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Influence of female reproductive anatomy on the outcome of sperm competition in *Drosophila melanogaster*

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Females as well as males can influence the outcome of sperm competition, and may do so through the anatomy of their reproductive tracts. Female *Drosophila melanogaster* store sperm in two morphologically distinct organs: a single seminal receptacle and, normally, two spermathecae. These organs have different temporal roles in sperm storage. To examine the association between sperm storage organ morphology and sperm competition, we used a mutant type of female with three spermathecae. Although the common measure of sperm competition, P_2, did not differ between females with two and three spermathecae, the pattern of sperm use over time indicated that female morphology did affect male reproductive success. The rate of offspring production by females with three spermathecae rose and fell more rapidly than by females with two spermathecae. If females remate or die before using up second male sperm, then second male reproductive success will be higher when they mate with females with three spermathecae. The results indicate that temporal patterns of sperm use as well as P_2 should be taken into account when measuring the outcome of sperm competition.

Keywords: spermathecae; sperm storage; temporal sperm use

1. INTRODUCTION

Since the inception of work on sperm competition (Parker 1970), studies of post-copulatory events have tended to concentrate on interactions between competing males. However, females too may influence patterns of sperm use (Walker 1980; Otronen *et al.* 1997; Ward 1998, 2000; Hellreigel & Bernasconi 2000). Various mechanisms have been suggested for ways in which multiply mated females may bias paternity in favour of particular males (Birkhead *et al.* 1993; Keller & Reeve 1995; Eberhard 1996). The complex morphology of female reproductive tracts in many species may reflect selection on females to influence the use of sperm from different males for fertilization (Walker 1980; Linley & Simmons 1981).

Females may affect the outcome of sperm competition through the utilization of different types of sperm storage organ (Pitnick *et al.* 1999). Female *Drosophila melanogaster* mate multiply (Imhof *et al.* 1998) and there is natural genetic variation between females for the degree of last-male sperm precedence (Clark & Begun 1998; Civetta & Clark 2000). Female flies can store sperm for up to two weeks after mating (Gilbert *et al.* 1981) and commonly store the sperm of two or more males simultaneously (e.g. Marks *et al.* 1988). They possess two morphologically distinct sperm storage organs: a seminal receptacle and, normally, two spermathecae. These organs have different temporal roles: sperm from the spermathecae are released sooner than are sperm from the seminal receptacle (Gilbert 1981).

The main aim of the study was to examine how female reproductive tract morphology affects sperm use, using mutant female *D. melanogaster* with three spermathecae rather than the normal two (figure 1). Sperm use can be estimated by counting the numbers of offspring sired by a male and, for two males mated to a single female, the outcome of sperm competition is generally measured using the index P_2 (second-male offspring/(first + second-male offspring)). However, this measure ignores the dynamics of sperm use. If a female mates more than twice, the second male's reproductive success will be affected by the rate at which second male's sperm are used for fertilization (Prout & Bundgaard 1977; Gilchrist & Partridge 1995). The seminal receptacle and spermathecae are thought to have different temporal roles in sperm storage (Gilbert 1981), so sperm use over time was also monitored. Temporal patterns of sperm use will provide an additional source of variation in male success during sperm competition if females die or remate before all the sperm of a previous male are used.

2. METHODS

(a) *Stocks and cultures*

(i) *Females with two and three spermathecae*

Females were derived from a line created by the mobilization of the *PlacW* insertion in the line *P{w + mC = lacW} l(3)j10B6^{10B6}/TM3*, from the Bloomington stock centre (stock number: 10175) during a mutagenesis screen. This line was cultured in vials containing 7 ml of maize–yeast medium (10 g agar, 85 g sugar, 60 g maize, 20 g yeast and 25 ml nipagin per litre of water) which was subsequently used throughout the experiment.

Dissection of the females revealed that about 30% of females possessed three spermathecae (3SP) rather than the normal two (2SP) (figure 1). However, as 2SP and 3SP females were also found in the original *PlacW* line, it is unlikely that the 3SP morphology was caused by mobilization of the *PlacW* to a new pos-

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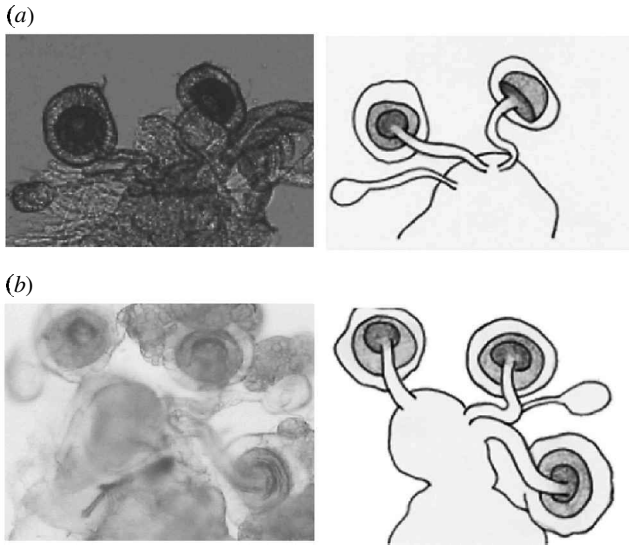

