PAUL DOUGLAS MACK
Sperm Competition and The Role of Females in *Drosophila melanogaster*
(Under the Direction of DANIEL PROMISLOW)

Although the phenomenon of sperm competition, the post-mating interaction of ejaculates within multiply mated females, has been studied extensively over the past 30 years, we still have much to learn about the mechanisms underlying sperm competition. Until very recently the majority of research has focused on male aspects of sperm competition. Here we add to the growing body of evidence for a significant female role in the outcome of sperm competition, specifically, and reproductive decisions, in general. First, we show that the ability of male *Drosophila melanogaster* to incapacitate stored sperm when they mate with previously mated females varies with male genotype and with the time interval since the female last mated. Second, we demonstrate the importance of the age of female *D. melanogaster* in the outcome of sperm competition. We explicitly tested the effect of age on the proportion of paternity achieved by the last male to mate with the female. However, our result indirectly supports the conclusion that female age may have more significant impacts on the reproductive success of previous (vs. last) males to mate with a female. Third, we test the hypothesis that first (e.g., prior) male paternity, or sperm defense, should be negatively correlated with the degree of relatedness between members of a mated pair in *D. melanogaster*. We provide the strongest evidence to date for the significance role of genetic relatedness on sperm competitive ability. In addition, taken together, our results from both the sperm incapacitation study and the relatedness study strongly suggest female genotype x male genotype interactions, although they do not explicitly test the hypothesis that such interactions exist. In addition to our investigations of sperm competition, we include a study of the age-specificity of novel mutations on male mating ability and fecundity. In that study, we detail the age-specific effect of cumulative novel mutations acting on both traits. Our result suggests that although age-specificity is present, the affect appears to be

transient. Overall, we demonstrate the importance of female age and genotype as well as male genotype, on the outcome of several important reproductive interactions.

INDEX WORDS: intersexual conflict, *Drosophila melanogaster*, sperm competition, accessory gland proteins, genetic relatedness, sperm selection, Aging, fecundity, male mating ability, mutation accumulation, senescence. female genotype, sperm offense, sperm defense, sperm incapacitation.

SPERM COMPETITION AND THE ROLE OF FEMALES IN *DROSOPHILA MELANOGASTER*

by

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DEDICATION

This work is dedicated to Corrie and David who generously urged me to pursue it.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Parker (1970) began a whole new field of evolutionary biology when he first suggested that males could continue competing with other males even after they had finished mating. Parker (1970) reasoned that males' should be selected for their ability to produce ejaculates (sperm and other components of the seminal fluid) that interact competitively with the ejaculates of other males who mate with the same female. Then a revolutionary concept, now well established, sperm competition has received so much attention since that two lengthy volumes summarizing all the work have been written thus far (Smith 1984; Birkhead and Moller 1998). In addition, the number of species for which females are known to mate multiply and store sperm has grown considerably since sperm competition was first hypothesized (Birkhead and Moller 1998). Indeed, genetic monogamy is now viewed as comparatively rare (Birkhead and Moller 1998, p.757). Thus, sperm competition is both common and widespread (Birkhead and Moller 1998).

For some arthropods, such as bushcrickets (Tettigoniadae), locusts (Locustidae), beetles (Coccinellidae), stalk-eyed flies (Diopsidae), and arachnids, especially spiders and mites, the first male to mate with a female sires most of the subsequent offspring, even after the female mates a second time (Simmons and Siva-Jothy 1998). For most other taxa, such as the majority of insects, and including *Drosophila*, the last male to mate sires most of the subsequent offspring, a phenomenon known as last male sperm precedence (Gromko *et al.* 1984; Birkhead and Moller 1998; Simmons and Siva-Jothy 1998). Many factors, in addition to mating order, are known to influence the male success at sperm competition. Various male-oriented parameters have been measured with respect to sperm competitive ability. Copulation duration (which could also be viewed as

a female-influenced parameter), for instance, has been measured in insects, including dragonflies (Michiels 1992), dung flies (Parker and Simmons 2000), and bushcrickets (Wedell 1998; Simmons and Achmann 2000). Copulation duration has also been studied rather intensively in the Drosophilidae (Gromko et al. 1984; Saul *et al.* 1988; Gilchrist, 2000; Bundgaard and Barker 2000). Other male parameters investigated are sperm transfer, effects of male size (Pitnick and Markow 1994; Zeh *et al.* 1997; Arnqvist and Danielsson 1999), time to mating, remating rate, sperm size and allocation (Pitnick and Markow 1994) etc. and, most significantly within the Drosophiladae and a few other insect taxa, accessory gland proteins (Acps).

More recently, investigators have begun to first suspect and then demonstrate that females play considerably more than the passive role they were once ascribed (Gowaty 1994). The suggestion that females might influence, possibly even control, the use of sperm was first made by Tyler (1948) over half a century ago. Several authors (Bedford 1965; Cohen 1969; Lloyd 1979) reiterated Tyler's (1948) original idea, before Thornhill (Thornhill 1983, 1984) formalized female influence during and after mating as cryptic female choice (CFC). More recently, Eberhard (Eberhard and Cordero 1995; Eberhard 1996, 2000) has extended our thinking on CFC considerably. Currently, the idea that females might be able to 'select' individual sperm from sperm they have stored is both controversial (Birkhead 1998) and hotly debated (Birkhead 2000; Eberhard 2000; Pitnick and Brown 2000).

Females of various species have now been shown to play a prominent role in the post-mating fate of sperm. For instance, females actively move sperm within their reproductive tract both during and after mating (Arthur *et al.* 1998; Bloch-Qazi *et al.* 1998; Hellriegel and Ward 1998; Hellriegel and Bernasconi 2000; Hosken and Ward 2000). Females have also been shown to store sperm non-randomly in many insects, including dung flies (Ward 1993; Otronen *et al.* 1997; Hellriegel and Bernasconi 2000), Dryomizid flies (Otronen and Siva-Jothy 1991; Otronen 1997), and fruit flies (Bressac

and HauschteckJungen 1996). Females have been shown to differentially store sperm in taxa other than insects as well (Zeh et al. 1997; Curach and Sunnucks 1999). Females are known to also use sperm differentially once stored (Siva-Jothy and Hooper 1996; Stockley and Simmons 1998), although they have not been shown, as yet, to select sperm *sensu strictu* (Birkhead 2000; Eberhard 2000; Pitnick and Brown 2000). A few studies provide suggestive evidence that females can select sperm to some extent, however (Olsson *et al.* 1996, 1997; Stockley, 1999, but see Stockley 1997)**,** but no consensus example yet exists (Birkhead, 2000; Bishop 1996; Birkhead 1998, but see Carre and Sardet 1984). Finally, females may also influence or control how long they mate with a male (Hellriegel and Bernasconi 2000, Elgar, 2000), in direct conflict with male interests (Birkhead, 1993; Eberhard, 1996).

It would be difficult to understate the importance of female influence or control over reproductive 'decisions' and allocations, especially when male and female interests are in conflict. The study of intersexual conflict has received continually increasing interest since Trivers (1972) classic paper first provided the fundamental argument and Dawkins (Dawkins 1976, Ch. 9) generalized that argument. Intersexual conflict may be a driving evolutionary force for polyandry (Arnqvist and Nilsson 2000), speciation (Parker and Partridge 1998; Arnqvist *et al.* 2000; Gavrilets 2000), female mate choice (Gavrilets *et al.* 2001; Moore *et al.* 2001), and male social dominance (Moore et al. 2001). Empirical evidence of intersexual conflict is abundant. Exposure to male Acps increases female mortality rates in *Drosophila* (Chapman *et al.* 1995; Rice 1996; Holland and Rice 1999). When males are allowed to evolve while female evolution is arrested, male features evolve that significantly reduce female fitness (Rice 1996). Recently, Holland and Rice (1998) proposed a form of directional antagonistic coevolution they term 'antagonistic seduction'. This sort of intersexual arms race is supported by theory as well (Holland and Rice 1998; Johnstone and Keller 2000, but see Rosenthal and Servedio 1999). Many of

the effects detailed above vary with both male and female genotype (Sgró *et al.* 1998; Clark *et al.* 1999; Civetta and Clark 2000).

Here we investigate female-oriented aspects of sperm competition to extend our general understanding of both sperm competition and its impact on broader evolutionary contexts such as speciation, etc. We show a significant impact of both female age and genetic relatedness to mates on the outcome of sperm competition. We also discuss intriguing evidence that is consistent with previous reports of significant male genotype x female genotype interactions (Clark et al. 1999). Male genotype x female genotype interactions have been implicated as both necessary and sufficient to demonstrate sperm selection by females, and thus, cryptic female choice (Birkhead 2000; Pitnick and Brown 2000).

CHAPTER 2

DIRECTION AND MAGNITUDE OF SPERM INCAPACITATION IN *DROSOPHILA MELANOGASTER* VARY BY STRAIN[1]

[1]Mack, P. D., B. A. Hammock, and D. E. L. Promislow. To be submitted to Journal of Evolutionary Biology.

Abstract

Past studies have suggested that *Drosophila* males can incapacitate sperm stored by females from previous mates. A recent study found no sperm incapacitation when females remated with XO males two days after the first mating, but significant sperm incapacitation when the interval between matings was seven days. We test the hypothesis that differences in sperm incapacitation between 2d and 7d intervals is a function of the advanced ages of 7d females relative to 2d females. In this study, we replicate the previous experiment using the same fly strains used previously (Ives) as well as a second laboratory strain, Canton-S, and a locally wild-caught strain. Contrary to our prediction that we would observe significant sperm incapacitation only in 7d females from the laboratory strains, we found significant effects in all three strains. In addition, we found significant sperm incapacitation when females remated only two days later for one strain (Canton-S) and two cases in which twice-mated females had significantly higher lifetime reproduction than control females. Typical laboratory culturing on a 2-week cycle may allow the accumulation of late-acting age-specific mutations (at ages older than 5-6 days) that render "older" females less able to respond to male ejaculate components. In addition, our results are consistent with increasing evidence that male genotype by female genotype interactions significantly affect the outcome of sperm competition.

Introduction

In many species, females mate with multiple males within a single breeding season (Smith 1984; Birkhead and Moller 1998). Females of these multiply mating species often store sperm, leading to intense sperm competition between males. In response to the challenges of sperm competition, males have evolved a range of mechanisms to maximize their own reproductive fitness (Waage 1979b; Ono *et al.* 1989; Lorch *et al.* 1993; Parker 1993; Pitnick and Markow 1994; LaMunyon and Ward 1998). These mechanisms include structural features on male intromittent organs that can remove

sperm from previous males (Waage 1979b), increased sperm counts (Harcourt *et al.* 1981; Pitnick and Markow 1994), and, in insects and potentially other taxa, accessory gland proteins that increase female rates of egg laying (Heifetz *et al.* 2001), decrease female receptivity to subsequent males (Manning 1962; Manning 1967; Miyatake *et al.* 1999), facilitate sperm storage (Neubaum and Wolfner 1999; Chapman *et al.* 2000), and incapacitate sperm from previous males (Harshman and Prout 1994; Holland and Rice 1999; Chapman et al. 2000; Gilchrist and Partridge 2000; Prout and Clark 2000). The high rates at which reproductive proteins evolve in insects and other organisms (Clark and Kao 1991; Lee *et al.* 1995; Metz and Palumbi 1996; Ferris *et al.* 1997; Wyckoff *et al.* 2000; Swanson *et al.* 2001) hint at the intense evolutionary conflict between males and females that may be played out in the female's reproductive tract. Until recently, most genetic studies of sperm competition focused on variation in male competitive ability (Gromko and Pyle 1978; Lewis and Austad 1994; Clark *et al.* 1995; Prout and Clark 1996; Hughes 1997; Wilson *et al.* 1997; Clark and Begun 1998; Snook 1998; Hosken and Ward 2000). We are now beginning to appreciate that the outcome of sperm competition depends critically on interactions between the male and female genotype (Clark et al. 1995; Clark and Begun 1998; Clark et al. 1999), and we are beginning to suspect that the resulting sperm utilization may depend more specifically on interactions between male ejaculate characteristics and chemical components of the female reproductive tract (Chapman et al. 2000; Heifetz et al. 2001).

In the fruit fly, *Drosophila melanogaster*, when a mated female remates with a second male, the second male sires the majority of her subsequent offspring (Gromko *et al*. 1984). Two independent forces appear to influence this pattern of second-male sperm precedence. First, sperm from the second male appear to displace sperm from the first male (Lefevre and Jonsson 1962; Gromko *et al*. 1984). But in addition, at least two studies have suggested seminal fluid from the second male appears to reduce the fertility of the first male's sperm (Scott and Richmond 1990; Harshman and Prout 1994), a

process now known as sperm incapacitation. However, these results conflict with earlier studies that did not show an effect of seminal fluid on first-male sperm competitive ability (Lefevre and Jonsson 1962; Gromko *et al.* 1984)

The discrepancy between these results was apparently resolved by Price *et al.* (1999), when they tested for an effect of seminal fluid on first-male sperm at two different time intervals prior to the second mating. In particular, they were interested in whether the duration that the first male's sperm remained in the female reproductive tract affected the ability of the second male to incapacitate those sperm. To test this idea, they used XO males, which have seminal fluid but no sperm. If a female mated with an XO male 2 d after her first mating, the seminal fluid from the second male had no effect on her fecundity. However, females that were mated with XO males 7 d after the first mating showed a marked reduction in offspring production compared with control females. Price *et al.* (1999) suggest that females might "gradually alter the physiology of the sperm they store" (Price *et al*. 1999, p. 451). They go on to suggest that if females have evolved this ability to alter sperm physiology, genetic variation for this trait may account for at least some of the observed variation among females (Clark and Begun 1998; Clark *et al*. 1999) in mediating the outcome of sperm competition. Price *et al.*'s (1999) study provides us with one intriguing possibility for how female reproductive physiology might influence the outcome of sperm competition**.**

We suggest an alternative explanation for the difference between females held for 2 d versus females held for 7 d before the second mating. Laboratory flies are typically kept in a two-week culture. Each generation of newly eclosed adult flies are placed in bottles with medium, allowed to lay eggs for several days, and then discarded. The next generation emerges some 8 - 12 d later, and at exactly two weeks, these adults are placed in fresh bottles, and the cycle starts anew. As Promislow and Tatar (1998) have pointed out, this process can potentially lead to relatively high rates of accumulation of mutations whose effects are seen only after 5-7 d. In two week culture, flies older than that have no

opportunity to contribute gametes to the next generation, and so these 'late-acting' (i.e. > 5 d) mutations are effectively invisible to selection. After hundreds of generations in culture, a very high load of late-acting mutations may accumulate. Given the absence of selection on mutations acting later than 5 d, a 10 or 12 d old fly may express a substantial load of deleterious genes. This phenomenon may explain, at least in part, why laboratory stocks are typically much shorter-lived than newly caught wild strains (Promislow and Tatar 1998; Sgrò and Partridge 2000). The same phenomenon may also account for the Price *et al.* (1999) observation that sperm incapacitation occurs only after a 7 d interval between matings. By 11 d of age, the female may already be senescent and simply no longer able to resist the effects of the XO male's accessory gland proteins.

We suggest that in Price *et al.*'s (Price et al. 1999) experiment 11-day-old females may already be senescing physiologically. To test this hypothesis, we repeated Price *et al.*'s (Price et al. 1999) experiment, but examined the effect of XO males on fecundity in the Ives strain used by Price *et al.* (Price et al. 1999), as well as in an additional lab-strain, Canton S, and in a recently wild-caught, long-lived strain. This long-lived strain should have a much lower load of late-acting deleterious mutations (Promislow and Tatar 1998). If this hypothesis is correct, then we would expect that the two lab strains would show an effect similar to that observed in Price *et al*'s (1999) original experiment, whereas the wild-caught strain would show no difference between the 2 d and 7 d intervals. Alternatively, if the phenomenon described by Price *et al.* (1999) is universal, then we would expect no difference between the three strains. Finally, if there is genetic variation among females not only in the outcome of sperm competition, as suggested by previous studies (Clark and Begun 1998; Clark et al. 1999), but also in the influence of incapacitation, we might expect each strain to respond differently to the treatment.

Materials and Methods

Drosophila stocks and husbandry

All strains were kept at 24° C on a 12/12 light-dark cycle on standard molasses-agar-cornmeal-killed yeast medium. Three of the strains used and a fourth strain used indirectly (Ives, bw^D, XO, and attached-X, attached-XY) were obtained from the Coyne lab (University of Chicago), and were identical to those that were used in the original Price *et al*. (1999) experiment. All females assayed were mated first with virgin males homozygous for the dominant eye-color marker, bw^D. Finally, we created XO males, which produce seminal fluid but no sperm, by mating virgin, 3-5 day old Ives females to males from an attached-X, attached-XY stock also obtained from the Coyne laboratory, also as in the Price *et al*. (1999) experiment.

In addition to these replicate strains, we tested two additional wild-type strains. Canton-S (CS) is a wild-type strain that has been in laboratory culture since the 1930's, and which we obtained from the *Drosophila* stock center in Bloomington, IN. The Georgia (GA) population is a wild-type strain derived from several hundred inseminated females collected from a peach orchard in Watkinsville, GA in August 1999. The GA population was maintained in a laboratory population cage with a density of approximately 5000 flies for approximately one year prior to the beginning of this experiment.

Matings

Within each of the three wild-type strains, fifteen female and ten male newly eclosed virgin flies were placed into 1/2-pint plastic bottles (Applied Scientific®) with 50 ml of yeasted fly medium for 48 h. Virgin females from each strain were collected under light CO_2 anesthesia approximately 12 days later, and held until they were 4-5 d old, except for Canton-S females, which, due to differences in developmental time, were 3-4 d old. We simultaneously collected virgin bw^D males in the same way. For of each of the three

female lines, we placed approximately 60 4-5 d old bw^D males and 30 wild-type females in each bottle at 630 h, and then allowed the flies to mate en masse for 4.5 h.

After mass mating, females were removed without anesthesia and placed individually in 8-dram vials with 5 ml of yeasted food. We checked these vials for presence of larvae 36 h after the first mating, and discarded any females that did not produce larvae, as they may not have mated initially. Remaining females were randomly assigned to one of the three treatments (Fig. 1): 2-day interval between first and second matings (2d), 7-day interval (7d), or control (no second mating).

