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

Parker, D. J.1,2,*, Zaborowska, J.1,3, Ritchie, M. G.1 and Vahed, K4.

1. Centre for Biological Diversity, University of St Andrews, St Andrews, Scotland KY16 9TH, U.K.
2. Department of Ecology and Evolution, University of Lausanne, Biophore, Lausanne, 1015, Switzerland.
3. Institute of Environmental Sciences, Jagiellonian University, Gronostajowa 7, 30-387 Kraków, Poland.
4. Environmental Sustainability Research Centre, University of Derby, Kedleston Road, Derby DE22 1GB, U.K.

* Corresponding author’s email address: djp39@st-andrews.ac.uk

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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
Introduction

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

The outcome of post-copulatory sexual selection, in terms of which male’s sperm is used to fertilise the majority of a multiply-mated female’s eggs, has usually been studied by mating females with two different males in a laboratory setting and is often expressed as the proportion of offspring sired by the last male to mate, or $P_2$ (Birkhead & Møller 1998; Simmons 2001). Laboratory-based studies have identified a wide range of factors that can determine variation in patterns of sperm use (Birkhead & Møller 1998; Simmons 2001, 2014; Droge-Young et al. 2016). Mating order is one such factor. In the majority of insect species, for example, the last male to mate with the female tends to fertilise the greater proportion of her eggs (i.e. there is last-male sperm precedence) (Simmons & Siva-Jothy 1998; Simmons 2001, 2014), although patterns of sperm precedence can vary widely, even between closely related species. In the bushcrickets or katydids (Orthoptera: Tettigoniidae), for example, reported patterns of sperm precedence in the lab range from first-male priority (Simmons & Achmann 2000), sperm mixing (Wedell 1991) to pronounced last-male sperm precedence (Helversen & Helversen 1991; Achmann et al. 1992; Vahed 1998). In some cases, mating order can affect the outcome of sperm precedence due to its effect on the relative positioning of sperm from different males in the female’s reproductive tract (Simmons & Siva-Jothy 1998; Droge-Young et al. 2016). It has been suggested that in insects, sperm from different males may sometimes become stratified within the female’s sperm stores as a result of their
elongated shape, leading to a “last in, first out” mechanism of sperm precedence (Simmons & Siva-Jothy 1998). In a few species, such as the dragonfly *Crocothemis erythraea* (Odonata: Libellulidae), males can influence the process of stratification using inflatable structures on their intromittant organ to push rival sperm to the back of the sperm storage organ prior to transferring their own sperm (Siva-Jothy 1988). Due to the difficulty of distinguishing sperm from different males within the female’s sperm stores, however, very few previous studies have been able to quantify the effect of the relative position of sperm on male fertilisation success (for examples, see Manier et al. 2010, 2013a, 2013b; Droge-Young et al. 2016).

In many animals, individual sperm do not mix freely within the reproductive tract of the female, but instead occur in discrete aggregations or bundles (spermatodesmata) or in capsules that enclose the sperm from individual males within the female’s sperm storage organ (spermatodoses, not to be confused with spermatophores, the packages males use to transfer sperm to the female) (Mann 1984, Higginson & Pitnick 2011, Fisher et al. 2014). Spermatodoses, or spermatodose-like structures, occur in numerous insect families in several orders including Orthoptera, Phthiraptera, Psocoptera, Thysanoptera, and Hemiptera (Vahed 2003; Marchini et al. 2012). In bushcrickets, spermatodoses are thought to form within the female’s spermatheca (sperm storage organ) from secretions that are transferred from the externally-attached spermatophore before the sperm mass (Vahed 2003). Because one spermatodose appears to be formed per mating and spermatodoses remain intact throughout the female’s adult life, spermatodose counts have been used to estimate the degree of polyandry in field-mated bushcrickets (Gwynne 1984; Vahed 2006, Vahed et al. 2011; Robson & Gwynne 2010; Kaňuch et al. 2013; Jarčuška & Kaňuch 2014). However, their influence on paternity has not been studied. In bushcrickets, each spermatodose has a spherical body with a double-layered outer wall surrounding a tightly coiled ball of sperm, arranged in feather-like spermatodesmata. Emerging from the body of the spermatodose is an elongated, tubular exit (Viscuso et al. 2002; Vahed 2003). In certain bushcricket species, such as *Pholidoptera griseoaptera*, the spermatodoses from different matings become stratified within the elongated spermatheca of the
female (Vahed 2003, Fig 1). It has been proposed that spermatodoses and other aggregations of sperm could function to block the exit of rival sperm from the spermatheca, while allowing the male to deploy his sperm strategically in a position closest to the exit of the spermatheca (Simmons & Siva-Jothy 1998; Vahed 2003); however this hypothesis has not been tested. This hypothesis predicts that a high level of last-male sperm precedence should occur in spermatodose-producing species.

