

1 **Postcopulatory inbreeding avoidance in guppies**

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23 **Abstract**

24 In many species the negative fitness effects of inbreeding have facilitated the  
25 evolution of a wide range of inbreeding avoidance mechanisms. While those  
26 operating prior to mating are well documented, evidence for postcopulatory  
27 mechanisms of inbreeding avoidance remain scarce. Here, we examine the potential  
28 for females to bias paternity in favour of unrelated males through postcopulatory  
29 inbreeding avoidance mechanisms in the guppy, *Poecilia reticulata*. To test this  
30 possibility, we used a series of artificial inseminations to deliver an equal number of  
31 sperm from a related (either full sibling or half sibling) and unrelated male to a female  
32 while statistically controlling for differences in sperm quality between rival ejaculates.  
33 This approach has the twofold advantages of (i) removing precopulatory processes  
34 that can bias paternity and (ii) accounting for differences in sperm number and quality  
35 between competing males. In this way we were able to focus exclusively on  
36 postcopulatory mechanisms of inbreeding avoidance and account for differences in  
37 sperm competitiveness between rival males. Under these carefully controlled  
38 conditions, we report a significant bias in paternity towards unrelated males, although  
39 this effect was only apparent when the related male was a full sibling. We also show  
40 that sperm competition generally favours males with highly viable sperm, and thus  
41 that some variance in sperm competitiveness can be attributed to difference in sperm  
42 quality. Our findings for postcopulatory inbreeding avoidance are consistent with  
43 prior work on guppies revealing that sperm competition success declines linearly with  
44 the level of relatedness, but also that such effects are only apparent at relatedness  
45 levels of full siblings or higher. Our study reveals evidence that postcopulatory  
46 processes offer an effective mechanism of inbreeding avoidance.

- 47 **Keywords:** sexual selection; relatedness; cryptic female choice; sperm competition;
- 48 genetic compatibility.

49 **Introduction**

50 Reproduction among close relatives can dramatically reduce offspring viability and  
51 fitness, a phenomenon known as inbreeding depression (Charlesworth and  
52 Charlesworth 1999; Keller and Waller 2002). To reduce the costs associated with  
53 inbreeding, many species exhibit a wide range of inbreeding avoidance mechanisms.  
54 For example, precopulatory mechanisms of inbreeding avoidance, such as dispersing  
55 away from natal territories and/or recognizing kin, can represent effective methods  
56 for reducing the likelihood of inbreeding (Pusey and Wolf 1996). However,  
57 postcopulatory inbreeding avoidance mechanisms can also evolve, particularly when  
58 the risks and costs of inbreeding are high, precopulatory inbreeding avoidance  
59 mechanisms are absent (e.g. Pitcher et al. 2008; Tan et al. 2012), or when  
60 precopulatory processes can be undermined through forced matings (Kokko and Ots  
61 2006). Thus, polyandrous females may avoid inbreeding depression by engaging in  
62 postcopulatory cryptic female choice to bias fertilizations towards unrelated males  
63 (Stockley et al. 1993; Zeh and Zeh 1996; 1997).

64 Postcopulatory inbreeding avoidance mechanisms may bias paternity in favour  
65 of genetically dissimilar males during competitive fertilizations. Accordingly, when  
66 inseminated with sperm from both related and unrelated males, cryptic female choice  
67 is thought to bias fertilization success towards unrelated males (Bishop 1996; Bishop  
68 et al. 1996; Olsson et al. 1996; Wilson et al. 1997; Stockley 1999; Kraaijeveld-Smit et  
69 al. 2002; Mack et al. 2002; Thuman and Griffith 2005; Jehle et al. 2007; Firman and  
70 Simmons 2008; Brekke et al. 2012, but for counter examples see Kleven et al. 2005;  
71 Sherman et al. 2008; Evans et al. 2008; Ala-Honkola et al. 2010; 2011).  
72 Mechanistically, females can bias paternity by influencing the number of sperm

73 accepted during mating (or stored following insemination) in favour of unrelated  
74 males (Pizzari et al. 2004; Welke and Schneider 2009; Bretman et al. 2004; Ala-  
75 Honkola et al. 2010; Tuni et al. 2013), or through interactive effects, for example  
76 between ovarian fluid and sperm performance, that differentially influence sperm  
77 quality in favour of unrelated sperm (Gasparini and Pilastro 2011; Butts et al. 2012).  
78 Despite promising support for the idea that cryptic female choice moderates  
79 inbreeding avoidance, very few studies have been able to exclude the influence of  
80 precopulatory inbreeding avoidance mechanisms (Pusey and Wolf 1996), or  
81 alternative postcopulatory processes, such as differential sperm allocation or  
82 investment by males based on genetic relatedness (Pizzari et al. 2004; Fitzpatrick et  
83 al. 2014). Moreover, most studies of postcopulatory inbreeding avoidance do not  
84 account for differences in sperm quality (i.e. sperm traits such as swimming speed,  
85 viability and morphology that predict fertilization success, Simmons and Fitzpatrick  
86 2012) between competing males. Indeed, only a single study (Denk et al. 2005), which  
87 failed to detect an effect of genetic relatedness on competitive fertilization success,  
88 controlled for differences in sperm quality between competing males when assessing  
89 postcopulatory inbreeding avoidance mechanisms. Consequently, examining the  
90 potential for postcopulatory inbreeding avoidance requires both the influence of  
91 cryptic female choice and sperm competition to be assessed simultaneously within an  
92 experimental framework that separates pre- from postcopulatory inbreeding  
93 avoidance mechanisms.

