1 **Paternity analysis of wild caught females shows that sperm package**
2 **size and placement influence fertilisation success in the bushcricket**
3 ***Pholidoptera griseoaptera***
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Abstract

In species where females store sperm, males may try to influence paternity by the strategic placement of sperm within the female's sperm storage organ. Sperm may be mixed or layered in storage organs and this can influence sperm use beyond a 'fair raffle'. In some insects, sperm from different matings is packaged into discrete packets (spermatodoses), which retain their integrity in the female's sperm storage organ (spermatheca), but little is known about how these may influence patterns of sperm use under natural mating conditions in wild populations. We examined the effect of the size and position of spermatodoses within the spermatheca and number of competing ejaculates on sperm use in female dark bushcrickets (*Pholidoptera griseoaptera*) that had mated under unmanipulated field conditions. Females were collected near the end of the mating season and seven hypervariable microsatellite loci were used to assign paternity of eggs laid in the laboratory. Females contained a median of 3 spermatodoses (range 1-6) and only 6 of the 36 females contained more than one spermatodose of the same genotype. Both the size and relative placement of the spermatodoses within the spermatheca had a significant effect on paternity, with a bias against smaller spermatodoses and those further from the single entrance/exit of the spermatheca. A higher number of competing males reduced the chances of siring offspring for each male. Hence both spermatodose size and relative placement in the spermatheca influence paternity success.

Keywords: polyandry, sperm competition, spermatodose, post-copulatory sexual selection, cryptic female choice

Running head: Sperm precedence in the field

52 **Introduction**

53

54 Polyandry (females mating with more than one male) is taxonomically widespread
55 (Simmons 2005; Taylor et al. 2014) and can result in intense post-copulatory sexual
56 selection, in the form of both sperm competition and cryptic female choice (Birkhead
57 & Møller 1998; Eberhard 1996, 2015; Simmons 2001, 2014; Arnqvist 2014). Sperm
58 competition (competition between the sperm of two or more males for the
59 fertilisation of the female's eggs) has resulted in numerous male adaptations to
60 maximise paternity, including traits that allow a male to displace or remove rival
61 sperm from the female's reproductive tract and to deter the female from mating
62 with other males (Birkhead & Møller 1998; Simmons 2001, 2014).

63

64 The outcome of post-copulatory sexual selection, in terms of which male's sperm is
65 used to fertilise the majority of a multiply-mated female's eggs, has usually been
66 studied by mating females with two different males in a laboratory setting and is
67 often expressed as the proportion of offspring sired by the last male to mate, or P_2
68 (Birkhead & Møller 1998; Simmons 2001). Laboratory-based studies have identified a
69 wide range of factors that can determine variation in patterns of sperm use
70 (Birkhead & Møller 1998; Simmons 2001, 2014; Droge-Young et al. 2016). Mating
71 order is one such factor. In the majority of insect species, for example, the last male
72 to mate with the female tends to fertilise the greater proportion of her eggs (i.e.
73 there is last-male sperm precedence) (Simmons & Siva-Jothy 1998; Simmons 2001,
74 2014), although patterns of sperm precedence can vary widely, even between
75 closely related species. In the bushcrickets or katydids (Orthoptera: Tettigoniidae),
76 for example, reported patterns of sperm precedence in the lab range from first-male
77 priority (Simmons & Achmann 2000), sperm mixing (Wedell 1991) to pronounced
78 last-male sperm precedence (Helversen & Helversen 1991; Achmann et al. 1992;
79 Vahed 1998). In some cases, mating order can affect the outcome of sperm
80 precedence due to its effect on the relative positioning of sperm from different
81 males in the female's reproductive tract (Simmons & Siva-Jothy 1998; Droge-Young
82 et al. 2016). It has been suggested that in insects, sperm from different males may
83 sometimes become stratified within the female's sperm stores as a result of their

84 elongated shape, leading to a "last in, first out" mechanism of sperm precedence
85 (Simmons & Siva-Jothy 1998). In a few species, such as the dragonfly *Crocothemis*
86 *erythraea* (Odonata: Libellulidae), males can influence the process of stratification
87 using inflatable structures on their intromittant organ to push rival sperm to the
88 back of the sperm storage organ prior to transferring their own sperm (Siva-Jothy
89 1988). Due to the difficulty of distinguishing sperm from different males within the
90 female's sperm stores, however, very few previous studies have been able to
91 quantify the effect of the relative position of sperm on male fertilisation success (for
92 examples, see Manier et al. 2010, 2013a, 2013b; Droge-Young et al. 2016).
93
94 In many animals, individual sperm do not mix freely within the reproductive tract of
95 the female, but instead occur in discrete aggregations or bundles
96 (spermatodesmata) or in capsules that enclose the sperm from individual males
97 within the female's sperm storage organ (spermatodoses, not to be confused with
98 spermatophores, the packages males use to transfer sperm to the female) (Mann
99 1984, Higginson & Pitnick 2011, Fisher et al. 2014). Spermatodoses, or
100 spermatodose-like structures, occur in numerous insect families in several orders
101 including Orthoptera, Phthiraptera, Psocoptera, Thysanoptera, and Hemiptera
102 (Vahed 2003; Marchini et al. 2012). In bushcrickets, spermatodoses are thought to
103 form within the female's spermatheca (sperm storage organ) from secretions that
104 are transferred from the externally-attached spermatophore before the sperm mass
105 (Vahed 2003). Because one spermatodose appears to be formed per mating and
106 spermatodoses remain intact throughout the female's adult life, spermatodose
107 counts have been used to estimate the degree of polyandry in field-mated
108 bushcrickets (Gwynne 1984; Vahed 2006, Vahed et al. 2011; Robson & Gwynne
109 2010; Kaňuch et al. 2013; Jarčuška & Kaňuch 2014). However, their influence on
110 paternity has not been studied. In bushcrickets, each spermatodose has a spherical
111 body with a double-layered outer wall surrounding a tightly coiled ball of sperm,
112 arranged in feather-like spermatodesmata. Emerging from the body of the
113 spermatodose is an elongated, tubular exit (Viscuso et al. 2002; Vahed 2003). In
114 certain bushcricket species, such as *Pholidoptera griseoaptera*, the spermatodoses
115 from different matings become stratified within the elongated spermatheca of the

