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Predicting variation in sperm precedence

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SUMMARY

Sperm competition theory predicts that males are adapted for success in sperm competition by the production of large numbers of sperm. This is supported by both inter- and intraspecific studies showing that males mating under high sperm competition risk increase investment in sperm production. Such an increase in sperm production is an advantage if sperm mix randomly or if sperm displacement occurs. When two males mate with the same female, the measurement of the proportion of eggs fertilized by the second male to mate (termed P_2) has been used to help elucidate sperm competition mechanisms. P_2 is usually quoted as a mean value, with little attention being paid to its variance, although P_2 estimates are notoriously variable. By predicting an expected variance for P_2, additional information on sperm competition mechanisms may be obtained. Here we present a technique for analysing the variance in P_2 when a given mechanism of P_2 is assumed. We apply this technique to P_2 data collected from *Plodia interpunctella* (Lepidoptera, Pyralidae), assuming a 'fair raffle' mechanism of sperm competition. We compare observed distributions of P_2 with theoretical distributions generated assuming random mixing of two ejaculates drawn randomly from a population of known mean and variance in sperm numbers. Ejaculates of known size were obtained by counting the number of sperm ejaculated by males mating for the first (large ejaculate) or second (small ejaculate) time. Females either received two small, or one small and one large ejaculate, and the distribution of P_2 (estimated using the sterile male technique) was compared with our theoretical predictions. The observed variance in P_2 was greater than our model prediction, thus we conclude that sperm from *P. interpunctella* do not mix randomly before fertilization.

1. INTRODUCTION

Sperm competition, which occurs when sperm from more than one male compete for a given set of eggs (Parker 1970*a*), is now recognized as being important in the evolution of male characteristics (e.g. Smith 1984; Birkhead & Møller 1992; Baker & Bellis 1995). For example, comparative studies have shown that males of species with high levels of sperm competition have relatively larger testes (Short 1979; Møller 1988*a*, *b*; Jennions & Passmore 1993; Gage 1994), and produce larger ejaculates (Svärd & Wiklund 1989; Wedell 1993) and more sperm (Møller 1988*a*). Theory predicts that the production of large numbers of sperm is an advantage in sperm competition (Parker 1982, 1984, 1993) if, for example, sperm mix randomly within the female (e.g. Martin *et al*. 1974; Simmons 1987; Wedell 1991) or displace previous males' ejaculates (Parker & Simmons 1991; Simmons & Parker 1992).

A male's fertilization success will generally increase with the number of his sperm relative to those of other males, depending on the underlying mechanism of sperm competition (Parker *et al*. 1990). The outcome of sperm competition is measured as the proportion of the eggs fertilized by the second of the two males to mate and is termed P_2 (Boorman & Parker 1976). Typically, a mean P_2 value for a species is quoted, and for a few species the mechanism behind the sperm precedence pattern is known. For example, in the odonate species *Calopteryx maculata* the mating male removes virtually all of the previous sperm, causing a P_2 value of nearly one (Waage 1979) and in the dungfly *Scatophaga stercoraria* the mating male appears to displace a proportion of the previous sperm with his own ejaculate leading to a mean P_2 of 0.8 (Parker 1970*b*; Parker & Simmons 1991; Simmons & Parker 1992). However, in many species P_2 values range from zero to one (see reviews in Lewis & Austad 1990; Simmons & Siva-Jothy 1997), and few studies have examined this intraspecific variation. In some studies that have done so, male body size has been an important factor, with larger males having increased success in sperm competition (Lewis & Austad 1990; LaMunyon & Eisner 1993*a*; C. Bissoondath & C. Wiklund, unpublished data); although this was not the case in studies by Svärd & McNeil (1994), Conner (1995) and Radwan (1996). Large males may have increased fertilization success by producing more sperm or larger spermatophores (LaMunyon & Eisner 1993*b*), or by producing sperm at a faster rate (Simmons & Parker 1992). Duration of copula may also effect fertilization success, for example, if sperm are transferred continuously during mating. Males that copulate for longer have higher fertilization success in some species (Dickinson 1986; Rubenstein 1989; Parker & Simmons 1991; Simmons & Parker 1992).

It is usually assumed implicitly in P_2 studies that when two males mate with the same female they both ejaculate the same number of sperm. However, this

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may not necessarily be the case. Several factors may influence how many sperm a male delivers, for example, in the moth *Plodia interpunctella* sperm numbers are reduced when males are reared on a restricted diet (Gage & Cook 1994). Additionally, males may vary sperm number according to mating history: in *P. interpunctella* males become depleted, producing fewer sperm on second and third matings compared with virgin matings (Gage & Cook 1994; Cook & Gage 1995). Sexually exhausted males of the bruchid beetle *Callosobruchus maculatus* achieve lower fertilization success than those on their first mating (Eady 1995). In contrast, male *Pieris rapae* butterflies increase sperm number from their first to second matings, possibly due to higher risk of sperm competition later in the flight season (Cook & Wedell 1996). Studies on a variety of species show that the number of sperm ejaculated by a male increases when sperm competition risk is high (Baker & Bellis 1989, 1993; Bellis *et al.* 1990; Gage 1991; Gage & Baker 1991; Simmons *et al.* 1993; Cook & Gage 1995). Males may increase the number of sperm they inseminate to increase the representation of their sperm in the fertilization set. Such an increase in sperm number as a result of mating with a non-virgin female could contribute towards the observed high values of P_2 seen in many species.

