Chapter 1

Introduction

Motivation for this study

Development is a key phase in the life-history of an organism since it influences traits affecting survival and reproduction from birth till death, and, thereby Darwinian fitness, especially in holometabolous insects in which pre-adult development is a major resource acquiring phase (Chippindale et al.,1994, 1997, 2003). Theory suggests that an 'ideal' life-history would be one that maximizes all fitness components. If there were no constraints on the evolution of traits, an ideal organism would take infinitesimally small time to develop and reach reproductive maturity, and keep reproducing infinitely at a high rate. However, such 'ideal' life histories are not seen in living organisms. Hence, trade-offs are central to the study of life-history evolution since these determine the constraints that prevent maximization of all fitness components simultaneously. All else being equal, an organism should develop and attain reproductive maturity as fast as possible since individuals with a shorter generation time would have a fitness advantage over those with a longer generation time (Roff, 1992). Consequently it has been of interest to try and understand the constraints on evolving rapid development to reproductive maturity. One approach to do this has been to select populations of *Drosophila melanogaster* for rapid pre-adult development, leading to overall reduction in the generation time, and examine the correlated evolution of various life-history related traits in such populations (Zwaan et al., 1995; Nunney, 1996; Chippindale et al., 1997; Prasad et al., 2000).

In our laboratory, four large *D. melanogaster* populations have been selected for rapid pre-adult development and early reproduction (FEJ populations: Prasad et al., 2000) for close to 540 generations and have also been studied for changes in diverse life-history and life-history related traits. A large body of work on *D. melanogaster* has focused on the study of genetic correlations among life-history related traits (Leroi et al., 1994; Archer et al., 2003; Chippindale et al., 2003; Phelan et al., 2003), and provided us a broad framework for understanding trade-offs. These studies have highlighted not only the lability of genetic correlations, but also how they are determined by a combination of historical genetic constraints and the ecology of the organism that can alter such relationships between fitness characteristics.

Our faster developing FEJ populations also exhibit many such trade-offs within and across different life-stages (Prasad et al., 2000, 2001; Shakarad et al., 2005). Selection for faster pre-adult development has resulted in a consequent decrease in body size, pre-adult viability, larval growth rate (Prasad et al., 2000), longevity, starvation resistance, fecundity (Prasad, 2004), competitive ability (Shakarad et al., 2005) and pathogen resistance (Ghosh-Modak, 2009) of the FEJ populations, compared to their ancestral controls.

Traditionally, study of such life-history traits in *Drosophila* evolution has largely focused on natural selection (reviewed in Prasad and Joshi, 2003), with relatively little attention given to how sexual selection could interact with natural selection in shaping the evolution of life-histories. Reproductive success, after all, depends on traits under both natural and sexual selection as reproduction involves an interaction between the two sexes, even if their evolutionary interests are not necessarily the same, resulting in sexual conflict. Sexual selection, whether viewed in the traditional way as Darwin (1871) proposed (of one sex selecting traits in the other), or how

Wallace (1889) discussed it (the ecology of the two sexes being different and therefore different selection pressures acting on them), is therefore an important force shaping the evolution of life-histories.

Given that males and females must cooperate, at least till the point of successful fertilization, it is reasonable to believe that the fitness interests of the two sexes would be inter-dependent and traits involved in reproduction that benefit both sexes would evolve. However, there is ample literature documenting the existence of traits that are beneficial only for one of the sexes, and are either neutral or harmful for the other, leading to a conflict between sexes (Parker, 1979; Arnqvist and Rowe, 2005). If the optimum trait values differ for the two sexes, traits that are shared between the sexes may be subject to intra-locus conflict whereas if the trait is expressed in only one of the sexes, but affects the fitness of the other, inter-locus conflict can ensue (reviewed in Chapman et al., 2003). Intra-locus conflict can sometimes be resolved through sex-limited expression of traits, leading to sexual dimorphism (Rice et al., 1984; Rhen, 2000). However, inter-locus sexual conflict usually results in sexually antagonistic coevolution and a consequent evolutionary 'arms-race' between the sexes, with adaptation in one sex leading to counter-adaptations in the other (Rice, 1996; Rice and Holland, 1997; Rice, 1998). Traits like parental investment in offspring, mating frequency and fecundity are commonly under inter-locus conflict (Lessells, 2006) and the relative costs and benefits of manipulative behaviour by one sex determine the optimum strategy for that sex.

In *D. melanogaster*, inter-locus conflict over mating has been extensively reported (reviewed in Chapman et al., 2003) with males being the protagonists in the conflict, manipulating their mates through various chemical means that not only enhance

male fitness, but also cause collateral reduction in female fitness. As long as the fitness advantage gained through this manipulation outweighs the fitness lost through harming the mate, the traits underlying such manipulation will be selected for, thereby driving selection for counter-adaptations in the female. In *D. melanogaster*, the manipulation of female reproductive behaviour by males is mediated by a number of accessory gland proteins (Acps) that are transferred by the male to the female during copulation (Chapman et al., 1995, 2001; Swanson et al., 2001). These Acps enhance male fitness through various means, e.g. by boosting immediate fecundity of the female (increasing the likelihood of most of her eggs being fertilized by that sperm), decreasing female receptivity to other males and improving self-sperm storage in the female tract (reviewed in Chapman et al., 2003).

Since the FEJ populations have been diverging from their ancestral control populations (JBs) for over 500 generations, possible reproductive isolation between these two types of populations has also been investigated (Ghosh and Joshi, 2012). In particular, the FEJ populations had diverged by over 60 hours (23%) from their controls in pre-adult development time (Ghosh-Modak, 2009), and it was hypothesized that this might render hybrids between the selected and control populations inviable. Although evidence for incipient reproductive isolation was found, it was not through post-zygotic isolating mechanisms, but rather through traits related to mating success and sexual conflict (Ghosh and Joshi, 2012). Control JB males were able to procure many matings with the FEJ females, but the FEJ females thereafter did not survive long. On the other hand, FEJ males were unable to secure many matings with JB females, regardless of whether or not the female had a choice of male from different populations (Ghosh and Joshi 2012).

One major factor contributing to these two mechanisms of incipient reproductive isolation is likely to be the significant size difference between the FEJ and JB flies. It is possible that the high mortality rate of the FEJ females after mating with JB males was due to physical damage and biochemical stress due to Acps incurred from mating with a much larger male (Pitnick and Garcia-Gonzales, 2002). Similarly, the inability of FEJ males to mate with the much larger JB female could be due to the size difference; JB females are almost twice as large as FEJ males (Ghosh-Modak, 2009). Size plays an important role in reproductive success of *D. melanogaster* females (Stearns, 1992; Roff, 2002) and males (Partridge and Farquhar, 1983; Partridge et al., 1987a; Markow, 1988; Markow and Ricker, 1992). Body size is also positively correlated with development time, growth rate, fecundity and longevity (reviewed in Prasad and Joshi, 2003) and size is therefore an important trait that is under both sexual selection and natural selection in *Drosophila.*

The pattern of female mortality that was observed by Ghosh and Joshi (2012) can be an outcome of not only body size differences but also changes in the levels of inter-locus sexual conflict in the FEJ populations. Smaller males are less harmful to females in terms of the mating stress they impose (Pitnick and Garcia-Gonzales, 2002), and females encountering less harmful males every generation would experience lower levels of sexual conflict. Evidence for a fitness cost to counter adaptations in females to manipulative harm by males has been obtained from studies manipulating levels of sexual conflict in populations of *Drosophila sp*., where selection for reduction in sexual conflict resulted in females from those populations becoming more susceptible to mating harm compared to populations where high levels of sexual conflict were maintained (Pitnick et al., 2001; Wigby and Chapman, 2004; Crudington et al., 2005; Kuijper et al., 2006). Additionally,

males and females in the FEJ populations may evolve reduced levels of manipulative ability and defence, respectively, in order to be able to develop faster because selection for rapid development in the FEJ populations is stronger than that for early reproduction. Since selection for fast pre-adult development occurs prior to any selection on reproductive traits, the onus on any genotype to become part of the breeding population by developing fast is greater than maintenance of high reproductive success. Therefore, both the evolution of reduced body size, and indirect selection pressures on FEJ populations as a consequence of a breeding ecology different from the controls, could be contributing to the evolution of pre-zygotic incipient reproductive isolation between FEJ and JB populations, highlighting the importance of studying the interaction between sexual selection and natural selection in the process of speciation (Wedell et al., 2006).

Outline of this study

In this thesis, I report results of preliminary studies directed towards understanding, in greater detail, changes in the sexual scenario in FEJ populations. To do so, I used a two-part approach to investigate (1) the role of body size difference between the FEJ and JB populations in mediating the pattern of post-mating mortality reported by Ghosh and Joshi (2012), and (2) differences in the overall breeding ecology of the FEJ and JB populations that may result from differences between the two selection regimes and cause different patterns of selection acting on traits involved in sexual conflict.

In the next chapter, I discuss the role of body size and its contribution to the potential reduction in the level of sexual conflict in the FEJ populations, using a size control for the JB populations. This size control was created by crowding the JB

populations as larvae to a degree sufficient to get an adult size close to that of the FEJ populations. Moreover, I also explored the potential role of differential mating success in giving rise to the increased mortality of the females after mating. Since the size difference between flies from FEJ and JB populations is significant, the JB males might be better at achieving forced matings with the FEJ females, thereby causing them to experience greater mating stress simply because JB males mate more often than FEJ males. In chapter 3, continuing with the line of investigation pursued in chapter 2, I discuss studies in which I examined two measures of mating-related fitness of males of the three types mentioned above: JB, FEJ and the Size controlled JBs.

In the fourth chapter, I discuss differences in the breeding ecology of the FEJ and JB populations during the period of yeasting two days prior to collection of eggs for the next generation. Conflict over optimum mating frequency between the two sexes (Parker, 2006) is believed to drive the subsequent manipulative behaviour that results in sexual conflict between the two sexes and mating frequency and level of inter-locus sexual conflict are positively correlated (Pitnick et al., 2001; Wigby and Chapman, 2004; Crudington et al., 2005; Kuijper et al., 2006). Therefore, I assessed courtship frequency and the number of matings in the 48 hours before egg collection for FEJ and JB populations. I also examined maturation time (time from eclosion to first mating) for the FEJ and JB populations, separately for males and females.

In the final chapter, I discuss the results obtained from the various experiments and the scope for future investigations into the possible evolutionary reduction of sexual conflict in the FEJ populations.

Experimental Populations

The studies reported in this thesis were performed on eight outbred *D. melanogaster* populations that have a common ancestry. Four of these populations were select for faster pre-adult development and early reproduction, and are referred to as the **FEJ**s (**F**aster developing, **E**arly reproducing, **J**B derived); the other four are ancestral controls, called the **JB**s (**J**oshi **B**aseline). The four control populations are descendants of a single wild-caught population of *D. melanogaster* described by Ives (1970), called the IV population. From this population, five **B** (**B**aseline) populations were derived, being maintained on a 14 day discrete generation cycle under constant light, 25^{o}C ± 1^{o}C and high humidity on banana-molasses food. After 360 generations from the derivation of B lines, another set of 5 populations were derived, one from each B population, called the **UU**s (**U**n-crowded as larvae, **U**n-crowded as adults) (Joshi and Mueller 1996) which were maintained on a 21 day discrete generation cycle, keeping all other aspects of maintenance the same. After about 170 generations of UU maintenance, the four JB populations were derived from the UU-1, 2, 3, and5 and maintained in a manner similar to the UUs in banana-jaggery food medium, in our laboratory in Bangalore, India (Sheeba et al., 1998).

For each replicate JB population, about 60-80 eggs are collected into 8-dram glass vials (2.2 cm diameter × 9.6 cm height) containing 6 ml of banana-jaggery food. Forty such vials are collected per population. On the 12th day after egg collection by which time all the flies from the JB vials have typically eclosed, flies are transferred to fresh food vials every alternate day. On the 18th day from egg collection, flies from all 40 vials are collected into a Plexiglas cage (25 cm × 20 cm × 15 cm) and provided with a petri plate of food medium with a generous smear of live yeast and acetic acid paste. After 3 days of yeasting, the flies are allowed to lay eggs for about

18 hours on banana-jaggery food. Eggs from this food plate are then collected for initiation of the next generation, thus cycling a generation every 21 days. Populations are maintained at a breeding adult number of 1500-1800 flies, under constant light, 25^{o}C ± 1^{o}C and about 90% relative humidity.