116 female (Vahed 2003, Fig 1). It has been proposed that spermatodoses and other
117 aggregations of sperm could function to block the exit of rival sperm from the
118 spermatheca, while allowing the male to deploy his sperm strategically in a position
119 closest to the exit of the spermatheca (Simmons & Siva-Jothy 1998; Vahed 2003);
120 however this hypothesis has not been tested. This hypothesis predicts that a high
121 level of last-male sperm precedence should occur in spermatodose-producing
122 species.
123
124 A further factor that can affect patterns of sperm use is relative ejaculate size
125 (Simmons 2001; 2014). Laboratory studies of a range of taxa have found that when a
126 female has mated with two different males, the relative amount of sperm received
127 from a given male determines the proportion of eggs that he subsequently fertilises
128 (Martin et al. 1974; Simmons 1987; Parker et al. 1990; Gage & Morrow 2003; but see
129 also Snook 2005). We are not aware of any previous studies that have examined the
130 effect of natural variation in ejaculate size on patterns of sperm use in field-mated
131 females.
132
133 Laboratory studies of factors associated with sperm precedence are unlikely to
134 reflect conditions experienced in the field, such as the females' natural number of
135 mates and natural re-mating intervals (Zeh & Zeh 1994; Simmons 2001; Lewis et al.
136 2005; Oneal & Knowles 2015). Zeh & Zeh (1994), for example, found that, in a
137 species of pseudoscorpion (*Cordylochernes scorpioides*), last-male sperm precedence
138 broke down when females were mated with more than two males. The nature of the
139 social group within which *Drosophila melanogaster* occur can also influence both the
140 remating rate and paternity of males in surprisingly complex ways (Billeter et al.
141 2012). The degree of polyandry and paternity skew (i.e. inequality among paternity
142 shares) can be quantified in females that have mated with multiple males under
143 natural field conditions using hypervariable molecular markers (Taylor et al. 2014),
144 including, for example, arthropods such as crickets and bushcrickets (Orthoptera:
145 Ensifera; Bretman & Tregenza 2005; Hockham et al. 2004; Simmons et al. 2007;
146 Simmons & Beveridge 2010; Turnell & Shaw 2015a, 2015b; Oneal & Knowles 2015).
147 Some studies of vertebrates, such as those of feral Soay Sheep, *Ovis aries* (Preston et

148 al. 2003), have additionally used direct observations of mating in the field to
149 examine factors that affect patterns of sperm use in field-mated females. In many
150 arthropod species, however, such field observations are often not practical due to
151 their small size, high mobility and/or cryptic nature. Consequently, very few previous
152 studies of arthropods (for examples, see Rodríguez-Munoz et al. 2010; Turnell &
153 Shaw 2015b) have been able to examine factors that affect patterns of sperm use in
154 females that have mated with multiple males under natural field conditions.
155
156 Here, by using a species in which sperm from different matings occur in discreet
157 aggregations (spermatodoses) within the spermatheca (the bushcricket *Pholidoptera*
158 *griseoaptera*), we were able to examine the influence of the position, size and
159 number of spermatodoses within the female spermatheca on patterns of sperm use
160 in females that had mated under un-manipulated, natural field conditions.
161
162 **Methods**
163 *The study species*
164 The dark bushcricket, *Pholidoptera griseoaptera* (DeGeer, 1773) is common and
165 widespread in Europe, where it is often associated with forest clearings, woodland
166 edges and hedgerows (Benton 2012). The eggs, which are laid in the summer and
167 autumn, hatch in either the spring of the following year or the one after (Hartley &
168 Warne 1972; Benton 2012). After passing through 6 to 7 nymphal instars, individuals
169 become adult in mid- to late July (Benton 2012; Kaňuch et al. 2015). The peak of
170 mating activity occurs in August (Kaňuch et al. 2015), but individuals can survive into
171 the late autumn (Benton 2012). Both sexes are flightless, but nevertheless have good
172 dispersal ability (Diekötter et al. 2010).
173
174 Males attract females by tegminal stridulation and both sexes mate multiple times
175 (Benton 2012; Kaňuch et al. 2015). In common with most other bushrickets, the
176 male transfers a large externally-visible spermatophore to the female towards the
177 end of copulation. The spermatophore represents approximately 11 % of male body
178 mass in this species and consists of two parts: the ampulla which contains the
179 ejaculate and the gelatinous spermatophylax which the female consumes during

180 ejaculate transfer (Vahed et al. 2014). As in other bushcrickets, both the male and
181 female enter a non-receptive sexual refractory period following each mating (Vahed
182 2007). The mean (± SE) sexual refractory period for females is 117.57 ± 15.62 hours,
183 while that for the males is 27.67 ± 6.94 hours (see Supporting information).
184
185 *Population sampling*
186 A total of 38 Female *P. griseoaptera* were collected from a field site near Silverton,
187 Devon, U.K., towards the end of the mating season from 5th – 12th September 2009.
188 The field site consisted of a 50m long stretch of roadside verge and hedge bank (grid
189 reference SS 95540 00570), at an altitude of approximately 43m above sea level.
190 Females were taken back to the lab and kept in separate cylindrical cages (17cm high
191 by 8cm in diameter). Each cage was provided with food in the form of wheat-germ,
192 together with young dock (*Rumex* sp.) and buttercup (*Ranunculus* sp.) leaves. A block
193 of flower-arranging "Oasis" polyurethane foam (Smithers-Oasis, USA), cut to 3cm X
194 8cm X 3cm, was provided as an oviposition medium. Females were allowed to lay
195 eggs for fourteen days before being frozen at -80°C until dissection and DNA
196 extraction. The eggs were extracted by crumbling the foam through a nylon sieve.
197 The mean number of eggs laid per female over the 2-week period was 56 (range: 21
198 – 85). Eggs from each female were placed in petri dishes containing damp cotton
199 wool, covered by a disc of filter paper. Eggs were maintained at 25°C for 3 months,
200 after which the degree of development of the embryos was scored. In *P.*
201 *griseoaptera*, eggs can either enter obligate winter diapause at the whole embryo
202 stage (in which the embryo occupies the whole of the egg and the eyes are clearly
203 visible towards the end of the egg), or as an early embryo (in which little embryonic
204 development is visible) (Hartley & Warne 1972). In our study, approximately 40 % of
205 viable eggs, on average, developed to the whole embryo stage after 3 months of
206 incubation, while the remainder were at the early embryo stage. There were very
207 few unviable eggs in our samples. Twenty whole-embryo eggs were collected at
208 random from each petri dish (i.e. from each female). Whole embryos were selected
209 simply to maximise the amount of DNA available. If sufficient whole-embryo eggs
210 were not available, eggs with early embryos were substituted. These were stored in
211 100% ethanol at -80°C prior to DNA extraction.

