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Directional postcopulatory sexual selection is associated with female sperm storage in Trinidadian guppies

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Female sperm storage (FSS) is taxonomically widespread and often associated with intense sperm competition, yet its consequences on postcopulatory sexual selection (PCSS) are poorly known. Theory predicts that FSS will reduce the strength of PCSS, because sperm characteristics favored before and after FSS may be traded-off, and opportunities for nondirectional PCSS should increase. We explored these questions in the guppy (*Poecilia reticulata*), by allowing females to mate multiply and by comparing the paternity pattern in two successive broods. Contrary to predictions, the variance in male fertilization success increased after FSS, driven by a change in male paternity share across broods. This change was positively associated with sperm velocity (measured before FSS) but not with the duration of FSS, indirectly suggesting that faster sperm were better in entering female storage organs, rather than in persisting within them. Other male traits, such as male size and orange color, heterozygosity, and relatedness to the female, did not influence paternity after FSS. These results indicate that processes associated with FSS tend to reinforce the strength of PCSS in guppies, rather than weaken it. Further work is necessary to test whether this pattern changes in case of more prolonged FSS.

KEY WORDS: **Cryptic female choice, genetic similarity, heterozygosity, poeciliids, sperm competition.**

Female sperm storage occurs when sperm are maintained inside female reproductive tract within sperm storage organs that allow the sperm to live significantly longer than in vitro or, within the female, outside these organs (Orr and Zuk 2012, 2014). This phenomenon is taxonomically widespread, as it has been reported in as phylogenetically distant species as molluscs (Evanno et al. 2005), annelids (Velando et al. 2008), arthropods (Page 1986; Diesel 1989; Simmons 2001), and vertebrates (e.g., Neubaum and Wolfner 1999; Pearse and Avise 2001; Sever 2002; Holt and Lloyd 2010; Kuehnel and Kupfer 2012). Sperm storage is probably advantageous for females in a number of circumstances, although it can also carry costs (Baer et al. 2006). For example, female sperm storage (FSS) may guarantee fertilization when population density is very low or it undergoes strong fluctuations making male–female encounters unpredictable (Deacon et al. 2011), or in those species in which the most favorable season for matings does not coincide with the best season for parturition (Birkhead and Møller 1993; Holt 2011; Orr and Brennan 2015).

It has also been suggested that FSS may have been selected as it increases opportunities for females to select mates at the postcopulatory level (Birkhead and Møller 1993). There are two nonmutually exclusive reasons why FSS is expected to affect postcopulatory sexual selection. Firstly, when prolonged sperm storage occurs, any insemination event, even outside female receptive period, can potentially result in fertilization, thus increasing the opportunities for sperm of multiple males to compete over the fertilization of the same set of eggs. Accordingly, sperm storage is typically associated with high levels of sperm competition in numerous taxa (Feldheim et al. 2004; Neff et al. 2008; Kleven et al. 2009; Calhim et al. 2011; Evans and Pilastro 2011; Liu and Avise 2011; Orr and Zuk 2013). Secondly, an intimate, prolonged contact between male gamete and female cells may increase the opportunities for cryptic female choice (Eberhard 1996).

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Cryptic female choice refers to female-mediated processes occurring during or after copulation that result in biased sperm use in favor of preferred or more compatible males (Thornhill 1983; Eberhard 1996).

High levels of sperm competition are usually associated with the production of more numerous (Stockley et al. 1997) and higher quality sperm (Snook 2005). Typically, when prolonged FSS is associated with high levels of sperm competition, divergent sperm phenotypes may be selected (Birkhead and Møller 1993; Orr and Zuk 2012). This is because sperm performance immediately after gamete release (which are important when freshly inseminated sperm from different males compete for the fertilization of the same eggs) are expected to be traded-off against sperm longevity. This, in turn, is likely to be important when sperm are stored for prolonged time in female storage organs and should affect fertilization success after female sperm storage. In particular, sperm competition typically favors fast swimming sperm and a positive correlation between competitive fertilization success and sperm velocity has been found in several internal fertilizers (e.g., Birkhead et al. 1999; Gage et al. 2004; Gasparini et al. 2010b; Boschetto et al. 2011). Sperm swimming is costly, however, and is expected to generate trade-off between sperm velocity and longevity (Ball and Parker 1996; Levitan 2000; Gage and Morrow 2003). This is because fast-swimming sperm are expected to have a short-life span in consequence of their higher consumption rate of energy reserves (Pizzari and Parker 2009). Furthermore, the higher metabolic rate associated with high swimming speed may also increase the production of oxygen radicals, which should accelerate sperm senescence and reduce sperm lifespan even when the female provides energetic resources to the stored sperm, that is in case of prolonged FSS (Blount et al. 2001; Pizzari and Parker 2009; Ribou and Reinhardt 2012). For these reasons, faster swimming sperm are expected to have a competitive fertilization advantage before FSS, but should be outperformed by slower sperm after FSS. Indeed, comparative evidence from passerine birds indicates that sperm swimming speed is positively correlated with the level of sperm competition and negatively associated with clutch size, a proxy for the duration of female sperm storage in these species (Kleven et al. 2009).

Despite the potential of female sperm storage to influence the outcome of postcopulatory sexual selection (PCSS) and the strong interest for this component of sexual selection in the last decades (Pizzari and Wedell 2013), the consequences of prolonged FSS on sperm phenotype have been rarely investigated since Birkhead and Møller's (1993) seminal paper. Evidence of divergent selection on sperm characteristics in relation to the level and timing of sperm competition in relation to FSS is often only indirect. For example, although last-male precedence is usually observed in sperm-storing species (Birkhead and Pizzari 2002), paternity share in current broods can also be biased toward sperm of males that mated with the female during previous ovarian cycles (Olsson et al. 2007; López-Sepulcre et al. 2013; Giraldo-Perez et al. 2015). This suggests that FSS may influence sperm competition outcome and that some males' sperm may be more efficient than others to survive within FSS organs or to retain a greater fertilization efficiency after FSS. In polymorphic lizards, male phenotype is associated with sperm competition success after prolonged FSS (Zamudio and Sinervo 2000; Olsson et al. 2009; Uller et al. 2013), suggesting a divergent selection for competitive fertilization success on freshly inseminated sperm and female stored sperm. However, sperm characteristics associated with fertilization success after FSS are not known in these lizards. More direct evidence comes from a study on swordtails (Smith 2012), which revealed that fast swimming sperm have a lower fertilization success after prolonged FSS, although the same study failed to evidence an advantage of sperm swimming speed when sperm were not stored prior to fertilization.

We investigated the consequences of FSS on sperm competition success in the guppy, *Poecilia reticulata*. Guppies are livebearing fish with internal fertilization and are an excellent model for unravelling the selective pressures acting on sperm phenotype during sperm storage, as sperm competition is very intense in this species (Evans and Pilastro 2011) and FSS can last several months (Winge 1937; Greven 2011). Prolonged FSS, combined with live birth, is thought to explain the invasiveness ability of this species (Deacon et al. 2011). Males can sire a significant proportion of the offspring months after copulation occurred, suggesting that, although freshly inseminated sperm usually get a greater share of paternity (Winge 1937), long-term stored sperm can outcompete, at least in some cases, freshly inseminated sperm, and significantly contribute to a male's reproductive fitness (López-Sepulcre et al. 2013).