A further factor that can affect patterns of sperm use is relative ejaculate size (Simmons 2001; 2014). Laboratory studies of a range of taxa have found that when a female has mated with two different males, the relative amount of sperm received from a given male determines the proportion of eggs that he subsequently fertilises (Martin et al. 1974; Simmons 1987; Parker et al. 1990; Gage & Morrow 2003; but see also Snook 2005). We are not aware of any previous studies that have examined the effect of natural variation in ejaculate size on patterns of sperm use in field-mated females.

Laboratory studies of factors associated with sperm precedence are unlikely to reflect conditions experienced in the field, such as the females’ natural number of mates and natural re-mating intervals (Zeh & Zeh 1994; Simmons 2001; Lewis et al. 2005; Oneal & Knowles 2015). Zeh & Zeh (1994), for example, found that, in a species of pseudoscorpion (Cordylochernes scorpioides), last-male sperm precedence broke down when females were mated with more than two males. The nature of the social group within which Drosophila melanogaster occur can also influence both the remating rate and paternity of males in surprisingly complex ways (Billeter et al. 2012). The degree of polyandry and paternity skew (i.e. inequality among paternity shares) can be quantified in females that have mated with multiple males under natural field conditions using hypervariable molecular markers (Taylor et al. 2014), including, for example, arthropods such as crickets and bushcrickets (Orthoptera: Ensifera; Bretman & Tregenza 2005; Hockham et al. 2004; Simmons et al. 2007; Simmons & Beveridge 2010; Turnell & Shaw 2015a, 2015b; Oneal & Knowles 2015).

Some studies of vertebrates, such as those of feral Soay Sheep, Ovis aries (Preston et
al. 2003), have additionally used direct observations of mating in the field to examine factors that affect patterns of sperm use in field-mated females. In many arthropod species, however, such field observations are often not practical due to their small size, high mobility and/or cryptic nature. Consequently, very few previous studies of arthropods (for examples, see Rodriguez-Munoz et al. 2010; Turnell & Shaw 2015b) have been able to examine factors that affect patterns of sperm use in females that have mated with multiple males under natural field conditions.

Here, by using a species in which sperm from different matings occur in discreet aggregations (spermatodoses) within the spermatheca (the bushcricket Pholidoptera griseoaptera), we were able to examine the influence of the position, size and number of spermatodoses within the female spermatheca on patterns of sperm use in females that had mated under un-manipulated, natural field conditions.

Methods

The study species

The dark bushcricket, Pholidoptera griseoaptera (DeGeer, 1773) is common and widespread in Europe, where it is often associated with forest clearings, woodland edges and hedgerows (Benton 2012). The eggs, which are laid in the summer and autumn, hatch in either the spring of the following year or the one after (Hartley & Warne 1972; Benton 2012). After passing through 6 to 7 nymphal instars, individuals become adult in mid- to late July (Benton 2012; Kaňuch et al. 2015). The peak of mating activity occurs in August (Kaňuch et al. 2015), but individuals can survive into the late autumn (Benton 2012). Both sexes are flightless, but nevertheless have good dispersal ability (Diekötter et al. 2010).

Males attract females by tegmental stridulation and both sexes mate multiple times (Benton 2012; Kaňuch et al. 2015). In common with most other bushrickets, the male transfers a large externally-visible spermatophore to the female towards the end of copulation. The spermatophore represents approximately 11 % of male body mass in this species and consists of two parts: the ampulla which contains the ejaculate and the gelatinous spermatophylax which the female consumes during
ejaculate transfer (Vahed et al. 2014). As in other bushcrickets, both the male and female enter a non-receptive sexual refractory period following each mating (Vahed 2007). The mean (± SE) sexual refractory period for females is 117.57 ± 15.62 hours, while that for the males is 27.67 ± 6.94 hours (see Supporting information).

