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Prepupation Behavior in *Drosophila*: Embedding

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A new D. Melanogaster *prepupation behavior, "embedding," is described. Prior to pupation, some larvae burrow through the agar and pupate at the end of the burrowed tunnel with the posterior end of their body embedded in the agar. Embedding behavior is studied in laboratory- and field-derived stocks under two light regimes and in two test dishes. The chromosomal constitution of the strains (in particular the third pair of autosomes) significantly affected differences in embedding behavior. Differences in embedding behavior were also affected by light regime but not by test dish.*

KEY WORDS: prepupation behavior; *Drosophila melanogaster*; embedding; chromosomal analysis.

INTRODUCTION

There are many laboratory studies of pupation site preference in *Drosophila* (Sokolowski, 1984; Sokolowski and Hansell, 1983; Ringo and Wood, 1982; Manning and Markow, 1981; Markow, 1979; for a review see Grossfield, 1978; Barker, 1971; de Souza *et al.*, 1970; Mensua, 1967; Sameoto and Miller, 1968; Sokal *et al.*, 1960). When *Drosophila* are reared in culture vials, larvae often pupate on the walls of the vials. Pupation height is measured as the distance from the top of the medium to the pupa. In many laboratories, differences in pupation site choice are measured as differences in pupation height.

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Sokal *et al.* (1960) and Sokolowski and Hansell (1983) showed that both genetic and environmental parameters influence differences in *D. melanogaster* pupation height. Pupation height differences are most likely under polygenic control (Markow, 1979; Sokolowski, 1984). Pupation height can be affected by density (Sokolowski and Hansell, 1983), moisture (Sameoto and Miller, 1968), temperature (Mensua, 1967), and light (Markow, 1981; Manning and Markow, 1981; Sokolowski and Hansell, 1983).

Sokolowski (1980, 1982a) identified a behavior genetic polymorphism in the locomotor component of larval foraging behavior in both laboratory and natural populations of *D. melanogaster*. In the present study, the same laboratory populations are used. Larvae of the "rover" morph have long foraging path lengths and traverse a large area compared with larvae of the "sitter" morph, which have relatively short path lengths and traverse a small area while foraging on a moist homogeneous food supply. A correlation between larval digging behavior and pupation height in these morphs was reported by Sokolowski (1982b) and Sokolowski and Hansell (1983). Rover larvae pupated higher and dug deeper into the medium than did sitter larvae. The second pair of autosomes contributed significantly to all of these preadult behaviors.

These larval foraging behaviors were measured in early third-instar larvae. At some time in the mid to late third instar, *Drosophila* larvae switch from food-related activities (foraging) to prepupation activities (wandering). This switch in motivation with respect to food can be quantified by measuring the tendency for a larva to remain on the feeding substrate (Sokolowski *et al.*, 1984). Larval behavior in the wandering phase culminates in a choice of pupation site.

The present study examines a new pupation site behavior observable when *Drosophila melanogaster* larvae are cultured in the following rearing environment. This environment consists of a weighed plug of nutrient medium placed on a layer of nonnutrient agar in a petri dish. Wandering larvae either traverse the surface of the agar and pupate or tunnel down into the agar. Larvae that tunnel usually embed their pupae (their posterior portion is below surface of the agar). This behavior is called embedding (Fig. 1).

The present study was designed to determine whether there were strain differences in embedding behavior and whether these differences were consistent over several different environments. Strains of *D. melanogaster* known to differ in larval foraging behavior and pupal height were used. Field strains which were derived from pupae found in different pupal microhabitats were also tested for embedding behavior.

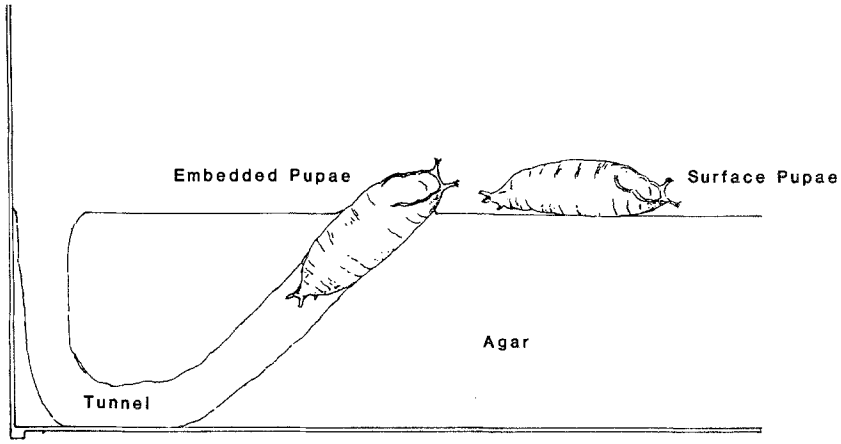

Fig. 1. The two phenotypic classes scored, embedded and nonembedded (surface) *Drosophila melanogaster* pupae. Prior to pupation some larvae tunnel into the agar and pupate at the end of the tunnel with their posterior end embedded in the agar.

