Università degli Studi di Padova
Department of Biology

Ph.D. COURSE IN: BIOSCIENCES

CURRICULUM: EVOLUTION, ECOLOGY AND CONSERVATION

SERIES XXXI

PHENOTYPIC PLASTICITY IN MALE SEXUALLY SELECTED TRAITS IN RESPONSE TO SOCIAL CUES

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ABSTRACT

The reproductive success of males is strongly influenced by their investment in costly sexually selected traits. Fitness returns, however, are often context-dependent and vary with demographic parameters such as sex ratio and population density. Under conditions of environmental variability, the ability to modulate reproductive decisions on the social context is highly beneficial. As a result, phenotypic plasticity of sexually selected traits is widespread. The aim of my study was threefold. Firstly, I worked to expand our current knowledge on phenotypic plasticity in sexually selected traits both empirically, through a test of the effect of female availability on male mating effort in the nursery web spider, and theoretically, through a literature review on the subject of strategic adjustments of ejaculate quality. Secondly, I evaluated costs and benefits of the anticipatory upregulation of sperm production observed in male guppies as a response to perceived mating opportunities. Finally, I investigated how post-copulatory processes may shape plastic male mate choice. The two species used in my study, the guppy, *Poecilia reticulata*, and the nursery web spider, *Pisaura mirabilis*, are particularly suited to investigate phenotypic plasticity of sexually selected traits because they both express costly reproductive traits and they experience environmental fluctuations in socio-sexual factors. When I explored the effect of female availability on male investment in pre- and post-copulatory traits, I found that *P. mirabilis* males do not respond to variations in this parameter. While males do not reduce their mating effort per partner as mating opportunities increase, they may respond instead by increasing their total reproductive budget. The literature review focusing on plasticity of ejaculate quality, besides showing the diversity of traits subject to adjustment and of stimuli triggering the response, highlighted the difficulty of estimating the fitness consequences of ejaculate plasticity because of the complexity of patterns of co-variation with other reproductive and non-reproductive traits. The experiments investigating costs and benefits of anticipatory ejaculate adjustments showed that the costs of plasticity are minor in guppies, as compared to the costs of phenotype. Furthermore, the trade-off between sperm production and pre-copulatory traits (courtship rate) appears to be stronger than the trade-off between sperm number and quality. Finally, my experiments on post-copulatory processes demonstrated a first male sperm precedence and an advantage of previous partners against novel ones in multiply mated female guppies. These findings brought an important contribution to the understanding of phenomena of male mate choice, such as mate choice copying, audience effect and Coolidge effect. In conclusion, the results of my study demonstrate how trade-offs between pre- and post-copulatory traits have crucial effects on costs and benefits of phenotypic plasticity in reproductive traits, highlighting the importance to adopt an integrative approach and to consider multiple traits and their interaction when studying sexual selection. My results also stress the need for a careful evaluation of episodes of post-copulatory selection when interpreting plasticity of both pre- and post-copulatory investment.

INTRODUCTION

Phenotypic plasticity

Phenotypic plasticity is defined as the ability of an individual or genotype to express different phenotypes depending on the environment (West-Eberhard 2003); this phenomenon is widespread across species and taxa. When individuals experience spatial or temporal fluctuations of environmental conditions and one single phenotype does not confer high fitness in all situations, context-dependent phenotype expression can be beneficial (Via et al. 1995). Phenotypic plasticity, however, is not necessarily adaptive, and can be as well neutral or maladaptive. Adaptive and maladaptive phenotypic plasticity, respectively by enhancing and reducing environmental tolerance, can have important consequences for evolution, although predictions about whether plasticity constrains or facilitates adaptation are conflicting (Ghalambor et al. 2007). By reducing relative fitness, non-adaptive plasticity is predicted to increase the strength of directional selection, and thus to accelerate phenotypic evolution (Ghalambor et al. 2015). Adaptive plasticity, on the one hand, may shield the genotype from the effects of selection and thus obstruct evolution, and on the other, it may facilitate evolution by increasing population persistence in new environments (Price et al. 2003). Linked to the latter scenario, phenotypic plasticity has recently received increased attention for its potential role in enhancing organisms' ability to cope with anthropogenic environmental change (e.g. Charmantier et al. 2008; Merilä and Hendry 2014; Seebacher et al. 2015; but see, Oostra et al. 2018).

According to West-Eberhard (2003), phenotypic plasticity includes morphological modifications, physiological and neural regulation, and changes in behavioral traits. However, there has been some debate about which phenomena should be included in the definition. In particular, authors disagree about whether reversible and irreversible responses should be both classified as cases of phenotypic plasticity (Piersma and Drent 2003; Ghalambor et al. 2010). One common point of view is that irreversible, developmental plasticity (i.e. the capacity of a genotype to adopt different developmental trajectories in different environments) is the only true form of phenotypic plasticity. Conversely, reversible responses, also referred to as physiological adaptation (Garland and Kelly 2006), activational plasticity (Snell-Rood 2013) or phenotypic flexibility (Piersma and Drent 2003), should be considered as a distinct phenomenon. While reversible and irreversible plasticity are likely to evolve under different conditions and may also differ concerning their costs and benefits and with regard to their consequences for phenotypic evolution (Snell-Rood 2013), it has been argued that in the absence of a biology-based demarcation between developmental and post-developmental phenomena, the distinction may be arbitrary (Fusco and Minelli 2010).

Keeping in mind this debate, I chose to adopt a broad definition of phenotypic plasticity, which includes reversible responses, and which, therefore, can be applied to the physiological changes and behavioral switches that represent the target of my experimental work. This choice is shared by several authors, who used the term to refer to ejaculate adjustments (e.g. Kelly and Jennions 2011; Simmons and Lovegrove 2017),

variations in behavioral strategies (e.g. Bretman et al. 2011; Mohorianu et al. 2017) and changes in mating preferences (e.g. Ghalambor et al. 2010; Rodríguez et al. 2013). Within this broad definition, plasticity phenomena are extremely varied. Here I will focus on cases in which individuals respond to social cues by actively modifying their physiology and behavior to express the optimal phenotype in the current or expected environment.

Phenotypic plasticity and sexual selection

Organisms invest large amounts of resources into reproduction, and although females have been traditionally considered the sex that invests more (Andersson 1994), traits and processes associated with male reproduction (i.e. sexually selected traits), including courtship, mate choice, sperm production and mating, are also costly (Dewsbury 1982; Kotiaho 2001; Scharf et al. 2013). Given that individuals normally possess a limited energetic budget devoted to reproduction, the costs associated with the expression of reproductive traits constrain males' ability to invest in other traits and functions, or in future mating events, resulting in resource allocation trade-offs (Stearns 1989; Parker et al. 2013). The costs, as well as the benefits, of investment into reproduction are often context-dependent, affected in particular by elements of the socio-sexual environment (Bretman et al. 2011). Since, in turn, socio-sexual conditions are often highly variable (Kasumovic et al. 2008), males benefit from tuning their reproductive effort based on the expected fitness returns, rather than investing maximally at all times. Plastic responses in reproductive decisions can bring an important contribution to individuals' fitness and, indeed, they are widespread across animal species (Wedell et al. 2002; Bretman et al. 2011). Phenotypic plasticity involves a number of different reproductive traits, including morphological (Immler et al. 2004; André et al. 2018), physiological (Firman et al. 2018; Burger et al. 2015), behavioral (Bretman et al. 2015; Royle et al. 2008) and life-history traits (Allen et al. 2007; Oddie and Reim 2002), and includes flexible decisions about optimal investment into reproductive traits.

Plastic investment involves several distinct decisions: on the one hand, males need to define how much to allocate to reproductive effort (i.e. the total reproductive budget), how much to allocate among different sexual traits (e.g. mate acquisition vs post-copulatory traits), and how to partition the reproductive budget among subsequent mating events. These types of plastic decisions differ substantially with regard to the trade-offs they generate. For decisions concerning resource allocation to a given mating event, trade-offs only constrain male ability to invest in subsequent matings (Kokko and Rankin 2006). In contrast, when the reproductive budget is fixed, investment in individual reproductive traits may be traded-off against each other (Simmons et al. 2017). Finally, adjustments of total resource allocation to reproduction may affect the expression of non-reproductive traits or male survival (Stearns 1989). As a result, the fitness consequences of adjusting resource allocation among sexual traits and mating events are difficult to predict due to the complex trade-offs that these adjustments may generate.

In addition, as resource limitation often prevents males from mating with every female they may encounter, important reproductive decisions also include mate choice. Although less common than female mate choice, male mating preferences are nonetheless frequently observed (Edward and Chapman 2011; Rosenthal 2017). There are some elements which may make females better partners under all circumstances, however, the benefits of mating with a female with given characteristics are often context dependent, resulting in plasticity in mate choice being beneficial (Qvarnström 2001). Male mating preferences, both in terms of preference function and of choosiness, are especially affected by social factors, such as competition (e.g. Audience effect, Plath et al. 2008), mating history (e.g. Coolidge effect, Dewsbury 1981) and mating opportunities (Kvarnemo and Simmons 1999).

The body of literature reporting plasticity in reproductive decisions is now very large and shows that the phenomenon is widespread. In particular, in the last decades, following the accumulation of evidence demonstrating that ejaculates are costly (Dewsbury 1982), that sperm depletion is a major risk for males (Wedell et al. 2002) and that optimal investment in ejaculates is context-dependent (Parker 1998), much research has focused on adjustments of ejaculate production and allocation (Kelly and Jennions 2011).

Within the context of ejaculate adjustments, similarly to what occurs for reproductive plasticity in general, males can make two types of decisions: they choose how much ejaculate to produce and of which quality (i.e. production plasticity) and how to allocate it across copulations (i.e. allocation plasticity) (Cattelan et al. 2018). Production plasticity enables males to limit energetic investment in the ejaculate when not necessary, hence saving energies for other reproductive and non-reproductive functions, while simultaneously allowing them to increase ejaculate availability when required and thus to avoid sperm depletion. Allocation plasticity enables males to tailor the portion of sperm and seminal fluid reserves transferred during a given copulation based on sperm competition, female quality and availability (Kelly and Jennions 2011).

Ejaculate plasticity is likely to be highly beneficial for males, but it is probably also constrained by costs (Bretman et al. 2011). For plasticity to be adaptive selection must favor different phenotypes in each context, with no single phenotype exhibiting superior fitness across all environments, and the costs of expressing plasticity must not exceed its benefits (Ghalambor et al. 2007). Costs of plasticity may be paid for having the ability to be plastic and for expressing plasticity, hence may be linked to the development, maintenance, and function of the sensory and regulatory machinery needed for modifying ejaculate production and allocation (i.e. intrinsic cost, Dewitt et al. 1998; Auld et al. 2010). Males also pay costs for expressing a phenotype (i.e. cost of phenotype, Murren et al. 2015), for example for upregulating sperm production. These costs are not directly linked to plasticity, as they are also paid by fixed individuals, but have important repercussions for plastic responses because they may constrain the expression of non-target traits (i.e. trade-offs, Stearns 1989). The costs of strategic ejaculate adjustments, paid in the form of trade-off with other pre- and post-copulatory traits, may be amplified whenever the response produced poorly matches the current

environmental conditions. Determining the adaptive value of strategic ejaculate adjustments, by measuring its costs and benefits in a range of environmental conditions, is crucial to understand when this phenomenon is expected to evolve.

AIM OF THE STUDY

The aim of my study was to investigate phenotypic plasticity of sexually selected traits through multiple approaches, mainly focusing on post-copulatory events. While the flexibility of reproductive decisions has been widely investigated, a great majority of studies has focused on male and female pre-copulatory strategies and on adjustments of ejaculate size. As a result, there are still areas that have received relatively less empirical attention. For example, in the field of sexual selection, studies aiming at quantifying the adaptive value of plastic responses are still relatively rare and usually focus on a single trait in a single context, ignoring potential effects of trade-offs and phenotype-environment mismatches. In addition, some cases of phenotypic plasticity in male mate choice have been traditionally interpreted based on assumptions on post-copulatory processes that would need to be explicitly tested. With my study, I aimed at improving our knowledge of this specific aspect of sexual selection. Throughout my project, I adopted an integrative approach, which simultaneously considers pre- and post-copulatory traits, as well as their interaction. My work will contribute to better understand the evolution of phenotypic plasticity of sexually selected traits and to detemrine which factors may promote or constrain its evolution. My project is organized in three sections, each with specific objectives.

1. Cases of phenotypic plasticity

Phenotypic plasticity of sexually selected traits has been shown to be widespread; it includes adjustments of morphological, physiological, behavioral and life-history traits and involves responses to a number of environmental stimuli (e.g. Rondeau and Sainte-Marie 2001; Oddie and Reim 2002; Brauer et al. 2007; Burger et al. 2015). Expanding our current knowledge of this phenomenon, by investigating how, in different species, multiple traits are affected by a diverse range of environmental conditions will contribute to understand the adaptive role of this phenomenon. In this section, I will firstly explore a potential case of phenotypic plasticity in a species, *Pisaura mirabilis*, which is known to express flexible behaviors (Tuni et al. 2017; Ghislandi et al. 2018), but for which responses to female availability have never been investigated. Variations in partner availability are known to potentially elicit two types of reactions. Males can either increase the total energetic budget devoted to reproduction and thus maintain a fixed resource allocation per mating event (e.g. Gage 1995), or can reduce allocation per partner in order to partition the reproductive budget when this cannot be enlarged (e.g. Warner et al. 1995). This experiment is designed to test the latter hypothesis.

Secondly, I will review available literature on strategic adjustments of ejaculate quality. In the last decades, scientific interest in plasticity of sperm numbers has raised, resulting in hundreds of studies being published (for a review see, Kelly and Jennions 2011; Delbarco-Trillo 2011). In contrast, despite evidence highlighting

its importance for sperm competition (Snook 2005), less is known about how ejaculate quality can be modified in response to social cues. In particular, beyond the scattered body of evidence showing that the phenomenon is common, a clear view on its prevalence and its consequences for male fitness is still missing. This study will contribute to gain a more comprehensive understanding of socially cued adjustments of ejaculate quality.

2. Costs and benefits of strategic ejaculate adjustments

The adaptive value of ejaculate adjustments has long been assumed, but in recent years, researchers have started to investigate the effects of these responses on male reproductive success. Evaluating the fitness consequences of ejaculate adjustments firstly requires measuring their intrinsic costs. Secondly, in order to test that each alternative phenotype is favored in the corresponding environment, it requires measuring how the up- and down-regulation of ejaculate production and allocation affects male reproductive success under a range of environmental conditions, and while accounting for the effects of trade-offs. To date, however, studies investigating the adaptive value of ejaculate plasticity have been limited to test whether an increase in investment in the ejaculate enhanced male fertilization success under conditions of sperm competition (Sakaluk and Müller 2008; Bretman et al. 2009; Barbosa 2012; Bartlett et al. 2017). In contrast, little effort has been done to quantify the intrinsic costs of ejaculate plasticity (but see, Firman et al. 2013) or to test the effect of increased ejaculate investment in multiple contexts and the potential contribution of trade-offs in affecting male overall fitness (but see, Bretman et al. 2013). Trade-offs between traits involved in mate acquisition and in post-copulatory competition, which are assumed to occur due to resource limitation, may represent a major constraint on the evolution of phenotypic plasticity, since high post-copulatory competitiveness is only beneficial after successfully mating and an increase in the first may compromise the latter. Investigating costs and benefits of socially cued ejaculate adjustments is crucial to understand why this form of plasticity is not ubiquitous and to clarify the circumstances under which it is expected to evolve. In this section, I will investigate costs and benefits of the strategic upregulation of sperm production induced in male guppies by exposure to females (Bozynski and Liley 2003). Firstly, I will measure the long-term costs associated with repeatedly responding to a fluctuating environment (i.e. intrinsic costs). I will then investigate how trade-offs involving other ejaculate traits and traits associated with mate acquisition affect male reproductive success under conditions of different female availability.

3. Corollary: the role of post-copulatory processes in phenotypic plasticity

The interpretation of phenomena of phenotypic plasticity and their adaptive value requires a deep understanding of how selective pressures act on reproductive traits and of how these pressures change depending on the context. In particular, clarifying mechanisms of post-copulatory selection is often crucial to comprehend which strategies may or may not be advantageous for males, both before and after mating,

in different social environments. However, the explanations invoked for cases of plasticity are sometimes based on assumptions, rather than on proved facts. In this section, I will investigate post-copulatory processes that will contribute to interpret cases of phenotypic plasticity in mate choice. The first experiment will test the effect of insemination order on male fertilization success in guppies. Patterns of sperm precedence play a central role in shaping male mating strategies, including the flexibility in mating preferences elicited by the presence of competitors and by their behavior (i.e. mate choice copying and audience effect, Auld and Godin 2015), the explanation of which has been controversial. In a second experiment, I will investigate whether previous exposure to ejaculates affects paternity success in subsequent copulations in multiply mated female guppies. Through a modulation of the immune response triggered by insemination, females may be able to favor either previous or novel partners. Such a mechanism could be involved in shaping costs and benefits of the preference for novel partners (i.e. Coolidge effect) frequently reported among males, and expressed both in terms of a decline of sexual interest (Dewsbury 1981) and in reduced sperm allocation (Pizzari et al. 2003) to previous partners. The results of this section will contribute to understanding the ultimate causes responsible for the evolution of such plastic behaviors.

STUDY SPECIES

Two species were used in this study: the guppy, *Poecilia reticulata*, and the nursery-web spider, *Pisaura mirabilis*.

The guppy, *Poecilia reticulata*

Guppies are small freshwater fish native to Central America (Houde 1997), belonging to the family of Poeciliidae. In the last two decades they have become a model species for sexual selection both at the pre- and post-copulatory level (Magurran 2005; Evans et al. 2011). Guppies are internal fertilizers: males transfer sperm into the female gonoduct through a modified anal fin, the gonopodium. Sperm are packaged in spermatozeugmata, or sperm bundles, each containing about 21,000 sperm cells, from which they are released after transfer into the female reproductive tract (Boschetto et al. 2011). Guppies are characterized by a sperm-only mating system and show strong sexual dimorphism with females presenting a mimetic coloration and males exhibiting a polymorphic color pattern (Houde 1997). Females are able to store sperm in the ovary, where these can remain viable for several months, nourished by cells of the ovarian epithelium (Jalabert et al. 1969; Gardiner 1978). Therefore, females can produce several successive broods using sperm stored after a single mating event (Winge 1937).

Males are sexually very active, performing up to one mating attempt per minute (Magurran and Seghers 1994), and alternatively adopt two mating tactics: they court females by performing sigmoid displays in order to obtain cooperative copulations or they attempt to forcibly inseminate females through gonopodial thrusting (Liley 1966; Houde 1997). All males flexibly switch between the two strategies, depending on the prevailing environmental conditions (see below). Cooperative copulations have higher insemination success, allowing males to deliver about three times more sperm than coercive copulations (Pilastro and Bisazza 1999; Matthews and Magurran 2000). Gonopodial thrusts, however, appear to be less energetically demanding than courtship displays (Devigili et al. 2013; Rahman et al. 2013) and allow males to overcome female resistance outside of the short receptivity window.

Both males and females express mating preferences. Males prefer large females as partners since these are on average more fecund than small females (Herdman et al. 2004). Females base their mate choice on male coloration, in particular the area of carotenoid coloration (Endler and Houde 1995; Evans et al. 2004), size (Reynolds and Gross 1992) and on sexual behavior, favoring males who court at higher rates (Kodric-Brown and Nicoletto 2001). In addition, both males and females show a mating preference for novel partners (.e. Coolidge effect, Kelley et al. 1999; Eakley and Houde 2004).

Since males and females are highly promiscuous (Houde 1997; Neff et al. 2008), post-copulatory sexual selection is intense in this species (Devigili et al. 2015b) and takes the form of sperm competition and cryptic female choice. The outcome of sperm competition is primarily determined by the number of sperm transferred during copulation, but is also affected by sperm velocity and viability (Boschetto et al. 2011; Cardozo et al. in prep). Females cryptically bias paternity to favor more colorful males, thus reinforcing pre-copulatory preferences (Pilastro et al. 2002; Pilastro et al. 2004). Cryptic female choice also involves selection based on male-female relatedness and MHC similarity (Johnson et al. 2010; Fitzpatrick and Evans 2014; Gasparini et al. 2015). Cryptic female choice is known to occur through at least two mechanisms: female control of copulation duration (which, in turn, determines the number of sperm transferred, Pilastro et al. 2007) and differential sperm activation by the ovarian fluid (Gasparini and Pilastro 2011; Gasparini et al. 2012). Insemination order also affects the outcome of sperm competition, with the last male to mate being favored when copulations occur in two successive reproductive cycles (Winge 1937; Gasparini et al. 2018), but also in the same one (Evans and Magurran 2001; Pitcher et al. 2003).

Guppies and phenotypic plasticity

Guppies are an excellent model species to study phenotypic plasticity, especially in relation to reproductive traits, since they live in a variable environment and express costly sexually selected traits. The habitat of wild guppy populations in Trinidad consists of small streams with a riffle-pool structure (Reznick et al. 1996), the architecture of which can be profoundly modified by seasonal flooding and drought, leading to pool fission and fusion (Grether et al. 2001). As a consequence, guppies experience frequent and intense environmental fluctuations of both abiotic and biotic factors, including water characteristics, predation intensity, food availability, population density and sex ratio (Grether et al.

2001; Pettersson et al. 2004; McKellar et al. 2009). These environmental factors, in turn, affect the fitness consequences of reproductive decisions and of male and female reproductive investment, which is conspicuous in this species. Several studies have documented costs (energetic costs or survival costs) associated with sperm production (Devigili et al. 2013; Gasparini et al. 2013; Rahman et al. 2013; Rahman et al. 2014a; Devigili et al. 2016), male sexual behavior (Godin 1995; Devigili et al. 2013; Rahman et al. 2013; Rahman et al. 2014b), male ornaments (Kodric-Brown 1989; Houde and Torio 1992; Godin and McDonough 2003), female choice and sexual behavior (Godin and Briggs 1996) and reproduction altogether (Reznick 1983). Indeed, guppies are characterized by marked plasticity in reproductive strategies, in response to abiotic and biotic factors.

Males have been shown to strategically adjust their sexual behavior, in particular relatively to the switch between courtship and coercive copulation attempts, in response to several physical factors, including light environment (Chapman et al. 2009) and water turbidity (Luyten and Liley 1985), and to predation (Godin 1995). However, it is the social environment the main factor affecting male mating strategies. Males indeed respond to the sex-ratio (Evans and Magurran 1999; Jirotkul 1999), to female receptivity (Magurran and Nowak 1991), to the presence of rivals (Auld et al. 2015), and to their previous experience in terms of female availability (Jordan and Brooks 2012).

Male and female sexual behavior is influenced by the social context also in terms of mating preferences. Not only females base their preferences on male comparison (Pilastro et al. 2004), but it has been shown that males and females copy other individuals' choices (Dugatkin 1992; Auld and Godin 2015) and males have been observed modifying their initial preferences in the presence of rivals (i.e. audience effect, Auld and Godin 2015).

Males are also plastic in sperm production, which is adjusted in response to expected mating opportunities: males increase ejaculate size and sperm velocity when maintained in the presence of females (Gasparini et al. 2009; Bozynski and Liley 2003; but see, Barrett et al. 2014). This enhanced investment in sperm number and velocity entails costs that are paid in terms of trade-offs with sperm viability (Cardozo et al. in prep), sexual behavior (Cattelan et al. 2016; Devigili et al. 2015a), lifetime growth (Jordan and Brooks 2010) and survival (Miller and Brooks 2005). Conversely, sperm production appears not to be affected by the presence of rivals (Evans 2009).

It is clear that guppies have adapted to a highly variable habitat, evolving mechanisms to respond to environmental changes and maximize the fitness returns of their investment into reproduction. However, since individual reproductive traits are often correlated, describing how traits interact with each other when their levels of expression change is crucial to understand how plasticity of sexually selected traits evolved and what are its consequences for male and female fitness.

The nursery web spider, *Pisaura mirabilis*

Nursery web spiders, *Pisaura mirabilis*, are members of the Pisauridae family found across the whole Palearctic region, where they inhabit a variety of habitats, from meadows to dunes and forests (World Spider Catalog 2017). Females produce a large egg sac that they carry around beneath their body; when the time for youngs to emerge approaches, the female deposits the egg sac on a leaf and protects it by spinning a silk 'nursery web' that owes the species its name. These spiders are characterized by a complex reproductive behavior with males offering a nuptial gift to females during courtship. The gift consists in a prey item, usually an insect, wrapped in several layers of silk, but males are also observed offering worthless gifts containing dry and empty insect exoskeletons or plant fragments wrapped in silk (Ghislandi et al. 2014).

Prey wrapping is elicited even in the absence of a female by sexual stimuli such as female silk (Albo et al. 2011a; Beyer et al. 2018), and males are typically observed wandering with a gift while searching for a female. The gift functions as a shield against female attacks during mating encounters and reduces the risk of pre-copulatory cannibalism (Toft and Albo 2016). The nuptial gift also greatly increases male mating success, although it is also possible for males to acquire copulations without one (Stålhandske 2001; Prokop and Maxwell 2009; Albo et al. 2011b). Gift content, being disguised by silk wrapping, does not appear to influence female likelihood to accept a male as a partner. Nuptial gifts are offered to the female during a characteristic courtship display which includes rubbing of the first and second pair of legs, vertical

stretching of the first pair of legs and gift offering, in which males bend backwards raising the first pair of legs and spread their pedipalps apart (Bristowe and Locket 1926; Nitzsche 2011). Courting males that are initially rejected by a female usually perform additional bouts of gift wrapping and are often eventually accepted (Bilde et al. 2007). Once the female had accepted the gift, by grasping it with her mouthparts, the copulation can begin. The male moves underneath the female in an antiparallel position to reach for the female epigyne where he logs one of his pedipalps and initiate sperm transfer. *P. mirabilis* males also typically engage in thanatosis, a striking death-feigning behavior performed when females, after accepting the gift, try to steal it from their partner before copulation is completed (Bilde et al. 2006). The male holds the gift with the chelicerae while he is been dragged around by the female; he revives and resumes copulation as soon as she stops. Thanatosis functions as an adaptive male mating strategy to overcome female resistance and to extend copulation duration (Hansen et al. 2008).

Females are polyandrous (Austad and Thornhill 1986; Drengsgaard and Toft 1999; Prokop and Maxwell 2009; Tuni and Bilde 2010) and store sperm from multiple males in the spermatheca to use them later for egg fertilization when an egg-sac is produced. Mating with multiple partners has been shown to confer indirect benefits to females by increasing the probability of oviposition and egg hatching success (Tuni et al. 2013). Post-copulatory sexual selection mainly depends on the control of copulation duration, which determines the number of sperm delivered to the female and fertilization rates (Stålhandske 2001; Albo et al. 2011b; Albo et al. 2013). Copulation duration appears to be primarily controlled by females (Stålhandske 2001; Albo et al. 2011b), and is influenced by gift donation. Males that offer a nuptial gift achieve longer copulations than males without a gift, leading to a higher number of fertilized eggs for gift-giving males (Drengsgaard and Toft 1999; Stålhandske 2001; Albo et al. 2011b). In addition, copulation duration is positively affected by gift size (Stålhandske 2001) and by the amount of silk applied to the gift (Lang 1996; but see, Albo et al. 2012), as silk wrapping facilitates male control over the gift during copulation and reduces the risk of females escaping with it before sperm transfer has been completed (Andersen et al. 2008). Gift content also influences copulation duration and sperm transfer, with males offering worthless donations obtaining shorter copulations (LeBas and Hockham 2005; Albo et al. 2011b). Besides controlling copulation duration, females appear to be able to exert some cryptic choice to favor gift-giving males also by regulating sperm storage, possibly through preferential sperm uptake during mating or differential sperm selection or ejection immediately after mating (Albo et al. 2013).

Nursery-web spiders and phenotypic plasticity

P. mirabilis males invest largely into reproduction. The costs involved in nuptial gift production are testified by the fact that males sometimes engage in courtship without a gift or with a worthless gift despite this behavior being associated with lower mating and/or fertilization success. Males spend time and energy in prey capture, release costly silk proteins for gift wrapping (Craig et al. 1999; Craig 2003) and are impaired in their movements by gift carrying (Albo et al. 2011a; Prokop and Maxwell 2012). Male feeding condition influences gift construction: males in better conditions produce gifts more frequently and use more silk than individuals in worse conditions (Albo et al. 2011a; but see, Lang 1996), but do not differ in terms of valuable vs worthless donations (Ghislandi et al. 2017). Courtship comprises of a set of conspicuous displays that may require considerable energetic investment and may expose males to predation. Similarly, thanatosis has been also proposed to be energetically costly, as an active performance may be needed by males to keep their legs stretched (Foelix 1996), and it has been shown that the ability to perform thanatosis was impaired in handicapped males (Hansen et al. 2008).

Because costs of mating are conspicuous in this species, males should be prudent in their reproductive decisions. Since the environmental factors that are likely to influence costs and benefits of male reproductive investment, such as prey availability, population density and operational sex-ratio, change during the mating season (Ghislandi et al. 2018), males are expected to be flexible in their mating effort and adjust their investment depending on the context to maximize fitness returns. Indeed, the gift-giving behavior (i.e. offering a genuine prey gift, a 'worthless' non-nutritious gift or no gift) of *P. mirabilis* males has been shown to be modified depending on seasonal variations in the abundance of preys and in the proportion of adult females (Ghislandi et al. 2018). Males also respond to sperm competition by reducing their investment into silk-wrapping and sperm transfer when exposed to rivals (Tuni et al. 2017). It is evident that male nursery-web spiders have the ability to modify their reproductive behavior to relevant environmental variables and thus represent an extremely well-suited species to study plasticity of sexually selected traits.

DISCUSSION

Phenotypically plastic investment is expected to evolve when trait expression is costly and the returns of investment in such traits depend on environmental conditions that are temporally or spatially variable. Phenotypic plasticity of sexually selected traits is associated in particular with variations in elements of the socio-sexual environment, such as competition for access to mates, partner quality and partner availability (Wedell et al. 2002; Bretman et al. 2011; Weir et al. 2011). The aim of this study was to investigate phenotypic plasticity in sexually selected traits, in order to contribute to understand its prevalence, to describe the diversity of traits subject to adjustments and that of environmental stimuli triggering responses, but in particular to quantify costs and benefits of plasticity, focusing on a specific phenomenon, namely the strategic adjustment of sperm production. My project was composed by three sections.

In the first section, I explored cases of phenotypic plasticity using both an empirical and a theoretical approach. Firstly, I investigated whether variations in the number of mating opportunities affected male mating effort in males of *Pisaura mirabilis*. Female availability is an important factor shaping reproductive decisions and can affect resource allocation to sexual traits in two directions. In fact, males may either reduce their mating effort per mating event, in order to partition the reproductive budget among multiple partners (Warner et al. 1995); or they may maintain allocation patterns fixed while enlarging the total reproductive budget (Koyama and Kamimura 2000). We found that when facing higher mating opportunities, males did not reduce their investment in sexual behaviors, but that they invested less in the construction of nuptial gifts when paired with relatively larger females. However, they may be able to adjust the total energetic budget devoted to reproduction by drawing resources from non-reproductive traits, a hypothesis that should be tested in future studies.

Then, I took into exam literature focusing on strategic adjustments of ejaculate quality in response to social stimuli. Ejaculate quality plays an important role in determining fertilization competitiveness (Snook 2005) and is also known to be costly (e.g. Devigili et al. 2013; Devigili et al. 2016). However, socially cued adjustments of ejaculate quality have received less attention than adjustments of sperm numbers. This review revealed that the phenomenon is widespread and diverse, but that a more systematic analysis of its prevalence, which could shed light on its importance relatively to sperm number responses, is still impossible due to the insufficiency, but above all the heterogeneity, of available studies. I highlighted, however, that ejaculate adjustments often impose trade-offs with other pre- and post-copulatory traits, which in turn, complicate the estimation of the fitness consequences of ejaculate plasticity.

The second section of my study aimed at investigating different types of costs of socially cued ejaculate plasticity. In particular, I focused on one case of plasticity that is the upregulation of sperm production elicited in male guppies by exposure to females (Bozynski and Liley 2003). Firstly, I investigated the long-term costs of repeatedly activating a plastic response when mating opportunities fluctuate continuously (i.e. intrinsic

costs of plasticity), and which could be paid in terms of reduced expression of reproductive and non-reproductive traits. The experiment showed that this plastic response is associated with limited intrinsic costs in guppies. This result is in line with previous studies in non-reproductive contexts, and suggests that selection may have eroded plasticity costs by favoring more efficient genetic variants (Dewitt et al. 1998).

Hence, I explored the phenotypic costs of this response, investigating how investment into the upregulation of sperm production affects other traits, due to trade-offs between components of the reproductive effort. In one experiment, I showed that males who have been exposed to females and who have consequently increased their investment into sperm production, display less intense sexual behavior, confirming previous results (Devigili et al. 2015a; Cattelan et al. 2016). This difference in sexual activity resulted in female-stimulated males having lower mating success when competing with a rival for access to females. In addition, having produced larger sperm reserves did not allow to transfer more sperm, but appeared to be beneficial in association with high mating rates, by reducing the risk of sperm depletion.

In another experiment, I investigated whether the upregulation of sperm production also determined within ejaculate trade-offs, and specifically trade-offs between sperm number and sperm ability to cope with aging during male sperm storage (i.e. sperm senescence). If these trade-offs result in sperm from female-stimulated males experiencing early senescence, they may become particularly costly in case of phenotype-environment mismatch, that is, when males upregulate sperm production because they anticipate high mating rates and instead do not have any opportunity to copulate, resulting in their sperm aging. Sperm senescence can have multiple deleterious effects on fitness (Jones and Elgar 2004; Levitas et al. 2005; Gasparini et al. 2017), and is known to affect guppies' sperm during male sperm storage (Gasparini et al. 2014). I found, however, that female-stimulated males did not suffer from increased sperm senescence, possibly because sperm production is very flexible and males quickly down-regulate it in the absence of females. Taken together, the results of these studies on costs of strategic ejaculate adjustments suggest that costs of plasticity are minor in guppies, as compared to the costs of the phenotype, and that the latter are primarily associated with trade-offs between sperm production and pre-copulatory traits rather than with trade-off between sperm number and quality.

The third section of my study represented a corollary to the core of my project, investigating processes of post-copulatory selection which may be involved in phenomena of plasticity in mate choice. Phenotypic plasticity of sexually selected traits does not only include adjustments of resource investment, but also, for example, context-dependent changes in mating preferences. Some of these variations in preference functions have been difficult to interpret, but may be explained by understanding how post-copulatory selection operates. In a first experiment, I studied patterns of sperm precedence as affected by insemination order. While in natural copulations guppies are characterized by a last male precedence (Evans and Magurran 2001), in my experiment the use of artificial insemination reversed the pattern and revealed a first male

precedence. Besides suggesting that the pattern observed in natural copulations probably originates from cryptic female choice (Pilastro et al. 2004) coupled with trading-up for male attractiveness (Pitcher et al. 2003), the discovery of first male precedence contributes to explain some male mating strategies. For example, it can explain the absence of male mate guarding in this species (Houde 1997) and male strong preference for virgin females (Guevara-Fiore et al. 2009). It also contributes to explain male mate choice copying and the audience effect (Auld and Godin 2015).

In the second experiment I tested whether female previous exposure to a male's ejaculate could affect his paternity success in subsequent copulations, by means of a modulation of the female immune response. I found that previous partners indeed benefitted from a paternity advantage, probably due to a male-specific suppression of the female immune response against the ejaculate, which may have evolved to protect the conceptus from being recognized as non-self and attacked by the mother's immune system. This mechanism could be involved in shaping costs and benefits of the Coolidge effect, and could contribute explaining the reduced sperm allocation to previous partners observed in some species (Pizzari et al. 2003; Reinhold et al. 2015).

CONCLUSIONS

Altogether, my results show that while phenotypic plasticity in sexually selected traits is widespread, trade-offs among traits associated with different components of male reproductive success are also common, and complicate the quantification of costs and benefits of plastic responses, and hence of their adaptive value. More specifically, with regard to guppies, I showed that the socially-cued strategic adjustment of sperm production does indeed result into post-copulatory benefits, but at a significant pre-copulatory cost. Furthermore, I showed that intrinsic costs of this form of plasticity are small as compared to those due to trade-offs with other traits. As expected for anticipatory plasticity, costs increase in case of phenotype-environment mismatch. While some evidence of the fertilization advantage provided by an increased investment in the ejaculate was already available for guppies (Cardozo et al. in prep) and for few other species (Sakaluk and Müller 2008; Bretman et al. 2009; Barbosa 2012; Bartlett et al. 2017), the existence of costs associated with this response explains why conditional upregulation of sperm production should be favored instead of fixed maximal investment across all contexts. This is one of the first studies quantifying the adaptive value of ejaculate plasticity by accounting for episodes of pre- and post-copulatory selection during multiple mating events (but see, Bretman et al. 2013). Also in light of the probable involvement of the post-copulatory processes I described in the onset of phenomena of mate choice plasticity, my results bring further support to the evidence that pre- and post-copulatory phenomena are inextricably linked and show that only by accounting for both it is possible to evaluate the adaptive meaning of plastic responses in reproductive traits.

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Manuscript 1a

Enough for everyone: *Pisaura mirabilis* males do not reduce their mating effort with increasing mating opportunities

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ABSTRACT

Male reproductive traits are costly. As individuals possess a finite energetic budget, resource allocation to one mating event constrains potential investment in further matings. Consequently, males of many species have evolved the ability to adjust their reproductive investment in response to elements of the social environment indicating future mating opportunities, such as partner availability. In particular, when female availability is high, male reproductive effort should be prudently partitioned among multiple partners to avoid resource depletion before mating opportunities have ceased. This theoretically applies to investment in pre-mating (e.g. courtship) and post-mating (e.g. sperm) traits, unless these are trade-off against each other. We tested this hypothesis using the spider, *Pisaura mirabilis*, a species characterised by nuptial gift-giving mating behaviour, which entails conspicuous costs for males. We manipulated male perception of mate availability by modifying the number of females they were exposed to and then recorded their investment to pre-mating traits (time allocated to gift construction and courtship effort) and traits at mating (copulation duration) with a female. Since gifts facilitate both, mate acquisition and sperm transfer, we expect males facing higher mating opportunities to reduce their investment in the traits targeted by our study. Contrary to expectations, males did not reduce their investment in the current partner when other females were present, suggesting lack of resource partitioning in response to variation in female availability. Males of this species may be able to increase their reproductive budget by drawing resources from food intake (i.e. consuming part of the gift prior to wrapping) or from non-reproductive traits (e.g. growth or immune defence). Interestingly, males silk-wrapped nuptial gifts for longer when mating with smaller females. Further studies would be required to test the effect of multiple matings on male growth and lifespan and to measure male food intake under different conditions of female availability. Moreover, a specifically designed experiment should explore the effect of female size on male preferences and resource allocation, and investigate potential benefits of male mate choice.

INTRODUCTION

Reproduction requires a conspicuous energetic investment. While it has been long assumed that males pay limited reproductive costs compared to females (Andersson 1994), it is now widely recognised that traits and processes associated with male reproduction, including courtship, mate choice, sperm production and mating *per se*, are often extremely costly (Dewsbury 1982; Kotiaho 2001; Scharf et al. 2013). This evidence has led to overcome the view, supported by Bateman's seminal study (1948), that males mate unlimitedly. Given that males possess a finite budget of resources, the costs associated with reproduction constrain males' ability to invest in other traits and functions, in the same mating event or in future ones, resulting in resource allocation trade-offs (Stearns 1989; Parker et al. 2013). Hence, while increasing the mating effort in a given mating event has the potential to increase the gain deriving from it, it may simultaneously reduce the opportunity for future copulations or their success (Kokko and Rankin 2006). In this scenario, males have evolved the ability to modulate their reproductive investment and allocate resources among individual mating events based on the expected fitness returns (Wedell et al. 2002; Bretman et al. 2011). Partner availability is one of the most relevant factors determining reproductive investment decisions as it affects the costs as well as the probability of success associated with searching and securing new partners (Parker 1974). Males may respond to an increase in the number of available partners, and hence mating opportunities, by modifying their resource allocation in two ways. On the one hand they may increase the total resource budget devoted to reproduction, for example by increasing investment in ejaculate production in the presence of females (Gage 1995; Koyama and Kamimura 2000; Bozynski and Liley 2003; Olsén et al. 2006). On the other hand, they may reduce their resource allocation per mating event (Warner et al. 1995), partitioning energetic resources among multiple partners. The latter could provide an efficient means to avoid resource depletion (e.g. time, energy, nuptial gifts, ejaculates) before mating opportunities have ceased (Proulx et al. 2002), and may ultimately allow males to mate with more partners.

