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Effect of male age on sperm traits and sperm competition success in the guppy (*Poecilia reticulata*)

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Abstract

Deleterious mutations can accumulate in the germline with age, decreasing the genetic quality of sperm and imposing a cost on female fitness. If these mutations also affect sperm competition ability or sperm production, then females will benefit from polyandry as it incites sperm competition and, consequently, minimizes the mutational load in the offspring. We tested this hypothesis in the guppy (*Poecilia reticulata*), a species characterized by polyandry and intense sperm competition, by investigating whether age affects post-copulatory male traits and sperm competition success. Females did not discriminate between old and young males in a mate choice experiment. While old males produced longer and slower sperm with larger reserves of strippable sperm, compared to young males, artificial insemination did not reveal any effect of age on sperm competition success. Altogether, these results do not support the hypothesis that polyandry evolved in response to costs associated with mating with old males in the guppy.

Introduction

Polyandry is a widespread phenomenon in nature, in spite of many associated potential costs, such as expenditure of time and energy, predation and transmission of sexual diseases or parasites (Stockley, 1997; Jennions & Petrie, 2000). Given these potential costs, it is not fully understood why females mate promiscuously since multiple matings are more beneficial to males than to females (Bateman, 1948). A number of different explanations have been proposed to account for polyandry, including adaptive and non adaptive hypotheses (Halliday & Arnold, 1987). Adaptive explanations involve a number of possible benefits for females that may outweigh or, at least, balance the costs of multiple matings. Belonging to this latter category are hypotheses that imply indirect (genetic) benefits, such as increased genetic diversity of the offspring or a reduced risk of genetic incompatibility (reviewed by Jennions & Petrie, 2000; Simmons, 2005). Polyandry may also benefit females by promoting sperm competition to enhance the probability of fertilizing her eggs with sperm of males that are better-than-average sperm competitors and hence benefit either by producing sons that are also superior sperm competitors, if sperm competitiveness has a heritable sire component (the 'sexually selected sperm hypothesis', Curtsinger, 1991; Keller & Reeve, 1995), or by producing higher quality offspring, if there is a positive covariation between sperm competitive ability and male genetic condition (the 'good sperm hypothesis', Madsen *et al.*, 1992; Yasui, 1997).

The quality of gametes, however, declines with age and male senescence has been suggested to have profound evolutionary and ecological consequences on gamete performance and fitness (see Pizzari *et al.*, 2008 for a recent review). In particular, new mutations have been shown to accumulate in the germline with age (Risch *et al.*, 1987; Glaser & Jabs, 2004), thereby decreasing the genetic quality of gametes (Hansen & Price, 1995; Pizzari *et al.*, 2008). Multiple mating by females could be favoured if (1) mating with old males is costly because their sperm are of lower genetic quality due to an accumulation of unfavourable mutations in the

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germline, and if (2) these mutations are also expressed at the haploid and/or sperm production stage and these mutations also affect sperm competitiveness by reducing sperm performance (Radwan, 2003). Thus, by mating with multiple males, females may reduce the risk that eggs will be fertilized by sperm of old (senescent) males.

The notion that mating with aged male may impose costs to the female somehow contradicts the view that male age is an honest signal of male genetic quality because old males have proven their superior ability to survive (Manning, 1985; Kokko & Lindstrom, 1996; Brooks & Kemp, 2001). While female preference for old males has been demonstrated in several species, some studies have challenged the universality of this preference (see Brooks & Kemp, 2001 for a review). For example, female bushcrickets (*Ephippiger ephippiger*) discriminate against old males, using song as a cue of male age (Ritchie *et al.*, 1995). Similarly, females of the lekking sandfly (*Lutzomyia longipalpis*) avoid mating with old males (Jones *et al.*, 2000). A variety of factors could promote the evolution of female discrimination against old males. For example, young individuals could be better adapted to the current environment than old males because their parents underwent selection more recently (Hansen & Price, 1995). However, there is growing evidence that male age affects particularly sperm production and quality (Cordero & Miller, 1992; Sharpe *et al.*, 2003). In humans, age is usually associated with diminished semen volume, sperm motility and proportion of morphologically normal sperm (reviewed in Kidd *et al.*, 2001).

Theory predicts that sperm quality should decline with age due to the accumulation of *de novo* mutations in the germline cells (Hansen & Price, 1995, 1999) because of the higher number of divisions that germ cells undergo (Drost & Lee, 1995; Radwan, 2003). Indirect evidence that sperm from old males may carry more deleterious mutations comes from the observation that mating with old males has negative effects on female fitness in terms of reduced female fecundity (Jones & Elgar, 2004; Jones *et al.*, 2007; Hale *et al.*, 2008) or developmental defects and reduced viability of offspring, mainly in humans (e.g. Wyrobek *et al.*, 2006; Pizzari *et al.*, 2008; Gavrilov & Gavrilova, 2009) but also in others animals (Price & Hansen, 1998; Serre & Robaire, 1998; Jones *et al.*, 2000). Recently, in mice, advanced paternal age has been shown to negatively affect survival time of offspring and weaning weight on F2 offspring, an observation in accord that sperm from old fathers carry deleterious mutation (Garcia-Palomares *et al.*, 2009).

