Original article

Analysis of larval behaviours underlying the pupation height phenotype in *Drosophila simulans* and *D melanogaster*

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Summary – Lines of *Drosophila melanogaster* and *D simulans*, previously selected for increased and decreased pupation height, have been investigated for larval correlated responses to selection. Larvae of all lines and species showed higher pupation sites when humidity increased from 40 to 100% RH. Pupation height measured in light and in dark revealed that only one out of the nine lines changed its phototactic behaviour during selection. In both species, the high pupation lines showed greater mobility than the corresponding controls. Selection for high pupation sites diminished the digging behaviour in *D simulans* but not in *D melanogaster*, whereas selection for low sites augmented the percentage of digging in *D melanogaster*. A different correspondence between pupation height and gravity response was found in each species. In *D simulans*, low lines were geopositive and high lines neutral, while low lines were neutral and high lines geonegative in *D melanogaster*. The results indicate that pupation height is a complex trait determined by other simpler behaviours, so that a given phenotype can be produced by different genetic systems. This question stresses the difficulty of deducing, on biological grounds, the meaning of the genetic architecture of a complex behavioural trait whose underlying basic mechanisms are unknown.

artificial selection / complex behavioural trait / correlated response / larval pupation height / *Drosophila*

Résumé – Analyse des comportements larvaires pour la hauteur de pupaison chez Drosophila simulans et D melanogaster. *Les réponses larvaires liées à la sélection ont été analysées dans des lignées de* Drosophila melanogaster *et de* D simulans *préalablement sélectionnées pour l'augmentation ou la diminution de la hauteur de pupaison. Les larves de toutes les lignées et espèces ont eu des sites plus élevés de pupaison quand l'humidité relative a augmenté de 40 à 100 %. La hauteur de pupaison mesurée à la lumière ou à l'obscurité a montré que seulement une des neuf lignées a changé son comportement phototactique pendant la sélection. Dans les deux espèces, les sites de pupaison plus élevés ont correspondu à une plus grande mobilité des larves. La sélection pour les sites élevés de pupaison a diminué le comportement de creusement chez* D simulans *mais pas chez* D melanogaster, *tandis que la sélection pour les sites bas a augmenté ce comportement chez* D melanogaster. *On n'a pas trouvé le même rapport entre hauteur de pupaison et*

réponse à la gravité chez les deux espèces. Chez D simulans, *les lignées basses ont été géopositives et les lignées hautes ont été neutres, tandis que, chez* D melanogaster, *les lignées basses ont été neutres et les lignées hautes géonégatives. Les résultats indiquent que la hauteur de pupaison est un caractère complexe déterminé par d'autres conduites plus simples, de sorte qu'un phénotype donné peut résulter de différents systèmes génétiques. On doit insister sur ce point si l'on veut obtenir, à partir de considérations biologiques, l'analyse de l'architecture génétique d'un caractère de comportement complexe, quand les mécanismes de bases ne sont pas connus.*

sélection artificielle / caractère complexe de comportement / réponse corrélée / hauteur de pupaison larvaire / drosophile

INTRODUCTION

There exists today a lot of information describing various aspects of *Drosophila* activities in both larvae and adults (see Grossfield, 1978; Spieth and Ringo, 1983). Most of the activities can be described as behaviours and in this sense have been studied from a genetic perspective. In some cases, however, the studies are unable to reveal the genetic basis underlying a specific behaviour, particularly when a trait is the result of several single behaviours (eg, taxes) acting in response to a set of stimuli simultaneously perceived by the organism.

We have studied in depth a pre-adult behaviour observed in *Drosophila melanogaster* and *D simulans*: the pupation behaviour. Once developing larvae reach the third stage, they leave the humid food searching for a dry site in which to pupate. In the laboratory, drosophila develop in glass vials with food at the bottom, and pupation occurs mostly on the vertical walls at varying heights. Pupation height is strongly affected by biotic and environmental variables, such as larval density (Sokal et al, 1960; Barker, 1971; Casares and Rubio, 1984; Casares and Carracedo, 1984a), light (Rizki and Davies, 1953; Markow, 1979; Manning and Markow, 1981), humidity (Sameoto and Miller, 1968; Casares and Carracedo, 1984b), etc, and also by genetic determinants (Markow, 1979; Ringo and Wood, 1983; Casares and Carracedo, 1986a, b; García-Florez et al, 1989). The interest of studying pupation behaviour in the laboratory is supported by the parallelism between the pupation sites observed in an orchard, and the corresponding height attained in the laboratory (Sokolowski, 1985; Sokolowski et al, 1985).