The adjustment of male reproductive investment as a function of partner availability has been observed in a wide range of taxa and has targeted several reproductive traits involved in both mate acquisition (pre-mating traits) and fertilisation (post-mating traits), corroborating the abovementioned theoretical predictions. It has been often documented how the time and energy spent in courtship positively correlate with male mating success (e.g. Vinnedge and Verrell 1998; Shamble et al. 2009) and how an increase in the time designated to copulation is beneficial in terms of fertilisation success (e.g. resulting from increased sperm transfer, Parker et al. 1990; removal of previous partners' sperm, Siva-Jothy 1987; delayed female re-mating, Mazzi et al. 2009; or cryptic female choice, Edvardsson and Arnqvist 2000). However, since such investment reduces the resources available to search for and obtain further copulations, it should be carefully allocated (Parker 1974; Weir et al. 2011). Indeed, male tree crickets (*Oecanthus nigricornis*) reduce their allocation to courtship food-gifts when experiencing relatively higher female encounter rates (Bussiere 2002; Bussiere et al. 2005), and

male guppies (*Poecilia reticulata*) invest more conspicuously in courtship displays when experiencing lower female availability (Jordan and Brooks 2012). Male dungflies (*Scatophaga stercoraria)* extend copulation duration in the current mating when males foresee fewer mating opportunities (Parker and Simmons 1994), and male walnut flies (*Rhagoletis juglandis)* perform shorter copulations with female-biased sex ratios (Alonso-Pimentel and Papaj 1996). Additionally, despite ejaculate size being the main predictor of fertilisation success, sperm depletion can potentially represent a major constraint for fitness when male mating rates rise (Wedell et al. 2002) and males should adjust the number of sperm they inseminate according to the probability and cost of acquiring further partners (Parker 1990a, b). Males of the bluehead wrasse (*Thalassoma bifasciatum*) characterised by higher mating success are for example known to strategically release fewer sperm per mating event than less successful males (Warner et al. 1995), and similar results have been obtained in a range of different species (Simmons et al. 1999; Cornwallis and Birkhead 2006; Worthington et al. 2013).

The strategic partitioning of male reproductive investment per mating as partner availability increases may play an important role in shaping mating systems. As a decrease in male reproductive investment reduces some of the benefits (e.g. sperm, nuptial gifts) obtained by females through copulation, it may, in fact, increase female likelihood to re-mate (favouring the evolution of polyandry), stimulate competition among females for access to males and/or ultimately promote sexual conflict (Stockley 1997; Sæther et al. 2001).

While evidence has now accumulated showing that males strategically adjust allocation to one or more reproductive traits when mating opportunities vary, little is known about the effect of partner availability on the whole set of pre- and post-mating traits. Theoretical models and empirical data predict that adjustments of the expenditure in pre- and post-mating traits in response to female availability should point to the same direction (namely, lower investment with increasing partner availability) (Parker 1974; Parker and Simmons 1994; Bussiere 2002). However, the presence of resource allocation trade-offs, common among reproductive traits (Parker 1998; Simmons and Emlen 2006; Simmons et al. 2017), may complicate the picture. Pre- and post-mating traits are indeed often negatively correlated (e.g. Simmons et al. 2010; Evans 2010; Durrant et al. 2016), hence an increased investment in mate acquisition (e.g., courtship) may lead to a decreased investment in traits associated with fertilisation success (e.g., ejaculate size). Furthermore, investment in some traits, such as nuptial gifts or sperm production, is more strictly constrained by a limited resource budget and cannot be promptly upregulated, while investment in more flexible traits, such as behaviours, could be increased by redirecting resources from other traits and activities (Bateman et al. 2001). Traits associated with a more constrained budget are hence expected to be more carefully partitioned among mating events. Since male fitness is ultimately determined by the interaction of the whole set of traits, it is crucial to explore simultaneously how male allocate resources to multiple pre- and post-copulatory traits depending on the social context (Devigili et al. 2015).

In this study, we investigated whether male investment into multiple pre- and post-mating traits is strategically adjusted in response to perceived partner availability in the gift-giving spider *Pisaura mirabilis*. Males of this species court females by donating a nuptial gift consisting of a prey (generally an insect) wrapped in silk, which is offered to the female through a series of courtship displays (Bristowe and Locket 1926). Gift-offering increases male mating success and prolongs copulation duration, as females feed on the gift during mating (Stålhandske 2001). Copulation duration in turn predicts the amount of sperm transferred by males (Albo et al. 2011b; Albo et al. 2013), providing gift-giving males with advantages in sperm competition in a system where females mate and store sperm from multiple partners (Drengsgaard and Toft 1999; Tuni et al. 2013). Males have additionally evolved a strategy to prolong copulations by performing thanatosis, a death-feigning posture that allows them to resume the mating position and avoid losing both the gift and the mating opportunity whenever the female interrupts copulation (Bilde et al. 2006; Hansen et al. 2008). Overall, mating carries costs for males in this species (Albo et al. 2011a); males spend time and energy in prey capture, release costly silk proteins for gift wrapping (Craig et al. 1999) and are impaired in their movements by gift carrying (Albo et al. 2011a; Prokop and Maxwell 2012). In this scenario, the ability to prudently partition reproductive resources among mating events may provide males with great fitness benefits. We know from previous studies that *P. mirabilis* males adjust their reproductive investment in response to the competitive environment by producing gifts of lower nutritional value with increasing male biased sex ratios (Ghislandi et al. 2018) and reducing silk-wrapping and sperm transfer when facing sperm competition risk (Tuni et al. 2017). Given that male reproductive success depends on both the number of mating partners achieved and the fertilisation outcome of each mating, and since mating opportunities fluctuate due to seasonal and local variations in the population sex ratio (Ghislandi et al. 2018), we expect male reproductive decisions to be influenced by partner availability.

To test this hypothesis we manipulated male perception of mating opportunities by varying partner availability. We exposed males to the presence of three females before and during courtship and copulation, and compared male allocation to reproduction, in terms of investment in gift-wrapping and courtship (pre-mating traits), copulation and thanatosis (post-mating traits), to that of males exposed to a single female. If males partition their reproductive resources among an increased number of partners we predict inidividuals exposed to multiple females to reduce their investment in mating with the current partner. Alternatively, males may be able to draw resources to the reproductive budget by reducing their allocation to non-reproductive traits. The resulting reproductive budget may be large enough not to need a parsimonious partitioning among multiple mating opportunities.

MATERIALS AND METHODS

Study organisms

Spiders (approx. 150) were collected as juveniles between September and October 2017 in the meadows surrounding the Ludwig Maximilians University's Biozentrum (LMU Munich, Germany) and were brought to the laboratory at the LMU where they were raised under natural photoperiod and room temperature (approximately 22°C). They were kept individually in vials (5 cm diameter, 10 cm height) topped with foam lids and supplied with a substrate of freshly collected moss. Moss was sprayed with water and spiders were fed three times a week with a mixed diet of fruit flies (*Drosophila* sp.), house flies (*Musca domestica*) and cricket nymphs (*Gryllus bimaculatus*; *Acheta domesticus*). Vials were checked daily for the presence of moulted exuviae in order to determine spider maturation to adulthood. Adult spiders (76 males and 71 females) were used in our experiments between 10 and 20 days after reaching adulthood. All animals were unmated.

Experimental design

Male spiders were randomly assigned to one of the two experimental treatments: in the "Low mating opportunities" (LMO) males (N=37) were exposed to one single female, the focal female with which the male was allowed to mate, and in the "High mating opportunities" (HMO) males (N=39) were exposed to four females, the focal female plus three stimulus females. We chose to use four females in the HMO treatment in order to provide a conspicuous difference with the LMO treatment, and, at the same time, a density of individuals similar to that observed in nature (Magris, M personal observation). While data on male mating

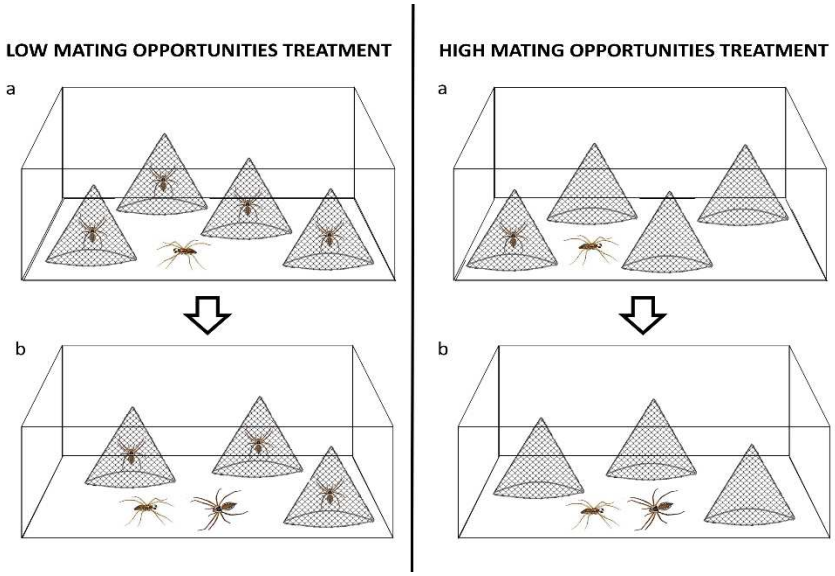

Figure 1. Graphic representation of the experimental set up showing the mesh cones delimiting females during gift-wrapping (a), and during courtship and copulation after the release of the focal female (b) in the two treatments.

rates are currently unavailable for this species, a previous laboratory study has shown that males can mate with four females within a short interval (i.e. four days, Tuni and Bilde 2010). Females were placed inside mesh cones allowing exchange of chemical and visual cues between the sexes, and even physical contact through the mesh while preventing aggressions and copulations (**Fig. 1**).

Prior to experiments males and females were weighed to the nearest 0.01g using a digital scale (KERN PKT, KERN & SOHN GmbH, Balingen, Germany). All focal females had been previously used as stimulus females; this allowed us to minimise the differences in social experience between females in the two treatments that could have affected female mating behaviour.

Experiments were conducted in a transparent plastic terrarium (30×18×20 cm) provided with a bottom sheet of absorbent paper. Mating trials were video-recorded using two web cameras (Logitech HD Pro Webcam C920) fixed at a distance of 40 cm from the terrarium, and each connected to a laptop. All time measurements of the behaviours recorded in the experiments were measured using a digital stopwatch (Conrad G-501) during the visual inspection of the video-recordings. In the LMO treatment the focal female was placed in the terrarium for 20 minutes in order to leave draglines, which are known to elicit male sexual arousal and gift-construction (Beyer et al. 2018). In the HMO treatment each of the four females was sequentially placed in the terrarium for five minutes, with the focal female always being the first to minimise her exposure to silk of other females. All females were then returned to their housing vials.

In order to construct a nuptial gift, the male was then given a live prey (house flies, on 4 occasions cricket nymphs) of known body mass (measured with a digital scale, see above) and size (body length was measured with a digital calliper, AEROSPACE, Beijing, China) inside his housing vial. After catching the prey, to reduce the risk of losing the prey during transfer, the male was given three minutes before he was transferred to the terrarium, and additional three minutes of habituation inside the terrarium before the females were introduced. In the LMO treatment the focal female occupied one mesh cone, while the other three were empty; in the HMO treatment, each of the four cones contained a female (**Fig. 1**). In both treatments, the male was gently pushed with a paintbrush to visit all 4 cones, in order to ensure he had perceived the presence (or absence) of all females, and was then allowed to move freely in the terrarium. Mesh cones and terraria were cleaned with ethanol (70%) and dried using paper towels after each trial in order to remove chemical cues.

<u>Pre-mating investment: silk wrapping of the gift and courtship effort</u>

Male silk wrapping of the prey started during the 3-minute habituation period in the terrarium or after the introduction of the females in the mesh cones. We measured the latency to silk wrapping as the time interval from gift acceptance to the start of wrapping and total silk-wrapping duration as the sum of the durations of

all wrapping sequences. Silk wrapping was considered completed when males had stopped wrapping for 10 consecutive minutes. Males that did not accept the prey or that did not wrap the prey within 60 min from catching it were returned to their vials and tested on the following day assigning them to the same treatment.

Once silk wrapping was completed the focal female was released from the mesh cone and the pair was left free to interact while we monitored male courtship behaviours. Courtship in *P. mirabilis* consists of a repeated series of stereotyped male displays including rubbing of the first and second pair of legs, vertical stretching of the first pair of legs and gift offering, in which males bend backwards raising the first pair of legs and spread their pedipalps (sperm transferring appendages) apart (Nitzsche 2011). We measured the latency to start courtship as the time interval from the first physical contact between the sexes to the start of any of the above mentioned courtship displays and the total duration of courtship displays as the sum of time spent performing each display. The total duration of the courtship trial was the time interval from the first contact between male and female until the females grasped the gift in their mouthparts. Hence we calculated male courtship effort as the proportion of time spent courting over the total duration of the courtship trial. If the males did not perform any courtship behaviour within 60 minutes the trial was terminated, the male was returned to his vials and tested on the following day assigning him to the same treatment. If the female did not accept the gift for 100 minutes the trial was interrupted and the individuals were not further used since extended social experience could potentially influence their behaviour in next trials. Data on gift construction for interrupted trials were included in the analyses, data on courtship duration were excluded.

Investment at mating: copulation duration and thanatosis

Once the female had grasped the gift with her mouthparts, the male moves underneath her in an antiparallel position to reach for the female epigyne (the female external genital opening) with one of his two pedipalps and transfers sperm into the female reproductive tract. A mating trial was considered successful if the male coupled a pedipalp with the epigyne. Interruptions of sperm transfer may be caused by males that decouple and switch to using the other pedipalp or by females that change position or try to run away with the gift. We noted for how long each palp (right or left) was being used and measured total copulation duration as the sum of all pedipalp insertion durations. Copulation duration was used as a proxy of the number of sperm transferred by males (Albo et al. 2011b; Albo et al. 2013).

Thanatosis is often observed whenever the female moves away during copulation. We measured the total duration in time (as the sum of all durations) males spent in the death feigning posture.

Copulation is terminated either with a conflict to keep hold of the gift or when either the male or the female separates from the gift. When the male lost the gift to the female, the mating trial was terminated as males without a gift are unlikely to remate (Albo et al. 2011b). If the female lost the gift to the male, the male was

allowed to resume courtship, after 60 min of inactivity the trial was terminated. If the male did not succeed at logging a palp in the epigyne, only data for gift-wrapping, copulation and thanatosis were included in the analyses, and the individuals were not used in other trials.

Statistical analyses

All analysis were conducted using RStudio (version 3.5.1). Weight and body length of the prey given to males for gift construction were compared between HMO and LMO groups with a t-test to ensure prey size did not differ between them. To test the effect of mating opportunities on the occurrence and duration of different behaviours we ran linear mixed-effects models (LMM and GLMM, using the "lmer" and the "glmer" functions, package lme4) and linear models (LM and GLM, using the "glm" function, package lme4), where treatment, male body mass and female body mass were entered as fixed effects. We included male body mass because it is known to affect male behaviour in this species (e.g. Albo et al. 2011a), and female body mass to investigate male mate choice, which in arthropods often consists in a preference for larger and more fecund females (Bonduriansky 2001). *Pre-mating investment.* To analyse latency to start wrapping and wrapping duration we used a LMM, where male identity was entered as a random effect to account for non-independence of the data collected from the same male (all males that wrapped the gift were included in this analysis, resulting in males not initiating courtship being re-tested). We tested the probability of males initiating courtship, by using a GLMM with binomial distribution and male identity as random effect. Latency to start courting and courtship effort were analysed with linear models. We included in this analysis all trials in which the female accepted the gift after being courted (excluding cases in which the female grabbed the gift without previous male courtship). For 11 trials we were unable to calculate courtship latency and courtship effort due to problems with video recordings.

Investment at mating. To test male copulation success (i.e. males succeeding or not to insert a palp in the female epigyne) we ran a GLMM with binomial distribution and male identity as random effect. All males that wrapped the gift were included in these analyses. Total copulation duration was analysed using a linear model. Occurrence of thanatosis among trials in which the female accepted the gift was analysed using a GLM with binomial distribution; when occurring, thanatosis duration was analysed using a linear model.

RESULTS

In 22 trials (13 LMO and 9 HMO) males did not accept the prey item or did not construct the gift; these trials were excluded from all subsequent analyses. Live prey given to males to construct gifts did not differ between treatments in terms of body mass (t-test, t=-0.32, df=72, p= 0.76, mean body mass (mg) ± SE, LMO 20.93 ± 0.92; HMO 21.35 ± 1.01), or body length (t-test, t= -0.44, df=71, p= 0.66, mean body length mass prey (mg) ± SE, LMO 8.14 ±0.87; HMO 8.05±0.84).

Pre-mating investment: silk wrapping of the gift and courtship effort

Results of the statistical analyses are shown in **Table 1**. Among the 67 males used, 8 were tested twice for wrapping (for a total of 75 mating trials). Mating opportunities did not influence latency to start wrapping (mean time interval (min) ± SE; LMO: 10.99 ± 1.83; HMO: 12.78 ± 1.99; **Table 1**), nor did male and female body mass (**Table 1**). Total wrapping duration did not differ between treatment (**Table 1**, **Fig. 2**) and according to male body mass (**Table 1**), but there was a tendency towards males wrapping for longer when paired with relatively smaller females (**Table 1**), and the effect became significant when non-significant factors were removed from the model (N=75, LMM: female weight, χ^2=4.75, P=0.029; **Fig. 3**).

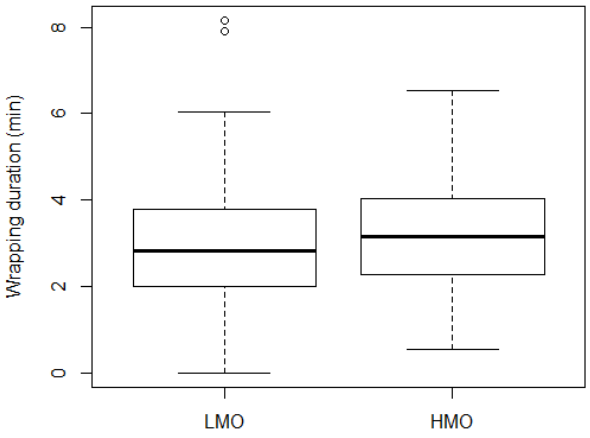

Figure 2. Mean silk-wrapping duration (min) of the nuptial gift of males experiencing low (LMO) and high (HMO) mating opportunities. Males are not affected by the treatment.

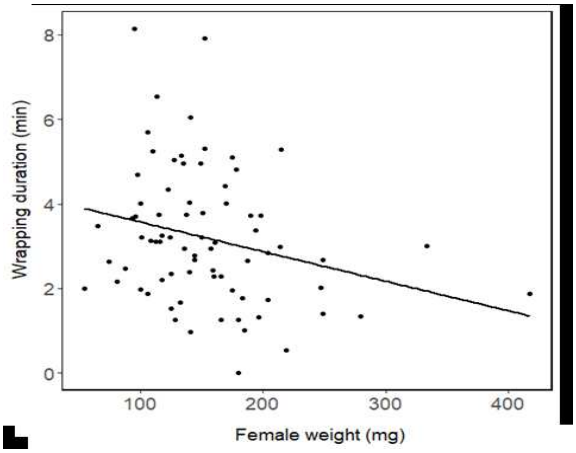

Figure 3. The amount of time males spent silk-wrapping their nuptial gifts covaries negatively with female body mass.

In some trials males constructed the gift but did not start courtship (N=7, 5 LMO and 2 HMO), while in others (4 trials, 2 LMO and 2 HMO) copulation occurred without previous courtship, as the female approached the male and bit the gift before he started courting (these trials were excluded from courtship analyses). The probability of initiating courtship was not affected by treatment, nor by male and female body mass (**Table 1**). Similarly, latency to start courting (mean time interval (min) ± SE; LMO: 9.07 ± 2.39; HMO: 11.96 ± 3.07; **Table 1**) and courtship effort (**Fig. 4**) were not affected by treatment, nor by male and female body mass (**Table 1**).

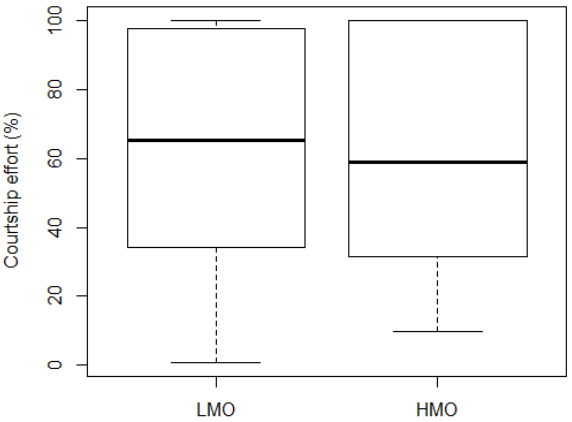

Figure 4. Courtship effort (calculated as the proportion of time spent courting over the total duration of the courtship trial) of males experiencing low (LMO) and high (HMO) mating opportunities. Males are not affected by the treatment.

Investment at mating: copulation success, copulation duration and thanatosis

In few trials male courtship was not effective and the female never accepted the gift (N=4, 1 LMO and 3 HMO). In other trials the female accepted the gift but copulation never took place because the female separated from the gift (N=1, 1 LMO) or stole the gift from the male before copulation began (N=4, 4 LMO). A total of 59 successful copulations were recorded (30 LMO and 29 HMO). Independently of the cause preventing copulation, there was no influence of treatment, nor of male and female body mass on copulation success (**Table 1**) and on copulation duration (**Table 1**, **Fig 5**). The occurrence of thanatosis and its duration (**Fig 6**) were also unaffected by treatment and individuals' body mass (**Table 1**).

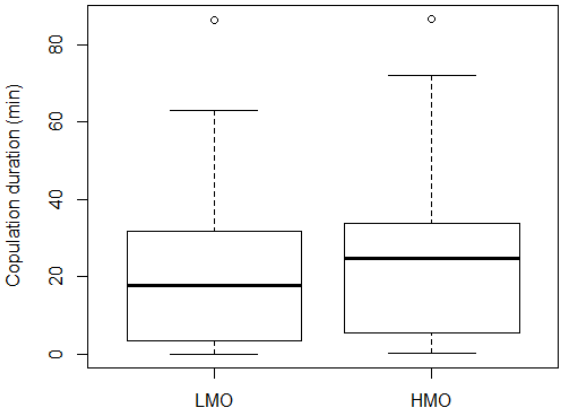

Figure 5. Mean copulation duration (min) of males experiencing low (LMO) and high (HMO) mating opportunities. Males are not affected by the treatment

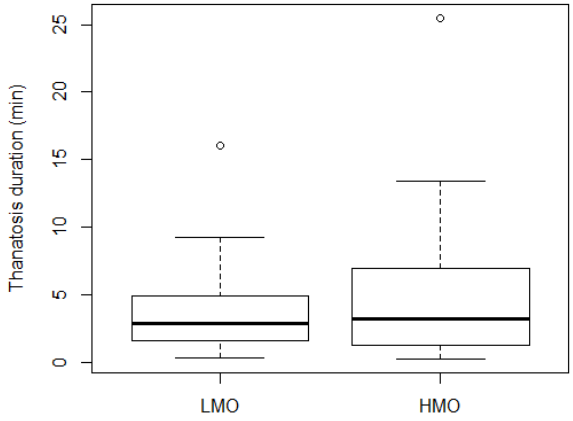

Figure 6. Mean thanatosis duration (min) of males experiencing low (LMO) and high (HMO) mating opportunities. Males are not affected by the treatment.

Table 1. Results of the statistical models showing the effect of treatment (males experiencing low, LMO, and high, HMO, mating opportunities), male and female body mass on male behaviours indicating male reproductive investment pre-mating and at mating. For details about each model see footnotes.

Response variable	*N*	Effect (Wald χ^2; *P*)			Statistical Model
		Treatment	Male body mass	Female body mass	
Pre-mating investment					
Latency to start wrapping	74	0.06; 0.800	0.08; 0.775	0.35;0.554	LMM[1]
Wrapping duration	75	0.26; 0.612	0.16; 0.685	3.77; 0.052	LMM[1]
Probability to start courtship	71	0.32; 0.569	1.52; 0.218	0.74; 0.390	GLMM[1,2]
Latency to start courtship	49	0.13; 0.715	0.04; 0.847	1.09; 0.296	LM
Courtship effort	49	0.47; 0.493	<0.01; 0.985	2.31; 0.129	LM
Investment at mating					
Copulation success	75	0.61; 0.435	0.52; 0.473	1.58; 0.208	GLMM[1,2]
Copulation duration	59	0.07; 0.797	0.19; 0.661	1.19; 0.276	LM
Thanatosis frequency	64	0.79; 0.374	2.29; 0.130	0.54; 0.461	GLM[2]
Thanatosis duration	43	0.90; 0.342	<0.01; 0.965	0.03; 0.870	LM

[1] Male identity included as random effect.
[2] Binomial distribution.

DISCUSSION

In our study we investigated whether males of the spider *Pisaura mirabilis* modify their allocation to reproductive traits when perceived mating opportunities vary, with the expectation that males partition their resources by reducing their investment in the current mating when the number of available partners increases. After manipulating male perception of mating opportunities by exposing them to either high or low female availability, we paired each male with a female and tested his mating effort in terms of pre-mating investment (gift wrapping, courtship) and investment at mating (copulation duration and thanatosis). Contrary to expectations, *P. mirabilis* males exposed to different female availability did not modify their investment in any of the multiple pre and post-mating traits that we measured. We found, however, an effect of female body mass on gift construction, with males wrapping for longer when paired with smaller females.

Our results indicate that male spiders do not respond differentially to cues of multiple females when it comes to reproductive investment. A possible explanation is that changes in this parameter may not be biologically relevant and male response may not have evolved. Sexually mature individuals of this species are usually found in high numbers in relatively small areas (Tuni, unpublished). Hence, males may experience little variation in female availability, which could be consistently high over the reproductive season. In this case, males may have been selected to allocate a fixed amount of resources per partner, in a way allowing them to achieve consistently high mating rates, and not to plastically adjust their investment in response to socio-sexual environmental cues. However, as shown in a field study involving our study population (Ghislandi et al. 2018), due to protandry, individuals of this species experience seasonal fluctuations in population composition such as density, sex ratio and proportion of mated vs non-mated females during their lifetime. Therefore, despite little is known about how individual movements may affect the time scale on which immediate female availability varies, short-term changes are likely to be involved, and hence potentially relevant.

The inability of our study to detect any effect of mating opportunities on male mating investment (pre-mating and at mating) may be explained as follows. Firstly, resource partitioning may be more pronounced in males under constrained energetic conditions (Van Noordwijk and de Jong 1986). All males used in our experiment had been reared in the laboratory under ad libitum diet conditions for at least 1.5 months prior being tested. High feeding regimes may have therefore reduced variability in male condition, providing males with sufficient resources for bearing costs of multiple courtships and matings. Secondly, while we did not reveal an effect of variation in female availability on our target reproductive traits (gift construction, courtship effort, copulation duration and thanatosis), we cannot exclude that males facing changes in mating opportunities modify their investment in the gift-giving strategy. *Pisaura mirabilis* males are known to adopt three alternative mating strategies: males can mate by courting females with a genuine gift consisting of a freshly caught arthropod prey of high nutritional content, with a nutritionally worthless gift consisting of silk-

wrapped prey remains and exoskeletons, or with no gift (Albo et al. 2011b; Ghislandi et al. 2014; Ghislandi et al. 2018). These tactics reflect a reduction in male energetic investment, from more costly genuine gifts to a cheaper "no-gift" strategy. Importantly, the frequency of these alternative tactics is shaped by seasonal changes not only in ecological factors, such as prey availability, but also in mating opportunities with males responding to an increase in female availability by increasing their mating effort (employing a gift-giving tactic, with either genuine or worthless gifts, Ghislandi et al. 2018). Future studies focusing on the male gift-giving strategy might provide better insight on the effect of female availability on male resource allocation. Thirdly, males may be sensitive to variations in mating opportunities but may respond by enlarging the energetic budget devoted to reproduction rather than by partitioning it, as documented in other species (e.g. Gage 1995; Bozynski and Liley 2003). This could be achieved in several ways. For example, males may be able to draw resources from non-reproductive functions, such as somatic growth or immune defence (Stearns 1989). Indeed these types of life history trade-offs, resulting in reduced body size or longevity, have been observed in a variety of species. In guppies (*Poecilia reticulata*) the increase in male reproductive investment elicited by female presence appears to come at the expenses of lifetime growth (Jordan and Brooks 2010) and adult survival (Miller and Brooks 2005), with many other studies highlighting analogous patterns (see e.g. Scharf et al. 2013 for a recent review on arthropods). *Pisaura mirabilis* males, could for example increase their energetic budget by increasing food intake. Despite sexually active males forgo feeding to wrap their captured prey in silk (Albo et al. 2009), we know that after catching a prey, *P. mirabilis* males partially feed on it prior to silk wrapping (Ghislandi et al. 2017). Our study was not designed to assess the extent of male feeding behaviour, but foreseeing high mating opportunities, a condition requiring conspicuous resources to allocate to mating effort, may induce males to consume a larger portion of the prey.

In addition, little we know about how likely males are to obtain copulations with females around them, that is, how proximity translates into mating opportunity. Mating opportunities may have required to be realized rather than only perceived to elicit an adjustment in male reproductive investment. The present experimental design, however, was chosen because monitoring male sexual investment across subsequent matings would have not allowed us to determine whether a reduction in resource allocation per partner was caused by strategic partitioning of resources across matings (Warner et al. 1995) or by resource depletion (Preston et al. 2001). Furthermore, potential variability in previous matings would have introduced undesired variance in male resource budget available for subsequent matings, as well as in male experience in terms of female attractiveness, aggressiveness and reluctance to mate, with consequences on following sexual interactions (e.g. Molina and Christenson 2008).

Finally, we cannot exclude that the lack of effect of female availability on male reproductive investment was due to our experimental design. We can firmly state that males assessed female presence, as we ensured that males interacted physically with all females and with their silk draglines, which are known to provide

males with information on female presence as they elicit male sexual behaviours (Beyer et al. 2018). Males may nevertheless need longer exposure to females to modify their strategies in response to the social information perceived, as suggested for a similar study reporting lack of partner availability effects on male reproductive investment in the spider *Argiope bruennichi* (Cory and Schneider 2018). Moreover, little is known about average number of partners males have under natural conditions, which could exceed the four mating opportunities used in our study for the HMO treatment. If this is the case, more extreme female availability may have been required to elicit a differential response.

When males pay high costs to reproduce and females vary in terms of fecundity, male mate choice could be expected to evolve (Edward and Chapman 2011). In addition to traditional pre-copulatory mate choice (i.e. to mate or not with a given female), males can also express their preference by varying the amount of resources allocated to a female on the basis of her quality (Bonduriansky 2001). Accordingly, we predicted that males would tailor their mating effort based on female body mass, which positively correlates with fecundity in this species (Austad and Thornhill 1986). We found that wrapping duration was indeed affected by female size (while other components of the mating effort were not), but, contrary to expectations, *P. mirabilis* males invested more in gift construction when paired with smaller females. This effect may stem from male or female behaviours and can have different explanations. Firstly, males may preferentially invest in copulations with smaller females, because it may be beneficial in terms of sperm competition. Since spiders can increase their weight during the season, body mass may be used by males as an indicator of female age, which, in turn, may predict their mating status. Young females are more likely to be virgin or to have mated with few partners, leading to a paternity advantage resulting from first male precedence (that has been recorded in this species, Drengsgaard and Toft 1999) or from reduced sperm competition intensity (Parker et al. 1996). Moreover, investment in small partners may be more rewarding for males because limited size dimorphism between the sexes may reduce the risk of prematurely losing the gift in a conflict with the female. Alternatively, the pattern may originate from small females being more reluctant to mate, and in males adding more silk layers on the gift to overcome female resistance.

In conclusion, we show that males of the gift-giving spider *P. mirabilis* do not adjust their investment into mating effort in response to changes in the number of mating opportunities, but invest more into silk wrapping when paired with smaller females. Males may respond to female availability by reducing investment in components of the mating effort that we did not measure, such as gift-giving strategies. Alternatively, our results may be explained if males do not need to partition the reproductive budget because they are able to enlarge it by drawing resources from non-reproductive traits or by increasing their food intake. To verify these hypothesis, future studies should test the effect of variations in female availability on i) gift giving strategies (i.e. mating with or without a gift, offering genuine or worthless donations) and the proportion of prey consumed prior wrapping, ii) male somatic growth, and iii) male lifespan. In addition,

experiments explicitly designed to test mate choice would be needed to confirm the observed pattern of size dependent wrapping effort and to investigate its potential value in terms of fitness.

ACKNOWLEDGEMENTS

We thank members of the Behavioural Ecology group at the LMU for help in collecting spiders and Katharina Platzdasch for assisting in collecting data. MM was supported by a Ph.D. fellowship from the University of Padova to conduct the study.

AUTHOR CONTRIBUTION

MM conceived the study, collected and analysed the data and wrote the manuscript, CT contributed to the study's design, implementation and to the writing of the manuscript.

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Manuscript 1b

Strategic adjustment of ejaculate quality in response to variation of the socio-sexual environment

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ABSTRACT

Strategic ejaculate adjustments occur when males modify their investment in the sperm and non-sperm component of the ejaculate according to the context. This strategy is expected to evolve when ejaculate production is costly, the returns of the investment in the ejaculate depend on the environment and environmental conditions are variable. While adjustments of sperm numbers have been widely documented, relatively less is known about how males modify ejaculate quality, despite evidence highlighting the importance of this trait for sperm competition. In this review we discuss and synthetize existing literature on strategic adjustments of ejaculate quality. We describe which ejaculate quality traits are most typically plastic and which environmental factors elicit such responses, focusing in particular on the socio-sexual environment. In addition, we summarize the available information on the timeframe within which such adjustments can occur and on the proximate mechanisms responsible for plasticity. We show that this phenomenon is widespread across taxa; it involves responses to several environmental factors and modifications of many ejaculate traits, with seminal fluid composition playing a central role, both as a trait *per se* and as proximate mechanism for sperm performance adjustments. We point out the circumstances that favor adjustments of ejaculate quality rather than of sperm numbers, and evaluate the fitness consequences of these responses, highlighting the complexity of patterns of co-variation with other reproductive and non-reproductive traits. Finally, we consider implications for male and female behavior. We highlight two areas of research on plasticity in ejaculate quality that may be particularly worth exploring further: 1) the proximate mechanisms responsible for plasticity; 2) the adaptive value of strategic ejaculate adjustments in relation with pre-copulatory plasticity.

INTRODUCTION

In polyandrous species male competition is not limited to mate acquisition, but continues after copulation. Sperm competition occurs when the ejaculates from different males compete for their fertilization (Parker 1970). Sperm competition is taxonomically widespread and it has been demonstrated to be a powerful force shaping behavioral, morphological and physiological traits (Birkhead and Møller 1998), acting particularly strongly on ejaculate characteristics (Birkhead et al. 2008). This form of post-copulatory selection, in fact, is expected to promote ejaculate adaptations aimed at enhancing sperm competitiveness and thus paternity success in polyandrous mating systems (Parker 1998). In many species, the relative number of competing sperms from rival males is the primary factor influencing paternity success under sperm competition and there is accumulating empirical evidence showing that sperm number evolves in response to sexual selection determined by sperm competition (Parker and Pizzari 2010). However, last decades of research have made increasingly apparent that ejaculate traits other than sperm number play an important role in determining fertilization competitiveness (Snook 2005; Simmons and Fitzpatrick 2012). Indeed, competitive fertilization success also depends on a series of ejaculate quality traits, such as, for example, seminal fluid composition, sperm velocity, viability and morphology. Consistently, sperm quality traits are positively correlated with the level of sperm competition across taxa (Hunter and Birkhead 2002; Gomendio et al. 2006; Immler et al. 2011; Montoto et al. 2011; delBarco-Trillo et al. 2016).

While sperm production has long been assumed to be limitless and almost costless, it is now recognized that it requires substantial resources (Dewsbury 1982; Van Voorhies 1992; Simmons 2011) and, therefore, that ejaculate size and quality are often condition dependent (Kidd et al. 2001; Morrow et al. 2008; Devigili et al. 2013), a characteristic also indicating that these traits have the potential to be expressed flexibly. As a consequence, males have been selected for the optimal use of their post-copulatory resources. Optimal post-copulatory investment, is not fixed and often depends on several socio-sexual factors, such as level of sperm competition, number of mating opportunities (or mating rate) and female quality, which typically vary during an individual's life. Under these circumstances, males of many species have

GLOSSARY

Ejaculate: the product of the testicles and the accessory glands, composed of sperm cells and seminal fluid.

Ejaculate quality: the fertilizing competitiveness of an ejaculate in cases of heterospermic inseminations after controlling for sperm number. It comprises a diverse range of sperm phenotypic traits, such as motility parameters, viability, morphological characteristics and metabolic rates, and seminal fluid composition.

Strategic ejaculate adjustment: a modification of sperm and/or non-sperm ejaculate components aimed at increasing fertilization success. It includes changes in sperm number and quality and in seminal fluid composition.

Ejaculate production plasticity: any adjustment of ejaculate size or quality occurring before ejaculation.

Ejaculate allocation plasticity: strategic use by males of variable portions of their ejaculate reserves across matings.

While it is shared by several authors, we are aware that use of these definitions may be controversial. The aim of this glossary is to facilitate the understanding of the present paper and not to impose general use of these definitions.

evolved the ability to strategically adjust their ejaculate investment in response to variations in the social environment (Kelly and Jennions 2011). While these adjustments are included by some authors in the definition of true phenotypic plasticity (e.g. Kelly and Jennions 2011; Simmons and Lovegrove 2017), they may be rather considered as forms of phenotypic flexibility by others (Piersma and Drent 2003) due to the fact that they are usually reversible (although cases of irreversible, developmental modifications also exist, e.g. Harris and Moore 2005; Janicke et al. 2016).

In the context of strategic ejaculate adjustments, males can engage in two types of plastic responses, as they can modify either ejaculate allocation (i.e. males use strategically their ejaculate reserves) or ejaculate production (i.e. males adjust strategically ejaculate production rate or ejaculate quality). When post-copulatory investment is fixed and cannot be up or downregulated (e.g. sperm are produced at a constant rate), males make decisions about how to allocate a fraction of this limited ejaculate budget to each mating partner (hereafter *allocation plasticity*), depending on the characteristics of the copulation event (Warner et al. 1995). Several models have been developed over the years describing how optimal ejaculate allocation strategies are conditioned by socio-sexual factors (e.g. risk and intensity of sperm competition, Parker et al. 1996; Parker et al. 1997; Parker 1998; number of mating opportunities, Abe and Kamimura 2015; partner quality, Reinhold et al. 2002; and partner mating status, Engqvist and Reinhold 2006; Ball and Parker 2007). After the first experimental demonstrations of strategic ejaculate allocation under risk (Gage 1991; Gage and Baker 1991) and intensity of sperm competition (Pilastro et al. 2002b), a large body of evidence on this phenomenon has now accumulated.

In other cases, males are able to modify the energetic budget devoted to ejaculate production and adjust their investment in ejaculate production (hereafter *production plasticity*) in response to environmental cues linked to sperm competition (Bjork et al. 2007; Firman et al. 2013) and mating opportunities (Gage 1995; Koyama and Kamimura 2000; Bozynski and Liley 2003; Olsén et al. 2006). Modifying ejaculate production allows males to limit energetic investment into this function when not necessary, while simultaneously avoiding sperm depletion before mating opportunities have ceased. We include in the term production plasticity not only changes that occur during spermatogenesis, but any modification of sperm or seminal fluid that occurs before ejaculation.

While allocation plasticity usually only allows males to adjust the number of sperm they transfer at each copulation (although they may modify the volume of seminal fluid they allocate, which may affect sperm performance, den Boer et al. 2010), production plasticity also allows to modify sperm velocity, viability and morphology, and to alter the quantity and composition of seminal fluids. In some species only one of these two types of adjustment can take place. For example males which mate one single time (e.g. Knoflach and van Harten 2001; Andrade and Banta 2002) do not need to partition their resources, and therefore will not make decisions about how to allocate the ejaculate, but only about how to produce it. Conversely, in

prospermatogenic species (i.e. in which males do not produce sperm during their adult life, Boivin et al. 2005) mating with multiple females, males will have limited possibility to adapt ejaculate production to the context; instead, their decisions about how to allocate available reserves will be crucial to prevent early sperm depletion (Martel et al. 2008; Schneider and Michalik 2011). Most species, however, would benefit from both processes and there is evidence that males respond to variations in environmental conditions by adjusting both ejaculate allocation and ejaculate production (e.g. in house mice, Ramm and Stockley 2007; Firman et al. 2013; and in fruit flies, Lüpold et al. 2010; Moatt et al. 2014).

Despite the frequent co-occurrence of these two forms of plasticity, we think that the distinction between allocation and production plasticity is useful because they differ substantially with regard to the type of trade-offs they generate. When plasticity involves allocation (i.e. temporal partitioning) of existing ejaculate resources, the investment in a given mating will only affect the post-copulatory success of that and the subsequent matings. In the case of production plasticity, in contrast, the increased ejaculate quality may negatively affect pre-copulatory investment (ornamentation, mate searching, courtship, male-male competition) and hence pre-copulatory success, and possibly survival (immunity, longevity) (see section: *Costs and benefits of strategic ejaculate adjustments*). As a result, the fitness consequences of allocation adjustments are relatively easier to predict than those of production plasticity which may be affected by trade-offs between mating and fertilization success.

OBJECTIVES

The aim of this review was to report on the state of the art in research on production plasticity, and in particular on socially cued strategic adjustments of ejaculate quality, focusing on these aspects: i) empirical evidence of phenotypic plasticity in ejaculate quality traits (sperm morphology and performance, and seminal liquid composition), ii) fitness consequences of adjustments of ejaculate production and quality, and iii) future directions in the study of ejaculate production plasticity. Simmons and Fitzpatrick (2012) and Fitzpatrick and Lüpold (2014) recently reviewed the effect of male-male interactions on adjustments of sperm quality; here we extended their work to include responses to all elements of the social environmental that have been shown to be relevant. We described which ejaculate traits can be modified, the possible timing of the adjustments and what is known about the proximate mechanisms that are involved.