MATERIALS AND METHODS

Laboratory Strains

Four strains isogenic for the second and third pairs of chomosomes used in this study were designated WW, WE, EE, and EW. The first letter of the two-letter designation denotes the second pair of chromosomes, whereas the second letter denotes the third pair of chromosomes. EW has the same second chromosome pair as EE but differs in having the same third chromosome pair as WW. These stocks were constructed by utilizing a standard breeding scheme that uses the presence of crossover suppressors to permit the substitution of intact second or third pairs of chromosomes from one strain to the other. A description of this technique and a detailed description of these stocks is given by Sokolowski (1980). Strains WW and WE were found to have a rover larval forager type and high pupal heights. Strains EE and EW have a sitter larval forager type and lower pupal heights (Sokolowski, 1980; Sokolowski and Hansell, 1983).

Field Strains

In the fall of 1983, *Drosophila melanogaster* pupae were collected from the following four microhabitats in a pear orchard: (1) on the upper surface of the fruit, on the skin; (2) on the lower surface of the fruit, on the skin; (3) under the fruit, on the ground; (4) under the fruit, in the

ground. Four strains of flies were derived from these pupae. They were called M1, M2, M3, and M4, respectively. Within 1 month (two generations) these four field strains were tested for the tendency of their larvae to embed their pupae. This was done in order to determine whether different larval prepupation behaviors in the field were related to differences in embedding behavior in the laboratory.

Rearing Environment

To determine the effect of the rearing environment on embedding behavior, two different test dishes were used, a large dish and a small one. The large dish (a 13.5-cm-diameter and 2.2-cm-high covered plastic petri dish) was filled to a depth of 0.5 cm with hot Sigma agar (prepared by combining 8 g of agar with 500 ml of distilled H_2O, boiling the solution for 5 min, then adding 5 ml of tegosept, a mold inhibitor). The agar was then flamed to eliminate bubbles, thereby ensuring a smooth surface texture for larval locomotion. A plug (2.5 cm in diameter and 0.5 cm high) of Parker's medium (a dead yeast agar medium) was placed on the surface of the agar and positioned in the center of the dish after the agar had cooled. The small dish (8.5 cm in diameter and 2.4 cm high) was prepared in a similar fashion to the large dish except that the food plug was 1.8 cm in diameter and 0.5 cm high (weighing 1.3 ± 0.05 g). A Kimwipe was placed under the lid of the small dish to control for condensation. All test dishes were placed on a black background.

Placing Larvae in Test Dishes

Two methods were used to seed the large and small test dishes with larvae. For the large dish, 25 early third-instar larvae (72 ± 2 h posthatching) were placed, using a paintbrush, onto the center of the food plug. For the first 72 h these larvae were cultured in a 8.5-cm-diameter by 1.4-cm-high covered petri dish filled to a depth of 0.5 cm with Parker's medium. In each dish, 100 larvae were grown at 24 ± 1°C under conditions of 12 h of light followed by 12 h of darkness (lights on at 0800 h) for 3 days. At this time, these third-instar larvae were exhaustively sampled from the culture dish and 25 larvae were randomly assigned to each large test dish. In the case of the small test dish, 25 first-instar larvae collected within 3 h of hatching [using a standard procedure (Sokolowski *et al.*, 1984)], were placed using a dissecting needle onto the center of each food plug. After all larvae had pupated, the phenotype of each pupa was scored.

Scoring the Behavioral Phenotype

An embedded pupa is the result of late third-instar larval behavior. The larva leaves the food and burrows into the agar, thereby leaving a visible tunnel. The larva pupates at the end of the tunnel with the posterior end of its body embedded (Fig. 1). The test dish enables one to score whether a pupa is embedded and witness the remaining tunnel in the agar. The pupae in the agar layer were clearly visible and could be unambiguously scored. All test dishes remained mold and bacteria free throughout these experiments.

Experimental Design

In the present study the effect of strain (the second and third chromosome contribution), the effect of rearing environment (large and small test dishes), and the effect of two light regimes (0:24 L:D or 12:12 L:D) on embedding behavior were studied. Each of the following treatments was replicated on 2 days (day effect).

I. Four large test dishes of each of the four strains (WW, WE, EE, and EW) were used in each of the two light regimes.

II. Four small test dishes of each of the four strains were used in each of the two light regimes.

The two dishes of all strains were randomly placed in an incubator at 24 ± 1°C with two fluorescent lights positioned 30 cm from the surface of the dishes. For the constant-dark treatment the test dishes were placed in the same incubator and covered with two layers of light-tight black felt.