The choice of a pupation site in the laboratory has important fitness repercussions, since mortality is very high for pupae located in the humid food (Sameoto and Miller, 1968; Wallace, 1974; Casares and Rubio, 1984). In this sense, we have found that populations of *D melanogaster* prefer to pupate on the vial walls and rather higher than populations of the sibling, sympatric species *D simulans*, which prefer to pupate in the food or neighbouring sites. This species difference, which greatly affects their pre-adult fitnesses (Casares and Rubio, 1984), must be maintained in nature by some kind of natural selection, as deduced from two independent artificial selection experiments, in which we have been able to increase pupation height in *D simulans* and to increase or decrease it in *D melanogaster* (Casares and Carracedo, 1986b; García-Florez et al, 1989).

The lines obtained after artificial selection are useful material with which to investigate the behavioural bases of the pupation behaviour. What are the factors

or behaviours involved in the observed high and low pupation height phenotypes? In the first place, one could suspect that artificial selection could have changed the larva's perception of humidity in the high and/or low pupation height lines, since humidity is the main factor accounting for pupation height in the laboratory (Sameoto and Miller, 1966, 1968; Mensua, 1967; Wallace, 1974). Also, perception of light (usually placed above the animal) and gravity could have been modified by selection, in this way affecting the displacement of the larvae to the top or the bottom of the vials. Finally, larval locomotion could be important in pupation behaviour because larvae from the high pupation lines must travel a greater distance than those from the low lines to reach the highest places in the vials.

In the present work we have examined the influence of humidity, light and gravity on the larval behaviour of lines selected for high and low pupation sites. We have also examined whether selection has brought about correlated changes in two other well-known larval behaviours: digging (Godoy-Herrera, 1978) and foraging (Sokolowski, 1980).

MATERIALS AND METHODS

The material consisted of lines of *D simulans* and *D melanogaster* from two experiments of selection for high (H) and low (L) pupation sites.

In *D simulans*, selection for increasing pupation height was successful, giving rise to the high lines SH1 and SH2 (Casares and Carracedo, 1986b) with means of approximately 110 mm of pupation height. Response to selection for decreasing pupation height was not statistically successful. The two low lines, SL1 and SL2, had mean pupation heights of about 25 mm. Selection was accomplished using 36 or more mating pairs per line along eight generations. The inbreeding coefficient of the resulting selection lines was calculated to be less than $F = 0.084$ in the high lines (Falconer, 1989) and even smaller in the low lines.

In *D melanogaster*, selection succeeded in both directions (García-Florez et al, 1989), the pupation heights of the high, low and control lines being around 120, 4 and 15 mm, respectively. The two high and two low selection lines are called MH1, MH2 and ML1, ML2, respectively. The base population MC is also used here as a control. In this species, selection was carried out using at least 48 mating pairs per line during 11 generations, and inbreeding of the selection lines was calculated as $F = 0.076$ for the high lines and smaller in the low lines.

In all experiments detailed below, the culture medium was prepared by boiling bakers' yeast (20%), sugar (5%) and agar (1.4%) in water, and adding propionic acid (0.5%) as a mold inhibitor.

Experiment I: influence of humidity

Virgin flies from each line were collected immediately after eclosion, then sexed and aged separately for 3 days. On day 4, mature males and females were allowed to mate, then groups of 15–20 females were placed for 6 h in petri dishes containing a thin layer of food to lay eggs. Around 9 000 pairs of flies were used. From the dishes, 75 newly emerged larvae (± 2 h) were collected with a lancet and seeded in vials with food. Development was at 21 °C.

It has been found that the higher the larval density, the greater the tendency of food to liquefy and release water as larvae eat it (Sokal et al, 1960; Sameoto and Miller, 1968; Wallace, 1974; Casares and Carracedo, 1984a). Because the vials exchange water with the outside, humidity on the inside can be controlled in an approximate manner by using plugs of different size and texture (Mensua, 1967; Casares and Carracedo, 1984b).

