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Ejaculates in competition:

a sperm race influenced by seminal fluid?

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RIASSUNTO

Negli ultimi quaranta anni, è emerso che i sistemi di accoppiamento nel mondo animale sono di tipo promiscuo o poliandrico molto più frequentemente di quanto non si credesse, rendendo necessarie una serie di considerazioni sulle implicazioni biologiche ed evolutive derivanti in particolare dal fatto che, in molte specie, le femmine si accoppiano con più di un maschio. La selezione sessuale è quel processo evolutivo che promuove la trasmissione dei geni che avvantaggiano dal punto di vista riproduttivo l'individuo che li esprime. Un sistema di accoppiamento poliandrico, o promiscuo, implica che la selezione sessuale possa proseguire anche dopo l'accoppiamento, fino al momento della fecondazione, e in alcuni casi anche successivamente. In questo contesto, il successo di fecondazione di un maschio non corrisponde necessariamente al suo successo di accoppiamento, ma può essere influenzato dai meccanismi di selettivi post-copulatori, quali la scelta criptica femminile e la competizione spermatica. Il primo meccanismo si realizza quando una femmina è in grado di favorire, "scegliere", l'eiaculato di un maschio rispetto a quello degli altri maschi con cui si è accoppiata, e la scelta è stata definita criptica perché avviene all'interno delle vie riproduttive femminili. La competizione spermatica è stata definita come la competizione tra gli eiaculati di due o più maschi per fecondare uno stesso gruppo di uova. Questo secondo meccanismo è stato studiato sia in specie a fecondazione interna che esterna, e spinge l'evoluzione di tutti quei tratti morfologici, comportamentali e fisiologici che possono avvantaggiare l'eiaculato di un maschio rispetto agli eiaculati rivali in un determinato contesto di competizione. La produzione di spermi e liquido seminale comporta dei costi, e i maschi possono variarne l'investimento per massimizzare il proprio successo riproduttivo a seconda del livello di competizione spermatica percepito. Gli studi sperimentali e i modelli teorici si sono da sempre concentrati su come la competizione spermatica possa modellare il numero e la qualità degli spermi prodotti. Recentemente, tuttavia, la ricerca scientifica ha mostrato come la competizione tra gli eiaculati possa coinvolgere anche il liquido seminale. Questo, infatti, spesso costituisce una parte consistente dell'intero eiaculato ed è fondamentale per il mantenimento e le prestazioni degli spermi. Inoltre, nelle specie a fecondazione interna, è stato dimostrato che può indirettamente influenzare il successo di fecondazione di un maschio spingendo ad esempio le femmine a deporre un maggior numero di uova, oppure riducendo la loro ricettività e tendenza a riaccoppiarsi per limitare la competizione con gli spermi di altri maschi. Solo negli ultimi anni è stata presa in considerazione la possibilità che il liquido seminale di un maschio potesse agire direttamente sul successo di fecondazione degli eiaculati rivali. Per esempio, uno studio comparativo ha dimostrato che nelle specie di api e formiche dove il livello di competizione è più alto, il liquido seminale di un maschio favorisce le prestazioni dei propri spermi, e in maniera minore quelle degli spermi dei maschi rivali. In altre specie di insetti, invece, il liquido seminale di un maschio aumenta allo stesso modo la performance di tutti gli spermi contemporaneamente presenti. Questi risultati

indicano che quando non si realizza un meccanismo di riconoscimento self/no-self tra gli spermi e il liquido seminale, le sue proprietà potrebbero essere sfruttate anche dagli spermi degli altri maschi in competizione. Di conseguenza, se, al momento dell'accoppiamento, un individuo riesce a stimare il grado di competizione che dovrà fronteggiare, potrebbe strategicamente regolare l'investimento nell'eiaculato a seconda che si trovi in una posizione favorita o sfavorita. I modelli teorici più recenti indicano che l'investimento nell'eiaculato può essere modellato su entrambe le sue componenti, a seconda di quanto spermi e liquido seminale influenzino il successo dell'intero eiaculato in un determinato contesto di competizione. Prima di questo progetto, le prove sperimentali a supporto delle predizioni derivate dai modelli sono state poche e i risultati contrastanti, probabilmente perché sia la ricerca teorica che quella sperimentale si sono prevalentemente concentrate su specie a fecondazione interna, dove è difficile separare il contributo di spermi e liquido seminale e distinguere tra gli eiaculati in competizione.

Nel mio progetto ho usato quindi come modello due specie di pesci Teleostei che presentano fecondazione esterna, il go, *Zosterisessor ophiocephalus* e il ghiozzo nero, *Gobius niger*, che hanno un sistema di accoppiamento simile e livelli di competizione spermatica confrontabili. In entrambe le specie i maschi adottano tattiche riproduttive alternative, con i maschi dominanti, territoriali, che scavano un nido all'interno del quale la femmina depone le uova, e i maschi opportunisti, sneaker, che cercano di sfruttare gli accoppiamenti dei maschi territoriali. I maschi sneaker, che si confrontano con un più alto rischio di competizione dovendo sempre competere almeno con l'eiaculato del maschio territoriale, producono un maggior numero di spermi e meno liquido seminale rispetto ai maschi territoriali. Nel go, le prestazioni degli spermi non differiscono tra le due tattiche, mentre nel ghiozzo nero gli spermi dei maschi sneaker sono più veloci, longevi e presentano un maggior contenuto in ATP. Le due specie costituiscono un buon modello anche perché il liquido seminale ha una diversa possibilità di entrare in gioco nella competizione tra gli eiaculati a causa di alcune differenze legate alle modalità di accoppiamento. Infatti, nel go, i maschi sneaker riescono ad entrare nel nido e a rilasciare il proprio eiaculato vicino a quello del maschio territoriale e alle uova e, di conseguenza, in questa specie il liquido seminale potrebbe avere un ruolo nell'influenzare la competizione tra gli eiaculati. Nel ghiozzo nero, invece, i maschi sneaker sono spesso costretti a rilasciare il proprio eiaculato all'ingresso del nido, e di conseguenza il poco liquido seminale prodotto va verosimilmente incontro ad una rapida diluizione, e il grado di interazione tra spermi e liquido seminale di maschi aventi tattiche opposte è di conseguenza minore rispetto a quanto non accada nel go.

Dato questo scenario, il mio progetto di dottorato ha voluto i) verificare, nelle due specie, quando e come il liquido seminale influenzi la competizione tra eiaculati, incrociando spermi e liquido seminale di maschi aventi la stessa o la tattica opposta e misurando le prestazioni degli spermi come velocità, vitalità e tramite test di fecondazione *in vitro*; ii) approfondire i meccanismi prossimi di interazione tra spermi e

liquido seminale nel go, dove il liquido potrebbe effettivamente pesare sulle prestazioni degli eiaculati di maschi aventi tattiche opposte; e iii) controllare, nel ghiozzo nero, se i parametri considerati per misurare le prestazioni di un eiaculato sono indicativi del suo reale successo di competizione, andando quindi a misurare il successo di fecondazione dei maschi territoriali sul campo, tramite nidi artificiali ed analisi di paternità. Nel go, questa distribuzione della paternità nei nidi naturali è già stata valutata in un precedente studio.

Nel go, è stato dimostrato un effetto tattica-dipendente del liquido seminale. Gli spermi dei maschi sneaker sono più veloci e mostrano un maggior successo di fecondazione quando è presente il liquido seminale di un maschio territoriale, mentre gli spermi dei maschi territoriali mostrano prestazioni inferiori, per gli stessi parametri, in presenza del liquido di un maschio sneaker, sempre rispetto ai valori misurati con il proprio liquido seminale. Un appropriato esperimento di controllo ha escluso che questi risultati siano dovuti ad un meccanismo di riconoscimento self/no-self tra spermi e liquido seminale.

L'indagine dei meccanismi prossimi alla base dell'interazione tra spermi e liquido seminale ha evidenziato che l'eiaculato differisce tra maschi aventi tattiche opposte sia nella composizione del liquido seminale, per quanto riguarda il contenuto e il profilo proteico (differenze qualitative e quantitative), sia nelle prestazioni degli spermi misurate in termini di tasso di consumo dell'ossigeno, un parametro metabolico importante ma raramente considerato. Inoltre, nonostante le analisi sulla funzionalità del liquido seminale siano solo preliminari, hanno già dimostrato come sia determinante la presenza della frazione non proteica (<3kDa) per la velocità degli spermi, nonostante anche la frazione proteica (>3kDa) contribuisca, seppur in maniera minore.

In questa specie, le prove di fecondazione *in vitro* rispecchiano i risultati emersi dai dati di velocità degli spermi, che di conseguenza risulta un buon indicatore della competitività dell'eiaculato. Considerando quanto emerso da uno studio precedente sulla distribuzione della paternità nei nidi naturali, sembra che il successo di fecondazione dei maschi territoriali sia determinato in ultimo dall'efficacia della difesa del nido, e cioè da quanto riescono a tenere a distanza i maschi sneaker durante l'accoppiamento. Infatti, il loro tasso di paternità correla positivamente con le loro dimensioni corporee.

Nel ghiozzo nero, dove un maschio sneaker rilascia il proprio eiaculato a distanza rispetto a quello del maschio territoriale, non emerge nessun effetto tattica-dipendente del liquido seminale. Nonostante il proprio liquido seminale migliori le prestazioni degli spermi nei maschi territoriali, gli spermi dei maschi sneaker rimangono ancora significativamente più veloci e vitali. Le prove di fecondazione *in vitro* non rispecchiano i dati emersi, e non emerge alcuna differenza significativa nel successo di fecondazione delle due tattiche, anche se questo potrebbe esser dovuto alla scarsa numerosità dei dati. Tuttavia, in questa specie gli spermi dei maschi territoriali presentano un moto significativamente più lineare rispetto ai maschi sneaker e quindi è possibile che il loro

nuoto sia in realtà più efficace, e possano percorrere la stessa distanza nello stesso tempo, nonostante gli spermi degli sneaker siano più veloci.
L'analisi della distribuzione della paternità su campo suggerisce che anche nel ghiozzo nero sia la distanza a cui gli sneaker rilasciano il proprio eiaculato a determinare il risultato della competizione tra gli eiaculati. Infatti, i primi risultati mostrano che il successo di fecondazione del maschio territoriale è minore in corrispondenza dell'entrata principale del nido, dove è più probabile che i maschi sneaker riescano ad avvicinarsi e fecondare parte delle uova. Il successo di fecondazione del maschio territoriale all'interno di un nido è comunque basso (<50%) se confrontato con quello del go (>70%) o di altre specie di pesci aventi un sistema di accoppiamento simile. Inoltre in due nidi, alcune uova sono risultate fecondate da maschi territoriali che difendevano nidi vicini. Apparentemente quindi i territoriali parassiterebbero occasionalmente i nidi di altri maschi aventi la stessa tattica. Se il risultato venisse confermato, la posizione dei maschi territoriali nella competizione tra eiaculati potrebbe non apparire più come il ruolo favorito, considerando che i maschi sneaker visitano sistematicamente più di un nido.

In entrambe le specie, il contesto spaziale in cui si realizza la competizione risulta decisivo. Il grado di interazione tra gli eiaculati di maschi aventi tattica opposta determina la possibilità che il liquido seminale possa esser sfruttato nell'ambito della competizione. Ne risulta che, nel go, dove il livello di interazione è alto, si crei l'opportunità per un maschio di sfruttare il liquido di un maschio avente tattica opposta, mentre nel ghiozzo nero, dove l'interazione è minima, i maschi siano costretti ad investire nel numero e qualità degli spermi prodotti o nella difesa del nido, a seconda della tattica.
Il mio progetto di dottorato dimostra che il liquido seminale può effettivamente influenzare la competizione tra gli eiaculati a seconda del contesto, e che uno dei principali fattori da tenere in considerazione è il grado di interazione tra eiaculati rivali. L'approccio multidisciplinare adottato ha permesso di fare luce anche sui meccanismi prossimi di interazione tra spermi e liquido seminale.

ABSTRACT

In the last forty years, the historical notion of monogamous females has been gradually eroded away, and female multiple mating is now look as a common and ubiquitous phenomenon in nature, triggering theoretical and experimental attention to its biological implications and evolutionary consequences. Sexual selection is the evolutionary process that favours the increase in frequency of the genes that confer a reproductive advantage to the individuals carrying them. Polyandry implies that sexual selection may persist even after the copulation up to the point of fertilization, and in some cases beyond. In this scenario, male mating not necessarily results in successful insemination, but depends on the outcome of post-copulatory sexual mechanisms influencing the fertilization success, namely cryptic female choice and sperm competition. Female choice is the possibility for females to non-randomly bias paternity, favouring the ejaculate of the best quality male, in order to maximize their fitness. On the other hand, male-male competition results in sperm competition when the ejaculates of two or more males compete to fertilize the same set of eggs, as it has been firstly defined by Geoffrey Parker. When ejaculates overlap in space and time, differences in characteristics that are key factors for the fertilization success may lead one ejaculate to overcome the rivals, generating differential males' reproductive success. This mechanism, investigated in both externally and internally fertilizing species, is a powerful evolutionary force moulding an amazing variety of behavioural, morphological and physiological traits. Ejaculates are costly to produce and, thus, sperm expenditure drawns the attention for how males, to increase their reproductive success, may modulate their investment in response to different sperm competition levels. Sperm competition is expected to influence those traits driving sperm fertilization capabilities in a specific context. To date, theoretical and empirical studies have primarily and only focused on how sperm characteristics, i.e. number and quality, affect the fertilization success of competing males.

Increasing evidence are suggesting that predictions on the outcome of sperm competition should not revolve only around the sperm component of the ejaculate. The seminal fluid often makes up a large part of an ejaculate and it may influence paternity success both directly and indirectly. Indeed, seminal fluid is already known to enhances sperm performance in several species as well as to indirectly influence paternity success, by decreasing female receptivity, increasing oviposition rate and forming mating plugs. Seminal fluids may also play a frontline role in sperm competition by directly affecting rivals' sperm performance. For instance, in promiscuous ants and bees, seminal fluid incapacitates the sperm of rival males, while in other insects, it improves equally the survival of own and other sperm. This suggests that, unless a self/non-self-recognition mechanism evolves, the function of seminal fluid to enhance own sperm performance can be exploited by the sperm of rival males. In particular, when a male can his reproductive role while mating with a female, if advantaged or disadvantaged, he could

strategically allocate his ejaculate to maximize the reproductive success. Theoretical analyses, still waiting for experimental tests, posit that selection should favour phenotypic plasticity in male expenditure on both sperm and seminal fluid components, specifically influencing that/those that affect more the ejaculate competitive weight. Clear evidence still lack, likely because, up to the present study, models and experimental works considered mostly internal fertilizers, where it is difficult to attribute sperm and seminal fluid to a specific individual.

I overcame these problems by using as model species two fishes with external fertilization, the grass goby (*Zosterisessor ophiocephalus*) and the black goby (*Gobius niger*) as they show a similar mating system and comparable levels of sperm competition, but potentially differ in the likelihood for seminal fluid to influence competition contexts. In both species males display territorial-sneaker mating tactics, where sperm competition risk varies according to the tactic adopted, with sneaker males experiencing the highest level producing a great number of sperm and less seminal fluid than territorial males. In the grass goby, sperm quality, in terms of velocity, viability and ATP content, does not vary between tactics, whereas, black goby sneakers produce sperm that are faster, more viable and richer in ATP than territorial males. In these two species, the dynamics of mating are potentially a crucial factor influencing the role of seminal fluid on the outcome of ejaculates competition. Indeed, grass goby sneakers enter inside the nest and may release their ejaculates in close proximity to those of the territorial male and to eggs. Thus, in this species I expected that the seminal fluid might have a competitive weigh, mediating sneaker and territorial ejaculates interplay. By contrast, in the black goby, sneakers are forced to release their ejaculate at the nest entrance and, thus, the opportunity for the mixing of territorial males' and sneakers' ejaculates does not occur or it is rare.

On the basis of these preconditions, my PhD project pointed to i) verify in both species when and how the seminal fluid affects sperm performances, in terms of velocity, viability of own and rival sperm, making combinations of sperm and seminal fluid within and between males adopting different tactics; ii) deepen, in the grass goby, the proximate mechanisms driving sperm and seminal fluid interplay; iii) evaluate if the results from sperm performance give reliable insights on their fertilization ability and on the outcome of ejaculates competition in nature. Therefore, I performed *in vitro* fertilization tests, applying the same experimental design used in sperm performance trials. Secondly, considering that the paternity success of the grass goby has been already investigated from natural nests in a previous work, I concentrated on the black goby. I evaluated the fertilization success in the field through artificial nests located in natural breeding sites, by using molecular parentage analyses.

In the grass goby, I found that sneaker's sperm increase their performance, both in terms of velocity and fertilization rate, in presence of territorial male's seminal fluid, while the performance of territorial male's sperm is decreased in presence of sneaker's

seminal fluid. Appropriate control experiments demonstrate that this effect is not mediated by a self/non-self recognition mechanism.

Investigating the proximate mechanisms driving sperm-seminal fluid cross interactions, we found that sneaker' and territorial male's ejaculates differ in seminal fluid protein content (quantitatively and qualitatively) and even in sperm quality, with sneaker sperm showing an higher oxygen consumption rate, a parameter rarely measured in sperm quality analyses. The deepening of sperm-seminal fluid proximate mechanisms is just at the beginning but I highlighted how the non-protein fraction of the seminal fluid (<3kDa) is crucial for sperm performance (velocity), despite even the protein fraction indicate a minor influence.

In this species, sperm velocity results are perfectly mirrored by *in vitro* fertilization tests, hence sperm velocity is a reliable indicator of ejaculate fertilization ability. Considering the paternity success recorded in the field during a previous work, it seems that it is the territorial males nest guarding that finally determines their fertilization success, and thus, the distance at which sneakers are forced to release their ejaculates. Indeed, territorial males fertilization success positively correlate with their body size.

In the black goby, where the ejaculates of competing males are released far from each other, seminal fluid does not affect the sperm performances of rival males, as expected. Despite the seminal fluid of territorial males significantly enhances their sperm speed, still sneaker sperm are significantly faster, regardless the seminal fluid present.

Results from *in vitro* fertilization tests, apparently do not mirror sperm performance, since sneaker and territorial males fertilization rates do not significantly differ, probably because the low number of trials. However, I evidenced that sneaker and territorial males sperm differ in their swimming mode, with territorial male sperm moving in a significantly more linear trajectory. As a consequence, it could take the same time to sneaker and territorial male sperm to travel the same distance, even if those of sneaker have an higher speed.

The analysis of the paternity distribution of territorial males in the field suggests that the distance at which sneakers are forced to release their ejaculates determines the number of eggs they fathered, as in the grass goby. Indeed, preliminary results from artificial nests in the field indicate that snakers stole more fertilizations close to the nest principal entrance, lowering the territorial male fertilization success in that area, but less in the rest of nest ceiling. However, territorial males parentage success is unexpectedly low, respect to that registered in the grass goby and across other fish species with a similar mating system. In addition, we found in two of four analysed nests few embryos sired by a neighbour territorial male. If the result would be confirmed by further analysis, it implies that territorial males may occasionally adopt sneaking behaviours, probably depending on the level of ejaculates competition determined by nests availability and male density. The territorial mating role would not appear as favoured as in other species with alternative mating tactics, especially considering that sneakers

visits more than one nest. Further studies should be addressed to the investigation of territorial male paternity success along the breeding season.

In both species, the spatial context in which the competition between ejaculates occurs proved to be important. The distance at which rival ejaculates are released determines the opportunity for the rival seminal fluid exploitation, and, consequently influences the strategy to maximize the fertilization success: through the number and/or the quality of the sperm, or taking advantage of the seminal fluid of a rival-tactic male.
The seminal fluid proven itself to be one of the factor that may tip the balance in the ejaculates competition scenarios, that need to be investigated with a comprehensive multidisciplinary approach.

INTRODUCTION

" […] a struggle between the males for the possession of females; the result is not death to the unsuccessful competitor, but few or no offspring " Darwin, 1859

1. Sexual selection

Sexual selection is the evolutionary process favouring the increase in frequency of genes that confer a reproductive advantage to the individuals carrying them (Darwin, 1859; Eberhard, 2009). Before genetic mechanisms came to light, Charles Darwin, reasoning on his idea of evolution by natural selection (Darwin, 1859; 1871), stressed out how some characters do not appear to directly favour the survival of their bearers. In his view, the origin and maintenance of traits such as the long colourful tails of some birds, not particularly fit to fly, the huge horns of some mammals, the animal's calls and displays possibly fascinating the potential partner but also conspicuous to predators, had to be attributed to a particular pressure: the sexual selection. The variability in the expression of sexual traits correlated with variance in mating success, and selection consequently arises through competition above mates or matings: *" the result is not death to the unsuccessful competitor but few or no offspring "* (Darwin, 1859).
The evolution and function of traits involved in sexual selection have been the primary focus of empirical and theoretical studies (Andersson, 1994; Jones and Ratterman, 2009; Shuster, 2009). Darwin proposed that sexual selection proceeds through two mechanisms: a) intra-sexual selection, i.e. the competition for mates occurring among the individuals of the same sex, usually males for the possession of females and/or resources related to reproduction, and b) intersexual selection, i.e. the mate choice performed by one sex on the individuals of the opposite sex. Although it has been shown that mate choice may occur in both sexes, the females are undoubtedly the sex most commonly choosing among the prospective mates (Cunningham and Birkhead, 1998; Andersson and Simmons, 2006). Male-male competition fosters the development of characters defined "weapons", giving an advantage in direct contests against rivals for mate acquisition (horns, claws, shields, badge of status etc.). The contests can take place under different ways, as scrambles to first find a mate, endurance to remain longer at a breeding site, fights over mates, that select for large size and weapons (Andersson, 1994). On the other side, female choice favours the development of traits defined as "ornaments"(colourful patches, refined vocal calls, courtship displays, etc..) whose exhibition catches females attention and preference. "Weapons" and "ornaments" have been together defined as "secondary sexual characteristics" (CSS) and advantage an individual over its rivals (during fights or courtship) without being directly involved in reproduction. Darwin distinguished them both from those sex differences directly employ in reproduction (as for gametes' production and transfer), namely "primary

sexual characteristics", and from sexual traits that differ between sexes due to ecological reasons, such as different feeding habits in males and females (Darwin, 1871; Andersson, 1994). Sexual selection fosters and models the evolution of CSS, even if Darwin first suggested that it may influence also some primary sexual traits, such as the shape of genitalia (Hosken and Stockley, 2004).

The understanding of intra-sexual selection was essentially complete since Darwin's work (Jones and Ratterman, 2009) and counts on several examples (Andersson, 1994), whereas the mechanisms underpinning inter-sexual selection were lately clarified, due to the scepticism that received the idea of female choice, at the beginning (Kirkpatrick, 1982; Andersson, 1982; Andersson, 1994). The choice of a partner involves costs, such as taking time away from feeding or higher exposure to predation risk, and consequently it must bring along some benefits to females. In several species, the expression of male CSS may signals the amount/quality of material resources that can be provided to females and/or offspring, increasing their fitness (direct benefits), such as for instance nuptial gifts to feed, resources linked to reproduction such as larger territories, more intense parental care, etc. (Andersson, 1994). Although choice for direct benefits conferred by males is very common, research on mate choice was heavily concentrated on species where direct benefits appear to be absent, as males don't make any material contribution to females or to the offspring, and contribute to reproduction just with their ejaculates. In these cases less straightforward models are required to explain female preference for more ornamented males. Chief among these explanations are the indicator or indirect-benefit models, such as the *good genes* model and the *runaway* model. The former proposes that the overblown sexual displays function as indicators of male quality (Trivers, 1972; Zahavi, 1975; Hamilton and Zuk, 1982; Andersson, 1994; Neff and Pitcher, 2005). Since extreme traits are costly to maintain, males that can afford greater displays, should excel also in survival, indicating an higher genetic makeup. Empirical evidence supporting this hypothesis have to demonstrate both the relationship between the male trait and his quality, and the hereditability of that trait (Neff and Pitcher, 2005). The so-called runaway model (Fisher, 1930; Kirkpatrick, 1986) posits if preference for a male trait has genetic components, consequently the offspring produced bears both the genes for choosiness and the genes for the male characteristic. The result may be a self-reinforcing selection process for extreme female preference and increasingly elaborated male traits, due to the genetic correlation. Direct evidence of this mechanisms are difficult, but modern molecular genetics coupled with evolutionary experiments offer new tools of investigations (Andersson and Simmons, 2006).

According to indirect benefits models, female choice acts as a directional selection and should drive the higher male traits expression to fixation. Although indirect genetic benefits of female choice are frequently reported, evidence are clearly showing that variance in CSS expression persists despite directional selection. As a possible resolution of this paradox (the *lek paradox*, Borgia, 1979; Taylor and Williams, 1982), Hamilton and Zuk (1982) proposed that the successful development of sexually selected traits signal

resistance to parasites: the host-parasite co-evolution entails a fluctuating selection that justify the continuous tailoring of these traits. Another hypothesis, dealing with the lek paradox, is the genic capture hypothesis (Rowe and Houle, 1996), based on the assumption that CSS depend on individual physical condition. Individual condition summarizes a large number of genetic loci, such as those involved in metabolism, muscular mass, nutrition, etc., thus condition dependence of CSS expression would maintain genetic variation despite of a persistent female choice.

Since Darwin set the stage, the study of sexual selection has received great attention and still the explanation of the detailed mechanisms by which sexually selected traits arise and vary represent one of the major challenges of biological researches. Overall, the individuals with the greater expression of CSS are expected to achieve the higher reproductive success. Intra and inter sexual selection may occur across most diverse mating systems, however, it is in species where the competition success is critical since few individuals monopolize the majority of matings (polygynous species) or in promiscuous species where mating success may be highly variable that "armaments" and "ornaments" are often markedly developed and sexual selection pressure is strong. Considering that sexual selection arises from differences in mating success it requires sexual reproduction but it does not necessarily requires different sexes.

However, it is exactly the different investment in gametes of male and females, namely "anisogamy", that is believed to have set the basis for the evolution of sex differences in morphology and behaviour (Trivers, 1972; Parker et al., 1972; Clutton-Brock and Parker, 1992). It has been suggested that once sexuality has arisen, anisogamy evolved from isogamy through disruptive selection on gamete size and number, with females producing few large and immobile eggs and male investing in tiny, numerous and mobile sperm (Parker et al., 1972; Gage and Morrow, 2003). In 1948, Bateman described the different strength of sexual selection in the two sexes through the relationship between mating success and offspring production. In particular, he compared fecundity versus the mating success, showing that the number of offspring sired by a male increases with the number of matings he achieves, while female fecundity does not significantly increases with the number of partners she copulates with. It is a question of quantity versus quality: whereas males could leave more descendants, a female could increase only offspring's quality. Consequently, it appears clear that a conflict between sexes lies on contrasting evolutionary interests in front of the diverse potential reproductive rate of the two sexes (Parker, 1979; 2006; Tregenza et al., 2006), since males' success is potentially higher and more variable than females. As a consequence, the intensity of sexual selection is higher on males than on females, leading to a strong male-male competition for the highest possible number of mates. The asymmetry between male and female reproductive interests was already noticed by Darwin, who described females as coy and choosy while males are more competitive and less exacting: "*the female, on the other hand, with the rarest exceptions, is less eager than the male*" (Darwin, 1871). This view partly accounts for the initial male-orientated approach of the

new born sexual selection theory. Indeed, at the beginning, the study of its mechanisms was likely influenced by a cultural bias due to the "Victorian age prudery", as T. Birkhead often highlighted (2010). The investigation of the different aspects of sexual reproduction was bound by the "common sense". Female mate choice found a wary and cold scientific audience in a period in which women did not have the voting right and even Darwin, along his dissertation about sexual selection mechanisms, seemed to strategically avoid unseemly topics. He was up-to date about process of insemination, sperm function and fertilization and even described extra-pair copulations (Darwin, 1871). Despite his wide overlook and detailed knowledge on mating systems, Darwin focused his research on pre-copulatory mechanisms of sexual selection, without probably never made a leap behind the mate acquisition, and thinking about the possibility that sexual selection could proceed after the insemination.