Figure 1. The spheroid, darkly pigmented cuticle of *Drosophila melanogaster* spermathecae from (*a*) a 2SP female and (*b*) a 3SP female. Samples were dissected on glass slide in PBS. Images were captured from a compound microscope (magnification × 250) using a video camera connected to a Macintosh computer.

ition in the genome. The genetic basis for this variation in spermathecal morphology was therefore unknown and naturally occurring. Dissection of mated females in phosphate-buffered saline (PBS) and examination under a compound microscope (magnification × 250) confirmed that the spermathecae of 2SP and 3SP females all contained sperm. 2SP and 3SP females were obtained from the same vials and experienced the same culture conditions.

(ii) *Wild-type*

Wild-type flies were derived from a collection made at Mas Canet (near Montpellier, France) in 1999. Flies were kept in the laboratory in population cages at 25 °C on a 12 L : 12 D regime. Each cage was supplied with three one-third of a pint glass bottles containing 70 ml sugar–yeast medium (100 g yeast, 100 g sugar, 27 g agar, 30 ml nipagin and 3 ml propionic acid per litre of water) per week, which were left in the cage for a total of four weeks.

(iii) *Brown dominant (bw^D)*

bw^D flies were obtained from the Umeå *Drosophila* stock centre (stock number: 40650) (February 2000), and were cultured in one-third of a pint glass bottles containing maize–yeast medium.

(iv) *Offspring phenotypes*

When mated to females described in § 2a(i), bw^D males sire phenotypically bw^D female offspring, distinguishable from wild-type offspring by their dark red eye colour. Because the females used in the experiment were homozygous for the *white* allele on the X chromosome (although partially rescued by the *PLacW* insertion), male offspring sired by wild-type or bw^D males all carried only the maternally derived *white* allele and were thus indistinguishable. The sex ratio was assumed to be 1 : 1 and estimates of bw^D and wild-type sperm use were made using female offspring only.

(b) *Experimental methods*

To determine the effect of spermathecal number on the outcome of sperm competition, 2SP and 3SP females were mated to either (i) bw^D then wild-type males, or to (ii) wild-type then bw^D males. All parental flies were reared at a standard density of 50 larvae per vial. Virgins were collected within 8 h of eclosion and placed in vials in single sex groups of 20. Approximately 260 7-day-old virgin females were anaesthetized using ice and aspirated into individual vials, and each was paired with one 4-day-old bw^D or wild-type male. Pairs were observed for the next 6 h and any that had not mated during this time were discarded. After each mating, males were removed. Seventy-two hours later, females that had previously mated with a wild-type male were given a 7-day-old bw^D male, and each female that had previously mated with a bw^D male was given a 7-day-old wild-type male. Pairs were observed for the next 9 h and any females that did not remate were again discarded. After each successful remating, the males were again removed and females were aspirated into fresh vials. After remating, 104 females had been mated first to bw^D then to wild-type males, and 82 had been mated first to wild-type then to bw^D males. Significantly fewer females mated first to wild-type males remated in the time allowed, compared with females mated in the opposite order ($\chi_{[1]} = 8.061$; $p = 0.0045$), probably because of a lower courtship ability among bw^D males.

After mating, the twice-mated females were transferred to new food with *ca.* 1.5 mg of live yeast. This was repeated every 2 days until the females ceased to produce offspring. To prevent crowding-induced mortality, the numbers of growing offspring were restricted to no more than 125 per vial. The offspring were counted and scored for eye colour on days 11 and 14 after the female parent had been introduced into each vial. This resulted in female offspring counts from each 2-day sample vial (n_r) representing the rate of use of bw^D and wild-type male sperm per 2-day period by parental females.

Parental females were transferred to Eppendorf tubes and frozen at −84 °C until dissection in PBS to determine whether they were 2SP or 3SP females. A small proportion of females resembled 3SP females but had two spermathecae that were fused or shared a single duct. For simplicity, only females with three distinct spermathecae were included in the dataset. The sample size was further reduced by deaths of the female parents before dissection, bringing the final sample sizes to 89 for females mated first to bw^D then to wild-type males, and 58 for females mated first to wild-type then to bw^D males.

