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TECHNICAL ADVANCES

The assessment of insemination success in yellow dung flies using competitive PCR

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Abstract

In spite of considerable interest in postcopulatory sexual selection, separating the effects of sperm competition from cryptic female choice remains difficult because mechanisms underlying postcopulatory processes are poorly understood. One methodological challenge is to quantify insemination success for individual males within the sperm stores of multiply mated females to discover how insemination translates into eventual paternity. Any proposed method must be applicable in organisms without extensive DNA sequence information (which include the majority of model species for sexual selection). Here, we describe the development and application of microsatellite competitive-multiplex-PCR for quantifying relative contributions to a small number of sperm in storage. We studied how DNA template characteristics affect PCR amplification of known concentrations of mixed DNA and generated regressions for correcting observations of allelic signal strength based on such characteristics. We used these methods to examine patterns of sperm storage in twice-mated female yellow dung flies, *Scathophaga stercoraria*. We confirm previous findings supporting sperm displacement and demonstrate that average paternity for the last mate accords with the mean proportion of sperm stored. We further find consistent skew in storage across spermathecae, with more last male sperm stored in the singlet spermatheca on one side of the body than in the doublet on the opposite side. We also show that the time between copulations may be important for effectively sorting sperm. Finally, we demonstrate that male size may influence the opportunity for sperm choice, suggesting future work to disentangle the roles of male competition and cryptic female choice.

Keywords: cryptic choice, DNA quantification, mate choice, paternity, sperm competition, sperm selection

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Introduction

Postcopulatory sexual selection remains a controversial subject partly because in contrast to precopulatory male competition and female choice, most postcopulatory selection is hidden from view within female reproductive tracts. As a result, the various influences of females and their multiple mates on postcopulatory sexual selection are difficult to disentangle, which makes separating the causes and consequences of male competition and female choice challenging (Birkhead 1998, 2000; Eberhard 2000; Pitnick & Brown 2000; Simmons 2001). This is especially true for the phenomenon of sperm selection, a contentious form of postcopulatory mate choice in which females use the sperm of certain males when fertilizing their eggs (Simmons & Siva-Jothy 1998; Simmons 2001). In many systems in which females store sperm from several males, convincing demonstrations of adaptive sperm selection could lead to evidence for sexual selection based on indirect benefits (e.g., good-genes mate choice), if for example the choice occurs in the absence of apparent direct natural selection on females (Brown *et al.* 1997; Bussière 2002). In addition, the accumulating evidence for sexual selection via sexual conflict makes inferring

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the selective basis for biases in sperm storage and use even more difficult (Arnqvist & Rowe 2005; Bussière *et al.* 2006), and there is widespread consensus that we need more information on the mechanisms through which biases are achieved before any conclusions can be drawn on the prevalence and importance of postcopulatory female choice in driving patterns of sexual selection (Birkhead & Moller 1998; Ward 2007).

Recent advances in using molecular markers to assign paternity have made studying the realized effects of postcopulatory processes relatively straightforward (Simmons 2001). However, linking paternity to the physiological mechanisms that affect it and the ultimate evolutionary causes of these mechanisms remains an elusive goal. Previously, some researchers have employed innovative techniques to track the contributions of different males to sperm in storage, including the use of transgenic lines of animals expressing green fluorescent protein (GFP, Civetta 1999), immunocytochemical approaches (Schärer *et al.* 2007), the radiolabelling of ejaculates (Simmons *et al.* 1999) or the use of phenotypic markers (Otronen *et al.* 1997; Hellriegel & Bernasconi 2000). Unfortunately, phenotypic markers such as sperm length are practically inferior to genetic markers such as microsatellites, as completely unambiguous assignment often turns out to be impossible. For example, in their study of sperm storage in yellow dung flies, Hellriegel & Bernasconi (2000) were only able to assign 79% of measured sperm to either of the two rival males based on length. In addition, it is often difficult to rule out the potentially confounding influence of most labelling techniques on sperm movement. However, the same molecular methods that currently allow the assignment of paternity can also be used to advance our knowledge of events within female sperm stores. Techniques based on allelic diversity have already been used to estimate the number of males contributing to mixed sperm stores in internally fertilizing species (Bretman & Tregenza 2005; Simmons *et al.* 2007) and have been used to quantify the relative sperm contributions in an externally fertilizing fish where sperm can be trapped on nylon membranes in the water column (Wooninck *et al.* 2000). Quantifying the relative sperm contributions of competing males with internal fertilization (in contrast to merely checking for their presence or absence as in allele counting) would be a further improvement in documenting the prevalence and importance of postcopulatory sexual selection.

Forensic scientists have been at the forefront of developing models for predicting the genotypes contributing to mixed DNA samples (Gill *et al.* 1998; Cowell *et al.* 2007). In the current paper, we describe a more straightforward exercise: to obtain information on the relative contributions of sires to female sperm stores when the genotypes of all adults are known. We used standard methods for genotyping microsatellites found in the mixed sperm of multiple sperm storage organs of female dung flies (see below). We used signal strength as an index of original DNA concentrations to study the effects of male and female phenotypes (e.g., body size) on sperm storage patterns within these organs. We corrected our estimates of signal strength for the effects of other variables thought to influence PCR amplification, namely the number and mean length of alleles in a run, the length of the focal allele, the number of alleles shorter than the focal allele and the number of alleles represented by a single peak (i.e., whether the focal individual is homozygous at that locus). Such techniques are readily transferable to other laboratories and study organisms for the purpose of studying postcopulatory sexual selection or other applications requiring the quantification of DNA within a mixed sample.