The four FEJ populations were derived from corresponding JB populations (first described in detail by Prasad et al., 2000). The maintenance regime is similar, except that since the FEJs are selected for faster pre-adult development, only around the first 25% of the eclosing flies per vial are collected to be part of the breeding pool for the next generation. To obtain a sufficiently large population size (1500-1800 flies per population) 120 vials are set up per replicate population. Vials are checked for the requisite number of flies every 2 hours from the time of darkening of pupae, and once about 15 flies have eclosed in a vial they are collected into a Plexiglas cage. The population in the cage is then provided with a generous smear of live yeast and acetic acid paste on a banana-jaggery food plate for 3 days. Flies are then allowed to lay eggs for a duration of only 1 hour after the yeasting period on banana-jaggery food (to obtained similar aged eggs to allow efficient selection on development time), from which eggs are collected to initiate the next generation. Chiefly, the FEJ maintenance differs from that of the JBs in having only the earliest eclosing flies being part of the breeding pool (selection on faster development), and in having egg collection for the next generation about 3 days after eclosion. Studies reported in this thesis were conducted between 510-540 generations of FEJ selection, when the generation time of the FEJs had come down to 10 days due to selection on faster development and early reproduction compared to their ancestral controls.

Each of the four FEJ populations has been derived from one JB populations (matched subscripts) such that ancestrally, FEJ_i is more closely related to JB_i population than other FEJ populations. Because ancestry here can be accounted for, selected and control populations that share the same numerical subscript have been treated as blocks in the statistical analyses.

Collection of Flies for Assays

Since the selected and control populations experience different maintenance regimes, non-genetic parental effects can be confounded with traits that have evolved in different populations. It is, therefore, important to control for this by rearing both control and selected populations under similar conditions for at least one full generation before assaying any trait. In this case, standardization of the populations was achieved by collecting around 60-80 eggs per vial, with 40 vials per JB population and 60 vials per FEJ population (egg-adult viability of FEJs is lower than JBs, and 60 vials are needed to obtain the requisite adult population size of ~1500 individuals). Flies are collected into a Plexiglas cage as soon as eclosion is complete in all the vials, which corresponds to the 7^{th} day from egg collection for FEJs and the 11^{th} from egg collection for JBs. Since there is a development time gap between the FEJs and JBs, FEJ egg collection is staggered to obtain both FEJ and JB standardized adult flies on the same day. Eggs from the standardized flies were collected to obtain individuals for assaying any trait from these populations thereafter.

Work reported in this thesis is from studies on adult traits, and therefore age of the FEJ and JB adults for assays was also synchronized by staggering egg collection of

the FEJs from the standardized populations according to the difference in the egg-to-adult development time between the selected and control populations.

Chapter 2

Introduction

Sexual selection can be an important force driving pre-zygotic reproductive isolation (Ritchie, 2007), and body size, a trait often under sexual selection (Partridge and Farquhar, 1983; Partridge et al., 1987a,b; Stearns, 1992; Markow and Ricker, 1992; Andersson, 1994; Byrne and Rice, 2006), can therefore drive reproductive isolation through differential mating patterns. Incompatibility in mating is one such mechanism that has been seen in the FEJ and JB populations: FEJ females housed with JB males show higher post-mating mortality than when they are housed with FEJ males (Ghosh and Joshi, 2012). Reduction in both body size and levels of inter-locus sexual conflict in the FEJ populations could be the cause for the female mortality pattern seen by Ghosh and Joshi (2012), as discussed below.

In many species, sexual conflict over mating frequency is prevalent, with a higher optimum mating frequency for males compared to females (Arnqvist and Nilsson, 2000; Lessells, 2006). In *D. melanogaster*, males increase mating related-fitness through manipulation of female physiology and behaviour via production of accessory gland proteins (Acps) that also negatively affect female fitness (Chapman et al., 1995). The level of Acp production can change with body size if a proportional change in reproductive investment occurs. Since there is a large body size difference between FEJ and JB flies (JB flies being twice as large: Ghosh-Modak (2009)), it is possible that the mating stress through physical damage, harassment and ejaculate volume (and therefore amount of Acps transferred) could result in the increased mortality of FEJ females mated with JB males. In light of this,

I checked post-mating female mortality of FEJ and JB flies, and a body size control created in JB background (hereafter Jc), when mated with FEJ, JB or Jc males, to assess the contribution of male body size to post-mating female mortality. Larger body size, moreover, could afford males the ability to secure forced mating with females, or to copulate for a longer duration. From work reported by Ghosh and Joshi (2012), it is evident that the presence of more males produces a higher mating stress, clearly highlighting that the number of matings and/or possibly persistent courtship could contribute to the reduction in survival probability of females after mating. I therefore also checked for differential mating success between these three types of males to see if the mortality results could be explained by different levels of mating by each type of male. FEJ flies are lethargic in comparison to JB flies, and such activity level differences between the FEJ and JB populations could also contribute to both differential mating success and harm to females mated to these three types of males via more efficient courtship ability of larger males (Partridge et al., 1987c; Partridge and Fowler, 1990).

It is also possible that FEJ females are less able to defend themselves against mating stress due to the fact that in their maintenance regime there is only a three day period between eclosion and egg collection, as compared to 10-11 days in JB cultures, and the FEJ females consequently probably experience far less courtship and mating than their JB counterparts. A correlation between the level of inter-locus sexual conflict and number of matings has been seen in *Drosophila* in a number of studies (Pitnick et al., 2001; Wigby and Chapman, 2004; Crudington et al., 2005). In this chapter, I focus on trying to assess contribution of body size reduction versus evolved genetic changes in FEJ female defence against manipulation by males in increasing female susceptibility to mating induced harm. Additionally, since it is the

male mating fitness and manipulative harm that would be driving both changes in the evolutionary responses of the females and our estimate of the level of inter-locus sexual conflict in the population, some assessment of the mating fitness of males was also made and is discussed in detail in the next chapter.

Materials and methods

Obtaining the body size control

The experiments described in this chapter and the next were performed with three sets of flies: the FEJ, JB and a body size control created for JB flies (Jc) by crowding them as larvae for one generation to a sufficient degree such that flies were small enough for their dry weight to not significantly differ from FEJ fly dry weight, as measured at the age at which these flies were used in the assay. For obtaining small JB flies (hereafter 'Jc'), a larval density of 220-230 eggs /1.5 ml of banana-jaggery food medium was used, whereas for FEJ and JB flies, egg collection was done as for the regular stock maintenance.

Collecting adults for the assays

Since all experiments assayed adult behaviour, egg collection from the different populations was staggered so as to obtain adults of the same age from eclosion at the same time. The development time difference between the FEJ and JB populations necessitated staggering egg collection from FEJ populations by 3 days after egg collection from the JB populations. Prior to assaying, eggs from standardized JB and FEJ populations were collected and then freshly eclosed flies were separated as virgins within 6 hours from eclosion using light carbon dioxide anesthetization. Thus, I ensured that eclosion began in all populations on the same day despite

differences in their pre-adult development time. Virgin males and females were subsequently housed in single sex vials and were aged for 3-4 days until the assays were performed. The following assays were performed between FEJ generation 513 and 537, JB generation 263 and 274.

Female mortality assay

An assay was performed to record the post-mating mortality of the three types of females under study (FEJ, JB and Jc) when paired with either FEJ, JB or Jc males as mates. Pairs of flies were set up in vials with 10 vials for each of the nine possible combinations of male and female type, using light carbon dioxide anesthetization. Flies were age matched, 3-4 day old virgin adults at the time of set up. The females were housed with the males for 50 hours after which the males were removed from the vials without anesthesia, and females were thereafter kept individually in vials to assess mortality by checking for female death once every 24 hours. The females were shifted to fresh food vials every alternate day for 14 days, at the end of which the cumulative mortality of the females was calculated at days 7 and 14 from setup.

Number of matings in 50 hours

Since the JB males are larger than the Jc or FEJ males, the increased mortality of females mated to JB males (Ghosh and Joshi, 2012) could be due to increased number of matings when housed with JB males rather than an intrinsically higher mate-harming ability of JB males. I therefore checked for differences in number of matings in the nine different combinations of male and female type. Similar to the set up described above for estimating post-mating female mortality, single pairs were set up when the virgin flies were 3-4 days old, and the number of successful matings in 50 hours recorded for each male-female combination. Ten vials were set

up per combination of male-female type and all vials were under continuous observation in a well illuminated environment without disturbance and a constant temperature of $25^{o}C \pm 1^{o}C$ for duration of 50 hours from setup. A mating was considered successful only when the mating pair was observed to be in copula for at least three minutes. Both the time of onset and termination of each mating was noted, allowing an estimation of the duration of each mating were noted. This permitted the calculation of total mating exposure of each female (total duration of being mated with) that could be a significant contributing factor in the stress experienced by females due to mating.

Female mortality (fixed number of matings)

As a final confirmation for the results obtained from the female mortality and the number of matings, I checked post-mating female mortality when the number of matings was fixed for all male-female type combinations at two matings, after which males were removed and 14^{th} day cumulative mortality rate was calculated for those females. Flies were separated as virgins and kept in single sex holding vials until 3-4 days of adult age. In all nine combinations of male and female type, 25 vials were set up, each with a single male-female pair. Vials were observed until two successful matings had been achieved in at least 10 vials per combination. Observations did not last for more than 24 hours since a sufficient number of replicate vials were obtained per regime within 24 hours. As soon as the second mating terminated in a vial, the male was removed without anesthesia and the female was kept for 14 days to check mortality once every day, with fresh food provided every alternate day. At the time of writing, due to time constraints, only blocks one, two and three of FEJ and JB populations had been assayed.

Dry weight measurement

On the day of assay set up, virgins were frozen at -20^{o}C for dry weight measurement later. Dry weight was measured for the FEJ and Jc males and females separately for each run of the different assays. For the measurement, 25 flies of each sex from each population were dried at 70^{o}C for 36 hours, with five replicate vials of five flies each. Flies from each vial were weighed three times and the mean dry weight was taken as one replicate value, to minimize variation due to machine error. These measurements were done to ensure that the body-size control being used, Jc, did not significantly differ from FEJs in body weight, thus permitting proper comparisons between them.

Statistical analyses

For both mortality rate assays, the arcsine square root transformed fraction of females that died by day 7 or day 14 was used for statistical analyses. For the assay on number of matings in 50 hours, the mean number of matings obtained across vials per male-female combination was used for analyses and the total mating exposure was calculated as the total time in hours that a female experienced mating, averaged across females per combination. For all traits, means across replicate vials were used for statistical analyses. Mixed model analysis of variance (ANOVA) was performed on these means with block as a random factor, and selection regime and sex as fixed factors for analysis of dry weights, or male and female type (FEJ, JB or Jc) as fixed factors for analysis of female mortality, mating number and mating exposure. All multiple pairwise comparisons were made by Tukey's HSD test at a 0.05 significance level. All analyses were implemented on Statistica^{TM} for Windows Release 5.0 B (StatSoft Inc., 1995).

Results

Female mortality

Cumulative female mortality through the first 7 or 14 days after exposure to males showed a similar pattern, with FEJ females mated with JB males showing substantially higher mortality than any other male- female type combination (Fig. 2.1, 2.3). The ANOVA on day 7 cumulative mortality revealed a significant main effect of female type, and multiple pair-wise comparisons (Tukey's HSD at 0.05 significance level) revealed that, on average, FEJ females suffered significantly greater mortality than either JB or Jc females (Table 2.1, 2.2a). Exactly the same pattern was seen for 14^{th} day cumulative mortality, too (Table 2.2, 2.4a). On an average, JB males induced substantially higher cumulative female mortality at both day 7 (Fig. 2.2b) and day 14 (Fig. 2.4 b), but the main effect of male type was only significant for day 14 cumulative mortality (Table 2.2). At both day 7 and day 14, there were significant interactions between male and female type (Tables 2.1, 2.2) and multiple pair-wise comparisons revealed that this was because FEJ females mated with JB males had significantly greater cumulative mortality than all other male- female type combinations at both day 7 (Fig 2.1) and day 14 (Fig. 2.3). The main effects of male type and female type are also likely to have been driven by this particular combination. Interestingly, FEJ female mortality was no greater when they mated with Jc males as compared to JB males, and Jc female mortality, similarly, was not different when mated with JB, Jc or FEJ males, although the trend of male type effects on female mortality tended to be opposite for JB and Jc females (Figs. 2.1, 2.3).

