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Female age and sperm competition: last-male precedence declines as female age increases

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Until very recently, most studies of sperm competition have focused on variation in male competitive ability. However, we now know that a number of reproductive traits, including oviposition rate, use of stored sperm and receptivity to mating, vary with female condition. Because females can play an active part in the movement of sperm within their reproductive tract, sperm competition may be influenced by female condition. Existing studies of sperm competition in fruitflies ignore the effects of female condition, using females that are 3–4 days old and in their reproductive prime. But condition will decline as a female senesces. Here, we examine the effect of female age on the outcome of sperm competition in three strains of the fruitfly, *Drosophila melanogaster*. Previous studies have shown that female age influences preference for mates and male ejaculation strategies. In this study, we find that when males are mated to females that are older than 17 days, last-male sperm precedence decreases significantly. These results could lead to a greater understanding of the physiological mechanisms that regulate the outcome of sperm competition.

Keywords: accessory gland proteins; Acps; *Drosophila melanogaster*; female condition; intersexual conflict; sperm competition

1. INTRODUCTION

Most studies of sperm competition have focused on variation in male competitive ability (Gromko & Pyle 1978; Clark *et al.* 1995; Snook 1998), diversity of male competitive structures (e.g. Waage 1979) and most recently, male accessory gland proteins (Acps) (reviewed in Chapman *et al.* 2000). Acps have been shown not only to increase female oviposition rates (Heifetz *et al.* 2000), facilitate sperm storage (Neubaum & Wolfner 1999; Chapman *et al.* 2000), and incapacitate sperm from previous males (Harshman & Prout 1994; Prout & Clark 2000), but also to reduce mating receptivity (Manning 1962; Leopold *et al.* 1971; Obata 1988; Eady 1995; Miyatake *et al.* 1999) and increase mortality rates (Chapman *et al.* 1995) in mated females. While males stand to benefit from each of these functions, some are clearly deleterious for females.

Females should be selected to counter male adaptations that decrease female fitness (Chapman *et al.* 1995; Rice 1996; Hellriegel & Bernasconi 2000). The stage is thereby set for sexually antagonistic coevolution, where females evolve means to reduce deleterious male effects and males, in turn, evolve new mechanisms to manipulate females (Rice 1996). Several lines of evidence indicate that antagonistic coevolution of this sort occurs. First, reproductive proteins in insects and other organisms evolve at an unusually rapid rate (e.g. Clark & Kao 1991; Palumbi 1999; Wyckoff *et al.* 2000; Swanson *et al.* 2001). Second, experiments that artificially increase (decrease) the strength of sexual selection on males lead to a concomitant decrease (increase) in female fitness (Rice 1998; Holland & Rice 1999). Third, comparative studies in insects have revealed that optimal mating rates for females are lower than those for males and that female fitness is reduced at male optima (Arnqvist & Nilsson 2000; Arnqvist & Rowe 2002). Finally, the ability of males of given genotypes to succeed in sperm competition with other males depends on the genotype of the female whose reproductive tract is the site of competition (Wilson *et al.* 1997; Clark & Begun 1998; Clark *et al.* 1999).

Although both sexes participate in this coevolutionary arms race, we know surprisingly little about how females respond to antagonistic male adaptations or about the extent to which female countermeasures influence reproductive interactions. What little we do know about the role of female variation is in terms of sexual selection. Results from recent studies point to the importance not only of variation in factors such as nutrient availability (e.g. Gromko & Gerhart 1984; Chapman & Partridge 1996), temperature (Ward 2000), mating history (Chapman *et al.* 1995; Rice 1996) and larval environment (Hodin & Riddiford 2000), but also female age. In a study of the cockroach (*Nauphoeta cinerea*), Moore & Moore (2001) found that older females were less choosy and exhibited lower reproductive quality than younger females. In dung flies (*Sepsis cynipsea*), Martin & Hosken (2002) found that males copulated for longer with older females than with younger ones. The study of age differences might be a simple yet particularly powerful way to study the effects of female variation on sexual selection.