In some species it may be difficult for females to avoid copulations with old males and, therefore, if sperm competitiveness declines with age and there are costs associated with mating with old males, then females may avoid having their eggs fertilized by old males by mating with multiple males (Radwan, 2003; see also Pizzari *et al.*, 2008). Females may, therefore, benefit from multiple mating because sperm competition decreases the likelihood of being fertilized by old males whose sperm can be burdened with deleterious mutations. There is evidence, mainly from studies on invertebrates, to support this argument. For example, selection experiments in the fruit fly (*Drosophila melanogaster*) have revealed that some aspects of sperm competitive ability decline with age (Service & Fales, 1993; Uglem *et al.*, 2001). Reduced sperm competition success associated with male age has also been demonstrated in the bulb mite *Rhizoglyphus robini* and in the spider *Pholcus phalangioides* (Schafer & Uhl, 2002; Radwan *et al.*, 2005). A recent study on the hide beetle (*Dermestes maculatus*) found that old males were poor competitors against intermediate-age males, although they were slightly favoured towards young males (Jones *et al.*, 2007). Studies on ejaculate characteristics suggest that an age-linked decline in sperm competitiveness may be a general phenomenon in vertebrates as well (Radwan, 2003; Pizzari *et al.*, 2008). However, whether male age affects fertilization success in a sperm competition context remains largely untested in vertebrates and the few studies published so fare yield contrasting results. For example, a recent study on the barn swallow (*Hirundo rustica*) reported a decline in sperm performance with age (Møller *et al.*, 2009). In contrast, sperm competition success *in vitro* did not correlate with male age in the sockeye salmon (*Oncorhynchus nerka*) (Hoysak *et al.*, 2004). Thus, more empirical data are necessary to better understand the role of male senescence and its associated decline in sperm competitiveness in explaining the evolution of polyandry, as proposed by Radwan (2003). This hypothesis may contribute to explaining the evolution of polyandry if the following predictions are verified: (1) females do not discriminate between young and old males at the precopulatory stage; (2) male success in sperm competition declines with age; (3) females suffer either reduced fecundity or produce lower quality offspring when mated with old males.

We investigated the first two predictions in the guppy, *P. reticulata*, a livebearing fish that has become a model for studies on sexual selection (Houde, 1997; Magurran, 2005). Females usually actively solicit copulations from more than one male over a single reproductive cycle (Pitcher *et al.*, 2003). Even in the absence of overt direct benefits, polyandrous mating appears to increase offspring fitness in guppies, but the mechanism producing this effect remains unknown (Evans & Magurran, 2000).

To investigate whether age effects on post-copulatory male traits and sperm competition success in the guppy may contribute to these previous results, we first tested whether female guppies discriminate between young and old males at the precopulatory stage. Second, we compared the ejaculate traits of two groups of male guppies whose age differed by a minimum of seven months. We counted the number of strippable sperm at rest and measured sperm velocity, viability and morphology, four

characteristics potentially associated with sperm competition success (Birkhead *et al.*, 1999; Levitan, 2000; Snook, 2005). Finally, we investigated and compared the sperm competition success of old and young males by artificial inseminating virgin females with the same number of sperm collected from young and old males and determining the paternity of the resulting broods.

Methods

General methods

The guppies used in this experiment were descendants of wild-caught fishes collected from the Tacarigua River in Trinidad (national grid reference PS 787 804), a high-predation site where guppies coexist with several predator species (Magurran & Seghers, 1994b). Virgin females were reared in single-sex tanks; males and post-partum females were reared in mixed-sex aquaria. The water temperature was maintained between 25 °C and 27 °C and illumination was set on a 12 h/12 h light/dark cycle (Philips TLD 36W fluorescent lamps). Fish were fed on a mixed diet of brine shrimp nauplii (*Artemia* sp.) and commercially prepared dry food (Duplarin).

Male age

We obtained males of known age by collecting newborn fish (age 1–3 days) from stock tanks and placing them in labelled tanks, each containing fish that were born in the same month. Thus, the age of all males used in the study was known to within 1 month. All males were raised under the same environmental and density conditions, with a sex ratio of 1 : 1, until they reached the age required for the experiment. The average age of young males was 5.27 months (SD = 0.91; range: 4–8 months) and that of old males was 14.08 months (SD = 1.18; range: 12–16 months); details of male age are reported for each experiment. Males become sexually mature at about 2 months (Houde, 1997) and 4-month-old individuals are therefore certainly fully sexually mature. Most of the studies on maximum lifetime have been conducted on females, but capture–recapture studies suggest that males surviving for more than 12 months are rare in nature (Olendorf *et al.*, 2006). Setting the age of old males at more than 12 months therefore allowed us to compare the maximum potential effect of age on sperm competition success.

Precopulatory female choice

We measured female precopulatory preference in relation to male age using a dichotomous choice experiment (Houde, 1997), in which a female (n = 40) could choose between two males differing in age. The young males used in these experiments were aged, on average, 5.43 months (SD = 1.38; range: 4–8 months, n = 20). Old males were, on average, 14.25 months old (SD = 1.00; range: 13–16 months, n = 20). The average difference between males was 8.82 months (range: 8–9). The choice tests followed the methods described by Bisazza & Pilastro (2000) and Evans *et al.* (2004a). Specifically, one male from each group was selected at random and individually placed in a compartment on either side of a central chamber (30 × 28 × 20 cm, with a capacity of ca. 12 L) containing a sexually naïve virgin female (n = 22) or a post-partum female (n = 18). Virgin and post-partum females are sexually receptive and are commonly used in mate choice experiments in this species (Houde, 1997; Pilastro *et al.*, 2007). The two male compartments (each measuring 9 × 28 × 20 cm, with a capacity of ca. 4 L) were individually illuminated with a fluorescent solar tube (18 W, True Lite1). The distance between the two males (30 cm) minimized the probability that males could see one another, thus avoiding the possible confounding influence of male–male interactions on female association patterns (Houde, 1997). The female's central chamber was divided into five equal sections (6 cm) by drawing on the front side with an indelible pen: a central 'no-choice' zone (three sectors of 6 cm each) and two 'choice zones' adjacent to the two male compartments (two sectors, each 6 cm). Opaque dividers, which could be raised and lowered from a remote location (behind a black cloth blind), prevented visual access by the female into the two side tanks during an initial acclimatization period (30 min).