212

213 *Dissection of spermatodoses*

214 After thawing, the spermatheca was dissected from the female and placed in a drop
215 of water in a Petri dish. The spermatheca itself was then dissected by removing the
216 spermathecal wall using mounted needles under a light-dissecting microscope,
217 working upwards from the exit of the spermatheca. Each spermatodose was
218 extracted as it emerged and the diameter of each spermatodose was measured. The
219 walls of the spermatodose are rigid and the diameter of the spermatodose does not
220 decrease as sperm exit. Consequently, spermatodose diameter is likely to reflect the
221 volume of sperm transferred by that male. The relative position of each
222 spermatodose within the spermatheca in relation to the opening of the
223 spermathecal exit was also recorded. Although spermathecal walls are flexible, the
224 spermatheca of this species is elongated, resulting in the stratification of
225 spermatodoses within the spermatheca (Fig. 1B). This allows us to determine the
226 order in which each spermatodose was deposited (Vahed 2003). For the statistical
227 analysis, the relative position of each spermatodose was recorded as "1" for the one
228 closest to the spermathecal opening (i.e. the last male to mate) and "0" for the one
229 furthest from the spermathecal opening (i.e. the first male to mate). If there were
230 more than two spermatodoses, the spermatodoses in between the two extreme
231 ends of the spermatheca were scored as fractions. For example, for four
232 spermatodoses, the order was recorded as: "0, 0.33, 0.67, 1" while for 5
233 spermatodoses the order was recorded as: "0, 0.25, 0.5, 0.75, 1" (Fig. 1B). Each
234 spermatodose was stored individually in an Eppendorf tube containing 100% ethanol
235 and maintained at -80°C prior to DNA extraction.

236

237 *DNA extraction*

238 For the females, we extracted DNA from 10-20 mg of hind-leg muscle tissue. For
239 offspring, we used whole embryos. DNA extraction from females and embryos was
240 conducted following standard molecular protocols. To extract DNA from
241 spermatodoses, we used a protocol adapted from Simmons et al. (2007), which
242 firstly removes DNA from any female cells that may be present in the sample, before

243 extracting male DNA from the spermatodose (for details see Supporting
244 information).
245
246 *Microsatellite analysis*
247 We used 6 microsatellite primer pair sequences from Arens et al. (2005), chosen on
248 the basis of their reported variability and fragment size. We used 5' fluorescent-dye
249 labeled/unlabeled primer pairs (Life Technologies) to allow multiplexing of
250 microsatellites (see Table 1). Note the same dye colour was used for WPG10-1 and
251 WPG1-28, and WPG2-30 and WPG8-2 as these can easily be distinguished as they
252 have different size ranges. Also note that primer pair WPG1-27 amplifies two
253 microsatellite loci as described in Arens et al. (2005) meaning that samples were
254 genotyped at a total of 7 microsatellite loci. Microsatellites were amplified with the
255 Qiagen Multiplex PCR kit following the manufacturer's instructions. The amount of
256 primer used for each microsatellite was optimized so that each product showed
257 similar amplification (final ratio used: WPG 10_1 : WPG 1_28 : WPG 2_30 : WPG 8_2
258 : WPG 2_15 : WPG 1_27 = 1.00 : 1.50 : 2.25 : 4.50 : 1.50 : 1.50). Microsatellites were
259 amplified using a G-Storm GS1 thermocycler with the following program: Denature
260 at 95˚C for 15 minutes, followed by 30 cycles at 94˚C for 2 minutes, 60˚C for 1.5
261 minutes, 72˚C for 1 min, followed by a final extension time of 30 minutes at 60˚C.
262 Extension products were resolved on an ABI 3730XL machine performed by
263 Edinburgh Genomics (https://genomics.ed.ac.uk/). Alleles were sized to an internal
264 size standard (GeneScan-500 LIZ; Applied Biosystems) using Peak Scanner v2.0
265 (Applied Biosystems), and corrected manually where necessary.
266
267 *Genotyping failure rate by loci*
268 1 spermatodose (from a total of 115) and 6 offspring (from a total of 693) were
269 unable to be genotyped at any of our microsatellite markers, and likely represent
270 DNA extraction failures. For the remaining samples 1 was genotyped only at 3 loci, 4
271 at 4 loci, with the remainder all being genotyped for at least five loci (mean number
272 of loci genotyped per individual = 6.31). The rate of genotyping success was not
273 uniform across loci, with some having a genotype success rate of near 100% whilst