3. RESULTS

(a) *Associations between total numbers of offspring, P_2, spermathecal morphology and male genotype*

Associations between total numbers (n_t) of bw^D and wild-type female offspring produced and spermathecal morphology were examined. For females mated first to bw^D then to wild-type males, total numbers of offspring sired by the first male to mate (first-male n_t) produced by 2SP females were compared with first-male n_t produced by 3SP females. Similar comparisons between 2SP and 3SP females were made for second-male n_t and for females mated in the opposite order. Kruskal–Wallis tests were used throughout, because n_t was distributed non-normally. For second-male n_t produced by females mated first to bw^D and then to wild-type males, 3SP females produced

Table 1. (*a*) Median total number of female offspring (n_t) sired by the first and second males to mate 2SP and 3SP females, from the time of the second mating until female sterility. Females were mated first to bw^D and then to wild-type males or first to wild-type and then to bw^D males. (*b*) Median P_2 values (first-male n_t/(first-male n_t + second-male n_t)) for 2SP and 3SP females mated first to bw^D and then to wild-type males or in the opposite order.

(*a*)

mating order	sired by	spermathecae	median n_t
bw^D, wild-type	first male	2	29
		3	13
	second male	2	91
		3	59
wild-type, bw^D	first male	2	42
		3	24
	second male	2	94
		3	82

(*b*)

mating order	spermathecae	median P_2
bw^D, wild-type	2	0.749
	3	0.867
wild-type, bw^D	2	0.723
	3	0.806

significantly fewer offspring than did 2SP females ($p = 0.0283$). Moreover, combining probabilities from all tests of significance (Sokal & Rohlf 1995) showed that, overall, 3SP females produced significantly fewer offspring than did 2SP females ($\chi^2_8 = 15.90$; $p = 0.0438$).

Kruskal–Wallis tests to examine differences in n_t sired by first and second males of different genotypes were carried out on another four comparisons. For 2SP females, first-male n_t produced by females mated first to bw^D then to wild-type males was compared with first-male n_t produced by females mated first to wild-type then to bw^D males. Similar comparisons were carried out for second-male n_t and for 3SP females. Females mated first to wild-type then bw^D males tended to produce more first- and second-male offspring than females mated in the opposite order (table 1*a*). However, this was only significant for first-male n_t produced by 2SP females ($p = 0.0371$) and combining probabilities from all four tests yielded no significant differences ($\chi^2_8 = 11.99$; $p = 0.1514$). Similar tests, and tests on their combined probabilities, were carried out on P_2 values (table 1*b*). This revealed no significant differences between 2SP and 3SP females (for combined probabilities, $\chi^2_4 = 4.14$; $p = 0.3866$), and no associations between P_2 and the genotype of the second male to mate (for combined probabilities, $\chi^2_4 = 4.82$; $p = 0.3061$).

(b) *Associations between offspring produced in 2-day samples, spermathecal morphology and male genotype*

All offspring were collected in 2-day samples and the number of female offspring per 2-day sample is referred to as n_r. The examples in figure 2 show first-male (figure 2*a*) and second-male (figure 2*b*) n_r over time produced by eight individual parental females in the experiment. Figure 3 shows bw^D and wild-type n_r production over time for all 147 parental females. We tested for an effect on these temporal patterns of spermathecal number and genotype of the first and second males to mate. In figure 4, n_r over time has been categorized by whether the parental female had 2SP (figure 4, columns *a–c*, rows (i) and (iii)) or 3SP (figure 4, columns *a–c*, rows (ii) and (iv)), and whether offspring were sired by the first (figure 4*a*) or second male (figure 4*b*) to mate. Females were either mated first to wild-type and then to bw^D males (figure 4*a–c*, (i) and (ii)) or to bw^D then wild-type males (figure 4*a–c*, (iii) and (iv)). Median numbers of offspring produced within each 2-day sample have been illustrated for clarity.

We examined the time (the 2-day sample) at which n_r reached a maximum (t_{rmax}). Kruskal–Wallis tests showed that for females mated first to bw^D and then to wild-type males, 3SP females had a lower t_{rmax} than did 2SP females, both for offspring sired by the first ($p = 0.0357$) and second ($p = 0.0387$) males. n_r therefore rose more rapidly for 3SP compared with 2SP females. In addition, t_{rmax} was lower for wild-type than for bw^D offspring, both for offspring sired by the first (2SP females: $p = 0.0001$; 3SP females: $p = 0.0055$) and second (2SP females: $p = 0.0001$; 3SP females: $p = 0.0231$) males to mate. Use of bw^D sperm was therefore delayed relative to wild-type.

