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Female sperm storage mediates post-copulatory costs and benefits of ejaculate anticipatory plasticity in the guppy

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Abstract

Males of many species evolved the capability of adjusting their ejaculate phenotype in response to social cues to match the expected mating conditions. When females store sperm for a prolonged time, the expected fitness return of plastic adjustments of ejaculate phenotype may depend on the interval between mating and fertilization. Although prolonged female sperm storage (FSS) increases the opportunity for sperm competition, as a consequence of the longer temporal overlap of ejaculates from several males, it may also create variable selective forces on ejaculate phenotype, for example by exposing trade-offs between sperm velocity and sperm survival. We evaluated the relationship between the plasticity of ejaculate quality and FSS in the guppy, *Poecilia reticulata*, a polyandrous live-bearing fish in which females store sperm for several months and where stored sperm contribute significantly to a male's lifelong reproductive success. In this species, males respond to the perception of future mating opportunities by increasing the quantity (number) and quality (swimming velocity) of ready-to-use sperm (an anticipatory response called 'sperm priming'). Here we investigated (a) the effect of sperm priming on in vitro sperm viability at stripping and its temporal decline (as an estimate of sperm survival), and (b) the in vivo competitive fertilization success in relation to female sperm storage using artificial insemination. As expected, sperm-primed males produced more numerous and faster sperm, but with a reduced in vitro sperm viability at stripping and after 4 hr, compared with their counterparts. Artificial insemination revealed that the small (nonsignificant) advantage of primed sperm when fertilization immediately follows insemination is reversed when eggs are fertilized by female-stored sperm, weeks after insemination. By suggesting a plastic trade-off between sperm velocity and viability, these results demonstrate that prolonged female sperm storage generates divergent selection pressures on ejaculate phenotype.

KEYWORDS

adaptive plasticity, cryptic female choice, sperm competition, sperm longevity, sperm priming, trade-off

Cardozo and Devigili contributed equally to this work.

Pietro Antonelli is undergraduate student.

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1 | INTRODUCTION

Polyandry is widespread in nature (Taylor, Price, & Wedell, 2014), and the resulting competition for egg fertilization (i.e., sperm competition) has strong evolutionary consequences (Parker, 1970; Parker & Birkhead, 2013). Selection on ejaculates to maximize fertilization success has driven a dramatic increase in testis investment (Baker & Shackelford, 2018; Møller & Briskie, 1995; Ramm, Parker, & Stockley, 2005; Rowley, Daly-Engel, & Fitzpatrick, 2018; Soulsbury, 2010), often accompanied by a diversification of sperm size, shape, structure and performance (Lüpold & Pitnick, 2018; Pizzari & Parker, 2009), as well as of other components of the ejaculate (Birkhead, Hosken, & Pitnick, 2009, Hopkins, Sepil, & Wigby, 2017, Poiani, 2006, see Simmons & Fitzpatrick, 2012 for a review). Ejaculate production entails significant costs (Dewsbury, 1982; Olsson, Madson, & Shine, 1997; Pitnick, 1996; Thomsen et al., 2006), and theory predicts that males should strategically allocate their ejaculate reserves across subsequent matings in response to the expected fitness return of each given mating (Ball & Parker, 1996; Parker, 2016; Parker, Ball, Stockley, & Gage, 1997; Parker & Pizzari, 2010). There has been accumulating empirical evidence that males strategically invest in their ejaculates as a response to different levels of sperm competition or female quality (Kelly & Jennions, 2011; Wedell, Gage, & Parker, 2002). Typically, ejaculate plastic responses include the number of the sperm produced and/or used during matings (Firman, Garcia-Gonzalez, Simmons, & Andre, 2018; Kelly & Jennions, 2011), sperm performances such as motility and velocity (Cornwallis & Birkhead, 2007; Gasparini, Peretti, & Pilastro, 2009; Kilgallon & Simmons, 2005; Rudolfsen, Figenschou, Folstad, Tveiten, & Figenschou, 2006), and changes in the composition of the seminal fluids (Bartlett, Steeves, Gemmell, & Rosengrave, 2017; Ramm et al., 2015; Simmons & Lovegrove, 2017; Sloan, Lovegrove, & Simmons, 2018; Wigby et al., 2009).

These changes in ejaculate composition are expected to confer a post-copulatory advantage, that is to increase competitive fertilization success under conditions that trigger a male's plastic response. Indeed, the few studies in which male ejaculate plasticity has been experimentally manipulated confirmed that strategically increased sperm or seminal fluid quality is associated with an increased fertilization success (e.g., Bartlett et al., 2017; Wigby et al., 2009). In internal fertilizers, however, the actual consequences of ejaculate adjustment on competitive fertilization success warrant further research, in particular when a substantial delay between insemination and fertilization can occur, as in species in which females store sperm for successive reproductive events (Orr & Brennan, 2015). As time between insemination and fertilization increases, the opportunity for male × male × female interactions to influence the outcome of sperm competition may increase (Firman, Gasparini, Manier, & Pizzari, 2017; Lüpold & Pitnick, 2018; Lupold et al., 2013). The effect of ejaculate plasticity on the interaction between competing ejaculates and female reproductive tract, however, is difficult to be predicted (Ala-Honkola & Manier, 2016; Manier et al., 2010). For example, prolonged female sperm storage (FSS) increases the probability that ejaculates from several males overlap at the time of fertilization (Birkhead & Moller, 1993; Orr & Brennan, 2015). One may predict that the competitive advantage of plastic adjustments of sperm quality is enhanced in species with FSS and associated high sperm competition. At the same time, prolonged FSS is expected to increase the opportunity for cryptic female choice, based, for example, on genetic similarity between the female and the competitors (Gasparini, Congiu, & Pilastro, 2015; Gasparini & Pilastro, 2011). Nondirectional cryptic female choice may reduce, or even cancel out the directional male benefits associated with increased ejaculate investment (Gillingham et al., 2009). Furthermore, prolonged FSS may result in stronger selection for sperm survival over time and weaker selection for sperm velocity, as these two components of sperm quality are often traded-off. Across passerine birds, for example, the sperm swimming speed is positively correlated with the risk of sperm competition and negatively associated with the duration of FSS (Kleven et al., 2009). Evidence of a trade-off between sperm swimming velocity and survival has also been found within species (Burness, Casselman, Schulte-Hostedde, Moyes, & Montgomerie, 2004; Levitan, 2000; Taborsky, Schütz, Goffinet, & van Doorn, 2018; Yamamoto et al., 2017), although positive correlations have also been found (Locatello, Rasotto, Evans, & Pilastro, 2006).

As a consequence of FSS, sperm with different phenotypes may have different fertilization success in relation to insemination timing and duration of storage (Clark & Aronson, 1951). For example, in the zebra finch *Taeniopygia guttata*, sperm length (which is closely correlated with swimming velocity) and timing of copulation (first or last) interact in influencing subsequent fertilization success (Hemmings & Birkhead, 2017). This interaction implies that plastic adjustment of ejaculate quality in response to transient social cues (like sperm competition level or mate availability) may have different fitness outcomes depending on the duration of FSS. Across passerine species, for example, sperm velocity declines, and sperm longevity is expected to increase, as the duration of FSS increases (Kleven et al., 2009). Within species, a negative correlation between sperm velocity (at the moment of ejaculation) and fertilization success after FSS has been found in the live-bearing fish *Xiphophorus nigrensis* (Smith, 2012).

We investigated how male strategic ejaculate investment and FSS interact in determining a male's fertilization success in the guppy (*Poecilia reticulata*). Guppies are live-bearing, internal fertilizing fish characterized by high levels of sperm competition and prolonged FSS (Evans & Pilastro, 2011; Schmidt, 1920). Males produce sperm bundles that can be used to perform heterospermic artificial inseminations in which the number of sperm transferred during insemination is carefully controlled (Boschetto, Gasparini, & Pilastro, 2011; Evans, Zane, Francescato, & Pilastro, 2003). Male guppies that are in visual contact with females increase the number ('ready-to-use sperm'; Bozynski & Liley, 2003) and the swimming velocity of strippable sperm (Gasparini et al., 2009), as compared to males that are isolated from females. This response is faster than the spermiogenesis cycle, that is within 3 days versus

approx. 1 month (Billard & Puissant, 1969), and is therefore defined 'sperm priming' (Bozynski & Liley, 2003). Since sperm number and velocity are predictors of competitive fertilization success in this species (Boschetto et al., 2011), this adjustment can be assumed to have positive effects on male sperm competitiveness. However, increasing the swimming velocity of sperm is expected to reduce its longevity, as faster sperm will use up their energy reserves at a higher rate (Ball & Parker, 1996; Levitan, 2000; Parker, 1998; Pizzari & Parker, 2009). A reduced sperm lifespan may also derive from the oxidative stress associated with a higher sperm cell metabolic activity (Reinhardt, 2007). Finally, increased cell maturation rate associated with sperm priming may reduce the efficiency of spermatogenesis, resulting in a higher incidence of sperm defects (Jewgenow et al., 2009; Neubauer, Jewgenow, Blottner, Wildt, & Pukazhenthi, 2004). In guppies, female-stored sperm tend to age and to be outcompeted by freshly inseminated sperm: paternity share is equal after simultaneous artificial insemination with equal sperm number from two males (Evans et al., 2003), it is biased towards the first male if the two inseminations are performed 1 day apart (Magris, Cardozo, Santi, Devigili, & Pilastro, 2017), but it becomes strongly biased towards the last male, if the two inseminations are 1 month, or more, apart (Gasparini, Daymond, & Evans, 2018; Schmidt, 1920). However, males can continue to fertilize eggs through their female-stored sperm months after their death (López-Sepulcre, Gordon, Paterson, Bentzen, & Reznick, 2013), suggesting that the sperm of some males may be able to survive for prolonged time in female sperm storage organs and occasionally outcompete more freshly inseminated sperm. Sperm velocity is associated with a greater fertilization success both at mating (Boschetto et al., 2011) and after 1 month of female storage (Devigili, Di Nisio, Grapputo, & Pilastro, 2016).