94 The guppy, *Poecilia reticulata*, is ideally suited to studies of postcopulatory  
95 inbreeding avoidance. Guppies are internally fertilizing, live-bearing freshwater fish  
96 characterized by high levels of polyandry (Neff et al. 2008; Evans and Pilastro 2011). In

97 their native tropical freshwater streams, guppies are likely to suffer periodic or  
98 sustained periods of elevated inbreeding risk, as small populations can often become  
99 isolated as water levels recede during the dry season (Griffiths and Magurran 1997).  
100 Indeed, molecular data indicate that as many as 16% of randomly chosen pairs of  
101 guppies in a Trinidadian stream reached levels of relatedness higher than those of  
102 half-siblings (Hain and Neff 2007). Moreover, inbreeding depression can be severe in  
103 guppies, and studies have shown that inbred offspring have reduced survival  
104 (Nakadate et al. 2003), vertebral deformities (Shikano et al. 2005), reduced  
105 reproductive rates (Zajitschek and Brooks 2008), and for males, reduced courtship,  
106 sexual colouration, sperm counts and semen quality (van Oosterhout et al. 2003;  
107 Mariette et al. 2006; Zajitschek et al. 2009). However, despite the high potential for  
108 inbreeding in wild populations, and associated costs, adult female guppies do not  
109 appear to practice precopulatory inbreeding avoidance through active mate choice  
110 (Viken et al. 2006; Pitcher et al. 2008; Zajitschek and Brooks 2008). Instead,  
111 polyandry, coupled with forced copulations by males (Magurran and Seghers 1994;  
112 Pilastro and Bisazza 1999), suggest that females may have to rely on postcopulatory  
113 inbreeding avoidance mechanisms (Gasparini and Pilastro 2011).

114 Here, we examine if genetic relatedness influences a males' competitive  
115 fertilization success using an established artificial insemination protocol (Evans et al.  
116 2003) to assess the potential for postcopulatory processes to influence relative  
117 paternity of related and unrelated males. Artificial insemination effectively uncouples  
118 pre- from postcopulatory inbreeding avoidance mechanisms and allows for rigorous  
119 control over sperm numbers between competing males, mating order effects, and  
120 differential sperm retention by females, all of which have the potential to influence

121 competitive fertilization success in guppies (Evans and Magurran 2001; Pilastro et al.  
122 2004). In addition, our protocol accounts for differences in components of sperm  
123 quality (e.g. velocity and viability) between rival males when assessing postcopulatory  
124 inbreeding avoidance mechanisms.

125

## 126 **Methods**

### 127 *Experimental animals and breeding protocols*

128 Experiments were performed on captive-bred guppies that were descendants (9-12  
129 generations) of wild-caught fish collected from Alligator Creek in Queensland,  
130 Australia. Stock populations were maintained in eight independent mixed-sex holding  
131 tanks, each containing ~300-400 guppies. To minimize inbreeding in the stock  
132 populations, random subsets of fish are transferred among holding tanks on a yearly  
133 basis. Full and half siblings were generated by randomly pairing a sexually mature  
134 male from one holding tank sequentially with two unrelated virgin females who  
135 originated from different holding tanks. Males and females were housed together for  
136 a five-day mating period in a 3L tank, after which males were removed from the tank  
137 and transferred to another 3L tank for a subsequent five-day mating period. Following  
138 the mating period, females were left undisturbed until broods were produced. This  
139 breeding protocol was repeated for 40 independent replicates (called 'family'  
140 hereafter). Offspring from each family were separated by sex at the earliest sign of  
141 sexual differentiation (when the male intromittent organ begins to develop) and  
142 reared in sex-specific family groups until reaching sexual maturity. The intention of  
143 this breeding design was to generate full and half male siblings for each family.  
144 However, due to a range of factors, including small brood sizes, the production of

145 mono-sex broods, the apparent absence of breeding and/or fertilizations during the  
146 mating period in some pairs, unsuccessful artificial inseminations, or a combination of  
147 these factors, we lacked sufficient replicates to evaluate both levels of relatedness for  
148 every family. Instead, we divided the families into two groups: a focal virgin female  
149 was either artificially inseminated with sperm from an unrelated male ( $R_0$ ) and a full  
150 sibling ( $R_{0.5}$ ) ( $R_{0.5} + R_0$ ;  $n=16$ ), or with sperm from an unrelated male ( $R_0$ ) and a half  
151 sibling ( $R_{0.25}$ ) ( $R_{0.25} + R_0$ ;  $n=14$ ). For all artificial inseminations, the unrelated male was  
152 chosen randomly from a different family generated through the breeding protocol  
153 described above, thus ensuring all males were housed in similar conditions. However,  
154 this design meant that some families (but not individuals from these family) were  
155 represented in both the full and half sibling treatments, which we account for in the  
156 statistical analyses (described below).

157

### 158 ***Sperm collection***

159 Male guppies produce sperm bundles (spermatozeugmata) that are easily collected  
160 manually (Matthews et al. 1997; Evans et al. 2003). Briefly, sexually mature males  
161 were anaesthetized and placed on a glass slide under low-power magnification (Evans  
162 2009). The ventral side of each male was dried before 60  $\mu$ l of an extender medium  
163 (207 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl<sub>2</sub>, 0.49 mM MgCl<sub>2</sub>, 0.41 mM MgSO<sub>4</sub>, and 10  
164 mM Tris with pH 7.5) was pipetted to the base of the male's gonopodium. The use of  
165 the extender medium ensured that sperm remained quiescent prior to analyses  
166 (Gardiner 1978). Gentle pressure was applied to the male's abdomen to release  
167 sperm bundles into the extender solution. For each male ( $R_{0.5}$ ,  $R_{0.25}$  and  $R_0$ ) sperm  
168 bundles were subdivided into four separate aliquots: 10 sperm bundles were



169 collected for artificial inseminations, 2-4 sperm bundles were collected for sperm  
170 velocity analyses, 10 bundles were collected to assay sperm viability and the  
171 remaining sperm bundles were used to assess sperm morphology. The order of sperm  
172 collection was randomized based on relatedness for each competitive dyad to avoid  
173 stripping order effects.

174

### 175 ***Artificial inseminations***

176 Ten sperm bundles from each of the two rival males in each trial (i.e.  $R_{0.5} + R_0$  and  $R_{0.25}$   
177 +  $R_0$ ) were added to an Eppendoff tube in 20  $\mu$ l of extender solution and gently mixed  
178 to ensure a homogenous distribution of sperm bundles. Because each sperm bundle  
179 contains  $\sim 2.7 \times 10^5$  sperm cells, performing competitive fertilization experiments with  
180 an equal number of sperm bundles from each male effectively controls for differences  
181 in sperm number between males (Evans et al. 2003). The mixed sperm bundles were  
182 taken up by a Drummond® microdispenser and artificially inseminated into an  
183 anaesthetised virgin focal female viewed under low-power magnification. Fin clip  
184 samples from females and males were collected and stored in absolute ethanol for  
185 subsequent paternity analyses. Females were left undisturbed in 3L tanks for  $\sim 10$   
186 weeks, during which up to two broods per female were obtained for paternity  
187 analyses. Offspring were euthanized within 48 hours of birth and placed in absolute  
188 ethanol for subsequent molecular analyses (sample sizes are reported below).