We focused here on socially-cued plasticity in ejaculate quality, i.e. sperm (size and performance) and seminal fluid quality traits for several reasons: firstly, while production plasticity can include both variation in ejaculate quality and quantity (e.g. sperm production rate), plasticity in sperm and seminal fluid quality by definition will involve production plasticity only. Secondly, despite the general consensus about the role of ejaculate quality traits in determining competitive fertilization, less empirical work is available focusing on strategic adjustment of ejaculate quality, although in more recent years this gap has started to be filled. Thirdly, theoretical models describing the expected relationships between relevant environmental factors

and sperm quality traits are less than a handful (but see Parker 1993; Cameron et al. 2007; Alonzo and Pizzari 2010; Parker et al. 2010; Engqvist 2012), and we hope that this review will promote more research on this specific aspect of post-copulatory plasticity.

Through an analysis of available studies, we tried to describe which circumstances promote the evolution of socially-cued strategic adjustments of ejaculate quality rather than of sperm numbers. Then we explored the adaptive consequences of socially-cued ejaculate adjustments, analyzing benefits and costs of this form of plasticity, with particular emphasis on trade-offs, and discussing their effect on male and female strategies. Finally, we highlighted areas that are still unexplored and provide suggestions for future research.

EVIDENCES OF PHENOTYPIC PLASTICITY IN EJACULATE QUALITY TRAITS

Quality traits subjected to adjustments

Ejaculate quality is defined as the fertilizing competitiveness of an ejaculate in cases of heterospermic inseminations after controlling for sperm number (Dziuk 1996; Birkhead and Møller 1998). It comprises a diverse range of sperm phenotypic traits, such as motility parameters, viability, morphological characteristics and metabolic rates, the importance of which for male fertilization success varies greatly across species (Snook 2005; Simmons and Fitzpatrick 2012). Intuitively, those traits that are the primary responsible for insemination success in a species and that are too costly to be constitutively expressed at high quality are also those that are more likely to be strategically adjusted in response to environmental changes.

Among traits of sperm motility, the most widely explored, in terms of responses to the social environment, are mean sperm velocity and percentage of motile sperms. Since faster and more motile ejaculates are usually more competitive (Snook 2005; Simmons and Fitzpatrick 2012; although evidence for a competitive advantage of slower sperm has been also reported, Lupold et al. 2012), males are expected to increase sperm velocity when facing higher levels of sperm competition or when mating with high quality females. Accordingly, in a number of species researchers have detected adjustments in sperm velocity (Vaz Serrano et al. 2006; Cornwallis and Birkhead 2007; Gasparini et al. 2009; Smith and Ryan 2011; Burger et al. 2015b) or in sperm motility (i.e. the proportion of moving sperms, Kilgallon and Simmons 2005; Crean and Marshall 2008; Fitzpatrick et al. 2008; Kustan et al. 2012; Jeannerat et al. 2017) in response to changes of the socio-sexual context.

Another important indicator of fertility is sperm viability, the percentage of live sperm in the ejaculate (Snook 2005). In some species sperm viability represents the main predictor of sperm competitiveness (e.g. in the cricket, *Teleogryllus oceanicus*, García-González and Simmons 2005) and, in insects, it has been found to co-vary with the intensity of sperm competition (Hunter and Birkhead 2002). Viability is indeed the target of strategic adjustments in response to social cues in a few species of insects (Montrose et al. 2008; Moatt et al. 2014) and in horses (Simmons et al. 2007; Jeannerat et al. 2018).

Ejaculate competitiveness is also affected by sperm morphometry, and while models predict that sperm length should generally be unrelated to sperm competition risk (Parker 1993; but see, Parker et al. 2010), adjustments of this trait to the social context have been recorded. The effect of sperm design on sperm competitiveness is mediated by its effect on other sperm traits, such as velocity, longevity and ability to displace competing sperms (Snook 2005; Immler et al. 2011; Simmons and Fitzpatrick 2012; Fitzpatrick and Lüpold 2014). Often it is the relative length of sperm constituent parts, rather than its total length, that is more relevant for sperm competition. For example, longer flagella may generate greater propulsion forces, but a larger midpiece may accommodate more mitochondria allowing the production of more energy to fuel motility and grant greater longevity (Snook 2005; Humphries et al. 2008; Pizzari and Parker 2009). However, while the direction of the effect of sperm velocity or viability on fertilizing success (when relevant) is largely consistent across species and taxa, the relation between sperm morphology and ejaculate competitiveness cannot be generalized (Immler and Birkhead 2007; Simmons and Fitzpatrick 2012) and the causes of intraspecific variability in these traits are for the most part poorly understood (Ward 1998; Hosken 2003), probably because sperm-female interactions play a major role in determining male fertilizing success (Humphries et al. 2008; Manier et al. 2010; Lüpold et al. 2013). Interspecific evidence seems more concordant to suggest a positive correlation between sperm length and sperm competition (e.g. Lüpold and Fitzpatrick 2015). Accordingly, in a species of ascidian, individuals exposed to high population density (linked to higher levels of sperm competition) produced sperm with larger heads (these sperm were also more motile, remained viable for longer, induced less polyspermy and, thus, had higher overall fertilizing success, Crean and Marshall 2008). Strategic sperm adjustments have been reported also for other morphometry traits, specifically for total sperm length (Janicke et al. 2016) and the relative size of the midpiece and of the flagellum (Immler et al. 2010).

In species characterized by heterospermy (the condition in which more than one discrete morphological or functional type of sperm is present within the same ejaculate, Pitnick et al. 2008), quality adjustments may also involve variations in the relative representation of the different sperm types. Usually, only one of the sperm types (referred to as 'eusperm' or 'eupyrene' sperm) is functional to egg fertilization and the role of the non-fertilizing sperm type (referred to as 'parasperm', or 'apyrene' and 'oligopyrene' sperm) has been reported to be related to sperm competition in several species (Hayakawa 2007). Adjustments of the proportion of eusperm and parasperm in the ejaculate have been described in response to different levels of sperm competition in various species, but their direction was not uniform. Higher levels of sperm competition, in fact, have been found to promote an increase (Cook and Gage 1995) or a decrease (He and Miyata 1997; Oppliger et al. 1998) in the proportion of eusperm. Under stronger sperm competition an increase in the proportion of eusperm is expected following predictions for sperm number adjustments, however predictions on the direction of adjustments in the number of parasperm are complicated by the fact that the function of these cells in sperm competition is less clear. It has been shown that parasperm enhance

eusperm survival in the female reproductive tract (Holman and Snook, 2008) and it has been proposed that they could play a role in in displacing or inactivating eusperm from previous matings or in delaying female re-mating (Silberglied et al. 1984). If parasperm were the major determinants of ejaculate competitiveness an increase in their representation in the ejaculate under strong sperm competition would be beneficial to males.

Among other sperm characteristics known to be relevant to fertilizing efficiency and competitiveness, sperm longevity, ATP content and proneness to oxidative stress have been shown to differ between males adopting different alternative reproductive strategies, possibly as a response to differences in the typical levels of sperm competition (Vladić and Jarvi 2001; Neff et al. 2003; Burness et al. 2004; Schulte-Hostedde and Burness 2005; Locatello et al. 2007; Jeannerat et al. 2017; Vladić et al. 2010 and see box: *Alternative reproductive tactics*).

Finally, non-sperm components of the ejaculate are known to affect sperm fertilization success. The seminal fluid contains a cocktail of compounds, covering a number of different functions (Poiani 2006). Possible functions can be divided into three categories: i) contributing to the process of fertilization by improving sperm performance; ii) impairing rival sperm competitiveness; iii) manipulating female behavior and physiology, by reducing female sexual receptivity, stimulating egg production and oviposition and providing nutrients. Since it is costly to produce (Simmons 2001; Friesen et al. 2015), seminal fluid represents a limited resource, which may become depleted when ample sperm reserves are still available for ejaculation (Linklater et al. 2007; Reinhardt et al. 2011). Therefore, socially-cued phenotypic plasticity in seminal fluid production should be also expected to evolve (Cameron et al. 2007; Alonzo and Pizzari 2010; Simmons and Fitzpatrick 2012). Attempts to predict the direction of socially-cued seminal fluid adjustments are complicated by two aspects. On the one hand, predictions must be component-specific, critically depending on the function of the component under consideration (Cameron et al. 2007). On the other, optimal strategies of seminal fluid investment may also be affected by investment in the sperm component of the ejaculate and by potential trade-offs among ejaculate components (Alonzo and Pizzari 2010; Perry et al. 2013). Nonetheless, some general trends can be inferred. When the risk of sperm competition is high and especially when mating with non-virgin females, males should reduce their investment in substances enhancing sperm performance or female fecundity and instead exploit the effects produced by seminal fluid of the female's previous mates (Hodgson and Hosken 2006; Alonzo and Pizzari 2010). Conversely, under the same circumstances, males are expected to increase their investment in compounds reducing female receptivity (but only if they mate first) or impairing rival sperm performance. Indeed, male flies, *D. melanogaster*, are able to tailor their seminal fluid composition to the expected level of sperm competition and to female quality (Wigby et al. 2009; Fedorka et al. 2011; Sirot et al. 2011; Wigby et al. 2016), following patterns that are consistent with predictions (Cameron et al. 2007; Alonzo and Pizzari 2010). Males of a number of species also respond to the socio-sexual context by adjusting the amount and quality of the

accessory substances in their spermatophores (Simmons 1993; Cook and Wedell 1996; Wedell and Cook 1999a; Wedell and Cook 1999b; Harris and Moore 2005; Zizzari et al. 2013). Evidence for adjustments of seminal fluid quantity and quality is now starting to accumulate (see also, Ramm et al. 2015; and indirect evidence from Sloan et al. 2018; Lemaitre et al. 2011; Bretman et al. 2015, who documented changes in seminal vescicles size as a developmental response to early exposure to rivals) and it is becoming apparent that seminal fluid adjustments are often responsible for mediating adjustments of sperm performance (see section: *Mechanisms responsible for strategic ejaculate adjustments*).

Relevant environmental stimuli

While abiotic and heterospecific factors might be involved in affecting ejaculate quality, often causing non-adaptive responses (Sherman et al. 2008; Devigili et al. 2013), we are focusing here exclusively on socio-sexual factors, which appear to be the most relevant variables inducing strategic plastic responses (Wedell et al. 2002; Kasumovic 2013). The socio-sexual factors inducing ejaculate adjustments are those that, i) affect the returns of male investment in the ejaculate, ii) are variable in time or space, and iii) are associated with reliable cues (Kasumovic 2013). The main relevant stimuli for plasticity in ejaculate traits are represented by variations in the strength of expected sperm competition, in number of perspective mating opportunities, in sex ratio (which may be a proxy for both changes in the level of expected sperm competition and mating opportunities) and in partner quality. However, the role of individual social stimuli critically depends on the species under consideration and on its mating system.

Predictions inferred from models on sperm numbers (Parker and Pizzari 2010) suggest that sperm competition should induce two types of adjustments: when immediate response are possible, ejaculate quality is expected to increase with sperm competition risk (the probability that a female will mate with more than one male) and to decrease with sperm competition intensity (the number of other males competing for fertilization). Indeed, male crickets, *Teleogryllus oceanicus,* have been found to increase their sperm viability when expecting to compete for fertilization with one male and to decrease it when sperm competition would involve two rivals (Simmons et al. 2007). Analogous adjustments of ejaculate quality to sperm competition risk have been observed in several other species (Cook and Gage 1995; Cook and Wedell 1996; Oppliger et al. 1998; Kilgallon and Simmons 2005; Moatt et al. 2014; Burger et al. 2015b). Manipulations of population density or sex-ratio have been used sometimes to investigate the effect of perceived sperm competition, showing that males produce higher quality ejaculates in larger or male-biased populations (He and Miyata 1997; Oppliger et al. 1998; Harris and Moore 2005; Janicke and Schärer 2010; Janicke et al. 2016). This approach, however, is associated with one major problem: as sex-ratio is composed of two variables, male density and female density, its variation affects both intra-sexual competition and mate availability (Alonso-Pimentel and Papaj 1996), which is also known to potentially affect male investment in the ejaculate (Reuter et al. 2008). Males, in fact, benefit from investing in the production of large and high quality ejaculates only

if chances to mate in the close future are high. Indeed, an increase in sperm quality as perspective mating opportunities raise has been documented for example in male guppies, who produce faster sperm when exposed to female cues (Gasparini et al. 2009). Since partners' quality may also have large effects on male reproductive success, males benefit from modulating their investment on female traits, and delivering higher quality ejaculates to more valuable females (in terms of fecundity, genetic quality or compatibility) in order to ensure fertilization of their eggs (cryptic male choice, sensu Engqvist and Sauer 2001). For example, in the fowl, *Gallus gallus*, males transfer more and faster sperm to more ornamented hens (Cornwallis and Birkhead 2007) and in the butterfly *Pieris rapae* males respond to female size (an indicator of fecundity, Wedell and Cook 1999a). However, since copulations with high-quality females are often associated with higher risk (and intensity) of sperm competition, understanding to which parameter males are responding may not be obvious (Simmons and Kvarnemo 1997; Parker and Pizzari 2010). Other female characteristics not directly associated with fecundity, such as reproductive status (females in fertile vs non-fertile phases, Dowling and Simmons 2012; Jeannerat et al. 2017), MHC profile (Jeannerat et al. 2018), and relatedness (Fitzpatrick et al. 2014) have been also shown to elicit ejaculate quality adjustments. Furthermore, in a Coolidge-effect scenario, female familiarity might be relevant too, resulting in males preferentially investing in the quality of the ejaculate transferred to novel females compared to females they have already mated with (as shown for sperm number, Pizzari et al. 2003). To date, however, empirical support for this hypothesis is lacking. Female mating status is also known to affect ejaculate quality (Simmons 1993; Wedell and Cook 1999a; Thomas and Simmons 2007); however, despite it being sometimes included among female quality traits, we believe that it should be more appropriately analyzed considering its consequences for sperm competition (Engqvist and Reinhold 2006; Kelly and Jennions 2011). Similar reasoning may be applied when considering male characteristics which also affect optimal ejaculate investment strategies (Parker and Pizzari 2010). Depending on own relative attractiveness or social status males are likely to face different levels of sperm competition, to have different mating opportunities and to incur different sperm production costs. Hence, when these characteristics are conditional and not genetically determined, they may be accompanied by conditional expression of ejaculate quality traits. For example, after definition of a dominance hierarchy, male salmons that become subordinate raise their ejaculate quality as a strategy to compensate for lower pre-copulatory success (Bartlett et al. 2017), but the opposite response is also observed, with individuals increasing their sperm quality after attaining a dominant status to match their increased mating opportunities (Fitzpatrick et al. 2008; Kustan et al. 2012). Analogous differences in ejaculate quality associated with male hierarchical status or male attractiveness have been observed in many other species (Koyama and Kamimura 2003; Thomas and Simmons 2009; Klaus et al. 2011; Worthington et al. 2013).

In addition, ejaculate quality adjustments are sometimes elicited by social experience *per se*, which is probably perceived as a cue of increased sperm competition (Montrose et al. 2008), or by rivals' competitiveness (Wedell and Cook 1999a; Immler et al. 2010; Smith and Ryan 2011). Finally, predictions

ALTERNATIVE REPRODUCTIVE TACTICS

In many species sexual selection has led to the evolution of alternative mating tactics (ARTs) within one sex, which usually involve a 'guarder' and a 'sneaker' tactic, and may involve tactic-specific morphological, physiological and behavioral adaptations (Parker 1990; Gross 1996). While ARTs can be genetically determined (i.e. polimorphisms, excluded from this review), they can also arise from differential developmental pathways and thus from phenotypic plasticity (i.e. poliphenisms, or conditional strategies, which will be considered here). Males adopting distinct reproductive strategies experience different socio-sexual conditions, in terms of sperm competition and mating opportunities, and, they adjust their pre- and post-copulatory copulatory investment accordingly (Parker 1990; Pizzari and Parker 2009). In particular, since sneakers always experience higher levels of sperm competition than guarders, they are expected to produce larger ejaculates (Parker 1990) containing higher quality sperm (Taborsky 1998). They are also expected to produce smaller amounts of seminal fluid (Cameron et al. 2007) and, specifically, to invest less in seminal fluid compounds that enhance female receptivity (Cameron et al. 2007) or sperm performance (as they can exploit the effect of guarders' seminal fluid, Locatello et al. 2013), and more in compounds that impair rival sperm performance (Locatello et al. 2013). Many studies have investigated how ejaculate quality varies between reproductive strategies, often confirming the predictions (Pizzari and Parker 2009). Sneakers' sperm have been found to be of higher quality in a number of species, being characterized by higher or different motility, viability, longevity and fertilizing competitiveness (Daye and Glebe 1984; Linhart 1984; de Fraipont et al. 1993; Gage et al. 1995; Simmons et al. 1999; Leach and Montgomerie 2000; Hoysak and Liley 2001; Uglem et al. 2001; Vladić and Jarvi 2001; Neff et al. 2003; Burness et al. 2004; Schulte-Hostedde and Burness 2005; Fitzpatrick et al. 2007; Locatello et al. 2007; Vladić et al. 2010; Iwata et al. 2011; Hirohashi and Iwata 2013; Hirohashi et al. 2013; Flannery et al. 2013; Hirohashi et al. 2016; Apostólico and Marian 2017a, b). Sneakers and guarders also produce seminal fluids that differ in terms of composition (Gombar et al. 2017) and for their effect on the performance of rival sperm (Locatello et al. 2013; Lewis and Pitcher 2016).

based on kin selection theory suggest that males may adjust their copulatory investment in response to the relatedness of their rival, transferring higher quality ejaculates to females that have first mated with a non-sibling male than to females mated to a related male. However, empirical studies testing this hypothesis failed to detect such an effect (Thomas and Simmons 2008).

Timing of adjustments

Adjustments of sperm quality also differ with regard to their timing relative to the onset of triggering environmental changes. Anticipatory plasticity occurs when individuals rely on current cues to predict future challenges and prepare to them, while reactive plasticity represents a response to current challenges

(Kasumovic 2013). The amplitude of the window, during which individuals are sensitive to environmental stimuli and are able to produce appropriate responses, may differ too (Fusco and Minelli 2010). Which type of phenotypic plasticity evolves in a population depends on the timing of environmental change relative to the latency between cue detection and the resulting response (Whitman 2009), which may vary with the mechanisms involved in ejaculate modifications. Obviously, one critical aspect is the duration of spermatogenesis and the extent to which sperm phenotype can be modified once spermatogenesis or spermiogenesis have already begun or even after they are completed. In general, while adjustments of sperm number may be immediately implemented (when they result from allocation plasticity), we may expect plasticity in sperm quality to be associated with longer latency because changes in sperm morphology or sperm energetics may only occur during sperm production and not afterwards (Burger et al. 2015a). This prediction is met in some species; for example, in horses, Burger and colleagues (2015a,b) did not record adjustments of sperm velocity after short exposure to relevant stimuli that was sufficient to induce adjustments in sperm numbers (28 days over a spermiogenesis period of 57 days), but detected them after longer treatments (56 days). Interestingly, later studies on stallions have shown that other ejaculate traits (i.e. sperm viability and motility) can be adjusted over shorter time intervals (Jeannerat et al. 2017; Jeannerat et al. 2018), showing how individual ejaculate traits within one species may require different time intervals to be modified, and suggesting that they may be adjusted in response to stimuli fluctuating with different frequency.

Indeed, socially-cued strategic adjustments of ejaculate quality across species have been documented to take place on the most diverse timescales, ranging from developmental plasticity (i.e. prior to sexual maturation) to immediate adjustments in allocation. In developmental, plasticity, the sensitivity window is limited to ontogeny and the phenotype induced is generally fixed (i.e. non-reversible) (West-Eberhard 2003). For example, population density during development, a proxy for the level of sperm competition during adult life, has been shown to influence ejaculate traits in several species (He and Miyata 1997; McNamara et al. 2010; Harris and Moore 2005; Janicke et al. 2016). In this case, ejaculate quality is shaped by the conditions encountered during the first phases of life, and changes in the environment occurring outside these periods of responsiveness may have little or no effect. In predictable environments, cues obtained during development can provide valuable information on the population-average socio-sexual conditions (Harris and Moore 2005), and may allow the most dramatic modifications, for example in terms of structure of the testicles (Gage 1995; Schärer and Vizoso 2007) and seminal vesicles (Lemaitre et al. 2011; although see, Brauer et al. 2007 for evidence of phenotypic plasticity in testes size at adulthood). On the other hand, relying on early cues to predict future context may also be risky: as developmental plasticity is irreversible, if the environment changes, the effects of past conditions may produce mismatches with current ones, with adverse consequences on reproductive success (Bateson et al. 2004; Kasumovic et al. 2011). When relevant environmental factors change frequently, it may be beneficial to maintain wider sensitivity windows and to

be able to respond faster. Evidence has shown not only that males can reversibly alter spermiogenesis in response to stimuli received during adult life (Burger et al. 2015b), but also that short treatments (i.e. over few days, when spermiogenesis is significantly longer) can be sufficient to produce differences in sperm characteristics. This suggests that mature sperm cells may maintain their plasticity before ejaculation (Reinhardt et al. 2015). These changes in sperm performance, also known as "sperm priming" (Bozynski and Liley 2003; Evans 2009), have been described in many species (Rudolfsen et al. 2006; Gasparini et al. 2009; Smith and Ryan 2011; Kustan et al. 2012; Bartlett et al. 2017). Sperm priming can be so fast that it allows instantaneous adjustments of sperm performance, for example in response to the immediate level of sperm competition or to female value (Kilgallon and Simmons 2005; Cornwallis and Birkhead 2007; Pizzari et al. 2007; Simmons et al. 2007; Thomas and Simmons 2007; Jeannerat et al. 2017; Jeannerat et al. 2018). Finally, sperm behavior can be plastically adjusted after ejaculation, as shown in leaf-cutter ants, in which sperm motility, velocity and linearity increase in the presence of rival male seminal fluid (Liberti et al. 2018) allowing the optimization of energetic investment in sperm mobility depending on the perceived level of sperm competition.

The timing of the adjustment is likely to result both from selective pressures dependent on the time frame on which relevant environmental conditions change and from constraints represented by latency in the responses, which is associated with the mechanism responsible for the adjustment (see below).

Mechanisms responsible for strategic ejaculate adjustments

Very little is known on how environmental stimuli are translated into signals for the testicles and accessory reproductive glands. In vertebrates, this process is mediated by the hypothalamus–pituitary–gonad axis (Francis et al. 1993), while the process has not been investigated in invertebrates. Proximate mechanisms of plasticity in ejaculate quality traits are still mostly unknown.

Changes in sperm morphology may represent the proximate cause of changes in sperm performance (i.e. bigger sperm may be faster and longer lived, Snook 2005; Fitzpatrick and Lüpold 2014). However, although it has been hypothesized that changes in the duration of the spermatogenic cycle or in the size of seminiferous tubules may be responsible for observed adjustments in sperm morphology (Lüpold et al. 2012), to our knowledge, how these factors may affect ejaculate plasticity has never been directly investigated. Changes in sperm performance can also result from modifications in sperm physiology (for example linked to their content in mythocondria, energy reserves and key metabolic enzymes, Amaral et al. 2013). These modifications are known to be responsible for differences in sperm performance within species between males adopting alternative mating strategies (Vladić and Jarvi 2001; Vladić et al. 2002; Burness et al. 2004; Burness et al. 2005; Hirohashi et al. 2016). Alterations of sperm morphology and sperm enzymatic and biochemical content are likely to occur during spermatogenesis and are expected to require at least several days. Alterations of spermatogenesis kinetics, for example, have been shown to be responsible for socially-

induced differences in sperm numbers in the hermaphroditic flatworm *M. lignano (Giannakara et al. 2016) and in mice (Ramm and Stockley 2009; Ramm et al. 2015; Firman et al. 2018).* However, in many cases sperm quality adjustments are achieved in timescales that are shorter than those required for spermatogenesis and even spermiogenesis (see section: *Timing of adjustments*), suggesting that they involve changes in the sperm cells occurring during last stages of maturation or after maturation. These rapid changes in sperm performance are likely to be mediated by seminal fluid composition or extra-gonadal milieu, rather than by sperm modifications *per se*. Changes in blood hormonal levels are often detected in association with changes in the social context (Immler et al. 2010; Burger et al. 2015b; Jeannerat et al. 2018). Since the secretory activity of accessory glands, which are responsible for the production of the seminal fluid, is under endocrine control (Poiani 2006 and references in it), changes in testosterone and corticosterone levels have the potential to affect the composition of the seminal fluid (McDowell et al. 1996), to which sperm are sensitive. In fact, the chemical environment of male reproductive tract and of seminal fluid, including factors as pH, content in proteins, monosaccharides, triglycerides, enzymes, hormones, ions (especially Ca^{2+}) and buffering acids and bases, has been shown to influence sperm performance (Poiani 2006). Males could rapidly control the volume of seminal fluid they allocate through mechanisms similar to those that consent adjustments in sperm numbers (den Boer et al. 2010), for example through differential contractions of the testicles or of the ejaculatory ducts (Rasotto and Shapiro 1998; Pound 1999). Furthermore, the composition of seminal fluids could be modified very quickly as its components turnover is notably more rapid than spermatogenesis (Cornwallis and O'Connor 2009; Claydon et al. 2012). As mentioned (see section: *Quality traits subjected to adjustments*), adjustments of seminal fluid composition have been shown to occur in response to social stimuli in several taxa (e.g. Wigby et al. 2009; Ramm et al. 2015; Simmons and Lovegrove 2017). Experiments employing reciprocal combinations of sperm and seminal fluid from males exposed to different contexts demonstrated that alterations of seminal fluid composition were responsible (even if not exclusively) for adjustments in sperm viability and velocity (Cornwallis and O'Connor 2009; Simmons and Beveridge 2011; Bartlett et al. 2017). In the fowl, for example, it has been proposed that alterations in the ionic characteristics of sperm maturation environment could affect mitochondrial ability to exchange Ca^{2+} and K^{+} which, in turn, could affect sperm performance (Cornwallis and Birkhead 2007; Cornwallis and O'Connor 2009); for humans, a substantial literature on the candidate compounds responsible for adjustments of sperm motility is available (for an overview see: Poiani 2006). Rudolfsen and colleagues (2006) suggested that testosterone level variations, triggered by social status changes, may produce an increase in seminal fluid pH, which in turn increases sperm content in cAMP. Moreover, the androgens immunosuppressive properties may protect sperm from immunological targeting in male reproductive tract and thus aid the production of high quality ejaculates.

The mechanisms by which males adjust the molecular composition of their seminal fluid within their own reproductive system are mostly unknown. However, evidence showing that in *D. melanogaster* at least some

components of the ejaculate appear to be transferred to the female sequentially (Lung and Wolfner 2001) suggests that a sort of allocation plasticity may be responsible for adjustments of seminal fluid composition occurring at ejaculation. Similarly, it has been suggested that sperm of different age, and possibly of different quality, may be located in distinct sites of the reproductive organs and that strategic duct contractions may allow to control which sperm are ejaculated in each context (Thomas and Simmons 2007). Furthermore, it has been proposed that the relative speed of spermatozoa may be enhanced by modulation of the mechanisms of ejaculation (Poiani 2006). Finally, Cook and Gage (1995) hypothesized that, in heterospermic species, males could be able to sort eusperm and parasperm, and thus control their relative representation in the ejaculate, based on the fact that eusperm are packaged in bundles while parasperm are free. Therefore, on all these cases, what appears to be a production plasticity may actually be an allocation plasticity.

FITNESS CONSEQUENCES OF ADJUSTMENTS OF SPERM PRODUCTION AND ALLOCATION

When are ejaculate quality adjustments expected to evolve?

The ecological, behavioral and life-history factors that favor the evolution of strategic adjustments in sperm production and allocation have been already discussed in previous works (e.g. Wedell et al. 2002). These considerations mostly apply also to the evolution of ejaculate quality adjustments, but which factors could induce males to adjust ejaculate quality together with or instead of sperm numbers? The first aspect to take into account is how relevant ejaculate quality is to male fertilization success. While the relative numerical contribution of sperm is often the most relevant factor determining the outcome of sperm competition (Parker and Pizzari 2010; Boschetto et al. 2011), in many other cases ejaculate quality is the main ejaculate trait affecting paternity shares (Snook 2005). In this latter case, individuals are expected to evolve strategic adjustments in ejaculate quality. Accordingly, in the cricket *Teleogryllus oceanicus*, in which fertilization success is determined by ejaculate quality, the level of sperm competition has been shown to elicit adjustments of sperm viability, while sperm numbers were unaffected (Simmons et al. 2007). Secondly, the production of consistently high quality ejaculates may be associated with higher costs than the production of large ejaculates (Perry and Rowe 2010), or may be constrained by seminal fluid supplies, when these mediate sperm performance (Linklater et al. 2007; Reinhardt et al. 2011). Finally, in many species the volume of ejaculate transferred at mating is controlled by females (Eberhard 1996), by determining copulation duration (Pilastro et al. 2007; Herberstein et al. 2011) or sperm discharge (Pizzari and Birkhead 2000; Peretti and Eberhard 2010). When females control the number of sperm transferred during copulation, adjustments of ejaculate quality may be an effective way to overcome female manipulation. Accordingly, in the guppy *Poecilia reticulata*, in which sperm number is relatively more important than sperm velocity in determining competitive fertilization success (Boschetto et al. 2011), and in which sperm transfer during copulation is largely under female control (Pilastro et al. 2002a; Pilastro et al. 2004; Pilastro et al. 2007), males adjust both

sperm number (Bozynski and Liley 2003) and velocity (Gasparini et al. 2009) in response to perceived mating opportunities.

But how common strategic modifications of ejaculate quality are, compared to adjustments of sperm numbers? Due to the fact that the latter have been way more often investigated, a direct comparison is not possible. Some studies have indeed investigated strategic responses of ejaculate quality and reported their absence; a likely positive publication bias, and the fact that negative results can be due to pitfalls in the experimental design (Engqvist and Reinhold 2005) or to insufficient statistical power (see the contrasting results of Janicke and Schärer 2010; Janicke et al. 2016), make it difficult to draw conclusions. Studies that investigated simultaneously strategic adjustments of both ejaculate size and quality, however, often found an effect on ejaculate size only (see for example: Koyama and Kamimura 2000; Lewis and Wedell 2009; Bonilla et al. 2011; Burger et al. 2015a; Firman et al. 2018), while the opposite has been less often reported (but see e.g. Thomas and Simmons 2007). If the above reported results reflect a real pattern, one may wonder why ejaculate quantity seems to be strategically adjusted more frequently than quality. A difference in canalization (and hence in potential for adaptive plasticity) between quantity and quality traits may be one reason: although condition dependence in sperm performance has been reported in several studies (Oppliger et al. 1998; Burness et al. 2004; Pizzari et al. 2007; Vermeulen et al. 2009; Arundell et al. 2014; Tuni et al. 2016), suggesting plasticity, sperm morphology is probably more tightly genetically controlled (e.g. Simmons and Kotiaho 2007; Simmons and Moore 2009; Gasparini et al. 2013). Alternatively, adjustments of sperm characteristics or seminal fluid composition may require more time to be attained (Burger et al. 2015a; Burger et al. 2015b), making more stringent the conditions under which social cues can be reliable and hence anticipatory plasticity is expected to evolve. Furthermore, it is possible that fitness functions of ejaculate quantity and quality have different shapes, dictating which of the two components may be more convenient for the male to allocate. For example, if we consider two common ejaculate quantity and quality traits such as sperm number and velocity, fertilization probability is likely to be linearly correlated with sperm number (Boschetto et al. 2011), whereas fertilization success may drop abruptly to 0 when sperm velocity is below a certain threshold (e.g. most sperm may die before reaching the fertilization site is they swim too slowly) and sperm velocity may rapidly reach its physical upper limits irrespective of male investment (Dresdner and Katz 1981).

Costs and benefits of ejaculate adjustments

The adaptive value of socially-cued ejaculate adjustments (both in terms of sperm number and of ejaculate quality) is usually assumed. This assumption is reasonable, because plastic adjustments often match the intermale or interspecific covariation between the same ejaculate traits and fitness. However, predictions about the adaptive value of socially-cued ejaculate adjustments may also represent a by-product of hormonal changes caused by social stimuli (Immler et al. 2010; Burger et al. 2015b; Jeannerat et al. 2018), and an

explicit test of their fitness consequences may therefore be necessary. Indeed, in recent years, studies investigating the effects of these responses on male reproductive success have started to accumulate. Plasticity is considered to be adaptive when plastic individuals have higher fitness than fixed ones. For this condition to be met each alternative phenotype must be favored in the corresponding environment, with no single phenotype exhibiting superior fitness across all environments, and the costs of expressing plasticity must not exceed the benefits (Ghalambor et al. 2007). Determining the adaptive value of strategic ejaculate adjustments, therefore, requires the quantification of the intrinsic cost of plasticity, that is the cost paid for having the ability to be plastic and for expressing plasticity (costs that are linked to the development, maintenance, and function of the sensory and regulatory machinery needed for plasticity, Dewitt et al. 1998; Auld et al. 2010). Furthermore, in order to test that each alternative phenotype is favored in the corresponding environment, it is necessary to investigate male reproductive success associated with ejaculate adjustments under a range of environmental conditions, not limited to the ones that elicited the response.

Up to the present, little empirical effort has been done to measure intrinsic costs of strategic ejaculate adjustments. Some indirect evidence has been provided by Firman et al. (2013), who compared two mice population differing for the level of sperm competition. They showed that phenotypic plasticity in sperm production was greater in the high sperm competition population, suggesting that this trait only evolves, or is maintained, when it is highly beneficial, possibly due to its intrinsic costs (Fusco and Minelli 2010). Future studies could employ experimental evolution and then compare lines differing for the level of environmental variability, to determine whether plasticity is retained when individuals experience consistent environmental conditions. However, in their attempt to measure the long-term effect of a repeated response to variations in female availability (which in guppies elicit changes in sperm production, Bozynski and Liley 2003), Magris et al. (2018) failed to detect a cost in terms of condition, survival and expression of sexually selected traits, suggesting that intrinsic costs of plasticity may be negligible in this species.

For what concerns the quantification of the consequences of ejaculate adjustments in terms of male reproductive success, few more studies are available. These have tested whether an increase in ejaculate production or allocation, triggered by cues of sperm competition, resulted in higher paternity shares when mating with polyandrous females (Sakaluk and Müller 2008; Bretman et al. 2009; Barbosa 2012; Bartlett et al. 2017). Interestingly, three of these studies demonstrated a fertilization advantage of the upregulated phenotype, while one did not (Sakaluk and Müller 2008). While this of course is a strong indication that the increase of ejaculate production or allocation elicited by high sperm competition or female availability is beneficial under these conditions, it may overestimate the benefits of socially-cued ejaculate plasticity. The evolution of ejaculate plasticity is expected when high investment in this trait is beneficial in one context but detrimental in the others, otherwise constitutively high investment should be selected for (Bretman et al.

2013). From here stems the need to test the fitness consequences of ejaculate adjustments by measuring the reproductive success of multiple alternative phenotypes in different conditions, that is, in case of phenotype-environment mismatches. This approach, however, has been rarely adopted (but see Bretman et al. 2013; Harvanek et al. 2017). Manipulating environmental stimuli to elicit ejaculate adjustments and measure mismatch costs carries two major problems: firstly, it may be difficult to maintain a mismatch when trait expression is highly flexible (individuals will quickly re-adjust to the new conditions), secondly changes in the environment may also induce adjustments in other non-target traits. One promising avenue to overcome these issues is to phenotypically engineer the function of reproductive traits, for example by employing RNA interference (e.g. Arbore et al. 2015; Ram et al. 2007). Compared to the manipulation of environmental stimuli this approach has the advantage to exclusively modify the expression of the trait(s) of interest, allowing to disentangle its fitness effects from those of other traits (Sekii et al., 2013). The use of RNAi also allows to discriminate between strategic adjustments elicited by an environmental stimulus and changes resulting from energetic constraints, that is from trade-offs with strategically modified traits. In fact, while for allocation plasticity trade-offs only concern resource distribution among subsequent matings (Smith et al. 2009), for production plasticity trade-offs may occur between individual ejaculate traits (Immler et al. 2011) or between pre- and post-copulatory traits (Simmons et al. 2017). Since male overall fitness is determined by his competitiveness during both pre- and post-copulatory selective episodes along his life (Andersson and Simmons 2006; Cornwallis and Birkhead 2008), it is crucial to consider the whole set of reproductive traits when evaluating the consequences of strategic ejaculate adjustments. Within the ejaculate, adjustments may generate for example trade-off between number and size (Oppliger et al. 1998; Vermeulen et al. 2009), or between velocity and longevity (Burness et al. 2004; Cardozo et al. in prep), but may also affect sperm integrity. Sperm priming responses are likely to be associated with increased cellular activity, which, in turn, might be physiologically demanding and thus affect the costs and the efficiency of germline maintenance (for a review see, Maklakov and Immler 2016). These effects may be highlighted by investigating the effect of increased ejaculate production on sperm mutations and senescence (Ramm and Schärer 2014). Evidence from a comparative study in rodent species, indeed, showed that sperm DNA fragmentation was positively associated with levels of sperm competition, possibly due to increased metabolism and faster rates of spermatogenesis (delBarco-Trillo et al. 2016). Trade-offs between individual ejaculate traits, in particular those between initial sperm performance and sperm senescence, may compromise ejaculate competitiveness, and may be responsible for discrepancies in the competitive fertilization success of alternative strategies that are sometimes observed between *in vitro* and *in vivo* trials (Fu et al. 2001; Neff et al. 2003).

Costs associated with early sperm senescence may be difficult to spot. For example, when using artificial insemination to compare the fertilization success of male guppies that have upregulated their sperm production in response to exposure to females, with that of males previously maintained in isolation, Cardozo

et al. (in prep) showed that the ejaculate adjustment allowed males to gain slightly higher paternity shares in the first brood produced by the female. Only when comparing paternity in the second brood it was possible to detect the cost of the response: female-exposed males had lower fertilization success, due to the drop in sperm viability occurring during female sperm storage.

Trade-offs between ejaculate traits and traits involved in mate acquisition are also expected (e.g. Ramm and Stockley 2009; Cattelan et al. 2016). Thus, an increased investment in ejaculate production may therefore negatively affect a male's attractiveness and/or his capability to compete with other males for access to females (Bretman et al. 2013). For example, male guppies that increase their sperm production and velocity, show a reduced courtship rate (Cattelan et al. 2016) and a reduced mating success when they must compete directly for mating with another males (*Manuscript 2b*). Since these pre-copulatory costs are sometimes subtle, a "total sexual selection" approach is therefore recommended to quantify them (Hunt et al. 2009; Evans and Garcia-Gonzalez 2016).

Further trade-offs may occur between current and future reproduction, and therefore, may only become apparent in medium to long-term experiments or when considering a series of subsequent matings, as shown by Bretman et al. (2013). In a similar way, benefits of the adjustments may only arise after several mating events. For example, if the primary advantage of increasing sperm production when foreseeing many mating opportunities consists in a reduced risk of sperm depletion, the response may only become beneficial in association with high mating rates (see *Manuscript 2b*). In addition, if increasing investment in the ejaculate is traded-off against male survival (Miller and Brooks 2005; Bretman et al. 2013; but see Moatt et al. 2013), costs may arise when measuring male lifetime reproductive success, but would be probably hidden if a short-term experimental approach is adopted.

Costs may also become evident in the subsequent generation. It is now well recognized that sperm senescence and other environmentally induced impairments of sperm performance, associated with heritable genetic and epigenetic damages, can affect zygote viability or offspring fitness (Soubry et al. 2014; Immler 2018; Macartney et al. 2018), and impair offspring's sperm quality (Stouder and Paoloni-Giacobino 2010; Gasparini et al. 2017). Socially cued strategic ejaculate adjustments could have the same consequences: for example, sperm with high initial performance could suffer from early senescence and this could affect offspring survival or performance. Indeed, Zajitschek et al. (2014) showed that in zebrafish the increase in sperm velocity and motility induced by exposure to high intensity of sperm competition, was associated with the production of offspring that hatched faster, but had reduced survival; similar patterns have been observed between alternative reproductive tactics in the whitefish, *Coregonus lavaretus, (Kekäläinen et al. 2015). These results suggest* that strategic ejaculate adjustments may indeed entail trans-generational costs. However, environment dependent adaptive trans-generational effects could also exist (Immler 2018): males may be able to facultatively modulate paternal effects based on their own

environment, to produce offspring with a phenotype that is advantageous in those specific conditions (anticipatory paternal effects, sensu Crean et al. 2013; Eisenberg 2011). If fluctuations in social conditions relevant for ejaculate adjustments occur on a timescale that is longer than generation length, anticipatory paternal effects may be selected for (Galloway and Etterson 2007). A study in the broadcast spawning ascidian *Styela plicata,* reported that offspring of males maintained at different population density, a factor known to elicit changes in sperm performance, were more likely to survive in environmental conditions that matched the environment their father experienced (Crean et al. 2013), and analogous anticipatory paternal effects have been described in a neriid fly, *Telostylinus angusticollis* (Adler and Bonduriansky 2013). Even more remarkably, it has been shown that in *D. melanogaster,* males experiencing risk of sperm competition mate for significantly longer and sire sons which also extend copulation duration compared to sons of males that experienced no risk, reporting for the first time the occurrence of adaptive paternal effects on offspring behavior (Dasgupta et al. 2016). Finally, evidence that the phenotype of a female's previous mate can influence her future offspring, sired by other males, possibly via the effects of seminal fluid (Crean et al. 2014), leads to the intriguing possibility that socially-cued seminal fluid adjustments could affect other males' fitness.