274 others were below 60% (Table 2). These loci were retained despite their high failure
275 rate as they still provided useful paternity information.
276
277 *Paternity analysis*
278 Paternity analysis was conducted using R package MasterBayes (version 2.52) in R (R
279 Core Team (2016), version 3.3.0). MasterBayes uses a Bayesian, consistent full-
280 probability model approach that allows paternity information and values of
281 parameters of interest to be estimated simultaneously (Hadfield et al. 2006). The
282 genotypes for the 7 microsatellite loci, along with phenotypic information for
283 relative mating order, and spermatodose size were provided to MasterBayes to
284 assign paternity to each offspring, and estimate the effect of relative mating order
285 and spermatodose size on the probability of siring offspring. MasterBayes was run
286 using default priors for 1,100,000 iterations with a burn-in of 100,000 iterations,
287 and thinning interval of 10. Drop-out and stochastic error rates were fixed at 0.005.
288 Mean values for the parameters of interest (relative mating order and
289 spermatodose size) were estimated from 100,000 MCMC samples from the
290 posterior distribution, which were also used to obtain a 95% credible interval
291 (highest posterior density interval) for these parameters.
292
293 To further examine these relationships, we used the offspring for which the
294 posterior probability of the most likely father was > 0.9. From this we calculated the
295 number of offspring each male sired as a proportion of those successfully assigned to
296 any father. In 6 of the females 2 of the spermatodoses in the female's spermatheca
297 had the same genotype, meaning offspring produced from spermatodoses with this
298 genotype could not be assigned to an individual spermatodose. As a result these
299 spermatodoses were discarded from subsequent analyses. Note that since the
300 number of offspring that were produced from either of these spermatodoses is
301 known, the correct proportion of offspring sired from the other spermatodoses in
302 the spermatheca could be correctly calculated and were thus retained in the GLM
303 analysis (below).
304

305 We then calculated paternity skew (sensu Pamillo & Crozier 1996) per female as
306 follows: paternity skew = (Total number of males - 1/ (Σx^2)) / (Total number of
307 males - 1), where x is proportion of offspring sired by a male. This measure of
308 paternity skew gives a value between 0 and 1 where 1 indicates a completely
309 unequal paternity share (one father sires all the offspring) and a value of 0 indicates
310 shared paternity (i.e. all fathers sire equal numbers of offspring). We then tested if
311 the observed paternity skew was significantly different than equal paternity (0) using
312 a one-sided, one-sample sign test in R (R Core Team (2016), version 3.3.0). Note, for
313 the calculation of paternity skew, females which had any offspring assigned to a
314 spermatodose with duplicate genotype in the same spermatheca (see above) were
315 discarded.
316
317 We then determined which factors influenced the proportion of offspring sired using
318 a quasi-poisson general linear model (GLM) in R (R Core Team (2016), version 3.3.0)
319 with the following terms: number of competing males, spermatodose size, and
320 relative mating order and all their possible interactions. Model simplification was
321 then conducted by dropping the highest least-significant term from the model until a
322 term had a p-value of < 0.05. Following this we then examined quadratic terms for
323 number of competing males, relative mating order, and spermatodose size by adding
324 these factors into the model one-by-one. If the added quadratic term was significant
325 ($p < 0.05$) it was retained.
326

327 **Results**

328 *Polyandry*

329 All of the 38 females collected in the study were found to have mated (i.e. showed
330 the presence of a spermatodose in the spermatheca) (mean number of
331 spermatodoses = 3.08; median = 3). However, 2 females were found to have mated
332 only once (Table 3) and thus were excluded from paternity analyses (below). We
333 found no correlation between number of spermatodoses and female size (pronotum
334 length) or fecundity (number of eggs laid) (r_s for pronotum length = 0.011, p = 0.95;
335 r_s for number of eggs laid = 0.209 , p = 0.21). Spermatodose size ranged from 0.50
336 mm to 1.4 mm in diameter (mean = 0.90 mm) and was not correlated with mating
337 order (r_s = 0.056, p = 0.555). There was no significant correlation between the
338 number of spermatodoses and either the diameter of the spermatodose nearest to
339 the blind end of the spermatheca (r_s = -0.163, p = 0.33) or mean spermatodose
340 diameter (r_s = - 0.144, p = 0.40).

341

342 *Paternity analysis*

343 Both relative mating order and spermatodose size have a significant effect on the
344 likelihood of siring offspring (Table 4). We found that the chance of siring offspring
345 increased with spermatodose size and male mating order (as inferred from relative
346 spermatodose position in the spermatheca), with males mating later in the mating
347 order siring more offspring. To examine these relationships in more depth, we
348 conducted additional analyses on those offspring for which the posterior probability
349 of the most likely father was > 0.9, which totalled 496 of the 693 offspring analysed.

350

351 Overall we found that paternity was highly skewed away from equal paternity
352 (median paternity skew = 0.92). Paternity skew was significantly higher than the
353 value expected for equal paternity (0) (one-sample sign test p-value = 3.559 10^{-08}).
354 This pattern was consistently found regardless of the number of competing males
355 (Fig. 2). The observed value of paternity skew was significantly higher than that
356 expected for equal paternity when the numbers of competing males was 2, 3, or 4
357 (one-sample sign test p-values = 0.0004, 0.0038, 0.0368 respectively) but not 5 or 6
358 (one-sample sign test p-values > 0.05) likely due to the small number of females in

359 these categories. Taken together these results show that paternity share is highly
360 skewed towards a small number of males.
361
362 To examine the possible causes of this paternity skew we then used a quasi-Poisson
363 GLM to determine the effect of the number of competing males, spermatodose size,
364 and relative mating order on the proportion of offspring sired. Results are
365 summarised in Table 5. Note fitting interactions between number of competing
366 males, spermatodose size and relative mating order were not significant ($p > 0.35$)
367 and so these terms were dropped. We also found that quadratic terms for
368 spermatodose size, and number of competing males were not significant ($p > 0.25$)
369 whereas such a term was significant for relative mating order (Table 5). Both a larger
370 spermatodose size, and being later in the mating order increased the chance of
371 siring offspring (Fig. 3A, 3B, Table 5). The effect of relative mating order followed a
372 quadratic curve, further penalising males early in the mating order. A higher number
373 of competing males reduced the chances of siring offspring (Fig. 3C, Table 5).
374
375 When assigning paternity to males we provided MasterBayes with phenotypic
376 information (mating order and spermatodose size). Since MasterBayes
377 simultaneously estimates the pedigree and the population-level parameters there
378 should be no bias from the use of this approach on our subsequent analysis to
379 examine the effects of mating order and spermatodose size on proportion of
380 offspring sired. To demonstrate this we repeated our analysis when paternity was
381 estimated without any phenotypic information (i.e. assigning paternity using only
382 genotypes). This approach produced very similar results to those described above
383 (Table S1, Supporting information).
384
385 Overall 44 out of 105 males (spermatodoses) produced 0 offspring. The proportion
386 of males that sired no offspring was higher in earlier mating males (proportion of
387 males siring no offspring when mating males last: 0.294, intermediate: 0.395, and
388 first: 0.576), however these differences were non-significant (logistic regression, $p >$
389 0.05). Interestingly, we found that when the last male to mate sired no offspring, the

male mating second-to-last sired most of the female’s offspring (mean proportion of offspring sired = 0.63).

