Genet. Res., Camb. (2005), **86**, *pp.* 107–114. *With 5 figures.* © 2005 Cambridge University Press
doi:10.1017/S0016672305007755 Printed in the United Kingdom

A QTL analysis of female variation contributing to refractoriness and sperm competition in *Drosophila melanogaster*

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(*Received 14 March 2005 and in revised form 16 August 2005*)

Summary

Sperm competition is an important fitness component in many animal groups. *Drosophila melanogaster* males exhibit substantial genetic variation for sperm competitive ability and females show considerable genetic variation for first versus second male sperm use. Currently, the forces responsible for maintaining genetic variation in sperm competition related phenotypes are receiving much attention. While several candidate genes contributing to the variation seen in male competitive ability are known, genes involved in female sperm use remain largely undiscovered. Without knowledge of the underlying genes, it will be difficult to distinguish between different models of sexual selection such as cryptic female choice and sexual conflict. We used quantitative trait locus (QTL) mapping to identify regions of the genome contributing to female propensity to use first or second male sperm, female refractoriness to re-mating, and early-life fertility. The most well supported markers influencing the phenotypes include *33F/34A* (P2), *57B* (refractoriness) and *23F/24A* (fertility). Between 10% and 15% of the phenotypic variance observed in these recombinant inbred lines was explained by these individual QTLs. More detailed investigation of the regions detected in this experiment may lead to the identification of genes responsible for the QTLs identified here.

1. Introduction

Reproductive success is not guaranteed upon mating. Post-mating sexual selection, in the forms of sperm competition, sexual conflict or cryptic female choice, may be as important in determining an individual's fitness as the more familiar pre-mating male–male competition and female preference (Parker, 1970). Examples of sperm competition are common in many animal groups suggesting that the evolutionary implications of post-mating interactions may be quite broad (Birkhead & Moller, 1998).

Research examining the genetics and physiology of sperm competition in *Drosophila melanogaster* suggests the potential for interesting evolutionary dynamics. Females mate multiply and store sperm in two types of storage organ: the paired spermathecae and the seminal receptacle (Harshman & Clark, 1998; Imhof *et al.*, 1998; Neubaum & Wolfner, 1999). In addition to receiving sperm, *D. melanogaster* females receive over 80 different seminal fluid proteins produced by the male accessory glands (Acps) (Swanson *et al.*, 2001), some of which have dramatic impacts on female behaviour and physiology (reviewed in Chapman, 2001; Wolfner, 2002). The underlying genetics of sperm competition are not well understood, though there is clearly abundant male-expressed genetic variation affecting the phenomenon (Clark *et al.*, 1995; Hughes, 1997). Natural variation at several *Acp* loci in *D. melanogaster* correlates with sperm defence and offence, as well as affecting female refractoriness (Clark *et al.*, 1995; Fiumera *et al.*, 2005). The female side has received less attention. Female genotype affects the outcome of sperm competition in *D. melanogaster* (Clark & Begun, 1998). This variation would be neutral if there were no other pleiotropic fitness effects associated with females 'preferring' first versus second male sperm. However, the situation is less clear if there are idiosyncratic male × female interaction effects. Such effects were documented in Clark *et al.* (1999). They showed that the outcome of sperm competition can depend on the particular

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combinations of male and female genotypes used in the experiment. In such a situation, the evolutionary dynamics could become very complicated (Clark, 2002). However complex the theoretical issues become, a more grounded empirical view of female-expressed variation affecting post-copulatory traits is necessary to stimulate progress in our understanding of male–female interactions. The recently reported results of Snook & Hosken (2004) suggest that the simple act of copulation can induce females to dump stored sperm, further highlighting the importance of female biology in *Drosophila* inter-sexual interactions.

We are also interested in natural variation affecting refractoriness to re-mating because such variation could have fitness consequences for both sexes. Most wild-caught *D. melanogaster* females have sperm from two or more males in their sperm storage organs (Imhof *et al*., 1998). Given that strong second male sperm precedence is typically observed in this species, male genotypes able to prevent or delay female re-mating could have increased fitness. From the female perspective, the optimum re-mating rate probably reflects a balance between the conflicting demands of avoiding sperm limitation and avoiding potential negative fitness effects of re-mating, which may include decreased lifespan (Chapman *et al*., 1995). Thus, some fraction of sexually antagonistic genetic variation could manifest as variation affecting refractoriness in natural *Drosophila* populations.