"Why would a male fly wait to court a female until after he has already achieved his evolutionary objective of copulating with her?" Eberhard, 2009

2. "The polyandry revolution": from pre-copulatory to post-copulatory sexual selection

One century later Darwin's detailed presentation of sexual selection mechanisms, other behaviours and morphological characteristics stood out to be barely understandable under the exclusive light of partner acquisition. Sexual traits, such as the peculiar shape of male and female genitalia, the mating plug, gluing female genital opening after the copulation, the differences in sperm morphology between and within species, or the mate guarding performed, in some species, by males after the copulation, caught researchers attention (Parker, 1970; 2006; Trivers, 1972; Birkhead, 2000; Birkhead et al., 2009). The historical notion of monogamous females, pair-bonded with the same male for life, or at least for a breeding season, has been gradually eroded away by the increasing evidence that multiple paternity is common in the natural litters, clutches, and broods of the most diverse taxa (Andersson, 1996; Taylor et al., 2014). Moreover, females of several species have been demonstrated to actively seek multiple copulation partners within a breeding cycle (Parker and Birkhead, 2013). As a result, female multiple mating is now accepted as an ubiquitous phenomenon in nature, triggering a great theoretical and experimental effort to highlight its evolutionary consequences (Taylor et al., 2014). A major implication of female promiscuity is that sexual selection may persist after the copulation up to the point of fertilization (Birkhead and Pizzarri 2002), entailing the ejaculates of more than one male to overlap in space and time. In 1970 G. Parker outlined the pattern of post-copulatory male-male competition, in insects, making the scientific community awake of the importance of mechanisms that occur after the copulation per se. Sexual selection theory widened after mating gave meaning to diverse traits and behaviours, such as mate guarding and mating plugs, as

males' expedients to avoid ejaculates competition. The keystone paper of Parker paved the way for further studies on post-copulatory sexual selection mechanisms, that since then is sparking interest in evolutionary biology research.

The benefits gained by males from mating multiply do not raise particular theoretical problems. Indeed, according to Darwin-Bateman paradigm (Darwin, 1871; Bateman, 1948; Dewsbury, 2005), males fitness is expected to steeply increase, compared to that of female, with number of mating acquired. This prediction is generated ultimately by anisogamy, with single sperm being less costly to produce respect to single egg and thus with male usually producing an enormously higher number of gametes than females. By contrast, the possible benefits that females may acquire in mating with more than one male are less intuitive. Bateman (1948) measured the reproductive success of male and female fruit fly *Drosophila melanogaster*. For a male, the more females he copulated with, the more offspring he fathered, but for females, reproductive success did not change regardless of the number of partners she had. However, through his experiments, Bateman had been forced to change the larval growth medium, keeping separately the results of male and female promiscuity tests. The tests where larvae had limited food, showed that females did in fact benefit, albeit not as much as males, from copulating with more than one partner. Female polyandry at the beginning was thought to be a non-adaptive by-product of the positive selection for promiscuity genes on males whereas, actually, in many species, females actively hunt for copulations with several males (Birkhead and Pizzari, 2002).

Possible direct benefits include nutrient acquisition, owing to nutrients in the ejaculate or nuptial gift, more paternal care and protection (Arnqvist and Nilsson, 2000; Birkhead and Pizzarri; 2002; Fisher et al., 2006). Polyandry can also improve female fertility, namely as increased egg production, reduced risk of male harassment, change partner for a better quality male, adequate sperm supply. The latest may be particularly important in externally fertilizing species, in which sperm numbers can be limiting, but also in internally fertilizing species, for example if insemination fails, or if males may become sperm depleted or allocate sperm strategically (Petrie et al., 1992; Reynolds 1996; Levitan, 1998). On the other hand, indirect benefits regard those advantages that are headed for the offspring, and post-copulatory mechanisms are more likely to increase the chances of finding 'good' or compatible genetic sires. Consistent with this, several empirical and theoretical studies have shown that polyandry can evolve in response to genetic incompatibility (Jenninson and Petrie, 2000; Zeh and Zeh, 2001; Tregenza and Wedell, 2000).

Clearly, promiscuity awareness brought into focus the post-mating variance in male paternity share rather than mating success, and fostered the switch from pre-copulatory to post-copulatory sexual selection. Copulation might not correspond to successful insemination and insemination might not result in proportional fertilization of the eggs, but the mating success of an individual may be influenced also between the copulation and fertilization.

Post-copulatory sexual selection takes shape under the mechanisms of cryptic female choice and sperm competition. The first one refers to the ability of a female to bias the fertilization success of the males that she copulates with (Eberhard, 1996), on the other hand sperm competition occurs whenever the ejaculates of two or more males compete to fertilize the same group of eggs (Parker, 1970). Cryptic female choice and sperm competition generate an amazing diversity of morphological, behavioural and physiological adaptations, that, by now, have been extensively demonstrated across numerous species and taxa (Moller and Briskie, 1995; Birkhead and Møller, 1998; Hosken and Stockley, 2004; Gomendio et al., 2006; Snook, 2005; Birkhead et al., 2009).

In general, female choice is directed towards those male traits, morphological, behavioural and physiological (ejaculates composition), that better enhance her reproductive success. However, males adaptations direct to favour the use of their own sperm may come in conflict with female interests on processes such as oviposition, sperm utilization, re-mating behaviours etc (Parker, 2006). For example, in the bluehead wrasse, *Thalassoma bifasciatum*, territorial males pair with spawning females throughout the day, and strategically reduce the sperm released at each mating to maximize the number of partners they can accept. As a results, not all females' eggs will be fertilized, as male fertilization success is 95-98% on average at each single mating (Warner et al., 1995). The conflict between sexes for the control of the fertilization outcome may lead to an arm race described by sexually antagonistic coevolution theory, where males' coercion drive females' responses to restore some control over who fertilizes their ova, resulting in additional selection on males to evolve counterstrategies (Holland and Rice, 1999; Hosken et al., 2001).

Furthermore, it's currently debated the extent to which post-insemination sexual selection may catalyst speciation. The potential for species divergence is dependent on the strength of inter-sexual conflict. In fact, post-copulatory sexual selection drives the evolution of many male and female reproductive traits that coevolved in a continuous cut and thrust, because a certain grade of cooperation is anyway required for sexual reproduction. The spread of alleles that allow one sex to reach the phenotypic optimum may not coincide with the other's interests, open to the possibility that a population goes towards reproductive isolation and then speciation. Moreover, speciation rate is relatively higher across clades that mainly show potential for post-copulatory sexual selection (Birkhead and Pizzari, 2002).

Recently, the study of post-copulatory sexual selection endeavoured the deepening proximate mechanisms driving cryptic female choice and ejaculates competition. The importance of sperm-egg compatibility was already known to be one of the factor determining male fertilization success, even in non-competition conditions (Kosman and Levitan, 2014). The possible mediation of seminal and, less, the ovarian fluid in the gametes interaction started to be considered in post-copulatory sexual selection mechanisms (Rosengrave et al., 2008; Gasparini and Pilastro, 2011). When and how their

presence may affect the male and female reproductive success are appealing questions, that triggered theoretical and experimental studies.

"Nevertheless, when we see many males pursuing the same female, we can hardly believe that the pairing is left to blind chance.." Darwin, 1871

3. Cryptic female choice

In pre-copulatory sexual selection, during male-male competition and female choice the contests or displays point out and select for best male qualities, in terms of direct (survival or reproductive resources) or indirect benefits (genes quality). In the last 30 year, the evidence that female multiple mating is common among species led up to think that the ability to bias the fertilization success of males a female copulated with may have evolved also after the copulation. The mechanism known as cryptic female choice has been clearly defined by Eberhard (1996), who drafted the potential strategies that females could adopt to favour the ejaculate of a certain male above others. Cryptic female choice, therefore, is the ability of a female to influence the paternity success of multiple partners after mating. It is 'cryptic' because the choice takes place hidden in female reproductive tract. However, copulation, insemination and the subsequent performance of an inseminated ejaculate depend on the complex interaction between male-driven and female-driven processes, the effects of which are difficult to disentangle (Lüpold et al., 2013). Moreover, in some species, male coupulatory behaviours may in turn mold female responsiveness. In the red flour beetle, *Tribolium castaneum*, males rub their legs on the lateral edges of the female wing cases; the intensity with which a male carries out this behaviour is positively correlated with the fertilizing success of his ejaculate in competition with that of a rival male (Edvardsson and Arnqvist, 2000). Cryptic female choice can result in directional or non-directional sexual selection. In directional cryptic female choice, females are expected to favour the ejaculates of the male that carries the phenotype preferred in pre-copulatory mate choice. In feral fowl, *Gallus gallus domesticus*, for example, females prefer socially dominant partners but cannot avoid some copulation with subdominant males. However, females expel ejaculates immediately after insemination, with a probability that is negatively correlated with the social status of a male (Pizzari et al., 2002). Instead, in non-directional cryptic female choice, the preference is directed towards the sperm of the males with compatible genotypes, regardless the phenotype borne. In *Scathophaga stercoraria*, females under experimental stable conditions favour fertilizations from genetically similar males, whereas if exposed to a more variable environment, generate heterozygous offspring, that, on average, might have a better chance to survive (Ward, 2000).

Thereby, cryptic female choice cannot be only a response to males harassment resulting from their higher reproductive rate. In fact, in some species females actively seek for multiple partners and gain some benefits in terms of reproductive success (direct as resources, indirect as genes quality). For example, a recent study of the cricket *Gryllus campestris* reported that, in a natural population, males and females show a positive and similar relationship between the number of mates and the lifetime reproductive success (Rodríguez-Muñoz et al., 2010).

For what concerns the mechanism of cryptic female choice, the action on different ejaculates may be random, where the effect is equal for all competing sperm, or selective, and sperm from different males are differently treated (Parker and Pizzari, 2010). Males may respond strategically allocating sperm in order to maximize their fertilization success, depending on the mechanisms of cryptic female choice. In particular, if the selection on ejaculates is random, the number of sperm allocated is expected to increase with the number of sperm killed by the female reproductive tract (Greeff and Parker, 2000). If female selection is biased towards preferred sperm, males that know to be in the favoured position are expected to allocate less sperm than disfavoured rivals, and, particularly, the theoretical models vary if favoured or disfavoured roles are fixed or random (Ball and Parker, 2003; Parker and Pizzari, 2010) (see section 4).

Cryptic female choice is difficult to be experimental demonstrated and how female may bias sperm utilization is still harshly discussed. Selection can favour female reproductive traits that are able to bias fertilizations towards either 'preferred' (Thornhill, 1983) or genetically compatible mates (Gasparini and Pilastro, 2011). The attention has been recently focused on the role of fluid and other egg chemical signals, already known to direct sperm race toward the eggs, and may potentially mediate sperm choice (Evans et al., 2013). Ovarian fluid has been demonstrated to upregulate sperm swimming velocity in salmonids (Butts et al., 2012; Galvano et al., 2013) and other externally fertilizing fishes (Elofsson et al., 2006). However, in the Chinook salmon, *Oncorhynchus tshawytscha*, the ovarian fluid has been indicated as a potential arbiter of cryptic female choice for genetically compatible mates (Rosengrave et al., 2008) but not through the influence on sperm swimming velocity that is the primer determinant of male fertilization success in this species (Evans et al., 2013).

Ovarian fluid may also play a role in avoiding hybridization, as demonstrated in the Atlantic salmon, *Salmo salar*, and the brown trout, *Salmo trutta*, where it promotes fertilization by the conspecific sperm. In particular, only conspecific ovarian fluid doubled sperm motile life span and straightened swimming trajectory, guaranteeing chemoattraction towards eggs (Yeates et al., 2013). Cryptic female choice can thus promote also reproductive isolation, influencing sperm swimming behaviours through ovarian fluid.

"One of the most fruitful directions for future research"
Parker, 1970

4. Ejaculates competition

Female polyandry leads up to think that male mating not necessarily results in successful insemination, especially when the ejaculates of rival males overlap in space and time. Sperm competition has been firstly defined by Geoffrey Parker as the competition between the ejaculates of two or more males to fertilize the same set of eggs (1970). To exclude the possible contribution of cryptic female choice, now a more strictly definition specified that sperm competition occurs when there is a direct action by a male or his semen on the sperm of another male (Eberhard, 2009). Sperm competition is investigated since forty years, in both externally and internally fertilizing species, proving to be highly widespread across taxa and standing out as powerful evolutionary force shaping males' behaviour, morphology and physiology.

Sperm competition may flow into two opposite selective pressures: influencing male abilities to prevent any female they copulate with from re-mating and/or to overcome any rival ejaculate already present. The arising male adaptations are referred to as 'defence' and 'offence' strategies according as they are aimed at avoiding rival ejaculates or at increasing fertilization success under direct competition, respectively. The *Drosophila pseudoobscura* species offers a good view of both mechanisms. Males transfer to female a set of seminal substances together with sperm. These consist of peptides, enzymes, prohormones (released from the males accessory glands) some of which deactivate the sperm already stored by female in their reproductive tract (offence), while others work as anti-aphrodisiac, preventing the female from re-mating (defence) (Birkheadd and Møller, 1998). One of the most common pre-insemination ruse adopted by males to prevent direct ejaculates competition is mate guarding, quite widespread in birds and insects. In some of these species, males remain close to the female, sometimes in genital contact, until the deposition of the eggs or up to the end of female receptive period (Parker, 1970). Another attempt to exclude competing ejaculates is represented by the application of mating plugs, that are known from almost all animal groups (Birkhead and Møller 1998). Mating plugs are made of diverse substances produced by accessory gland and turn the male loose from guarding the partner, since they mechanically block female genital openings after copulation, interfering with female polyandry (Birkhead and Møller, 1998; Simmons, 2001; Simmons and Fitzpatrick, 2012). A prolonged copulation too may allow to exclude a direct ejaculate competition, being particularly efficient when ì) mating precedes immediately the eggs' fertilization; ìì) the last male to mate fathers the majority of the offspring; ìîì) the male density is high and rivals' rejection is effective in preventing 'take-over' behaviours (Parker, 1970; Birkhead and Møller, 1998).

To increase fertilization success in direct ejaculate competition males may scrape out rival sperm from the female reproductive tract (Simmons, 2001), displacing and/or replacing it (e.g. Gack and Peschke, 1994) with their ejaculate (Diesel, 1990). The active removing of other males' ejaculates is, in some species, associated with morphological adaptations of male copulatory organs, such as spines or hooks (Crudgington and Siva-Jothy, 2000). However, the trait more commonly shaped by sperm competition is ejaculate investment, representing the first and the last mean for gaining fertilizations (Simmons and Fitzpatrick, 2012). The work on ejaculate allocation in response to sperm competition has been mainly focused on the sperm component and only recently the possible variability of seminal fluid has begun to be investigated.

"Sperm: solders in the battle for fertilization"
Simmons and Fitzpatrick, 2012

4.1 The Sperm

The evolutionary responses in sperm investment focused on i) sperm expenditure (e.g. sperm ejaculated at a given reproductive event) under different sperm competition scenarios and ii) sperm quality characteristics.

Male sperm expenditure

The initial sperm competition models referred to sperm expenditure as ejaculate expenditure, ascribing predictions on sperm investment to the whole ejaculate. According to them, ejaculates are costly to be produced and males may run out of sperm and seminal fluid for successive copulations, so they have to strategically allocate their ejaculate in order to maximize the fertilization success during successive matings and according with the competition conditions. In detail, a male is expected to fine-tune his ejaculate investment depending on the fertilization success he could aim to, that is determined by female quality and sperm competition context (Birkhead and Møller, 1998; Parker and Pizzari, 2010) (Fig.1).

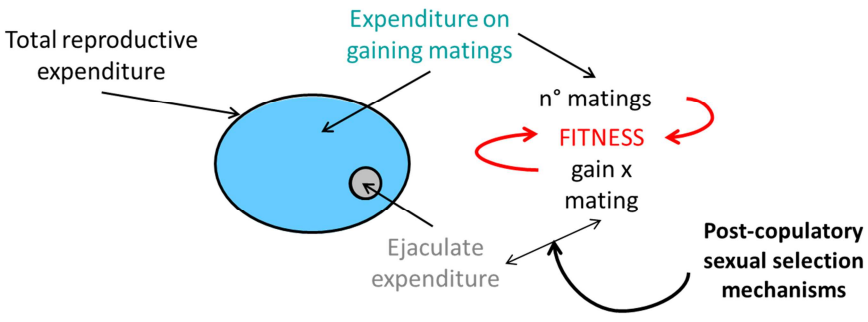

Fig.1 Representation of the different contributions to male reproductive investment (reviewed from Birkhead and Møller, 1998)

The mechanisms by which sperm compete vary in different species and determine how ejaculates contrast against each other. In fact, competition scenarios may range from "fair raffles", where all males have the same chance to fertilize a given set of eggs, to volumetric displacement, where the ejaculate of a male displaces the rival ones. A key factor is represented by the arena available for ejaculates competition. Therefore, in species with external fertilization there are minimal space constraints, while internal fertilizers are limited by the size of female storage organs (Parker and Pizzari, 2010).

Historically, first pioneering theoretical models regard "fair raffle" scenarios. They were developed by Parker to predict how males should respond i) to the probability that females mate with more than one male, defined as sperm competition risk (Parker, 1970), and ii) to an increasing number of rival ejaculates, named as sperm competition intensity (Parker et al., 1996; 1997; Parker, 1998). Risk models work at low levels of sperm competition, when females are supposed to mate with just two males, while intensity models are designed for competitive scenarios with more than two rival males. According to these models, as the extent of sperm competition boosts, males' investment in the ejaculate should increases across species. Thus, in monogamous mating systems males are expected to invest low in the ejaculate, maximizing their survival to advantage of future reproduction, while the enhancement of the ejaculate expenditure is predicted as the degree of polyandry increases. Extensive empirical evidence, supporting theoretical predictions, came from comparative studies, across both species and populations of the most diverse taxa, showing that an increase in the level of sperm competition is paralleled by a greater ejaculate investment as judged by relative testis size and sperm number (Harcourt et al., 1995; Gage, 1994; Stockley et al., 1997; Birkhead and Møller 1998; Hosken and Ward, 2001).

When resources, mates, or locations can be monopolized, males may adopt alternative reproductive tactics (ARTs), where individuals that have an advantaged reproductive condition (dominant, territorial), for instance, due to their size or status, control the majority of the matings or of the reproductive resources. By contrast, males that, due to a disfavoured condition, cannot fight for and court females adopt opportunistic behaviours, that may involve agonistic behaviours and conflict (sneaker, streaker males), but also cooperation among competitors and/or with the dominant male (satellite males) (e.g., subordinate males pay a cost to dominant males to access to a part of fertilizable eggs) (Taborky, 2001). As a consequence, opportunistic males has always to face at least with the ejaculate of one male: sneakers/streakers/satellites with that of the dominant/territorial one and group spawners with those of the others (Taborsky, 2001). Alternative male mating tactics, then, generate different levels of sperm competition, that may lead to different strategies in the ejaculate allocation. Opportunistic males, playing the tactic associated with the highest level, have been proved to invest relatively more in sperm than do males that experience lower risk (e.g. Shapiro et al., 1994; Gage et al., 1995; Locatello et al., 2006; Fitzpatrick et al., 2007; Montgomerie and Fitzpatrick, 2009; Petersen and Mazzoldi, 2010). Support to

theoretical models came also from evolutionary experimental studies, showing that testis size responds to variation of the sperm competition level perceived. For instance, in the yellow dung fly, *Scathopaga stercoraria* (Hosken and Ward, 2001), *Drosophila melanogaster* (Pitnick et al., 2001) and the dung beetle, *Onthophagus taurus* (Simmons and Garcı´a-Gonza´lez, 2008), experimental populations forced to monogamous matings show the decrease of the testis size respect to populations reproducing in polyandrous conditions, over multiple generations.

When several ejaculates compete simultaneously for the same set of eggs, theoretical models predict that an individual male facing variable levels of sperm competition among successive spawns or matings should release fewer sperm as the estimated number of competitors at a given spawning increases above two, because of diminished returns from providing more sperm (the so-called ‘‘intensity model’’; Parker et al., 1996). This counterintuitive males' response is due to the fact that in spawns with several competitors the chances of encountering an unfertilized egg are too low to favour the release of additional sperm. In other words, if a male can strategically allocate sperm among spawns, an increase in output will profit more when the intensity of sperm competition is low. This prediction was demonstrated across different species (Simmons and Kvarnemo, 1997; Thomas and Simmons, 2008), for instance, in two gobies species with alternative mating tactics, the grass and the black goby (*Zosterisessor ophiocephalus*; *Gobius niger*). In both species, it has been experimentally showed that the sperm expenditure of a sneaker peaked when, in addition to the dominant male, only another sneaker was present, then decreased when other two-four additional sneakers participated to the same mating (Pilastro et al., 2002). Sneakers, however, during the experiments, were always competing with the territorial male, that could be considered as an additional competitor. Therefore, the situation was partly different from the fair lottery envisaged in the theoretical models (Parker et al., 1996), and territorial males do not seem to similarly vary sperm investment, but respond increasing aggressive behaviours. Sneakers' sperm allocation strategy is apparently influenced only by the presence of other males adopting the same tactic. If the fraction of eggs cuckolded by sneakers in a spawning does not increase with the number of sneakers, these results are perfectly consistent with the pattern outlined by Parker's intensity model.

Most of theoretical predictions about sperm allocation strategy (sperm expenditure on a given ejaculate) assumed that ejaculates competition is played on the basis of a “fair raffle”, where all rivals compete on equal terms. However, looking at different mating systems, more than one factor can bias competition conditions, resulting in a favoured or disfavoured mating positions or roles. Following analysis concerned the way in which “unfairness” in the fertilisation raffle would affect sperm allocation. Ball and Parker (2003) modelled the possible influence of cryptic female choice on male response. In fact, female action may not only indiscriminately test all ejaculates present, but rather select for specific characteristics, in terms of direct (reproductive resources)

or indirect benefits (offspring's quality). As a result, males would stand in a favoured or disfavoured mating role, depending on the female choice. This hypothesis generate two different scenarios conditioned by assumptions on male mating position, if fixed or random. In the random roles version, all males are equal, since they can mate both in the favoured or disfavoured position. Individuals are expected to allocate more sperm when mating in the favoured position. On the other side, in the constant role version, males are unequal, and the advantaged role always allocates less sperm than the disfavoured one. Roles may reflect the order of mating, first or second male to mate with a same female. In many insect species, for example, the last male to mate fathered the majority of the eggs, due to sperm displacement mechanisms against previously inseminated ejaculates (Parker, 1970; Birkhead and Møller, 1998; Simmons, 2001; Parker and Pizzari, 2010). Alternatively, male roles may be fixed by the male phenotype (Gage et al., 1995; Cunningham and Birkhead, 1998; Taborsky, 1998). A perfect example is offered by species with alternative mating tactics (ARTs). In fact, as previously said, sneaker males mate always in a disfavoured position since they have to face always at least with the territorial male ejaculate. In this sperm competition context, a given male phenotype always faces a given role, and the adopted tactic defines the sperm allocation strategy (Mazzoldi et al., 2000; Vladić and Järvi, 2001; Rasotto and Mazzoldi, 2002; Neff et al., 2003; Rudolfsen et al., 2006). It has to be highlighted that a key factor for model predictions is whether or not males can assess if they are mating in favoured or disfavoured role: in species whit ARTs the sperm expenditure response is fixed by the male phenotype, offering a clear model to test other possible influences on sperm ejaculate investment.

Sperm quality traits

According to theoretical predictions, the outcome of ejaculates competition is mediated largely by the investment in sperm number and in its strategic allocation among different matings (Birkhead and Møller, 1998; Wedell et al., 2002). However, the relative sperm number alone does not always account for the male fertilization success, as suggested disparate studies, where the variance in paternity success among males could not be explained solely by ejaculates size (Birkhead and Møller, 1998; Simmons, 2001). As a consequence, excluded female influence, attention was focused on sperm quality traits, such as sperm size, longevity, viability, mobility, and velocity all potentially affecting fertilization efficiency (Birkhead et al., 1999; Simmons et al., 2003; Gage et al., 2004; Casselman et al., 2006; Lupold et al., 2012; Simmons and Fitzpatrick, 2012). These parameters were known to vary among males, but only recently began to be analysed in relation to sperm competition level, after controlling for sperm number (Birkhead and Møller, 1998; Snook, 2005; Immler et al., 2010; Fitzpatrick and Baer, 2011). Sperm quality traits, affecting male competitiveness, have been reported to vary not only between external and internal fertilizers, but even among species adopting the same mating strategy, up to differ, at intraspecific level, also between males adopting

alternative mating strategies (Froman et al., 2002; Stoltz and Neff, 2006; Locatello et al., 2007). Sperm competition is thus expected to influence those traits driving sperm fertilization capabilities depending on a specific context and the dynamics of fertilization.

Sperm size and morphology strongly vary across species (Cummins and Woodall, 1985; Gage, 1994) and within species (Ward, 1998; Morrow and Gage, 2001). Within *Drosophila* spp., sperm length exhibits over a 100-fold difference between species (Snook, 1997). Indeed, sperm competition might influence sperm size since longer sperm are expected to have faster sperm swimming velocity (Gomendio and Roldan, 1991; Byrne et al., 2003), or increased survival (Parker, 1998). Few species of *Drosophila* show unexpected egg-like giant sperm: males produce few and the largest known sperm, the direction of post-copulatory sexual selection is reversed from the traditional male strategy to produce many tiny gametes (Pizzari, 2006). Analysis highlighted that in these species females present higher variability in reproductive success together with a stronger selection for remating behaviours, both similar to males' values. In fact, if males inseminate few giant gametes some eggs may fail to be fertilize pushing females to mate with more males to ensure a sufficient sperm supply. Sperm size has been proved to be driven by the evolution of females storage organs in *Drosophila melanogaster*, and in particular large storage organs may select for longer sperm (Miller and Pitnick, 2002; Bjork and Pitnick, 2006). However, sperm morphology show an inconsistent relationship with fertilization success and sperm competition level, even if the negative relationship between sperm length and fertilization success is prevalent in studies using natural matings despite *in vitro* fertilization tests (Simmons and Fitzpatrick, 2012).