As females age, virtually all demographic, behavioural and physiological parameters eventually decline in quality (Finch 1990; Rose 1991). Prior studies of insect sperm competition either ignore female age (e.g. Danielsson 2001) or employ females that have just reached sexual maturity (e.g. Saul & McCombs 1993; Edvardsson & Arnqvist 2000). Among 20 years of sperm competition studies in *Drosophila melanogaster*, females ranged from 3–6 days of age (e.g. Gromko & Pyle 1978; Letsinger & Gromko 1985; Clark & Begun 1998). Previous work indicates that females play an active part in sperm manipulation by assisting sperm transport within their reproductive tracts (e.g. Arthur *et al.* 1998; Bloch-Qazi *et*

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al. 1998; Hellriegel & Bernasconi 2000). If measures of sperm competitive ability change with female age, this would provide further evidence that females play an active physiological or behavioural part in determining the outcome of sperm competition.

At present, there are, to our knowledge, no existing models to predict how patterns of sperm competition should be affected by female age. In *D. melanogaster*, when a female mates with multiple males, the last male to inseminate the female typically sires most of the offspring. Males and females both play an important part in determining the extent of last-male paternity (Wilson *et al.* 1997; Clark *et al.* 1999). As a female senesces, she may exert less influence over the dynamics of sperm competition. In particular, she may lose her ability to affect the proportion of her eggs that are fertilized by stored versus newly inseminated sperm. Which of these two processes is most affected by female age will determine the degree of last-male sperm precedence.

By studying how female age affects sperm competition, we may be able to develop a more complete picture of the dynamics of sperm competition, and more importantly, to understand just how active a part the female plays. To that end, the following study, to our knowledge, provides the first assessment of how the outcome of sperm competition varies among females of different ages.

2. METHODS

To determine the effects of female age on sperm competition, we assayed last-male paternity (P2) for three female ages using three inbred female strains and three wild-type male genotypes. We calculated P2 as the proportion of the total offspring sired by the second male to mate in a two-male sequence; conversely, the proportion of total offspring sired by the first male to mate, which we consider briefly below, is referred to as 'P1' (Boorman & Parker 1976).

(a) Drosophila *stocks*

The wild Georgia line of flies (GA98 strain), from which we derived males for our sperm competition assay, was generated from approximately 250 inseminated females collected from a peach orchard in Watkinsville, GA, USA, in August 1998. We maintained this population in a 30 l Plexiglas population cage with overlapping generations at a density of approximately 5000 individuals for six months before the start of the experiment. The inbred strains, 79L, 67L and 58S, are from a set of 98 recombinant inbred (RI) lines developed for quantitative trait loci mapping by T. F. C. Mackay at NCSU, Raleigh, NC, USA (Nuzhdin *et al.* 1998). The bw^D line was kindly provided by Dr Jerry Coyne, University of Chicago. Flies were maintained throughout the experiment on a standard yeast–agar–cornmeal–molasses medium at 24 °C on a 12 L : 12 D cycle. Except where otherwise noted, all individuals were first collected under light CO_2 anaesthesia, then maintained in 8 dram vials at a density of 20 flies per vial on 5 ml of standard medium, with live yeast added.

(b) *Production of females: generating females of different ages*

Outbred lines are, by definition, genetically heterogeneous. If allelic variation is related to survival rates, then different age classes may not be random samples of their initial cohort (Vaupel & Yashin 1985; Service 2000). By using RI lines, we minimized the possibility that differences in P2 among females of different ages could be confounded by genetic effects.