We tested the prediction that increased sperm production and velocity entails sperm viability and survival costs, affecting fertilization success after FSS. To this end, we maintained two groups of male guppies either in visual and chemical contact with females (female-present group, FP hereafter) or in a completely female-deprived environment for 5 days (female-absent group, FA), a condition in which males have, on average, a lower sperm number and slower swimming sperm (Bozynski & Liley, 2003; Cattelan, Evans, Pilastro, & Gasparini, 2016; Cattelan & Pilastro, 2018; Devigili, Doldán-Martelli, & Pilastro, 2015; Gasparini et al., 2009). Firstly, we assessed the effect of sperm priming on in vitro sperm viability at stripping and 4 hr after sperm activation (as measure of sperm survival, modified from Gasparini & Evans, 2013). Secondly, we artificially inseminated virgin females with an equal number of sperm bundles from pairs of males. Each pair consisted of one randomly chosen male from each experimental group (FP and FA). We then assessed the effect of sperm priming on paternity share in the first brood, whose eggs are fertilized by freshly inseminated sperm, with that obtained in the subsequent brood, which is separated temporally by *circa* 1 month and whose eggs are therefore fertilized by female-stored sperm.

2 | MATERIALS AND METHODS

2.1 | Study species

The guppies used in this experiment were descendants of wild-caught fish from the lower part of Tacarigua River, Trinidad. Males and females used in the experiments derived from large stock tanks (150 L), each containing approx. 50 individuals of each sex. Individuals have been rotated across tanks during all storage period in order to maintain an adequate outbreeding (see Devigili et al., 2016, for an estimate of heterozygosity in the laboratory population). Fish were raised under controlled temperature (26 ± 1°C) and illumination (12:12-hr light/dark cycle) conditions and fed twice daily on a mixed diet of brine shrimp nauplii and dry food. Virgin females used for the artificial insemination experiment (see below) were obtained by separating females from males as soon as they could be sexed (around 2 months) and maintaining them in single-sex tanks until sexually mature (approx. at 4 months).

2.2 | Experimental design

We used an experimental design based on the presence of stimulus females to trigger a 'sperm priming' response, following a standard protocol (Bozynski & Liley, 2003; Cattelan et al., 2016; Gasparini et al., 2009). Adult males originated from stocks were isolated individually for 5 days ($N = 100$). After this period, males were stripped to remove previous sperm reserves and were randomly assigned to one of the two experimental groups, namely 'female present' (FP) and 'female absent' (FA). To this end, we used experimental aquaria that were divided into two compartments by a central, transparent wall to allow visual contact with females. Each male was placed individually in one of the two compartments. The other compartment either contained three females (FP) or was left empty (FA). The dividing wall was provided with small holes to allow chemical interaction with the females (where present). After 5 days, males were anaesthetized and sperm bundles were collected for artificial insemination and ejaculate quality assays, following established protocols (for further details, see Magris et al., 2017). In the guppy, full spermatogenesis lasts 5 weeks (Billard & Escaffre, 1969) but the males' priming response is quick, and 3 days is sufficient to induce a response (Gasparini et al., 2009). All males ($N = 100$) were digitally photographed to measure body size and coloration following established methods (Cattelan, Di Nisio, & Pilastro, 2018). Males assigned to the two groups were not different in size or coloration (see Supporting Information). A subsample was used for ejaculate traits analysis ($N = 78$) and/or for artificial insemination, according to the quantity of ejaculate available from each male. To this end, we randomly paired two males, one from each experimental group ($N = 70$). From these 35 male dyads, we obtained paternity data for 32 dyads ($N = 64$).

2.3 | Ejaculate quality assays

We estimated the effect of treatment on sperm number (no. of strippable bundles), sperm velocity and sperm viability on 48 males used for artificial insemination (see below) and on further 30 males that were not used for artificial insemination. All males went through either the FP or the FA treatment. We obtained an estimate of sperm number from 75 males (FP, $N = 37$; FA, $N = 38$), of sperm velocity from 65 males (FP, $N = 33$; FA, $N = 32$), and of sperm viability from 74 males (FP, $N = 37$; FA, $N = 37$). Sample size differed across ejaculate quality traits for logistic reasons (e.g. too few sperm bundles available).

2.3.1 | Sperm number

We took a digital photograph of the total bundles stripped on a black glass slide, and we subsequently used ImageJ software (available at http://rsb.info.nih.gov/ij/) to count the bundles. Each bundle contains an average of 22,000 sperm, and there is a significant correlation ($R^2 = .96$) between sperm number and number of bundles (Gasparini, Devigili, Dosselli, & Pilastro, 2013). Counting the number of bundles of each male allowed us to estimate the number of strippable sperm available to the males.

2.3.2 | Sperm velocity

Sperm swimming velocity was estimated at stripping and after 4 hr of incubation in activating solution (150 mM of KCl). To estimate sperm velocity at stripping, three bundles were collected from each freshly stripped ejaculate and placed on a glass slide containing 3 μl of activating solution and gently covered with a coverslip (Gasparini et al., 2009). Glass slides were previously coated with polyvinyl alcohol (PVA, 1%) to reduce sperm sticking to the glass surface. The swimming velocity of the sperm leaving the bundles was analysed using a Ceros Sperm Tracker (v. 12.3; Hamilton Thorne Research). For each male, measurements of sperm velocity, taken blind of the experimental group, were based on approximately 100 motile sperm and include average path velocity (VAP), straight-line velocity (VSL) and curvilinear velocity (VCL). The threshold values defining static cells were predetermined at 25 μm/s for VAP and 20 μm/s for VSL (Gasparini et al., 2009). To estimate sperm velocity after 4-hr incubation, 50 sperm bundles collected from the freshly stripped ejaculate were incubated in 30 μl of activating solution containing 40% 150mM KCl and 60% NaCl (Billard & Cosson, 1990) and maintained at 26 ± 1°C. After incubation, 3 μl of sperm in KCl-NaCl solution was placed on a glass slide with a coverslip and sperm velocity parameters were measured again as above. VAP, VSL and VCL were highly positively correlated (Pearson > 0.87, $p < .001$), and we thus present result for VAP.

2.3.3 | Sperm viability and survival

An aliquot of 6 μl of the incubation solution containing the sperm was taken immediately after sperm activation and stained with a live–dead kit (L-7011; Molecular Probes). This kit contains two dyes. The green dye stains live cells, whereas dead and damaged cells are stained red. The proportion of live sperm (stained green) was calculated over approximately 100 sperm cells per analysis, following an established procedure (Gasparini, Marino, Boschetto, & Pilastro, 2010). Sperm survival was estimated repeating the sperm viability assay on another 6 μl aliquot of the incubation solution 4 hr after activation.

2.4 | Artificial insemination and paternity analysis

At the end of the experimental treatment, we randomly paired one FA and one FP male and used collected ejaculate to artificially inseminate one virgin female with equal number of sperm bundles from two males, following established protocols (Evans et al., 2003; Gasparini & Evans, 2013; Gasparini & Pilastro, 2011). Artificial insemination (AI) allowed us to control for precopulatory processes (equalizing the confounding effect of mating order; Magris et al., 2017) and number of inseminated sperm (which affect competitive fertilization success; Boschetto et al., 2011). Briefly, after obtaining the ejaculate from one FA and one FP male (see Matthews, Evans, & Magurran, 1997), we collected 10 bundles from each male, mixed them in a small volume of saline solution (NaCl 0.9%) and used the 20 bundles to artificially inseminate one virgin female. After insemination, females were isolated in 8-L tanks with abundant vegetation and checked for offspring production at least three times a day, minimizing the effect of cannibalism on fry. Sixteen females did not produce any offspring, and five females produced a single offspring and were therefore excluded from the paternity analysis. Five females produced a brood >45 days after insemination. These may be second broods if the first one was miscarried or the fry were stillborn (a common phenomenon in poecilids, e.g., Mukherjee, Heithaus, Trexler, Ray-Mukherjee, & Vaudo, 2014), or first broods with a slow developing rate. Since it is not possible to ascertain with confidence the length of FSS, these broods were excluded from the paternity analysis. We obtained offspring from 36 females, but only 20 of them produced a second brood within 45 days after the first brood was delivered.