Sexual selection in yellow dung flies

Yellow dung flies, *Scathophaga stercoraria* (L.), are a model system for studying sexual selection. Males aggregate on and around dung pats to which gravid females fly in order to lay their eggs (Parker 1970b). Although male interactions seem to drive precopulatory sexual selection (Parker 1970a,b), females appear to retain significant control over insemination (Hosken *et al.* 2001); thanks partly to their elaborate reproductive morphology (Hosken *et al.* 1999; Arthur *et al.* 2008). As in many of the Diptera, female yellow dung flies have multiple spermathecae (sperm storage organs) into which males cannot directly insert sperm (Hosken 1999; Hosken *et al.* 1999; Simmons *et al.* 1999; Hosken & Ward 2000). Instead, males deposit sperm at the entrance of the three spermathecal ducts (Hosken *et al.* 1999), arranged into a solitary singlet spermatheca on one side of the body, and a doublet pair on the opposite side. The singlet and doublet spermathecae appear to have independent musculature in live preparations (our own unpublished observations), and thus could potentially assist in sorting sperm for subsequent sperm choice during oviposition (Hellriegel & Bernasconi 2000).

A bias in sperm storage has been demonstrated for *Dryomyza* flies (Otronen 1997), although demonstrating a similar pattern in *S. stercoraria* has been a challenge (Otronen *et al.* 1997). Part of the reason for this is undoubtedly that males exert a strong influence on the outcome of insemination, and there is strong selection on both copula duration, which covaries with insemination success (Parker 1970a; Simmons & Parker 1992; Parker & Simmons 1994), and on the displacement of rival sperm (Parker & Simmons 1991; Simmons & Parker 1992; Simmons *et al.* 1999). Furthermore, the phenotype of sperm themselves seems to exert an influence on storage patterns in a complex way (Otronen *et al.* 1997). Nevertheless there is

substantial variation in how male traits influence sperm competition success (Simmons & Siva-Jothy 1998). This variation could be partly due to a female influence on paternity, presumably to favour the ejaculates of some males over others (Ward 1998, 2000), although this premise remains controversial (Simmons *et al.* 1996; Simmons 2001). Compelling evidence that postcopulatory female choice occurs comes from research showing that female experience of environmental conditions influences the siring success of mates in a way that is consistent with adaptive sperm selection (Ward 2000). Even in this well-studied system, however, exactly how sperm sorting and selection occurs is not yet clear, and the events occurring between insemination and fertilization are most often deduced from patterns of paternity instead of direct observations of sperm movement and storage. As a consequence, the tremendous theoretical work on postcopulatory sexual selection in this species (e.g., Parker 1974, 1992; Hellriegel & Ward 1998) cannot yet be thoroughly evaluated because we lack strong evidence of the mechanisms of sperm storage and use within the female reproductive tract. One of the greatest impediments to understand cryptic female choice in this and other systems relates to the challenge in identifying paternal contributions to sperm stores and the bias in sperm storage across spermathecae.

In yellow dung flies, average P2 (the proportion of paternity assigned to the second of two copulating males) is typically reported to be near 0.8 although there is considerable variation among individuals and across studies (Parker 1970a; Simmons & Parker 1992; Otronen *et al.* 1997; Simmons & Siva-Jothy 1998; Simmons *et al.* 1999; Hellriegel & Bernasconi 2000; Ward 2007). In the focal population, average P2 is reported to be slightly lower than 0.8 (i.e., 0.74 ± 0.09 in Ward 2000), although typically within the margin of error. In addition to developing our methods for amplifying and quantifying paternal contributions to sperm stores, we sought to determine whether this typical P2 value accords with average S2 (the proportion of stored sperm assigned to the second of two copulating males) across spermathecae, i.e., is paternity broadly consistent with a fair raffle among stored sperm? We further examined which male or female phenotypic characters helped to explain any variation in S2 that could account for large variance in observed P2 in this species. In addition, we tested for general patterns in sperm storage bias consistent with either the singlet or doublet being the preferred site of storage for males having a particular phenotype. Finally, we experimentally manipulated female storage time between successive matings, a factor that has been previously shown to influence the outcome of postcopulatory sexual selection (Hellriegel & Bernasconi 2000), and observed its effects on biases in sperm storage across spermathecae.

Materials and methods

Animal husbandry and laboratory matings

All the flies involved in this series of investigations were F3 or F4 descendants of adults collected in the field from dung pats in Fehraltorf, Switzerland and reared using a standard laboratory protocol for dung flies (Ward 1993; Blanckenhorn *et al.* 2009). We used adults who had matured for a minimum of 10 days posteclosion in all mating experiments.

We transferred single males from their housing containers to clean vials (28.5 mm diameter × 95 mm tall) and subsequently introduced a single virgin female selected haphazardly, observing the pair to ensure that copulation occurred and noting its duration. We then introduced each mated female to a second virgin male either 1 or 24 h after the completion of the initial copulation, once again noting its duration. We did not provide dung in either mating arena because we wanted to study sperm storage patterns without the complications introduced by differential sperm usage during oviposition. We allowed all females to hold sperm in storage for a full 24 h after the second copulation before freezing them at −80 °C. Although mating on dung pats in the wild is typically followed quickly by oviposition, mating also occurs away from the dung (Parker 1971; Parker *et al.* 1993), and sperm are retained in storage between oviposition bouts. As a consequence, our sperm storage time and the absence of dung during mating reflect the natural situation in at least a subset of wild females. Mating partners were chosen at random without prior screening of microsatellite genotypes.