189

### 190 ***Sperm quality analyses***

191 Sperm quality was assessed for each male by quantifying sperm swimming speed,  
192 viability, and morphology. These are biologically important measures of sperm quality

193 that are associated with fertilization success in non-competitive and competitive  
194 fertilizations in guppies and other taxa (reviewed by Simmons and Fitzpatrick 2012). A  
195 3  $\mu$ l aliquot of the extender medium containing two sperm bundles was placed on an  
196 individual well of a 12-cell multi-test slide (MP Biomedicals, Aurora, OH), previously  
197 coated with 1% polyvinyl alcohol to reduce sperm sticking to the glass (Wilson-Leedy  
198 and Ingermann 2007). These quiescent sperm samples were then activated with 3  $\mu$ l  
199 of a 150 mM KCL solution (Billard et al. 1990) containing 2mg/L BSA to further prevent  
200 sperm from sticking to the slide (Pitcher et al. 2007). Computer-assisted sperm  
201 analysis (CASA) was used to assess sperm swimming speed for each sample using a  
202 CEROS Sperm Tracker (Hamilton Thorne Research, Beverly, MA, USA) under 100x  
203 magnification using the threshold values described in Evans (2009). Two sperm  
204 velocity measures were determined for each male from different sperm  
205 subpopulations within a sample and the mean of these values was used for analyses.  
206 Sperm velocity measures were based on  $68 \pm 7.6$  (mean  $\pm$  SE) motile sperm tracks per  
207 sample. CASA produces several highly co-linear measures of sperm velocity (Simpson  
208 et al. 2013), including the average path velocity (VAP), which estimates the smoothed  
209 path velocity, the curvilinear velocity (VCL), which is the actual velocity of the sperm  
210 over the path, and the straight-line velocity (VSL), which measures the distance  
211 between the start and end point of the path. To incorporate as much information as  
212 possible in our analyses, these co-linear sperm velocity measures were collapsed  
213 using principal component analyses (PCA) into a single principal component (PC1)  
214 with an eigenvalue of 2.59, which explained 86.4% of the variance in sperm swimming  
215 speed. PC1 was used as a measure of sperm swimming speed in all analyses (note,  
216 however, that we obtained qualitatively similar results when using any of the

217 individual measures of sperm velocity). We were unable to quantify sperm swimming  
218 speed for one male in the full sibling/unrelated male cross due to an experimental  
219 handling error and consequently removed this replicate from analyses of sperm  
220 quality (see below).

221 Sperm viability (i.e. the proportion of live sperm in the male's ejaculate) was  
222 determined using a live/dead sperm viability assay (Invitrogen, Molecular probes).  
223 This assay uses fluorescent dyes that stain live sperm green with the membrane-  
224 permeant nucleic acid stain SYBR-14 and dead sperm red with propidium iodide.  
225 Sperm bundles (10 bundles in 20  $\mu$ l of the extender medium) were broken up by  
226 thoroughly vortexing the sample. A 1:50 dilution of the SYBR-14 (1 mM) stain was  
227 added to a 10  $\mu$ l sample of the sperm/extender solution. Samples were left in the  
228 dark for 10 min, followed by the addition of 2  $\mu$ l of 2.4 mM propidium iodide and an  
229 additional incubation in the dark for 10 min. Samples were viewed under a Leica DM  
230 1000 fluorescence microscope under 400x magnification and the number of live and  
231 dead sperm were counted from 200 sperm per sample as an estimate of sperm  
232 viability.

233 To assess sperm morphology, sperm head, midpiece and flagellum length were  
234 measured from 20 sperm per male. Sperm were digitally photographed using a Leica  
235 DFC320 camera fitted to Leica DM 1000 phase contrast microscope under 400x  
236 magnification. Sperm lengths were measured using ImageJ (v1.37) and mean values  
237 were used in statistical analyses.

238 We did not expect any systematic differences in sperm quality measures  
239 between related and unrelated males given the breeding protocols outlined above.  
240 Nevertheless, we were concerned that any such systematic differences in sperm

241 quality generated by chance could bias our results. Therefore, we compared each of  
242 the sperm traits measured between related and unrelated males to account for any  
243 unintentional source of bias in our analyses and found no difference between  
244 competing related and unrelated males in any of the sperm traits assessed in this  
245 study (Table S1).

246

### 247 ***Parentage analysis***

248 Genomic DNA was extracted from adult and offspring tissue samples using a standard  
249 salting-out protocol (Patwary et al. 1994). Paternity was determined by scoring up to  
250 five polymorphic microsatellite loci that have been optimised previously for guppies:  
251 TTA, KonD15, KonD21, Pret46, Pr39 (GenBank accession numbers AF164205,  
252 AF368429, AF368430, AB100334 and AF467903, respectively). PCR amplifications  
253 were performed on a GeneAmp PCR System 2700 Thermocycler (Applied Biosystems,  
254 CA, USA) following methods described in Gasparini et al. (2010a). Amplified fragments  
255 were separated using an ABI 3100 sequencer (ABI PRISM, Applied Biosystems) and  
256 PCR products were visualized using GeneMarker v1.91 (<http://www.softgenetics.com>).  
257 Paternity was assigned to putative fathers, incorporating the known genotype of the  
258 mother, using CERVUS v3.0.6 (Kalinowski et al. 2007). A total of 235 offspring were  
259 scored (mean  $\pm$  SE:  $7.8 \pm 0.9$  per female,  $n=30$ , range 3-21). Families where only  
260 considered in the analyses if more than two offspring were assigned to either of the  
261 competing males; this criterion removed one family (consisting of three offspring  
262 from the  $R_{0.5} + R_0$  male treatment) from the analyses. Our final sample size therefore  
263 comprised 232 offspring. CERVUS assigned paternity to 93% ( $n=216$ ) of the offspring  
264 at the strict (95%) level of confidence and assigned an additional two offspring (from

265 one brood) at the relaxed (80%) level of confidence. Our results remained  
266 qualitatively similar when we restricted our analysis to include only offspring assigned  
267 using the strict confidence level and when we included males assigned at both levels  
268 of confidence. Consequently, we only present data from the strict level of confidence.  
269 Seventeen offspring, from eight crosses, could not be assigned to either of the  
270 putative fathers (i.e. confidence <80%) and were therefore excluded from our  
271 analyses.