Following the settlement period the opaque dividers were raised, allowing the female visual access into the two adjacent compartments. As a measure of female preference for either male, side association data were collected during a 30-min observation period. Specifically, the female's position (in or out of either of the two choice zones) was recorded every 5 s throughout the 30-min period, providing 360 point samples per test female (Houde, 1997). The investigator recorded the female's position every 5 s using a timer set to 'beep' every 5 s. To control for possible side bias in the preference measures, young and old males were alternated between the left and right sides of the central chamber on consecutive trials. Following each trial, the males were anaesthetized and photographed using a digital camera as described below.

Ejaculate quality assays (sperm number, velocity, morphology and viability)

We assessed sperm number in 51 males (young = 27, old = 24). The average age of young males was 5.63 months (SD = 0.09; range: 5–6 months) and that of old males was 13.41 months (SD = 1.45; range: 12–15 months). Males were kept in isolation for at least 3 days before sperm collection in order to ensure that they had fully replenished sperm reserves (Evans *et al.*, 2004a), and that sperm numbers and velocity were not

influenced by the differences in the social context. This is because male guppies respond quickly (i.e. 3 days) to the perceived mating opportunities by priming more sperm with higher swimming speed (Bozynski & Liley, 2003; Gasparini *et al.*, 2009). Sperm were manually stripped following Matthews *et al.* (1997) and Evans *et al.* (2003). Briefly, after anaesthetization in a bath containing water and MS-222 (Tricaine Methanesulfonate), each male was placed on a Petri dish under a low-power dissection microscope with a drop of physiological solution (0.9% NaCl). The gonopodium (the intromittent organ) was rotated forward and gentle pressure was applied to the side of the abdomen. This action releases sperm that are packaged in bundles (Kuckuck & Greven, 1997), each containing about 27 000 individual sperm cells (Billard, 1969; Evans *et al.*, 2004b). The whole ejaculate was collected with a pipette and diluted in an appropriate volume of physiological solution. Solutions of free sperm, obtained by vortex samples for about 1 min, were counted in an 'improved Neubauer chamber' haematocytometer (Pilastro *et al.*, 2002; Evans *et al.*, 2004b).

We measured sperm velocity and morphology for 86 males (young = 43, old = 43) that were subsequently used for artificial inseminations. The young males in the experiment were, on average, 4.97 months old (SD = 0.55; range: 4–6 months) and the old males were 14.32 months old (SD = 0.46; range: 14–15 months). Sperm were stripped from each male following an established protocol (see above). Sperm velocity was measured following the procedure described by Locatello *et al.* (2006). A two-step procedure was followed to ensure simultaneous activation of all sperm cells (Billard & Cosson, 1992). First, we placed 20 spermatozeugmata into 10 μL of extender medium (207 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.41 mM $MgSO_4$, 10 mM Tris, pH 7.5) in which sperm remain quiescent (Gardiner, 1978). The sample was maintained at 3–5 °C until required for the motility analyses (within 2 h of collection), at which point it was warmed to 26 °C and activated with a 40 μL solution of 150 mM KCl and 2 mg mL^{-1} bovine serum albumin (Billard & Cosson, 1990). The Hamilton-Thorne computer-aided semen analyser (Hamilton-Thorne Research, Beverly, MA, USA) was used to assess motility parameters. An activating solution was added and then the sperm bundles were gently broken to induce motility, by mixing the solution with a micro-pipette. Five μL samples were placed in disposable 12-μm deep microcell chambers and analysed. Sperm velocity measures were based on an average of 108.58 ± 8.96 motile sperm. Velocity analyses were performed on two sub-samples of the ejaculate of each male and the mean was used in the final analysis. The following parameters were measured: path velocity (VAP: the average velocity of the smoothed cell path in $\mu m\ s^{-1}$) and progressive velocity (VSL: the average velocity measured in a straight line from the beginning to the end of the track in $\mu m\ s^{-1}$). The threshold values defining static cells were predetermined at 20 $\mu m\ s^{-1}$ for VAP, and 15 $\mu m\ s^{-1}$ for VSL. These two motility measures provide an estimate of progressive velocity and have been shown to correlate well with fertilization rates in various vertebrate species (Froman & Feltmann, 2000; Rurangwa *et al.*, 2004). We found that VAP and VSL were highly correlated ($r = 0.998$, $P < 0.001$, $n = 84$), and gave nearly identical results in subsequent analyses. We have therefore presented only the VAP results.

To analyse sperm morphology we incubated free sperm, collected from the males used in the sperm velocity analysis, in a solution of 1% Rose Bengal for 20 min. Dyed samples were then viewed under 1000 × magnification and photographed with a digital camera (Olympus DP10, Japan). We analysed 20 sperm for each male using an image analysis software (Image Tool) and measured the length (μm) of three different spermatozoon components: head, midpiece, and flagellum.