Altogether, these considerations show how important it is, in order to reliably measure the adaptive value of plastic responses, to study the effect of ejaculate adjustments in long-term studies, carried out under the most natural conditions possible, while accounting for trade-offs with non-target pre- and post-copulatory traits, and while considering the potential effect of competition for partners on reproductive outcomes.

Consequences of strategic ejaculate adjustments for male and female behavior

The evidence that males, in addition to ejaculate size are also able to adjust ejaculate quality, represents a further challenge to the universality of Bateman's paradigm of choosy females and promiscuous males, based on the assumption that ejaculates are cheap and unlimited (Bateman 1948; Tang-Martinez and Ryder 2005; Parker and Birkhead 2013; but see: Dewsbury 2005). The description of cryptic female choice has produced a shift in sexual selection research from a focus on male reproductive tactics to greater consideration of the female perspective, suggesting that females may have the ultimate control over post-copulatory decisions (Eberhard 1996). The discovery that males are able to strategically adjust ejaculate investment, adds a new weapon to the sexual conflict, providing males with further means to influence fertilization patterns and to reduce the scope for cryptic female choice (Bonilla et al. 2011). Adjustments of ejaculate quality in particular may allow males to overcome female strategies related to the control of number of sperm received or retained during copulation (Smith and Ryan 2010). Moreover, male prudent allocation in terms of sperm numbers, by potentially generating sperm limitation in females, may play a role in the evolution of polyandry and may promote female competition for access to mates (Stockley 1997; Sæther et al. 2001; Bocedi and Reid 2016). At the same time, the ability of males to modify their allocation of seminal gifts (nutrients, fertility

enhancing compounds and immunostimulatory or antibiotic factors that are present in the ejaculate and that confer benefits to females, Poiani 2006; Gwynne 2008), depending on partner mating status (Sirot et al. 2011), will decrease the direct benefits obtained by females, and may thus reduce their keenness to re-mate. In addition, status-dependent allocation may select against chemical advertisement of female mating status (Thomas 2011) and, conversely, promote the evolution of female strategies aimed at concealing their mating history (as suggested for males, Wedell and Ritchie 2004).

The transgenerational effects of ejaculate adjustments may further complicate the scenario of sexual conflicts. When these imply a reduction in offspring fitness, females may be selected to recognize male behaviors signaling ejaculate adjustments in order to avoid mating with these males. Alternatively, females may choose to increase parental allocation to the offspring sired by males who have adjusted sperm production in the attempt to compensate for their predicted lower viability (Gowaty 2003). In contrast, when adaptive paternal effects are associated with ejaculate adjustments, females would benefit from recognizing males who are responding to social cues and from preferentially mate with them.

The study of the costs of socially-cued strategic ejaculate adjustments may also have important practical implications, for example for the treatment of human infertility and for the animal breeding industry.

FUTURE DIRECTIONS

While strategic adjustments of ejaculate quality have been shown to occur in many species, more studies will be necessary to compare the relative importance (or frequency) of socially cued adjustments in ejaculate quality traits vs. sperm number (Delbarco-Trillo 2011). Ejaculate quality plasticity ("production plasticity" in our definition) is likely to involve short-term trade-offs that need to be considered. Future research should aim at evaluating the consequences of strategic ejaculate adjustments accounting for both pre- and post-copulatory competitiveness. In this context, researchers should account for the presence of trade-offs occurring between individual ejaculate quality traits (e.g. sperm velocity vs. longevity or DNA integrity), and between pre- and post-copulatory traits. Following Fitzpatrick and Lupold (2014) and Simmons and Fitzpatrick (2012), future studies should address the problem of strategic ejaculate adjustments considering the ejaculate as a whole, using a multivariate analytical approach and directly testing fertilizations success (see for example Bartlett et al. 2017).

Furthermore, it is becoming increasingly apparent that *in vivo* and *in vitro* experiments investigating ejaculate competitiveness may lead to contrasting results (e.g. compare results from: Neff et al. 2003; Schulte-Hostedde and Burness 2005; Stoltz and Neff 2006). Measuring ejaculate quality in non-competitive contexts might be limiting as sperm competitive success depends on ejaculate-ejaculate and ejaculate-female interactions (Simmons and Fitzpatrick 2012; Fitzpatrick and Lüpold 2014). For example, it has been demonstrated that seminal fluids affect rivals' success (Nguyen and Moehring 2018) and that seminal fluids from different individuals vary in the way in which they affect rival ejaculates and they are affected by them

(Simmons and Beveridge 2011; Lupold et al. 2012; Locatello et al. 2013; Lewis and Pitcher 2016). Similarly, processes of cryptic female choice may play a role too, since differences in fertilizing competitiveness resulting from adjustments of sperm quality may be affected by interactions with female ovarian secretions, as shown for males adopting alternative strategies (Alonzo et al. 2016; Lehnert et al. 2017). These mechanisms may be responsible for the observed differences in fertilizing efficiency between competitive and non-competitive contexts (Vladić et al. 2010).

It should be also acknowledged that ejaculate quality adjustments are likely to be traded-off with pre-copulatory traits with potential effects on male mating success (see section: *Costs and benefits of ejaculate adjustments*). These considerations show that, if we aim at measuring the fitness associated with alternative strategies of ejaculate investment, we need to adopt an integrated approach in which the ejaculate is treated as a unit, female- and rival-mediated effects are taken into account and potential trade-offs affecting male mating success are recognized.

Another important objective for future studies within the field of ejaculate quality adjustments should be to understand the underlying mechanisms that enable such phenotypic modulations. Firstly, investigating the neurological and/or hormonal signaling pathways that allow perceived environmental stimuli to be translated into signals for the testicles and accessory reproductive glands represents a promising avenue, since very little is known on the subject, especially in invertebrates. Secondly, research should address the mechanisms responsible for alterations in the ejaculate once the signal has been received. Evidence is accumulating on the central role of the seminal fluid on facultative modulation of sperm performance (Cornwallis and O'Connor 2009; Simmons and Beveridge 2011; Bartlett et al. 2017) and the current development of proteomic methods represents a useful tool to further explore this subject (Wigby et al. 2009; Fedorka et al. 2011; Sirot et al. 2011; Ramm et al. 2015). More work will be needed to determine how seminal fluid composition changes and to explore other potential mechanisms responsible for alterations of sperm phenotype, and in particular of sperm morphometry.

The discovery of cryptic female choice after that of sperm competition, followed by strategic ejaculate adjustment including mechanisms of cryptic male choice, has uncovered increasingly sophisticated mechanisms adopted by males and females to dominate the sexual conflict. An intriguing possibility that stems from this consideration and that would be worth exploring, is that females could be able to adjust the composition of their ovarian fluid to the socio-sexual context, in a similar way to males with the ejaculate. Evidence indeed exists showing that the ovarian fluid of receptive female guppies enhances sperm velocity more sharply than that of unreceptive females (Gasparini et al. 2012); this process has been proposed as a mechanism to disfavor undesired sperm received during the unreceptive period, likely through forced copulations. Moreover, females may partly limit the scope for facultative ejaculate modulation, if they could interfere with ejaculate transfer and composition (Perry et al. 2013). This could be possible for example in

species in which ejaculate components are transferred in a fixed order, and thus females could differentially accept components by strategically terminating copulation.

Finally, due to its relevance for male and female fitness, the modulation of paternal effects associated with ejaculate quality adjustments represents a fascinating area deserving further attention in the future (see also, Immler 2018).

CONCLUSIONS

We have shown that strategic adjustments of ejaculate quality occur in response to variations in the socio-sexual environment in many taxa, but we are convinced that more work is needed in this field to understand the prevalence of the phenomenon and its relevance relatively to strategic adjustments of sperm numbers. We have also shown that the subject of ejaculate quality modifications is extremely heterogeneous in terms of type of adjusted traits, triggering stimuli, timing and mechanisms. While this aspect had limited the scope for meta-analyses and theoretical modelling, the accumulation of more studies may contribute to shed light on general patterns in strategic post-copulatory plasticity. Finally, we have highlighted that ejaculate quality adjustments may have complex implications in the sexual selection process due to trade-offs occurring at different levels, from mate acquisition to fertilization and offspring phenotype. Exploring these implications could bring an important contribution not only to our understanding of the evolution of strategic post-copulatory allocation, but also to more applied fields such as reproductive biology.

FUNDING

This work was supported by a grant from the University of Padova (PRAT 2012 no. CPDA 120105) to A.P. and a Ph.D. fellowship from the University of Padova to M.M.

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Behavioral Ecology

The official journal of the
ISBE
International Society for Behavioral Ecology

Behavioral Ecology (2018), 00(00), 1–11. doi:10.1093/beheco/ary087

Original Article

Quick-change artists: male guppies pay no cost to repeatedly adjust their sexual strategies

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Received 17 February 2018; revised 21 May 2018; editorial decision 23 May 2018; accepted 25 May 2018.

Sexually selected traits involved in mate acquisition and fertilization success are usually costly and males often plastically adjust their reproductive investment in response to social conditions. Phenotypic plasticity in male sexual traits is generally assumed to be adaptive, yet its costs are rarely investigated. Male guppies (*Poecilia reticulata*) adjust their ejaculate production and sexual behavior in response to perceived mating opportunities. In natural populations, mating opportunities can fluctuate continuously, and the iterated activation of plastic responses may impose a cost on males. To determine such costs, we experimentally manipulated male social environment by exposing males either to a constant number of females, or to weekly oscillations in female number. We measured traits linked to condition and reproductive success throughout male life. We found no significant difference in the expression of these traits nor in male lifespan between the 2 groups. Our results suggest that male guppies pay negligible costs for the iterated activation of plastic responses, possibly as a consequence of selection to minimize them.

Key words: **phenotypic plasticity, plasticity costs, Poecilia reticulata, sexual selection.**

INTRODUCTION

Phenotypic plasticity is defined as the ability of an individual or genotype to express different phenotypes depending on the environment (West-Eberhard 2003). Phenotypic plasticity is widespread and can involve morphological modifications, physiological and neural regulation, or behavioral changes (Fusco and Minelli 2010). Theoretical models predict that phenotypic plasticity should evolve when populations are exposed to variable, but predictable, environments and when the mean fitness of plastic individuals is greater than that of fixed individuals across the relevant fluctuations in environmental conditions (Ghalambor et al. 2007; Kasumovic et al. 2011). Therefore, for plastic individuals to show higher fitness, it is necessary not only that selection favors different phenotypes in each environment and that no single phenotype exhibits superior fitness across all environments, but also that the costs of expressing plasticity do not exceed its benefits (Ghalambor et al. 2007). In a heterogeneous environment, the evolution of "perfect" or "infinite" plasticity would represent the optimal response for individuals, enabling them to express the best trait value in each environment. Such response, however, is extremely rare, if not completely absent in natural populations, suggesting the existence of costs constraining the evolution of plasticity (Dewitt et al. 1998; Callahan et al. 2008).

Costs associated with the expression of a plastic phenotype can be related to the development, maintenance, and function of the sensory and regulatory machinery needed for plasticity, including the genetic material encoding such machinery (Dewitt et al. 1998; Auld et al. 2010). When the plastic response is, at least to some extent, reversible, plasticity costs may increase with the frequency of environmental changes. In a highly variable environment, however, selection is expected to progressively erode such costs, as individuals that pay high costs to produce plastic responses should have lower fitness. Over evolutionary time, costs of plasticity may be reduced to such an extent to make them difficult to detect empirically (Dewitt et al. 1998; Scheiner and Berrigan 1998). According to this prediction, most of the studies exploring costs of plasticity have found only mild costs or have failed to find any (for reviews and meta-analyses see: Van Kleunen and Fischer 2005; Van Buskirk and Steiner 2009; Murren et al. 2015).

Although females have been traditionally considered the sex that invests more in reproduction, traits and processes associated with male reproduction (i.e. sexually selected traits), including courtship, mate choice, sperm production, and mating, are also costly (Dewsbury 1982; Scharf et al. 2013). The costs, as well as the benefits, associated with male reproductive decisions are often affected by the context, with the conspecific socio-sexual environment playing the most important role in shaping the selective forces operating on individuals (Bretman et al. 2011). As social conditions are often highly variable (Kasumovic et al. 2008), plastic responses in reproductive allocation to such variation are expected to bring an important contribute to individuals' fitness. The expression of sexually

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selected traits is indeed characterized by high levels of plasticity (Bretman et al. 2011) and factors such as population sex ratio, number of mating opportunities, presence and number of rivals, and partner quality have been found to elicit plastic responses in male reproductive investment (for reviews see: Cotton et al. 2006; Harris and Uller 2009; Bretman et al. 2011; Kasumovic et al. 2011; Kelly and Jennions 2011; Engqvist and Taborsky 2016).

Probably because the adaptive value of phenotypic plasticity in male reproductive allocation seems obvious, costs and benefits of plastic responses have rarely been quantified in this context. Rather than costs and benefits of plasticity per se (i.e. costs and benefits of a more plastic phenotype as compared with those of a less plastic one), costs and benefits of enhancing a sexual trait's expression in response to a given social cue (i.e. cost and benefit of the phenotype, sensu Murren et al. 2015) have been more frequently measured (e.g. Sakaluk and Müller 2008; Bretman et al. 2009; Barbosa 2012; Bretman et al. 2013; Moatt et al. 2013). There is, however, some indirect evidence of intrinsic costs of phenotypic plasticity in male reproductive traits, coming from studies that compared the ability of producing plastic responses of different populations. Males from populations characterized by lower, and more consistent, female availability (Carroll and Corneli 1995) or by lower levels of competition for mates (Firman et al. 2013) appeared to have a reduced ability to plastically respond to the social context, suggesting that phenotypic plasticity evolves, or is maintained, only in populations in which environmental variability is high (Fusco and Minelli 2010; although a lack of benefits associated with mutational degradation could also be responsible for this pattern, Masel et al. 2007; Auld et al. 2010). More direct evidence of the costs of plasticity has been provided by Fraser et al. (2014), who analyzed patterns of gene expression associated with male alternative mating behavior in the sailfin molly (*Poecilia latipinna*). In this fish species, some genotypes are plastic and respond to changes in the social environment by switching from courtship displays to coercive mating attempts and vice versa. Other genotypes have instead a fixed mating strategy and do not display plasticity. The study showed that changes in brain gene expression (which mediate the behavioral response) associated with environmental variations were significantly greater in plastic individuals than in fixed individuals who did not respond. Similar results have been found in the three-spined stickleback (Bukhari et al. 2017). If conspicuous changes in gene expression are energetically demanding (Stoebel et al. 2008), we could expect plasticity to entail substantial physiological costs.

We tested this hypothesis by investigating the lifetime cost associated with adaptive plasticity in sexually selected male traits in the guppy, *Poecilia reticulata*, a species closely related to the sailfin molly and characterized by analogous alternative mating tactics. Guppies are freshwater fish with internal fertilization, characterized by high levels of male and female promiscuity (Magurran 2005). Male reproductive success is influenced mostly by male body coloration, sexual behavior, and ejaculate quality (Houde 1997; Magurran 2005; Devigili et al. 2015b). Males are sexually very active and can perform up to 1 mating attempt per minute (Magurran and Seghers 1994). As females mate multiply (Evans and Magurran 2000; Neff et al. 2008; Devigili et al. 2015b), post-copulatory sexual selection is intense (Devigili et al. 2015b) and fertilization success is influenced by the number and the swimming velocity of sperm transferred during copulation (Boschetto et al. 2011). Similarly to sailfin mollies, male guppies alternatively adopt 2 mating tactics: they court the female by performing sigmoid displays in order to obtain cooperative copulations or they attempt to forcibly inseminate the female through gonopodial thrusting (Liley 1966; Houde 1997; Pilastro and Bisazza 1999). However, among guppies, all males are characterized by plasticity in mating strategies, which are affected by a number of environmental factors (Luyten and Liley 1991; Reynolds et al. 1993; Godin 1995; Chapman et al. 2009) and especially by the social environment (Evans and Magurran 1999; Guevara-Fiore et al. 2009; Kiritome et al. 2012). In natural conditions, the social environment of this species is highly variable, due to intense fluctuations in population density and sex ratio (Pettersson et al. 2004; McKellar et al. 2009). Males respond to increased density and male-biased sex ratio by decreasing their courtship rate and increasing their attempts to sneak copulations through gonopodial thrusting (Jirotkul 1999). This plasticity in male reproductive decisions is not limited to mating strategies, as male guppies also adjust their sperm investment in response to perceived mating opportunities. Males maintained in visual and chemical contact with females produce larger ejaculates and faster sperm (Bozynski and Liley 2003; Gasparini et al. 2009), as compared with males that have been isolated from females. Due to energetic trade-offs, this adjustment affects male sexual behavior causing the former males to preferentially rely on gonopodial thrusting when interacting with females, rather than on more costly courtship displays (Cattelan et al. 2016; but see Devigili et al. 2013 for the absence of this trade-off under dietary restriction). This plastic response in sperm production and sexual behavior to perceived mating opportunities occurs within few days (3–7) and it is fully reversible (Cattelan et al. 2016). These results highlight the presence of a cost of the phenotype: the increase in sperm production stimulated by female presence, in fact, is associated with a reduced courtship rate and analogous socially cued changes in male reproductive effort have been shown to reduce male lifetime growth (Jordan and Brooks 2010). Conversely, we do not know anything about the costs of flexibility per se. If the activation of this set of plastic responses induced by fluctuating mating opportunities is indeed energetically demanding (possibly due to conspicuous gene regulation costs, as shown in analogous contexts in the related species *P. latipinna* by Fraser et al. 2014), then these costs might be amplified in case of iterated responses. As in natural guppy populations male mating opportunities fluctuate continuously (Pettersson et al. 2004), exploring this hypothesis would contribute to understanding the evolution of this type of socially cued anticipatory plasticity (Bretman et al. 2011; Delbarco-Trillo 2011; Kelly and Jennions 2011).

To this end, males were randomly sorted into groups of 4 and exposed to differing social environments: in the control treatment males interacted with a constant number of females (4 females) and in the plastic treatment the number of females was weekly alternated between 0 and 8. We determined the effect of the variable number of mating opportunities on male lifespan. At different time points during the treatment, we also measured male condition and the expression of sexually selected traits. If males exposed to the plastic treatment were paying a cost for the iterated activation of plastic responses, we expected them to have an earlier senescence and a reduced lifespan.

MATERIALS AND METHODS

Study animals

Guppies used in this experiment were descendants of wild-caught fish from the Lower Tacarigua river, Trinidad. They were maintained in our laboratory in large stock tanks (150-L tanks

containing approximately 150 individuals of all age classes) with a balanced sex ratio and in which outbreeding was assured by periodically moving individuals across different stocks. The bottom of the tanks was covered with mixed color gravel and the tanks were provided with aquatic plants and algae. All experimental fish were maintained under controlled temperature and lighting conditions (26 ± 1 °C; 12:12 h light:dark cycle, Philips TLD 36W fluorescent lamps). All fish were fed ad libitum twice a day with a mixed diet of brine shrimp nauplii (*Artemia salina*) and commercially prepared flake food (Duplarin) (see Pilastro et al. 2007, for details on fish maintenance). Both males and females used in the experiment were collected from stock tanks. All fish were sexually mature (at least 4 months old) when employed in the experiments.

Experimental design

The fish were collected from stock tanks and individually isolated for 1 week before being tested (see below) and randomly placed in the treatment tanks. Treatment tanks consisted of 125-L tanks divided by 2 opaque partitions into 3 equal sections. Each section contained 4 individual males, which constituted a replicate. Replicates were then randomly assigned either to the plastic treatment ($N = 14$ replicates, 56 males) or to the control treatment ($N = 12$ replicates, 48 males), which differed in the number of mating opportunities males were exposed to. Males of the plastic treatment were housed with 8 females for 1 week and with no females for the following week (the same group of females was used throughout the experiment). Males of the control treatment were housed with 4 females, and 2 groups of 4 females were alternated every week in each control replicate. Each female group switched weekly between 2 male groups of the same treatment, so that they were always housed with males. Males of the plastic and the control treatment therefore interacted with the same total number of individual females (8) throughout the experiment, but the number of females simultaneously interacting with the males varied in the 2 groups (always 4 in controls, from 0 to 8 in the plastic treatment). The treatment continued until all males died. The pictures taken before the beginning of the treatment allowed us to individually recognize each male throughout the experiment thanks to their unique coloration patterns. When an experimental male or a female died in a replicate, it was replaced with another individual to maintain the original sex ratio. However, substitute males were not considered in subsequent analysis.

At different time points during the experiment, we measured a wide suite of male traits (Table 1 and Figure 1) that are known to be associated with male reproductive success (Houde 1997; Magurran 2005; Boschetto et al. 2011; Brooks and Postma 2011; Devigili et al. 2015b) and to be condition dependent (e.g. Gasparini et al. 2013). Furthermore, we measured male performance in a simulated predator evasion test (hereafter capture test, Evans and Magurran 2000; Gasparini et al. 2013) and male lifespan. In particular, 1) before the beginning of the treatment (hereafter, 0 months), we performed capture tests aimed at measuring male general condition; we stripped the males to determine their sperm reserves; finally, we took a digital photograph for body morphological analyses. 2) At 9 weeks (hereafter, 2 months), we conducted behavioral observations on the groups of males of each replicate in their treatment tanks to measure male sexual and inter-male aggressive behavior during the treatment (hereafter, replicate observations). 3) At 13 weeks, females were removed from the all males' tanks to equalize recent mating history and to allow males to restore their sperm reserves. One week later, (hereafter, 3 months) we performed observations of the sexual behavior of each individual male in standardized conditions and we repeated the capture tests. We subsequently isolated males in individual tanks for 2 days before stripping their ejaculates to estimate sperm reserves (number of sperm), sperm velocity, and sperm viability. We took another photograph before returning the males to their treatment tanks. 4) At 27 weeks (hereafter, 6 months), we repeated all tests performed at 14 weeks, following the same protocol. Afterwards, we returned males to their treatment tanks and the experimental treatment was continued as above until each male's death. During this last part of the experiment, we progressively pooled the replicates of the same treatment group to maintain constant the sex ratio. Only males that survived until the 3-month measurements were included in the analyses.

Sperm collection

Sperm were collected from each male following an established procedure (Evans et al. 2003). Males were anaesthetized by immersion in a solution of fish anaesthetic MS222 (0.5 g/L) and placed on a slide under a stereomicroscope (ZEISS Stemi 2000-C). One milliliter of saline solution (NaCl 0.9%) was also placed on the slide to favor sperm collection. The gonopodium was repeatedly swung back and forward and then a gentle pressure was applied on the abdomen to allow sperm release. Sperm in this species are packaged in discrete units, called spermatozeugmata or sperm bundles, that can be easily collected with a pipette. The slide with the sperm bundles was photographed under the stereomicroscope (magnification ×6.5, Canon EOS 450D camera). Sperm bundles were then split into different aliquots for subsequent analyses (sperm-velocity and sperm-viability analyses, see the Sperm number, sperm velocity, and sperm viability section).

Table 1
Timing of tests performed during the experiment

Test	0 months	2 months	3 months	6 months
Male morphology	$N = 79$		$N = 76$	$N = 47$
Capture test	$N = 79$		$N = 79$	$N = 48$
Sperm number	$N = 79$		$N = 75$	$N = 44$
Sperm velocity			$N = 76$	$N = 47$
Sperm viability			$N = 74$	$N = 47$
Male sexual behavior (measured individually in standardized condition)			$N = 79$	$N = 48$
Male sexual behavior (measured in groups—replicate observations)		$N = 104$		

Empty cells indicate that the test has not been performed at that time point. The sample size changes across measurements due to mortality (males that died before the 3-month tests were also excluded from the 0-month dataset). In some cases, we were unable to complete 1 or more tests on an individual, because the individual died during testing or because of problems in the experimental procedures. This led to differences in sample size across tests and measurement. All males were included in the analyses of the replicate observations.

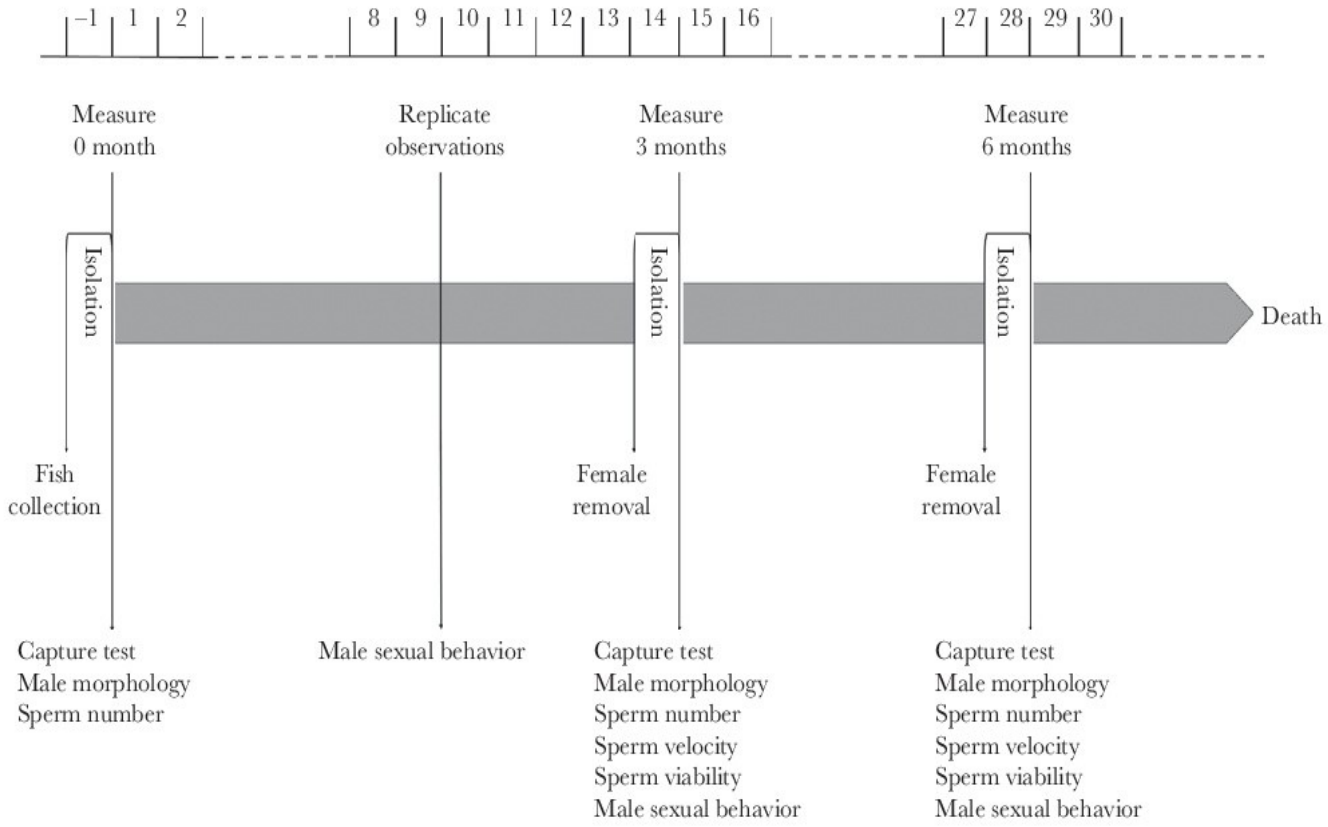

Figure 1
Schematic diagram of the experimental design showing the timing of the different measures. The experimental treatment (gray band) was carried out from Week 1 until the individuals' death.

Sperm number, sperm velocity, and sperm viability

The number of bundles produced by the male was measured from the photograph taken after stripping (using ImageJ software, http://rsbweb.nih.gov/ij/download.html; accessed 7 June 2018).

For sperm-velocity assays, intact sperm bundles from each male were collected with a Drummond pipette and placed on a multi-well slide into 3 µL of activating medium (150 mM of KCl and 2 mg/mL bovine serum albumin, see Billard and Cosson 1990). Swimming velocity of individual sperm was measured as they were moving away from the opening bundles and was recorded using a Hamilton Thorne computer-aided sperm analyzer (CEROS, Hamilton Thorne Research, Beverly, MA). Sperm velocity includes 3 commonly used parameters: average path velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s), and straight line velocity (VSL, µm/s). The threshold between static and motile cells was set at VAP = 25 µm/s, VSL = 20 µm/s and velocity was recorded only for motile sperms. The 3 measures of sperm velocity are highly correlated (Devigili et al. 2015b; Magris et al. 2017) and VAP is the most common measure of sperm velocity used in guppies (e.g. Evans 2011; Barrett et al. 2014; Fitzpatrick et al. 2014); we therefore considered only VAP in our subsequent analyses. Sperm viability was assessed using the live/dead Sperm Viability Kit (L-7011; Molecular Probes Inc., OR), a fluorescence-based assay, which includes a membrane-permeant nucleic acid stain (SYBR14) which labels live sperm with green and a membrane-impermeant stain (propidium iodide) which labels dead or damaged sperm with red (only cells with intact membrane were considered viable). We measured viability immediately after sperm-velocity analyses. Fifty sperm bundles were collected from each male and transferred into a 0.5-mL Eppendorf tube containing 40 µL of saline solution. Sperm bundles were then broken by vortexing samples for 90 s. Six microliter of the mixture were transferred to an empty 0.5-mL Eppendorf tube, to which we added 2 µL of SYBR14 stain and, after 9 min, 2 µL of propidium iodide. All the operations involving the stains were carried out in low illumination conditions in order to prevent damage to the UV-sensitive compounds. The sample was placed on a microscopic slide and gently covered with a coverslip. Fluorescent images of samples were recorded using a ×20 objective on a Leica 5000 B microscope (Leica Microsystems, Wetzlar, Germany) with a digital camera (DFC480; Leica Microsystems, UK) and stored using Leica IM500 image-manager software. The proportions of live and dead spermatozoa were then assessed from images using the software Image J.

Male morphology: body size and color pattern

After sperm collection, each male was photographed under the stereomicroscope (magnification ×6.5, Canon EOS 450D camera) on millimetre paper for calibration. The images were then analyzed using ImageJ software to measure total area (including caudal fin but excluding dorsal fin), body area (excluding dorsal and caudal fins), and coloration. We measured 3 main components of the coloration pattern: surface area of orange spots (orange, yellow, and red, representing all the area of carotenoid and pteridine spots; hereafter: orange), melanistic spots (only including permanent black spots and not fuzzy black areas; hereafter: melanistic), and structural iridescent spots (combined measures of blue, green, violet, silver, and white; hereafter: iridescent) (Evans et al. 2003; Devigili et al. 2015b).

Observations of male sexual behaviour during the treatment (replicate observations)

After 9 weeks (±1 week) from the beginning of the treatment, behavioral observations were carried out on the groups of males in their treatment tanks. Previous studies describing adjustments of male sexual behavior or sperm production in response to the number of mating opportunities involved treatments that lasted significantly less than ours (i.e. between 3 and 7 days: Bozynski and Liley 2003; Gasparini et al. 2009; Cattelan et al. 2016; and see Barrett et al. 2014, that did not find an effect in sperm production). To test whether males in the plastic treatment still responded to the higher mating opportunities when females were present after longer experimental manipulation, we conducted behavioral observations of male sexual behavior in the treatment tanks two months after the beginning of the experiment (i.e. during the 10th week of the experiment). We then compared the sexual behavior of plastic males (while housed with 8 females) with that of control males (housed with 4 females) in their treatment tanks. Since half of plastic replicates started the treatment with 8 females and half with 0 females, the replicates that in the 10th week were housed with no females were observed in the following week (when they were housed with 8 females). Each of the 4 males in a replicate was observed for 5 min during which we recorded the number of courtship displays (sigmoid displays, where the male positions himself in front of the female in an s-shaped posture and quivers) and forced mating attempts (gonopodial thrusts, where the male attempts to coercively inseminate the female) (Liley 1966). In addition, we recorded the total time each male spent interacting and swimming within 2 body lengths from the female as a measure of the male's overall sexual interest in the female (hereafter: following, Cattelan et al. 2016). Finally, we noticed the number of performed and received intra-sexual aggressions (i.e. chasing and nipping behaviors). A subset of individuals ($N = 27$) was also observed when housed with 0 females (data shown only for aggressions).

Observation of individual male sexual behavior in standardized conditions

After 3 and 6 months, we also measured, blind of male treatment, each male's sexual behavior in a standardized context (Devigili et al. 2015a). To this end, each male was placed individually in a 30-L tank (with multicolor gravel on the bottom) and allowed to interact freely with a gravid female that was not familiar to the male. Gravid females are not sexually receptive (Houde 1997), this allowed us to minimize the effect that female receptivity may have on male sexual behavior (Houde 1997; Evans et al. 2002). The observations lasted 15 min, during which we recorded the number of sigmoid displays, gonopodial thrusts, and the duration of following. No successful copulation attempt was recorded during the behavioral trials. Each female was used for 2 trials, one with a plastic and one with a control male, in an alternated order, to equalize female effects between treatments.

Predator evasion capability: capture test

The capture test is an established protocol, previously used in other experiments with guppies (e.g. Gasparini et al. 2013), which estimates the individual's predator evasion capability. As this is a condition-dependent trait, male performance in the test can be used as a proxy of his condition. The capture test was carried out on the same day of the observation of sexual behavior, after having given the male time to recover (at least 30 min). All the tests were performed blind to male treatment by the same operator. The male was reintroduced in the tank previously used for the observation of sexual behavior and allowed to acclimatize for between 1 and 5 min. The operator then gently introduced a small hand net in the tank and started chasing the male with the net at a constant speed. The test ended when the fish slowed down and was caught in the net. The time between introduction of the net and capture was recorded using a chronometer. Despite its simplicity, capture test has a high repeatability and reflects male condition ($R = 0.68 \pm 0.13$ SE, $P < 0.001$, Gasparini et al. 2013).

Statistical analyses

All statistical analyses were performed with RStudio (version 3.5.0) and SPSS (version 23, IBM, Armonk, NY). Since the 3 components of male sexual behavior measured in replicate observations (i.e. sigmoid displays, gonopodial thrusts, and following) were positively correlated, we combined them using principal component analysis (PCA). Four males (1 plastic and 3 control males) did not engage in any sexual behavior and were excluded from the analysis. A single principal component (PC, explaining 56% of the total), representing overall sexual activity, was obtained (Supplementary Table S1).

PC scores of the 2 groups were compared using a t test. The number of aggressions was not normally distributed even after transformation and was thus analyzed using a Mann–Whitney test. Additionally, we ran an Anova test to compare aggression rates in the 3 conditions (0, 4, and 8 females) simultaneously (we included in the analysis only a subsample of the individuals housed with 8 females, specifically, those that had not been observed with 0 females).

To test the effect of our treatment, we ran linear mixed-effects models (LMM) and generalized linear mixed-effects models (GLMM), using the "glmer" function (package lme4). The experimental tank in which each 4-male group was housed during the experiment did not explain any difference in subsequent analyses (see Supplementary Table S2) and was therefore excluded from all models. Therefore, we considered each male as an independent statistical observation. The response variables can be subgrouped in 4 categories: male morphology including: a) total area, b) orange, c) melanistic, d) iridescent; general condition: e) duration of the capture test; ejaculate traits: f) sperm number, g) sperm velocity, h) sperm viability (binomial); sexual behavior i) PC1, j) PC2 (see below). All models included treatment, time and their interaction as fixed effects and male identity as random effect. To account for correlations between body size and traits, male total area was added as covariate in models b–d and male body area was added as covariate in model f. Sperm viability was analyzed using a GLMM with binomial distribution. Since the model was overdispersed we added an observation level random effect to account for overdispersion. All the other traits were analyzed using LMMs.

To analyze male sexual behavior in the standardized tests, we used the same procedure as above (for the replicate observations) and reduced the variables describing sexual behavior (i.e. sigmoid displays, gonopodial thrusts and following) using PCA. One male did not engage in any sexual behavior and was excluded from the analysis. We obtained 2 PCs, explaining together the 79% of the total variance, that were used for further analyses. PC1 accounted for the overall sexual activity (41% of the variance) and PC2 accounted for the frequency of sigmoid displays relatively to gonopodial thrusts (37% of the variance) (Supplementary Table S3). Positive PC1 scores represent individuals with overall higher sexual activity, whereas positive PC2 scores represented males that

performed a higher proportion of sigmoid displays. PC1 and PC2 were analyzed using LMMs (see above).

For all models, the statistical significance of fixed effects and interactions was assessed based on the 95% credible intervals (CI) around the mean (β). We used the "sim" function (package arm) to simulate the posterior distribution of the model parameters and values were extracted based on 2000 simulations. We consider an effect to be significant when the 95% CI did not overlap 0. We used visual assessment of the residuals to evaluate model fit.

We used Mann–Whitney tests to compare males' lifespan in the 2 treatments. Means and their SEs are given. All probabilities are 2 tailed.

Ethical note

Our study was approved by the Ethics committee of the University of Padova and by the Italian Ministry of Health (permits no. 72 /2017 and 12 /2014) and thus meets the guidelines for the care and use of research animals of the Animal Behavior Society and of the Italian Government.

RESULTS

Male behavior measured in groups—replicate observations

Males housed with 8 females (N = 55) engaged in significantly more intense sexual behavior than males housed with 4 females (N = 45) as expressed by PC scores (t test: $t = -4.009$, $P < 0.001$, 95% CI = −1.47, −0.51). Male–male aggression rates did not differ significantly in the 2 groups (Mann–Whitney test: W = 1267, P = 0.592). (See Figure 2 and Supplementary Table S4 for details on the descriptive statistics on male sexual behavior and aggressions). These results confirmed that, 2 months after the beginning of the experiment, males in the plastic group continued to show an increased sexual activity when 8 females were present as compared to that of their control counterparts in the presence of 4 females. Aggressions were significantly more frequent when males were housed without females (Anova: F = 15.16, $P < 0.01$, post hoc comparisons, with Tukey corrections: 0–4, $P < 0.001$; 0–8: $P < 0.001$).

Male traits measured along the experiment

We documented a change over time in the expression of some of male sexual traits (Table 2 and Figure 3). Males grew during the 6-month treatment (β = 2.111; 95% CI = 1.59, 2.62) and increased their sperm production (β = 23.571; 95% CI = 1.89, 45.31). Male general condition worsened during the treatment as shown by a decrease in the duration of the capture test (β = −0.151; 95% CI = −0.22, −0.09). Sperm viability was also reduced from 3 to 6 months (β = −0.178; 95% CI = −0.31, −0.05) and there was a trend, although not significant, towards a reduction of sperm velocity (β = −1.272; 95% CI = −3.19, 0.75). The extension of the color spots also did not change with time, only iridescent spots tended (not significantly) to increase over time (orange: β = −0.033, 95% CI = −0.19, 0.12; melanistic: β = −0.004, 95% CI = −0.05, 0.04; iridescent: β = 0.134, 95% CI = −0.07, 0.34). Analogously, male sexual activity was not affected by time, although both overall intensity (PC1, β = −0.133, 95% CI = −0.35, 0.07), and the frequency of sigmoid displays relative to that of gonopodial thrusts (PC2: β = −0.171, 95% CI = −0.36, 0.03) showed a tendency towards a (nonsignificant) decrease. Treatment did not affect the expression of any of the male traits considered here (Treatment: 95% CI overlapped 0), nor the rate with which traits changed over time (Interaction

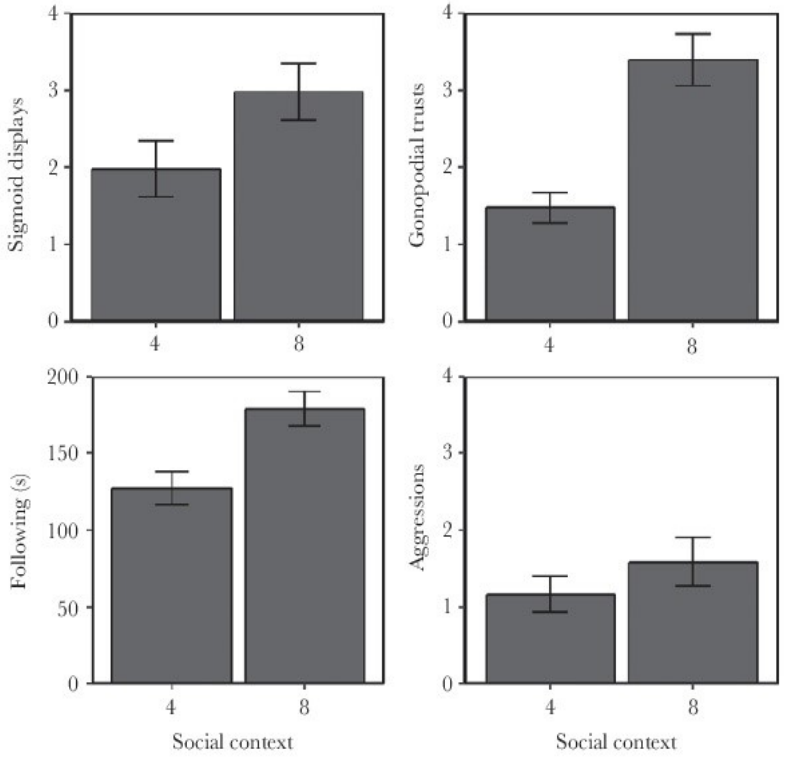

Figure 2
Male behavior as recorded 2 months after the start of the experiment in the treatment tanks in relation to the number of females in the tank (control = 4 females; plastic = 8 females). Means ± SE are given.