At two days or seven days after the initial mass mating (for 2d and 7d females, respectively), we placed two XO males and a single female into a vial with 5 ml of standard medium between 730 h and 830 h. To ensure that females had mated with one of the XO males, we observed females continuously using a scan sampling technique (Altmann 1974). We did not consider females to have mated unless they copulated for longer than 10 minutes. For the 2d treatment, most females mated early in the day, though we continued our observations until 2330 h. Females for the 7d treatment were placed together with males between 630 h and 700 h and mated somewhat more readily; in this case, we stopped observing females at 1800 h. Control females were not exposed to XO males.

Immediately after this second mating, all females were transferred to fresh, yeasted food vials. We transferred control females to fresh vials at approximately the same time that mated females were transferred.

Lifetime Reproduction Assay

To measure lifetime reproduction, we transferred females to fresh yeasted food vials every three days as long as they were producing fertile eggs. In the case of 2d females, the second transfer occurred after a two-day interval so that subsequently females from all treatments could be transferred on the same day. We randomized placement of vials

across treatments within and across strains to account for micro-environmental variation within incubators.

Each vial was kept in the incubator for 16 d, at which time we inverted the vials and placed them at 4 °C. This allowed us to delay counting the number of offspring until the entire experiment was complete. The number of females successfully assayed varied amongst the different strains and treatment types (between 15 and 59 individuals; see Table 2.1 for details).

Statistical Methods

We tested for differences in lifetime reproduction between 2d, 7d, and control females using four different statistical methods. First, simply compared total progeny counts for female as in the Price *et al.* (1999) experiment. We used a square-root transformation to normalize these data, then compared treatments for each interval type within each strain. However, we used a two-tailed t-test for making the comparisons, in contrast to Price *et al*. (Price et al. 1999). For this test, we excluded all replicates that lacked data for any of the 3-day intervals and excluded any females that died or were lost during the assay (Table 2.1).

Visual inspection of the data suggested that not only did total offspring production decline with age of the female, but that the difference between treatment and control groups sometimes differed among age-classes. If we ignore the fact that fecundity drops with age, we run the risk of obtaining inflated error variances for mean lifetime fecundity. To control for the effect of age, we performed a second statistical test we where we compared offspring production between treatment and control groups using a two-factor ANOVA, with age interval and treatment type treated as fixed factors. To reduce the influence of age intervals with few females and comparatively high variance, we included only age intervals with data for 10 or more females. For this test, we standardized the

square root transformed data to compare mean progeny/female/day, then carried out the analysis in JMP 4.0.2 (SAS Institute 2000).

The difficulty with this last approach is that the sample sizes diminish over the course of the experiment, as females stop laying eggs. To account for this decrease in sample size, we carried out a third statistical test using a permutation approach (Manly 1997). Within each female line and for each treatment group, we calculated the average number of progeny for each age class. We then calculated the sum of all these averages to obtain an average estimate for total lifetime production. To test for statistically significant differences in these summed averages between treatments, we randomly reassigned individuals to treatment (within an interval), then recalculated the summed averages. We repeated this procedure 10,000 times to obtain an estimate of the distribution of such values. The P-value was calculated as the proportion of randomized summed averages that were greater than (or less than) the original summed average.

Finally, because we also wished to test for differences among the two mating interval types (2d and 7d) we conducted a second two-factor ANOVA on the, where we treated both mating interval and treatment as fixed factors. We calculated the mean progeny per female per day on square root transformed progeny numbers as with the first ANOVA (above).

Results

In each of the three experiments, we tested the degree of sperm incapacitation caused by spermless XO males either 2 d or 7 d after initial mating. We found distinctly different results in each case. The different statistical methods we employed yield generally similar results (all results reported in Tables 2 and 3). In the following, we discuss results from each line separately, focusing primarily on results from the ANOVAs.

Experiment 1: Ives

The first experiment attempted to replicate Price *et al.*'s (1999) experiment as closely as possible. As with the original study, we found no significant effect of XO seminal fluid on lifetime reproduction in Ives females mated to XO males 2 d after the initial mating to bw^D males (Fig. 2.2a, Table 2.2; $F_{1,510} = 0.128$, P = 0.721). Females that were held for 7 d and then mated to XO males had a marginally significant increase in lifetime reproduction (Fig. 2.2b, Table 2.2**,** $F_{1,545} = 3.693$, P = 0.055). Note that this contrasts with Price *et al*.'s (1999) result, where offspring number was reduced by the presence of XO seminal fluid. The slight increase was maintained for as long as the females continued to lay eggs—in this case, more than a month (Figure 2.3b). While we obtained similar results from each of the three methods of analysis, the results did differ slightly (Table 2.2, $F_{1,545} = 3.693$, P = 0.055; $t_{84} = 2.252$, P = 0.027; permutation: P = 0.119). When we considered both mating interval types together in an ANOVA without controlling for the effects of age (Table 2.3), we found no effect due to treatment, but a marginally significant interaction between treatment and mating interval (P = 0.0558). The significant interaction reflects the significant and unexpectedly reversed effect in 7d but not 2d females.

Experiment 2: Canton-S (CS)

Based on analysis of total lifetime reproduction, females that experienced a 2 d delay between matings showed a significant increase compared with control females (Fig. 2.2b, Table 2.2**,** $t_{50} = 2.125$, P = 0.0386). When we controlled for the effects of age, this effect was even stronger (Fig. 2.3c, Table 2.2, $F_{1,247} = 26.598$, P = 0.0036). By contrast, females that experienced a 7 d delay between the two matings produced lower numbers of offspring than control females (Fig. 2.2b, 3d). While generally consistent with the pattern observed by Price *et al.* (1999), the difference among treatments at 7 d was not significant (Table 2.2; $F_{1,104} = 1.781$, P = 0.185; $t_{47} = -1.780$, P = 0.082; permutation, P =

0.0846). In addition, the highly significant treatment x mating interval interaction revealed by the results of the ANOVA we performed over the combined CS data (Table 2.3, P = 0.0069) is also consistent with a strong effect at only one mating interval (in this case, the 2d treatment). As with Experiment 1, these differences persisted throughout the females' short reproductive lifespan (Fig. 2.3d).

Experiment 3: Georgia (GA)

XO males had no effect on female reproduction when mated 2 d after a female's first mating (Fig. 2.2c, 3e, Table 2.2; $t_{67} = -0.691$, P = 0.49; $F_{1,552} = 0.430$, P = 0.552). Among females held for 7 d between matings, mating with XO males led to a significant reduction in offspring numbers (Fig. 2.3f, Table 2.2; $F_{1,490} = 5.244$, P = 0.0224). This pattern held for five of the first six age intervals, after which there was no significant difference in 7 d females between mated and control individuals (Fig. 2.3f). By the sixth age interval, the total number of flies still laying eggs was quite small, resulting in little statistical power. Results varied somewhat among the methods of analysis in this case. When we considered total lifetime reproduction without controlling for the effects of age, the difference between control and XO-mated females was not significant ($t_{75} = -1.373$, P = 0.174). In our permutation analysis, the difference in lifetime reproduction between treatment and control females was marginally significant (P = 0.0492) but not after correction for multiple tests. The ANOVA on the combined GA data did not yield a significant interaction between treatment and mating interval (Table 2.3; $F_{1,142} = 0.212$, P = 0.646).

Discussion

In their study of sperm incapacitation in *D. melanogaster*, Price *et al*. (1999) found that females mated to spermless, XO males 7 d after a first mating showed a marked reduction in lifetime fecundity. Females mated 2 d after their first mating did not

experience this reduction. Sperm stored in the female for 2 d appeared to be resistant to the effects of seminal fluid from a subsequent male. However, Price *et al*. (1999) suggest that after 7 d, females may have carried out some sort of physiological manipulation of stored sperm such that seminal fluid from a subsequent male could incapacitate the stored sperm. Furthermore, they observed the same pattern in *D. simulans*, suggesting that this pattern is common.

In our introduction to this paper, we suggested an alternative explanation for Price *et al*.'s (1999) results. Recently, researchers have pointed out that when fly strains are kept for hundreds of generations in 2-week culture, females of those strains older than 5-6 d are sheltered from the effects of selection (Promislow and Tatar 1998) and so accumulate late-acting germ-line mutations. Here, 'late-acting' refers to mutations whose effects are confined to adult ages greater than 5 or 6 d. In the Price *et al*. (1999) experiment, females in the 7 d group are 11 d old by the time of their second mating, and may already be suffering from significant physiological effects of senescence. These senescent females may be less able to withstand the manipulative effects of seminal fluid from XO males (Rice 1996; Holland and Rice 1999). In separate experiments from our laboratory we have observed that last male sperm precedence (Gromko et al. 1984) decreases as females age (Mack *et al*. submitted).

On the basis of this argument, we predicted that each of the two short-lived lab strains (IV and CS) would show a marked reduction in offspring production in females mated to XO males after 7 d. In contrast, we predicted that GA99 females should experience no such reduction. Our results fail to support this hypothesis. While CS 7 d flies showed a pattern similar to that observed by Price *et al*. (1999), GA 7 d females had a smaller but still significant decrease in progeny production after mating with XO males. Surprisingly, in the Ives lines, there was a marginally significant increase in progeny production among females mated to XO males. This last result is in direct contrast to what Price *et al*. (1999) observed using the same lines, and similar to what we observed in 2 d CS females,

where those females that were mated to XO males had a marked increase in lifetime fecundity compared with control females.

These results raise several important questions. First, why might we obtain different results from Price *et al* (1999) in our experiment with Ives females? Certainly this result is surprising, but not altogether unexpected. Collaborators who replicate an experiment in different laboratories sometimes observe significant location effects (Clark and Begun 1998) or observe a significant interaction between location and some other factor of interest (Clark et al. 1999) even when the experiments are conducted simultaneously. Moreover, *Drosophila* researchers commonly perform fitness assays in blocks (Hughes and Charlesworth 1994; Hughes 1997; Clark et al. 1999) and must analyze the data for block effects (Sokal and Rohlf 1995) or for gene by environment interactions, a type of block effect (Falconer and Mackay 1996, p.131). We note however, that we observed a reversal of sign and not just a difference in magnitude of the effect originally observed by Price *et al*. (1999). While this is surprising, it is not entirely unexpected. If we replicated the experiment 100 times, we could expect to observe a result outside any measurable trend a small percentage of the time (Palmer 2000). Given that the experiment in question has been replicated only five times - twice by Price in *D. melanogaster* and *D. simulans*, it is difficult to say under the current circumstances, which of the two observed results, Price *et al*. (1999) and our experiment, truly reflect the fundamental trend. In fact, in a pilot experiment conducted for the experiment we present here, we observed the same result for the Ives line as we have observed in the full experiment (P. Mack, unpublished data). And, although we replicated the design of the Price *et al*. (1999) experiment, our study differs from Price *et al*. (1999) in having somewhat larger sample sizes and different statistical analyses.

If we have not simply observed a rare contradictory result in a series of replications, as described above, we must account for the fact that a second mating with a sterile male increased female fecundity, contrary to expectation, as with CS 2 d and IV 7 d females.

Male *Drosophila* transfer several accessory gland proteins (Acps) in their ejaculate, including two, Acp26Aa and Acp70A, that stimulate oviposition (Herndon and Wolfner 1995; Wolfner 1997). This increase is caused by elevated oocyte release (Heifetz *et al.* 2000), and lasts for a period of up to 11 days in the presence of sperm (Kalb *et al.* 1993). A female that mates a second time therefore receives a double dose of Acp26Aa and Acp70A, while control females receive only a single dose. When the interval between matings is extended to 2 d, the increased oocyte stimulation might be expected to extend for a period of 13-14 d, and 18-19 d when the interval between matings is 7 d.

Given our hypothetical explanation above, we would expect to see a similar trend across both treatments and all strains, but we do not. What could account for our failure to observe the expected pattern? While we have not tested specifically for intersexual conflict here, the results are consistent with such a pattern. CS females have not evolved in concert with IV males and thus may not have evolved to resist paying the costs due to antagonistic coevolution as suggested by prior work measuring fitness traits (Rice 1996; Holland and Rice 1999) and consistent with estimates of rapid evolution in reproductive proteins for both mammals and *D. melanogaster* (Swanson et al. 2001; in press).

Alternatively, the variation we see may have been due to gene by environment (GxE) interactions. There is now substantial evidence for the important role that GxE interactions play on fitness components in *Drosophila* (Mackay 2001). Our results could be viewed as the outcome of GxE interactions, where male-derived ejaculate molecules are the gene products and the female reproductive tract and genome represent the environment in which these molecules act.

Conclusion

It is tempting to promote intersexual conflict as a driving force behind the patterns we observe here and to cite the growing body of theoretical and empirical evidence that

female reproductive processes reflect antagonistic rather than mutual coevolution (e.g. Gowaty 1996). However, the present experiment provides no such direct test, although our observations do not preclude the role of intersexual conflict. Our results suggest that the existence of a general trend will only be revealed after the original experiment is replicated both in different fly strains and in different laboratories. Clearly, we cannot accept the universality of the original Price *et al.* (Price et al. 1999) result without repeated support. If further replication does not reveal a general trend, future work must seek to better understand both the underlying complexity of the reproductive playing field and the dynamics that might lead to the variable patterns we observe.

Table 2.1. Sample sizes by strain and treatment. Numbers in parentheses refer to sample sizes for the t-test for lifetime reproduction. Numbers in brackets indicate ages considered for the ANOVA analysis.

Strain	2d	2d Control	7d	7d Control
IV	27(16) [1-8]	54(43)	48(43) [3-10]	50(43)
CS	18(16) [1-5]	41(36)	16(15) [3-4]	40(34)
GA	29(23) [1-8]	59(46)	38(31) [3-10]	58(46)

Table 2.2. P-values for each analytical method by strain and treatment. The symbols †, *, **, *** indicate marginally significant, significant, highly significant, and very highly significant P-values respectively.

Strain	ANOVA	Permutation	t-test
IV 2d	n.s.	n.s.	n.s.
7d	0.055†	0.119	0.027*
CS 2d	0.038 *	0.0004 ***	0.039*
7d	n.s.	0.019 *	0.082
GA 2d	n.s.	n.s.	n.s.
7d	0.022*	0.085†	n.s.

Table 2.3. ANOVA table by female strain; two-factor model with both factors fixed.

Strain	Factor	d.f.	MS	F	P
IV	Treatment	1	3.204	0.661	n.s.
	Mating Interval	1	206.167	42.532	<0.0001***
	Interval x Treatment	1	18.028	3.719	0.0058**
	Error	141	4.847		
CS	Treatment	1	0.403	0.093	n.s.
	Mating Interval	1	468.217	107.750	<0.0001***
	Interval x Treatment	1	33.079	7.612	0.0069**
	Error	97	4.345		
GA	Treatment	1	8.563	2.091	n.s.
	Mating Interval	1	337.839	82.509	<0.0001***
	Interval x Treatment	1	0.869	0.212	n.s.
	Error	142	4.095		

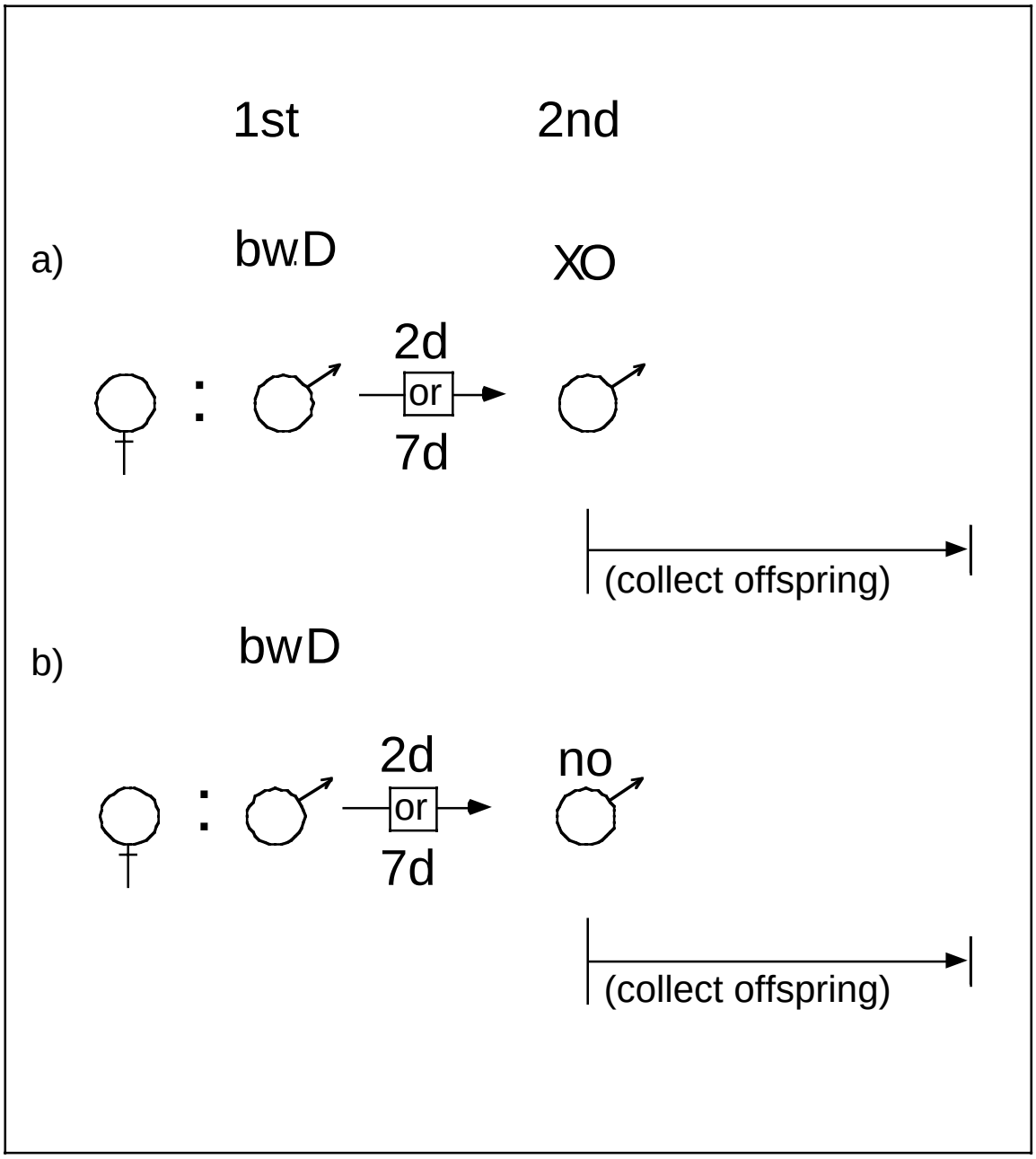

Figure 2.1. Study design: 1st and 2nd refer to matings (or lack of) for each female treatment. Offspring production/collection continued until females stopped producing fertile eggs.