We also assessed sperm viability, using the live/dead Sperm Viability Kit (L-7011; Molecular Probes Inc., OR, USA) in 39 males (young = 18, old = 21). Average ages were 5.11 months for young males (SD = 0.74; range: 4–6 months) and 14.15 months for old males (SD = 9.05; range: 13–16 months). A membrane-permeant nucleic acid stain (SYBR14) labelled live sperm with green and a membrane-impermeant stain (propidium iodide) labelled dead or damaged sperm with red: only cells with the membrane intact were considered viable. We measured viability immediately after sperm extraction (hereafter: 0 h) and again after 5 h (5h). The solution of free sperm was stained according to the manufacturer's protocol. The sample was placed on a microscopic slide and gently covered with a coverslip. Fluorescent images of samples were recorded using a × 40 objective with a digital camera (DFC480; Leica Microsystems, UK) and stored using Leica IM500 image-manager software. The proportions of live and dead spermatozoa (coloured green and red respectively) were then assessed from images of at least 100 sperm for each male. After sperm stripping, each male was photographed as described below.

Artificial insemination

Sexually naïve, 6-month-old virgin female guppies were artificially inseminated (see Clark, 1950; Evans *et al.*, 2003) with equal numbers of sperm from two males differing in age. Males used for artificial insemination were isolated from females three days prior to the trials in order to ensure that their sperm reserves were replenished (Pilastro & Bisazza, 1999). Considering that sperm senescence can occur with advancing age of either the male (premeiotic sperm senescence) or of the sperm cell (post-meiotic, Pizzari *et al.*, 2008), we tried to minimize the effects of post-meiotic sperm senescence (which may be unrelated to male age) by giving the two groups the same mating opportunities before the experiment (see also the discussion). Furthermore, the use of artificial

insemination allowed us to control behavioural and mating order effects on sperm competition success (Evans & Magurran, 2001; Pitcher *et al.*, 2003). We used 43 pairs of males. These males had previously been used for sperm assays of velocity and morphology. The average age of young males was 4.97 months (SD = 0.55; range: 4–6 months) and that of old males 14.32 months (SD = 0.46; range: 14–15 months), with an average age difference of 9.35 months (range: 8–10 months). Males with similar body colour spots were paired in dyads, to control for possible influences of phenotypic traits in fertilization success (Evans *et al.*, 2003; Locatello *et al.*, 2006). Males were stripped as described above. Part of the stripped sperm bundles was used to estimate the number of sperm per bundle using an 'improved Neubauer chamber' haematocytometer (see Pilastro *et al.*, 2002; Evans *et al.*, 2004b) and the rest of the bundles was used for the artificial inseminations. Afterwards, each male was photographed (see below), its caudal fin was clipped for paternity analysis, and it was revived in fresh water. The same number of sperm bundles from each dyad of males was used to inseminate two unrelated, virgin females. The procedure for artificial inseminations followed an established protocol (Evans *et al.*, 2003). Each female was anaesthetized in a water bath containing a mild dose of MS-222 and placed in a polystyrene 'cradle' with the genital pore exposed. A Drummond micropipette was used to transfer 20 sperm bundles – 10 from a young and ten from an old male – suspended in 3 μL of physiological solution (0.9% NaCl) into the female's gonoduct. Immediately after insemination, females were revived in a 5 L plastic container (containing conditioned fresh water, gravel, aquatic weed and an air stone), in which they remained isolated until they produced their first brood. Offspring were killed using an excess of MS-222 and stored at −80 °C until used for paternity analysis. Adult males and females were kept in a post-treatment tank for 2 weeks after fin clipping. Mortality rate during this period was similar to that observed in our stock fishes (< 2%).

Measurement of male colour pattern and body size

To measure male colour patterns, individual males were anaesthetized in a water bath containing MS222 and photographed using a digital camera (Nikon Coolpix 4300, Japan). UTHSCSA Image Tool (University of Texas Health Science Center, San Antonio, TX; http://ddsdx.uthscsa.edu/dig/download.html) was then used to estimate the body area of each male (including caudal fin but excluding dorsal fin) as well as the surface area of orange, yellow, and red spots (hereafter: orange) and the standard length (distance from the snout to the tip of the caudal peduncle = SL). Black spots (encompassing fuzzy black lines) and iridescent spots (combined measures of blue, green, purple, and white) were also included in the analysis because these colours are known to influence female mating preferences in some populations (e.g. Brooks, 1996; Kodric-Brown & Nicoletto, 1996). In the population used for this study, however, only the area of the orange spots is positively correlated with sperm competition success and sperm quality (Evans *et al.*, 2003; Locatello *et al.*, 2006). Colour measures were made on the left side of each male's body. To control for the effects of body size, the relative area of each colour pigment was used in the analyses.