Effect	*df*	MS	*F*	*P*
Female	2	0.3379	5.160	0.0496
Male	2	0.2009	4.210	0.0720
Female × Male	4	0.1667	5.365	0.0103

Table 2.1 Results of ANOVA performed on the arcsine square root transformed cumulative female mortality by day 7, with male type and female type being fixed factors. In this design random factors and interactions are not tested for significance and have been omitted for brevity.

Effect	*df*	MS	*F*	*P*
Female	2	0.8065	37.35	0.0041
Male	2	0.1726	6.976	0.0271
Female × Male	4	0.1954	8.594	0.0088

Table 2.2 Results of ANOVA performed on the arcsine square root transformed cumulative female mortality by day 14, with male type and female type as fixed factors. In this design random factors and interactions are not tested for significance and have been omitted for brevity.

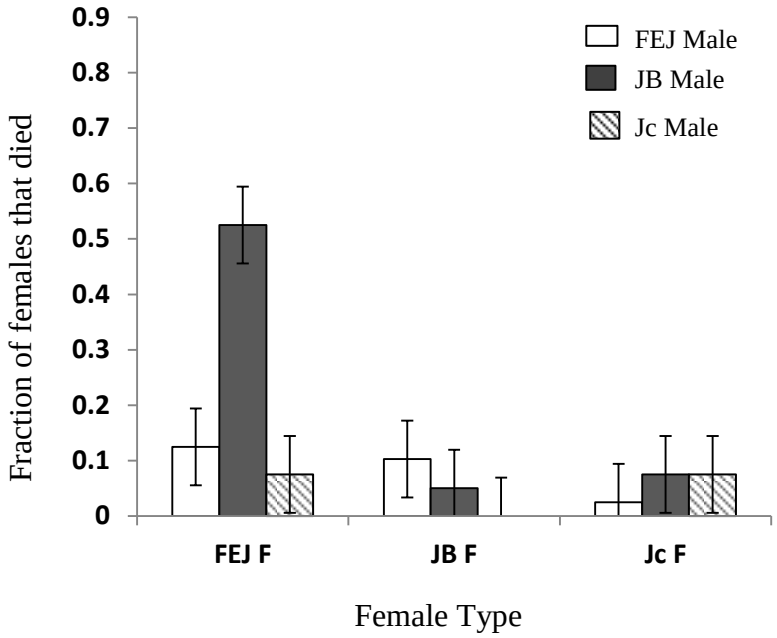

Fig 2.1 Mean fraction of females that died over the first 7 days from set up in the female mortality assay. Error bars are 95% confidence intervals around means of four blocks allowing for visual hypothesis testing.

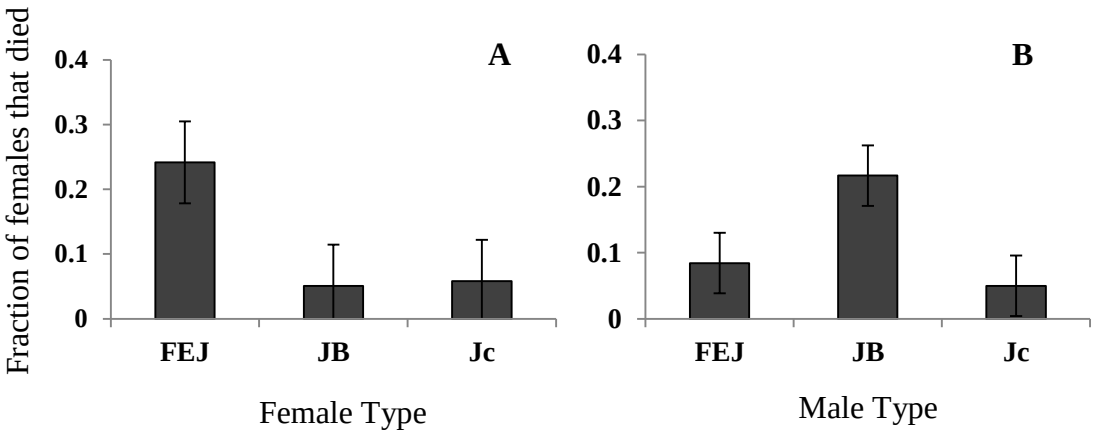

Fig. 2.2 Mean fraction of females that died over the first 7 days from set up of the female mortality assay showing (A) main effect of female, and (B) main effect of male. Error bars are 95% confidence intervals, allowing for visual hypothesis testing.

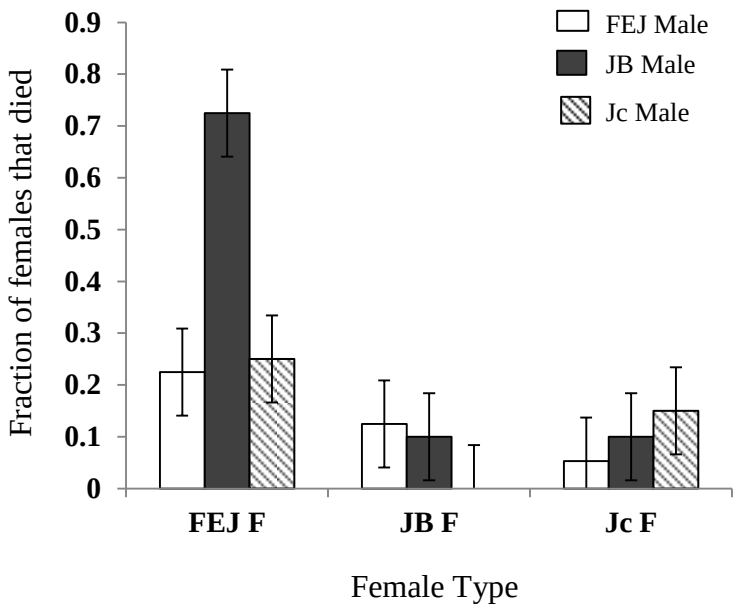

Fig. 2.3 Mean fraction of females that died over the first 14 days from set up of the female mortality assay. Error bars are 95% confidence intervals allowing for visual hypothesis testing.

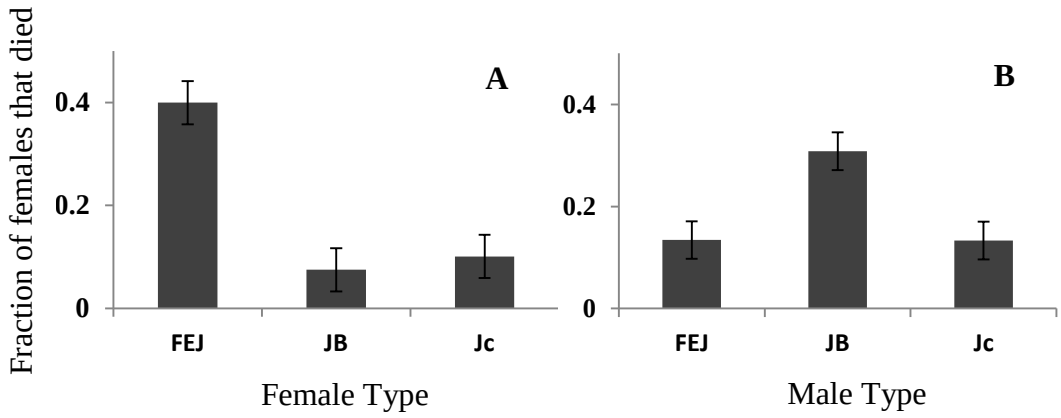

Fig. 2.4 Mean fraction of females that died over the first 14 days from set up of the female mortality assay showing (A) main effect of female, and (B) main effect of male. Error bars are 95% confidence intervals, allowing for visual hypothesis testing.

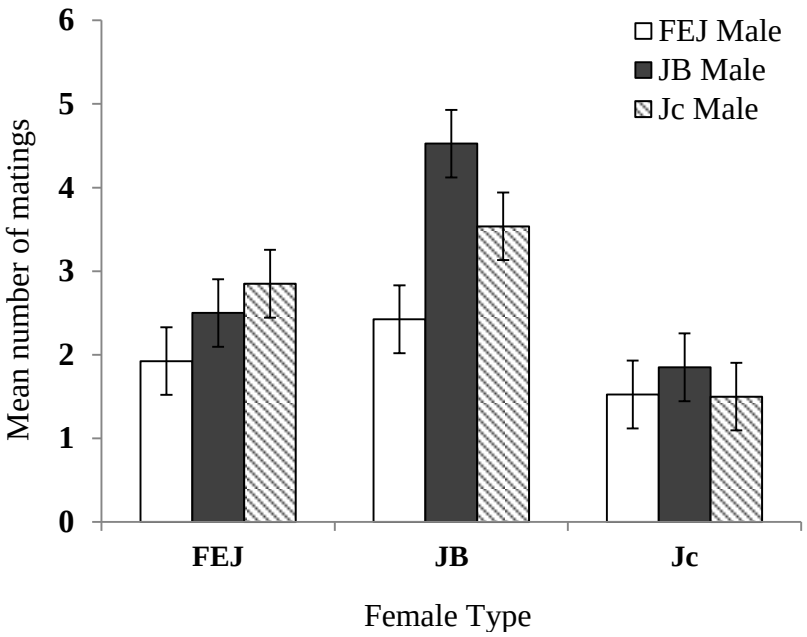

Fig. 2.5 Mean number of matings as recorded in 50 hours of females being housed with FEJ, JB or Jc males. Error bars are 95% confidence intervals and can therefore be used for visual hypothesis testing.

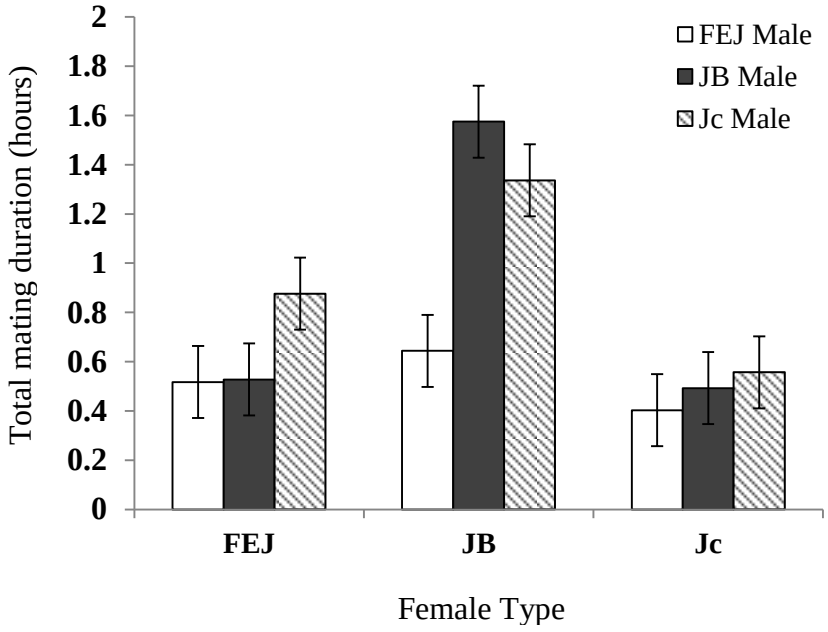

Fig. 2.6 Total exposure to mating as experienced by females when housed for 50 hours with FEJ, JB or Jc males. Error bars are 95% confidence intervals, facilitating visual hypothesis testing.