Any differences between 2SP and 3SP females in the numbers of offspring produced during any 2-day sample will indicate a difference in the temporal patterns of n_r. Kruskal–Wallis tests were carried out on n_r (each 2-day sample separately), comparing the same groups as for tests carried out on n_t. After correcting for multiple comparisons, the critical *p*-value for these tests was 0.0071. For second-male offspring produced by females mated first to bw^D and then to wild-type males, n_r on days 6 and 8 was significantly lower for 3SP females than for 2SP females (table 2*a*; figure 4*b*(i) and (ii)). There were similar patterns for first-male offspring, although the main difference (for n_r collected on day 8) was marginally non-significant ($p = 0.0081$; figure 4*a*(i) and (ii)). Similar trends for females mated to males in the opposite order were not significant (table 2*a*; figure 4*a–c* (iii) and (iv)). These results indicate that the falling phase of n_r over time is

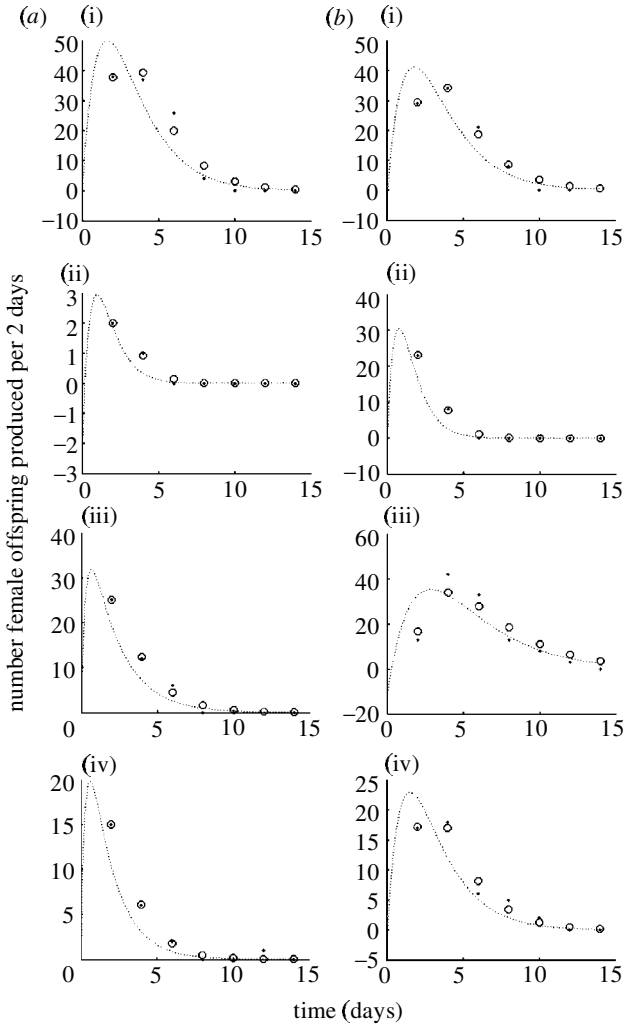

Figure 2. Numbers of female offspring (indicated by dots) produced per 2-day sample (n_r) over time (in days), by eight different parental females, sired by the (*a*) first or (*b*) second male to mate. Dotted lines represent the best-fit two-compartment model to n_r over time (see Appendix A). Open circles represent the integral of the dotted line over 2 days, indicating the model's estimation of n_r from the best-fit parameters. Graphs show n_r over time for: (i) 2SP females mated first to bw^D then to wild-type males; (ii) 3SP females mated first to bw^D then to wild-type males; (iii) 2SP females mated first to wild-type then to bw^D males; and (iv) 3SP females mated first to wild-type then to bw^D males.

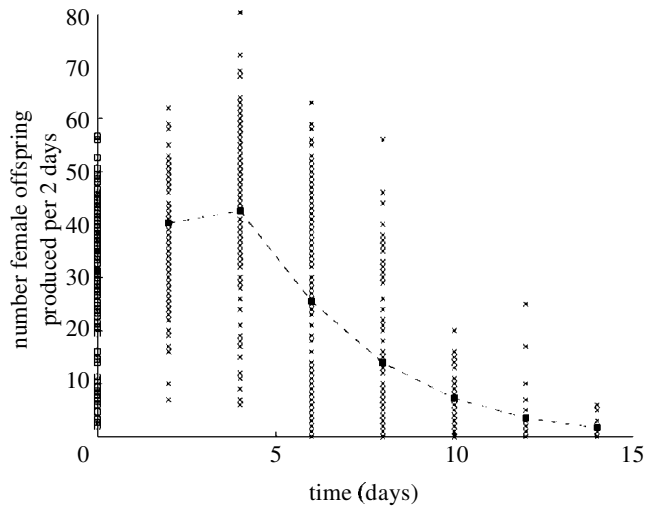

Figure 3. Numbers of female offspring (crosses) produced per 2-day sample (n_r) over time (in days) by each female parent, bw^D and wild-type offspring shown as separate data-points. Open squares (at day 0) indicate the numbers of first-male offspring produced by each female parent during the equivalent time interval before the second mating occurred. The model was fitted to n_r over time for each individual female parent, bw^D and wild-type males separately. The median parameter values from these fits were taken and used in the model to produce re-estimated 'model' values of n_r for each 2-day sample (closed squares).