In the present experiment, we established three different humidity levels – low, intermediate and high – as follows: we used vials 25 mm in diameter and 200 mm in length, with standard bakers' yeast food (25 mm in height). Low humidity was achieved using unplugged vials that facilitated the interchange of water with the climatic chamber in which all the vials were kept (40% RH); humidity in these vials was low, as deduced from the dryness of the vial walls during pupation. To achieve high humidity, some vials were plugged under pressure with foam dishes 35 mm in diameter, through which water interchange with the outside was difficult. During pupation in these vials, a thin layer of water covering the walls to a height of around 90 mm above the food was observed, suggesting that relative humidity inside the vials was close to 100%, a value which was similar to that observed in the selection experiments from which the lines originated. Intermediate humidity was accomplished by plugging vials with foam dishes 25 mm in diameter, the water layer attaining here around 15 mm in height and relative humidity supposedly being between 40 and 100%. Sixteen vials were used for each line and humidity.

Nine days after seeding, all larvae had pupated and the height of the pupae in the vials was measured with a transparent plastic cylinder that was laterally printed with marking ink into 10 mm divisions. The cylinder was externally attached to the vials, allowing a quick classification of pupae into different height classes (Casares and Carracedo, 1986a). The mean value of pupation height per vial was used as the raw measure in all statistical analyses.

Experiment II: influence of light

Cages ($40 \times 40 \times 30$ cm) made of wood (two sides) and glass (four sides) were used. A hole 8 cm in diameter was made in the two wooden sides. In half of the cages, the glass walls were perfectly sealed with matt black paper, so that total darkness was achieved inside. In the other cages, light penetrated through the glass. Forty-two vials (10×2 cm) per line were seeded with five first instar larvae following the procedure detailed in experiment I. Half of the vials were introduced through the hole into the lighted cage and the other half into the dark cage. The holes were plugged with dense foam cylinders. The cages were incubated side by side in a chamber at 21 °C with constant illumination. Pupation height was measured as in experiment I.

Experiment III: larval mobility

Adult flies from each line were sexed and aged as detailed earlier. Eight pairs of mature, 3-day-old virgin flies were put into a vial (12×2.5 cm) with food and allowed to mate and oviposit for 24 h and then discarded. Five days later, one larva of the third instar was carefully picked up, washed in distilled water for a few seconds, dried in absorbent paper and placed in the center of a petri dish (10 cm in diameter).

Previously, a thin layer of agar medium (1.2% agar in distilled water) slightly stained with methylene blue had been poured into the dishes. Larvae were left for 15 s to recover from manipulation. Mobility was measured in individual larvae by the number of forward and backward movements in 60 s following the methodology of Sokolowski (1980).

Observations were carried out under a stereomicroscope at 21 °C. Fifty *D simulans* larvae and 70 *D melanogaster* were scored from each line.

## Experiment IV: digging behaviour and effect of gravity

Third instar larvae were obtained and petri dishes prepared as described in experiment III, with the difference that a softer medium with 1% agar was used here to facilitate larval digging. Two tests were performed simultaneously. In one test, five larvae were placed in the centre of the dish, observed for 60 s and classified as diggers or non-diggers. Two hundred larvae per line were assayed. In the other test, all things were similar, except that once the five larvae were placed in the dish, this was inverted and put upside down under the microscope; in this case, observations were made through the bottom wall of the dish. One hundred larvae per line were measured in this test. Thus in one test digging was favoured by gravity, while in the other test the opposite was true.

RESULTS

Experiment I: influence of humidity

No differences in larval to adult viability and development time were detected between the three humidities. The two replications of each selection line in each humidity were compared by a Student's *t*-test. Since all comparisons were non-significant (data not shown), the two replications were pooled. The pupation height values so obtained are given in figure 1. In both species and in all lines, height was much greater at increasing humidities. Within each line, comparisons of pupation height between humidities were carried out by one-way analyses of variance (Sokal and Rohlf, 1981). All tests were significant (results are not shown), as could be expected from the large differences in mean values and the small standard errors that appear in figure 1. Not all lines responded to increased humidity with the same intensity. To prove this, we carried out a two-way analysis of variance with lines and humidities as the sources of variation. As expected, the line × humidity interaction was significant (MS interaction = 1 996.7 with 16 df; MS error 61.2 with 405 df; $F = 32.6$, $P < 0.001$).

