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Elucidating the Behavioral Phenotype of *Drosophila melanogaster* Larvae: Correlations Between Larval Foraging Strategies and Pupation Height

M. B. Sokolowski[1,2] and R. I. C. Hansell[1]

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Larvae which demonstrated long trails covering a large area while feeding (rover *foragers) pupated significantly higher than those covering a relatively small area and exhibiting short paths* (sitter *foragers). Pupation height and density of larvae per vial were positively correlated. Under the condition of equal larval density per vial,* rovers *were found to pupate significantly higher than* sitter *larval foragers. The effect of three light regimes (constant light, constant darkness, and 12 h light followed by 12 h dark) indicated a more complex relationship between pupation height and larval foraging behavior.*

KEY WORDS: foraging behavior; pupation heights; larval; *Drosophila melanogaster.*

INTRODUCTION

Drosophila larvae spend most of their lives foraging for food (Bakker, 1961; Ohnishi, 1979; Sewell *et al.*, 1975; Burnet *et al.*, 1977). A *Drosophila* larva feeds by shoveling food with its mouth hooks and moves by alternately extending its anterior and retracting its posterior end. Foraging behavior reflects the relative amounts of feeding (shoveling) and locomotor (crawling) behavior performed. Sokolowski (1980a, 1981, 1982a,

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[1] Department of Zoology, University of Toronto, Toronto, Ontario, Canada M5S 1A1.
[2] Present address: Department of Biology, York University, Downsview, Ontario, Canada M3J 1P3.

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b, c) identified a behavioral polymorphism in *Drosophila melanogaster* larval foraging patterns: a *rover* larva traversed a large area while foraging on a yeast-covered petri dish, whereas a *sitter* larva covered a relatively smaller area. Genetic analysis using chromosomal manipulation showed that differences in these forager types could be attributed to the second chromosomes of this species (Sokolowski, 1980).

When larvae have reached their minimum weight for pupation (late third instar), they begin to search for a pupation site (Bakker, 1961). In the laboratory, they often utilize the walls of culture bottles as pupation sites. In one of the few studies of a natural population, McCoy (1962) observed that the larvae of *D. melanogaster* pupated on the dry skins of fruits, on the soil surface, or approximately 0.5 in. below the soil surface. In his review of nonsexual behavior of *Drosophila*, Grossfield (1978) summarized the results of experiments performed on pupation heights (usually measured as the distance between the top of the medium and the pupa). Mensua (1967) found that pupation height increased with increasing temperature (from 13 to 25°C), but at 29°C pupation height was drastically reduced. Sameoto and Miller (1968) found that increased moisture raised pupation heights. Sokolowski (1981) found that pupation height increased with increasing larval density.

The effects of a variety of physical and biological factors on pupation site choice were tested in a study of Sokal *et al.* (1960). They concluded that there were significant developmental, as well as gene–environment interactions, affecting pupation site choice. Sokal *et al.* also failed to find any correlation between pupation site and larval behavior patterns. Whereas some authors have suggested polygenic control of pupation, de Souza *et al.* (1970) found that a single major gene was responsible for larval choice of pupation site in *D. willistoni*. The allele for pupating outside the food cup was dominant over the allele for pupating inside. These differences in the choice of pupation site were correlated with adult activity levels.

The sibling species *D. melanogaster* and *D. simulans* have been the subjects of investigations involving their differential resource utilization (see Parsons, 1975, for an extensive review; Bos *et al.*, 1977; Hoenigsberg, 1968; Kawanishi and Watanabe, 1978; Matheson and Parsons, 1975; McKenzie and McKechnie, 1979; Moth and Barker, 1977; Sameoto and Miller, 1968; Sokolowski and Hansell, 1983; Sokolowski *et al.*, 1983; Tantaway and Soliman, 1968). Larval pupation height is a widely studied behavior in these species. Sameoto and Miller (1966) and Barker (1971) found that larvae of *D. simulans* tended to pupate on the surface of the medium, compared with larvae of *D. melanogaster*, which tended to pupate on the sides of the vials. In contrast to the study of Sokal *et al.*

(1960), Barker (1971) found that *D. melanogaster* larvae showed no change in pupation site with increased larval density, whereas *D.* simulans larvae tended to prefer more peripheral pupation sites. *D. melanogaster* pupated away from a light source; the position of the light source had no effect on the pupation of *D. simulans*.