2.5 | DNA extraction and amplification

We collected a tissue sample from the females from which we obtained at least two offspring in the first brood ($n = 36$) and from their potential sires ($n = 64$, i.e., males used for inseminating females that produce no broods were not genotyped). Samples were stored in a freezer at −20°C until DNA extraction. Fin clips were obtained from anaesthetized males after ejaculate collection, and from females (if they produced at least one brood) 60 days after AI. Offspring were euthanized in MS222 and stored in 99% ethanol until DNA was extracted from half of their body.

Genomic DNA was extracted using CHELEX (Walsh, Metzger, & Higuchi, 1991) for offspring and salting out for adult fins (Miller, Dykes, & Polesky, 1988). Paternity has been assigned using two microsatellite markers: TTA (GenBank accession number: AF164205; Taylor, Sanny, & Breden, 1999) and AGAT11 (BV097141; Olendorf, Reudi, & Hughes, 2004; see Supporting Information for PCR details). Paternity was assigned using CERVUS v 3.0 (www.fieldgenetics.com/pages/home.jsp; Kalinowski, Taper, & Marshall, 2007; Marshall, Slate, Kruuk, & Pemberton, 1998). The data set included 561 offspring (from 36 females, 16 of which produced only the first brood and 20 of which produced two broods) of which 478 (i.e., 85%) were all assigned to one of the two competing FP-FA males with >95% confidence probability (see Devigili et al., 2016, for more details; Devigili, Evans, Di Nisio, & Pilastro, 2015). The average paternity assignment success for each female was 88%.

2.6 | Statistical analysis

Size and orange coloration (proportion of orange, arcsine-square-root-transformed) of males, and sperm production (number of bundles, log-transformed) were compared between FA and FP treatments using Student's *t* test. Difference between treatments (FA and FP) and time (at stripping and after 4 hr) in sperm velocity was tested using a linear mixed model in which male identity was entered as random factor, and experimental group (two levels: FA and FP), time (two levels: at stripping and after 4 hr) and their interaction as fixed factors. For sperm viability, we used a generalized linear mixed model (GLMM) with binomial distribution (logit link function) in which the total number of sperm observed was the binomial total and the number of viable sperm was the dependent variable; male identity was entered as random factor, and experimental group (two levels: FA and FP), time (two levels: at stripping and after 4 hr) and their interactions as fixed factors. To fit the model and estimate the parameters, we used the Laplace approximation of the log-likelihood. We corrected for a slight overdispersion (dispersion parameter of the initial model = 1.74) by adding an observation-level random effect with a separate level for each individual measurement (dispersion parameter of the final model = 1.00) (Browne, Subramanian, Jones, & Goldstein, 2005).

To compare the paternity share of FA and FP males, we first calculated the mean proportion of offspring sired by the FP male in the first (pFP_1) and in the second brood (pFP_2) and their difference (pFP_2-pFP_1, hereafter ΔpFP). We statistically compared the change in fertilization success between first and second broods only for those male dyads for which we had both broods ($N = 20$). Following Devigili et al. (2016), we first calculated, for each of the 20 male dyads, the observed mean paternity share of FP males in the first and in the second brood (pFP_{mean}). Secondly, we generated a simulated paternity distribution in the first and in the second broods assuming that each FP male had the same probability to sire an offspring in the two broods, which was set to be equal to his observed pFP_{mean}. For each simulation, the resulting *p*FP in the first and the second brood (pFP_{1sim} and pFP_{2sim}, respectively) were calculated for each of the male dyads (i.e., based on each dyad's observed pFP_{mean} and brood sizes). The observed difference in paternity across broods ΔpFP was compared with the null distribution of differences obtained from the simulated paternity pattern (i.e., pFP_{1sim}-pFP_{2sim}), given the observed brood size, thus accounting for binomial errors associated with differences in brood sizes. To this end, a Monte Carlo routine was iterated 10,000 times and the observed statistic (ΔpFP) was compared with the distribution of the simulated statistic (ΔpFP_{sim}). *p* values were derived from the proportion of ΔpFP_{sim} values that were larger or smaller than ΔpFP. Alpha level was set at .05. We used the same procedure to calculate the standardized variance in pFP_1 and pFP_2 [var *p*FP/(mean *p*FP)^2, hereafter var*p*FP] and to compare their observed difference with the distribution obtained for the pFP_{1sim} and pFP_{2sim} values. The standardized variance in *p*FP (often noted as *I*) represents an estimation of the opportunity for selection (Arnold & Wade, 1984; Crow, 1958; Jones, 2009) and allows to compare the strength of post-copulatory sexual selection before and after FSS (Devigili et al., 2016). Simulated distributions and Monte Carlo routines were performed in Windows Excel 2016 using PopTools 3.2.5 (Hood, 2011). Proportions were arcsine-transformed before statistical analyses (Sokal & Rohlf, 1995).

3 | RESULTS

3.1 | Effect of sperm priming on sperm quality in vitro

As expected, FP males had larger sperm reserves. FP males had, on average, 38.0% more sperm bundles as compared to FA males (FP: 253.4 ± 23.34 *SE*; FA: 183.7 ± 14.21 *SE*; Student's $t = 2.59$, $p = .012$; Figure S1). FP males also produced faster sperm, both at stripping and 4 hr after activation (Table 1, Figure S2). Sperm velocity significantly declined after 4 hr of incubation, although the decline in velocity did not differ between groups (Table 1). In particular, sperm of FP males were on average 7.2% faster at stripping than sperm of FA males. Four hours after sperm activation, the difference in sperm velocity was reduced, on average, to 3.9%, still in favour of FP males. In contrast, FP males showed a significant lower sperm viability both at stripping and 4 hr after activation, as compared to their FA counterparts (Table 1, Figure S3). Considering that the proportion of live sperm differed between the two groups, we calculated the number of live sperm as the product between the number of stripped sperm and the estimated proportion of live sperm. After correction for viability, the numerical advantage of FP at stripping and 4 hr after activation was equal to 28.9% and 24.2%, respectively (as compared to the 38.0% numerical advantage estimated without correcting for sperm viability).

3.2 | Effect of sperm priming on competitive fertilization success in vivo

Average brood size (considering only offspring whose paternity was successfully assigned) was 8.42 ± 0.66 *SE* for the first brood

TABLE 1 Effect of sperm priming on sperm velocity (VAP µm/s) and viability (% live sperm) at stripping and 4 hr after sperm activation

Sperm trait	FP	FA	Treatment	Time	Treatment*time
VAP					
At stripping	104.2 ± 15.51	97.17 ± 8.99	$F_{1,613} = 5.07$, $p = .028$	$F_{1,63} = 19.99$, $p < .001$	$F_{1,61} = 0.81$, $p = .371$
After 4 hr	93.75 ± 12.90	90.21 ± 10.69			
N	33	32			
Viability					
At stripping	77.98 ± 14.22	83.46 ± 9.79	$z = 2.03$, $p = .042$	$z = -3.035$, $p < .001$	$z = 0.580$, $p = .562$
After 4 hr	70.34 ± 16.50	77.96 ± 12.12			
N	37	37			

Note: Mean ± *SD* are given. For sperm velocities, significance of the fixed effects was tested in a linear mixed model. For sperm viability, significance of the fixed effects was tested in a generalized linear mixed-effects model with binomial distribution and logit link function (overdispersion = 1.00). Male identity was entered as random factor in both models.

Abbreviations: FA, female absent; FP, female present.

