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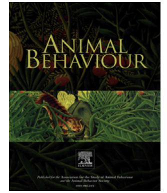

Sperm-limited males save ejaculates for future matings when competing with superior rivals

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Adjusting ejaculates to sperm competition can lead to sperm limitation. Particularly in polygynous species, males may face a trade-off between investing sperm in current or future mating opportunities. The optimal sperm allocation decision should depend on the relative intensity of sperm competition experienced in a mating sequence. Here we asked how males respond to this trade-off in polygynous fish with alternative male mating tactics, intense sperm competition and sperm limitation. Large bourgeois males of the shell-brooding cichlid *Lamprologus callipterus* build nests consisting of empty snail shells, in which females spawn and raise offspring. During spawning, nest males release ejaculates into the shell opening. Genetically distinct, parasitic dwarf males enter shells during spawning to fertilize the eggs from inside the shell. These dwarf males were previously shown to be superior sperm competitors to nest males. Here we showed that when spawning with several females simultaneously, nest males reduced the spawning duration for each clutch and the number of ejaculations per female tended to decrease, reflecting sperm limitation. Experimental exposure of nest males to sperm competition with dwarf males reduced the number and duration of ejaculations by roughly half. Hence, when exposed to competition with a superior rival, nest males did not increase their sperm expenditure as predicted by sperm competition risk models, but in fact saved sperm for future mating opportunities as predicted by sperm competition intensity theory. This seems to be adaptive because of the considerable sperm demands in this species, which is partly due to their high degree of polygyny.
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Sperm competition, where sperm of two or more males compete for the fertilization of eggs (Parker, 1970), can cause behavioural and morphological adaptations in males. Adaptive adjustment to sperm competition includes prolonged copulations (Schöfl & Taborsky, 2002), mate guarding and an increase in copulation frequency (Birkhead, 1998), sperm displacement (Parker & Simmons, 1994), the development of copulatory plugs (Dunham & Rudolf, 2009) and breakage of copulatory organs (Snow, Abdel-Mesih, & Andrade, 2006). Males may monopolize either limited resources for breeding or females directly to prevent rival males from gaining access to females (bourgeois tactic; Taborsky, 1997; Taborsky & Brockmann, 2010). As a consequence of this mating monopolization, male reproductive success is typically strongly skewed, and alternative reproductive tactics (ARTs; Oliveira, Taborsky, & Brockmann, 2008) may evolve, where parasitic males invest relatively more in testes than bourgeois males due to their higher risk of sperm competition (Byrne, Roberts, & Simmons, 2002; Stockley, Gage, Parker, & Møller, 1997; Taborsky, 1998).

Adaptations to sperm competition have been modelled extensively, with two types of game theoretical approaches considering how males should respond to either sperm competition risk (SCR; whether it occurs or not, Kelly & Jennions, 2011; Parker, 1998) or sperm competition intensity (SCI; number of ejaculates competing for a set of ova, Parker, Ball, Stockley, & Gage, 1996, 1997). SCR models predict that ejaculate expenditure increases when males are exposed to a single competitor, which has been confirmed by empirical data (for review see Wedell, Gage, & Parker, 2002). SCI models predict that ejaculate expenditure decreases with an increasing number of competitors because males save sperm for better future spawning opportunities, which has received less empirical support (for reviews see Engqvist & Reinhold, 2005; Kelly & Jennions, 2011; Wedell et al., 2002). These models imply that, in general, if males can succeed in sperm competition they should increase ejaculate expenditure, whereas if they have little chance of succeeding against superior competitors, they should reduce ejaculate expenditure to save sperm for future matings. This may be

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particularly important in polygynous species, where both mating frequency and sperm competition can lead to sperm limitation (Wedell et al., 2002). Additional causes of sperm limitation (Shapiro & Giraldeau, 1996) include low fertilization efficiency, large clutch size and high reproductive costs for males (Wedell et al., 2002).

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

It is possible to disentangle the influence of sperm competition and sperm limitation on sperm and ejaculate characteristics by manipulating the degrees of sperm competition and polygyny independently of each other in species that combine polygyny with the existence of ARTs. To test for an influence of sperm limitation, one can determine how males adjust ejaculation characteristics to the number of females they mate with at a time. To test for an influence of sperm competition, ejaculation characteristics of bourgeois males mating either alone or together with parasitic males can be compared. However, in externally fertilizing species releasing their gametes into the water, it is difficult to determine ejaculation characteristics under different experimental conditions (Shapiro, Marconato, & Yoshikawa, 1994).

In the cichlid fish *Lamprologus callipterus*, large bourgeois males construct and defend nests consisting of empty snail shells, in which the much smaller females breed (henceforth called 'nest males'; Schütz & Taborsky, 2005). Females lay clutches containing on average 95 eggs, which are deposited one by one inside a shell at intervals of more than 2 min (Schütz, Heg-Bachar, Taborsky, & Heg, 2012). Each egg needs to be fertilized by a separate ejaculate, leading to a total spawning duration of nearly 7 h on average (Schütz et al., 2012) and to severe sperm limitation of nest males (Schütz, Pachler, Ripmeester, Goffinet, & Taborsky, 2010). The latter reduce the number of sperm released per ejaculate drastically after 5 h of continuous spawning, even when spawning with only one female (Schütz et al., 2010) and even though eggs are deposited at a similar rate over the whole duration of a spawning (Schütz et al., 2012).