396 **Discussion**

397

398 Here we have examined the influence of spermatodose size and placement on
399 paternity in field-collected samples of *P. griseoaptera*. Paternity share was highly
400 skewed with typically only one or two males siring the majority of a female's
401 offspring. Both the size and relative order of the spermatodoses within the
402 spermatheca had a significant effect on paternity, with a bias against smaller
403 spermatodoses and those further from the single entrance/exit of the spermatheca.
404 As expected, a higher number of competing males also reduced the chances of siring
405 offspring for each male. While previous studies of orthopteran insects have used
406 microsatellite analysis to estimate the degree of polyandry and paternity skew in
407 field-mated females (Bretman & Tregenza 2005; Hockham et al. 2004; Simmons et
408 al. 2007; Simmons & Beveridge 2010; Turnell & Shaw 2015a, 2015b; Oneal &
409 Knowles 2015), none of these have used the relative position of sperm within the
410 female's reproductive tract to predict the pattern of sperm use. Even if laboratory
411 based studies and other taxa are included, the number of previous studies that have
412 been able to relate directly the relative position of sperm within the female's
413 reproductive tract to sperm use by the female are very limited (Droge-Young et al.
414 2016). Manier et al. (2010, 2013a, 2013b) and Droge-Young et al. (2016), for
415 example, used transgenic lines with fluorescent-tagged sperm heads to resolve
416 mechanisms of competitive fertilisation success in *Drosophila spp* and *Tribolium*
417 *casteneum*, respectively, in a laboratory setting.

418

419 A further novel aspect of the present study was that, in the absence of field
420 observations, we were able to determine for each female the extent of repeated as
421 opposed to multiple mating. Our results indicated that there was a very low
422 frequency of repeated mating with the same male (only 6 out of 36 females
423 contained 2 spermatodoses of the same genotype, and no females contained >2
424 spermatodoses of the same genotype). Furthermore, there was only one case of a
425 female that appeared to have mated twice with the same male in two successive
426 matings (note that this may be considered a conservative estimate, since it is
427 possible that two males could share the same genotype). This low remating rate

428 could be a result of the 5-day long sexual refractory period in the female (Supporting
429 information) as a male that mates with a female is likely to have moved on by the
430 time the female is ready to mate again. The low level of repeated mating with the
431 same male could also reflect female choice (Ivy et al. 2005; Weddle et al. 2013).
432 Laboratory mate choice trials in Gryllid crickets, such as *Gryllodes sigillatus*, have
433 demonstrated that females actively avoid copulating with previous mates,
434 presumably in order to obtain any benefits from mating with different males (see Ivy
435 et al. 2005; Weddle et al. 2013).
436
437 The relationship between spermatodose position within the spermatheca and
438 paternity in the present study was best explained by a quadratic curve; while
439 spermatodoses furthest away from the opening of the spermatheca were generally
440 less successful in achieving paternity, there were diminishing returns of being
441 positioned closer to the spermathecal opening. This pattern is not entirely consistent
442 with the hypothesis that spermatodoses allow the male to block the exit of rival
443 sperm already present within the spermatheca (Simmons & Siva-Jothy 1998), which
444 would predict paternity to be very strongly skewed in favour of the last male to
445 mate. Sperm from all spermatodoses, even those at the distal end of the
446 spermatheca (i.e. from male that mated first), achieved some paternity.
447
448 Because sperm in storage were examined, some mechanisms of sperm precedence
449 can be ruled out, such as the removal or ejection of sperm from previous males
450 (Simmons & Siva-Jothy 1998; Simmons 2001). It is, however, possible that females
451 may have used up a greater proportion of sperm from earlier matings by the time
452 they were collected. Furthermore, in common with virtually all other studies of
453 sperm precedence, the possibility that post-meiotic sperm-ageing might have
454 contributed to the patterns of sperm use observed cannot be ruled out (Pizzari et al.
455 2008). The likely time that sperm were in storage in proportion to the female's
456 lifespan was relatively short, however. The median number of matings for females in
457 the present study was 3. Given that females have a sexual refractory period of 5 days
458 (Supporting information), that the majority of mating in this species occurs in
459 August, and that females were collected in early September, a reasonable estimate

460 of the time that sperm had been in storage in the spermatheca would be in the
461 region of 10-20 days. In contrast, the adult lifespan of the female is likely to be three
462 to four months or more; females can frequently survive and continue to lay eggs into
463 October and November, or even later (Hartley & Warne 1972; Benton 2013).
464
465 The only data available on sperm precedence in another tettigoniid species that
466 produces spermatodoses examined patterns of sperm precedence of female
467 *Decticus verrucivorus* (which is in the same sub-family as *P. griseoaptera*) that had
468 mated with two different males in a laboratory setting (Wedell 1991). Results were
469 consistent with a "fair raffle" (Parker 1990) and, unlike in the present study, no bias
470 against the use of sperm from the first male to mate was reported. It is possible that
471 depletion or ageing of sperm from the first mating could have been more
472 pronounced in our study in comparison to that of Wedell (1991), which could have
473 contributed to the observed fertilisation bias against earlier spermatodoses. Future
474 work comparing paternity patterns in both the field and lab will help to resolve these
475 issues.
476
477 Unexpectedly, approximately one third of the spermatodoses closest to the
478 exit/entrance of the spermatheca sired no offspring. In many insects, mating failures
479 are known to occur (Greenway & Shuker 2015). Such failures are often interpreted
480 as resulting from a failure to transfer sperm to the female's sperm storage organs,
481 which was clearly not the case here. When dissecting spermatodoses, it was
482 apparent that some still appeared to be full of a large ball of tightly coiled
483 spermatodesmata, while others appeared to be almost empty (Vahed 2003). It is
484 possible that spermatodoses do not begin to release their content immediately, but
485 that there is a delay. Even if discharge from the spermatodoses does begin soon
486 after their transfer, those from the females' most recent mates would have had less
487 time to discharge their content into the spermatheca, perhaps accounting for the
488 relatively high proportion of offspring sired by sperm from spermatodoses in the
489 second-to-last mating position in these families. The mechanism by which sperm are
490 released from spermatodoses and the rate at which they are discharged is currently
491 unknown (Vahed 2003). A further possible reason why sperm from spermatodoses

