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

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

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

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

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

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

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

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

METHODS

Study Animals

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

Sperm Collection

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

Artificial Insemination

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

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

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

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

Sperm Velocity Analysis

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

Paternity Assignment

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

Body Size and Coloration

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

Statistical Analyses

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

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

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

Ethical Note

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

RESULTS

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

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

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

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

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

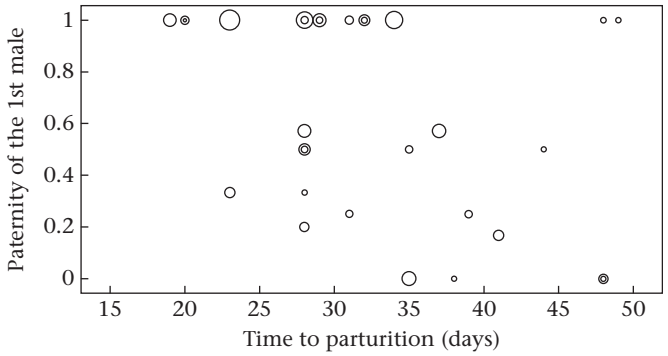

Figure 1. Proportion of offspring sired by the first male in relation to the interval between insemination and parturition. The size of the points is proportional to the brood size (number of offspring assigned to one of the sires; range 2–10).

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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

Conclusions

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

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Supplementary Material

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APPENDIX

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

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

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

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

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

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

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