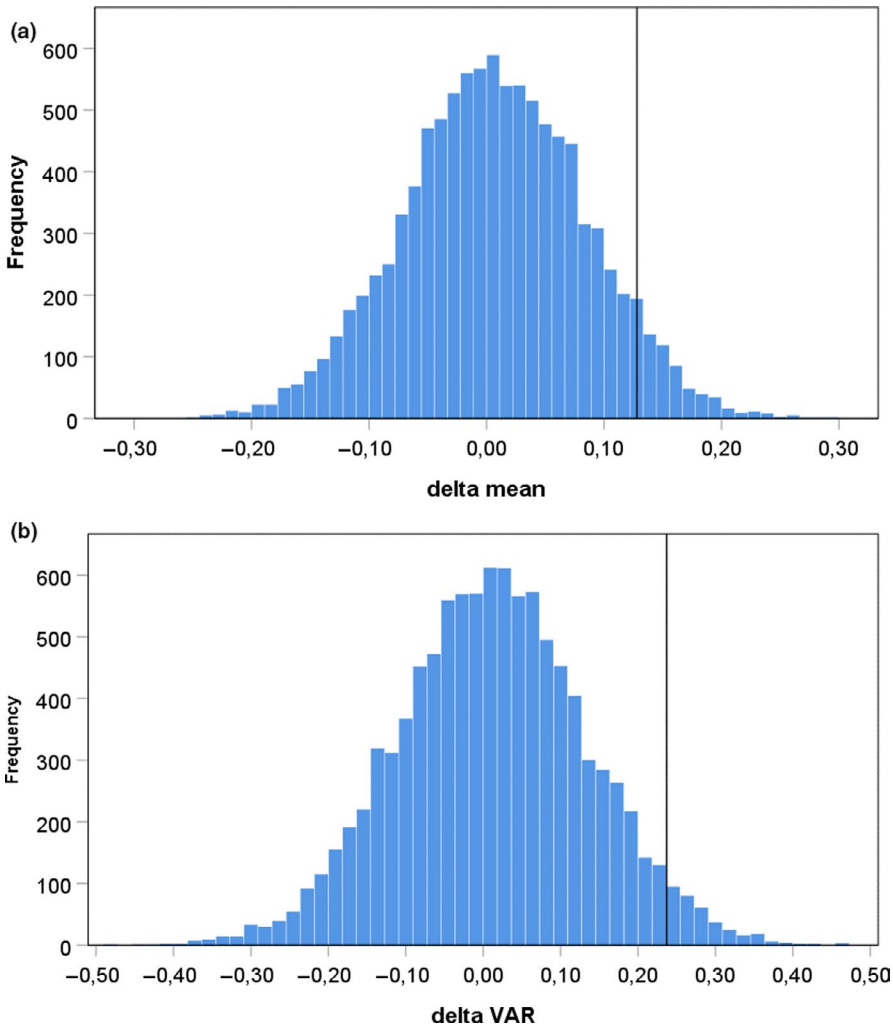

FIGURE 1 Observed difference in (a) the mean fertilization success across broods and (b) the standardized variance in fertilization success across broods. Histograms represent the expected distribution of the differences assuming equal probability to fertilize the eggs before and after FSS (=in the first and in the second brood). For each male dyad, the expected fertilization success was calculated as the across-broods mean. Vertical lines represent the observed difference

(Min = 2, Max = 17, *n* broods = 36, *n* offspring = 303) and 8.75 ± 0.98 *SE* for the second brood (Min = 2, Max = 16, *n* broods = 20, *n* offspring = 175), which is in line with brood sizes obtained in paternity studies with this guppy population (Devigili, Doldán-Martelli, et al., 2015). The mean proportion of offspring sired by FP males was 54.5% ± 4.98 *SE* in the first brood (*n* broods = 36), and 42.0% ± 5.62 SE in the second brood (*n* broods = 20). When considering only the 20 male dyads for which we also had the second brood, pFP_1 was 50.1% ± 7.46 *SE*. Δ*p*FP was therefore equal to −8.1%, and this difference was larger in magnitude than expected if *p*FP was the same in the two broods ($p = .044$, 10,000 iterations; Figure 1a). The standardized variance in fertilization success was larger in the first brood

TABLE 2 Effect of sperm priming and female sperm storage (first or second brood) on paternity share (mean and variance) of FP males

	First brood	*SE*	Second brood	*SE*	*p*
Mean fertilization rate (FP male)	0.501	0.075	0.420	0.058	.041*
Variance in fertilization rate	0.425	0.164	0.188	0.044	.021

Note: Each female ($n = 20$) was artificially inseminated with equal number of sperm bundles from two different males, one FP (female present, $n = 20$) and one FA (female absent, $n = 20$), and paternity was assigned on the first two consecutive broods. Differences between means and variances were tested using a permutation test in which the same fertilization success was expected across broods (see Methods). Standard errors of variance estimates were obtained using a bootstrap procedure.

*Permutation test was conducted after square root transformation of the proportions.

(var$p$FP$_1$ = 0.352) than in the second brood (var$p$FP$_2$ = 0.128), and the difference was larger than expected if var$p$FP was the same in the two broods ($p = .018$, 10,000 iterations; Figure 1b, Table 2).

4 | DISCUSSION

As predicted on the results of previous studies (Bozynski & Liley, 2003; Gasparini et al., 2009), we found that male guppies, when previously maintained in visual and chemical contact with females, increased the number (+38%) and the swimming velocity (VAP, +7.2%) of strippable sperm as compared to males that were isolated from females. The velocity of sperm after exposure to female stimulus was transient. Four hours after stripping, sperm velocity decreased and the difference with the sperm obtained from female-deprived males became nonsignificant. Our analysis of sperm viability highlighted an intrinsic post-copulatory cost associated with sperm priming: in FP males, the proportion of viable sperm was significantly lower both at stripping and 4h after activation, as compared to their FA counterparts (see Figure S1 and Table 1). If only alive sperm were counted, FP males still were at a numerical advantage over their FA counterparts, but the magnitude of this advantage (+28.9%) was lower than that estimated from total sperm count (+38.0%). Four hours after sperm activation, FP males' numerical advantage was further reduced to +24%. Our results from the in vitro measurements suggest that the increment of sperm number and velocity in response to the perceived mating opportunities is associated with a sperm viability cost both at stripping and after 4 hr. This may influence male fitness of wild individuals considering that natural sex ratio may vary between 0.2 and 0.9.

The results of our artificial insemination experiment allowed to compare the pFP before and after prolonged FSS. The eggs of the first brood are fertilized after artificial insemination, when the female was ovulating, or by sperm that were stored by the female for a short period (up to 5 days), which corresponds to the estimated ovulation cycle in virgin guppies (Rosenthal, 1952). In contrast, the eggs of the second brood were fertilized by sperm that was stored in the female reproductive tract for 3–4 weeks, which corresponds to the inter-brood interval (Magurran, 2005). In the first brood, competitive fertilization success, after inseminating the same number of sperm bundles from the two males, matched the result predicted from the in vitro measures of sperm velocity and viability: FP males with primed, faster sperm had a slight (although not significant) advantage. After a prolonged FSS, paternity share switched towards the FA males, matching their higher in vitro measures of sperm survival. The change in mean fertilization success between the first and the second brood was accompanied by a decrease in the standardized variance in pFP, indicating that FSS attenuated the strength of post-copulatory sexual selection. We will first discuss the mechanisms that may be responsible for the decreased viability and survival of sperm associated with sperm priming and how these may have impacted a male's fertilization success. Secondly, we will discuss the evolutionary consequences of the interaction between sperm priming and FSS on post-copulatory sexual selection in guppies.

The negative effect of sperm priming on sperm viability may have several, not mutually exclusive, explanations. For example, the reduced viability 4 hr after activation may be a consequence of the enhanced velocity and the expected trade-off between sperm performance and longevity (Levitan, 2000). A faster sperm cell senescence rate associated with enhanced velocity, however, does not explain our observation that sperm viability was reduced at stripping, before sperm swimming activity started. Spermatogenesis requires approximately 1 month in the guppy (Billard & Escaffre, 1969). The increase in the number of 'ready' sperm must therefore result from an acceleration of the last stages of spermiation (Bozynski & Liley, 2003), as our male treatment lasted only 5 days. A trade-off between sperm production rate and sperm quality has been reported in domestic cats, in which the proportion of abnormal spermatozoa is positively associated with increased sperm output (Neubauer et al., 2004). This effect is thought to be a consequence of reduced cell apoptosis during spermatogenesis (Jewgenow et al., 2009). Determining whether the negative relation between sperm maturation rate and proportion of viable sperm observed in guppies is associated with reduced apoptosis will require further investigation.

The trade-off with spermiation rate and sperm velocity may not be the only explanation for the observed reduction in sperm viability: the presence of the females during the treatment induces a higher sexual activity (courtship displays, male locomotor activity) in the males (Miller & Brooks, 2005), which may indirectly affect sperm viability, for example by elevating the oxidative stress in the FP males (Reinhardt & Ribou, 2013). Guppy males exposed to females have a reduced lifespan as compared to isolated males (Miller & Brooks, 2005), indicating a substantial cost of this pre- and post-copulatory plastic response. Consistent with this hypothesis, male houbara bustards (*Chlamydotis undulata*) with an elevated display rate suffer a faster senescence rate of their ejaculate quality (Preston, Saint Jalme, Hingrat, Lacroix, & Sorci, 2011). However,

other studies failed to find an effect of male precopulatory sexual effort on the oxidative stress in the testes (e.g., Garratt et al., 2012; Sloan et al., 2018). Furthermore, germline and sperm cells are particularly well protected from oxidative damage in guppies, as suggested by the relatively low senescence rate of ejaculate quality in this species (Gasparini, Devigili, & Pilastro, 2019). However, in the zebrafish *Danio rerio*, a high male–male competition environment leads males to produce faster swimming sperm, at a cost of DNA integrity and offspring fitness (Silva et al., 2019; Zajitschek, Hotzy, Zajitschek, & Immler, 2014), suggesting that this protection is incomplete. To distinguish between these alternative explanations, it would be necessary to experimentally manipulate male courtship effort independently from sperm priming.