Here, we report the results of a quantitative trait locus (QTL) experiment on the female component of second male sperm precedence, refractoriness to re-mating and early-life fertility, using a set of 125 recombinant inbred lines (RILs) that have been scored for *roo* element markers. The measurement comprising our estimate of the female component of sperm competition is the average P2, which is the proportion of offspring fathered by the second male, among doubly mated females for each RIL. We use the percentage of females per RIL that do not re-mate as our estimate for refractoriness to re-mating. Finally, our proxy for early-life fertility (hereafter, fertility) is the average total number of offspring eclosed per female over a 10 day egg-laying period. The markers are densely distributed with one present every 2 cM on average (S. Nuzhdin). We use single-marker analysis and composite interval mapping to locate regions of the genome harbouring variation that contributes to these phenotypes.

2. Materials and methods

(i) Drosophila *stocks*

The RILs used in this study were generated from the offspring of two wild-caught fertilized females (Winters, CA). One virgin daughter of one wild-caught female was crossed with one son of the other wild-caught female (Kopp *et al*., 2003). Recombinant F2 genomes were then isogenized by 25 generations of full-sib mating. At each locus, up to four alleles segregated among RILs because the parents were not isogenic or inbred, thus, these lines capture more natural variation than standard RILs constructed from two inbred strains. Females from each RIL were mated to bw^D males and wild-type males derived from inbred line WI-98 (S. Nuzhdin).

(ii) *Molecular markers*

Roo element positions, determined by S. Nuzhdin, were used as markers. The methods have been described previously and will be discussed here only briefly (Kopp *et al*., 2003). Five individuals were genotyped for each line and the marker was recorded as present (1) if detected in all larvae and absent (0) if detected in none. If the marker was present in some of the larvae but not all, it was recorded as segregating and considered as missing data in all analyses. One hundred and fifty-two markers and a large inversion on 3R (~89EF; 96A) segregated between parental chromosomes. The marker density is one per 2 cM, on average (S. Nuzhdin).

(iii) *Mating protocol*

Over the course of 3 days, 15 virgin females from each of 125 RILs were collected under carbon dioxide anaesthesia and housed by genotype in vials with standard medium and yeast. Two days after virgin collection was completed, females were aspirated individually into vials. When females were 4–6 days old, the first mating occurred. At dawn, two bw^D males were aspirated into each female's vial. With four people working, this process took 2 h. Within 20 min of aspiration of males into a vial, that vial was placed on its side and observed for copulating pairs. Each vial was examined approximately every 15 min and the time was recorded for each observed mating. If a mating was observed within the first 4 h of pairing, males were removed by aspiration and discarded. If a mating was not observed, males were removed within 8 h of pairing with females. Regardless of mating status, females remained in this vial (hereafter, vial 1) for 48 h from the point when males were first deposited.

At the 48th hour, two males from wild-type isogenic line WI-98 were aspirated into each vial to provide an opportunity for re-mating. Again, vials were observed for copulations and the time of mating was recorded. Each vial was examined every 15 min for a copulating pair over a 7·5 h duration. Within 1·5 h of the last observation taken, males were

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removed from vials in which copulations were observed and these females were transferred to another vial (hereafter, vial 2) using light carbon dioxide anaesthesia. All females not observed to mate during the 7·5 h observation period were left in vial 1 overnight with males to increase the number of doubly mated females. The following morning, these females were transferred to vial 2 under light carbon dioxide anaesthesia and the males were discarded. Females in both categories (observed to re-mate and not observed to re-mate) were left in vial 2 for 4 days at which point they were transferred to vial 3 and left for a final 4 days.