Another factor affecting pupation site choice is the relationship between larval density and strain-dependent viability changes due to crowding (Alvarez *et al.*, 1979; Kearsey and Kojima, 1968; Mishima, 1964). Experiments comparing the viability of the *ebony*^{11} strain with various wild-type strains (Jacobs, 1961) are of particular importance to the present study. Moree (1952) measured the relationship between degree of crowding and viability in e^{11} and wild strains. He used the term viability to represent the number of progeny emerging after a 4-day egg-laying period. He found that when crowding was low, the wild and ebony strains had equal viabilities. However, with increased crowding the viability of e^{11} decreased. Jones and Barker (1966) also found a decreased viability in e^{11} under crowded conditions during intraspecific competition (with a wild strain). Dawood and Strickberger (1964, 1969) performed viability studies similar to those of Moree (1952) and also found that the frequency of ebony homozygotes decreased with increased larval crowding. The frequency of +/e heterozygotes increased compared to that of the wild homozygotes, when crowding and homozygosity of the wild type strains were high. Weisbrot (1966) showed genetic differences in average larval survivorship (using a density of 40 larvae/5 ml of medium) when comparing ebony homozygotes with two different wild-type stocks.

The results of pupation site studies tend to vary with biotic factors such as the density, age structure, and genetic architecture of the population or species studied. Abiotic factors that affect pupation height include moisture, temperature, and light. The results of selection and other genetic analyses have led to differing views of the genetic basis for the choice of pupation site. Models include polygenic and single-gene inheritance, as well as little or no genetic control of this behavior. Certainly, these results are dependent upon experimental conditions. The methods by which pupation site choice is measured are of primary importance when comparing the results of these studies. Surprisingly, reviews of this subject rarely take this into account. Some authors used discrete methods (central versus peripheral, inside versus outside pupation), whereas others used continuous measurements (distance of the pupae from the top of the medium). Finally, strain-dependent viability responses to crowding are likely to confound the data.

In the following experiment, a continuous measurement of pupation height has been chosen in order to investigate whether there was any

correlation between the choice of pupation sites by *Drosophila* larvae and their foraging behavior.

METHODS

The four stocks isogenic for the second and third pairs of chromosomes used in this study were designated W_2W_3, W_2E_3, E_2E_3, and E_2W_3. A breeding scheme that utilizes the presence of crossover suppressors to permit the substitution of intact second or third chromosome pairs from one stock into another is described by Sokolowski (1980). The chromosome-substituted stocks would be E_2W_3 and W_2E_3. E_2W_3 would have the same second chromosome pair as E_2E_3 but differ in having the third chromosome pair from W_2W_3. E_2E_3 and W_2E_3 both carried recessive alleles for the gene for ebony body color (e^{11}) on their third chromosomes.

Vials 9.5 cm high and 2 cm in diameter were filled with a standard nonliving Brewers yeast–agar medium. Each vial was filled with medium to a depth of 2 cm and was plugged with a cotton ball. Each set of replicates was prepared from the same batch of fresh medium. The medium was autoclaved for 20 min. The flies were cultured at 22 ± 1°C, a relative humidity of approximately 60%, and a light cycle of 12 h light (from overhead lights) and 12 h dark; the lights were turned on at 8:00 AM.

In the first experiment neither the number of eggs per vial nor the number of first-instar larvae which hatched from these eggs was controlled. One female and one male (5–10 days old) were allowed to mate and lay eggs for 96 h. In the second experiment the number of eggs present in each vial was controlled. Vials were prepared by allowing fifty 5- to 10-day-old flies of each stock to lay eggs on a spoon covered with a standard nonliving Brewers yeast–agar medium. After 20–24 h, 20 freshly laid eggs were collected from the spoon using a needle and placed on the surface in the center of the medium in each vial. Since differential egg hatchabilities also were found to affect density, in the third experiment the number of first-instar larvae per vial was controlled. Approximately 10 freshly hatched larvae were placed and positioned as above in each vial using a needle. When all larvae had pupated, a flexible ruler was used to measure the distance from the top of the medium to the spot between the spiracles of each pupa. This measurement was termed pupal height and was used in all experiments.