In this study, we compared the paternity pattern in the first and the second brood of females that previously mated with multiple mates and were subsequently isolated from males until they produced two broods. Interbrood interval is approximately one month in this species (Magurran 2005). The eggs of the first clutch were therefore fertilized by freshly inseminated sperm, whereas the eggs of the second clutch were fertilized by sperm stored in the female ovary, on average, one month after having fertilized the eggs of the first clutch. Considering that the first and the second brood were fertilized by the same pool of sperm transferred during the initial matings, the change in the proportion of offspring sired by each male in the two successive broods can be explained by processes occurring during FSS. We first analyzed the change in the paternity share between the first and the second brood and tested whether, on average, it was larger than expected by the binomial error associated with small brood sizes. A significant deviation from the null binomial error distribution would indicate that some males' sperm have a relatively better

(or worse) fertilization performance after female sperm storage than others. As a second step, we related the change in paternity between the first and the second brood with male and sperm traits that have been shown to be associated with sperm competition success before FSS in guppies, namely sperm velocity (Boschetto et al. 2011), and male color and size (Evans et al. 2003). The general prediction was that traits associated with fertilization success in the absence of female sperm storage should be negatively correlated with the relative fertilization success after female storage, due to the expected trade-offs between sperm performance before and after FSS (Birkhead and Møller 1993). In particular, our first prediction was that in vitro sperm velocity, which is positively correlated with competitive fertilization success before FSS (Boschetto et al. 2011), should be negatively correlated with competitive fertilization success after FSS, due to the expected trade-off between sperm velocity and longevity (Levitan 2000). Secondly, we considered male body length and the degree of body orange coloration, which are negatively (body size) and positively (relative area of male orange spots on the body) associated with competitive fertilization success before FSS (Evans et al. 2003). Although in this case it is more difficult to envisage a mechanism that may generate a negative correlation between these male precopulatory traits and the relative fertilization success after FSS, we maintain the general prediction that postcopulatory success before and after FSS are expected to be traded off. Thus, a male that produces an ejaculate that is optimal for competitive fertilization before FFS (e.g., a small male with large orange spots), is expected to have a poorer postcopulatory success after FSS (Birkhead and Møller 1993). It has to be considered, however, that the expected trade-off between fertilization success before and after FSS may be obscured, or even reversed, by female-mediated processes associated with sperm storage. For example, if faster sperm are more efficient in entering the FSS organs (knob-shaped micropockets, Potter and Kramer 2000; Kobayashi and Iwamatsu 2002), and their survival within these organs is hereafter guaranteed by female nourishment (Greven 2011), it may predict a positive correlation between sperm velocity and competitive fertilization success after FSS.

Finally, we considered two further traits that may influence the outcome of competitive fertilizations in guppies. Firstly, is has been proposed that FSS may enhance the efficiency of cryptic female choice for less related males (Orr and Zuk 2014). In particular, male guppies that are unrelated to the mated female have a sperm competition advantage over males that are close kin (Gasparini and Pilastro 2011; Fitzpatrick and Evans 2014). This bias in paternity toward unrelated males is only significant when the related male is a full sibling (Fitzpatrick and Evans 2014). However, in previous experiments, the effect of genetic similarity between male and female on competitive fertilization success has been investigated only in first brood. Whether the genetic similarity between partners affects competitive fertilization after FSS in guppies is presently unknown. Sperm found within female gonoduct often have their head embedded in the apical ends of the epithelial cells and are surrounded by abundant cells of the immune system (Campuzano-Caballero and Uribe 2014). Considering this prolonged and intimate contact between sperm and female occurring during FSS, genetic similarity between male and female may affect cryptic female choice and finally male fertilization success more strongly than it is observed without FSS. We therefore predicted that genetic similarity may be associated with a decreased fertilization success after FSS. To test this prediction, we genotyped males and females used in this study at 11 neutral microsatellite loci and tested whether genetic similarity at these loci was negatively correlated with the change in paternity after FSS. Secondly, inbred, homozygous guppies have a reduced sperm competitiveness (Zajitschek et al. 2009) and heterozygosity is associated with increased sperm quality in other species (Fitzpatrick and Evans 2009). It therefore seems reasonable to assume that sperm from heterozygous males will increase their fertilization advantage after prolonged FSS. We tested whether male heterozygosity, estimated at 11 microsatellites loci, was positively associated with the change in competitive fertilization success after prolonged FSS.

Materials and Methods

EXPERIMENTAL FISH AND MATING DESIGN

The guppies used in this experiment were descendants of wild-caught fish collected from the lower part of Tacarigua River in Trinidad (Trinidad national grid reference: PS 787,804; coordinates: N10°40.736', W061°19.168'). Laboratory stock and all experimental fish were maintained under constant temperature and lighting conditions (26 ± 1°C; 12:12 h light/dark cycle) and fed twice daily on a mixed diet of brine shrimp nauplii (*Artemia salina*) and commercial fish dry food. Males used in the experiments derived from large stock tanks (150 l), each containing approx. 50 individuals of each sex that were allowed to breed freely. Experimental females were reared in single-sex tanks and were therefore virgin when entered into the experiment. The adults used in the experiment were 4–6 months old and were all sexually mature. Six males were randomly assigned to each of 10 experimental tanks, and allowed to settle for three days, to ensure that they entered the mating trials with fully replenished sperm stores. At the end of the acclimation period, eight randomly chosen virgin females were put into each experimental tank, for a total of 60 males and 80 females. Within each tank, the six males and the eight females were allowed to freely interact for seven days. At the end of this period, females were isolated in 2-l tanks until they produced two successive broods. At this point, tissue sample (fin clips from the mother and from

Table 1. Number of males and females used in this study.

No. of mates	Females	Males
1	—	12
2	19	15
3	10	10
4	11	5
5	1	5
Total	41	47

After an initial mating period of 7 days (see also Fig. 1), females were isolated from males until they produced two successive broods.

the offspring of the second brood and whole bodies of the offspring of the first brood) were collected and preserved in absolute ethanol for subsequent paternity analysis. Seventy-two females produced the first brood and related paternity data have been used in a previous study aimed at investigating the contribution of pre- and postcopulatory processes to overall variance in male reproductive success and its association with sexually selected traits (Devigili et al. 2015). Fifty of these females produced two broods and more than one offspring per brood. Since we were interested in the change in fertilization success between the first and the second brood, we included in the present study only females that produced two broods, at least one of which was sired by two or more males ($n = 41$). The remaining nine females, which had all their offspring sired by a single male, were excluded from the analyses since we do not know whether they mated multiply (i.e., whether sperm competition occurred). Offspring from the second brood were raised to the age (45–60 days) at which sexes start to differentiate (Houde 1997) for an estimation of their growth rate and sex ratio for a parallel study. Since some individuals died before sexual maturation, we excluded any influence of selective mortality by performing our analyses on the entire dataset (no. of broods = 41) and on a restricted datasets containing only broods in which a maximum of one offspring died (no. of broods = 29; mean number of genotyped offspring per brood was 10.21 ± 1.01 SE, range = 2–23). Our results were qualitatively identical (see below), indicating that postbirth mortality did not influence our conclusions. In total 47 males produced at least one offspring in one first or second brood (Table 1).