This experiment shows that larvae of both species perceive and respond to changes in humidity regardless of their having a phenotype for high or low pupation sites. Although between-line differences were generally greater at the highest humidity, differences persisted at a relative humidity as low as 40% indicating that differences in pupation height are not due merely to the ability to perceive and respond to high humidities.
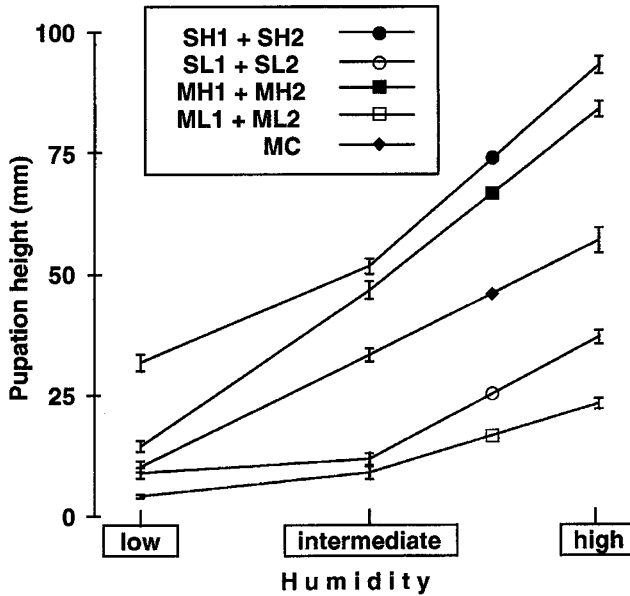
Fig 1. Means and standard errors of pupation height of the different lines at low, intermediate and high humidities. The values of the two replicated lines have been pooled. Each value is based on 32 observations except for the MC line (16).

Experiment II: influence of light

Figure 2 shows the height attained by the larvae in light and darkness. Pupation height was almost unaffected by light. Only one out of the nine lines (the ML1 line) showed a statistically different height in the two environments (t-test $= 3.74$; df $= 40$; $P < 0.001$). This result strongly contrasts with that of Markow (1979), who stated that most populations of *D melanogaster* and *D simulans* exhibit higher pupation sites in dark conditions. On the other hand, Schnebel and Grossfield (1986) found that *D melanogaster* pupated higher in the light and that *D simulans* was unaffected by this factor. The discrepancies between these authors and between their results and ours are probably due to the use of different methodologies or strains. Markow used different chambers for measuring pupation height under dark and light conditions. Whether humidity, which is undoubtedly the main factor affecting pupation height (Sameoto and Miller 1966; 1968; Mensua, 1967; Wallace, 1974; Casares and Carracedo, 1984b), was the same in the two chambers is unknown. The same problem might apply in the Schnebel and Grossfield methodology, for these authors used the same chamber for measuring the two conditions but darkness was accomplished in a box inside the chamber with the consequent risk of a difference in humidity. In contrast, as described above, our light and dark environments were produced simultaneously, in the same type of chamber, at the same temperature and especially with the same humidity level.
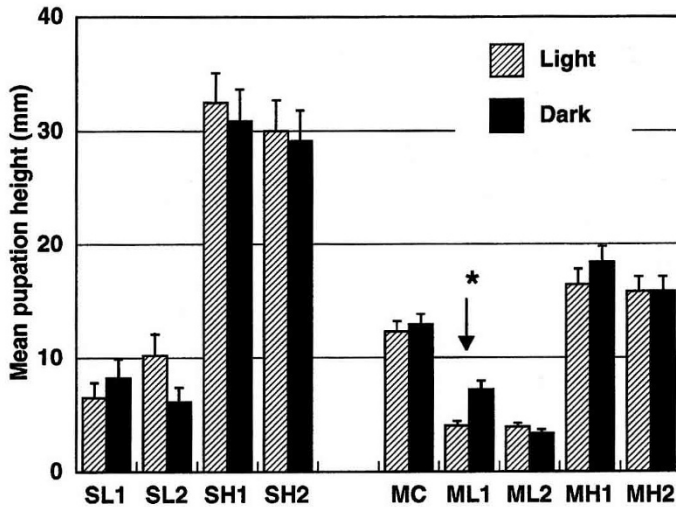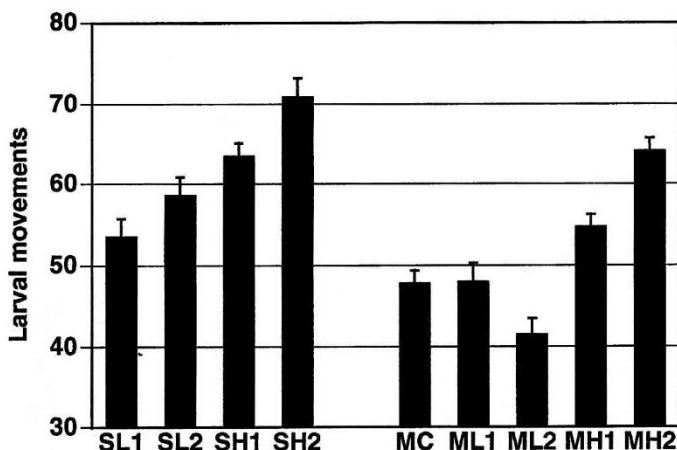
Fig 2. Means and standard errors of pupation height of the different lines measured under light and dark conditions. Comparisons between the two conditions were carried out for each line by *t*-tests. The asterisk indicates significant differences under the two conditions at the 5% level.