Table 2

Effect of treatment and time on the expression of target traits

Response variable	Effect	β Estimate	SE	95% CI
Morphological traits				
a) Total area (mm^2)[a,b]				
	Treatment	0.141	1.880	−3.35, 3.86
	Time	2.111	0.264	**1.59, 2.62**
	Treatment * Time	0.506	0.353	−0.20, 1.18
b) Area of orange spots (mm^2)[a,b]				
	Treatment	−0.001	0.488	−0.94, 0.98
	Time	−0.033	0.083	−0.19, 0.12
	Treatment * Time	−0.023	0.098	−0.21, 0.17
	Total area (covariate)	0.041	0.019	**0.00, 0.08**
c) Area of melanistic spots (mm^2)[a,b]				
	Treatment	−0.021	0.145	−0.31, 0.27
	Time	−0.004	0.023	−0.05, 0.04
	Treatment * Time	0.039	0.027	−0.01, 0.09
	Total area (covariate)	0.019	0.005	**0.01, 0.03**
d) Iridescent (mm^2)[a,b]				
	Treatment	0.690	0.485	−0.27, 1.64
	Time	0.134	0.107	−0.07, 0.34
	Treatment * Time	−0.061	0.131	−0.32, 0.20
	Total area (covariate)	0.210	0.020	**0.17, 0.25**
General condition				
e) Capture test duration (s)[a,b]				
	Treatment	−25.451	10.691	**−45.91, −5.41**
	Time	−8.227	1.897	**−11.86, −4.37**
	Treatment * Time	3.063	2.518	−2.05, 7.96
Ejaculate traits				
f) Sperm number[a,b]				
	Treatment	−22.560	49.503	−117.68, 77.41
	Time	23.571	11.133	**1.89, 45.31**
	Treatment * Time	13.917	13.571	−14.23, 40.50
	Body area (covariate)	16.780	3.169	**10.48, 22.85**
g) Sperm velocity[b]				
	Treatment	0.125	5.679	−11.26, 11.07
	Time	−1.272	0.967	−3.19, 0.75
	Treatment * Time	−0.702	1.296	−3.28, 1.81
h) Sperm viability[c]				
	Treatment	0.057	0.399	−0.74, 0.84
	Time	−0.178	0.067	**−0.31, −0.05**
	Treatment * Time	0.019	0.091	−0.16, 0.20
Sexual behavior				
i) PC1: overall sexual activity[b]				
	Treatment	−0.136	0.608	−1.42, 1.13
	Time	−0.133	0.106	−0.35, 0.07
	Treatment * Time	0.060	0.140	−0.22, 0.35
j) PC2: male mating tactic[b]				
	Treatment	−0.204	0.567	−1.26, 0.94
	Time	−0.171	0.099	−0.36, 0.03
	Treatment * Time	0.016	0.131	−0.25, 0.27

Results of the linear mixed-effects model (LMM) and the generalized linear mixed-effects model (GLMM). All models included treatment, time (months 0, 3, and 6), and their interaction as fixed effects and male identity as random effect.

Significant effects are in bold.

[a]Traits measured at all 3 time points. The other traits were measured twice, 3 and 6 months after the beginning of the treatment.

[b]LMM.

[c]GLMM with Binomial distribution. Overdispersion was corrected by creating a variable that adds a progressive number to each data point and entering it as random effect.

treatment × time: 95% CI overlapped 0). The GLMM highlighted a significant effect of treatment on capture time (β = −0.530; 95% CI = −0.90, −0.16), due to control males having initially a longer capture time. Although there was a trend towards a steeper decrease in capture time in control males (Interaction treatment × time: β = 0.081; 95% CI = −0.004, 0.17), the effect was not significant. Males' lifespan did not differ between the 2 treatments (Plastic: mean = 199 ± 17 days, Control: mean = 186 ± 18 days; Mann–Whitney test: $W = 1252$, $P = 0.55$).

DISCUSSION

Male guppies show anticipative phenotypic plasticity in their sperm production and sexual behavior in response to the number of expected mating opportunities (Bozynski and Liley 2003; Gasparini et al. 2009; Cattelan et al. 2016). Although anticipative phenotypic plasticity is assumed to be associated with fitness benefits, it probably also imposes costs, yet these costs are rarely quantified. The work from Fraser et al. (2014) on the closely related species, *P. latipinna*,

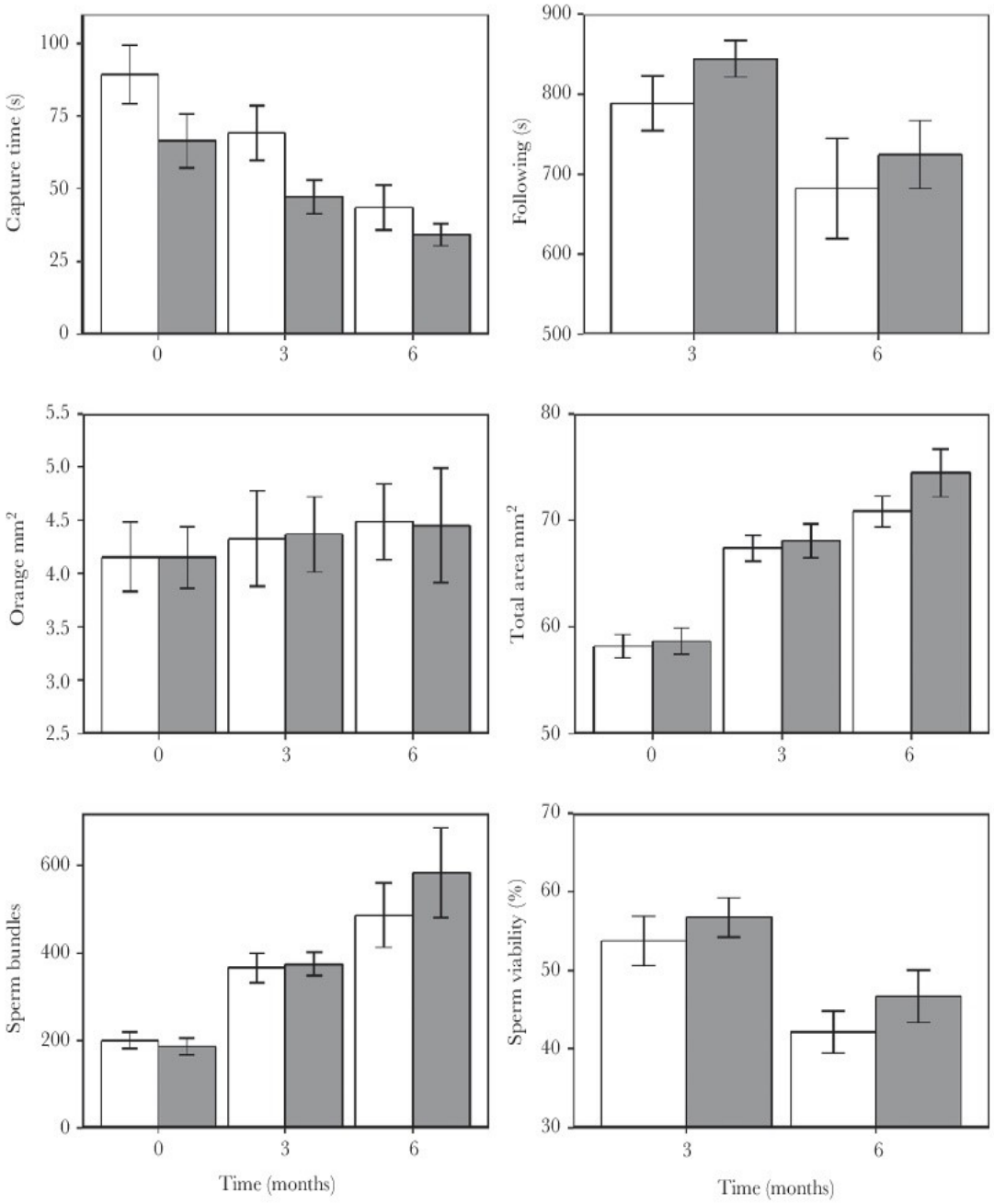

Figure 3
Effect of treatment and time on the expression of target traits (mean ± SE); open bars represent the control group and solid bars represent the plastic group.

had shown that similar socially cued responses were accompanied by substantial changes in brain gene expression, which, in turn, are likely to be associated with energetic costs. To explicitly test this hypothesis, we manipulated the number of females encountered by male guppies during their lifetime simulating 2 conditions: constant mating opportunities, in which the sex ratio was 4M:4F throughout the experiment, and variable mating opportunities, in which the sex ratio oscillated regularly between 4M:0F and 4M:8F. While keeping constant the total number of females encountered by each male during his lifetime, this experimental manipulation was aimed at inducing a more variable male reproductive investment in the latter group as compared with the former one. Indeed, observations conducted during the experiment confirmed that males were sexually more active in the presence of 8 females than in the presence of 4 females and that familiarity did not suppress their sexual interest. Males in the plastic treatment showed almost no sexual activity when females were not in the tank (although sigmoid displays were sometimes directed towards other males), indicating that mean male sexual investment in the control group was intermediate between the mean sexual behavior observed in the plastic group in response to female absence/presence. We did not quantify sperm production during the treatment, as there is overwhelming evidence that males increase their sperm production in response to female stimulus (Bozynski and Liley 2003; Cattelan et al. 2016; and see Aspbury and Gabor 2004, for similar effects in closely related species) and we preferred to minimize disturbance during the experiment. In addition, a reliable comparison of sperm production during the treatment would not have been possible since the size of sperm reserves is not only determined by strategic sperm production in response to the number of mating opporunities, but also depends on male recent mating history, which in turn is affected by the number of females males are housed with. These results

also indicate that a prolonged variation in mating opportunities did not induce habituation, at least 9 weeks after the beginning of the experiment when the observations were carried out.

We documented changes in some traits across subsequent measures: males increased in body size but showed significant senescence for some sexual traits and for their capability to escape a simulated predator. This was expected, as the experiment (lasting 6 months) covered a considerable part of the lifespan of the individuals (male life expectancy rarely exceeds 12 months, both in nature, Olendorf et al. 2006; and in the lab, Miller and Brooks 2005; Gasparini et al. 2010; Devigili et al. 2015a). Indeed, males were captured more easily as they aged and ejaculate traits were also affected by male age. In fact, sperm production increased, and sperm viability decreased, whereas sperm velocity remained consistent. The intensity of sexual behavior did not change over the 6 months, as the frequency of sigmoid displays relative to that of gonopodial thrusts.

Although the effect of age on some traits was apparent, growth and senescence rates did not differ between treatments, suggesting that the repeated activation of plastic responses is not associated with relevant costs, at least from what we could assess from their effect on the traits we measured. We are aware that our analysis did not include all male traits (although it comprised of a high number of traits associated with pre and postcopulatory reproductive success and with condition, including lifespan) and that plasticity activation costs may have negative effects on traits that we did not consider. We also acknowledge that our conclusion may be limited to the manipulation we operated and that more extreme stimulations may have imposed plasticity activation costs. Nontheless, our findings are consistent with the results of previous studies that have investigated costs of plasticity in nonreproductive contexts suggesting that these costs are usually negligible compared with costs of the phenotype (Van Kleunen and Fischer 2005; Van Buskirk and Steiner 2009; Murren et al. 2015). Anticipatory plasticity is a widespread phenomenon in the context of sexual selection, but benefits are often just assumed (Bretman et al. 2011) and costs are rarely measured directly. Furthermore, the rare studies available so far have focused mainly on the costs and benefits of the phenotype, rather than on those of plasticity per se (Sakaluk and Müller 2008; Bretman et al. 2009; Barbosa 2012; Bretman et al. 2013; Moatt et al. 2013; Cattelan et al. 2016). Indirect evidence that the ability of plastically responding to changes in the social environment entail costs comes from the comparison of populations exposed to different levels of sexual competition. Anticipatory plasticity in male sexual investment appears to be reduced in the populations characterized by lower or more constant female availability or male competition, suggesting the presence of costs (Carroll and Corneli 1995; Firman et al. 2013). The same pattern, however, may also arise from mutational degradation, that has been shown to determine, in nonsexual traits, the loss of plasticity when it is not under selection (Masel et al. 2007). An interesting study on the sailfin molly, a species closely related to the guppy, has shown that the switch between alternative mating strategies requires extensive changes in gene expression (Fraser et al. 2014), which are probably associated with energetic costs (Stoebel et al. 2008). It is therefore probable that guppies pay analogous transcriptional regulation costs associated with their plastic responses. Our results indirectly suggest, however, that these costs may be minor, as they apparently did not negatively affect male fitness. Clearly, to draw a comprehensive picture, we will need to investigate both short- and long-term costs of plasticity in more species.

Costs of plasticity are predicted to be moderate because natural selection should favor the most efficient allelic variants for plasticity, those associated with smaller costs (Dewitt et al. 1998). Physiological costs of producing a plastic response, however, might still be maintained in the population, as long as they are exceeded by benefits, because of a lack of genetic variability in plasticity costs, or when they are unavoidable (i.e. intrinsically constrained). For example, when the genetic, physiological, and cognitive machinery responsible for the expression of anticipatory plasticity is costly to be maintained (Scheiner and Berrigan 1998), adaptive plasticity may entail costs irrespective of whether the plastic response is produced or not. The cost of possessing the machinery that allows organisms to produce a plastic response differs from the cost of producing the plastic response; these 2 types of costs, although not mutually exclusive, require 2 different experimental approaches to be quantified. In the first case, genotypes differing in the degree of plasticity should be compared; in the second case, experimental manipulation of the expression of plasticity should be used (DeWitt 1998; Van Buskirk and Steiner 2009). Our experimental manipulation was aimed at testing the latter and, specifically, the long-term effects of the costs of the activation of the anticipatory plastic response to varying social-sexual context (e.g. Fraser et al. 2014; Bukhari et al. 2017). Although our results seem to be in line with previous work suggesting that costs of plasticity might be intrinsically small (Van Kleunen and Fischer 2005; Van Buskirk and Steiner 2009; Murren et al. 2015), we cannot exclude completely that our experimental design prevented us from detecting them.

Males of the plastic treatment were maintained without females for half of their time. During these periods, sexual behavior was strongly reduced and rest from sexual activity might have compensated for the costs of plasticity activation. Whether this is the case is difficult to say, as sexual behavior was not completely suppressed in female-deprived males, which sometimes engaged in sigmoid displays towards other males (in a range of 0–3 sigmoid displays per 5-min observation, data not shown). In addition, we have shown that female-deprived males engaged in male–male aggressive behavior more frequently than their control counterparts.

Alternatively, the costs of plasticity may be apparent only under stressful conditions, such as, for example, resource limitation (Agrawal 2001; Steinger et al. 2003; Van Buskirk and Steiner 2009). Our males were fed ad libitum, and it is possible that costs of plasticity may be exposed under less favorable conditions, like under a restricted diet (Devigili et al. 2013). Even if this was the case, we might conclude that the costs of plastically adjusting sexual investment upwards and downwards are minor as compared to the costs of the phenotype (i.e. of maintaining a constantly upwardly regulated sexual investment). Indeed, experiments similar to ours in which males were constantly exposed to females or were female deprived have highlighted significant costs of the phenotype (sensu Murren et al. 2015) both in the short term (Cattelan et al. 2016) and over a male's lifetime (Miller and Brooks 2005).

In conclusion, our results indicate that, despite the potential costs associated with the activation of an anticipatory sexual response to a fluctuating social context (Fraser et al. 2014; Bukhari et al. 2017), male guppies exposed to varying mating opportunities did not show any negative effect on a large suite of fitness-related traits, including traits linked to condition (capability to escape from a simulated predator) and longevity, and sexual traits known to affect mating and fertilization success (Boschetto et al. 2011; Devigili et al. 2015b). Artificial selection for male anticipatory plasticity or quantitative genetic studies may be used to estimate 1) the genetic

variation in the magnitude and/or the latency of male anticipatory sexual plasticity and 2) the difference in fitness between more plastic and less plastic genotypes both under constant and variable levels of mating opportunities (Dewitt et al. 1998; Auld et al. 2010). The investigation of the costs of plasticity, including those determined by phenotype–environment mismatches caused by low reliability of environmental information or latency in the response (Agrawal 2001), will be necessary to improve our understanding of the evolution of anticipatory adaptive plasticity in male reproductive strategies.

SUPPLEMENTARY MATERIAL

Supplementary data are available at *Behavioral Ecology* online.

FUNDING

This work was supported by a grant from the University of Padova (PRAT 2012 no. CPDA 120105) to A.P. and a Ph.D. fellowship from the University of Padova to M.M.

We thank Isabella Zanata for logistic support during behavioral trials and Alexander Hutfluss for helpful discussion on the statistical methods. We would like to thank John Fitzpatrick and 2 anonymous Referees for their helpful suggestions on a previous version of the manuscript.

Data accessibility: Analyses reported in this article can be reproduced using the data provided by Magris et al. (2018).

Handling editor: John Fitzpatrick

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Manuscript 2b

Short blankets: trade-offs and the benefits of strategic sperm adjustments

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ABSTRACT

In polyandrous species males invest significant resources in producing large and high quality ejaculates. As sperm are costly, males are expected to modulate their ejaculate investment in order to anticipate future mating conditions (e.g. level of sperm competition or mating opportunities), a capability that has been demonstrated in several species. While this plasticity in male reproductive strategies is likely adaptive, its fitness consequences have been rarely investigated. Male guppies (*Poecilia reticulata*) adjust their ejaculate production and sexual behavior on expected mating opportunities: males maintained in contact with females produce more numerous and faster sperm but reduce their sexual behavior (and hence their attractiveness) in comparison with isolated males. As reproductive success results from the combination of mating and fertilization success, costs and benefits of male plasticity at the pre-and post-copulatory level must be explicitly quantified. Using a repeated-measure design, we tested whether males, previously exposed or not to female stimuli, differed in their mating and insemination success. In a first experiment we allowed each male to mate in sequence with 6 sexually-receptive females; in a second experiment we allowed two males, one of each treatment, to interact simultaneously with two females in sequence. Previously isolated males engaged in more intense sexual behavior, but progressively reduced their mating effort as compared to males previously exposed to females. Due to their less intense sexual behavior, female-stimulated males suffered from a reduction in mating success, when competing for access to a female with a male that had been previously isolated. However, the upregulation of sperm production elicited by exposure to females reduced the risk of sperm depletion after multiple copulations. These data suggest that the plastic response to female presence is only beneficial when mating opportunities are high, while, probably due to trade-offs between pre- and post-copulatory traits which appear to compromise male ability to obtain copulations, it is detrimental when mating opportunities are rare. Our results show how the effects of plastic responses on male reproductive success should always be explicitly tested rather than assumed. Since trade-offs can affect the fitness consequences of reproductive investment adjustments, a comprehensive approach, accounting for the whole set of reproductive traits, should be always used when investigating costs and benefits of plasticity.

INTRODUCTION

Ejaculate production is costly (Dewsbury 1982; Thomsen et al. 2006; Hayward and Gillooly 2011) and since the fitness returns of investment into sperm and seminal fluids are often context dependent, males have evolved the ability of strategically adjusting ejaculate production and allocation according to the social environment (Wedell et al. 2002; Kelly and Jennions 2011). This form of socially cued anticipatory plasticity (Kasumovic et al. 2011) allows males to strategically partition available sperm reserves among individual copulations and to modify sperm production in order to limit energetic investment when not necessary while simultaneously enabling them to increase ejaculate availability if required (Wedell et al. 2002; Parker and Pizzari 2010). Strategic adjustments involve a variety of ejaculate traits, such as sperm number, sperm quality and seminal fluid composition, and are triggered mainly by variations in perceived levels of sperm competition, in partner availability and in partner quality (Wedell et al. 2002).

The existence of plasticity in ejaculate investment suggests that such response is adaptive, otherwise it would have been removed from populations in the presence of intense sexual selection (Bretman et al. 2011), and that producing large, high quality ejaculates must be costly, otherwise males would invest maximally at all times (Bretman et al. 2013). Investigating the costs of responding is crucial to understand why plasticity is not ubiquitous and to clarify the circumstances under which it is expected to evolve. In fact, not only some species and populations are flexible while others are not (e.g. Firman et al. 2013), but the same individuals may respond to a given environmental stimulus and not to others, despite these being probably all relevant for the efficiency of reproductive strategies (e.g. Bozynski and Liley 2003; Evans 2009; Pardo et al. 2018). These differences in plasticity may be explained by differences in costs and benefits of plasticity (Bretman et al. 2011). While ejaculate quality adjustment can result in higher paternity shares in competitive fertilizations (Sakaluk and Müller 2008; Bretman et al. 2009; Barbosa 2012; Bartlett et al. 2017), these may indirectly affect pre-copulatory competitiveness due to trade-offs between resources allocated to ejaculate production and traits associated with mate acquisition (e.g. Ramm and Stockley 2009; Cattelan et al. 2016). If the costs of upregulating sperm production are such as to impair male ability to obtain copulations, they may constrain the evolution of this response, as high post-copulatory competitiveness is only beneficial if males succeed at mating. Because a male's reproductive fitness is determined by his competitiveness during both pre- and post-copulatory selective episodes (Andersson and Simmons 2006; Cornwallis and Birkhead 2008), it is crucial to consider the whole set of reproductive traits when evaluating the consequences of strategic ejaculate adjustments.

Most studies on ejaculate plasticity test the competitiveness of alternative ejaculate phenotypes in a single context (usually the one that elicited the upregulation of ejaculate investment, i.e. high sperm competition, Bretman et al. 2009; Barbosa 2012), and rarely investigate how their relative fitness may vary in different environments (but see, Bretman et al. 2013). The costs of socially-cued anticipatory ejaculate adjustments

may be amplified in case of phenotype-environment mismatches, that is when the wrong response is produced (Harvanek et al. 2017).

Here we will use the guppy, *Poecilia reticulata*, to examine the consequences of anticipatory sperm adjustments on both male mating and insemination success. Guppies are live-bearing freshwater fish with internal fertilization (Magurran 2005). Both males and females mate with multiple partners, thus sperm competition plays a central role in their mating system (Evans and Pilastro 2011). Male reproductive success is determined, at the pre-copulatory level by female choice, which is based on male body coloration, male size and sexual behavior and, to a lesser extent, by male-male competition (Houde 1997). Males adopt two alternative mating strategies: they court females by performing sigmoid displays in order to obtain cooperative copulations or use gonopodial thrusting to attempt to forcibly inseminate females (Liley 1966; Houde 1997). At the post-copulatory level, both sperm competition and cryptic female choice occur. The outcome of the first is determined by the number and the swimming velocity of sperm transferred during copulation (Boschetto et al. 2011), while the second is influenced by male attractiveness (Pilastro et al. 2004; Pilastro et al. 2007), but also by male-female relatedness and MHC similarity (Gasparini and Pilastro 2011; Fitzpatrick and Evans 2014; Gasparini et al. 2015). In guppies, male reproductive strategies are largely flexible and depend on elements of the social environment, such as population density and sex ratio, which typically fluctuate both in space and time (Grether et al. 2001; Pettersson et al. 2004; McKellar et al. 2009). Males plastically switch between the two alternative mating strategies in response to the social context (Evans and Magurran 1999; Jirotkul 1999; Guevara-Fiore et al. 2009; Kiritome et al. 2012). Males' reproductive plasticity involves ejaculate investment, which is rapidly (i.e. over three days) adjusted to the perceived mating opportunities: males have larger sperm reserves and faster sperm when exposed to female stimuli ('sperm priming', Bozynski and Liley 2003; Gasparini et al. 2009). Ejaculate production and sexual behavior are known to be energetically demanding in this species (Devigili et al. 2013; Gasparini et al. 2013). Accordingly, upregulating sperm production imposes costs, as suggested by the reduction in male lifetime growth (Jordan and Brooks 2010), male longevity (Miller and Brooks 2005), and sperm viability (Cardozo et al. in prep) recorded in males maintained in visual contact with females as compared to males that are isolated from females. Trade-offs originating from these costs also involve male sexual behavior. In fact, exposure to females has been shown to be associated with a reduced investment in traits linked to mate acquisition, with males switching from costly courtship displays to less expensive gonopodial thrusting to obtain copulations (Devigili et al. 2015; Cattelan et al. 2016). These results suggest that fertilization success may be traded-off against mating success in the guppy. However, it is not known whether strategic ejaculate production actually affects, and in which measure, male pre- and post-copulatory success. In order to have a direct measure of male mating and insemination success associated with strategic ejaculate production we manipulated male perception of mating opportunities (high mating opportunities, HMO, or low mating opportunities, LMO) by keeping them in visual contact with three females (HMO males) or isolated from females (LMO males)

(Bozynski and Liley 2003; Gasparini et al. 2009). In a first experiment, each male was allowed, after the seven-day treatment, to mate with 3 pairs of virgin females encountered in sequence (6 females in total). In a second experiment, two males, one from each treatment, competed for access to a virgin female in two consecutive trials (2 females in total). Each experimental male underwent both treatments in random order. In both experiments, we recorded male sexual behavior, mating success and insemination success (i.e. the number of sperm transferred to the female during copulation and, for Experiment 1, the number of residual sperm available to males after their last copulation) and compared them within male across treatments.

MATERIALS AND METHODS

Study animals

We used descendants of wild-caught fish from the Lower Tacarigua river, Trinidad. In 2013 these guppies originated a large self-sustaining population maintained in semi-natural conditions in the tropical freshwater pool (46 x 4.4 x 0.4 m) of the greenhouse of the Botanical Garden of the University of Padova. In the laboratory, fish were maintained in large stock tanks with a balanced sex ratio and in which outbreeding was assured by periodically moving individuals across different stocks. The tanks were provided with aquatic plants and algae and their bottom was covered with mixed color gravel. Laboratory stock and all experimental fish were maintained under controlled temperature and lighting conditions (26 ± 1°C; 12: 12 h light/dark cycle, Philips TLD 36W fluorescent lamps). All fish were fed ad libitum twice a day a mixed diet of brine shrimp nauplii (*Artemia salina*) and commercially prepared flake food (Duplarin). Males used in the experiment were collected from stock tanks, whereas virgin females were kept in single-sex tanks. All fish were sexually mature (at least four months old) when used for the experiments.

Experimental design

Experiment 1

Male guppies (N=25) were collected from stock tanks and transferred to the isolation tanks in groups of four, to prevent sexual interactions and to avoid at the same time the stress produced by social isolation. The isolation tanks consisted of 80-l tanks divided by two opaque partitions into three equal sections, which contained aquatic plants and had a gravel-covered bottom; the same tanks were used later as treatment tanks. After 7 days, males were collected and stripped to empty their sperm reserves and equalize their initial conditions and hence individually placed in their treatment tank. Individuals were randomly assigned to either one of two treatments: “high mating opportunities” (HMO) or “low mating opportunities” (LMO). In the HMO treatment the male was housed in the treatment tank together with 3 non-virgin stimulus females, while in the LMO treatment the male was alone in the tank. The females were placed in a transparent perforated plastic drinks bottle (12 cm diameter), which allows both visual and olfactory contact with the male, but prevents any physical interaction (Cattelan et al. 2016). The bottle was left empty in the LMO

treatment. Twice a week, the stimulus females were moved among males of the HMO treatment, to potentiate the effect of the treatment (males tend to decrease their sexual interest for females as they become familiar and show renewed sexual interest when introduced to new sexual partners for the so called "Coolidge effect": Beach and Jordan 1956; Kelley et al. 1999). The use of non-virgin (and therefore most likely non-receptive) females, allowed us to minimize possible differences among males attributable to variation in female responsiveness. The treatment lasted 7 days; at the end of it, males underwent the mating trials. Mating trials took place in tanks similar to the isolation tanks.

The focal male was transferred to a tank where two virgin females had been acclimatizing for 7 days. The male interacted with the females for 45 minutes, during which we carried out observations of sexual behavior. At the end of the trial, the females were removed from the tanks and we proceeded to recover the inseminated sperm from their genital tract (see below). The male was allowed to recover for 45 minutes before being transferred to the tank of the second pair of females. The procedure was repeated as described above for a total number of 3 mating trials (6 females), at the end of which the male was stripped of his sperm reserves and photographed (see below). The male was then assigned to the alternative treatment and underwent a second series of mating trials.

Experiment 2

Similarly to Experiment 1, males underwent HMO and LMO treatments. While the type of stimulation and its aim were the same in the two experiments, some details of the treatment were different, but had been previously shown to be equally effective (Cardozo et al. in prep). Each experimental block consisted in two randomly chosen males and 4 virgin females. Briefly, after collection from the stock tanks, males were transferred to 1-liter individual tanks, where they were maintained for 7 days, after which they were stripped (see below), in order to equalize their initial sperm reserves. Males were then transferred to the treatment tanks, which consisted of 8-liter tanks, with aquatic plants and gravel-covered bottom. In each tank we placed either a bottle (see above) containing 2 non-virgin stock females (HMO treatment) or an empty bottle (LMO treatment). The treatment lasted 7 days, at the end of which males underwent the mating trials. Two males, one for each treatment, were transferred together to a 15-liter observation tank with gravel-covered bottom where one virgin female had been acclimatizing for 30 minutes. The tank also contained some juvenile individuals (<1 month), whose presence contributed to reducing the stress experienced by females by creating a more natural environment. Usually nor the males or the females directly interacted with the juveniles. Behavioral observations were carried out over a period of 30 minutes during which individuals were free to interact (see below). At the end of the trial, the female was transferred to an individual tank and subsequently underwent the sperm-recovery procedure (see below). The males were transferred to an individual tank and allowed to recover for 30 minutes, during which a second virgin female acclimatized in the observation tank. A second mating trial was carried out following the same procedure as the first. At the

end of the second mating trial the males were stripped to empty their sperm reserves before being assigned to the alternative treatment and then going through a second series of mating trials which took place as previously described.

Observation of sexual behavior

During the mating trials we recorded the total time (min) each male spent interacting and swimming within two body lengths of a female as a measure of the male's overall sexual interest in the female (hereafter: following duration, Cattelan et al. 2016). We also recorded the total number of courtship displays (sigmoid displays, SD, where the male positions himself in front of the female in an s-shaped posture and quivers) and forced mating attempts (gonopodial thrusts, GT, where the male attempts to coercively inseminate the female) performed by each male (Liley 1966). The focus of the behavioral observations was on males, therefore in Experiment 1 we did not take into account which female was the recipient of male sexual interest and mating attempts, and in Experiment 2 we recorded separately the sexual behaviors performed by each male. We also noticed all successful copulations (and the identity of the individuals involved).

Recovery of inseminated sperm from female genital tract and sperm count

To extract sperm from the female's gonoduct we followed established protocols (Pilastro and Bisazza 1999; Pilastro et al. 2007). The procedure was performed between 30 and 60 minutes after copulation. We anaesthetized the female by immersion in a solution of fish anesthetic MS222 (0.5 g/liter) and then we placed her on a special polystyrene support, to expose the gonopore. The procedure was carried out under a stereomicroscope (magnification 25X, ZEISS Stemi 2000-C). With a Drummond micropipette, we injected 3 µl of saline solution (NaCl 0.9%) into the female's gonoduct, then retrieved it and placed it in a 0.5 ml Eppendorf sample tube. We repeated this operation five times to ensure the recovery of all sperm present in the gonoduct. With a Gilson pipette, we measured the volume of the solution drained from the female and we diluted it 1:5 in saline solution. We counted the sperm contained in the solution using an 'improved Neubauer chamber' haematocytometer under an optical microscope (at 400X magnification) (Pilastro et al. 2002; Pilastro et al. 2007). The reliability of this technique was proved in previous studies (Pilastro et al. 2007), which demonstrated that the number of sperm retrieved from the female is significantly correlated with the number of sperm inseminated. In experiment 2, sperm were not recovered from the female if she had copulated with both males since it would have been impossible to distinguish each male's contribution.

Sperm collection from males and sperm count

At the end of the third mating trial we stripped males to collect residual sperm. Following an established procedure (Evans et al. 2003), we anaesthetized the individual (with the same procedure used for the females) and placed him on a slide under a stereomicroscope (magnification 6.5X). We also placed 1 µl of saline solution (NaCl 0.9%) on the slide to favor sperm collection. We swung the gonopodium back and

forward and then applied a gentle pressure on the abdomen to allow sperm release. Male guppies produce sperm packaged in discrete units, called spermatozeugmata or sperm bundles. We photographed the sperm bundles on the slide under the stereomicroscope (magnification 6.5X, Canon EOS 450D camera). The number of bundles produced by the male was measured from the photograph (using ImageJ software, http://rsbweb.nih.gov/ij/download.html).

Individual morphology: body size and color pattern

In experiment 1, after the stripping procedure or the sperm recovery procedure, males were photographed under the stereomicroscope (magnification 6.5 X, Canon EOS 450D camera) on millimeter paper for calibration. The images were then analyzed using ImageJ software to measure: body area (excluding both caudal and dorsal fin) and, in males, surface area of carotenoid spots (orange, yellow, and red, hereafter: orange).

Statistical analyses

Statistical analyses were performed in SPSS (version 21, IBM, Armonk, NY) and in Excel using Poptools (Hood 2011). Mean and their standard errors given in the figures are calculated from the raw data and have illustrative purposes only.

Sexual behavior

To test the effect of our treatment on male sexual behavior we ran Generalized Estimating Equations (GEE). The number of total copulation attempts was log transformed and analyzed with a normal distribution, the proportion of sigmoid displays on total copulation attempts was analyzed with a binomial distribution and a logit link function and following duration was analyzed with a normal distribution. In the analyses of Experiment 1, for all three variables we entered in the GEE (which included male identity as repeated subject factor, treatment and trial as within-subject factors): a) treatment (HMO, LMO), b) trial (1, 2, 3), c) mating success during the current trial (hereafter current partners, 0, 1, 2), d) mating success during previous trials (hereafter previous partners, 0-4) and e) interactions between treatment and each other factor. Male mating success in current and previous trials were entered in the model because of their expected effect on male sexual behavior: copulations may reduce male subsequent sexual behavior due to a decreased motivation to mate again with the same partner (Kelley et al. 1999) and to the reduction of male sperm reserves (which are positively correlated with male sexual activity, Matthews 1997), but interacting with non-responsive female may also reduce male sexual activity. In the analyses of Experiment 2, we entered treatment (HMO, LMO), trial (1, 2) and their interaction in the GEEs (which included male identity and pair identity as repeated subject factors, treatment and trial as within-subject factors). For all analyses, we then used a backward stepwise elimination procedure to exclude nonsignificant terms, starting from nonsignificant interactions (only results from the final models are shown).

Mating success

We ran a GEE with a multinomial error distribution and a cumulative logit function to analyze male mating success in each trial (0, 1, 2) of Experiment 1. Treatment, trial, body area (covariate), orange area (covariate) and the interaction between each variable and treatment were entered as predictors. Male morphology was entered in the model to account for its effect on female mate choice (Houde 1997). We used a backward stepwise elimination procedure to exclude nonsignificant terms. We compared male total mating success (the sum of male mating success in each of the three trials) in the two conditions using a Wilcoxon test.

In the Experiment 2, each pair of males was tested twice, reversing each male's treatment between trials. For example, in pair 1, male A was previously exposed to HMO and male B to LMO, whereas in the successive trials male A was previously exposed to LMO and male B to HMO. Different individual males (2) and females (4) were used for each experimental block (no. of blocks = 27). We determined each male's mating success as the number of females he mated with during the trials. When a male mated with two females, we considered mating priority (i.e. which of the two males mated first). This is because the first male to mate has a post-copulatory advantage (Magris et al. 2017) and mating order will reflect male relative pre-copulatory competitiveness. Since mating success of one male in each block was not independent from the success of the other male, we treated each block as an observation. We therefore calculated, for each block the proportion of matings obtained by males in the LMO condition over the total number of matings. If treatment did not affect male mating success the expected proportion is 0.5 (i.e. males have an equal probability to mate in the two roles). To test this hypothesis we randomized, within each block, the mating success of the males in relation to their treatment using the "Shuffle" function in PopTools (Hood 2011) to obtain a null distribution of mean mating success using a Monte Carlo simulation with 10,000 iterations. We then compared the observed mean mating success of males from the LMO treatment with the expected distribution assuming equal mating probabilities. Our prediction was that the observed mean mating success of males after LMO treatment was significantly larger than 0.5.

Insemination success

Number of inseminated sperm was log transformed. For the analysis of data from Experiment 1, we ran a GEE (which included male identity as repeated subject factor, treatment and mating rank as within-subject factors) with normal distribution, in which treatment, mating rank (covariate, indicating the order of the current mating to account for the number of females previously inseminated by the male in the whole mating trial, 1-6), male body area (covariate), orange area (covariate) and interaction between treatment and the other variables, were entered as predictors. Male morphology was included as it is known to affect male insemination efficiency (Pilastro and Bisazza 1999) and cryptic female choice (Pilastro et al. 2004). Again, nonsignificant terms were excluded from the final model. To analyze data from Experiment 2, we ran a GEE (including pair identity and male identity as repeated subject factors, treatment and mating rank as within-

subject factors) with normal distribution, which included treatment, mating rank (1 or 2) and their interaction.

The number of residual sperm bundles (log transformed) was analyzed with a GEE with normal distribution and in which we entered treatment, mating success and their interaction as fixed factors.

RESULTS

Experiment 1

A total of 25 males and 300 females were included in the study. We observed 160 copulations, and all males copulated at least once in both conditions. Results from the sperm-recovery procedure allowed us to check the accuracy of the observer in recording copulations during the behavioral observations. Of the 140 females that had not been observed copulating, 2 were found to have sperm in their reproductive tract (1.4%). From 5 females out of the 160 that we observed copulating, we did not retrieve any sperm (3.1%). In 3 cases the copulations that did not deliver any sperm were the last performed by the males, after which the stripping procedure revealed that they did not to have any residual sperm (leading to the conclusion that these males had already exhausted their sperm reserves when they copulated with the last partners). The other 2 cases probably originated from sloppy copulations during which the male failed to transfer any sperm even though his reserves were not empty. All observed copulations (including those which did not result in sperm transfer) and cases where sperm were retrieved even if copulation had not been observed were included when evaluating male mating success. Copulations resulting in no sperm transfer were excluded from the analysis of insemination success. Results of the models are shown in **Table 1**.

Sexual behavior

The number of copulation attempts decreased from the first to third trial and was negatively correlated with trial number and with mating success in the previous trials, but only the latter interacted significantly with treatment (**Table 1**), with males in the LMO treatment experiencing a steeper decline as the number of previous partners increased. Similarly, the proportion of sigmoid displays declined in subsequent trials and was affected by the number of current partners; this effect was significantly different between treatments, with males in the LMO treatment producing fewer SD in the trials in which their mating success was higher (**Table 1** and **Fig. 1a**). Instead. The number of previous partners did not affect the proportion of sigmoid displays. Time following the females declined from the first trial onwards and this decline was steeper in the LMO condition (**Table 1** and **Fig. 1b**). Mating success (previous or current) did not affect following duration.

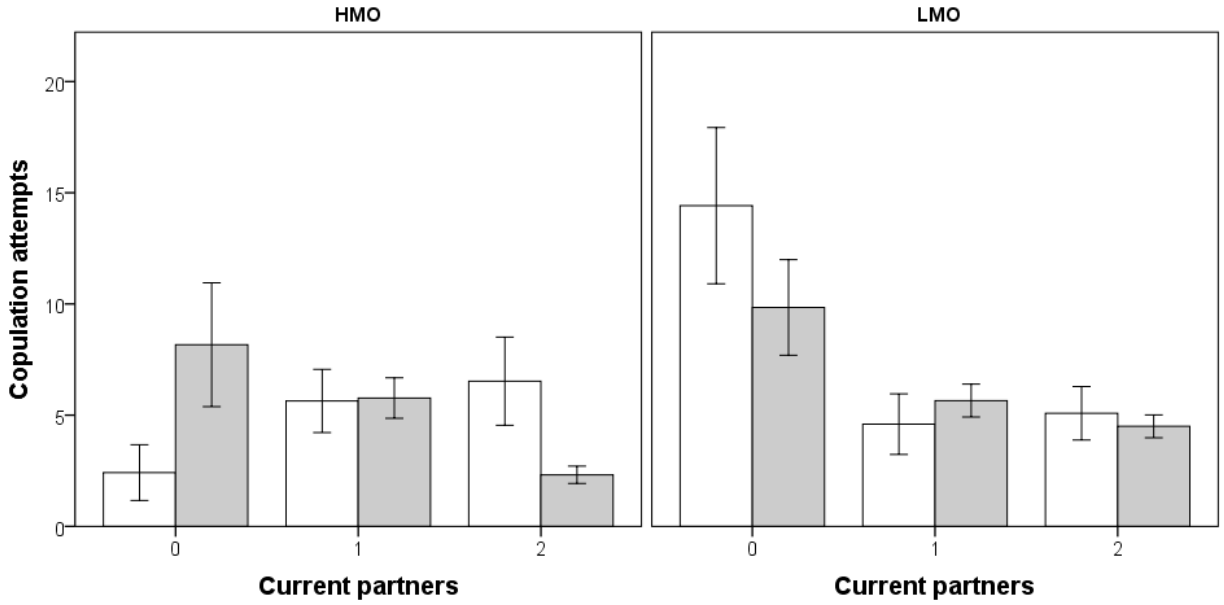

Figure 1a. Effect of treatment and mating success during the current mating trial on the number of sigmoid displays (white bars) and of gonopodial thrusts (gray bars). N=25 males, repeated measures.

