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Research Paper

Genetic and Behavioral Analysis of Natural Variation in *Drosophila melanogaster* Pupation Position

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KEY WORDS

Drosophila, behavior, pupation, wandering, QTL, quantitative, genetics

ABBREVIATIONS

QTL	quantitative trait locus/loci
RI	recombinant inbred
LR	likelihood ratio
5-HT	serotonin

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ABSTRACT

Drosophila melanogaster pupae are exposed to many biotic and abiotic dangers while immobilized during several days of metamorphosis. As a passive defense mechanism, appropriate pupation site selection represents an important mitigation of these threats. Pupation site selection is sensitive to genetic and environmental influences, but the specific mechanisms of the behavior are largely unknown. Using a set of 76 recombinant inbred strains we identify a single quantitative trait locus, at polytene position 56A01-C11, associated with pupation site variation. We furthermore present a detailed investigation into the wandering behaviors of two strains expressing different pupation position tendencies, and identify behavioral differences. Larvae from a strain that tends to pupate relatively far from the food also tend to travel significantly farther from the media during wandering. We did not observe consistent differences in either the number or duration of wandering forays made by near or far pupating strains. The ability of larvae to integrate several internal and external environmental cues while choosing a contextually appropriate pupation site, and specifically, the variation in this ability, presents a very interesting behavioral phenotype in this highly tractable genetic model organism.

INTRODUCTION

Drosophila melanogaster larvae inhabit a challenging world. They must avoid perils such as parasites, predators, and various abiotic dangers, including excessive heat, cold, moisture, and dryness, as they forage for adequate food for the timely completion of development. Toward the end of the third larval instar, the animals cease feeding, become less negatively phototactic, and initiate wandering behaviors preceding pupation and metamorphosis into adult flies.[1,2] Wandering larvae often leave the food source to make forays into the surrounding environs. After approximately 6–8 hours (at 25°C) of wandering, the larva begins pupariation (a developmental stage immediately preceding pupation when the spiracles evert, the larva becomes immobilized, and cuticle tanning is initiated). Pupation, beginning with larval/pupal apolysis, follows 4–6 hours later.[1,3,4] During the four days of metamorphosis, the animal remains at risk from many of the same perils faced as a larva, except that it is now immobilized inside its pupal case. The location selected for pupation can critically impact survival in various environmental conditions. For example, in drier conditions animals that pupate closer to the moist food source have a higher survival rate than those who move further afield. Conversely, in damp environments animals pupating close to the food have lower survival, perhaps due to increased microbial attack, or to drowning and suffocation as other larvae churn up and liquefy the substrate.[5,6] Therefore, it is important that larvae correctly assess their environments and select contextually appropriate pupation positions in order to reduce their risk.

Natural variability in the *D. melanogaster* pupation position, usually measured relative to the food source, has been described and previous studies have observed the influences of various environmental factors such as light, humidity, temperature, and crowding.[5-14] Notably, genetic factors also strongly influence the tendency to pupate near or far from the food in a given environmental context.[10,15-19] Several studies have also reported a sex effect with males pupating on average further from the food than females.[17,20,21] The genes and cellular mechanisms that interact to influence this natural behavioral variation are not known, but previous studies have demonstrated effects linked to the three major chromosomes.[16-18] Potential cellular mechanisms could include those affecting the sensitivity, response, tolerance or resistance to environmental variables of significance to pupation site selection, such as temperature, light and humidity.

Genetic variation for pupation site selection within populations of *D. melanogaster* may have arisen in response to their habitat, which is highly variable in time and space and where, under certain conditions, ideal pupation sites may be limited. For example, the localized areas around fruit colonized by larvae can vary dramatically as parts of the fruit are consumed, rot, or dry. Harsh conditions present challenges to the wandering larva as competition for the preferred pupation sites increases. In a crowded environment, larvae that pupate near the fruit may face greater risks either from drowning, suffocation, or rot, as subsequent larvae wander and pupate on top of them. Furthermore, larvae that pupate in groups might face greater risks from pupal parasitoids. Conversely, in drier conditions, larvae pupating too far from the fruit will be more likely to desiccate. Genetic variation, however, ensures that some individuals will be able to make more effective use of a wider variety of available sites.

A correlation between pupation distance and larval developmental rate has been reported,[21] and experiments with selected lines have yielded the observation that a variety of larval behaviors, such as mobility, digging rate, and geotaxis, change as correlated responses to selection for pupation distance.[22] These observations indicate that pupation distance is not likely a simple behavior, but rather that it is a manifestation of the integration of several simpler behavioral responses. Studies of pupation distance, and the related "embedding" behavior,[23] have used a variety of assays, however, all of these methods focus on the endpoint of wandering behaviors: the final pupation position. By also studying the wandering behavior of strains predisposed to pupate near or far from the food we may gain insight into this behavioral integration. For example, do near or far pupators spend more or less time closer to or farther away from the media while wandering?

Here, we present the results of our mapping of quantitative trait loci (QTL) affecting variation in pupation position using a mapping population of 76 recombinant inbred (RI) lines. In addition, we present a detailed description of the wandering behaviors of two RI lines expressing different pupation position phenotypes. Our results and techniques may be used in future studies to initiate a more highly detailed comparison of the wandering behavior, which will provide insight into the ability of larvae to assess and balance various risks and benefits as they select their optimal pupation position.

METHODS

Fly strains used. Recombinant inbred (RI) strains. The RI strains were generated by, and are described in, Nuzhdin et al.[24] Briefly, two unrelated parental lines, Oregon R and 2b, each with multiple *roo* transposable element insertions (45 and 47 respectively) spread across the three major chromosomes, were crossed. The Oregon R-derived chromosome 4 was marked with the recessive mutation *spa*^{pol}. F_1 progeny were backcrossed to 2b and the resulting progeny were randomly intermated for 4 generations. At generation 5, random pairs were isolated and mated and the RI lines were produced by 25 generations of full-sib matings within their progeny followed by a further 10 generations of small mass matings of 20 pairs each.[24] The presence or absence of each of the 92 *roo* elements or the *spa* mutation provides information regarding the ancestral origin of the corresponding chromosomal regions in the RI lines. Thus, *spa* and the *roo* elements serve as molecular markers for QTL mapping. The mean pupation positions of 76 RI lines were assayed, of which, 4 far pupating (stains 2, 12, 16, and 43) and 2 near pupating (strains 31 and 70) lines were retested for confirmation. More detailed individual assays of wandering and pupation behaviors were carried out on larvae from RI strains 43 and 70.