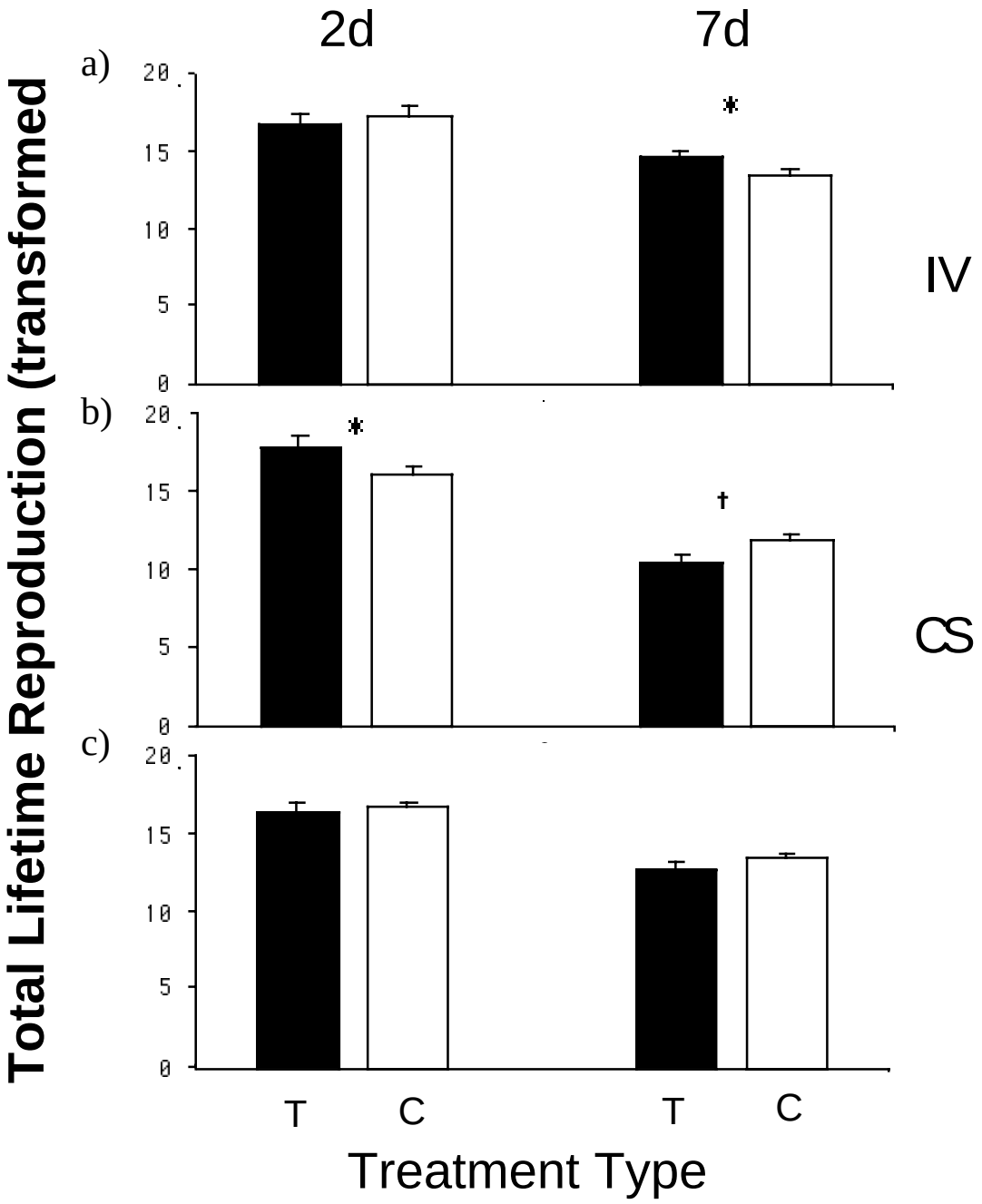

Figure 2.2. Mean values for square root transformed total lifetime reproduction in IV 2d, 7d (a), CS 2d, 7d (b), and GA 2d, 7d (c). Filled bars represent treatment females (mated to XO males); unfilled bars represent control females (not remated). Significance symbols reflect t-test analyses only.

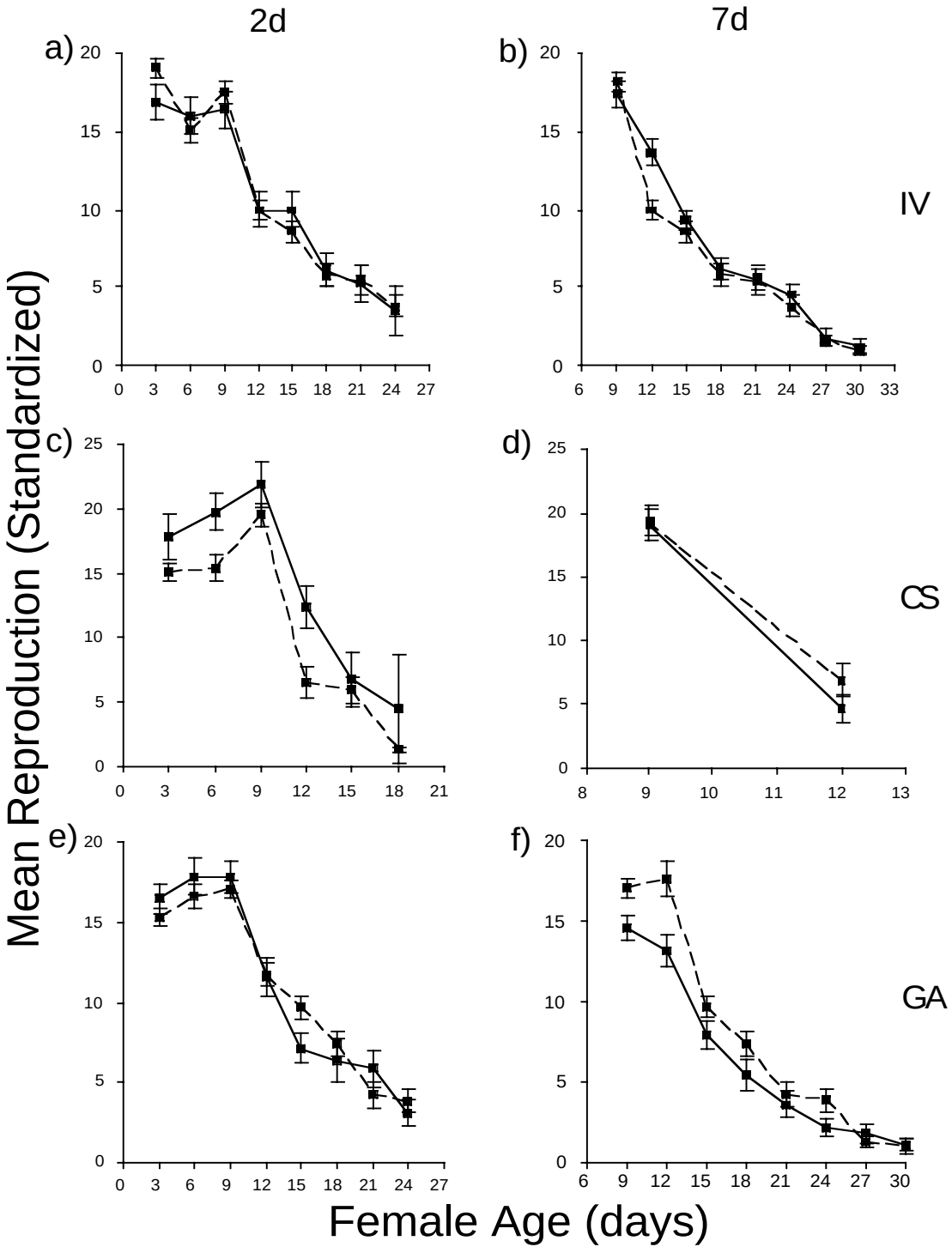

Figure 2.3. Mean lifetime reproduction expressed in standardized format as mean square root transformed offspring per day for: a) Ives (IV) 2d; b), Ives 7d; c) Canton-S (CS) 2d; d) CS 7d; e) Georgia (GA) 2d; f) GA 7d. Dashed lines denote control treatments; solid lines denote treatment females (mated to XO males).

CHAPTER 3

FEMALE AGE AND SPERM COMPETITION: LAST MALE PRECEDENCE DECLINES AS FEMALE AGE INCREASES[2]

[2]Mack, P. D., N. K. Priest, and D. E. L. Promislow. To be submitted to Proceedings of the Royal Society of London Series B-Biological Sciences.

Abstract

Although we still have much to learn about the mechanisms underlying sperm competition, last male sperm precedence has been thoroughly documented in the majority of taxa examined, including insects. Until very recently, however, the majority of research has focused on male aspects of sperm competition. We now know that a number of reproductive traits, such as oviposition rate, use of stored sperm, and receptivity to mating, vary with female environment and female quality or state and that these factors can, in turn, affect the outcome of sperm competition. And while female age has been shown to influence female preference for mates, we know little about how age-related changes in female quality or physiological state affect the outcome of sperm competition. Here, we examine the effect of female age on the outcome of sperm competition in three different strains of the fruit fly, *Drosophila melanogaster*. We show that last male sperm precedence decreases significantly when males are mated to females older than 17 days of age relative to females of 3-4 days old, the age of females typically used in sperm competition assays.

Introduction

In many species, females mate with multiple males within a single breeding season (Smith 1984; Birkhead and Moller 1998). Females of these species frequently store sperm, so males encounter intense sperm competition. In response to the challenges of sperm competition, males have evolved a range of mechanisms to maximize their own reproductive fitness (Waage 1979b; Ono et al. 1989; Lorch et al. 1993; Parker 1993; Pitnick and Markow 1994; LaMunyon and Ward 1998). In addition to well known structural features (e.g., sperm removal devices on intromittent organs – Waage 1979a),

many organisms have also evolved biochemical "weapons". During mating, male insects transfer accessory gland proteins that increase female oviposition rates (Heifetz et al. 2000), decrease female receptivity to subsequent males (Manning 1962, 1967; Leopold *et al.* 1971; Obata 1988; Eady 1995; Miyatake et al. 1999), facilitate sperm storage (Neubaum and Wolfner 1999; Chapman et al. 2000), and incapacitate sperm from previous males (Harshman and Prout 1994; Gilchrist and Partridge 1995; Holland and Rice 1999; Chapman et al. 2000; Prout and Clark 2000). The high rates at which reproductive proteins evolve in insects and other organisms (Clark and Kao 1991; Civetta and Singh 1995; Lee et al. 1995; Metz and Palumbi 1996; Ferris et al. 1997; Wyckoff et al. 2000; Swanson et al. 2001) hint at the intense intersexual evolutionary conflict waged in the female reproductive tract. Until recently, however, most genetic studies of sperm competition have focused on variation in male competitive ability (Gromko and Pyle 1978; Lewis and Austad 1994; Clark et al. 1995; Prout and Clark 1996; Hughes 1997; Wilson et al. 1997; Clark and Begun 1998; Snook 1998; Clark et al. 1999; Hosken 1999; Hosken and Ward 2000). We are now beginning to appreciate that the outcome of reproductive interactions depends critically on female state (Chapman and Partridge 1996), and on interactions between male and female genotype (Clark et al. 1995; Wilson et al. 1997; Clark and Begun 1998; Clark et al. 1999; Civetta and Clark 2000; Sawby and Hughes 2001). Females maintained in nutrient-enriched environments increase their rates of oviposition, use of stored sperm, and mating (Gromko and Gerhart 1984; Trevitt *et al.* 1988; Chapman *et al.* 1994; Chapman and Partridge 1996). Ward (2000) showed that female environment influences the reproductive success of male yellow dung fly (*Scathophaga stercoraria*) differing at the *Pgm* locus. In a related experiment, female size and morphology (number of sperm storage sites) significantly influences the outcome of sperm competition in dung flies (Ward 2000). It seems likely that sperm utilization depends, at least in part, on interactions between male ejaculate characteristics and female physiological state (Chapman et al. 2000; Heifetz et al. 2001).

We already know that female genotype plays a significant role in the outcome of sperm competition in several insect species (Wilson et al. 1997; Clark and Begun 1998; Clark et al. 1999). Female *Drosophila melanogaster* and yellow dung flies not only physically assist the transfer of sperm from the site of deposition to storage organs or fertilization sites (e.g. Otronen and Siva-Jothy 1991; Ward 1993; Eady 1994; Arthur et al. 1998; Bloch-Qazi et al. 1998; Simmons *et al.* 1999; Hellriegel and Bernasconi 2000), but also respond to a number of chemical cues provided by males in the form of various accessory gland proteins (Acps) (reviewed in Wolfner *et al.* 1997 and Neubaum and Wolfner 1999). Chapman *et al*. (1995), showed that exposure to male Acps increases female mortality rates. Taken together with the results from the work of Rice (1996, 1998) and colleagues (Holland and Rice 1999), these results suggest that sexually antagonistic physiological interactions between male Acps and female biomolecules are not only plausible, but likely. Unfortunately, given our current level of understanding of sperm competition processes, we cannot predict how changes in female condition will affect the outcome of sperm competition. However, given the existing evidence for antagonism cited above, we strongly suspect that variation in female state should play an important role.

Female condition may depend on such factors as mating history (Chapman et al. 1995; Rice 1996), larval development conditions (Hodin and Riddiford 2000), environmental features such as the availability of nutrients (Chapman and Partridge 1996; Hodin and Riddiford 2000), and even maternal and grandmaternal effects (Hercus and Hoffmann 2000). One such parameter that has been overlooked in past work on sperm competition is female age. Theory suggests that intrinsic factors, such as reproductive quality, should influence mate choice (Parker 1983; Partridge and Endler 1987) and that females of reproductive quality below a certain threshold should be less choosy than females above that threshold. Although Parker's (1983) ESS model assumes that female reproductive quality is constant, reproductive quality is likely to vary with age (Moore and Moore in

press). For example, recent studies have shown that older female guppies (Kodric-Brown and Nicoletto 2001) and female cockroaches (Moore and Moore in press) are less choosy than younger females. And in moths, males invest fewer sperm in older virgin females relative to the number of sperm transferred to very young virgins (Cook and Gage 1995).

Do females also experience an age-specific reduction in their ability to influence the outcome of sperm competition? Previous studies of sperm competition in insects have either failed to control for female age (Lewis and Austad 1994; Kotrba 1996; Sakaluk and K. 1996; Wilson et al. 1997; Danielsson 2001), or utilized females only over a brief interval after they have reached sexual maturity (Saul and McCombs 1993; Wedell and Cook 1998; Edvardsson and Arnqvist 2000). In a survey of studies over the past 20 years that examined sperm competition in *D. melanogaster*, we found that, in all but one case, females used were between 3 and 5 d old (Gromko and Pyle 1978; Gromko and Newport 1988; Chapman et al. 1995; Clark et al. 1995; Clark and Begun 1998; Clark et al. 1999). In the one exception, females ranged up until 6 d of age (Letsinger and Gromko 1985).

Little is known about age-structure in wild populations of *Drosophila*, but it is likely that many females continue to mate at later ages. Insects of many species undergo reproductive diapause (Tatar *et al.* 2001), including the Drosophiladae (Kimura 1988; Saunders and Gilbert 1990; Williams and Sokolowski 1993), often as a means of overwintering. Consequently, it is likely that female *D. melanogaster* of relatively advanced ages make up a significant proportion of the breeding population into the latter stages of population increase in the spring.

Mated *D. melanogaster* females undergo a refractory period of 1-3 days immediately after mating. During that time they are unreceptive to courtship. When a female then remates with a second male, most of her subsequent offspring are sired that last male. In laboratory experiments on sperm competition, last male paternity is characteristically expressed as 'P2', the proportion of the total offspring sired by the second male to mate in a two-male sequence. To our knowledge, no prior study has examined the effect of

female age on the outcome of sperm competition. Until recently, researchers have focused primarily on the male role in the outcome of sperm competition, ignoring the female physiological parameters that might affect sperm use. We need to consider the genetic and physiological state of both males and females if we are to develop a more complete picture of the dynamics of sperm competition. To that end, we assay sperm competition (P2) on females from three different inbred strains at three different female ages, to determine how female age affects the outcome.

Materials & Methods

Mated *D. melanogaster* females undergo a refractory period of 1-3 days immediately after mating. During that time they are unreceptive to courtship. When a female then remates with a second male, most of her subsequent offspring are sired that last male. In laboratory experiments on sperm competition, last male paternity is characteristically expressed as 'P2', the proportion of the total offspring sired by the second male to mate in a two-male sequence. To determine the effects of maternal age on P2, we examined sperm competition over three female ages using three inbred female strains and three wild type male genotypes. In addition, we measured the relative competitive ability of larvae sired by males of the marker genotype and two of the three wild type male genotypes to control for differential survival in the sperm competition assay.

Drosophila Stocks

The GA98 strain was generated from ~250 inseminated females collected from a peach orchard in Watkinsville, GA, in August 1998. We maintained this population in a 30-liter plexiglass population cage with overlapping generations at a density of approximately 5000 individuals for 6 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 QTL mapping by T. F. C. Mackay at NCSU, Raleigh NC (Nuzhdin *et al.*

1998). The RI lines were derived from crosses between inbred derivatives of the Oregon RC (Nuzhdin *et al.* 1996) and 2b strains (Pasyukova and Nuzhdin 1993), both of which had been in laboratory culture for at least a decade. Throughout the experiments the flies were maintained at 24° C. on a 12:12 light/dark cycle. Before the start of both experiments, the strains were cultured in plastic half-pint bottles at a density of approximately 250 eggs/bottle for two generations. All flies were maintained on standard yeast-agar-cornmeal-molasses medium, with propionic acid added as a preservative.