The fertilization success of FP males in the first brood (short FSS) was higher than that of FA males, although not significantly so. This is expected, as we used an equal number of sperm bundles from the two males to inseminate the female, thereby controlling for the larger sperm reserves available to the FP males. Considering that FP males' sperm had a lower proportion of live sperm at stripping, this result suggests that the higher swimming velocity of FP males' sperm compensated for the reduced sperm viability (i.e., the total number of alive sperm). The fertilization success of FP males, however, faded, after prolonged FSS, to 42%. Since each virgin female was artificially inseminated only once, the two broods were fertilized by sperm from the same initial pool. Considering that the average inter-brood interval in this species is about 1 month, the sperm that fertilized the second brood were stored by the females 1 month more than the sperm that fertilized the first brood. These results suggest that sperm from FP males have a reduced survival or a more rapid senescence in their competitive fertilization success within the FSS organs as compared to the sperm from FA males. An alternative explanation for the reduced fertilization success of FP males in the second brood is related to sperm depletion, rather than a loss of competitiveness after sperm storage. If more sperm from FP males have been used earlier to fertilize the first brood, less sperm may have entered the FSS organs, resulting in a lower sperm number available to fertilize the second brood. This explanation, however, seems less likely. For the artificial inseminations, we used 10 bundles from each male, corresponding to approximately 220,000 sperm per male (Gasparini et al., 2013). Sperm bundles immediately dissolve in the female ovarian fluid (Cardozo & Pilastro, 2018), and sperm start moving towards fertilization and storage sites. Even if the sperm from a single male fertilized all the eggs in the most numerous brood (17 offspring), only a small proportion of the available sperm was necessary to fertilize all eggs. Indeed, results from previous studies demonstrate that two bundles are enough to fertilize all female eggs (Pilastro, Gasparini, Boschetto, & Evans, 2008). Furthermore, under this scenario one would expect a negative correlation between fertilization success in the first and in the second brood. In contrast, we observed a positive, although not significant, correlation ($r = .14$, $N = 20$, $p = .57$).

The combination of our in vitro and in vivo assays indicates that the strategic up-regulation of sperm production and sperm velocity is therefore associated with a reduced sperm competitiveness after prolonged FSS. A similar result has been found in the swordtail *Xiphophorus nigrensis*, in which sperm velocity at insemination is negatively correlated with competitive fertilization after FSS (Smith, 2012). Interestingly, in this same guppy population, the opposite pattern is found when males are maintained in the same conditions before mating (i.e., there is not differential level of sperm priming among males). The mean fertilization success of males producing fast swimming sperm increases between the first and the second brood (Devigili et al., 2016). The results from the present study suggest that the sperm priming response (i.e., the increased number and velocity of ready sperm) to female stimuli revealed a trade-off between sperm velocity and sperm viability–survival that was not evident when males are all in the same condition (Devigili et al., 2016). Alternatively, the increased sperm velocity and reduced sperm survival (and fertilization success after FSS) may represent two independent effects of our experimental manipulation. Whatever the mechanism behind our observations, it appears that the plastic adjustment of some components of ejaculate quality (number and velocity of ready sperm) comes at a significant cost of sperm competitiveness after FSS.

Sperm priming also reversed the effect of FSS on the opportunity of post-copulatory sexual selection. Although in Devigili et al. (2016) the opportunity of post-copulatory sexual selection increased with FSS (i.e., second broods had a larger variance in fertilization success than first broods), here we found the opposite pattern, and var*p*FP decreased between first and second brood. This suggests that sperm priming is expected to increase the variance in male post-copulatory success when fertilization rapidly follows insemination, as it increases the phenotypic variance in ejaculate quality. In contrast, FFS appears to attenuate the opportunity for post-copulatory selection, probably by exposing a sperm velocity/survival trade-off. The results obtained in a similar experiment in which the fertilization success of unmanipulated male guppies has been compared across subsequent broods (Gasparini & Evans, 2018) are in agreement with our prediction. The standardized variance in the proportion of offspring fertilized by the second male (*P*2) in the first and in the second brood, calculated from the data by Gasparini and Evans (2018), is 0.258 and 0.214 ($n = 18$), respectively. These values appear to be intermediate between the standardized variances in the first and the second brood found in our study (0.425 and 0.188, respectively). Irrespective of the mechanism determining the reduced sperm viability and fertilization success after FSS associated with sperm priming response, our results can explain why, in natural guppy populations, posthumous fertilizations can contribute to up to 25% of male reproductive success (López-Sepulcre et al., 2013). This also implies that the sperm of some males may increase their relative fertilization success after prolonged FSS, despite more freshly inseminated sperm having been shown to outcompete previously stored sperm (Gasparini et al., 2018; Schmidt, 1920). The evidence that sperm priming reduces competitive fertilization success after prolonged FSS may contribute explaining why fertilization success can change substantially over successive broods (López-Sepulcre et al., 2013).

FSS influences post-copulatory sexual selection in the guppy in a complex way. Timing of mating is important, but male precedence (i.e., the effect of insemination order on male competitive fertilization success) depends on the time elapsed between copulations: after artificial inseminations, first male precedence is observed if the inseminations from two males are 1 day apart (Magris et al., 2017), whereas there is a strong second male precedence when the second insemination occurs 1 month apart (Gasparini et al., 2018; Grove, 1980). Turnovers in paternity, however, can also occur, with sperm stored for months still contributing to a significant proportion of offspring sired several breeding cycles after copulation occurred (Hildemann & Wagner, 1954; López-Sepulcre et al., 2013). Results from the present study suggest that paternity succession may also be influenced by the interaction between sperm priming and FSS.

5 | CONCLUSIONS

In our study, we shed light, for the first time, on the interaction between anticipatory ejaculate plasticity and FSS. As predicted, improved ejaculate quality comes at a cost in sperm viability that negatively affects fertilization success after FSS. These results highlight the importance of considering all the potential fitness consequences of a strategic adjustment of ejaculate quality in response to a changing social environment (Dore et al., 2018; Kelly & Jennions, 2011; Magris, Chimetto, Rizzi, & Pilastro, 2018). Although ejaculate anticipatory responses are obviously expected to increase overall male reproductive fitness, they also imply costs (e.g., Bretman, Fricke, & Chapman, 2009; Silva et al., 2019). Previous work suggests that sperm priming can negatively affect male mating success (Cattelan et al., 2016; Devigili, Doldán-Martelli, et al., 2015), although this cost has never been quantified in the guppy. Here, we showed that sperm priming, one of the most common forms of male response to variable socio-sexual conditions (Bartlett et al., 2017; Bretman, Gage, & Chapman, 2011; Kelly & Jennions, 2011), may also entail intrinsic post-copulatory costs and that these costs are contingent to the duration of FSS. Our results highlight the importance to consider long-term consequences of the strategic adjustments of ejaculate components in species with FSS (Orr & Brennan, 2015). The phenomenon of sperm competition was first described in guppies, 100 years ago (Schmidt, 1920), in an experiment in which competitive fertilization success was found to be dependent on the duration of FSS: 'In the peculiar state of things here described I have had recourse to the assumption that the fresh spermatozoa are more agile than the older stock which had been stored for varying lengths of time in the genital duct of the female, and are thus unable to compete with the former. …The fact that we can, by pairing a female simultaneously with two males of different form, produce, in one and the same brood, offspring belonging to the same forms, seems to support this explanation of the selection which takes place among the spermatozoa in the genital duct'. (Schmidt, 1920, p. 8–9). From the results of the first study on sperm competition, it appears evident that FSS provides scope to influence competitive fertilization success (Birkhead & Møller, 1993). The way FFS interacts with sperm phenotype is complex and not easy to be investigated (Hemmings & Birkhead, 2017; Lupold et al., 2013; Manier et al., 2010). Our results suggest that ejaculate plasticity adds further complexity to post-copulatory processes mediated by FSS, making sperm storage a fascinating avenue for future research.

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AUTHORS' CONTRIBUTIONS

G.C. and A.P. conceived the study and organized the experiment with A.D. G.C., A.D. and P.A. performed the experiment. A.D, G.C. and A.P. analysed the results, and all authors contributed in their interpretation. A.D. and A.P. wrote the manuscript, and all the authors contributed to the final version and approved it for publication.