MALE COLOR PATTERN AND SPERM VELOCITY

At the end of the mating period, sires were allowed to rest in isolation for five days. Prior to ejaculate collection, males were anesthetized with MS222 (Tricaine methanesulfonate) and photographed using a Canon EOS 450D with a millimeter ruler for calibration. We used image analysis software (ImageTool: http://ddsdx.uthscsa.edu/dig/download.html) to estimate the standard length (distance from the snout to the end of the tail, SL) and the relative area of carotenoid spots (including orange, yellow and red, hereafter "orange spots"). After the photograph, each anesthetized male was immediately placed on a glass slide under a stereomicroscope. The gonopodium was swung forward, and gentle pressure was applied to the side of the abdomen, close to the base of the gonopodium, to release sperm bundles (Matthews et al. 1997). Immediately after sperm collection, sperm velocity was assessed following an established protocol (Gasparini et al. 2013). Briefly, a sperm-activating solution of 150 mM/l KCl and 4 mg/ml bovine serum albumin was added to the sperm bundles and the swimming velocity of the sperm moving away from the opening bundles was estimated on a mean of 194.4 sperm (SE = 8.99, range: 26–302). We used a Hamilton-Thorne CEROS sperm tracker to estimate the sperm velocity using the following parameters: frame rate 60 Hz; no. of frames 30; threshold value for static cells 25 μm/s (Gasparini et al. 2009; Gasparini et al. 2013). We obtained three sperm velocity estimates (μm/s), namely VAP, which is the average velocity of sperm cells over a smoothed cell path, curvilinear velocity (VCL), and straight line velocity (VSL), which are strongly positively correlated in this guppy population (see results), are significantly repeatable (Gasparini et al. 2009) and predict competitive fertilization success (Boschetto et al. 2011).

PATERNITY ANALYSES

Total genomic DNA was extracted from half caudal fin of adult guppies using the salting out method (Miller et al. 1988) and from small fry using the Chelex 100 resin protocol (Walsh et al. 1991). Paternity was assessed with five high variable microsatellites markers (see Table 2). All offspring could unequivocally be assigned to putative sires according to allele sharing.

GENETIC SIMILARITY BETWEEN MALES AND FEMALES AND MALE HETEROZYGOSITY

We genotyped adult males and females for 11 microsatellites markers, including the five loci used for the paternity analysis (Table 2). Male heterozygosity was calculated as the proportion of the observed heterozygous loci. Genetic similarity between male and female was estimated, according to the methods proposed by Queller and Goodnight (1989), Ritland (1996), and Lynch and Ritland (1999), using GenAlex 6.5 (Peakall and Smouse 2012).

STATISTICAL METHODS

Pattern of paternity in the first and the second brood

Variation in the proportion of offspring sired by males between first and second brood (i.e., sperm competition success) is expected to occur by chance alone, due to binomial error associated with small brood sizes. To test whether this change was larger than expected by chance (i.e., selection may have occurred) we generated an expected distribution of sperm competition success by using a randomization approach (e.g., Garcia-Gonzalez 2008;

Table 2. Microsatellite markers used for paternity assignment (underlined) and for genome-wide genetic similarity and heterozygosity estimate.

N° GeneBank	Name	No. alleles	Range	Reference
AF164205	TTA	13	102–163	(Taylor et al. 1999)
AF368429	Kond15	14	244–296	(Seckinger et al. 2002)
AF467904	Pr40	9	244–298	(Becher et al. 2002)
AF467905	Pr80	10	142–168	(Becher et al. 2002)
AF467907	Pr171	21	269–385	(Becher et al. 2002)
AF467908	Pr172	11	147–202	(Becher et al. 2002)
BV097141	AGAT11	21	240–371	(Olendorf et al. 2004)
DQ855573	G43	21	247–393	(Shen et al. 2007)
DQ855596	G208	7	220–324	(Shen et al. 2007)
DQ855605	G289	17	282–315	(Shen et al. 2007)
DQ855611	G339	7	129–219	(Shen et al. 2007)

Garcia-Gonzalez and Evans 2011). We first calculated, for each of the 41 females, the observed mean sperm competition success of the males they mated with in the first and in the second brood (observed SCS_{mean}). Secondly, we generated a simulated paternity distribution in the first and in the second broods assuming that each male had (within each female) an expected probability to sire one of the offspring equal to the observed SCS_{mean}. For each simulation, the resulting SCS in the first and the second brood (simulated SCS_1 and SCS_2, respectively) was calculated for each of the males that mated with any given female. The observed pattern in paternity across broods was compared to the null distribution obtained from the simulated paternity pattern (derived from the simulated SCS_1 and SCS_2), given the observed brood sizes (i.e., due to the intrinsic binomial error associated with small brood sizes). To this end, a Monte Carlo simulation routine was created in Windows Excel 2007 using PopTools 3.2.5 (Hood 2011). The routine was iterated 10,000 times and the observed statistic was compared with the distribution of the simulated statistic. *P* values were derived from the proportion of the simulated statistics that were larger or smaller than the observed one. Alfa level was set at 0.05. Since the statistics were calculated for each female, the sample size was = 41 (i.e., the number of females). To compare the variation in SCS across broods we divided the variance by the squared mean in SCS (hereafter I_{SCS}) to obtain a dimensionless, standardized measure of variation in male fertilization success (Crow 1958; Arnold and Wade 1984). Standardized variances are usually adopted as an index of the opportunities for selection in sexual selection studies (Jones 2009). We also expressed the variation in SCS as coefficient of variation (ratio of the standard deviation to the mean multiplied by 100), which is another commonly used standardized measure of the dispersion. Most of the males in this study produced offspring with more than one female (Table 1). To test whether some of these males were better-than-average in sperm competition after FSS, we estimated the variance component due to male and female identity in the SCS across broods using a generalized linear mixed model. In this model brood (first or second) was the fixed factor, female identity, male identity, and the interaction between male identity and brood number were the random factors. The statistical significance of the variance components was tested using a Wald-*z* test, using SPSS ver. 22.