To obtain virgin females for assay, we first expanded the three RI lines (hereafter 'genotypes') for two generations in plastic bottles containing *ca.* 50 ml of medium. Larval density was maintained at *ca.* 250 larvae bottle^{-1} throughout population expansion. We then collected and held virgin females in a single 3.8 l clear plastic jar for each genotype, at initial densities of 1000 females jar^{-1}. Each jar was supplied with fresh food every other day. We collected at two-week intervals to obtain the following age classes: 3.5 days, 17 days and 31 days. Data on the age distribution of female flies in natural populations were not available, hence we elected to sample across a range of ages at which females continue to reproduce under laboratory conditions (D. Promislow, personal observation). For a more detailed description of the methods used to culture females, see Priest *et al.* (2002).

(c) *Production of males*

To test for interactions between female age and male genotype and for interactions between female genotype and male genotype, we created three different male genotypes. Each wild-type male genotype (MT1–3) was created by crossing two different extraction lines initially derived from the GA98 base population via standard techniques (Ashburner 1989). Males resulting from each cross were identically heterozygous for both chromosomes II and III and these males were subsequently used to mate secondly in the sperm competition assay. We created a similar genotype (identically heterozygous for chromosomes II and III), but homozygous for the dominant marker, bw^D, for use as the competing first male in our assays. We obtained this competitor (C) genotype by crossing two extraction lines derived from a subpopulation bearing bw^D on the GA98 background.

To measure P2, we mated each female first to a C male and again 3 days later to a male of one of the three male genotypes (MT1–3) assigned at random.

(d) *Sperm competition assay*

We assayed P2 in virgin females from each of the three female genotypes simultaneously. Initial matings took place *en masse* in plastic half-pint bottles. At *ca.* 17.00 on day 0, we placed approximately 40 virgin females of the same genotype and age into a bottle, then added 60–65 virgin 3.5–4.5 day-old C males. All bottles were held at 24 °C for 3 h, then we transferred females individually to vials. Prior to the second mating, we excluded all unmated females.

Seventy-two hours later, we placed two males of the same genotype, assigned at random, into the vial with each female and allowed them to remain together overnight. We then transferred all females to a fresh vial (vial 1). Females were transferred to fresh food vials two more times, at 3 days (vial 2) and 6 days (vial 3). We collected offspring from each vial 16 days after set-up, then calculated P2. All flies were handled, collected and transferred during this assay without using anaesthesia.

Finally, because estimates of P2 could be influenced by viability differences between the larvae sired by the two types of male used in the assay, we carried out a separate larval competition assay. Larvae sired by either MT1 or MT2 males that had mated singly to females of one of the three age classes were raised with larvae produced similarly in matings of C males (MT3 males were not tested). Fifty larvae of each type were

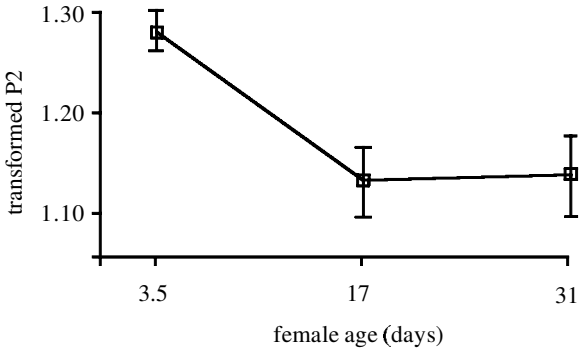

Figure 1. Mean arcsine-transformed P2 estimates by female age; all female strains are combined.

placed in each assay vial with 20 vials per assay; we subsequently tested for departure from a 1 : 1 ratio in the adults that eclosed.

(e) *Statistical analyses*

Because our measures of P2 were distributed non-normally, we carried out an arcsine square root transformation. To test for effects of female age on P2 within each line, we conducted two-factor ANCOVAs, with female age as the covariate and male genotype as a random effect. To test for the possibility of an interaction between female genotype and male genotype, we combined the three inbred line datasets and performed an ANCOVA with female age as a fixed continuous effect, and both female genotype and male genotype as random effects.