Behavioral assays. Group assay. Due to the large number of fly strains assayed (76 RI strains and 2 controls), testing was spread over four consecutive days. Each strain was randomly assigned for assay on one of the four test days and roughly 25% of the strains were prepared on each day. Larvae were selected and placed in vials as described below. All test vials were randomized across strains and housed in the same set of vial racks in the same incubator. Vials containing media alone were used as place-holders for those strains not yet prepared, and were replaced by the relevant test vials, once prepared.

Pupation position was measured as described in Bauer and Sokolowski.[16] For each strain, pupation distances were tested by placing 10 first instar larvae (0–4 hours post-hatch) into glass vials containing 6mL of carefully poured (such that there were no bubbles in the media and no media on the vial walls) standard yeast-agar (1.3% agar) culture media. The media was not punctured as the larvae were placed on the surface, and each vial was plugged with a cotton ball, leaving approximately 65 mm between the bottom of the cotton plug and the food surface. Five vials were prepared per strain and, as mentioned above, the vials were randomized in racks, across strains (and therefore, days). The cotton plugs were marked with different colors indicating the preparation day, thereby facilitating identification and reducing the disturbance of younger cultures while the sexes of pupae set on earlier days were recorded. All test vials were kept in the same incubator with lights attached directly above each shelf. A row of vials containing only media was placed around the outer edge of the racks, ensuring that all test vials were surrounded by other vials containing media. This reduces the exposure of the outermost test vials to direct light, thereby reducing vial position affects due to lighting. The vials were left undisturbed in an incubator at 25°C, 12:12 hour light:dark cycle, with lights on at 08:00, until pupation. Once all the larvae had pupated, the distances between the anterior pupal spiracles and the food surface were measured, and mean distances were calculated for each strain. Strain means were then used in the QTL analysis. QTL analysis was also performed on strain means calculated from the mean vial means and this method identified peak QTL likelihood scores at the same position as that found using the global means. Pupal sexes were recorded by observing the presence or absence of sex combs on the developing pupae.

Individual assay. This more detailed study of wandering behavior was performed on two RI strains, 43 and 70. These strains exhibited robust viability in culture and test conditions as well as consistently divergent pupation position phenotypes. Though these strains are consistently divergent, they do not exhibit the most extreme pupation phenotypes (Fig. 1). Larvae from strain 43 pupate farther from the food whereas strain 70 larvae pupate more near. The *roo* element marker patterns indicate genetic variation between these strains at the relevant chromosomal region (50F–57C).

For each strain (43 and 70), fifty 0 to 4 hour old larvae were placed in a 60 mm Petri dish containing 15 mL standard yeast-agar-sucrose media and were left to develop to mid-third instar (about 96 hours, at 25°C). A fine paintbrush was used to individually transfer the mid-third instar larvae into 10 x 75 mm glass vials containing 0.8 mL of fresh, carefully poured, standard yeast-agar-sucrose medium. A large sterile dissection pin was used to pierce the media immediately before placing the larva in each vial facilitating larval entry into the food. The vials were stopped with cotton and the

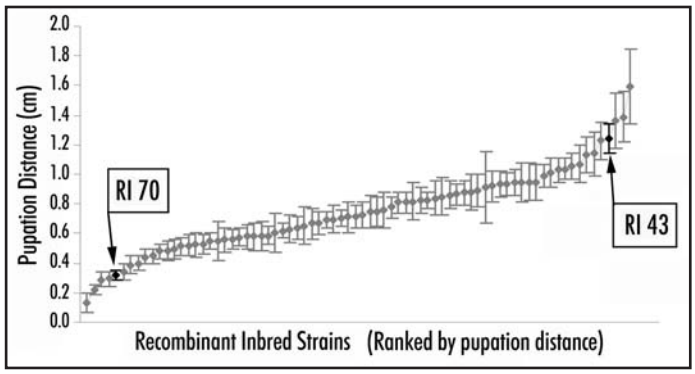

Figure 1. Variability of pupation position selection among 76 RI lines. The response distribution was normal (Shapiro-Wilk/rankit plot, p = 0.9843). Two RI strains, one near and one far pupator (strains 70 and 43, respectively, as labeled and plotted in black), were chosen for the detailed analysis of wandering behaviors.

cotton was moistened with 0.1 mL of distilled water to reduce dehydration. Alternating vials with larvae from either near or far pupating strains were placed upright in single rows and back lit with a uniform diffuse red light (photographic safelight) in a small room with a humidifier (50–65% relative humidity) and a 12:12 hour light:dark cycle. Indirect white light was provided by a single 48" fluorescent tube mounted under the testing bench. Thus, the apparatus was exposed to 12L:12D of light reflected from the white walls and ceiling of the room. This helped to reduce glare and alleviated complications with photographic exposure as the light switched on and off. A digital camera was used to record images at a rate of 10 frames per minute until all the larvae had pupated. Progress was monitored remotely by uploading 1 frame per minute to a web page, thus the animals were not disturbed during wandering. The recorded frames were transcoded into video to be played back at 30 frames per second (transcoding software from www.transcoding.org, and QuickTime software from www.apple.com/quicktime were used to make the movies). Thus, it is possible to condense 3–4 days of behavior into videos of about 15 minutes duration. The Dynamic Image Analysis System (DIAS)[25] (www.geocities.com/solltech/dias) was then used to track and analyze the movements of the wandering larvae. The horizontal (X) and vertical (Y) coordinates of the larval centers of area per video frame were recorded and used in our analysis. Several small computer programs were written for use in our analysis (filtering and assembly of dataset, calculation of number, duration, and maximum height of forays, etc.), the source code for these is available upon request.

QTL analysis. The genomic marker positions (i.e., the cytological insertion sites of the *roo* transposable elements) were mapped relative to each other using the recombination frequencies derived from the set of RI lines used in this study relative to the parental strains (Oregon R and 2b). QTL analysis was performed using QTL Cartographer (v. 1.17e) software[26,27] much as described in Viera et al.,[28] but with the single trait of pupation distance. Specifically, a Kosambi mapping function was applied to the recombination map, composite interval mapping[29] was used, and the likelihood ratio (LR) was calculated as LR = -2 ln (L_0/L_1) where L_0/L_1 is the ratio of the likelihood under the null hypothesis (no QTL in the interval) to the likelihood under the alternate hypothesis (that a QTL is present in the interval), and conditioning window of 10cM was used. The 5% threshold of significance was determined by 10,000 random permutations of the data[30,31] (for further reference see the QTL Cartographer users manual[27]).