**Population sampling**

A total of 38 Female *P. griseoaptera* were collected from a field site near Silverton, Devon, U.K., towards the end of the mating season from 5\textsuperscript{th} – 12\textsuperscript{th} September 2009. The field site consisted of a 50m long stretch of roadside verge and hedge bank (grid reference SS 95540 00570), at an altitude of approximately 43m above sea level. Females were taken back to the lab and kept in separate cylindrical cages (17cm high by 8cm in diameter). Each cage was provided with food in the form of wheat-germ, together with young dock (*Rumex* sp.) and buttercup (*Ranunculus* sp.) leaves. A block of flower-arranging “Oasis” polyurethane foam (Smithers-Oasis, USA), cut to 3cm X 8cm X 3cm, was provided as an oviposition medium. Females were allowed to lay eggs for fourteen days before being frozen at -80\textdegree C until dissection and DNA extraction. The eggs were extracted by crumbling the foam through a nylon sieve. The mean number of eggs laid per female over the 2-week period was 56 (range: 21 – 85). Eggs from each female were placed in petri dishes containing damp cotton wool, covered by a disc of filter paper. Eggs were maintained at 25\textdegree C for 3 months, after which the degree of development of the embryos was scored. In *P. griseoaptera*, eggs can either enter obligate winter diapause at the whole embryo stage (in which the embryo occupies the whole of the egg and the eyes are clearly visible towards the end of the egg), or as an early embryo (in which little embryonic development is visible) (Hartley & Warne 1972). In our study, approximately 40 % of viable eggs, on average, developed to the whole embryo stage after 3 months of incubation, while the remainder were at the early embryo stage. There were very few unviable eggs in our samples. Twenty whole-embryo eggs were collected at random from each petri dish (i.e. from each female). Whole embryos were selected simply to maximise the amount of DNA available. If sufficient whole-embryo eggs were not available, eggs with early embryos were substituted. These were stored in 100% ethanol at -80\textdegree C prior to DNA extraction.
Dissection of spermatodoses

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

DNA extraction

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

**Microsatellite analysis**

We used 6 microsatellite primer pair sequences from Arens et al. (2005), chosen on the basis of their reported variability and fragment size. We used 5′ fluorescent-dye labeled/unlabeled primer pairs (Life Technologies) to allow multiplexing of microsatellites (see Table 1). Note the same dye colour was used for WPG10-1 and WPG1-28, and WPG2-30 and WPG8-2 as these can easily be distinguished as they have different size ranges. Also note that primer pair WPG1-27 amplifies two microsatellite loci as described in Arens et al. (2005) meaning that samples were genotyped at a total of 7 microsatellite loci. Microsatellites were amplified with the Qiagen Multiplex PCR kit following the manufacturer’s instructions. The amount of primer used for each microsatellite was optimized so that each product showed similar amplification (final ratio used: WPG 10_1 : WPG 1_28 : WPG 2_30 : WPG 8_2 : WPG 2_15 : WPG 1_27 = 1.00 : 1.50 : 2.25 : 4.50 : 1.50 : 1.50). Microsatellites were amplified using a G-Storm GS1 thermocycler with the following program: Denature at 95°C for 15 minutes, followed by 30 cycles at 94°C for 2 minutes, 60°C for 1.5 minutes, 72°C for 1 min, followed by a final extension time of 30 minutes at 60°C.

Extension products were resolved on an ABI 3730XL machine performed by Edinburgh Genomics (https://genomics.ed.ac.uk/). Alleles were sized to an internal size standard (GeneScan-500 LIZ; Applied Biosystems) using Peak Scanner v2.0 (Applied Biosystems), and corrected manually where necessary.

**Genotyping failure rate by loci**

1 spermatodose (from a total of 115) and 6 offspring (from a total of 693) were unable to be genotyped at any of our microsatellite markers, and likely represent DNA extraction failures. For the remaining samples 1 was genotyped only at 3 loci, 4 at 4 loci, with the remainder all being genotyped for at least five loci (mean number of loci genotyped per individual = 6.31). The rate of genotyping success was not uniform across loci, with some having a genotype success rate of near 100% whilst
others were below 60% (Table 2). These loci were retained despite their high failure rate as they still provided useful paternity information.