272

### 273 ***Statistical analyses***

274 To determine if paternity differed between related and unrelated males, we used a  
275 randomization test to compare the observed paternity share of the unrelated males  
276 against a null expectation that assumes relatedness does not bias sperm use (i.e.  
277 paternity probability = 0.5). Specifically, the observed difference in paternity success  
278 between related and unrelated males was compared to the expected difference in  
279 paternity success between related and unrelated males for the given brood size under  
280 the assumption that competing males had an equal probability of fertilizing eggs.  
281 Random binomial probabilities were calculated and resampled 100,000 times to  
282 obtain an expected distribution against which the observed binomial distributions  
283 from each replicate were tested. Randomization tests were performed for the  $R_{0.5} +$   
284  $R_0$  and  $R_{0.25} + R_0$  crosses separately using an R script written by W. Black.

285 To determine the relationship between sperm quality, relatedness and  
286 paternity success we used a generalized linear mixed model (GLMM) with a binomial  
287 error distribution and a logit link function. Paternity was coded from the related  
288 males perspective, with offspring sired by the related male coded as successes and

289 offspring sired by the unrelated male coded as failures. All crosses were assessed in a  
290 single model with difference in sperm trait values (i.e. sperm head length, midpiece  
291 length, flagellum length, swimming speed PC1, and viability) between the related and  
292 unrelated male (i.e. relate male sperm – unrelated male sperm traits) in each cross  
293 added as covariates, the relatedness level of the related male (i.e. full sibling or half  
294 sibling) included as a fixed effect, and all interactions between each of the sperm  
295 traits and the relatedness level. To account for the use of individuals from the same  
296 families in the artificial inseminations at different levels of relatedness we included  
297 family identity as a random effect in the model. Attempts to reduce the model by  
298 removing non-significant interaction terms degraded the model fit, evidenced by  
299 elevated Akaike weight (AIC) values in the reduced model compared to the full model  
300 (full model:  $df = 14$ ,  $AIC = 104.50$ ; reduced model:  $df = 9$ ,  $AIC = 559.32$ ). Consequently,  
301 we only consider the full model in the analyses. All analyses were performed in R  
302 v3.0.1 (R Development Core Team 2013). GLMM analyses were performed using the  
303 *glmer* function in the *lme4* package (Bates et al. 2014). Significant values from the  
304 glmer model were calculated from Type II Wald chi square tests using the ‘Anova’  
305 function in the *car* package. Overdispersion was assessed using the function  
306 *overdisp\_fun* in R, and this analysis revealed that overdispersion was not an issue in  
307 the model.

308

## 309 **Results**

310 Paternity analyses revealed that fertilization success was skewed towards unrelated  
311 males, but only when females were inseminated with sperm from full siblings and an  
312 unrelated male competitor (i.e.  $R_{0.5} + R_0$ ). When females were artificially inseminated

313 with an equal number of sperm from a full sibling and unrelated male, mean ( $\pm$ SE)  
314 paternity percentage was  $40.6 \pm 8.7\%$  for  $R_{0.5}$  males. The mean difference in paternity  
315 between  $R_{0.5}$  and  $R_0$  males was  $-18.9\%$  ( $n = 15$ , Figure 1), which differs significantly  
316 from the null expectation of equal paternity between competing males (random  
317 binomial probability,  $p = 0.038$ ). In contrast, when females were artificially  
318 inseminated with sperm from a half sibling and unrelated male, mean ( $\pm$ SE) paternity  
319 percentage was  $52.2 \pm 6.3\%$  for  $R_{0.25}$  males. The mean difference in paternity between  
320  $R_{0.25}$  and  $R_0$  males was  $4.3\%$  ( $n = 13$ , Figure 1), which did not differ from the null  
321 expectation ( $p = 0.35$ ).

322 The paternity skew against related males observed in the  $R_{0.5} + R_0$  treatment could  
323 have been generated either by postcopulatory mechanisms of inbreeding avoidance  
324 (e.g. cryptic female choice in favour of unrelated sperm) and/or sperm competition  
325 (i.e. differences in sperm quality between competing males where sperm quality was  
326 consistently higher in unrelated males). To distinguish between these possibilities we  
327 determined whether the proportion of offspring sired by the related male ( $R_{0.5}$  and  
328  $R_{0.25}$ ) was influenced by sperm quality while also controlling for the relatedness level  
329 of the competing males. Relatedness level and difference in sperm viability between  
330 males significantly influenced paternity success of related males in the full and  
331 reduced model (Table 1, Figure 2). However, the significant relatedness level x sperm  
332 viability interaction terms in the model revealed that while males with greater sperm  
333 viability sired more offspring in both relatedness treatments, the effect of sperm  
334 viability on competitive fertilization success was influenced by the level of relatedness  
335 of male competitors (Figure 2). Specifically, the effect of sperm viability on  
336 competitive fertilization success is more pronounced in the full sibling treatment than

337 the half-sibling treatment. Compared with the half sibling treatment, full sibling males  
338 sired more offspring when they had high sperm viability (relative to unrelated males)  
339 and fewer offspring when they had lower sperm viability (relative to unrelated males)  
340 in competitive fertilizations (Figure 2). No other sperm quality traits measured  
341 predicted paternity success (Table 1). Thus, this analysis supports the paternity results  
342 from our randomization tests while demonstrating the importance of considering  
343 complex interactions between sperm traits, in this case sperm viability, and levels of  
344 relatedness when examining paternity success.

345

## 346 **Discussion**

347 Our results demonstrate that competitive fertilization success was biased toward  
348 unrelated male guppies, but only when females were inseminated with sperm from a  
349 full sibling ( $R_{0.5}$ ) and an unrelated male ( $R_0$ ). In contrast, competitive fertilization  
350 success was not influenced by genetic relatedness when females were artificially  
351 inseminated with sperm from a half sibling ( $R_{0.25}$ ) and an unrelated male ( $R_0$ ). As our  
352 experimental design prevented precopulatory mechanisms from biasing paternity and  
353 controlled for postcopulatory processes known to influence competitive fertilization  
354 success (i.e. mating order effects, differences in sperm number and quality), our  
355 results provide strong support for the idea of postcopulatory inbreeding avoidance in  
356 guppies. These findings support recent work showing that female guppies use  
357 postcopulatory processes to bias paternity towards unrelated males following  
358 artificial insemination by full sibling and non-sibling males (Gasparini and Pilastro  
359 2011) or highly inbred (four generations of full sibling matings) and outbred males  
360 (Zajitschek et al. 2009). However, our data also demonstrate that paternity biasing in