Quantitative complementation. Quantitative complementation is a technique that facilitates the mapping of QTL to relatively small genomic regions.[32] Briefly, after QTL mapping identifies a candidate genomic region, heterozygous strains with balanced deficiencies in the area of interest are crossed to strains expressing divergent phenotypes. The phenotypes of the two progeny types (heterozygous with either the deficiency or balancer chromosome) are then assessed. A strain by chromosome (deficiency or balancer) interaction such that the phenotypic differences are greater in the progeny carrying the deficiency compared to those with the balancer chromosome, indicates that the deficiency uncovers a relevant genomic region. The use of overlapping deficiency strains permits relatively high resolution mapping of the QTL. Quantitative complementation was performed with a set of eight deficiency strains acquired from the Bloomington stock center. The names and genotypes of the deficiency strains are shown in Table 1.

Genetic crosses were performed to balance the deficiency chromosomes over a GFP-marked *CyO* chromosome from Bloomington Stock Center strain *w**; In(2LR)*noc*^{4L} *Sco*^{rv9R} *b*^{1}/*CyO*, P{*w*$^{+mC}$=*Act*GFP} JMR1 (FlyBase ID: FBst0300816). Males from these *CyO*-GFP balanced deficiency strains were then crossed to virgin females from near or far pupating strains (RI strains 70 or 43, respectively) to generate larvae for group testing.

Statistical analysis. Statistical analyses were performed using SAS software,[33] with the exception of the Shapiro-Wilk/rankit plot for which we used Statistix (www.statistix.com). To test for significant

Table 1 **Deficiency strains used in the quantitative complementation of the pupation position QTL, ordered by breakpoints**

Symbol (FlyBase ID)	Df Breakpoints	Full Stock Genotype
Df(2R)Jp5 (FBst0003519)	52A13-14;52F10-11	*w*^{1118}; *Df(2R)Jp5*/CyO, P{*ry*$^{+t7.2}$=*sev*Ras1.V12}FK1
Df(2R)vg89e88 (FBst0006359)	52B3-C1;53E2-F01	*Tp(2;3)vg89e88*, *vg*^{89e88}/In(2L)*Cy*, In(2R)*Cy*, *Cy*^{1}*Bl*^{1}*L*^{4}
Df(2R)robl-c (FBst0005680)	54B17-C4;54C1-4	*Df(2R)robl-c*/CyO, *y*$^{+}$
Df(2R)RM2-1 (FBst0005426)	54F2;56A1	*w**; *Df(2R)RM2-1*/CyO, P{*ry*$^{+t7.2}$=*sev*Ras1.V12}FK1
Df(2R)P34† (FBst0000757)	55E2-4;56C1-11	*y*^{1} *w**/*Dp(1;Y)y*$^{+}$; *Df(2R)P34*/CyO
Df(2R)AA21 (FBst0003467)	56F9-11;57D11-12	*Df(2R)AA21*, *c*^{1}*px*^{1}*sp*^{1}/SM1
Df(2R)XE3030 (FBst0005442)	57C2;58C	*Df(2R)XE3030*/CyO, P{*ry*$^{+t7.2}$=*sev*Ras1.V12}FK1
Df(2R)59AD (FBst0003909)	59A1-3;59D1-4	*w**; *Df(2R)59AD*/SM1

†Failed to complement pupation position QTL.

interactions in the quantitative complementation tests, analysis of variance (ANOVA) was performed with chromosome (balancer or deficiency) and strain (near or far pupators) as factors, with vial number nested. ANOVA was also used to analyze pupation distances and wandering behaviors. Differences were identified using the Student-Newman-Keuls (SNK) test.[33] A 5% level of significance was used for all tests.

RESULTS

QTL mapping. Pupation assays were performed on 76 RI lines, which originated from two parental strains: 2b and Oregon R.[24] The pupation position phenotypes of the RI lines were analyzed. Normally distributed between-strain variation was observed (Fig. 1) (Shapiro-Wilk/rankit plot, W = 0.9843).[34] Several near and far pupating RI lines were chosen, based on their phenotype in our assay, for retesting, and their relative performance in the retests were consistent with the original QTL assay results (data not shown).

We observed that larval sex has a significant effect on pupation position, in agreement with previous studies.[16,17,21] In the RI lines, male larvae, on average, pupate significantly further from the food than do females (Fig. 2). There was no significant sex by strain interaction (2 way ANOVA, $p_{strain} < 0.0001$, $p_{sex} < 0.0001$, $p_{strain \times sex} < 0.171$).

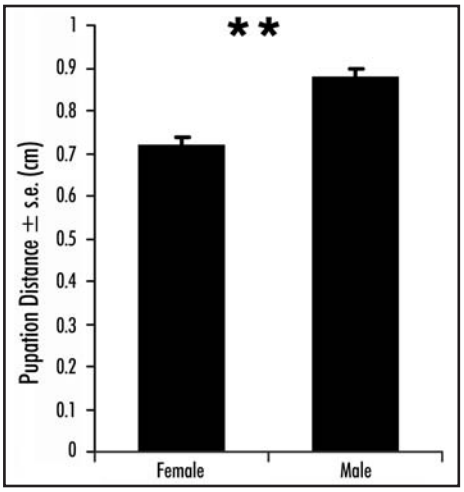

Figure 2. Across the 76 RI strains, male larvae pupate significantly further from the food than do females. Since there was no significant sex × strain interaction, male and female data were pooled for the QTL analysis (**denotes significance, ANOVA, $p_{strain} < 0.0001$, $p_{sex} < 0.0001$, $p_{strain \times sex} < 0.171$).

We used composite interval mapping to identify one significant QTL (Fig. 3). The LR statistic, which indicates the likelihood that a QTL is present at a specific genomic location, is plotted against recombination maps of the three major chromosomes. Based on the recombination map derived from the recombination that occurred during the construction of the RI lines, chromosome 2 is separated into two linkage groups between polytene positions 50F and 57C. The 5% level of significance for the LR, calculated from 10,000 random permutations of the dataset, was 12.12, and was exceeded at one point near the genomic marker at polytene position 57C, at the border of the second chromosome 2 linkage group. We therefore have a one sided curve indicating that the peak lies either at 57C or somewhere between 50F and 57C, but, based on the LR plot, likely closer to 57C.