Correlation between change in paternity between first and second brood and male traits

In order to identify male traits associated with sperm competition success after FSS, we used the same procedure described above to calculate the correlation between the change in paternity across broods with sperm and male characteristics. We considered male traits that have been demonstrated to predict competitive fertilization success, namely male body size and colors (Evans et al. 2003) and sperm velocity (Boschetto et al. 2011). We also considered male heterozygosity, which has been shown to covary with male coloration in guppies (Herdegen et al. 2014) and with sperm quality in other species (Fitzpatrick and Evans 2009). Practically, we calculated, for each of 41 females, the mean trait value (e.g., sperm velocity) of the males that were competing in the first and the second brood (the number of males competing "within" each female ranged from 2 to 5, Table 1). We then calculated, for each male within female, his relative trait value as the difference between his trait value and the mean observed for the males competing within that given female (hence, "relative trait," see Fig. 1). Once obtained the relative trait values, we calculated, within each female, the correlation between the relative male trait and the observed change in paternity between the first and the second brood. Finally, we calculated the mean correlation coefficient by averaging the values obtained from our dataset of 41 females. The resulting mean correlation coefficient was compared with the distribution of simulated mean correlation coefficients obtained by assuming a constant fertilization probability across broods (as above). One male had a particularly low relative sperm velocity

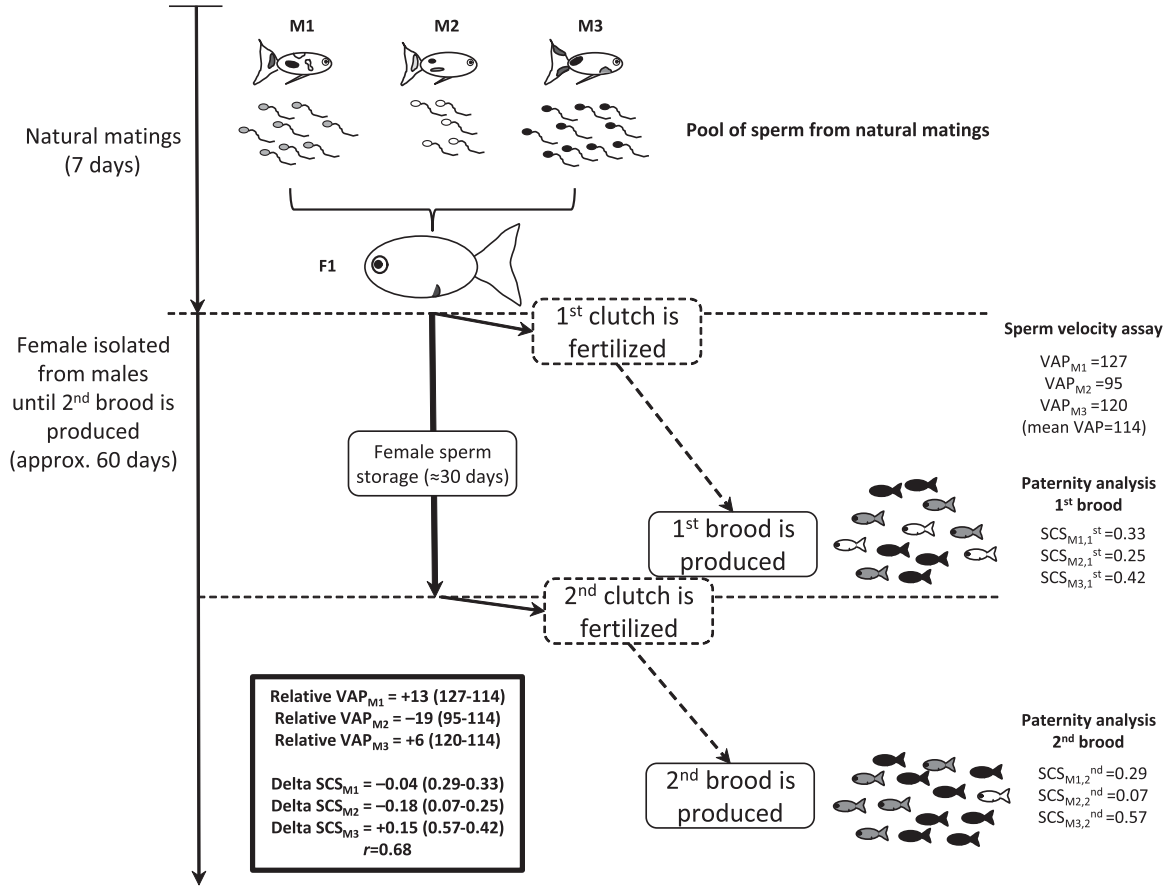

Figure 1. **Graphic description of the experimental design. In the hypothetical example depicted here, a female mated with three males and produced two successive broods. For each of the three males the absolute and relative sperm velocity, the change in SCS across broods, and their correlation, are reported. This procedure was applied to the 41 females considered in this study. Offspring paternity is graphically indicated by the color of the sperm of the sire.**

(relative VAP = –42). Omitting this outlier from the analyses did not affect any of the results.

Effect of genetic similarity on SCS

The same procedure as above was used to test whether genetic similarity between mates was associated with the change in SCS between first and second brood. Since the genetic similarity between mates is estimated by comparing the genotype of the sire with that of the female (and hence it was already "standardized" within female), we used the observed genetic similarity values between sires and female without any further transformation. In particular, we calculated, within each female, a correlation coefficient between the genetic similarity and the change in SCS across broods (delta SCS). To test whether the genetic similarity between female and sires was significantly associated with the change in SCS we first calculated the mean correlation coefficient (by averaging the 41 correlation coefficients observed within each female) and compared it with the distribution of the simulated correlation coefficients obtained using the same Monte Carlo procedure described above. This procedure was repeated for the three measures of genetic similarity used (see above). The other statistical analyses were performed using GenStat v.16.0 (Payne and Arnold 2003).

RESULTS

Our dataset included the first and the second brood of 41 females and 47 sires, for a total of 117 male–female fertilization events (including fertilization failures) for each of the two broods. The distribution of SCS in the two broods is presented in Figure 2. Mean brood size, mean time from matings to first brood and interbrood interval (i.e., the time elapsed between the first and the second brood), total number of offspring genotyped, and the number of sires in the first and in the second brood are summarized on Table 3.

Pattern of paternity in the first and the second brood

The standardized variance in SCS_{mean} (I_{SCS}) increased from 0.766, in the first brood, to 1.145 in the second brood (observed

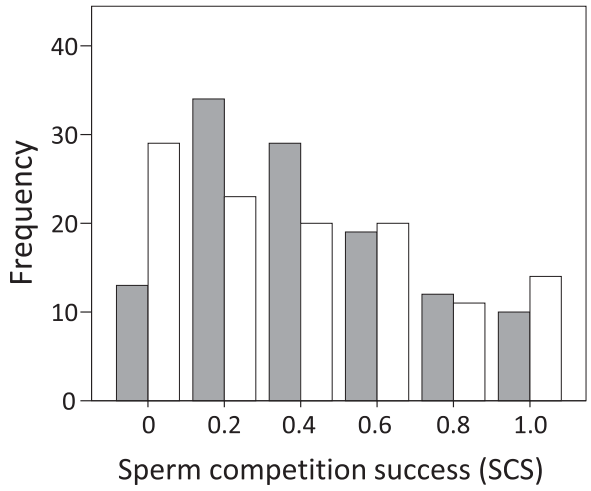

Figure 2. Distribution of sperm competition success in the first (shaded bars) and the second brood (open bars).

