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# ISOLATION OF LARVAL BEHAVIORAL MUTANTS IN *DROSOPHILA MELANOGASTER*

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Genetic loci that influence behavior are often difficult to identify and localize in part due to the quantitative nature of behavioral phenotypes. Previous studies had found an association between pupal lethality and altered larval behavior for mutants of several genes. To facilitate the identification and localization of new mutants that influence larval foraging (movement in the presence of food) and general locomotion (movement in the absence of food) behaviors we identified and then screened a collection of strains carrying pupal-lethal mutations for alterations in these larval behaviors. When the lethal mutation segregated with the behavioral alteration this permitted the mapping of the behavioral locus. Nine new loci on the second chromosome were found to affect larval behavior. Of these, seven loci affected foraging and two affected locomotion. Analyses of these new loci will lead to further understanding of the mechanistic bases of larval behavior.

*Keywords:* locomotion; foraging behavior; genetic screen; chemical mutagenesis; P-element mutants; pupal-lethal

## INTRODUCTION

The induction, localization and characterization of mutations which modify the behavior of *D. melanogaster* (Benzer 1973; Hall 1985; Dubnau and Tully 1998) has provided insight into the biochemical basis for several

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behaviors of the adult fly. Notable examples include the discovery that a cAMP messenger system plays a major role in associative learning mechanisms (Davis 1996; Carew 1996; Dubnau and Tully 1998), the elucidation of the role of G-protein mediated signal transduction cascades in adult photo transduction (Zucker 1992, 1996) and the importance of the Per protein in the maintenance of circadian rhythms (Hall 1998).

Relative to *D. melanogaster* adult behavior, the genetic loci and biochemical pathways involved in larval behaviors have received little attention (Ball *et al.*, 1985; Kernan *et al.*, 1994; Heiman *et al.*, 1996; Inestrosa *et al.*, 1996; Osborne *et al.*, 1997). Larval locomotion is accomplished by posterior muscular contractions and anterior extensions of the body and head resulting in telescoping peristaltic movements (Green *et al.*, 1983; Berrigan and Pepin 1995). On a nutritive substrate larvae combine such locomotion with the shoveling of food by successive extensions and retractions of their mouth hooks. Locomotion in conjunction with feeding has been defined as foraging behavior (Sokolowski, 1980; Green *et al.*, 1983).

Since an underlying theme of the larval behavioral repertoire is locomotion in the search for food (Parsons 1980; Green *et al.*, 1983), we are interested in identifying genes which influence larval locomotion while feeding (foraging) as well as genes that influence larval locomotion in a non-nutritive environment (locomotion). The first genetic variants of larval foraging behavior were isolated from studies of natural populations (Sokolowski 1980). The *foraging (for)* gene affects the locomotory component of larval foraging behavior. Larval foraging behavior is assayed by measuring the distance (path length) a larva travels while foraging on a yeast and water paste during a five minute test interval (Sokolowski 1980). *for* was identified as a major gene responsible for the naturally occurring rover/sitter *D. melanogaster* foraging polymorphism (de Belle *et al.*, 1989). Allelic variation at *for* results in two foraging phenotypes in both natural and laboratory populations (de Belle and Sokolowski 1987; de Belle *et al.*, 1989). Larvae with a dominant rover allele ($for^R$) exhibit long foraging path lengths while larvae homozygous for the recessive sitter allele ($for^s$) exhibit significantly shorter paths (Sokolowski 1980; de Belle *et al.*, 1989). Expression of this polymorphism is conditional on the presence of a nutritive substrate. Rover and sitter locomotion do not differ on non-nutritive substrates, nor do the variants differ in developmental time, feeding rate or body size when food is not limited (Sokolowski *et al.*, 1984; Graf

and Sokolowski 1989; Sokolowski and Hansell 1992). Osborne *et al.*, (1997) demonstrated that *for* corresponds to the gene *dg2* (Kalderon and Rubin 1989). This gene encodes a cGMP-dependent protein kinase (PKG) thereby implicating PKG activity and the cGMP second messenger system in the regulation of food search behavior in *D. melanogaster* (Sokolowski 1998; Sokolowski 1999; Sokolowski and Riedl 1999). Other genes known to influence foraging behavior are *Chaser* (*Csr*), a dominant suppressor of $for^{s}$ (Pereira *et al.*, 1995) and *no-bridge* (*nob*) and *ellipsoid body* (*ebo*) both suppressors of $for^{R}$ (Varnam *et al.*, 1996); their gene products have not yet been determined. Further genetic dissection of this phenotype will aid in uncovering other components of this PKG signaling pathway as well as any additional pathways involved in food search behavior.