It is also clear that a large amount of unexplained variation in P_2 occurs within the majority of insect species (Lewis & Austad 1990; Simmons & Siva-Jothy 1997). We suggest the following approach for analysing intraspecific variation in P_2: (i) assume a given mechanism of sperm competition; (ii) examine the variation in sperm numbers delivered during mating by the population of males used for the sperm competition study; (iii) given (i), use (ii) to predict the variation in P_2; (iv) compare the observed and predicted P_2 distributions: if there is a fit, we have circumstantial evidence for the causes of variation in P_2 (although we may not know why number of sperm transferred varies within the sample of males used in the experiment). If observed and predicted distributions differ, we can claim that the mechanism of sperm competition is unlikely to be the one we have assumed under (i).

In the present paper we demonstrate this method by applying it to data collected on the pyralid moth *Plodia interpunctella* (Hübner). P_2 in *P. interpunctella* is 0.68 (Gwynne 1984, using data from Brower 1975); a value that superficially indicates high sperm mixing. Sperm numbers in *P. interpunctella* are quite variable (Gage & Cook 1994; Cook & Gage 1995), and males mating with already mated females increase the number of sperm they ejaculate compared to those copulating with virgin females (Cook & Gage 1995). From a knowledge of the mean and variance of sperm numbers in each ejaculate we predict what the distribution of P_2 values would be if the sperm mix randomly within the female storage organ, and we test this hypothesis against observed distributions of P_2.

2. THEORETICAL BACKGROUND

We first derive probability distributions of the P_2 values that would arise if the sperm from two ejaculates drawn from populations of known mean and variance in sperm number mixed randomly and if each sperm has an equal chance of being selected for fertilization.

We competed 'large' and 'small' ejaculates of known mean and variance in sperm numbers (figure 1*a*; for methods see §3*b*). Enhancing the variation in

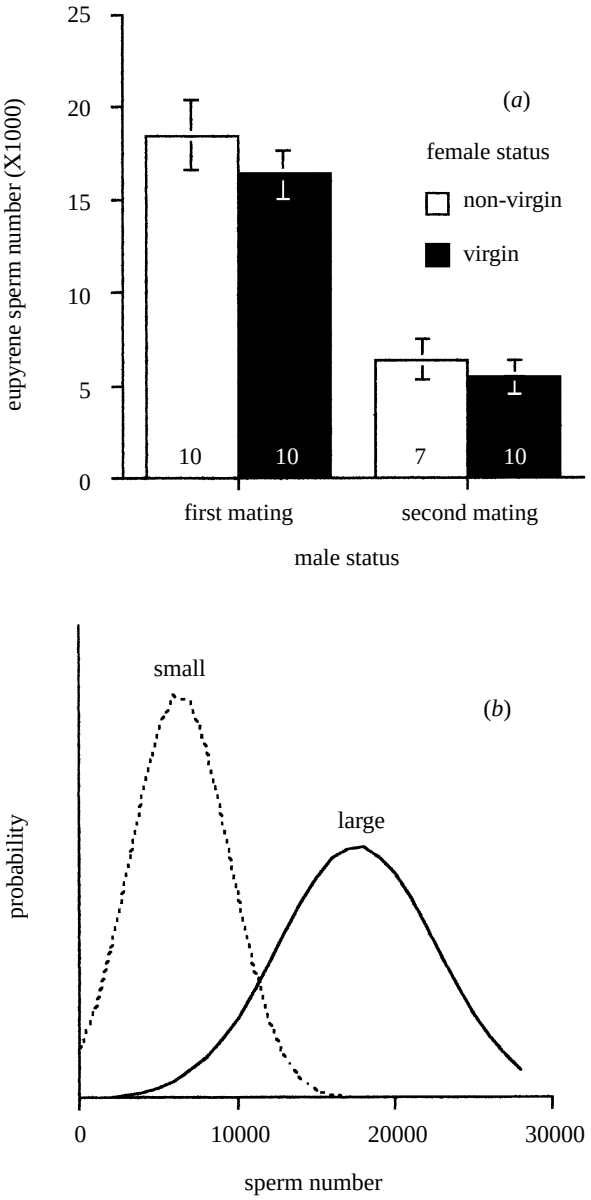

Figure 1. (*a*) The mean number of eupyrene sperm (bars are standard errors; numbers at bases of bars are sample sizes) produced by males on their first ('large' ejaculate) and second ('small' ejaculate) matings when mating with females that were either non-virgin (having previously received a 'small' ejaculate) or virgin. Apyrene numbers are not shown as they are not used in the model, however the pattern is the same. (*b*) As males provide both virgin and non-virgin females with similar numbers of eupyrene sperm, normal plots for 'large' (males' first matings; $\bar{X} = 17075$, s.d. = 5006) and 'small' (males' second matings; $\bar{X} = 5873$, s.d. = 3095) ejaculates are fitted from the combined data; these sperm number distributions are then used in the model (§2).

sperm numbers between the two competing males increases the power of the test for fit between predicted and observed distributions. From fitted normal probability plots (figure 1*b*), the probability (q) of male 1 producing an ejaculate of size n_1, and the probability (r) of male 2 producing an ejaculate of size n_2 can be calculated. The probability of these two ejaculates meeting each other in the female tract is therefore qn_1rn_2. If the sperm then mix randomly and each sperm has an equal chance of being picked for fertilization, the proportion of the eggs to be fertilized by the second male is the same as the proportion of his sperm in the female tract, that is:

$$P_2 = \frac{n_2}{n_1 + n_2} \quad (1)$$

i.e. the 'fair raffle' principle of Parker *et al.* (1990).