In some cases, the arcsine transformation did not adequately normalize the distribution of P2. Thus, we also carried out non-parametric Kruskal–Wallis one-way analyses of variance (corrected for ties), with female age and male genotype as single factors in separate analyses for each female genotype. All parametric analyses were done using JMP (SAS Institute 2000). The non-parametric analyses were performed using STATVIEW (Abacus Concepts 1994). In all cases, we included only females for which we had a complete set of data (vials 1–3).

3. RESULTS

While the conclusions and associated p-values from our parametric and non-parametric analyses agree closely, the appropriate non-parametric tests do not allow us to test for interactions between factors. Thus, we present the results of both parametric and non-parametric analyses here. Because we found no significant viability differences among larvae sired by competing males within any of the different female age–male genotype pairings tested (3.5 days: $t_{38} = -1.67$, $p = 0.10$; 17 days: $t_{38} = -0.46$, $p = 0.64$; 31 days: $t_{36} = 1.30$, $p = 0.20$), it was not necessary to adjust the raw data prior to transformation.

In each of the three experiments, we tested for differences in P2 values among female age groups (3.5 days, 17 days and 31 days) and also among females mated to each of the three different male genotypes (MT1–3).

(a) *Effect of female age and genotype*

Estimates of mean P2 were significantly higher in the youngest females than in the older two female age classes (figure 1; table 1) when all female genotypes were combined. When we considered individual female genotypes separately, 67L and 58S females exhibited declines that were highly and marginally significant, respectively (figure 2; table 2). The decline observed in 67L females remained significant after Bonferroni correction (Holm 1979; Rice 1989). The 79L females showed a non-significant decline (table 2).

Female genotype had a significant effect on P2 (figure 2; table 1). We also observed a significant interaction between male and female genotype (table 1).

(b) *Effect of male genotype*

We found significant differences among the three male genotypes assayed, with MT1 males having higher P2 values than MT2 or MT3 males in all female genetic backgrounds (figure 3). However, this difference was significant only for males mated to 58S and 67L females (table 2) and remained so after correction for multiple tests.

(c) *Female age by male genotype interaction*

We found no single female genotype in which there was a significant interaction between female age and male genotype (figure 3; $F_{2,133} = 0.115$, $p = 0.89$; $F_{2,144} = 2.336$, $p = 0.101$; $F_{2,89} = 1.10$, $p = 0.34$, for 58S, 67L and 79L, respectively). However, the female age × male genotype interaction among female genotypes combined, while not significant (table 1), indicated that we might have detected a significant interaction given increased sample size and statistical power.

4. DISCUSSION

Our results demonstrate that the strength of last-male sperm precedence declines in older females, and thus indicates strongly that the outcome of sperm competition may depend on the condition of the female involved. In addition, in line with previous studies (Wilson *et al.* 1997; Clark & Begun 1998; Clark *et al.* 1999), we found significant variation for P2 among male genotypes, and a significant female genotype × male genotype interaction.

What are the possible mechanisms that could lead to a change in P2 with female age? As stated earlier (§ 1), the outcome of sperm competition depends upon the fate of both stored and newly received sperm. Because these two types of sperm may be affected by different processes in the female reproductive tract, the sperm type most affected by female age will determine how P2 changes. We can envisage both adaptive and non-adaptive hypotheses that might explain our results, and outcomes that are determined by changes in either female or male behaviour.

One non-adaptive explanation for the observed change in P2 is that as females age, they lose the ability to take up newly received sperm in the seminal receptacle due to the general physiological decline that results from senescence. Alternatively, an age-related decrease in P2 may occur if females respond adaptively to their diminishing residual reproductive value. Because life expectancy decreases with age in flies, older females are less likely to survive long enough to remate than younger ones. In the event that a younger female remates with a higher-quality male, she can increase fitness if she uses more of this newly received sperm relative to the amount that she would use if that male were of comparable quality to her previous

Table 1. Influence on P2: lines combined.
(Lines and vials combined: ANCOVA F and p-values, and female age as the covariate. Data arcsine-transformed prior to analysis. All significant terms ($p < 0.05$) are in bold.)