more rapid for 3SP compared with 2SP females. The analysis also revealed differences in temporal patterns of n_r associated with male genotype. 2SP females mated to males in different orders differed significantly in first-male n_r (collected on days 2 and 4) and second-male n_r (collected on day 2) (table 2*b*; figure 4*a–c* (i) and (iii)). Although similar trends for 3SP females were non-significant, these differences indicate that bw^D n_r over time was delayed relative to wild-type n_r over time.

(c) *Associations between the temporal patterns of offspring production, spermathecal morphology and male genotype*

To summarize temporal patterns of sperm use, a model was fitted to n_r over time for each parental female separately (see examples in figure 2). Differences in temporal patterns of sperm use between groups (figure 4) can be determined by statistical analysis of the best-fit parameters of the model. In a previous study, passive sperm loss in the zebra finch *Taeniopygia guttata* was adequately described using a one-compartment model (Lessells & Birkhead 1990; Colegrave *et al.* 1995). By contrast, the rising and falling phases of n_r over time for females in the present experiment (figures 2 and 3) mean that a one-compartment model was not adequate (see Appendix A). A two-compartment model was therefore used to describe the temporal patterns of first- and second-male n_r for each individual female (see Appendix A). Kruskal–Wallis tests were used to determine if the model parameters were significantly affected by spermathecal morphology and male genotype.

A low value of parameter τ_1 corresponds to a rapid initial falling phase of n_r over time (see Appendix A). For females mated first to bw^D then to wild-type males, parameter τ_1 was significantly lower for 3SP than for 2SP females (table 3*a*) for both first- (not shown) and second-male n_r over time (figure 4*a*,*b* (i) and (ii)). The same trend was seen for females mated in the opposite order (figure 4*a–c* (iii) and (iv)), although the differences here were non-significant (table 3*a*). Combining probabilities from each of the independent tests of significance, showed that, overall, τ_1 was consistently significantly lower for 3SP females than for 2SP females ($\chi^2_8 = 20.82$; $p = 0.0076$). Parameter τ_2 is associated with both the rising phase and (less strongly) with the long-term falling phase of n_r over time (see Appendix A). For females mated first to bw^D and then to wild-type males, parameter τ_2 was lower for 3SP compared to 2SP females (table 3*a*). Moreover, combining probabilities across all Kruskal–Wallis tests carried out on τ_2 (second-male offspring shown in table 3*a*),