Paternity analysis

Paternity analysis was performed following Evans *et al.* (2003). Genomic DNA was extracted from the whole body tissue of newborns and from the caudal fin of adults using a Chelex protocol (Walsh *et al.*, 1991). The tissue samples obtained from the mothers, the potential fathers and all of the offspring (n = 542 from 34 broods) were collected and stored in a freezer at −80 °C until analysis. Three microsatellite markers were used to assign paternity, including TTA (Genbank accession numbers: AF164205; Taylor *et al.*, 1999), Pooc-G49 (AF026459; Parker *et al.*, 1998) and Pr92 (AF467906; Becher *et al.*, 2002). Polymerase chain reaction amplifications were performed on a GeneAmp® PCR System 2700 Thermocycler (Applied Biosystems, CA, USA). The PCR was performed in 15 μL reaction volumes with 1.2 μL $MgCl_2$, 0.525 μL dNTPs, 0.15 μL of each primer, 1.5 μL Taq buffer, 0.08 μL Taq DNA polymerase (Promega) and 2.4 μL DNA template. The cycling protocol included an initial denaturation step at 95 °C for 1 min, followed by 27 cycles of 10 s denaturation at 95 °C, 30 s annealing at 52 °C (60 °C for Parker), extension at 72 °C for 30 s, and a final extension for 5 min at 72 °C. Amplified fragments were separated by electrophoresis on an ABI 3100 sequencer (ABI PRISM, Applied Biosystems), using 400 HD ROX (Perkin-Elmer, Applied Biosystems) as a size standard (http://www.bmr-genomics.com). PCR products were visualized using Genotyper software (v. 3.7, Applied Biosystems) and paternity was assigned to all offspring according to allele sharing between putative sires, mother and offspring.

Statistical analyses

Statistical analyses were performed using SPSS (version 15.0, SPSS Inc., Chicago, IL, USA). If not otherwise stated, mean ± SD are reported. All probabilities are two-tailed. Data were checked for normality and appropriate transformation was adopted when necessary. Proportion data were arcsine square root transformed before analysis. In the ANCOVA analyses, we first tested for homogeneity of slopes and then applied a standard main effects analysis or a separate slopes analysis as required. A generalized linear model with binomial errors and logit link function was then used to determine whether male age, size and colour pattern, as well as sperm traits

accounted for deviance in paternity. For this purpose, either the young or the old male was randomly labelled (A or B) and, for each family, the success of male B (the number of offspring sired by male B) and the total number of offspring were entered as the binomial response variables. Predictor variables, representing the age of male B and the differences in the phenotypic trait measurements taken from the two males per family (male B trait minus male A trait), were fitted into the model. Since paternity data were overdispersed, appropriate overdispersion correction was adopted according to Williams (1982). Initially, the full model included all possible explanatory variables; the term with the least significant probability was then excluded in a stepwise procedure. The deviance increase of the generalized linear model, resulting from the removal of each term, was tested against chi-square distribution. We removed all terms whose exclusion did not cause a significant increase in deviation.

To assess the probability of accepting the null hypothesis when it was false (i.e. that young males indeed have an advantage in sperm competition) we generated 1000 random datasets, using the Resample function in Poptools 3.0 (http://www.cse.csiro.au/poptools) for increasing values of fertilization advantage of young males and compared the observed distribution of the logistic regression coefficients in the randomized dataset with the observed value (see results). Briefly, we randomly assigned, for each of the 34 broods, the paternity to the young male based on an expected fertilization success ranging from the binomial null expectation (0.5) towards increasing bias values of young male fertilization success (0.53).

Results

Colour pattern and male body size

We measured the colour pattern and male body size of all the males involved in the experiments and we found that old males were larger on average than their young counterparts (SL: old males = 18.87 ± 1.36 mm; young = 18.00 ± 1.28 mm; $t_{252} = 5.301$, $P < 0.001$). In contrast, the relative area of coloured spots (orange, black and iridescent) did not show significant difference for any of the characteristics measured (all $P > 0.90$). Similar results were obtained using the residuals of the regression of the area of the colour spots on total body area (all $P > 0.201$).

Precopulatory choice

Females actively chose between the two males, spending an average of 67.8 ± 14.9% of their time in the choice sectors (Fig. 1). Females spent an average of 31.93 ± 15.38% of the total time in the sector near the old male and 35.81 ± 14.24% of the total time near the young male (paired *t*-test: $t_{39} = 0.96$, $P = 0.34$). As shown in Table 1, no effect of male age on female choice was evident when we controlled for differences in male size, male colour pattern and female status (virgin vs. post-partum).

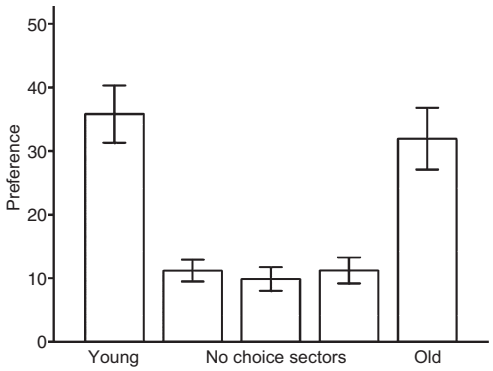

Fig. 1 Proximity female preference for old and young males. Bars represent the proportion of time (mean ± SE) spent by the females ($n = 40$) in each of the five virtual sectors in which the female tank was subdivided (the three central were considered 'no choice zone', and the two adjacent to male's compartment 'choice zone').