Except where otherwise noted, all individuals were first collected under light CO_2 anesthesia, then maintained in 8-dram vials at a density of 20 flies per vial on 5 ml of standard molasses-agar-cornmeal-killed yeast medium, with live yeast added. We carried out all expansions in plastic half-pint bottles containing approximately 50 ml of yeasted medium.

Generating females of different ages

The use of outbred lines to study female age effects has one drawback. Outbred lines are, by definition, genetically heterogeneous. We might expect that individuals that survive to late ages are, in genetic terms, a non-random sample of the initial population (Vaupel and Yashin 1985). Thus, differences in P2 among females of different ages could be confounded both by genetic effects and by changes in female physiological state. We reduced that risk by using inbred lines. Though genetically identical within strains, females may still differ in viability due to physiological variation.

We designed the experiment to enable simultaneous sperm competition assays of females of different ages and genotypes. The development time of each strain was measured in pilot trials, and the timing of the set-up of each strain was adjusted so that parental age was identical for all strains. For each of the genotypes, we collected parental (P) flies five days after they started emerging from a set of 24 bottles. For each P cohort, we held the flies at a density of 1000/jar in three 3.8-L clear plastic jars (modified from

Fukui and Kirscher 1992). We designed the jars with a 10x10 cm screen and a hard plastic top with three holes for food vials which we changed every other day. After one week, we isolated male and female P flies from the holding jars, placed them in 96 new food bottles at a density of 12 pairs/bottle, and allowed them to lay eggs for two days. The offspring (F1) of this cross emerged 8-10 days later; we collected virgin F1 females from this cohort for each of the three strains over a two-day period.

After collecting virgin flies, we maintained three jars for each sex and genotype with initial densities of 1000 flies per jar. We repeated this P-F1 generation procedure every two weeks, until we had holding jars with virgin females that were approximately 1, 3, and 5 weeks old. The actual ages (in days) at the time of assay were 3.5 (FA1), 17 (FA2), and 31 (FA3) d (note: FA1 and FA2 were 18 and 32 d, respectively for 67L females).

Production of Males

Males used in the experiment were derived from the GA98 population described above. We used standard techniques (Ashburner 1989) to isolate chromosomes II and III in homozygous state to produce the four extraction used in the present experiment lines (PCE lines 112, 234, 338, and 348). In addition, we backcrossed the dominant marker, bw^D, into a subpopulation (bw^D98) of the outbred population for 8 generations prior to extraction. We used two such marked extraction lines, C238 and C302, to generate marked males for use in this experiment.

To measure second male sperm precedence (P2), we mated each female first to a competitor (C) male with a dominant genetic marker (bw^D), and then mated the female again three days later to a wild type male. Accordingly, we generated three identically heterozygous tester male genotypes and one identically heterozygous bw^D marker line by setting up the following crosses, PCE 112 (male) x PCE 338 (female); PCE 234 (male) x PCE 112 (female); PCE 348 (male) x PCE 234 (female) to produce the tester males

(MT1-3). Similarly, we crossed the marked lines, C302 (males) and C128 (females), to produce competitor (C) males, homozygous for bw^D.

Sperm Competition Assay

We assayed P2 in each of the three female lines (genotypes) simultaneously. We set up the initial mating of the sperm competition assay *en masse* in plastic half-pint bottles. At ~ 1700 hrs, approximately 40 virgin females of a single female strain, age, and male treatment type were placed in a bottle with 60-65 virgin C males aged 3.5-4.5 d without using anesthesia. We set up a single bottle for each of the 27 female strain x female age x male treatment combinations over a one hour period (with one exception, see below). All bottles were held at 24 °C with lights on for 2 hr, then placed under dim laboratory light at room temperature to simulate twilight for one additional hour, after which we removed the females. All flies were placed briefly on ice while females were removed and placed individually in vials containing yeasted medium. Females for one of the female strain-age combinations, 58S – FA3, were set up the same day, but at ~2400 hr, and allowed to mate overnight. These females were removed from bottles after 9.5 hr and placed in vials containing yeasted medium.

We held all single females grouped by genotype in single trays. The trays were then arranged in random order at 24 °C for 3 d. We screened all females and eliminated all vials that did not contain larvae after 2.5 d.

Seventy-two hours after the first mating, we randomly assigned females to one of the three male genotypes for the second mating, and added two males of the assigned genotype to each female vial. Males were chilled on ice for no more than three minutes prior to transfer. We removed all males the following morning and transferred all females to fresh vials containing 5 ml standard medium and a single grain of yeast. We transferred all females to fresh yeasted vials twice, after 3 d, and then after 6 d, so that,

for most females, we had 3 vials of offspring from which to assay sperm competition by the end of the experiment.

We collected offspring from each vial by first inverting the vial 16 days after setup, then placing the inverted vials at -20 °C overnight. We then transferred frozen progeny to standard microfuge tubes and sorted and counted all offspring by eye color phenotype to assign paternity. We calculated P2 as the proportion of offspring sired by the second male out of the total progeny array (Boorman and Parker 1976).

Statistical Analyses

Given the highly non-normal distribution typical of measures of P2, we conducted both parametric and non-parametric analyses of the sperm competition data. All parametric analyses were done using JMP (SAS Institute 2000) on arcsine square root transformed P2 and all non-parametric analyses were done using Statview (Abacus Concepts 1994). For both parametric and non-parametric analyses, we included only females for which we had obtained data over the full 9 days of offspring collection.

To test for effects of female age on P2 within each line, we conducted two-factor ANCOVAs, with female age as a fixed, continuous effect and male genotype as a random effect. In addition, because we wanted to test for the possibility of an interaction between female line and male genotype, we combined the three inbred line datasets and carried out an ANCOVA with female line as a random effect in addition to female age and male genotype as fixed. In our non-parametric analyses, we performed Kruskal-Wallis (K-W) non-parametric one-way analyses of variance, testing female age and male genotype as single factors in separate analyses for each female genotype.

Results

We report the results for both types of analyses, parametric and non-parametric, for three reasons. First, with one exception, both the conclusions and the associated p-values

from each method agree very closely. Second, ANOVA results obtained from large data sets, such as ours, are robust with respect to the assumption of normality (Sokal and Rohlf 1995, p.407), yet such data sets are more likely to fail tests of normality such as the Shapiro-Wilks test. Finally, while non-parametric tests circumvent the assumption of normality, they do not allow us to test for interactions between factors.

In each of the three experiments, we tested for differences in P2 values among female age groups (FA1, 2, and 3) and also among females mated to each of the three different male genotypes (MT 1, 2, and 3). Overall, we found a highly significant negative correlation between female age and P2, regardless of the statistical analysis employed. While the effects of female age varied somewhat among the different female inbred lines, they were significant or marginally significant in two of the three experiments. In addition, we found no significant differences in viability among larvae resulting from the different female age-male genotype pairings (Mack, data not shown). Hence we report P2 values without adjustment. We also present results from K-W tests (H-values) for differences among mean P2 for each female age after correction for ties.

Effect of Female Age

Estimates of mean P2 were significantly higher in the youngest females than in the older two female age classes (Fig. 3.1,Table 3.1, $F_{1,368} = 20.896$, $P < 0.0001$). Female genotype had a significant effect on P2 as well (Fig. 3.1, Table 3.1, $F_{2,368} = 13.655$, $P = 0.0093$), but was only marginally significant after applying a table-wide sequential Bonferroni correction (Holm 1979; Rice 1989). Of the three interaction combinations, only male genotype by female line was marginally significant after sequential Bonferroni correction (Table 3.2, $F_{4,368} = 3.002$, $P = 0.024$). Within each of the three lines, estimates of mean P2 over all vials combined decreased with female (Fig. 3.2). This decline was highly significant for 67L females (Fig. 3.2, Table 3.2; $H = 16.583$, $P = 0.0003$; $F_{1,144} = 15.525$, $P = 0.00012$) and marginally significant for 58S (Fig. 3.2, Table 3.2, H= 5.793,

P = 0.0552; $F_{1,133}$ = 4.173, P = 0.043). Our analyses for 79L females initially yielded conflicting results due to the presence of outliers (Fig. 3.2; Table 3.2; H = 2.746, P = 0.25; Table 3.2, $F_{1,89}$ = 5.271, P = 0.024). The analyses agreed, however, when we repeated the analysis excluding a single one of the outliers ($F_{2,88}$ = 3.1075, P = 0.08). The significance of the results does not differ after sequential Bonferroni correction.

Effect of Male Genotype

MT1 males achieved higher P2 values than either MT2 or MT3 for all 3 female lines (Fig.3.3). This difference was highly significant for males mated to 58S females (Fig. 3.3, Table 3.2, H = 13.391, P = 0.0012; $F_{1,133}$ = 17.83, P < 0.0001). Males mated to 67L females also differed among genotypes in both the non-parametric analysis (Fig. 3.3, Table 3.2, H = 26.068, P < 0.0001) and the parametric analysis, but only after correcting for unequal variance (Bartlett's test: $B_{2,142}$ = 9.17, P = 0.0001;Welch ANOVA: $F_{2,87.25}$ = 6.82, P = 0.0017). Mean P2 did not differ significantly among males mated to 79L females (Fig. 3.3, Table 3.2; H = 3.295, P = 0.193; $F_{2,89}$ = 1.947, P = 0.182). All significant results remained so after correction for multiple tests.

Female Age by Male Genotype Interaction

We found no female line in which there was a significant interaction between female age and male genotype (Fig. 3.3, $F_{2,133}$ = 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).

Discussion

In each of our three experiments (58S, 67L, and 79L) we found evidence for a decreasing second male advantage (lower mean P2) in older females. We also found a significant difference among male genotypes (MT) for P2, and that this trend was consistent across all female lines. There was no evidence, however, for a significant male genotype by female genotype interaction.

Why might last male sperm precedence decline as females age? It has been suggested that older *Drosophila* males might have reduced overall function in their reproductive tract (T. Markow, pers. comm.). If we apply the same reasoning to females, we would predict higher P2 values since females would be less efficient at using sperm stored from prior mates. However, we do not observe higher mean P2 values in any of the cases assayed. In fact, there is no *a priori* reason to suspect that either component of sperm competition, offense (P2) or defense (P1), should be influenced equally by change in female age, or any other parameter of female physiology. Although the exact mechanisms underlying sperm defense (P1) and sperm offense (P2) remain unknown, it does appear that the two processes are independent of each other (Clark et al. 1995; Civetta and Clark 2000; Sawby and Hughes 2001). Clark *et al*. (1995) found no significant correlation between estimates of P1 and P2 in an assay of over a 100 chromosome extraction lines. Using a subset of the same extraction lines, Civetta and Clark (2000) found that females mated to male genotypes with high mean P1 had relatively higher mortality rates. In contrast, there was no correlation between P2 and female mortality rate (but see Sawby and Hughes 2001). If P1 and P2 are, in fact, independent, females may respond

differentially to each underlying mechanism or set of mechanisms, with a resulting change in last male sperm precedence.

Several proteins, especially Acps, are likely candidates to mediate such an effect in *Drosophila* and other insects, if one exists, since they influence both sperm storage (Neubaum and Wolfner 1997; Neubaum and Wolfner 1999; Chapman et al. 2000) and oviposition rate (Heifetz et al. 2000; Heifetz et al. 2001). A female-specific age-related response to either protein could produce the effect we observed.

What are the implications for a finding that last male sperm precedence declines as a function of female age? Because sperm competition assays are almost universally conducted using females at or very near sexual maturity, and likely at their individual peak of overall physical condition, mean P2 values that are reported in the primary literature and later compiled in comprehensive reviews (e.g., Simmons and Siva-Jothy 1998), may not be representative of females in general, but only of the youngest females. Indeed, with the exception of a few species such as *D. melanogaster* (Clark et al. 1995; Hughes 1997; Clark and Begun 1998), too little attention is paid to intraspecific variation in sperm competition parameters (Lewis and Austad 1990; Simmons and Siva-Jothy 1998, p.346). Yet published means for little studied species and genera are still used in comparative studies (Walker 1980; Gwynne 1984; Ridley 1989; Simmons and Siva-Jothy 1998). Such studies may reach erroneous conclusions if they lack accurate estimates of the true variation in sperm competitive ability within and among the taxa considered.

In addition, if we know how male reproductive success may vary as a function of the age of his mate, we may construct more realistic models of how males allocate reproductive effort and choose mates. Using molecular techniques, Imhof *et al*. (1998),

concluded that female *D. melanogaster* obtained from a wild population had mated with a minimum of 4-6 mates. As a result, most males will not obtain last male sperm precedence and may achieve a far more significant proportion of their overall reproductive success by being successful at sperm defense as "first" males (P1) than by being adept at sperm offense (P2). Mean P1 estimates in species that exhibit last male sperm precedence are comparatively low, of course, but may still be important. In our experiment, mean P1 estimates for C males nearly doubled when mated to FA2 and FA3 females than to the youngest (FA1) females (Fig.4). This increase, from 0.12 to 0.23, was highly significant, just as the decrease in P2 over the same age span was ($H = 6.666$, $P = 0.036$; $F_{2,368} = 9.234$, $P = 0.00012$).

Male strategies for ejaculate partioning might be affected by changes in expected paternity when mating with older females. As noted earlier, female *D. melanogaster*, as well as many other insect species undergo reproductive diapause (Tatar and Yin 2001), hence older females may make up a much larger component of wild populations of insects than is currently appreciated. When mating with older females, males can expect to either achieve higher "first" male paternity or lower last male sperm precedence relative to their expectations from mating with young females. It is possible that males may even maximize paternity by preferring to mate with older females. By doing so, males could expect to gain greater fitness since the increased expected P1 would counterbalance their rare loss of paternity through lower expected P2. The finding that P1 is positively correlated with female mortality rate while P2 is not (Civetta and Clark 2000) raises the intriguing possibility that P1 mechanisms are more strongly selected than processes underlying P2 (Rice 1996). We do not yet know what cost males would

pay in offspring fitness by mating with older females, but recent work with *D. melanogaster* (Priest *et al.* submitted) shows that older females have offspring that survive significantly longer than offspring from younger females. The Priest et al. (submitted) result suggests that, for at least some strains of *D. melanogaster* males who mate with older females avoid some of the potential fitness costs.

Despite the intensity of investigative efforts directed at understanding the mechanisms of sperm competition in various taxa, especially in *Drosophila* sp., much still remains to be learned before we have a clear picture of the discrete processes involved in sperm offense (P2) and sperm defense (P1) (Birkhead and Moller 1998; Simmons and Siva-Jothy 1998). Indeed, we do not even know to what extent last male sperm precedence should be viewed as an adaptive trait (Gromko et al. 1984), or an artifact of correlated evolution with some other aspect of *Drosophila* reproductive biology. By themselves, high mean P2 values do not necessarily reflect strong selection on sperm competitive ability, since high P2 values would also result when male-female processes interact to reduce the temporal or functional overlap of male ejaculates, and thereby minimize actual sperm 'competition' (Simmons and Siva-Jothy 1998, p. 344). In fact, intermediate P2 values are the more likely result of intense sperm competition (Simmons and Siva-Jothy 1998), precisely what we observed in the older age classes of females in the present experiment. This suggests that sperm competition may be even more intense in older females than among females of the ages typically assayed.

By examining the patterns of sperm competition at other ages in representative taxa, we may learn considerably more about mechanisms 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 and female genotype interact with female age and physiological state. Specifically, we need to know in greater detail how male ejaculate components, such as Acps in *Drosophila* and other insects, moderate female physiology and whether or not such effects are age-specific. Future work in other taxa should address not only the effect of female age, but also other aspects of female condition, such as parasite load, general immunological state, and maternal and grandmaternal effects (Hercus and Hoffmann 2000). We also need to investigate various features of the female environment, such as resource availability, seasonality, climate, etc. As we improve our ability to measure gene expression via the use of microarray techniques, we will be able to conduct more sophisticated screens of which genes are expressed in response to mating and which of those genes are most critical in the outcome of sperm competition. This latter approach, united with conventional investigations of sperm precedence, is likely to provide a more powerful route to understanding the dynamics of intersexual conflict.

Table 3.1. ANOVA: arcsin transformed p2; all vials combined.

Factor	df	F	P
Female Age	1	20.896	<<<0.0001
Male Genotype	2	4.425	0.073
Female Line	2	13.655	0.009
Female Line x Female Age	2	3.814	0.118
Female Line x Male Genotype	4	3.002	0.024
Male Genotype x Female Age	2	5.438	0.069
Male Genotype x Female Age x Female Line	4	0.450	0.77

Table 3.2. Individual Lines, arcsine transformed P2 values, all vials combined: Kruskal-Wallis H and associated P-values; ANOVAF and P-values.

Line	Factor	df	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				

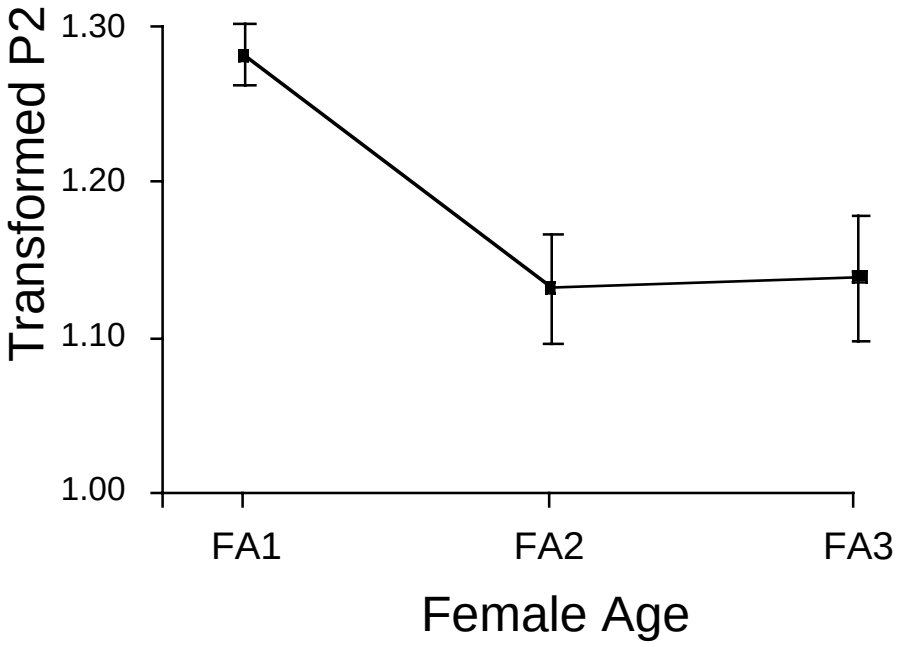

Figure 3.1 Mean arcsine transformed P2 estimates by female age, all female strains combined. Actual ages in days (d): FA1 - 3-4 d; FA2 - 17 d; FA3 - 31 d.