The number of vials and the number of pupal heights measured for each stock in the first experiment were as follows: W_2W_3 (14 vials; 657 pupae), E_2E_3 (14; 255), W_2E_3 (9; 187) and E_2W_3 (16; 566). In the second experiment 10 vials were used for the two former stocks (219 W_2W_3 and 65 E_2E_3 pupae were counted), 13 vials and 170 pupae were counted for

W_2E_3, and 12 vials and 202 pupae were counted for E_2W_3. In the third experiment 105 pupae in 13 vials of W_2W_3 and 120 pupae in 13 vials of E_2E_3, 76 pupae in 12 vials of W_2E_3, and 165 pupae in 18 vials of E_2W_3 were scored.

The fecundity results of experiment 1 demonstrated that there were stock-specific differences in the number of adult progeny produced. In order to determine whether pupal mortality influenced these results, one female and one male fly (5–10 days old) were allowed to mate and lay eggs for 96 h. This was repeated using 20 vials of W_2W_3, 19 vials of E_2E_3, 18 vials of W_2E_3, and 20 vials of E_2W_3. Both the number of pupae and the number of flies which eclosed from the pupae were recorded.

All the previous experiments were performed using a light cycle of 12 h of light followed by 12 h of darkness. Another set of experiments was designed to measure the pupal heights of the four stocks under two other illumination conditions, constant light (LL) as compared to constant darkness (DD). Approximately 10 freshly hatched larvae were collected and placed in each vial using the methods described for experiment 3. Twenty vials for each stock (W_2W_3, E_2E_3, W_2E_3, E_2W_3) were prepared in this manner. Approximately 10 of the vials for each stock were incubated under LL, whereas the other 10 were reared in DD. One hundred and two pupae in 10 vials of the W_2W_3 stock, 99 pupae of the E_2E_3 stock, 63 pupae of the W_2E_3 stock, and 94 pupae of the E_2W_3 stock were scored for the LL condition. Ninety-six pupae in 10 vials for the W_2W_3 stock, 97 pupae in 10 vials for the E_2E_3 stock, 66 pupae in 8 vials for the W_2E_3 stock, and 94 pupae in 10 vials for the E_2W_3 stock were scored for the DD condition.

RESULTS

The relationship between pupation height and larval density per vial for all four of the stocks used in experiment 1 is summarized in Fig. 1. The mean pupal heights $\pm$SE were 2.2 $\pm$ 0.03 for W_2W_3 pupae and 1.4 $\pm$ 0.05 for E_2E_3 pupae. These means were significantly different (Z = 13.3, $P < 0.001$, N = 657 for W_2W_3 and N = 255 for E_2E_3). The W_2E_3 *rover* stock tended to pupate higher than the E_2W_3 *sitter* stock. The mean pupal height was 1.92 $\pm$ 0.07 for W_2E_3 and 1.38 $\pm$ 0.03 for E_2W_3. These means were significantly different (Z = 7.3, $P < 0.001$, N = 187 for W_2E_3 and N = 566 for E_2W_3). Unfortunately, there was also a large difference in the fecundity and/or egg hatchability and/or larval viability in these two stocks. Consequently the resulting density of larvae per vial in these two stocks confounded the results.