Effect of male age on sperm traits

Old males had larger reserves of strippable sperm than their young counterparts (young males, $7.00 \times 10^6 \pm 3.71 \times 10^6$, $n = 27$; old males, $10.40 \times 10^6 \pm 6.30 \times 10^6$, $n = 24$; $t_{51} = 2.374$, $P = 0.022$). Our results remained substantially unchanged after statistically controlling for differences in body size and the size of the colour spots (ANCOVA, age, $F_{1,45} = 4.036$, $P = 0.051$; all covariates, $P > 0.098$; after log transformation, marginal means: young males, 6.794 ± 0.048 SE, $n = 27$; old males, 6.940 ± 0.051 SE, $n = 24$). The results also revealed that sperm morphology was influenced by age, with young males having significantly shorter sperm (i.e. with a shorter flagellum) than old males (Table 2). Age also influenced sperm velocity, with young males having faster sperm than old males, although the difference was marginally nonsignificant (Table 3). Sperm viability

Table 1 Repeated measure ANOVA on female proximity preference in relation to male age and phenotype.

	d.f.	Mean square	*F*	*P*
Age	1	0.113	2.603	0.116
Male body size (SL)	1	0.073	1.692	0.202
Relative area of orange spots*	1	0.018	0.423	0.520
Relative area of black spots*	1	0.063	1.446	0.238
Relative area of iridescent spots*	1	0.012	0.270	0.607
Female status	1	0.036	0.825	0.370
Error	34	0.043		

*After arcsine transformation.

showed a significant decrease over time after stripping (proportion of live sperm: 0 h = 85.99% ± 9.64; after 5 h = 69.79% ± 25.92; paired *t*-test: $t_{38} = 2.942$, $P = 0.006$), but did not differ between old and young, both immediately following sperm extraction ($t_{37} = 1.159$, $P = 0.25$), and 5 h later ($t_{37} = 0.243$, $P = 0.81$).

Sperm competition success

We obtained 34 broods from the 43 virgin females that were artificially inseminated. Mean brood size was 15.94 ± 6.84 (range: 6–29, total no. of offspring = 542). Within male dyads that produced offspring ($n = 34$), young males showed faster sperm (old males = 45.08 μm s^{-1} ± 14.48; young males = 52.57 μm s^{-1} ± 18.43; paired *t*-test $t_{33} = 2.33$, $P = 0.026$) and shorter flagellum length (old males = 45.99 μm ± 1.76, young males = 44.65 μm ± 2.52 paired *t*-test $t_{33} = 2.719$, $P = 0.01$) than old males. In contrast, the number of sperm per bundle ($\times 10^6$) did not differ significantly within the male dyads (old males = 11.912 ± 6.745; young males = 11.514 ± 6.715; paired *t*-test $t_{33} = 0.381$, $P = 0.71$). The young males sired 252/542 offspring (mean proportion of offspring sired by a young male = 0.48 ± 0.21, $n = 34$). We found no significant advantage of young males in fertilization success (logistic regression, age: $b = -0.154 \pm 0.288$, Wald chi-square = 0.286, $P = 0.59$; corrected for overdispersion, Phi = 0.1123; factor: age [young as reference level]). The results did not change once other male phenotypic traits possibly influencing fertilization success (relative size of male colour spots, sperm velocity, sperm morphology and no. of sperm per bundle) were added to the model (see Table 4). The power of our dataset to reveal a sperm competition advantage of young males was estimated by comparing the observed regression coefficient ($b = -0.154$) with the distributions of regression coefficients obtained from 1000 randomized datasets generated by assuming a progressively increasing fertilization success of the young males, ranging between 0.50 and 0.53. The results indicate that the observed regression coefficient was unlikely to occur if young male's fertilization success was equal or greater than 0.51 (Fig. 2).

Table 2 General linear model of flagellum length in relation to male age and phenotype.

	d.f.	*b*	SE	*F*	*P*
Model	5			5.396	0.000
Intercept	1	43.978	4.016	120.349	0.000
age	1	1.760	0.513	11.760	0.001
Body size (SL)	1	0.160	0.174	0.844	0.361
Relative area of orange spots*	1	4.112	3.989	1.063	0.306
Relative area of black spots*	1	−5.353	3.021	3.140	0.080
Relative area of iridescent spots*	1	−10.882	3.054	12.694	0.001
Error	77				

*After arcsine transformation.

Table 3 General linear model of sperm velocity (VAP) in relation to male age and phenotype.

	d.f.	*b*	SE	*F*	*P*
Model	5			1.638	0.160
Intercept	1	29.123	31.550	0.637	0.427
Age	1	−7.160	4.019	3.173	0.079
Body size (SL)	1	1.084	1.365	0.630	0.430
Relative area of orange spots*	1	9.530	31.303	0.093	0.762
Relative area of black spots*	1	20.373	23.727	0.737	0.393
Relative area of iridescent spots*	1	23.291	23.889	0.951	0.333
Error	78				

*After arcsine transformation.

Discussion

Male guppies provide no resources during mating, nor do they defend territories against rival males (Houde, 1997). Furthermore, females can store sperm for several months and produce several consecutive litters from a single copulation (Constantz, 1984). Sexually receptive female guppies regularly solicit matings from several males (Houde, 1997; Pitcher *et al.*, 2003) even though polyandry leads to a reduction in foraging efficiency (Magurran & Seghers, 1994a) and increased vulnerability to predation (Magurran & Nowak, 1991). Therefore, to evolve, polyandry is expected to be associated with substantial benefits for females. Indeed, some of these benefits have been identified, as polyandrous females have a shorter

Table 4 Logistic regression model of fertilization success (proportion of offspring sired by one male) in relation to male age, body size and relative size of body colour spots.

	b	SE	Wald chi-square	*P*
Body size (SL)	0.104	0.103	1.020	0.313
Orange	−0.0077	0.032	0.058	0.810
Black	−0.0059	0.0456	0.017	0.897
Iridescent	0.1008	0.0599	2.832	0.092
VAP	−0.0081	0.00909	0.784	0.376
Sperm per bundle	−0.000031	0.000032	0.932	0.334
Sperm length	−0.0394	0.0745	0.280	0.597
Age*	−0.139	0.48	0.084	0.772

*A backward selection of the predictors in which age was forced into the model did not select any significant predictor. Overdispersion was corrected for by using the Williams procedure [full model, $\varphi = 0.1236$ (Williams, 1982)].