Paternity analysis

Paternity analysis was conducted using R package MasterBayes (version 2.52) in R (R Core Team (2016), version 3.3.0). MasterBayes uses a Bayesian, consistent full-probability model approach that allows paternity information and values of parameters of interest to be estimated simultaneously (Hadfield et al. 2006). The genotypes for the 7 microsatellite loci, along with phenotypic information for relative mating order, and spermatodose size were provided to MasterBayes to assign paternity to each offspring, and estimate the effect of relative mating order and spermatodose size on the probability of siring offspring. MasterBayes was run using default priors for 1,100,000 iterations with a burn-in of 100,000 iterations, and thinning interval of 10. Drop-out and stochastic error rates were fixed at 0.005. Mean values for the parameters of interest (relative mating order and spermatodose size) were estimated from 100,000 MCMC samples from the posterior distribution, which were also used to obtain a 95% credible interval (highest posterior density interval) for these parameters.

To further examine these relationships, we used the offspring for which the posterior probability of the most likely father was > 0.9. From this we calculated the number of offspring each male sired as a proportion of those successfully assigned to any father. In 6 of the females 2 of the spermatodoses in the female’s spermatheca had the same genotype, meaning offspring produced from spermatodoses with this genotype could not be assigned to an individual spermatodose. As a result these spermatodoses were discarded from subsequent analyses. Note that since the number of offspring that were produced from either of these spermatodoses is known, the correct proportion of offspring sired from the other spermatodoses in the spermatheca could be correctly calculated and were thus retained in the GLM analysis (below).
We then calculated paternity skew (sensu Pamillo & Crozier 1996) per female as follows: paternity skew = (Total number of males - 1/ (Σx²)) / (Total number of males - 1), where x is proportion of offspring sired by a male. This measure of paternity skew gives a value between 0 and 1 where 1 indicates a completely unequal paternity share (one father sires all the offspring) and a value of 0 indicates shared paternity (i.e. all fathers sire equal numbers of offspring). We then tested if the observed paternity skew was significantly different than equal paternity (0) using a one-sided, one-sample sign test in R (R Core Team (2016), version 3.3.0). Note, for the calculation of paternity skew, females which had any offspring assigned to a spermatodose with duplicate genotype in the same spermatheca (see above) were discarded.

We then determined which factors influenced the proportion of offspring sired using a quasi-poisson general linear model (GLM) in R (R Core Team (2016), version 3.3.0) with the following terms: number of competing males, spermatodose size, and relative mating order and all their possible interactions. Model simplification was then conducted by dropping the highest least-significant term from the model until a term had a p-value of < 0.05. Following this we then examined quadratic terms for number of competing males, relative mating order, and spermatodose size by adding these factors into the model one-by-one. If the added quadratic term was significant (p < 0.05) it was retained.
Results

Polyandry

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

Paternity analysis

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

Overall we found that paternity was highly skewed away from equal paternity (median paternity skew = 0.92). Paternity skew was significantly higher than the value expected for equal paternity (0) (one-sample sign test p-value = 3.559 $10^{-08}$). This pattern was consistently found regardless of the number of competing males (Fig. 2). The observed value of paternity skew was significantly higher than that expected for equal paternity when the numbers of competing males was 2, 3, or 4 (one-sample sign test p-values = 0.0004, 0.0038, 0.0368 respectively) but not 5 or 6 (one-sample sign test p-values > 0.05) likely due to the small number of females in
these categories. Taken together these results show that paternity share is highly skewed towards a small number of males.

To examine the possible causes of this paternity skew we then used a quasi-Poisson GLM to determine the effect of the number of competing males, spermatodose size, and relative mating order on the proportion of offspring sired. Results are summarised in Table 5. Note fitting interactions between number of competing males, spermatodose size and relative mating order were not significant (p > 0.35) and so these terms were dropped. We also found that quadratic terms for spermatodose size, and number of competing males were not significant (p > 0.25) whereas such a term was significant for relative mating order (Table 5). Both a larger spermatodose size, and being later in the mating order increased the chance of siring offspring (Fig. 3A, 3B, Table 5). The effect of relative mating order followed a quadratic curve, further penalising males early in the mating order. A higher number of competing males reduced the chances of siring offspring (Fig. 3C, Table 5).