Table 3. Number of offspring produced at first and second parturition by 41 females (total offspring genotyped = 715), duration of the gestation (time from the beginning of the experiment to parturition for the first brood, and interbrood interval for the second brood), and number of sires per brood.

	Mean	SE	Range
First brood			
Brood size at birth	8.02	0.52	2–16
Gestation (days)*	34.0	1.66	21–60
No. of sires per brood	2.54	0.14	1–5
Second brood			
Brood size at birth	10.8	0.78	2–24
Gestation (days)*	25.6	0.70	21–50
No. of sires per brood	2.15	0.11	1–4

*Gestation represents the number of days elapsed between the day the females were isolated after the mating period and the day the first brood was delivered, and the interbrood interval for the second brood.

difference = 0.379). The observed difference was significantly larger than expected assuming equal SCS within male across females (mean simulated difference in I_{SCS} = 0.026 ± 0.109 SE, $P = 0.003$, Fig. 3). Effect size and Cohen's d of the difference in I_{SCS}, using the method for paired groups that accounts for correlation between groups (Dunlap et al. 1996), were 0.332 and 0.494, respectively, corresponding to a small-to-medium effect size (Cohen 1988). Similar results were obtained when coefficients of variation were used (CV in SCS, 1st brood = 77.9% ± 6.3 SE; 2nd brood = 95.4% ± 7.7 SE; difference in CV = 17.5%, $P = 0.012$).

Males produced offspring with a lower number of females in the second brood compared to the first brood (no. of females per male, first brood 2.23 ± 0.197 SE, second brood, 1.89 ± 0.211 SE; observed difference = –0.34, mean difference under

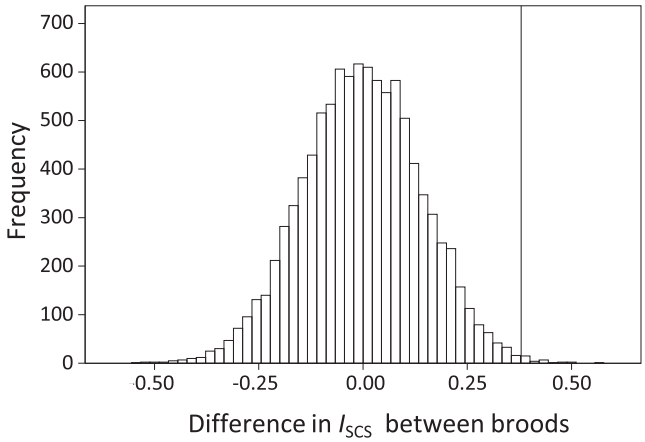

Figure 3. Simulated versus observed difference in I_{SCS}. Positive differences indicate that opportunities for postcopulatory sexual selection were greater in the second than in the first brood. The vertical line represents the observed difference in (I_{SCS}).

expected equal SCS, 0.046 ± 0.100 SE, $P < 0.0001$, Monte Carlo simulation). In 29 cases, a male did not obtain any offspring in the second brood from females with which he obtained offspring in the first brood (Fig. 2). The opposite phenomenon (i.e., males obtaining paternity in the second brood from females with which they did not sire offspring in the first brood) occurred in 13 cases (Chi squared = 6.095 d.f. = 1, $P = 0.014$). This is a conservative probability as the stochastic variation in paternity (and hence the probability of a complete fertilization failure by one male) associated with binomial error should decrease in the second broods, which were larger, on average, than first broods. The higher incidence of null paternity in the second broods resulted in a lower mean number of sires per brood in the second as compared to the first brood (Table 3, $z = 2.446$, $P = 0.014$, related sample Wilcoxon sign rank test). This higher incidence of these "fertilization failures" in the second brood certainly contributed to the higher I_{SCS} observed in the second broods. In contrast, in our simulation in which a male's expected fertilization success was constant across broods (and equal to his observed mean SCS in the first and the second brood), the frequency of fertilization failures did not differ, on average, between the two broods ($P = 0.28$).

Male identity explained a significant proportion of the variance in SCS both across females (variance component, male identity: 0.908 ± 0.280, $z = 3.243$, $P < 0.001$) and, within female, across broods (female identity: 0.149 ± 0.074, $z = 2.019$, $P = 0.043$; interaction between male identity and brood number: 0.227 ± 0.103, $z = 2.205$, $P = 0.027$; generalized linear-mixed model, dependent variable: number of offspring sired, binomial total: brood size). The effect of the interaction between male and female identity could not be estimated in our model, due to the low number of cases in which the same male produced offspring with several females (Table 1). As a result, mean male SCS in the

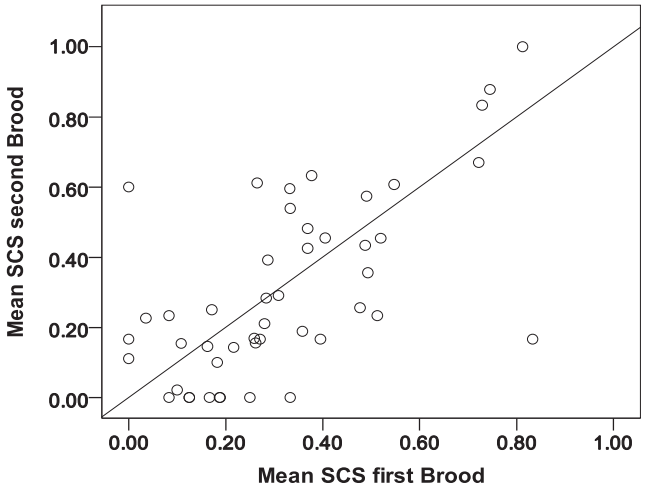

Figure 4. **Mean sperm competition success (SCS) of the 47 males considered in this study in the first and in the second brood. The line represents identical SCS values across the two broods.**

first and in the second brood were significantly correlated ($r = 0.644$, $P < 0.001$, $N = 47$, Fig. 4). Interestingly, this correlation coefficient, although highly statistically significant, was smaller than the correlation coefficient obtained when the expected SCS across broods was assumed to be constant and equal to the mean SCS in the two broods (expected $r = 0.79 \pm 0.074$ SE, difference between predicted and observed correlation coefficient $= 0.149$, $P = 0.036$), confirming that the change in SCS between broods was larger than expected by chance alone.

