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RESEARCH ARTICLE

Seminal fluid enhances competitiveness of territorial males' sperm in a fish with alternative male reproductive tactics

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ABSTRACT

The most common adaptation to sperm competition in males is represented by an increase in the sperm number and/or quality released at mating, to raise their probability of egg fertilization. However, rapidly mounting evidence highlights that seminal fluid may directly influence the competitive fertilization success of a male by affecting either own and/or rival sperm performance. In the black goby, *Gobius niger*, an external fertilizer with guard-sneaker mating tactics and high sperm competition level, sneaker ejaculates contain less seminal fluid and more sperm, that are also of better quality, than those of territorial males. However, territorial males gain a higher paternity success inside natural nests. Here, we ask whether the seminal fluid can contribute to the reproductive success of territorial males by enhancing their sperm performance and/or by decreasing that of sneaker males. Using sperm and seminal fluid manipulation and *in vitro* fertilization tests, we found that own seminal fluid influences the velocity and fertilization ability of sperm only in territorial males, making them as fast as those of sneakers and with a similar fertilization rate. Moreover, both sneaker and territorial sperm remain unaffected by the seminal fluid of rival males. Thus, black goby males respond to the different level of sperm competition faced by differential allocation of sperm and non-sperm components of the ejaculate, with sneakers primarily investing in sperm of intrinsic high quality and territorial males relying on the effect of seminal fluid to increase the lower intrinsic quality of their sperm.

KEY WORDS: Sperm competition, Seminal fluid, Ejaculates interaction, Alternative reproductive tactics

INTRODUCTION

Sperm competition, occurring whenever two or more males compete to fertilize a given set of eggs (Parker, 1970), is the major selective force shaping ejaculate investment and expenditure per mating (Birkhead and Møller, 1998; Simmons, 2001; Birkhead and Pizzari, 2002; Pizzari and Parker, 2007). Extensive research has focused on differences in sperm investment across species and populations, consistently showing that higher levels of sperm competition select for an increased sperm number and/or quality in terms of viability, velocity and longevity (Simmons, 2001; Birkhead and Pizzari, 2002; Snook, 2005; Simmons and Fitzpatrick, 2012). Moreover, males are able to strategically adjust their ejaculate expenditure in successive matings, according to the level of sperm competition faced. Indeed, males may vary the amount of sperm released in each mating event depending on the number of competitors (Shapiro et al., 1994; Pilastro et al., 2002; Pizzari et al., 2003; del Barco-Trillo and Ferkin, 2004; Velando et al., 2008), and they may release sperm of higher quality when they perceive a threat from rival males or when they encounter females of greater quality (Rudolfsen et al., 2006; Cornwallis and Birkhead, 2007; Smith and Ryan, 2011). Because sperm performance is not only the result of its intrinsic quality, being also determined by the interaction with the seminal fluid (Poiani, 2006), the rapid variation in sperm quality across different mating events is likely due to a rapid change and modulation of the quantity and composition of the non-sperm components of the ejaculate (Cornwallis and O'Connor, 2009; Simmons and Fitzpatrick, 2012; Bartlett et al., 2017; Simmons and Lovegrove, 2017).