In *L. callipterus*, two distinct parasitic male types exist: sneaker males opportunistically try to enter territories where nest males are spawning to fertilize eggs by releasing ejaculates into the shell opening when the nest male is inattentive. This tactic is transitional and performed by males of the nest male type (Schütz, Parker, Taborsky, & Sato, 2006). Parasitic dwarf males constitute a genetically distinct male morph (Wirtz Ocana, Meidl, Bonfils, & Taborsky, 2014) remaining small throughout life (Taborsky, 2001). They try to enter shells in which a female is spawning to fertilize the eggs from inside the shell. If they enter successfully, dwarf males stay in the shell during the whole spawning event, and therefore they are in much closer vicinity to the female and eggs during laying than nest males, i.e. they are in a privileged position to fertilize the eggs (Sato, Hirose, Taborsky, & Kimura, 2004). This contrasts with most other species, where bourgeois males are usually much closer to eggs than parasitic males (Taborsky, 2008; Taborsky, Oliveira, & Brockmann, 2008). Thus, spawning of a nest male with a parasitic dwarf male resembles a loaded raffle (Parker, 1990a) where dwarf males have a fertilization advantage, which is revealed also by the much greater fertilization success of dwarf males than nest males in nature (Wirtz Ocana et al., 2014). Theory predicts that the unprivileged male type (here the nest male) should compensate for his disadvantage by investing more in the present ejaculate than the privileged male type (here the dwarf male; Parker, 1990a). Hence, in accordance with sperm competition risk models, nest males should increase their reproductive effort when spawning with a parasitic dwarf male (Parker, 1998). Alternatively, nest males might decrease ejaculate expenditure in such competitive situations to save sperm for future spawning opportunities without participating dwarf males, as predicted by sperm competition intensity models, especially if they involve highly loaded raffles (Parker et al., 1996, 1997).

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

Here we aimed to clarify the relative roles of sperm limitation and sperm competition for shaping ejaculation characteristics of *L. callipterus* nest males. Specifically, we asked how much they invest in ejaculates in relation to increasing sperm limitation and sperm competition risks. We compared nest male sperm and ejaculation characteristics when spawning (1) with one or more females simultaneously to test for the influence of sperm limitation, and (2) with or without a parasitic dwarf male present to test for the influence of sperm competition.

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

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

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

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

METHODS

Study Species

Lamprologus callipterus is a cichlid fish endemic to Lake Tanganyika, which shows the greatest sexual size dimorphism among animals, with males being bigger than females (Schütz & Taborsky, 2000). Two genetically determined ARTs exist. (1) Nest males: after passing a size threshold of 9 cm standard length (Schütz & Taborsky, 2005), these males build and defend nests consisting of clumps of empty snail shells, in which females lay eggs and care for the brood for 10–14 days (Sato, 1994; Sato et al., 2004; Schütz & Taborsky, 2000). Nest males collect empty snail shells from the vicinity of their nests or steal them from neighbouring males (Mitchell, Wirtz Ocana, & Taborsky, 2014; Sato, 1994). Since nest males hardly feed when defending a territory, their condition decreases during territory maintenance until their territories are taken over by larger or heavier males (Sato, 1994; Schütz et al., 2010). Males pursuing this life history pathway may also adopt a parasitic mating tactic, particularly when they are still too small to successfully defend a nest. These 'sneaker' males try to enter a nest owner's territory during spawning to fertilize eggs by releasing ejaculates into the shell opening (Sato et al., 2004). (2) Dwarf males: these males remain even smaller than females throughout life (Taborsky, 2001; Wirtz Ocana et al., 2014); in the field, they weigh on average only 2.5% of nest males (Sato et al., 2004). Dwarf males try to enter a shell with a spawning female inside by wriggling past the female in order to enter the inner whorl of the shell, from where they can fertilize the eggs (Sato et al., 2004). If successful in their attempt to enter the shell, they fertilize the majority of the eggs from this privileged position (Wirtz Ocana et al., 2014).

Holding Conditions for Fish in the Laboratory

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

Female Visits and Sperm Competition in Nature

To check for the potential risk of sperm competition and sperm limitation, we determined how many females and males of different tactics are found around territories at Lake Tanganyika. As the spawning activity in this species varies significantly with the lunar cycle (Nakai, Yanagisawa, Sato, Niimura, & Gashagaza, 1990), we attempted to monitor nests throughout the different phases of the moon cycle. During two field seasons, seven nests at Wonzye Point (1995) and four nests at Kasakalawe Point (1997) were continuously recorded via underwater video-cameras from new moon to 1 week after full moon. In 10 of these nests, spawning was recorded. All videos were analysed for the entire daylight period (0600–1800 hours). From these video recordings, we determined the number of females, nest male intruders (mainly neighbouring males that try to steal shells or to take over the nest), sneaker males and dwarf males (males trying to steal fertilizations) entering a nest per day, and we calculated averages per moon day.

We divided the moon cycle into 3-day intervals (seven intervals for 3 weeks of recording) and, with chi-square tests, checked whether the frequency of dwarf plus sneaker males, nest male intruders and visiting females differed from equal distributions. Additionally, we used chi-square tests to check whether the frequency of dwarf plus sneaker males and of nest male intruders differed from the frequency of females visiting a nest per day. For statistical analyses, we used the software package IBM SPSS statistics 22 (IBM, Armonk, NY, U.S.A.) and tested for two-tailed probabilities. For the linear mixed-effect model (LMM) of sperm number per ejaculation in relation to time of sperm release we used the package 'lme4' of the software R 3.2.2. (R Core Team, 2015).