The mean pupal counts per vial during the 96-h egg-laying period for

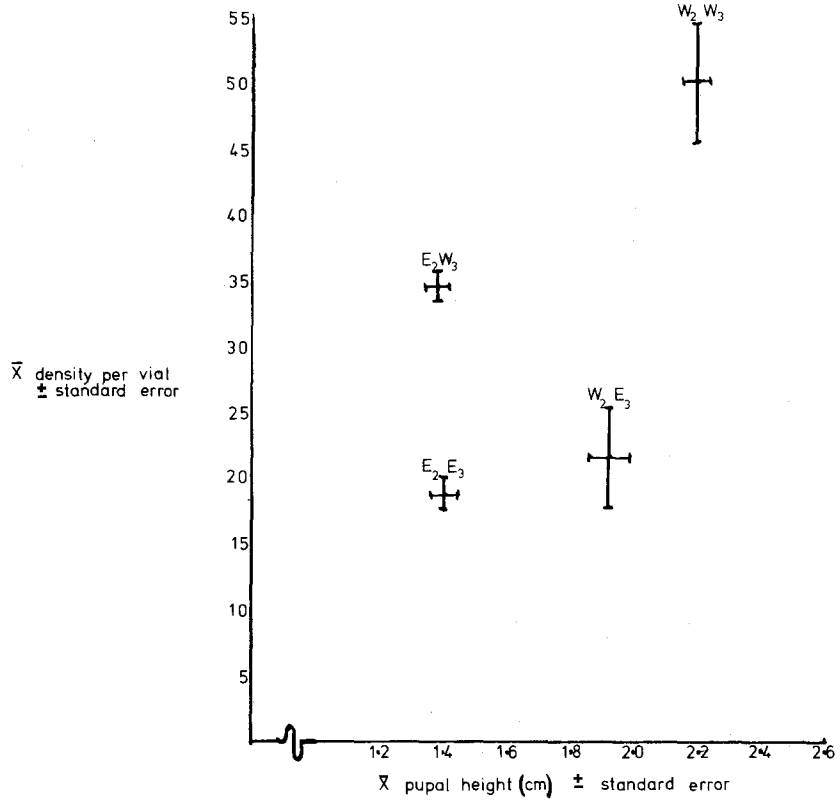

Fig. 1. The relationship between pupation height and larval density per vial for all four of the stocks of *Drosophila melanogaster* used in experiment 1 are summarized. The mean pupal height ± SE for all the pupae measured per stock is presented on the horizontal axis. The overall mean density of pupae per vial ± SE is shown on the vertical axis.

each stock were approximately 47 for W_2W_3, 23 for W_2E_3, 18 for E_2E_3, and 36 for E_2W_3. The E_2E_3 and W_2E_3 stocks that share the E_3 chromosomes did not differ in their mean pupal counts/vial ($t = 0.9$, Student's t test). They had significantly ($P < 0.02$, Student's t test) lower densities than did the W_2W_3 and E_2W_3 stocks. W_2W_3 had a significantly higher ($t = 2.8$, $P < 0.01$) pupal density than did E_2W_3. Table I shows the results of the experiment designed to measure pupal survivorship in these stocks. The four stocks differed in the number of pupae produced (a composite of the number of eggs laid, egg hatchability, and larval survivorship to pupa) as a result of the 96-h egg-laying period, rather than in pupal survivorship. The variability in the data from experiment 1 is illustrated in Fig. 2, showing the mean pupal height per vial plotted against the number of pupae per vial for each vial measured.

Table I. Fecundity and Pupal Survivorship in *Drosophila melanogaster*

Stock	Number of vials	Mean number of pupae/vial ± SE	Mean number of flies/vial	96 pupal survival
W_2W_3	20	104.5 ± 22.6	102.6	98.1
E_2E_3	20	93.0 ± 25.8	90.4	97.1
E_2W_3	19	67.4 ± 21.7	63.0	93.4
W_2E_3	18	24.2 ± 16.7	21.7	89.5

It was thought that the additional data resulting from experiment 2, in which the number of eggs placed in each vial was fixed, would illustrate the effect of forager type on pupal height under equal density conditions. However, in experiment 2, the four stocks tested each still had a different number of pupae per vial. This was probably the result of both differential egg hatchability and/or differential larval survivorship. W_2W_3 had the