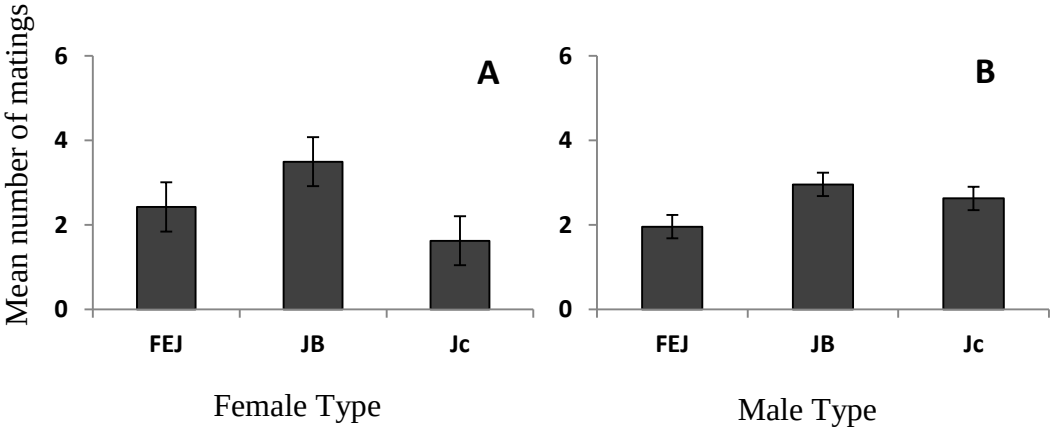

Fig. 2.7 Mean number of matings in 50 hours of exposure showing (A) main effect of female type, and (B) main effect of male type. Error bars are 95% confidence intervals and can therefore be used for visual hypothesis testing.

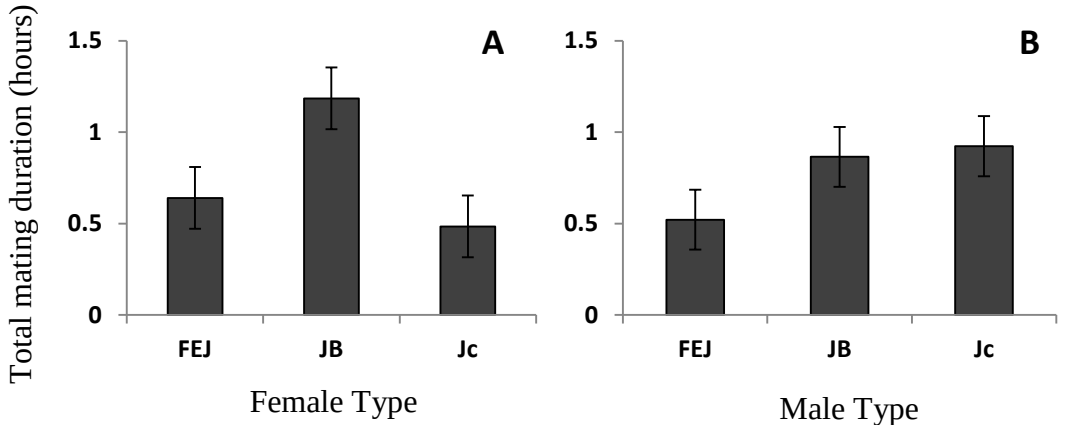

Fig. 2.8 Total exposure to mating in 50 hours showing (A) main effect of female type, and (B) main effect of male type. Error bars are 95% confidence intervals and can therefore be used for visual hypothesis testing.

Mating exposure in 50 hours

ANOVA results showed significant effects of female type, male type and male type × female type interaction for mean number of matings in 50 hours of exposure (Table 2.3). JB females with JB males showed maximum number of matings, significantly higher than the other combinations, as revealed by pairwise

comparisons (Fig. 2.5). This was mirrored when the total duration of mating (total exposure to mating) was analyzed with the main effects of female type, male type and male type × female type interactions being significant (Table 2.4) (Fig. 2.7). Trends also revealed that Jc females, in general, were mated less than JB females, although this was significantly different only when total mating duration was checked (Fig. 2.7) (Table 2.4). This might be indicative of male mate preference, where larger females have been shown to be preferred (Anderson 1994, Byrne and Rice 2006), or better ability of the Jc females to avoid being force-mated with. The higher activity levels of Jc females is a more likely cause, since a single female is available for the male to mate with, and therefore choice is unlikely to be exercised in the absence of another female for comparison. Jc males were significantly more successful at mating compared to FEJ males in terms of both the mean number of matings achieved (Fig. 2.6 (B)) and total duration of mating exposure (Fig. 2.8 (B)) and did not significantly differ from JB males in either measure of male mating success. This can be indicative of better quality of Jc males compared to FEJ males, even though they are of the same small size.

Female mortality (fixed number of matings)

When mortality of females was checked after allowing only two matings per female, a significant main effect of female type was seen, with the FEJ females showing greater mortality than Jc or JB females (Fig. 2.9) ($F_{4,2}$=10.25, P=0.026). Although no significant male type × female type interaction was observed, the trends did indicate higher FEJ female mortality when exposed to JB males (Fig. 2.9), consistent with results from both the mating exposure (in 50 hours) and female mortality assays.

Effect	*df*	MS	*F*	*P*
Female	2	10.567	17.70	0.0030
Male	2	3.1161	23.06	0.0015
Female × Male	4	1.1619	4.004	0.0273

Table 2.3 Results from ANOVA performed on mean number of matings in 50 hours, with male and female type as fixed factors. In this design random factors and interactions are not tested for significance and have been omitted for brevity.

Effect	*df*	MS	*F*	*P*
Female	2	1.625	32.15	0.0006
Male	2	0.565	11.80	0.0083
Female × Male	4	0.280	7.385	0.0030

Table 2.4 Results from the ANOVA performed on total duration of matings exposure in 50 hours, with male and female type as fixed factors. In this design random factors and interactions are not tested for significance and have been omitted for brevity.

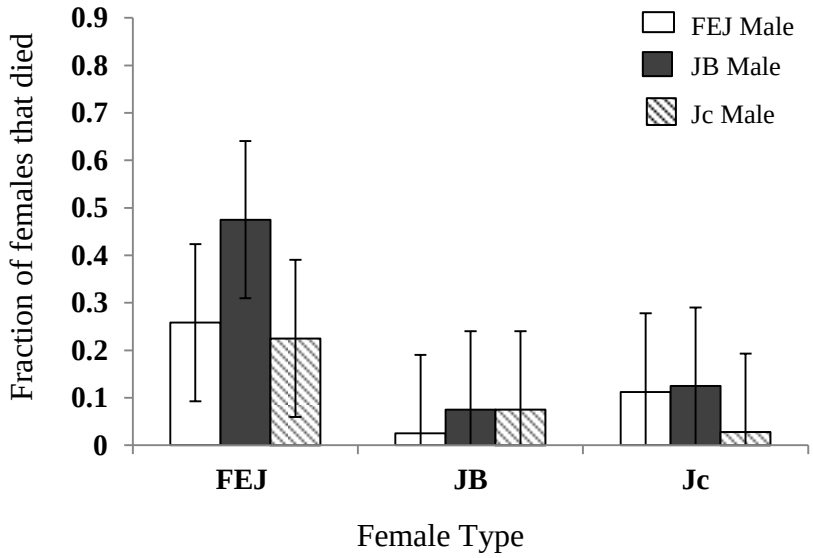

Fig. 2.9 Fraction of females that died 14 days after exposure to two matings. Error bars are 95% confidence intervals, allowing for visual hypothesis testing.

Dry weight

No significant differences were seen in sex specific dry weights between FEJ and Jc flies used for the female mortality assay, mating exposure assay or female mortality with fixed mating exposure assay (data not shown) indicating that Jc flies were of similar size compared to their FEJ counterparts in all the assays.

Effect	*df*	MS	*F*	*P*
Female	2	0.7123	10.25	0.0266
Male	2	0.0734	1.741	0.2856
Female × Male	4	0.0518	2.660	0.1113

Table 2.5 Results of ANOVA performed on cumulative mortality over the first 14 days from set up in the female mortality assay with exposure to only 2 matings. Female and male types were treated as fixed factors.

Discussion

Post-mating mortality of females in *D. melanogaster* populations is dependent on the condition of the males in terms of their mate-manipulative ability that, in turn, is likely to be correlated with body size (Chapman et al., 1995; Pitnick and Garcia-Gonzales 2002). When I exposed FEJ females to JB males for a short duration (50 hours), a significant increase in post-mating mortality of females was seen as compared to when they mated with either FEJ or Jc males. The significant main effects of male and female type are likely to have been driven by the significantly higher post-mating mortality rate of the FEJ female with JB male combination (Figs. 2.1, 2.3), as revealed by pair wise comparisons. Jc females were not as susceptible to mating stress as FEJ females since they did not show increased mortality compared to the larger JB females (Fig 2.2 (A), 2.4 (A)), indicating that being a smaller female per se did not result in increased post-mating mortality. It had been previously observed that FEJ females did not appear to suffer any visible physical damage after mating with the large JB males (Ghosh and Joshi, 2012), which is corroborated by the current results of Jc females not suffering higher mortality. Moreover, Jc females are smaller than JB females due to just a single generation of crowding, as compared to the FEJ females which have evolved along with smaller, potentially less harmful FEJ males for many generations. The defence mechanisms to cope with biochemical aspects of mating stress are, thus, likely to be retained in Jc females, whereas they might have been reduced in FEJ females. This can potentially explain the lack of change in female susceptibility to mating induced harm in Jc females.

Reduction in size per se, however, did seem to affect the degree of post-mating harm that males are able to cause females, since smaller Jc males were no more harmful

than FEJ males (Fig 2.2 (B), 2.4 (B)). This could be indicative of reduced seminal fluid production (assuming a proportional reduction with body size) and a consequent reduction in Acp quantity. However, assessment of the mating fitness of a male through the reduction in the survival probability of a female that it mates with assumes a direct correspondence of manipulative harm with male size, which might not necessarily be the case.

The ability to forcibly acquire a mating is likely to be related to the size and energy levels of males, and therefore it is possible that JB males (due to larger size) and Jc males (possibly due to higher activity levels) might mate longer and more often than FEJ males. Similarly JB and Jc females could perhaps avoid being forcibly mated with, resulting in differential mating stress being imposed on JB, Jc and FEJ females. The exposure to mating in experiments reported here was much less than had been tested earlier (Ghosh and Joshi, 2012), where females had been housed with multiple males throughout the assay duration of two weeks and, therefore, it was unlikely that mating exposure per se could give rise to high FEJ female mortality in my experiment. Nevertheless, I checked the number of matings and total duration of mating in the various male-female combinations in the 50 hour duration of mating exposure to eliminate this possibility and found the trends in mating to not correspond with mortality of females (Fig. 2.5). Similarly, the trends in total duration of mating (total exposure) also did not correspond with the trends in mortality data (Fig. 2.6). I also checked for female mortality when each female had been exposed to only two matings (Fig. 2.9), which additionally indicated no correlation of mating exposure with increased mortality, thus confirming that differential mating success in different male-female combinations was not the cause of high FEJ female mortality after mating with JB males.

The results from these experiments indicate a significant contribution of body size to the mating stress imposed by males, since Jc male lethality to females was not different from that of FEJ males. However, studying the mating success of these males in terms of the number of matings achieved, as well as the total duration for which they were able to mate, Jc males were not significantly different from the JB males but significantly more successful than the FEJ males (Figs. 2.7 (B), 2.8 (B)). This could be purely due to higher activity and energy levels (FEJ males being lethargic in comparison), and potentially indicates better courtship ability and increased ability to manipulate females into mating in the Jc males, compared to FEJ males. In *D. melanogaster*, sperm transfer usually occurs within the first 3-5 minutes of copulation, the remaining copulation duration being spent in transferring Acps to the female reproductive tract. Therefore, the total duration of mating can be indicative of the ability of the males to manipulate the females through chemical means. Jc males clearly mated for much longer than the FEJ males and, therefore, potentially transferred more Acps compared to the FEJ males, even when scaled by the quantity per ejaculate which might be less due to reduced body size. Despite this, since no increase was observed in the FEJ female mortality when mated with Jc males, as compared to FEJ males, we are forced to speculate that perhaps the seminal fluid output is not responsible for imposing greater mating stress, but rather JB males may differ in other features like kind of Acp production, courtship rates and mate-manipulative ability (see Chapter 3).

It is important to note here that Jc flies had been obtained for this experiment through larval crowding for one generation, which results in resource limitation in the pre-adult stage and a consequent reduction in adult body size. Given that these individuals have experienced a relatively stressful environment as larvae, its

consequences on the adult mating behaviour cannot be disregarded, and potentially the Jc flies may be intrinsically different from the JB flies in respects other than size as well. Although Edward and Chapman (2012) have shown that larval crowding does not significantly affect the mating behaviour of *D. melanogaster* males for a wide range of larval crowding till body size gets affected, we know that courtship rates are significantly reduced as a consequence of larval crowding in *D. melanogaster* (N. G. Prasad personal communication). This might partly explain differences that are seen between Jc and JB males that are unlikely to be due to body size per se, and further detailed investigation into the effects of larval crowding on adult male and female mating behaviour is required.

