with the surrounding tissue. The
378 removed tissue regrew fully within a period of 4 to 8 weeks. For the field work at Lake Tanganyika,
379 permission was granted by the Fisheries Department of the Ministry of Agriculture and Co-
380 operatives of Zambia. The laboratory work was licensed by the Veterinärdienst, Amt für
381 Landwirtschaft und Natur des Kantons Bern (license number: 17/09).

382

383 **Results**

384

385 Female visits and sperm competition in nature

386

387 On average, 22.31 females visited a nest per day between new moon and one week after full moon
388 (range: 6.7-109.2, Fig. 1). During the same period, the mean number of nest male intruders was
389 16.14 per day and nest (range: 3.6-26.0; mainly neighbouring nest males that were expelled by the
390 nest owner), whereas the corresponding number of sneakers entering the nest was on average 0.83
391 (range: 0-5.4) and that of dwarf males 0.38 (range: 0-2.14, Fig. 1).

392

393 During the moon cycle, which was separated into seven 3-day periods, the frequencies of visiting
394 females ($X_6 = 16.74$, $P = 0.010$) and of reproductive parasites (dwarf and sneaker males combined)
395 differed significantly from an equal distribution ($X_6 = 20.871$, $P=0.002$), but the frequency of nest
396 male intrusions did not ($X_6 = 8.27$, $P=0.205$). Furthermore, the distribution of visiting females
397 differed significantly from the distribution of parasitic males attempting to participate in spawning
398 ($X_6 = 15.597$, $P=0.016$) and from the distribution of nest male intrusions ($X_6 = 21.5$, $P=0.0015$).

399

400 Adjustment of ejaculations to spawning with multiple females

401

402 Nest males were spawning with up to four females simultaneously (28 spawnings corresponding to
403 28 different females, recorded in 10 monitored nests). The total spawning duration of a clutch
404 significantly depended on the number of females spawning at a time (GLM, $F_{1,3} = 4.536$, $P =$
405 0.015), whereas it was not related to male identity ($df=8$, $F=1.265$, $p=0.321$). The number of
406 ejaculations per female neither seemed to be influenced by the number of simultaneously spawning
407 females (GLM, $F_{1,3} = 0.811$, $P = 0.505$) nor by male identity ($F_{1,8} = 1.296$, $P = 0.310$). However,
408 viewed on a continuous scale, the number of ejaculations per female tended to decline with
409 increasing numbers of simultaneously spawning females (Spearman rank correlation, $r_s = -0.353$, N
410 $= 28$, $P = 0.065$, see Fig. 2).

411

412 Sperm release rate versus the duration of ejaculations

413

414 Both the average number of sperm per ejaculation (Spearman rank correlation, $r_s = 0.905$, $N = 8$, P
415 $= 0.002$, Fig. 3a) and the sperm release rate (sperm number per ejaculate / duration of ejaculation
416 bout in s, Spearman rank correlation, $r_s = 0.881$, $N = 8$, $P = 0.004$, Fig. 3b) correlated positively
417 with the total number of sperm a male released during spawning. The mean duration of ejaculation
418 bouts related neither to the total number of sperm (Spearman rank correlation, $r_s = 0.143$, $N = 8$, $P =$
419 0.736) nor to the number of sperm released per ejaculate (Spearman's rank correlation, $r_s = -0.024$,
420 $N = 8$, $P = 0.955$, Fig. 3c).

421

422 The number of ejaculations serves as a good proxy for the total number of sperm released since (i)
423 there is a positive correlation between the number of ejaculations and the total number of sperm
424 released (Spearman rank correlation, $r_s = 0.857$, $N = 8$, $P = 0.007$) and (ii) the number of sperm per
425 ejaculation remains rather constant over the majority of spawning time. During the first 5 hours of
426 spawning, time showed no significant effect on the number of sperm per ejaculate (model

427 comparison with likelihood ratio test of the two models with and without time as fixed factor, χ^2
428 = 0.742, df = 1, p = 0.389).