361 guppies is sensitive to the genetic relatedness of the competing males and is only  
362 evident in matings involving full siblings (also see Zajitschek et al. 2009). Similar  
363 inbreeding avoidance mechanisms appear to be in place in wild populations. For  
364 example, Johnson et al. (2010) reported that unrelated males sired more offspring in  
365 clutches from multiply mated female Trinidadian guppies, although this field result  
366 could have resulted potentially from both pre- and postcopulatory processes.  
367 Inbreeding avoidance mechanisms specifically targeting brothers are likely beneficial  
368 for female guppies as a single generation of full sibling inbreeding can reduce juvenile  
369 survival (Nakadate et al. 2003) and delay the onset of sexual maturity (Pitcher et al.  
370 2008) and reduce male courtship behaviours (Mariette et al. 2006). Consequently,  
371 multiple mating by females coupled with postcopulatory inbreeding avoidance  
372 mechanisms likely mitigate inbreeding depression in guppies.

373 The extensive use of guppies as a model system for studying postcopulatory  
374 sexual selection and inbreeding offers a rare opportunity to evaluate how variance in  
375 male relatedness influences inbreeding avoidance exclusively at the postcopulatory  
376 level. Together with the present study, seven other studies have used artificial  
377 inseminations to assess sperm competitiveness in males of varying levels of  
378 relatedness (Evans et al. 2003; Evans et al. 2008; Evans and Rutstein 2008; Zajitschek  
379 et al. 2009; Gasparini et al. 2010a; Gasparini and Pilastro 2011; Boschatto et al. 2011).  
380 Examining these studies together revealed two important patterns. First, significant  
381 biasing of paternity in favour of unrelated males is only observed in artificial  
382 inseminations involving relatedness levels of full sibling and above in guppies (e.g. this  
383 study, Zajitschek et al. 2009; Gasparini and Pilastro 2011). Second, when we  
384 combined these studies to examine the relationship between related male paternity

385 and level of relatedness (see Supporting Information for details), we observed a  
386 significant decrease in paternity success of related males as male relatedness to  
387 females increased (linear regression:  $n = 5$ ,  $r = 0.91$ ,  $p = 0.03$ , Figure 3). Thus, in  
388 guppies postcopulatory inbreeding avoidance appears to be exaggerated when  
389 inbreeding risk is elevated. A similar pattern of reduced paternity of related males  
390 with increasing relatedness was observed in *Drosophila melanogaster*, where full  
391 sibling males exhibited lower competitive paternity success than half siblings, cousins  
392 and unrelated males (Mack et al. 2002; but see Ala-Honkola et al. 2011). Moreover,  
393 negative relationships between genetic similarity (i.e. band sharing) between males  
394 and females and a males' paternity success in the sand lizard *Lacerta agilis* (Olsson et  
395 al. 1996), ascidian *Diplosoma listerianum* (Bishop et al. 1996), and the marsupial  
396 *Antechinus agilis* (Kraaijeveld-Smit et al. 2002) also suggest that cryptic female choice  
397 for unrelated sperm intensifies as relatedness between males and females increases.  
398 However, the accumulated evidence from guppies (Figure 3) represents the first  
399 evidence for a graded inbreeding avoidance response that can be attributed solely to  
400 postcopulatory processes. This graded response may stem from the relative costs and  
401 benefits of inbreeding, as females should only avoid inbreeding when the costs are  
402 high (i.e. in full sibling matings), while potentially reaping inclusive fitness benefits  
403 when the costs are low (i.e. in matings involving relatedness levels of half siblings or  
404 lower) (Kokko and Ots 2006).

405 Our analysis accounted for differences in sperm quality between competitors  
406 when testing for postcopulatory inbreeding avoidance. However, this analysis also  
407 sheds light on how variation in sperm quality among males influences competitive  
408 fertilization success. Our results revealed that sperm viability is an important

409 predictor of competitive fertilization success in guppies, although levels of relatedness  
410 of the competing males modulated this effect. Although sperm viability is commonly  
411 expected to influence male fertility and to be shaped by sperm competition, there is  
412 relatively little evidence that sperm viability influences competitive fertilization  
413 success (Simmons and Fitzpatrick 2012). For example, sperm viability predicts  
414 competitive fertilization success in the cricket *Teleogryllus oceanicus* (Garcia-Gonzalez  
415 and Simmons 2005) and the swordtail *Xiphophorus nigrensis* (Smith 2012), but in two  
416 species of frogs (*Crinia georgiana* and *Litoria peronii*) sperm viability does not  
417 influence male fertility under competitive conditions (Sherman et al. 2008; 2009;  
418 Dziminski et al. 2009). Therefore, our findings contribute towards a relatively scarce  
419 body of literature revealing the importance of sperm viability during sperm  
420 competition. Interestingly, however, the remaining sperm traits assessed in this study  
421 (sperm morphology and velocity) did not predict sperm competitiveness. This is  
422 surprising as sperm viability is negatively genetically correlated with sperm  
423 morphology (head, flagellum and total length) in the study population (Evans 2011).  
424 Yet, our finding is partially in keeping with recent work on guppies and other poeciliid  
425 fishes that did not find an effect of sperm morphology and only weak effects of sperm  
426 swimming speed on competitive fertilization success (Gasparini et al 2010b;  
427 Boschetto et al. 2011; Smith 2012). Nevertheless, it remains unclear why sperm  
428 morphology and swimming speed appear to be unrelated to male fertility in this and  
429 other studies of poeciliid fishes, as there is growing evidence that these sperm traits,  
430 particularly sperm swimming speed, predict male fertilization success during sperm  
431 competition (Simmons and Fitzpatrick 2012).