Computer simulations (using Minitab, Release 9) were carried out to generate P_2 distributions that would occur as a result of sperm number variation: (i) A matrix was constructed to calculate the probabilities of all the possible combinations of pairs of ejaculates from males 1 and 2. The simulations used a range of 1000–12000 sperm (increment 1000 sperm) for 'small' ejaculates, and a range of 2000–24000 sperm (increment 2000 sperm) for 'large' ejaculates (these sperm number ranges were selected to cover those shown in figure 1*b*). (ii) A corresponding matrix was constructed to calculate the P_2 values that would occur if the two ejaculates met (from equation (1)). (iii) Each P_2 value therefore has an associated probability in the corresponding matrix. P_2 values were assigned into the categories: 0–0.05, 0.05–0.1, ... 0.95–1.0. The probabilities from the corresponding matrix were summed for each category of P_2. This generates theoretical P_2 distributions that occur if the proportion of eggs fertilized by the second male was purely a result of random sperm mixing (see line on figure 2).

3. MATERIALS AND METHODS

(*a*) *Rearing technique*

Plodia interpunctella was cultured in constant condition rooms at 25 (±2) °C with a 16L:8D photoperiod. Larvae were reared on a diet of bran midlings, glycerol and yeast in a 10:1:1 ratio and at density of about 100 eggs per 400 ml of medium. Virgin animals were obtained by isolating individual 5th instar larvae from the stock culture in 30 ml vials with a small amount of food medium.

(*b*) *Sperm counts*

Lepidoptera produce two types of sperm, a fertile 'eupyrene' form and a non-fertile, anucleate 'apyrene' form. The function of the apyrene sperm type is unknown (although they may influence female sexual receptivity: Silberglied *et al.* 1984; Cook & Gage 1995), therefore their numbers are not incorporated in the model.

We manipulated ejaculate size by allowing given males to mate for the first ('large' ejaculate) or second time ('small' ejaculate); male *P. interpunctella* predictably produce ejaculates with decreasing numbers of sperm over successive matings (Gage & Cook 1994; Cook & Gage 1995). Male *P. interpunctella* have been shown to vary the size of their ejaculate depending on whether females are virgin or non-virgin (Cook & Gage 1995), therefore the number of sperm produced by males on their first and second matings to both virgin and non-virgin females was ascertained. Females, 3–4 days old, were mated either once or twice to 3–4-day-old males. Singly-mated females received either a small or a large ejaculate, and the number of sperm in the spermatophore were counted using established protocols (Gage & Cook 1994; Cook & Gage 1995). Doubly-mated females all received a small ejaculate at the first mating. After 36 h, females were mated to a male on either his first or second mating, and the number of sperm transferred were counted. *P. interpunctella* are more active during the dark cycle. However, virgin females mate readily in the light cycle. All first matings were therefore carried out in the light cycle. Non-virgin females are generally more reluctant to mate during the light cycle, so for second matings, pairs were observed throughout the dark cycle. About half of the females remated under these conditions, therefore twice as many doubly-mated as singly-mated treatments were set up.

In contrast to previous results (Cook & Gage 1995: see § 5*a*), female mating status (virgin or not) had no effect on the number of sperm transferred by males (figure 1*a*; first mating: eupyrene, $F_{1,18} = 0.43$; n.s.; apyrene, $F_{1,18} = 0.05$, n.s; second mating: eupyrene, $F_{1,15} = 0.25$, n.s.; apyrene, $F_{1,15} = 0.35$, n.s). The mean and variance in the numbers of sperm in a 'small' (male on his second mating) and a 'large' (male on his first mating) ejaculate were calculated. As there were no significant differences in the number of sperm produced by males mating with virgin or non-virgin females, the data for each size of ejaculate were combined to estimate the parameters. The distributions are not significantly different from normal (the data were highly correlated with their normal scores, equivalent to a Shapiro–Wilk test (Minitab Reference Manual 1991); large ejaculates, $r = 0.978$, $p > 0.1$, $n = 17$; small ejaculates, $r = 0.992$, $p > 0.1$, $n = 20$).

To check that irradiated and normal males did not differ in the number of sperm they transfer, virgin 1- or 2-day-old irradiated and normal males were mated and the number of sperm they ejaculated was counted. The irradiated and normal males did not differ in the number of eupyrene sperm ejaculated (normal: $\bar{X} \pm$ s.e. $= 11676 \pm 1058$, $n = 18$; sterile: $\bar{X} = 12996 \pm 1031$, $n = 17$; $F_{1,33} = 0.80$, n.s.), but irradiated males ejaculated more apyrene sperm than normal males (normal: $\bar{X} = 117207 \pm 10776$, $n = 18$; sterile: $\bar{X} = 155654 \pm 11088$, $n = 17$; $F_{1,33} = 6.18$, $p = 0.018$).