Conventional recombination mapping techniques are difficult to apply to genes which have quantitative behavioral phenotypes (de Belle *et al.*, 1989). These phenotypes are often modified by the pleiotropic effects of genetic markers, differing genetic backgrounds between strains, and environmental variation (de Belle and Sokolowski 1989). Thus, even in the instances where quantitative larval behavioral phenotypes show single gene inheritance patterns (for example *for*), it has been necessary to tag/mark the phenotype with a qualitative trait in order to map its position on a chromosome as required for further genetic and molecular analysis.

In order to localize *for*, de Belle *et al.*, (1989) developed an approach called "lethal tagging". Recessive lethal mutations were generated which caused a change in behavior from rover to sitter and coincident pupal lethality. These lethals appear to be allelic to *for* and were used with deletion mapping to localize *for* to the chromosomal region 24A3–5. Further, in addition to their effects on behavior, several mutations which influence larval mechanoreception (Kernan *et al.*, 1994) and an allele of the *lethal(2)thin* gene which influences larval locomotion (Ball *et al.*, 1985), also cause death during pupal development. The pupal lethal phase of these mutations/tags is an important feature as, unlike embryonic or larval lethal mutations, they allow for the investigation of the effects of mutations upon the behavior of the larvae (i.e. homozygotes survive through all larval instars).

We chose to screen a collection of lethal mutations on the second chromosome of *D. melanogaster* for those which conferred death during pupal development and concomitantly affected larval foraging or locomotor behavior. By first selecting for pupal-lethal mutations, once a behavioral variant was identified, we ensured that the behavioral alteration also car-

ried a discrete genetic "tag" (lethality) linked to any observed quantitative behavioral phenotype thereby facilitating the mapping of the locus.

## MATERIALS AND METHODS

### Strains and Chromosomes

Eight hundred and ninety-six *D. melanogaster* lines with recessive lethal mutations on the second chromosome were obtained from an ethylmethane sulfonate (EMS) mutagenesis of $ry^{+5}$ flies performed by Humphreys *et al.* (1996) and one hundred seventy-six lethals generated by P-element insertional mutagenesis were obtained from the Karpen and Spradling (1992) collection. The $ry^{+5}$ strain was homozygous for the $for^{R}$ rover allele. A low dose (12 mM) of EMS was used to obtain lines carrying an average of one lethal mutation per chromosome (Humphreys *et al.*, 1996). These recessive lethal chromosomes are maintained as balanced heterozygotes with *In(2LR)SM1*, $al^2$ *Cy* $cn^2$ $sp^2$, hereafter referred to as *SM1*. All chromosome-2 deficiencies were obtained from the Bloomington Stock Center. All mutations are described in Lindsley and Zimm (1992) unless otherwise referenced here. Flies were housed in plastic bottles containing 45 ml of standard fly medium and were kept at 25±1°C on a 12L:12D light cycle with lights on at 0800 hours (standard conditions).

### Identification of Pupal Lethal Lines

All of the balanced lethal lines were screened for those in which homozygosity for the lethal bearing chromosome conferred lethality during pupal development. To accomplish this 25, 5–7 day old flies were placed in vials containing 10 ml of standard fly medium and kept at 25°C (for 24 hrs) after which the adult flies were removed. After seven days the earliest and latest stages of pupal development were recorded for each vial using a WILD Herrbrugg light microscope. The pupae that had not eclosed after 14 days were marked on the vial and these pupae were observed once daily for five days. Stage of lethality (as described in Bainbridge and Bownes 1981) was recorded as the latest recognizable developmental stage reached by more than 50% of previously marked pupae before their death was evident (ie. histolysis, dehydration, darkening of tissues). A minimum of 20%

of the pupating progeny failed to eclose from all balanced lethal lines identified as carrying a pupal lethal mutation.