Adjustment of Ejaculations to Spawning with Multiple Females

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

From continuous video recordings in the field (see above), we could identify the exact start and end of laying a clutch for 28 individual females spawning in different shells (each female spawns in a separate shell in this species; see Schütz et al., 2012 for details and methods). To check whether the two variables differ between males spawning with one, two, three or four females at a time, we used general linear models (GLM) with the number of simultaneously spawning females as a fixed factor and male ID as a random factor. Additionally, with Spearman rank correlation analyses we tested whether the two variables were related to the number of females with which a nest male was concurrently spawning.

Sperm Release Rate Versus the Duration of Ejaculations

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

When a female was ready to spawn (showing an extended whitish belly), the experiment started at 0900 hours on the following morning. The other three females were taken out of the compartment, the male was transferred from the small into the

large compartment, and continuous video recording was started. To collect the sperm released by the male, we prepared shells by attaching a silicone tube (inside diameter = 2 mm) 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 (see Schütz et al., 2012). The amount of water contained in the tube itself was 5 ml, and the mean ± SD shell volume was 15.4 ± 0.84 ml. We took 30 ml for each water sample to ensure that the complete tube plus shell volumes were collected for each extraction. The water sample was immediately mixed with the same amount of 0.1 M 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 to stain the head of the spermatozoa before passing the sample through a Millipore filter (0.22 μm pore size) under vacuum. The filter paper was dried and cleared with immersion oil. Sperm were counted under a light microscope at a magnification of 400× in an area of 0.185 × 0.185 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 = 160.61 mm^2; therefore, the sperm count was multiplied by 4692.65).

Water samples of ejaculates were taken at intervals of about 10 min, 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 (ejaculations/min) for each male. The number of sperm per ejaculate divided by the duration of ejaculation bouts gave the mean sperm release rate for each ejaculation (number of sperm/s).

With Spearman rank correlation analyses we tested whether the ejaculation bout duration correlated with the number of sperm released per ejaculate, and whether the total number of sperm a male released during spawning correlated with the mean number of sperm released per ejaculation, the sperm release rate and the mean ejaculation bout duration. We also used Spearman rank correlation analyses to see whether the total number of sperm correlated with the number of ejaculations. Furthermore, we tested whether the number of sperm released per ejaculation was constant throughout a spawning during the first 5 h of spawning. For this purpose, we standardized the data from the eight males as follows: [time] = time of ejaculation sample minus time of first sperm release; [sperm per ejaculation] = sperm of ejaculation sample/average sperm per ejaculation. We calculated an LMM (Bates, Mächler, Bolker, & Walker, 2014) with time as a fixed factor, male ID as a random factor and sperm number per ejaculate as a dependent variable. The dependent variable was reciprocally transformed as $\frac{1}{\sqrt{\text{sperm per ejaculation}+0.5}}$. The residuals of the model were checked for normal distribution by visual inspection of the Q–Q plot, and the Kolmogorov–Smirnov and Shapiro normality tests. The significance of time was tested using a likelihood ratio test between the full model that included time as a fixed effect and a null model in which the fixed effect was excluded.

Response of Nest Males to Sperm Competition with Dwarf Males

We checked whether nest males increased their ejaculate investment when faced with a dwarf male competitor or whether instead they conserved sperm for future spawning opportunities. Before the experiment, 24 large nest males (>9 cm; to ensure that they were able to transport shells; Schütz & Taborsky, 2005) were placed individually into 200-litre experimental tanks and given 1 week to acclimatize. At the same time, dwarf males were held in a 200-litre storage tank and stimulated with unusable shells and two females. Females that had not reproduced for at least 8 weeks were transferred to a 400-litre stimulation tank, in which a nest male was present to stimulate them. When they showed signs of being ready to spawn, two such gravid females were placed in a separate compartment of the experimental tanks (approximately a quarter of the tank, separated from the nest male by a clear partition) for 2 days to allow for acclimatization. A video camera was set up in front of the experimental tank to record the nest and surroundings (approximately a third of the experimental tank). Experimental tanks were protected with black curtains from visual disturbance by the observer.

On the day of the experiment, the partition was removed and three manipulated shells were put into the tank at 0900 hours. The experimental snail shells had a hole (ca. 0.5 × 1 cm) covered with Plexiglas, where normally the dwarf male's body is located during spawning (approximately in the second whorl). This Plexiglas 'window' was fixed to the shell with a rubber band. Tanks were checked every 30 min for courtship activity.

Approximately 3 h after the first ejaculation of the nest male (i.e. approximately 2 h after the first egg had been laid; see Schütz et al., 2012), we opened the shell's Plexiglas window and added a dwarf male head first into the shell ($N = 12$). The window was closed again with the rubber band, and the shell was put back into the nest at the same position it had been before handling. If spawning did not start until 1200, the trial was aborted (i.e. the manipulated shells were taken out), and the procedure was started again on the next day to ensure that 9 h of light were available for the spawning. In the control treatment ($N = 8$), the procedure was the same (tank equipment, timing, shell handling, camera installation, etc.), except that no dwarf male was added into the shell during shell handling.

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

Fifteen successfully manipulated spawnings ($N = 7$ of the experimental treatment, $N = 8$ of the control treatment) could be completely video recorded and analysed using Observer 5.0 (Noldus Information Technology, Wageningen, The Netherlands). We could not analyse the videos of five spawnings of the experimental treatment because of video system malfunction. We continuously noted the rate of three behaviours known to be of key importance during spawning (Schütz et al., 2012): 'mouthing', when the nest male opens and closes his mouth within the entrance of a shell containing a female; 'ejaculation', when the male stays motionless with his genial papilla over the shell entrance; and 'sniffing', which corresponds to mouthing into an empty shell. Additionally, the duration of each ejaculation bout (as an estimate of sperm release duration), the number of ejaculations and the time the nest male stayed in his nest were determined over the whole spawning event. The latency from handling until the first ejaculation by the nest male was determined for the experimental and control group to check

whether handling affected the two groups differently. A different latency in the experimental and control treatments would indicate different disturbance levels of nest males and females between the two groups. All broods from the experimental treatments (i.e. with a dwarf male contributing to spawning; $N = 12$) were tested for paternity using microsatellite analysis (for details of DNA sampling and analysis see the Appendix). The aim was to analyse at least 20 individuals per brood, if the clutch was large enough.