To date, most studies on the role of seminal fluid in the context of sperm competition have focused on its effects on female physiology and behaviour in internal fertilizers (Simmons and Fitzpatrick, 2012; Perry et al., 2013). In these species, seminal fluid proteins indirectly influence male fertilization success by decreasing female receptivity, forming mating plugs, increasing sperm storage/uptake rate or stimulating oviposition rate (Chapman, 2001; Ram and Wolfner, 2007; Ramm et al., 2009, 2015; Wigby et al., 2009; Fedorka et al., 2011; Yamane et al., 2015). Accordingly, males are able to adjust the composition of their seminal fluid in response to the perceived risk of sperm competition (Wigby et al., 2009; Sirot et al., 2011). However, a primary function of seminal fluid is to guarantee male fertility, by contributing to sperm capacitation, viability, velocity, nourishment and defence along the potentially hostile female reproductive tract (Poiani, 2006; Simmons and Fitzpatrick, 2012; Perry et al., 2013). Thus, in competitive contexts, the modulation of seminal fluid amount and/or composition might confer a fitness benefit to males by directly affecting their own and/or rival sperm performance (Hodgson and Hosken, 2006; Cameron et al., 2007). Evidence that short-term changes in own sperm performance, in response to sperm competition risk, are mediated by variations in own seminal fluid is recently increasing. A study on the Chinook salmon, *Onchorhynchus tshawytscha*, where male social status was experimentally manipulated, shows that the rapid increase in sperm speed and fertilization success in males exposed to a higher risk of sperm competition is driven by seminal fluid (Bartlett et al., 2017). Moreover, in both the house mouse, *Mus musculus*, and the Australian field cricket, *Teleogryllus oceanicus*, the expression of genes related to sperm fertilization ability increased in males exposed to cues of sperm competition, such as rival scent or calls (Ramm et al., 2015; Simmons and Lovegrove, 2017). In addition, a few studies have documented a direct effect of seminal fluid on rival males' sperm performance. In stalk-eyed flies and polyandrous ants and bees, whose females exhibit a high re-mating rate, seminal fluid impairs the viability of rival sperm (den

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Received 11 December 2017; Accepted 22 May 2018

Boer et al., 2010; Fry and Wilkinson, 2004). A similar effect was documented in two fish species with alternative male reproductive tactics (ARTs). In the grass goby, *Zosterisessor ophiocephalus*, where rival males come in close proximity to each other and to females, and thus their ejaculates commonly overlap, sneaker males release a seminal fluid that decreases territorial male sperm performance (Locatello et al., 2013). In addition, grass goby sneakers significantly enhance their fertilization ability by exploiting the seminal fluid of territorial males (Locatello et al., 2013). A slight negative effect of the seminal fluid of opportunistic jack males on the velocity of dominant males' sperm was recently found also in the Chinook salmon, *Onchorhynchus tshawytscha* (Lewis and Pitcher, 2017). Collectively, these findings indicate that seminal fluid components, by affecting sperm performance, may indeed vary in response to sperm competition risk and that the outcome of sperm competition may not exclusively be determined by ejaculate quality per se, but also by the interactions with rival ejaculates.

In the black goby, *Gobius niger* Linnaeus 1758, a demersal fish with external fertilization, ARTs and male parental care (Mazzoldi and Rasotto, 2002; Rasotto and Mazzoldi, 2002), sperm competition is very intense and up to six sneakers can be observed during spawning (Poli, 2015). Sneaker ejaculates contain on average 10-fold more sperm than those of territorial males, which, by contrast, have 10-fold more seminal fluid than those of sneakers (Rasotto and Mazzoldi, 2002; Poli, 2015). Moreover, sneakers' sperm (when assayed in a control solution) performs better than that of territorial males, in terms of velocity, viability and ATP content (Locatello et al., 2007). Despite sneakers' sperm overcoming that of territorial males in number and quality, territorial males father on average 70% of the young in their nest (Poli, 2015). Several factors may contribute to the higher paternity success of territorial males. Territorial males perform mate guarding, often forcing sneakers to release ejaculate at the nest entrance, rather than deep inside, leading to a rapid dilution of sperm in water (Mazzoldi, 1999; Poli, 2015; Movie 1). Female ovarian fluid may influence territorial male sperm performance, as recently documented in another fish species with ARTs (Alonzo et al., 2016). Another possibility is that territorial males produce a seminal fluid that increases their own sperm performance and/or negatively influences the sperm performance of sneakers.

Here, we evaluate whether seminal fluid investment in this species may contribute to the differences in the reproductive success of males adopting alternative tactics. We analyse sperm velocity and fertilization success of sneaker and territorial males' sperm in their own seminal fluid. If the territorial male reproductive success observed in the field is influenced by the effect of seminal fluid on own sperm performance, we expect territorial male sperm velocity and fertilization ability to overcome or, at least equate, those of sneaker sperm. Moreover, to control for a possible effect of the rival ejaculate interactions, we measured sperm performance by separating sperm and seminal fluid components of ejaculates and making reciprocal combinations between males adopting different tactics.