Dissections

We isolated stored ejaculates from previously frozen females that had been dehydrated in ethanol for a minimum of 24 h before dissection (Tripet *et al.* 2001). We carefully removed the posterior portion of the female reproductive tract (including the common oviduct, spermathecae, accessory glands and copulatory bursa) from the rest of the female by grasping the genital valves in forceps and tearing them from the abdomen. We separated dehydrated, and thus solidified, sperm 'pellets' from female spermathecal tissue using *very* finely sharpened dissecting tweezers viewed under a quality binocular microscope (Leica MZ-12, Leica Microsystems GmbH, Wetzlar, Germany). We took great care to extract the entire ejaculate, and although some sperm may have been missed, this quantity relative to the extracted sperm mass is likely to be trivial. Each sperm pellet was transferred separately to a buffer solution (ATL buffer from the QIAamp® DNA Micro Kit, Qiagen; see below). The three sperm pellets from each female, which each

originated from a different spermatheca, were amplified and analysed separately to study the skew in sperm storage across spermathecae. In our analyses, we distinguish the singlet spermatheca (regardless of the side of the body on which it is found) from the middle and outer doublet spermathecae (Hosken *et al.* 1999). We also measured hind tibia length of all animals as an index of body size.

Extraction, amplification and analysis of DNA

We used DNeasy® Tissue Kits (Qiagen AG, Switzerland) to extract DNA from the heads of all flies. We used a special kit designed for use with forensic amounts of DNA sample (QIAamp® DNA Micro Kit, Qiagen AG, Switzerland) to extract the potentially very low number of DNA copies from sperm pellets. We followed the recommended protocols, including adding carrier RNA to buffer AL (1 μL dissolved carrier RNA in 200 μL buffer AL), and the minimum recommended amount of elution buffer AE (20 μL) when extracting DNA from sperm pellets to retain the highest possible concentration of DNA. We then used the QIAGEN® Multiplex PCR Kit to simultaneously amplify four microsatellite loci: SsCa17, SsCa24, SsCa26 (Garner *et al.* 2000) and SsCa30 (Demont *et al.* 2008). Total PCR reaction volume for the heads was 6 μL: 1μL DNA template, 3 μL QIAGEN Multiplex PCR Master Mix, 1.4 μL distilled water and 0.6 μL microsatellite primer mix (100 μM). Total PCR reaction volume for the sperm was 24 μL retaining the mixing ratio from the heads (e.g., DNA template and all other volumes four times higher than for the heads). Cycling conditions for the heads were as follows: 95 °C for 15 min, then 27 cycles of 94 °C for 30 s, 60 °C for 3 min and 72 °C for 45 s and finally 60 °C for 30 min. Cycling conditions for the sperm DNA were the same with one modification: 30 cycles instead of 27 to allow for the lower initial template concentration. It should be noted that using these conditions large stutter bands are usually not produced. Of these four loci, one (SsCa17) was not sufficiently polymorphic in our samples to correct adequately using the procedure described below. So for the remainder of the paper we shall focus on the other three loci. PCR products were separated on a capillary sequencer (Applied Biosystems 3730 DNA Analyzer), and the output was analysed using Applied Biosystems GeneMapper® software. Each PCR amplification from template DNA was performed in triplicate to check the repeatability of our observations.

Data collection

The response variable for most of the results discussed below is the relative signal intensity of a male's alleles in an amplified subsample of the DNA extracted from the sperm pellet. Once the adult genotypes were known, we were able to select informative alleles that could provide information on the ratio of DNA concentrations belonging to each of two putative sires. We counted as informative only alleles unique to one of the males (i.e., an informative allele could be shared by neither the rival male nor the female, even though in many cases we could find very little evidence of female DNA at other loci because our dehydration and dissection successfully separated the sperm pellet from female tissue, see results below). We also avoided using data from the SsCa30 locus in which either male had only a single allele, because previous work using this locus for paternity and population genetics analyses revealed the presence of null alleles at this locus (Hosken *et al.* 2001; Demont *et al.* 2008). As this locus is highly variable, we discarded data from only seven pairs of males at this locus; in all other cases, both males possessed two visible alleles and their contributions to mixed sperm could not have been underestimated as a result of null alleles. Signal strength was assessed as the area of an individual peak rather than peak height because for very intense peaks we often observed a greater peak width (see Fig. 1).

Correcting estimates of signal strength

Many factors besides the initial concentrations of alleles (e.g., allele length) may contribute to the observed signal strength of a particular allele after PCR (see e.g., Suzuki & Giovanni 1996; Haberl & Tautz 1999; Lion 2003). We corrected measures of relative peak intensity for each of our microsatellite loci using linear mixed models of signal strength on several allele characteristics that we reasoned might influence signal strength. These models were computed using controlled mixtures of DNA from genotyped adults. We used 61 adult samples to study the effects of allele characteristics on genotyping signal strengths. These samples were chosen to cover the range of allele sizes and combinations found in the main study. For each sample, we estimated DNA concentration in the extraction twice for each of two independently drawn samples using an Eppendorf BioPhotometer (Eppendorf AG). Repeated measurements of the same subsample were highly consistent, as evidenced by a very strong correlation in estimated concentrations ($r = 0.998$, $n = 122$), and the correlation across independently drawn samples from the same individual was only slightly lower ($r = 0.991$, $n = 61$). Of the 61 samples measured, nine had concentrations lower than 30 μg/mL and were discarded. We diluted the remaining 52 samples to a standard concentration of 30 μg/mL dsDNA. Subsequently, we haphazardly selected 96 pairings of two of these individuals that would provide information on a minimum of two loci. We mixed together the DNA in seven ratios: 0.0625, 0.125, 0.25, 0.5, 0.75, 0.875 and 0.9375. (Our protocol for diluting the