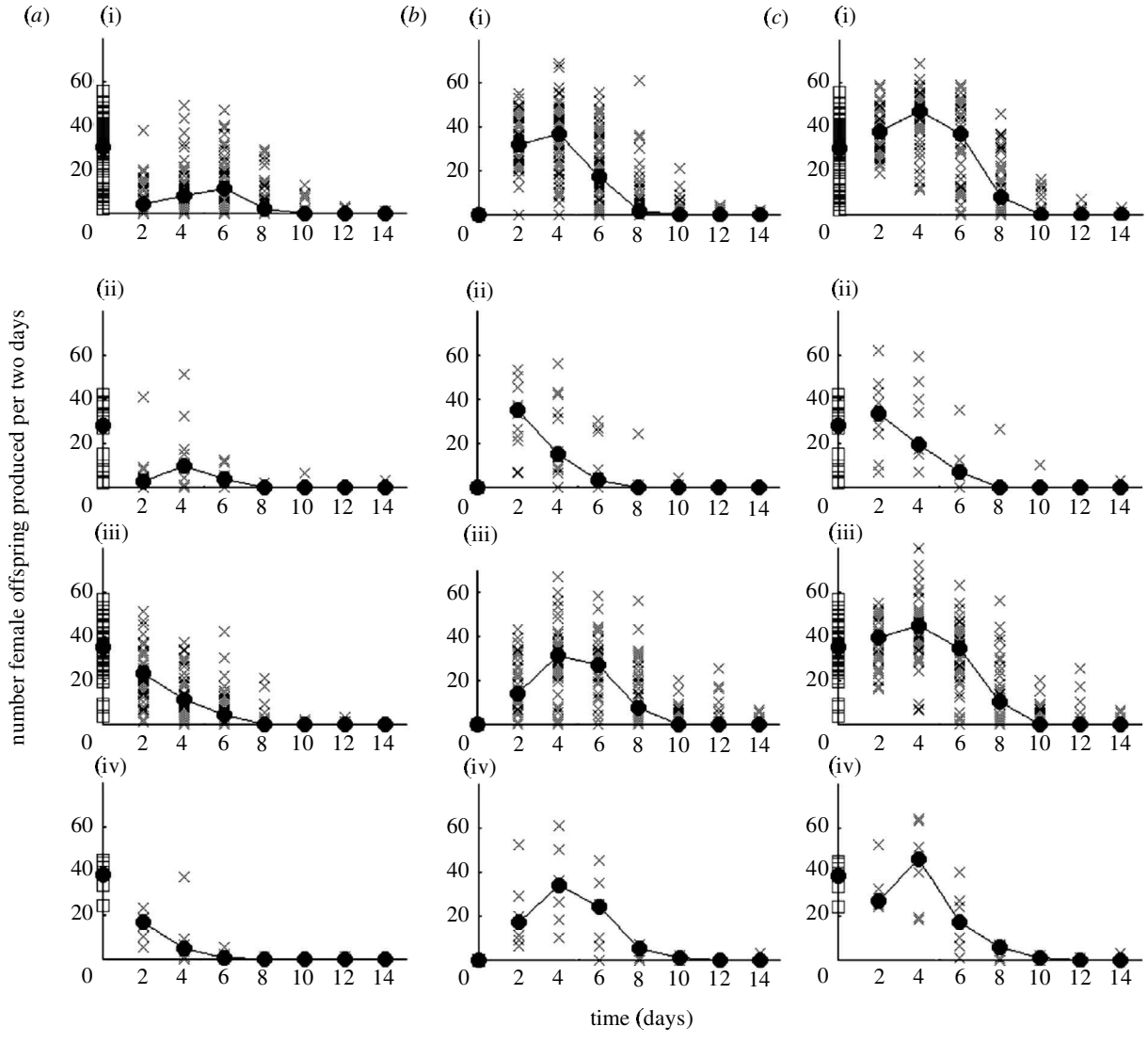

Figure 4. Numbers of female offspring (crosses) produced per 2-day sample (n_r) over time (in days) by each female parent. Graphs show n_r over time sired by (*a*) the first male to mate, (*b*) the second male to mate, and (*c*) the offspring sired by both first and second males. On each graph, filled circles indicate the median n_r across females within time samples. Lines joining the filled circles represent the median n_r over time. Graphs in (*a*) also include the numbers of offspring sired by the first male before the second mating occurred (filled squares) during the equivalent time sample. Graphs show n_r over time for: (i) 2SP females mated first to bw^D then to wild-type males; (ii) 3SP females mated first to bw^D then to wild-type males; (iii) 2SP females mated first to wild-type then to bw^D males; and (iv) 3SP females mated first to wild-type then to bw^D males.

showed that, overall, τ_2 was significantly lower for 3SP females than for 2SP females ($\chi^2_8 = 16.60$; $p = 0.0346$). Thus, these results support the finding that 3SP females have faster rising and falling phases of n_r over time than females with 2SP (figure 4).

There were also significant differences in the temporal patterns of bw^D and wild-type offspring production. Combining probabilities resulting from tests carried out on first- and second-male offspring and across 2SP and 3SP females (table 3*b*), showed that parameter τ_1 was significantly higher for bw^D than for wild-type males ($\chi^2_8 = 54.02$; $p < 0.0001$). The same was true for parameter τ_2 ($\chi^2_8 = 49.05$; $p < 0.0001$). This confirms that for first- and second-male offspring, bw^D n_r over time was delayed relative to wild-type n_r over time (figure 4). Parameter τ_3 also relates to the rising phase, but corresponds most strongly to the overall magnitude of the curves (see Appendix A), where a higher value of τ_3 corresponds to a lower overall magnitude of the curve. Again, combining probabilities from independent tests of significance (table 3*b*), showed that parameter τ_3 was significantly higher for bw^D as compared with wild-type n_r over time ($\chi^2_8 = 28.20$; $p = 0.0004$). This is therefore consistent with previous tests carried out on n_t, where for 2SP females bw^D first males sired fewer total offspring than did wild-type first males.

4. DISCUSSION

(a) *Associations between sperm use and spermathecal morphology*

The results show that analysis of temporal patterns of offspring production can reveal features of sperm competition that are missed by tests carried out on P_2 or on the total numbers of offspring produced. One of the main findings was that the rate of offspring production by females with three spermathecae (3SP) increased and decreased

Table 2. Median n_r produced during 2-day samples from time of remating until female sterility.
(Significant p-values (after correcting for multiple comparisons, critical value $p = 0.0071$) are marked with *. p-values result from Kruskal–Wallis tests carried out on each measurement of n_r separately to compare (*a*) offspring production by 2SP and 3SP females (results for second-male offspring only are shown here) and (*b*) offspring sired by the first and second males of bw^D or wild-type genotype (offspring produced by 2SP females only are shown here).)