Quantitative complementation[32] was used to further resolve the location of the QTL. Strains carrying various genomic deficiencies for the region between 52A-59D were used, but not all regions were uncovered by this deficiency collection (Table 1). Significant strain

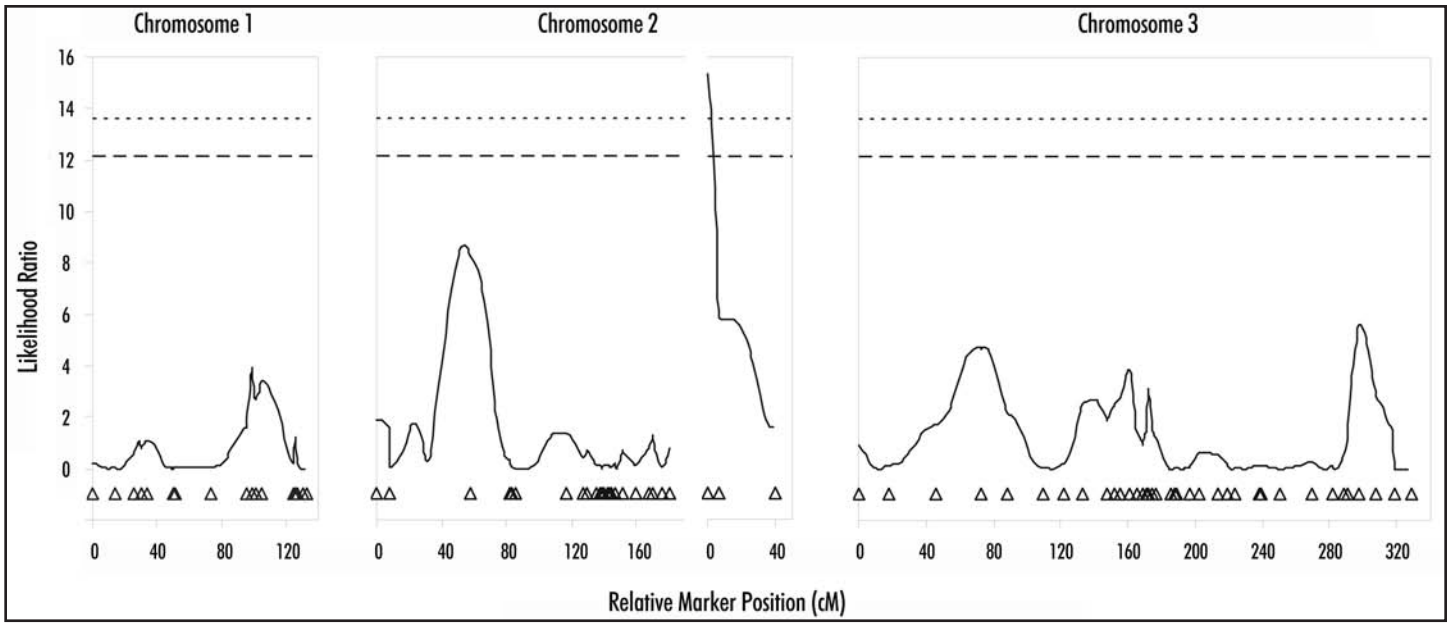

Figure 3. Results of the QTL analysis of pupation position variation among 76 RI strains. The genome is divided into the three major chromosomes, represented by four plots indicating the linkage groups (more than 50cM separates two adjacent genomic markers on chromosome 2). The relative positions of the *roo* transposable elements used as genomic markers are indicated by the triangles positioned along the horizontal axis. The distributions of the *roo* elements among the RI strains were used to generate the recombination map. Plotted against this map is the likelihood ratio (LR) as calculated by composite interval mapping.[29] The LR is an indicator of the correlation between the behavior and variation at each genomic position. The long- and short- dashed lines across the plots indicate the 5% and 2.5% levels of significance, respectively. These significance thresholds were determined from 10,000 random permutations of the dataset. One significant QTL was detected on the left arm of chromosome 2.

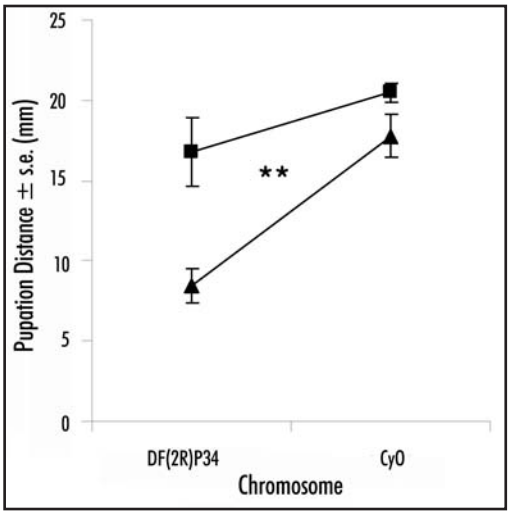

Figure 4. Interaction plot from the quantitative complementation analysis between *Df(2R)P34* (see Table 1 for the full genetic name) and near and far pupating RI lines (strains 70 and 43, respectively). Males carrying the deficiency balanced over a GFP-marked CyO were crossed to females from the near (▲) and far (■) pupating strains and the pupation position of the progeny were tested. Significantly greater differences were observed between the near and far derived progeny carrying the deficiency than between those inheriting the balancer chromosome (2-way ANOVA, $P_{strain \times chromosome} < 0.0321$). Such an interaction effect between the RI strain and tester (Df or CyO) chromosome effects indicates the presence of an influential genomic region within the deleted segment. Using a series of deficiencies, we resolved the relevant region to polytene position 56A01-C11.

by chromosome interaction was observed with deficiency strain *Df(2R)P34* (breakpoints at 55E2-4 and 56C1-11) (Fig. 4), but not with overlapping deficiency strain *Df(2R)RM2-1* (breakpoints at 54F2 and 56A1) indicating the presence of a QTL between 56A1 and 56C11.

Individual assay. To further investigate the nature of the behavioral differences between strains that pupate near or far from the food we developed an assay to analyze in detail the wandering behaviors of individual larvae. Two phenotypically divergent RI lines (43 and 70, see Fig. 1) were chosen for this study. Individual mid-third instar larvae were placed in small glass test tubes and their movements were photographed at 6-second intervals until all larvae had pupated. The images were then compiled into MPEG videos and the movements were analyzed. Figure 5A shows the final frame of one such movie. Individuals derived from far or near pupating strains were placed in alternating vials and the larvae and pupae can be seen through the sides of the vials.