Sperm longevity and/or viability are important for animal fertility (Dziuk, 1996; Linhart et al., 2005), but results about their relation with the level of sperm competition are not uniform, due to the lack of studies. However, sperm viability seems to influence sperm fertilization success in competition contexts. In fact, a comparative study across insects found that promiscuous species produce more-viable sperm than monogamous species (Hunter and Birkhead, 2005), and sperm viability is positively related to sperm competition among Australian wrens (Rowe and Pruett-Jones, 2011). Among external fertilizing fish species with ARTs, sneaker males show lower sperm viability than territorial ones in the bluegill sunfish *Lepomis macrochirus*, (Burness et al., 2004) whereas they have higher sperm viability in the Atlantic salmon *Salmo salar* (Gage et al. 1995) and in the corkwing wrasse *Symphodus melops* (Uglem et al., 2001).

Sperm velocity and mobility appear to be positively related to fertilization success in both competitive and non-competitive scenarios. Comparative studies found out that species experiencing greater sperm competition level have faster and more motile sperm, in fish, birds and mammals (Froman et al., 2002; Gage et al., 2004; Birkhead et al., 1999; Malo et al., 2005). Moreover, few studies reported that in species with ARTs, sneakers sperm swim faster (Locatello et al., 2007; Montgomerie and Fitzpatrick, 2009;

Pitnick et al., 2009a). In *Gallus gallus domesticus* sperm mobility positively correlates with fertilization success: in this species subordinate males, that mate in a disadvantageous role, show higher sperm swimming velocity than dominant ones (Froman et al., 2002). Similarly, also different investment in sperm velocity has been shown in the Artic charr *Salvelinus alpinus* where males that become dominant produce less sperm with lower velocity (Rudolfsen et al., 2006). A positive relation between sperm velocity and ejaculate fertilization success has been demonstrated also in the Atlantic salmon *Salmo salar* (Gage et al. 2004) but with no difference between males exhibiting different mating tactics (Vladi and Järvi, 2001). Intuitively, faster sperm are expected to be more competitive, since they may reach the eggs more quickly than slower ones. However, in a recent work on *Peromyscus maniculatus* it has been found that the increasing velocity of sperm is not due to a change in speed, but rather to a travel with a more direct path (Fisher et al., 2014). The subtle difference lays in how sperm motility is evaluated, if considering the motion in a flat surface or in a three-dimensional space. If the higher speed follows through a motion that is far to be along a linear path, the whole travelling could be less efficient than swimming slower but along a more linear way. The study, combining mathematical and experimental analysis, reveals the importance of the relationship between swimming mode and sperm velocity, suggesting that sperm movement may interact with evolutionary selective pressures in competitive environments. Path trajectory, measured as linearity (LIN), has already been found to be related to fertilization success and under post-copulatory sexual selection pressure, in different species. In the chinook salmon, *Oncorhynchus tshawytscha*, females differentially enhance the swimming speed of sperm of preferred males through ovarian fluid mediation, and path linearity show a positive correlation (Roserngrave et al., 2008). Similarly, in the Arctic charr (*Salvelinus alpinus*) the presence of ovarian fluid increases sperm longevity, swimming speed and linearity of sperm trajectory (Turner and Montgomerie, 2002). In the three-spined stickleback *Gasterosteus aculeatus*, sexual ornamentation (breeding coloration) was found to positively correlate with sperm velocity and linearity and suggested to act as a possible proxy for male's fertilisation ability (Mehlis et al., 2013). Sperm linear movement might be particularly relevant in external fertilizer species, in which the competition arena is not limited by the female reproductive tract, and reaching the eggs faster and in the smaller space can be crucial for the male fertilization success.

In addition, sperm quality traits show a remarkably plasticity in response to variable competition environment. In the Artic charr, *Salvelinus alpinus*, and in the fowl, *Gallus gallus*, changes in social status are accompanied by a reduction of sperm velocity of the new dominant male respect to socially subordinate males, due to the reduced levels of sperm competition (Rudolfsen et al., 2006; Cornwallis and Birkhead, 2007). Instead, in the swordtail *Xiphophorous nigrensis*, males adopt alternative mating tactics and sneakers strategically enhance their velocity when paired with other sneaker males (Smith and Ryan, 2011). Males of the Gouldian finches, *Erythrura gouldiae*, are able to

strategically adjust sperm morphometry (sperm midpiece and flagellum lengths) to variation in post-copulatory selective pressures, produced by modification of social conditions (Immler et al., 2010).

Since resources allocated to reproduction are limited and sperm number must exceed the threshold value required for reproduction, trade-off are expected between sperm number and quality traits and among quality traits, themselves (Parker, 1993; Ball and Parker, 1996; Moore et al. 2004). However, whereas increased sperm number appears to be an almost ubiquitous solution to sperm competition, it remains undetermined whether there are patterns to describe the response of sperm quality traits to different sperm competition scenarios (Fitzpatrick and Lupold, 2014). Up to now, empirical evidences cannot be summarized through a unique model since they are contrasting and sometimes differing from theoretical predictions (Stockley et al., 1997; Simmons and Fitzpatrick, 2012; Smith, 2012). The discrepancy between theory and results could be due to several factors, such as i) incorrectly associations between sperm traits (i.e. phenotypic correlations not always allow to infer genetic relationships); ii) the use of different sperm competition measures, iii) possible female influences, for example the influence of ovarian fluid deserves attention since the effect it may have on sperm traits; iv) experimental conditions can also bias the correlation of quality traits with the sperm fertilization success and the relationships between traits themselves (Snook, 2005). In fact, the contribution of sperm number and/or different quality traits to the male final fertilization success could be complex to disentangle in a given competition scenario. Attention should be paid to the context in which competition takes place, especially in external fertilizing species, where ejaculates can be blended at different degree, depending on the environment and the dynamics of fertilization. In the bluegill *Lepomis macrochirus*, for instance, competition trials revealed that sperm from males mating in a disfavoured role (sneakers), based on proximity to the eggs and timing of sperm release, have an advantage over those from males mating in a favourite role (territorials). In particular, sperm number strongly influences fertilization success, but, independently from sperm number, sperm from sneakers have a competitive advantage over those from territorials, apparently not depending on the sperm quality traits measured in the experiment (Stoltz and Neff, 2006).

''Despite this […] complexity 'ejaculate' and 'sperm' are frequently used synonymously in the literature'' Perry et al., 2013

4.2 Seminal fluid

Since recently the study of sperm competition selective pressure on ejaculate investment has mainly focused on sperm production, allocation, and quality, with the terms 'ejaculate' and 'sperm' frequently used as synonymous (Wedell et al., 2002).

However, the ejaculate consists of gametes and seminal fluid, a biochemically complex mixture of proteins, peptides, salts and sugars, defensive compounds, lipids, water, and microbes (Poiani, 2006) and the sperm component alone cannot fully describe the functionality of the whole ejaculate. To date, increasing evidence are suggesting that the predictions on the outcome of sperm competition should not revolve only around the sperm component, but the biological function and the molecular make-up of the seminal fluid have to be included (Pojani, 2006; Chapman, 2008; Wigby et al., 2009; Simmons and Fitzpatick, 2012; Perry et al., 2013). Seminal fluid often makes up a large part of an ejaculate and plays crucial role in male fertilization success. In fact, the seminal fluid takes part in different fitness-relevant processes, such as sperm fertilization ability, sperm storage and egg fertilization, by controlling pH, nourishing and protecting sperm inside the female reproductive tract and storage organs and favouring sperm movement (Chapman, 2001; Alavi and Cosson , 2005; Wolfner, 1997; 2002). On the whole, seminal fluid may influence paternity success both i) directly, by enhancing male sperm performance (Poiani, 2006), and ii) indirectly, affecting females' physiology (Poiani, 2006; Wigby et al., 2009).

Direct effects on male fertilization success

Seminal fluid contains sugars and other compounds that are important for sperm maintenance and nourishment (reviewed by Gillott, 1996; Poiani, 2006). More recently, the attention has been focused on its proteic components (Simmons and Fitzpatrick, 2012) as seminal fluid proteins (sfps) have been proved to directly influence sperm viability (den Boer et al., 2008; Simmons and Beveridge, 2011) and motility (Poiani, 2006) in different species. Moreover, proteins are involved in sperm capacitation through female genital tract, in enhancing sperm survival, and in modulating sperm-eggs interactions (Clark et al., 2006). Other proteins are involved in sperm storage mechanisms, for instance, large glycoproteins were identified with this function in insects (Wolfner, 1997; 2002). Particularly, in insects, male accessory glands secrete molecules helping the sperm transfer to the spermathecal while other proteins contribute to sperm motility (Gillot, 1996; Chapman, 2001). Seminal fluid proteins and proteases with inhibiting capacity, components of the immune systems and molecules have been demonstrated to contribute to sperm protection, against microbial attacks and oxidative damages, across all taxa (Chapman, 2001; Pilch and Mann, 2006; Baer et al., 2009 a; Avila et al., 2011). For instance, some of these compounds work as scavengers of reactive oxygen species in birds and mammals (Breque et al., 2003; Chen et al., 2003; Avila et al., 2011).

Seminal fluid provides also an immunoregulatory function, that has to be fine balanced between costs and benefits to males: an excessive immune activity direct to sperm defence risks to lead to autoimmunity and male infertility (Poiani, 2006). Sperm protection may be particularly awkward inside the female reproductive tract. In fact, in internal fertilizers, one of the mainly seminal fluid function to guarantee sperm

fertilization abilities is responding to the potentially immune reaction of the female reproductive tract towards sperm, that can be recognised as foreign bodies (Birkhead et al., 1993; Poiani, 2002). Furthermore, since producing an acid medium is a primary female defence that do not facilitate sperm swimming, the seminal fluid has buffering capabilities. In fact, even the pH of the surrounding environment may be crucial for sperm efficiency: in humans, values are maintained above 7 by vesicles (prostasomes) secreted by the prostatic gland into the seminal fluid (Arienti et al., 1999). The control of pH values, may be essential also to enhance sperm motility inside male reproductive tract (Poiani, 2006). In external fertilizers, seminal components contribute to form a microenvironment that maintain elevated osmolality and pH around sperm, essential for sperm performance.

The conservation of sperm performance is critical to guarantee the male fertilization success, and seminal fluid demonstrated to influence diverse sperm quality traits across species in internal as well as in external fertilizers. For instance, sperm viability is coordinated by semen components in cricket, *Teleogryllus oceanicus* (Simmons and Beveridge, 2011) but also in the rainbow trout*, Oncorhyncus mykiss* (Lahnsteiner et al., 2003). In both species, the seminal fluid proteins prolong and stabilize sperm viability and, in particular, in the rainbow trout, the protein fraction <50 kDa accounts for significantly higher sperm motility rates and swimming velocity. In the honeybee, *Apis mellifera*, seminal fluid strongly affect sperm viability for a period comparable to the sperm storage process in the queen (King et al., 2010). Sperm viability is enhanced by seminal fluid enzymes in fruit flies and honeybees (Ramn and Wolfner, 2007; Baer et al., 2009b). There are indications that sperm might benefit from the presence of seminal fluid proteins that bind cations and fatty acids, whose adjustment is fundamental to the osmotic pressure balance and against damages to the sperm membrane (King et al., 2011). It was demonstrated that the sfps integrity and presence is essential, and their specific effect cannot be replicated with other seminal fluid compounds, as sugars. Moreover, few studies tried to elucidate how variations in sfps profile may influence male fertilization ability. In the honeybee *Apis mellifera*, sfps significantly vary in their abundance across genetic lineages, probably due to differences in animal genotypes, even if they were not investigated in relation to male reproductive success (Baer et al., 2012). In the field cricket, *T. oceanicus*, males present age dependent sperm competitive ability that was found to be determined by changes in sfps abundance and gene expression. In particular, ontogenetic increase in sfps quantity are associated with increasing sperm viability and male competitive fertilization success, paralleled by accessory glands swelling and regression in testis size (Simmons et al., 2014). Up to now, detected sfps variations in seminal fluid composition concern their abundance and none qualitative difference emerged in the seminal fluid protein profile across males of the same species.

Seminal fluid amount appears to vary, at intra-specific level, in relation to mating dynamics and female quality. In demersal fish spawners where egg deposition lasts for

several hours, dominant males release viscous ejaculates, in form of mucous trails that slowly dilute in water, releasing sperm for prolonged period of time, whereas males parasitizing dominant males' spawn release ejaculates poor in seminal fluid and rich in sperm (Scaggiante et al., 1999; Mazzoldi and Rasotto, 2002). In the fowl, *Gallus gallus*, dominant males allocate larger ejaculates to more attractive females, increasing sperm velocity through the higher seminal fluid expenditure (Cornwallis and O'Connor, 2009).

Indirect effects on male fertilization success

The seminal fluid composition may play a role in sexual conflict mechanisms, since some components have been recognised to manipulate female reproductive physiology and behaviours, thus indirectly increasing male paternity success (Baer et al., 2001; Simmons, 2001; Poiani, 2006; Ramn and Wolfner, 2007; Avila et al., 2011). In detail, the seminal fluid may influence female fecundity/ovulation and receptivity/re-mating behaviours.

Proteins secreted by male accessory gland have been found to induce egg-laying, while other seminal fluid compounds appear to increase both egg maturation and oviposition rate in both insects and vertebrates (Gillott, 2003; Chapman et al., 1995; Chapman, 2001; Poiani, 2006; Avila et al., 2011). *Drosphila melanogaster*'s seminal fluid contains a broad array of sfps. Among these, ovulin and sex peptide have been recognised to play an important role in male mating success, stimulating egg production and oviposition (Herdon and Wolfenr, 1995; Liu and Kubli, 2003) and suppressing female receptivity in order to exclude future rivals from mating (Liu and Kubli, 2003), respectively. On the other side, female receptivity is directly modulate by few seminal fluid proteins in drosophilids, while in a moth seminal fluid contains a peptide that act suppressing females pheromones for few hours, making them unreceptive to males (Chapman et al., 1995; Hartmann and Loher, 1996; Chapman, 2001). In *Drosophila suzukii* it was found a substance produced by male accessory glands that have the double function of stimulating females ovulation and contemporary suppressing their receptivity towards males (Ohashi et al., 1991).

The inhibition of female re-mating behaviour driven by seminal fluid occurs in several invertebrates and vertebrates, with, a great array of molecules both arranging mating plugs and decreasing female receptivity after the copulation (Avila et al., 2011). Mating plugs formation may involve both proteins or fatty acids, and comparative evidence indicate that seminal coagulation is an evolutionary product of sperm competition. Indeed, in primates the degree of clotting is higher in species with higher degrees of polyandry (Dixson and Anderson, 2002) and a similar results emerged in rodents. This last study elegantly shows a positive correlation between testis mass and seminal vesicles development (Ramn et al., 2005), but, getting in greater detail, it also highlights that the molecular mass of a specific sfps correlates with the relative testis size across species. The increased molecular mass of the seminal protein results from selection for an increased number of its cross-linking sites that leads to the formation of more thick

copulatory plugs in species with higher level of ejaculates competition (Ramn et al., 2009).

Semen components, due to the diversity of their functions, are subject to overlapping evolutionary pressures that range from sexual conflict to post-copulatory sexual selection mechanisms (Froman et al., 2002)(fig.2). For instance, the immunosuppressive effect on females to protect sperm inside their reproductive tract may conflict with females interest to defend themselves against sexually transmitted pathogens. As a consequence, the enhancement of the females immunity may in turn promote selection on ejaculate quality and sperm competitiveness (Birkhead et al., 1993; Alonzo and Pizzari, 2010). Poiani, in his key review (2006), suggested a co-adaptive model where seminal fluid functionality is influenced by sperm competition pressure combined with male and female defence mechanisms against pathogens. Males are expected to balance their attempts to overcame females defences without compromising future offspring survival. The hypothesis seems to be supported by the evidence that in species without parental care, such as many insects, seminal fluid causes more severe effect to female survival, even if comparative studies still lack.

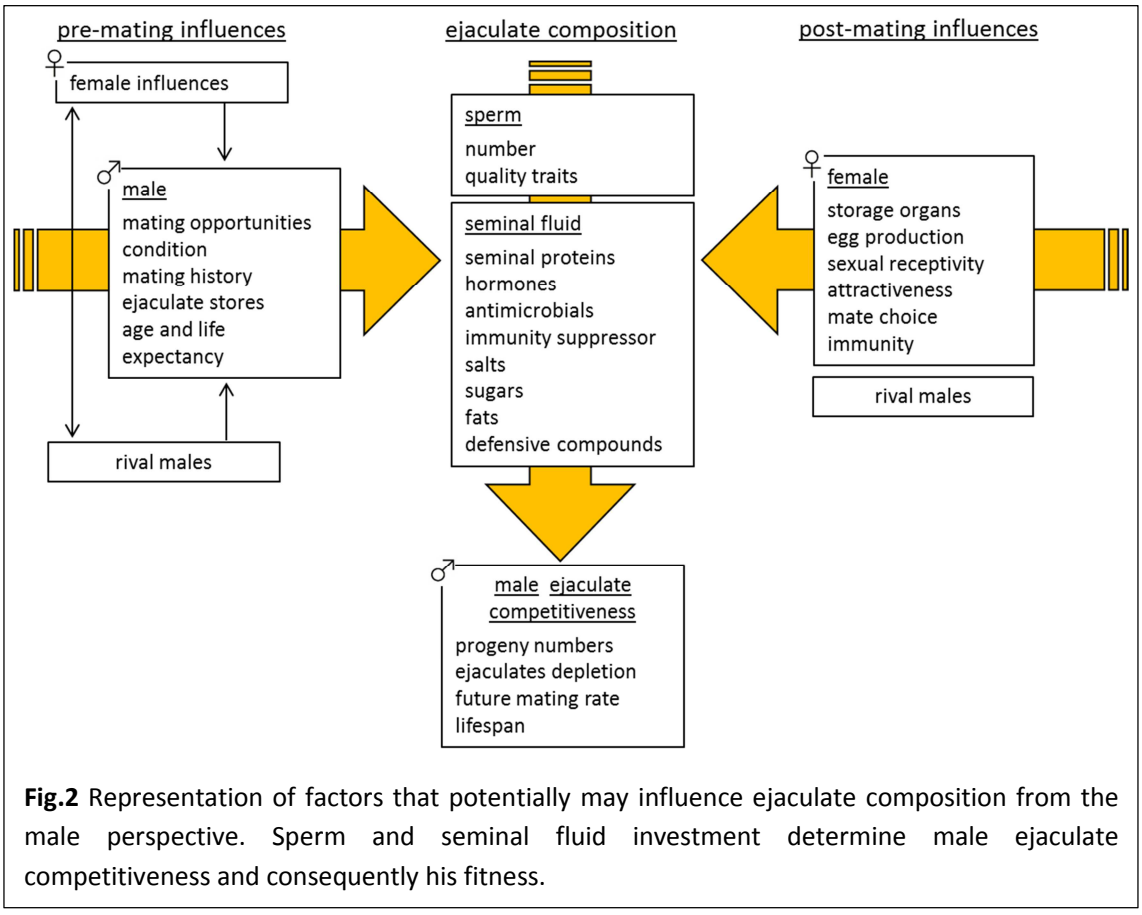

Fig.2 Representation of factors that potentially may influence ejaculate composition from the male perspective. Sperm and seminal fluid investment determine male ejaculate competitiveness and consequently his fitness.

5. Seminal fluid in competition contexts

Aside from female influences, the pivotal contribution of the seminal fluid to both sperm performances and sperm-egg interactions makes ejaculate composition a potential tool to sway male fertilization success under sperm competition conditions. The contribution of the seminal fluid may potentially produce two opposite effects, enhancing or alternatively lowering the performance of rivals' sperm. However, the few studies that investigated how seminal fluid may mediate rival ejaculates interplay came to conflicting results, that for the great part led to hypothesis rather than evidences. In addition, data arose exclusively from species with internal fertilization, where the contribute of rival males' ejaculates and the relative weight of sperm and seminal fluid are difficult to disentangle.

Historically, the first hypothesis on the role played by seminal fluid in competition contexts claimed that semen components are able to incapacitate rival males' ejaculates, killing or generally impeding sperm from fertilization (Harshman and Prout, 1994). Although evidence supporting sperm incapacitation emerged (Price et al., 1999), alternative explanations of the results were never definitively excluded, preventing from coming to a reliable and grounded demonstration of the hypothesis but triggering an heated debate. Indeed, sperm incapacitation was firstly supposed in *D. melanogaster* where females re-mated with males that transfer just seminal fluid produce less progeny than control females (Harshman and Prout, 1994). However, sperm storage was not directly controlled during the trial, leaving open the possibility that other processes occurred, such as sperm dumping by the female (Snook and Hosken, 2004). Sperm dumping happens when females actively release stored sperm from their reproductive tract after copulation with a second male, without any contribution of incoming sperm nor seminal fluid. The results emerged in *Drosophila* are probably caused by sperm ageing during sperm storage, rather than by killing seminal fluid properties (Snook and Hosken, 2004). In general, it seems more likely that males caused sperm dumping or females may differentially eject sperm previously stored. Sperm incapacitation has been proposed, but never proved, to occur in the human species too, as seminal fluid contains different types of leucocytes and cytokines, elements that might play an "allospermicidal" function (Huleihel et al., 1999; Poiani, 2006).

More recently, the possible seminal fluid detrimental effect on rival males' sperm emerged from a comparative study across monandrous versus polyandrous ants and bees. In these species, seminal fluid enhances own sperm viability, but the seminal fluid produced in promiscuous species has a lower effect on the viability of rivals' sperm compared to that on own sperm (den Boer et al., 2010). However, these results suggested the occurrence of a self/no-self recognition mechanism between sperm and

seminal fluid, rather than exactly a negative action on rival ejaculates. The presence of molecules with immunostimulatory properties (Poiani, 2006) apparently constitutes a contradiction to the down-regulation action of the semen components against female immune response. It has been hypothesized that these compounds may become active inside female tract acting against incoming rival ejaculates. However, in *Drosophila melanogaster*, clearly emerged that the lethal effect of males' seminal fluid on females is a by-product of sperm competition, since in populations where males were experimentally forced to monogamous matings for several generations, they evolved to be less harmful to their mates (Holland and Rice, 1999).

Even though most of these results are ambiguous and arise exclusively from species with internal fertilization, male investment in seminal fluid clearly has to be included in sperm competition models framework. Sperm and seminal components differ in their costs and benefits to males, that likely depend on the social and ecological context. Thus, males are expected to adjust their ejaculate composition at multiple levels, in relation to past and future copulations, to other males' strategy, and to the environmental context, with differences between species and populations.

The most recent theoretical analyses, still built on species with internal fertilization, considered that the optimal ejaculate composition for males based on two major assumptions: 1) a greater investment in sperm returns greater paternity (according with the "fair raffle" principle of sperm competition game theory) and 2) an investment in non-sperm components enhances female fecundity. Thus, males are expected to invest less in components enhancing female fecundity as the level of sperm competition increases. However, this primary prediction is expected to vary in relation to 1) the function of ejaculate components on the outcome of sperm competition, i.e. the relative weight of sperm and seminal fluid in influencing ejaculate competitiveness in a given competition scenario; 2) the male mating order or role; 3) the female re-mating rate (Hodgson and Hosken, 2006; Cameron et al., 2007; Alonzo and Pizzari, 2010).

1) In their models, Cameron et al. (2007), for the first time, pay attention to both sperm and non-sperm components of the ejaculates, defining the possible scenarios of their relative variation. If sperm numbers determine sperm competitive success, then males mating in the favoured role are expected to invest more in all ejaculate components, to maximize their paternity success. Whereas, when non-sperm components enhance sperm performance, advantaged males should invest less in sperm, enhancing non-sperm components (Cameron et al., 2007). By contrast, males disadvantaged in competition are expected to always allocate more in the sperm component (fig. 3, modified from Cameron et al., 2007).

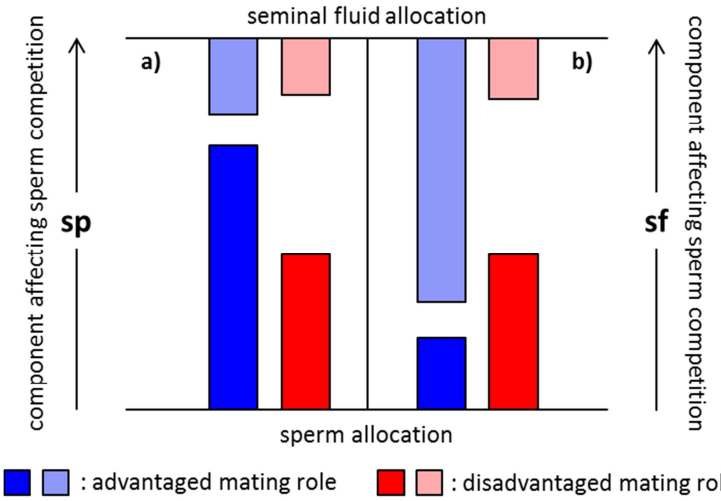

Fig.3 Optimal seminal fluid investment depending on the competition context and mating role: when **a)** sperm production (sp) weight on sperm competition outcome advantaged males (blue) allocate more in sperm than in seminal fluid and vice versa when **b)** the seminal fluid (sf) mainly influences ejaculates competition. Disfavoured males (red) always invest more in sperm production.

Looking at the empirical data, sperm number allocation in relation to sperm competition grounds on considerable evidence. The variation in sperm quality traits, instead, does not reveal a common pattern, since results are often unclear or contrasting, especially when looking for possible trade-offs between sperm number and quality (Snook, 2005). This suggest that seminal fluid may be a possible mediator of variation in sperm quality traits, thus directly influencing ejaculate competitiveness. A good example highlighting how sperm and seminal fluid may differently weight on the fertilization success according to competition scenario is to compare what occurs in the Atlantic salmon, *Salmo salar*, an in the bluegill, *Lepomis macrochirus* (Gage et al., 2004; Stoltz and Neff, 2006). In the Atlantic salmon, a species with alternative mating strategies, sperm number does not explain variation in fertilization success of competing males while sperm velocity accounts for the differences in sperm competitiveness (Gage et al., 2004). Instead, in the bluegill, *Lepomis macrochirus*, where males display three alternative mating tactics, the sperm number explains most of the differences in the fertilization success (Stoltz and Neff, 2006). Intriguing, the difference has been suggested to arise from the different ecology of the two species (Stoltz and Neff, 2006). Indeed, the Atlantic salmon spawns in flowing rivers, and fertilization tests were performed in a funnel stream, in order to resemble natural fertilization conditions, while the bluegill prefers lake environments, spawning in comparatively more calm waters and with females taking few hours to lay all their eggs. The conditions in which competition takes place may in turn be one of the factors that may influence male reproductive investment, favouring those traits that more affect sperm performance in a specific fertilization environment. In the bluegill situation, in which low water flow reduce ejaculate dilution, to outnumber sperm of rival males became the major task, whereas in the Atlantic salmon, where the high water flow rapidly dilute both ejaculates, sperm velocity is a crucial factor to gain fertilization success over competitors.