432 Our results, and those summarized from recent studies of guppies (Figure 3),  
433 highlight the importance of postcopulatory inbreeding avoidance mechanisms as a  
434 means of mitigating inbreeding. Although we cannot entirely rule out the possibility  
435 that differences in ejaculate traits (not measured here) may have contributed towards  
436 our findings, we suggest that our results are more consistent with the notion that  
437 females exert some form of postcopulatory selection that favours unrelated males.  
438 One potential mechanism to account for the paternity biases detected in our study is  
439 that offspring arising from consanguineous matings (i.e.  $r > 0.5$ ) exhibit impaired  
440 survival compared to those sired by unrelated parents. However, in the case of  
441 guppies, this explanation seems unlikely as Gasparini and Pilastro (2011)  
442 demonstrated that under non-competitive fertilization conditions females produced  
443 equivalent sized broods when artificially inseminated with sperm from full siblings or  
444 unrelated males. Instead, we suggest that our findings are more likely to be explained  
445 by fertilization biases that favour sperm from unrelated males when they compete to  
446 fertilize eggs. This conclusion is supported by the previous observation that female  
447 guppies exert cryptic female choice via the differential action of their ovarian fluid on  
448 the sperm swimming velocity of ejaculates from related and unrelated males  
449 (Gasparini and Pilastro 2011). Moreover, Gasparini and Pilastro (2011) showed that  
450 under conditions of sperm competition, this differential effect of ovarian fluid on  
451 sperm velocity generated a significant bias in paternity in favour of unrelated males,  
452 leading them to hypothesize that such effects may be attributable to interactions  
453 between peptides in the ovarian fluid and sperm membrane (e.g. major  
454 histocompatibility (MHC) peptides). Indeed, the MHC complex mediates fertilization  
455 success in mice (e.g. Wedekind et al. 1996; Rulicke et al. 1998) and may play a similar

456 role in guppies. Thus, avenues for future examination would be to experimentally  
457 validate the role of MHC in postcopulatory inbreeding avoidance mechanisms in  
458 guppies and to assess if the graded response in paternity biasing observed across  
459 various levels of relatedness is mirrored by increasing effects of ovarian fluid on  
460 sperm performance as relatedness increased. Elucidating the mechanisms driving  
461 paternity biasing based on relatedness promises to be both a challenging and  
462 stimulating future research endeavour.

463

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475

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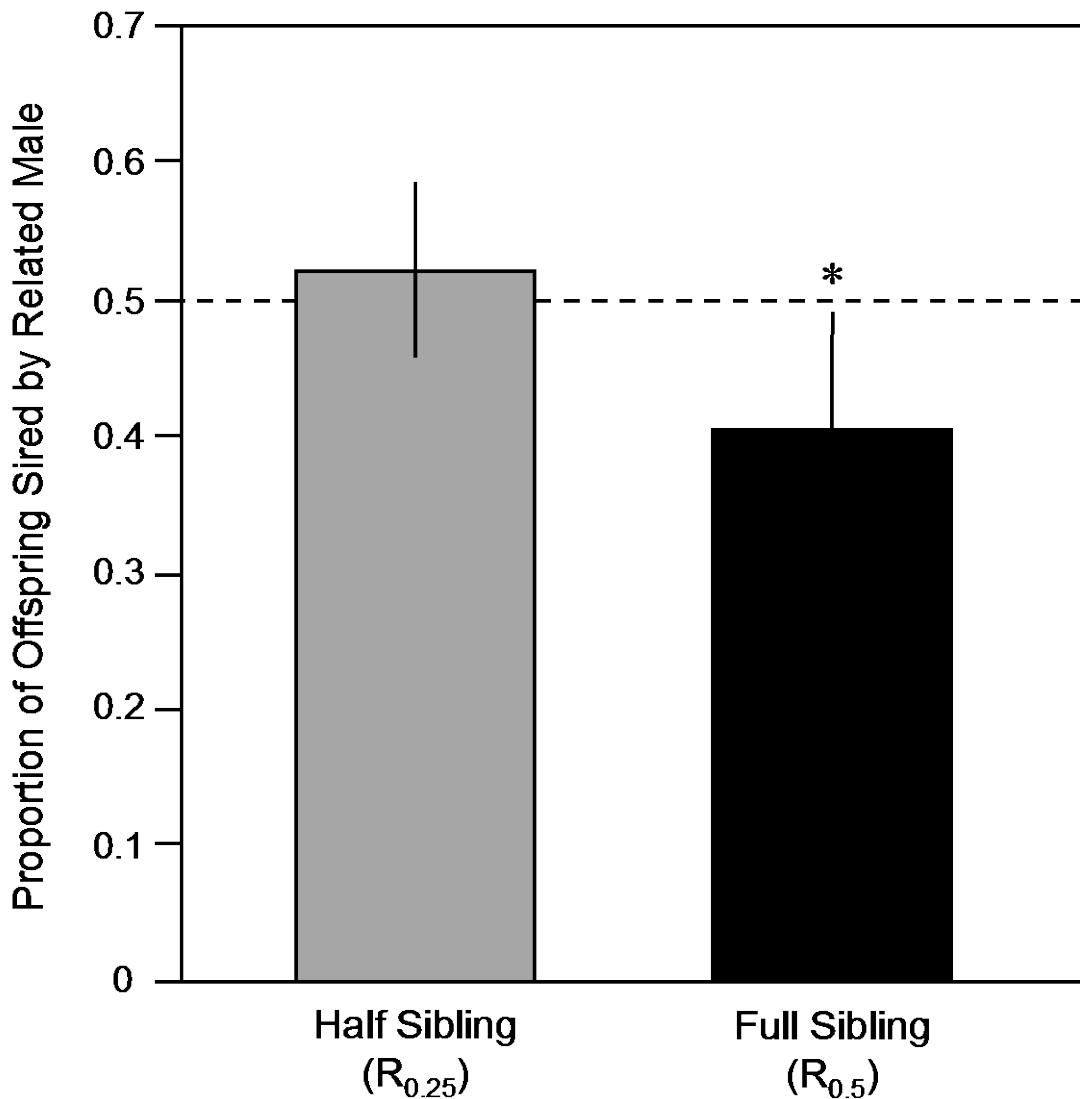
729 **Table 1.** The proportion of offspring sired by related males in relation to differences  
730 in sperm traits between the competing males and level of male relatedness.  
731 Relatedness refers to the level of relatedness of the related sperm competitor ( $R_{0.5}$  or  
732  $R_{0.25}$ ). Test parameters ( $\chi^2$ ) and significance levels (p) are generated from generalized  
733 linear mixed-effects models. Significant parameter values are presented in bold text.  
734