(*c*) $\mathbf{P_2}$ *experiment*

To estimate P_2, two males were mated with the same female and the number of offspring sired by each of the males was counted. We used the sterile male technique: males were exposed to a sublethal doses of irradiation

so that although they still produced sperm that were capable of fertilizing the egg, the resulting embryos suffered lethal mutations in the early stages of development. In a double mating, eggs that failed to hatch were therefore attributed to the sterile male and those that hatched to the normal male. There are two disadvantages with this technique: first, there may be differences in the competitiveness of normal and sterile male's sperm and to control for this the mating order was reversed (Parker 1970*a*); second, paternity assignment can be ambiguous, as a proportion of eggs that are fertilized by a normal male usually fail to hatch and some of the progeny from a sterile male may develop. The proportion of hatching eggs attributed to each male from the double matings was therefore corrected using the mean fertility of control females mating with either two normal or two sterile males. Two equations have been published to incorporate the correction factors (Boorman & Parker 1976; Sillén-Tullberg 1981). However, these are algebraically equivalent (Appendix 1) and we used the following form to calculate P_N, the proportion of eggs fertilized by the normal (i.e. non-irradiated) male:

$$P_N = \frac{(x-z)}{(p-z)} \qquad (2)$$

where x is the proportion of eggs that hatch from the sterile–normal and normal–sterile matings, p is the proportion hatching after two normal matings and z is the proportion hatching after two sterile matings.

Females each received two ejaculates, with all females first receiving a small ejaculate, as such females remate more readily. For their second mating, females received either a large ejaculate or another small ejaculate. Experimental females were mated alternately to sterile and to normal males, and vice versa, and controls were mated to two sterile or two normal males, giving rise to eight categories.

All the adults eclosing over a 2-day period were collected and sexed. Half the males were irradiated on the same day using 350 Gy (at a rate of 4.5 Gy min^{-1}) from a gamma radiation source at the John Moores University, Liverpool (a dose sufficient to induce 98% sterility in the small–small and 95% sterility in the small–large sterile controls). Males were randomly assigned first or second male roles. Females were either assigned to experimental or to control categories, or they were used to deplete the males.

Males (both sterile and normal) that were to be in the first male role were mated for the first time one day prior to the experiment in order to deplete them. For females' first matings, females were placed with 'first males' (now on their second mating) and after copulation, males were removed. The following day any female that had laid more than about 20 eggs was discarded from the experiment. Females were then given the opportunity to mate for the second time (and were later dissected upon death to ascertain the number of matings from spermatophore counts; Drummond 1984), with a male transferring either a 'small' ejaculate (i.e. already mated on the previous day) or a 'large' ejaculate (i.e. virgin). For second matings, pairs were housed together for 24 h (to allow maximum opportunity for remating), and the second male was removed the following morning. Females that had mated only once or (as occurred very rarely) more than twice were removed from the analysis. Males were placed in the freezer after their mating opportunity and after the experiment male size was estimated (see §3*d*). Males and females were all 3–4 days old on females' first matings and 4–5 days old on females' second matings.

After females were separated from their 'second males', they were isolated in 30 ml plastic tubes and eggs were collected every 2 days for the duration of the female lifespan. *P. interpunctella* do not require a substrate on which to oviposit; eggs were either found loose in the tube or adhered to the walls. Up to 70 eggs (depending on how many had been laid) were sampled randomly and sprinkled in a small Petri dish on sticky paper, allowing larvae to hatch and preventing them from escaping, eating the egg cases or cannibalizing siblings. After 5 days, the numbers of unhatched and empty egg cases were counted using a binocular microscope and the proportion of eggs to hatch was calculated.

The data for the proportion of hatching eggs were divided into 'early' (those laid in the first 2 days) and 'late' (laid in the remainder of the female's life). The corrected proportion of eggs fertilized by the normal male (P_N; equation (2)) was calculated for early and late egg collections separately. The mean proportions of viable eggs from normal–normal controls (p) were 0.94 (early eggs) and 0.75 (late eggs) for 'small–small' treatments, and 0.94 (early eggs) and 0.68 (late eggs) for 'small–large' treatments. The mean proportions of viable eggs from the sterile–sterile controls (z) were 0.02 (early eggs) and 0.01 (late eggs) for 'small–small' treatments, and 0.05 (early eggs) and 0 (late eggs) for 'small–large' treatments. In the case of sterile–normal matings, P_N is the P_2 value. For normal–sterile matings, values of P_N are P_1 values (the proportion eggs fertilized by the first male) and were therefore converted to P_2 values by subtracting them from one.

The resulting P_2 dataset had some values of less than zero and some of greater than one. The values less than zero were caused by some of the sterile–sterile controls being more fertile than the experimentals (i.e. when $z > x$). Values greater than one were caused by some of the normal–normal controls being less fertile than the experimentals (i.e. when $p < x$). The datasets were therefore transformed so that the data lay in the range 0 to 1 as follows:

$$\text{Corrected } P_{2i} = \frac{(P_{2i} - P_{2min})}{(P_{2max} - P_{2min})} \qquad (3)$$

where P_{2i} are individual P_2 values, P_{2min} is the lowest and P_{2max} the highest P_2 value in the dataset.