The embedding behavior of the field strains was studied in the small test dishes. Five replicates for each of the four field-derived populations were tested simultaneously in each of the two light regimes. The rearing and testing procedures were identical to those used for the laboratory strains.

RESULTS

Pupae were scored on the food, on the agar, or embedded in the agar. For pupae off food, the proportion embedded was calculated. These proportions were transformed using an arcsin square-root transformation corrected for small sample size (Bishop *et al.*, 1975).

$$X = 0.5(\arcsin \sqrt{n_e/n_t + 1} + \arcsin \sqrt{n_e + 1/n_t + 1}),$$

where n_e is the number of embedded pupae in the dish and n_t is the total number of pupae off the food plug.

Table I. Transformed (Arcsin Square Root) Mean Proportion of Embedded *Drosophila melanogaster* Pupae

	Large dish		Small dish	
Strain	Day 1	Day 2	Day 1	Day 2
12:12 L:D				
WW	1.07	1.03	0.97	1.26
EW	0.95	0.92	1.23	1.27
WE	0.41	0.31	0.79	0.74
EE	0.77	0.94	0.75	1.06
0:24 L:D				
WW	0.72	0.65	0.59	1.03
EW	1.03	0.89	0.92	0.96
WE	0.54	0.44	0.54	0.62
EE	0.82	0.94	0.89	0.80

The transformed data were analyzed by fitting a linear normal model with fixed and random effects [nested mixed-model ANOVA (Sokol and Rolf, 1969)]. The fixed factors were strain (decomposed by second and third chromosomes), light regime (light), and larval test dish (environment). The random factors were the day the experiment was set up (day), which was nested within light and environment, and the replicate dishes (replicates).

The transformed means for each fixed treatment are shown in Table I. The linear normal model used assumes that the variance around the treatment means is uncorrelated with, and equal for, each treatment. The sum of the squared normalized deviations within treatments should therefore be distributed as a chi-square with 3 degrees of freedom. The largest of the 32 test statistics is 8.52, which is not significant (for $N = 32$).

Figure 2 is a plot of the ranked observed test statistics against those expected from a chi-square with 3 degrees of freedom. The dashed line represents the expected relationship. This indicates that the distribution of our within-treatment deviations is consistent with the assumptions of the model. Therefore, the parameters of our mixed-model ANOVA were fitted. The results are shown in Table II. Both the days and the strain-by-days variance components were significant. This necessitated testing the effects of light and environment against the days component, as well as all effects involving strain with the strain-by-days component.

In Table II, significant fixed effects are associated with all three strain related contrasts. Ninety percent of the variance between strains is explained by differences in the second and third chromosomes. The only other significant effect is a third chromosome-by-light interaction. On the

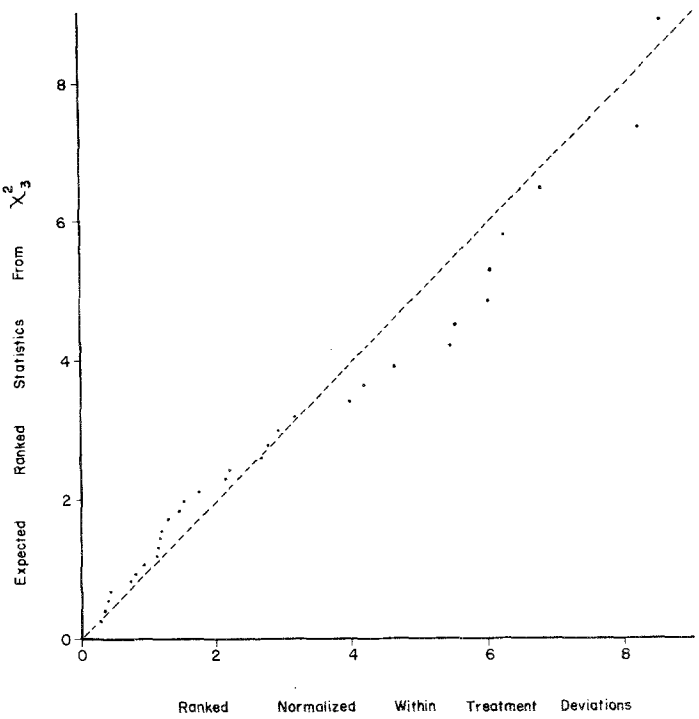

Fig. 2. A plot of the ranked observed test statistics against those expected from a chi-square with 3 degrees of freedom. The dashed line represents the expeted relationship.

average, strains with a W pair of third chromosomes embed less under a 0:24 L:D cycle than under a 12:12 L:D cycle. The effect of the rearing environment (small and large test dish) was not significant, nor were any interaction components involving environment.

