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Evolutionary biology

Black goby territorial males adjust their ejaculate's characteristics in response to the presence of sneakers

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In many species, males can rapidly adjust their ejaculate performance in response to changing levels of sperm competition, an ability that is probably mediated by seminal fluid adaptive plasticity. In the black goby, *Gobius niger*, territorial males attach viscous ejaculate trails to the nest roof, from which sperm are slowly released into the water during the long-lasting spawning events. Sneaker males release their sperm in the vicinity of the nest, and territorial males try to keep them at a distance by patrolling their territory. We show here that territorial males' ejaculate trails released a higher proportion of their sperm in the presence of a single sneaker, but this proportion decreased when there were three sneakers, an effect that is most likely mediated by a change in the seminal fluid composition. Field observations showed that when multiple sneaking attempts occurred, territorial males spent more time outside the nest, suggesting that ejaculation rate and territory defence are traded-off. Altogether, these results suggest that the adjustment of sperm release from the ejaculate may be strategic, guaranteeing a more continuous concentration of the territorial male's sperm in the nest, although at a lower level, when he is engaged in prolonged territory defence outside the nest.

1. Introduction

Sperm competition, occurring when females mate with multiple males during the same breeding event, is recognized as a pervasive selective force influencing ejaculate production and its strategic allocation [1]. Increased levels of competition are typically associated with an enhanced ejaculate investment [1–3] resulting in the production of high sperm number and performance [1,2,4–7]. Moreover, since the cost of ejaculate production is not trivial, a prudent ejaculate allocation with respect to the value of a mating event is also predicted to occur at the intraspecific level [1,3]. In particular, individual males should increase the ejaculate expenditure at mating when faced with an immediate risk of sperm competition (i.e. the probability that the female will mate with another male) [8]. By contrast, when the intensity of sperm competition (i.e. the average number of potential female's partners) increases, the ejaculate expenditure should decrease, as the paternity payoff diminishes [9–10]. This fine-scale, rapid adjustment of sperm number and/or quality in relation to the perceived level of sperm competition occurs in several species [11–13]. Although theoretical and empirical research has focused mainly on the sperm component of the ejaculate, there is increasing evidence that the seminal fluid can be plastically adjusted in response to variable competition levels, driving competitive fertilization success by influencing sperm performance or affecting female fertility [14–20].

In many cases, males also respond to cues of increased levels of sperm competition behaviourally, for example by increasing their mate-guarding effort, or their territory defence against intruders, likely reducing the level of sperm competition [21]. These behaviours are, therefore, expected to influence male allocation into post-copulatory traits [22]. Under such circumstances, the adjustments of the ejaculate characteristics in response to different levels of sperm competition may not follow those predicted by classical fair-raffle risk and intensity models of optimal ejaculate allocation [8–10], as they will depend on the interaction between pre- and post-copulatory episodes of sexual selection [23]. These interactions are not easy to predict, as they may idiosyncratically depend, for example, on trade-offs between mate guarding and mating rate. The integration of pre- and post-copulatory episodes of sexual selection has been relatively little investigated theoretically in the context of sperm economics [1] and more empirical work is necessary to refine theory. We, therefore, investigated the ejaculate plasticity in response to the level of sperm competition in territorial males of the black goby, *Gobius niger*, a species with guard–sneaker mating tactics. Black goby territorial males produce ejaculates containing approximately 10-fold more seminal fluid and fewer sperm than those of sneakers [24,25]. During a spawning event, territorial males partition their ejaculate into trails that are attached to the nest roof. The large mucous component of seminal fluid influences the timing of sperm release from the ejaculate trail into the water: females attach their eggs to the nest roof, and the sperm, continuously released from the ejaculate trails into the water, can fertilize the eggs that are sequentially laid over several hours [24,26]. Viscous ejaculates free parental males from staying in close contact with the female during egg deposition and allow them to guard the nest without interrupting the fertilization process [24–27]. By contrast, sneakers release their ejaculates containing little seminal fluid and high sperm numbers as close as they can to the nest entrance [24–27]. Sneakers strategically allocate their sperm expenditure according to the number of other competing sneakers [28], whereas territorial males adjust their nest-guarding effort to keep sneakers away from the nest entrance, but do not adjust their overall sperm expenditure [29,30]. These previous studies, however, did not consider the seminal fluid component of a territorial male's response to the level of sperm competition. By modulating seminal fluid composition, territorial males might regulate the rate of sperm release from the ejaculate trails in response to the change of the guarding/sperm release pattern associated with the changing level of sperm competition faced. In particular, we expect that in the presence of a competitor, the rate of sperm release from the ejaculate trail should be increased, as compared with the absence of sneakers, to better compete with the numerous sneaker sperm, if mate guarding does not interfere with the pattern of ejaculate trail deposition. By contrast, when sneaking pressure increases and territorial nest guarding increases accordingly [29,30], territorial males should produce ejaculate trails with a slower sperm release compared with that in the presence of only one competitor. This would guarantee a steady supply of sperm during the prolonged engagement in nest defence.