Interestingly, when the larvae were removed from their group-reared conditions and placed into the individual vials, there was often a period of increased locomotion on the sides of the vials. This increased activity is similar in both near and far pupating strains and sometimes persisted for more than an hour after the transfer (Figs. 5B and 6).

Wandering behavior is initiated approximately 6–8 hours before pupariation (Fig. 6), and is characterized by the many forays from the food onto the side of the glass (Figs. 5C and 6). Here, a wandering foray is defined as a bout of locomotion during which the larva is visible above the surface of the media. Foray number, duration, and the maximum distance from the food achieved per foray are used as metrics for comparison. Speed and total distance traveled could not be accurately quantified because only one camera was used and movement parallel to the camera's line of sight could not be

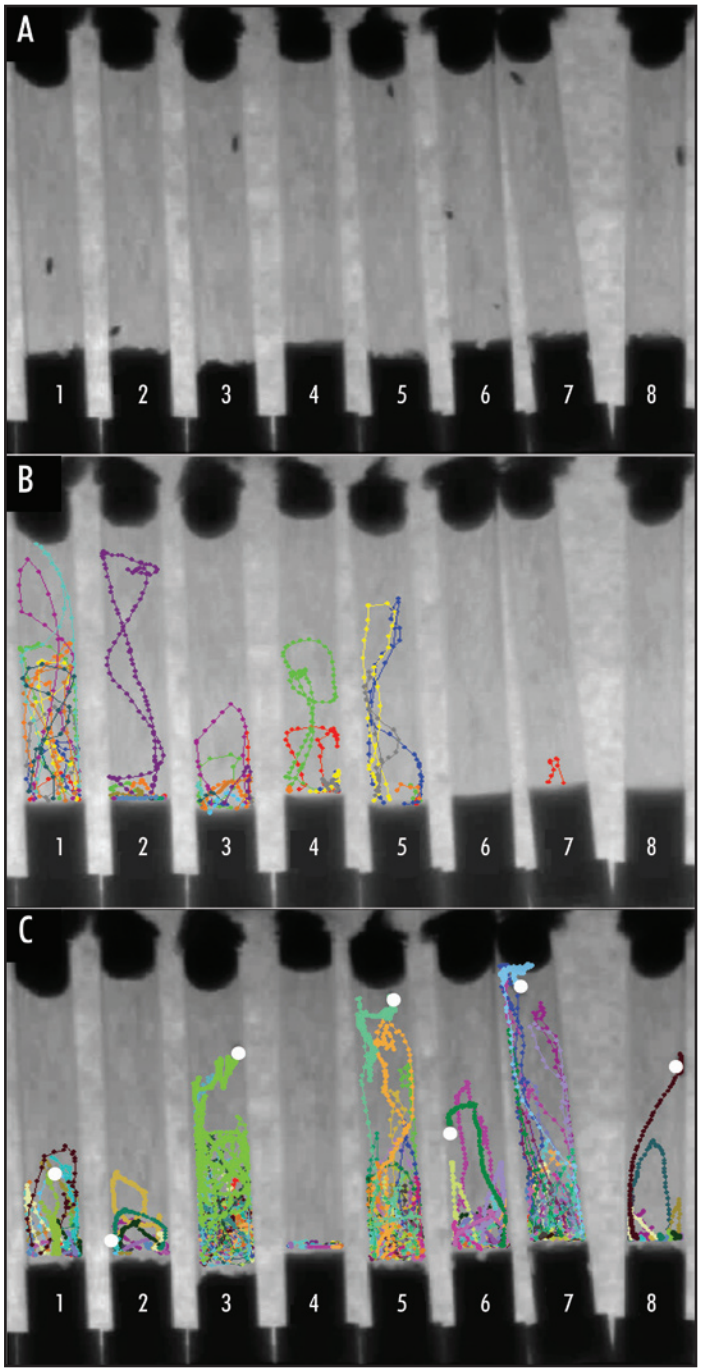

Figure 5. Images recorded during the individual pupation distance assay. Mid-third instar larvae are placed individually into vials and their wandering behavior is filmed. (A) The final frame of the recording, the pupae are clearly visible on the vial walls. Vials 1, 3, 5 and 7 contain genetically far pupators (from RI strain 43), while vials 2, 4, 6 and 8 have genetically close pupators (RI Strain 70). (B) Upon being placed in the vials there is an initial burst of activity during which we observed no significant differences between the strains. The colored lines represent the larval paths; each color is an individual foray out of the food. For reference, the paths are overlaid on a frame from early in the video. These paths were all collected between frames 1-3000, representing the first five hours of recording. (C) Several hours later, wandering begins and the behavioral differences between the near and far-pupating lines become more apparent. Generally, the far pupators move further from the food during wandering forays. In this figure, the DIAS-regenerated wandering paths are overlaid on the final video frame, and the pupation positions are indicated by the white circles.