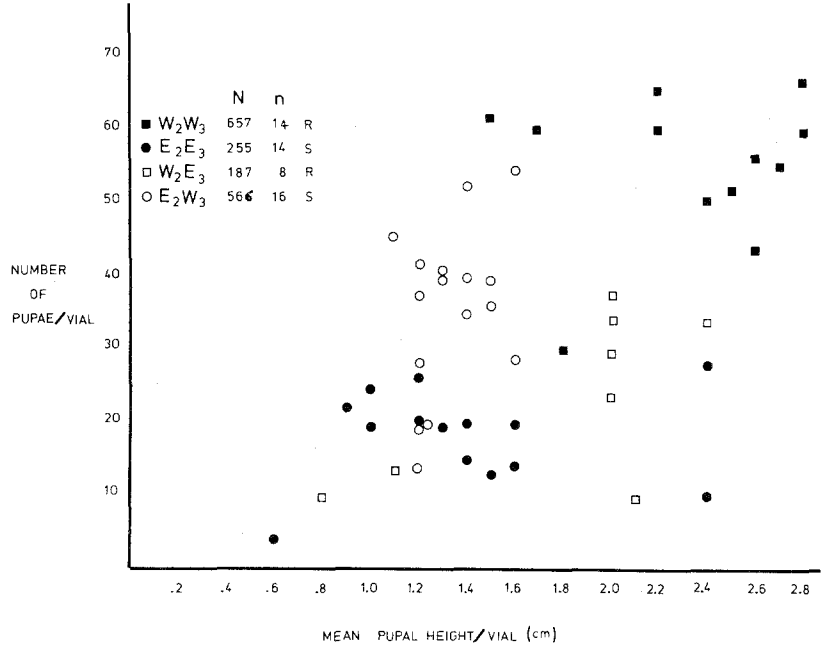

Fig. 2. The mean pupal height per vial is plotted against the number of pupae per vial for each vial measured in experiment 1. The key shows the four stocks of *Drosophila melanogaster* illustrated, the number of pupae measured (N), the number of vials measured (n), and whether the larvae in the vial were *rover* (R) or *sitter* (S) larval foragers. Squares represent stocks whose larvae are *rovers*, whereas circles represent stocks of *sitter* larval foragers.

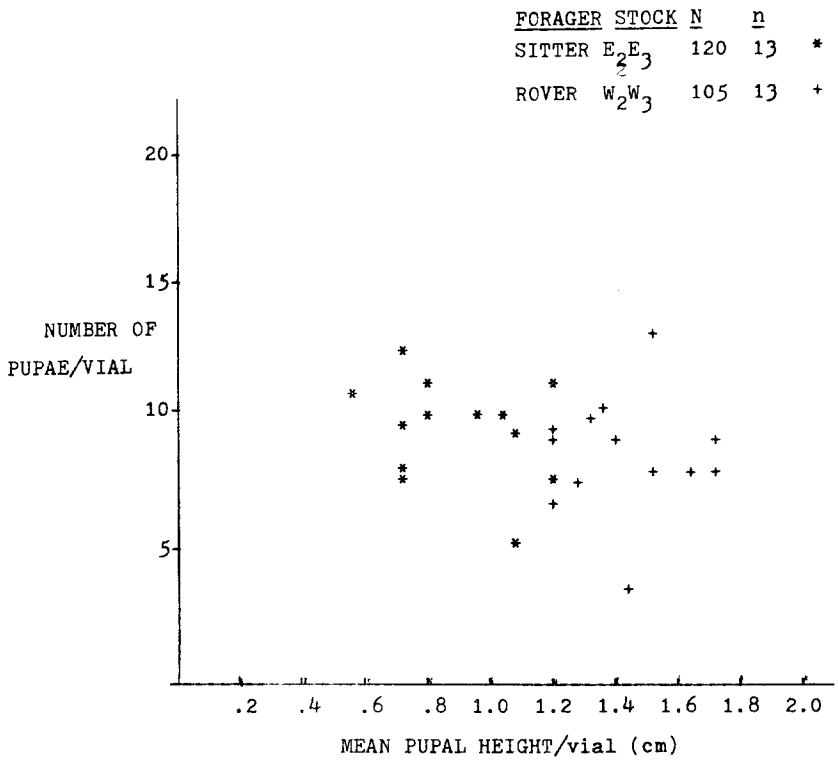

Fig. 3. The relationship between larval density per vial and mean pupal height per vial for each of the 13 vials of W_2W_3 (*rover*) and the 13 vials of E_2E_3 (*sitter*) stocks of *Drosophila melanogaster* measured in experiment 3.

highest mean number of pupae per vial (21.9), and E_2E_3 the lowest (6.5) of the four stocks. W_2E_3 and E_2W_3 had intermediate mean numbers of pupae per vial (13.1 and 16.8, respectively) and these numbers were not significantly different from each other.

Further analysis of the interaction between the two characters density and forager type is attained by using a discriminant analysis (Sokal and Rolf, 1969) which uses forager type and density to discriminate pupation heights (dependent variable). Forager type significantly affected pupation height ($F = 10.9$; the degrees of freedom for the F statistic were 2 and 81 for the regression and residual variance) even when density was kept constant. Density also contributed significantly ($F = 17.6$; the degrees of freedom for the F statistic were 2 and 81 for the regression and residual variance) to stock differences in pupation height when forager type was held constant.