In order to make offspring counts from each vial at the same relative time-point in the experiment, vials were turned upside down and put at −80 °C after all offspring had emerged. We had previously confirmed that bw^D and wild-type progeny are easily distinguished when frozen. Offspring counts from vial 1 were used to confirm a mating with a bw^D male and to contribute to estimates of fertility. Offspring counts from vials 2 and 3 were used to estimate P2 and fertility. During aspiration and removal of males and during the three vial transfers, dead females were recorded. Previous experiments using bw^D and wild-type stocks revealed only minor effects of viability variation on estimates of sperm use (Clark & Begun, 1998; Clark *et al*., 1999). Therefore, viability variation was not estimated.

(iv) *Estimation of P2, refractoriness and fertility*

Observed mating times and numbers of bw^D and wild-type offspring in vials 1, 2 and 3 were recorded for 1860 females (127 179 offspring scored). However, some data were excluded to provide more reliable estimates of line means for measures of fertility, refractoriness and P2. Females with fewer than 10 offspring in vials 2 and 3 were excluded (330 females, nearly half of which had died before the transfer to vial 2). We also excluded females that produced no bw^D offspring in vials 1+2+3, even if these females were observed to mate with bw^D males (194 females). Finally, all lines represented by fewer than 5 females were also removed, resulting in a total of 118 RILs examined, 1255 females assayed and 105 953 offspring scored. These data were used for fertility estimates, and then further modified for estimates of P2 and refractoriness as described below.

Fertility: Line means for the total number of offspring eclosed across vials 1, 2 and 3 per female were calculated. We consider this trait a surrogate for fertility although it is a composite trait comprised of at least egg-laying rate and egg-to-adult viability. Each line was represented by an average of 10·6 females (range 6–15).

P2: To obtain an accurate estimate of P2, we took additional conservative measures to remove females that may not have mated twice. Females that did not mate to bw^D males were removed as described above. Females that never produced any wild-type offspring were also removed (343 females). Again, some lines were represented by too few females to provide a reliable estimate of P2, so we excluded lines represented by fewer than four females (12 lines removed, resulting in 106 RILs examined, 892 females assayed and 77 696 offspring scored).

Refractoriness: We calculated the percentage of females per genotype that mated with bw^D males but did not re-mate with wild-type males as a proxy for refractoriness, similar to Clark *et al*. (1995). Thus, refractoriness is a property of a group of females (in our case, a group of replicated genotypes).

(v) *Single-marker analysis*

F statistics for the effects of single-markers and the segregating inversion on each trait were computed using the GLM procedure in SAS (release 8.02, SAS Institute). A SAS macro written by S. Nuzhdin was used to permute the trait data respective to the marker data of each RIL. After each permutation, an *F* test was completed for every marker and the *P* value of the most significant marker was retained. Following 1000 permutations, the 10th, 50th and 100th most significant *P* values were retained representing the $\alpha = 0{\cdot}01$, 0·05 and 0·1 experimentwise levels respectively (Churchill & Doerge, 1994).

(vi) *Composite interval mapping*

The original parental lines used to create the RILs examined here were not genotyped. Thus, the original (ancestral) linkage groups were estimated by inference from residual 'linkage disequilibrium' between markers (Kopp *et al*., 2003; Mezey *et al*., 2005). Mezey *et al*. (2005) estimated recombination distances between markers taking into account the original linkage groups, thereby modifying the RILs to conform to the Ri2 design in QTL Cartographer (Wang *et al*., 2003). The hypothesis tested in this design using these lines is thus whether an allele in the linkage group being tested affects the phenotype differently from the weighted sum of the other linkage groups (up to three different alleles) (for details see Mezey *et al*., 2005). If a particular marker appeared to be present in more than one parental linkage group, it was excluded from the analysis (35/152 markers excluded). Two of the re-created parental linkage groups for the third chromosome were nearly identical and were thus combined; one of the X chromosome linkage groups only contained three markers and was excluded. This results in two linkage groups

Table 1. *Summary statistics of the trait data*

Trait	No. of lines	No. of individuals	Av. no. of individuals per line	Mean	Median
P2	106	892	8·41	0·803	0·943
Refractoriness	118	1255	10·63	27%	24%
Fertility	118	1255	10·63	83·2	81·8