Here, we tested this prediction by manipulating the number of sneaker males surrounding the nest of territorial black goby males during simulated matings and estimating the rate of sperm release from the ejaculate trails produced under different sperm competition levels. The experimental manipulation was coupled to field video recordings aimed at verifying the pattern of sneaking pressure and its effect on territorial guarding.

2. Material and methods

Fish were captured in the Venetian lagoon (Adriatic Sea) by divers during the breeding season (May–August 2020) (45°15′24.8″ N, 12°17′42.4″ E). Territorial males were individually transferred to 84 l experimental tanks; sneaker males and females were maintained in 60 l common stocking tanks until the experiment (2 days after capture). The experimental tank was partitioned into a central sector, housing the territorial male with a nest, and two side sectors (figure 1). On the first experimental day, a female was added to one of the lateral compartments of the tank two times per day for 60 min each time, to keep the territorial male stimulated to produce the ejaculate. On the second day, the territorial male was randomly allocated to one of the following three experimental treatments: (a) absence of competition, with no sneaker in the tank ($n = 18$); (b) low competition, with one sneaker in one lateral compartment for 2 h ($n = 18$) and (c) high competition, with three sneakers in one lateral compartment for 2 h ($n = 19$) (figure 1). Sneakers were added randomly to the left or to the right compartment. A female was introduced in the empty side compartment (or in one random side for the no-competition treatment) in the last 30 min of the trial to simulate the natural spawning environment. After the treatment, each territorial male was anaesthetized in a water solution of MS 222 (tricaine sulfate, Sandoz) (0.5 g l^{-1}), measured (SL: the distance between the snout and the base of the tail) and weighed (g). Male viscous ejaculate (3 cm length) was then collected on a piece of acetate sheet of standard length by gentle pressure on the male abdomen. The acetate sheet was carefully deposited in a beaker containing 80 ml of filtered seawater and the ejaculate left to dissolve for 30 min. After 30 min, the sheet was removed and the residual ejaculate rinsed in another beaker with 80 ml of filtered seawater. The two solutions were stained following [25] for sperm count. As an index of sperm release rate, we measured the proportion of sperm released in 30 min over the total sperm in the ejaculate trail. Following [24], sperm release peaks within the first 60 min after the ejaculate trail is stripped and at least 20% of sperm are released in the first 30 min (figure 2). All individuals were released unharmed to the site of collection at the end of the experiment.

In the field, 16 territorial nests were video-recorded (GoPro HERO4) in a 10 cm radius area around the entrance. Recordings lasted 10 or 20 min depending on environmental conditions. From videos, we quantified the frequency of sneaking attempts and the time interval between successive events, as well as the frequency of territorial male exits in response to sneaking attempts.

The effect of the treatment on the total number of sperm (log-transformed) in the ejaculate was analysed with a linear model (LM) (model's residuals followed normality, Shapiro–Wilk $p = 0.151$), whereas for the proportion of sperm released from the ejaculate, we used a generalized linear model (GLM) with quasi-binomial error distribution (logit link) to account for overdispersion. The effect of the number of sneaking attempts per minute on territorial males' exits was analysed with a GLM with quasi-Poisson error distribution (log link) to account for overdispersion. The total observation time (log) was added as an offset in order to model the response variable per time of observation.

3. Results

The number of sneakers affected the rate of sperm release from ejaculate trails ($F_{2,52} = 8.091$, $p < 0.001$; figure 2), which