MATERIALS AND METHODS

Animal sampling and gamete collection

Black goby males were collected by SCUBA diving in the Venetian Lagoon during their breeding season (June–August 2015). Each male was anaesthetized in a water solution of MS-222 (tricaine methanesulphonate; Sandoz) and categorized as territorial or sneaker on the basis of standard length (SL; distance between the snout and the base of the tail), sexual traits expression (black nuptial coloration and elongation of the first dorsal fin) and ejaculate characteristics, i.e. the amount of sperm (higher in sneaker males) and seminal fluid (higher in territorial males) (Rasotto and Mazzoldi, 2002). Ejaculate was collected with a Gilson pipette by gently squeezing the abdomen of the anaesthetized male. Samples were centrifuged at 13,300 ***g*** for 3 min at 4°C to separate sperm from the supernatant seminal fluid (mean±s.d. seminal fluid volume, sneaker: 3.58±2.28 µl; territorial: 42.47±22.29 µl). Sperm were then resuspended in inactivating medium (in g l^{-1}: 3.5 NaCl, 0.11 KCl, 0.39 $CaCl_2$, 1.23 $MgCl_2$, 1.68 $NaHCO_3$, glucose 0.08, pH 7.7) (Fauvel et al., 1999) (medium volume range: sneaker=50–300 µl; territorial=40–100 µl). The inactivating medium volume was individually adjusted in order to standardize for sperm concentration in inactivated samples ($42.03\times10^3\pm0.35$ sperm μl^{-1}, mean±s.d.). Sperm concentration was assessed with an improved Neubauer chamber haemocytometer. Sperm and seminal fluid were maintained at 3–5°C until analysis (within 1 h of collection).

Eggs were released from anaesthetized, ready-to-spawn females through a gentle pressure on their swollen abdomen, and collected on acetate sheets onto which they adhere. Acetate sheets with eggs were maintained in filtered seawater until fertilization trials were performed, within a few minutes of collection. All individuals were released, unharmed, at the site of collection.

Ethical standards

Sampling and experimental procedures were approved by the animal ethics committee of the University of Padova (CEASA, permission no. 35/2011).

Experimental design

In order to test the effect of seminal fluid on the sperm performance of sneaker and territorial males, the velocity of each male's sperm was tested with: (1) no seminal fluid, (2) the male's own seminal fluid or (3) the fluid of another male adopting the opposite tactic. Sperm velocity tests were performed on sneakers (N=33; SL range=4.5–6.9 cm) and territorial males (N=44; SL range=8.2–12.2 cm) by an operator blind to the identity of the subject. Because 1 µl of seminal fluid per treatment was used (see 'Sperm velocity tests', below), the minimum volume needed to test the effect of each male's seminal fluid on both their own sperm and on sperm of a rival male was 2 µl. However, only 10 out of the 33 sneakers produced 2 µl of seminal fluid; therefore, the sperm of all 44 territorial males could be tested in the absence of seminal fluid and with their own seminal fluid, but only 10 of these 44 territorial samples were tested also with seminal fluid of a male adopting the opposite tactic (i.e. sneaker).

A set of *in vitro* fertilization trials, aimed at verifying that sperm velocity was a reliable predictor of fertilization success, was performed with sperm of 16 territorial and 18 sneaker males in their own seminal fluid.

Sperm velocity test

A volume of 7 µl of sperm in the inactivating solution was activated with 15 µl of filtered seawater (21±1°C, containing 2 mg ml^{-1} of bovine serum albumin; Billard et al., 1995) and incubated for 2 min without any seminal fluid, with 1 µl of the male's own fluid or with 1 µl of the fluid of a male adopting the opposite tactic.