429

430 Response of nest males to sperm competition with dwarf males

431

432 *Nest male behaviour*

433

434 Repeated measures ANOVAs determined that the ejaculation duration and the number of
435 ejaculations per female differed before and after adding a dwarf male into the shell, and that this
436 difference was due to the treatment (comparing treatment with control conditions; table 1, Fig. 4).
437 After experimentally adding a dwarf male, the duration of ejaculation bouts per female dropped by
438 61.5% and the number of ejaculations per female declined by 40.5% in comparison to the
439 experimental control (Fig. 4, right panels, A: dwarf versus control). The mouthing and sniffing
440 rates, and the time the nest male stayed inside the nest also differed between before and after the
441 shell manipulation, but this was not explained by the treatment (table 1). The latency to the first
442 ejaculation occurring after handling did not differ between the treatment and control groups (Mann-
443 Whitney U test: $U = 25$, $N_1 = 7$, $N_2 = 8$, $P = 0.779$), suggesting that handling *per se* did not affect
444 the two experimental groups differently.

445

446 *Paternity*

447

448 The percentage of larvae the dwarf male sired correlated positively with the percentage of total
449 spawning time for which it had been in the shell during spawning (Spearman rank correlation, $r_s =$
450 0.867, $N = 7$, $P = 0.012$), whereas it did not correlate with the clutch size (Spearman rank
451 correlation, $r_s = -0.078$, $N = 12$, $P = 0.809$). Microsatellite analyses showed that dwarf male

452 paternity ranged between zero and 100 percent ($\bar{x} = 13.6\%$; see Appendix, table A1). In 6 of 12
453 cases the dwarf male did not fertilize any eggs.

454

455 **Discussion**

456

457 Long-term video surveys of *L. callipterus* nests in the field revealed that many neighbouring nest
458 males intrude into a territory during spawning, mainly trying to steal shells or fertilisations (Maan
459 & Taborsky, 2008). In addition, sneaker and dwarf males enter the nests during spawning. Although
460 sneaker males occasionally succeed in releasing sperm into a shell where a female is laying eggs,
461 their fertilisation success is very low in comparison to dwarf males (Wirtz Ocana et al., 2014),
462 because due to the time pattern of egg release (Schütz et al., 2012) they can fertilise only one egg
463 per ejaculation. Thus, whereas dwarf males outcompete nest males in fertilisations, sperm
464 competition with sneaker males is of minor importance (Wirtz Ocana et al., 2014). The distributions
465 of intruding males and nest visiting females differed from each other during the moon cycle.
466 Therefore, nest males face different levels of sperm competition during their territory holding
467 period, which should select for an ability to adjust ejaculation characteristics to the current risk of
468 sperm competition.

469

470 The total spawning duration a nest male spent for each clutch declined with an increasing number of
471 simultaneously spawning females, whereas male identity had no obvious effect. Additionally, the
472 number of ejaculations per spawning tended to decrease with increasing numbers of simultaneously
473 spawning females. Since the number of ejaculations is positively correlated with the total number of
474 sperm released, and the number of sperm per ejaculation remains constant over the majority of
475 spawning time (see also Schütz et al., 2010), the ejaculation number per female is a good predictor
476 for the number of sperm released per female. These changes in spawning behaviour likely reflect

477 sperm depletion in males, which was revealed also when nest males spawned with single females
478 for longer periods than five h (Schütz et al., 2010). Sperm depletion of males may lead to a decrease
479 in female productivity (Dunn, Andrews, Ingrey, Riley, & Wedell, 2006) and to sexual conflict over
480 the size and distribution of ejaculates (Diaz, Haydon, & Lindstrom, 2010; Smith, Pateman-Jones,
481 Zieba, Przybylski, & Reichard, 2009). Female choice may hence be influenced by potential sperm
482 limitation of males. In *L. callipterus*, females may reduce the risk of suffering from nest male sperm
483 limitation by choosing large nest owners (Maan & Taborsky, 2008), and by enabling dwarf males to
484 enter the shell during later phases of spawning, when nest males are already sperm depleted.

485

486 Our laboratory experiments revealed that the mean number of sperm released per ejaculate and the
487 sperm release rate were good predictors for the total number of sperm released for a whole clutch.
488 However, the mean duration of ejaculation bouts did not significantly correlate with the number of
489 sperm released per ejaculation or with the total number of sperm released per spawning. This might
490 suggest that our estimate of the actual ejaculation duration as derived from the amount of time the
491 male stays motionless with his genial papilla over the shell entrance may be inaccurate. Nest males
492 may not release sperm during the whole time holding this position, and therefore the number of
493 ejaculations per female is a better measure for male sperm expenditure than ejaculation bout
494 duration.