(*a*)

			days						
sired by	mating order	spermathecae	2	4	6	8	10	12	14
second male	bw^D, wild-type	2SP	32	35	16	1	0	0	0
		3SP	34	13.5	3	0	0	0	0
		p-value	0.9681	0.0098	0.0013*	0.0030*	0.3428	0.0864	0.2818
second male	wild-type, bw^D	2SP	13	29	26	4	0	0	0
		3SP	17	34	24	5	1	0	0
		p-value	0.4506	0.4722	0.7419	0.3642	0.8194	0.1785	0.5382

(*b*)

			days						
spermathecae	sired by	mating order	2	4	6	8	10	12	14
2SP	first male	bw^D, wild-type	2	5	4	0	0	0	0
		wild-type, bw^D	22	10.5	3.5	0	0	0	0
		p-value	0.0001*	0.0055*	0.4415	0.0969	0.2852	0.9505	0.4058
2SP	second male	bw^D, wild-type	32	35.5	16.5	1	0	0	0
		wild-type, bw^D	13	29	26	4	0	0	0
		p-value	0.0001*	0.0396	0.3087	0.0664	0.0843	0.2283	0.5149

Table 3. Median values for best-fit parameters of the two-compartment model (see Appendix A) to n_r over time.
(p-values are the result of Kruskal–Wallis tests carried out on the best-fit parameters to compare n_r over time for: (*a*) 2SP and 3SP females (results for second-male n_r only are shown here); and (*b*) offspring sired by the first and second males of different genotypes (results for 2SP females only are shown here). Significant p-values are marked with *.)

(*a*)

			parameters				
sired by	mating order	spermathecae	ν_1	ν_2	τ_1	τ_2	τ_3
second male	bw^D, wild-type	median for 2SP	410 000	5×10^{-5}	1.590	1.560	5420
		median for 3SP	922 000	0.0031	1.050	1.150	8510
		p-value	0.9359	0.4847	0.0031*	0.0334*	0.9556
second male	wild-type, bw^D	median for 2SP	157 500	−0.0006	2.250	2.240	4430
		median for 3SP	124 000	−2.27	2.050	1.780	368
		p-value	0.6494	0.8202	0.6862	0.3247	0.3372

(*b*)

			parameters				
spermathecae	sired by	mating order	ν_1	ν_2	τ_1	τ_2	τ_3
2SP	first male	bw^D	2×10^6	−0.223	2.59	2.41	134 000
		wild-type	21 600	0.0007	1.145	1.005	21.95
		p-value	0.0003	0.0002*	0.0001*	0.0001*	0.0001*
2SP	second male	bw^D	157 500	−0.0006	2.25	2.24	4430
		wild-type	410 000	0.0005	1.59	1.56	5420
		p-value	0.6336	0.1466*	0.0001*	0.0002*	0.8837

more rapidly than for females with two (2SP), although this difference was significant only for females mated first to bw^D then to wild-type males. These temporal patterns are important because, if a female dies or remates with a third male, second male reproductive success will be higher if his sperm is used faster (Anderson 1945; Prout & Bundgaard 1977; Gilchrist & Partridge 1995). Thus, analysis of temporal patterns of sperm use can provide insights into the potential effects of traits on male reproductive success.

The rapid rise and fall of the rate of offspring production for 3SP compared with 2SP females may have been due to the faster release of sperm from three spermathecae with three ducts compared with two spermathecae and two ducts. This could result in the loss of coordination between sperm release, ovulation and fertilization. An earlier study (Bouletreau-Merle 1977) showed that the removal of one spermatheca from 2SP females also resulted in a rapid falling phase of offspring production. The present results are therefore consistent with two alternative hypotheses. The first is that 3SP females had reproductive tract defects that could not be detected under the microscope but represented a system functionally equivalent to the one-spermathecal system. This would result in a reduction of sperm storage capacity and perhaps a reduction in the ability to maintain viable sperm for fertilization longer than 4 days (Anderson 1945; Filosi & Perotti 1975). A second idea is that a careful balance is required between sperm storage, release and fertilization mechanisms, reflecting a more coordinated system than previously thought. Under both hypotheses, females with unusual spermathecal morphology may rely more for sperm storage on the seminal receptacle, which fills and releases sperm sooner than do the spermathecae (Gilbert 1981).

(b) *Associations between sperm use and first and second male genotype*

Male genotype also affected the temporal pattern of sperm use. Comparisons across mating orders of the numbers of first-male offspring produced before the second mating with those produced after the second mating indicates that bw^D males were less able to displace first-male sperm. The reduced displacement of wild-type first-male sperm meant that maximum first-male offspring production occurred significantly later for bw^D compared with wild-type offspring, and this was consistent with the significantly higher values of the model parameter τ_2 in this group. Poor displacement by bw^D males is consistent with direct observations of sperm storage in doubly mated females, and may be an effect either of genetic background or of bw^D itself (Civetta 1999).