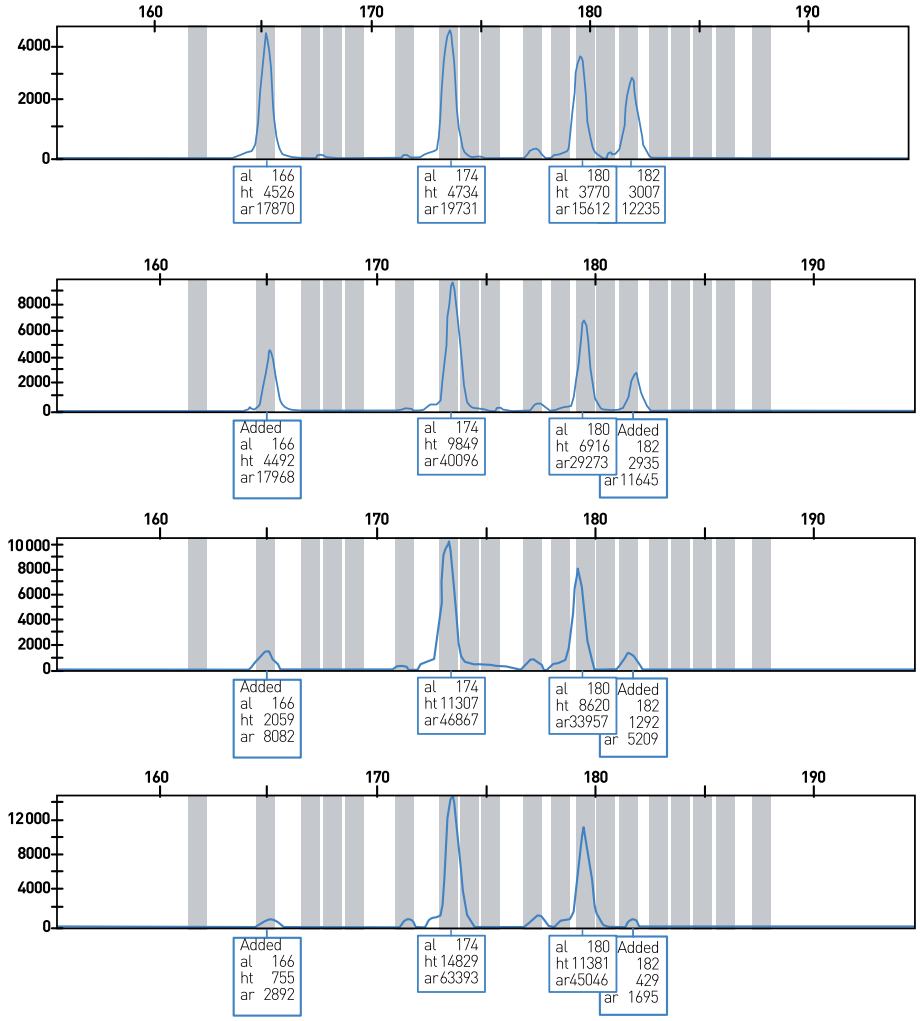

Fig. 1 Electropherogram from ABI GeneMapper software illustrating how we assessed peak intensity in four different PCR reactions. DNA from two heterozygous genotypes (one having alleles 174 and 180 for locus SsCa30 and the other having alleles 166 and 182) were mixed together in four different ratios, shown from top to bottom of the figure as follows: 1:1 (mimicking an S2 for the pair above of 0.5), 3:1 (S2 = 0.25), 7:1 (S2 = 0.125) and 15:1 (S2 = 0.0625). The peaks under the line illustrate signal strength for each of the alleles (possible alleles for this locus are shaded in light grey), and the signal strength can be quantified using peak height or area (denoted by ht and ar, respectively, in the box below each peak).

concentration for this series of mixtures produced two independent mixtures at a ratio of 0.5 for each block of eight mixtures.) This range was chosen to reflect the possible variation in sperm ratios within female spermathecae. We then observed how signal strength for different alleles varied according to initial concentration and several specific properties of the particular PCR and sequencing run. Based on discussions with colleagues and our observations of the behaviour of signal strength in heterozygotes, we included the following allele characters in our linear models: initial relative allelic concentration (from the controlled mixtures), number of alleles being amplified in the reaction, number of shorter alleles (relative to the focal allele) amplified in the reaction, mean length (in bases) of alleles in the reaction, relative length of the focal allele (focal allele length - mean allele length) and whether the focal allele was homozygous in the focal individual or not. The resulting equations could then be solved for initial allelic concentration, and the rearranged equations used to correct observed values of signal strength for experimental runs in which the starting DNA concentration was unknown.