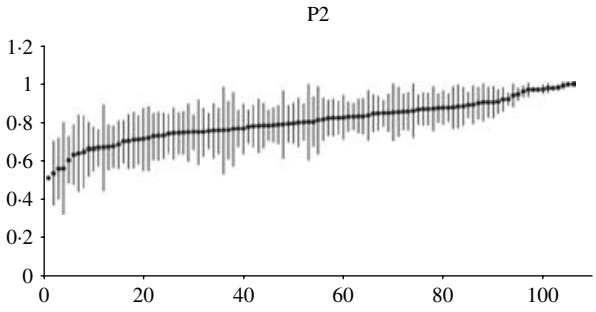

Fig. 1. P2 means and standard errors for 106 lines.

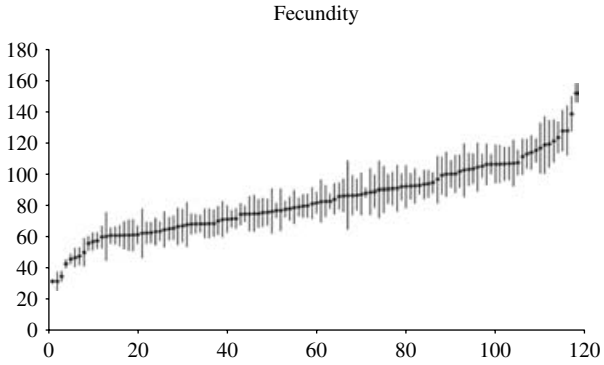

Fig. 2. Mean fertility and standard errors for 118 lines.

for the X, four for the second and three for the third chromosome. Each unique parental linkage group was compared with the others to test whether the focal allele differed in effect from the sum of the effects of the other linkage groups. This results in nine likelihood profiles for each trait.

Traits were analysed using Composite Interval Mapping (CIM) model 6 in QTL Cartographer version 2.0 (Wang *et al*., 2003). One thousand random permutations were run for each phenotype on each linkage group to determine the likelihood ratio significance levels. Various combinations of window sizes (5, 10, 20, 30) and control marker numbers (2, 5), including forward–backward regression estimation, were explored and the results were largely robust to changes.

3. Results

(i) *Mating protocol*

Re-matings were observed for 25% of females; 4% of re-mating females were actually observed to re-mate twice (1% of all females). One line (RIL 33) represented 33% of all doubly re-mating females.

(ii) *Overview of phenotypic variation*

Summary statistics were estimated using JMP (version 5.0, SAS Institute) and are presented in Table 1.

P2: Average P2 across the lines was 0·80, with line means ranging from 0·51 to 1. The residuals of P2 were not normally distributed and so were unsuitable for ANOVA ($P<0{\cdot}0000$, Shapiro–Wilk W-test). Log and angular transformations did not resolve the issue ($P<0{\cdot}0000$, Shapiro–Wilk W-test). A non-parametric Kruskal–Wallis test was not significant (df 105, $\chi^2=124$, $P=0{\cdot}098$). Line means and standard errors for P2 are presented in Fig. 1.

Refractoriness: On average, 27% of females per line were refractory (i.e. did not re-mate), with line means ranging from 0 to 90%. Some females scored as refractory may have re-mated with wild-type males but not used wild-type male sperm. In fact, 21 females were observed to re-mate but did not produce any wild-type offspring, suggesting that some females may not have stored or used any second male sperm, although sterile males or unsuccessful sperm transfer are equally plausible explanations. Therefore, our refractory phenotype may be a mixture of refractoriness to re-mating and other factors affecting post-copulatory processes (e.g. lack of sperm transfer). Non-normally distributed residuals motivated a Kruskal–Wallis test in place of a parametric ANOVA. This test revealed highly significant line effects (df=117, $\chi^2=284$, $P<0{\cdot}0001$).

Fertility: The average 10 day (vials 1, 2 and 3) fertility across the lines was 83 offspring. A highly significant line effect was also detected for fertility by ANOVA (df=117, $R^2=0{\cdot}33$, $P<0{\cdot}0001$). Line means and standard errors for fertility are presented in Fig. 2.