When assigning paternity to males we provided MasterBayes with phenotypic information (mating order and spermatodose size). Since MasterBayes simultaneously estimates the pedigree and the population-level parameters there should be no bias from the use of this approach on our subsequent analysis to examine the effects of mating order and spermatodose size on proportion of offspring sired. To demonstrate this we repeated our analysis when paternity was estimated without any phenotypic information (i.e. assigning paternity using only genotypes). This approach produced very similar results to those described above (Table S1, Supporting information).

Overall 44 out of 105 males (spermatodoses) produced 0 offspring. The proportion of males that sired no offspring was higher in earlier mating males (proportion of males siring no offspring when mating males last: 0.294, intermediate: 0.395, and first: 0.576), however these differences were non-significant (logistic regression, p > 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).
Discussion

Here we have examined the influence of spermatodose size and placement on paternity in field-collected samples of *P. griseoaptera*. Paternity share was highly skewed with typically only one or two males siring the majority of a female’s offspring. Both the size and relative order 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. As expected, a higher number of competing males also reduced the chances of siring offspring for each male. While previous studies of orthopteran insects have used microsatellite analysis to estimate the degree of polyandry and paternity skew in field-mated females (Bretman & Tregenza 2005; Hockham et al. 2004; Simmons et al. 2007; Simmons & Beveridge 2010; Turnell & Shaw 2015a, 2015b; Oneal & Knowles 2015), none of these have used the relative position of sperm within the female’s reproductive tract to predict the pattern of sperm use. Even if laboratory based studies and other taxa are included, the number of previous studies that have been able to relate directly the relative position of sperm within the female’s reproductive tract to sperm use by the female are very limited (Droge-Young et al. 2016). Manier et al. (2010, 2013a, 2013b) and Droge-Young et al. (2016), for example, used transgenic lines with fluorescent-tagged sperm heads to resolve mechanisms of competitive fertilisation success in *Drosophila spp* and *Tribolium castaneum*, respectively, in a laboratory setting.

A further novel aspect of the present study was that, in the absence of field observations, we were able to determine for each female the extent of repeated as opposed to multiple mating. Our results indicated that there was a very low frequency of repeated mating with the same male (only 6 out of 36 females contained 2 spermatodoses of the same genotype, and no females contained >2 spermatodoses of the same genotype). Furthermore, there was only one case of a female that appeared to have mated twice with the same male in two successive matings (note that this may be considered a conservative estimate, since it is possible that two males could share the same genotype). This low remating rate
could be a result of the 5-day long sexual refractory period in the female (Supporting information) as a male that mates with a female is likely to have moved on by the time the female is ready to mate again. The low level of repeated mating with the same male could also reflect female choice (Ivy et al. 2005; Weddle et al. 2013). Laboratory mate choice trials in Gryllid crickets, such as *Gryllodes sigillatus*, have demonstrated that females actively avoid copulating with previous mates, presumably in order to obtain any benefits from mating with different males (see Ivy et al. 2005; Weddle et al. 2013).

The relationship between spermatodose position within the spermatheca and paternity in the present study was best explained by a quadratic curve; while spermatodoses furthest away from the opening of the spermatheca were generally less successful in achieving paternity, there were diminishing returns of being positioned closer to the spermathecal opening. This pattern is not entirely consistent with the hypothesis that spermatodoses allow the male to block the exit of rival sperm already present within the spermatheca (Simmons & Siva-Jothy 1998), which would predict paternity to be very strongly skewed in favour of the last male to mate. Sperm from all spermatodoses, even those at the distal end of the spermatheca (i.e. from male that mated first), achieved some paternity.