Because in this species, sperm remain motile for more than 30 min (Locatello et al., 2007), a 2 min incubation ensures that they are not exhausted before performance measurements. Sperm velocity was measured with an CEROS Sperm Tracker (Hamilton Thorne Research, Beverly, MA, USA) placing 3 µl of each activated

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Table 1. Results of linear mixed model on curvilinear sperm velocity (V_{CL}, μm s^{-1}) of sneaker (*N*=33) and territorial (*N*=44) male black gobies

	Lower	Estimate	Upper	*t*-value	*P* (>|*t*|)
(Intercept)	4.755	4.83	4.905	127.876	**<0.001**
Tactic: territorial versus sneaker	−0.215	−0.115	−0.016	−2.316	**<0.05**
Treatment: no fluid versus own fluid	−0.015	0.054	0.125	1.548	0.124
Treatment: own fluid versus opposite fluid	−0.043	0.027	0.098	0.773	0.441
Interaction: tactic×treatment no fluid versus own fluid	−0.269	−0.176	−0.083	−3.76	**<0.001**
Interaction: tactic×treatment own fluid versus opposite fluid	−0.159	−0.028	0.103	−0.425	0.672

Approximate 95% confidence intervals of parameter estimates are reported. The *P*-values of significant effects are highlighted in bold.

sample in separate wells on a 12-well multi-test slide, and covering with a coverslip (MP Biomedicals, Aurora, OH, USA) previously coated with a polyvinyl alcohol solution (1%; Sigma-Aldrich, St Louis, MO, USA) to avoid sperm sticking to the glass slide (Wilson-Leedy and Ingermann, 2007). We focused on curvilinear velocity (V_{CL}, μm s^{-1}), as this measure is a reliable predictor of the fertilization success in many external fertilizers, including the grass goby (Au et al., 2002; Casselman et al., 2006; Locatello et al., 2013).

In vitro fertilization trials

In order to perform *in vitro* fertilization trials, sperm were activated in their own seminal fluid, as in sperm velocity tests (see above), and an amount of sperm solution containing 2×10^5 sperm cells was diluted to 50 μl with filtered seawater, to standardize the volume used in each test. For each fertilization trial we used 139.41±55.48 eggs (mean±s.d.) collected on acetate sheets from three different females, to minimize potential male-by-female interactions at fertilization (Fitzpatrick et al., 2012; Locatello et al., 2013). The number of eggs used did not differ between trials on sneaker and territorial males (*t*-test: t=−1.73, d.f.=30, P=0.094). The acetate sheets with eggs, one for each female, were placed on the bottom of a glass beaker containing 175 ml of filtered seawater, corresponding to a 3 cm of depth, namely the average depth of natural nests (Poli, 2015). Sperm were homogeneously distributed on the water surface with a Gilson pipette and after 15 min, the acetate sheets were removed, gently rinsed and placed in a new glass beaker with clean filtered seawater and oxygen supply at a temperature of 21±1°C. The percentage of fertilized eggs was checked 4 h later when the complete lifting of chorion and the first stages of cellular division can be clearly distinguished (Locatello et al., 2013).

Statistical analyses

Statistical analyses were performed using R version 3.2.4 (https://www.r-project.org/). The 'pastecs' and 'nortest' packages were used for descriptive statistics (https://CRAN.R-project.org/package=pastecs; https://CRAN.R-project.org/package=nortest). Prior to analyses, data were checked for normality and homogeneity of variance following the Kolmogorov–Smirnov and Bartlett's test, respectively. Sperm velocity data were log transformed, whereas fertilization success data were arcsine square root transformed. The effect of the treatment on sperm velocity (V_{CL}; μm s^{-1}) was analysed using a linear mixed model (LMM), with restricted maximum likelihood estimation (REML), using the 'nlme' package (https://CRAN.R-project.org/package=nlme). We included sperm velocity as the dependent variable, and seminal fluid treatment (no seminal fluid, own seminal fluid or opposite tactic seminal fluid), male tactic (sneaker or territorial) and the interaction between tactic and treatment as fixed factors. To account for multiple measures from the same male, male identity (nested within tactic) was included as a random factor with estimation of random intercepts for each subject. The diagnostic plots of the LMM (Figs S1–S3) did not evidence substantial concerns with residuals distribution and model fitting. *Post hoc* comparisons of interest, following LMM, were performed through two-tailed *t*-tests for independent samples when comparing treatments between groups, and through two-tailed paired *t*-tests when comparing treatment within groups. *P*-values were adjusted for multiple testing following Benjamin and Hochberg (1995).