No significant correlations were found between line mean estimates of P2, fertility and refractoriness (data not presented).

(iii) *Single-marker analysis*

No effect of the third chromosome inversion was detected on any trait (data not presented).

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Single-marker analysis indicated one marginally significant ($P<0{\cdot}1$, experiment-wise) marker-trait association for the trait P2. Lines with a *roo* element at this position ($n=24$) have an average P2 of 0·73, while lines without the marker ($n=79$) have a mean P2 of 0·82. Variation at this marker accounts for 12% of the phenotypic variance; however, it is important to note that this estimate may only hold for the particular lines examined here. It is possible that in a different set of RILs or in a natural population, this QTL may not exist or, if it did, it may explain a very different proportion of the variance observed in the trait.

Refractoriness is influenced by variation on the second chromosome spanning *51A–57B*. The four significant (or marginally significant) markers in this region (*51A*, $P<0{\cdot}05$; *54A*, $P<0{\cdot}1$; *54C*, $P<0{\cdot}05$; and *57B*, $P<0{\cdot}01$) are all from the same linkage group (2·2). However, another marker in this linkage group (*55C*) is not significant, perhaps indicating the presence of two QTLs in this region, one to the right of *55C* and one to the left of *55C*. *57B*, the most significant single marker for refractoriness, accounted for 14% of the variance observed in these lines. Lines with a *roo* element at this position ($n=40$) show an average refractoriness of 38%, while the lines without the marker ($n=76$) show an average refractoriness of 22%. Variation at marker positions *51A* and *54C* each accounted for about 11% of the phenotypic variance observed in these lines. Two marker locations on the X chromosome, *8C* and *9A*, were marginally significantly ($P<0{\cdot}1$) associated with refractoriness.

We detected significant associations of fertility variation with markers at *23F/24A* and *56E* ($P<0{\cdot}05$ for both). The presence of a marker at *23F/24A* ($n=26$) is associated with an average fertility of 97·9 offspring compared with an average of 79·5 offspring in lines without the marker ($n=92$). The presence of a marker at *56E* ($n=73$) is associated with an average fertility of 88·7 offspring compared with an average of 73·6 offspring in lines ($n=42$) without the marker. Variation at *23F/24A* and *56E* accounts for 12% and 11% of the variance observed in these lines, respectively.

(iv) *CIM analyses*

For CIM analyses, each trait has a different estimated threshold above which markers are significant. Additionally, each linkage group has a different number of markers. The traits that had significant results using a window size of 30, two control markers, the Kosambi map function and an experiment-wise significance level of $P<0{\cdot}01$ are presented separately for each linkage group in Figs. 3–5. These parameter values were chosen to be consistent with other work on these lines (e.g. Mezey *et al.*, 2005). The likelihood ratio scores and permutation-based threshold scores for significant markers are available upon request. The results of this analysis largely overlap with the single-marker analysis; however, CIM detects several additional QTLs.

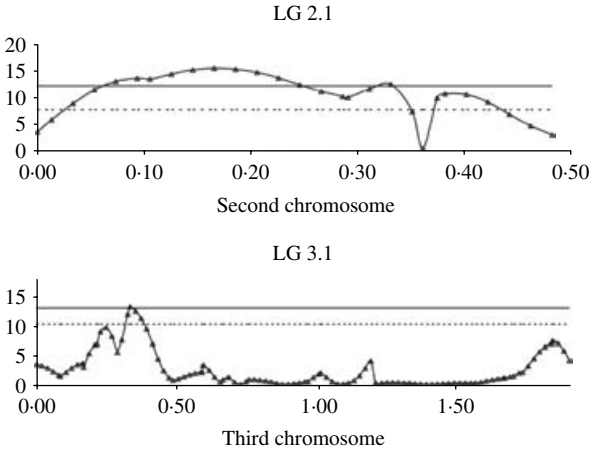

Fig. 3. CIM linkage groups that contain significant interval associations with P2 ($P<0{\cdot}01$). LOD scores are represented on the *y*-axis. The *x*-axis represents the chromosome labelled (in morgans). The upper unbroken line represents the 0·01 threshold and the lower dotted line represents the 0·05 threshold as determined by 1000 permutations.