(*d*) *Measurement of male size*

Male size was estimated by measuring the length of the forewing. Accurate measurements were obtained by removing the scales from wings (following the

technique given in Reid 1976) and mounting the wings on glass slides. The length between the wing margin/vein one junction and the point of wing insertion for both left and right wings was measured using an eye piece graticule at × 15 magnification. The mean of the two measurements was taken.

(*e*) *Analysis*

To test whether the observed distribution of P_2 values was the same as the predicted P_2 distributions shown in figure 2, the data for the four treatments were first converted to the same categories of P_2 values as the theoretical P_2 distributions (0–0.05, 0.05–0.1, … 0.95–1.0). The cumulative observed distributions were then compared with the cumulative theoretical P_2 probability distributions using Kolmogorov–Smirnov goodness-of-fit tests. Two-sample Kolmogorov–Smirnov tests were used to test for differences between pairs of observed distributions. These non-parametric tests have the null hypothesis that the observed and expected (goodness-of-fit test) or the two observed (two-sample test) distributions have identical shapes, and are sensitive to differences in location, dispersion and skewness (Sokal & Rohlf 1995).

4. RESULTS

Mean P_2 values vary from 0.34 to 0.88 (table 1) suggesting some sperm mixing (cf. Brower 1975). However, expressing the data as means gives little indication of the true pattern of sperm precedence; the frequency distributions of P_2 are shown in figure 2. Although the distributions in figure 2 show the two mating orders combined for simplicity, Kolmogorov–Smirnov goodness-of-fit tests were carried out on the mating orders separately. The expected and observed distributions differ significantly when a small ejaculate is followed by a large ejaculate, for both early (normal–sterile: $D_{0.5} = 0.55$, $n = 17$, $p < 0.01$; sterile–normal: $D_{0.5} = 0.71$, $n = 19$, $p < 0.01$) and late egg collections (normal–sterile: $D_{0.5} = 0.46$, $n = 19$, $p < 0.01$; sterile–normal: $D_{0.5} = 0.37$, $n = 23$, $p < 0.01$). Similarly, expected and observed distributions differ significantly when a small ejaculate is followed by another small ejaculate, in both early (normal–sterile: $D_{0.5} = 0.56$, $n = 12$, $p < 0.01$; sterile–normal: $D_{0.5} = 0.7$, $n = 18$, $p < 0.01$) and late egg collections (normal–sterile $D_{0.5} = 0.7$, $n = 15$, $p < 0.01$; sterile–normal: $D_{0.5} = 0.39$, $n = 16$, $p < 0.01$).

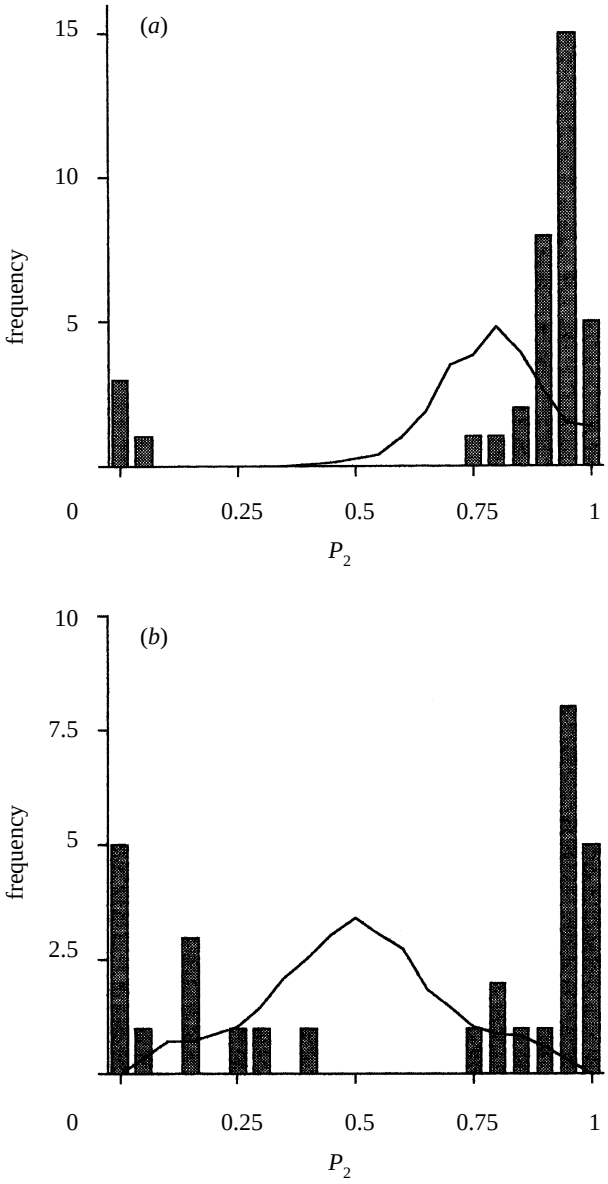

Figure 2. Expected P_2 if ejaculates of known mean and variance in sperm number (from figure 1*b*) mix randomly (line) compared with the observed data (bars) from eggs laid early in the female's life (for both mating orders combined). (*a*) Sperm from a large ejaculate competing with sperm from an initial small ejaculate, and (*b*) sperm from a small ejaculate competing with that of an initial small ejaculate.