Table II. *Drosophila melanogaster* Pupation Heights in Constant Light and Constant Darkness

Stock	Forager type	Body color	Light (LL) $\bar{X} \pm$ SE	Light (LL) N	Dark (DD) $\bar{X} \pm$ SE	Dark (DD) N	$[\bar{X}(\text{LL}) + \bar{X}(\text{DD})]/2$
W_2W_3	*Rover*	Wild	1.36 ± 0.05	102	1.53 ± 0.08	96	1.44
W_2E_3	*Rover*	Ebony	1.53 ± 0.10	63	1.44 ± 0.09	66	1.48
E_2E_3	*Sitter*	Ebony	1.43 ± 0.07	99	1.05 ± 0.07	97	1.24
E_2W_3	*Sitter*	Wild	1.26 ± 0.06	94	1.47 ± 0.09	94	1.36

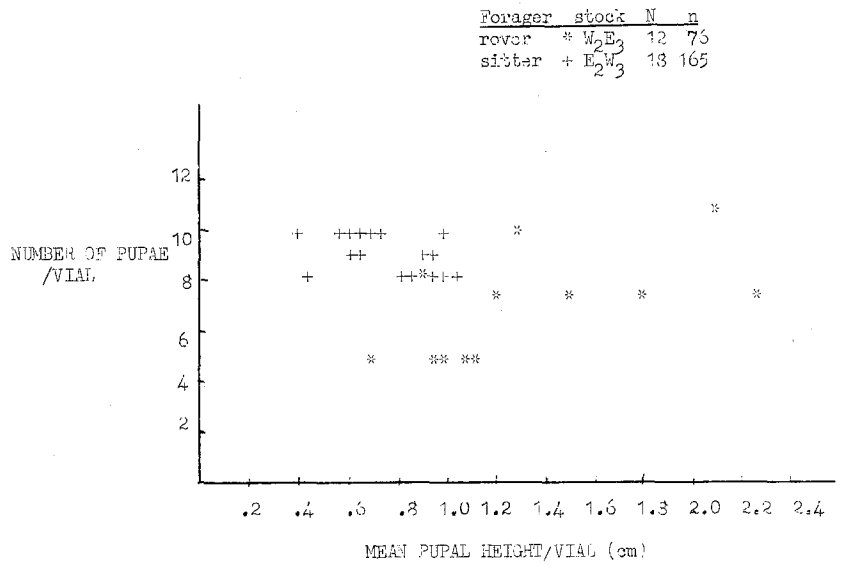

Fig. 4. The relationship between larval density per vial and mean pupal height per vial for each of the 12 vials of W_2E_3 (*rover*) and the 18 vials of E_2W_3 (*sitter*) stocks of *Drosophila melanogaster*.

In experiment 3, larval density was successfully controlled. By seeding vials with freshly hatched larvae instead of eggs, the stock-specific egg hatchability was no longer a factor affecting the number of pupae per vial. Differential larval survivorship, the only factor affecting the density per vial in experiment 3 was minimal at densities of 10 larvae/vial in these stocks. Consequently, this technique is superior to those used in experiments 1 and 2. Figure 3 shows the relationship between larval density per vial and mean pupal height per vial for each of the 13 vials of the W_2W_3 (*rover*) and E_2E_3 (*sitter*) stocks ($t = 7.5$, $P < 0.005$, Student's t test). At equal larval densities, *rover* larvae clearly pupated higher than *sitters*. The same conclusions can be drawn from comparisons of the reconstructed stocks, W_2E_3 and E_2W_3 ($t = 5.1$, $P < 0.005$, Student's t test), found in Fig. 4.

Table II illustrates the stock specific results when pupation heights were measured in constant light (LL) and in constant dark (DD). In LL, E_2W_3 larvae pupated lower than did those of the other stocks. In DD, E_2E_3 larvae pupated significantly ($P < 0.05$, Z score) lower than did those of the other stocks. Larvae of E_2E_3 pupated significantly lower ($Z = 3.6$, $P = 0.05$) in DD than in LL. $[\bar{X}(\text{LL}) + \bar{X}(\text{DD})]/2$ shows the average effect of LL and DD treatments.