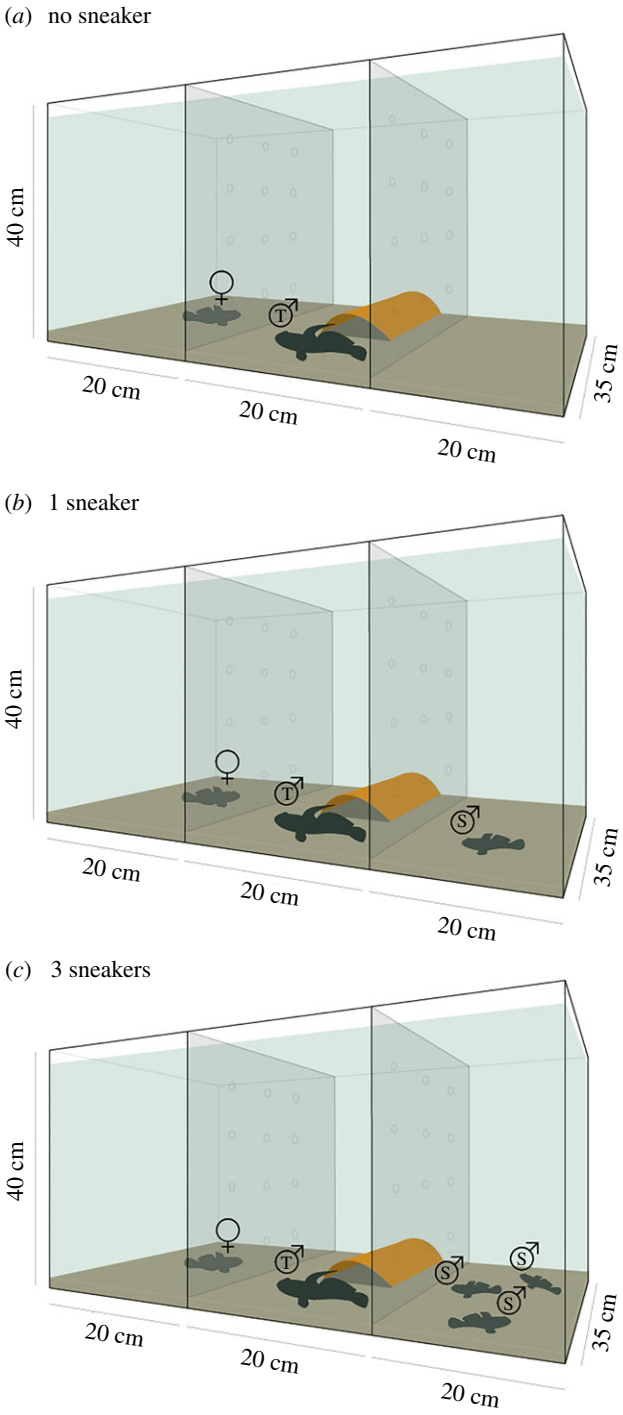

Figure 1. Experimental tank. The territorial focal male (T) occupied the central compartment furnished with a nest (half cut PVC pipe, 5 cm diameter × 16 cm length). Each T experienced one of three treatments: (*a*) absence of competition (no sneaker in the tank, $n = 18$); (*b*) low competition (one sneaker in the tank, $n = 18$) and (*c*) high competition (three sneakers in the tank, $n = 19$). A female was introduced in the other lateral compartment in the last 30 min of the treatment.

increased in the presence of one sneaker and decreased, at the level of the no-sneaker treatment, when three sneakers were present (*post hoc* test, table 1). The effect remained significant after excluding one outlier from the treatment with one sneaker ($F_{2,51} = 6.722$, $p < 0.01$). The total number of sperm in the stripped ejaculate, in contrast, was not influenced by the number of sneakers ($F_{2,52} = 0.628$, $p = 0.537$. Mean ± s.e.: no-sneakers = $3.12 \times 10^5 \pm 0.74 \times 10^5$; one sneaker = $2.38 \times 10^5 \pm 0.5.7 \times 10^5$; three sneakers = $3.14 \times 10^5 \pm 0.78 \times 10^5$). Results on the inspection of homogeneity of territorial male size,

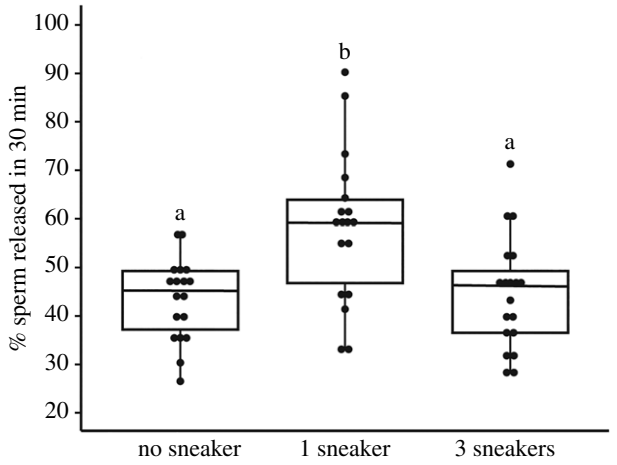

Figure 2. Effect of the number of competitor sneakers on the percentage of sperm released from the territorial ejaculate in 30 min. Different letters indicate significant differences between groups. Boxplots (first to third quartile) with median line and whiskers (1.5 interquantile range).

weight and condition among experimental groups are reported in electronic supplementary material, table S1.