To analyse the role of the dwarf male's presence (treatment: present; control: absent), we subdivided the behavioural recordings into 10 min intervals and calculated means (of rates and durations) per interval for each behavioural variable, starting 1 h after the first ejaculation. As more and more spawnings end as spawning time increases, only the first 30 intervals of 10 min were analysed to keep sample size constant (12 intervals before and 18 intervals after handling the shell). From these intervals, we calculated means of behaviours shown before and after the experimental manipulation. With repeated measures ANOVAs, we tested whether the number of ejaculations per female, the ejaculation duration, the time the nest male was in the nest, and the sniffing and mouthing rates differed before and after the manipulation (within-subjects variables), and whether a difference was due to the treatment effect (between-subjects factor; treatment versus control). We used a Mann–Whitney U test to compare the latency until the first ejaculation by the nest male after handling, between treatment and control conditions. Paternity results were compared with the percentage of total spawning time for which the dwarf male was inside the shell, and with total clutch size.

Ethical Note

Prior to the laboratory experiments, fish were held in mixed groups of various sizes in tanks between 200 and 500 litres. They were fed twice per day on 6 days of the week as in the experiments. Each individual was used only once for an experiment. After the experiments, fish were placed back into the tanks from which they had been taken. In accordance with the Veterinary office of the Kanton Bern granting permission for our experiments, fish were not anaesthetized for fin clipping in order to reduce stress and potential negative effects of anaesthesia. Fin clips were taken from the dorsal and anal fins by removing one fin ray with the surrounding tissue. The removed tissue regrew fully within 4–8 weeks. For the field work at Lake Tanganyika, permission was granted by the Fisheries Department of the Ministry of Agriculture and Co-operatives of Zambia. The laboratory work was licensed by the Veterinärdienst, Amt für Landwirtschaft und Natur des Kantons Bern (licence number: 17/09).

RESULTS

Female Visits and Sperm Competition in Nature

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

During the moon cycle, which was separated into seven 3-day periods, the frequencies of visiting females ($\chi^2_6 = 16.74$, $P = 0.010$) and of reproductive parasites (dwarf and sneaker males combined) differed significantly from an equal distribution ($\chi^2_6 = 20.871$, $P = 0.002$), but the frequency of nest male intrusions did not ($\chi^2_6 = 8.27$, $P = 0.205$). Furthermore, the distribution of visiting females differed significantly from the distribution of