Intervals in two linkage groups are associated with variation for P2 (Fig. 3). The significant interval in LG 2.1 actually spans *22C–48D*; however, marker *33F/34A* is the location of greatest significance, in accordance with the single-marker analysis. CIM also detects an association of variation in LG 3.1 at interval *67D–70C* with P2.

Refractoriness shows significant associations with intervals on all the chromosomes (Fig. 4). QTLs in the interval *51A–57B* (LG 2.2) are consistent with the results from the single-marker analysis. The X chromosome region, *8D–9A* (LG X.2), was marginally significant in the single-marker analysis. CIM results indicate a contribution of variation at *34EF–44C* (LG 2.4), *87B–E* (LG 3.1) and *77B–77D* (LG 3.23). These three regions were not significant in the single-marker analysis.

Fertility QTLs were detected on chromosome 2 in three of the four linkage groups (Fig. 5). The interval spanning *22A–24A*, including the significant single marker *23F/24A*, is significant in linkage group 2.2. Additional significant intervals include *28C–42B* (LG 2.3) and *30B–34EF* (LG 2.4). Composite interval mapping using published recombination distances instead of inferred ancestral structure of the RILs provides qualitatively similar results to the single-marker analysis (results not presented).

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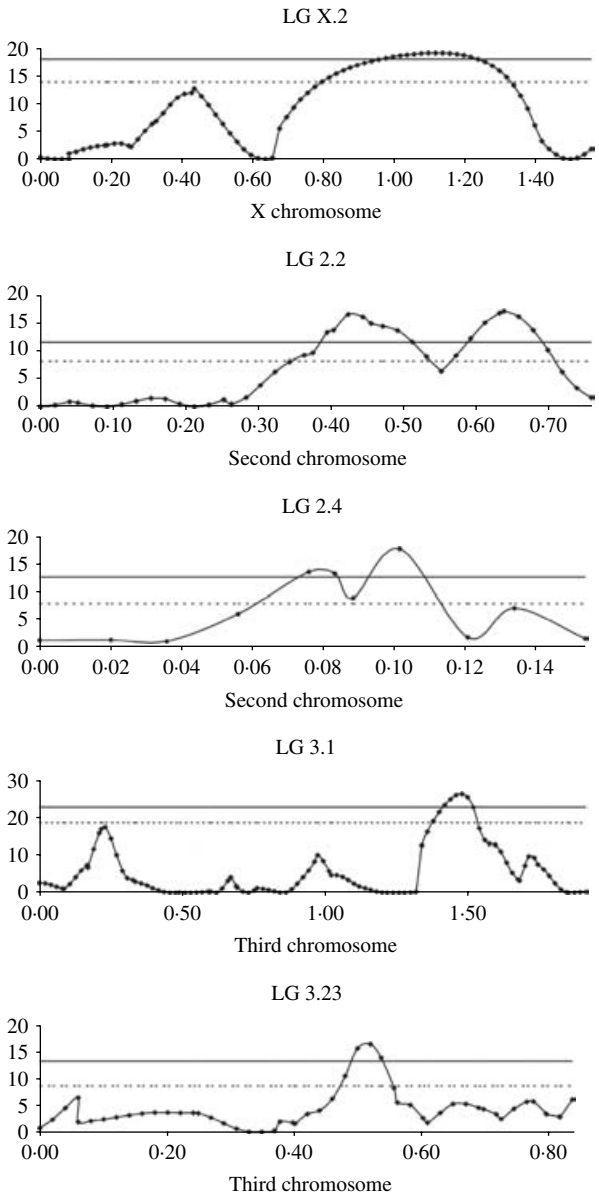

Fig. 4. CIM linkage groups containing significant associations with refractoriness ($P<0{\cdot}01$).