In conclusion, although size seems to partly modulate the mating capability of male flies, Jc males still fare better than FEJ males (see also Chapter 3) but do not cause any greater mortality in females post-mating compared to FEJ males. Thus lethality to females of the males seems to correspond well with size of the males, even though other aspects of mating success do not. Females, however, are no more susceptible to mate-induced harm when smaller, indicating the possibility that it is a genetic change in the female susceptibility to mating stress brought about by selection that has made FEJ females less capable of counteracting manipulative harm compared to JB females. Since FEJ females do seem to have evolved lower defences, it is indicative of possible reduction in levels of inter-locus sexual conflict in the FEJ populations.

Chapter 3

Introduction

In chapter 2, the possible contribution of body size to the increased post-mating mortality of FEJ females mated with JB males was explored in detail. In this chapter, I enlarge the scope of the experiments reported in chapter 2 by assessing the contribution of body size to mating related fitness of males, in order to better understand the role that body size divergence has played in possibly affecting sexual conflict in the FEJ populations, compared to their ancestral controls.

The effect of body size on sexual behaviour in *Drosophila* has been well documented, showing that male competitive ability in terms of mating and fertilization success is often directly correlated with size (Partridge and Farquhar 1983; Partridge et al., 1987a,b). The reduction in survival probability and egg output of females after mating with large males has also been documented (Pitnick and Garcia-Gonzalez, 2002; Friberg and Arnqvist, 2003). Reduction in body size of the FEJ populations is, therefore, likely to result in changes in the levels of sexual selection and conflict. As their exposure is routinely only to small males that are less harmful to females compared to their ancestral counterparts, females in the FEJ populations can probably afford to reduce defences against mating related stress, perhaps freeing up energy resources that might translate into enhanced fecundity, an important fitness trait that is reduced in FEJ females due to their small size and low lipid content (Prasad 2004). Moreover, reduced male size might also affect the realized mating frequencies in the FEJ population, which could also affect levels of sexual conflict.

Here, I report results from experiments in which I allowed a female to mate with two males in succession in order to examine components of the mating fitness of males by assessing (1) their ability to secure a mating with already mated females, and (2) their ability to keep a female they have mated with non-receptive to other males, by estimating time to re-mating. Time to re-mating /refractory period can be affected by the size of the second male, possibly through female choice (Pitnick et al., 1991), and is independent of the size of the first mate, while the ability to keep a mate non-receptive can depend on the quality and quantity of accessory gland proteins transferred by the first mate which, in turn, can be dependent on body size (Chapman et al., 1995).

Materials and methods

Refractory period assay

The purpose of performing this assay was to assess two components of male mating fitness, namely the ability to re-mate with an already mated female, and the ability to keep a female non-receptive to a second male after mating. For this purpose, a common female background was chosen to avoid confounding effects of using females from any of the populations being studied, as evolved males from that population have an advantage in reproductive interactions, having co-evolved with females from the same populations for many generations. Scarlet eye (SE) mutant outbred populations that have been maintained in the laboratory for about 70 generations were used for this purpose. These populations were created by introgressing the SE gene into an MGB background. The MGB populations were created by mixing the four replicate JB populations and then re-deriving five new outbreeding populations from the resulting four-way hybrid populations.

For assessing the ability to mate with an already mated female, one virgin male and one virgin female each from the SE population were placed in vials using light anesthetization with carbon dioxide. Immediately after the first successful mating (minimum of three minutes in copula) was achieved, the male was replaced (without using anesthesia) with a virgin experimental male (FEJ, JB or Jc). Creation of Jc flies has been described in detail in Chapter 2. Initially, 15 such vials were set up for each of the three regimes, and second mates were introduced into vials in which the first mating happened within four hours from setup. Two indices were used to assess the ability of JB, FEJ and Jc males to mate with a previously mated female: (1) the proportion of vials in which a second successful mating was not achieved (refractoriness), and (2) the time taken from introduction of the second mate till successful mating (refractory period). The ability to secure a mating with an already mated female can involve making the female receptive for a second mating through either forced copulation /coercion or successful courtship. In this assay, however, it was not possible to differentiate between these two mechanisms of second-male mating success.

To assess the ability of JB, FEJ and Jc males to keep the female they mated with non-receptive to a second male, a similar assay was carried out except that the first mate for the virgin SE female was the experimental male (FEJ, JB or Jc), and the virgin SE male was used as the second mate. Fifteen such vials were initially set up using light carbon dioxide anesthetization. The second mate virgin SE males were replaced for the first males without anesthesia in vials in which a successful mating was achieved within six hours. As in the previous case, refractoriness and refractory period were estimated. For both assays, the total observation period for both matings to occur was eight hours.

To ensure that Jc flies were appropriate body size controls, dry weights of FEJ and Jc males and females were estimated, as described in chapter 2. These assays were performed between FEJ generation 513 and 519, JB generation 263 and 266, and SE generation 60 and 63.

Statistical analyses

For all analyses, FEJ, JB and Jc populations with the same numerical subscript were treated as random blocks for the ANOVA. Mean refractory period across females for each block was subjected to mixed model ANOVA with male type as a fixed factor. Refractoriness data were fractional data and therefore subjected to arcsine square root transformation and then subjected to a mixed model ANOVA. Separate analyses were done for assays in which experimental males (JB, FEJ or Jc) were the first or second mate, respectively.

Results

Ability to keep a mate non-receptive after mating

When the first mate was the experimental male (JB, FEJ or Jc), the ANOVA on mean refractory period showed a significant effect of male type ($F_{2,6}$=11.79, P=0.008) (Fig 3.1). JB males kept their mates non-receptive for a significantly longer time compared to the FEJ males, with Jc males being intermediate between FEJ and JB and significantly different from both (Tukey's HSD at 0.05 significance level). Similarly, the fraction of females that did not re-mate (refractoriness) was significantly higher in case the first mates were JB males, showing their ability to prevent mating of their mates with other males ($F_{2,6}$=44.14, P=0.0002) (Fig. 3.3). However, the Jc males did not significantly differ from the FEJ males in

refractoriness they induced. Thus both these indices indicate a significant contribution of both genetic background and body size on the differences in male mating fitness between FEJ and JB populations.

Ability to re-mate with an already mated female

When the second mate was the experimental male, the ANOVA on mean refractory period showed no significant effect of male type (FEJ, JB or Jc) ($F_{2,6}$=0.068, P=0.934) (Fig. 3.2). However there was a significant main effect of male type on refractoriness ($F_{2,6}$=6.476, P=0.031) (Fig. 3.4). Pairwise comparisons revealed that the fraction of females which did not mate a second time was significantly less when the second mates were JB compared to FEJ and Jc males, indicating that the large JB males were more successful at re-mating with an already mated female, compared to the smaller FEJ or Jc males. FEJ males were the least successful at obtaining mating with an already mated female, with the Jc males being intermediate between JB and FEJ (Fig 3.4).

Dry weight comparison of males

The dry weight of males, as measured at the age when the assay was performed was not significantly different between the FEJ and the Jc populations ($F_{1,3}$=0.281, P=0.632) allowing us to consider them equivalent in body size.

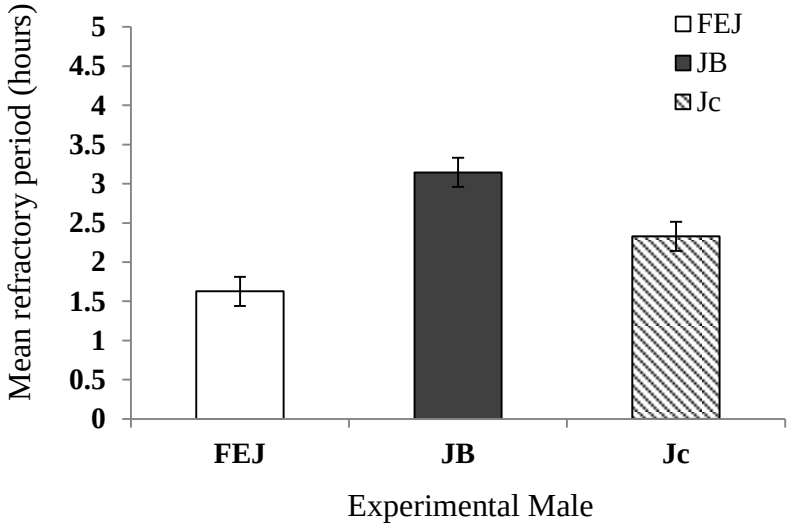

Fig 3.1 Mean refractory period: time till successful mating from introduction of second mates, first mates being FEJ, JB or Jc virgin males. The ability of the three types of males to keep mates non-receptive was being tested. Error bars represent 95% confidence intervals around the mean of four replicate populations and therefore can be used for visual hypothesis testing.

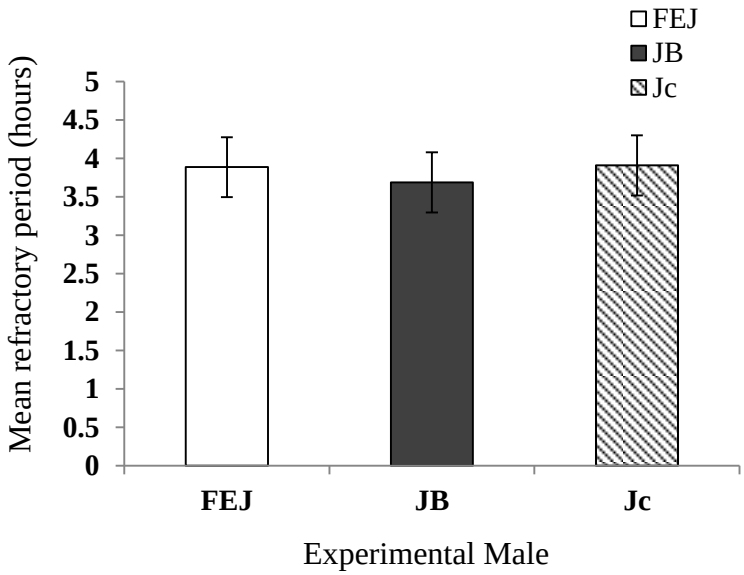

Fig 3.2 Mean refractory period: time till successful mating from introduction of second mates (FEJ, JB or Jc virgin males). The ability of the three types of males to mate with an already mated female was being tested. Error bars represent 95%

confidence intervals around the mean of four replicate populations and therefore can be used for visual hypothesis testing.

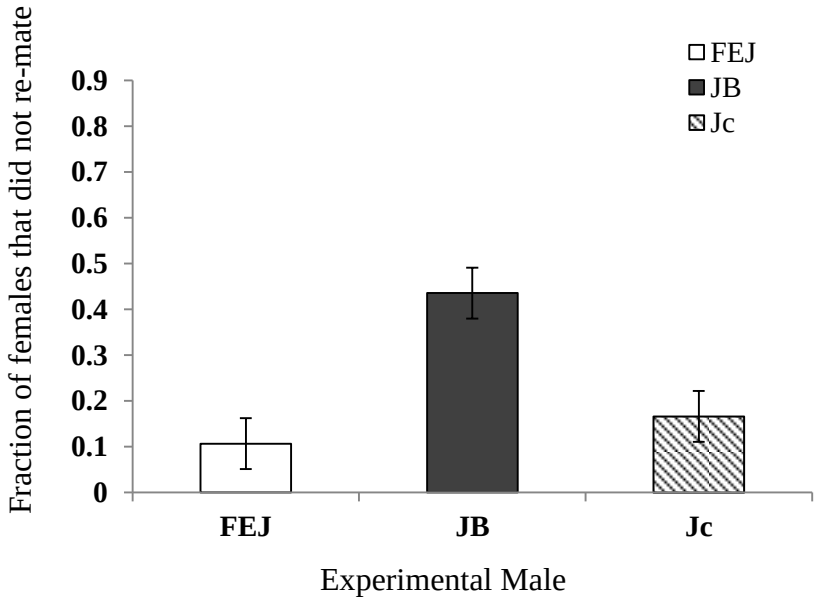

Fig. 3.3 Refractoriness: the fraction of females that did not re-mate when ability to keep a mate non-receptive was being tested with JB, FEJ or Jc males being the first mate. Error bars are 95% confidence intervals around the 4 population means, allowing visual hypothesis testing.