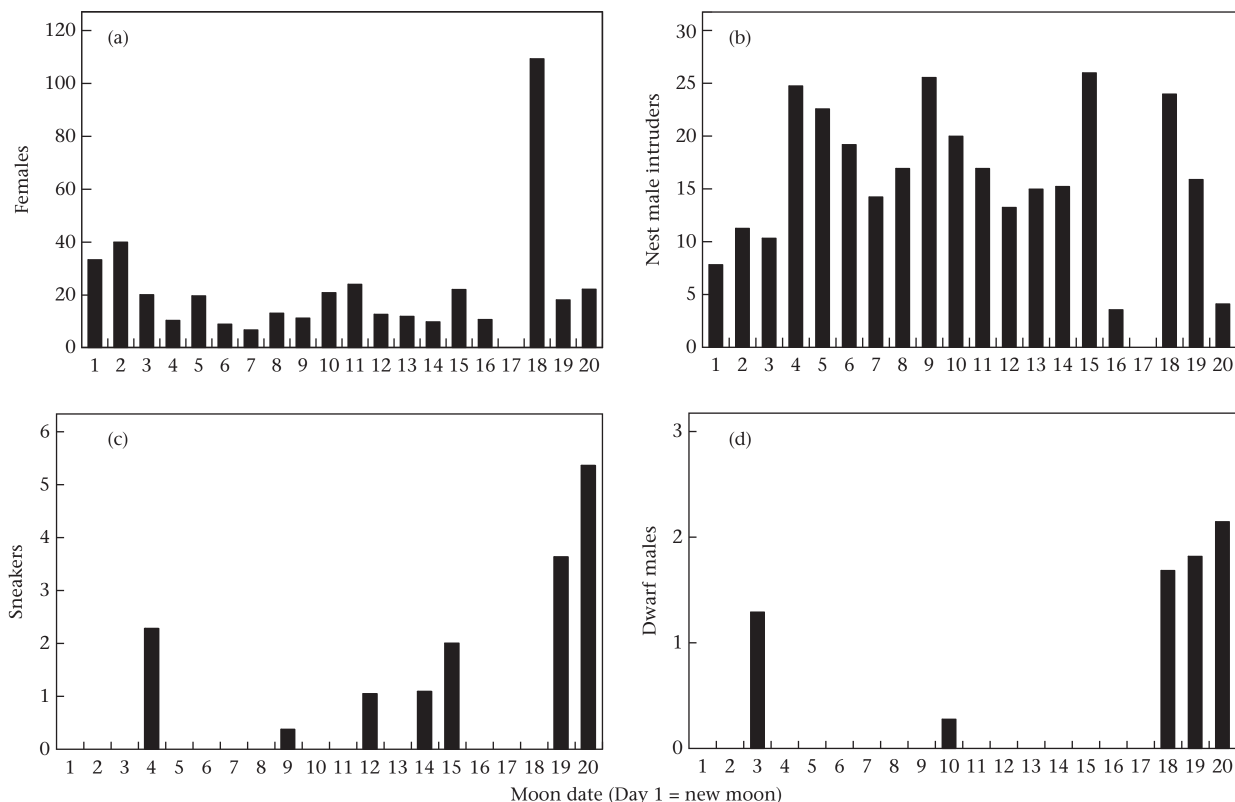

Figure 1. Frequency of (a) females, (b) nest male intruders, (c) sneakers and (d) dwarf males entering a nest per day. Day 1 = new moon, day 15 = full moon; on day 17 no data were available. Note the different scales on the ordinate.

parasitic males attempting to participate in spawning ($\chi^2_6 = 15.597$, $P = 0.016$) and from the distribution of nest male intrusions ($\chi^2_6 = 21.5$, $P = 0.002$).

Adjustment of Ejaculations to Spawning with Multiple Females

Nest males spawned with up to four females simultaneously (28 spawnings corresponding to 28 different females, recorded in 10 monitored nests). The total spawning duration of a clutch depended significantly on the number of females spawning at a time (GLM: $F_{1,3} = 4.536$, $P = 0.015$), whereas it was not related to male identity ($F_{1,8} = 1.265$, $P = 0.321$). The number of ejaculations per female did not seem to be influenced by the number of simultaneously spawning females (GLM: $F_{1,3} = 0.811$, $P = 0.505$) or by male identity ($F_{1,8} = 1.296$, $P = 0.310$). However, viewed on a continuous scale, the number of ejaculations per female tended to decline with increasing numbers of simultaneously spawning females (Spearman rank correlation: $r_S = -0.353$, $N = 28$, $P = 0.065$; Fig. 2).

Sperm Release Rate Versus the Duration of Ejaculations

Both the average number of sperm per ejaculation (Spearman rank correlation: $r_S = 0.905$, $N = 8$, $P = 0.002$; Fig. 3a) and the sperm release rate (sperm number per ejaculate/duration (s) of ejaculation bout; Spearman rank correlation: $r_S = 0.881$, $N = 8$, $P = 0.004$; Fig. 3b) correlated positively with the total number of sperm a male released during spawning. The mean duration of

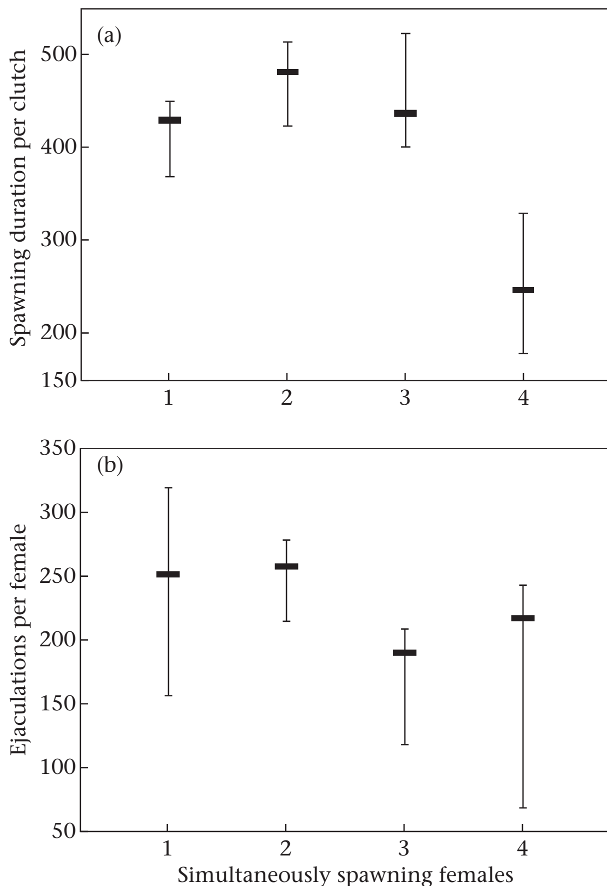

Figure 2. Spawning characteristics of nest males when spawning with one, two, three or four females (medians, first and third quartiles). (a) Spawning duration per clutch (min), (b) ejaculations per female.