An important consequence of the effect of seminal fluid in improving own sperm performance, is that in competition contexts, when rival ejaculates overlap due to sperm competition, this positive effect potentially may be transmitted to all the sperm present, thus enhancing the rival males' fertilization success. As a result, sperm of a male may benefit of the presence of the previous male' seminal fluid with implications for sperm competition outcome and male ejaculate expenditure strategy. Indeed, unless a self/non-self-recognition mechanism evolves (Holman, 2009), theory predicts that the function of seminal fluid to enhance or protect own sperm can be exploited by the sperm of rival males, that may reduce their own expenditure (Hodgson and Hosken, 2006). Here, it should be emphasized that the ejaculate overlapping may not occur only in internal fertilizers, as even in externally fertilizing species, when the fertilization dynamics allow the ejaculates of competing males to mixed adequately, sperm seminal fluid interactions between rivals can be expected to sway the males' relative fertilization success. The cross interaction of sperm and seminal fluids of rival males influence the outcome of sperm competition was not experimentally tested (before the present Thesis) but there is evidence that seminal fluid enhance both own and rival sperm performance. Indeed, in *Drosophila* and in the field cricket *Teleogryllus oceanicus*, it has been demonstrated that not only the seminal fluid does not have any killing ability, but, instead, it improves equally the survival of both own and rival males sperm (Holman, 2009; Simmons and Beveridge, 2011).

2) Optimal ejaculate allocation is expected to also critically depends on males mating order. Then, predictions on male investment vary weather a male can assess in which reproductive role he is mating, if advantaged or disadvantaged. For instance, in the field cricket *Teleogryllus oceanicus* males adjust sperm viability to the perceived level of both the risk and the intensity of sperm competition. In fact, males are able not only to detect if a female is already mated but also the number of partners she had accepted, on the base of chemical signals left behind by the males. Therefore, males produce ejaculates with increased sperm viability till they can gain a payoff, that is when the number of competitor is at least two, and then save sperm quality investment (Simmons et al., 2007a; Thomas and Simmons, 2007; 2008). Males that can alternatively stand in the advantageous or disadvantageous role have to vary their ejaculate investment assessing the competition position at each mating. In species with alternative mating tactics, instead, the mating role is fixed by the adopted male phenotype. Then, males investment in sperm and seminal fluid components is expected to be tactic dependent.

3) In internal fertilizers, males are expected to invest less in components enhancing female fecundity as the level of sperm competition increases (Alonzo and Pizzari, 2010; Perry et al., 2013). However, males may lowered their investment in seminal fluid even when its compounds stimulate females fecundity, if responses such as egg-laying have been maximally stimulated by previous partners. In *Drosophila*, experimental variations

of the sperm competition level revealed that at its higher values males respond increasing both the size and productivity of the accessory glands, where seminal fluid is produced (Crudgington et al., 2009). A similar response of male accessory glands to sperm competition perceived level was found in promiscuous mammals (Lemaitre et al., 2011). Moreover, in *Drosophila*, males prolong mating duration when exposed to rivals: as a result they would transfer a larger amount of seminal fluid (Wigby et al., 2009). Male flies can fine-tune their seminal fluid expenditure even beyond and adjust the amount of singular semen components depending on female reproductive value. Indeed, males have been found to boost the fecundity-stimulating Sfp (ovulin) concentration in the seminal fluid when mating with a virgin respect to an already mated female, but to not vary the transfer of sex peptide, inhibiting receptivity (Sirot et al., 2011).

Concurrently to the development of theoretical models, an important work has been trying to shed light on the proximate mechanisms that drive sperm-seminal fluid interaction, mainly focusing on the role of seminal fluid proteins (sfps). Since sfps are already known to mediate sperm quality traits (see above), they have been the first target also in the study of sperm-seminal fluid interplay in competition contexts. Both females and males reproductive proteins, indeed, showed the signature of rapid evolution across a wide range of taxa, ranging from invertebrates to mammals (Clark and Dell, 2006; Schumacher et al., 2013), and genes that encode for reproductive proteins on the average show higher evolutionary rate respect to others (Simmons and Fitzpatrick, 2012). There are increasing evidence that sperm competition and female promiscuity are some of the steering factors of their evolutionary divergence. Post-copulatory sexual selection will drive adaptive variation in seminal fluid, and sperm, proteins that improve male fertilization success (Simmons et al., 2012), as species with greater selection pressures showed higher protein divergence (Dorus et al., 2004; Wagstaff and Begun, 2007; Ramn et al., 2009).

The study of seminal fluid proteins mainly proceeds through identification and hypothesis about their function in relation to male fertilization success. In the last years, data about seminal fluid proteome of both vertebrate and invertebrate species exponentially rose, even if the functionality of known proteins is difficult to disentangle and often is far to be clarified. In fact, except for *Drosophila* spp, sfps specific action is rarely tested directly on sperm (Lahnsteiner et al., 2003) and indications about their role emerged indirectly through differences in paternity or fertilization success of ejaculates that vary in their seminal fluid composition, or alternatively through comparative studies across species. For instance, in the cricket *Teleogyllus oceanicus*, the seminal fluid of males, that invest more in accessory gland, improves sperm viability (Simmons and Bevedrige, 2011) and both own and rival offspring viability (García-González and Simmons, 2005a; García-González and Simmons, 2007).

Experimental evidence highlight the importance of widening the taxonomic breath of seminal fluid biochemistry, considering the whole composition. In fact, also other components such as sugars, fatty acids and salts, are important for sperm nourishment and performance and may be differently allocated depending on species and competition contexts. There are indications that sperm might benefit from the presence of seminal fluid proteins that bind cations and fatty acids, whose adjustment is fundamental to the osmotic pressure balance and against damages to the sperm membrane (King et al., 2011). Moreover, in rodents, a sperm calcium channel protein, that is essential for motility and fertilization ability, was found to evolve directly under sperm competition pressure (Vicens et al., 2014). Therefore, the relative abundance and reserves of small compounds, such as ions, that interact with sfps or directly with sperm, might be meaningful.

Post-copulatory sexual selection is a potent broker of rapid molecular evolution, and even if proteins are the main target of ongoing studies, the contribute of all non-sperm components to the whole ejaculate functionality cannot be overlooked to draw the evolutionary trajectories of male ejaculate allocation across species.

6. Two fish species as a model to test the possible influence of the seminal fluid in sperm competition outcome

The theoretical analyses posit that, in ejaculates competition contexts, the optimal ejaculate composition for males depends on a) ejaculate components functionality, where either sperm or seminal fluid may enhance the whole ejaculate competitiveness; b) males' mating order or role (Cameron et al. 2007). Experimental evidence still lack, likely because it is difficult, in internal fertilizers, to attribute the ejaculate components, i.e. sperm and seminal fluid to a specific individual. Conditions for ejaculate competition are reasonably common in natural mating systems, but it's not always easy to correctly disentangle the pressures on ejaculate functionality and to distinguish the favoured or disfavoured mating role.

Two goby species, the grass goby, *Zosterisessor ophiocephlus* (Pallas, 1822), and the black goby, *Gobius niger* L., appear a good model to experimentally verify if seminal fluid allocation may varies in relation to its weight on ejaculate competitiveness and with ejaculates competition level. Indeed, the two species: a) show similar reproductive modalities and mating system, b) present ejaculates competition, with its risk varying among males, due to male alternative reproductive tactics, and c) potentially differ in the likelihood for seminal fluid to influence ejaculate competitiveness. Moreover, both species are external fertilizers, a condition that facilitates to experimentally separate the contribution of sperm and seminal fluid to male fertilization success in competition context.

The grass goby, *Zosterisessor ophiocephlus* (Pallas, 1822), and the black goby, *Gobius niger* L., are two coastal marine species (fig. 4); the former inhabiting the seagrass meadows of *Zostera spp*. *(Z. marina* and *Z. nolti*) in shallow brackish water, while the latter preferring sandy environments (Mazzoldi et al., 2000; Mazzoldi and Rasotto, 2002). These gobies are external fertilizers, egg deposition last for several hours, and ejaculate are released in form of sperm trails, bands of mucins that, slowly dissolving in seawater, continue to release active sperm over a long period of time (Marconato et al., 1996; Mazzoldi et al., 2005). In both species males exhibit alternative mating tactics (Gandolfi et al., 1991; Mazzoldi et al., 2000; Rasotto and Mazzoldi, 2002). During the breeding season, larger/older dominant individuals build and defend a nest, where they court females, mate, and provide parental care to eggs (hereafter territorial males). By contrast, smaller/younger mature males, disadvantaged in nest construction and females attraction, mate opportunistically by sneaking inside territorial male nest when females are laying eggs. In addition to age, size and reproductive behaviour, territorial and sneaker males differ in other traits related to fertilization. Territorial males, typically show a lower investment in the sperm component and an higher mucins production than sneaker males (Scaggiante et al., 1999; Rasotto and Mazzoldi, 2002). Ejaculates of sneakers contains on average 5.2 times the number of sperm of those of territorial males in the grass goby and 10.4 in the black goby (Scaggiante et al., 1999; Mazzoldi et al., 2000; Rasotto and Mazzoldi, 2002), whereas the smaller territorial male ejaculate contains, on average, in both species, ten times more seminal fluid than the greater sneaker one (unpublished data). As a result of these differences, ejaculates released by territorial and sneaker perform differently. Territorial males lay sperm as trails, rubbing the urogenital papilla on the ceiling and on the walls of the nest. Ejaculated trails slowly release active sperm into the water, and the duration of a sperm trail is positively correlated with its mucin content and can last several hours (Scaggiante et al., 1999; Mazzoldi et al., 2000; Rasotto and Mazzoldi, 2002). Instead, sneaker ejaculates, being poorer in mucins, release most of their sperm immediately (Mazzoldi et al., 2000; Rasotto and Mazzoldi, 2002). This variability in sperm and seminal fluid allocation, mirrors the different sperm competition risk faced by males depending on the adopted tactic, with sneakers that cope with greater risk since they have always to compete at least with the ejaculate of the territorial male, that, instead, may succeed in excluding rivals from the mating. Thus, the greater seminal fluid allocation of territorial male guarantees a low but steady supply of active sperm, allowing them to invest more in the nest defence, whereas sneakers' higher sperm production maximize their possibility to stole fertilizations to the territorial at the time of spawning. In these species, then, the greater investment in the seminal fluid component of the ejaculate evolved not in order to influence female physiology, as postulated by theoretical models built on internal fertilizers (Cameron et al., 2007). Up to the work of this Thesis, the higher seminal fluid production appeared a response that indirectly increase the territorial males fertilization success, allowing them to face the attempts of sneakers to enter inside the nest, without

considering the possibility that the seminal fluid play a direct role in the rival ejaculates interplay.

a)  **b)** 

Fig. 4 a) *Zosterisessor ophiocephalus*, the grass goby and **b) *Gobius niger***, the black goby

Despite the similar mating system, these two gobies differ in nest conformation, a characteristic that, apparently, influences sneaking dynamics. In the grass goby, territorial males dig a deep and wide burrow under the sea grass rhizomes; nests are usually multi-chambered and often provided with up to three openings, allowing territorial males to escape from predators and to generate a water flow favouring egg fanning (Mazzoldi et al., 2000). However, the multiple entrances of grass goby nest, make easier for sneakers to overcome territorial male guard, remaining inside the nest and having the opportunity to release their ejaculate in close proximity both to the eggs and the territorial male sperm trails (Mazzoldi et al. 2000). In fact, up to five sneakers males can be found inside a single nest. Black goby nests, instead, consist of small cavities under rocks or in the mud along sloping seabed, but may also be artificial substrates, such as empty cans (Mazzoldi and Rasotto, 2002; Rasotto and Mazzoldi, 2002) (Fig.5). Regardless the type of substrate used, black goby nests usually present at most two narrow entrances and territorial males easily force sneakers to release their ejaculate at the nest entrance, far from eggs and susceptible of a rapid dilution (Mazzoldi 1999). The difference between the grass and the black goby in sneaking dynamics is mirrored in sperm number and performances. Indeed, both longevity and sperm number are lower in the grass goby than in the black goby, as expected if black goby sperm undergo a more rapid dispersion (Locatello et al., 2007). Moreover, in the grass goby, sneaker and territorial males do not differ in their sperm performance in terms of velocity, longevity and ATP content, whereas black goby sneakers produce sperm that are faster, more viable and richer in ATP than those of territorial males (Locatello et al., 2007).

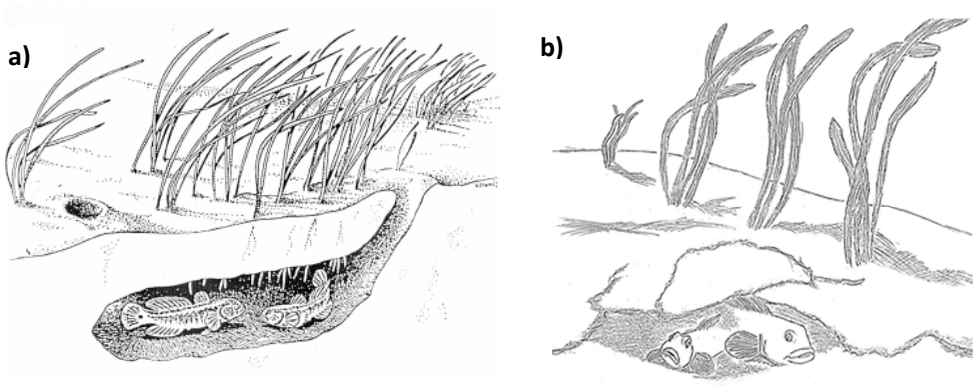

Fig. 5 Drawings of the nests of **a)** *Zosterisessor ophiocephalus* and **b)** *Gobius niger*

Summarizing, the grass and the black goby: i) show a similar mating system and comparable levels of sperm competition, ii) display alternative mating tactics, with sperm competition risk varying with the tactic adopted by males, iii) ejaculate composition, both in terms of sperm number and seminal fluid amount, mirrors sperm competition risk; iv) their sneaking dynamics differently affects the degree of rival ejaculates interplay in the two species, thus setting different opportunities for the seminal fluid to influence sperm competition outcome. On the basis of these characteristics, and according to the theoretical models (Cameron et al., 2007), seminal fluid may be expected to play a role in sperm competition context, influencing ejaculate competitiveness only in the grass goby, where the ejaculate of territorial and sneakers males may, in fact, come in contact. In this case, it can be hypothesized that seminal fluid may be exploited or may negatively affect sperm competitiveness of rival males, according to male mating tactics, and it may consequently vary not only in quantity but also in quality between sneaker and territorial males.

7. Aims of the Thesis

My PhD project, using the grass goby and the black goby as study species, was aimed at verifying a) if seminal fluid differently enhance ejaculate competitiveness in these two species; b) if and how seminal fluid allocation differ according to male mating tactics (Fig.6). In particular, the project was focused on three main questions:

1. Does the seminal fluid influence sperm performance? (Paper I and Paper III)

To verify if the seminal fluid affects the performance of both own and rival male sperm I measured, in both the study species, the sperm performances, in terms of velocity and viability, by separating the sperm and seminal fluid components of ejaculates and making reciprocal combinations within and between males using different tactics. With

respect to this question, I expected, in both species, that seminal fluid would affect own sperm performances. Instead, I had different expectations for the two species on the influence of seminal fluid on rival sperm:

- in the **grass goby**, where the ejaculates of males adopting alternative mating tactics have similar performances and may be released in close proximity (Locatello et al., 2007), the seminal fluid of a male might influence the performance of the sperm released by males adopting a different tactic, overall affecting the outcomes of sperm competition. I expected that i) the seminal fluid of territorial males could be exploited by opportunistic males, to enhance the performance of their own sperm or, otherwise, ii) territorial males' seminal fluid could impair sneakers' sperm, to counteract their numerical superiority, and/or iii) sneaker seminal fluid might have a detrimental effect on territorial male sperm.
- in the **black goby**, where the ejaculates of competing males are released far from each other, I did not expect the seminal fluid to influence the outcome of sperm competition.

2. How does seminal fluid affect sperm performance? The proximate mechanisms driving sperm seminal fluid-sperm interactions. (Paper II)

As I expected that, in the grass goby, the seminal fluid would influence sperm performance, I deepened the possible mechanisms regulating sperm-seminal fluid interplay. If a tactic specific influence of the seminal fluid were emerged, this could be due by a ì) qualitative/quantitative difference in seminal fluid composition, and/or ìì) a difference in sperm quality for a parameter not yet measured. Consequently I analysed the seminal fluid composition, in terms of glucose and protein concentration, of both territorial and sneaker males. With respect to sperm quality, I evaluated the oxygen consumption rate, again on both the sperm of territorial and sneaker males. This parameter is not often measured, despite mitochondrial morphology and functionality have been already demonstrated to influence to sperm motility characteristics (Lupold et al., 2009; Suquet et al., 2012).

3. Does the fertilization success mirror sperm performance? (Paper I, Paper III and IV)

I performed *in vitro* fertilization tests in both species (Paper I and III), repeating the same sperm and seminal fluid reciprocal combinations designed to address the first question on the effect of seminal fluid on sperm quality (velocity, viability). Indeed, it was crucial to verify that any variation caused by the seminal fluid on sperm performance was mirrored on the sperm fertilization ability. I expected that in both species, *in vitro* fertilization results mirrored the results of sperm quality tests, as sperm velocity has been demonstrated to be a fair indicator of sperm fertilization success, in both internal and external fertilizer (Birkhead et al., 1999; Gage et al., 2004; Snook, 2005).

Moreover, to evaluate if the results of *in vitro* fertilization tests give reliable insights on the ejaculate competition in nature and considering that grass goby paternity success

in natural condition has been already investigated (Pujolar et al., 2012), I concentrated my attention on the black goby fertilization success in the field. As in this species, sneakers release their ejaculate at nest entrance I expected that their fertilization success might decrease along the nest length, with the eggs closest to the entrance showing the highest sneaker paternity rate. To test this hypothesis I recorded fertilization success, in artificial nests located in natural breeding sites, by using molecular parentage analyses (Paper IV).

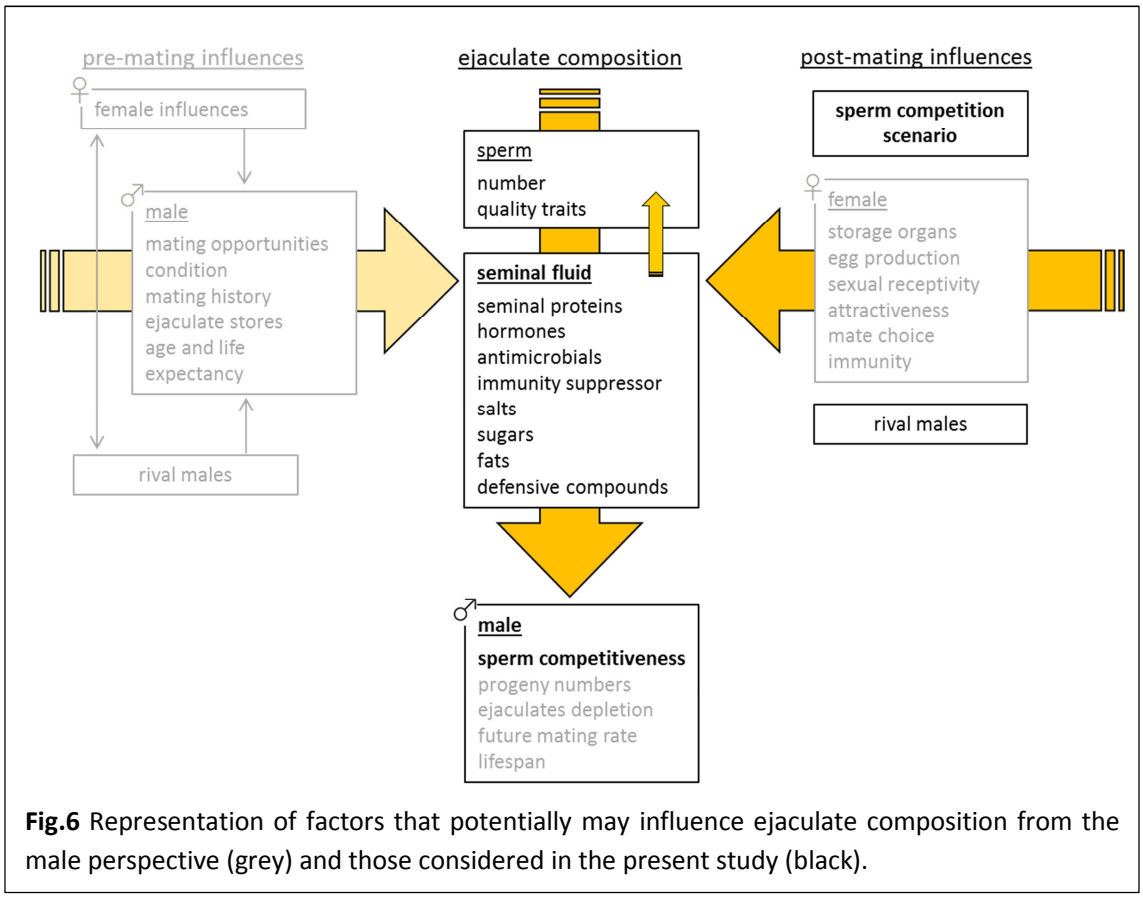

Fig.6 Representation of factors that potentially may influence ejaculate composition from the male perspective (grey) and those considered in the present study (black).

PAPERS' EXTENDED ABSTRACTS

The findings of the studies I have conducted on the grass goby and the black goby, are organized in four papers. I summarized below the content of these papers, following the three specific questions addressed by the project.

Question #1: Does the seminal fluid influence sperm performance in the two species?
Zosterisessor ophiocephalus, Paper I; *Gobius niger*, Paper II

Paper I: study species *Zosterisessor ophiocephalus*

"Tactic specific-differences in seminal fluid influence sperm performance"
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Proceedings of the Royal Society of London B vol. 280 no. 1755 2013 doi:10.1098/rspb.2012.2891

In this species the seminal fluid is expected to influence the outcome of ejaculates competition, as sneakers males enter inside nests and may be able to release their ejaculates in close proximity to those of territorial males. In this scenario, a potential high level of sperm-seminal fluid cross interactions may occur inside the nest. To test this prediction, we separated sperm from seminal fluid of sneakers (n=24) and territorial males (n=20) to evaluate the effect of seminal fluid on the performance of the sperm (velocity and fertilization rate) of the two male types. The *in vitro* fertilization tests, would allow to control if sperm performance mirrors sperm fertilization success (question #3). We performed reciprocal combination, within and between male employing alternative tactics, of sperm of both male types. We also simulated the conditions of natural competition by using a mixture of sneaker and territorial males' seminal fluids.
The results demonstrate how seminal fluid not only enhances sperm performance, but has a tactic dependent effect on sperm of males displaying the opposite tactic. Indeed, while sperm of sneaker and territorial males did not differ in their performance when they interacted with only their own seminal fluid, sperm of sneakers increased their velocity and fertilization rate in presence of territorial males' seminal fluid. In contrast, sneaker seminal fluid had a detrimental effect on the performance of territorial males' sperm. Sperm velocity was unaffected by the seminal fluid of males employing the same tactic, suggesting that seminal fluid's effect on opposite-tactic sperm is not based on a self/non-self recognition mechanism. Summarizing, the performance of territorial and sneaker males' sperm in their own seminal fluid, as well as in that of other males performing the same tactic, is similar but when the fluid of a male employing a different tactic is present, sperm performance goes in opposite directions. This suggests that ì) the seminal fluids of sneaker and territorial males vary in composition and ìi) the sperm released by territorial and sneaker males differ in quality.

The consistency of results on sperm velocity and *in vitro* fertilization tests make us comfortable in stating that, in this species, fertilization success mirrors sperm performance (question #3). Overall, this study shows that cross interactions of sperm and seminal fluid may influence the fertilization success of competing ejaculates with males strategically modulating allocation in both sperm and seminal fluid in response to sperm competition risk.

Paper II: study species *Gobius niger*

"Seminal fluid does not mediate ejaculates competition in the black goby"

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In this species the seminal fluid is not expected to influence the outcome of ejaculates competition, as sneaker males do not usually enter inside nests but release their ejaculates at the nest entrance. The design of this study was similar to that performed to study the effect of seminal fluid in the grass goby. Indeed, we separated sperm from seminal fluid of sneakers (n=35 and territorial males (n=44) to evaluate the effect of seminal fluid on the performance of the sperm (velocity and fertilization rate) of the two male types. The *in vitro* fertilization tests, would allow to control if sperm performance mirrors sperm fertilization success (question #3). We performed reciprocal combinations of sperm and seminal fluid within and between male employing alternative tactics. However, we did not test sperm performance in a mixture of sneaker and territorial males' seminal fluids, since in natural condition the mixing of ejaculates never occurs or it is a rare event. As sneakers' ejaculates are extremely poor in seminal fluid (ten time less than territorial males on the average) and are released at the nest entrance, their sperm may come in contact with the territorial male seminal fluid diluting inside the nest, but the seminal fluids of two male types should not mix.

The findings support the expectation, as the seminal fluid does not affect sperm performance of opposite tactic males. Indeed, even if territorial males' seminal fluid enhances their own sperm velocity, sneaker sperm still are faster and more viable in their own seminal fluid. Cross interactions between sperm and seminal fluid of males adopting opposite tactics do not reveal any effect on ejaculate competitiveness. In this species, *in vitro* fertilization trials did not reveal any significant difference between sneaker and territorial males, thus not reflecting what recorded by sperm velocity tests. However, it cannot be excluded that this finding is affected by the high variability of the results and the relative low number of the tests (n= 12 territorials; 12 sneakers). Interestingly, sneaker and territorial males' sperm vary in the swimming mode when evaluated as linearity (LIN), with territorial male sperm moving in a significantly more linear trajectory, a difference never previously highlighted, nor found in the grass goby. This difference suggests that sneaker and territorial male sperm could cover the same distance in a similar time, even if those of sneaker have an higher linear velocity (VCL).