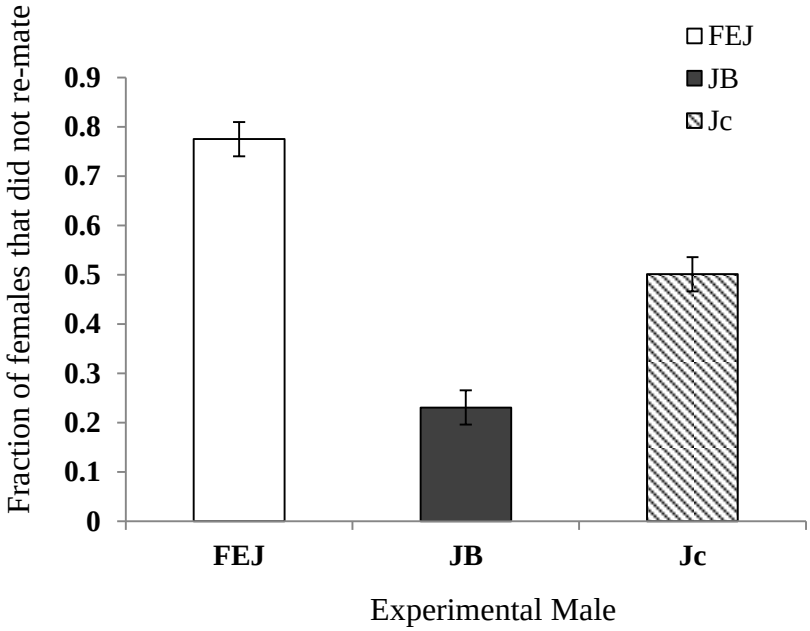

Fig 3.4 Refractoriness: the fraction of females that did not re-mate when ability to mate with an already mated female was being tested, experimental male being the second mate. Error bars are 95% confidence intervals around the 4 population means.

Discussion

Results from the assays performed to assess the number of matings achieved by males in 50 hours, discussed in the previous chapter (Fig.2.5) indicated that FEJ male mating success in terms of the number of matings and total duration in copula is less than the Jc male mating success, regardless of the identity of the female. When we looked at two aspects of male mating success in these populations assessing the ability of males to manipulate the re-mating interval of females, our earlier results were corroborated showing that Jc males were intermediate between JB and FEJ males in securing second matings as well as in keeping their mates non-receptive. This implicates both body size per se, and genetic constitution in

mediating differences in male reproductive behaviour between the FEJ and JB populations.

FEJs usually show very low activity levels and movement compared to the JBs when kept as adults in cages (Avani Mital, personal observation). Perhaps FEJ males are less effective in courting females due to their low activity levels compared to the JB controls. However, as it was not possible to differentiate between forced mating and successful courtship in these assays, it is also possible that the larger JB males might be successful in forcing copulation with the SE females, although this explanation would not explain the higher mating success of Jc males as compared to FEJ males. Moreover, it is possible that the FEJ populations have also diverged from their ancestral controls in courtship song production, which might lend JB males an advantage since SE populations have essentially been created in a JB-like background. Sexual selection mediating such divergence might result in courtship incompatibility between flies from different populations resulting in poor mating success of FEJ males with SE females. These data also suggest that the earlier observation that FEJ males were unsuccessful in getting many matings with JB females (Ghosh and Joshi, 2012) is likely to be due to divergence between JB and FEJ populations in sexually selected traits in addition to the size difference between them. If the differences in mating success were solely due to size we would expect the mating success of Jc to be similar to FEJ males rather than intermediate between FEJ males and JB males (Figs. 3.1, 3.4)

Since inter-locus sexual conflict over mating and fertilization success is largely mediated by manipulative behaviour by the males, a change in the mating fitness of males could potentially drive evolutionary changes in the FEJ populations through

reduced sexual conflict. Specifically, the ability to keep a mate non-receptive in *Drosophila* has been shown to be mediated through accessory gland proteins that affect female fitness in various ways (reviewed in Chapman et al., 2003). In these assays, a behavioural read-out for reduction in mate-manipulative male traits in the FEJ populations was taken by looking at their ability to keep their mates non-receptive, and this was found to be significantly less than both JB and Jc (Fig. 3.1). This observation is consistent with the notion that FEJ males have evolved to be less harmful to females, potentially leading to the possible co-evolutionary changes in FEJ females towards reduced defence against manipulation by these males.

Although pre-adult and adult stages are morphologically disjunct in *Drosophila*, they are nevertheless connected through resource accumulation because of which changes during development can affect fitness-related traits in the adults (Chippindale et al., 1996, Prasad and Joshi, 2003) A critical point here is the time course of selection in the FEJs compared to JBs. FEJs flies become part of the breeding population only if they are among the first 15%- 20% to eclose, their fitness being zero otherwise. Consequently, mating success is under selection only conditional upon rapid development and, thus, selection is stronger on development time than on reproduction related traits. Therefore, fitness advantage that might be gained through increased mating success might have been foregone in the FEJ males for fast development. Consequently, with less manipulative males in the population, inter-locus conflict over mating rates is likely to have gone down in the FEJ populations resulting in counter-adaptations to male-induced harm in the FEJ females also being reduced, as any resources thus saved could be used to increase fecundity.

Thus, the results reported in this chapter support the possibility of reduced levels of inter-locus sexual conflict in the FEJ populations, mediated by both direct (through reduced manipulative ability of males) and indirect (by potentially affecting mating and courtship frequencies in the populations) effects of the evolutionary reduction of body size in the FEJ populations as a correlated response to selection for rapid pre-adult development.

Chapter 4

Introduction

Selection for specific traits can result in correlated changes in other traits either due to genetic correlations among traits or as a result of indirect selection. When selecting for a single trait, the population often experiences indirect selection pressures on other traits that might be by-products of the population's response to selection. Trait evolution as an outcome of indirect selection pressures has been reported in many earlier laboratory selection experiments (reviewed by Harshman and Hoffmann, 2000; Prasad and Joshi, 2003).

Conflict over optimum mating rate between the sexes is expected (Parker, 1979) and can result in one sex evolving to manipulate the other in a manner so as to move the mating rate towards its own optimum (Parker and Partridge, 1998; Arnqvist and Nilsson, 2000). Additional inter-locus sexual conflict over fertilization success can also occur as the fitness consequences for the two sexes might differ with respect to the proportion of eggs of a female that will be fertilized by sperm from a specific male. The level of inter-locus sexual conflict, thus, can be an outcome of sexually antagonistic co-evolution in which one sex initiates increased conflict through manipulation of its mate, driving counter-adaptations in the other sex. In principle, however, sexual conflict levels may also evolve downwards if fitness costs associated with maintaining such manipulative behaviour outweigh the fitness advantage gained through such manipulation (Lessells, 2006). In *Drosophila.*, manipulative traits in males (typically mediated through Acps) have been shown to increase male fitness, while reducing female fitness (Chapman et al., 1995). Thus, in situations where manipulative behaviour by males is known to cause harm to

females, the degree to which males can manipulate (harm) their mates can be used as a surrogate measure of male reproductive fitness. Male-female antagonistic co-evolution has been studied through selection experiments in which the degree of intra-sexual male-male competition was varied by enforcing promiscuity /monogamy (Pitnick et al., 2001; Crudington et al., 2005), or altering operational sex ratios (Wigby and Chapman, 2004). Males from a promiscuous mating environment are exposed to greater intra-sexual competition, and evolve to have increased fitness causing increased harm to mates (Pitnick et al., 2001; Wigby and Chapman, 2004; Crudington et al., 2005). Consequently, females from such populations also evolve to be better able to resist the harm caused by mating with such males through counter-adaptations.

These studies highlight two important aspects related to sexual conflict that can drive changes in mating fitness of the sexes: (1) levels of intra-sexual competition in males can drive selection for manipulative (possibly harmful) traits that increase male mating fitness, and (2) females can then evolve counter-adaptations which might be costly, as indicated by the rapid loss of such defences by females when the selection pressure for their maintenance is removed (Holland and Rice, 1999; Pitnick et al., 2001; Wigby and Chapman, 2004; Crudington and Snook et al., 2005).

In our laboratory, *D. melanogaster* populations selected for rapid pre-adult development and early reproduction (FEJs) potentially may have been subjected to unintended indirect selection pressures that affect sexual conflict. Since eggs in the FEJ populations are collected 3 days after eclosion compared to 10-11 days after eclosion in the control JB populations, there is potentially substantial difference in opportunities for re-mating between the FEJ and JB populations as the time available

for repeated mating before eggs are collected for starting the next generation is much smaller in the FEJs compared to the JBs. Allowing for a reproductively immature period after eclosion, when mating is not possible, the FEJ populations have a little over two days of possibly sexual activity before the eggs for the next generation are collected (Prasad, 2004). As *Drosophila* females have a refractory period after mating when they are not receptive to males, this effectively means that re-mating opportunities might be much less in the FEJ populations, potentially altering the pattern and strength of sexual selection acting on the two types of populations. Moreover, FEJ flies also move sluggishly, fly less and are much smaller than JB flies, all of which could further contribute towards reducing the likelihood of repeated matings in the FEJ populations.

Mating rate in the FEJ populations is, therefore, likely to be much lower than in the JB controls. Given the importance of inter-male competition in affecting levels of inter-locus sexual conflict (Holland and Rice, 1999; Pitnick et al., 2001; Wigby and Chapman, 2004; Crudington et al., 2005), if the frequency of mating is low in the FEJ populations compared to the JBs, then the intra-sexual competition experienced by FEJ males is likely to be much less than JB males. Earlier work in our lab has shown an overall reduction in the expression levels of Acp (Accessory gland protein) genes in the FEJ populations (Satish, 2010; P. Dey and A. Joshi, unpublished data). As Acps are believed to mediate manipulation of mated females by male *Drosophila* (Chapman et al., 1995) low Acp gene expression in FEJ males may indicate relaxation of selection on FEJ females to maintain defences against Acp-mediated manipulation, resulting in the evolution in decreased levels of inter-locus sexual conflict in the FEJ populations. Body size is also an important factor affecting sexual selection, reproductive behaviour and egg output in *Drosophila*

(Patridge and Farquhar, 1983; Markow and Ricker, 1992; Stearns, 1992; Roff, 2002). Consequently, the smaller body size of FEJ males might also affect sexual behaviour. Females in *D. melanogaster* are known to select larger males for mating (Pitnick et al., 1991), which are also known to harm the females to a greater extent than smaller males (Pitnick and Garcia-Gonzalez, 2002).

In this chapter I explore possible differences in the breeding ecology in terms of the levels of courtship experienced by the FEJ and JB flies in the populations during their regular stock maintenance that might drive a reduction in the level of inter-locus sexual conflict in the FEJ populations. In the previous chapters I discussed the possible contribution of body size reduction in mediating evolution of lower inter-locus sexual conflict in the FEJ populations. Here, I report results on the observation of courtship frequency and mating rate in cage populations. Apart from the actual act of mating, courtship itself can also impose stress on the females through harassment (Partridge et al., 1990). I also examined maturation time (time from eclosion to first mating) for males and females in the FEJ and JB populations.

Materials and methods

Maturation time

Maturation time of adult flies can be defined as the time from eclosion to first successful mating (Prasad, 2004). Although sexual maturation may be considered to have been achieved when the first event of courtship is exhibited, a similar behavioural marker for females is not available and, therefore, time till first successful mating (minimum copulation duration of 3 minutes) was scored. Virgin flies were collected two days prior to the setup of this assay to provide sexually mature mates for the freshly eclosed experimental flies collected over a one hour

time window. To estimate the maturation time for females, freshly eclosed female flies were paired with mature males, and vice-versa for virgin males, in individual vials, with 20 replicate vials per sex × selection regime combination. All four blocks were assayed separately between generations 511-513 of FEJ selection, corresponding to, generation 262-263 of JB populations. No alternate mate was provided, unlike in the study of Prasad (2004) to avoid any confounding effects of intra-sexual competition on time till first successful mating. Carbon dioxide anesthetization was used for the setting up of vials which were then kept undisturbed on a white background on a table under constant illumination at $25^{o}C \pm 1^{o}C$.