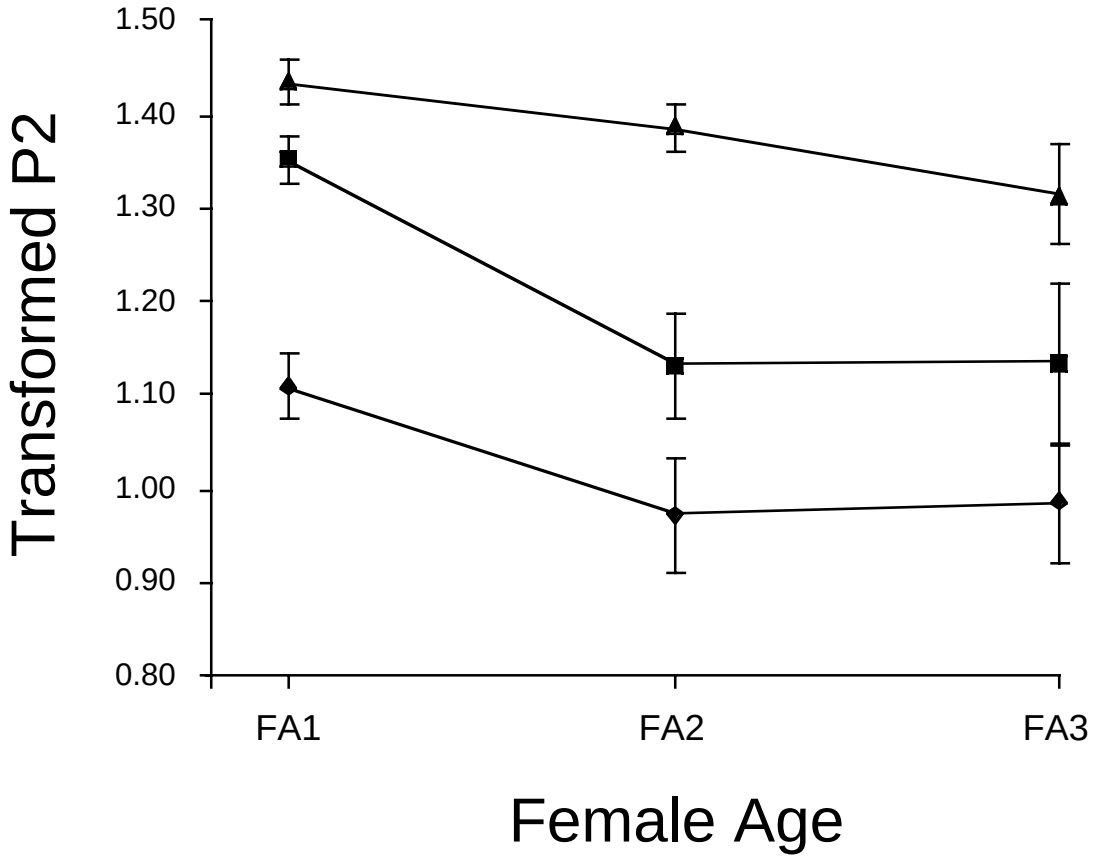

Figure 3.2. Mean arcsine transformed P2 estimates by age, each female line separate. Individual lines depict female strain: diamonds (◆) represent data from males mated to 58S females; squares (■) - 67L females; triangles (▲) - 79L females. Actual ages in days (d): FA1 - 3-4 d; FA2 - 17 d; FA3 - 31 d.

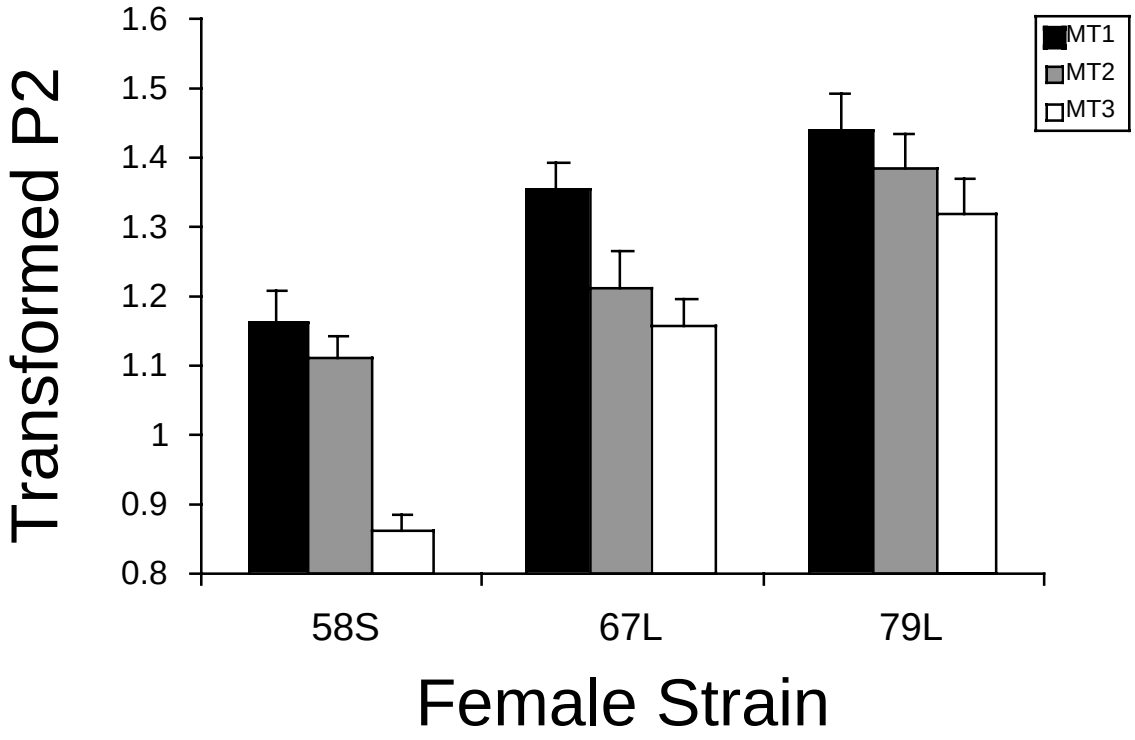

Figure 3.3 Mean arcsine transformed P2 by female line, male genotype (MT) separate. Individual bars depict means for each MT: black bar - MT1; grey bar - MT2; white bar - MT3.

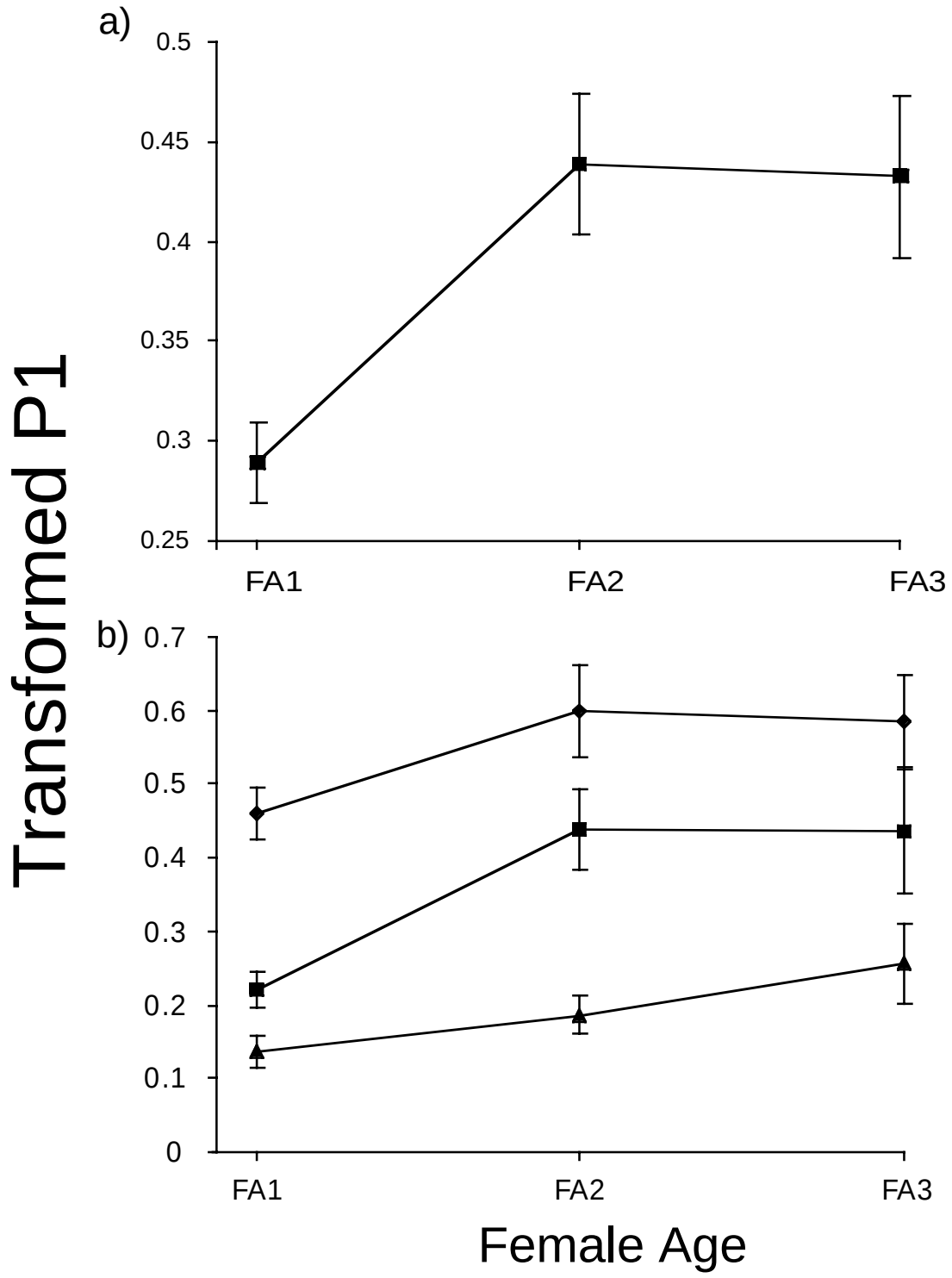

Figure 3.4 Mean arcsine transformed P1 estimates for C males mated to females of all three ages (FA1-3): a) female strains combined; b) female strains separate. Individual lines depict female strain: diamonds (◆) represent data from males mated to 58S females; squares (■) - 67L females; triangles (▲) - 79L females. Actual ages in days (d): FA1 - 3-4 d; FA2 - 17 d; FA3 - 31 d.

CHAPTER 4

SPERM COMPETITIVE ABILITY AND GENETIC RELATEDNESS IN *DROSOPHILA MELANOGASTER*: SIMILARITY BREEDS CONTEMPT[3]

[3]Mack, P. D., N. K. Priest, and D. E. L. Promislow. To be submitted to Evolution.

Abstract

A negative correlation between sperm competitive ability and genetic relatedness would suggest that females can bias their use of sperm of unrelated males relative to sperm provided by related males. Few prior studies of sperm competition have examined whether females can 'screen' sperm at levels of resolution beyond recognition of conspecificity and have yielded equivocal results. To address these previous equivocal results, we created male *Drosophila melanogaster* of four different levels of relatedness to mate with females, then measured the sperm competitive ability of those males in comparison to a standardized competitor. We show that sperm competitive ability is negatively corrrelated with relatedness, as hypothesized, and provide the strongest evidence to date that females bias sperm usage against sperm obtained from related males.

Introduction

Both multiple mating by females and storage of sperm from previous matings are widespread amongst a diverse array of animal and plant taxa (Birkhead and Moller 1998). As a result, post-mating competition between males, or sperm (pollen) competition (Boorman and Parker 1976) is the rule and, conversely, sexual or genetic monogamy is comparatively rare (Birkhead and Moller 1998, p.757). This sperm competition forces males to keep evolutionary pace by continually adapting new defensive and/or offensive weapons, such as the accessory gland proteins found in *Drosophila* and other insect species (Wolfner 1997).

A male’s attempts to improve his competitive ability against other males may exact a cost on females; female *D. melanogaster* exposed to male ejaculate components suffer increased mortality rates (Chapman et al. 1995). Exposure to male ejaculate components

also reduces female receptivity to subsequent mates in many species (Manning 1962, 1967; Leopold et al. 1971; Obata 1988; Eady 1995; Miyatake et al. 1999), so females may also pay an additional fitness cost in missed opportunities to mate with high quality males. Females who evolve a mechanism to reduce or avoid such costs should have a selective advantage over females who do not.

Females might avoid such costs by exerting physiological control over the success (non-random use) of stored sperm, an idea that has been a matter of consideration for many years (Tyler 1948; Bedford 1965; Cohen 1969; Lloyd 1979; Birkhead and Moller 1993). This potential for females to bias reproduction, now known as cryptic female choice (Thornhill 1984; Eberhard 1985; Eberhard 1996) has received more attention in recent years. Sperm selection by females remains the most controversial aspect of cryptic female choice, as well as the most difficult to demonstrate convincingly (Birkhead 1998; Pitnick and Brown 2000, but see Eberhard, 2000 #954). Prior evidence suggests that females may be able to bias usage of sperm obtained from heterospecific males (Markow 1997; Price 1997; Alipaz *et al.* 2001). Females have also been shown to avoid using sperm received from genetically incompatible males (Zeh and Zeh 1996; Zeh and Zeh 1997; Stockley 1999; Alipaz et al. 2001, but see Stockley, 1997).

And while females of many plant species have been shown to avoid pollen obtained from related individuals relative to unrelated competitors (Willson and Burley 1983; Delph and Havens 1998), similar attempts in animal taxa have, thus far, produced equivocal results (Olsson et al. 1996; Stockley 1997, 1999). Olsson *et al*. (1996; 1997) found that male sand lizards (*Lacerta agilis*) with a higher genetic similarity to their mate sire a lower proportion of the resulting offspring than more distantly related males. While Birkhead (Birkhead 1998) described Olsson *et al*.'s (1996) study as some of the best evidence to date for sperm selection, he suggested that the negative correlation between relatedness and paternity may have been due to the high level of inbreeding in the lizard population studied. In a study of the common shrew, *Sorex araneus,* Stockley (1997)

found no difference in relative fertilization success among pairings of different levels of relatedness. And, in a study of the decorated field cricket, *Gryllodes supplicans*, Stockley (1999) found that males mated to related females tended to achieve lower first-male paternity than unrelated males, but this relationship was not statistically significant.

Prior studies of relatedness and sperm competition employed study organisms characterized both by relatively long generation times and a comparative lack of phenotypic markers. Consequently, these same studies were constrained by rather small sample sizes. In contrast, the short generation-time (8-12 days) and capacity to control genetic relatedness among lineages with relative ease, make *Drosophila melanogaster* an ideal system for examining the effects of genetic relatedness on sperm competition. Here we use *D. melanogaster* to provide strong evidence that genetic relatedness influences sperm competitive ability.

Materials and Methods

In the fruit fly, *D. melanogaster*, females undergo a post-mating refractory period of 3-5 days, during which they are unreceptive to courtship by subsequent males (Markow 1996). When a female remates with a second male, that male sires the majority of her subsequent offspring. This phenomenon is referred to as last male sperm precedence and is characteristically expressed as P2, the proportion of the total offspring sired by the second male to mate in a two-male sequence (Boorman and Parker 1976). Conversely, the proportion of total offspring sired by the first male, in 'defense' against the newly received sperm from the last male, is referred to as P1 (Boorman and Parker 1976).

To assay the effect of relatedness on the outcome of sperm competition, we mated females to full sibs (R1), half-sibs (R2), cousins (R3), or unrelated (R0) males. We mated virgin females first to a male, randomly assigned from one of the four male treatments, and then, after 2 d, remated her to an unrelated competitor (C) male homozygous for the bw^D allele (details below).

Females mated to a related male may produce fewer offspring due to the effects of inbreeding on embryonic development and larval viability (Keller 1998). To control for this, we measured male fertility in each of the four relatedness treatments.

Drosophila Stocks

We generated all wild type flies used in these experiments from a base population of wild-caught mated females (GA), collected in a peach orchard in Watkinsville, GA in August 2000. At that time, we set up 150 isofemale (I) lines and subsequently used the majority of these 150 I lines to generate the experimental males and females for our experiment. Except where noted, all expansions, crosses, collections, and matings were conducted as follows. We set up all crosses (to produce all individuals for assay, as well as the sperm competition matings themselves) with a single male and a single female, then collected their offspring under light CO_2 anesthesia 10-12 days (d) later. Progeny were maintained in single-sex 8-dram vials containing approximately 5 ml of standard molasses-agar-cornmeal-killed yeast medium, at a density of 20 individuals per vial at 24 °C on a 12/12 light-dark cycle. We added a single grain of active yeast to the surface of the medium in each vial.

Generation of Experimental Males and Females

To carry out this experiment, we generated males of four different relatedness levels – full sib (R1, r = 0.5) , half-sib (R2, r = 0.25), cousin (R3, r = 0.125), and unrelated (R0, r = 0) – to females (hereafter, focal females) in each of eleven different lineages (blocks). To produce males of the four different relatedness levels relative to focal females, we mated a single male (male *i*) to each of two unrelated females each from a different outbred line (Fig. 4.1). We collected both male and female offspring from the first female (female *a*) for use as sibling (R1) males and focal females, respectively. Similarly, we used the male offspring of the second female, female *b*, for the half-sib (R2) treatment. In

addition, we mated a sibling male of male *i* to a female (female *c*) from a third unrelated outbred line to produce males for the cousin (R3) treatment. Finally, to produce males for the unrelated (R0) treatment, we mated a male and female, each from separate outbred lines different from any other outbred line already described. For each set of crosses as just described we used a total of six outbred lines. For each set of six lines, we set up four replicate sets of the crosses just outlined. Hereafter we refer to each set of identical replicates as a block. We repeated this setup with eleven independent blocks; no outbred line was used more than once to ensure that all blocks were independent from each other. In addition, to avoid the effects of larval density on sperm production (Gage 1995), we kept larval densities low by transferring individual females to fresh vials at 24 hr intervals.