492 closest to the exit of the spermatheca did not always achieve highest paternity
493 relates to the position of the elongated spermatodose tube (through which sperm
494 exit the spermatodose). Vahed (2003) observed that in *P. griseoaptera*, in 50% of
495 cases, the spermatodose tube of the spermatodose nearest to the spermathecal exit
496 was oriented away from the exit rather than towards it.
497
498 In some cricket species, there is compelling evidence that the female can bias the
499 use of sperm from selected males by controlling not only the duration of attachment
500 of an externally-attached spermatophore, but also the uptake of sperm to the
501 spermatheca (Vahed 2015). Whether or not the female can influence the discharge
502 of sperm from spermatodoses as a further mechanism of cryptic female choice
503 deserves further investigation. There is also evidence that females might be able
504 exert control over the differential storage and use of sperm from their mates by
505 digesting stored sperm. In some bushcrickets, for example, spermolytic activity has
506 been found within the lumen of the duct of the spermatheca (Viscuso et al. 1996;
507 Brundo et al. 2011). It has been proposed that the walls of the spermatodoses may
508 function to protect the male's sperm from such spermolytic activity within the
509 spermatheca (Vahed 2003), that is, spermatodoses may be the result of inter-sexual
510 conflict over the fate of stored sperm, and sperm in older spermatodoses may be
511 more degraded as well as further away from the spermathecal opening.
512
513 We found that sperm from larger spermatodoses had a greater chance of siring
514 offspring. This is consistent with other sperm competition studies of various taxa,
515 which have demonstrated that when a female has mated with two different males
516 the relative number of sperm from each male predicts the paternity of her offspring
517 (Martin et al. 1974, Simmons 1987; Parker et al. 1990; Wedell 1991; Gage & Morrow
518 2003; Bretman et al. 2009). Spermatodose size is highly likely to reflect sperm
519 number: when full, the sperm occur in a tightly-coiled ball which takes up most of
520 the spherical body of the spermatodose (Vahed 2003). The transfer of larger
521 volumes of ejaculate does not only benefit the male by increasing his representation
522 in the female's sperm stores. Evidence suggests that in many insects, including
523 bushcrickets, substances in the ejaculate are also transferred that delay the female

524 from re-mating in a dose-dependent manner (Gillott 2003). This effect might also be
525 triggered by an increase in the physical 'fullness' of the spermatheca. In *P.*
526 *griseoaptera*, Jarčuška & Kaňuch (2014) found that the mean size of spermatodoses
527 within the spermatheca predicts the number of spermatodoses received over the
528 female's lifetime, suggesting that females that had received a larger ejaculate
529 subsequently mated with fewer males. We were unable to confirm this relationship
530 using our data set, although it should be noted that the sample size of females was
531 smaller than in Jarčuška & Kaňuch's (2014) study. The benefit to a male of delaying
532 or deterring his mate from remating was demonstrated in the present study: we
533 found that the proportion of offspring sired by each male declined with the number
534 of competing males. Simmons & Beveridge (2010) found a similar pattern in the field
535 cricket *Teleogryllus oceanicus* that had mated in the field.
536
537 It is possible that the influence of spermatodose order on paternity varies with
538 differences in polyandry. In *P. griseoptera*, we found that females contained up to 6
539 spermatodoses (median = 3), however the number of spermatodoses per female (i.e.
540 the degree of polyandry) is considerably greater than this in some bushcrickets
541 (Vahed 2006). In *Platycleis affinis*, for example, females contained up to 23
542 spermatodoses, while in *Anonconotus* spp, females contain up to 44 (Vahed 2006).
543 Examining the influence of spermatodose order on paternity in such highly
544 polyandrous species would be challenging but potentially useful. In addition, the
545 lifetime degree of polyandry is known to vary between populations (e.g. clinal
546 variation in remating rate is seen in *Drosophila pseudoobscura* (Price et al. 2008) and
547 *Metrioptera roeselii* (Kaňuch et al. 2013)). The techniques used here could be used
548 to compare how mating order affects sperm precedence between different
549 populations, which could provide a novel means of testing models of ejaculate
550 allocation (e.g. Parker 1990, 1998).
551
552 By using a species in which sperm from different matings occur within discreet
553 aggregations (spermatodoses), we were able to examine the effects of the order of
554 sperm deposition from different males within the female's sperm storage organ and
555 of ejaculate size, on male fertilisation success in females that had mated under

556 natural field conditions. The approach used here is likely to be generalizable to
557 other taxa in which sperm form discrete aggregations, but perhaps also to taxa for
558 which the stratification of sperm due to mating order may be more cryptic. Future
559 work to examine the influence of sperm aggregation on paternity are needed to
560 examine this, in particular from species in which sperm aggregations are less discreet
561 (for examples, see Mann 1984, Higginson & Pitnick 2011, Fisher et al. 2014).
562
563
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565
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824
825

826 **Data Accessibility**
827
828 All data is available in Dryad:
829 http://datadryad.org/review?doi=doi:10.5061/dryad.6t3dn
830

831 **Tables**

832

833

834 **Table 1. Properties of the of the six microsatellite markers used in the paternity**
835 **analysis (For primer sequences, see Arens et al. (2005))**

Locus Name	Number of alleles	Length (bp)	Dye-label
WPG10-1	3	123-129	VIC
WPG1-28	32	267-543	VIC
WPG2-30	3	147-174	PET
WPG8-2	9	217-286	PET
WPG2-15	7	240-258	FAM
WPG1-27 (a)*	3	189-229	NED
WPG1-27 (b)*	14	268-307	NED

836 * Note primer pair WPG1-27 amplifies 2 microsatellite loci (Arens et al. 2005) (denoted a and b here).