<b>Parameter</b>	<b><math>\chi^2</math></b>	<b>p</b>
Sperm head length	2.37	0.12
Sperm midpiece length	1.16	0.28
Sperm flagellum length	1.94	0.16
Sperm viability	14.88	<b>&lt;0.001</b>
Sperm swimming speed (PC1)	0.00	0.99
Relatedness	14.52	<b>&lt;0.001</b>
Relatedness x Sperm head length	1.16	0.28
Relatedness x Sperm midpiece length	0.00	0.99
Relatedness x Sperm flagellum length	0.01	0.91
Relatedness x Sperm viability	10.20	<b>&lt;0.01</b>
Relatedness x Sperm swimming speed (PC1)	1.58	0.21

735

736

737 **Figure 1.**

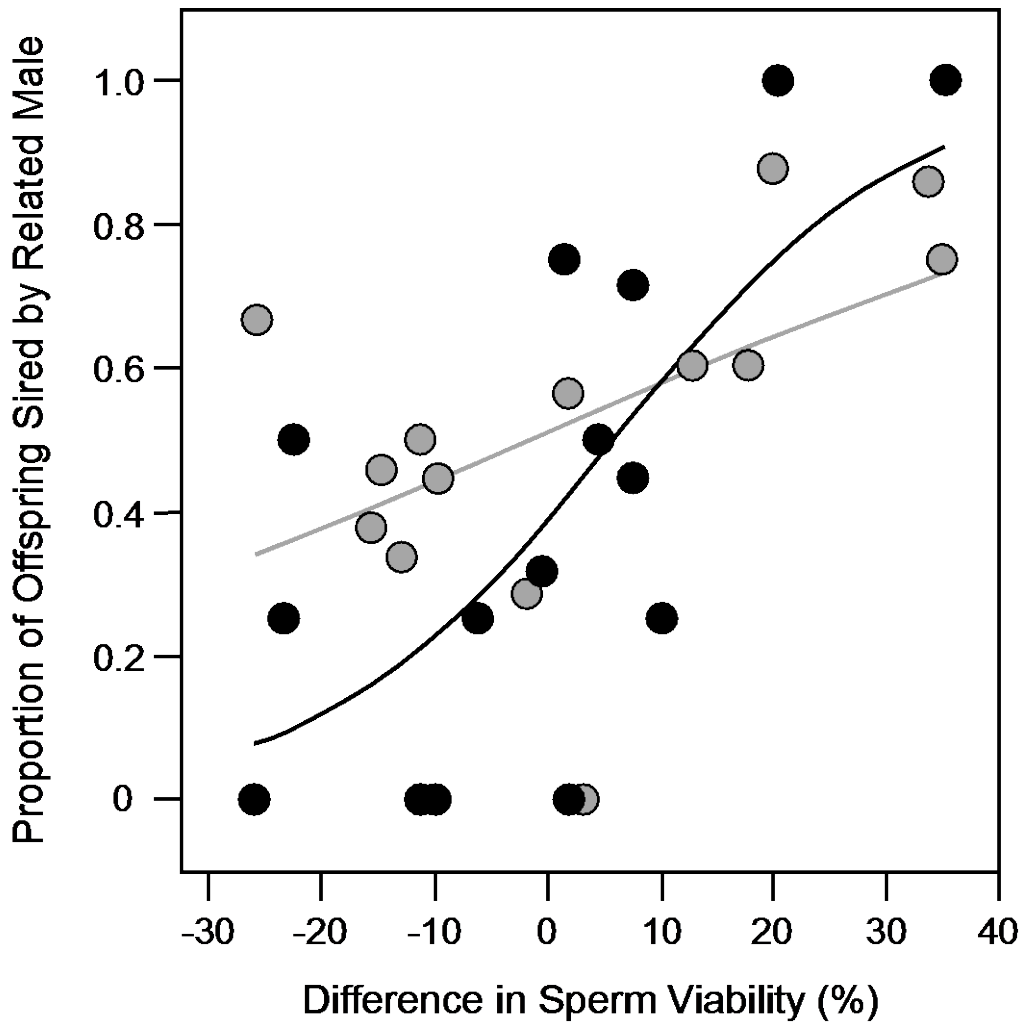


738

739 **Figure 1.** Proportion of offspring sired (mean  $\pm$  SE) by related males when females  
740 were artificially inseminated with equal numbers of sperm from a related and  
741 unrelated male. Males were related at the level of half sibling ( $R_{0.25}$ ) or full ( $R_{0.5}$ )  
742 sibling, resulting in artificial inseminations of half sibling and unrelated male ( $R_{0.25} +$   
743  $R_0$ ) or a full sibling and unrelated male ( $R_{0.5} + R_0$ ). The dotted line represents the null  
744 expectation of equal paternity between competing males. The \* indicates a significant  
745 difference in paternity success between males based on randomization tests.