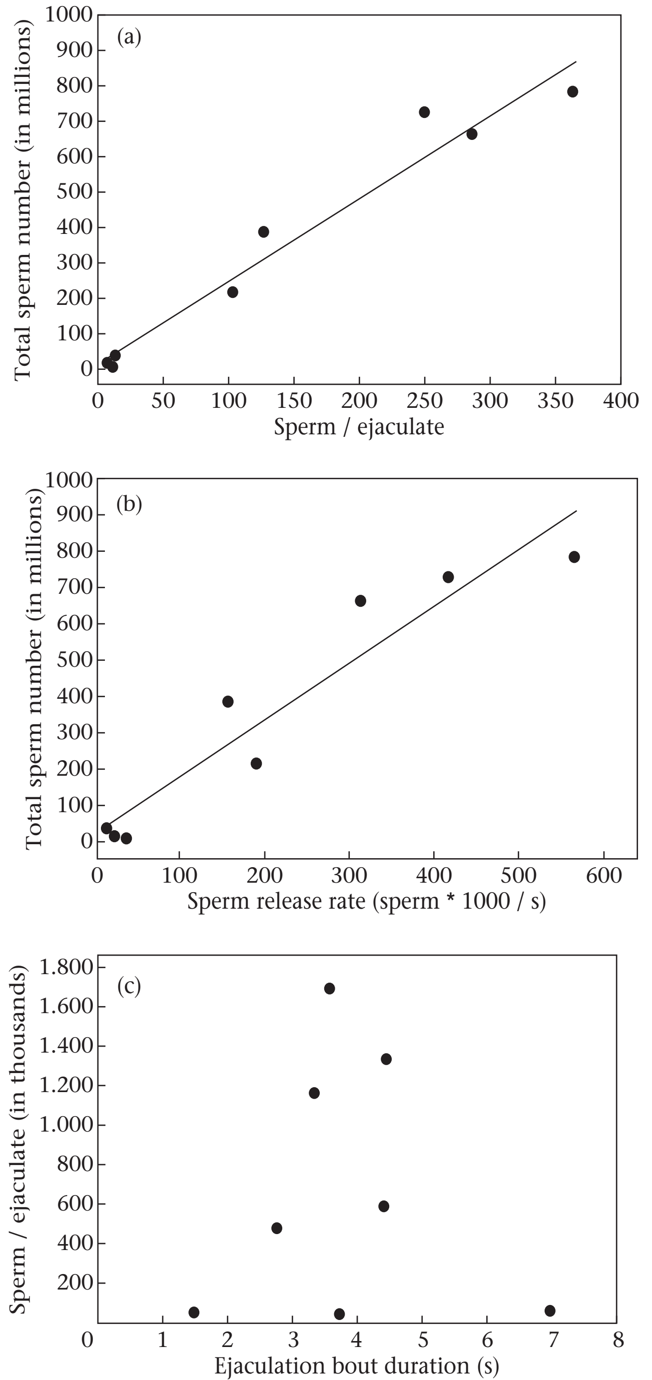

Figure 3. Relationships between different ejaculate and sperm release characteristics. (a) Mean number of sperm released per ejaculation and total number of sperm released during spawning; (b) sperm release rate (sperm number/s) and total number of sperm released during spawning; (c) mean duration of ejaculation bouts and number of sperm released per ejaculate. Lines show significant linear regressions.

ejaculation bouts was not related to either the total number of sperm (Spearman rank correlation: $r_I = 0.143$, $N = 8$, $P = 0.736$) or the number of sperm released per ejaculate (Spearman rank correlation: $r_S = -0.024$, $N = 8$, $P = 0.955$; Fig. 3c).

The number of ejaculations serves as a good proxy for the total number of sperm released since (1) there is a positive correlation between the number of ejaculations and the total number of sperm

released (Spearman rank correlation: $r_S = 0.857$, $N = 8$, $P = 0.007$) and (2) the number of sperm per ejaculation remains rather constant over most of the spawning time. During the first 5 h of spawning, time showed no significant effect on the number of sperm per ejaculate (model comparison with likelihood ratio test of the two models with and without time as fixed factor, $\chi^2_1 = 0.742$, $P = 0.389$).

Table 1
Differences in behaviours before and after manipulation of a shell with a spawning female inside, and treatment effect (experimental addition of a dwarf male versus control manipulation)

	Variable		Variable*treatment	
	$F_{1,13}$	P	$F_{1,13}$	P
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

The table shows results of repeated measures ANOVAs. Significant differences are highlighted in bold.