### Assays of Larval Foraging Behavior

The larval foraging behavior of our pupal lethal mutants was quantified using the foraging assay described in Pereira *et al.*, (1995) and briefly outlined here. The locomotory component of larval foraging behavior was measured by placing individual third instar larvae (96 ± 2 hours in age) into the center of a circular well (8.5 cm in diameter) coated with a thin homogeneous layer of yeast paste (distilled water and Fleischmann's bakers' yeast in a 2:1 ratio by weight). Each well was then covered with a Petri-dish lid to reduce evaporation. The larvae were then allowed to forage for 5 minutes, after which time the paths traveled were traced onto the Petri lids, the lengths of these paths were termed the "foraging path-lengths". After testing, the paths were numbered and the larvae were placed individually in correspondingly numbered 3 ml glass vials containing 1 ml of standard fly medium and kept for 7 days under standard conditions. The path lengths of only those larvae which exhibited death during pupal development were used to calculate the mean path length for the homozygous mutant larvae. These path lengths were compared statistically to those of the concurrently tested control $ry^{+5}$ strain.

Homozygous mutant larvae whose path lengths differed significantly from those of the control were classified as putative foraging mutants and were retested. As most behavioral traits are strongly influenced by environmental conditions it is common for the absolute differences between mean path lengths to vary across different test days (Graf and Sokolowski 1989; Sokolowski 1992). Hence, only path lengths of larvae tested on the same day were statistically compared.

### Tests of General Larval Locomotion

The foraging phenotype is dependent upon the presence of a nutritive substrate. That is, the mean path lengths of foraging mutants and wildtype larvae do not differ on a non-nutritive substrate (Sokolowski and Hansell 1992). To separate foraging mutants from general locomotory mutants, the path lengths of the putative foraging mutants were measured on a

non-nutritive agar substrate. Individual third instar larvae were placed in the center of Petri-dishes (8.5 cm diameter, 1.4 cm height) coated with 10 mL of hardened 3.2% agar (Sokolowski and Hansell 1992; Pereira *et al.*, 1995). The dishes were covered with Petri-dish lids and the distance traveled by each larva in a 5 min test interval was measured. Each larva was reared after testing as described above. The path lengths of only those larvae which did not survive pupation were used to calculate the mean path length for the homozygous mutant larvae of each putative foraging mutant. The path lengths were statistically analyzed in comparison with the path lengths of the concurrently tested control strains as described above.

### Statistics

Path lengths were analyzed using one-way analyses of variance (one-way ANOVA). To determine which strains exhibited significantly different path lengths, the Student-Newman-Keuls (SNK) test was performed as an *a posteriori* test (Zar 1984).

### Genetic Mapping

The EMS- and P-element-induced pupal-lethal mutations were localized by deficiency mapping using 39 second chromosome deficiencies which span approximately 65% of the second chromosome euchromatin. Pair-wise tests of complementation were also done between mutants that localized within a given deficiency. A minimum of 50 adult progeny were scored for each complementation cross.

## RESULTS AND DISCUSSION

Of the 896 lethal lines generated by EMS mutagenesis (Humphreys *et al.*, 1996), 88 were classified as pupal-lethal of these the homozygous mutant larvae of 18 exhibited mean foraging path lengths which were significantly shorter than the $ry^{+5}$ control strain. Of the 176 lethal lines generated by P-element insertional mutagenesis, 25 were classified as

pupal-lethal. Of these, the homozygous mutant larvae of one exhibited mean foraging path lengths significantly shorter than the $for^R$ control strain.

The foraging phenotype is dependent upon the presence of a nutritive substrate. That is, the mean path lengths of mutants in foraging behavior will only differ significantly from wild type larvae in the presence of food. To distinguish true foraging behavior mutants from general locomotory mutants, we assessed the locomotion of these 19 putative foraging mutants in the absence of food. This identified 7 mutants whose mean path lengths on a non-nutritive agar substrate were significantly shorter than those of the corresponding control strain. These mutants were classified as general locomotory mutants. The distance traveled on agar by the remaining 12 mutants did not differ significantly from that of the appropriate control strains. Therefore, these lines were classified as foraging mutants. The foraging path lengths of the homozygous larvae were compared to those of their heterozygous sibs (*mutation/SM5* or *CyO*) and were found to be significantly shorter; pathlengths of heterozygous larvae did not differ significantly from the wild-type $ry^{+5}$ controls, indicating that the mutant behavioral phenotypes are recessive (see below).