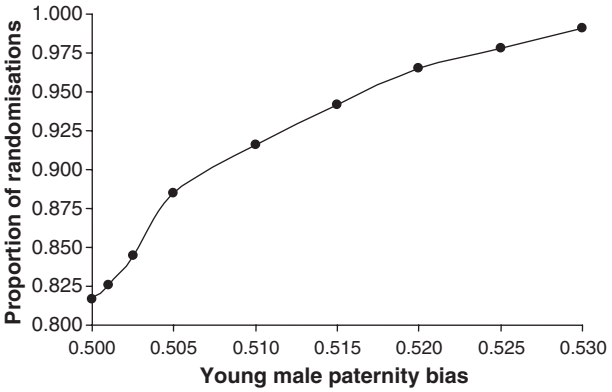

Fig. 2 Power to detect increasing young-male advantage in sperm competition expressed as the proportion (out of 1000 randomized dataset for each expected fertilization bias) of logistic regression coefficients that were larger than the observed value ($b = -0.154$). *X* axis represent expected biases of young male sperm competition success.

gestation time and produce offspring of larger body size and greater antipredator ability than their singly-mated counterparts (Evans & Magurran, 2000; Ojanguren *et al.*, 2005). These results suggest that females, by mating multiply, increase their short-term fitness, although it is still unclear which mechanism underlies these effects (but see Ojanguren & Magurran, 2007; Head *et al.*, 2008).

It has been suggested that polyandry may serve to reduce the costs imposed to the female by male senescence (Radwan, 2003; Pizzari *et al.*, 2008). In species in which it is difficult to determine the relative age of a potential mate, or to evade copulations with old males (for example due to forced copulations), Radwan (2003) proposed that polyandry may be selected as a strategy to avoid, via sperm competition, fertilization by old males – whose sperm can be burdened with deleterious mutations – and therefore minimize negative consequences on offspring fitness. If these deleterious mutations carried by sperm, or any other phenotypically mediated effects of age on ejaculate traits, lead to lower fertilization success in a sperm competition context (due to lower sperm quality or to reduced sperm production), then young males are predicted to be favoured in sperm competition over old males.

The results of our study do not lend support to the prediction that sperm competition success is influenced by male age in the guppy. We found that female guppies do not discriminate between males of different age at the precopulatory stage; furthermore females receive a high number of unwanted copulations during their lives (Magurran & Seghers, 1994b; Pilastro & Bisazza, 1999) and their capability to favour young males at the precopulatory stage therefore appears limited. In contrast to other species (e.g. Hansen & Price, 1995), male age does not seem an important criterion in female mate choice in guppies. Indeed, the three main traits on which females base their choice (body colour, courtship rate and body size, Houde, 1997) show little change, if any, with age: male courtship rate does not change with age in this species (Miller & Brooks, 2005); although male coloration (proportion of body area covered by colour spots) increases with age between 3 and 5 months (Miller & Brooks, 2005), it probably remains constant thereafter, as suggested by the similar size of colour spots in the two groups of males of our sample. Females did not show a preference for young or old males after we statistically controlled for phenotypic differences (colour pattern and body size Evans *et al.*, 2004a).

The analysis of the ejaculate characteristics considered to influence sperm competition success, namely sperm number, viability, velocity and morphology (reviewed in Snook, 2005), yielded contrasting results. We found significant differences between young and old males in three of four measured traits. Male age had no effect on sperm viability, measured as the proportion of live sperm immediately after stripping and after five hours. Old males had larger reserves of strippable sperm and produced longer sperm with lower, *in vitro*, swimming speed as compared to their young counterparts. While elevated sperm velocity is generally associated with higher fertilization rates in invertebrates (Levitan, 2000; Kupriyanova & Havenhand, 2002), fish (Gage *et al.*, 2004; Casselman *et al.*, 2006), birds (Birkhead *et al.*, 1999) and mammals (Malo *et al.*, 2005), whether sperm size is important in determining fertilization success is less clear (Humphries *et al.*, 2008). The production of longer sperm by older individuals can be due to ageing, as found by Green (2003) in the rove beetle (*Aleochara bilineata*), in which it has been shown that sperm length increases with age. Whatever the link between sperm morphology, sperm velocity and fertilization success, however, their net effect on sperm competition success was null, as young males did not fertilize a higher proportion of the eggs. The results of our Monte-Carlo simulation indicated that the observed fertilization success of young males ($P_Y = 0.48$) is unlikely to occur if the actual fertilization success of the young males was greater than 0.51. Thus, even if a sperm competition advantage of young males cannot be statistically excluded, our results suggest that it is, at most, marginal. Given that we formed male dyads in which we limited as far as possible the differences in male phenotype, which have been shown to influence sperm competition success in this population (body size and size of orange spots, Evans *et al.*, 2003), we can conclude that, at least in this population, male age plays a minor role in sperm competition success.