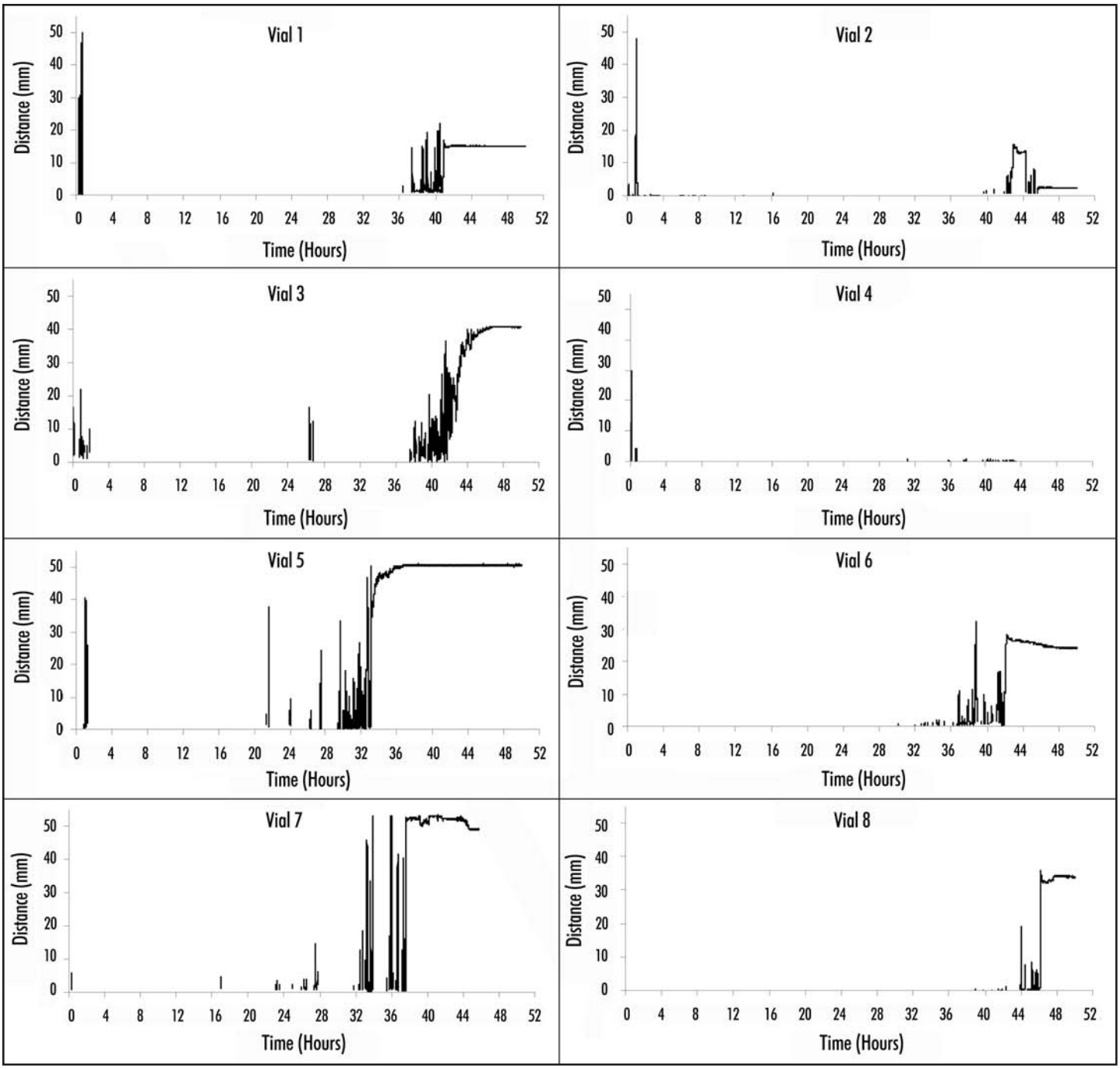

Figure 6. DIAS-derived behavioral profile of the four far (vials 1, 3, 5 and 7) and near (vials 2, 4, 6 and 8) pupating larvae introduced in Figure 5. The distance of the larvae from the food (mm) is plotted at six second intervals. Differences in the distance moved from the food per foray, and the tendency of the far pupators to wander and pupariate earlier than the near pupators become apparent.

accurately measured. Individuals from the far pupating strain (RI 43) range significantly further from the food during wandering forays than do larvae from the near pupating RI strain, 70 (Fig. 7A). As time to pupation decreases, larvae of both the near and far pupating strains tend to range further from the food during forays (Fig. 7A). For each plot in (Fig. 7), time "0" represents the time that the final foray was initiated, i.e., the last time the larvae contacted the medium before pupation.

The near and far pupating strains do not significantly differ in the duration of wandering forays and in both variants the duration of forays increases as pupation approaches (Fig. 7A). Although the far pupators made more wandering forays than did the larvae from the near pupating strain (Fig. 7C), this difference was not always observed in replicate tests. This difference is decreased in the final hours preceding pupation. Interestingly, after correcting for the absolute number of forays, the distribution of forays was very similar during wandering by near and far pupators, with about 25% of forays occurring in the final hour before pupariation (Fig. 7D).

As in the group assay employed for the QTL analysis above, a significant difference in the mean pupation positions of the near and far pupating strains was also observed using the individual assay (far pupating strain: mean = 37.75, s.e.m. = 4.32, n = 8; near

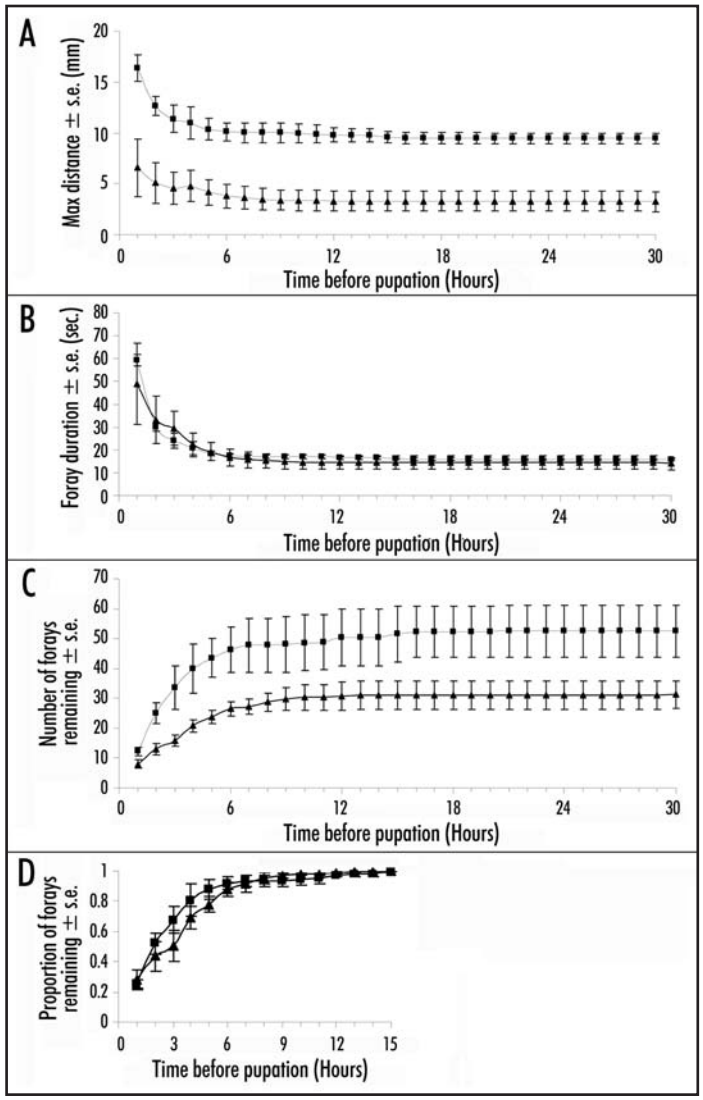

Figure 7. Comparisons of the wandering behavior of near (▲) and far (■) pupators. The horizontal axis is a reversed time scale in hours from the time of the final foray during which the animal pupariates. Each point indicates the mean value of all remaining forays between that time point and time "0", the beginning of the final foray. (A) Far pupators move significantly further away from the food media during wandering. In both strains, the maximum distance reached from the media increases as time to pupation diminishes. (B) Interestingly the amount of time spent wandering on the vial wall was not different between the near and far pupators. Note the increase in foray duration approaching pupation. (C) The number of wandering forays was higher in the far pupators, although this observation was not always significant across replicate tests, the trend was consistent (data not shown). The majority of forays are made in the final 6–8 hours prior to pupation. (D) After converting the foray numbers into proportions of forays remaining 15 hours before pupation, the distribution of forays made by near and far pupators are not different, with about 25% of the forays made in each of the final two hours preceding pupariation.