Correlation between change in paternity and male traits

Summary statistics of the pre- and postcopulatory traits of the 47 males used in this study are listed on Table 4. We first tested the association between sperm velocity (VAP) and the fertilization success of female-stored sperm by calculating, within each female, the correlation between the difference in SCS between first and second brood (delta SCS), and the relative swimming velocity of sperm of the males she mated with (VAP). Delta SCS and relative VAP were positively correlated ($r = 0.321 \pm 0.124$ SE, $n = 41$, Fig. 5). This correlation coefficient was significantly larger than expected if SCS was constant across broods (expected $r = -0.003 \pm 0.133$ SE, $P = 0.007$) and remained significant when we considered only second broods in which a maximum of one offspring died before paternity assignment (observed $r = 0.274 \pm 0.149$ SE; expected $r = -0.002 \pm 0.139$ SE, $P = 0.024$, $N = 29$). Similar results were obtained when we estimated the relative sperm velocity from a composite measure of sperm velocity (scores from a Principal Component Analysis; PC1, factor loadings: VAP = 0.993, VSL = 0.967, VCL = 0.925; variance explained = 92.3%; observed $r = 0.338 \pm 0.124$ SE; simulated

Table 4. **Characteristics of the males ($n = 47$) considered in this study: in vitro sperm velocity (VAP, μm s-1), relative area of orange spots (% of body area), body size (SL, mm), heterozygosity and genetic similarity calculated on the 117 comparisons between 41 females and their sires.**

	Mean	SE	Range
Sperm velocity (VAP, $\mu m\ s^{-1}$)[§]	84.9	2.57	20–119.8
Orange spots (%)	12.98	0.81	2.96–28.67
Body size (mm)	18.19	0.19	15.14–21.26
Heterozygosity	0.72	0.03	0.273–1.00
Genetic similarity (QG)[*]	0.0121	0.0152	–0.307–0.575
Genetic similarity (RI)[**]	0.0032	0.0055	–0.064–0.323
Genetic similarity (LR)[***]	0.0051	0.0108	–0.159–0.682

[§]One male had alive but nearly immotile sperm. Its velocity has therefore been set to 25 $\mu m\ s^{-1}$, the minimum threshold set up by the CASA program (see methods).

[*]Genetic similarity calculated according to Queller and Goodnight (1989).

[**]Genetic similarity calculated according to Ritland (1996).

[***]Genetic similarity calculated according to Lynch and Ritland (1999).

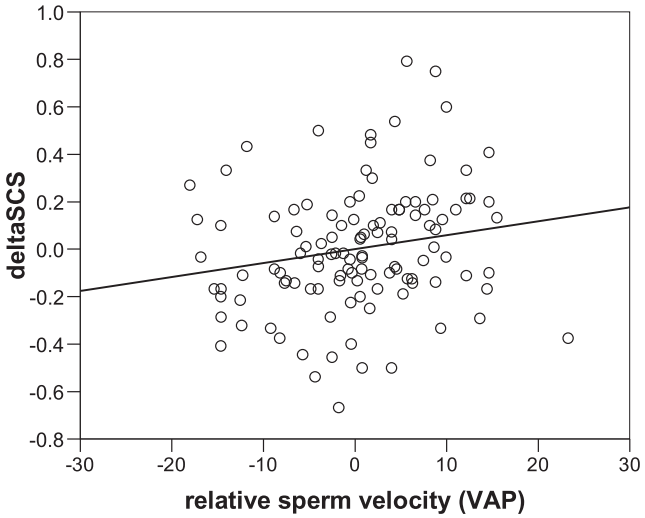

Figure 5. **Relationship between sperm velocity and the change in sperm competition success across broods (SCS_2–SCS_1). Sperm velocity is expressed as the difference between the VAP of a male and the mean VAP of the males sharing paternity with him with a given female.**

$r = 0.005 \pm 0.132$ SE, $P = 0.004$). The correlation between relative PC1 and delta SCS remained substantially unchanged in the restricted dataset (observed $r = 0.299 \pm 0.150$ SE; expected $r = 0.003 \pm 0.144$ SE, $P = 0.016$, $N = 29$). Sperm velocity (VAP) was not significantly associated with the male SCS in the first brood ($r = -0.16$, $P = 0.29$, $N = 47$).

Following the same procedure as above, we tested for significant correlations between delta SCS and other male traits

Table 5. Results of a multiple regression model in which the change in sperm competition success (SCS) between the first and the second brood was the dependent variable, and male body length (SL), body color (% of body area covered by yellow, red, and orange spots), sperm velocity (VAP), genetic similarity between male and female and male heterozygosity, (estimated at 11 microsatellite loci), were the predictors.

Predictor	*B*	SD	*P*
Genetic similarity*	0.0129	0.1896	0.300
Sperm velocity (VAP)	**0.0061**	**0.0027**	**0.016**
Male heterozygosity	–0.1264	0.1846	0.720
Male body size (SL)	0.0181	0.0245	0.269
Male color (orange)	–0.0042	0.0074	0.691

*Genetic similarity based on Queller and Goodnight (1989). Results were similar when based on other genetic similarity indexes (see Methods). Significance of the effects in the model was tested using a Monte Carlo simulation (10,000 iterations) in which, within male, the expected SCS was constant across broods.

potentially associated with sperm competitiveness, namely the relative area of orange spots, male body size (standard length, SL), and heterozygosity (Table 4). Male relative coloration and body size were not significantly correlated with the change in sperm competition success (SL: observed $r = 0.088 \pm 0.125$ SE; expected $r = 0.006 \pm 0.129$ SE, $P = 0.27$; orange: observed $r = -0.012 \pm 0.128$ SE; expected $r = 0.012 \pm 0.134$ SE, $P = 0.57$). Similarly, we found no effect of relative male heterozygosity on delta SCS (observed $r = -0.039 \pm 0.130$ SE, simulated $r = -0.006 \pm 0.131$ SE, $P = 0.60$).

Effect of genetic similarity on SCS

Genetic similarities between females and sires are reported on Table 4. On average, the correlation between a sire's genetic similarity with the female and his change in SCS across broods was negative, but not significant (RI, mean observed $r = -0.161 \pm 0.129$ SE, $N = 41$, mean expected $r = -0.0079 \pm 0.134$ SE, $P = 0.128$; LR, mean observed $r = -0.172 \pm 0.126$ SE, mean expected $r = -0.0070 \pm 0.133$ SE, $P = 0.108$; QG, mean observed $r = -0.107 \pm 0.126$ SE, mean expected $r = -0.009 \pm 0.133$ SE, $P = 0.231$). Finally, we tested simultaneously the effect of all predictors considered above in a multiple regression model. The comparison of the observed multiple regression coefficients with the simulated distribution confirmed that sperm velocity was the only significant predictor of delta SCS (Table 5).

Effect of the duration of FSS on the change in SCS across broods

The duration of the female sperm storage, which corresponded to the time elapsed between the initial matings and the time when the first brood was delivered (which when the second brood is fertilized (Magurran 2005) varied between 21 and 60 days (Table 3). To test whether the duration of sperm storage was associated with the change in SCS across broods, we calculated, for each female, the difference in I_{SCS} between first and second brood and correlated this change with the gestation time. If the increased I_{SCS} across broods is a function of the length of sperm storage, a positive correlation between the differences in I_{SCS} (I_{SCS} 2nd brood – I_{SCS} 1st brood) should be observed. In contrast, we observed a negative correlation, although not significant, between the change in I_{SCS} and the duration of sperm storage ($r = -0.22$, $P = 0.17$, $N = 41$).

Furthermore, we tested whether the strength of the positive correlation between relative sperm velocity and delta SCS within each female was influenced by the duration of the FSS. If the advantage of males producing faster sperm was associated with the duration of the sperm storage, we should observe a stronger correlation between relative sperm velocity and the change in SCS in those females in which sperm storage was longer. We found no association between the strength of the correlation between relative sperm velocity and change in SCS, and the duration of sperm storage ($r = 0.065$, $P = 0.69$, $N = 41$).