To confirm that the lethal mutations of the behavioral mutants were responsible for the observed behavioral phenotypes we needed to show that the behavioral phenotypes of the mutants co-localized to the region of the lethal mutations. We were able to accomplish this for 9 of the aforementioned 19 putative behavioral mutants using the deficiencies (Table I). The P-element mutant, *scribbler* (*sbb*), mapped to chromosome arm *2L* and showed altered locomotion. One of the EMS mutants, *pokey* (*pky*), showed reduction in locomotion and was mapped to chromosome arm *2R*, as were two novel foraging-specific mutations: *homebody* (*hby*) and *lackadaisical* (*lkd*). The other five novel mutants: *lingerer* (*lnr*), *lounger* (*lgr*), *loiterer* (*ltr*), *lazybones* (*lzb*), and $caboose^{fg}$ ($cbs^{fg}$), demonstrated foraging-specific defects and mapped to arm *2L*.

Table I shows the cytological locations and path length data of these 9 behavioral mutants. Since the mutants were tested in groups on different days, the absolute differences between the mean path lengths reported in Table I may vary. However, the relative differences between each mutant and its appropriate control line(s) remain consistent.

TABLE I Mean foraging and locomotor path lengths of larval behavior mutants

| *Mutant* | *Mean Foraging Path Length (cm) on yeast* | | *Mean Locomotor Path Length (cm) on agar* | | *Phenotype* | *Cytogenetic Location* |
|---|---|---|---|---|---|---|
| | *Mutant* | *Control* | *Mutant* | *Control* | | |
| *lingerer (lnr)* | 7.18 ± 0.50 (41) | 10.15 ± 0.77 (37) | 14.21 ± 0.71 (23) | 16.23 ± 1.0 (12) | fg | 37F05–38A01; 39D03-E01 |
| *lazybones (lzb)* | 5.67 ± 0.64 (20) | 9.13 ± 0.51 (46) | 15.14 ± 1.4 (11) | 20.24 ± 1.2 (13) | fg | 38A06-B01; 39D03-E01 |
| *lounger (lgr)* | 4.25 ± 0.57 (10) | 9.74 ± 0.67 (39) | 15.63 ± 1.4 (10) | 19.08 ± 1.3 (10) | fg | 27C02–09; 28B03-04 |
| *loiterer (ltr)* | 6.28 ± 1.0 (18) | 11.44 ± 0.48 (27) | 17.43 ± 0.20 (10) | 20.50 ± 0.14 (24) | fg | 36A08–09; 36E01-02 |
| *homebody (hby)* | 4.50 ± 0.42 (22) | 9.13 ± 0.51 (46) | 10.16 ± 0.66 (10) | 13.68 ± 1.0 (19) | fg | 57B04; 58B |
| *lackadaisical (lkd)* | 4.51 ± 0.48 (14) | 9.13 ± 0.51 (46) | 9.60 ± 0.90 (10) | 13.68 ± 1.0 (19) | fg | 57B04; 58B |
| *caboose$^{fg}$ (cbs$^{fg}$)* | 3.84 ± 0.57 (12) | 9.14 ± 0.57 (28) | 15.45 ± 1.0 (15) | 20.24 ± 1.2 (13) | fg | 21A01; 21B07–08 |
| *pokey (pky)* | 4.59 ± 0.47 (16) | 9.13 ± 0.51 (46) | 12.45 ± 0.86 (13) | 16.23 ± 1.0 (12) | loc | 42E-44C |
| *scribbler (sbb)* | 3.57 ± 0.40 (19) | 10.92 ± 0.53 (37) | 6.31 ± 0.70 (11) | 13.68 ± 1.0 (19) | loc | 55C01–02 |