Table 1. *Mean* P_2 *values, standard deviations and sample sizes for all treatments in both early (the first 2 days of egg laying) and late (eggs laid subsequently) egg collections*

(The range of P_2 values was 0 to 1 in all cases. Females each received two ejaculates: ø is a small ejaculate from an irradiated male, o is small ejaculate from a normal male, Ø is large ejaculate from an irradiated male and O is large ejaculate from a normal male.)

treatment		early			late		
first	second	$\bar{X}$	s.d	*n*	$\bar{X}$	s.d.	*n*
o	Ø	0.78	0.37	17	0.78	0.33	19
ø	O	0.88	0.22	19	0.75	0.27	23
o	ø	0.34	0.37	12	0.78	0.38	15
ø	o	0.80	0.32	18	0.46	0.38	16

Two-sample Kolmogorov–Smirnov tests between pairs of observed distributions were also carried out. Mating order (whether the irradiated male mated first or second) significantly affects the distribution of P_2 values when two small ejaculates compete (in both early and late clutches), but not when a small ejaculate is followed by a large one (table 2). The effect of ejaculate size on P_2 is ambiguous. Although it appears

Table 2. *Kolmogorov-Smirnov two-sample tests to compare the distributions of* P_2 *between the different mating orders and ejaculate sizes*

(Females each receive two ejaculates: ø is a small ejaculate from an irradiated male; o is small, normal; Ø is large, irradiated and O is large, normal. Early eggs were those collected during the first 2 days of oviposition and late eggs were all those collected subsequently. For sample sizes, see table 1.)

	early		late	
	D_{max}	p	D_{max}	p
mating order effects				
o Ø and ø O	0.42	n.s.	0.37	n.s.
o ø and ø o	0.64	< 0.001*	0.55	0.012*
ejaculate size effects				
o Ø and o ø	0.57	0.013*	0.21	N.S.
ø O and ø o	0.28	N.S.	0.51	0.01*

* Remain significant at an experiment-wise error rate of 0.05 following the sequential Bonferroni technique (Rice 1989).

Table 3. *Spearman's rank correlations between relative male size (male 2/male 1) and* P_2

(Females each receive two ejaculates (treatment): ø is a small ejaculate from an irradiated male; o is small, normal; Ø is large, irradiated and O is large, normal. Early eggs were those collected during the first 2 days of oviposition and late eggs were all those collected subsequently.)

treatment		early			late		
first	second	n	r_s	p	n	r_s	p
o	Ø	16	0.10	n.s.	14	0.13	n.s.
ø	O	17	0.23	n.s.	20	−0.12	n.s.
o	ø	11	−0.48	n.s.	11	−0.43	n.s.
ø	o	17	0.06	n.s.	15	−0.13	n.s.

from figure 2 that large ejaculates are more successful, when the data are analysed for mating order separately, no clear pattern emerges. Instead, the distributions seem more strongly affected by mating order and whether eggs are laid early or late in the female lifespan (table 2). Fertilization success in this species does not appear to be related to male size (table 3).

5. DISCUSSION

(*a*) *Experimental*

The observed distributions of sperm numbers (figure 1) show a large amount of variation and, if the sperm were competing as in the fair raffle model (Parker *et al.* 1990), our model predicts a corresponding large variation in the P_2 (figure 2). For example, in the case of an initial small ejaculate competing with a later large ejaculate, P_2 would be expected to vary from 0.5 to 1 (figure 2*a*). The observed distributions of P_2 values vary from 0 to 1. However, the shapes of the distributions do not fit with the model based on the 'fair raffle' hypothesis (Parker *et al.* 1990).

Here the number of sperm transferred by each male was known; however, several factors make it difficult to assess accurately whether these sperm are mixing randomly in the female storage organs. Fertilization success may become biased towards the second male to mate if the female oviposits between the two matings. This was controlled by discarding females that laid more than 20 eggs. Sperm from the first mating may also be passively lost from storage (Tsubaki & Yamagishi 1991; Eady 1994). Although the time between the two matings was as short as possible, sufficient time had to be allowed for the sperm to travel to the storage organs allowing (i) enough room in the bursa for another spermatophore, and (ii) the female to become receptive (the presence of a full spermatophore in the bursa delays female remating; Sugawara 1979).

In other invertebrate studies that examine intraspecific variation in P_2, male size has been important in determining fertilization success. In two lepidopteran species, large males transfer larger spermatophores and have greater fertilization success (LaMunyon & Eisner 1993*b*; C. Bissoondath & C. Wiklund, unpublished data). Larger males of the dungfly *Scatophaga stercoraria* transfer sperm at a faster rate but copulate for a compensatory shorter time; thus their P_2 is the same as that of small males (Simmons & Parker 1992). However, in *P. interpunctella*, large males do not transfer more sperm (unpublished data) and, in common with another lepidopteran (Svärd & McNeil 1994), male size does not correlate with P_2. Interestingly, in the mite *Rhizoglyphus robini*, fertilization success correlates only with sperm size and not with sperm numbers or male size (Radwan 1996).

Several explanations have been put forward to explain observed P_2 patterns in Lepidoptera. In species where females remate very rapidly it has been suggested that the second male's spermatophore may displace that of the first so that few of the first male's sperm reach the spermatheca (Drummond 1984). This is unlikely to be the case here, as females were allowed adequate time (24 h) between each mating for sperm to be transported to the storage organs. Another suggestion is that the pattern of second male precedence could be caused by the tubular shape of the spermatheca (Walker 1980). The second male may have an advantage because the sperm from the previous males is displaced backwards. If sperm displacement occurs, larger ejaculates may displace more of the previous sperm. This could account for the more consistent second male advantage when the male produces a large ejaculate rather than a small one (figure 2*a* compared with 2*b*).