factor	d.f.	F	p
female age	1	20.896	**< 0.0001**
male genotype	2	4.425	0.073
female genotype	2	13.655	**0.009**
female genotype × female age	2	3.814	0.118
female genotype × male genotype	4	3.002	**0.024**
male genotype × female age	2	5.438	0.069
male genotype × female age × female genotype	4	0.450	0.77

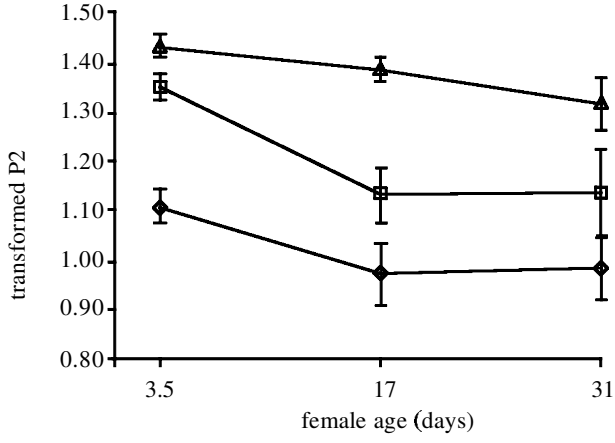

Figure 2. Mean arcsine-transformed P2 estimates by age, each female genotype is separate. Individual lines depict the female strain: diamonds, data from males mated to 58S females; squares, data from males mated to 67L females; triangles, data from males mated to 79L females.

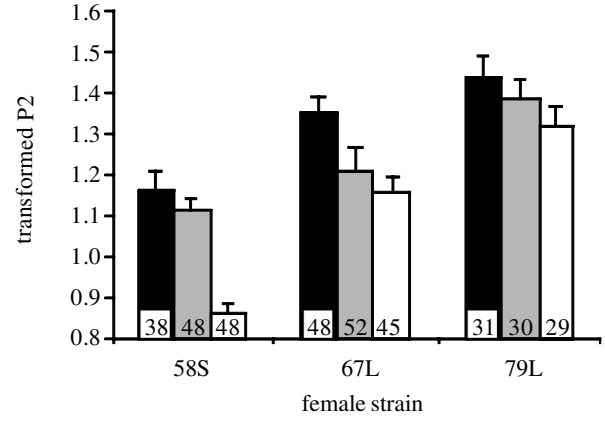

Figure 3. Mean arcsine-transformed P2 by female genotype, MTs separate. Individual bars depict means for each MT: black bars, MT1; grey bars, MT2; white bars, MT3. Numbers at the base of each bar indicate sample size.

Table 2. Influence on P2: individual lines.
(Individual lines, all vials combined: Kruskal–Wallis H and associated p-values; ANCOVA F and p-values, and female age as the covariate. Data arcsine-transformed prior to analysis. All significant terms ($p < 0.05$) are in bold.)

line	factor	d.f.	H	p	F	p
58S	female age	1	5.793	0.055	4.173	**0.043**
	male genotype	2	13.391	**0.0012**	17.831	**< 0.0001**
	error	128				
67L	female age	1	16.583	**0.0003**	15.526	**0.00012**
	male genotype	2	10.066	**0.0065**	3.130	0.146
	error	139				
79L	female age	1	2.746	0.25	5.271	**0.024**
	male genotype	2	3.295	0.19	1.947	0.18
	error	89				

mate. By contrast, it may be optimal for older females to use all available sperm. This would explain our observation that P2 values in younger females were higher than in older females.