Because sperm in storage were examined, some mechanisms of sperm precedence can be ruled out, such as the removal or ejection of sperm from previous males (Simmons & Siva-Jothy 1998; Simmons 2001). It is, however, possible that females may have used up a greater proportion of sperm from earlier matings by the time they were collected. Furthermore, in common with virtually all other studies of sperm precedence, the possibility that post-meiotic sperm-ageing might have contributed to the patterns of sperm use observed cannot be ruled out (Pizzari et al. 2008). The likely time that sperm were in storage in proportion to the female’s lifespan was relatively short, however. The median number of matings for females in the present study was 3. Given that females have a sexual refractory period of 5 days (Supporting information), that the majority of mating in this species occurs in August, and that females were collected in early September, a reasonable estimate
of the time that sperm had been in storage in the spermatheca would be in the region of 10-20 days. In contrast, the adult lifespan of the female is likely to be three to four months or more; females can frequently survive and continue to lay eggs into October and November, or even later (Hartley & Warne 1972; Benton 2013).

The only data available on sperm precedence in another tettigoniid species that produces spermatodoses examined patterns of sperm precedence of female Decticus verrucivorus (which is in the same sub-family as P. griseoaptera) that had mated with two different males in a laboratory setting (Wedell 1991). Results were consistent with a “fair raffle” (Parker 1990) and, unlike in the present study, no bias against the use of sperm from the first male to mate was reported. It is possible that depletion or ageing of sperm from the first mating could have been more pronounced in our study in comparison to that of Wedell (1991), which could have contributed to the observed fertilisation bias against earlier spermatodoses. Future work comparing paternity patterns in both the field and lab will help to resolve these issues.

Unexpectedly, approximately one third of the spermatodoses closest to the exit/entrance of the spermatheca sired no offspring. In many insects, mating failures are known to occur (Greenway & Shuker 2015). Such failures are often interpreted as resulting from a failure to transfer sperm to the female’s sperm storage organs, which was clearly not the case here. When dissecting spermatodoses, it was apparent that some still appeared to be full of a large ball of tightly coiled spermatodesmata, while others appeared to be almost empty (Vahed 2003). It is possible that spermatodoses do not begin to release their content immediately, but that there is a delay. Even if discharge from the spermatodoses does begin soon after their transfer, those from the females’ most recent mates would have had less time to discharge their content into the spermatheca, perhaps accounting for the relatively high proportion of offspring sired by sperm from spermatodoses in the second-to-last mating position in these families. The mechanism by which sperm are released from spermatodoses and the rate at which they are discharged is currently unknown (Vahed 2003). A further possible reason why sperm from spermatodoses
closest to the exit of the spermatheca did not always achieve highest paternity
relates to the position of the elongated spermatodose tube (through which sperm
exit the spermatodose). Vahed (2003) observed that in *P. griseoaptera*, in 50% of
cases, the spermatodose tube of the spermatodose nearest to the spermathecal exit
was oriented away from the exit rather than towards it.

In some cricket species, there is compelling evidence that the female can bias the
use of sperm from selected males by controlling not only the duration of attachment
of an externally-attached spermatophore, but also the uptake of sperm to the
spermatheca (Vahed 2015). Whether or not the female can influence the discharge
of sperm from spermatodoses as a further mechanism of cryptic female choice
deserves further investigation. There is also evidence that females might be able
exert control over the differential storage and use of sperm from their mates by
digesting stored sperm. In some bushcrickets, for example, spermolytic activity has
been found within the lumen of the duct of the spermatheca (Viscuso et al. 1996;
Brundo et al. 2011). It has been proposed that the walls of the spermatodoses may
function to protect the male’s sperm from such spermolytic activity within the
spermatheca (Vahed 2003), that is, spermatodoses may be the result of inter-sexual
conflict over the fate of stored sperm, and sperm in older spermatodoses may be
more degraded as well as further away from the spermathecal opening.