For second-male offspring too, the rising phase of bw^D offspring production was slower than for wild-type offspring, a delay that could be associated with the reduced ability of bw^D males to displace first-male sperm. However, values of parameter τ_1, describing the falling phase of offspring production, indicated that bw^D males continued to sire offspring for longer than did wild-type males (also see figure 4). Although bw^D second males did not suffer in total offspring numbers produced (table 1*a*), this delay in the temporal pattern of sperm use is important. It indicates that the reproductive success of bw^D compared with wild-type males could have been compromised had females mated a third time.

Sperm use by females and the extent to which males affect female post-mating physiology are areas of intensive current interest. This study shows that the temporal pattern of sperm use is potentially an important determinant of success in sperm competition. We would not have seen such a complete picture of events had our analysis been restricted to the total numbers of offspring produced.

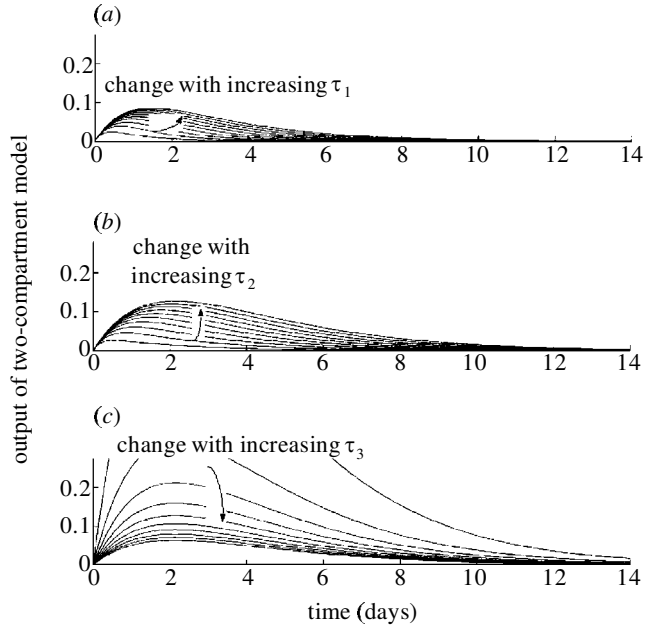

Figure 5. The shape of the curve defined by the two-compartment model over time undergoes changes according to small changes in parameters (*a*) τ_1, (*b*) τ_2 and (*c*) τ_3. Arrows indicate the change in the curve as the value of each of the parameters is increased.

Methods for carrying out analysis of the temporal patterns of sperm use will facilitate future investigation in this area.

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APPENDIX A

Graphs of n_r over time produced by eight individual females are shown in figure 2. A two-compartment model was fitted to these temporal patterns of n_r for each female, bw^D and wild-type males separately. In figure 2, the model is illustrated by the dotted line and was integrated to produce re-estimated values of n_r (open circles). Although the two-compartment model is unnecessarily complex for fitting n_r in the example in figure 2*a*(iii), it is able to fit the rising and falling phases seen in, for example, figure 2*b*(i). Figure 3 shows bw^D and wild-type n_r over time for all 147 females in a single graph.

The differential equations that describe the two-compartment model are as follows:

$$\frac{d\nu}{dt}(1) = \frac{(\nu_2 - \nu_1)}{\tau_1}, \tag{A 1}$$

$$\frac{d\nu}{dt}(2) = -\left(\frac{\nu_2}{\tau_2}\right) - \left(\frac{(\nu_2 - \nu_1)}{\tau_3}\right). \tag{A 2}$$

A fitting program (MATLAB, v. 6.0.0.88; release 12) was used to search for the values of parameters of the two-compartment model (ν_1, ν_2, τ_1, τ_2, τ_3) that minimized the residuals (using MATLAB function *fminsearch*) between empirical values of n_r and model estimates of n_r. Parameter τ_1 (figure 5) is a time constant strongly associated

with the initial falling phase of the curve, where a higher value of τ_1 corresponds to a longer initial falling phase. Parameter τ_2 (figure 5) is associated with both the rising and long-term falling phases, and again, the higher the value of this parameter the longer these phases and flatter the overall appearance of the curve. Because it corresponds to both the rising and falling phases, τ_2 should not necessarily be taken as evidence of significant differences in the rising phase without supporting evidence from independent tests. Parameter τ_3 (figure 5) also relates to the rising phase of the curve, but more strongly influences the overall magnitude of the curves. Thus, a higher value of τ_3 corresponds to a longer rising phase, but more strongly corresponds to a lower overall magnitude of the curve.

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