It is hard to explain the occurrence of occasional low P_2 values in our data. For example, three females out of 37 in the small–large treatment apparently used sperm from the first male (the small ejaculate) to fertilize all their eggs (figure 2*a*). In these cases it is possible that (i) the female was sterile (and her eggs therefore

attributed to the sterile male), but this is unlikely to account for many low P_2 values, as complete infertility in normal matings is rare (of 197 females double-mated to normal males, only one female was completely infertile; unpublished data); or (ii) transfer of sperm from the second spermatophore failed. Although, upon post mortem, data from females whose spermatophore had obviously not drained were omitted, it is possible that we were not always able to detect failures of sperm transport. An alternative explanation for such 'all or none' P_2 patterns (that are more apparent in some other species: Retnakaran 1974; LaMunyon & Eisner 1993*a*; Svärd & McNeil 1994) is that female Lepidoptera exert choice over ejaculates (LaMunyon & Eisner 1993*a*, *b*). As females appear to control the movement of sperm from the bursa to the spermatheca (Tschudi-Rein & Benz 1990; LaMunyon & Eisner 1993*a*), they may also have the potential to select an individual male's sperm.

Sterilization treatment has a significant effect on P_2 when two small ejaculates compete (table 2). This difference in sperm competitiveness does not seem to occur when males are on their first mating (producing a large ejaculate) as second males transferring large ejaculates do equally well (table 2). However, on a male's second mating (small ejaculate), irradiation seems to cause the sperm to be considerably less competitive than that of the normal male (tables 1 and 2). Sterilization may have a greater effect on the sperm used in the second ejaculate if these sperm had been still developing at the time of the irradiation dose. However, this is not consistent with the apparent reversal of the sperm precedence pattern later in the female's life, with sperm from the sterile male being used (table 1).

Another effect of the sterilization treatment was the production of significantly more apyrene (non-fertile), but not eupyrene (fertile), sperm by sterile males than normal males in first ejaculates (§3b). Male *P. interpunctella* appear to have a mechanism whereby they change the number of apyrene sperm relative to eupyrene sperm depending on female quality (Cook & Gage 1995). It is possible that if the irradiation treatment damaged this mechanism there would be a resulting change in apyrene sperm numbers.

In this study, variation in P_2 cannot be explained by an increase in sperm numbers ejaculated by the second male to mate in response to sperm competition risk (figure 1*a*). In contrast, Cook and Gage (1995) found that the number of eupyrene sperm ejaculated by male *P. interpunctella* depended on sperm competition risk: males increased the number of sperm when mating with non-virgin females. There are two possible reasons why we find no such effect in the present study: (i) in the previous study males used both female age and mating status as cues to determine how many sperm to ejaculate, whereas here we use females of an intermediate age; and (ii) previously males were found to ejaculate larger numbers of sperm to females that already contained a 'large' ejaculate compared to those that contained 'small' ejaculates, whereas in the present study, females' first matings were always to males producing a 'small' ejaculate.