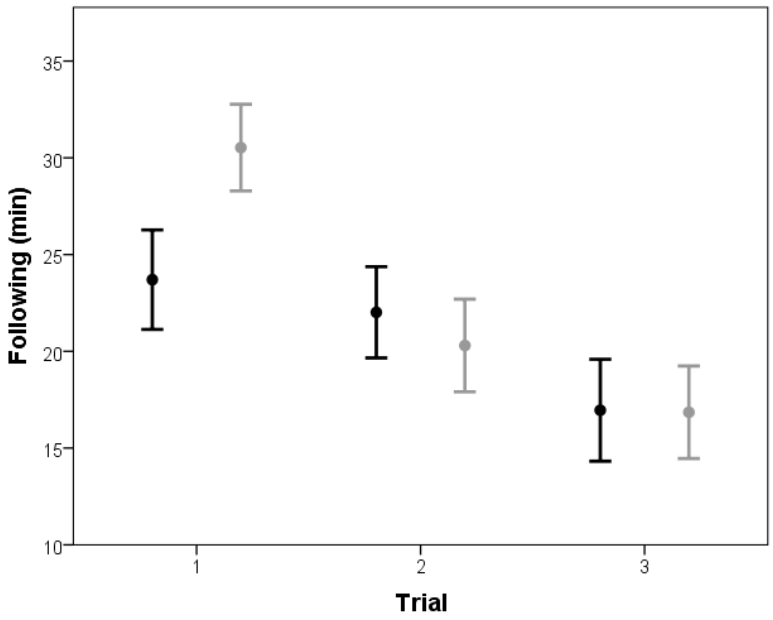

Figure 1b. Effect of treatment on following time in subsequent mating trials (mean ± se). N=25 males, repeated measures. HMO treatment is labelled in black; HMO treatment in gray.

Mating success

Male mating success declined in the third trial, but the decline was not affected by treatment (**Table 1**). Orange area positively influenced male mating success (although the result was only marginally significant),

while male body area did not. Overall mating success in the two conditions did not differ between treatments (HMO: 3.28±0.26, LMO: 3.20±0.29, Wilcoxon test: Z=-0.058, P=0.593), and was instead correlated within male (ρ=0.455, P=0.022).

Insemination success

The number of sperm inseminated decreased as the number of previous copulations increased, and the trend was similar between treatments (**Table 1** and **Fig. 2a**). The number of inseminated sperm was also affected by male size, with larger males inseminating on average more sperm, but not by male coloration. There was no difference between HMO and LMO treatments in the number of sperm inseminated during first copulation (HMO: 413,826±67,836, LMO: 484,522±79,478; paired t-test: t=-0.741, P=0.467); however there was a within male, between treatments correlation in the total and in the mean number of sperm inseminated (ρ=0.515, P=0.008; ρ=0.503, P=0.012, respectively).

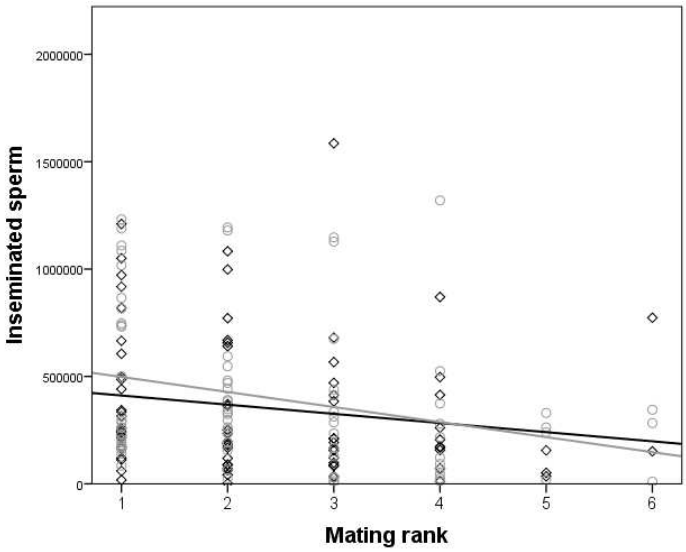

Figure 2a. Effect of treatment and mating rank on the number of sperm inseminated into each partner. Black diamonds represent the HMO treatment and gray circles represent the LMO treatment. Lines represent least-square regression fit and are presented for graphic purposes only. N=157 copulations.

The number of residual sperm bundles declined as male mating success increased, and this effect was stronger in males from the LMO treatment (**Table 1** and **Fig. 2b**). Males from the HMO treatment depleted their sperm reserves on average after 4.0±0.39 copulations, while male in the LMO treatment after 3.6±0.48.

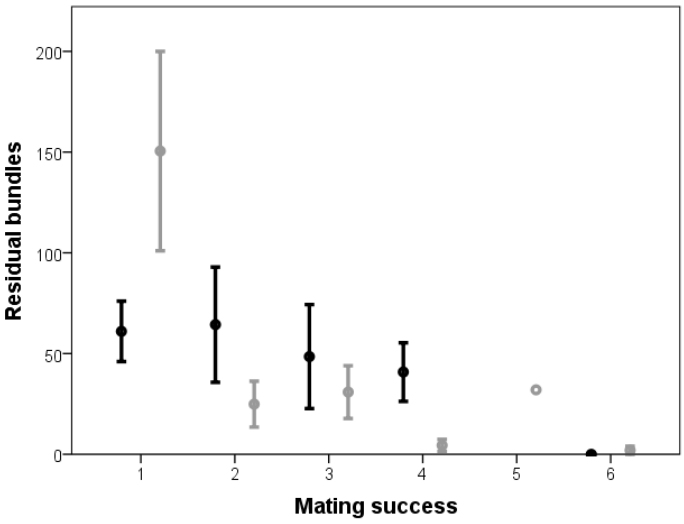

Figure 2b. Effect of treatment and mating success on the number of bundles stripped from males at the end of the trials (mean ± se). N=25 males, repeated measures. HMO treatment is labelled in black; HMO treatment in gray.

Table 1. Results of the GEEs that show the effect of treatment, trial and/or mating history on male sexual behavior, mating success and insemination success (for details see footnotes).

Response variable	Effect	Wald χ^2	*df*	*P*
Sexual behavior				
a) Total sexual attempts (SD+GT)[1,2]	Intercept	348.771	1	<0.001
	Treatment	0.412	1	0.521
	Trial	7.916	2	**0.019**
	Previous partners	4.324	4	0.364
	Treatment * Previous partners	16.262	4	**0.003**
b) Proportion of sigmoid displays[3]	Intercept	0.491	1	0.483
	Treatment	0.357	1	0.550
	Trial	9.228	2	**0.010**
	Current partners	10.147	2	**0.006**
	Treatment * Current partners	20.209	2	**<0.001**
c) Following (min) [2]	Intercept	223.114	1	<0.001
	Treatment	0.648	1	0.421
	Trial	20.053	2	**<0.001**
	Treatment * Trial	8.088	2	**0.018**
Mating success [4]				
	Treatment	0.002	1	0.967
	Trial	12.785	2	**0.002**
	Treatment * Trial	2.576	2	0.276
	Orange area (covariate)	3.821	1	**0.051**
Insemination success				
a) Inseminated sperm[1,2]	Intercept	691.784	1	<0.001
	Treatment	2.527	1	0.112
	Mating rank (covariate)	20.835	1	**<0.001**
	Treatment * Mating rank	1.069	1	0.301
	Body area (covariate)	10.840	1	**0.001**
b) Residual bundles[1,2]	Intercept	284.038	1	<0.001
	Treatment	1.921	1	0.166
	Mating success	291.304	5	**<0.001**
	Treatment * Mating success	14.420	4	**0.006**

Significant effects are in bold.

[1] Log transformed.

[2] Normal distribution.

[3] Binomial distribution with logit function.

[4] Multinomial distribution with cumulative logit function.

Experiment 2

A total of 27 pairs of males were tested in the two conditions and 108 females were involved in the experiment. For five blocks, behavioral data were not available, reducing the sample size for the analysis of behavioral traits to 22. Four pairs of males were excluded from the analysis of mating success, because none of the two individuals mated in either of the two conditions. We observed 40 copulations; in 1 case we did not retrieve any sperm from females who have been observed copulating. This case was included in the

analysis of mating success but was excluded from the analysis of insemination success (see below). In 5 cases both males copulated with the same female (i.e. 10 copulations), hence sperm were not extracted from the female. In other 2 cases we did not perform the sperm recovery procedure. The final dataset for insemination success included 27 copulations, involving 23 different males.

Sexual behavior

Males in the LMO treatment performed more copulation attempts (GEE, treatment: χ^2=13.365, P<0.001) and a higher proportion of sigmoid displays (GEE, treatment: χ^2=3.844, P=0.050, **Fig. 3a**) than males in the HMO treatment, while trial and its interaction treatment did not affect these variables (P>0.05). Following duration was affected by trial (GEE, trial: χ^2=5.539, P=0.019), but did not differ between treatments (GEE, treatment: χ^2=0.813, P=0.367, **Fig. 3b**).

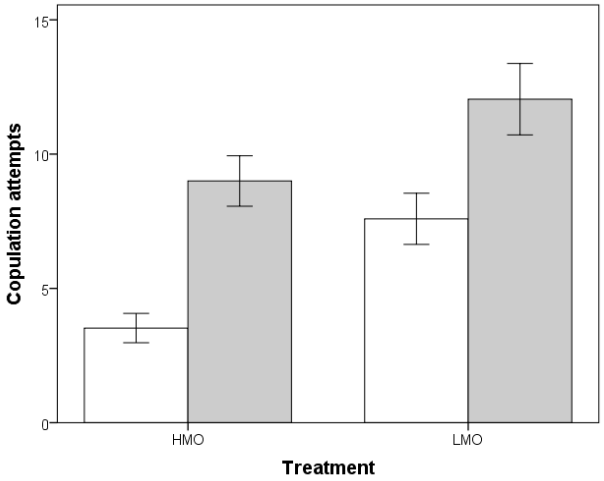

Figure 3a. Effect of treatment on the number of sigmoid displays (white bars) and gonopodial thrusts (gray bars). N=22 pairs of males, repeated measures.

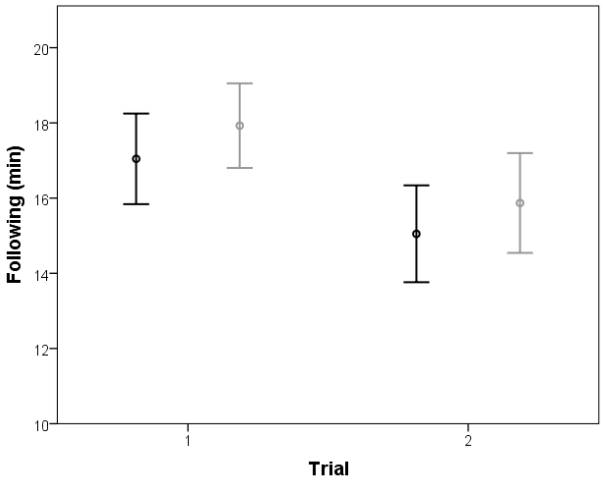

Figure 3b. Effect of treatment on following time in subsequent mating trials (mean ± se). N=22 pairs of males, repeated measures. HMO treatment is labelled in black; HMO treatment in gray.

Mating success

The overall mating success of the males (considering only the first copulation) summed up to 35 females, 13 in the HMO conditions and 22 in the LMO condition. The mean proportion of matings obtained by males after LMO treatment was 0.685 ± 0.086 SE (N=23). This proportion was significantly larger than expected if the probability of mating was the same in the two conditions (P=0.022, Monte Carlo simulation, 10,000 iterations, **Fig. 4**).

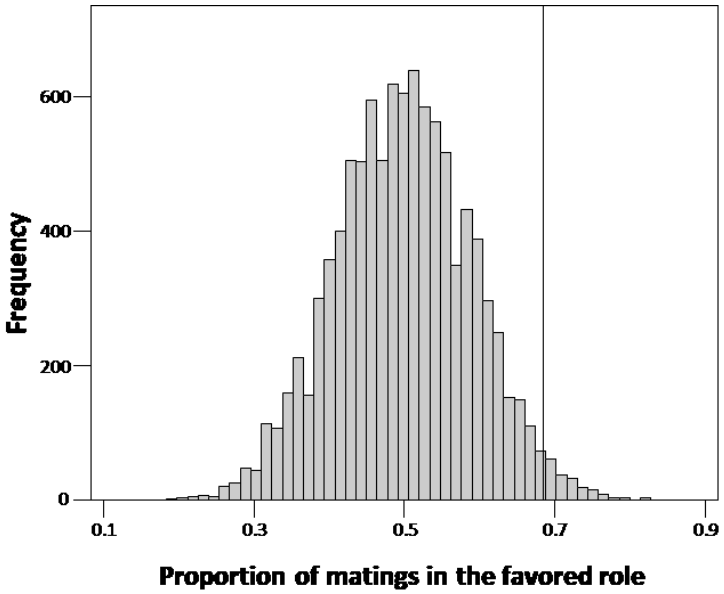

Figure 4. Distribution of the proportion of matings expected assuming equal probability of mating in the two roles obtained in a Montecarlo simulation (10,000 iterations) in which the mating success in the two roles (LMO and HMO) was randomized. The vertical line represents the observed proportion of matings obtained by the males in the LMO role.

Insemination success

The statistical model was unable to calculate the effect of the interaction between treatment and mating rank (first or second copulation). This was because data on number of sperm inseminated in the second copulation of a male were only available for 2 males. When we excluded the interaction we found that nor treatment or mating rank affected insemination success (GEE, treatment: χ^2=0.811, P=0.368; mating rank: χ^2=0.034, P=0.853; **Fig. 5**).

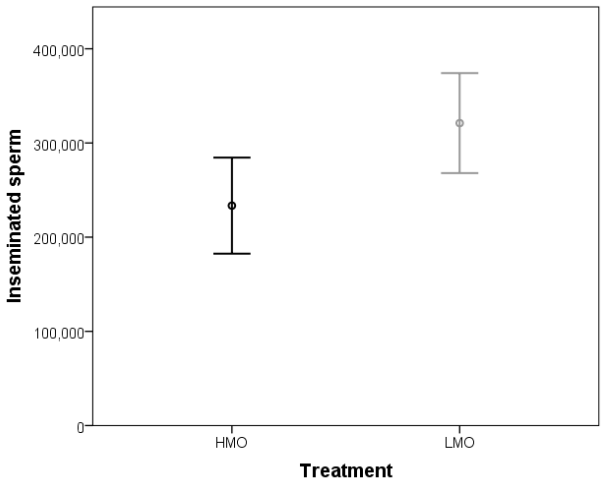

Figure 5. Effect of treatment on the number of sperm inseminated into each partner. N=27 copulations.

DISCUSSION

Plasticity in ejaculate production is a widespread phenomenon and its frequency may support the view that it is beneficial for male fitness, however, explicit tests should always be performed to confirm this assumption. To test the adaptive value of strategic ejaculate adjustments, it is important to evaluate the benefits as well as the costs associated with the response. While there is some evidence suggesting that the upregulation of sperm production and allocation can provide a fertilization advantage under conditions of sperm competition (Sakaluk and Müller 2008; Bretman et al. 2009; Barbosa 2012; Bartlett et al. 2017), an

increased investment in post-copulatory traits may also impose costs, in particular in expensive traits associated with mate acquisition (Parker 1998; Ramm and Stockley 2009). Our experiment investigated how, in male guppies, a female-stimulated upregulation of sperm production affects male mating and insemination success, both in the absence and in the presence of a rival. We confirmed previous results showing that after being exposed to females (HMO), males reduce their sexual behavior compared to when they have been kept isolated (LMO). When tested in multiple trials (Experiment 1), the difference in sexual behavior between treatments decreases in later encounters and as matings accumulate, due to a steeper decline in the intensity of sexual behavior recorded among males of the LMO treatment. These differences in sexual behavior did not affect, however, male mating success in the absence of competitors. Despite their larger sperm reserves, males responding to female presence did not transfer larger ejaculates. The post-copulatory advantage of the upregulation of sperm production became apparent only after several copulations, in the form of a reduced risk of sperm depletion (i.e. larger residual sperm reserves).

Exposure to females was negatively associated with the expression of all the three sexual behaviors that we measured (sigmoid displays, gonopodial thrusts and following). While this effect had been previously described (Devigili et al. 2015; Cattelan et al. 2016), it was not known how male mating history could interact with the response to female presence to influence male sexual activity. Indeed, we observed that, as copulations accumulated, males reduced their investment in sexual behavior and that such decline was steeper in the LMO condition, resulting in males from the two treatments expressing similar sexual activity after mating with several partners. However, mating success differentially affected individual sexual behaviors. The number of total copulation attempts was influenced by male mating success in previous trials. This pattern was expected because copulation attempts are known to positively correlate with number of available sperm (Matthews 1997; Pilastro and Bisazza 1999), which obviously decreases after copulation. The proportion of sigmoid displays, instead, depended on number of partners during the current trial, with males in the LMO treatment performing fewer sigmoid displays in trials with higher mating success and the opposite being the case for their counterparts. This result may indicate that males in the LMO treatment were more persistent than their counterparts when encountering reluctant females. Following duration, in contrast, was unaffected by male mating history, but decreased in subsequent trials (also in Experiment 2), suggesting it is the behavior more strictly constrained by energy depletion. Copulation attempts also decreased across subsequent mating trials in Experiment 1, although novel females were introduced at each new trial, suggesting that the decline was not caused by habituation (i.e. Coolidge effect, Kelley et al. 1999), but rather by exhaustion. These results provide further support to the evidence that sexual behavior is highly costly for male guppies (Devigili et al. 2013; Rahman et al. 2013). In Experiment 1, mating rate decreased in subsequent trials in both treatments, probably as a result of the reduction in sexual behavior intensity.

Mating success was significantly influenced by treatment in Experiment 2, when two males directly competed for the access to females. This result confirms previous findings by Bretman et al. (2013) in *D. melanogaster*,

and more generally confirms that choosiness is amplified in binary choice tests as compared to no-choice tests (Edward 2015)), in particular if males can directly interact (Hunt et al. 2009).

We detected no effect of treatment on patterns of sperm insemination in either experiment and we found, instead, that the number of sperm inseminated was repeatable between treatments and was positively affected by male size (as previously shown, Pilastro and Bisazza 1999). This suggests that insemination success is associated with male intrinsic quality and the perception of it by females, who probably have a strong control over sperm transfer during copulation (Pilastro et al. 2004; Pilastro et al. 2007). We also found that sperm transfer decreased in subsequent copulations as a result of progressive depletion of sperm reserves. Two alternative predictions could have been made concerning the effect of treatment on insemination patterns. On the one hand, since the number of sperm delivered positively correlates with the amount of sperm available (Pilastro and Bisazza 1999; but see Evans and Magurran 2001), female-stimulated males, which have greater sperm reserves, could have been expected to deliver larger ejaculates per copulation. On the other hand, the opposite pattern could have been observed if female-stimulated males, which were foreseeing multiple mating opportunities, reduced sperm allocation per female to strategically partition their sperm reserves (Warner et al. 1995). In addition, males from the LMO treatment could have been expected to inseminate larger ejaculates due to cryptic female choice based on courtship rates, since female guppies are known to have a post-copulatory preference for males courting at higher rates (Evans and Magurran 2001) and to be able to manipulate the amount of sperm received during copulation to favor preferred partners (Pilastro et al. 2007). While strategic partitioning coupled with larger ejaculates in males from the HMO treatment could have determined the similar insemination success observed in the two treatments, this appears unlikely. In fact, the observation that, as matings followed, the amount of sperm transferred at copulation progressively decreased (and that the pattern was similar between treatments), suggests that male guppies, including when they anticipate high female availability, do not strategically partition ejaculates across subsequent copulations.

Despite this lack of strategical allocation, the upregulation of sperm production provided males from the HMO treatment with a reduced risk of sperm depletion, which enables them to potentially achieve higher mating rates. From this point of view, guppies seem less sophisticated than fruit flies: Linklater et al. (2007) compared ejaculate allocation strategies between two lines of *D. melanogaster* that had an evolutionary history of maintenance at either male- or female-biased sex ratios. Similarly to us, they found that the female-biased group benefited from a reduced risk of ejaculate depletion, but the two groups of males also differed in the rate of decline in ejaculate allocation across subsequent matings.

Altogether, our study indicates that male response triggered by exposure to females entails a significant cost in terms of reduced mating success. Such reduction is likely to originate from a trade-off between sperm production and male sexual behavior. Our results have important consequences for the interpretation of this

form of plasticity. Firstly, they contribute to explain why male guppies modify sperm production to respond to changes in female availability, but appear not to be sensitive to variations in the level of sperm competition (Evans 2009), a social factor that is widely recognized as a strong stimulus for ejaculate adjustments (Kelly and Jennions 2011). The fact that the increased investment in post-copulatory traits appears to compromise male ability to obtain copulations when another male is present suggests that competition for access to females may cancel the potential benefits associated with this response. Secondly, when the reduction in sexual activity associated with sperm upregulation was first observed in guppies (Devigili et al. 2015), it has been proposed that besides representing a trade-off, it may alternatively reflect a change in the optimal reproductive strategy associated with different mate encounter rates. Indeed, the finding that male guppies adjust their courtship effort on their recent history, as this serves as a proxy for the likelihood of encountering mates in the future (Jordan and Brooks 2012), could have supported this hypothesis. However, our discovery that the switch in behavior reduces male pre-copulatory competitiveness provides instead support for the trade-off hypothesis.

If these ejaculate adjustments are costly and provide limited benefits, why this response evolved and why it was maintained in a species characterized by intense sexual selection? One explanation is that sperm depletion may represent a major constraint for male reproductive success (Preston et al. 2001; Sato 2018). In addition, in the wild, under harsher conditions and a more restricted diet, sperm reserves may be smaller and therefore may be exhausted after fewer matings compared to what we documented here (although resource limitation may have opposite effects on the benefits of ejaculate adjustments by amplifying trade-offs). Indeed, sperm depletion has been shown to be widespread across multiple taxa (Wedell et al. 2002) and to represent a powerful force shaping male and female strategies (Gray 1997; Sæther et al. 2001), and in particular sperm economy (Shapiro et al. 1994).

This is one of the few studies explicitly investigating the costs and benefits of strategic ejaculate adjustments on both pre- and post-copulatory traits and testing their ultimate effect on male reproductive success (Bretman et al. 2013). Our results demonstrate that costs and benefits of increased investment in the ejaculate are strongly context-dependent, a fundamental condition for the evolution of plasticity. Indeed, the female-stimulated upregulation of sperm production appears to be detrimental when mating opportunities are rare due to impaired ability to acquire mates (i.e. in case of mismatch), and to be, instead, beneficial with high female availability, when the disadvantage in terms of sexual behavior is reduced and the decreased risk of sperm depletion becomes apparent.

ETHICAL NOTE

Our study was approved by the Ethics committee of the University of Padova and by the Italian Ministry of Health (permit no. 72 /2017) and thus meets the guidelines for the care and use of research animals of the Italian Government.

ACKNOWLEDGEMENTS

We thank members of the Behavioural Ecology group at the University of Padova for logistic support in fish husbandry. This work was supported by a grant from the University of Padova (PRAT 2012 no. CPDA 120105) to A.P. and a Ph.D. fellowship from the University of Padova to M.M.

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Manuscript 2c

Strategic ejaculate adjustments and mismatches: are males paying sperm senescence costs?

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ABSTRACT

In many species, males show anticipatory plasticity for sperm production, which they adjust to match perceived mating opportunities. While the strategic adjustment of sperm production is likely to be beneficial, it may be also associated with costs, including those arising from the expression of a phenotype that is poorly matched to the conditions that males will subsequently experience. Mismatch costs are exacerbated by trade-offs between investment in the ejaculate and investment in other traits. Trade-offs, in fact, may determine a decrease in male competitiveness, due to impaired ability to obtain copulations or to reduced ejaculate quality. We explored mismatch costs using male guppies, which are known to increase sperm production, but reduce their investment in sexual behavior, when maintained in the presence of females. Increasing ejaculate size in the absence of females could impose costs that may be paid when an opportunity to mate eventually arises and that may be due to different processes, including increased sperm senescence. To explore mismatch costs, firstly we induced two groups of males to differentiate their sperm production by exposing them or not to female stimuli. Then we isolated them to prevent matings and have their sperm ageing. Finally, we compared ejaculate quality between the groups. Contrary to expectations, we found that female-stimulated males did not suffer from increased sperm senescence. These costs are probably minimized by the fact that sperm production is very plastic and can be quickly re-adjusted to a new environment. Other types of mismatch costs may be more relevant, for example in relation to trade-offs with sexual behavior.

INTRODUCTION

When females copulate with more than one male, competition to fertilize their eggs occurs among the ejaculates of different partners (Parker 1970). Sperm competition selects for increased sperm numbers and higher ejaculate quality (Parker 1998; Pizzari and Parker 2009). It is now widely recognized that producing large and high quality ejaculates is costly for males who have a limited resource budget to partition among different functions and among reproductive traits and processes (Dewsbury 1982; Van Voorhies 1992; Simmons 2011). As a consequence, males of many species have evolved the ability to modulate their ejaculate production and allocation depending on the context, in particular on the socio-sexual environment (Wedell et al. 2002; Kelly and Jennions 2011; Delbarco-Trillo 2011). Adjustments of investment in the ejaculate have been recorded in response to the perceived level of sperm competition, to partner quality and to the number of mating opportunities (for a review see, Kelly and Jennions 2011). An increase in ejaculate size and quality requires an energetic investment that is often paid by individuals in terms of trade-offs with reproductive and non-reproductive traits (Ramm and Stockley 2009; Bretman et al. 2013). Trade-offs can also involve other ejaculate traits; for example, an increase in sperm number can cause a reduction in sperm size (Oppliger et al. 1998) and viability (Cardozo et al. in prep). Moreover, the upregulation of sperm production could anticipate or exacerbate sperm senescence (Pizzari et al. 2008). Sperm senescence occurs as sperm age and progressively accumulate damage (Reinhardt 2007; Pizzari et al. 2008). It is caused by a number of factors, but mainly by thermodynamic damage and oxidative stress due to the accumulation of reactive oxygen species (Pizzari and Parker 2009). Sperm senescence can have multiple deleterious effects on fitness, impairing sperm performance (Levitas et al. 2005; Gasparini et al. 2014), fertilization ability (Jones and Elgar 2004; Jurema et al. 2005), offspring viability (White et al. 2008; Tan et al. 2013) and offspring reproductive competitiveness (Gasparini et al. 2017). Several pieces of evidence suggest that the upregulation of sperm numbers and velocity stimulated by social factors may determine early sperm senescence, supporting theoretical models predicting trade-offs between initial sperm swimming speed and sperm longevity (Ball and Parker 1996). Firstly, an across-species comparison showed that increased spermatogenesis rates (predicted by higher levels of sperm competition) are associated with increased DNA fragmentation (delBarco-Trillo et al. 2016) and intraspecific comparisons have highlighted inverse relationships between sperm velocity and sperm longevity (Levitan 2000; Helfenstein et al. 2008). In addition, in arctic charrs and in bluegills, sperm from subordinate/sneaker males, which swim faster than those of dominant/parental males, also show a faster decrease in sperm velocity after activation, suggesting a faster short-term ageing (Burness 2004; Haugland et al. 2009

While ejaculate allocation can be determined instantaneously whenever a relevant stimulus arises (e.g. Kilgallon and Simmons 2005; Cornwallis and Birkhead 2007), alterations of sperm production are usually associated with more substantial lag-time between sensing and responding to environmental cues. Lag-time can produce temporary phenotype–environment mismatches with consequent fitness costs (Dewitt et al.

1998) (see below). Therefore, to produce their responses at the right time, males need to rely on indirect cues predicting impending environmental shifts and initiate phenotypic change before the appearance of relevant environmental factors (anticipatory plasticity, Kasumovic et al. 2011). However, environmental cues may not always be reliable and individuals may misinterpret them, again resulting in phenotype-environment mismatches, with consequent fitness costs (Dewitt et al. 1998; Harvanek et al. 2017). The most obvious costs associated with mismatches originate if individuals downregulate their sperm production when the context would have required a large investment, resulting in reduced sperm competitiveness and increased risk of sperm depletion (Bretman et al. 2012; *Manuscript 2b*). However, upregulating sperm production in contexts that do not require it can also be costly. Firstly, because the increased investment does not provide any fertilization return and thus represents a waste of resources. Secondly, because mismatch costs may be exacerbated if the upregulation of target traits constrains the expression of non-target traits (i.e. trade-offs). The expression of the wrong phenotype will result in males who have upregulated their sperm production gaining no fertilization benefit, and instead potentially suffering from reduced competitiveness due to trade-offs.

Investigating mismatch costs requires testing alternative phenotypes across the various environments in which each phenotype is purported to be adaptive (Dewitt et al. 1998). To do so, individuals should be stimulated to produce different phenotypes by manipulating their perception of the environment, and then transplanted in the opposite environment to compare their fitness (Kasumovic et al. 2011). Empirical evidence has shown that failing to produce the inducible phenotype that would be beneficial in a given environment could generate costly mismatches. For example, under conditions of high sperm competition, males not upregulating their ejaculate investment gain lower paternity shares (Bretman et al. 2009; Barbosa 2012). Currently, little is known about mismatch costs in reproductive contexts (but see, Harvanek et al. 2017). Here we will use the guppy to test potential costs of phenotype-environment mismatches associated with strategic sperm production. Specifically, we will explore the costs, in terms of ejaculate competitivity, that may be paid by males when they upregulate sperm production and subsequently do not mate, resulting in ejaculate senescence.

Guppies are small freshwater fishes native to central America, characterized by high levels of sexual activity and promiscuity (Magurran 2005). Male reproductive success is influenced mostly by male body coloration, sexual behavior and ejaculate quality (Houde 1997; Magurran 2005; Devigili et al. 2015). Male guppies plastically switch between two alternative mating strategies: they perform courtship displays (i.e. sigmoid displays) to obtain cooperative copulations or they engage in gonopodial thrusts to forcibly inseminate the female (Liley 1966; Houde 1997; Pilastro and Bisazza 1999). Since females mate with multiple partners (Evans and Magurran 2001), sperm competition is intense (Devigili et al. 2015), with male fertilization success being affected by sperm numbers and velocity (Boschetto et al. 2011). As the socio-sexual environment this species experiences is highly variable (Pettersson et al. 2004; McKellar et al. 2009), and as sperm production has

been shown to be costly (Devigili et al. 2013; Cattelan et al. 2016; Devigili et al. 2016), males have been selected to develop plasticity in reproductive strategies. Male guppies modulate their sexual behavior according to population density and sex ratio (Jirotkul 1999). They also adjust sperm production in response to perceived mating opportunities: when maintained in visual and chemical contact with females, they produce larger ejaculates and faster sperm (Bozynski and Liley 2003; Gasparini et al. 2009). This response appears to involve the last stages of spermatogenesis since adjustments can occur in as short as 3 days (Bozynski and Liley 2003; Gasparini et al. 2009), while spermatogenesis is known to require 36 days (22 days for spermatocytogenesis and 14 days combined for meiosis and spermiogenesis) in this species (Billard 1968; Billard 1969). These sperm adjustments entail phenotypic costs paid as trade-offs with male lifetime growth (Jordan and Brooks 2010), survival (Miller and Brooks 2005) and sexual behavior (Cattelan et al. 2016). In addition, a recent study has reported that the upregulation of sperm production is associated with a reduction of sperm viability and a decrease in competitive fertilization success after female sperm storage, suggesting a reduced lifespan of stored sperm (Cardozo et al. in prep). This may occur because faster sperm use up their energy reserves at higher rates or increase the production of intracellular oxygen radicals (ROS) and thus the risk of oxidative damage, hence reducing their lifespan (Reinhardt 2007; Pizzari and Parker 2009). Alternatively, increased spermatogenesis rates may reduce the efficiency of meiosis or sperm maturation, resulting in a higher incidence of sperm defects and DNA fragmentation (Jewgenow et al. 2009; delBarco-Trillo et al. 2016). Independently of its proximate causes, upregulating ejaculate investment may also affect sperm senescence rate before ejaculation, during male sperm storage. When sperm production is upregulated but there is no opportunity to mate, the post-copulatory benefits of the response cannot be realized, (*Manuscript 2b*). The relevance of this phenomenon may not be limited to phenotype-environment mismatches. An analogous scenario may involve unattractive males who correctly increase their sperm production in the presence of many females, but then do not succeed at mating.

Here we took a step further and tested potential senescence costs arising in case of phenotype-environment mismatch. We exposed a group of males to stimuli from three females and while males form another group were kept isolated, in order to induce the first to increase their sperm production, and the latter to keep it consistently low. Then we removed the females and left each male isolated simulating a context where no mating opportunity arises. Stimulated males experienced a mismatch between the conditions they had anticipated (many mating opportunities) and those they encountered (no mating opportunity). Finally, we tested ejaculate traits. Since prolonged male sperm storage is known to negatively affect sperm quality in this species (Gasparini et al. 2014), we predicted that, after the experimental treatment, all males would have slower and less viable sperm, but that the effect would be stronger in stimulated males compared to their counterparts. This would result from two phenomena. Firstly, stimulated males would have filled sperm reserves quickly after being exposed to the females, while non-stimulated males maintaining spermatogenesis at low rates would have filled them gradually over a longer interval, resulting in sperm of

the former being older than sperm of the latter. Secondly, the increase in sperm production rates may have caused sperm from stimulated males to experience earlier, or more severe, senescence.

MATERIALS AND METHODS

Study animals

Fishes used in this study were descendants of wild-caught fish from the Lower Tacarigua river (Trinidad) that live in semi-natural conditions in a large pool (46 x 4.4 m, h. 0.4 m) at the Botanical garden of the University of Padova. In the laboratory, they were maintained in large stock tanks (150-litre tanks containing approximately 150 individuals of all age classes). We maintained a balanced sex ratio in the stock tanks and ensured outbreeding by periodically moving individuals across different stocks. The bottom of the tanks was covered with mixed color gravel and the tanks were provided with aquatic plants and algae. We maintained water temperature between 25 and 27 °C and we set illumination on a 12:12 h light:dark cycle (Philips TLD 36W florescent lamps). We fed all fish twice a day, ad libitum, with a mixed diet of brine shrimp nauplii, *Artemia salina*, and commercially prepared flake food (Duplarin; see Magris et al. 2017 for details on fish maintenance). For this experiment, we collected the males from tanks containing individuals of known age, and we collected stimulus females from stock tanks. All fish were sexually mature (at least 4 months old) when used for the experiments, males were four to five months old.

Experimental design

We collected the males from their tanks and we stripped them to empty their sperm reserves (Strip 0). We then isolated males in individual tanks (1 l) for 7 days, to allow them to replenish their sperm reserves. We stripped males again (Strip 1) and analyzed their ejaculate traits (see below, Measure 1), before randomly assigning them to one of two treatments. Treatment tanks had a capacity of 8 l tanks and were divided in two halves by a transparent, perforated partition that allowed visual and olfactory contact between the two parts, but prevented physical interactions and copulations. We covered the bottom of the tanks with gravel and placed java moss in the water. In the high mating opportunities treatment (HMO), we placed one male on one side of the partition and 3 stimulus females on the other side. In the low mating opportunities treatment (LMO), we placed one male in one half of the tanks, while the other half was left empty. These treatments are known to produce differences in ejaculate traits between HMO and LMO, since female presence induces males to produce more and faster sperm (Bozynski and Liley 2003; Gasparini et al. 2009). After 3 days we removed the stimulus females and we kept males isolated in their treatment tanks for other 9 days (see Fig 2). This phase simulated a context where no mating opportunity arises. Finally, we stripped males again (Strip 2) and analyzed ejaculate traits (Measure 2).

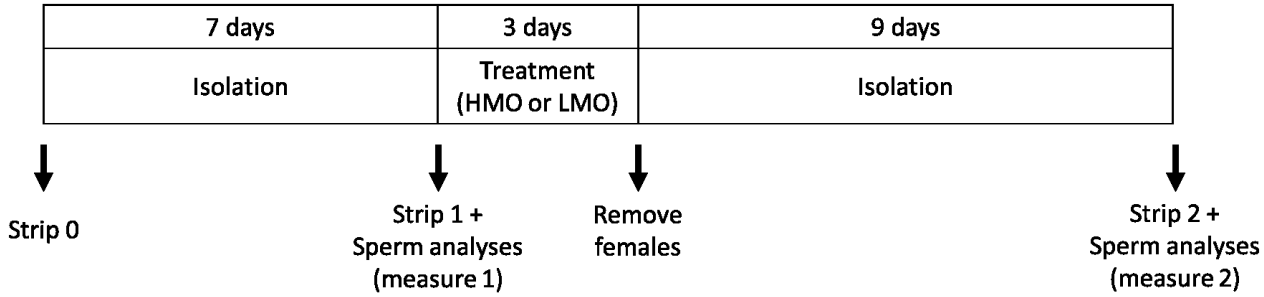

Figure 1. Schematic representation of the experimental design.

Sperm Collection

We collected sperm from each male following an established procedure (Evans et al. 2003). We anaesthetized a male by immersion in a MS222 solution and placed him on a slide under a stereomicroscope. Then, we added 1 ml of saline solution (NaCl 0.9%) to favor sperm collection. To perform sperm stripping we repeatedly swung the gonopodium back and forward and then gently applied pressure on the male's abdomen, to cause sperm release. We then transferred the male back to its individual tank where it was revived in conditioned water. Sperm in this species are packaged in discrete units, called spermatozeugmata or sperm bundles, which can be easily collected with a pipette. The slide with the sperm bundles was photographed under the stereomicroscope (magnification 6.5 X, Canon EOS 450D camera) for subsequent sperm count. We then split the ejaculate into different aliquots for sperm velocity (3 bundles, see below) and sperm viability (50 bundles) analysis (see below).

Sperm number, sperm velocity and sperm viability

We measured the number of bundles produced by the male from the photograph taken after stripping (using ImageJ software, http://rsbweb.nih.gov/ij/download.html).

To perform the sperm velocity assays, we used a Drummond pipette to collect 3 intact sperm bundles from each male and place them on a multi-well slide into 3 µl of activating medium (150 mM of KCl and 2 mg/ml bovine serum albumin, see Billard and Cosson 1990). We measured swimming velocity of the sperms as they were moving away from the opening bundles and we recorded it using a Hamilton-Thorne computer-aided semen analyzer (CASA: CEROS, Hamilton-Thorne Research, Beverly, MA, USA). The CASA takes three sperm velocity parameters: average path velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s) and straight line velocity (VSL, µm/s). The threshold between static and motile cells was set at VAP =25 µm/s, VSL= 20 µm/s and velocity was recorded only for motile sperms.

To assess sperm viability we used the live⁄dead Sperm Viability Kit (L-7011; Molecular Probes Inc., OR, USA), a fluorescence-based assay, which includes a membrane-permeant nucleic acid stain (SYBR14) which labels live sperm with green and a membrane-impermeant stain (propidium iodide) which labels dead or damaged sperm with red (only cells with intact membrane were considered viable). We measured viability immediately

after sperm velocity analyses. We collected 50 sperm bundles from each male and transferred them into a 0.5-ml Eppendorf tube containing 40 µl of saline solution. We broke the sperm bundles by vortexing the samples for 90 seconds. We transferred 6 µl of the mixture to an empty 0.5 ml Eppendorf tube, to which we added 2 µl of SYBR14 stain and, after 9 minutes, 2 µl of propidium iodide. We carried out all the operations involving the stains in low illumination conditions in order to prevent damages to the UV-sensitive compounds. We placed the sample on a microscopic slide and gently covered it with a coverslip. We recorded fluorescent images of samples using a X20 objective on a Leica 5000 B microscope (Leica Microsystems, Wetzlar, Germany) with a digital camera (DFC480; Leica Microsystems, UK) and we stored them using Leica IM500 image-manager software. We then assessed the proportions of live and dead sperm from images, using the software Image J.

Statistical analyses

We performed all statistical analyses with R Studio (version 3.2.5). We tested the correlation across the three measures of sperm velocity. Confirming previous data (Devigili et al. 2015; Magris et al. 2017), these parameters were highly correlated (VAP-VSL: $R=0.95$; $p<0.01$; VAP-VCL: $R=0.66$, $p<0.01$; VCL-VSL: $R=0.47$, $p<0.01$). Since VAP is the most common measure of sperm velocity used in guppies (e.g. Evans 2011; Barrett et al. 2014; Fitzpatrick et al. 2014); we considered only VAP in our subsequent analyses. To test the effect of our treatment on sperm number and velocity we used the "lmer" function (package lme4) to run linear mixed-effects models (LMM). To analyze sperm viability we used the function "glmer" to run generalized linear mixed-effects models (GLMM) with binomial distribution. All models included treatment, order of measure (1 or 2) and their interaction as fixed effects and male identity as random effect. The binomial model was checked for overdispersion and an observation level random effect was added to account for it.

For all models, the statistical significance of fixed effects and interactions was assessed based on the 95% credible intervals (CI) around the mean (β). We used the "sim" function (package arm) to simulate the posterior distribution of the model parameters and values were extracted based on 2000 simulations. We consider an effect to be significant when the 95% CI did not overlap zero. We used visual assessment of the residuals to evaluate model fit.

RESULTS

Sperm number increased from measure 1 to measure 2, but there was no effect of treatment on this change (**Table 1**). Sperm velocity and viability were not affected by treatment and did not change between measure 1 and measure 2 (**Table 1**, **Fig 2**).

Table 1. Effect of treatment and measure number on the expression of target traits: results of the generalized linear mixed-effects model (GLMM). All models included treatment, measure (first and second) and their interaction as fixed effects and male identity as random effect (for details see footnotes).