By necessity, we collected virgin females from *a* females over a 3-day period prior to their use in the assay. As a result, females varied from 3-5 days in age. We pooled the females prior to the assay to randomize age variation across treatments. We collected sufficient males of all levels of relatedness over a 2-day collection period, then pooled by level within each block. Males were randomly selected from pooled groups within block for assay.

Competitor Male Production

To produce C males with the bw^D marker phenotype, we used two chromosome extraction lines derived from a base population collected in 1998 at the same site as the GA flies. Each of the two parent lines, C128 and C302, was identically homozygous for unique combinations of chromosomes II and III, as well as homozygous for bw^D. For details, see Mack *et al*. (submitted). We produced identically heterozygous C males by placing 15 virgin 2-4 day old C128 females and 12 virgin 2-4 day old C302 males in each of 72 half-pint bottles, each with approximately 50 ml standard medium and several grains of live yeast applied to the surface. We collected virgin males over a 2-day period

beginning five days prior to their use in the sperm competition assay. As with our focal females and related males, we pooled C males over the two days of collection to minimize variation due to age.

Sperm Competition Assay

We first mated randomly selected focal females to treatment males by combining a single focal female from block *i* in a vial with two 2-4 d old block *i* males from one of the four treatment groups. Note that for mating between females and sibs (R1), we used males reared in separate vials from females to avoid common rearing environment effects (Kim, submitted). For each block, we created ten replicate vials per treatment. We began first matings at 1600h. We exposed focal females to treatment males for approximately four hours, and then removed the males. Focal females were left in the original vials until the second mating of the sequence. Beginning at 1900h two days after the first mating, we placed two C males aged 4-5 d with each female overnight. The following day, we removed all C males and transferred each female to a new vial (v1) containing fresh medium between 900-1000h. Females were transferred two additional times to fresh vials (v2 and v3) at 3 and 6 days.

Sixteen days after each vial set was created, we collected all offspring and stored them at -80 °C. Individual progeny were subsequently sorted by eye color to assign paternity and counted. Vials 1-3 were counted and scored separately to test for differences in P2 among vial sets although we found no qualitative differences between analyses of P1 over all vials combined and P1 from vial 1 only (data not shown). We excluded replicates for which we did not have a complete set of 3 vials. Sample sizes for each treatment by block are reported in Table 4.1.

Egg-Adult Viability Control

We also tested for possible differences in fertility and larval viability among males of different levels of relatedness to focal females. We placed single 4-5 d old males of block *i* and treatment R_j in a vial with a single focal female beginning at the same time as the sperm competition matings, 2000h. We transferred male-female pairs to fresh food vials 24 h later, and began counting eggs the following morning. Male-female pairs were removed when approximately 30 eggs had been oviposited or after 24 h if females had oviposited fewer than 30 eggs. We counted the total number of eggs within 2 hours after removal of adult flies. All resulting progeny were collected and stored at -80 °C 16 d later. We measured egg-adult viability as the relative proportion of eclosed adults to eggs oviposited.

For this assay, we attempted to set up five replicate vials for each treatment-block combination ($R_{j,}$ $block_i$), but some $R_{j,}$ $block_i$ combinations did not produce sufficient males. As a result, not all treatment types were represented in each block. Although our sample size for the egg-adult viability control were not equal across all blocks, we were able to increase overall sample size per treatment type by creating more than five replicates in blocks which produced treatment males beyond what we initially acquired. We report sample sizes in Table 4.2.

Statistical Analysis

To adjust our counts of progeny sired by each type of male, we divided the number of wild type offspring by the mean egg-adult viability (f_i), pooled across blocks, for males of relatedness R_i (Table 4.3). Similarly, we divided the number of bw^D offspring by the mean egg-adult viability for C males (Table 4.3, $f_C = 0.867$). We used these adjusted counts to calculate an 'adjusted P1' that was non-normally distributed. To more closely approximate normality, we used a modified Box-Cox transformation,

$$Y' = [(Y + c)^{\lambda} - 1]/\lambda \quad (1)$$

where $\lambda = 0.2253$, $c = 0.2605$ (Sokal and Rohlf 1995, p.418). We obtained the λ-value from a Box-Cox maximization program. We selected *c* to maximize the Shapiro-Wilks value with the selected λ-value. Model residuals from adjusted P1 data were normally distributed after Box-Cox transformation (Shapiro-Wilks value = 0.983, P = 0.56). We then used ANCOVA in JMP 4.0.2 (SAS Institute 2000) with relatedness and fecundity (adjusted total flies) as continuous factors, and block as a random factor. In a separate ANCOVA, we tested for an effect of relatedness on egg-adult viability, with relatedness as a fixed, continuous factor and block as a random discrete variable. We excluded all replicates for which no bw^D offspring were counted, since we could not verify that the female mated C males in those cases (n = 16).

Results

Egg-adult Viability

We observed significantly lower egg-adult viability when R1 males mated to sibling females compared to egg-adult viability measured for males of all other relatedness levels (Table 4.3; $F_{1,110} = 19.394$, $P < 0.0001$). Neither block nor the block by relatedness interaction were significant (Table 4.3). Because egg-adult viability did not differ among blocks within treatments, we were able to pool egg-adult viability values across blocks to calculate 'adjusted P1' as described previously.

Effect of Relatedness

Relatedness is significantly negatively correlated with P1. Of the four levels assayed, R1 males that mated to a sibling female achieved significantly lower P1 values (Fig. 4.2, Table 4.4; $F_{1,204} = 7.81$, $P = 0.0057$) than more distantly related males. Furthermore, although R2 males did not appear to achieve significantly different P1 values from either R3 or R4 (unrelated) males, P1 did show a general increase as relatedness decreased as

we hypothesized (Fig. 4.2). Because P1 is a ratio, we were concerned that the observed negative correlation between relatedness and P1 was actually caused by the relationship between relatedness and total fecundity. In fact, we did find that P1 was negatively correlated with total fecundity (Table 4.4; $F_{1,204} = 17.702$, $P < 0.0001$), but there was no relationship between fecundity and relatedness (Table 4.5; $F_{1,204} = 0.0659$, $P = 0.79$). Both relatedness and total fecundity remained significantly correlated with P1 after sequential Bonferroni correction (Holm 1979; Rice 1989).

Discussion

Our data support the hypothesis that sperm competitive ability (in this case, sperm defense or P1) is inversely correlated to the level of relatedness between the female and her mate. We have shown that P1 values for male siblings (R1) of the focal female are significantly lower than those of other males and that relatedness level in general is a significant predictor of P1. Furthermore, our study is based on sample sizes an order of magnitude larger than any previous study of this type (Stockley 1997, 1999).

Several prior studies have demonstrated a cost of inbreeding. When related individuals mate, offspring fitness, and, thus, reproductive fitness, are reduced. Here we show that females who mate with related males also use proportionally less of that male's sperm relative to sperm of subsequent mates. Thus, related males pay a two-fold cost via a reduction in paternity and a reduction in fitness of the resulting offspring. As a result, selection should act on both males and females to avoid mating with close relatives. Bias against the sperm of related males may, in fact, provide a post-mating mechanism for females to reduce the cost of inbreeding. If a virgin female encounters a related male after reaching sexual maturity, she could mate with that male to ensure that she has sperm in storage. If she subsequently encounters a second, unrelated male, she could mate with him and preferentially use his sperm over the related male's. We must rely on future work to determine whether or not females can facultatively sequester sperm from related

males. If so females must be able to recognize related males. Recent evidence suggests that females can bias mate choice by recognizing kin in both mice (Manning *et al.* 1992; Penn and Potts 1999) and flies (Kim, submitted).

Our results demonstrate that female *D. melanogaster* have evolved a more fine-grained mechanism for selecting sperm than has been shown in prior work (Birkhead 1998; Pitnick and Brown 2000). We know from earlier investigations that females could bias sperm use against sperm of both heterospecific (Price 1997; Price *et al.* 2000; Shapiro 2000; Alipaz et al. 2001; Price *et al.* 2001; reviewed in Howard *et al.* 1998) or genetically incompatible males (Palumbi 1994; Olsson et al. 1996; Zeh and Zeh 1996; Zeh and Zeh 1997; Alipaz et al. 2001). Here we show that female *D. melanogaster* have evolved the ability to avoid sperm of genetically similar males and, thus, may have taken an additional step toward higher resolution sperm selection (sensu Birkhead 1998; Birkhead 2000; Pitnick and Brown 2000).

However, it remains to be seen whether females can select sperm from the most preferable or highest quality males and not just simply avoid sperm on the basis of relatedness or conspecificity. To demonstrate sperm selection definitively, we need to better understand the proximate mechanisms by which females and males influence reproductive decisions in three fundamental ways. First, we need to know how males influence the amount of sperm transferred and the manner in which it is stored in the female. Second, how do females manage and utilize the sperm they receive? Third, we need to know to what extent, if any, female animals can bias resource allocation to embryos sired by non-preferred or lower quality mates. Any of these processes could influence the outcome of sperm competition (Birkhead 1998; Birkhead 2000; Pitnick and Brown 2000).

As we extend our ability to employ genetic markers beyond those now in use in model systems such as *D. melanogaster*, we will be able to assess whether or not sperm choice

exists, and how common it may be. Future studies must attempt to delineate a mechanism by which females bias sperm selection, at whatever scale it occurs.

Table 4.1. Sperm competition assay sample sizes by treatment and block.

Block/	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**11**	**12**	**Total**
Relatedness												
0.5	5	5	7	1	5	5	5	5	5	3	6	52
0.25	8	7	4	3	6	5	6	7	7	6	5	64
0.125	9	4	5	2	4	6	5	5	5	2	5	52
0	4	3	6	3	5	7	6 9	5	3	7	58	

Table 4.2. Egg-to-adult viability assay: sample sizes by treatment and block.

Block/	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**11**	**12**	**Total**
Relatedness												
0.5	2	1	1	5	2	4	3	2	4	3	3	30
0.25	2	2	2	5	1	3	3	1	3	2	3	27
0.125	2	1	1	5	2	4	3	2	4	1	3	28
0	2	2	1	5	2	4	3	-	3	2	3	27

Table 4.3. a) Egg-adult viability estimates by relatedness level; b) ANCOVA on egg-adult viability with relatedness and fecundity as fixed and continuous factors and block as a random factor.

a)	**Relatedness**		**Mean**	**s.e.**
	0.5	(sib)	0.772	0.045
	0.25	(half-sib)	0.941	0.017
	0.125	(cousin)	0.929	0.025
	0	(unrelated)	0.949	0.012
	C	(competitor)	0.867	0.037

b) **Factor**	**df**	**F**	**P**
Relatedness	1	16.980	<0.0001 ***
Block	10	0.962	0.48
Error	141		

Table 4.4. ANCOVA on adjusted P1 estimates with relatedness and fecundity as fixed, continuous factors and block random.

Factor	df	F	P
Relatedness	1	12.046	<0.001 ***
Block	10	0.0323	0.999
Fecundity	1	21.437	<0.001 ***
Relatedness x block	10	2.457	0.0083 **
Fecundity x block	10	1.311	0.23
Relatedness x fecundity	1	0.048	0.83
Error	192		

Table 4.5. ANCOVA on total fecundity estimates for all vials combined with relatedness as fixed and continuous and block random.

Factor	df	F	P
Relatedness	1	0.066	0.80
Block	10	0.092	0.997
Relatedness x block	10	1.761	0.07 †
Error	204		

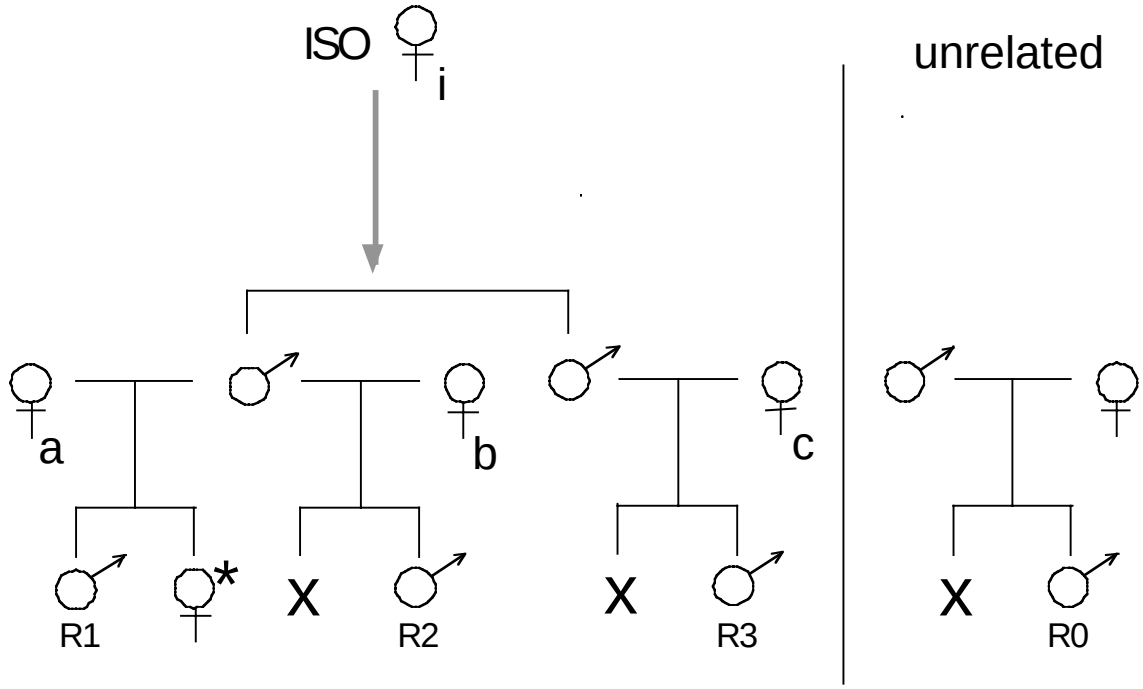

Figure 4.1. Crossing design employed to generate males of relatedness levels R0-3. All females and males used are from unrelated isofemale lines except for the full sib male offspring from the female designated isofemale *i*.. Symbols *a*, *b*, *c*, and *d* indicate females selected for production of treatment males R1 (r = 0.125), R2 (r = 0.25), R3 (r = 0.5), and R0 (r = 0); the "*" designates the focal female.

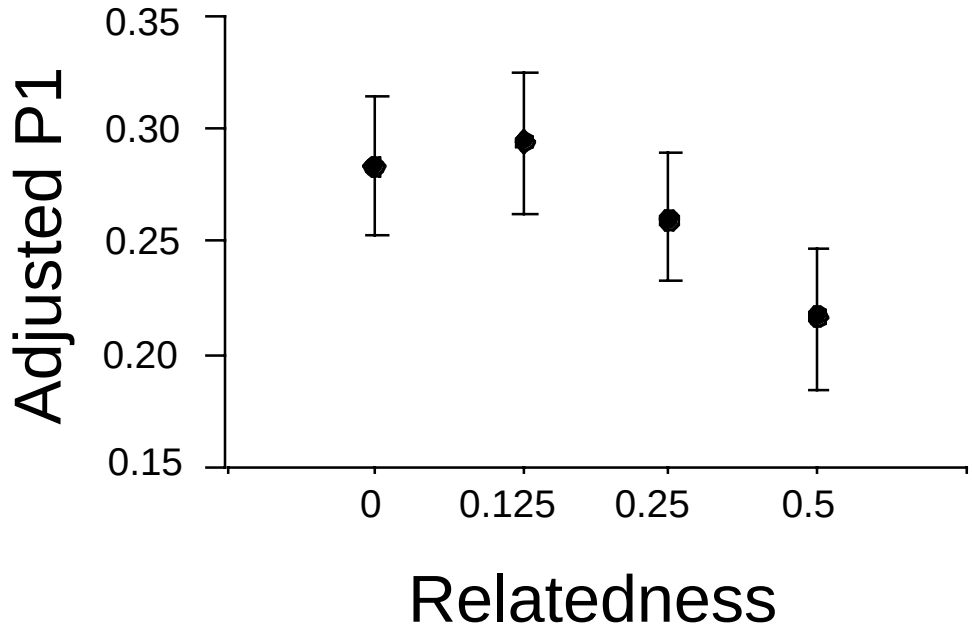

Figure 4.2. Mean adjusted P1 estimates by relatedness level.

CHAPTER 5

AGE-SPECIFIC EFFECTS OF NOVEL MUTATIONS IN *DROSOPHILA MELANOGASTER*: FECUNDITY AND MALE MATING ABILITY[4]

[4]Mack, P. D., V. K. Lester, and D. E. L. Promislow. In press. Genetica
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Abstract

Evolutionary theories of senescence assume that mutations with age-specific effects exist, yet until now, there has been little experimental evidence to support this assumption. In this study, we allowed mutations to accumulate in an outbred, wild population of *Drosophila melanogaster* to test for age-specific differences in both male mating ability and fecundity. We assayed for age-specific effects of mutations after ten, 20, and 30 generations of mutation accumulation. For mating ability, we found the strongest effects of mutations in the first half of the life span after 20 generations, and at nearly all ages by generation 30. These results are qualitatively consistent with results from a companion study in which age-specific mortality was assayed on the same lines of *D. melanogaster*. By contrast, effects of fecundity were confined to late ages after 20 generations of mutation accumulation, but by generation 30, as with male mating ability, effects of novel mutations were distributed across all age classes. We discuss several possible explanations for the differences that we observe between generations within traits, and among traits, and the relevance for these patterns to models of aging as well as models of mate choice and sexual selection.