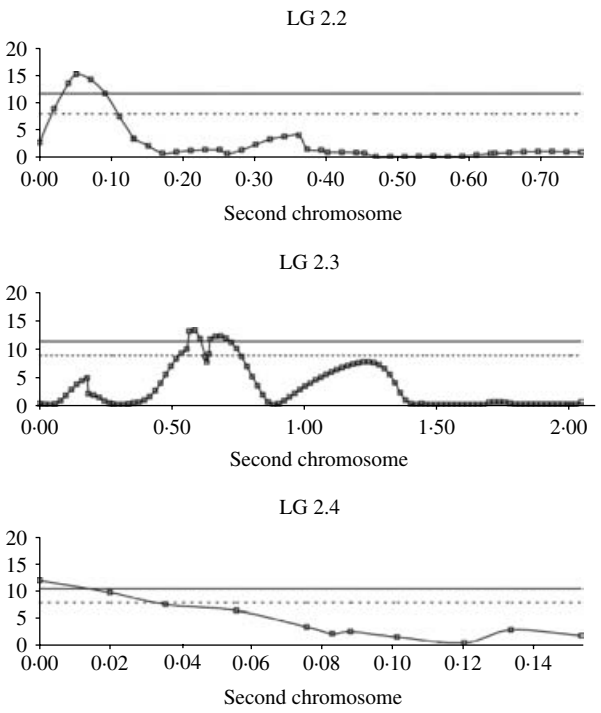

Fig. 5. CIM linkage groups that contain significant interval associations with fertility ($P<0{\cdot}01$).

4. Discussion

Despite its potential importance in understanding the population genetics of male–female interactions in *Drosophila*, genetic analysis of female-expressed post-copulatory variation has been limited. We used densely marked recombinant inbred lines to detect chromosomal regions associated with genetic variation contributing to female refractoriness to re-mating, female components of sperm competition and fertility in *D. melanogaster*. Given the inherent difficulty of measuring these phenotypes, two of which reflect complicated interactions between individuals, it seems likely that the QTLs identified here are associated with mutations of fairly large effect.

Considering the single-marker and CIM analyses in combination, our results indicate that P2 is probably influenced by variation near *33F/34A*, a region containing 78 genes, and *67F–69A*, a region containing over 200 genes. A previous analysis of genetic variation for female components of sperm competition localized effects to whole chromosomes and detected a marginally significant effect of chromosome 2 on P2, concordant with our mapping results (Civetta & Clark, 2000). While P2 is relatively convenient to measure, it probably reflects a complicated and heterogeneous set of biological processes. For example, artificial selection for increased and decreased seminal receptacle and sperm length in *D. melanogaster* also affected the outcome of sperm competition (Miller & Pitnick, 2002). P2 was strongly influenced by an interaction between sperm length and seminal receptacle length yet no consistent seminal receptacle length effect alone was found (Miller & Pitnick, 2002).

Though P2 estimates the proportion of progeny sired by the second male, the nature of male–female interactions means that female interactions with first males may have major effects on P2. For example, *Acp36DE* is important for proper sperm storage (Bertram *et al*., 1996; Bloch Qazi & Wolfner, 2003). Males homozygous for a null allele showed dramatic declines in fertility and sperm competitive ability compared with wild-type males (Bertram *et al*., 1996; Chapman *et al*., 2000). In principle, female-expressed genetic variation interacting with *Acp36DE* variation could affect the amount or efficiency of first-male sperm storage, thereby affecting P2. Detailed investigation of female phenotypes, especially those

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associated with the more extreme RILs identified in our experiment, may provide insight into the aspects of female biology affecting sperm use in doubly mated females. An important caveat to the results presented here is that while we have attempted to emulate the standard sperm competition design used in most published studies, the design is artificial. Therefore, the results could change dramatically with, for example, a different time period between matings or a different length of exposure to males.