Response variable	Effect	β *Estimate*	*SE*	*95% CI*
Sperm number[1]				
	Treatment	17.920	46.781	-75.45, 108.61
	Time	104.909	18.883	**67.02, 143.05**
	Treatment * Time	10.674	26.142	-42.42, 62.19
Sperm velocity[1]				
	Treatment	3.296	5.836	-8.58, 15.08
	Time	3.898	2.604	-1.32, 9.40
	Treatment * Time	0.144	3.605	-7.09, 7.37
Sperm viability[2]				
	Treatment	0.048	0.458	-0.84, 1.00
	Time	-0.073	0.207	-0.47, 0.33
	Treatment * Time	0.112	0.286	-0.47, 0.67

Significant effects are in bold.

[1] LMM (Gaussian distribution).

[2] GLMM with Binomial distribution. Overdispersion was corrected by creating a variable that adds a progressive number to each data point and entering it as random effect.

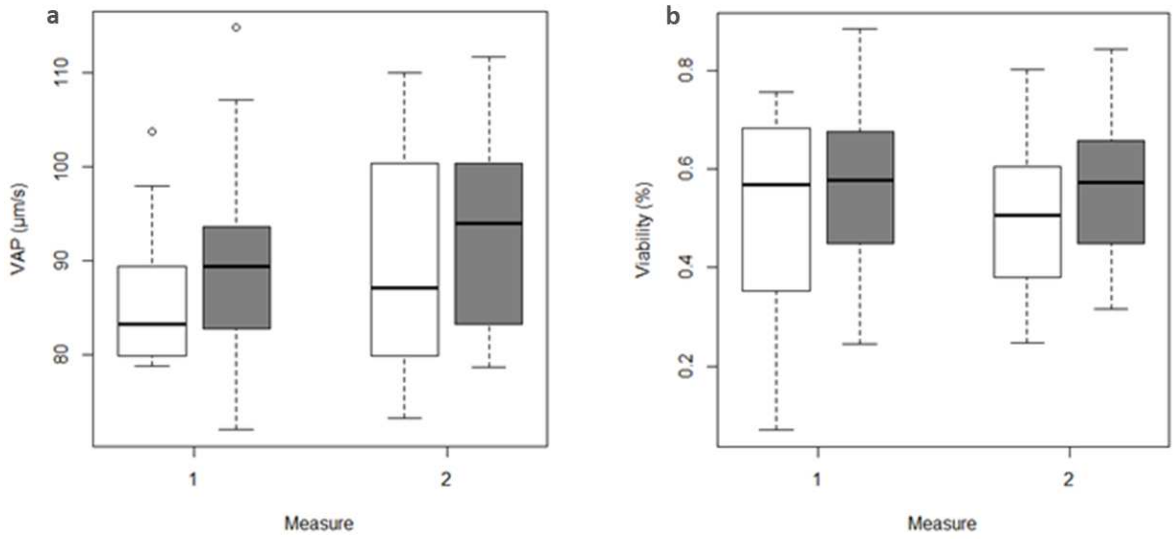

Figure 5. Effect of treatment and measure number on sperm velocity (a) and viability (b) (mean ± SE); open bars represent the HMO group and solid bars represent the LMO group.

DISCUSSION

Male guppies show anticipatory plasticity for sperm production: they increase the number of sperm and their velocity when anticipating many opportunities to mate (Bozynski and Liley 2003; Gasparini et al. 2009). The increase in investment is costly and it has been shown that males trade it off with the viability of freshly ejaculated and female-stored sperm (Cardozo et al. in prep). These costs may be further exacerbated when males express a phenotype that is not correctly matched to the environment. One of such costs may be that stored sperm may senesce at a faster rate than the stored sperm of males that did not burst their sperm production. We tested the hypothesis that a mismatch occurring when males upregulate their sperm

production, but subsequently do not have opportunities to mate, increases senescence rates of male-stored sperm. In contrast with our expectations, we found that sperm from males which were exposed to female stimuli, and thus should have upregulated their sperm production, did not show more pronounced signs of senescence after male storage.

In guppies this plastic response to perceived mating opportunities occurs within few days (3-7) and it is fully reversible (Cattelan et al. 2016). In our experiment, males of the two groups were exposed to a different environment for 3 days, but then experienced the same conditions of isolation for 9 days. Since this response in sperm production is plastic, males of the high mating opportunities treatment probably adjusted quickly to the new conditions when females were removed. At the time when ejaculates were collected for Measure 2, males will have downregulated sperm production for nine days. It is possible that sperm produced during 9 days of isolation outnumbered those produced during the 3 days of treatment, masking a potential effect of the plastic response on sperm senescence. The lack of effect may have also another explanation. While short-term plasticity in sperm number, velocity and viability in response to female presence has been repeatedly demonstrated in different experiments and populations (Gasparini et al. 2009; Bozynski and Liley 2003; Cattelan et al. 2016; Cardozo et al. in prep.), these effects become particularly evident using a paired design (i.e. each individual male experiences both female presence and deprivation). This is because there is a large inter-male variation in ejaculate quality (e.g. Cattelan et al. 2018), that exceeds the magnitude of the intra-male plastic response. For logistic reasons we did not follow a paired design (that will have required to quadruplicate the experimental design used by Gasparini et al. 2014), but one possibility is that the senescence in male-stored sperm associated with plasticity in response to female presence may have been obscured by this inter-individual variation in overall ejaculate quality. The third possibility is that, although senescence of male-stored sperm does occur in this species, its rate is not affected by ejaculate plasticity. Whatever the explanation of our results, we can conclude that post-copulatory costs of mismatches are probably minor as compared to pre-copulatory consequences of the ejaculate anticipatory plasticity (*Manuscript 2b).*

In the present study we did not detect a negative effect of male sperm storage on sperm quality, in contrast with Gasparini et al. (2014). In our study, the comparison of sperm quality was between 7 and 12-day old sperm, while they compared sperm that were 3 and 9 days old. If most sperm senescence takes place between 3 and 7 days, we might have sampled already "old" sperm in both measures. Indeed, in our experiment, sperm velocity as recorded in the first measure (7 days old) was lower than that measured in other experiments employing the same population (a direct comparison with Gasparini et al. 2014 would not be appropriate since fish came from two different populations) for 3-days-old sperm ($VAP_{7\ days}$=86.96±1.29; $VAP_{3\ days}$=95.48±1.92) (Magris et al., 2017).

In conclusion, we found that, in guppies, phenotype-environment mismatches arising in the context of strategic sperm adjustments do not entail measurable costs in terms of sperm senescence when males are prevented from mating. In fact, sperm stored by males which have upregulated their sperm production in response to a perceived increase in mating opportunities, and do not subsequently encounter any female, do not show a faster rate of senescence compared to their control counterparts.

ETHICAL NOTE

Our study was approved by the Ethics committee of the University of Padova and by the Italian Ministry of Health (permit no. 72 /2017) and thus meets the guidelines for the care and use of research animals of the Italian Government.

ACKNOWLEDGEMENTS

We thank members of the Behavioural Ecology group at the University of Padova for logistic support in fish maintenance. This work was supported by a grant from the University of Padova (PRAT 2012 no. CPDA 120105) to A.P. and a Ph.D. fellowship from the University of Padova to M.M.

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Animal Behaviour 131 (2017) 45–55

Contents lists available at ScienceDirect

Animal Behaviour

journal homepage: www.elsevier.com/locate/anbehav

Artificial insemination unveils a first-male fertilization advantage in the guppy

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ARTICLE INFO

Article history:
Received 12 December 2016
Initial acceptance 27 February 2017
Final acceptance 9 June 2017

MS. number: 16-01063R

Keywords:
cryptic female choice
Poecilia reticulata
postcopulatory sexual selection
sperm competition
sperm precedence
trade-up

Several factors are involved in determining the outcome of sperm competition. In addition to sperm number, sperm quality and male phenotype, insemination order is often associated with skewed paternity share. Patterns of sperm precedence can be produced by the mechanics of sperm storage and fertilization, or by active processes under male or female control. However, as males and females always interact during copulation, it is difficult to identify the mechanism responsible. The Trinidadian guppy, *Poecilia reticulata*, is a polyandric species characterized by last-male sperm precedence in natural matings. During such matings, females allow attractive males to inseminate more sperm by controlling copulation duration. We used artificial insemination to clarify the extent to which female control of sperm transfer influences the observed pattern of sperm precedence in this species. This technique allowed us to experimentally manipulate the number of sperm transferred and the timing of insemination. We found a significant first-male fertilization advantage. This advantage, however, declined as the time between insemination and parturition increased. Presumably, the anatomy and the physiology of the female genital tract favour egg fertilization by the first ejaculate inseminated, whereas sperm mixing is likely to be responsible for the reduction in first-male advantage associated with longer insemination–parturition intervals. Our results suggest that the last-male precedence detected after two consecutive natural matings is caused by cryptic female preference for attractive males associated with a female trading-up strategy (i.e. the second male is more frequently more attractive than the first male), rather than by insemination order per se. As the pattern of sperm precedence has important consequences for male reproductive strategies (for example mate guarding and male mate choice copying), unravelling its dynamic represents an important contribution to understanding the sexual behaviour of this model species.

Sperm competition occurs when a female mates with more than one male during the same reproductive cycle (Parker, 1970). Many factors related to male attractiveness, ejaculate characteristics and male–female genetic compatibility are known to affect paternity patterns under sperm competition (Fitzpatrick & Lüpold, 2014). Studies covering several animal groups with internal fertilization have shown that insemination order is often involved as well, with fertilization success biased in favour of either the first or the last mate (Birkhead & Hunter, 1990). Such patterns of sperm precedence (SP) have important implications for male postcopulatory success, as they influence, in turn, both male and female precopulatory strategies for increasing reproductive success and avoiding the costs of mating (Birkhead & Hunter, 1990). For example, last-male precedence (LMP) is usually associated with mate guarding and prolonged copulation (Parker, 1970), whereas first-male precedence (FMP) can lead to the evolution of a strong male preference for virgin females (Eberhard, Guzmàn-Gòmez, & Catley, 1993) and eventually to extreme male strategies such as traumatic inseminations observed in bed bugs, *Cimex lectularius* (Stutt & Siva-Jothy, 2001) and patrolling for about-to-emerge females in Dawson's burrowing bees, *Amegilla dawsoni* (Houston, 1991). In polyandrous species, SP is therefore crucial to understand the adaptive value of mating system dynamics in the two sexes.

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http://dx.doi.org/10.1016/j.anbehav.2017.07.009
0003-3472/

LMP is observed in most insects and birds (Birkhead, 1987; Danielsson, 1998; Parker, 1970; Simmons, 2001). In contrast, FMP is less widespread (e.g. Birkhead & Pringle, 1986; Elagoze, Poirie, & Periquet, 1995; Jones, Adams, & Arnold, 2002), but seems extremely common in spiders (Austad, 1984; Uhl, 2002). In other taxa, such as mammals, where sperm usually remain viable in female reproductive tracts for a very short time (Ginsberg & Huck, 1989), there are no general sperm precedence patterns and male fertilization success therefore largely results from the interaction between mating time/order and timing of ovulation (Birkhead & Hunter, 1990). The influence of insemination order on paternity shares is a subject that has been largely neglected in internal fertilizing fishes, with the only exception of guppies, *Poecilia reticulata* (Evans & Magurran, 2001; Neff & Wahl, 2004; Pitcher, Neff, Rodd, & Rowe, 2003).

Patterns of SP, related to insemination order, can result from different mechanisms, often interacting with one another to produce the fertilization outcome. The patterns of SP are determined by the interaction between the ejaculate and the female reproductive tracts and sperm storage organs (Walker, 1980), and are often linked to female anatomy. For example, FMP can be produced by mechanical constraints when one male's ejaculate serves as a physical impediment and limits sperm transfer by subsequent males, or when the ejaculates stratify and the first sperm to enter are in a more advantageous position for subsequent fertilizations ('first in, first out'; Austad, 1982; Uhl, 2002). Alternatively, first males can also bias paternity in their favour by placing mating plugs in the female genital openings and thus preventing or limiting the efficiency of subsequent inseminations (Masumoto, 1993; Parker, 1970). Finally, first-male advantage can result from active processes under female control, when females get most of their sperm stores from the first mate, and then 'top off' their storage organs with smaller quantities of sperm from additional mates (Jones et al., 2002). Similarly, LMP may result from different processes. LMP is typically observed when ejaculates form layers within the female sperm storage organs and the uppermost layer, derived from the last copulation, is in a favoured position to fertilize eggs ('last in, first out'; Birkhead & Hunter, 1990). In this case, last-male advantage may decrease with the time elapsed between insemination and fertilization, as a result of sperm mixing. LMP may also derive from the gradual loss of sperm from the female reproductive tract over time ('passive sperm loss'). Because of such loss, the proportion of the initial number of sperm stored after a copulation will progressively decrease with time and, if males transfer ejaculates of similar size, the first male will be disadvantaged (Lessells & Birkhead, 1990). In this case, last-male advantage will increase with the time elapsed since the previous copulations. 'Sperm senescence' can produce the same pattern: when successive inseminations occur, sperm from the first male will be older than those from subsequent copulations and may thus have reduced competitive fertilizing potential (Snook & Hosken, 2004; Tsubaki & Yamagishi, 1991; Winge, 1937). It has also been proposed that last males may take advantage of the prior 'buffering' of the hostile environment of the female reproductive tract by previous males' ejaculates, which could reduce their sperm mortality (Hodgson & Hosken, 2006). Alternatively, sperm can be displaced from the female reproductive tract by the 'flushing out' of one ejaculate by a subsequent one, or through an active removal operated by the last male during copulation (Birkhead & Hunter, 1990). Indeed, males of several species have evolved copulatory organs provided with specialized structures to scoop or brush out previously stored sperm (Cordero-Rivera, 2016; Waage, 1979; Wada, Takegaki, Mori, & Natsukari, 2005). LMP may also derive from the incapacitation of competitor's sperm when the seminal fluid from the most recent copulation interferes with the survival or fertilization capability of previously stored sperm (den Boer, Baer, & Boomsma, 2010). Finally, cryptic choice allows females to influence the outcome of sperm competition by favouring one male's sperm over another's both through differential discharge (Pizzari & Birkhead, 2000; Snook & Hosken, 2004) and transport to storage and fertilization sites (Bloch Qazi, Aprille, & Lewis, 1998; Tregenza & Wedell, 2002; and for recent reviews on cryptic female choice mechanisms see Firman, Gasparini, Manier, & Pizzari, 2017; Peretti & Aisenberg, 2015). When cryptic female choice is concordant with mate choice (i.e. it favours attractive males also at the postmating level, Pizzari & Birkhead, 2000), it may obscure otherwise expected sperm precedence patterns, for example by masking the effect of passive sperm loss from the female sperm storage organs or the senescence of stored sperm, or may itself generate a sperm precedence pattern, for example when matings with the most attractive males occur more frequently in a given order (Pitcher et al., 2003).

Understanding which mechanisms are responsible for the pattern of sperm precedence observed in a species is not straightforward: recognizing interactions of the ejaculate with the female reproductive tract or discriminating between male and female influence is complicated by the fact that they interact during copulation and several mechanisms often occur simultaneously (Manier et al., 2013). The use of artificial insemination can represent a useful tool to overcome this issue: by excluding male–female behavioural interactions before and during copulation, it has the power to highlight processes related to the mechanics of storage and fertilization. Furthermore, it allows the experimental manipulation of the number of sperm transferred and the temporal pattern of insemination (Bonnier & Trulsson, 1939), thus controlling for adjustments of male sperm allocation and female ejaculate manipulation influenced, for example, by male phenotype or the sociosexual context (Ala-Honkola & Manier, 2016; Kelly & Jennions, 2011; Pizzari & Birkhead, 2000). Successfully performed for the first time in the late 1700s on a bitch by Lazzaro Spallanzani (Foote, 2002), artificial insemination has been largely developed for the animal breeding industry first (bees, Watson, 1928; cattle, Salisbury & VanDemark, 1961; poultry, Bonnier & Trulsson, 1939; Lake & Stewart, 1978) and for conservation biology later (e.g. peregrine falcon, *Falco peregrinus*, Blanco, Wildt, Hofle, Voelker, & Donoghue, 2009; giant panda, *Ailuropoda melanoleuca*, Masui et al., 1989; chimpanzee, *Pan troglodytes*, Matsubayashi, Kumazaki, & Kamanaka, 1985), and is now performed on species as different as insects (Baer & Schmid-Hempel, 2000; Davis, 1965), garter snakes, *Thamnophis marcianus* (Quinn, Blasedel, & Platz, 1989), skates, *Raja eglanteria* (Luer, Walsh, Bodine, & Wyffels, 2007) and hamsters, *Mesocricetus auratus* (Smith, Koyanagi, & Yanagimachi, 1987). Artificial insemination has also been used to study sperm competition, for example in mice (Musialek, 1969), birds (Bonnier & Trulsson, 1939; Brillard & Bakst, 1990) and poeciliid fishes (Clark, 1950; Evans, Zane, Francescato, & Pilastro, 2003; Gasparini, Simmons, Beveridge, & Evans, 2010; Lodi, 1981), and it has been decisive in understanding the effect of insemination order on competitive fertilization success, in the domestic fowl, *Gallus gallus* (Birkhead, Wishart, & Biggins, 1995; Compton, Van Krey, & Siegel, 1978), the mallard, *Anas platyrhynchos* (Cheng, Burns, & McKinney, 1983), and the honeybee, *Apis mellifera* (Moritz, 1986). In these species, artificial insemination has produced the same patterns of sperm precedence as those from natural copulations, suggesting that they are determined by mechanics of sperm storage and fertilization rather than female behaviours.

Guppies are small, freshwater, live-bearing, internally fertilizing fish native to Venezuela and Trinidad (Magurran, 2005). Females show a mating preference for males with high courtship display rates and large areas of orange coloration (Houde, 1997). Female

choice, however, can be undermined by coercive copulations performed by males (Evans & Pilastro, 2011; Houde, 1997). As females mate promiscuously (Evans & Magurran, 2000) and they can store viable sperm for months (Greven, 2011; López-Sepulcre, Gordon, Paterson, Bentzen, & Reznick, 2013; Winge, 1937), postcopulatory sexual selection is intense in this species (Devigili, Evans, Di Nisio, & Pilastro, 2015; Evans & Pilastro, 2011; Hain & Neff, 2007; Neff, Pitcher, & Ramnarine, 2008). Male fertilization success is affected by several factors including sperm quality (e.g. sperm velocity and viability) and number (Boschetto, Gasparini, & Pilastro, 2011). The number of sperm transferred during copulation is controlled to some extent by females, which favour more attractive males (Pilastro, Mandelli, Gasparini, Dadda, & Bisazza, 2007; Pilastro, Simonato, Bisazza, & Evans, 2004). Male–female relatedness and MHC similarity also affect male fertilization success through mechanisms of cryptic female choice (Fitzpatrick & Evans, 2014; Gasparini & Pilastro, 2011; Gasparini, Congiu, & Pilastro, 2015). Finally, the outcome of sperm competition is influenced by insemination order as well, with the last male to mate being favoured when copulations occur not only in two successive reproductive cycles (Grove, 1980; Hildemann & Wagner, 1954; Winge, 1937) but also in the same one (Evans & Magurran, 2001; Neff & Wahl, 2004; Pitcher et al., 2003). This observed pattern of LMP, however, derives from natural copulations and thus does not allow us to disentangle male- and female-mediated effects. Indeed, LMP may also be explained by passive sperm loss or sperm senescence. Pitcher et al. (2003), however, showed that females adopt a strategy known as 'trading-up': they mate less selectively with a first male in order to ensure fertilization, but they become increasingly choosy with each successive mating opportunity. Experimental evidence indicates that females cryptically bias insemination success in favour of the most attractive male (Pilastro et al., 2004, 2007). This provides an alternative mechanism, mediated by female mating strategy (Pitcher et al., 2003), that may be responsible for the observed LMP. While these results may support the hypothesis that LMP is determined by female-mediated processes, conclusive evidence about the role of insemination order per se on fertilization success is still lacking. Here we used artificial insemination to isolate the effect of mating order from other behaviourally mediated female effects on sperm precedence in the guppy. We predicted that, once males are randomly allocated to the first or the last male role, and behavioural female effects on sperm transfer are controlled, the advantage of the last male should be reduced compared to that observed after natural copulations. If cryptic female choice is the only mechanism of sperm precedence operating in this species, we expected that insemination order would not affect fertilization success. Alternatively, we predicted a residual LMP effect if passive sperm loss, sperm senescence or last in – first out dynamics dominate the storage and the utilization of the inseminated sperm. In contrast, FMP should be observed under a first in – first out scenario.

METHODS

Study Animals

This study was conducted in May–October 2015 at the Biology Department of the University of Padova. We used descendants of wild-caught fish from the Lower Tacarigua river (Trinidad) that were maintained in our laboratory in large stock tanks (150-litre tanks containing approximately 150 individuals of all age classes) with a balanced sex ratio and in which outbreeding was ensured by periodically moving individuals across different stocks characterized by similar individual density. The bottom of the tanks was covered with mixed colour gravel and the tanks were provided with aquatic plants and algae. Water temperature was maintained between 25 and 27 °C and illumination was set on a 12:12 h light:dark cycle (Philips TLD 36W florescent lamps). All fish were fed ad libitum twice a day on a mixed diet of brine shrimp nauplii, *Artemia salina*, and commercially prepared flake food (Duplarin; see Pilastro et al., 2007 for details on fish maintenance). Males used in the experiment were collected from stock tanks, whereas virgin females were reared in single-sex tanks (capacity = 50 litres, containing approximately 25 individuals). All fish were sexually mature (at least 4 months old) when used for the experiments.

Sperm Collection

After having been collected from the stock tanks, males ($N = 56$) were stripped to equalize their initial sperm reserves. Males were then isolated for 3 days, during which they were exposed to visual and olfactory cues from three females (which were kept on the other side of a perforated, transparent, partition) to ensure complete replenishment of sperm reserves (Bozynski & Liley, 2003). Sperm were collected from each male following an established procedure (Evans et al., 2003). Briefly, males were anaesthetized using MS222 and placed on a slide under a stereomicroscope. Then, 1 ml of saline solution (NaCl 0.9%, kept at room temperature, about 22 °C, as was the slide) was placed on the slide to favour sperm collection. Sperm stripping was performed by repeatedly swinging the gonopodium back and forward and then applying gentle pressure on the male's abdomen. Sperm in this species are packaged in discrete units, called spermatozeugmata or sperm bundles, which can be easily collected with a pipette. The ejaculate was split into different aliquots for subsequent sperm velocity analysis (three bundles, see below) and for the artificial insemination of females (five or 40 bundles, see below).

Artificial Insemination

Males were paired at random and, within each pair, randomly assigned to the first or the second role, depending on the order in which they were used for the artificial insemination of two females per pair. For each male pair, two virgin females were randomly labelled A and B. Female A was inseminated with five bundles from the first male and, after 24 h, with five bundles from the second male; female B was inseminated with 40 bundles from the first male and, after 24 h, with 40 bundles from the second male. Within a pair, each male was tested in the same role with the two females. Artificial inseminations were performed following the protocol described in Evans et al. (2003). Briefly, for each male pair (number of male pairs, $N = 28$) two virgin females (total number of females, $N = 56$) were anaesthetized, placed under a stereomicroscope and, using a thin plastic tip fitted to a micropipette, artificially inseminated with five (female A) or 40 (female B) sperm bundles freshly collected from the first male in 2 µl of saline solution. Immediately after insemination, females were revived in conditioned water and then transferred to individual tanks. The procedure was repeated 24 h after the first insemination, using the same number of bundles (five for female A and 40 for female B) freshly collected from the second male in the pair. The role in each male pair was assigned randomly. Intervals and ejaculate sizes were chosen to match the most common situation observed in natural conditions. We chose a 24 h interval between the two inseminations because it allowed us to compare our results with those obtained from natural copulations (Pitcher et al., 2003) and therefore to test our prediction that LMP derives from behaviourally mediated directional cryptic female choice. Furthermore, this interval is also biologically relevant. While in the wild the interval between two consecutive copulations may be shorter than 24 h (Evans & Magurran, 2001), females are sexually receptive over a much longer period, usually 3–5 days

after parturition, during which they mate with several males (Houde, 1997; Magurran, 2005). An interval of minutes or hours may not be the most representative of the variation in the interval between successive matings under natural conditions. Indeed, unpublished results from our guppy population suggest that most matings probably occur 1 day apart (Cattelan, Morbiato, & Pilastro, 2015). In an experiment in which females could mate with males for 1 h per day on 5 consecutive days we found that seven of the 79 females that copulated with two or more males mated exclusively within 1 h (8.9%), 48 mated both within 1 h and within 1 or more days (69.6%) and 17 only within 1 day or more (21.5%). Among those females that did not mate within the same hour, the most frequent interval between consecutive copulations was 1 day (39%), followed by 2 (31%), 3 (21%) and 4 days (9%).

The bundle numbers with which females were inseminated (either five or 40) correspond approximately to 105 000 and 840 000 sperm, assuming an average content of 21 000 sperm/bundle (Boschetto et al., 2011) and cover the range of variation in the number of sperm transferred during natural matings (Pilastro et al., 2007). By inseminating either small (five plus five) or large ejaculates (40 + 40) we aimed to test whether the absolute numbers of sperm transferred during copulation influenced sperm precedence patterns, as may happen when female storage organs are saturated by a previous male's sperm. Females remained in isolation until they produced a brood (females that had not given birth to any offspring after 60 days following artificial insemination were returned to postexperimental tanks and excluded from the analyses). The interval between insemination and parturition was recorded as it is important to estimate the timing of fertilization and the duration of sperm storage before fertilization.

A tissue sample was collected from each male and female by fin clipping (males were fin clipped after sperm collection, whereas females after parturition; fin clipping was performed under anaesthesia) and stored in absolute ethanol at −20 °C. Newborn fish were humanely euthanized with an excess of anaesthetic (MS222) and their entire body was preserved as for the adults' fins until required for DNA analysis.

Sperm Velocity Analysis

Intact sperm bundles from each male ($N = 56$) were collected with a micropipette and placed on a multiwell slide into 3 µl of activating medium (150 mM KCl and 2 mg/ml bovine serum albumin, see Billard & Cosson, 1990). The velocity of the sperm moving away from the opening bundle was recorded using a Hamilton–Thorne computer-aided semen analyser (CEROS, Hamilton–Thorne Research, Beverly, MA, U.S.A.). The sperm velocity of motile cells included three commonly used parameters: VAP (average path velocity, µm/s), VCL (curvilinear velocity, µm/s) and VSL (straight line velocity, µm/s). The threshold between static and motile cells was set at VAP = 25 µm/s, VSL = 20 µm/s. Sperm velocity measures were based on an average of 156.91 ± 10.07 SE motile sperm tracks from at least three bundles. As in previous studies, VAP was highly correlated with both VCL and VSL (product-moment Pearson correlation coefficient: $r > 0.76$, $P < 0.001$). VAP is the most common measure of sperm velocity used in guppies (e.g. Barrett, Evans, & Gasparini, 2014; Devigili et al., 2015; Evans & Pilastro, 2011; Gasparini & Pilastro, 2011). We therefore considered only VAP in our subsequent analyses, although results were similar for VCL and VSL (not shown).

Paternity Assignment

Genomic DNA was extracted from offspring tissues using the Chelex protocol (Walsh, Metzger, & Higuchi, 1991), and from adult fin clips using a salting-out protocol, which ensures high extraction efficiency from small tissue samples (Miller, Dykes, & Polesky, 1988). Polymerase chain reactions (PCRs) were performed on a Thermal Cycler (mod. 2720, Applied Biosystems, Foster City, CA, U.S.A.) to amplify two microsatellite markers (TTA and AGAT11; GenBank numbers: AF164205 and BV097141). The PCR was performed in 10.5 µl reaction volumes with 0.6 µl $MgCl_2$, 1 µl dNTPs, 0.14 µl of each primer (0.14 µl forward + 0.14 µl reverse), 2 µl Taq buffer, 0.1 µl Taq DNA polymerase (Promega) and 1.5 µl DNA template. The cycling protocol included an initial denaturation step at 94 °C for 3 min, followed by 30 cycles of 30 s denaturation at 94 °C, 30 s annealing at 55 °C, extension at 72 °C for 60 s and a final extension for 5 min at 72 °C. Amplified fragments were then separated by electrophoresis on an ABI 3100/3700 sequencer (ABI PRISM, Applied Biosystems), using 400 HD ROX (Perkin-Elmer) as a size standard. PCR products were visualized using Peak Scanner software v. 1.0 (www.appliedbiosystems.com) and paternity was assigned according to allele sharing between putative sires, mother and offspring.

Body Size and Coloration

Previous studies have demonstrated that colourful males produce more competitive ejaculates (Locatello, Rasotto, Evans, & Pilastro, 2006) and have higher sperm competitiveness (Evans et al., 2003). We therefore measured male body size and colour pattern to statistically control whether differences in male attractiveness influenced the paternity share in our experiment. To this end, we took a digital photograph (Canon 450D camera, equipped with Canon EFS 60 mm MACRO lens and circular flashlight) of the left side of each male, along with a scale for calibration (Devigili, Di Nisio, Grapputo, & Pilastro, 2016). Total body area (including the caudal fin), standard length (from the snout to the base of the tail fin) and the area of colour spots were measured using ImageJ software (http://imagej.nih.gov/ij/download.html). Three main components of these colour patterns were considered: the area of (1) orange pigmentation (including red and yellow, representing all the area of carotenoid and pteridine spots), (2) melanistic black spots and (3) the iridescent structural colours, which include white, blue, green, silver and violet (Devigili et al., 2015; Evans et al., 2003). Colour spot area was subsequently standardized to body area (%), to obtain the percentage of orange, melanistic, iridescent and total colour.

Statistical Analyses

Male morphology (body size and coloration) and sperm velocity were compared between the first and second males with a *t* test to ensure the two groups did not differ intrinsically for these traits. We tested both whether first and second groups of males differed (independent-sample *t* test) and whether there was a statistical difference between the first and second male within a pair (paired *t* test). The two tests gave substantially identical results and for the sake of brevity we present here only the independent-sample statistics. We then compared the observed distribution of fertilization success of males with the expected distribution due to simple binomial error and equal probability of siring an offspring for the two males. In particular, we compared the occurrence of broods sired entirely by one of the two males with the expected occurrence under equal expected fertilization success given the observed brood sizes, using Poptools' function dBinomialDev (http://www.poptools.org/functions/) to generate the expected paternity share and iterated the procedure 10 000 times using a Monte Carlo analysis. To investigate the effect of male insemination order (first or second), we ran a generalized linear mixed model

(GLMM) with a binomial error distribution and a logit link function in which the number of offspring sired by the first male on the total assigned offspring of the brood was the dependent variable (with Satterthwaite approximation to calculate denominator degrees of freedom, using the glmer function in the lme4 R package, The R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org). Male identity and male pair identity were entered as random factors to control for statistical nonindependence of the relative sperm competition success of the same pair of males across females when both artificially inseminated females gave birth to offspring, as male competitive fertilization shows significant repeatability in this species (Evans & Rutstein, 2008).

We then evaluated whether the number of sperm bundles inseminated (which were equal within a male pair and could vary between five and 40 bundles for each male), male sperm velocity and time between artificial insemination and parturition affected the observed paternity pattern. To this end, we ran a second GLMM including, along with the same random and fixed factors as for the initial model, sperm velocity (VAP; average sperm velocity of both competing males was included), number of bundles inseminated and interval (days) between insemination and parturition, as covariates. We then used a backward stepwise elimination procedure to exclude nonsignificant terms, starting from nonsignificant interactions. At each step, we checked that the exclusion of predictors did not result in a significant increase in deviance using a log-likelihood ratio test (twice the difference in the log-likelihood between models was compared with a chi-square distribution with 1 degree of freedom). Predictors were removed only when the log-likelihood ratio test was nonsignificant. Finally, we tested whether the results from the final model were influenced by male morphology traits (namely, standard length, percentage of orange, percentage of melanistic and percentage of iridescent), which were previously shown to correlate with fertilization success in this guppy population (Evans et al., 2003). If not otherwise stated, means and their SEs are given. All probabilities are two tailed. The statistical analyses were conducted using SPSS (version 23, IBM, Armonk, NY, U.S.A.) and R (version 3.2.5).

Ethical Note

This experiment was conducted according to the Italian legal requirements and was approved by the Ethics committee of the University of Padova (permit no. 12 /2014). As they were descendants of wild-caught fish maintained in our facilities, no transport of the experimental fish was necessary. We used the lowest number of individuals necessary to achieve the aims of the experiment (56 males and 56 females). To this end, the required sample size was calculated by estimating the effect size from a pilot study, while fixing $\alpha = 0.05$ and aiming at a power = 0.8. Experimental animals were isolated during some steps of the experiment. Males were physically isolated but maintained in visual contact with females for 3 days to ensure sperm reserve replenishment before ejaculate collection (Bozynski & Liley, 2003). After 3 days of isolation males resume their normal behaviour as soon as they are returned to stock tanks (e.g. Cattelan, Evans, Pilastro, & Gasparini, 2016). Females were isolated after insemination until they gave birth or up to 60 days. Isolation was necessary for females to prevent further copulations and to correctly assign the offspring to their mother, something that could not be achieved if females were housed together. Isolated females were kept in visual contact with other females to minimize the stress and recovered their normal social behaviour when they were returned to stock tanks. The fish were fully anaesthetized (by immersion in a solution of fish anaesthetic MS222, 0.5 g/litre) before all experimental procedures (sperm extraction, artificial insemination, phenotypic measurements and fin clipping). Anaesthesia, which was conducted by an expert operator and followed established procedures (e.g. Gasparini et al., 2015), lasted 2–5 min. All individuals fully recovered their normal behaviour within 10–15 min after being revived in conditioned water. The pregnancy success (about 60%) was slightly lower than that obtained in previous studies conducted in our laboratory (M. Magris, G. Cardozo, F. Santi, A. Devigili & A. Pilastro, personal observation), but it was in the range of that reported for other artificial insemination experiments (e.g. from ca. 50%, Gasparini, Marino, Boschetto, & Pilastro, 2010; Gasparini et al., 2010, to ca. 85%, Gasparini et al., 2015). We had no evidence that the double insemination negatively affected the females, as we did not observe any sign of stress or any postinsemination mortality in the females. Clipped fins regrew completely in about 2 weeks. All fin-clipped individuals recovered fully from anaesthesia and were returned to temporary postexperimental tanks where they were monitored for signs of stress. The mortality rate in post-experimental tanks was similar to that observed in stock tanks, suggesting that manipulation (artificial insemination and fin clipping) did not significantly affect the subsequent survival of experimental fish. Only offspring were euthanized for DNA analysis (with an excess of anaesthetic, MS222) because fin clipping would not have provided a sufficient tissue sample for DNA extraction, whereas all adults were eventually returned to the stock tanks.

RESULTS

In total, 34 of the 56 initially inseminated females produced a brood (14 from the five plus five bundles group and 20 from the 40 + 40 group) for a total of 252 offspring (mean brood size: 7.41 ± 0.66 offspring, range 1–16). Two broods (nine offspring in total), produced by two females, could not be genotyped because of problems with DNA extraction/preservation, and 12 offspring could not be assigned to a sire. The final sample comprised 32 broods and 231 offspring (95.1% of the initial offspring sample). These 32 broods were produced by females that had been inseminated by 28 different pairs of males (i.e. in four cases the same pair of males inseminated two females). Mean time to parturition among these remaining 32 females was 32.94 ± 1.51 days (range 19–49 days, for details see Supplementary material Table S1).

There was no significant difference in the mean sperm velocity between first and second males (for details see Appendix Table A1 and Supplementary material Table S2). Similar results were obtained when we considered the other measures of sperm velocity (VCL, VSL). First and second males did not differ in any of the measured morphology traits (for details see Appendix Table A1 and Supplementary material Table S2). Similar results were obtained when the first and second male within each pair were compared using a paired *t* test (data not shown).

Broods entirely sired by one male (20 cases, 16 of which were sired by the first male) were more frequent than expected under simple binomial error ($P < 0.001$, assuming equal probability of siring the offspring, Monte Carlo simulation), indicating that the observed paternity deviated from fair raffle expectations. Insemination order significantly affected paternity share, with first males having an advantage over second males (GLMM, dependent variable: proportion of offspring sired by first male: log-likelihood = −98.6, $b = 3.68 \pm 1.22$ SE, $z = -3.026$, $P = 0.003$; random factor: male identity: variance component = 10.72 ± 3.28 SD). The mean proportion of offspring sired by the first male (P_{1st}) was 0.71 ± 0.103 SE.

When the other factors that may influence paternity share in guppies were entered in the GLMM, we found that neither VAP nor number of sperm bundles, nor their interaction with insemination order, significantly affected paternity share. In contrast, time to

parturition, and its interaction with insemination order, significantly predicted a male's fertilization success (Table 1). In particular, the advantage of the first males over the second males declined as time from insemination to parturition increased (Fig. 1). When morphological traits (standard length, percentage of orange, percentage of melanistic and percentage of iridescent) were included in this final model we found that they did not predict paternity shares ($P > 0.07$), either alone or in interaction with the other predictors (role and time to parturition); only the percentage of orange approached significance (see Appendix Table A2).

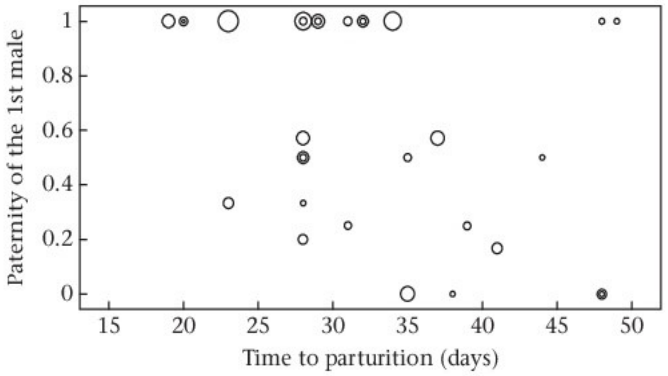

Figure 1. Proportion of offspring sired by the first male in relation to the interval between insemination and parturition. The size of the points is proportional to the brood size (number of offspring assigned to one of the sires; range 2–10).

DISCUSSION

When a female guppy mates sequentially with two males, paternity is usually biased towards the second male (Evans & Magurran, 2001; Neff & Wahl, 2004; Pitcher et al., 2003). Second males, in particular, sire all or most offspring, while the first male rarely gets most of the fertilizations (Evans & Magurran, 2001), possibly as a consequence of females biasing the number of sperm transferred during copulation to favour the most attractive male (Pilastro et al., 2007). When behavioural interactions between partners and potential differences in ejaculate size between males were removed using artificial insemination, the last-male precedence detected after natural copulations was completely reversed. In our experiment, the paternity share, although more equally distributed between the two males than observed after natural copulations (Evans & Magurran, 2001), was significantly biased towards the first male. The simplest explanation for these contradictory results is that LMP observed after natural copulations derives from the combined effect of (1) cryptic female choice for more attractive males (Pilastro et al., 2004) and (2) the positive association between mating order and male attractiveness (Pitcher et al., 2003). In guppies, virgin females are less choosy when they mate for the first time than in subsequent matings, and their probability of remating increases if the second male is more attractive than the first (Pitcher et al., 2003). If females encounter males in random order with respect to their attractiveness, and if the probability that previously mated females mate with a second male depends on its attractiveness relative to the first, the second male to mate is expected to be more attractive, on average, than the first. Female guppies cryptically favour more attractive males, by controlling copulation duration, which, in turn, determines the number of sperm transferred by males (Pilastro et al., 2004, 2007). Second males are therefore expected to inseminate, on average, more sperm than the first males and, since sperm number is the primary postcopulatory predictor of sperm competition success (Boschetto et al., 2011), to sire a larger proportion of the offspring. Colourful, attractive males have been reported to produce more competitive ejaculates (Evans et al., 2003; Locatello et al., 2006), a factor that may further bias paternity towards the second male. A role of cryptic female choice in explaining LMP after natural copulations was first proposed by Evans and Magurran (2001), who also proposed using artificial insemination to test whether LMP persists when females are denied the choice of partners. Our results confirm their hypothesis and allow us to confidently ascribe patterns of LMP observed after natural copulations to female control of the number of sperm transferred during copulation (Pilastro et al., 2007), possibly in association with female sperm ejection, which is also occasionally observed (I. Zanata and M. Magris, personal observation).

Table 1
GLMM on the proportion of offspring sired by the first male

Dependent variable	Fixed effect	Estimate	SE	z	P
Proportion of offspring sired by first male	Intercept	1.7780	0.7550	2.355	**0.019**
	Order	−3.5560[a]	1.1704	−3.038	**0.002**
	Time to parturition	−1.7869	0.7665	−2.331	**0.020**
	Order*time to parturition	3.5739	1.1676	3.061	**0.002**

We report the final model after elimination of the nonsignificant terms. GLMM: dependent variable: proportion of offspring sired by first male: log-likelihood = −92.3. Fixed factors: insemination order; time to parturition; insemination order * time to parturition. Random factors: male identity; pair identity. Binomial error distribution. Significant effects are in bold.

[a] Estimated effect with first male as reference level.

While confirming that cryptic female choice influences LMP after natural copulations, our results revealed, perhaps surprisingly, that purely postcopulatory processes occurring independently from male–female behavioural interactions before and during copulation do favour the first male to mate. Among the other factors that may have influenced the observed paternity pattern, sperm velocity, the total number of sperm inseminated and male attractiveness did not significantly influence paternity share, with the exclusion of the size of orange spots which was positively correlated with fertilization success (although this effect did not reach statistical significance). We also found that the advantage of the first male decreased with an increase in the time between insemination and parturition, which is expected to covary with the duration of sperm storage. We discuss these results in turn below.