The *in vitro* fertilization success of territorial and sneaker sperm in their own seminal fluid was compared through an independent-samples *t*-test.

RESULTS

The LMM on sperm velocity evidenced a significant interaction between tactic and treatment, with a significantly higher effect of seminal fluid (with respect to the basic level: no seminal fluid) in territorial males (Table 1, Fig. 1). Indeed, the within-tactic *post hoc* comparisons showed a significant difference in sperm velocity when comparing territorial males' sperm in the absence of seminal fluid and in the presence of their own fluid (paired *t*-test: d.f.=43, t=3.60, adjusted P<0.01). On the contrary, this difference was non-significant in sneaker males (paired *t*-test: d.f.=32, t=1.41, adjusted P=0.30). In both sneaker and territorial males, sperm velocity was not affected by the seminal fluid of a male adopting the opposite

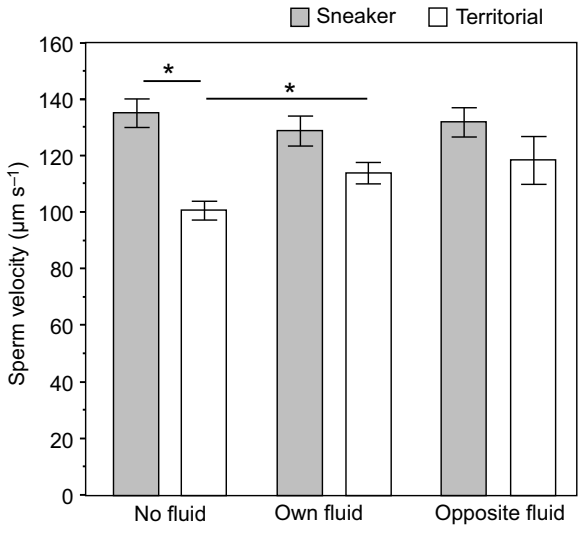

Fig. 1. Curvilinear sperm velocity (V_{CL}, μm s^{-1}; mean±s.e.m.) of sneaker (grey bars) and territorial (white bars) male black gobies. Data are from sperm with no seminal fluid (sneaker *N*=33; territorial *N*=44), own seminal fluid (sneaker *N*=33; territorial *N*=44) and opposite tactic seminal fluid (sneaker *N*=33; territorial *N*=10). Asterisks indicate significant differences (adjusted P<0.05).

tactic (paired *t*-test: sneaker d.f.=32, t=1.07, adjusted P=0.34; territorial d.f.=9, t=0.56, adjusted P=0.30; Fig. 1).

Sneakers' sperm showed a mean velocity higher than that of territorial males (see the significant effect of the tactic in Table 1; Fig. 1) only in seawater (*post hoc t*-test: d.f.=75, t=6.20, adjusted P<0.001), whereas this difference was no longer significant in the presence of seminal fluid, both the male's own (*t*-test: d.f.=75, t=2.21, adjusted P=0.08) and that of a male adopting the opposite tactic (*t*-test: d.f.=9, t=0.56, adjusted P=0.30).

The *in vitro* fertilization tests revealed that fertilization success of sperm in their own seminal fluid did not differ between sneaker and territorial males (*t*-test: d.f.=32, t=1.45, P=0.16).