495

496 Males seem to increase the number of sperm released during a spawning mostly through a higher
497 sperm release speed rather than by increasing the duration of ejaculation bouts. This can be
498 explained by the peculiar spawning condition in *L. callipterus*, where each egg has to be fertilised
499 by a separate ejaculate of the nest male over a period of many hours (Schütz et al., 2012). In
500 principle, males have three possibilities to raise the total sperm number and to increase their
501 fertilisation probability. They may increase the ejaculation frequency, the duration of ejaculation

bouts, or the sperm release rate. However, since females obviously determine the timing of egg laying (adopting quite regular intervals of 2 minutes between subsequent eggs; Schütz et al., 2012), nest males have to assume this pattern of gamete release. Thus, nest males would not enhance fertilization chances by increasing the ejaculation frequency or the duration of ejaculation bouts, hence raising the sperm release rate per ejaculate seems to be their best option (i.e. per egg to be fertilized).

Inducing sperm competition experimentally showed that nest males did not increase their sperm expenditure as predicted by sperm competition risk models (Parker, 1990b; Parker, 1998), but rather decreased it as predicted by sperm competition intensity theory (Parker et al., 1996; Parker et al., 1997; Wedell et al., 2002). Adding a dwarf male into a shell when a nest male was spawning resulted in 61.5% shorter ejaculation bout durations of the nest male, and in 40.5% fewer ejaculations per spawning female than in the control experiment. Apparently, nest males decrease their ejaculate expenditure on average by roughly 50% when dwarf males are present to save sperm for better future spawning opportunities. The mouthing and sniffing rates and the time the nest male stayed inside the nest also differed between the periods before and after the manipulation, but these differences were not explained by the treatment effect, so they reflected rather a response to the manipulation.

Modulation of ejaculate investment by which males increase the sperm number released when sperm competition risk is high has been observed in several species with internal fertilization and sperm storage (Wedell et al., 2002). For example, with increasing perceived risk of sperm competition, males increased the amount of sperm transferred in two cricket species, *Acheta domesticus* and *Gryllodes supplicans* (Gage & Barnard, 1996), in *Drosophila melanogaster* (Moatt, Dytham, & Thom, 2014), in the South American fruit fly *Anastrepha fraterculus* (Abraham, Teresa,

527 & Perez-Staples, 2015), and in the lekking lesser wax moth, *Achroia grisella* (Jarrige, Riemann,
528 Goubault, & Schmoll, 2015). In horses, *Equus caballus*, stallions experiencing a high risk of sperm
529 competition ejaculated more sperm after exposure to mares that had previous contact to other
530 stallions than males that experienced a low risk of sperm competition (no previous exposure to
531 other stallions; Burger, Dolivo, & Wedekind, 2015).

532

533 Regarding species with a fertilization mode similar to that of *L. callipterus*, ejaculation
534 characteristics have been shown to be adjusted to the presence of rival males in two species of
535 bitterlings. These fish are spawning into the gill chambers of living mussels with fertilization taking
536 place inside the gill cavity, resembling a female reproductive tract in internally fertilizing species
537 (Smith, Warren, Rouchet, & Reichard, 2014). When faced with rival male ejaculations, male
538 Chinese rose bitterling, *Rhodeus ocellatus*, decreased the overall ejaculation rate, but released
539 sperm into the mussels more frequently (Smith et al., 2014). In contrast to *L. callipterus*, territorial
540 male European bitterling, *Rhodeus sericeus*, increased ejaculate expenditure when competing with a
541 rival male in comparison to situations when spawning alone (Smith, Reichard, & Jurajda, 2003).
542 They decreased ejaculate expenditure, however, with an increasing numbers of competitors (Smith
543 et al., 2003), confirming two predictions of sperm competition risk and intensity models (Parker,
544 1990b; Stockley et al., 1997). In contrast to parasitic dwarf males in *L. callipterus*, which are in a
545 superior spawning position inside the shell, in bitterlings it has been assumed that sperm
546 competition between guarders and sneakers resembles a fair raffle, where each male fertilizes a
547 number of eggs proportional to his contribution of sperm (Smith & Reichard, 2013). We assume
548 that due to the unprivileged position of *L. callipterus* nest males when spawning with dwarf male
549 participation (Wirtz Ocana et al., 2014), nest males are unable to compensate for offspring loss to
550 dwarf males efficiently by increasing sperm numbers. Thus, when spawning with parasitic males
551 present inside the shell, due to the highly loaded raffle (Parker et al., 1997) in favour of the parasite

552 it seems advantageous for nest males to conserve sperm for better spawning opportunities in the
553 future, without dwarf male participation.