Male sperm precedence can be determined by factors occurring (1) at the formation of the 'fertilization set' (the population of sperm potentially competing to fertilize eggs, sensu Parker, Simmons, & Kirk, 1990) and/or (2) when sperm in the fertilization set are used for fertilization (Ala-Honkola & Manier, 2016). In internal fertilizing species with female sperm storage, copulations occur sequentially and the definition of the fertilization set competing for a batch of eggs depends on the timing of copulations with respect to the timing of fertilizations. In principle, one possible explanation for first-male sperm precedence may therefore be that some of the females already had their eggs ready to be fertilized when they were artificially inseminated with the sperm from the first male. As the female gonoduct is a few millimetres long (Greven, 2011) and sperm swimming speed in vitro is approximately 100 μm/s (Gasparini, Andreatta, & Pilastro, 2012), when the sperm of the second male were inseminated, all (or most) eggs would have already been fertilized by the first male. Indeed, the first male sired all the offspring of 16 of 32 females, most of which (14) produced their brood within 35 days after insemination. This number greatly exceeds that expected under fair raffle, which would be, on average, equal to 4.3 females out of 32 (95%

confidence interval = 1–8; Monte Carlo simulation). By contrast, in later broods, paternity share was more equally distributed or even slightly biased towards the second male. Although this explanation cannot be ruled out, we think that it is unlikely that the observed FMP can exclusively be attributed to fertilizations occurring before the female was inseminated with the sperm of the second male. First, egg fertilization is not perfectly synchronous in guppies and has been estimated to occur over 3–8 days (Martyn, Weigel, & Dreyer, 2006; Thibault & Schultz, 1978). This period is significantly longer than the interval between two successive artificial inseminations that we used in this study (24 h). Second, if most of the virgin females have ready-to-fertilize eggs, a first-male sperm precedence would be expected also when natural copulations occur at the same 24 h interval. However, a study in which females copulated in succession with two males 24 h apart resulted in a last-male fertilization advantage (Pitcher et al., 2003).

If we can exclude active female choice and differences in male intrinsic competitiveness related to morphology or ejaculate traits, what is the mechanism responsible for the observed FMP? In other species, FMP is usually associated with specific characteristics of the female reproductive tract (Birkhead & Hunter, 1990). For example, spiders possess spermathecae with separate ducts for the passage of sperm during insemination and fertilization, a reproductive morphology favouring a mechanism of 'first in–first out' (Uhl, 2002). Sperm retention and storage occur at different sites in female guppies (see also Potter & Kramer, 2000 for other poeciliid species): (1) the gonoduct and the ovarian lumen and (2) ovarian micropockets. In the gonoduct and in the ovarian lumen spermatozoa are found either in close contact with the apical ends of the epithelial cells (Campuzano-Caballero & Uribe, 2014) or within their cytoplasm (Jalabert, Billard, & Escaffre, 1969). This intimate contact may permit long-term sperm preservation. Spermatozoa are also found in the synaptic knob-shaped micropockets (SSP) expanding from the ovarian cavity to the follicle's surface; these are probably the sites of sperm entry at fertilization and are likely to be involved in short-term storage (Kobayashi & Iwamatsu, 2002). The SSPs are constituted by a short invagination with a narrow entrance enlarging in proximity with the follicles. This shape may prevent mixing of ejaculates from subsequent inseminations, leaving the first male's sperm in the most advantageous position, closer to the follicle's surface. This advantage, however, is not permanent, as we observed that second males increased their paternity share as time to parturition increased. At least two, potentially co-occurring mechanisms could explain this latter result. First, persistence of first-male sperm near the fertilization site or within storage organs may be reduced with time due to sperm ageing (a longevity advantage of the second male's sperm has been reported for more temporally separated insemination events: Grove, 1980; Winge, 1937). Alternatively, second-male sperm may move progressively closer to the fertilization site and mix with first-male sperm, reducing the latter's initial advantage. The second explanation seems more plausible, since the difference in age between the sperm from first and second males should become relatively less important as the time between insemination and fertilization increases. Further studies exploring the localization of spermatozoa in the female reproductive tracts at different time points, coupled with sperm labelling (e.g. Lymbery, Kennington, & Evans, 2016; Lüpold et al., 2012), would be very helpful to clarify the origin of the observed pattern of first-male precedence.

Previous studies in this guppy population revealed that colourful males produce more competitive ejaculates (Locatello et al., 2006) and have a fertilization advantage when females are simultaneously inseminated with a mix of equal numbers of sperm bundles from two males (Evans et al., 2003). While we found that the extension of males' orange spots was nearly significant in predicting paternity shares ($P = 0.07$), confirming this previous evidence, we also demonstrated that these differences in male ejaculate quality did not influence the observed FMP pattern. By showing that first and second males did not differ significantly in colour pattern and that the effect of mating order did not change after statistically controlling for phenotypic differences between males, we demonstrate that the effect of mating order on paternity patterns was not spuriously determined by differences in males' intrinsic fertilizing competitiveness. Moreover, in contrast to previous investigations which found a positive correlation between sperm velocity and paternity share both in the first (Boschetto et al., 2011) and in the second brood (Devigili et al., 2016; i.e. after prolonged female sperm storage), in the present study we found no evidence that higher sperm velocity was associated with a greater paternity share. These results may indirectly indicate that the effect of mating order overrides the effect of individual differences in sperm velocity. Alternatively, this explanation may be due to a lack of statistical power: phenotypic variation in sperm velocity in our sample was slightly smaller (SD = 11.7) than in the previous two studies, in which SD was 18.6 (Boschetto et al., 2011) and 17.6 (Devigili et al., 2016). More extreme differences in sperm velocity between males may be necessary to detect a significant correlation between paternity shares and VAP. Finally, we did not find any significant effect of the total number of sperm inseminated on paternity share. The observation that a significant FMP is also observed when both males compete with relatively few bundles (a five-bundle ejaculation size is in the low range for guppies: Pilastro & Bisazza, 1999; Pilastro et al., 2004, 2007) indirectly suggests that a small ejaculate is capable of saturating sperm storage organs close to fertilization sites, or, alternatively, to stratify in the proximal part of the gonoduct and to limit the access of the second male's sperm to the fertilization sites, at least initially. Clearly, while our results were useful in unveiling paternity patterns associated with insemination order, they say little about the underlying mechanisms.

Independently of the proximate mechanism responsible for the observed first-male precedence, the first males' intrinsic fertilization advantage has important consequences for our interpretation of male and female mating strategies. From the female's point of view, the intrinsic fertilization advantage of first males constrains, at least to some extent, her capability to cryptically bias paternity towards the second male using a trade-up strategy (Evans & Magurran, 2001; Pitcher et al., 2003). The combined effect of timing of copulation and ejaculate size should be experimentally tested to quantify the limits within which females can efficiently trade up in subsequent mate choice. In addition, the positive correlation between the time elapsed between insemination and parturition (which, in turn, probably depends on the timing of ovulation) raises the intriguing possibility that females may be able to bias paternity towards the last male by delaying ovulation, as observed in arthropods (Peretti & Aisenberg, 2015). Female guppies are known to shorten the time between mating and parturition in response to predation risk, although it is not known whether this resulted from anticipated egg maturation or shorter brood retention (Evans, Gasparini, & Pilastro, 2007).

From the point of view of male behaviour, the implications of FMP for male mating strategies may be more profound. FMP accords with the observation that male mate guarding is virtually absent in this species (Houde, 1997; Magurran, 2005), with male preference for virgin females (Guevara-Fiore, Skinner, & Watt, 2009), even though they are less fecund, on average, than nonvirgin females (Devigili et al., 2016), and with the preference for unfamiliar females (Kelley, Graves, & Magurran, 1999; 'Coolidge effect', described in guppies by; Jordan & Brooks, 2010). In addition, FMP may be particularly relevant for the interpretation of male

mate choice copying which has been observed in guppies (Auld & Godin, 2015; but see Dosen & Montgomerie, 2004) and other poeciliids (Bierbach, Kronmarck, Hennige-Schulz, Stadler, & Plath, 2011; Witte & Ryan, 2002). Mate choice copying is usually assumed to be associated with a fertilization advantage of the last male: LMP, in fact, would (at least partly) compensate for the increased sperm competition risk associated with this strategy (Bierbach et al., 2011; Witte & Ryan, 2002). Our results indirectly indicate that male mate choice copying cannot be explained by a sperm competition advantage of the last male, but must be associated with some precopulatory benefits. For example, males may use other males' behaviour to locate sexually receptive (i.e. virgin and postpartum) females, which are a small proportion of the adult females (Houde, 1997). Similarly, the so-called 'audience effect' (the observation that male guppies alter their initial mate preference when other males are observing their interaction with the female) is often interpreted assuming LMP (Auld & Godin, 2015; Auld, Jeswiet, & Godin, 2015; Zajonc, 1965). For example, the male's reduced sexual interest towards the initially preferred female has been interpreted as an attempt by the focal male to deceive bystander males about the quality of his initially preferred female ('deception hypothesis', Auld & Godin, 2015; Plath, Richter, Tiedemann, & Schlupp, 2008). Results from a theoretical model suggest that male deception is an evolutionarily stable strategy only when associated with strong LMP (Castellano, Friard, & Pilastro, 2016). LMP, in fact, would reduce the costs associated with the failure of deception: if the bystander male does not copy the deceiving choice of the focal male and instead mates with the female initially preferred by the latter, the cheating male will only be able to mate as second male. If LMP occurs, even when the cheating strategy fails, the cheating male may still expect to have a sperm competition advantage. Conversely, if the first mate is favoured, the cheating male will either mate with the lower quality female or compete in the disadvantaged role for the higher quality female. In the light of the above-mentioned model by Castellano et al. (2016), the deception hypothesis seems an unlikely explanation of the audience effect in the guppy. It has been suggested that this reversal of mating preferences may be aimed at reducing competition for mating ('flexible decision hypothesis', Auld & Godin, 2015; Castellano et al., 2016). Male competition can be direct (e.g. male–male contests) or indirect (mediated by female choice for the most attractive male). In a comprehensive study on 10 poeciliid species, Bierbach et al. (2013) found no correlation between the strength of the audience effect and the level of male–male aggression, suggesting that the audience effect may not be explained by an attempt to reduce the costs of aggressive interactions. However, male–male competition can be subtle and take the form of covert agonistic behaviour, such as jockeying for position behind females and occasional displacements of other courting males (Kodric-Brown, 1993). Alternatively, males may reverse their initial preference switching to the less attractive female to avoid being directly compared by the female with the bystander male, especially when the latter is phenotypically attractive. Indeed, female perception of a male's attractiveness is influenced by the comparison with other males (Pilastro et al., 2004) and males choose the female to court by the relative quality of the surrounding competitors (Gasparini, Serena, & Pilastro, 2013). Auld, Ramnarine, and Godin (2017) showed that male guppies altered their initial mate preferences to a greater extent when the audience males were larger than they were, but that they were unaffected by the audience males' ornamentation. Since female guppies prefer larger males (Houde, 1997) and body size may be a predictor of male competitiveness, Auld et al.'s results may be explained by focal males avoiding more competitive and/or more attractive competitors.

Conclusions

Our study unveiled the effect of insemination order on sperm competition success in guppies. We showed that, when behavioural interactions between partners were controlled using artificial insemination, the first ejaculate was significantly favoured over the second in terms of fertilization success (mean $P_{1st} = 0.71$). The anatomy of the female reproductive tracts and storage organs probably promotes ejaculate stratification, with the first male's sperm in a more favourable position to fertilize the eggs. FMP declined progressively with longer intervals between insemination and parturition, suggesting increased sperm mixing. To unequivocally identify the mechanisms generating the observed pattern of FMP further investigation is required. Our results imply that the LMP observed after natural copulations in the guppy is determined by the combination of females' trade-up mating strategy and cryptic preference for attractive males, rather than by insemination order per se. The postcopulatory advantage of being the first to mate with a female should be considered when interpreting the adaptive function of the audience effect and of male mate choice copying adopted by male guppies and, potentially, by other poeciliids.

Acknowledgments

We thank two anonymous referees and the Editor for their useful comments on the manuscript and Mari Carmen Uribe and Juan Carlos Campuzano-Caballero for their fruitful insights on the anatomy of the female guppy's reproductive tract. M.M. was supported by a Ph.D. fellowship from the University of Padova. G.C. was supported by an external scholarship of the Consejo Nacional de Investigaciones Científicas y Técnicas de Argentina (CONICET) 2014-2015. This study was funded by a grant from the University of Padova: PRAT 2012 no. CPDA 120105 to A.P.

Supplementary Material

Supplementary material associated with this article is available, in the online version, at http://dx.doi.org/10.1016/j.anbehav.2017.07.009.

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APPENDIX

Table A1
Sperm traits and male morphological traits in the two groups (first and second males): descriptive statistics and comparisons between groups

Trait	Role	*N*	Mean	SD	*F* (Levene test equality variances)	*P*	*t*	*df*	*P*
VAP (μm/s)	1st	28	95.48	10.19	1.695	0.199	0.106	54	0.916
	2nd	28	95.14	13.50					
SL (mm)	1st	28	16.37	1.44	0.168	0.683	0.513	54	0.610
	2nd	28	16.56	1.35					
Orange (% of body area)	1st	28	7.32	3.51	0.017	0.898	0.202	54	0.841
	2nd	28	7.14	3.35					
Iridescent (% of body area)	1st	28	10.18	3.36	2.267	0.138	0.077	54	0.939
	2nd	28	10.26	4.43					
Melanistic (% of body area)	1st	28	1.69	0.74	0.852	0.360	0.260	54	0.796
	2nd	28	1.74	0.65					

Comparison of first and second males showed that they did not differ systematically in sperm velocity, size or ornamentation (*t* test: $P > 0.05$). The two groups also had similar variances (Levene test: $P > 0.05$).

Table A2
GLMM: effect of insemination order, time to parturition and male orange coloration on proportion of offspring sired

Dependent variable	Fixed effect	Estimate	SE	*z*	*P*
Proportion of offspring sired by first male	Intercept	1.8020	0.7192	2.505	**0.012**
	Order	−3.6036[a]	1.1155	−3.231	**0.001**
	Time to parturition	−1.8317	0.7392	−2.478	**0.013**
	Percentage of orange	0.9251	0.5106	1.812	0.070
	Order * time to parturition	3.6061	1.1210	3.271	**0.001**

When proportion of orange was included in the model, insemination order remained the primary predictor, with first-male precedence still diminishing as the interval between insemination and parturition increased. The percentage of orange was only marginally significant and was nonsignificant in its interaction with the other predictors. We report the final model after elimination of the nonsignificant terms. GLMM: dependent variable: proportion of offspring sired by first male: log-likelihood = −90.5. Fixed factors: insemination order; time to parturition; percentage of orange; insemination order * time to parturition. Random factors: male identity; pair identity. Binomial error distribution. Significant effects are in bold.

[a] Estimated effect with first male as reference level.

Manuscript 3b

Can previous exposure to ejaculates affect paternity success in multiple mated females?

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ABSTRACT

In a variable environment females could benefit from diversifying their offspring genotypes. Pre-copulatory strategies promoting offspring genetic diversity include polyandry and preference for novel partners. Female guppies (*Poecilia reticulata*), polyandrous livebearing fish with internal fertilization, and prolonged female sperm storage, display both behaviors, but since their mating preferences are frequently overridden by male coercion, post-copulatory processes might also be expected. Internal fertilization and gestation might give raise to immune-mediated bias in female sperm usage: previous exposure to a male's ejaculate may prime the female immune system resulting in a stronger immune response against sperm from that individual and thus in a paternity advantage of the novel mate. However, if insemination elicits a male-specific downregulation of the mother's immune system aimed at protecting embryos, the familiar partner may be instead favored. We used artificial insemination to test these alternatives: a first homospermic artificial insemination was followed, in the next reproductive cycle, by a heterospermic artificial insemination with equal numbers of sperms obtained from the previous and a novel male. Paternity analyses on the offspring revealed an advantage of the male whose ejaculate the female had been primed to during the previous reproductive cycle. These results highlight a potential for female insemination history to affect the outcome of sperm competition in subsequent copulations. The effect likely results from a male-specific suppression of anti-ejaculate or anti-embryo immune response, which has been observed in several mammals and which may have evolved under natural selection to avoid the risk of embryos being recognized as non-self and attacked by the female's immune system. These results add to the large body of evidence showing that male reproductive success, rather than being fixed, is largely context dependent, influenced by a variety of social and environmental factors, including female mating history.

INTRODUCTION

Polyandry has been suggested to increase female fitness through several mechanisms, including allowing females to gain indirect benefits by having their eggs fertilized by sperm from genetically superior or more compatible sires (Tregenza and Wedell 2000; Zeh and Zeh 2001, 2003) or by increasing offspring genetic diversity (Jennions 2000). On one hand, polyandry can enhance within-individual offspring genetic variability, which can improve offspring survival and performance (Garant et al. 2005). On the other, it can promote among-individual offspring diversity, which, in turn, can limit sibling competition (Ridley 1993; Griffiths and Armstrong 2001), inbreeding costs (in case of sibling mating, Cornell and Tregenza 2007) and may predispose offspring to a wider array of environmental conditions (i.e. bet-hedging, Yasui 1998). Since the beneficial effects of multiple mating only arise when mating with different partners, females (in particular when sperm from previous matings are stored by females for prolonged periods) are expected to develop discriminating behaviors against previous mates (Ivy et al. 2005), analogous to those observed in males (i.e. Coolidge effect, Dewsbury 1981). Indeed, female pre-copulatory preferences for novel partners have been reported in several species (e.g. Zeh et al. 1998; Archer and Elgar 1999; Eakley and Houde 2004; Vega-Trejo et al. 2014). In contrast, little is known about post-copulatory mechanisms favoring novel mates (but see, Gershman 2009; Gershman and Sakaluk 2010), despite their potential role to re-gain control over offspring paternity when pre-copulatory mate choice is constrained or not efficient (e.g. because females are unable to recognize previous partners or forced copulations occur, Birkhead 1998; Pizzari and Birkhead 2000). Interestingly, cryptic female choice based on partner novelty may be involved in determining the elevated fertilization success of extra-pair copulations (Birkhead et al. 1988) and in selecting for male preference for novel mates over previous ones if previous partners penalize their sperm, they may gain higher paternity shares by searching for new mates instead). A post-copulatory bias towards novel mates may occur through different forms of cryptic female choice, including sperm choice, which, in turn, may result from an enhanced immune response against the ejaculate of previous partners. Immunity has been recognized to have far-reaching evolutionary consequences for its association with reproduction (Lawniczak et al. 2007), but most studies have focused on the trade-off between immunity and costly reproductive traits. However, a more direct role of immunity on sexual selection processes has also been recently proposed (Ghaderi et al. 2011; Morrow and Innocenti 2012; Kekäläinen and Evans 2018), supported by the discovery that females tend to have more powerful immune responses than males (Arizza et al. 2013). When the ejaculate comes in contact with the female reproductive tract, it often causes a cascade of effects in females, including changes in their immune status (McGraw et al. 2004; Shoemaker et al. 2006). As it has been documented in humans and other mammals, both sperm and seminal fluid trigger immune responses in females (e.g. Denison et al. 1999; Robertson et al. 2002; Schuberth et al. 2008). While ejaculate-triggered female immune responses were first seen as an adaptive response (‘pre-emptive strike’) against pathogens that may be transmitted with the ejaculate (Peng et al. 2005), it may have evolved instead as a barrier to sperm which enables post-mating

choice. Such hostile environment may filter sperm based on their ability to survive the female-derived immunological attack and may therefore allow females to eliminate damaged sperm cells and to select partners producing high quality ejaculates (Birkhead et al. 1993; Lawniczak et al. 2007; Schuberth et al. 2008). If immune responses are male-specific, they may further allow females to bias paternity towards males with higher genetic quality or compatibility (Zeh and Zeh 1997; Ziegler et al. 2005). Indeed, there is evidence of individual variation across human males in terms of strength of the inflammatory response they elicit in the female reproductive tract following insemination (Sharkey et al. 2007) and there are polymorphisms in the genes regulating the antibody- or cell-mediated immune responses (Witkin et al. 2000). Based on the above evidence, immune responses to the ejaculate may also result in female insemination history affecting sperm competition in next copulation events. Previous exposure to a male's ejaculate may, in fact, prime the female immune system, making subsequent immune responses against the ejaculate from the same male more effective. This process could bias fertilization resulting in an advantage of novel mates. An opposite pattern, however, may also be predicted. In humans, where the phenomenon is well studied, exposure to the partner ejaculate previous to fertilization is important for the establishment and maintenance of a viable pregnancy and for its optimal outcome (Robertson and Sharkey 2016). In this context, previous exposure can help establish a tolerance to non-self antigens of the ejaculate, so that, subsequent fertilizations may be favored. While immunity has been suggested to be involved in sperm choice (Zeh and Zeh 1997; Kekäläinen and Evans 2018), to our knowledge, its role on post-copulatory preferences for novel partners has never been investigated. To shed light on this phenomenon, we used guppies (*Poecilia reticulata*). The guppy is a small tropical fish characterized by internal fertilization and viviparity (Magurran 2005). In the small streams of Trinidad which represent their habitat, guppies experience frequent and intense fluctuations of both abiotic and biotic factors (Grether et al. 2001; Pettersson et al. 2004). In such variable environment a bet-hedging strategy of producing a high number of diversified offspring may be beneficial (Yasui 1998). Indeed, guppies adopt mating strategies that contribute to enhance offspring diversity: they are characterized by marked promiscuity which leads to high levels of multiple paternity (Evans and Magurran 2000; Hain and Neff 2007; Neff et al. 2008) and by a mating preference for novel partners over previous ones, observed both in males (Kelley et al. 1999) and females (Hughes et al. 1999; Eakley and Houde 2004). In contrast, little is known about post-copulatory mechanisms that might be involved in promoting variation among offspring, and that might play a central role considering the high occurrence of coercive copulation in this species (Magurran and Seghers 1994). Cryptic female choice is known to take place in guppies; by controlling copulation duration females manipulate the amount of sperm they receive from males based on their attractiveness, and through sperm choice they bias male fertilization success based on partner relatedness (Gasparini and Pilastro 2011; Fitzpatrick and Evans 2014) and MHC similarity (Gasparini et al. 2015). It has been shown that the bias towards unrelated males is mediated by a differential activation of sperm by the ovarian fluid (Gasparini and Pilastro 2011), and while the mechanism responsible for sperm recognition is currently unknown, the

immune system is a good candidate to mediate such responses. If immunity was involved in sperm choice in the guppy, it may also determine patterns of paternity bias based on female mating history with a given male. Another important characteristic of the guppy reproductive system is that females store sperm from multiple males for several months and are therefore able to fertilize several successive broods after a single mating (Schmidt 1919; Lopez-Sepulcre et al. 2013; Devigili et al. 2016). Whether in a given reproductive cycle sperm of a male are favoured in the competition with those of other males in relation to their mating history with the female, however, it is not known. We tested whether this occurs using artificial insemination to experimentally control the timing of insemination and the number of sperm inseminated. Briefly, using a paired-block design, we inseminated a virgin female with a small amount of sperm from one single male and, after she had given birth, performed a heterospermic insemination with equal (and larger) numbers of sperm obtained from the previous and a novel male. We then collected and genotyped the offspring from the second brood to assign paternity and determine if competitive fertilization success was affected by previous exposure to one of the male's ejaculate.

MATERIALS AND METHODS

Study Animals

All fish were descendants of wild-caught guppies collected in 2002 from the Lower Tacarigua River in Trinidad that live in semi-natural conditions in a large pool (46 x 4.4 m, h. 0.4 m) at the Botanical garden of the University of Padova. Before the experiments, in the laboratory, fish were maintained in stock aquaria (ca. 150 fish/tank) with a balanced sex ratio and in which outbreeding was ensured by periodically moving individuals across different stocks. Water temperature was maintained between 25°C and 27°C and illumination was set on a 12 h/12 h light/dark cycle (Philips TLD 36W florescent lamps). Fish were fed ad libitum twice a day on a mixed diet of brine shrimp nauplii, *Artemia salina*, and commercially prepared flake food (Duplarin). The bottom of the tanks was covered with mixed color gravel and the tanks were provided with aquatic plants and algae. Males used in the experiment were collected from stock tanks, whereas virgin females were reared in single-sex tanks. All fish were sexually mature (at least 4 months old) when used for the experiments. In guppies, sperm are released in bundles that contains approximately 21,000 sperm each (Boschetto et al. 2011). Sperm bundles can be collected from males by applying a gentle pressure on their abdomen under a dissection microscope and bundles can be individually counted and used to artificially inseminate a known number of sperm (see Evans et al. 2003 for details on methods). Female guppies produce one brood every 20-30 days (Magurran 2005). A few days after parturition a new batch of eggs is ready to be fertilized from previously stored or from freshly inseminated sperm. Timing of insemination affects the outcome of sperm competition: the first male has an advantage over the second male if the two inseminations occur one day apart (Magris et al. 2017). In contrast, second male sperm has nearly a 100%

success when the first insemination has occurred one month, or more before the second (Schmidt 1919; Gasparini et al. 2018).

Experimental Overview

We conducted a paired design, with each block consisting of two virgin females (female 1 and 2) and two males (male A and B), in a fully reciprocal experimental design. Each female was randomly assigned to one or the other male (male A or male B, **Fig. 1**), and artificially inseminated with 5 sperm bundles from the male (approx. 105,000 sperm cells, hereafter monospermic inseminations). Females were then left in isolation to give birth to their first brood. Offspring were counted and released in stock tanks. The day after parturition, females were artificially inseminated using a mix of equal numbers of sperm bundles from the two males of the block (10 bundles from each male, 20 bundles in total, approx. 420,000 sperm; hereafter heterospermic inseminations). Each female was therefore inseminated just before their second ovulation with an equal quantity of sperm from the first male (hereafter "familiar" partner) and from a new male (hereafter "unfamiliar" partner), to control for the effect of insemination order on paternity shares (Magris et al. 2017). Offspring resulting from the heterospermic insemination were collected for paternity analysis. We obtained a total of 18 blocks (each of which consisted of 2 males, 2 females and 2 broods).

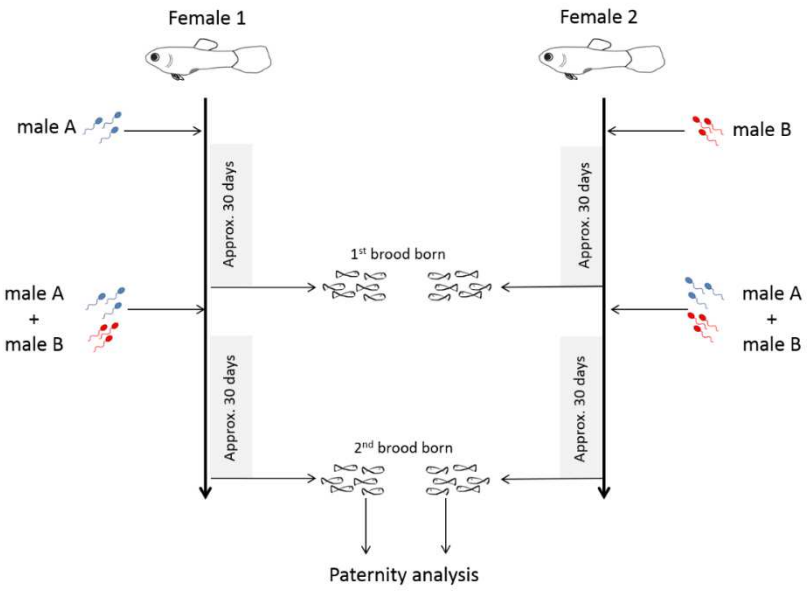

Figure 1. Schematic representation of the experimental design. For simplicity, the figure depicts only one out of the 18 blocks conducted. Each block consisted of two virgin females (female 1 and 2) and two males (male A and B). Artificial inseminations (see text) were used to control for the number of sperm inseminated.

Artificial Inseminations

Artificial insemination (AI) technique was used to inseminate virgin females (6 months old) following a standard procedure (e.g. Gasparini and Pilastro 2011). Briefly, males were anaesthetized with MS222 and

sperm collected by applying a gentle pressure on their abdomen. Females were similarly anaesthetized and inseminated using a micropipette with a known amount of sperm bundles. For the first insemination we used 5 bundles, while in the second 10 bundles from each male were used. Five bundles were used to ensure fertilization and achieve the 'priming' goal but, at the same time, to minimize the possibility of sperm storage across cycles that could have biased the paternity share in the second brood toward the first male due to unbalanced sperm number (see also discussion). Ten bundles were used because this number is in the range of sperm transferred during a natural copulation (Pilastro et al. 2007).

Paternity analysis

Newborns were euthanized with an excess of anaesthetic (MS222), and fin clips were collected from all adults at the end of the experiment. Tissues were preserved in absolute ethanol until required for DNA analysis. Genomic DNA was extracted from offspring tissues using a Chelex protocol (Walsh et al. 1991) and from the tissue of the adults using a standard salting-out protocol (Patwary et al. 1994). Two highly variable microsatellite markers (TTA and Agat11; GenBank number AF164205 and BV097141, respectively) were used to assign paternity. PCRs were performed following previous protocols (Gasparini and Pilastro 2011). PCR products were analysed on an ABI 3100/3700 sequencer (Applied Biosystems) and visualized using the software Gene Marker (SoftGenetics). Paternity was assigned on broods with at least 3 offspring using the software CERVUS v 3.0 (Marshall et al. 1998; Kalinowski et al. 2007), with strict confidence of 95%.

Statistical analysis

Analyses were performed in R v. 3.3 (R Development Core Team 2016) and in Excel using Poptools (Hood 2011). One out of the 18 blocks was excluded because males were genotypically too similar to reliably assign paternity to the offspring, one block was excluded because the DNA sample of one of the two males was degraded, and one block was excluded because one of the two females produced only two offspring. The final dataset therefore included a total of n=15 complete blocks (30 males, 30 females and 342 offspring from 30 broods, see below).

To test whether unfamiliar partners obtained higher paternity shares than familiar partners we first calculated the proportion of offspring shared by male A in the two conditions (familiar and unfamiliar), and the average difference between the two conditions (Δobs = observed fertilization success as unfamiliar mate minus observed fertilization success as familiar mate). Using the function dBinomialDev (http://www.poptools.org/functions/) in Poptools, we generated the expected proportion of offspring sired by male A in the two broods assuming that the probability was the same in the two broods (i.e. deviations from the expected difference =0 are due to binomial error only). This procedure was iterated 10,000 times using a MonteCarlo simulation to generate a null distribution of the difference in fertilization success in the familiar and unfamiliar role. We compared the observed mean difference in fertilization success of the males

in the two conditions with the null distribution. Probability that the observed difference is larger than 0 can be derived from the number of simulations in which the observed difference was larger than the simulated one over the total number of simulations. Since males are intrinsically different in their sperm competition success (Evans and Rutstein 2008), the expected fertilization success differed for each block of males and was equal to the observed mean fertilization success in the two roles. Note that results did not change when the same probability (0.5) to fertilize the eggs was assigned to all blocks' males (data not shown). Means are reported with their standard error (SE).

RESULTS

Mean brood size increased from the first to the second brood (first: 7.39 ± 0.74, second: 10.81 ± 0.81, paired t-test: t_{35}= 4.259, P<0.001). Paternity was assigned to 342 out of 348 newborns obtained from heterospermic AIs (98.3%). The proportion of offspring sired by male A was 0.55 ± 0.069 in the familiar role and 0.41 ± 0.061 in the unfamiliar role (**Fig. 2**). The difference in fertilization success between the two roles (0.14) was larger than expected under simple binomial error, assuming equal probability of siring the offspring in the two roles (P=0.007, Monte Carlo simulation, **Fig. 3**).

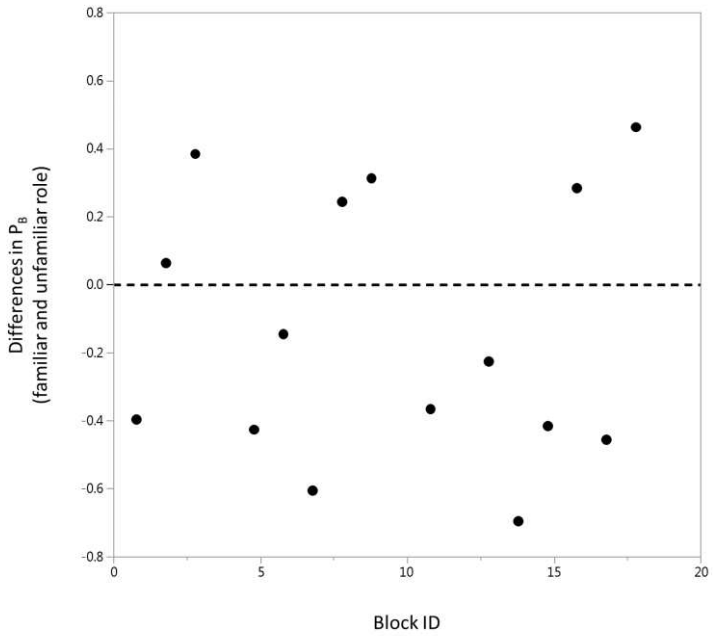

Figure 2. Differences in paternity of male B when in the unfamiliar and in the familiar role. Positive value indicates that the male sired more offspring in the unfamiliar role, negative values indicates that the male sired more offspring in the familiar role.

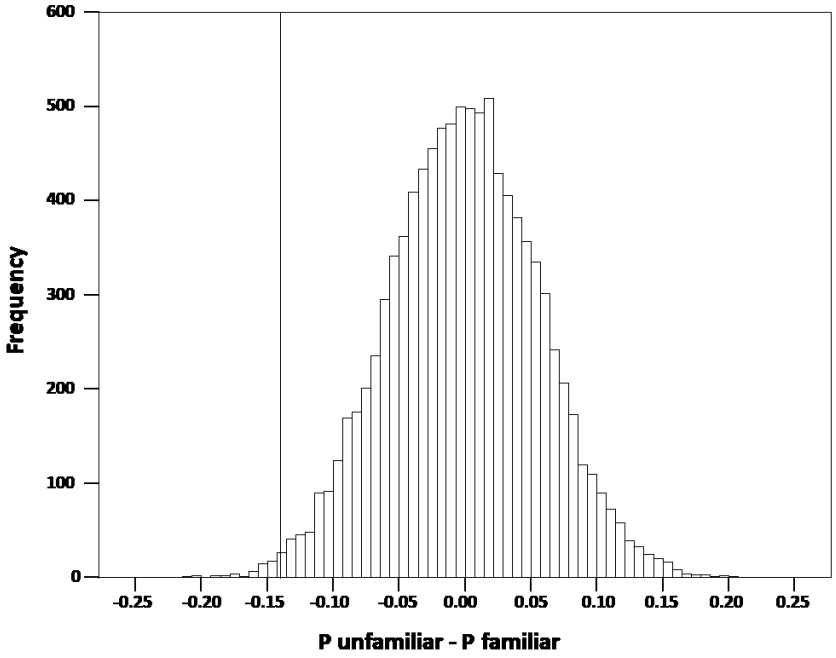

Figure 3. Distribution of the difference in paternity shares between the unfamiliar and the familiar partner as produced by the Montecarlo simulation (10,000 iterations) under simple binomial error, assuming equal probability of siring an offspring in the two roles. The difference of -0.14 obtained in our experiment is also shown in the graph.

DISCUSSION

Post-copulatory selection in guppies favors unrelated partners (Gasparini and Pilastro 2011; Fitzpatrick and Evans 2014) and MHC-similar males (Gasparini et al. 2015), suggesting that the immune system may be involved in sperm choice in this species. The effect of the immune system on familiar sperm fertilization success may be negative, if sperm are perceived as antigens and a specific immune response is developed by the female against them in successive inseminations, or positive, if the immune system is specifically downregulated at fertilization or during subsequent gestation. Our results suggest that paternity is biased towards the familiar male and therefore support the second hypothesis.

Considering that female guppies are able to store viable sperm for several months (Greven 2011; Lopez-Sepulcre et al. 2013), it might be argued that the advantage of the familiar mate observed in our experiment could result from the presence, in the female storage organs, of sperm from the first insemination. Although this possibility cannot be completely ruled out, the 'carry-over' effect of sperm storage from the first artificial insemination is unlikely to explain the difference observed in the present experiment (14%) for two reasons: i) the number of sperm bundles used in the first (homospermic) insemination was lower (5) than those used for the heterospermic insemination (10+10), and ii) fresh sperm nearly outcompete completely the stored sperm even when equal number of bundles are artificially inseminated in the first and in the second insemination (99%, Gasparini et al. 2018). Indeed, assuming conservatively that stored sperm explained 1% of the paternity in favor of the familiar male as in Gasparini et al. (2018, not considering the numerical

difference between first and second insemination in our study) still the advantage of the familiar male was larger than expected by chance (P=0.04, MonteCarlo simulation).

Our results are coherent with recent work reporting a down regulation of female immune system following insemination in several species (Lawniczak et al. 2007; Robertson 2007). In mammals this down regulation has been shown to be an important step to the successful implantation of the fertilized eggs and the initiation of a viable pregnancy (Robertson and Sharkey 2016). Exposure to a male's ejaculate constitutes a 'priming' event, helping to establish a state of immune tolerance to the male's antigens that will protect the developing embryos from the mother's immune system, and may therefore be associated with the evolution of livebearing. In this scenario, the immune tolerance elicited by exposure to the ejaculate may also promote the suppression of anti-sperm immunity in a male-specific way. However, rather than by previous contact with sperm, the male-specific suppression of against-sperm immunity may be also triggered by mother-embryos interactions during the first pregnancy, especially because this clearly involves a more intimate contact. An experimental design using inactivated sperm in the first insemination could help to shed light on the mechanisms involved in the phenomenon.

The mechanisms by which the female's immune system may mediate the observed process have yet to be described. However, seminal fluid, ovarian fluid and egg secretions are all known to contain various immune molecules, including immune system signaling molecules (such as cytokines and chemokines) and major histocompatibility complex (MHC) antigens, that can regulate sperm motility and chemotaxis, with possible effects on sperm fertilization competitiveness (Rizzo et al. 2009; Shimada et al. 2013; Caballero-Campo et al. 2014; Dahl et al. 2014). MHC peptides represent optimal candidates for mediating male-specific sperm recognition, as they are involved in mediating the male-specific nature of the immune suppression elicited by ejaculates (Robertson et al. 2009), and as MHC based fertilization biases have been described in several species (Skarstein et al. 2005; Yeates et al. 2009; Gessner et al. 2017), including guppies (Gasparini et al. 2015). Alternatively, the observed paternity bias may arise from a differential embryo survival: embryos sired by a male which the female has been more intensely primed to (during two insemination events and during the first pregnancy), may in fact benefit from increased survival rates. While this hypothesis is theoretically supported as the ejaculate-elicited immune suppression evolved to protect the conceptus from the mother's immune response, previous work suggests that embryo mortality is probably rare in the guppy (Gasparini and Pilastro 2011).

In terms of natural selection the benefits of suppressing immunity against paternally derived antigens are obvious if the process positively affects embryo survival. However, whether its effect on the competitiveness of subsequent ejaculates simply represents a by-product of protecting embryos or is adaptive per se is difficult to say. A potential role of this process in the context of sexual selection is puzzling as it contrasts with female pre-copulatory preferences for novel partners (Hughes et al. 1999; Eakley and Houde 2004).

Analogously, from the male perspective, the phenomenon also reduces the benefits resulting from male preference for novel partners (Kelley et al. 1999), as these matings would grant males lower paternity shares. It should be noticed, however, that the beneficial effect of previous insemination on male fertilization competitiveness was observed when female choice was prevented and males competed with equal amount of sperm. Female preferences for novel partners displayed before and possibly during copulation (as female guppies can control the amount of sperm they receive to favor preferred males, Pilastro et al. 2004; Pilastro et al. 2007) may limit the opportunity for the immune tolerance effect to produce significant biases in paternity. However, if the phenomenon is widespread it may contribute to explain the reduced ejaculate allocation to previous partners observed in some species (Pizzari et al. 2003; Spence et al. 2012; Reinhold et al. 2015); when males mate in the favored role (i.e. familiar), they may not need to allocate large ejaculates to gain high paternity shares.

In conclusion, our results provide the first evidence that female insemination history has the potential to affect the outcome of sperm competition in subsequent copulations, resulting in an advantage of the ejaculate which the female had been primed to during the previous reproductive cycle. The effect likely results from a male-specific suppression of the anti-ejaculate immune response, which may have evolved under natural selection to avoid the risk of embryos being recognized as non-self and attacked. These results add to the large body of evidence showing that male reproductive success, rather than being fixed, is largely context dependent, influenced by a variety of social and environmental factors, including female mating history. Further studies may investigate gene expression after insemination to understand in detail if and how immunity is involved in the process.

ETHICAL NOTE

Our study was approved by the Ethics committee of the University of Padova and by the Italian Ministry of Health (permit no. 12 /2014) and thus meets the guidelines for the care and use of research animals of the Italian Government.

ACKNOWLEDGEMENTS

We thank members of the Behavioural Ecology group at the University of Padova for logistic support in fish husbandry. This work was supported by a Marie Curie International Outgoing Fellowship to C.G. (grant no. 272613), an external scholarship of the Consejo Nacional de Investigaciones Científicas y Tecnicas de Argentina (CONICET) 2014-2015 to GC, a Ph.D. fellowship from the University of Padova to M.M and a grant from the University of Padova (PRAT 2012 no. CPDA 120105) to A.P.

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