837

838 **Table 2. Percentage genotyping success for the microsatellite loci used in the**
839 **paternity analysis**

Microsatellite	Samples genotyped (N)	Samples genotyped (%)
WPG10-1	837	100.0
WPG1-28	835	99.8
WPG2-30	834	99.6
WPG8-2	488	58.6
WPG2-15	836	99.9
WPG1-27 (a)	647	77.5
WPG1-27 (b)	808	96.6

840

841

842 **Table 3. Number of spermatodoses present in females**

Number of spermatodoses	Number of females
0	0
1	2
2	13
3	10
4	8
5	3
6	2

843

844

845

846

847

848 **Table 4. Parameter estimates from MasterBayes using a 100,000 MCMC samples**
849 **from the posterior distribution, showing the effect of relative spermatodose order**
850 **with the spermatheca and spermatodose diameter on the likelihood of siring**
851 **offspring (HPD = Highest Posterior Density).**

Parameter	**Posterior mean (95% HPD)**
Relative mating order	0.793 (0.544-1.042)
Spermatodose diameter	8.164 (6.910-9.417)

852

853
854
855 **Table 5. Parameter estimates from the best-fitting quasi-Poisson GLM, showing the**
856 **effects of relative spermatodose order within the spermatheca, number of**
857 **competing males, and spermatodose diameter on paternity**

Coefficients	Estimate	t value	p-value
Relative order	3.65	3.09	0.0026
$(\text{Relative order})^2$	-2.70	-2.52	0.0132
Number of competing males	-0.39	-3.51	0.0007
Spermatodose diameter	2.75	4.01	0.0001

858

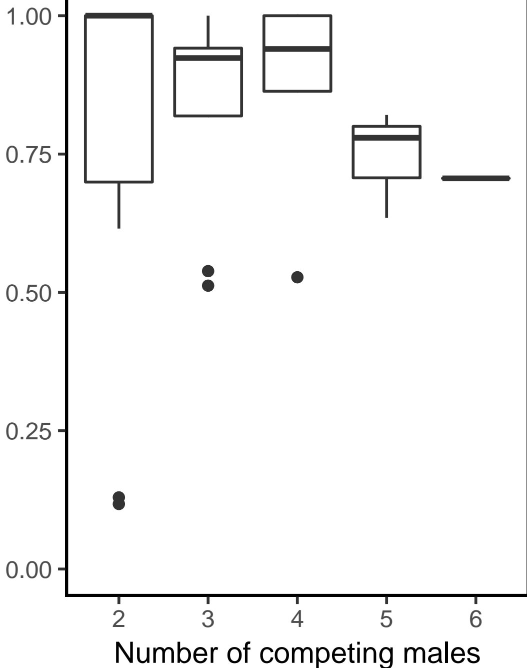
859 **Figure Legends:**
860
861 Fig. 1. (A) Photograph of dissected spermatodoses from *P. griseoaptera* (mean
862 diameter = 0.90 mm). (B) Schematic diagram of a longitudinal section through
863 the spermatheca in *P. griseoaptera*, showing how the relative position of each
864 spermatodose within the spermatheca was scored.
865
866 Fig. 2. Paternity skew for different numbers of competing males. A value of 1
867 indicates all a female's offspring are sired by one male whereas a value of 0
868 indicates all males sire the same number of a female's offspring.
869
870 Fig. 3. The relationships between the proportion of offspring sired by a given
871 male and: (A) the position of the male's spermatodose within the spermatheca (0
872 = furthest from the single exit/entrance, 1 = closest to the entrance/exit); (B).
873 the diameter (in mm) of the male's spermatodose and (C) the number of
874 competing males (see also Table 5). Note points were jittered along the X-axis to
875 aid visualisation of overlapping points.
876
877

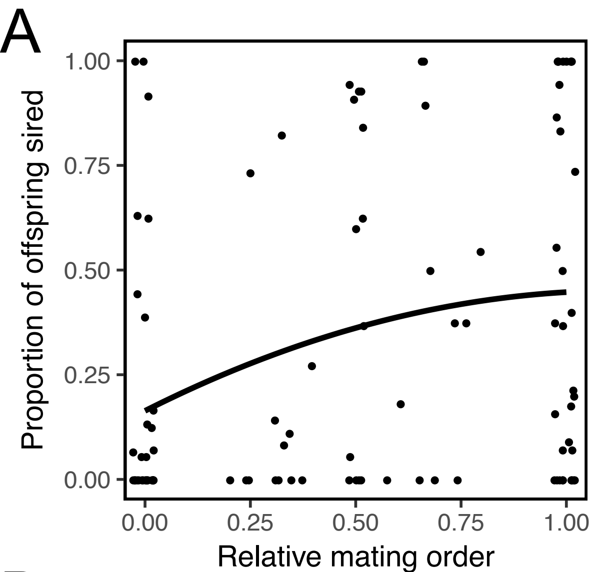
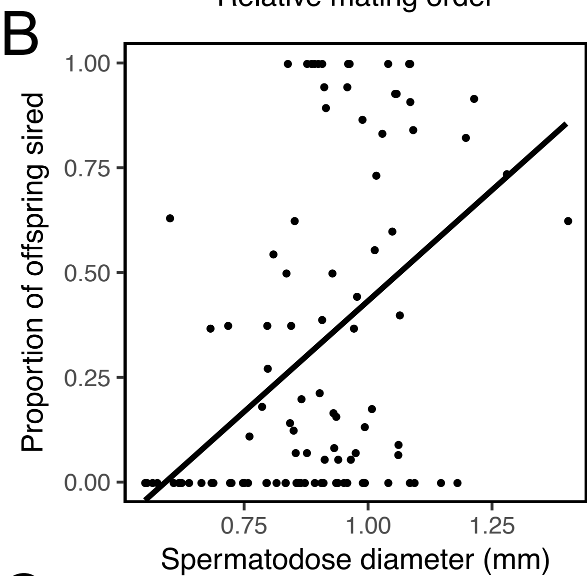
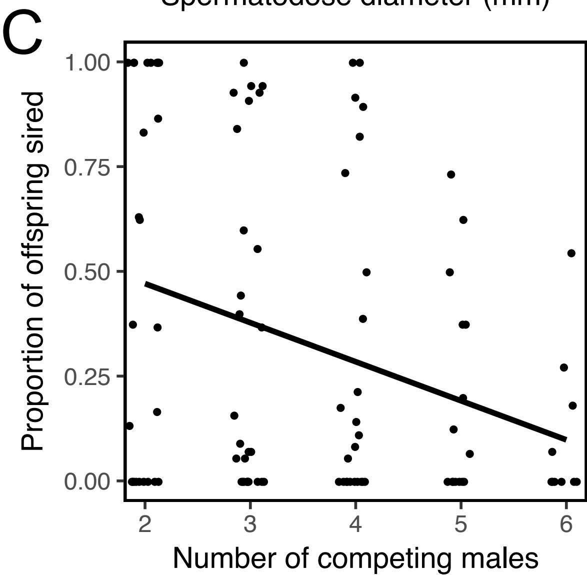
A