554

555 Paternity analysis showed that the proportion of offspring sired by the dwarf male correlated highly
556 with the percentage of spawning time the latter was inside the shell, whereas it did not relate to
557 clutch size. In our lab experiment the relative reproductive success of dwarf males was considerably
558 smaller than dwarf male success in the natural situation (Wirtz Ocana et al., 2014). In half of the 12
559 broods where a dwarf male was experimentally added into the shell they did not sire any offspring,
560 and the average percentage of larvae a dwarf male sired was 13.6%. In contrast, all of 10 broods
561 with dwarf male participation collected in the field contained dwarf male offspring (range: 15.6–
562 100%), and the majority of offspring were sired by the dwarf male in those cases ($\chi^2 = 77.6\%$,
563 Wirtz Ocana et al., 2014). This difference in dwarf male success between field and experimental lab
564 situation was probably due to the influence of our manipulation, by which dwarf males were forced
565 into their position inside the shell. In nature, successful wriggling into a shell and dwarf male
566 participation in spawning depend on the relative sizes of the dwarf male, the female and the shell
567 (Sato et al., 2004). To participate in spawning in our laboratory experiment, dwarf males had no
568 choice between shells or females, thus important parameters might not have been appropriate for
569 them to behave normally. However, our data show that nest males responded strongly to the
570 presence of dwarf males in the shell during spawning, which enabled us to scrutinize this response
571 with respect to adjustments in their ejaculate release.

572

573 Earlier studies showed that the average spawning duration was 6.9 h in the field (Schütz et al.,
574 2012). However, the number of sperm released per ejaculate of nest males dropped sharply by 88%
575 on average after 5 h of spawning, even when males were spawning with only one female at a time
576 (Schütz et al., 2010), which suggests sperm depletion. If nest males are limited to fertilize eggs laid

577 during the last 1.9 h of spawning, they might loose up to 27.5% (1.9 / 6.9) of offspring per brood
578 due to sperm limitation, and probably considerably more when spawning with several females
579 simultaneously. Our study showed that nest males decrease ejaculate expenditure by roughly 50%
580 when dwarf males are present. Since nest males sired on average only 22.4% of offspring when
581 dwarf males succeeded to enter a shell in nature (Wirtz Ocana et al., 2014), they might do better by
582 saving sperm when competing with a rival in a highly disadvantaged position. In spawnings without
583 dwarf male participation, nest males sire virtually all offspring (Wirtz Ocana et al., 2014). Our
584 study showed that in the field, nest males spawned with only one female at a time in 62.9% of all
585 cases, simultaneously with two females in 14.3% of cases, and with 3 and 4 females in 11.4% of
586 cases each. Thus, the probability to spawn with more than one female at a time corresponds to about
587 37%, which substantially increases the possibility to make use of sperm saved from spawnings with
588 dwarf male participation.

589

590 To our knowledge, this is the first study showing that ejaculate expenditure decreases in the
591 presence of a single rival male in externally fertilising fish. When a parasitic dwarf male
592 participates in spawning, nest males reduce ejaculate expenditure by roughly 50%, which is
593 contrary to predictions from ESS models of responses to sperm competition risk (Parker, 1990a;
594 Parker, 1998). Apparently, nest males save sperm for future mating opportunities as predicted by
595 sperm competition intensity models (Parker, Ball, Stockley, & Gage, 1996; 1997). Results from this
596 and previous studies combined suggest that this is due to the unprivileged position of *L. callipterus*
597 nest males when spawning with parasitic dwarf males, which differs from the competition between
598 bourgeois and parasitic males during spawning in most other species. Nest males are highly sperm
599 limited even when spawning with only one female, and their ejaculate expenditure decreases
600 significantly when spawning with more than two females simultaneously. Obviously, in *L.*
601 *callipterus* nest males the proposed trade-off between investing in the current or in future matings

602 (Wedell et al., 2002) is solved by reducing ejaculate expenditure when in competition with superior
603 dwarf males and thereby conserving sperm for future spawning opportunities without dwarf males.