Response of Nest Males to Sperm Competition with Dwarf Males

Nest male behaviour

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

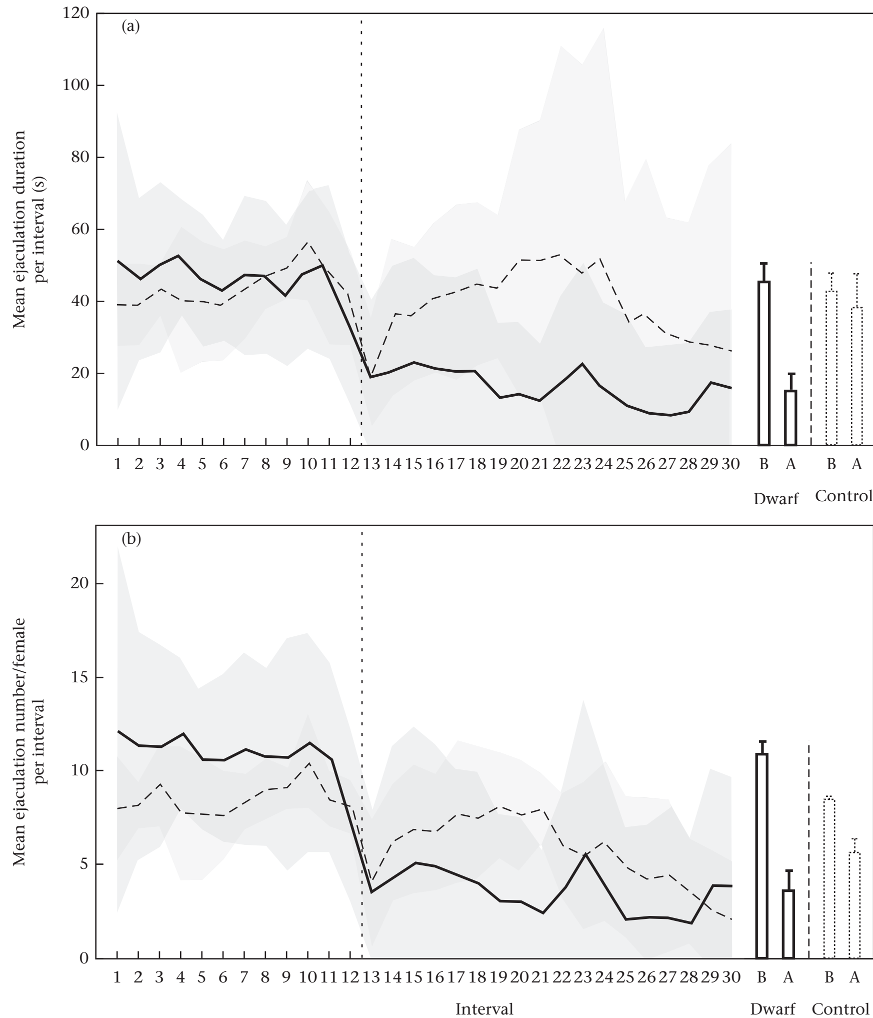

Figure 4. Nest male behaviour before and after adding a dwarf male. (a) Mean duration of ejaculation bouts; (b) ejaculation frequency, i.e. ejaculation number per female. Averages per 10 min observation interval are shown on the left-hand side, where the vertical dotted lines represent the time of handling. Lines show mean intervals (continuous line: dwarf male treatment; dotted line: control treatment), grey areas show standard deviations (very light grey: control treatment; light grey: dwarf male treatment; grey: intersection between control and dwarf male treatments). On the right-hand side averages of behaviours are shown before handling (B, intervals 1–12) and after handling (A, intervals 13–30) in the dwarf male and control treatments (means and SDs).

suggesting that handling per se did not affect the two experimental groups differently.

Paternity

The percentage of larvae the dwarf male sired correlated positively with the percentage of total spawning time for which it had been in the shell during spawning (Spearman rank correlation: $r_S = 0.867$, $N = 7$, $P = 0.012$), whereas it did not correlate with the clutch size (Spearman rank correlation: $r_S = -0.078$, $N = 12$, $P = 0.809$). Microsatellite analyses showed that dwarf male paternity ranged between zero and 100% (mean = 13.6%; see Appendix). In six of 12 cases the dwarf male did not fertilize any eggs.

DISCUSSION

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

The total spawning duration a nest male spent for each clutch declined with an increasing number of simultaneously spawning females, whereas male identity had no obvious effect. Additionally, the number of ejaculations per spawning tended to decrease with increasing numbers of simultaneously spawning females. Since the number of ejaculations is positively correlated with the total number of sperm released, and the number of sperm per ejaculation remains constant over most of the spawning time (see also Schütz et al., 2010), the ejaculation number per female is a good predictor of the number of sperm released per female. These changes in spawning behaviour probably reflect sperm depletion in males, which was revealed also when nest males spawned with single females for longer than 5 h (Schütz et al., 2010). Sperm depletion of males may lead to a decrease in female productivity (Dunn, Andrews, Ingrey, Riley, & Wedell, 2006) and to sexual conflict over the size and distribution of ejaculates (Diaz, Haydon, & Lindstrom, 2010; Smith, Pateman-Jones, Zieba, Przybylski, & Reichard, 2009). Female choice may hence be influenced by potential sperm limitation of males. In *L. callipterus*, females may reduce the risk of suffering from nest male sperm limitation by choosing large nest owners (Maan & Taborsky, 2008), and by enabling dwarf males to enter the shell during later phases of spawning, when nest males are already sperm depleted.

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

Males seem to increase the number of sperm released during a spawning mostly through a higher sperm release speed rather than by increasing the duration of ejaculation bouts. This can be explained by the peculiar spawning condition in *L. callipterus*, where each egg has to be fertilized by a separate ejaculate of the nest male over a period of many hours (Schütz et al., 2012). In principle, males have three possibilities to raise the total sperm number and to increase their fertilization 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 regular intervals of 2 min between 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, 1998), but rather decreased it as predicted by sperm competition intensity theory (Parker et al., 1996, 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 treatment. 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 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, & Perez-Staples, 2015), and in the lekking lesser wax moth, *Achroia grisella* (Jarrige, Riemann, Goubault, & Schmoll, 2015). In horses, *Equus caballus*, stallions experiencing a high risk of sperm competition ejaculated more sperm after exposure to mares that had previous contact with other stallions than males that experienced a low risk of sperm competition (no previous exposure to other stallions; Burger, Dolivo, & Wedekind, 2015).

Regarding species with a fertilization mode similar to that of *L. callipterus*, ejaculation characteristics have been shown to be adjusted to the presence of rival males in two species of bitterlings. These fish spawn into the gill chambers of living mussels with fertilization taking place inside the gill cavity, resembling a female reproductive tract in internally fertilizing species (Smith, Warren, Rouchet, & Reichard, 2014). When faced with rival male ejaculations, male Chinese rose bitterling, *Rhodeus ocellatus*, decreased the overall ejaculation rate, but released sperm into the mussels more frequently (Smith et al., 2014). In contrast to *L. callipterus*, territorial male European bitterling, *Rhodeus sericeus*, increased ejaculate expenditure when competing with a rival male in comparison to situations when spawning alone (Smith, Reichard, & Jurajda, 2003). They decreased ejaculate expenditure, however, with increasing

numbers of competitors (Smith et al., 2003), confirming two predictions of sperm competition risk and intensity models (Parker, 1990b; Stockley et al., 1997). In contrast to parasitic dwarf males in *L. callipterus*, which are in a superior spawning position inside the shell, in bitterlings it has been assumed that sperm competition between guarders and sneakers resembles a fair raffle, where each male fertilizes a number of eggs proportional to his contribution of sperm (Smith & Reichard, 2013). We assume that due to the unprivileged position of *L. callipterus* nest males when spawning with dwarf male participation (Wirtz Ocana et al., 2014), nest males are unable to compensate efficiently for offspring loss to dwarf males by increasing sperm numbers. Thus, when spawning with parasitic males present inside the shell, owing to the highly loaded raffle (Parker et al., 1997) in favour of the parasite, it seems advantageous for nest males to conserve sperm for better spawning opportunities in the future, without dwarf male participation.