Question # 2: How seminal fluid affect sperm performance?
Zosterisessor ophiocephalus, Paper III

Paper III: study species *Zosterisessor ophiocephalus*

"Proximate mechanisms driving sperm-seminal fluid cross interactions in ejaculates competition"

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In the grass goby, *Zosterisessor ophiocephalus*, we highlighted that seminal fluid enhances sperm performance, with a tactic dependent effect on sperm of males displaying the opposite tactic. Indeed, while sperm of sneaker and territorial males do not differ in their velocity and fertilization rate when only their own seminal fluid is present, sneakers' ejaculates increased their performance when interact with territorial males' seminal fluid. In contrast, sneaker seminal fluid had a detrimental effect on the performance of territorial males' ejaculates.
These findings (paper I) brought out that i) sneakers' sperm make the most of territorial male seminal fluid, more than own territorial sperm, and so they must be endowed of an higher quality for a parameter not previously measured, and ii) seminal fluid composition vary between the two tactics. Indeed, literature data indicate that grass goby sneakers' sperm do not differ from those of territorial males in terms of morphology, velocity, viability, longevity and ATP content.
Thus, in this study we a) measured a sperm performance parameter not commonly recorded in sperm competition contexts that is the sperm oxygen consumption rate, and b) analysed seminal fluid composition. Results outlined that a) sneakers' sperm showed higher oxygen consumption rate and b) seminal fluid composition does not differ in the glucose content, whereas territorial seminal fluid presents an higher protein concentration. In addition, the protein profiles of sneaker and territorial seminal fluid disclosed both qualitative and quantitative differences. The proteins we identified from sneaker profile were serotransferrin, lysozyme C, and a parvalbumin-like protein. All of them were found in the seminal fluid of other fish species, and they are supposed to take part in sperm protection from oxidative damages and pathogens, antimicrobial function and being part of the Ca^{2+}-mediated mechanism of sperm activation, respectively. Moreover, to investigate which components of seminal fluid might be involved in impairing territorial males' sperm and enhancing sneaker males' sperm, the seminal fluid was divided in protein (>3kDa) and non-protein (<3kDa) components and we proceeded to evaluate their relative effect on the velocity of both territorial and sneaker sperm, with fractions isolated from both their and opposite tactic seminal fluid.
Among sneakers, emerged that 1) the non-protein fraction (whose major components are glucose, ions but the presence of small peptides cannot be excluded) marks the differences between treatments, since their absence significantly lowered sperm

velocity irrespective of the protein fraction effect; 2) the presence/absence of the protein fraction appears to influence the mean sperm velocity, but the difference is not significant, possibly because of the limited sample size (n= 17 tested with same tactic seminal fluid; 15 tested with opposite tactic seminal fluid).
My results outlined how the effect of compounds other than proteins, has to be considered looking at the whole ejaculate performance. In particular, we suggest that additional analysis have to be addressed to the investigation of the seminal fluid ionic composition. Indeed, in fishes, ions such as Na+, K+ and Ca2+ and their relative abundance are pivotal for the maintenance of seminal fluid pH and osmotic pressure that in turn influence sperm activation and motility. Samples are not enough to shed light on the tactic dependent effect of the seminal fluid, but we highlighted how studies on the seminal fluid functionality have to be prudent in looking at the protein fraction as representative of the whole seminal fluid contribution to ejaculate performance.

Question # 3: Does the fertilization success mirror sperm performance?

This issue was investigated, in lab condition in both the grass goby (paper I) and the black goby (paper II). However, while for the grass goby, paternity rate in the field is already known (Pujolar et al., 2012), allowing to discuss the pattern emerging form natural and captivity condition, information on the black goby fertilization success in the field were lacking. In paper IV are presented the results of the study aimed at filling this gap.

Paper IV: study species *Gobius niger*

"Multiple paternity and fertilization success of territorial males in the black goby (*Gobius niger* L.)"

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In the black goby large territorial males defend and court females from nest sites, while small sneaker males spawn opportunistically, releasing their ejaculate at nest entrance. The ejaculate investment, in both sperm and seminal fluid, differs in male adopting alternative mating tactics with sneakers releasing ejaculates poorer in seminal fluid but with higher sperm number and quality than territorial males. Seminal fluid enhances sperm performance of territorial males (paper II) but still sneakers' sperm remain faster. Sneaker ejaculates performance are apparently superior and territorial males strengthen aggressive behaviours in response to sperm competition intensity, without adjusting sperm investment. The aim of this study was to clarify how the apparently higher competitiveness of sneaker sperm respect to territorial ones affects the paternity sharing between the two tactics inside natural nests. The territorial mate guarding was

expected to contrast the higher quality of sneaker ejaculates. In particular, territorial males should fathered the majority of the eggs, as found in other fish species with similar mating system, and, since sneakers are halted at the entrance of the nest, their paternity success is expected to decrease along the nest's depth.

Parentage assignment of 301 eggs from 4 different nests revealed a level of sperm competition higher than that registered in the grass goby and other fish species with alternative reproductive tactics and nest defence. We found that black goby territorial paternity success never exceeded the 50%, and was on average 30,6%, whereas the paternity success of the first more successful opportunistic males was 23,6%, and the 13,9% on the average. Our results suggest that the efficiency of territorial male nest guarding is crucial for his parentage success, in particular, the distance at which sneakers are forced to release their ejaculates influences the number of eggs he fathered. Indeed, we found that the parentage success of the territorial male is lower in correspondence of the principal nest opening, where eggs are likely more exposed to sneaking attempts. We did not find any correlation between this trend and territorial body size but the number of candidate sneakers weights on the fertilization success in the different nest areas. Therefore, the pattern appears to be anyway influenced by the male nest defence ability and/or his quality (ejaculate's performance). However, these results are derived from four nests, that amount to 301 embryos, and we need to increase the sample size to address more robust conclusions, measuring also the sneakers parentage pattern. Indeed, we need to clarify if it is strictly the number of sneakers or also how much each of them goes deep into the nest that influence the fertilization success of the two male phenotypes.

PAPERS' COLLECTION

Paper I

"Tactic specific-differences in seminal fluid influence sperm performance"

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Paper II

"Seminal fluid does not mediate ejaculates competition in the black goby"

Poli Federica[1], Locatello Lisa[1], Rasotto Maria Berica[1]

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Paper III

"Proximate mechanisms driving sperm-seminal fluid cross interactions in ejaculates competition"

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Paper IV

"Multiple paternity and fertilization success of territorial males in the black goby (*Gobius niger* L.)"

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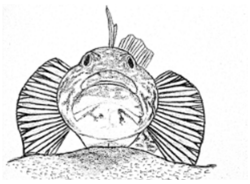
Paper I



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Tactic-specific differences in seminal fluid influence sperm performance

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Seminal fluid often makes up a large part of an ejaculate, yet most empirical and theoretical studies on sperm competition have focused on how sperm characteristics (number and quality) affect fertilization success. However, seminal fluid influences own sperm performance and may potentially influence the outcome of sperm competition, by also affecting that of rivals. As a consequence males may be expected to allocate their investment in both sperm and seminal fluid in relation to the potential level of competition. Grass goby (*Zosterisessor ophiocephalus*) is an external fertilizer with guard-sneaker mating tactics, where sperm competition risk varies according to the tactic adopted. Here, we experimentally manipulated grass goby ejaculates by separately combining sperm and seminal fluid from territorial and sneaker males. While sperm of sneaker and territorial males did not differ in their performance when they interacted with their own seminal fluid only, sperm of sneakers increased their velocity and fertilization rate in the presence of territorial males' seminal fluid. By contrast, sneaker males' seminal fluid had a detrimental effect on the performance of territorial males' sperm. Sperm velocity was unaffected by the seminal fluid of males employing the same tactic, suggesting that seminal fluid's effect on rival-tactic sperm is not based on a self/non-self recognition mechanism. Our findings show that cross interactions of sperm and seminal fluid may influence the fertilization success of competing ejaculates with males investing in both sperm and seminal fluid in response to sperm competition risk.

1. Introduction

Sperm competition, occurring whenever the sperm of rival males compete to fertilize the same group of eggs [1], is a widespread phenomenon and a powerful evolutionary force shaping male behaviour, morphology and physiology [2–4]. The most common adaptation to sperm competition in males is represented by an increase in sperm expenditure at mating to increase their probability of egg fertilization [2–5]. Indeed, comparative studies across both species and populations show that an increase in the level of sperm competition is paralleled by a greater ejaculate investment as judged by relative testis size, sperm number and sperm quality [2,6,7]. For example, in species in which male alternative reproductive tactics (ARTs) experience different levels of sperm competition, opportunistic males, playing the tactic associated with the higher level, release more sperm, which can be also faster and/or more viable, than males experiencing lower risk [8–12]. Moreover, males have been shown to rapidly adjust their ejaculate expenditure, in terms of sperm number and/or quality, when the level of sperm competition varies among successive matings, as well as in relation to social status and female quality [13–17].

To date, theoretical and empirical studies on the effects of sperm competition have primarily focused on sperm number and quality [2,18]. However, a substantial portion of the ejaculate is made up by the seminal fluid, which may indirectly influence paternity success by affecting female reproductive success [19]. Indeed, seminal fluid contains substances that decrease female receptivity, increase oviposition rate and form mating plugs [19,20], and males are capable of adjusting their amount in response to the perceived level of sperm competition [21]. Some studies have recently shown that seminal fluids may play a more direct role in sperm competition by affecting rivals'

sperm performance. Indeed, in promiscuous ants and bees seminal fluid incapacitates the sperm of rival males [22], while in other insects it improves equally the survival of own and other sperm [23,24]. This suggests that, unless a self/non-self recognition mechanism evolves [23], the function of seminal fluid to enhance own sperm performance can be exploited by the sperm of rival males [25].

Although the conditions for male parasitism of rival ejaculates are reasonably common in natural mating systems, the idea that males may gain fertilization advantage in allocating their seminal fluid investment in relation to mating order or role [25,26] still lacks experimental evidence, probably because it is difficult, in internal fertilizers, to attribute the seminal fluid to a specific individual. Here, we overcome this problem by using the grass goby, *Zosterisessor ophiocephalus* (Pallas), a fish species with external fertilization and guard-sneaker mating tactics [27]. In this species, territorial males, during the breeding season, dig and defend their nest, court females and perform parental care to the egg while sneakers parasitize the spawnings of territorial males [27,28]. Territorial males release viscous ejaculates (sperm trails) on the nest ceiling, where eggs are laid both before and during egg deposition [27,28]. These ejaculates slowly dilute in seawater, thus releasing active sperm [27]. Sneaker males enter inside a nest when spawning occurs and release their ejaculate in proximity to those of territorial males and to eggs [28]. Sperm competition is intense, but in approximately 30 per cent of spawning, territorial males do not mate in competition while sneaker males always do [27]. Territorial males' ejaculates contain more seminal fluid and fewer sperm than those of sneakers [27,28], but sperm quality, in terms of velocity, viability and adenosine-5′-triphosphate (ATP) content, does not vary with tactic (when assayed in a saline solution) [10]. It may be envisaged that in species with ARTs the seminal fluid of territorial males could be exploited by opportunistic males, to enhance the performance of their own sperm. Otherwise, territorial males' seminal fluid could have a detrimental effect on sneakers' sperm, to counteract their numerical superiority.

We tested these predictions by analysing whether seminal fluid affects performance, in terms of velocity and fertilization success, of own and rival sperm, according to the mating tactic employed by the male. We measured sperm performance by separating the sperm and seminal fluid components of ejaculates and making reciprocal combinations within and between males using different tactics. We also simulated the conditions of natural competition by using a mixture of the seminal fluids of sneaker and of territorial males.

2. Material and methods

(a) Animal sampling and handling

Males and females were collected in the Venetian Lagoon during their breeding season (April–June), and kept in separate tanks under artificial light (14 L : 10 D). Water (20°C) was changed daily and fish were fed with fresh food. Each male was anaesthetized in a water solution of MS 222 (tricaine sulphate; Sandoz), his standard length (SL; distance between the snout and the base of the tail) was measured and his ejaculate was collected. Each male was categorized as territorial or sneaker on the basis of their size and the characteristics of their sperm trails, which are white in sneaker males, owing to the high sperm content, and dense and opaque in territorial ones, owing to the high mucin content and low amount of sperm [28].

(b) Gamete collection and ejaculate processing

Ejaculate was obtained through a gentle pressure on the abdomen of anaesthetized males and collected with a Gilson pipette. Ejaculate samples were centrifuged at 13.300*g* for 3 min at 4°C to separate sperm from the supernatant seminal fluid (mean fluid volume: territorial: 199 µl ± 123 s.d.; sneaker: 19 µl ± 16 s.d.). Sperm cells were then re-suspended in an extender inactivating medium (3.5 g l^{-1} NaCl, 0.11 g l^{-1} KCl, 0.39 g l^{-1} $CaCl_2$, 1.23 g l^{-1} $MgCl_2$, 1.68 g l^{-1} $NaHCO_3$, 0.08 g l^{-1} glucose, pH 7.7) [29] and maintained at 3–5°C until analysis (within 1 h of collection). As the number of sperm varies among males and is significantly higher in sneakers than territorials, the volume of inactivating solution was individually adjusted (range: sneaker, 70–800 µl; territorial, 45–500 µl), in order to standardize for sperm concentration in inactivated samples (76.069 ± 970 s.d. sperm μl^{-1}). Sperm concentration was checked with an improved Neubauer chamber haemocytometer. Seminal fluid was also maintained at 3–5°C until analysis. Eggs were obtained from previously anaesthetized ready-to-spawn females, through a gentle pressure on their swollen abdomen, and collected on acetate sheets onto which they adhere. Immature eggs do not adhere well to the sheets, and thus these samples were easily detected and discarded. Acetate sheets with eggs were maintained in filtered seawater until the trials (within a few minutes of collection). All individuals were released, unharmed, at the site of collection.

(c) Sperm velocity measurement

Ten microlitres of sperm were taken from inactivated samples and activated by adding 20 µl of filtered seawater at 20°C ± 1°C, containing 2 mg ml^{-1} of bovine serum albumin. Activated sperm samples were then incubated for 2 min without seminal fluid or with 1.5 µl of different seminal fluid solutions (see §2e). In this species, eggs remain fertilizable for several hours and sperm are active for more than 30 min [10,27], thus 2 min of incubation represents a conservative time to guarantee they are not exhausted before performance measurements. Three microlitres of samples were then placed in separate wells on a 12-well multitest slide (MP Biomedicals, Aurora, OH) previously coated with 1 per cent polyvinyl alcohol (Sigma-Aldrich), to avoid sperm sticking to the glass slide [30], and covered with a coverslip. Sperm velocity was measured using a CEROS Sperm Tracker (Hamilton Thorne Research, Beverly, MA). Mean speed measurements were based on 76.31 ± 28.05 (mean ± s.d.) sperm tracks per sample. Among sperm velocity measures we focused our analyses on curvilinear velocity (VCL), as this measure is positively correlated with fertilization success in many external fertilizers [31,32].

(d) *In vitro* fertilization

For each male, subsamples of 10 µl of sperm were activated with the addition of 20 µl of marine filtered seawater and incubated for 2 min with 1.5 µl of different seminal fluids (see §2e). A volume of sperm solution containing 8×10^5 sperm cells was then taken, diluted to 50 µl with filtered seawater, and used for fertilization trials, which were performed by placing an acetate sheet with a pool of eggs collected from three different females on the bottom of a glass beaker containing 500 ml of filtered seawater. Eggs were pooled with the intent of minimizing the potential male-by-female interaction effects at fertilization [33]. A new group of three females was used for each male, while the same three females gave the pools of eggs for the different treatments performed on each male. The three batches of eggs from each female were distributed randomly among treatments with respect to collection order from female. Sperm were homogeneously deposited on the water surface with a Gilson pipette (distance from surface to bottom = 8.5 cm). After 15 min, the acetate sheet was extracted, gently washed and placed in a

new glass beaker with clean filtered seawater and oxygen supply. The percentage of fertilized eggs of each pool was checked 4 h later, when one could clearly distinguish the complete lifting of chorion and the first stages of cellular division. For each trial, 273 ± 47 (mean ± s.d.) eggs were used.

(e) Experimental design

(i) Effect of seminal fluid on sperm performance

In a first experiment, we evaluated the effect of the seminal fluid, released by males performing different tactics, on sperm velocity. The sperm of each territorial male ($n = 20$; SL range: 13.7–18.5 cm) was tested after incubation in its own seminal fluid or with that of a sneaker male. Conversely, the sperm of each sneaker male ($n = 20$; SL range: 6.4–9.3 cm) was incubated either in its own seminal fluid or in that of a territorial male. We also recorded sperm velocity after incubation with the seminal fluid of a male adopting the same tactic to further check if any effect of another male's seminal fluid was specifically owing to interaction with the fluid of a male adopting a different tactic or more generally to the interaction with the fluid of any rival male.

In a second experiment, we compared the sperm velocity of territorial and sneaker males in mixed seminal fluids, a situation closer to that occurring in nature, where ejaculates compete. Sneakers' seminal fluid was diluted 10-fold in filtered seawater before use to match the natural seminal fluid concentration of sneaker and territorial males (see §2*b*). Following the procedure described above, the velocity of the sperm of 24 territorial males (SL range: 13.9–19.0 cm) was measured either in its own seminal fluid or after incubation in a 1 : 1 mixture of its own seminal fluid and the diluted (1 : 10) fluid of a sneaker male. Similarly, the velocity of the sperm of 24 sneaker males (SL range: 6.2–9.4 cm) was measured after incubation in its own seminal fluid (diluted 1 : 10) and in a mixture (1 : 1) of its own (diluted 1 : 10) and the seminal fluid of a territorial male.

Moreover, on all individuals, employed in both experiments, sperm velocity was preliminarily measured also after incubation in activating solution, without any seminal fluid, and results were then compared with sperm velocity after incubation in each male's own seminal fluid. This allowed to determine sperm velocity in the absence of any seminal fluid and to prove the general positive effect of males' own seminal fluid (see details in the electronic supplementary material).

(ii) Effect of the interaction between sperm and seminal fluid on fertilization efficiency

Grass goby sneakers may enter the nest, releasing their ejaculates in proximity to territorial male' ejaculate and to eggs [27,28]. Thus, as long as a sneaker succeeds in entering the nest, his sperm and seminal fluid mix with those of territorial males. In this scenario, fertilization tests performed on either sneakers or territorial males' sperm in the presence of a mixture of both males' seminal fluids may give a reliable indication of the fertilization success in a context of competition.

The effect of a mixture of territorial and sneaker males' fluid on sperm fertilization rate was determined, *in vitro*, for 10 territorial (SL range: 13.1–16.6 cm) and nine sneaker males (SL range: 5.8–8.5 cm). We recorded fertilization rates of territorial male sperm after incubation in his own seminal fluid or in the mixture (1 : 1) of his own seminal fluid with that of a sneaker male (after 10-fold dilution in filtered sea water). The same procedure was applied to the sperm of sneaker males, incubated with their own seminal fluid (diluted 1 : 10) or with a mixture (1 : 1) of their own seminal fluid (diluted 1 : 10) and that of a territorial male.

(f) Statistical analyses

Fertilization data were arcsine square root-transformed prior to analysis. Data are reported as mean standard deviation (s.d.). Normality and homogeneity of variance were checked following Kolmogorov–Smirnov and Bartlett's tests, respectively. Effect of treatment (seminal fluid) on performance and fertilization rate of territorials' and sneakers' sperm were analysed using a univariate ANOVA for repeated measure (generalized linear model). The treatment with different seminal fluids was used as within-subject factor, and the male tactic as between-subject factor. Post hoc comparisons of interest were performed through *t*-test for independent samples when comparing treatments between groups, and through paired *t*-test when comparing treatment within groups. *p*-values were adjusted for multiple testing following Benjamini and Hochberg method. Statistical tests were performed using STATISTICA v. 7.0. Data have been deposited in the Dryad Repository [34].

3. Results

(a) Effect of seminal fluid on sperm performance

The results of the first experiment, where sperm experienced both own seminal fluid and that of a male performing the same or a different tactic, showed a significant effect of treatment on sperm velocity, in opposite directions depending on the adopted tactic (repeated measures ANOVA: male tactic, $F_{1,31} = 0.70$, $p = 0.41$, treatment, $F_{2,62} = 5.14$, $p = 0.008$; tactic × treatment, $F_{2,62} = 3.60$, $p = 0.033$). Indeed, the sperm of sneaker males proved faster when incubated in territorial males' fluid than in their own (paired *t*-test: $t = -2.9$, adjusted $p = 0.01$; figure 1*a*), while territorial males' sperm proved slower when exposed to sneakers' fluid than when incubated in their own fluid (paired *t*-test: $t = 2.97$, adjusted $p = 0.01$; figure 1*a*). This reduced performance of territorial males' sperm cannot be ascribed to the dilution of their seminal fluid in the mixed tactic treatment (see details in the electronic supplementary material). A between-tactics comparison showed that sneakers' sperm in territorial fluid performed better than territorial males' sperm in sneakers' fluid (*t*-test: $t = -2.7$, adjusted $p = 0.01$; figure 1*a*). Moreover, the velocity of both territorial and sneakers' sperm was not affected by the seminal fluid of a male adopting the same tactic (paired *t*-test: sneakers, $t = 0.45$, $p = 0.66$, $n = 20$; territorial, $t = 1.4$, $p = 0.18$, $n = 13$), suggesting that the effect of seminal fluid on sperm performance is tactic-dependent and not due to the interaction with non-self seminal fluid.

In the second experiment, where seminal fluids of two male types were mixed, we again observed a significant effect on sperm velocity depending on the tactic, adopted by males (repeated measures ANOVA: male tactic, $F_{1,46} = 2.87$, $p = 0.1$, treatment, $F_{1,46} = 0.17$, $p = 0.68$; tactic × treatment, $F_{1,46} = 23.63$, $p < 0.001$; figure 1*b*). The sperm of sneaker males were found to be faster with the addition of territorial males' fluid (paired *t*-test: $t = -2.45$, adjusted $p = 0.022$; figure 1*b*), while territorial males' sperm were slowed by the addition of a sneaker's fluid (paired *t*-test: $t = 6.29$, adjusted $p < 0.001$; figure 1*b*). The between-tactics comparison confirmed the results of the first experimental set. Indeed, sneakers' sperm exposed to own plus territorial males' fluid swam significantly faster than territorial males' sperm in the presence of own plus sneakers' fluid (*t*-test: $t = 3.23$, adjusted $p = 0.0036$; figure 1*b*).

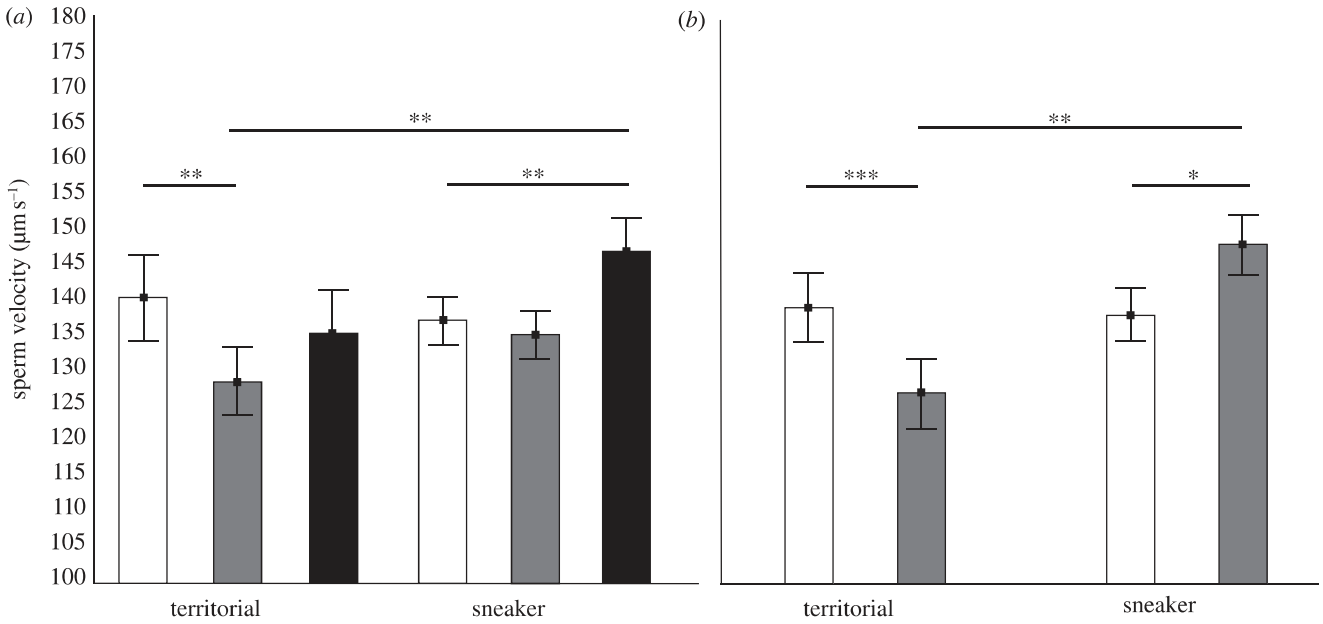

Figure 1. Velocity of territorial and sneaker males' sperm after incubation in different seminal fluids (mean ± s.e.m.). (*a*) Sperm tested in the presence of own fluid or with the fluid of a male performing the same or a rival tactic ($n = 20$ territorial, 20 sneaker; white bars, own fluid; grey bars, other sneaker fluid; black bars, other territorial fluid). (*b*) Sperm tested in the presence of only own fluid or with the addition of rival-tactic male fluid ($n = 24$ territorial, 24 sneaker; sneaker's fluid pre-diluted 1 : 10; white bars, own fluid; grey bars, own + rival-tactic male fluid). *Adjusted $p < 0.05$; **adjusted $p \leq 0.01$; ***adjusted $p \leq 0.001$.

(b) Effect of the interaction between sperm and seminal fluid on fertilization efficiency

In vitro fertilization experiments showed an effect of the treatment on the fertilization efficiency of both territorial and sneaker males, in opposing directions (repeated measures ANOVA: male tactic, $F_{1,17} = 1.49$, $p = 0.24$; treatment, $F_{1,17} = 0.03$, $p = 0.87$; tactic × treatment, $F_{1,17} = 13.13$, $p = 0.002$; figure 2). In particular, sneaker males' sperm showed significantly higher fertilization percentages with the addition of territorial males' fluid (paired *t*-test: $t = -3.10$, adjusted $p = 0.043$; figure 2), while territorial males' sperm had significantly lower fertilization rates when sneaker males' fluid was added (paired *t*-test: $t = 2.36$, adjusted $p = 0.043$; figure 2). The between-tactics comparison showed that sneakers' sperm had a significantly higher fertilization rate in the presence of his own seminal fluid mixed with that of a territorial male, when compared with that of territorial male's sperm in the presence of his own seminal fluid mixed with that of a sneaker male (*t*-test: $t = 2.32$, adjusted $p = 0.043$; figure 2). The observed results did not change when also considering the number of eggs as a covariate in the model (repeated measures ANOVA: number of eggs, $F_{1,17} = 0.30$, $p = 0.59$; male tactic, $F_{1,17} = 1.47$, $p = 0.24$; treatment, $F_{1,17} = 0.01$, $p = 0.93$; tactic × treatment, $F_{1,17} = 12.25$, $p = 0.003$).