Males, too, could determine the outcome of P2 as females age. In a recent study on dung flies (*S. cynipsea*), Martin & Hosken (2002) compared copulation duration when males were paired with either young or old females, and as either first or second males to mate. They found that with young females, first matings were significantly shorter than second matings and that first males appeared to transfer fewer sperm than second males. In older females, first males mated for as long as second males. Martin & Hosken (2002) argued that male *S. cynipsea* are treating younger females as virgins and older females as non-virgins. If this pattern is also true in the wild, males mating with older females are more likely to experience sperm competition so they extend copulation to transfer more sperm. One could thus argue that an age-related increase in copulation duration among first males could have led to the decline in P2 that we observed. The likelihood of this explanation will depend on the relationship

between copulation duration and transfer of sperm and/or Acps (Gilchrist & Partridge 2000).

Finally, our results may be due to differences in the strength of selection that has shaped defensive mechanisms (P1) versus offensive mechanisms (P2) over evolutionary time. In the wild, female *Drosophila* typically carry viable sperm from multiple males (Anderson 1974; Harshman & Clark 1998; Imhof *et al.* 1998, but see Gromko & Markow 1993). Thus, only the last of such mates achieves the large share of paternity that typifies P2, at least in *Drosophila*. If males achieve most of their paternity in competition with ejaculates from subsequent rivals, then the mechanisms underlying P1 should be under much stronger selection than the mechanisms that mediate P2. There is some indirect empirical support for stronger selection on P1 than P2. When assaying P2 in *D. pseudoobscura*, Turner & Anderson (1984) found that first male genotype was a significant factor in determining P2 while second male genotype was not. If stronger selection on P1 than P2 is a more general phenomenon, and if the mechanisms underlying P1 and P2 are at least partially independent, as appears to be the case (Clark *et al.* 1995; Civetta & Clark 2000; Sawby & Hughes 2001), we might expect P1 to be less variable than P2 in the changing environment of an ageing female reproductive tract.

Do our results apply in natural populations? Although we have no data on age structure in wild populations of *D. melanogaster*, recently caught wild strains live in excess of 100 days in the laboratory (Linnen *et al.* 2001) and remain fertile for close to two months (D. Promislow, personal observation). In addition, females undergo reproductive diapause as a means of overwintering (e.g. Saunders & Gilbert 1990; reviewed in Tatar *et al.* 2001). Thus, older female *D. melanogaster* may comprise a significant proportion of the actively breeding females in spring populations. The interaction between age structure and sexual selection may influence the genetic structure of populations. To the extent that genetic structure in a population depends on variance in male mating success (Nunney 1999), our results imply that matings with older females, where variance in male mating success is reduced, might actually increase effective population size. The results presented here also point to the general need for age-structured models of sexual selection. Despite numerous calls for a general age-structured model of mate choice and sexual selection (Partridge & Endler 1987; Promislow *et al.* 1992; Svensson & Sheldon 1998), only recently have theoreticians turned their attention to this problem. Most efforts have focused on female preference for older versus younger males (Hansen & Price 1995; Kokko & Lindström 1996; Kokko 1998; Beck *et al.* 2002). These models do not consider the possibility that optimal behaviour in both males and females may change as the female ages (e.g. Engqvist & Sauer 2002).

In addition, the age-related decline in P2 that we observed has several important implications for future studies of sperm competition. First, experimental efforts should be directed at testing the alternative hypotheses presented above to determine which of these is most likely to explain our observations. Second, it would be of great interest to know whether or not the age-related decline in P2 that we observed here is a general phenomenon and, if so, why, as high P2 values have been found in over 70% of all species tested so far (Simmons & Siva-Jothy 1998). Third, models of mate choice and sperm competition that incorporate age-related changes in P1 or P2 could prove fruitful. Recent studies of maternal age effects indicate that males who mate with older females may produce fitter offspring (Priest *et al.* 2002). Thus, the effects of female age on sperm competition may also turn out to be an important factor in optimal male mating strategies. Finally, because the effects of female age may reflect the more general influence of female condition on the outcome of sperm competition, further progress in understanding the mechanisms of sperm precedence and how they have evolved will require a more complete view of how male genotype × female genotype effects are influenced by a female's physiological state.

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As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.