604

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606

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611

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740 **Appendix: Details of the paternity analysis**

741

742 Genomic DNA was extracted from ethanol-preserved fin clip samples (1-2mm² each) and whole
743 larvae with a manual 96-well format DNA extraction protocol on the basis of a magnetic separation
744 technique (White, Braeden, Creswell, & Smith, 1998). According to the protocol of the Wizard®
745 Genomic DNA Purification Kit (Technical Manual No. TM050; Promega), tissue lysis was
746 performed in a Lysis-Buffer consisting of Nuclei Lysis Solution (Promega), 0.5M EDTA (Sigma-
747 Aldrich) and Proteinase K (Qiagen AG). By adding MagneSil® Paramagnetic Particles (Promega;
748 (White et al. 1998) to the lysate, DNA was captured in solution and washed 2-3 times with 80%
749 ethanol with the aid of a magnetic separator (MagnaBot®96 Magnetic Separation Device, Promega,
750 Cat.# V8151) to eliminate residual contaminations. DNA was finally eluted in 50-100µl of distilled
751 water.

752

753 Eleven microsatellite primer pairs (loci NP007, NP773, ULI2, Pzeb3, Pzeb4, TmoM5, TmoM13,
754 TmoM25, TmoM27, UME003 and UNH154; see Wirtz Ocana et al. 2014) were multiplexed in one
755 polymerase chain reaction using the QIAGEN Multiplex PCR Kit (Qiagen). PCR reactions were
756 carried out in a 10µl volume containing 1-2µl of the genomic DNA, 1x QIAGEN Multiplex PCR
757 Master Mix (consisting of QIAGEN Multiplex PCR buffer with a final concentration of 3 mM
758 MgCl₂, dNTP mix, and HotStarTaq DNA polymerase), 0.1 µM of locus-specific 5' fluorescent-
759 labelled forward primer [fluorescent dyes: 6-FAM, HEX (Microsynth), NED and PET (Applied
760 Biosystems)], and non-labelled reverse primers.

761

762 Amplification was done in a 96-well GeneAmp® PCR System 9700 (Applied Biosystems) using
763 the following cycling protocol: 15 min at 95°C; 35 cycles consisting of 30 s at 95°C, 3 min at 57°C,
764 and 1 min at 72°C, followed by a final 15-min extension at 72°C. Fluorescent PCR fragments were

765 visualised by capillary electrophoresis on an ABI PRISM® 3100 Genetic Analyzer. Genotypes
766 were scored automatically using GeneMapper software version 3.7 (Applied Biosystems) against an
767 internal size standard (GeneScan 500 LIZ, Applied Biosystems) and revised manually to ensure
768 genotyping consistency.

769

770 Since the present experimental groups consisted of breeding females with their offspring and only 2
771 potential fathers (1 nest male and 1 dwarf male), maternity was always certain, and therefore
772 assignment of paternity was very simple, performed visually via allelic matching at all 11 loci. For
773 all of the offspring tested, parentage could be assigned unambiguously, there were 0 mismatching
774 alleles with the respective parents.

775

776 **Tables**

777

778 **Table 1:** Differences in behaviours before and after manipulation of a shell with a spawning female
779 inside, and treatment effect (experimental addition of a dwarf male versus control manipulation):
780 Results of repeated measures ANOVAs. Significant differences are highlighted in bold.

781

	Variable		Variable × treatment	
	<i>F</i> _{1,13}	<i>P</i>	<i>F</i> _{1,13}	<i>P</i>
Ejaculation duration	12.203	0.004	6.67	0.023
Ejaculations / female	38.129	0.001	7.809	0.015
Mouthing	22.677	0.001	0.004	0.95
Sniffing	5.366	0.037	1.81	0.202
Time in nest	7.401	0.017	3.075	0.103

782

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Figure 1

Figure 1

Figure 2

Figure 2

Figure 3

Figure 3

Figure 4

Figure 4