pupating strain: mean = 9.71, s.e.m. = 5.34, n = 7; ANOVA/SNK, $p < 0.002$). Furthermore, results from this test are also consistent with an earlier report that far pupators tend to pupariate earlier than near pupators.[22] Far pupators pupariated at a mean time of approximately 40 hours after recording began, compared with 45 hours for the near pupators (Fig. 6).

DISCUSSION

Diverse forces have conspired in the selection of variation in prepupal wandering and pupation site selection behaviors. Wandering larvae perceive and respond to a variety of cues and their response is ultimately manifest in the pupation position. Environmental cues such as humidity, temperature, and light are integrated into the position choice and in some conditions the probability of survival is increased by pupating nearer the food while in others, it is advantageous to move further away.[19,35] Thus, a final, and potentially critical, act of the larva is to find a suitable pupation position.

Natural variation in pupation site selection is affected by genes, the environment, and gene-by-environment interactions.[10,15-19] Towards the ultimate goal of understanding the integrations and interactions of these effects to produce and/or maintain phenotypic variation, it is helpful to focus on the genetic and environmental components separately. Various environmental effects have been described in previous studies.[5-14] This study is presented as a further step toward identifying the genetic contributors to natural variation in wandering and pupation site selection behaviors. The precise mechanisms and their implications for gene-by-environmental interactions will be more easily understood once the genes contributing to the variation have been identified.

QTL analysis. Even under the controlled environmental conditions of laboratory assays, larval pupation position remains variable and the predisposition to pupate near or far from the media is heritable. Previous studies revealed that larvae derived from wild-collected pupae also show variation in their pupation position in a grouped pupation assay similar to that used in this paper.[15] Importantly, this variation was consistent with the location from which the founding pupae were collected: larvae descended from pupae that were found on food in the wild tended to pupate nearer the food in the lab assay compared to those derived from wild pupae collected further from the food. These observations indicate a correlation between the natural pupation position variation and that observed in the laboratory assay.

Our observation of a significant sex effect on pupation position is consistent with previously published studies.[17,20,21] In two other studies pupal sex was reported but the sex effects were either not quantified or were not significant, nevertheless, the males tended to pupate further from the food than the females.[8,16] The existence of this trend even across the myriad fly strains and pupation position assays used may be an indication of the robustness of a sex effect. The mechanisms affecting this sexual dimorphism in pupation behavior are not clear. It could, for example, be that male and female larvae differ in their sensitivity, response, resistance, or tolerance of the various environmental cues associated with site selection (e.g., temperature, light, humidity).[36,37] Sex differences in pupation site selection may have arisen via correlated response to selection. For example, adult males are selected in part based on their ability to produce a courtship song.[38] This song is produced by rhythmic wing vibrations and thus, wing structure is important. By pupating slightly further from the food, males may increase the probability of eclosing into cleaner and drier conditions thereby facilitating proper wing inflation and song production. Alternatively, differences in pupation site selection may reflect differences in the environmental conditions needed for male vs. female metamorphosis. Future studies will address these hypotheses.

Our QTL analysis identifies a single genomic region where variation was correlated with the pupation position phenotype. Due to the

Table 2 **Identified or predicted genes mapped to the sequence within the candidate pupation position QTL (56A1-C11)**

Symbol	Location	Selected Annotated Biological Processes*
sano	55F8-56A1	
prod	56A2-56A2	lymph gland development
CG15107	56A2-56A2	
Topors	56A2-56A2	protein ubiquitination
CG18605	56A2-56A2	
CG15105	56A2-56A3	cell proliferation; protein ubiquitination; regulation of transcription from RNA polymerase II promoter;
5-HT1B	56B1-56B1	serotonin receptor (1B)
CG30126	56B1-56B1	
CG15115	56B1-56B1	
CG15116	56B2-56B2	defense response; response to stress, toxins
CG15109	56B2-56B2	
5-HT1A	56B2-56B5	serotonin receptor (1A)
CG30125	56B3-56B3	
CG15117	56B5-56B5	carbohydrate metabolism; proteolysis
botv	56B5-56B5	segment polarity determination; N-acetylglucosamine metabolism
CG15118	56B5-56B6	
CG15111	56B5-56B5	
ena	56B5-56C1	actin filament organization; axon guidance
CG10737	56C1-56C1	diacylglycerol binding; intracellular signaling
CG7097	56C1-56C4	JNK cascade; induction of apoptosis; protein amino acid phosphorylation
CG7137	56C4-56C4	
cora	56C4-56C4	cytoskeleton organization and biogenesis; regulation of tracheal tube size
wbl	56C4-56C4	exocytosis; dorsal/ventral axis specification
CG33454	56C4-56C4	
CG33453	56C5-56C5	
CG7229	56C6-56C6	MAPKKK cascade
rib	56C6-56C6	Malpighian tubule morphogenesis; CNS development
CG11906	56C6-56C6	nucleic acid binding; Zn-finger
CG10476	56C6-56C6	
CG10474	56C7-56C7	amino acid catabolism; glycoprotein catabolism
CG18606	56C8-56C8	
CG18607	56C8-56C8	
FK506-bp2	56C8-56C8	protein folding
mip40	56C8-56C8	chorion gene amplification; negative regulation of transcription from RNA polymerase II promoter
Tab2	56C8-56C9	positive regulation of I-kappaB kinase/NF-kappaB and JNK cascades
CG7461	56C9-56C10	acyl-CoA metabolism; electron transport
endoB	56C10-56C10	endocytosis; transmission of nerve impulse
Rgk1	56C10-56C11	small GTPase signal transduction
CG30127	56C11-56D1	