ETHICAL APPROVAL

This experiment was conducted according to the Italian legal requirements and was approved by the ethics committee of the University of Padova (permit no. CEASA #12/2014). See Supporting Information for more details.

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REFERENCES

Ala-Honkola, O., & Manier, M. K. (2016). Multiple mechanisms of cryptic female choice act on intraspecific male variation in *Drosophila simulans*. *Behavioral Ecology and Sociobiology*, *70*, 519–532. https://doi.org/10.1007/s00265-016-2069-3

Arnold, S. J., & Wade, M. J. (1984). On the measurement of natural and sexual selection: Theory. *Evolution*, *38*, 709–719. https://doi.org/10.1111/j.1558-5646.1984.tb00344.x

Baker, R. R., & Shackelford, T. K. (2018). A comparison of paternity data and relative testes size as measures of level of sperm competition in the Hominoidea. *American Journal of Physical Anthropology*, *165*, 421–443. https://doi.org/10.1002/ajpa.23360

Ball, M. A., & Parker, G. A. (1996). Sperm competition games: External fertilization and "adaptive" infertility. *Journal of Theoretical Biology*, *180*, 141–150.

Bartlett, M. J., Steeves, T. E., Gemmell, N. J., & Rosengrave, P. C. (2017). Sperm competition risk drives rapid ejaculate adjustments mediated by seminal fluid. *Elife*, *6*, e28811. https://doi.org/10.7554/eLife.28811

Billard, R., & Cosson, M. P. (1990). The energetics of fish sperm motility. In C. Gagnon (Ed.), *Controls of sperm motility: Biological and clinical aspects* (pp. 155–173). Boca Raton, FL: CRC Press.

Billard, R., & Escaffre, A. (1969). La spermatogenèse de *Poecilia reticulata*. I. Estimation du nombre de générations goniales et rendement de la spermatogenèse. *Annales de Biologie Animale Biochimie Biophysique*, *9*, 251–271.

Billard, R., & Puissant, C. (1969). La spermatogenèse de *Poecilia reticulata*. II. La production spermatogénétique. *Annales de Biologie Animale Biochimie Biophysique*, *9*, 307–313. https://doi.org/10.1051/rnd:19690301

Birkhead, T. R., Hosken, D. J., & Pitnick, S. (2009). *Sperm biology: An evolutionary perspective*. London, UK: Academic Press.

Birkhead, T. R., & Moller, A. P. (1993). Sexual Selection and the temporal separation of reproductive events – Sperm storage data from reptiles, birds and mammals. *Biological Journal of the Linnean Society*, *50*, 295–311. https://doi.org/10.1111/j.1095-8312.1993.tb00933.x

Boschetto, C., Gasparini, C., & Pilastro, A. (2011). Sperm number and velocity affect sperm competition success in the guppy (*Poecilia reticulata*). *Behavioral Ecology and Sociobiology*, *65*, 813–821. https://doi.org/10.1007/s00265-010-1085-y

Bozynski, C. C., & Liley, N. R. (2003). The effect of female presence on spermiation, and of male sexual activity on 'ready' sperm in the male guppy. *Animal Behaviour*, *65*, 53–58.

Bretman, A., Fricke, C., & Chapman, T. (2009). Plastic responses of male *Drosophila melanogaster* to the level of sperm competition increase male reproductive fitness. *Proceedings of the Royal Society B: Biological Sciences*, *276*, 1705–1711.

Bretman, A., Gage, M. J. G., & Chapman, T. (2011). Quick-change artists: Male plastic behavioural responses to rivals. *Trends in Ecology and Evolution*, *26*, 467–473. https://doi.org/10.1016/j.tree.2011.05.002

Browne, W. J., Subramanian, S. V., Jones, K., & Goldstein, H. (2005). Variance partitioning in multilevel logistic models that exhibit overdispersion. *Journal of the Royal Statistical Society Series A-Statistics in Society*, *168*, 599–613. https://doi.org/10.1111/j.1467-985X.2004.00365.x

Burness, G., Casselman, S. J., Schulte-Hostedde, A. I., Moyes, C. D., & Montgomerie, R. (2004). Sperm swimming speed and energetics vary with sperm competition risk in bluegill (*Lepomis macrochirus*). *Behavioral Ecology and Sociobiology*, *56*, 65–70. https://doi.org/10.1007/s00265-003-0752-7

Cardozo, G., & Pilastro, A. (2018). Female nutritional condition affects ovarian fluid quality in guppies. *Biology Letters*, *14*. https://doi.org/10.1098/rsbl.2018.0122

Cattelan, S., Di Nisio, A., & Pilastro, A. (2018). Stabilizing selection on sperm number revealed by artificial selection and experimental evolution. *Evolution*, *72*(3), 698–706.

Cattelan, S., Evans, J. P., Pilastro, A., & Gasparini, C. (2016). The effect of sperm production and mate availability on patterns of alternative mating tactics in the guppy. *Animal Behaviour*, *112*, 105–110. https://doi.org/10.1016/j.anbehav.2015.11.024

Cattelan, S., & Pilastro, A. (2018). Sperm priming response to perceived mating opportunities is reduced in male guppies with high baseline sperm production. *Current Zoology*, *64*, 205–211. https://doi.org/10.1093/cz/zoy008

Clark, E., & Aronson, L. R. (1951). Sexual behavior in the guppy, *Lebistes reticulatus* (Peters). *Zoologica*, *36*, 49–66.

Cornwallis, C. K., & Birkhead, T. R. (2007). Changes in sperm quality and numbers in response to experimental manipulation of male social status and female attractiveness. *American Naturalist*, *170*, 758–770. https://doi.org/10.1086/521955

Crow, J. F. (1958). Some possibilities for measuring selection intensities in man. *Human Biology*, *30*, 1–13.

Devigili, A., Di Nisio, A., Grapputo, A., & Pilastro, A. (2016). Directional postcopulatory sexual selection is associated with female sperm storage in Trinidadian guppies. *Evolution*, *70*, 1829–1843. https://doi.org/10.1111/evo.12989

Devigili, A., Doldán-Martelli, V., & Pilastro, A. (2015). Exploring simultaneous allocation to mating effort, sperm production, and body growth in male guppies. *Behavioral Ecology*, *26*, 1203–1211.

Devigili, A., Evans, J. P., Di Nisio, A., & Pilastro, A. (2015). Multivariate selection drives concordant patterns of pre- and postcopulatory sexual selection in a livebearing fish. *Nature Communications*, *6*, 8291. https://doi.org/10.1038/ncomms9291

Dewsbury, D. A. (1982). Ejaculate cost and male choice. *The American Naturalist*, *119*, 601–610. https://doi.org/10.1086/283938

Dore, A. A., McDowall, L., Rouse, J., Bretman, A., Gage, M. J. G., & Chapman, T. (2018). The role of complex cues in social and reproductive plasticity. *Behavioral Ecology and Sociobiology*, *72*, 124. https://doi.org/10.1007/s00265-018-2539-x

Evans, J. P., & Pilastro, A. (2011). Postcopulatory sexual selection. In J. P. Evans, A. Pilastro, & I. Schlupp (Eds.), *Ecology and evolution of Poeciliid fishes* (pp. 197–208). Chicago, IL: Chicago University Press.

Evans, J. P., Zane, L., Francescato, S., & Pilastro, A. (2003). Directional postcopulatory sexual selection revealed by artificial insemination. *Nature*, *421*, 360–363. https://doi.org/10.1038/nature01367

Firman, R. C., Garcia-Gonzalez, F., Simmons, L. W., & Andre, G. I. (2018). A competitive environment influences sperm production, but not testes tissue composition, in house mice. *Journal of Evolutionary Biology*, *31*, 1647–1654. https://doi.org/10.1111/jeb.13360

Firman, R. C., Gasparini, C., Manier, M. K., & Pizzari, T. (2017). Postmating female control: 20 years of cryptic female choice. *Trends in Ecology and Evolution*, *32*, 368–382. https://doi.org/10.1016/j.tree.2017.02.010

Garratt, M., McArdle, F., Stockley, P., Vasilaki, A., Beynon, R. J., Jackson, M. J., & Hurst, J. L. (2012). Tissue-dependent changes in oxidative damage with male reproductive effort in house mice. *Functional Ecology*, *26*, 423–433. https://doi.org/10.1111/j.1365-2435.2011.01952.x

Gasparini, C., Congiu, L., & Pilastro, A. (2015). Major histocompatibility complex similarity and sexual selection: Different does not always mean attractive. *Molecular Ecology*, *24*, 4286–4295. https://doi.org/10.1111/mec.13222

Gasparini, C., Daymond, E., & Evans, J. P. (2018). Extreme fertilization bias towards freshly inseminated sperm in a species exhibiting prolonged female sperm storage. *Royal Society Open Science*, *5*, 172195. https://doi.org/10.1098/rsos.172195

Gasparini, C., Devigili, A., Dosselli, R., & Pilastro, A. (2013). Pattern of inbreeding depression, condition dependence, and additive genetic variance in Trinidadian guppy ejaculate traits. *Ecology and Evolution*, *3*, 4940–4953.