The embedding behavior of the four field-derived strains was tested in the small test dishes under the two light regimes. There was no simple relationship between the pupal microhabitat of the four strains and their tendency to embed. Strains M1 and M3 showed the highest levels of embedding behavior in both light regimes. An ANOVA which tested the effect of strain, light, and strain by light on embedding behavior indicated a significant strain effect [$F_{(3,16)} = 3.48$, $P < 0.05$] and a highly significant light effect [$F_{(1,16)} = 11.18$, $P < 0.001$]. There was no significant strain-by-light interaction [$F_{(3,16)} = 0.12$, $P > 0.05$]. All of the field strains showed a similar decrease in embedding behavior under conditions of constant darkness.

Table II. ANOVA of the Effect of Strain (Second and Third Chromosome), Day, Light, and Test Dish Environment on the Embedding Behavior of *Drosophila melanogaster* Larvae

Source	df	MSL	$F^{(m,n)}$		$P <$
Strain	3				
2nd		1.466	14.37	(1, 12)	0.005
3rd	1	2.144	21.01	(1, 12)	0.005
2 × 3	1	0.374	6.84	(1, 12)	0.025
Days	4	0.0767	2.85	(4, 96)	0.050
Strain × days	12	0.0545	2.02	(12, 96)	0.050
Light (l)	1	0.5349	6.97	(1, 4)	NS
Strain × light (s × 1)	3		3.21	(3, 12)	
2 × 1	1	0.0836	1.53	(1, 12)	NS
3 × 1	1	0.3760	6.89	(1, 12)	0.025
2 × 3 × 1	1	0.0665	1.22	(1, 12)	NS
Environment (e)	1	0.4976	6.49	(1, 4)	NS
Strain × environment	3		1.55	(3, 12)	
2 × e	1	0.0723			NS
3 × e	1	0.0007			NS
2 × 3 × e	1	0.1822	3.34	(1, 12)	NS
Light × environment	1	0.2267	2.95	(1, 4)	NS
Strain × light × environment	3				
2 × 1 × e	1	0.0119	0.22	(1, 12)	NS
3 × 1 × e	1	0.0086	0.16	(1, 12)	NS
2 × 3 × 1 × e	1	0.1854	3.39	(1,12)	NS
Error	96	0.0269			

DISCUSSION

The purpose of this study was twofold: first, to find out if differences in the prepupation behavior that we call embedding had a genetic component and, second, to determine how consistent strain differences in embedding behavior were when larvae were cultured in different larval rearing environments.

There appears to be a genetic component to differences in embedding behavior. In 14 of 16 comparisons, strains with a W third pair of chromosomes showed more embedding than strains with an E pair of third chromosomes. Differences in embedding behavior were also affected by light. All differences in embedding behavior could not be related to chromosomal composition. The phenotypic score for embedding behavior is robust for different rearing environments (large and small test dish) relative to differences between strains (Table II).

There was a significant effect associated with differences in light regimes in laboratory strains with a W pair of third chromosomes. Under conditions of constant darkness, the strain WW showed the largest decrease in the phenotypic score for embedding. In the field-derived stocks

larvae reared in constant darkness also showed less embedding behavior than in 12:12 L:D.

Larval forager type, pupal height, and larval digging behavior have been related to the second pair of chromosomes in these laboratory strains (Sokolowski, 1984). The third pair of chromosomes had the largest effect on embedding behavior. Embedding behavior is distinctive and well defined in these preparations. Variability in embedding behavior is probably due to processes that come into play before the larva starts tunneling. Larvae probably process information from the environment which influences whether they tunnel or not. Variability in embedding behavior would then be a measure of variability in this process.

Embedding behavior is strain dependent. Differences in embedding behavior indicate genetic components to this process. Strain-by-environment (light) interactions indicate that the effects of changes in the environment on this process are not constant or relatively proportional across strains.

We have shown that the phenotypic score for embedding behavior is less affected by different rearing environments than it is by chromosomal differences between strains. There is a strong genetic component to this score when measured in a fixed environment on a single day (i.e., error term, Table II). This is the analysis usually followed in order to produce the largest estimates of the genetic contribution to phenotypic variability. If, however, this is the only measure of variability, then it is difficult to extend the results obtained in the laboratory to predictions about the behavior in the field. Variation in embedding behavior is not found only in laboratory stocks; strains derived from different field microhabitats showed variation in embedding behavior.

Why might variations in embedding behavior be maintained in natural populations? Embedding behavior may change the probability of pupal dessication and may also insulate a pupa from temperature fluctuations. It is also possible that embedding behavior is related to pupal parasitism pressure. Larvae that embed may be less parasitized by pupal parasitoids than nonembedded or exposed larvae. We are presently testing this hypothesis.

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