B

Mating order	Relative position
1st	0
2nd	0.25
3rd	0.5
4th	0.75
5th	1

Spermethecal opening


Reproductive skew
1.00
0.75
0.50
0.25
0.00
2
3
4
5
6
Number of competing males


A
Proportion of offspring sired
1.00
0.75
0.50
0.25
0.00
0.00
0.25
0.50
0.75
1.00
Relative mating order

B
Proportion of offspring sired
1.00
0.75
0.50
0.25
0.00
0.75
1.00
1.25
Spermatodose diameter (mm)

C
Proportion of offspring sired
1.00
0.75
0.50
0.25
0.00
2
3
4
5
6
Number of competing males

Supplemental materials

Extraction of spermatodose samples

Spermatodose samples taken from -80°C were defrosted, centrifuged for 5 min at 13,000 rpm, and the supernatant of ethanol discarded. The pellet was then washed by adding 1 ml of 10 mM Tris pH 8.0, vortexing the sample, centrifuging at 13,000 rpm, and then removing the supernatant. The washing step was then repeated. 350 μl of DNA extraction buffer (50 mM Tris-HCl pH 8.0, 50 mM EDTA, 100 mM NaCl, 1% SDS) and 2.5 μl of 20 mg/ml Proteinase K then was added to each sample, incubated for 30 min at 37°C, centrifuged for 5 min at 13,000 rpm, and supernatant discarded. Pellets were then washed twice with 1 ml of 10 mM Tris pH 8.0, as described previously. This step removes DNA from any female cells that may be present in the spermatodose sample. Sperm cells are resistant to this treatment as sperm head proteins contain disulphide bridges. To extract DNA from the sperm pellet, we added 330 μl DNA extraction buffer, 2.5 μl of 20 mg/ml Proteinase K, and 20 μl of 1 M DTT (dithiothreitol) to the pellet. This mix was then incubated for 3 hours at 56°C, before adding 2.5 μl of 10 mg/ml RNase A and incubating for 15 minutes at 37°C. Samples were then left to cool, before adding 150 μl of 5 M NaCl, vortexing gently, and centrifuging for 10 min at 13,000 rpm. The supernatant was transferred to a new Eppendorf tube, before adding 500 μl of cold 100% isopropanol and mixing by inversion. Samples were centrifuged at 13,000 rpm for 10 minutes before removing the supernatant. The DNA pellet for each sample was then washed with 600 μl 70% ethanol twice before resuspending the DNA in 10 μl Milli-Q water.

Sexual refractory period

Methods

In addition to the 38 females used in the paternity analysis, a further 10 males and 10 females were also collected from the same site at the same time of year for behavioural observations. These were maintained in captivity as described in the main methods section. All individuals were maintained separately. Pairs were set up by introducing a male into the female's container at 9.00 h. The container was observed at regular intervals of approximately 15 min until mating occurred, after which the original male was removed and replaced with a different male. The time taken for the female to consume the spermatophylax fully was also noted. The male was left in the females' cage until 21.00h, after which it was replaced in its own cage. On each subsequent day, the procedure was repeated with a different male until mating occurred. Sexual refractory period data was obtained for 7 females.

42 Three of the males that were used to determine the female's sexual refractory
43 period were each moved to a cage containing a different female within an hour
44 after the end of copulation. Each cage was observed at regular intervals as
45 described above. If mating did not occur, the female was removed at 21.00h and
46 a new female was placed in the male's cage the following morning at 9.00h. The
47 procedure was repeated until mating occurred.
48
49 ***Results***
50 The mean (± SE) sexual refractory period for the females was 117.57 ± 15.62
51 hours (n = 7), while that for the males was 27.67 ± 6.94 hours (n = 3). Females
52 took 248.8 ± 21.4 min (n = 4) to consume the spermatophylax fully, after which
53 they removed and consumed the ampulla of the spermatophore.
54
55
56 **Table S1. Parameter estimates from the best-fitting quasi-Poisson GLM, showing**
57 **the effects of relative spermatodose order within the spermatheca, number of**
58 **competing males, and spermatodose diameter on paternity when paternity was**
59 **assigned independently of any phenotypic information (see**

Coefficients	Estimate	t value	p-value
Relative order	4.08	3.40	0.0010
(Relative order)^2	-3.09	-2.86	0.0052
Number of competing males	-0.40	-3.50	0.0007
Spermatodose diameter	2.59	3.73	0.0003

60
61
62