Gasparini, C., Devigili, A., & Pilastro, A. (2019). Sexual selection and ageing: Interplay between pre- and post-copulatory traits senescence in the guppy. *Proceedings of the Royal Society B: Biological Sciences*, *286*, 20182873. https://doi.org/10.1098/rspb.2018.2873

Gasparini, C., & Evans, J. P. (2013). Ovarian fluid mediates the temporal decline in sperm viability in a fish with sperm storage. *PLoS One*, *8*, e64431. https://doi.org/10.1371/journal.pone.0064431

Gasparini, C., & Evans, J. P. (2018). Female control over multiple matings increases the opportunity for postcopulatory sexual selection. *Proceedings of the Royal Society B: Biological Sciences*, 285. https://doi.org/10.1098/rspb.2018.1505

Gasparini, C., Marino, I. A. M., Boschetto, C., & Pilastro, A. (2010). Effect of male age on sperm traits and sperm competition success in the guppy (*Poecilia reticulata*). *Journal of Evolutionary Biology*, *23*, 124–135.

Gasparini, C., Peretti, A. V., & Pilastro, A. (2009). Female presence influences sperm velocity in the guppy. *Biology Letters*, *5*, 792–794. https://doi.org/10.1098/rsbl.2009.0413

Gasparini, C., & Pilastro, A. (2011). Cryptic female preference for genetically unrelated males is mediated by ovarian fluid in the guppy. *Proceedings of the Royal Society B: Biological Sciences*, *278*, 2495–2501. https://doi.org/10.1098/rspb.2010.2369

Gillingham, M. A. F., Richardson, D. S., Løvlie, H., Moynihan, A., Worley, K., & Pizzari, T. (2009). Cryptic preference for MHC-dissimilar females in male red junglefowl, *Gallus gallus*. *Proceedings of the Royal Society B: Biological Sciences*, *276*, 1083–1092.

Grove, B. D. (1980). *An analysis of intraovarian sperm interactions in the guppy*, Poecilia reticulata. Department of Zoology, Master of Science – MSc. University of British Columbia, Vancouver, Canada.

Hemmings, N., & Birkhead, T. (2017). Differential sperm storage by female zebra finches *Taeniopygia guttata*. *Proceedings of the Royal Society B: Biological Sciences*, 284.

Hildemann, W. H., & Wagner, E. D. (1954). Intraspecific sperm competition in *Lebistes reticulatus*. *The American Naturalist*, *88*, 87–91. https://doi.org/10.1086/281813

Hood, G. M. (2011). *PopTools version 3.2.5*. Available on the internet. Retrieved from http://www.poptools.org

Hopkins, B., Sepil, I., & Wigby, S. (2017). Seminal fluid. *Current Biology*, *27*, R404–R405. https://doi.org/10.1016/j.cub.2017.03.063

Jewgenow, K., Neubauer, K., Blottner, S., Schoen, J., Wildt, D. E., & Pukazhenthi, B. S. (2009). Reduced germ cell apoptosis during spermatogenesis in the teratospermic domestic cat. *Journal of Andrology*, *30*, 460–468. https://doi.org/10.2164/jandrol.108.006726

Jones, A. G. (2009). On the opportunity for sexual selection, the Bateman gradient and the maximum intensity of sexual selection. *Evolution*, *63*, 1673–1684.

Kalinowski, S. T., Taper, M. L., & Marshall, T. C. (2007). Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology*, *16*, 1099–1106. https://doi.org/10.1111/j.1365-294X.2007.03089.x

Kelly, C. D., & Jennions, M. D. (2011). Sexual selection and sperm quantity: Meta analyses of strategic ejaculation. *Biological Reviews*, *88*, 863–884. https://doi.org/10.1111/j.1469-185X.2011.00175.x

Kilgallon, S. J., & Simmons, L. W. (2005). Image content influences men's semen quality. *Biology Letters*, *1*, 253–255. https://doi.org/10.1098/rsbl.2005.0324

Kleven, O., Fossøy, F., Laskemoen, T., Robertson, R. J., Rudolfsen, G., & Lifjeld, J. T. (2009). Comparative evidence for the evolution of sperm swimming speed by sperm competition and female sperm storage duration in passerine birds. *Evolution*, *63*, 2466–2473. https://doi.org/10.1111/j.1558-5646.2009.00725.x

Levitan, D. R. (2000). Sperm velocity and longevity trade off each other and influence fertilization in the sea urchin *Lytechinus variegatus*. *Proceedings of the Royal Society B: Biological Sciences*, *267*, 531–534.

Locatello, L., Rasotto, M. B., Evans, J. P., & Pilastro, A. (2006). Colourful male guppies produce faster and more viable sperm. *Journal of Evolutionary Biology*, *19*, 1595–1602. https://doi.org/10.1111/j.1420-9101.2006.01117.x

López-Sepulcre, A., Gordon, S. P., Paterson, I. G., Bentzen, P., & Reznick, D. N. (2013). Beyond lifetime reproductive success: The posthumous reproductive dynamics of male Trinidadian guppies. *Proceedings of the Royal Society B: Biological Sciences*, *280*, 20131116. https://doi.org/10.1098/rspb.2013.1116

Lüpold, S., & Pitnick, S. (2018). Sperm form and function: What do we know about the role of sexual selection? *Reproduction*, *155*, R229–R243. https://doi.org/10.1530/REP-17-0536

Lupold, S., Pitnick, S., Berben, K. S., Blengini, C. S., Belote, J. M., & Manier, M. K. (2013). Female mediation of competitive fertilization success in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America*, *110*, 10693–10698. https://doi.org/10.1073/pnas.1300954110

Magris, M., Cardozo, G., Santi, F., Devigili, A., & Pilastro, A. (2017). Artificial insemination unveils a first-male fertilization advantage in the guppy. *Animal Behaviour*, *131*, 45–55. https://doi.org/10.1016/j.anbehav.2017.07.009

Magris, M., Chimetto, G., Rizzi, S., & Pilastro, A. (2018). Quick-change artists: Male guppies pay no cost to repeatedly adjust their sexual strategies. *Behavioral Ecology*, *29*, 1113–1123. https://doi.org/10.1093/beheco/ary087

Magurran, A. E. (2005). *Evolutionary ecology: The trinidadian guppy*. Oxford, UK: Oxford University Press.

Manier, M. K., Belote, J. M., Berben, K. S., Novikov, D., Stuart, W. T., & Pitnick, S. (2010). Resolving mechanisms of competitive fertilization success in *Drosophila melanogaster*. *Science*, *328*, 354–357. https://doi.org/10.1126/science.1187096

Marshall, T. C., Slate, J., Kruuk, L. E. B., & Pemberton, J. M. (1998). Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology*, *7*, 639–655. https://doi.org/10.1046/j.1365-294x.1998.00374.x

Matthews, I. M., Evans, J. P., & Magurran, A. E. (1997). Male display rate reveals ejaculate characteristics in the Trinidadian guppy *Poecilia reticulata*. *P. Proceedings of the Royal Society B: Biological Sciences*, *264*, 695–700.

Miller, L. K., & Brooks, R. (2005). The effects of genotype, age, and social environment on male ornamentation, mating behavior, and attractiveness. *Evolution*, *59*, 2414–2425.

Miller, S. A., Dykes, D. D., & Polesky, H. F. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research*, *16*, 1215. https://doi.org/10.1093/nar/16.3.1215

Møller, A. P., & Briskie, J. V. (1995). Extra pair paternity, sperm competition and the evolution of testis size in birds. *Behavioral Ecology and Sociobiology*, *36*, 357–365.

Mukherjee, S., Heithaus, M. R., Trexler, J. C., Ray-Mukherjee, J., & Vaudo, J. (2014). Perceived risk of predation affects reproductive life-history traits in *Gambusia holbrooki*, but not in *Heterandria formosa*. *PLoS One*, *9*, e88832.

Neubauer, K., Jewgenow, K., Blottner, S., Wildt, D. E., & Pukazhenthi, B. S. (2004). Quantity rather than quality in teratospermic males: A histomorphometric and flow cytometric evaluation of spermatogenesis in the domestic cat (*Felis catus*). *Biology of Reproduction*, *71*, 1517–1524.