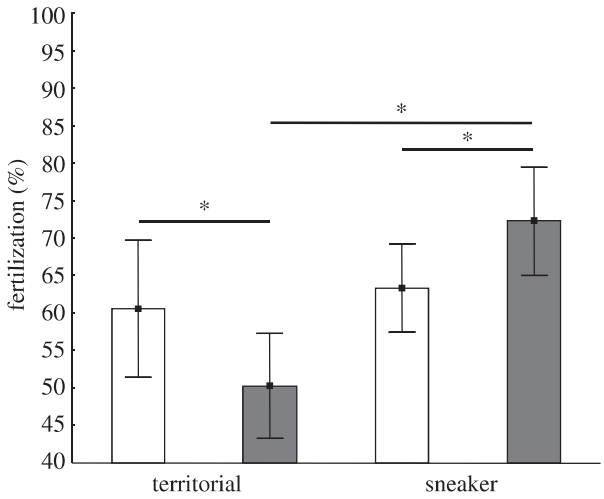

Figure 2. Fertilization percentage (mean ± s.e.m.) of territorial ($n = 10$) and sneaker ($n = 9$) males' sperm after incubation in own seminal fluid and in a mix of own + fluid of a rival-tactic male. The seminal fluid of sneaker males was diluted prior to mixing (1 : 10). *Adjusted $p < 0.05$. Statistics on arcsine square root-transformed data. White bars, own fluid; grey bars, own + rival-tactic male fluid.

4. Discussion

We found that the seminal fluid differently affects the sperm performance of other males in terms of velocity and fertilization success, in relation to the tactic adopted by males. Indeed, the performance of territorial males' sperm is negatively affected by the seminal fluid of sneaker males, while sneakers' sperm perform significantly better in the presence of territorial males' seminal fluid. Thus, sneakers, always mating in competition, enhance their fertilization success by exploiting the seminal fluid of territorial males, and decrease that of territorial males by altering their investment in seminal fluid. These results highlight that male allocate their ejaculate investment in both sperm [2,18] and non-sperm components in response to sperm competition risk, and add an important observation to the understanding of ejaculate evolution.

Grass goby ejaculates vary in both sperm number and seminal fluid amount according to the mating tactic adopted by males, with those of territorial males being richer in seminal fluid and poorer in sperm than those of sneakers [27,28]. However, the variation in seminal fluid volume does not solely account for the entire pattern emerging from our study. Indeed, the performance of territorial and sneaker

males' sperm in their own seminal fluid, as well as in that of other males performing the same tactic, is similar (see also the electronic supplementary material), but when the fluid of a male employing a different tactic is present, sperm performance goes in opposite directions. Moreover, in a mixture of territorial and sneaker males' seminal fluids, sneakers' sperm are faster than territorial males'. This suggests that: (i) the detrimental effect of sneakers seminal fluid on territorial males' sperm is not due to a self/non-self process, but rather to a variation in composition; and (ii) the sperm released by territorial and sneaker males differ in quality. However, how the composition of seminal fluid changes with ejaculate size in males adopting ARTs and which components affect sperm velocity are still unknown.

A substantial amount of research documents the influence of different seminal fluid components in stimulating sperm capacitation, enhancing sperm speed and viability, and providing nourishment to sperm, particularly in mammals and insects (for review, see [19]). Moreover, an intra-specific variation in seminal fluid composition in relation to ejaculate size has been proposed to occur in fowls, where seminal fluid taken from the large ejaculate released by dominant males increases sperm velocity, while that of the smaller ejaculates released by subdominant males decreases it [35]. Much less is known about fish, but seminal fluid proteins with molecular weight less than 50 kDa, monosaccharides, and triglycerides seem to affect sperm viability and speed [36,37]. The seminal fluid of grass goby territorial males might be richer in these compounds to sustain the fertilizing ability of their sperm. Territorial males' sperm are embedded in an abundant and viscous seminal fluid that, by slowly dispersing and diluting in seawater, allows a steady supply of sperm during spawns lasting, on average, 8 h [27,28]. Territorial males begin to lay their ejaculates before egg deposition, while sneakers release theirs in proximity to eggs [27]. Thus, sneakers' sperm may exploit the seminal fluid of the territorial males' ejaculates. Sperm of external fertilizers has to deal with hostile environmental conditions, in particular with the different water osmolarity compared with that of their seminal fluid [38], and the exploitation of rival seminal fluid could reduce the cost of ejaculate production for sneakers [25]. A similar tactic-dependent investment in non-sperm components of the ejaculate probably occurs in numerous fish species, including salmonids, blennies, wrasses, damselfishes, sunfishes, cichlids and other gobies [39], where parasitic males mate in the presence of a territorial male, and thus might exploit their seminal fluid. This expectation is supported by the observation that in those species where seminal fluid is produced by specific accessory organs, these are more developed in territorial than in opportunistic males [37,40–42].

Still, grass goby sneakers' sperm appear to be of higher quality than those of territorial males. An increase in sperm quality in response to higher sperm competition risk has been documented in several species [8,10,15,43–46]. However, grass goby sneakers' sperm do not differ from those of territorial males in the performance parameters commonly recorded, including morphology, velocity, viability, longevity and ATP content [10,11]. This suggests that differences in sperm quality between tactics can only be detected in the presence of a mixture of seminal fluids, an approach not considered in previous studies. It is unknown which physiological feature allows sneakers' sperm to make the best of territorial males' seminal fluid. Similarly, it is unknown which components of seminal fluid might be involved in impairing territorial males' sperm and enhancing sneaker males' sperm. Both aspects need to be elucidated to understand the proximate mechanisms driving sperm–seminal fluid interaction in this species.

Regardless of the physiological mechanisms by which males alter sperm and seminal fluid quality, a general pattern can be derived from these results. Grass goby territorial males, not necessarily mating in competition, bias their ejaculate allocation to seminal fluid, having to guarantee fertilization and to limit sperm waste during the long-lasting spawning [27,28]. By contrast, sneakers, taking advantage of territorial males' seminal fluid, lower the quality of their own fluid, and may increase the investment in sperm number [28] and quality. This may have important implications for the evolution of ejaculate composition and allocation strategies. Indeed, the outcome of sperm competition may not only be determined by sperm quality or number [47], as seminal fluid may also affect sperm performance. The importance of considering the effect of sperm–seminal fluid interactions in response to sperm competition has been stressed by theoretical studies [25,26]. Our results, in line with these expectations, underline that further experimentation to understand the evolution of ejaculate allocation strategies cannot neglect the effect of seminal fluid on sperm fertilization success.

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Paper II

“Seminal fluid does not mediate ejaculates competition in the black goby”

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Abstract

Increasing evidence are suggesting that predictions on the outcome of sperm competition should not revolve only around the investment in sperm number and quality. Indeed, sperm competition is expected to influence those traits driving sperm fertilization capabilities in a specific context. Seminal fluid contribution may depend on the degree of interactions between sperm and seminal fluids of rival males’ ejaculates, that in turn is swayed by species-specific mating dynamics and fertilization mode. The black goby (*Gobius niger*) is an external fertilizer with territorial-sneaker mating tactics, where sperm competition risk varies depending on the tactic adopted. Territorial males address their investment to enhance aggressive behaviours at higher levels of sperm competition, forcing sneakers to release their ejaculates almost at the nest entrance. On the other side, sneakers produce more sperm that are faster, more viable and with higher ATP content than those of territorial males. As mating dynamics makes the mixing of sneakers and territorial males’ ejaculates very unlikely or rare, ejaculate competitiveness in this species is expected to be influenced by sperm number and or/quality and not by seminal fluid. To test this prediction, we experimentally manipulated black goby ejaculates, by separately combining sperm and seminal fluid from opposite tactics males, and analysed sperm performance in terms of velocity, viability, and fertilization success. Own seminal fluid increases sperm velocity and viability only in territorial male ejaculates, but sneaker sperm still remain faster and more viable than territorial males ones. Both velocity and viability of sneakers and territorial males’ sperm were not affected by the seminal fluid of other males, regardless the tactic they adopted. However, territorial males’ sperm move with a significantly more linear trajectory than those of sneaker males. The better performance of sneaker sperm, in velocity and viability, are not mirrored by fertilization rate *in vitro*, as both sneakers and territorial males’ sperm appear to have similar ability. We suggest that the difference in sperm movement trajectory might account for the observed fertilization rates, as sneaker and territorial male sperm could cover the same distance in a similar time, even if sneaker sperm have an higher linear velocity. Overall these findings confirm that ejaculate competitiveness in this species is shaped by sperm and not by seminal fluid.

Introduction

In the last forty years, the historical notion of monogamous females, pair-bonded with the same male for life, has been gradually eroded away by the increasing evidences of multiple paternity in natural litters, clutches, and broods of diverse taxa (Andersson, 1996; Taylor et al., 2014). As a result, female multiple mating is now addressed as a common and ubiquitous phenomenon in nature, that has relevant biological implications at multiple scales, triggering a great theoretical and experimental attention to its evolutionary consequences (Taylor et al.,2014). Polyandry implies that sexual selection may persist even after the copulation up to the point of fertilization, and in some cases beyond (Birkhead and Pizzarri, 2002). In this scenario, male mating not necessarily results in successful insemination, but depends on the outcome of post-copulatory sexual mechanisms influencing paternity. Among these, sperm competition occurs when the ejaculates of two or more males compete to fertilize the same set of eggs, as it has been firstly defined by Geoffrey Parker (Parker, 1970). When ejaculates overlap in space and time, differences in characteristics that are key factors for the fertilization success may lead one ejaculate to overcome the rivals, generating differential males' reproductive success. This mechanism, investigated in both externally and internally fertilizing species, is a powerful evolutionary force moulding an amazing variety of behavioural, morphological and physiological traits (Birkhead and Moller, 1998; Birkhead and Pizzari, 2002). The most widespread adaptation to sperm competition in males is represented by an increase in sperm expenditure to enhance their probability of egg fertilization. Ejaculates are costly to produce, thus males may modulate their investment in response to different sperm competition contexts, to maximize their reproductive success. Indeed, comparative studies, across both species and populations, show that increasing levels of sperm competition drive a greater ejaculate investment as assessed by relative testis size, sperm number and sperm quality (Stockley et al., 1997; Birkhead and Møller, 1998; Snook, 2005; Simmons and Fitzpatrick, 2012; Lupold et al., 2012). For example, in species with alternative reproductive tactics (ARTs), males experience different levels of sperm competition depending on the adopted phenotype. Opportunistic males face with the highest levels and respond releasing more sperm, that can be also faster and/or more viable, than dominant males, playing the lower risk tactic (Gage et al., 1995; Stoltz and Neff, 2006; Locatello et al., 2007).

To date, theoretical and empirical studies have primarily focused on how sperm characteristics, i.e. number and quality, affect the fertilization success of competing males (Birkhead and Møller, 1998; Snook, 2005). However, increasing evidence are suggesting that predictions on the outcome of sperm competition should not revolve only around the sperm component of the ejaculate. The seminal fluid often makes up a large part of an ejaculate and it may influence paternity success both directly and indirectly. Indeed, seminal fluid is already known to enhance sperm performance in

several species (Pojani, 2006), as well as to indirectly influence paternity success, by decreasing female receptivity, increasing oviposition rate and forming mating plugs (Poiani, 2006; Wigby et al., 2009). Seminal fluid may also play a frontline role in sperm competition by directly affecting rivals' sperm performance. For instance, in promiscuous ants and bees, seminal fluid incapacitates the sperm of rival males (den Boer et al., 2010), while in other insects, it improves equally the survival of own and other sperm (Holman, 2009; Simmons and Beveridge, 2011). This suggests that, unless a self/non-self-recognition mechanism evolves (Holman, 2009), the function of seminal fluid to enhance own sperm performance can be exploited by the sperm of rival males (Hodgson and Hosken, 2006). In particular, when a male can assess in which reproductive role he is mating with a female, if advantaged or disadvantaged, he could strategically allocate its ejaculate to maximize his reproductive success. Theoretical analyses, still waiting for experimental tests, posit that selection should favour phenotypic plasticity in male expenditure on both sperm and seminal fluid components, specifically influencing that/those component/s that affect more the ejaculate competitive weight (Cameron et al., 2007).

Conditions for male parasitism of rival ejaculates are reasonably common in natural mating systems, but it is not always easy to distinguish the favoured or disfavoured mating role and to correctly disentangle sperm competition pressures. Recently, in the grass goby, a species with ARTs *(Zosterisessor ophiocephalus*), it has been demonstrated that sneaker sperm not only take advantage of the territorial males seminal fluid, but also their ejaculates have a detrimental effect on territorial male fertilization success (Locatello et al., 2013). The opportunity for sneakers to exploit territorial male seminal fluid likely arises from the spatial context in which ejaculates competition occurs. Indeed, grass goby sneakers enter inside the nest and may release their ejaculates in close proximity to those of the territorial male and to the eggs, setting the conditions for rival sperm-seminal fluid interplay. A potential crucial factor influencing the possibility that the seminal fluid weights on the outcome of ejaculates competition is exactly the degree of rival ejaculates interaction (Cameron et al., 2007), but experimental evidences are still lacking.

The black goby (*Gobius niger*) is a good model species to test if where the interaction between rival ejaculates is unlikely, seminal fluid does not contribute to post-copulatory competitiveness, being excluded/reduced the opportunity for the exploitation of rivals' seminal fluid and/or for the impairing of rivals' sperm. This species shows a mating system similar to that of the grass goby, with guard-sneaker mating tactics, and comparable levels of sperm competition, but potentially differ in the likelihood for seminal fluid to influence competition contexts, due to some differences in the mating dynamics. Indeed, in the black goby, sneakers are forced to release their ejaculate at the nest entrance and, thus, the opportunity for the mixing of territorial males' and sneakers' ejaculates does not occur or it is rare (Taborsky, 1998; Locatello et al., 2007). Moreover, differently from the grass goby, sperm quality investment vary between the

two tactics, with sneaker producing sperm that are faster, more viable and whose ATP content is higher than territorial males, as well as more numerous (Locatello et al., 2007). However, sperm quality traits have been recorded without their seminal fluid (Locatello et al., 2007). To verify if sperm performance is representative of the whole ejaculate, we experimentally manipulated black goby ejaculates, and measured sperm viability and motility (velocity and path linearity) in presence of their own seminal fluid. Indeed, we may expect the seminal fluid to influence the performance of own sperm.

We do not expect the seminal fluid to influence the outcome of sperm competition in this species, since the ejaculates of competing males are released far from each other and sneaker males greatly address their ejaculates expenditure towards sperm number and quality. However, while the low amount of seminal fluid released by sneakers likely entailed a rapid dilution (Mazzoldi, 1999), territorial trails slowly dissolve inside the nest and close to the eggs and consequently we can not a priori exclude that the seminal fluid of territorial males may affect sneaker sperm. Therefore, we added a treatment for sneakers sperm, incubating them with the seminal fluid of a territorial male.

Finally, we verified that the results emerged from sperm viability and motility were mirrored by sperm fertilization rates, evaluated under the same treatments.

Materials and methods

Animal sampling and handling

Animals were collected in the Venetian Lagoon during their breeding season (June-August) and separately maintained in tanks under artificial light (14 L : 10 D), daily change of water (24°C) and fresh feeding. Before ejaculate sampling, males were anaesthetized in a water solution of MS 222 (Tricaine sulfate, Sandoz), and their body measures were collected (standard length, SL: distance between the snout and the base of the tail). Each male was assigned to sneaker and territorial category on the basis of their size, the development of secondary sexual traits (nuptial coloration and first ray's elongation of the first dorsal fin) and the characteristics of sperm trails, as already described (Mazzoldi et al., 2000; Mazzoldi and Rasotto, 2002; Locatello et al., 2013).

Gamete collection and ejaculate processing

Ejaculate was obtained through a gentle pressure on the abdomen of anaesthetized males and collected with a Gilson pipette directly from the urogenital papilla. Ejaculate samples were centrifuged at 13,300 g for 3 min at 4°C to separate sperm from the supernatant seminal fluid. Sperm were then re-suspended in an inactivating solution (3.5 g L^{-1} NaCl, 0.11 g L^{-1} KCl, 0.39 g L^{-1} $CaCl_2$, 1.23g L^{-1} $MgCl_2$, 1.68 gL^{-1} $NaHCO_3$, glucose 0.08 g L^{-1}, pH 7.7) (Fauvel et al., 1999). As the number of sperm varies among males and is significantly higher in sneakers than territorials, the volume of inactivating solution was individually adjusted in order to reach the same sperm concentration for all inactivated samples for sneaker and territorial males. Sperm concentration was evaluated with an improved Neubauer chamber haemocytometer. Sperm and seminal

fluid separate samples were maintained at 3–5°C until analysis (within 1 h of collection). Eggs were obtained from previously anaesthetized ready-to-spawn females, through a gentle pressure on their swollen abdomen, and collected on acetate sheets onto which they adhere. Immature eggs do not well adhere to the sheets, and thus these samples were easily detected and discarded. Acetate sheets with eggs were maintained in filtered sea water until the trials (within a few minutes of collection). All individuals were released, unharmed, at the site of collection.

Sperm viability

The proportion of living sperm immediately after activation and after 30 minutes was estimated for both tactics (n=6 sneaker, 6 territorials), since it was demonstrated that sperm speed declined similarly in both tactics after this period, probably affecting overall sperm performance (Locatello et al., 2007). Sperm were incubated with their own seminal fluid and subsamples of 10 µL were placed on a glass slide and observed under a light microscope, maintaining room temperature at 24±1°C. Sperm were considered viable if they show head or tail movements (Locatello et al., 2008).

Sperm motility measures

The analyses were performed on 79 black gobies (territorials: SL range= 8.2–12.2 cm n=44; sneakers: SL range= 4.5–6.6 cm, n= 35). Sperm were activated by adding filtered sea water at 24°C ± 1°C containing 2 mg/mL of BSA. Seven microliters of sperm were taken from inactivated samples and activated by adding 15 µL of filtered sea water, at 24°C+1°C, containing 2 mg mL of bovine serum albumin. Activated sperm samples were then incubated for 2 min without seminal fluid or with 1 µL of different seminal fluid, depending on the treatment (Locatello et al., 2007). Sperm velocity was measured with an IVOS Sperm Tracker (Hamilton Thorne Research, Beverly, MA) placing three microliters of activated samples in separate wells on a 12-well multitest slide and covering with a coverslip (MP Biomedicals, Aurora, OH, USA) previously coated with polyvinyl alcohol solution (1%; Sigma-Aldrich) to avoid sperm sticking to the glass slide (Wilson-Leedy and Ingermann, 2007). Among sperm motility different measures we focused on curvilinear velocity (VCL), as this measures is a reliable clue of the fertilization success in many external fertilizers (Au et al., 2002; Casselman et al., 2006). In addition, we evaluated also the straightness of sperm swimming trajectory, through path linearity (VSL/VCL; VSL=straight-line speed): its value varies between 0 and 1, where 0 means the sperm started and ended at the same location while 1 implies the sperm progress in a straight line (Kime et al., 2001; Stoltz and Neff, 2006; Fisher et al., 2014).

In vitro fertilization trials

For each male (n=12 territorials; n=12 sneakers), subsamples of 7 µL of sperm were activated as for sperm speed tests. A volume of sperm solution containing $2x10^5$ sperm cells was then diluted to 50 mL with filtered sea water, and used for fertilization trials, in

order to standardize the volume for each test. Acetate sheets with a pool of eggs collected from three different females were placed on the bottom of a glass beaker containing filtered sea water. Eggs were pooled to minimize the potential female influences at fertilization (Fitzpatrick et al., 2012; Locatello et al., 2013). Sperm were homogeneously distributed on the water surface with a Gilson pipette and the distance from surface to bottom was 3 cm, that was the mean height of nests recorded in the field. After 15 min, the acetate sheet was extracted, washed and placed in a new glass beaker with clean filtered sea water and oxygen supply. The percentage of fertilized eggs of each pool 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). For each trial, 149.83±53 (mean+s.d.) eggs were used.

Experimental design

Sperm velocity and motility. The effect of seminal fluid on sperm velocity was evaluated comparing sperm velocity with and without their own seminal fluid (n=44 territorials; n=35 sneakers). In this species we did not expect that the seminal fluid of sneaker males affect territorial sperm performance, since they are forced to release their ejaculate at the nest entrance sperm and seminal fluid entailing a rapid dilution (Mazzoldi, 1999). Through a preliminary experiment we compared territorial sperm velocity in their own seminal fluid and with that of a sneaker male (n=10 territorials). On the other side, the seminal fluid of territorial males may affect sneaker sperm, since territorial trails slowly dissolve inside the nest and close to the eggs and consequently sneaker sperm are likely to encounter territorial ejaculates. Therefore, we added a treatment for sneakers sperm, incubating them with the seminal fluid of a territorial male.

In vitro fertilization trials. Fertilization success of sneaker (n=12) and territorial males (n=12) was evaluated with their own seminal fluid. As in sperm velocity tests, sneaker sperm fertilization rate was evaluated also in the presence of a territorial male seminal fluid.

Statistical analyses

Normality was checked following Kolmogorov–Smirnov. Data are reported as mean standard error (s.e.). Fertilization rates were arcsine square root transformed prior to analyses.

Effect of treatment (seminal fluid) on performance of territorials' and sneakers' sperm were analysed using linear mixed model (with restricted maximum likelihood estimation REML) in SPSS 21. We included, depending on the experiment, seminal fluid treatments (without/with own/ with territorial seminal fluid) measured as velocity (VCL) or path linearity (LIN) or fertilization rate as the dependent variable, and tactic (sneaker or territorial) as a fixed factor. To account for repeated measures on individual males, male identity was included as a random factor with estimate of random intercepts for each subject. Tests of the fixed effects were followed by post-hoc pairwise comparisons and

p-values were adjusted for multiple comparisons following Benjamini and Hochberg method (Benjamini and Hochberg, 1995).

Results

Seminal fluid effect on sperm viability

Viability tests confirmed the past values obtained in salt water without the seminal fluid (Locatello et al., 2007), with sneaker sperm showing significantly higher rates than territorial males, both in absence and in presence of their own seminal fluid (linear mixed model: p=0.033 F=6.14).

Seminal fluid effect on sperm speed and path linearity

The preliminary experiment confirmed that sneaker seminal fluid does not influence territorial sperm performance neither in terms of velocity (VCL; test T for dependent samples: t=0.49 p=0.63) nor linearity (LIN; test T for dependent samples: t=0.65 p=0.53). Velocity (VCL) results confirmed the expectations, and despite the fact that own seminal fluid increases the sperm performance only in territorial males (linear mixed model, none, own versus opposite tactic seminal fluid VCL: tactic p<0.001 F=20.02; treatment p=0.543 F=1.015; interaction tactic x treatment p<0.001 F=13.04), sneaker sperm are still faster than territorial ones (adjusted p=0.04). Moreover, sneaker sperm velocity is not affected by territorial males seminal fluid and are still faster than territorial sperm in their own seminal fluid (adjusted p<0.001) (fig. 3a). The sperm, instead, differ between the male of the two tactic, for the type of motion, regardless the presence of seminal fluid, with territorial males' sperm having higher linearity values (linear mixed model: LIN tactic p=0.001 F=11.69; treatment p=0.528 F=0.40; tactics x treatment p=0.42 F=0.67). In addition, results do not change when sperm are tested with the seminal fluid of a male having the opposite tactic (fig. 3b).

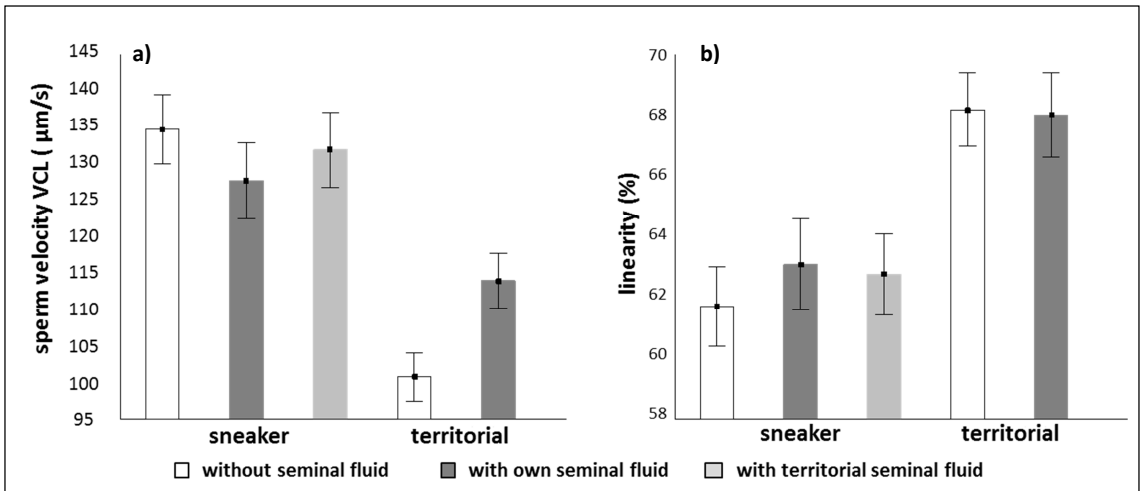


Fig. 3 **a)** velocity and **b)** linearity (mean ± s.e.; n=44 territorials; 35 sneakers) of territorial and sneaker males' sperm after incubation in different seminal fluids. Sperm tested in the presence of none fluid, own fluid and sneaker with the territorial male seminal fluid. Sneaker sperm have significantly higher velocity (VCL), while territorial males displays higher linearity values, in all treatments.

In vitro fertilization trials

Fertilization rates measured for sneaker and territorial males did not mirror sperm speed results, the fertilization success do not significantly vary between the two tactics. However, it cannot be excluded that this finding is affected by the high variability of the results and the relative low number of the tests (linear mixed model: fertilization rate tactic p=0.96 F=0.003; treatment p=0.34 F=0.97).

Discussion

In the black goby we found that seminal fluid of territorial males significantly enhance their sperm performance, but sneaker sperm are faster and more viable, regardless the presence of their own seminal fluid. Sneaker sperm might have the opportunity to exploit territorial males seminal fluid, as grass goby sneaker do (Locatello et al., 2013). In fact, even if they are necessarily released at distance due to the tight territorial mate guarding, while swimming towards the eggs attached to the nest ceiling they likely come in contact with territorial ejaculate, that slowly dissolves from trials laid to the nest walls. However, we did not find any effect of territorial male seminal fluid on sneaker sperm speed, one of the key predictor of sperm fertilization success in external fertilizers (Snook, 2005; Locatello et al., 2013; Mehlis and Bakker, 2014). In addition, seminal fluid do not sway the relative sperm viability, with sneaker sperm more viable than those of territorial males, confirming what observed in a previous study (Locatello et al., 2007). Thus, the ejaculate competitiveness of sneakers males is based on sperm number and quality, without any substantial contribute of seminal fluid. Nevertheless, *in vitro* fertilization trials did not mirror sperm performance results, as sneaker and territorial ejaculates do not differ in their fertilization success, nor territorial male seminal fluid significantly affect sneaker fertilization rate. Although we cannot exclude that the number of fertilization trials may be insufficient to highlight an asymmetry in the proportion of eggs fertilized by the two male phenotypes, an explanation of the observed fertilization rates might reside in the different swimming mode performed by sneakers and territorial males' sperm .