Statistical analyses

We estimated the effect of allele characteristics on peak signal strength with mixed models (using the lme function in the nlme package for R, Pinheiro *et al.*, 2008) of

arcsine square-root transformed proportions of informative allele signal observed. The fixed effects included first and higher order terms for the initial DNA concentrations (based on the mixture in question) and the allele characteristics described above, while the random effects were the PCR run nested within the particular pairing of individual samples. We compared higher order models (including quadratic and cubic terms) that would allow for nonlinear changes in the observed signal strengths as the ratios of alleles contributing to the PCR run changed with first order models (allowing only linear trends) by computing the Bayesian Information Criterion (BIC). BIC is a penalized log-likelihood measure that quantifies goodness-of-fit for a model but trades-off model fit with the number of parameters included, and tends to favour simpler models than rival methods such as the Akaike Information Criterion (Burnham & Anderson 2004). This was ideal for our purposes as we sought a simple model that predicted the effects of allele properties on signal strength, but in which the explanatory variable 'initial DNA concentration' could be easily isolated from other terms to convert observations of relative signal strength in experimental PCR runs to an estimate of the relative starting concentration of alleles.

After correcting signal strengths as described above, we adjusted estimates of DNA ratios if one of the males had more informative copies of DNA than the other (e.g., if the first male had two informative alleles but the rival had only one, we halved the corrected signal strength of the first male's allele before calculating the ratio to get a fair estimation of each male's contribution to the ejaculate stores). These data represented our indices of S2 (the fraction of sperm stored within a spermatheca that belongs to the second of two males mated to a female, which is analogous to P2 values in paternity studies). In a few cases, the corrections produced estimates of S2 that were slightly higher than 1; these estimates were adjusted to a value of 1 (i.e., complete second male priority) before proceeding. We then transformed all DNA ratio data using arcsine square-root transformations. None of these transformed S2 distributions (of data or residuals) showed any evidence of significant deviations from normality after transformation (all Kolmogorov–Smirnov tests: $P > 0.10$). As we had up to three estimates of relative concentration from each spermatheca (from the three independently evaluated microsatellite loci), we were able to assess the repeatability of our estimates before and after correction using ANOVA (Becker 1992). The transformed S2 ratio from all informative loci for each spermatheca was used in our subsequent assessment of factors affecting skew in sperm storage.

We used one-sample *t*-tests on our experiment-wide transformed S2 values against the expected values of 0.8 and 0.5 (following arcsine square-root transformation), from previous findings of paternity in this species and models of sperm storage featuring no displacement respectively. We then built linear mixed effects models as above to study the within female skew in storage across spermathecae and the between-female effects of treatment (time interval between matings) on overall S2. The fixed effects included the time interval between mating (1 or 24 h), the spermathecal identity, the hind tibia lengths of the female and both males and the duration of both copulations, while the random effects were the locus for which the data were collected nested within spermatheca nested within female. To further study what influenced biases in sperm storage across the singlet and doublet (which arguably represents the opportunity for sperm selection), for each female, we then calculated the difference between the singlet S2 value and the mean of the doublet S2 values. We then modelled the effects of behavioural and morphological attributes of the females and their mates on this index of bias. In both cases, the significance of individual terms was not sensitive to the structure of the model, and consequently we present the full model including nonsignificant terms in our results. Statistical analyses were conducted using SPSS (Anonymous, 2005) and R (R Development Core Team, 2008).

Results

Correcting PCR runs

We conducted 768 PCRs of mixed DNA (96 pairs × 8 concentration mixes), but because not all pairings had informative alleles for all loci, the number of informative runs and peaks for each set of regressions differs. The observed ratios of alleles consistently overestimated the fraction of DNA represented by alleles present in low concentrations and conversely underestimated alleles present at high concentrations, although in general the uncorrected estimates were reasonably close to predicted values (see Table S1). We therefore used a series of linear mixed models to improve the correspondence between predicted and observed ratios of allele signal strengths. We summarize the comparisons between higher order and first order models in supplementary Table S2. For all three loci, the linear first order model had the highest BIC weight.

We summarize the parameter estimates for these linear first order models in Table 1. For all three loci, the number of alleles shorter than the focal allele and the heterozygosity of the focal allele were associated with relatively high coefficients, indicating an influence on the predicted signal strength. Heterozygous alleles had higher observed signal strength than half the predicted value of a single homozygous allele, while a greater

Table 1 Summaries for first order linear regressions of observed signal strength on various allele properties for three microsatellite loci (Demont *et al*. 2008; Garner *et al*. 2000) in yellow dung flies

			Regression parameter estimates for original equations					
Locus	No. informative mixtures (/96)	No. informative PCR runs	Intercept	Transformed initial DNA concentration	No. shorter alleles in run	Relative allele length	Focal allele heterozygosity	Total no. alleles visible in run
SsCa24	79	594	0.066	0.891	−0.075	0.005	0.015	0.017
SsCa26	65	487	0.050	0.909	−0.033	−0.001	0.040	−0.008
SsCa30	86	642	0.019	0.939	−0.032	−0.002	0.033	0.000

The three original equations for correcting observed proportion signal strength were based on the original parameters, but the rearranged equations were used to calculate the initial DNA concentration (thus the coefficients in the equation are the inverse of the parameter estimates listed above). For example, the rearranged equation for SsCa24 is as follows: transformed initial [DNA] = −0.074 + 1.122*observed proportion signal + 0.084*no. shorter alleles – 0.006*relative allele length – 0.016 * heterozygosity s.019*no. alleles in run.

number of shorter alleles dampened the observed signal strength. The total number of competing alleles typically had a small coefficient, but for SsCa24 large numbers of alleles seemed to slightly increase predicted signal strength. Relative allele length was associated with only small parameter estimates for all three loci.