746

747 **Figure 2.**

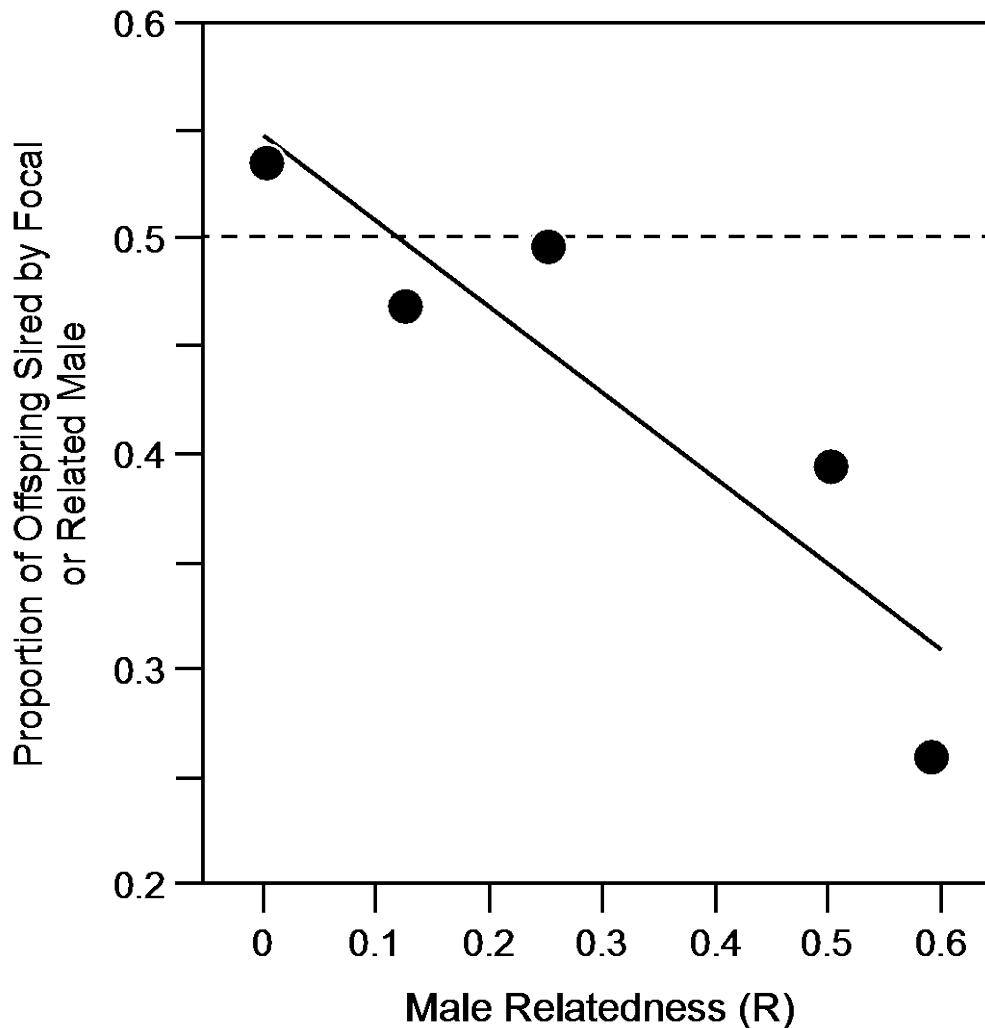


748

749 **Figure 2.** The relationship between the proportion of offspring sired by a half sibling  
750 ( $R_{0.25}$ , grey circle and fit line) or full sibling ( $R_{0.5}$ , black circle and fit line) male when in  
751 sperm competition with an unrelated male and the difference in sperm viability (%)  
752 between the related and unrelated males.

753

754 **Figure 3.**



755

756 **Figure 3.** Paternity success (mean  $\pm$  SE) of focal males in studies where female guppies  
757 were artificially inseminated with equal numbers of sperm from two unrelated males  
758 ( $R_0 + R_0$ ) or from an unrelated and related male at varying degrees of relatedness (first  
759 cousin:  $R_{0.125} + R_0$ ; half sibling:  $R_{0.25} + R_0$ ; full sibling:  $R_{0.5} + R_0$ ; four generations of full  
760 sibling mating:  $R_{0.59} + R_0$ ). When females were artificially inseminated with sperm from  
761 two unrelated males ( $R_0 + R_0$ ) the mean paternity success of a randomly chosen male  
762 is presented. When females were artificially inseminated with sperm from a related  
763 and unrelated male the mean paternity success of the related male is presented. The  
764 dotted line represents the null expectation of equal paternity between competing

765 males. See Supporting Information for details on how data was extracted from

766 published work and combined in this analysis.

767



## 768 **Postcopulatory inbreeding avoidance in guppies**

769 John L. Fitzpatrick and Jonathan P. Evans

770

### 771 **Supporting Information**

772

#### 773 **Appendix S1: Additional Methodological Details**

774 Data from the present study and seven other recent studies using artificial  
775 inseminations to assess sperm competitiveness in males of varying levels of  
776 relatedness were combined. Published studies include: Evans et al. (2003; 2008),  
777 Evans and Rutstein (2008), Zajitschek et al. (2009), Gasparini et al. (2010a), Gasparini  
778 and Pilastro (2011), and Boschatto et al. (2011). In all cases, females were artificially  
779 inseminated with equal numbers of sperm from two males, with the focal male in  
780 each artificial insemination exhibiting varying levels of relatedness with the female.  
781 Sperm competitiveness was assessed from two unrelated males in four studies ( $R_0 +$   
782  $R_0$ : Evans et al. 2003; Evans and Rutstein 2008; Gasparini et al. 2010a; Boschatto et al.  
783 2011), a first cousin and unrelated male in one study ( $R_{0.125} + R_0$ : Evans et al. 2008), a  
784 half sibling and unrelated male in two studies ( $R_{0.25} + R_0$ : this study, Zajitschek et al.  
785 2009), a full sibling and unrelated male in two studies ( $R_{0.5} + R_0$ : this study, Gasparini  
786 and Pilastro 2011), and males following four generations of full sibling mating and an  
787 unrelated male in one study ( $R_{0.59} + R_0$ : Zajitschek et al. 2009). In cases where only one  
788 study was available we used the mean paternity of focal or related males in our  
789 analyses. In cases where two or more studies were available we generated a mean  
790 values by calculated a sample size weighted mean from each study. When mean

791 values were not presented in the text but were plotted instead, data was extracted  
792 from published figures using GraphClick® (Ariozona software, [http://www.arizona-  
software.ch/graphclick/](http://www.arizona-<br/>793 software.ch/graphclick/)).

794 **Table S1.** Comparison between sperm traits of related and unrelated males  
 795 competing to fertilize eggs in artificial inseminations. Paired t-tests were used to  
 796 compare sperm traits between the unrelated male in the artificial insemination and  
 797 the a) related males from the  $R_{0.5}$  or  $R_{0.25}$  treatments. Differences in sperm traits  
 798 between the unrelated male and the related male were also examined for the b)  $R_{0.5}$   
 799 and c)  $R_{0.25}$  relatedness treatment separately.

800

Comparison	Sperm trait	t	df	p
<b>a) All males: <math>R_{0.5}</math> and <math>R_{0.25}</math> treatments</b>				
Related vs. Unrelated	Sperm viability	0.69	25	0.49
	Sperm head length	0.82	25	0.42
	Sperm midpiece length	1.81	25	0.08
	Sperm flagellum length	-0.48	25	0.64
	Sperm swimming speed (PC1)	-0.18	25	0.86
<b>b) <math>R_{0.5}</math> treatment</b>				
Related vs. Unrelated	Sperm viability	-0.19	13	0.85
	Sperm head length	0.49	13	0.63
	Sperm midpiece length	1.05	13	0.31
	Sperm flagellum length	-0.55	13	0.59
	Sperm swimming speed (PC1)	-1.21	13	0.25
<b>b) <math>R_{0.25}</math> treatment</b>				
Related vs. Unrelated	Sperm viability	1.06	11	0.31
	Sperm head length	0.68	11	0.51
	Sperm midpiece length	2.03	11	0.07
	Sperm flagellum length	-0.12	11	0.91
	Sperm swimming speed (PC1)	0.74	11	0.48

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