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

Earlier studies showed that the average spawning duration was 6.9 h in the field (Schütz et al., 2012). However, the number of sperm released per ejaculate of nest males dropped sharply by 88% on average after 5 h of spawning, even when males spawned with only one female at a time (Schütz et al., 2010), which suggests sperm depletion. If nest males are limited to fertilizing eggs laid during the last 1.9 h of spawning, they might lose up to 27.5% (1.9/6.9) of offspring per brood due to sperm limitation, and probably considerably more when spawning with several females simultaneously. Our study showed that nest males decrease ejaculate expenditure by roughly 50% when dwarf males are present. Since nest males sired on average only 22.4% of offspring when dwarf males successfully entered a shell in nature (Wirtz Ocana et al., 2014), they might do better by saving sperm when competing with a rival in a highly disadvantaged position. In spawnings without dwarf male participation, nest males sire virtually all offspring (Wirtz Ocana et al., 2014). Our study showed that in the field, nest males spawned with only one female at a time in 62.9% of all cases, simultaneously with two females in 14.3% of cases, and with three and four females in 11.4% of cases each. Thus, the probability of spawning with more than one female at a time corresponds to about 37%, which substantially increases the possibility of using sperm saved from spawnings with dwarf male participation.

To our knowledge, this is the first study showing that ejaculate expenditure decreases in the presence of a single rival male in externally fertilizing fish. When a parasitic dwarf male participates in spawning, nest males reduce ejaculate expenditure by roughly 50%, which is contrary to predictions from evolutionarily stable strategy models of responses to sperm competition risk (Parker, 1990a, 1998). Apparently, nest males save sperm for future mating opportunities as predicted by sperm competition intensity models (Parker et al., 1996, 1997). Results from this and previous studies combined suggest that this is due to the unprivileged position of *L. callipterus* nest males when spawning with parasitic dwarf males, which differs from the competition between bourgeois and parasitic males during spawning in most other species. Nest males are highly sperm limited even when spawning with only one female, and their ejaculate expenditure decreases significantly when spawning with more than two females simultaneously. Obviously, in *L. callipterus* nest males the proposed trade-off between investing in the current or in future matings (Wedell et al., 2002) is solved by reducing ejaculate expenditure when in competition with superior dwarf males and thereby conserving sperm for future spawning opportunities without dwarf males.

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Appendix: Details of the paternity analysis

Genomic DNA was extracted from ethanol-preserved fin clip samples (1–2 mm^2 each) and whole larvae with a manual 96-well format DNA extraction protocol on the basis of a magnetic separation technique (White, Braeden, Creswell, & Smith, 1998). In line with the protocol of the Wizard Genomic DNA Purification Kit (Technical Manual No. TM050; Promega, Madison, WI, U.S.A.), tissue lysis was performed in a lysis buffer consisting of Nuclei Lysis Solution (Promega), 0.5 M EDTA (Sigma-Aldrich, St Louis, MI, U.S.A.) and Proteinase K (Qiagen AG, Hilden, Germany). By adding MagneSil Paramagnetic Particles (Promega; White et al., 1998) to the lysate, DNA was captured in solution and washed two or three times with 80% ethanol with the aid of a magnetic separator (MagnaBot 96 Magnetic Separation Device, Promega, Cat. No. V8151) to eliminate residual contamination. DNA was finally eluted in 50–100 μl of distilled water.

Eleven microsatellite primer pairs (loci NP007, NP773, ULI2, Pzeb3, Pzeb4, TmoM5, TmoM13, TmoM25, TmoM27, UME003 and UNH154; see Wirtz Ocana et al., 2014) were multiplexed in one polymerase chain reaction using the QIAGEN Multiplex PCR Kit (Qiagen). PCR reactions were carried out in a 10 μl volume containing 1–2 μl of the genomic DNA, 1x QIAGEN Multiplex PCR Master Mix (consisting of QIAGEN Multiplex PCR buffer with a final concentration of 3 mM $MgCl_2$, dNTP mix and HotStarTaq DNA polymerase), 0.1 μM of locus-specific 5′ fluorescent-labelled forward primer [fluorescent dyes: 6-FAM, HEX (Microsynth, Balgach, Switzerland), NED and PET (Applied Biosystems, Foster City, CA, U.S.A.)] and nonlabelled reverse primers.

Amplification was done in a 96-well GeneAmp PCR System 9700 (Applied Biosystems) using the following cycling protocol: 15 min at 95 °C; 35 cycles consisting of 30 s at 95 °C, 3 min at 57 °C and 1 min at 72 °C, followed by a final 15 min extension at 72 °C. Fluorescent PCR fragments were visualized by capillary electrophoresis on an ABI PRISM 3100 Genetic Analyzer. Genotypes were scored automatically using GeneMapper software version 3.7 (Applied Biosystems) against an internal size standard (GeneScan 500 LIZ, Applied Biosystems) and revised manually to ensure genotyping consistency.

Since the experimental groups consisted of breeding females with their offspring and only two potential fathers (one nest male and one dwarf male), maternity was always certain, and therefore assignment of paternity was very simple, performed visually via allelic matching at all 11 loci. For all offspring tested, parentage could be assigned unambiguously; there were no mismatching alleles with the respective parents.