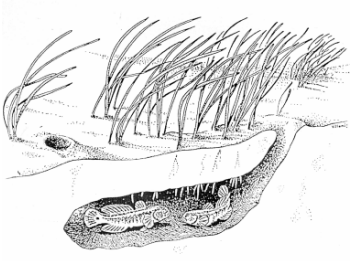
Indeed, territorial males' sperm move with a straighter trajectory compared to sneakers' ones, as shown by their higher linearity values. As a consequence, sneaker and territorial males' sperm might travel the same distance taking on the same time even if those of sneaker have an higher linear velocity (VCL). Indeed, if the higher speed follows through a motion that is far to be along a linear path, the whole travelling could be less efficient than swimming slower but along in a more linear path. In a recent work on *Peromyscus maniculatus* it has been found that the higher sperm velocity (VCL) of more competitive ejaculates is not due to a change in speed, but rather to travelling with a more direct path (Fisher et al., 2014). The sperm path linearity has rarely been proved to influence sperm fertilization rate and results are often contrasting (Froman et al., 1999; Stoltz and Neff, 2006; Fisher et al., 2014). However, this motility measure

could account for the similar fertilization rates we have recorded in sneaker and territorial males' sperm, even if our sample size is too limited to draw a robust conclusion. Future studies would be needed to clarify the relationship between sperm linearity and fertilization ability in this species.

In addition, sperm swimming trajectory could be a key factor in external fertilizers, where it may be essential to distinguish among sperm speed measured along the three-dimensional path trajectory, namely sperm velocity, and the different sperm speed measures calculated on a two-dimensional plane.

In the black goby, as well as in the grass goby, the spatial context in which the competition between ejaculates occurs proved to be important. The distance at which rival ejaculates are released influences the strategy to maximize the fertilization success: through the number and/or the quality of the sperm, or taking advantage of the seminal fluid of a rival-tactic male. Increasing evidence brought out the importance of seminal fluid across competition contexts. The comparison between the grass and the black goby scenarios suggests that the degree of ejaculates interplay determined by mating dynamics and the spatial context is an important factor driving males' allocation in ejaculate components .

Paper III



"Proximate mechanisms driving sperm-seminal fluid cross interactions in ejaculates competition"

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Abstract

To date, increasing evidence outlined that predictions on the outcome of sperm competition should not revolve only around the sperm component of the ejaculate and point at the seminal fluid as a new possible player in the ejaculates competition game. The study of seminal fluid influence on the outcome of ejaculates competition proceeds analysing if and when it may sway rival ejaculates competitiveness and, on the other side, how proximate mechanisms drive sperm-seminal fluid interactions.
In the grass goby, *Zosterisessor ophiocephalus*, a species with alternative mating tactics, it has been demonstrated that while own seminal fluid increase sperm velocity in both male phenotypes, the seminal fluid of the opposite tactic impairs territorial males' sperm and enhances sneaker males' sperm (velocity and fertilization rate). These findings brought out that i) sneakers' sperm appear to be of higher quality than those of territorial males for a parameter not previously measured and ii) seminal fluid composition differ between the two tactics. Thus, in this study we evaluated sperm performance recording their oxygen consumption rate, a parameter not commonly recorded in sperm competition contexts, and b) analysed seminal fluid composition. Results outlined that a) sneakers' sperm present higher oxygen consumption rate and b) seminal fluid composition do not differ in the glucose content, whereas territorial seminal fluid presented a higher protein concentration, with both qualitative and quantitative differences between sneaker and territorial protein profiles. The proteins we identified from both tactics seminal fluid correspond to others found in the seminal fluid of other fish species, and they are supposed to take part in sperm protection from oxidative damages and pathogens, antimicrobial function and being part of the Ca^{2+} mediated mechanism of sperm activation. In addition, we investigated which components of seminal fluid might account for the tactic dependent effect found. The seminal fluid was divided in protein (>3kDa) and non-protein (<3kDa) components and we proceeded to evaluate their relative effect on the velocity of both territorial and sneaker sperm, with fractions isolated from both their and opposite tactic seminal fluid.
Among sneakers, emerged that 1) the non-protein fraction (whose major components are glucose, ions but the presence of small peptides cannot be excluded) marks the differences between treatments, since their absence significantly lowered sperm velocity irrespective of the protein fraction effect; 2) the presence/absence of the protein fraction appears to influence the mean sperm velocity, but the difference is not significant, possibly because of the limited sample size

Introduction

Ejaculates consist of sperm and seminal fluid but, until recently, only the first component was considered when studying male fertilization success in the context of post-copulatory sexual selection. In the last few years, the seminal fluid function and molecular make-up received great scientific attention as they appear to influence a wide range of fitness-determinant processes, being critical for male fertility. Indeed, seminal fluid constitutes a biochemically complex mixture crucial for ejaculates functionality, enabling sperm fertilization ability, maintaining sperm during storage in internal fertilizers (Gillot, 1996; Chapman, 2001; Wolfner, 1997; 2002; Froman, 2003; King et al., 2010), and enhancing sperm performance in both external and internal fertilizers (Lahnsteiner et al., 2003; Poiani, 2006; Simmons and Beveridge, 2011). Seminal components may also indirectly influence paternity success, by influencing female physiology (Pojani 2006; Wigby et al., 2009) or by affecting rivals' sperm performance. For instance, in promiscuous ants and bees, seminal fluid incapacitates the sperm of rival males (den Boer et al., 2010), while in other insects, it improves equally the survival of own and other sperm (Holman, 2009; Simmons and Beveridge, 2011). This suggests that, unless a self/non-self-recognition mechanism evolves (Holman, 2009), the function of seminal fluid to enhance own sperm performance can be exploited by the sperm of rival males (Hodgson and Hosken, 2006).

Theoretical analyses posit that selection should favour phenotypic plasticity in male expenditure on both sperm and seminal fluid components, specifically influencing that/those that affect more the whole ejaculate competitiveness (Cameron et al., 2007). Male allocation can thus differently works on sperm and/or seminal fluid components to maximize ejaculate fertilization success. An increase in sperm investment, in terms of sperm number and/or quality, is a widespread phenomenon occurring, at both inter- and intra-specific level, in relation to sperm competition risk (Harcourt et al., 1995; Gage, 1994; Stockley et al., 1997; Birkhead and Møller 1998; Hosken and Ward, 2001). By contrast, variation in seminal fluid composition in relation to ejaculate competition has been shown in *Drosophila melanogaster*, where males are capable of adjusting the amount of specific seminal fluid proteins in response to the perceived level of competition (Wigby et al., 2009).

In the grass goby, *Zosterisessor ophiocephalus*, males exhibit guard-sneaker mating tactics with sneaker males releasing ejaculates richer in sperm but poorer in seminal fluid than territorial males (Scaggiante et al., 1999; Mazzoldi et al., 2000). It has been recently demonstrated that seminal fluid mediates rival ejaculates interplay (Locatello et al., 2013). Indeed, while sperm of sneaker and territorial males do not differ in their velocity and fertilization rate when only their own seminal fluid is present, sneakers' ejaculates increased their performance when interact with territorial males' seminal fluid. In contrast, sneaker seminal fluid had a detrimental effect on the performance of territorial males' ejaculates.

These results evidenced that both sperm quality and seminal fluid composition are involved in the proximate mechanism driving sperm seminal fluid interaction. Indeed, i) sneaker sperm must be endowed of an higher quality, since they take advantage of territorial male seminal fluid more than own territorial sperm, and ii) the seminal fluid composition has to differ between the two male tactics, as it was excluded a self/non-self recognition mechanism between sperm and seminal fluid (Locatello et al., 2013). Therefore, we evaluated sperm quality recording sperm oxygen consumption rate, a parameter not often measured, despite mitochondrial morphology and functionality have been already demonstrated to influence sperm motility characteristics in other species (Lupold et al., 2009; Suquet et al., 2012). Secondly, we deepened seminal fluid composition measuring a) glucose amount, that constitute an energetic reserve for sperm (Poiani, 2006) and it was already found in fish seminal fluid (Lahsteiner et al., 1997; Aramli et al., 2013), and b) protein total concentration looking at possible qualitative and quantitative differences between the two tactics protein profiles. Proteins are the main target of studies that are trying to shed light on the mechanisms regulating sperm-seminal fluid interplay, even in competition contexts. Indeed, both seminal fluid and sperm proteins proved to be under post-copulatory sexual selection pressure, that drives adaptive variations to improve male fertilization success (Dorus et al., 2004; Ramn et al., 2009; Simmons et al., 2013). Finally, to investigate the functionality of different seminal fluid components, we tested the effect of protein versus non-protein fractions of seminal fluid (isolated from the same and the opposite tactic) on sperm curvilinear velocity (VCL), a parameter proved to be a reliable predictor of sperm fertilization ability (Locatello et al., 2013). We aimed not only to detect the differences between the two tactic seminal fluid composition, but also to clarify which components of the seminal fluid account for its tactic dependent effect on sperm of different male phenotypes.

Materials and methods

Animal sampling and handling

Animals were collected in the Venetian Lagoon during their breeding season (March–June) and maintained in tanks under artificial light (14 L : 10 D), daily change of water (20°C) and fresh feeding. Before the collection of ejaculate sampling, males were anaesthetized in a water solution of MS 222 (Tricaine sulfate, Sandoz), and their body measures were recorded (standard length, SL: distance between the snout and the base of the tail). Each male was assigned to sneaker and territorial category on the basis of its size and the characteristics of sperm trails, as already described (Mazzoldi et al., 2000; Mazzoldi and Rasotto 2002; Locatello et al., 2013).

Gamete collection and ejaculate processing

Ejaculate was obtained through a gentle pressure on the abdomen of anaesthetized males and collected with a Gilson pipette directly from the urogenital papilla. Ejaculate

samples were centrifuged at 13.300g for 3 min at 4°C to separate sperm from the supernatant seminal fluid. Sperm were then re-suspended in an inactivating solution (3.5 g L^{-1} NaCl, 0.11 g L^{-1} KCl, 0.39 g L^{-1} $CaCl_2$, 1.23g L^{-1} $MgCl_2$, 1.68g L^{-1} $NaHCO_3$, glucose 0.08 g L^{-1}, pH 7.7) (Fauvel et al., 1999). As the number of sperm varies among males and is significantly higher in sneakers than territorials, the volume of inactivating solution was individually adjusted, in order to obtain the same sperm concentration for both tactics, controlled with an improved Neubauer chamber haemocytometer (around 76.069±970 s.d. sperm mL^{-1}). Sperm and seminal fluid separate samples were maintained at 3–5°C until analysis (within 1 h of collection). Seminal fluid samples collected to perform the analysis of the components and functionality tests were stored at -80°C, immediately after being separated from the sperm component. Sperm were activated adding to ten microlitres of inactivated samples 20 µL of filtered sea water, at 20°C±1°C, containing 2 mg mL^{-1} of bovine serum albumin. Activated sperm samples were then incubated for 2 min before performing any analysis (Locatello et al., 2007). All individuals were released, unharmed, at the site of collection.

Sperm quality

Through a micro sensor oxygen meter (Microx TX2-AOT) we recorded the oxygen consumption of sperm. Sperm were separated from seminal fluid and then activated with filtered salt water at a standard concentration for microliter. Seminal fluid was not added to the treatment because we wanted to isolate the sperm respiration performance, without any contribute of possible nourishment or enhancing components contained in the seminal fluid. The oxygen consumption was recorded for 30 minutes, and then calculated as oxygen consumption rate per minute for one million of sperm (N=24 territorial; N=22 sneaker).

Seminal fluid composition and function

Seminal fluid composition

We recorded glucose and protein content from seminal fluid samples (previously frozen at -80°C). The glucose content was measured through quantichrom glucose assay kit (DIGL 100) (N=10 territorials; N=8 sneakers), whereas protein concentration was detected with bicinchoninic acid protein assay kit (BCA Sigma-Aldrich) (N=17 territorial; 17 sneaker).

To highlight the possible qualitative differences in proteins profiles between the two tactics we set up a protocol to analyze protein composition starting from a pool of seminal fluids collected from different males, separately for each tactic. We measured protein concentration of a given pool, in order to compare sneaker and territorial profiles in the subsequent analysis, without any bias due to different protein concentration. We performed polyacrylamide gel electrophoresis (SDS-PAGE; 4-20%) without pre-treating seminal fluid samples. Then, to identify, through mass determination, the proteins differing in the seminal fluids of sneaker and territorial males, we isolated from gels and processed bands of interest and then compared the

sequences obtained with those available in databases (software Mascot Search Engine version 2.2.4). Pool of seminal fluids collected from different males, separately for each tactic, were analyzed by SDS-PAGE using a Mini-PROTEAN TGX precast 4-20% resolving gels (Bio-rad). The bands were visualized by Colloidal Coomassie G-250 Staining. We loaded 24 µg of total protein amount from sneaker or territorial samples into each well. The proteins relative to bands, that resulted different (presence or amount) between the two tactics, were analyzed by mass spectrometry. Excised from the gel, spots were washed with 50% v/v acetonitrile (ACN) in 0.1 M NH_4HCO_3, and vacuum-dried. The proteins were reduced for 30 min at 56°C with 10 mM DTT in 0.1 M NH_4HCO_3. After cooling, the DTT solution was immediately replaced with 55 mM iodoacetamide in 0.1 M NH_4HCO_3 to alkylate the free SH groups for 20 min at 25°C in the dark. After washing with 50% ACN in 0.1 M NH_4HCO_3, the dried gel pieces were swollen in 15 µL of digestion buffer containing 25 mM NH_4HCO_3 and 12.5 ng/µL trypsin (Promega, Madison, WI, USA) and incubated overnight at 37°C. Tryptic peptides were extracted according to the protocol described by Kim et al. . Peptide mixtures were then analysed by LC-MS/MS on a 6520 Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) coupled to a chip-based chromatographic interface. A Large Capacity Chip (C18, 150 µm × 75 µm) with an enrichment column (C18. 9 mm, 160 nL volume) was used to separate peptides at a flow rate of 0.3 µL/min. Water/formic acid 0.1% and acetonitrile/formic acid 0.1% were used as eluents A and B, respectively. The chromatographic separation was achieved with a gradient of B from 5% to 50% in 20 min. Raw data files were converted into Mascot Generic Format (MGF) with MassHunter Qualitative Analysis Software version B.03.01 (Agilent Technologies) and analyzed with Mascot Search Engine version 2.2.4 (Matrix Science). MS/MS spectra were searched against the SwissProt database (version 2013-04, 539829 sequences). Enzyme specificity was set to trypsin/P with 1 missed cleavage, using a mass tolerance window of 1.2 Da for peptides and 0.6 Da for fragment ion matches. Carbamidomethylation of cysteine was set as fixed modification and methionine oxidation as variable modification. Proteins were considered as positive hits if at least 2 peptides per protein were identified with high confidence ($p < 0.05$).

Function of seminal fluid components

To investigate which seminal fluid component/s account for the observed influence on the sperm performances of sneakers and territorial males we compared the effect of protein and non-protein fractions (ions, metabolites and small peptides) on sperm velocity (VCL). Pools of seminal fluids of sneakers' and territorial' males were divided in two parts, one was kept intact (treatment 1, P=pool), while the other was boiled for 3 minutes at 100°C (treatment 2, Pb=pool boiled), to eliminate protein biological activity by denaturation (King et al., 2011). The intact pool was split in two parts, one of them was filtered through Millipore Ultracel® centrifugal filter device with a 3 kDa cut off membrane in order to isolate just the proteins (treatment 3, Pf=protein fraction). Then, half of the filtered pool was boiled (treatment 4, Pfb= protein fraction boiled). We referred to as "the protein fraction" (proteins above 3 kDa) and the "non-protein

fraction" (peptides and other small compounds below 3 kDa) because the smallest seminal fluid protein identified so far around 10 kDa (den Boer et al., 2009) would not pass through the membrane, including those hypothesized to be involved in sperm performance.

The curvilinear velocity (VCL) of sneakers' and territorials' sperm were compared i) without their seminal fluid; ii) with their own seminal fluid; iii) with the four seminal fluid fractions (P; Pb; Pf; Pfb) isolated from the seminal fluid of same tactic males and, in a second experiment, from opposite tactic males. Sperm were activated (see, *Gamete collection and ejaculate processing*) in order to maintain always the same seminal fluid total concentration at each treatment. Hence, it was added 1.5 µL of seminal fluid for the treatment "own fluid", "P" and "Pb" (see, Locatello et al., 2007; Locatello et al., 2013), whereas for the other treatments the volume of the solution was adjusted considering the specific dilution factor derived from the seminal fluid filtration and separation in the "protein" and "non-protein" fractions. Sperm velocity (VCL) was measured with an IVOS Sperm Tracker (Hamilton Thorne Research, Beverly, MA) placing and covering with a coverslip three microlitres of activated samples in separate wells on a 12-well multitest slide (MP Biomedicals, Aurora, OH, USA) previously coated with polyvinyl alcohol solution (1%; Sigma-Aldrich) to avoid sperm sticking to the glass slide (Wilson-Leedy and Ingermann, 2007).

Statistical analyses

Normality was checked following Kolmogorov–Smirnov. Effect of treatment (without seminal fluid; with own seminal fluid; fractions P; Pb; Pf; Pfb) on performance of territorials' and sneakers' sperm were analysed using linear mixed model (with restricted maximum likelihood estimation REML) in SPSS 21. We included, depending on the experiment, seminal fluid treatments measured as velocity (VCL) as the dependent variable, and tactic (sneaker or territorial) as a fixed factor. To account for repeated measures on individual males, male identity was included as a random factor with estimate of random intercepts for each subject.

Results

(i) Sperm quality

In the first 2 minutes of treatment, the oxygen consumption rate of sneaker sperm was significantly higher than that of territorial ones (T-test: mean territorial 0,12/sneaker 0,27 nmol/min/10^6sperm; t= 2,89; p=0,006). We firstly evaluated the first two minutes, as in the velocity tests performed by Locatello et al. 2013, the analysis last for the same time. However, also considering the first 5 minutes, the consumption rate is still higher for sneaker sperm (T-test: mean territorial 0,11/ sneaker 0,18 nmol/min/10^6sperm; t =-2,21; p=0,032), while there is no difference in the performance of sneakers and territorial males' sperm when the whole test time, i.e. 30 min (T-test: territorial 0,074 /sneaker 0,09 nmol/min/10^6sperm; t= 1,45; p=0,16) is taken into account.

(ii) Seminal fluid composition

Sneaker and territorial males' seminal fluids did not differ in glucose content (T-test: mean territorial 5.56/sneaker 6.45 mg/dL; t=-0.32 p=0.75) but showed a significant difference in the protein content, with territorial male presenting the higher values (T-test: mean territorial 9.15/sneaker 6.00 mg/mL t=2.34 p=0.026). Considering possible variations in the protein profile between the two tactics, polyacrylamide gel electrophoresis (SDS-PAGE; 4-20%) showed that sneaker and territorial males' protein profiles differ both quantitatively and qualitatively (fig. 1). A preliminary analysis comparing the band patterns of "fresh" samples, i.e. immediately after ejaculates collection, with samples stored at -80°C, did not revealed any effect of freezing. The protein profile analysis was repeated running different seminal fluid samples of sneaker and territorial males, collected from both single individuals and grouped as pools and pattern resulted highly repeatable among both tactics samples (see the representative gel with four pool samples of territorial a) and sneaker b) seminal fluids in fig.1).

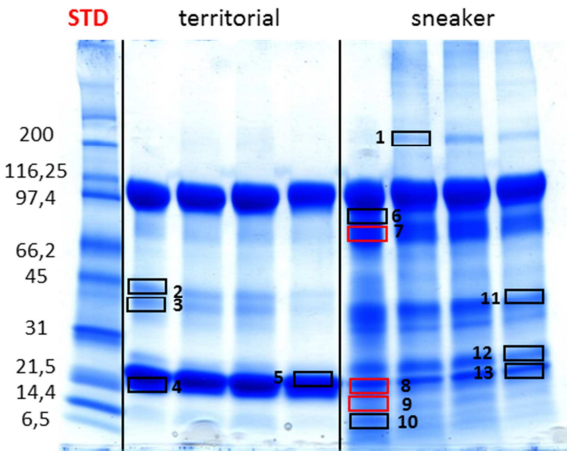

Fig. 1 Representative polyacrylamide gel electrophoresis (SDS-PAGE 4-20%) of **a) territorial** and **b) sneaker** males different seminal fluid pools. **Red** squares highlight the bands already identified (see tab.1), whereas **black** squares represent unknown bands.

Through mass determination and sequences analysis (MASCOT software) we identified 3 bands from sneaker seminal fluid and territorial; their identification is summarised in tab. 1.

Tab.1 Proteins already identified and corresponding bands (red squares in fig 1.); protein identification with the highest score from analysis of Q-TOF data using in-house MASCOT. All files were searched against the[1]NCBI nr database;[2]SwissProt database (after taxonomy filter).

spot	accession n° a	protein name	theoretical mass(kDa)	score b	peptides c	sequence coverage (%)
b9	PRVA_CYPCA[2]	parvalbumin alpha [*Cyprinus carpio*]	11,5	259/	5(5)	45.0
b8	LYSC_PSEMX[1]	lysozyme C [*Psetta maxima*]	16,3	132/	2(2)	14.7
b7	TRFE_ORYLA[1]	serotransferrin [*Oryzias latipes*]	76,4	91/	2(2)	4.3

In addition to the identified bands we evidenced in the territorial seminal fluid that proteins corresponding to bands in position n° 1 may be glycosylated, since after the seminal fluid sample was treated with the glycosidase, these bands shifted their position along the gel (fig.2).

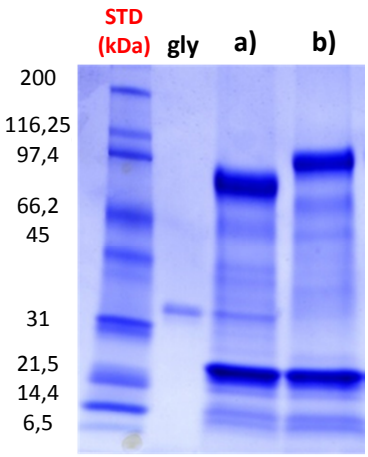

Fig.2 Samples of territorial male seminal fluid **a)**treated with glycosidase PNGase F(**gly**) and **b)** pure.

This result candidate the band in that position as a possible mucin-like protein. The presence of mucous substances in the grass goby seminal fluid has already been inferred (Scaggiante et al., 1999), on the basis of ejaculate histological staining and dilution behaviour. However, their specific chemical nature is complex to resolve across species, since mucins are difficult to handle for their identification and characterization via proteomic applications due to their heavily glycosylated nature (up to 90% carbohydrate by weight), high molecular weight and size (Kesimer and Sheehan, 2012).

iii) Function of seminal fluid components

The different treatments allowed to evaluate both separately and simultaneously how protein and non-protein fraction respectively affect sperm velocity (tab.2).

Tab.2 The absence **(0)** or the contribution **(1)** of protein and non-protein fraction are indicated for each treatment.

treatment	protein fraction (>3kDa)	non protein fraction (<3kDa)
no seminal fluid	0	0
own seminal fluid	1	1
pool (P)	1	1
pool boiled (Pb)	0	1
protein fraction (Pf)	1	0
protein fraction boiled (Pfb)	0	0

Statistical analysis were applied only to sneaker sperm' results, since the low number and high variability of the results obtained with territorial male sperm does now allowed to perform a reliable analysis. Overall we found that the non-protein fraction of both own and territorial males' seminal fluid strongly marks the differences between treatments, significantly enhancing sperm performance both with the same tactic seminal fluid fractions (when non protein fraction is present, sperm velocity VCL >11,4% on average respect to other treatments) and the opposite tactic ones (when non protein fraction is absent, sperm velocity VCL >14,1% on average respect to other treatments) (linear mixed model results: non protein fraction in tab.3). Moreover, the protein fraction, of both own (with protein fraction sperm velocity VCL >3,9% on average) and territorial males' seminal fluid (with protein fraction sperm velocity VCL >6,8% on average), seems to influence the mean velocity of the sperm too, but this result is not statistically significant (linear mixed model results: protein fraction in tab.3).

Tab.3 Results of linear mixed models for sneaker sperm tested with seminal fluid fractions from **same tactic** and **b) opposite tactic** pools.

a) same tactic sf	**F**	**p**
treatment	0.25	0.86
protein fraction	1.44	0.23
non protein fraction	34.85	<0.0001
b) opposite tactic sf	**F**	**p**
treatment	1.333	0.27
protein fraction	3.495	0.07
non protein fraction	49.317	<0.0001

It is noteworthy, looking at the velocity pattern among treatments, that: i) sperm performance is not affected by the freezing of the seminal fluid nor by the fact that seminal fluids are pooled from different males, since velocity do not vary between treatments "own" and "P"; ii) sperm performance do not decrease with Pb treatment respect to the presence of own seminal fluid or the P treatment; iii) sperm velocity is lowered by the Pf treatment but remains higher than the total absence of seminal fluid; iv) the Pfb treatment control for the effective exclusion of proteins functionality. In particular, the Pfb treatment worked as a control of the filtration method, since we expected that sperm velocity does not vary from the absence of the seminal fluid, since small compounds and metabolites were eliminated through filtration and proteins are denatured.

Discussion

In this study we tried to disentangle the proximate mechanisms that drive sperm seminal fluid interaction in the ejaculate competition scenario provided by the grass goby, where a tactic dependent effect of the seminal fluid on sperm competitiveness has

been already demonstrated (Locatello et al., 2013). We provide evidence that the two male phenotypes differ both in the sperm quality and in the seminal fluid composition.

Respect to sperm quality traits, we investigated the mitochondrial function registering sperm oxygen consumption rate on the same sperm number to evaluate sperm respiration activity (Froman and Kirby, 2005). We found that sneaker sperm present an oxygen consumption rate higher than territorial males during the first two and five minutes after activation, but after thirty minutes the rate does not differ between the two tactics. This last observation is in agreement with data existent in literature that show sperm velocity equally decline after thirty minutes in both sneaker and territorial males (Locatello et al., 2007). This results indicate that the higher sneaker sperm performance in territorial male seminal fluid could be grounded on a greater efficiency of their sperm mitochondrial respiration. In fish, sperm respiration activity was demonstrated to be one of the best descriptive variable of their fertilization (Lahnsteiner et al., 1998).