DISCUSSION

This study provides strong support for the hypothesis that gene–environmental interactions influence pupation height in *D. melanogaster.* Other authors have studied inter- and intraspecific variability of pupation heights in *Drosophila* (e.g., Sokal *et al.*, 1960; Markow, 1979; de Souza *et al.*, 1970), but none has shown the effect of different pairs of chromosomes on pupation height. Furthermore, although several of these authors have suggested that the choice of pupation site may be correlated with larval behavior patterns, this is the first reasonably comprehensive study of the problem.

The type of foraging performed by the larva was shown to have a significant effect on pupation height. The results of experiment 3 showed that this effect was independent of the density of larvae per vial. What can phenotypic correlations between two behaviors, in this case larval foraging behavior and pupation heights, reflect about their underlying genetic systems? The first possibility is that the two behaviors are associated by linkage, that is, genes associated with *roving* or *sitting* are located on the same chromosome(s) as those of pupation height. The tighter the association between *roving* and high pupal heights, and between *sitting* and low pupal heights, the stronger the correlation and the closer the linkage between the genes associated with these behaviors. In *D. melanogaster*, which has only four pairs of chromosomes, there is a good chance that these behaviors may be associated by linkage. The second possibility is that the correlation between larval foraging behavior and pupation heights reflects the same sets of genes which have pleiotropic effects. In the case of pleiotropy, larvae may have sets of genes which influence activity levels or responsiveness. *Roving*, high pupation heights, and digging deeper into the medium (Sokolowski, 1982) may reflect higher activity levels in *rovers* as compared to *sitters*. We (present study; Sokolowski, 1980) have shown that differences in *roving* and *sitting* and pupation height are due to differences in the second chromosomes in these strains, thus indicating that the genes influencing these behaviors are linked. Additional research is required to determine if pupation heights are affected by the same or closely linked genetic systems as larval crawling and digging behavior, (Sokolowski, 1980, 1982). Specifically, by measuring the foraging path length and pupation height of individual larvae from "*rover* by *sitter*" crosses, it may be possible to examine further the question of whether these behavioral correlations reflect linkage and/or pleiotropy.

Factors which influence larval fitness include foraging ability (crawl-

ing, shoveling, and digging behavior), resistance to harmful physical and biological factors (i.e., dryness, bacterial infections), and choice of a suitable pupation site. In experiments 1 and 2, E_2E_3 and W_2E_3 had significantly lower mean densities per vial than did W_2W_3, and E_2W_3 had significantly lower mean densities per vial than did W_2W_3, and E_2W_3. The presence of the recessive gene ebony in its homozygous form apparently reduces both egg viability and adult fecundity. It was highly unlikely that foraging ability was contributing to the differences in mean density per vial in experiment 1, since W_2W_3 (wild) and W_2E_3 (ebony) were *rover* larval foragers and E_2E_3 (ebony) and E_2W_3 (wild) were *sitter* larval foragers.

The genetic control of pupation height in *Drosophila* is difficult to study because of the large range of physical factors which affect this trait and strain differences in response to differing sets of experimental conditions. For example, both W_2W_3 and W_2E_3 *rover* stocks show no significant difference in pupation height in constant light (LL) compared to constant darkness (DD) (Table II). Each *sitter* stock shows differences in LL compared to DD. E_2E_3 *sitter* larvae pupated significantly lower in DD than in LL; E_2W_3 *sitter* larvae pupated lower in LL than in DD. From experiments 1–3, which were conducted under a 12L:12D light regime, we conclude that *sitters* (E_2E_3 and E_2W_3) pupate lower than *rovers* W_2W_3 and W_2E_3). It is possible that the pupation height measured in these experiments was, in part, the result of a "cumulative" effect of both LL and DD on this behavior.

However, it may be stated that under the specific experimental conditions used in these experiments, (1) there appear to be strong second-chromosomal affects on pupation height, (2) pupation height increases with larval density, and (3) there is a strong correlation between larval foraging patterns and pupation site choice in the stocks, indicating that *rover* larvae pupate higher than *sitter* larvae.

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