Repeatability of competitive PCR

In the main study, we attempted to mate 60 females to two males each (30 for each sperm storage time treatment). Of these 60, two females failed to mate with one of their assigned mates and an additional four females had four spermathecae (as is found in a small fraction of wild-type flies, see Ward 2000). All were removed from the analysis to simplify its interpretation. A further 10 females were removed from the analysis because one of the three specimens in a mating triad were lost before DNA extraction ($n = 2$) or because of failures in removing all three spermathecae without damaging them ($n = 8$). The remaining 44 females were evenly split amongst the two sperm storage time treatments.

Even among the subset of preparations for which our dissection notes did not indicate any obvious contamination by female tissue ($n = 38$), there was sometimes evidence of female DNA in the fragment analysis runs (in 15/38 cases, female contributions to peak areas exceeded 10% of the total signal area for a genotyping run). Almost all of these samples were conducted early in the sequence of dissections, and our ability to remove contaminating female tissue improved over time (in the last samples we find nearly no contaminating female tissue at all). In any case, the magnitude of female contamination was usually rather low (mean 12.0 ± 2.4% of signal in a PCR run).

The relative signal strengths of alleles from the same locus across replicate PCR and genotyping runs were highly repeatable (repeatability = 0.91). This confirms that for a given mixed sample of DNA, our PCR conditions were sufficiently consistent to provide a reliable estimate of the original DNA concentration. However, the uncorrected estimates obtained across different loci within the same spermatheca were less consistent (repeatability = 0.71), indicating that the particular characteristics of a competitive PCR run play a substantial role in determining the correspondence of signal strength to starting DNA concentrations. By correcting our estimates of signal strength using equations derived from the parameter estimates described in Table 1, we were able to increase the repeatability across loci to 0.77. Results from tests based on a single locus were qualitatively the same as those for multiple loci. The loss of power was attributable to the fewer degrees of freedom afforded analyses of individual loci compared with the model in which locus was nested within spermatheca. All of the results reported below are therefore those for the corrected dataset using all three loci.

Sperm storage patterns in doubly mated yellow dung flies

One-sample *t*-tests revealed that our experiment-wide findings of S2 averaged across spermathecae were significantly different from 0.5 as would be expected in a situation without sperm displacement ($t = 5.883$, 43 df, $P < 0.001$; see Fig. 2 for mean S2 values across treatments). By contrast, these same mean values were not significantly different from 0.8 ($t = -0.837$, 43 df, $P = 0.407$), supporting previous reports that sperm displacement occurs during copulation, and that on average for this species, paternity is assigned in proportion to the relative number of sperm in storage. However, there was considerable variation in both the overall level of sperm storage priority accorded for second males, and

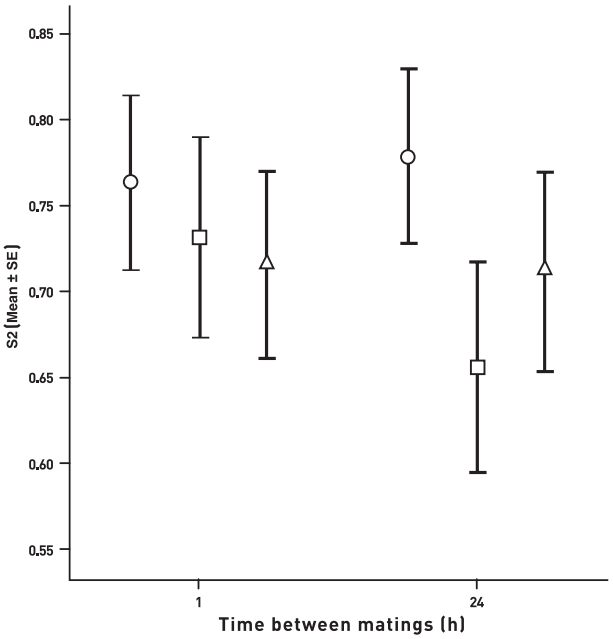

Fig. 2 The mean (± SE) proportion of second male sperm in storage in each of the three spermathecae (circles: singlet spermatheca; squares: middle doublet spermatheca; triangles: outer doublet spermatheca) as a function of the number of hours elapsed between matings.

also substantial variation in the patterns of storage across the spermathecae.

To explore this variation, we modelled changes in S2 across spermathecae as summarized in Table 2. Several sources of variance significantly explained variation in S2 across spermathecae and across females. The significant effect of spermatheca indicates a consistently lower S2 estimate for the doublet spermathecae compared with the singlet (see Fig. 2). The duration of the second male's copula (in seconds) has a significant positive effect on S2 across spermathecae, ($\beta = 0.0001052 \pm$ SE 0.0000363, $P = 0.0063$). As in a previous analysis of paternity (Parker & Simmons 1991), the copula duration of the first male did not contribute significantly, nor did the size of the female or of either male. We detected a significant interaction between spermathecal identity and the time interval between matings. This interaction indicates a significant effect of sperm storage time on the skew in S2 across spermathecae: when females are given 24 h between matings, the difference in S2 value between the singlet and middle doublet is more pronounced than it is when the second male mates only 1 h following the first (see Fig. 2).

This mixed effects model examined systematic skew in sperm storage patterns across all three spermathecae. However, we were also interested in exploring sperm storage skew across the singlet and doublet specifically, as this might represent the opportunity for choice given independent female control over the singlet vs. the doublet spermathecae. We computed the difference between the singlet and the mean S2 for the doublet and used a univariate analysis to explore covariance between this difference and the time between matings as well as behavioural or morphological covariates (see Table 3). The only significant contributor to changes in the skew across the singlet and doublet spermathecae was the time interval between matings, with greater skew occurring when copulations were separated by 24 h than when they were only 1 h apart (1 h skew: 0.043 ± 0.033, 24 skew: 0.132 ± SE 0.024). The hind tibia length of the second male exerted a marginally nonsignificant effect, in which larger second males tended to promote greater skew in sperm storage ($\beta = 0.104 \pm$ SE 0.050).