Discussion

Our analysis of the relative sperm competition success after FSS provided four main results: (1) in agreement with previous studies, we found that some males have a significantly higher overall SCS than others; despite this relative constancy in SCS across broods, we found that (2) FSS was associated with a significant change in SCS and that some males were more affected than others; furthermore, (3) the standardized variance in SCS was larger in the second brood than in the first, suggesting that FSS increases the opportunities for PCSS on males; finally, (4) our analyses revealed a competitive fertilization advantage, after FSS, of males producing faster sperm. We will discuss these results in the light of our knowledge of sperm selection in female storage organs in the guppy and in other internal fertilizing species, and present two selection mechanisms that may explain our findings.

The analysis of the pattern of SCS in the first and the second brood revealed that some males were significantly better sperm competitors than others. This conclusion is supported by our linear-mixed model analysis, revealing a significant effect of male identity on the overall SCS, and by the significant correlation between the mean male SCS in the first and the second brood (Fig. 4). This conclusion agrees with previous studies on the same (Devigili et al. 2015), and on another guppy population (Evans and Rutstein 2008). Although some males were therefore better sperm competitors, the variation in paternity share after FSS exceeded that expected as a simple consequence of stochastic binomial variation. Indeed, a significant component of this

variation in SCS across broods was explained by male identity, as indicated by the significant interaction between male identity and brood number in our generalized linear-mixed model analysis. This suggests that some males had relatively higher fertilization success than others after FSS. The interaction between male and female identity may also have influenced the outcome of sperm competition after FSS (Rosengrave et al. 2008). Unfortunately, the structure of our data did not allow to estimate this effect because most of the males mated with one or two females, and, when multiply mated, competed with different males across females (Table 1). To detect such interactions it would be necessary to compare the SCS of the same male across several females and against the same competitors (Garcia-Gonzalez and Evans 2011).

The standardized variance in fertilization success (I_{SCS}), as well as the coefficient of variation (CV), were significantly larger in the second brood than in the first brood. This significant increase and the significant effect of male identity on the change in SCS across broods indicate that FSS creates further opportunities for PCSS, as predicted theoretically (Birkhead and Møller 1993). The females used in this study were initially virgin and were allowed to mate with up to six males for one week. Afterwards, females were isolated from males and the two successive clutches of eggs produced were therefore fertilized by sperm deriving from these initial matings. Thus, sperm from this initial pool contributed to the observed paternity pattern in the two successive broods. Since the copulations were natural, the initial variance in SCS was likely due to differences in the number of sperm delivered during mating and, to a lesser extent, differences in sperm velocity between different males (Boschetto et al. 2011). Accordingly, in studies in which only two males competed for fertilizations, I_{SCS} was equal to 0.42 when differences for sperm number were not controlled for, that is following natural copulation (data from Fig. 1 in Evans and Magurran 2001). In contrast, this value dropped to 0.19, when equal numbers of sperm were used to artificially inseminate the female (Gasparini et al. 2010a). This latter value represents the opportunities for PCSS on sperm quality and is slightly smaller than the difference in I_{SCS} observed between the first and the second brood in the present study. Although these I_{SCS} values are not directly comparable due to the different number of competing males (two in the above studies and up to five in the present study), they suggest that FSS generates opportunity for postcopulatory sexual selection on sperm quality that is similar, in its effect size, to that observed for the same trait before FSS. This accords with the observation that in natural guppy populations a significant proportion (13.5%) of the offspring produced by the females derives from fertilizations by sperm that have been stored in the female for up to 10 months (López-Sepulcre et al. 2013). Moreover, guppies often live in ephemeral and isolated ponds where females have reduced opportunities of encountering mating partners (Magurran 2005). FSS is therefore likely to affect significantly male fitness and thus selection on males. In guppies, postcopulatory processes explain a substantial component of the variance in male reproductive success (Devigili et al. 2015), as observed in other polyandrous species (e.g., Collet et al. 2012; Droge-Young et al. 2012; Turnell and Shaw 2015). Our results therefore extend these findings to the postcopulatory processes occurring during and after FSS and concur with other empirical evidence from this fish family (e.g., Smith 2012; López-Sepulcre et al. 2013).

It has to be noted that the estimated change of SCS associated with FSS may be overestimated because we were unable to consider the cases in which SCS did not change across broods. In particular, we excluded from our initial sample of 50 females that produced two broods, nine females in which all the offspring were sired by a single male. If these females were actually double mated, SCS occurred and did not change across broods. Therefore, the exclusion of these females may have led to an overestimation of the mean change in SCS across broods. In particular, assuming that all of them were double mated (one male with SCS = 1 and the other with SCS = 0), the difference of CV in SCS across broods would decrease from 17.5 to 14.4%. While this possibility cannot be excluded, it has to be noted that in this guppy population the mean number of mating partners per female (estimated from behavioral observation) has been found to be 2.53 (Devigili et al. 2015). This value is remarkably similar to the number of sires per brood observed in our 50-females sample (2.52), suggesting that the nine females excluded may actually have really mated with a single male. Thus, although variances in SCS estimated only from paternity are intrinsically biased (Collet et al. 2014), we think that our observed change in SCS across broods is unlikely to be largely overestimated.

As a second step, we investigated which male traits were associated to the variation in SCS across broods. The general prediction was that traits that are positively correlated with competitive fertilization success before female sperm storage should be negatively associated with competitive fertilization success after female sperm storage (Birkhead and Møller 1993). We considered those traits that were previously shown to influence SCS in guppies, namely the relative area of orange spots (Evans et al. 2003) and sperm velocity (positive correlation) (Boschetto et al. 2011) and body size (negative correlation) (Evans et al. 2003). Furthermore, we extended our analysis to male heterozygosity and genetic similarity between male and female. In guppies, male heterozygosity is positively correlated with orange coloration (Herdegen et al. 2014) and may therefore be associated, either directly or indirectly, with SCS after FSS. Postcopulatory sexual selection should favor males that are genetically more dissimilar from the female. While evidence of this selection has been found only when males that are closely related with the female compete with unrelated males (Gasparini and Pilastro 2011; Fitzpatrick

and Evans 2014), the protracted intimate contact between female soma and male sperm during prolonged FSS may increase the efficiency of postcopulatory selection in favor of genetically dissimilar males (but see Gasparini et al. 2015). In contrast with these predictions, male body size, color, heterozygosity, and genetic similarity to the female did not predict the fertilization success of female stored sperm. Surprisingly, we found that the difference in paternity share between the first and the second brood was positively correlated with a male's sperm velocity. This result is unexpected, as theoretical and empirical work would lead to predict that faster sperm should have a reduced longevity (Ball and Parker 1996; Levitan 2000). Indeed, evidence that sperm velocity is associated with a reduced sperm longevity has been found in external fertilizing fish (e.g., Burness et al. 2004). Furthermore, in the poeciliid *Xiphophorus nigrensis*, sperm velocity is negatively correlated with sperm competition success as the time between insemination and parturition increases (Smith 2012), possibly indicating an accelerated sperm cell senescence associated with the high metabolic activity of fast swimming sperm (Ribou and Reinhardt 2012).