We found that sperm from larger spermatodoses had a greater chance of siring
offspring. This is consistent with other sperm competition studies of various taxa,
which have demonstrated that when a female has mated with two different males
the relative number of sperm from each male predicts the paternity of her offspring
(Martin et al. 1974, Simmons 1987; Parker et al. 1990; Wedell 1991; Gage & Morrow
2003; Bretman et al. 2009). Spermatodose size is highly likely to reflect sperm
number: when full, the sperm occur in a tightly-coiled ball which takes up most of
the spherical body of the spermatodose (Vahed 2003). The transfer of larger
volumes of ejaculate does not only benefit the male by increasing his representation
in the female’s sperm stores. Evidence suggests that in many insects, including
bushcrickets, substances in the ejaculate are also transferred that delay the female
from re-mating in a dose-dependent manner (Gillott 2003). This effect might also be triggered by an increase in the physical ‘fullness’ of the spermatheca. In *P. griseoaptera*, Jarčuška & Kaňuch (2014) found that the mean size of spermatodoses within the spermatheca predicts the number of spermatodoses received over the female’s lifetime, suggesting that females that had received a larger ejaculate subsequently mated with fewer males. We were unable to confirm this relationship using our data set, although it should be noted that the sample size of females was smaller than in Jarčuška & Kaňuch’s (2014) study. The benefit to a male of delaying or deterring his mate from remating was demonstrated in the present study: we found that the proportion of offspring sired by each male declined with the number of competing males. Simmons & Beveridge (2010) found a similar pattern in the field cricket *Teleogryllus oceanicus* that had mated in the field.

It is possible that the influence of spermatodose order on paternity varies with differences in polyandry. In *P. griseoaptera*, we found that females contained up to 6 spermatodoses (median = 3), however the number of spermatodoses per female (i.e. the degree of polyandry) is considerably greater than this in some bushcrickets (Vahed 2006). In *Platycleis affinis*, for example, females contained up to 23 spermatodoses, while in *Anonconotus* spp, females contain up to 44 (Vahed 2006). Examining the influence of spermatodose order on paternity in such highly polyandrous species would be challenging but potentially useful. In addition, the lifetime degree of polyandry is known to vary between populations (e.g. clinal variation in remating rate is seen in *Drosophila pseudoobscura* (Price et al. 2008) and *Metrioptera roeselii* (Kaňuch et al. 2013)). The techniques used here could be used to compare how mating order affects sperm precedence between different populations, which could provide a novel means of testing models of ejaculate allocation (e.g. Parker 1990, 1998).

By using a species in which sperm from different matings occur within discreet aggregations (spermatodoses), we were able to examine the effects of the order of sperm deposition from different males within the female’s sperm storage organ and of ejaculate size, on male fertilisation success in females that had mated under
natural field conditions. The approach used here is likely to be generalizable to other taxa in which sperm form discrete aggregations, but perhaps also to taxa for which the stratification of sperm due to mating order may be more cryptic. Future work to examine the influence of sperm aggregation on paternity are needed to examine this, in particular from species in which sperm aggregations are less discreet (for examples, see Mann 1984, Higginson & Pitnick 2011, Fisher et al. 2014).

Acknowledgments

We thank Jarrod Hadfield for assistance using MasterBayes, Sonia Pascoal and Tanya Sneddon for help with multiplexing PCRs, and Jeff Graves for advice. We are grateful to Paul Eady and three anonymous referees for helpful comments on the manuscript. This work was funded by the University of Derby (KV), NERC (DJP), and Erasmus and Erasmus Plus (JZ).
References


Data Accessibility

All data is available in Dryad:
http://datadryad.org/review?doi=doi:10.5061/dryad.6t3dn
### Table 1. Properties of the six microsatellite markers used in the paternity analysis (For primer sequences, see Arens et al. (2005))

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

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

### Table 2. Percentage genotyping success for the microsatellite loci used in the paternity analysis

<table>
<thead>
<tr>
<th>Microsatellite</th>
<th>Samples genotyped (N)</th>
<th>Samples genotyped (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WPG10-1</td>
<td>837</td>
<td>100.0</td>
</tr>
<tr>
<td>WPG1-28</td>
<td>835</td>
<td>99.8</td>
</tr>
<tr>
<td>WPG2-30</td>
<td>834</td>
<td>99.6</td>
</tr>
<tr>
<td>WPG8-2</td>
<td>488</td>
<td>58.6</td>
</tr>
<tr>
<td>WPG2-15</td>
<td>836</td>
<td>99.9</td>
</tr>
<tr>
<td>WPG1-27 (a)</td>
<td>647</td>
<td>77.5</td>
</tr>
<tr>
<td>WPG1-27 (b)</td>
<td>808</td>
<td>96.6</td>
</tr>
</tbody>
</table>