Breeding ecology assay

Since the purpose of this study was to better understand the breeding ecology of FEJ and JB populations during regular stock maintenance, it was necessary to try to the assay setup with the actual maintenance regime. Therefore, flies used in the assay were not subjected to standardization to eliminate non-genetic parental effects. For observing courtship and mating, I chose to focus on the time when flies would normally be given a yeast supplement for a few days prior to egg-collection. Although there is a large age difference between the FEJ and JB flies at the time that they are provided yeast supplement, that is potentially the most relevant window in terms of mating that is likely to contribute eggs to the next generation. Moreover, yeast has been shown to enhance the courtship and copulation frequencies in *D. melanogaster* populations (N. G. Prasad, personal communication). Because it was not feasible to observe more than 200 flies per population for scoring courtship and mating, 100 males and 100 females were put in a Plexiglas cage (20 cm × 15 cm × 12.5 cm), with three replicate cages per population. In order to keep the density of flies in the cage relatively close to the stock maintenance cages, I used cages half the

volume of cages used for regular stock maintenance The first 25% of flies eclosing from each vial of FEJs (at generations 535 and 536), and all flies eclosing from the JB vials (at generation 274) were collected into cages from which 100 males and 100 females per assay cage were aspirated out, and put ino the smaller observation cages. FEJ egg collection was staggered relative to JBs so as to obtain freshly eclosed FEJ flies at the same time when JB flies were already 18 days from egg collection, such that FEJ and JB flies of different ages, corresponding to their respective yeasting periods, could be assayed at the same time. Once all the observation cages were set up, yeast supplement was provided on small Petri-plates with banana-jaggery food medium. Observations were started 3 hours after yeasting to allow the flies to acclimate to the observation cages.

For taking courtship observations, 1 minute per check (see below) was spent on each cage during which the entire cage was scanned and visual observations were taken for the number of males seen performing any courtship related behaviour toward a female. The number of courtship events was recorded only from the male perspective; the female responses (rejection /allowance) were not scored. Various behaviour patterns noted under courtship included: orientation toward a female, wing extension and vibration (courtship song), chasing, licking and attempted copulation. Immediately after the check in which observations were made for courtship, a scan (< one minute) was done to record the number of pairs seen in the act of copulation.

Observations were taken over a 2 day period in batches of 3 hour duration, during which 3 checks were performed spanning over those 3 hours. Thus, a total of 24 such checks were performed and 24 minutes of observation and 24 scans were obtained for courtship and mating, respectively. The number of dead flies was

recorded prior to each check to be able to estimate the frequency of courtship and mating. Twice over the period of those two days, dead flies were aspirated out of the cage, and all cages were disturbed similarly irrespective of the presence or absence of dead flies. During the entire observation period, the cages were kept undisturbed on a table with a white background under constant illumination at $25^{o}C \pm 1^{o}C$.

Statistical analyses

For maturation time, the mean of time in hours from eclosion to first successful mating was compared for JB and FEJ males and females. Courtship frequency was estimated as the average across checks of the number of courtship events seen in a cage at each check divided by the number of males in that cage at the time. For mating number, mean of number of copulation pairs seen per cage across scans was calculated. Mixed model ANOVA was performed on courtship frequency and mating number and maturation time, treating blocks as a random factor and selection regime and sex (for maturation time) as fixed factors crossed with block.

Results

Maturation time

Overall, the mean maturation time for FEJ flies was significantly greater than for JB flies (Fig 3.2) ($F_{1,3}$=166.648, p=0.01018). In FEJ, males and females had similar maturation times but JB males had a greater maturation time than JB females (Fig. 3.1) although the selection regime × sex interaction was not significant ($F_{1,3}$=8.484, P=0.0616). Although the trend of FEJ maturation time being greater than JB maturation time is consistent with earlier results (Prasad, 2004) the mean time till maturation is about 5 hours greater for both FEJ and JB as compared to the study of

Prasad (2004). Given the sensitivity of estimating time till an event that can be sensitive to anesthetization dose or presence or absence of alternate mates, it is not surprising to find such differences. Overall, if FEJ flies take around 24 hours from eclosion to first mating, the FEJ flies essentially have only 2 days to obtain matings, compared with the 10-11 days available for JBs in their maintenance regimes.

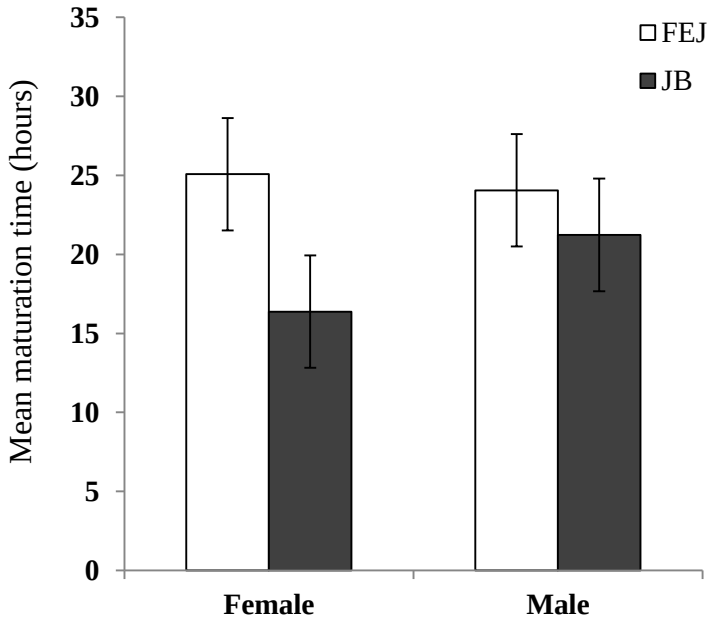

Fig. 3.1 Mean time between eclosion and first mating for FEJ and JB males and females. Error bars represent 95% confidence intervals around means of 4 replicate blocks.

Effect	*df*	MS	*F*	*P*
Sex	1	16.281	33.68	0.0101
Selection	1	137.50	116.64	0.001
Sex x Selection	1	36.829	8.484	0.0618

Table 3.1 Results of ANOVA on time from eclosion till first mating, with sex and selection regime as fixed factors. In this design random factors and interactions cannot be tested for significance and have therefore been omitted for brevity.

Effect	*df*	MS	*F*	*P*
Courtship frequency	1	0.8724	8.490	0.0618
Mating number	1	0.4888	378.14	0.0002

Table 3.2 Summary of results of ANOVA on courtship frequency and mating number with selection regime as a fixed factor. The main effect of selection regime is shown for each trait. In this design, random factors and interactions cannot be tested for significance and have therefore been omitted for brevity.

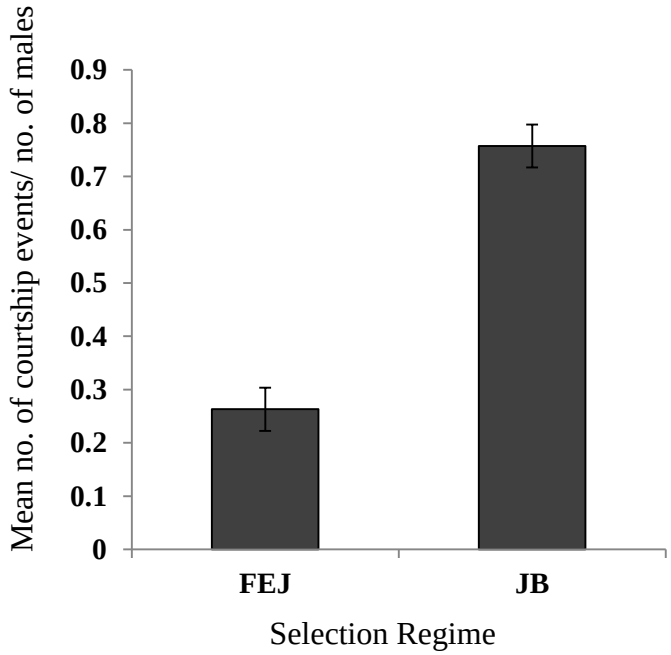

Fig. 3.2 Means number of courtship events per male for FEJs and JBs. Error bars represent 95% confidence intervals around the means of 4 replicate blocks, allowing visual hypothesis testing.

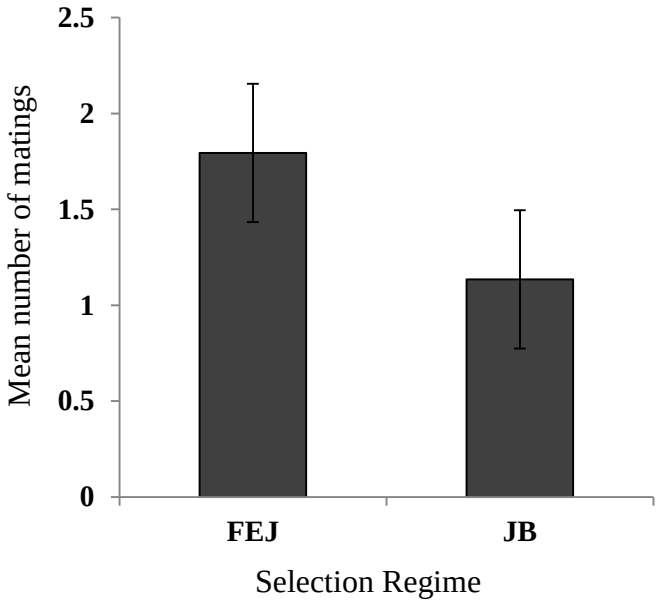

Fig. 3.3 Mean number of matings per scan observed across 2 days of yeasting from 100 mating pairs. Error bars represent 95% confidence intervals around the means of 4 replicate blocks, allowing visual hypothesis testing.

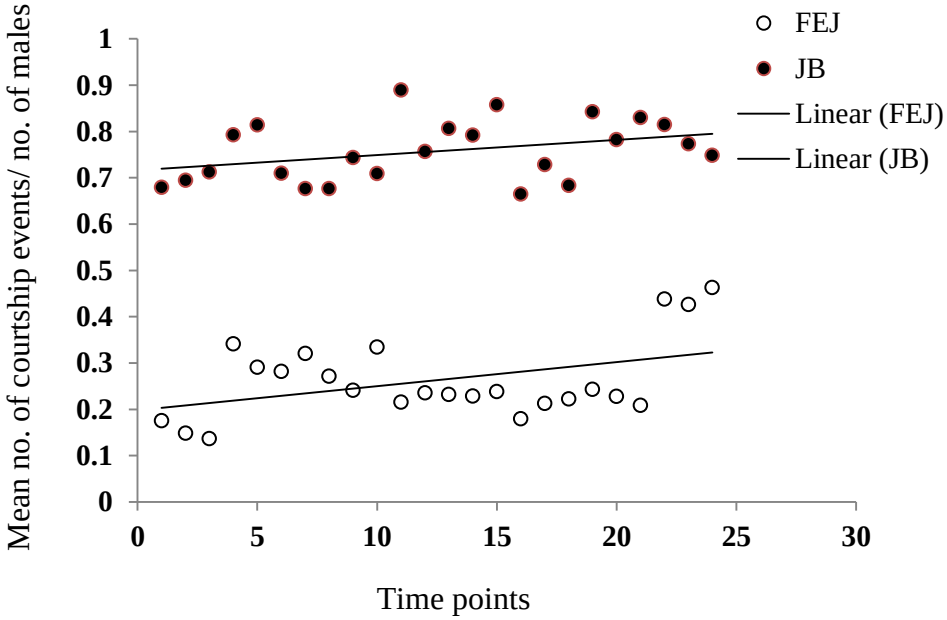

Fig 3.4 Plot of courtship frequencies changing across time points (checks) over the course of 48 hours that the observations were taken.