Discussion

Sperm storage in yellow dung flies

In the present study, we successfully employed competitive PCR to study biases in sperm storage across the sperm storage organs of female yellow dung flies. As a

Table 2 Summary of the linear mixed model for transformed proportion of second male sperm in storage as a function of the time interval between matings, the spermatheca and behavioural and morphological covariates

Source	Numerator (df)	Denominator (df)	*F* value	*P* value
Time interval between matings	1	37	0.0005	0.9829
Spermatheca	2	84	9.7821	0.0002
Time interval × Spermatheca	2	84	3.7976	0.0264
Female hind tibia length	1	37	2.6761	0.1103
First male hind tibia length	1	37	1.2675	0.2675
Second male hind tibia length	1	37	0.0690	0.7943
First male copula duration	1	37	0.5250	0.4733
Second male copula duration	1	37	8.4056	0.0063

The model included the locus that provided a given estimate nested within the spermatheca nested within the female as random effects

Table 3 Analysis of variance of the effect on time between mating and morphology on the difference in S2 values between a female's singlet spermatheca vs. her doublet spermatheca

Source	df	MS	*F* value	*P* value
Time interval between matings	1	0.0863	4.708	0.037
Female hind tibia length	1	0.0012	0.066	0.798
First male hind tibia length	1	0.0002	0.008	0.928
Second male hind tibia length	1	0.0687	3.746	0.061
First male copula duration	1	0.0072	0.393	0.534
Second male copula duration	1	0.0098	0.532	0.470
Error	37	0.0183		

broad measure of second male success, we found that overall S2 across spermathecae was consistent with the general pattern of last male paternity (P2) in this species, which confirms previous findings suggesting sperm displacement by the last male (Parker 1970a; Parker & Simmons 1991; Simmons *et al*. 1999) over alternatives such as stratification within the sperm stores of females. We note that P2 can be highly variable within this species, ranging from complete first male precedence to complete last male precedence (Otronen *et al*. 1997; Simmons *et al*. 1999; Hellriegel & Bernasconi 2000), and that we also found substantial variation in S2 that is as yet unexplained. We encourage future work that directly links the empirical observations of contributions to stored sperm within the spermathecae and paternity.

The significant spermathecal identity effect (Table 2) supports consistency across females in the pattern of sperm storage for the second male, which accords with previous work on a dryomyzid fly (Otronen 1997). Our observations of skew across spermathecae in S2 values, with the singlet typically having higher S2 values than in either doublet spermatheca (Fig. 2) imply one of two scenarios, which are not mutually exclusive: (1) there is a level of consistency in female influence on the patterns of sperm storage; or (2) the second males consistently fill spermathecae in the same order, with the singlet being filled first. However, this second scenario would predict an interaction between the second male's copula duration and spermathecal identity, which our data do not support. While this failure to detect an effect could conceivably be a result of low power (we cannot rule out that such an interaction would require more sensitive techniques than the one employed here), there is no trend in the data suggesting that an increase in sampling would produce a significant interaction.

In *Dryomyza anilis*, males use their abdominal claspers to tap the females' abdomens during copulation, and more sperm move into the singlet spermatheca when this behaviour occurs. Furthermore, females preferentially use sperm from the singlet during oviposition, so males who tap more gain an advantage over rivals (Otronen 1997). In *S. stercoraria*, previous work revealed a more complex relationship between male size, sperm morphology, mating order and the bias in sperm storage which did not suggest a single consistent site of storage for preferred sperm (Otronen *et al*. 1997). Our results suggest that a consistent bias may be observed in *S. stercoraria* under some conditions. If this is true, one might predict that the contents of the singlet and doublet spermathecae are not equally likely to be used during fertilization. Alternatively, the presence of multiple sperm storage organs containing different mixtures of competing ejaculates may allow females some control over paternity even if all sperm stores are used equally. In this case, the sorting that occurred during copulation would be critical in defining the relative success of different males. A final intriguing possibility is that sperm are segregated so that they can be sorted by age, although there is no evidence that sperm function declines with age (Bernasconi *et al*. 2002). More work on the relative contributions of each sperm store to fertilization will be needed to address these questions.

Unlike the study by Otronen *et al*. (1997), our analysis does not suggest any other phenotypic male character that relates to this skew, but we acknowledge that we measured only a single aspect of male morphology, and that our study was primarily designed to observe the effect of storage time between matings rather than male phenotypic characters. The significant interaction between the time interval between matings and spermathecal identity supports a role for females in sperm sorting, although the extent to which this pattern is the result of adaptations specifically evolved in the context of mate choice remains unknown. In nature, sperm are stored between oviposition bouts that can be separated by weeks, but male contests on the oviposition resource can often result in successive copulations separated by mere minutes, typically followed immediately by a bout of oviposition (Parker 1971). Although our experimental flies were prevented from ovipositing, our results nevertheless suggest that in the latter instance, sperm storage may be less skewed, and from the perspective of the last mate, therefore less at risk of any female sperm choice that counteracts the typical last male advantage in this species.