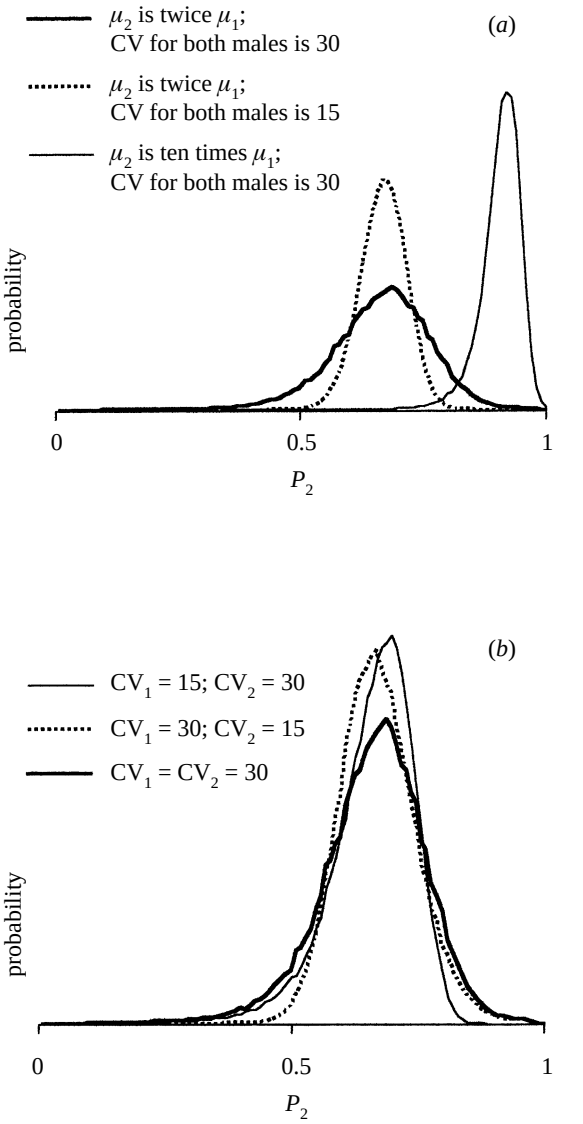

Figure 3. The effect of altering the mean and variance of sperm numbers on the predicted P_2. (*a*) The coefficient of variation (CV) is the same for both males. Increasing the mean size of a second male's ejaculate (thin line) or reducing the variation in both males' ejaculates (broken line) decreases the predicted variation in P_2. μ_1 and μ_2 are the mean sperm numbers for the first and second male respectively. (*b*) When the mean numbers of sperm ejaculated by both males are kept constant (here the second male's ejaculate is twice that of the first male's), reducing the CV of either male (thin line and broken line) decreases the predicted variation in P_2. CV_1 and CV_2 are the coefficients of variation for sperm number for the first and second male respectively.

(*b*) *Theoretical*

In principle it seems that the technique we have applied could be used in all sperm competition studies to provide evidence for various mechanisms of sperm competition. Our technique relies on estimating the population variance in sperm numbers. However, caution is needed as variation due to sampling occurs at two levels. There is for most studies (i) an error associated with subsampling sperm to deduce an individual male's sperm count, and (ii) an error associated with sampling males to deduce the population variance. The present study assumes no error in

measuring an individual's sperm count; this is a reasonable assumption in this case because the total number of eupyrene sperm is counted directly (see Gage & Cook 1994). However, most sperm counts rely on subsampling and under such circumstances appropriate ANOVA techniques should be used to estimate the population (inter-individual) variance in sperm numbers.

It is useful to obtain some feel for the behaviour of our model under random sperm mixing. Figure 3 shows some predicted P_2 distributions when means and coefficients of variation of sperm numbers of two competing ejaculates are varied. The second male always has the larger ejaculate. The main effects are (i) reducing the coefficient of variation at a given sperm number reduces the predicted variation in P_2 (figure 3*a*); (ii) increasing the difference in mean sperm number at a given coefficient of variation reduces the predicted variation as well as increasing P_2 (figure 3*a*); and (iii) reducing the coefficient of variation of either of the males reduces the predicted variation in P_2, although this effect is small relative to the effect of changing sperm numbers (figure 3*b*).

(*c*) *Conclusion*

Our study demonstrates convincingly that sperm competition does not occur by random mixing, following a 'fair raffle' (Parker *et al*. 1990) in *P. interpunctella*. We could investigate further possible mechanisms of sperm competition (e.g. sperm displacement or stratification) using the same technique. To do this, we would simply insert the appropriate alternative form into equation (1): the rest of the method for generating the predicted distributions would then proceed in an identical fashion. The equation for sperm displacement with instant random mixing is given in Parker and Simmons (1991, equation (11)). However, the strong bimodal distribution of P_2 is suggestive of female choice of ejaculates. In principle, female preferential use of sperm could also be modelled by our technique, as P_2 is likely still to depend (although less strongly) on sperm numbers. To do this we would need to quantify the bias algebraically and substitute this definition into equation (1).

This paper is, in essence, a starting point for further analyses of P_2 data. We suggest that variance in sperm numbers can be used routinely to predict P_2 distributions; a test against the observed distribution then gives a more sensitive test than using simply the mean P_2.

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APPENDIX 1

Two equations have been published to incorporate the correction factors when using the sterile male technique to calculate P_2 (Boorman & Parker 1976; Sillén-Tullberg 1981). Boorman and Parker (1976) showed that from the sterile–normal and normal–sterile matings, the proportion of eggs that hatch (x) is adjusted using the fertility after two normal matings (p) and two sterile matings (z) to give the proportion of eggs fertilized by the irradiated male (P_R):

$$P_R = \left(1 - \frac{x}{p}\right) + \left(\frac{z}{p} * \frac{1 - \left(\frac{x}{p}\right)}{1 - \left(\frac{z}{p}\right)}\right). \quad \text{(A 1)}$$

Sillén-Tullberg (1981) published an equation where the mean proportion of eggs fertilized by the normal male (P_N) is calculated by:

$$P_N = \frac{(x - z)}{(p - z)}. \quad \text{(A 2)}$$

The eggs not fertilized by the normal male are attributed to the sterile male, so P_R (the proportion of eggs fertilized by the sterile male) therefore will be (from equation (A 2)):

$$P_R = 1 - \frac{(x - z)}{(p - z)}. \quad \text{(A 3)}$$

It can be shown that equations (A 1) and (A 3) are algebraically equivalent. By substituting the terms x/p and z/p from (A 1) for e and f respectively, equation (A 1) becomes:

$$P_R = (1-e)+f\frac{(1-e)}{(1-f)} \tag{A 4}$$

and simplifies to:

$$P_R = \frac{(1-e)}{(1-f)} \tag{A 5}$$

and so:

$$P_R(1-f) = 1-e. \tag{A 6}$$

Substituting e and f back to x/p and z/p it can be shown that:

$$P_R-\left(\frac{zP_R}{p}\right) = 1-\frac{x}{p} \tag{A 7}$$

which, when multiplied by p becomes:

$$P_R(p-z) = p-x \tag{A 8}$$

This is the same as:

$$P_R(p-z) = (p-z)-(x-z) \tag{A 9}$$

which rearranges to form equation (A 3).