*Where known, from http://flybase.org/. [39]

relative paucity of genomic markers in the region of interest (Fig. 3), our analysis identified a one-sided peak in the likelihood ratio plot. The result of this was a lower resolution of the QTL than would otherwise have been expected. Nonetheless, our results confirm and further refine previous reports of a strong chromosome 2 influence on pupation position variation as observed across different strains and assays.[16-18]

Using quantitative complementation we further resolved the QTL to region 56A01-C11, an area containing approximately 39 annotated genes (Table 2).[39] Although many of these loci are of unknown function, several present intriguing candidate genes that we plan to investigate further in our ongoing studies. Examples of such candidates are the serotonin (5-HT) receptor types 1A and 1B genes, respectively located at 56B2-5 and 56B1. Type 1 5-HT receptors

have been implicated in mammalian hydration behaviors,[40,41] and in the honeybee, *Apis melifera*, treatment with 5-HT significantly reduces the proboscis extension response to water vapor, whereas cotreatment with 5-HT receptor antagonists suppress the effect.[42] These observations are notable because humidity or moisture has long been implicated as a significant factor in *D. melanogaster* pupation position.[5,8,21,43] Furthermore, a recent study of crayfish primary sensory neurons demonstrates an interaction between the signaling of 5-HT and the ecdysteroid, 20-hydroxyecdysone,[44] which is also a major molting and pupation hormone in *Drosophila*.[4,45,46]

There are likely many loci influencing natural variation in pupation position besides the QTL identified in this study. The nature of our QTL search restricts our results to identification of loci, which varied between the two strains used to generate the RI lines. These founding strains were not previously selected for near or far pupation positions as would be ideal to maximize the relevant genetic variation. Therefore, we have identified only a minimum number of the loci affecting pupation position behaviors. Besides chromosome 2, previous studies have attributed significant influences on pupation position to chromosomes 3 and *X*.[16-18]

Individual pupation position assay. Two strains classified as either near or far pupators in the group assay (RI strains 70 and 43, respectively) continued to express similar pupation site preference phenotypes when tested individually. This indicates that the variation is not necessarily dependent on group phenomena, such as encountering other individuals or their trails. Nevertheless, because the test larvae were not raised completely in isolation, we cannot exclude the influence of group effects earlier in development.

Our results are also consistent with a previous observation that far pupators pupariate earlier than near pupators.[22] Casares and Caracedo[21] suggest possible explanations for this observation, such as: decreased humidity in the vials over time, or impoverishment of the media leading to locomotory weaknesses in the later wanderers. In our individual assay, larvae complete larval development, wander, and pupate in solitude, thereby reducing the probability of nutritional deprivation as the cause of near pupation. Though we cannot exclude the possibility that humidity inside the individual vials decreases over time, we attempted to minimize this by maintaining a flow of humidified air across the vials throughout the test and by moistening the cotton used to plug the vials at the outset. This relationship of developmental time to pupation site is intriguing in light of the observation that males, which tend to have longer developmental times,[1,4] nevertheless pupate farther from the food than do females.[17,20,21] This provides evidence that the link between developmental time and pupation site is likely not solely mediated by environmental changes such as decreased humidity or food quality.

After placing the mid-third instar larvae into individual vials, we observed an interesting initial behavior. The larvae rarely resumed foraging behaviors immediately; rather, they exhibited a period of rapid locomotion on the vial wall. This was despite our attempts to facilitate larval entry into the media by punching a fresh a hole into the food prior to adding a larva. This activity may be an escape response induced by the trauma of being moved, but the duration of the response is of interest. As seen in Figure 6, the increased locomotion was often observed over an hour after the larvae had been placed in the vials. This observation may have implications for other studies of larval behavior where an acclimatization period of a few minutes is typically perceived as adequate.

Previous studies of pupation behaviors have tended to focus on variations in the final position chosen for metamorphosis, or, as in the case of embedding behavior, on the very final moments of wandering.[23] It has hitherto been unclear how differences in wandering behaviors may be related to the final pupation position. Using time-lapse photography to record and analyze wandering behaviors, we observed significant variation in the wandering phenotypes of two RI strains expressing preferences to pupate either relatively near or far from the food. In fact, the mean distance moved from the media during wandering forays can be a more reliable identifier of an individual's strain origin than is their final pupation position (Fig. 5C).

Although they differ significantly in the mean distance moved away from the media, the near and far pupators do not significantly differ in the number, distribution, or duration of wandering forays. Speed of crawling, and overall distance moved could not be measured accurately due to the nature of the camera setup and the lack of information about movement in the plane parallel to the line of sight. The observation that far pupators move significantly further from the media whereas the foray durations do not differ may indicate either that the far pupators move faster or that the near pupators move equally fast but only over a region near the media. Notably, it has been previously reported that strains selected for far pupation position also exhibit increased larval mobility.[22] Future studies will help clarify this issue.

The onset of wandering is distinguished by a marked increase in the occurrence of forays onto the vial wall and occurs approximately 6–8 hours before the final foray (during which the larva pupates and does not return to the food) (Fig. 6). In both strains, wandering forays are usually relatively brief excursions onto the vial wall averaging only a few seconds in time, but increasing both in mean duration and mean maximum distance from the media as the time of pupariation approaches (Fig. 7). This series of excursions may enable the larvae to test the environmental conditions outside the media. For example, a larva may assess its rate of water loss during the forays and then rehydrate itself upon returning to the media before the next excursion. Light and humidity have been demonstrated to influence *Drosophila* pupation position.[5,22] Light may be used in assessing both predation/parasite risk and moisture levels, i.e., brightness may indicate an open, more desiccating environment, thus inducing pupation nearer to the media.[47] Future studies will investigate the relative light sensitivity and desiccation tolerance of larvae and pupae from near and far pupating strains. Furthermore, it will be important to assay the wandering phenotypes of other, unrelated, high and low pupating strains to test the conclusions presented above.

Natural variation in pupation position has an impact on survival. Some individuals are predisposed to pupate farther from food in a given environment and previous studies have shown that larvae are able to assess risks and modify their behavior to suit the environment in a way that increases the odds of survival.[5] How larvae assess and balance conflicting behavioral strategies (for example, the simultaneous avoidance of desiccation and drowning or rot) in order to establish the most environmentally appropriate pupation site, and how differences in the perceived appropriateness of any given site are genetically influenced, remain to be elucidated.

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