The lack of correlation between sperm speed and sperm competition success may be surprising, as previous studies using this population have found that colourful males produce faster sperm (Locatello *et al.*, 2006) and

have greater success in sperm competition (Evans *et al.*, 2003). There are several, not mutually exclusive explanations for these results. First, the advantage of having faster sperm (found in young males) may be offset by that of producing longer sperm (found in old males), and the combination of the two characteristics masks any fertilization bias towards one or the other group. Second, the correlation between sperm competition success and sperm velocity or morphology may be blurred by other factors, such as genetic similarity between partners (although work on another population of guppies suggests that genetic similarity between males and females has no effect on sperm competition success, Evans *et al.*, 2008). Most important, however, may be that the within-pair difference in the size of the orange spots was as twice as large in Evans *et al.* (2003) (4.2% ± 3.18 SD), as in this study (2.0% ± 2.18 SD). Probably as a consequence of this, the standardized deviance in paternity from the binomial distribution (i.e. deviance of the null model divided by d.f.) was as twice as large in Evans *et al.* (2003) as in the study presented here (5.73 and 2.88, respectively). Our experimental design may have therefore be appropriated to detect differences in fertilization success related to male age but had consequently little power to reveal any association between male phenotype and sperm competition success.

Contrary to the prediction by Radwan (2003), old males produced more sperm than young males, suggesting that in natural copulations they may even be at an advantage over young males, although sperm transfer ability in relation to male age needs further investigations. We can rule out that the observed difference depended on differences in body size between age groups (Pilastro & Bisazza, 1999; Pitcher & Evans, 2001; Evans *et al.*, 2002), as this difference remained significant after statistically controlling for differences in body size. This result suggests that either sperm production increases with age or survival rate and sperm production are positively correlated. Findings by Evans *et al.* (2002) support the former explanation, as sperm reserves increase with age from sexual maturity up to the six months, but a longitudinal study would be necessary to discriminate between these two explanations.

The only other study, to our knowledge, that has investigated the effect of male age on sperm competition success in a fish species, the salmon *Oncorhynchus nerka*, also found no effect of male age (Hoysak *et al.*, 2004). In this species male age is related to alternative reproductive phenotypes (sneakers and guarders) with expected differences in sperm investment. However, paternity analysis in competitive mating experiments did not indicate that sperm performance declines with age in this fish. Sperm performance actually increased with age in another fish, the bass *Morone saxatilis*, although information on sperm competition success is not available for this species (Vuthiphandchai & Zohar, 1999). It has to be borne in mind that male age is often associated with different mating histories which, in turn, may affect a male's sperm competitiveness (e.g. Jones & Elgar, 2004), irrespective of his mutational load. Considering this, a deterioration of sperm competition success with age may not necessarily be taken as a support of premeiotic sperm senescence (*sensu* Pizzari *et al.*, 2008). In the guppy it is impossible to disentangle the effect of previous mating history from that of ageing *per se*, yet age-related differences in mating history may not be relevant for the hypothesis tested here. Indeed, polyandry may evolve in response to an increased mutation load in the germline of senescent males only if old males, under natural conditions, have reduced sperm competitiveness. We maintained fish in conditions (as regards to mating rate) that are similar to those they experience in natural populations (Magurran, 2005), and we can therefore conclude that sperm competition is unlikely to serve as a mechanism reducing fertilizations by old males in guppies. Furthermore, sperm traits are mainly influenced by short-term fluctuations of mating opportunities (Bozynski & Liley, 2003; Gasparini *et al.*, 2009) and the conditions in which males were maintained during their lives and immediately before the experiment should ensure that these effects were controlled for. Whether, and in which direction, long-term mating history affects sperm traits in guppies is not known and this is certainly avenue for further research.

In conclusion, our study highlighted three main findings. First, females do not discriminate among mates in relation to male age. Second, we found that male age significantly influences sperm morphology, velocity and number (but not viability), although not necessarily in the expected direction. Third, these differences in sperm quality traits did not affect sperm competition success which did not decline significantly with age. Our findings that old males had larger sperm reserves than their younger counterparts, suggest that old males may even have a sperm competition advantage in natural copulations. Altogether, our findings do not support the hypothesis that polyandry evolved in guppies to avoid deleterious mutations carried by older males. Although only a few fish species have been studied so far, it does not seems that sperm competition success decline with age (Vuthiphandchai & Zohar, 1999; Hoysak *et al.*, 2004; this study). This pattern contrasts with evidence found in invertebrates (Service & Fales, 1993; Uglem *et al.*, 2001; Schafer & Uhl, 2002; Radwan *et al.*, 2005; Jones *et al.*, 2007) and other vertebrates such as birds and mammals (Serre & Robaire, 1998; Kidd *et al.*, 2001; Møller *et al.*, 2009), in which a decline in both sperm quality and sperm competition success with age seems a general phenomenon. Guppies are characterized by high levels of sperm competition, with more than three sires per brood observed in natural populations (Neff *et al.*, 2008), and sperm production is continuous throughout adult life (Magurran, 2005). Male biased mutation load is expected to be particularly pronounced

under such circumstances (Bartosch-Harlid *et al*., 2003; Ellegren, 2007; Pizzari *et al*., 2008) and its therefore somewhat surprising that we did not find any evidence of senescence in sperm competitiveness. From a life-history perspective, because of the cumulative effects of mortality, selection will be stronger on younger individuals and deleterious mutations that are expressed late in life are under relatively relaxed selection, thus generating the phenomenon of senescence (Medawar, 1952). However, if males face a high level of sperm competition success at any mating, then selective pressures on sperm competitiveness may actually be very strong throughout a male's reproductive life and may explain the results observed in the guppy (and possibly in other fishes). Despite the lack of effect of male age on sperm competition success in the guppy, it is still possible that fertilizations by old males impose a cost to females represented by the production of offspring with lower genetic quality, possibly generating intersexual conflict in this species (Pizzari *et al*., 2008), an hypothesis that warrants further investigation.

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