We detected regions on each of the major chromosomes affecting refractoriness. Fukui & Gromko (1991a) carried out an artificial selection experiment on two different base populations for slow and fast re-mating rates. They found a large effect of the second chromosome on re-mating speeds in one population, and an effect of all three chromosomes in the other population (Fukui & Gromko, 1991a). A genetic mapping analysis located the second chromosome region to the right of *welt* (*55B*) (Fukui & Gromko, 1991b). In our experiment the region near *57B* is the most significant region harbouring variation contributing to refractoriness, perhaps representing variation at the same locus artificially selected in Fukui & Gromko's experiment. There are 500 genes in the *55–57* region including 11 receptors of various sorts. Female-expressed receptors in this interval are plausible candidate genes for the refractoriness QTL revealed in our experiment because Sex Peptide (SP) is thought to bind to specific membrane-bound receptors in the reproductive tract and in the nervous system of females (Ottiger *et al*., 2000; Ding *et al*., 2003). SP, an Acp transferred to females during mating, represses female receptivity to re-mating and stimulates oogenesis (Chen *et al*., 1988; Aigaki *et al*., 1991). In two recent studies using targeted knockout and RNA*i*, SP was shown to be the major protein responsible for both the short- and long-term maintenance of these phenotypes in females (Liu & Kubli, 2003; Chapman *et al*., 2003). SP is transferred to females both as free protein in the seminal fluid and bound to sperm tails (Liu & Kubli, 2003). The maintenance of increased oogenesis and refractoriness is probably due to the release of sperm-bound SP when a sperm fertilizes an egg (Liu & Kubli, 2003).

Refractoriness to re-mating appears tightly coupled with ovulation in many studies. In a screen of *P*-element lines, Ejima *et al*. (2001) found several mutants showing spontaneous virgin ovulation and increased virgin rejection behaviour. A deficiency over the region *87C1–3* failed to complement this mutant. In our study, another region associated with refractoriness is *87B–E*, again perhaps indicating that variation at the same locus detected by Ejima *et al*., (2001) contributes to refractoriness. Fuyama (1995) examined females homozygous for a *lozenge* mutation that causes virgins to ovulate at abnormally high levels. He also found these females to show reduced receptivity to mating and proposed that SP induces ovulation and that the induction of ovulation decreases receptivity. Lozenge is located at *8D*, near one of our marginally significant intervals for refractoriness (*8D–9A*), perhaps indicating that variation at this locus contributes to refractoriness to re-mating or ovulation rates.

The most significant region associated with variation for fertility (*23F/24A*) contains only 38 genes. Recently, non-circadian expression of the clock gene *timeless* in the ovaries of female flies has been determined to be important for fertility (Beaver *et al*., 2003). *Timeless* therefore stands out as a gene in this region that may harbour genetic variation contributing to fertility.

QTL studies often speak only indirectly to the forces maintaining genetic variation in populations. This is a result of potential biases associated with creation of mapping resources (e.g. fixing mutants of large effect in selection lines), biases associated with analyses (e.g. detecting alleles of large effect), or small samples of genetic variation ($n=4$ genomes maximally here) that are problematic for reliable estimation of population parameters. While we do not yet have a good sense of what forces are most important in maintaining genetic variation seen here and in previous work, our analyses reveal several chromosomal regions containing variation that affects post-mating phenotypes in this population of flies. It is possible that different QTLs would be discovered in flies from geographically distant populations or by examining RILs created from parents that had been artificially selected in divergent directions for the phenotypes of interest. However, the chromosomal regions determined to harbour variation affecting refractoriness to re-mating and the outcome of sperm competition in this study are progress towards identifying important genes. A better understanding of the genetics and biology of female components of post-mating interactions is a fundamental step towards revealing the co-evolutionary dynamics of the sexes (Civetta, 2003). More detailed investigation of the underlying phenotypes associated with the extreme RIL genotypes measured here, as well as potential identification of the genes responsible for the QTL detected in this experiment, should deepen our understanding of male–female interactions in *Drosophila*.

We thank Doug Grubb, Corbin Jones, Heather Lindfors and Laura Rodriguez for help with pairing and observing flies. We also thank Jason Mezey, Sergey Nuzhdin, David Pot and Todd Schlenke for valuable discussions and H. Allen Orr and two anonymous reviewers for useful comments on the manuscript. Funding was provided by National Institutes of Health and National Science Foundation grants to D.J.B. and an NSF Dissertation

Improvement Grant and a Center for Population Biology Research Award to M.K.N.L.

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