The positive covariation between relative sperm swimming velocity and SCS after FSS may have several, nonmutually exclusive explanations. Even though evolutionary trade-offs between two costly traits are inevitable, the sign of their within-species phenotypic correlation depends on the variance in the total resources allocated to the traits (van Noordwijk and de Jong 1986; Reznick et al. 2000). Indeed, in the guppy population used in our experiment, colorful males produce sperm that, in vitro, swim faster and live longer (Locatello et al. 2006), supporting the notion that some males produce overall higher quality ejaculates (i.e., whose sperm are both faster and live longer). Alternatively, fast sperm may be more efficient in reaching, and persisting, within the female sperm storage organs. For example, domestic fowl males producing highly motile sperm fertilize an increasing proportion of the eggs after prolonged FSS (Pizzari et al. 2008). This is probably because sperm are flushed out from the female sperm storage tubules when their swimming velocity is insufficient to contrast the fluid current generated by glandular secretion (Froman 2003). It has to be noted, however, that this phenomenon is not universal. In *Drosophila melanogaster*, for example, slow swimming sperm have a greater probability to remain within female storage organs as compared to fast swimming sperm (Lüpold et al. 2012). In both examples, however, sperm remain active during FSS whereas in guppies, as well as in other species with prolonged FSS (Orr and Zuk 2012), female-stored sperm are immotile and are thought to be nourished by the female (Greven 2011). In guppies, female stored sperm are associated with specific epithelial cells (SACs) lining the oviduct, either within deep surface pits and pockets (synaptic knob-shaped micropockets), or incorporated within the cytoplasm of the SACs (Potter and Kramer 2000; Kobayashi and Iwamatsu 2002). Therefore, sperm velocity should not affect the persistence of sperm within guppy female storage organs. Rather, a sperm's swimming velocity may affect its capability to enter female storage organs, as observed in turkeys (Donoghue et al. 1998). In guppies, the number of sperm reaching the ovary (and hence determining the fertilization success in the first brood) is mainly proportional to the number of sperm transferred during copulation and, to a lesser degree, to sperm swimming velocity (Boschetto et al. 2011). If female-stored sperm derive from the pool of sperm reaching the ovary, but faster sperm are more likely to enter the female storage organs, they will be further overrepresented in the pool of sperm competing to fertilize the subsequent clutches. In contrast, it seems unlikely that sperm velocity per se affects fertilization success after release from the storage organs, as stored sperm are nearly in direct contact with the immature eggs (Winge 1937; Kobayashi and Iwamatsu 2002).

A second mechanism that may link sperm swimming velocity with fertilization success after FSS is represented by oxidative damages. While enhanced metabolic activity, such as that associated with high sperm swimming velocity, is expected to increase the oxidative damage of the sperm cells (Dowling and Simmons 2009), empirical, correlative evidence does not fully support this prediction. For example, highly motile sperm are not susceptible to nuclear DNA denaturation in domestic turkeys (Donoghue et al. 1998), and sperm velocity is negatively correlated with DNA fragmentation in humans (Irvine et al. 2000). Colorful male guppies produce sperm that, in vitro, swim faster, live longer (Locatello et al. 2006; Gasparini et al. 2009) and have a higher competitive fertilization success (Evans et al. 2003). Although studies on oxidative damages in sperm are lacking for guppies, evidence that sperm from colorful males are also better protected from oxidative damages has been found in birds (Helfenstein et al. 2010). We can only speculate about the mechanism responsible for the positive covariation between sperm velocity and fertilization success of female-stored sperm. However, the lack of correlation between the duration of FSS and the change in paternity across broods on the one hand, and the significant association between sperm velocity and change in SCS across broods on the other hand, suggest a difference in the capability to reach/enter storage organs, rather than a difference in survival during FSS. Indeed, in poeciliids female stored sperm are immotile and nourished by the female (Greven 2011) and the expected trade-off between velocity and longevity may therefore be attenuated in these species. Techniques for color-marking the sperm with vital dyes (Lymbery et al. 2016) may allow to directly test whether faster sperm are overrepresented within female storage organs.

Our prediction that male heterozygosity may have influenced the fertilization success of female-stored sperm was not supported by our results. Male heterozygosity is associated with fertilization success in other species (Fitzpatrick and Evans

2009) and inbreeding has been shown to affect ejaculate quality (Zajitschek and Brooks 2010; Gasparini et al. 2013) and sperm competition success in guppies (Zajitschek et al. 2009). However, this effect becomes evident only at high levels of inbreeding (Zajitschek et al. 2009). The level of heterozygosity found in this study was comparable to that observed in studies based on wild guppy populations (López-Sepulcre et al. 2013; Herdegen et al. 2014), indicating that our lab population was not characterized by elevated inbreeding. The lack of association between heterozygosity and the relative competitive fertilization success in the second brood therefore suggests that female sperm storage does not increase the strength of the PCSS for heterozygous males, at least when populations are outbred. Similarly, we did not find any effect of male–female genetic similarity on fertilization success. Our prediction of a negative correlation between male–female genetic relatedness and the fertilization success of stored sperm was based on the observation that, during female storage, sperm are in close physical and physiological association with female cells lining the ovary (Greven 2011). This close association during sperm storage should facilitate recognition in favor of sperm from unrelated males, a phenomenon that also occurs before FSS, although effective only when full-sibs and unrelated males are competing (Gasparini and Pilastro 2011; Fitzpatrick and Evans 2014). In contrast, the correlation between genetic similarity and change in SCS across broods, although negative, was not statistically significant. This result suggests that prolonged FSS may not improve the cryptic female choice for the sperm from distantly related males, at least when the overall genetic similarity (i.e., based neutral microsatellite markers) is considered. It has been recently demonstrated that female guppies bias fertilization toward males that are more similar to them at major histocompatibility (MHC) class IIB genes (Gasparini et al. 2015). We did not have information about the genetic similarity at this locus and the role of MHC in the fertilization dynamics associated with FSS clearly deserves further investigation.

In conclusion, we found no evidence of a trade-off between sperm velocity and sperm fertilization competitiveness after FSS. Rather, our results suggest that fast swimming sperm maintain their fertilization advantage over successive fertilization events. Postcopulatory sexual selection associated with prolonged FSS (i.e., >3 weeks after insemination) acts concordantly with the temporally preceding episode of PCSS, occurring within a few days after insemination (Boschetto et al. 2011), and with the other episodes of sexual selection occurring before and at copulation. Indeed, in this guppy population sperm velocity is positively correlated with male coloration (Locatello et al. 2006), which in turn predicts male mating success (Evans et al. 2004), male insemination success (Pilastro et al. 2002, 2004, 2007), and competitive fertilization success (Evans et al. 2003). Altogether, these results suggest that the positive covariation between pre- and postcopulatory reproductive success observed in guppies (Devigili et al. 2015) further extends to include the processes associated with FSS.

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DATA ARCHIVING

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