We note that the average difference in S2 across spermathecae represents one potential aspect of sexual selection, but does not capture the opportunity for individual females to exercise choice if the doublet spermathecae operate as an integrated unit. Our second analysis therefore reduced the S2 values in the three spermathecae to the difference in S2 within the singlet and the doublet, with large positive values indicating relative high S2 in the singlet and vice-versa. Once again, the time interval

between matings influenced the skew across the singlet and doublet spermathecae, but in addition the model included a marginally nonsignificant effect of the second male's hind tibia length, suggesting that the opportunity for exercising sperm choice is the greatest when there is more time between matings and when the second male is large. While clearly tentative given its nonsignificance, the effect of male size in this instance deserves more study, as it is consistent with both female preferences for large males or alternatively size-dependent differences in success in sperm transfer that occur in the absence of active female mate choice.

Our results, which do not suffer from many of the technical shortcomings associated with previous methods for estimating relative contributions to sperm stores (see Introduction), contribute to increasing evidence that females may have a role in biasing sperm use in yellow dung flies. An obvious next step in understanding the mechanisms affecting sperm sorting and fertilization is to relate patterns within sperm stores to eventual paternity. For example, separately amplifying portions of the sperm pellet (i.e., proximate to or distal from the spermathecal duct) could provide a thorough test for sorting within individual sperm stores. As our methods are invasive (involving sacrificing the females in question) the necessary experiments will require careful consideration of events occurring between sperm storage and fertilization. Such work will doubtless clarify the intricate coevolutionary relationships underlying the remarkable animal diversity in reproductive morphology and biochemistry.

Methodological findings

Our approach to quantifying mixed DNA does not depend on an assumption that all alleles amplify equally well, but rather that allele characteristics affect amplification in a predictable way. Even before correcting the ratio of signal strength for allele properties, the signals provide reasonably accurate estimates of the starting concentrations of DNA (see Table S1). Correcting raw scores of signal strength using observations of allele amplification in controlled mixtures substantially improved our ability to replicate estimates of S2 across different loci (repeatability across loci increased from 0.71 to 0.77 after correcting for allele properties). Our methods are sufficiently well resolved to study variation in sperm storage within a single female in spite of the fact that we have restricted our correction to a linear model, the unexplained variation in signal amplitude across loci and the relatively low number of sperm copies obtainable from individual sperm storage organs of female dung flies. This repeatability may be insufficient for some applications, but we note that when specimens are not as heavily affected by sampling error, the repeatability may increase. Ongoing work that has adapted the protocol for crickets has been very successful and demonstrated higher repeatabilities of up to 0.82 across loci and 0.96 across replicate PCR runs (M.D. Hall, L.F. Bussière, M. Demont, P.I. Ward, and R. Brooks, unpublished data). Many model systems have a large number of microsatellite markers available, and using a larger number of loci should also increase the accuracy of the estimates, although we were unable to test this given small number of loci we studied. Our method does not require the extensive genomic knowledge that would be needed to develop a suitable array of SNP markers for real-time PCR (e.g., Wilkening *et al.* 2005), nor is it limited to documenting gene presence/absence conditions as for example used in XY-FISH protocols for quantifying chimerism after cell transplantation (Buño *et al.* 2005). As portfolios of microsatellite markers are now developed for a large number of species, these techniques have the potential to be widely applied.

We note that correcting for allelic characteristics using controlled mixtures is time-consuming and associated with moderate costs. Furthermore, as is evident in differences across loci within this system, these corrections will need to be carried out independently every time a new series of markers are to be used for quantifying the relative DNA contribution to a mixed sample. It may also be the case that the relationship between specific characteristics of an allele and the signal strength observed changes across different instruments in different laboratories, and so we advise basic good laboratory practice, where all measures of controlled mixtures used for correcting estimates of allele areas be conducted on the same instruments with the same reagents as those used for amplifying and assessing the mixtures of interest themselves.

We restricted our analyses to instances in which the contribution of a male could be unambiguously inferred because the male possessed a unique allele (not shared by the other male or by the female) at that locus. In principle, it would be possible to estimate male contributions by subtracting the estimated female contamination (on the basis of female alleles that are unique at the same or a different locus). It may also be possible to combine information from multiple loci to infer male contributions to peaks shared by the males themselves. In this first application of our newly developed methods, we wanted to keep the analysis as simple as possible given the number of alleles to which we had access. The necessity to exploit all the information available from amplifications of mixed DNA will depend on the number of loci available, their level of polymorphism and the confidence with which researchers can rule out female contamination of samples. In our study, the fact that in early dissections we were inconsistently able to

completely isolate sperm from female tissue (although we did improve in this skill over time) and the availability of alternate loci at which contributions could be unambiguously assessed suggested a conservative approach.

We hope that these statistical methods for assessing contributions to mixed sperm stores will be useful in other contexts in addition to studies of postcopulatory sexual selection. The wide availability of microsatellites for many study systems makes this approach feasible across a wide range of organisms, and the limited technical requirements and relatively low-cost of fragment analysis would allow its implementation for a number of applications, including for example the assessment of the fraction of self-fertilizing pollen on the stigmata of plants, determining the ploidy level of individuals, the diet composition of planktivores and bacteriotrophs and measuring the success of different parasitic strains within individuals.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 The mean values and variances of uncorrected observed signal ratios given different initial concentrations. The expected concentrations were derived from the original mixture, taking into account the total number of alleles in the PCR run

Table S2 Schwarz's Bayesian Information Criterion (BIC) for linear mixed models predicting the observed signal strength for alleles as a function of allele properties (listed in Table 1). For each locus, we compare a linear model with models including quadratic and cubic terms for the original DNA concentration. We rank the models based on the BIC scores, and include the BIC weight, which is the probability that a given model is the best among alternatives

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