In the field, we observed from 1 to 29 sneaking attempts per nest, with an average of 0.4 ± 0.4 (mean ± s.d.) sneaking attempts per minute in the 16 nests. The multiple sneaking attempts were sequential, with a time interval of 53 ± 68 s (mean ± s.d.). Of the total 209 events registered in all the 16 nests, the majority occurred at time intervals greater than than 10 s, 52 occurred at time intervals of less than 10 s, 23 of which were less than 5 s. Moreover, territorial males increased the number of exits per minute when the number of sneaking attempts per minute increased (estimate = 1.216 ± 0.286 s.e., $t = 4.248$, $p < 0.001$, 95% CI = 0.636–1.766).

4. Discussion

Our results suggest that black goby territorial males appear to tailor the rate of sperm release from the ejaculate trails attached to the nest roof in response to the level of sperm competition with sneaker males. In particular, in the presence of one sneaker, the rate of sperm release from the ejaculate trail increased, as compared to when no sneakers were present, while it did not change with three competitor sneakers. Moreover, field observations showed that attempts of intrusion from many sneakers occurred sequentially and territorial males responded to increasing sneaking attempts by leaving the nest more frequently to keep sneakers away.

When the levels of pre-copulatory and post-copulatory sexual competition correlate, such as when there is a simultaneous increase of male–male contests and sperm competition level, territorial males may rely more on nest defence than on increased sperm expenditure, if the former is energetically less expensive and/or more efficient in reducing cuckoldry than the latter [29,31–34]. Black goby territorial males increase their engagement in nest defence as the number of sneakers approaching the nest increases ([29,30], present results), without varying their overall sperm expenditure/mating ([29], present results). However, when engaged in more intense nest defence, territorials leave the nest more frequently to keep sneakers away and may be impeded from attaining an ejaculate trail deposition rate that maximizes their fertilization success during the

Table 1. Quasi-binomial GLM testing the effect of the number of sneakers on the percentage of sperm released from the ejaculate of territorial males in 30 min. Estimates with standard error, *t*-statistic, *p*-value (Bonferroni adjusted) and 95% confidence interval are presented for each between-group comparison. Significant effects ($p < 0.01$ and the 95% CI not overlapping zero) are highlighted in bold.

	estimate	s.e.	*t*	*p*	95% CI
no sneaker versus one sneaker	−0.591	0.166	−3.554	**<0.01**	**−0.918 to −0.266**
no sneaker versus three sneakers	−0.036	0.164	−0.219	0.974	−0.357 to 0.285
one sneaker versus three sneakers	0.555	0.164	3.387	**<0.01**	**0.235 to 0.878**

prolonged spawning, which can last hours. Our results indirectly suggest that the trade-off between the rate of ejaculate deposition and nest guarding may be at least partly resolved by adjusting the rate at which sperm are released from the viscous ejaculate trail. In principle, this adjustment may prevent sperm depletion of ejaculate trails when territorial males are engaged in nest defence, and allow a continuous release of sperm, although at a lower rate. By contrast, when only one sneaker is nearby, nest guarding may not interfere with the pattern of ejaculate deposition, and a more fluid ejaculate may guarantee to territorial males a faster release of sperm to face the increased risk of competition.

Our observations concur with recent evidence of the crucial role that seminal fluid plasticity has in driving sperm performance responses to cues of variable levels of competition [16–20]. The results of our experiments on black goby territorial males indicate that seminal fluid plasticity may be integrated with pre-copulatory behavioural plasticity in responding to variable levels of perceived sperm competition. These results add a further level of complexity to the role that seminal fluid has in driving the outcome of sperm competition between territorial and sneaker males [24–26,35]. While plasticity in the seminal fluid viscosity is the most obvious explanation for the observed pattern of sperm release from the ejaculate trail, the mechanism through which this is attained has not been explored yet. Rapid changes in ejaculate viscosity might involve the adjustment of the quantity of mucins added to the seminal fluid, or the modulation of proteolytic enzymes acting in mucin breakdown. Clearly, further investigations will be necessary to identify the mechanisms underlying the observed ejaculate plasticity. Regardless of the mechanisms involved, our results highlight the importance of integrating pre- and post-copulatory episodes of sexual selection, as well as considering the non-sperm ejaculate components in order to refine theories of ejaculate economics [1].

Ethics. Sampling and experimental procedures were approved by the animal ethics committee of the University of Padova (OPBA, permission no. 30/2015).

Data accessibility. Raw data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.gb5mkkwpq [36].

Authors' contributions. L.L. participated in study design, data collection and analysis, and wrote the manuscript. O.B. collected laboratory data and revised the manuscript. F.P. collected field data and revised the manuscript. A.P. and M.B.R. participated in study design and wrote the manuscript. All authors gave final approval for publication and agree to be held accountable for the work performed herein.

Competing interests. We declare we have no competing interests.

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