Olendorf, R., Reudi, B., & Hughes, K. A. (2004). Primers for 12 polymorphic microsatellite DNA loci from the guppy (*Poecilia reticulata*). *Molecular Ecology Notes*, *4*, 668–671. https://doi.org/10.1111/j.1471-8286.2004.00777.x

Olsson, M., Madson, T., & Shine, R. (1997). Is sperm really so cheap? Costs of reproduction in male adders, *Vipera berus*. *Proceedings of the Royal Society B: Biological Sciences*, *264*, 455–459.

Orr, T. J., & Brennan, P. L. R. (2015). Sperm storage: Distinguishing selective processes and evaluating criteria. *Trends in Ecology and Evolution*, *30*, 261–272. https://doi.org/10.1016/j.tree.2015.03.006

Parker, G. A. (1970). Sperm competition and its evolutionary effect on copula duration in fly *Scatophaga stercoraria*. *Journal of Insect Physiology*, *16*, 1301–1328.

Parker, G. A. (1998). Sperm competition and the evolution of ejaculates: Towards a theory base. In T. R. Birkhead, & A. P. Møller (Eds.), *Sperm competition and sexual selection* (pp. 1–54). London, UK: Academic Press.

Parker, G. A. (2016). The evolution of expenditure on testes. *Journal of Zoology*, *298*, 3–19. https://doi.org/10.1111/jzo.12297

Parker, G. A., Ball, M. A., Stockley, P., & Gage, M. J. G. (1997). Sperm competition games: A prospective analysis of risk assessment.

Proceedings of the Royal Society B: Biological Sciences, *264*, 1793–1802. https://doi.org/10.1098/rspb.1997.0249

Parker, G. A., & Birkhead, T. R. (2013). Polyandry: The history of a revolution. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *368*, 20120335. https://doi.org/10.1098/rstb.2012.0335

Parker, G. A., & Pizzari, T. (2010). Sperm competition and ejaculate economics. *Biological Reviews*, *85*, 897–934. https://doi.org/10.1111/j.1469-185X.2010.00140.x

Pilastro, A., Gasparini, C., Boschetto, C., & Evans, J. P. (2008). Colorful male guppies do not provide females with fecundity benefits. *Behavioral Ecology*, *19*, 374–381. https://doi.org/10.1093/beheco/arm140

Pitnick, S. (1996). Investment in testes and the cost of making long sperm in *Drosophila*. *The American Naturalist*, *148*, 57–80. https://doi.org/10.1086/285911

Pizzari, T., & Parker, G. A. (2009). Sperm competition and sperm phenotype. In R. B. Tim, J. H. David, & P. Scott (Eds.), *Sperm biology* (pp. 207–245). London, UK: Academic Press.

Poiani, A. (2006). Complexity of seminal fluid: A review. *Behavioral Ecology and Sociobiology*, *60*, 289–310. https://doi.org/10.1007/s00265-006-0178-0

Preston, B. T., Saint Jalme, M., Hingrat, Y., Lacroix, F., & Sorci, G. (2011). Sexually extravagant males age more rapidly. *Ecology Letters*, *14*, 1017–1024. https://doi.org/10.1111/j.1461-0248.2011.01668.x

Ramm, S., Edward, D., Claydon, A., Hammond, D., Brownridge, P., Hurst, J., … Stockley, P. (2015). Sperm competition risk drives plasticity in seminal fluid composition. *BMC Biology*, *13*, 87. https://doi.org/10.1186/s12915-015-0197-2

Ramm, S. A., Parker, G. A., & Stockley, P. (2005). Sperm competition and the evolution of male reproductive anatomy in rodents. *Proceedings of the Royal Society B: Biological Sciences*, *272*, 949–955. https://doi.org/10.1098/rspb.2004.3048

Reinhardt, K. (2007). Evolutionary consequences of sperm cell aging. *Quarterly Review of Biology*, *82*, 375–393. https://doi.org/10.1086/522811

Reinhardt, K., & Ribou, A. C. (2013). Females become infertile as the stored sperm's oxygen radicals increase. *Scientific Reports*, *3*, 2888. https://doi.org/10.1038/srep02888

Rosenthal, H. L. (1952). Observations on reproduction of the poeciliid *Lebistes reticulatus* (Peters). *Biological Bulletin*, *102*, 30–38. https://doi.org/10.2307/1538621

Rowley, A. G., Daly-Engel, T. S., & Fitzpatrick, J. L. (2018). Testes size increases with sperm competition risk and intensity in bony fish and sharks. *Behavioral Ecology*, 364–371. https://doi.org/10.1093/beheco/ary174

Rudolfsen, G., Figenschou, L., Folstad, I., Tveiten, H., & Figenschou, M. (2006). Rapid adjustments of sperm characteristics in relation to social status. *Proceedings of the Royal Society B: Biological Sciences*, *273*, 325–332. https://doi.org/10.1098/rspb.2005.3305

Schmidt, J. (1920). Racial investigations IV. The genetic behaviour of a secondary sexual character. *Compt. R. Tr. Lab. Carlsberg*, *14*, 1–23.

Silva, W. T. A. F., Sáez-Espinosa, P., Torijo-Boix, S., Romero, A., Devaux, C., Durieux, M., … Immler, S. (2019). The effects of male social environment on sperm phenotype and genome integrity. *Journal of Evolutionary Biology*, *32*, 535–544. https://doi.org/10.1111/jeb.13435

Simmons, L. W., & Fitzpatrick, J. L. (2012). Sperm wars and the evolution of male fertility. *Reproduction*, *144*, 519–534. https://doi.org/10.1530/REP-12-0285

Simmons, L. W., & Lovegrove, M. (2017). Socially cued seminal fluid gene expression mediates responses in ejaculate quality to sperm competition risk. *Proceedings of the Royal Society B: Biological Sciences*, *284*, 20171486. https://doi.org/10.1098/rspb.2017.1486

Sloan, N. S., Lovegrove, M., & Simmons, L. W. (2018). Social manipulation of sperm competition intensity reduces seminal fluid gene expression. *Biology Letters*, *14*, 20170659. https://doi.org/10.1098/rsbl.2017.0659

Smith, C. C. (2012). Opposing effects of sperm viability and velocity on the outcome of sperm competition. *Behavioral Ecology*, *23*, 820–826. https://doi.org/10.1093/beheco/ars036

Sokal, R. R., & Rohlf, F. J. (1995). *Biometrics*. San Francisco, CA: Freeman.

Soulsbury, C. D. (2010). Genetic patterns of paternity and testes size in mammals. *PLoS One*, *5*, e9581. https://doi.org/10.1371/journal.pone.0009581

Taborsky, M., Schütz, D., Goffinet, O., & van Doorn, G. S. (2018). Alternative male morphs solve sperm performance/longevity trade-off in opposite directions. *Science Advances*, *4*, eaap8563. https://doi.org/10.1126/sciadv.aap8563

Taylor, J. S., Sanny, J. S. P., & Breden, F. (1999). Microsatellite allele size homoplasy in the guppy (*Poecilia reticulata*). *Journal of Molecular Evolution*, *48*, 245–247. https://doi.org/10.1007/BF03356596

Taylor, M. L., Price, T. A. R., & Wedell, N. (2014). Polyandry in nature: A global analysis. *Trends in Ecology and Evolution*, *29*, 376–383. https://doi.org/10.1016/j.tree.2014.04.005

Thomsen, R., Soltis, J., Matsubara, M., Matsubayashi, K., Onuma, M., & Takenaka, O. (2006). How costly are ejaculates for Japanese macaques? *Primates*, *47*, 272–274. https://doi.org/10.1007/s10329-005-0171-7

Walsh, P. S., Metzger, D. A., & Higuchi, R. (1991). Chelex-100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *BioTechniques*, *10*, 506–513.

Wedell, N., Gage, M. J. G., & Parker, G. A. (2002). Sperm competition, male prudence and sperm-limited females. *Trends in Ecology and Evolution*, *17*, 313–320.

Wigby, S., Sirot, L. K., Linklater, J. R., Buehner, N., Calboli, F. C. F., Bretman, A., … Chapman, T. (2009). Seminal fluid protein allocation and male reproductive success. *Current Biology*, *19*, 751–757. https://doi.org/10.1016/j.cub.2009.03.036

Yamamoto, T., Hirohashi, N., Fujiwara, E., Suzuki, T., Maruta, H., Omiya, H., & Kitanishi, S. (2017). Relationships between body size and secondary sexual characters, and sperm characters in male Dolly Varden char (*Salvelinus malma*). *Ecology of Freshwater Fish*, *26*, 397–402.

Zajitschek, S., Hotzy, C., Zajitschek, F., & Immler, S. (2014). Short-term variation in sperm competition causes sperm-mediated epigenetic effects on early offspring performance in the zebrafish. *Proceedings of the Royal Society B: Biological Sciences*, *281*, 20140422. https://doi.org/10.1098/rspb.2014.0422

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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