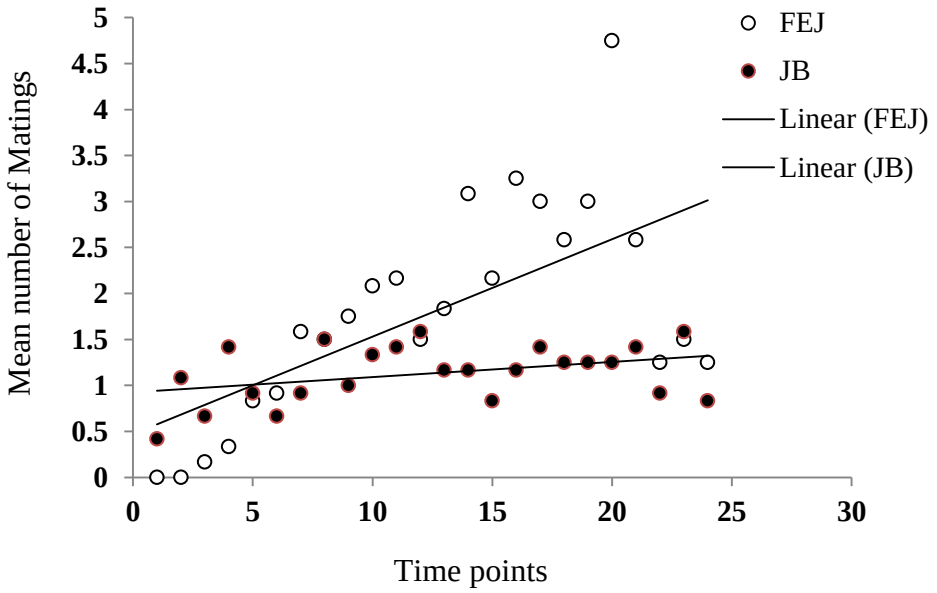

Fig 3.5 Plot of number of matings seen per scan changing across time points (scans) over the 48 hour duration that the observations were taken.

Courtship frequency

Mean courtship frequency was significantly lower for the FEJ populations compared to JB populations ($F_{1,3}$=378.147, P=0.0002) (Fig. 3.2) perhaps indicating a lower mating drive or reduced energy levels in the FEJ populations. The trend across time points indicated a slight increase in courtship frequency with time (Fig. 3.4).

Mating number

The number of matings did not significantly differ between the FEJ and JB cages, with the mean number of matings per scan being 1 ($F_{1,3}$=8.4903, P=.0618) (Fig. 3.3). However over the course of the 48 hours of observation, FEJ matings per scan increased mating whereas it remained relatively constant for the JBs (Fig. 3.5).

Discussion

The results clearly suggest that although levels of courtship exhibited by FEJ males are considerably lower than JBs, there was not much difference in the number of matings between the JB and FEJ populations. Although an index for estimating amount of courtship required for mating to take place has not been calculated, given the non-significant difference between the mating numbers, and the huge difference in courtships rates, probably much less courtship is needed for mating to take place in the FEJ populations compared to the JBs. This may indicate the evolution of reduced 'choosiness' (sexual selection) by the FEJ females, perhaps due to the short time available from eclosion till egg collection in the FEJ populations as compared to the JB controls. It is harder to interpret the mating frequency data for the JB populations as it is possible that the JB females will lay eggs on day 21 that are fertilized by sperm from matings experienced before day 18, when the yeast supplement is first made available to the JB flies in their normal maintenance regime. It seems likely, though, that JB adults will have more opportunity for repeated mating compared to the FEJ adults, due to the much longer time from eclosion to egg collection in the JB maintenance regime. The notion that there might be lower levels of re-mating and reduced likelihood of sexual selection in the FEJ populations is also supported by an earlier observation that the variance in male mating success in 14 day discrete generation cycle populations of *D. melanogaster* shows a Poisson distribution (Joshi et al., 2000).

The maturation time for the FEJs is seen to be greater than for JBs in this study, as also seen by Prasad (2004). Anesthetization with carbon dioxide can potentially increase the time till first mating (Barron, 2000) and, thus, the absolute values of

maturation time may be an over-estimate. However it is unlikely that carbon dioxide affects JB and FEJ flies differentially, as I did not observe any differences in recovery time between the JB and FEJ flies.

Lessells (2006) describes the various optimum strategies for each sex under a conflict scenario over a trait and the conditions under which harmful manipulative behaviour is expected to evolve. Sexual conflict over mating stems from the fact that the optimum mating rate and re-mating duration are expected to be different for the two sexes owing to differential investment made per progeny. One of the sexes is expected to invest more in few progeny, while the other is expected to invest less, but in a larger number of progeny. Commonly, females maximize their reproductive fitness through few matings, large investment per egg, and maximal possible egg output over their life-time. Males on the other hand invest in ensuring that most eggs laid by a female are fertilized by their sperm, and by obtaining as many matings as possible. Due to this, there is conflict over the optimum mating rate between the two sexes, with the female optimum usually being lower than that for the males. Since mating requires participation of both sexes, males are expected to manipulate their mates to secure matings at rates sub-optimal for females (higher than female optimum) , while females are expected to evolve traits to resist such manipulation thereby bringing the mating rate down, closer to their optimum, but sub-optimal for males, resulting in sexually antagonistic co-evolution. This basal conflict, purely due to differing optima for the two sexes with respect to mating rate, has been described as the 'conflict load' (Lessells, 2006). However, when such manipulative behaviour results in collateral damage to the mate, it can affect the fitness of both sexes. Therefore, harmful manipulative behaviour is conditional upon the fitness loss of the sex exhibiting this behaviour (through harm to the mate) being outweighed by the

advantage gained through manipulation. The sex suffering the harm then is expected to evolve resistance traits, depending on whether the cost of resistance is greater or smaller than the fitness lost through harm. This fitness cost due to mate harming is over and above the basal conflict load.

In case of the FEJ populations, it seems that levels of sexual conflict might have evolved to become lower in respects of both conflict load as well as additional conflict due to harm. With the window of re-mating opportunity being small for the FEJ populations, both males and females might have relatively similar optima for mating frequency for two reasons: (1) in case of females, optimum mating frequency might be higher due to the time constraint, affording them the chance to mate multiply, and (2) the males might have reduced mating frequencies due to energy and size constraints, reducing their optimum.

Given the frequency of courtship, the number of matings achieved by the FEJs is quite high, possibly indicating a reduction in female choosiness (lower sexual selection). Low levels of courtship (decreased investment in stimulatory behaviour toward the females) might indicate a lower threshold of acceptance of these females. Since the FEJs do not have much time to mate before egg lay, it is likely that the more choosy females have been selected against over generations. This might also increase the optimum mating rate of the females in FEJ populations. However, the stress of multiple mating with relatively more harmful males could result in such females suffering a cost, possibly being unable to contribute sufficiently to the next generation. If this were the case, there would then be indirect sexual selection on the males to become less harmful toward their mates, thus reducing their mate-manipulative ability. Overall, it seems likely that the FEJs have evolved lower levels of inter-locus sexual conflict partly as a result of reduced size of FEJ males

(Chapters 2,3) and partly due to the reduced time window (~2 days) available for mating prior to egg collection in the FEJ maintenance regime.

Chapter 5

In *D. melanogaster*, sexual conflict commonly occurs over mating (Bateman, 1948), and arises as a consequence of differential investment in progeny made by the sexes, that in turn affects the fitness costs and benefits of re-mating for males and females (Arnqvist and Nilsson, 2000). Number of matings can influence the level of inter-locus sexual conflict in a population by affecting the level of male-induced female harm that, in turn, is an outcome of the level of competition experienced by males. Moreover, large body size can influence mating rate (Rice et al., 2006) and also the harm caused to females, either through mating (Pitnick and Garcia-Gonzales, 2012) or through increased courtship rate (Partridge and Fowler 1990), thus affecting sexual conflict levels.

In the current thesis, I investigated a possible reduction in the levels of inter-locus sexual conflict in the FEJ and JB populations, speculated upon by Ghosh and Joshi (2012), by assessing the breeding scenario experienced by these flies during their regular maintenance to get an idea about the mating and courtship frequencies experienced by these flies. I also assessed the contribution of size divergence per se between the FEJ and JB populations to the levels of inter-locus sexual conflict in these populations, via differential male mating success and male-induced female harm. These experiments were conceived of as a preliminary follow-up to the serendipitous observations of very high female mortality in the FEJs after mating with JB males, leading to a suggestion that the FEJs may have evolved reduced levels of inter-locus sexual conflict (Ghosh and Joshi, 2012). The results of my experiments provide some support for this notion and point the way to future directions of study.

Body size contributed significantly to the degree of male-induced female harm (as assessed by post-mating female mortality), since the mortality rate assay showed Jc (small like FEJs but genetically JB) and FEJ males to be equally lethal towards females (Chapter 2). However, size contributed only partly to the mating and mate-manipulative ability of males (Chapters 2, 3). Data from the mating exposure assay (Chapter 2) showed a significant difference between the JB and FEJ males in the numbers and duration of mating, while Jc males did not differ from the JB males in this respect, possibly due to differences between the JB and FEJ genetic constitutions that affect their activity levels. The differences in mating-related fitness between the FEJ and JB populations were also mirrored in the JB and FEJ courtship rate data that showed JB males to be courting females much more than FEJ males (Chapter 4). Additionally, the mating fitness of males, as assessed from their ability to manipulate female re-mating interval (which involves Acps: reviewed in Chapman et al., 2003) also indicated that size by itself did not reduce male mating fitness, and genetic constitution significantly contributed towards it (Chapter 3). Thus, my study provides evidence for the contribution of both body size and genetic constitution to the differences in male reproductive behaviour between the JB and FEJ populations.

Even though there was no significant difference in FEJ female mortality when mated with either FEJ or Jc males, differences in the mating efficiency of FEJ and Jc males need not translate into increased female mortality, since loss in female fitness can occur through increased stress susceptibility as a consequence of an immediate fecundity boost (Salmon et al., 2001; Wang et al., 2001). Although I did not check female fecundity, Ghosh and Joshi (2012) have shown increased FEJ fecundity when they mate with JB males compared to when they mate with FEJ males, which can

impose additional costs on the fitness of the females, and this may not be completely size-dependent.

All this together hints at a reduction in competitive mating fitness of FEJ males, due to both size reduction and genetic changes arising as a result of selection and, in turn, possible driving the coevolution of inter-locus conflict between males and females down to a lower level of antagonism. The contribution of genetic constitution may be varied, most likely affecting male behaviour through activity and energy level differences between JB and FEJ males. However, further investigations into mate-manipulative ability (types of Acps produced) of FEJ, JB and Jc males are required since this was only partly addressed through a behavioural read-out in the refractory period assay (Chapter 3). Moreover, the body size allometry of FEJ and Jc males might vary due to the different modes of size reduction (genetic versus through phenotypic manipulation, respectively). In this respect, the size of the accessory glands in FEJ and Jc males also need to be investigated.

Various studies have earlier shown reduction in mating frequency to be correlated with decreased male vigour (due to decreased male-male competition for mates) and a consequent reduction in the level of harm and manipulation that the females from such selected populations experience (Pitnick et al., 2001; Wigby and Chapman, 2004; Crudington et al., 2005). Mating frequency is expected to be different between the FEJ and JB populations due to the much shorter (three days) time available for mating between the FEJ compared to JB populations (11 days). Although that is not evident from the mean mating number data (Chapter 4), that has only been checked for the duration two days prior to egg collection, when JB populations have already been mating for 6-7 additional days compared to FEJ populations. Further

exploration of the number of matings in the entire duration from eclosion to egg collection in both FEJ and JB populations, and some estimation of the reproductive output from these matings, is likely to be of interest.

One line of investigation that could shed more light on the potential reduction in level of inter-locus sexual conflict in the FEJ populations would be to assess the optimum mating rates for males and females in these populations. My own personal observations from the breeding ecology assay suggest a possible increase in the willingness of the FEJ females to mate, given that the mating rate seen was the same in both JB and FEJ cages even though the courtship rates for the FEJ flies were much lower. With practically only two days available for mating before eggs are collected for initiation of the next generation, it is possible that to attain their optimum number of matings, the FEJ females have a higher mating rate. It has been suggested that sexual selection can occur via female resistance to harmful male traits rather than through preferences (Holland and Rice, 1998). In the FEJ populations, female resistance to re-mating might be lower if the harm experienced by the females is less, as an outcome of male body size reduction.

Overall, the FEJ populations seem to have diverged from the JB population in not only life-history related traits, but also in traits affecting reproductive success. As correlated responses to selection for rapid pre-adult development and early reproduction, adult traits affecting sexual selection and conflict also appear to have diverged between FEJ and JB populations, and are already known to contribute to incipient pre-zygotic reproductive isolation between them (Ghosh and Joshi, 2012). In light of this, other behavioural changes relating to mating success can be explored in the FEJ populations; courtship song for instance, which can contribute to mating incompatibility. A change in sexual selection seems to have occurred in the FEJ

populations either as a by-product of selection for rapid pre-adult development (that resulted in body size reduction), or as an indirect selective force due to selection for early reproduction, or some combination of both.

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