DISCUSSION

Our study shows that seminal fluid does contribute to the paternity success of black goby territorial males by directly influencing their own sperm performance. Indeed, the more abundant seminal fluid of territorial males enhances the velocity of their own sperm, making it as fast as that of sneakers. Conversely, sneaker seminal fluid does not affect the performance of their own sperm. However, contrary to what occurs in other species with ARTs (Lewis and Pitcher, 2017; Locatello et al., 2013), seminal fluid does not affect rival sperm performance, suggesting that, in this species, the cross-interaction of rival ejaculates does not influence the outcome of sperm competition. The finding that territorial males' sperm with the addition of their own fluid reaches the velocity of sneakers' sperm is reinforced by the results of fertilization trials, besides confirming that sperm velocity is a reliable predictor of fertilization ability. Considering that the sperm of territorial males increase velocity only in their own seminal fluid, in this species, seminal fluid appears to vary among males adopting different tactics, not only in quantity but also in quality. Indeed, if the quality of the seminal fluid produced by the two male types was similar in terms of composition, we should have also recorded a significant increase of territorial males' sperm velocity when exposed to sneakers' seminal fluid. Thus, while seminal fluid composition of territorial males clearly affects sperm velocity, which is crucial to fertilization ability, the function of sneaker seminal fluid might be limited to prevent sperm motility when sperm are still in the sperm duct, and to initiate it at the proper time when sperm are released (Alavi and Cosson, 2006; Poiani, 2006). Our results also confirm the intrinsic higher quality of sneakers' sperm (Locatello et al., 2007), as in the absence of any fluid the velocity is significantly higher than that of territorial male sperm. This performance does not significantly vary when seminal fluid is present, regardless of whether it is a male's own seminal fluid or that of a rival male. This suggests that the intrinsic quality reached by sneakers' sperm could not be further increased, explaining why their performance remains unaffected even by territorial males' seminal fluid.

The functional differences of the seminal fluid of sneaker and territorial males strongly suggest a tactic-specific composition that, contrary to what occurs in the grass goby (Locatello et al., 2013) and the Chinook salmon (Lewis and Pitcher, 2017), does not include 'offensive' components, i.e. substances negatively affecting rival sperm. Substantial evidence documents the role of different seminal fluid components in sperm activation, speed and viability, particularly in mammals and insects (Poiani, 2006; Ramm et al., 2015; Simmons and Lovegrove, 2017). Much less is known in fish, but ion content of the medium appears to be crucial for sperm activation (Alavi and Cosson, 2006), whereas proteins, monosaccharides and triglycerides appear to affect sperm viability and speed (Lahnsteiner et al., 2004; Lahnsteiner, 2007). Support for the role of seminal fluid protein abundance in the plasticity of sperm performances in response to sperm competition risk comes not only from the house mouse and the Australian field cricket (Ramm et al., 2015; Simmons and Lovegrove, 2017) but also from the Chinook salmon (Gombar et al., 2017) and the grass goby (F.P., unpublished data), where the seminal fluid protein profile differs in relation to male tactic. Accordingly, in the black goby, the seminal fluid of territorial males might be richer in protein content, to enhance the fertilization ability of their sperm, whereas that of sneaker males might be poorer in protein but endowed with an ionic composition adequate to properly regulate sperm activation.