Introduction

Mutations provide the fundamental source of genetic variation on which natural selection acts. The rate at which mutations occur has far-reaching implications for a variety of biological phenomena, including the evolution of sex (Kondrashov, 1988; Kondrashov, 1994), patterns of mate choice and sexual selection (Iwasa, Pomiankowski & Nee, 1991; Pomiankowski, Iwasa & Nee, 1991; Hansen & Price, 1995; Kokko & Lindström, 1996; Beck & Powell, 2000), the risk of population extinction (Lynch et al., 1993; Lande, 1994; Lynch, Conery & Burger, 1995b; Lynch, Conery & Burger, 1995a),

and the evolution of senescence (Medawar, 1946; Medawar, 1952; Charlesworth, 1990). The role of mutations in senescence is complex. Patterns of aging will depend not only the mutation rate, but on the distribution of mutational effects, the age-specificity of individual mutations, the nature of epistatic interactions, and the degree to which novel mutations act pleiotropically across age classes.

In his Mutation Accumulation (MA) model for the evolution senescence, Medawar (1952) placed novel mutations at the center of aging theory. Specifically, he noted that the strength of natural selection declines with age. If a mutation that reduces survival or fecundity has effects confined to very late ages, it will experience little or no selection, and so may be maintained at relatively high frequency in the population. In contrast, a mutation of equal magnitude, but with effects confined to early ages, would soon be removed from the population by natural selection. The extent to which deleterious germ-line mutations accumulate over evolutionary time will be proportional to their age of action. This process can thus lead to an age-specific decrease in fitness components.

Mathematical models of mutation accumulation and aging make specific assumptions about the way in which these mutations are expressed (e.g. Hamilton, 1966; Charlesworth, 1990). For example, Charlesworth's model assumes that mutations affect fitness at only one age, and that a mutation is equally likely to affect any one age-class. A novel mutation that decreases fitness equally at all ages will be removed by selection. Charlesworth's (1990) model predicts that additive genetic variance for fitness components should increase with age (see also Rose & Charlesworth, 1981b). And while some work supports this prediction (Kosuda, 1985; Hughes & Charlesworth, 1994), other studies have found either no change with age (Rose & Charlesworth, 1981a), or an age-related decline in genetic variance with age (Promislow et al., 1996; Tatar et al., 1996; Shaw et al., 1999). As a consequence, we have argued previously (Promislow et al., 1996) that we need to test the basic assumptions underlying the MA model of senescence,

including not only the rate and magnitude of novel mutations, but also the age-distribution of their effects.

Over the past decade, researchers have attempted to measure rates of deleterious mutations in a variety of organisms, including bacteria (Kibota & Lynch, 1996; LeClerc et al., 1996; Blattner et al., 1997; Jacobs & Grogan, 1997; Matic et al., 1997), yeast (Drake, 1991; Goffeau et al., 1996), nematodes (Keightley & Caballero, 1997; Vassilieva & Lynch, 1999), plants (Schultz, Lynch & Willis, 1999; Willis, 1999), and starting much further back, fruit flies (Mukai, 1964; Mukai et al., 1972; Ohnishi, 1977; Keightley, 1994; Hughes, 1995b; Keightley, 1996). In some of this work, researchers have also tried to incorporate estimates for the distribution of the magnitude of effects of these novel mutations (Garcia-Dorado, Monedero & Lopez-Fanjul, 1998; Keightley, 1998; Fry et al., 1999). However, little has been done to determine the age-distribution of the effects of these mutations — information that is critical if we are to understand the role that novel mutations play not only in the evolution of aging, but in the overall structure of an organism's life history strategy.

Only one study has set out to determine the degree to which spontaneous germ-line mutations affect fitness components (specifically, mortality) at different ages (Pletcher, Houle & Curtsinger, 1998). They found significant effects of mutation on mortality at early and middle ages, but no effects for either sex past 37 d. Although Pletcher, Houle & Curtsinger's (1998) work provided an important first look at the age-specific nature of deleterious mutations influencing mortality, two limitations of their study inspired our own work. First, the lines they used were derived from lab strains, and so may have suffered from high mutation load (see Yampolsky, Pearse & Promislow, in press). More relevant to the present study, the previous study did not consider the effect of mutations on other age-related traits. In particular, here we are interested in mutational effects on sex-specific fitness traits, including female fecundity and male mating ability.

Quantitative genetic studies that have looked simultaneously at mortality and fecundity (Promislow et al., 1996; Tatar et al., 1996) or mortality and male mating ability (Hughes, 1995a) suggest that genetic structure for these traits can be quite different. In light of these studies and that of Pletcher and colleagues, here we extend questions asked by these prior studies to determine the age-specificity of novel mutations acting on two important behavioral aspects of fitness—female fecundity and male mating ability. Mutation plays a central role in some models of mating behavior and sexual selection (e.g. Pomiankowski, Iwasa & Nee, 1991; Beck & Powell, 2000). Information derived from the present study may allow us to develop more complete models on the role of mutation in mate choice.

To allow mutations to accumulate, we use a relatively new approach known as the Middle Class Neighborhood (MCN) (Shabalina, Yampolsky & Kondrashov, 1997). The MCN approach allows us to accumulate mutations in newly-caught wild populations of flies. This approach circumvents the problem of accumulating mutations on a genetic background that is already virtually saturated with late-acting mutations that have accumulated in the lab (see Yampolsky, Pearse & Promislow, in press). Taken together, our studies of fecundity, male mating ability, and larval and adult mortality provide the first comprehensive picture of how novel mutations might influence behavioral and demographic traits related to fitness throughout the life course.

Materials and Methods

Drosophila Lines

We created an outbred population of *Drosophila melanogaster* from approximately one hundred mated females obtained at the University of Georgia Horticultural Farm in Watkinsville, GA in August 1997. Flies were brought to the lab, placed singly in 8-dram vials, and allowed to lay eggs for several days. We created 200 separate F_1 families from these vials. Throughout the experiment, we maintained flies on standard

molasses/agar/cornmeal/yeast medium with propionic acid added to inhibit fungal growth.

To provide a standard male genotype against which to assay male mating ability of the experimental and control flies, we created a separate marker strain that was homozygous for the recessive, sparkling eye mutant (spa^{pol}). We obtained the spa^{pol} mutant from the Bloomington stock center, and crossed these flies with the outbred, wild-caught strain, isolating spa^{pol}-homozygous F_2 progeny, which had 50% Georgia background and 50% spa^{pol} stock background. This line was used as a standard against which to measure male mating ability. We maintained the sparkling line as a stock culture in 8-dram shell vials on a 2-week culture cycle at a minimum population size of 400. During assay periods, we expanded the spa^{pol} population to produce approximately 600 virgin males and 500 virgin females.

Mutation Accumulation

After one generation in the laboratory, we used our wild-caught flies to create two mutation accumulation lines and two control lines. We allowed mutations to accumulate by maintaining them in the Middle Class Neighborhood (MCN, see Shabalina, Yampolsky & Kondrashov, 1997) which effectively removes the force of selection acting on the population. Each mutation accumulation line consisted of one hundred families, and each family contributed exactly one son and one daughter to the next generation. The effective population size in the MCN is approximately 4*N*, where *N* is the number of individuals in the population (Crow & Kimura, 1970), and all but lethal and semi-lethal mutations are free to spread through the population in the absence of selection. The two mutation accumulation lines (MCN1 and MCN2) were kept at 25° C and allowed to breed at 2-3 d old, so that generation time was approximately 11 d.

At the same time that we set up MCN1 and MCN2, we created two control lines, C1 and C2. These lines were maintained in a similar regime, except that we kept the

temperature at 15° C. In this case, generation time was approximately 100 d. Over the course of the experiment, we ran through thirty generations in the MCN lines, but only five generations in the control lines. After 21 generations of mutation accumulation, we returned to the original site where we collected our wild flies to collect a third control line (C3). See Yampolsky, Pearse & Promislow (in press) for a more complete discussion of the methods, benefits and drawbacks of the Middle Class Neighborhood approach to accumulating mutations.

Assays

After ten, 20 and 30 generations of mutation accumulation, we measured age-specific fecundity and male mating ability in MCN and control lines. Flies from each of the MCN and control lines underwent one generation of expansion before the actual assay (see Yampolsky, Pearse & Promislow, in press).

Male Mating Ability

Male mating ability (MMA) provides a comprehensive measure of the ability of wild-type males, in competition with a separate marker strain of males, to sire offspring. It may include the effects of female preference and male courtship ability, sperm competition, and larval viability. To assay MMA in our experiment, we competed wild type (focal) males from each of the experimental lines against spa^{pol} males. We repeated these assays for a series of wild-type males of different ages competed against four-day-old spa^{pol} males. Offspring of matings between wild-type MCN or control males and spa^{pol} females will be wild-type, while offspring of spa^{pol} female _ spa^{pol} male matings will have the sparkling eye phenotype. Thus, the ratio of wild type to spa^{pol} offspring provides an estimate of male mating ability, and the relative proportion of wild-type offspring in MCN lines versus control lines provides an estimate of the effect of *de novo* mutations on male mating ability.

At each of generations 10, 20, and 30, we randomly selected several hundred offspring from each MCN and control line, and expanded them in 1/2-pint plastic bottles (Applied Scientific) on standard molasses/cornmeal/agar medium for one generation. From this expansion, we collected approximately 2000 virgin males and females over a 2-day period, under light CO_2 anesthetic. We held all virgin males in 8-dram shell vials at approximately 20 individuals/vial at 24° C on a 12:12 light-dark cycle. We carried out mating assays at approximately ten-day intervals (actual ages are shown in Figure 1) until all males had either been used once or had died.

For each assay, we placed five virgin males from one of the MCN or control lines with five 2-3 day old virgin *spa*^{pol} males in an 8-dram shell vial (vial 1) with 5 mg food. No anesthesia was used for this procedure. Approximately 24 h later, at 1400 h, we transferred five 3-4 d old *spa*^{pol} virgin females into the vial with the ten males. After 24 h, we transferred all flies to a second vial (vial 2). We removed adults from vial 2 after 24 h and discarded them. After we removed adults from the vials, we placed the vials at 24° C on a 12:12 photoperiod. There were fifteen replicate vials for each MCN and each control line. We collected progeny from each replicate vial twice, 13 d and 18 d after mated flies were removed, and stored the collected progeny at -80° C for subsequent counting.

We repeated this process for a series of male ages, using 15 replicate assay vials for each line, and for each age class. At generation 30, we included 15 additional replicates for the C3 line for each age class. The exact ages at which males were assayed varied slightly among the three assay periods. In general, we conducted the first assay when the control and experimental males were 4-5 d old (actual ages are shown in Figure 1).

Additional Controls for MMA

MCN males at later ages (generally > 35 d) were more likely to die prior to transfer to vial 2 than Control males, possibly due to the cost of male courtship and mating (Partridge, Green & Fowler, 1987). To test for an effect due to this increased male

mortality on MMA, we assayed a subset of vials where only females were transferred to vial 2. Whether males were present or absent in vial 2 had no significant effect on measures of MMA (data not shown).

Fecundity

To measure fecundity at generations 10 and 20, we placed single females with single mature GA males that had been maintained in population cages throughout the experiment, and allowed the pairs to mate in 8-dram shell vials. After 24 h, we transferred females singly to fresh vials and allowed them to lay eggs for 24 h. We froze eggs on the food surface by adding a small amount of liquid nitrogen to each vial; this arrested egg development and facilitated counting. Frozen eggs were then counted under a dissecting microscope. We repeated the transfer and freezing every three days, throughout each female's lifespan. New, 3-5 d old males were added to females at intervals of approximately two weeks to ensure an adequate supply of sperm for the females. If a male died, he was replaced by a new male of the same age.

We carried out the first egg counts when the females were three days old, and then conducted subsequent counts at approximately three-day intervals. When egg counts began to level off or decline for the majority of females, we extended the interval between counts to seven or eight days. We continued the egg counts as long as any females were producing fertile eggs. We measured fecundity on a total of 37, 28 and 48 different days for generations 10, 20, and 30 respectively. To avoid counter bias, all vials were coded so that the egg counter did not know the source of the vial.

At generations 10 and 20, mortality rates in females were relatively high. To reduce female mortality at generation 30, we did not keep males continuously housed with females. Initially, we placed 24-48 h old females in a vial with two 3-5 d old wild-type males for a 24-hour period. We then transferred the female alone to a new vial to lay eggs for 24 h, after which she was transferred to a fresh vial. We placed one new wild-type

male with each female 24-hours prior to each subsequent count period. We removed males after the 24 h period and transferred the female to a fresh laying vial for 24 hours. After the transition to seven-day intervals between counts, females spent six days alone in vials until the next pair of males was introduced. As a result of the reduced exposure to males, females lived longer and we were able to assay females at considerably greater ages during the generation 30 assay than for the two prior assays. As with the MMA estimates, we included the wild control line, C3, during generation 30 along with the other lines.

Statistical Methods

To test for significant differences in MMA between MCN and control lines at different male ages, we used a general linear model of the form

$$P_{ijk} = \upsilon + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk} \quad (1)$$

where P_{ijk} is the proportion of offspring with wild type eye color observed at age *i* for treatment *j* in vial *k*, α is the age effect, β is the treatment effect, $\alpha\beta$ the interaction term, and ε is the error term.

To detect the effect of age-specific mutations on MMA, we compared the results from MCN males to those of Control males at several ages within each of generations 10, 20 and 30 separately. In each case, we considered replicate lines within a treatment as a random effect. If mutations with age-specific effects on MMA had occurred, MCN lines should differ from Control lines at some ages but not at others. Due to uncertainty about the distribution of the error term, we analyzed the MMA data using two different ANOVA approaches. In one, we assumed that the error variance was normally distributed, while in the other, we assumed a binomial distribution. Statistical results from the two methods agreed in all cases, so only those assuming a normal error term will be reported here. To correct for multiple comparisons, we used a sequential Bonferroni test (Holm, 1979).

We also wished to test for age-specific differences in fecundity. Here we also compared the mean fecundity of MCN females to that of Control females at several ages within a generation, looking for differences at some ages but not others as the telltale sign of age-specific deleterious mutations. Data for age-specific fecundity were neither normally distributed nor balanced. To mitigate these problems, we used a standard randomization approach to test for significant differences between the MCN and control regimes (Manly, 1997). More specifically, we permuted the existing data set 2000 times, redistributing the data for each vial at random among lines, and then recalculating our test statistic—the difference between the mean fecundity of the MCN and Control lines. We used these mean values to generate a distribution against which to compare our observed difference. The significance value of these differences was given by the proportion of randomized test statistics that were greater than or equal to the observed test statistic.

Results

Male Mating Ability

For all assays, there were no significant differences between vials 1 and 2, so data from the two sets of vials were pooled.

Results from the standard ANOVA showed no significant effects of mutation accumulation at generation 10, but MMA for Control lines was significantly greater than MMA for the MCN lines at generations 20 and 30 (Table 5.1). However, we were particularly interested in the age-specific effects of mutations as seen at specific ages. The patterns for MMA turn out to be quite different than what was observed for age-specific mortality (Yampolsky, Pearse & Promislow, in press).

At generation 10, there was already some evidence for mutational effects (Figure 5.1). At five of six ages, the average MMA was greater in control lines than in MCN lines. However, only two ages (5 d and 44 d) exhibited significant differences and these were no longer significant after correcting for multiple comparisons.

Males in generation 20 showed a substantial increase in mutational effects for male mating ability throughout the life span (Figure 5.1), though the magnitude of the effects appeared to be greater in the first half of the life span. By age 42, there was no significant decrease in MMA in the MCN lines. At age 52, the difference between MCN and control lines was significant (Figure 5.1), but the effect appeared to be primarily due to Control line 1, since the other control line had lower mating ability than MCN1 (see Figure 5.1).

Effects of *de novo* mutations on male mating ability appeared to be even stronger after 30 generations of mutation accumulation, and were distributed throughout all ages classes. Male mating ability was higher for controls than for MCN males for all ages, as expected. All ages showed significant differences, except the oldest age, 68 d. At this last age, as mean MMA approached zero (0.041 $\pm$ 0.013, mean $\pm$ s.e.), we had little statistical power to detect significant differences.

We also tested for differences between C1, C2, and C3. Mean MMA for C3 was significantly greater than the means for C1 and C2 only at 38 d ($p<.0001$). This difference was still significant after Bonferroni correction. We reanalyzed the data without C3 at all ages, but found little qualitative difference from the analysis including C3. MMA for MCN was still significantly less than Control MMA at all ages up to, but not including 57d (after Bonferroni correction).

Fecundity

Based on our randomization tests, novel mutations did not appear to have as strong an effect on fecundity as on male mating ability. However, this may have been due in part to the relatively high female mortality in generations 10 and 20, where females had continuous contact with relatively young and vigorous males (e.g. Partridge, Green & Fowler, 1987). This high mortality led, in turn, to relatively small sample sizes and limited statistical power. There were no differences between MCN and control lines at generation 10 (Figure 5.2, P-values all > 0.5 except for age 13 d, P = 0.113). By

generation 20, the last three age classes showed the expected pattern of decreased fecundity in the MCN lines (P-values = 0.011, 0.041, and 0.078 for ages 20, 24, and 29 d, respectively). However, after controlling for multiple comparisons, none of these differences was statistically significant (Figure 5.2).

By generation 30, we begin to see consistent treatment differences in fecundity at several age classes. Before controlling for multiple comparisons, five out of nine age classes showed either significant or marginally significant decreases in fecundity in MCN lines. As with male mating ability, these effects were distributed throughout the life cycle, with significant or marginally significant effects at ages four, seven, and 14 d, and ages 31 and 47 d (P-values = 0.0055, 0.094, 0.0795, 0.015 and 0.044 for ages 4, 7, 14, 32, and 48 d, respectively). After the Bonferroni correction, only age four days remained significant ($P < 0.05$).

Discussion

Age-specificity of novel mutations

Models for the evolution of senescence (Charlesworth, 1990) make explicit assumptions about the age at which novel mutations act, but until now we have known very little about the age-specificity of *de novo* mutations. In this study, we have found some evidence for a bias in favor of early-acting mutations at generation 20 for MMA, but by generation 30 there appeared to be little or no age-specificity to the effects. The pattern for fecundity at generation 20 was quite different from that for MMA. There was no effect of mutations early in life. We observed a substantial decrease in MCN line fecundity at intermediate to late ages, though the effect was only significant at one age. The high mortality rates in the fecundity analysis at generation 20 preclude us from being able to know what might have happened at very late ages. By generation 30, as with male mating ability, mutations affecting fecundity were relatively evenly distributed across age classes.