### Table 3. Number of spermatodoses present in females

<table>
<thead>
<tr>
<th>Number of spermatodoses</th>
<th>Number of females</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
</tr>
</tbody>
</table>
Table 4. Parameter estimates from MasterBayes using a 100,000 MCMC samples from the posterior distribution, showing the effect of relative spermatodose order with the spermatheca and spermatodose diameter on the likelihood of siring offspring (HPD = Highest Posterior Density).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Posterior mean (95% HPD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative mating order</td>
<td>0.793 (0.544-1.042)</td>
</tr>
<tr>
<td>Spermatodose diameter</td>
<td>8.164 (6.910-9.417)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Coefficients</th>
<th>Estimate</th>
<th>t value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative order</td>
<td>3.65</td>
<td>3.09</td>
<td>0.0026</td>
</tr>
<tr>
<td>(Relative order)$^2$</td>
<td>-2.70</td>
<td>-2.52</td>
<td>0.0132</td>
</tr>
<tr>
<td>Number of competing males</td>
<td>-0.39</td>
<td>-3.51</td>
<td>0.0007</td>
</tr>
<tr>
<td>Spermatodose diameter</td>
<td>2.75</td>
<td>4.01</td>
<td>0.0001</td>
</tr>
</tbody>
</table>
**Figure Legends:**

Fig. 1. (A) Photograph of dissected spermatodoses from *P. griseoaptera* (mean diameter = 0.90 mm). (B) Schematic diagram of a longitudinal section through the spermatheca in *P. griseoaptera*, showing how the relative position of each spermatodose within the spermatheca was scored.

Fig. 2. Paternity skew for different numbers of competing males. A value of 1 indicates all a female’s offspring are sired by one male whereas a value of 0 indicates all males sire the same number of a female’s offspring.

Fig. 3. The relationships between the proportion of offspring sired by a given male and: (A) the position of the male’s spermatodose within the spermatheca (0 = furthest from the single exit/entrance, 1 = closest to the entrance/exit); (B) the diameter (in mm) of the male’s spermatodose and (C) the number of competing males (see also Table 5). Note points were jittered along the X-axis to aid visualisation of overlapping points.
Mating order

<table>
<thead>
<tr>
<th>Mating order</th>
<th>Relative position</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>0</td>
</tr>
<tr>
<td>2nd</td>
<td>0.25</td>
</tr>
<tr>
<td>3rd</td>
<td>0.5</td>
</tr>
<tr>
<td>4th</td>
<td>0.75</td>
</tr>
<tr>
<td>5th</td>
<td>1</td>
</tr>
</tbody>
</table>

Spermethecal opening
Reproductive skew vs. 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.
Three of the males that were used to determine the female's sexual refractory period were each moved to a cage containing a different female within an hour after the end of copulation. Each cage was observed at regular intervals as described above. If mating did not occur, the female was removed at 21.00h and a new female was placed in the male’s cage the following morning at 9.00h. The procedure was repeated until mating occurred.

**Results**

The mean (± SE) sexual refractory period for the females was 117.57 ± 15.62 hours (n = 7), while that for the males was 27.67 ± 6.94 hours (n = 3). Females took 248.8 ± 21.4 min (n = 4) to consume the spermatophylax fully, after which they removed and consumed the ampulla of the spermatophore.

**Table S1. Parameter estimates from the best-fitting quasi-Poisson GLM, showing the effects of relative spermatodose order within the spermatheca, number of competing males, and spermatodose diameter on paternity when paternity was assigned independently of any phenotypic information (see**

<table>
<thead>
<tr>
<th>Coefficients</th>
<th>Estimate</th>
<th>t value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative order</td>
<td>4.08</td>
<td>3.40</td>
<td>0.0010</td>
</tr>
<tr>
<td>(Relative order)$^2$</td>
<td>-3.09</td>
<td>-2.86</td>
<td>0.0052</td>
</tr>
<tr>
<td>Number of competing males</td>
<td>-0.40</td>
<td>-3.50</td>
<td>0.0007</td>
</tr>
<tr>
<td>Spermatodose diameter</td>
<td>2.59</td>
<td>3.73</td>
<td>0.0003</td>
</tr>
</tbody>
</table>