The similar sperm performance recorded among black goby males adopting different tactics appears to be based on different mechanisms, with sneakers producing sperm of intrinsic high quality and territorial males relying on the effect of seminal fluid to increase the lower intrinsic quality of their sperm. These results shed new light to our understanding of how males invest in sperm and seminal fluid in response to sperm competition risk and mating order or role. In particular, our findings support the theoretical expectations that the relative allocation depends on which ejaculate components more strongly influence the sperm competitiveness (Cameron et al., 2007; Perry et al., 2013). Black goby sneakers always mate in competition and in a disfavoured position, often being forced by territorial males to ejaculate at the nest entrance, 3–4 cm away from the female, with a resulting rapid dilution of sperm in seawater (Mazzoldi, 1999; Poli, 2015). Moreover, inside the nest, the sneaker sperm experiences an environment in which the territorial male seminal fluid is diluted several thousand times (average territorial fluid volume: 42.4 µl, average nest volume: 669 cm^3; present study; Poli, 2015). This mating dynamic strongly reduces the opportunity for the interaction between rival ejaculates, making it unlikely for sneakers to either impair rival sperm with their seminal fluid or to exploit rival seminal fluid for increasing their own sperm performance, as it occurs in other fish species with ARTs (Locatello et al., 2013; Lewis and Pitcher, 2017). In such a scenario, sneakers enhance their sperm competitiveness primarily by investing in sperm number and intrinsic quality (Rasotto and Mazzoldi, 2002; Locatello et al., 2007; present study). By contrast, territorial males, suffering a lower level of sperm competition and producing a lower number of sperm than sneakers (Mazzoldi, 1999; Poli, 2015), may increase the overall competitiveness of their sperm by investing more in the seminal fluid. The seminal fluid of territorial males is rich in mucins, making the ejaculate a viscous band (also referred to as 'sperm trail') that slowly dissolves into the water, allowing a constant release of sperm spanning several hours (Rasotto and Mazzoldi, 2002). By laying sperm trails, territorial males do not need to stay close to females over the entire spawning period, and can defend the nest from competitors while active sperm are still being released inside the nest. Therefore, in addition to enhanced sperm velocity levelling off the differences between the intrinsic performance of their sperm compared with that of sneakers, the seminal fluid components of territorial males also shape how sperm are released, increasing, as a result, the overall ejaculate longevity and, indirectly, favouring nest defence effectiveness (Mazzoldi and Rasotto, 2002). Despite identical longevity for free sperm (Mazzoldi, 1999), black goby males have functionally polymorphic spawns, with territorial males that, thanks to seminal fluid mucins, have fewer but longer-functioning sperm per spawn. Thus, territorial males' seminal fluid contributes to their paternity success by both indirectly favouring nest guarding effectiveness and directly enhancing the performance of their own sperm.

To fully evaluate the higher fertilization success of black goby territorial males, the effect of female ovarian fluid on sperm performance needs to be investigated. A great intra-specific variability in the influence of ovarian fluid on sperm velocity or viability has been documented in several fish species (Urbach et al., 2005; Rosengrave et al., 2009; Butts et al., 2012; Galvano et al., 2013), but when the sperm of males adopting different tactics was analysed, ovarian fluid appeared to equally influence all sperm types (Alonzo et al., 2016; Makiguchi et al., 2016; Lehnert et al., 2017). Despite having the same effect on the sperm of all male phenotypes, ovarian fluid may still favour the fertilization success of a specific male phenotype when sperm differ in their intrinsic quality or experience a different ovarian fluid concentration (Lahnsteiner, 2002; Alonzo et al., 2016). In the black goby, considering the spatial and temporal positions of the ejaculates released by sneakers and territorial males with respect to the female laying eggs, ovarian fluid could potentially affect more the performance of territorial male sperm than that of sneaker male sperm, which are released further.

The exploration of seminal fluid composition, function and variability in relation to sperm competition level is just at the beginning (Poiani, 2006; Simmons et al., 2012; Locatello et al., 2013; Perry et al., 2013; Ramm et al., 2015; Lewis and Pitcher, 2017; Simmons and Lovegrove, 2017). The present findings suggest that more information on spawning dynamics and, in particular, on ejaculate release and opportunity for interactions should be collected in order to achieve a deeper understanding of the variability of seminal fluid composition and function in response to sperm competition risk.

Acknowledgements
We thank Andrea Sambo for invaluable and constant technical support, and the students who helped in the fieldwork. We thank the anonymous reviewers of the manuscript for their comments and suggestions.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: F.P., L.L., M.B.R.; Methodology: F.P., L.L., M.B.R.; Software: F.P.; Validation: F.P.; Formal analysis: F.P., L.L.; Investigation: F.P., L.L.; Data curation: F.P., L.L.; Writing - original draft: F.P., L.L., M.B.R.; Writing - review & editing: F.P., L.L., M.B.R.; Visualization: F.P., L.L.; Supervision: M.B.R.; Project administration: M.B.R.; Funding acquisition: M.B.R.

Funding
This research was funded by the University of Padova ('Fondi di ricerca ex–60%' to M.B.R.). The funder had no role in the study design, data collection and analysis, decision to publish or preparation of the manuscript.

Data availability
All data are available from the Figshare repository: http://dx.doi.org/10.6084/m9.figshare.5985229.

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.175976.supplemental

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