The data suggest that the apparent distribution of age-specific effects of *de novo* mutations may depend on the duration of mutation accumulation. Initially, perhaps one or a few mutations exhibit age-specific effects. If the distribution of age-specific effects is fairly uniform across all ages, then after sufficient time has passed, we might expect the age-specific effects to combine to give the appearance of no age-specific effect. In this study, mutational effects for male mating ability appear to exhibit age-specificity at generation 10. At generation 20, the age-specific effects are clear, and significant. By generation 30, there was little or no signal of age-specificity, perhaps due to a uniform distribution of age-specific effects as discussed above.

If we consider these results together with the results on mortality from recent work from our laboratory (Yampolsky, Pearse & Promislow, in press), and earlier work by Pletcher, Houle & Curtsinger (1998), the most consistent pattern that emerges is that of variability. Each trait examined within each study shows a different pattern. In their analysis of 29 inbred mutation accumulation lines, Pletcher, Houle & Curtsigner (1998) observed an increase in variance among lines for mortality rates at ages 15 and 21 d, and in females only. Yampolsky, Pearse & Promislow (in press) reported significantly increased mortality rates for MCN over Control flies restricted to ages < 50 days. The results of these two mortality studies, along with our analysis of male mating ability, suggest that novel mutations tend to have early-acting effects. In contrast, our analysis of fecundity data suggests that if there are age-specific effects of novel mutations on fecundity, they tend to occur at intermediate ages. In all cases, the apparent age-specific effects for mortality, male mating ability, and fecundity were transient. By 30 generations, all age-classes showed effects of novel mutations. In light of these results, it may be worth building theoretical models of aging that allow for different age-distributions of novel mutations, with the additional possibility that novel mutations have qualitatively different effects on mortality and fecundity.

In addition, several models of mate choice and sexual selection have included mutation as a critical parameter (Iwasa, Pomiankowski & Nee, 1991; Pomiankowski, Iwasa & Nee, 1991; Hansen & Price, 1995; Kokko & Lindström, 1996; Beck & Powell, 2000). Our results suggest that there may be strong age-biases in the effects of novel mutations on mating ability. It would be interesting to develop theoretical models to determine the effects of such age-biases on age-specific mate preference (e.g. Kokko & Lindström, 1996; Beck & Powell, 2000) and exaggerated secondary sexual characteristics (e.g. Iwasa, Pomiankowski & Nee, 1991; Pomiankowski, Iwasa & Nee, 1991).

Alternative explanations

There are at least four alternative explanations that might explain the variation in age-specific mutational effects among studies as well as the variation in effects among traits we observed in our own study. One possibility is that in studies of adult fitness components, we are observing the effects of rare mutations of relatively large effect. If we were to repeat the study, we might find that initial mutational effects were confined to late ages, rather than early ones. Second, gene-by-environment interaction for novel mutations (Fry, Heinsohn & Mackay, 1996) may account for the differences in mutational effects between mortality (Yampolsky, Pearse & Promislow, in press), and mating ability and fecundity (this study). Subtle differences between the environments in which flies were maintained when we assayed each of these three traits may have been sufficient to give rise to differences in patterns of age-specific mutational effects. Gene-by-environment interaction may also account for differences in the same trait among generations. In addition, the difference we observed between males and females is consistent not only with Pletcher, Houle & Curtsinger (1998), who found a stronger effect in females than in males, but also with quantitative trait locus (QTL) mapping work by Nuzhdin et al. (1997), who identified several QTLs that exhibited sex-specific effects.

Third, the patterns we observed may have been due to variation in magnitude, sex-specificity, or age-specific rate of accumulation of novel mutations, or to some combination of these three factors. Although the challenge is an experimentally daunting one, future studies are needed not only to determine the age-specificity of novel mutations, but also the degree to which the distribution of the magnitude of these mutations may differ among age classes.

Finally, the fact that patterns of mutational effects differed among traits may also be due to the nature of the genetic pathways involved in these traits. Work by Rogina and Helfand (Helfand, Blake & Rogina, 1995; Rogina & Helfand, 1995; Rogina & Helfand, 1996) has shown a multiplicity of age-specific patterns of gene expression. In their work, they found that expression of some genes increased over the life span, others decreased monotonically, and still others appeared to oscillate in expression intensity, most notably at later ages (> 45 d) (Rogina & Helfand, 1996).

In the present study, we were able to detect mutational effects as late as 57 d for male mating ability, and 47 d for fecundity, suggesting that our flies did not have an inordinately high genetic load of late-acting deleterious mutations. Nonetheless, we recognize that these mutation accumulation studies are still far from ideal. The most obvious problem with earlier mutation accumulation studies is that they used lab-adapted strains, which may have had very high loads of late-acting deleterious alleles. Given this unusually high mutation load for late-age alleles, such experiments may tell us little about the effect of mutations under natural conditions. The Middle Class Neighborhood (Shabalina, Yampolsky & Kondrashov, 1997) provides an alternative approach that allows us to measure the effects of *de novo* mutations on a natural, outbred genetic background. However, this approach is not without its own problems. First, the controls that we use in this study are imperfect. The control lines were maintained at a generation time almost ten times longer than in the MCN lines. If the rate at which novel mutations accumulate is proportional to generation time (Lynch et al., 1999), this control should be

fairly effective. But if mutation accumulation is proportional to calendar age, there is no reason to expect mutations to accumulate more slowly in the controls than in the MCN lines. In addition, although mortality rates were substantially lower at 15° C, there was still some mortality in the control, so it is possible that they were actually improving over time due to selection. Similarly, by the time the control lines were brought back to 24° C and used to create the next line, some of the flies were no longer fertile, giving further opportunity for selection. However, the similarity of C1 and C2 with the newly obtained C3 at generation 30 suggests that the effects of selection were minimal. Thus, while Shabalina et al. (1997) rightly point out the merit of using genetically heterogeneous, wild-caught lines to test for the effects of novel mutations, the approach increases the likelihood that selection will influence the outcome. An additional challenge arises when we try to assay for age-specific effects of mutations using the MCN. In the classic, inbred-line approach (e.g. Pletcher, Houle & Curtsinger, 1998), flies within a given line are genetically homogeneous. However, in the MCN, flies are outbred, so there is substantial opportunity for differential mortality among 'high quality' and 'low quality' genotypes within a line over the course of a single assay (Yampolsky, Pearse & Promislow, in press). The dynamics of differential mortality due to heterogeneity can lead to complex patterns when comparing lines with different average quality (Vaupel & Yashin, 1985). Taken together, each of these factors could lead to underestimates of mutational effects, particularly at late ages.

In addition to the adverse consequences of selection on standing genetic variance, there may also have been non-genetic maternal age effects. Early literature on maternal age effects suggests that survival rates may be substantially lower in offspring of older mothers (Lansing, 1947). To try to mitigate this effect in our control lines, we expanded the lines for one generation at 24° C, and then used the offspring of these flies for subsequent assays, to minimize non-genetic maternal effects. It is still possible, however, that our results were influenced by non-genetic grandparental effects.

Experimental controls

While there is no ideal control in the MCN approach, some studies have successfully created controls by freezing embryos in liquid nitrogen and then reviving them at a later date. However, the technique is not always successful, and rates of revival decline as embryos spend more time in nitrogen. Recent work on adult diapause (M. Tatar, pers. comm.) suggests that this may be one way to extend generation time to at least three months with no mortality whatsoever. Further work still needs to be done to determine the maximum duration for which adults can be put into diapause, and also whether there are conditions at which all populations will go into diapause, or only ones from high-latitude environments, as suggested by Williams and Sokolowski (1993).

Notwithstanding the many cautionary notes that these complex experiments demand**,** the patterns we identify remain intriguing. Comprehensive, biologically realistic models for evolutionary theories of aging will benefit from realistic information about the magnitude and age-specificity of *de novo* mutations. Consistent with earlier work (Pletcher, Houle & Curtsinger, 1998) using a quite different approach, our work suggests that there is a bias in favor of mutations with early-acting effects. However, in contrast to Pletcher, Houle & Curtsinger, we also find late-acting effects. Whether this is due to the effects of diminishing returns epistasis in their lines, or chance fixation of rare, late-acting mutations in our lines remains to be determined. As techniques improve for accumulating mutations in natural genetic backgrounds, and in particular, for maintaining appropriate controls, we should begin to see a clearer picture emerge for the distribution of magnitude and age-specificity of novel deleterious mutations. These results should, in turn, provide the necessary information to construct powerful evolutionary models for a range of important biological phenomena.

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Table 5.1. ANOVA table for % wild-type progeny as a function of age and treatment for generations 10, 20, and 30**.**

Gen.	Factor	df	SS	MS	F	P
10	Age	5	12.8676	2.574	104.87	0.0001
	Treatment	3	0.0939	0.031	1.28	0.28
	Age*Treatment	15	0.7346	0.049	2.00	0.0151
20	Age	4	10.247	2.5617	61.93	0.0001
	Treatment	3	2.662	0.8873	21.45	0.0001
	Age*Treatment	12	0.9017	0.0751	1.82	0.0455
30	Age	6	34.5791	5.7632	225.05	0.0001
	Treatment	4	3.3962	0.8491	33.16	0.0001
	Age*Treatment	24	2.2301	0.0929	3.63	0.0001

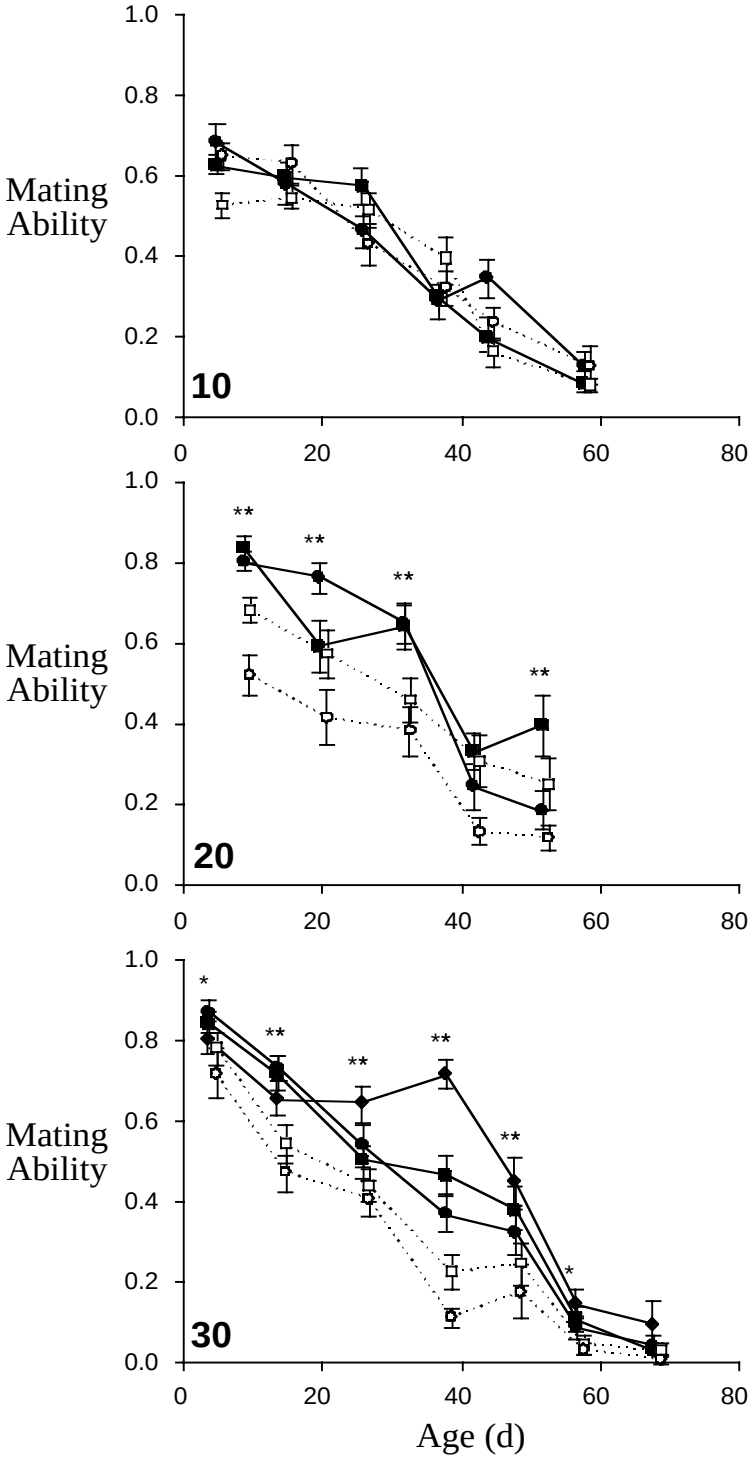

Figure 5.1. Male mating ability, measured as per cent wild type offspring out of total progeny, as a function of age, for MCN1 (dashed, open square) and MCN2 (dashed, open circle) and control lines C1 (solid, filled square) and C2 (solid, filled circle). Control 3, shown as a solid line with a filled diamond in the third figure, is the wild-caught control line established for the generation 30 assay only. Numbers in the bottom left-hand corner refer to the number of generations of mutation accumulation.

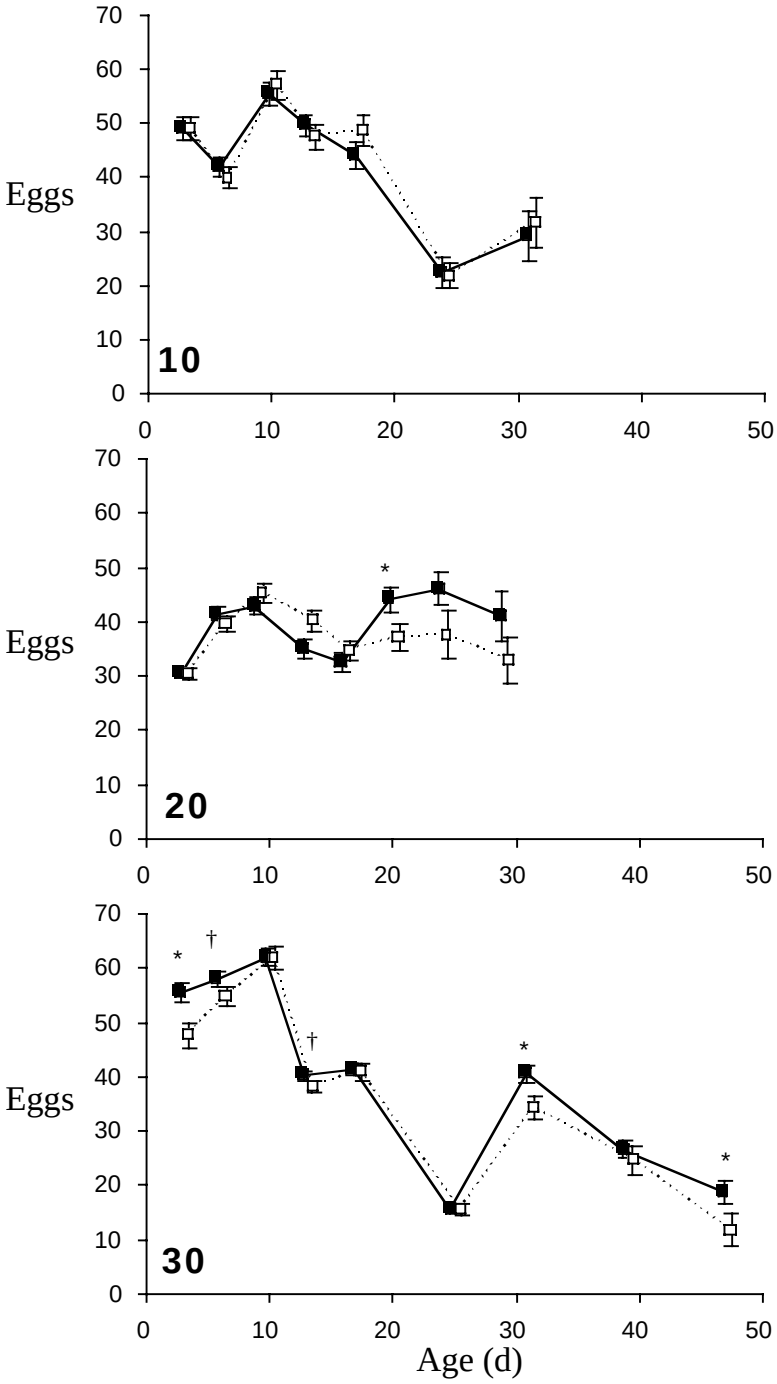

Figure 5.2. Fecundity as a function of age, averaged over control lines (filled squares) and mutation accumulation lines (open square), for 10, 20 and 30 generations of mutation accumulation. Flies that laid no eggs at a particular are not included in these figures (see text). Numbers in the bottom left-hand corner refer to the number of generations of mutation accumulation.

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CHAPTER 6

CONCLUSION

Within the context provided by pre-existing evidence for the important role that females play in reproductive interactions, we have extended the list of female parameters that significantly impact sperm competition, and thus, reproductive fitness. We provide the most significant evidence, to date, in support of the important role for both female age and genetic relatedness of females to their mates on the outcome of sperm competition. First, we show that when male *D. melanogaster* mate with females of ages greater than 5 days, the proportion of subsequent progeny they sire is significantly reduced when compared with males mated to 3-5 day old females. Females older than 5 days of age exceed the age of females used in all previous assays of sperm competition in *Drosophila* we are aware of. Second, we provide the strongest evidence to date that females of any taxon avoid using sperm received from related males relative to sperm of unrelated males. While it is unclear at this point if the underlying mechanism is facultative or passive, our result suggests that, whatever the mechanism, *D. melanogaster* females have evolved a mechanism that is a significant step closer to high resolution sperm selection. Third, we show that the ejaculates of male *D. melanogaster* can influence females to avoid the use of stored sperm from prior males, but that the extent of the effect varies with the interval between matings. Moreover, the mating interval effect as well as the direction of the effect can reverse as a function of female genotype; that is, our result suggests female genotype by male genotype interaction is important. Finally, we present evidence for age-specific phenotypic effects of novel mutations on both male mating ability and fecundity in *D. melanogaster*.

As a result, we have significantly clarified the important role that females play in the resolution of potentially antagonistic reproductive interactions between females and males.

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