A different sperm respiration rate may derive from a different number or morphology of mitochondria, or alternatively from differences with respect to the mitochondrial chain. Mitochondria influence on sperm performance was firstly investigated considering their number in relation to the sperm midpiece size, namely the mitochondria sheath of the sperm where the metabolism of cyclic AMP catalysed ATP (Bedford and Hoskins, 1990). Theoretical models predict that sperm with a greater midpiece size might contain more mitochondria and consequently provide more energy for powering the flagellum, without considering the glycolytic support (Cardullo and Baltz, 1991). Evidence that sperm with a longer midpiece produce more ATP were found in the Atlantic salmon, *Salmo salar* (Vladić et al., 2002), and in the domestic fowl, *Gallus domesticus*, where sperm velocity also positively correlates with the rate of ATP synthesis (Froman and Feltmann 1998). Absolute midpiece size was also positively related to sperm velocity in sperm competition contexts, in comparative studies that expect enlarged midpiece sizes in species under intense sperm competition, but evidences are contrasting (Johnson and Briskie, 1999; Immler and Birkhead, 2007). However, in the grass goby morphological difference among sperm parts were not found between the tactics in previous studies (Locatello et al., 2007), and we thus do not expect that sperm morphology or the relative proportion of different parts affect their velocity, as demonstrated across other species (Lupold et al., 2009), even if changes in the sperm morphology (shrinkage of mitochondria and vacuoles observed in the midpiece) may take place after sperm activation, lowering mitochondrial activity (Suquet et al., 2012). Among fish species, the number of mitochondria is variable ranging from the single ring-shaped mitochondria of Salmonidae spermatozoa up to more than six in Blenniidae and Labridae (Lahnsteiner and Patzner, 2008). However, the number or arrangement of mitochondria was never found to account for any functional differences in sperm energetic capacity (Bobe and Labbé, 2009). Secondly, sperm respiration may diverge due to variations in the mitochondrial respiratory chain. In humans, an increase in sperm motility requires a

parallel increase in mitochondrial respiratory capacity, supporting the fundamental role played by mitochondrial oxidative phosphorylation in sperm motility to produce ATP (Ferramosca et al., 2012). We suggest that a difference in the mitochondrial chain components may explain the asymmetry in the sperm respiratory rate that we detected for the first time in a species with alternative mating tactics, even if further analysis are required. Indeed, the improved ability of sneaker sperm may alternatively ground on a higher ADP/ATP ratio (Perchec et al., 1995), that would correspond to higher energetic potential. Otherwise, the characteristics of sperm membrane have been also considered among quality traits, in fact, it is involved in sperm motility and viability, in processes that mediate the gametes fusion, and protects sperm from oxidative damages, overall influencing the fertilization success (Delbarco-Trillo and Roldan, 2014). Considering protection from reactive oxygen species, the fatty-acid composition of membrane phospholipids is crucial against damages, such as lipid peroxidation, that would cause sperm loss of motility and structural damage with negative effect on capacitation and fusion with the oocyte (Wathes et al., 2007; White, 1993). Oxidative damage boosts with higher sperm metabolism, and, consequently may be related to higher levels of sperm competition that select for increasing sperm velocity, as grass goby sneaker sperm showed. Consequently, a sperm membrane less prone to oxidative damages may allow to boost sperm velocity, as found in a comparative analysis in mammals (Delbarco-Trillo and Roldan, 2014).

On the other side, we found that also the seminal fluid composition differ between the two tactics and may be involved in the sperm seminal fluid competitive interactions. The deepening of the seminal fluid functionality indicates the “non-protein” (<3kDa) fraction as essential for sneaker sperm velocity in the grass goby, since only its presence drives sperm velocity to values comparable with those measured with the complete seminal fluid, even when sperm are tested with the opposite tactic seminal fluid. This result brought out as the effect of the small fraction of the seminal fluid, namely peptides, salts and other small compounds, has to be taken in consideration evaluating sperm motility. We suggest that additional analysis have to be addressed to the investigation of the seminal fluid ionic composition in both tactics. Indeed, in fishes, ions such as Na^+, K^+ and Ca^{2+} and their relative abundance are pivotal for the maintenance of seminal fluid pH and osmotic pressure that in turn influence sperm activation and motility (Alavi and Cosson, 2005; 2006). Hwang and Idler (1969) suggested a correlation between the seminal plasma Na^+/K^+ ratio and sperm fertility in the Atlantic salmon, *Salmo salar*, and also the long last motility of sperm in other species (Alavi and Cosson, 2006). The influx and efflux of these ions inside sperm cells governs the motility mechanisms of axonemes through ion channels activity (Alavi and Cosson, 2008) or mediate sperm activation (Morisawa, 2008). Samples are not enough to shed light on the tactic dependent effect of the seminal fluid but we highlight how studies on the seminal fluid functionality have to be prudent in looking at the protein fraction as representative of the whole seminal fluid contribution to ejaculate performance. The

analysis of proteins profiles deserves the same attention. Indeed, the preliminary analysis of seminal fluid functionality performed on sneakers found that protein fraction anyway contribute to sperm velocity. Genes that encode for reproductive proteins on the average show higher evolutionary rate respect to others (Simmons and Fitzpatrick, 2012). There are increasing evidence that sperm competition and female promiscuity are some of the steering factors of their evolutionary divergence. Post-copulatory sexual selection will drive adaptive variation in seminal fluid, sperm, and proteins that improve male fertilization success (Simmons et al., 2013), as species with greater selection pressures showed higher protein divergence (Dorus et al., 2004; Wagstaff and Begun, 2007; Ramn et al., 2009). In addition, the seminal fluid proteins abundance and gene expression demonstrated plasticity even within the same species. Indeed, in the field cricket, *Teleogryllus oceanicus,* the protein profile of the seminal fluid revealed quantitative ontogenetic changes. This variation is paralleled by a variation of the ejaculate fertilization success that increases as male crickets aged. The increased ejaculate competitiveness is suggested to be dependent on seminal fluid chemistry (Simmons et al., 2014). In the grass goby, the switch from sneaker to territorial phenotype is related to the ontogenetic life cycle, even the social context may have some influences (Scaggiante et al., 2004), and the protein profiles of sneaker and territorial males differ both qualitatively and quantitatively making intriguing to identify the diverse proteins and their functionality. Up to now, we discriminated only few proteins that are common at the two tactic protein patterns. The matching of the sequences was particularly difficult due to the absence of the species genome. However, our results are robust, since the identified protein were found in the seminal fluid of other fish species. Serotransferrin is supposed to be involved in sperm protection from oxidative damage and pathogens, in *Cyprinus carpio* and *Gallus gallus* (Dietrich et al., 2014; Marzoni et al., 2013), whereas having bacteriolytic activity in sea urchin, *Paracentrotus lividus* (Stabili and Canicatti, 1994). The lysozyme activity, in addition to the well-known antimicrobial action, positively correlated with sperm motility parameters, and improved the viability of spermatozoa *in vitro* in fish species (Giacomello et al., 2006; Lahnsteiner and Radnera, 2010). Finally, a parvalbumin-like protein was suggested to be important part of the Ca^{2+}-mediated mechanism of sperm activation, among fish species (Dietrich et al., 2014). Considering the mating dynamics of the grass goby, sperm protection through bacteriolytic activity of serotransferrin together with the lysozyme C may be functional for territorial males in the eggs protection, an activity of great adaptive value for *Z. ophiocephalus*, which lays eggs in mud nests, as was previously suggested for sperm-duct gland mucins (Giacomello et al., 2008). Ca^{2+} mediation in sperm activation processes and protection from oxidative damages agree with the finding of both the importance of small seminal fluid compounds and the different sperm oxygen consumption rate between the two tactics.

Our results represent a first attempt to clarify sperm seminal fluid proximate interactions mechanisms considering the contribution of both ejaculate components and

their reciprocal interplay, in agreement with the most recent theoretical models that highlight the importance of considering ejaculates as functional units (Fitzpatrick et al., 2012d; Simmons and Fitzpatrick, 2012).

DISCUSSION

The findings of my PhD project contribute to shed light on the evolution of male allocation in sperm and seminal fluid in the context of ejaculates competition. The two study species, the grass goby, *Z. ophiocephalus*, and the black goby, *G. niger*, present similar mating systems but different competition contexts, thus providing a good model to test the expectations of recent theoretical models on the variation of optimal ejaculate composition, in sperm and seminal fluid, in relation to male advantage in competition contests and male mating order or role (Cameron et al., 2007). These gobies are external fertilizers, males' mating roles are easily recognizable by their phenotypes, and it is possible to experimentally manipulate sperm and seminal fluid components to investigate separately their influence on the ejaculate competitiveness.

The positive relationship between male investment in sperm number and/or quality and the sperm competition level, has been widely documented, both within and among species (Harcourt et al., 1995; Gage, 1994; Stockley et al., 1997; Birkhead & Møller 1998; Snook, 2005; Simmons and Fitzpatrick, 2012). By contrast, the allocation on seminal fluid has been, until recently, a neglected aspect of ejaculate investment in competition contexts. Thus, I focused my attention to it and, using a multidisciplinary approach, I developed my PhD project proceeding through three main questions.

1. Does the seminal fluid influence sperm performance?

For what concerns the possible influence of the seminal fluid in ejaculates competition contexts, my results appear to support the theoretical models positing that variation in seminal fluid allocation in relation to sperm competition is expected only if seminal fluid components may influence the ejaculate competitiveness (Cameron et al., 2007). In both my study species, the spatial context in which the competition between ejaculates occurs proved to be important. Indeed, in externally fertilizing species, sperm competition conditions range from near complete male mate monopolization to large spawning assemblages (e.g. in fishes Stockley et al., 1997). The success in sperm competition is predicted to depend on male ejaculate investment, but the whole probability of fertilization during spawning may be influenced also by the proximity of males to the eggs during ejaculation and by the concurrence of sperm and eggs release (Taborsky, 1998; Fitzpatrick et al., 2012). Together these aspects determine the degree of ejaculates overlapping in space and time, varying the scenario and the factors that mainly influence the outcome of ejaculates competition.

In the grass goby, where ejaculates of sneakers and territorial males may come in close contact, I observed a tactic dependent effect of the seminal fluid, with sneaker sperm not only taking advantage of the territorial males' seminal fluid, but also sneakers' seminal fluid having a detrimental effect on territorial male fertilization success (Locatello et al., 2013; *Z. ophiocephalus*; Paper, I). Appropriate control experiments demonstrate that the seminal fluid effect is not mediated by a self/non-self

recognition mechanism. By contrast, in the black goby, where the ejaculates of competing males are released far from each other, seminal fluid, as expected, does not affect the sperm performances of rival males. Although the seminal fluid of territorial males significantly enhances their sperm velocity, still sneaker sperm are significantly faster, regardless the seminal fluid present. Black goby sneakers seem to devote their ejaculate investment in the sperm component, increasing sperm production and strengthening sperm quality traits (velocity, viability, ATP content) (Locatello et al., 2007). Thus, sperm performance appears to be the main factor determining the fertilization success of sneakers' ejaculates. They have to cover a greater distance and to outcompete the ejaculate of the territorial male, already present and closer to the eggs. The competition arena makes less convenient for sneaker males to enhance their sperm performance through both their seminal fluid, that would rapidly be diluted, or alternatively through that of territorial males, since if sperm performance would be less effective they may not even arrive at mixing with the territorial male ejaculate and to the eggs (*Gobius niger*; Paper II).

The main message highlighted by these results is that, in a scenario of competition, the ejaculates' mix matters, as it may influence the male fertilization success, allowing either the exploitation of competitors' seminal fluid and/or the impairment of their sperm. A variation in fertilization success when the seminal fluids of rival males or of males adopting rival tactics mix (here documented in the grass goby) is expected to occur in internal fertilizers where the overlapping of ejaculates is reasonably common (Perry et al, 2013). Direct evidence are still lacking but indirect ones arise from insect where a detrimental effect of seminal fluid on rival males' sperm or variation in seminal fluid composition in relation to the perceived level of sperm competition (den Boer et al., 2010; Wigby et al., 2009) have been documented. However, conditions for ejaculate mixing are not exclusive of internal fertilizers as several external fertilizers exhibit ARTs or spawn in group (Levitan, 1998; Petersen and Warner, 1998). Indications on the possible occurrence of a strategic allocation in seminal fluid according to the level of sperm competition, come from numerous teleost species. In the great majority of gobies (Miller, 1992; Mazzoldi et al., 2005) seminal fluid is produced in male accessory structures, defined seminal vesicles or sperm duct glands, showing a conspicuous variability both among (Fishelson, 1991; Mazzoldi et al., 2005) and within species (Scaggiante et al., 1999; Rasotto and Mazzoldi, 2002; Drilling and Grober, 2005). Intraspecific analyses of the male reproductive apparatus and ejaculate characteristics have demonstrated that the development of accessory structures is positively related to the amount of seminal fluid released. In particular, in goby species with ARTs (including the grass goby and the black goby), the differences in ejaculate composition, in terms of sperm number and amount of seminal fluid, among males are paralleled by differences in testis size and accessory structure size and function, with territorial males having smaller testes and more developed accessory structures, filled with secretions, than opportunistic males, which accessory organs are used to store sperm rather than to

produce seminal fluid (Mazzoldi, 1999; Scaggiante et al., 1999; Rasotto and Mazzoldi, 2002; Drilling and Grober, 2005). A similar morpho-functional pattern, with a tactic-dependent investment in non-sperm components of the ejaculate, has been documented in several teleost fish, such as in salmonids, blennies, wrasses, damselfishes, sunfishes, cichlids (Taborsky, 2008). Thus, it can be expected that in these species too, if the mating dynamics opens the way for ejaculate mixing, as it occurs in the grass goby, opportunistic males could exploit the seminal fluid of dominant males and/or impair the performance of dominant males' sperm with their seminal fluid.

2. How seminal fluid affect sperm performance? The proximate mechanisms driving sperm seminal fluid-sperm interactions

With respect to the proximate mechanisms driving sperm-seminal fluid interactions in the grass goby, we found that the ejaculates of male adopting alternative tactics differ in both i) sperm quality (in term of oxygen consumption rate) and ii) seminal fluid composition (quality and quantity of protein content). Furthermore, investigating seminal fluid functionality, we detected iii) a different weight of seminal fluid components on sneaker sperm performance.

i) ***Sperm quality***

Among fish species, the number or arrangement of mitochondria are variable (Lahnsteiner and Patzner, 2008), but were never found to account for any functional differences in sperm energetic capacity and performance (Bobe and Labbé, 2009). In fish, sperm respiration activity was demonstrated to be one of the best descriptive variable of their fertilization success (Lahnsteiner et al., 1998). We investigate the mitochondrial function registering sperm oxygen consumption rate on the same sperm number, as a measure of sperm respiratory activity (Froman and Kirby, 2005), and we found that sneaker sperm present higher values than territorial ones. Sperm morphology do not differ between the two tactics (Locatello et al., 2007), looking in particular at the midpiece length, namely the mitochondria sheath of the sperm, where the metabolism of cyclic AMP catalysed ATP (Bedford and Hoskins, 1990). Therefore, a difference in the mitochondrial size or number seems unlikely, and we suggest that variations in the mitochondrial chain components may explain the asymmetry in the sperm respiratory rate that we detected for the first time among males exhibiting alternative reproductive tactics.

ii) ***Seminal fluid composition***

We found that protein profiles differ both qualitatively and quantitatively between sneaker and territorial males. The protein profiles appear to undergo to both qualitatively and quantitatively variations during the switch from sneaker to territorial phenotype, a scenario never registered before, considering that in the grass goby alternative mating tactics are related to the ontogenetic life cycle (Scaggiante et al.,

2004). The seminal fluid proteins abundance demonstrated a comparable plasticity within the same species only in the field cricket, *Teleogryllus oceanicus.* In this species, however, the protein profile of the seminal fluid revealed ontogenetic changes between young and older males, but only in terms of quantity. This variation is paralleled by a variation of the ejaculate fertilization success that increases as male crickets aged. The increased ejaculate competitiveness is suggested to be dependent on seminal fluid chemistry (Simmons et al., 2014). Our results set the condition to verify if the change in seminal fluid composition between alternative mating tactics account for its tactic dependent effect on sperm performance and therefore if it is driven by ejaculates competition pressure.

Up to now, we discriminated only few proteins from sneakers' seminal fluid. The matching of the sequences was particularly difficult due to the absence of the genome of the species. However, our results are robust, and the identified proteins were found in the seminal fluid of other fish species (*Z. ophiocephalus*; Paper II). Serotransferrin is supposed to be involved in sperm protection from oxidative damage and pathogens, in *Cyprinus carpio* and *Gallus gallus* (Dietrich et al., 2014), whereas having bacteriolytic activity in sea urchin, *Paracentrotus lividus* (Stabili and Canicatti, 1994). The lysozyme activity, in addition to the well-known antimicrobial action, positively correlated with sperm motility parameters, and improved the viability of spermatozoa *in vitro* in fish species (Giacomello et al., 2006; Lahnsteiner and Radnera, 2010). Finally, a parvalbumin-like protein was suggested to be important part of the Ca^{2+}mediated mechanism of sperm activation, among fish species (Dietrich et al., 2010; Dietrich et al., 2011). Considering the mating dynamics of the grass goby, sperm protection through bacteriolytic activity of serotransferrin together with the lysozyme C may be functional for territorial males in the eggs protection, an activity of great adaptive value for *Z. ophiocephalus*, which lays eggs in mud nests, as was previously suggested for sperm-duct gland mucins (Giacomello et al., 2008).

iii) ***Seminal fluid functionality***

Despite the great attention that proteins involved in sexual selection mechanisms have recently received, our results highlight that other seminal fluid components (Poiani, 2006) have to be considered. Indeed, the deepening of the seminal fluid functionality indicates that in the grass goby, "non-protein" (<3kDa) fraction of the seminal fluid, namely peptides, salts and other small compounds, is essential for sperm velocity, since only its presence drives sperm velocity to values comparable with those measured with the complete seminal fluid, even when sperm are tested with the opposite tactic seminal fluid. Up to now, data analysed We suggest that additional analysis have to be addressed to the investigation of the seminal fluid ionic composition in both tactics. In fish species, ions such as Na^+, K^+ and Ca^{2+} and their relative abundance are pivotal for the maintenance of seminal fluid pH and osmotic pressure that in turn influence sperm activation and motility (Alavi and Cosson, 2006). Hwang and Idler (1969) suggested a correlation between the seminal fluid Na^+/K^+ ratio and sperm fertility in the Atlantic

salmon, *Salmo salar*, whereas in other species their relative abundance influences the long last motility of sperm (Alavi and Cosson, 2006). The influx and efflux of these ions inside sperm cells governs the motility mechanisms of axonemes through ion channels activity (Alavi and Cosson, 2008) or mediate sperm activation (Morisawa, 2008). Therefore, studies on the seminal fluid functionality have to be prudent in looking at the protein fraction as representative of the whole seminal fluid contribution to the ejaculate performance.

Secondly, considering the functionality of seminal fluid proteins (Sfps), if future analysis would confirm the positive effect of protein on sperm velocity, our results would be consistent with findings arising from other species. Indeed, sfps have been proved to directly contribute to sperm viability and motility in both internal and external fertilizers, and their influence has been demonstrated also among fishes (Tram and Wolfner, 1999; Lahnsteiner et al., 2003; Alavi and Cosson, 2006; King et al., 2011; Simmons and Beveridge, 2011; Simmons et al., 2013). Sfps are among the most evolutionary divergent proteins in almost all taxa (Clark et al., 2006), and their divergence is steeper across species where post-copulatory sexual selection pressure is higher (Dorus et al., 2004; Wagstaff and Begun, 2007; Ramn et al., 2009). In particular, they are proved to be involved in ejaculates competition contexts (Wolfner, 2002; Ramn et al., 2009; Simmons et al., 2013). However, evidence came from few genetically well-characterized species, and sfps identification and functionality lack the taxonomic breadth to outline the general patterns driving their evolution in the context of post-copulatory sexual selection (Simmons et al., 2013).

Our results represent a first attempt to clarify sperm seminal fluid proximate interactions mechanisms considering the contribution of both ejaculate components and their reciprocal interplay, in agreement with the most recent theoretical models that highlight the importance of considering ejaculates as functional units (Fitzpatrick et al., 2012d; Simmons and Fitzpatrick, 2012).

We suggest that ambiguous competition contexts previously investigated where it was not clear if sperm number or quality determine the outcome of ejaculates competition (Simmons et al., 2003; Snook, 2005; Stoltz and Neff, 2006; Simmons et al., 2007) should be reconsidered in the light of possible interactions among all the ejaculates components, to overcome the sperm specific focus that the study of post-copulatory sexual selection employed for years.

For example, in the bluegill *Lepomis macrochirus*, opportunistic males have two morphs: smallest and younger individuals are sneakers, that are recognised and kept away from territorials, while males of medium size and age (looking like a female), named satellites, intrude between the territorial male and the female, spawning in the position nearest to the eggs. Curiously, although satellites mate from the most favourable place and sneakers are more distant respect to territorial males, competition trials revealed that both opportunistic males have a competitive advantage respect to territorial males independent from sperm number. Therefore, authors suggest that

some other aspect of sperm quality not measured must contribute to the increased competitiveness of sperm from sneakers (Stoltz and Neff, 2006). However, not just sperm characteristics but also seminal fluid contribution should be considered in future studies, since the different distances at which males mate lead up to different competition arenas, where ejaculates mix at diverse degree: consequently sperm and seminal fluid may diversely affect the outcome of sperm competition in this species, as we demonstrated for the black and the grass goby. In the field cricket, *Teleogryllus oceanicus*, males adjust the quality of their ejaculates in relation to the perceived level of competition varying the percentage of live sperm. The mechanism by which they modulate ejaculate quality suggest the mediation of seminal fluid composition (Simmons et al., 2007).

Future studies should consider firstly the mating dynamics and the degree of rival ejaculates interplay to assess which components, sperm and/or seminal fluid components weight more on the whole ejaculate competitiveness, without overlook their relative interactions that make the ejaculate a functional unit. We show how a multidisciplinary approach is necessary to outline a scenario as complete as possible.

3. Does the fertilization success mirror sperm performance?

This third question was investigated in both species trough *in vitro* fertilization trials, mirroring the experimental design adopted for sperm performance tests. Moreover, we evaluated the paternity success of sneaker and territorial males in the field, in the black goby, as for the grass goby this aspect has been recently analysed (Pujolar et al, 2012). We found that:

- in **the grass goby**, the fertilization tests perfectly reflect the results emerged from sperm performances analysis, and bring out the same tactic dependent effect of the seminal fluid (Locatello et al., 2013; *Z. ophiocephalus*; Paper I). In natural nests territorial males sire approximately 75% of the progeny, with larger males showing the higher fertilization (Pujolar et al., 2012). Grass goby territorial males respond to increased levels of competition increasing the attacks against sneakers (Scaggiante et al, 2005). Thus, larger males, likely more effective in nest defense, may overcome sneaker competitiveness and gain an higher paternity success. Territorial males able to keep sneakers far from the eggs and their ejaculates, prevent the exploitation of their seminal fluid and the impairment of their sperm by the sneakers' seminal fluid. Therefore, even if sneakers are advantaged by the mix of ejaculates, the distance at which there are constrained by the territorial males appears, at last, to determine the outcome of ejaculates competition;

- in **the black goby**, *in vitro* fertilization trails do not appear to mirror sperm performance. We cannot exclude that the limited number of trials may be insufficient to bring out an asymmetry in the proportion of eggs fertilized by the two male phenotypes. However, sneaker and territorial males sperm, indeed, differ in their swimming

trajectory, with territorial male sperm moving more linearly. As a consequence, it could take the same time to sneaker and territorial male sperm to travel the same distance, even if those of sneaker have an higher velocity. In a recent work on *Peromyscus maniculatus* it has been found that the increasing sperm velocity is not due to a change in velocity, but rather because of travelling with a more direct path (Fisher et al., 2014). The subtle difference lays in how sperm motility is evaluated, if considering the motion in a flat surface or in a three-dimensional space. If the higher velocity follows through a motion that is far to be along a linear path, the whole travelling could be less efficient than swimming slower but along in a more linear way (Fitzpatrick et al., 2012). This hypothesis could explain the similar fertilization rate found between sneaker and territorial males in the black goby, even if the sample size is too low to draw a robust conclusion (*Gobius niger*; Paper I).

The analysis of the paternity distribution of black goby territorial males in the field suggests that the straighten of territorial male nest guarding is crucial for its parentage success. Indeed, preliminary results, from artificial nests in the field, indicate that sneakers fertilize more eggs than territorial males in proximity to the nest main entrance, while in the rest of nest territorial males' paternity is higher. Many studies have quantified the relative fertilization success of alternative reproductive males phenotypes, showing that territorial males usually outcompete sneakers, but, in most fish species, some degree of cuckoldry was observed (Coleman and Jones, 2011). However, the parentage success of territorial males is unexpectedly low, respect to that registered in the grass goby and across other fish species with a similar mating system (Coleman and Jones, 2011). In the grass goby the fertilization success of the territorial male (>70%) resulted among the highest among species with ARTs and nest defence (Pujolar et al., 2012). By contrast, in the black goby the observed proportions of eggs fertilized by sneakers (ranging from 61,36 to 78,41%) is three times the mean cuckoldry success (20%) registered across species with a similar mating system (Coleman and Jones, 2011), and similar values have been found only in the sand goby (Jones et al., 2001). The paternity success seems highly variable among sneakers, with two-three males sharing the large part of cuckolded eggs, with up to six fathers estimated per nest (the territorial male excluded). The number of estimated mothers per nest is 12 on average, above the 3.1 mean registered across other fish species with male uniparental care and nest defence (Coleman and Jones, 2011).

Overall, the degree of multiple mating appear extremely high in the black goby, in both sexes. In particular, the high number of males that fathered the eggs indicate that the strength of competition among rival ejaculates has to be great. We did not detect any nest take over event, but we recorded in two of four analysed nests few embryos sired by a neighbour territorial male (*Gobius niger*; Paper II). If the results would be confirmed by further analysis, they involves that territorial males may occasionally adopt sneaking behaviours, probably depending on the level of ejaculates competition determined by the nests availability and male density (Bessert et al., 2007). The

territorial mating role do not appear as favoured as in other species with alternative mating tactics, especially considering that sneakers visits more than one nest. Indeed, the degree of cuckoldry is expected to reduce the efficiency of territorial males to monopolize access to females through nest-guarding (Petersen and Warner, 1998; Jones et al., 2001). Further studies should be addressed to the investigation of territorial male paternity success along the breeding season to better quantify the relative paternity success of the two male phenotypes.

These findings evidence the importance of relating any measure about sperm performance to their fertilization ability and to the competition context, before drawing a conclusion on the whole ejaculate competitiveness.

It would be interesting to widen the range of competition scenarios across different species, in order to outline the general patterns that drive male allocation towards seminal fluid and its different components (Froman et al., 2002; Fitzpatrick et al., 2012). The findings of my PhD project, noteworthy, derived from species with external fertilization, when theoretical and experimental efforts are in the majority limited to internal fertilizers. We pointed out i) the investigation of the spatial context as an important factor that influence the degree of rival ejaculates interplay, and ii) to the deepening of the proximate mechanisms that underlie sperm seminal fluid interactions, that cannot overlook the contribute of all the seminal fluid components.

In both species, territorial males do not vary their sperm expenditure as the competition level increases, how sneaker males do (Pilastro et al., 2002), but nest guarding efficiency appears crucial for their fertilization success. Considering that i) the seminal fluid enhances the performance of territorial males and ii) that the seminal fluid content correlates with the duration of ejaculate trails, that slowly dissolving guarantee a steady sperm supply to the eggs allowing territorial males to defend the nest, we hypothesized that territorial males may vary their seminal fluid expenditure depending on the level of sperm competition. The strategic allocation of seminal fluid quantity and quality in relation to female quality or sperm competition level has been recently proved in some species (Cornwallis and O'Connor, 2009; Sirot et al., 2011), but it has never been proposed for species with external fertilization.

Our results place my PhD project among the recent studies trying to shed light on factors that influence the phenotypic plasticity in ejaculate components allocation, and on proximate mechanisms underlying sperm and seminal fluid interplay, under post-copulatory sexual selection pressure.

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