All mean foraging path lengths of homozygous mutant larvae are significantly shorter (one- way ANOVA) than the control strain, $ry^{+5}$ ($p \leq 0.005$). The mean path lengths of *pokey* (*pky*) and *scribbler* (*sbb*) on the non-nutritive agar surface are significantly shorter (ANOVA, $P \leq 0.005$) than the control strain and, as shown in column six by the designation "loc", this mutant is classified as a general locomotion mutant. The remaining mutants (whose mean path lengths on agar do not differ significantly from those of the control strain) were classified as foraging mutants ("fg"). Path lengths are means ± standard error, with numbers of larvae in parentheses The *sbb* cytogenetic location was provided by the Berkley Drosophila Genome Project (BDGP); the BDGP ID for *sbb* is *l(2)03432*.

To verify that the lethal mutations we mapped were also responsible for the observed behavioral phenotypes we performed complementation analyses for foraging behavior with the deficiencies to which the lethal phenotypes map. Deficiency heterozygotes of the 8 cytologically localized EMS mutants were tested. Figure 1 shows that the foraging behavior phenotypes (path length) of the hemizygotes of the pupal lethals tested did not differ significantly from those of larvae homozygous for the corresponding recessive lethal but were significantly shorter than the path lengths of their heterozygous sibs (*lethal/Balancer*), which, in turn, were not significantly different from those of the wild-type ($ry^{+5}$) control. The behavior of *lgr* may present one exception where, although the hemizygotes' mean path length was shorter than the heterozygotes', they were not significantly so. No balanced heterozygotes displayed behavior differing significantly from wild-type. Therefore, the foraging behavior phenotype of the homozygous pupal lethal larvae of each line co-localized with the lethal mutation to the same chromosomal region with the possible exception of *lgr*. In *lgr*'s case the lethality was co-localized but the behavior may not have been. *lgr* may carry two distinct mutations, one affecting lethality and another, behavior.

*sbb* larvae moved during the 5 minute test time on agar but tended to remain within a small localized area of the test plate, making their paths difficult to decipher. To better quantify scribbler behavior we superimosed a 6mm grid over the locomotion trail and counted the number of squares entered by individual larvae during the behavior test on agar. Homozygous *sbb* larvae entered significantly fewer grid squares than did their heterozygous sibs (*sbb/CyO*) (Figure 2) or larvae of the wild-type control strain which did not differ from the heterozygotes (data not shown).

We found that while homozygous mutant larvae of the line $cbs^{fg}$ exhibited altered foraging behavior, the other pupal lethal allele of this locus ($cbs^{wt}$) did not influence this larval behavior. The *cbs* alleles did not complement for lethality and were uncovered by the same deletion with respect to pupal lethality. The behavioral defect associated with the $cbs^{fg}$ allele was also uncovered by the deletion.

The nine genes we identified appear to influence both larval behavior and pupal viability, suggesting each of the genes has pleiotropic effects. This pleiotropy may have resulted from, for example, the production of multiple transcripts from a single gene, a single transcript that has a dual role at different times in development or in independent cells, or from two separate genes with overlapping promoters. Once molecularly mapped, the

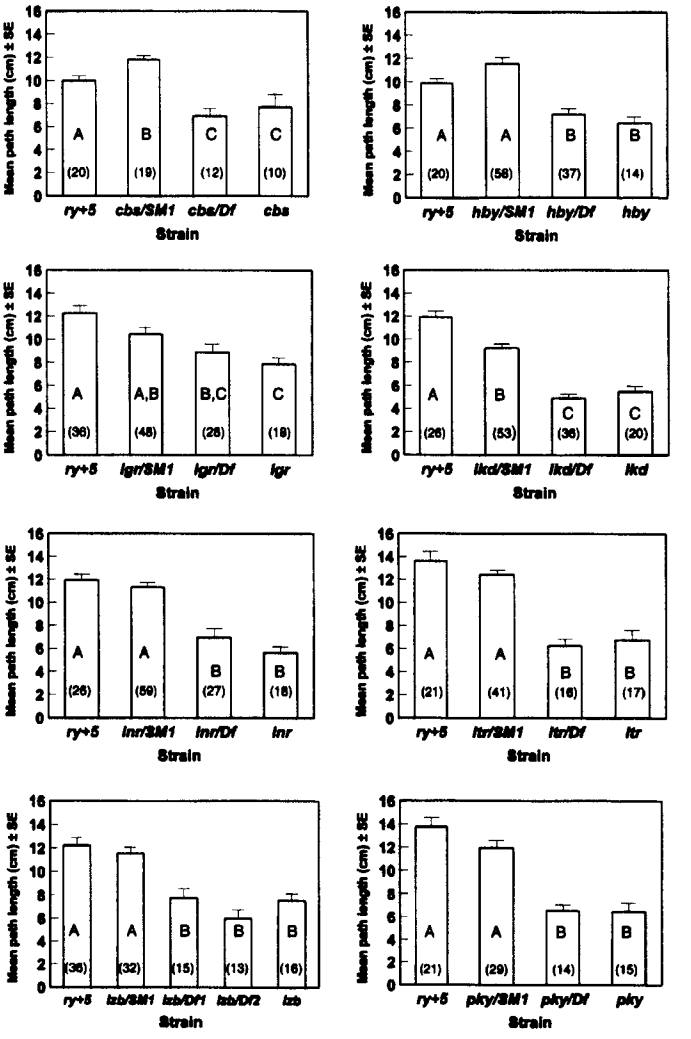


FIGURE 1 Larval path lengths of foraging and locomotory mutants on yeast. The foraging path lengths of hemizygotes of *lnr, lgr, pky, ltr, hby, lzb, lkd,* and $cbs^{fg}$, do not differ significantly from those of larvae homozygous for the corresponding recessive lethal mutation and are significantly shorter than the path lengths of their heterozygous sibs (balanced over *SM1*) (excepting *lgr*, where the difference was not significant) and the $ry^{+5}$ control strain (one-way ANOVA, P≤0.001). Means are presented ± standard error with number of larvae in parentheses. Different letters represent different Student Neuman Keuls (SNK, p=0.05) mean groupings. The breakpoints of the deficiencies used to localize each of the EMS mutants are listed in column 7 of Table I

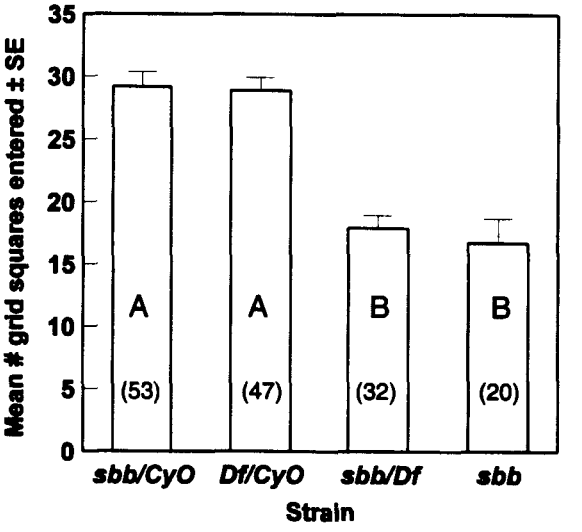


FIGURE 2 The *scribbler* (*sbb*) locomotor phenotype on agar is uncovered by the deficiency *Df(2R)Pc4* (*Df*). The number of grid squares entered by hemizygous (*sbb/Df*) larvae is significantly fewer than the number entered by *sbb/CyO* larvae but does not differ significantly from that of *sbb* homozygotes (one-way ANOVA, $P \leq 0.0001$). Means are presented ± standard errors with the number of larvae in parentheses. Different letters represent different SNK mean groupings ($p=0.05$)

lethal phenotypes of the aforementioned lesions should aid in the elucidation of the region(s) of these loci essential for viability and for wild type behavior.

This study has demonstrated that the seemingly simple foraging and locomotor behaviors of *D. melanogaster* larvae are influenced by a number of genes. We have localized and isolated mutants in 9 of these genetic loci. The selection of pupal lethal mutations affecting larval behavior reduced the difficulties of the direct selection and localization of mutations influencing such quantitative behavioral phenotypes. The successful identification of a collection of loci which influence larval foraging or locomotor behavior suggests that the characterization of the corresponding gene products will facilitate the definition of pathways which are involved in various components of larval food search behavior (ie. locomotion, searching, foraging).

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