The conclusion of this experiment is that, with the exception of the ML1 line, the larvae of the selection lines have a similar phototactic response during pupation. Therefore, the observed between-line differences in pupation height are independent of the presence or absence of light. Concerning the ML1 line, the response to selection for low pupation height was due, at least partly, to indirect selection for a diminished phototactic behaviour.

Experiment III: larval mobility

The means and standard errors of larval mobility of the different lines and species are depicted in figure 3. One-way analyses of variance demonstrated significant between-line differences in both species (MS between $= 2\,739$ with 3 df; within $= 237$ with 196 df; $F = 11.56$; $P < 0.01$ for *D simulans*; between $= 5\,178$ with 4 df; within $= 245$ with 345 df; $F = 21$; $P < 0.01$ for *D melanogaster*).

The between-line differences found, although significant, are not so great as those found in the 'rover-sitter' larval polymorphism described by Sokolowski (1980), who found that the crawling scores for sitters and rovers were around 35 and 140, respectively, in 6 min of observation. Since our scores are between 40 and 70 in only 1 min, it is clear that all our lines display the rover behaviour, although this result must be taken with caution as our substrate differs from that of Sokolowski in composition and density. However, Bauer and Sokolowski (1984) state that classification of larvae into only two mobility classes (rover/sitter) is an oversimplification that does not correspond with the mobility scores found in natural populations.

Fig 3. Means and standard errors of the number of forward and backward larval movements in 60 s found in the different lines. Values are based on 50 and 70 observations in the *D simulans* and *D melanogaster* lines, respectively.

A contrast between the high and low lines of *D simulans* by Scheffe's method (Sokal and Rohlf, 1981) gave a significant result (contrast $\pm 95\%$ confidence limits: 11.15 ± 6.14) and the same result for *D melanogaster* when comparing the control and the two high lines (13.18 ± 7.10). Therefore, the high pupating lines of both species show greater mobility than the corresponding controls. This is an important result because larvae pupating in the highest sites of the vial must travel a longer distance than those pupating in the lowest ones, which suggests that locomotion could play a significant role in this larval displacement. The same applies to the comparatively small mobility showed by the low pupation line ML2.

Experiment IV: digging behaviour and effect of gravity

The percentages of diggers of the different lines are shown in figure 4. It is clear that, in *D simulans*, digging was more frequent in the low than in the high lines: a STP procedure for homogeneity (Sokal and Rohlf, 1981) applied to the four percentages of digging in normal food gave the following:

SH2   SH1   SL1   SL2

where two lines not joined by a straight line are different at a 5% or lower probability level. Thus, selection for increasing pupation height in *D simulans* was accompanied by a clear decrease in the number of digger larvae.

In contrast, the digging behaviour of the high lines of *D melanogaster* was more similar to the control line than that of the low lines:

MH1   MH2   MC   ML2   ML1
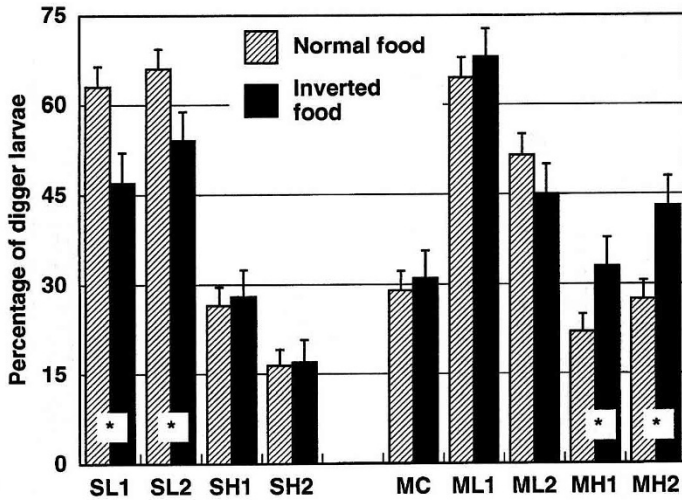
Fig 4. Average percentages of digger larvae observed with food below (normal food) or above (inverted food) the animal in the different lines. Comparisons between the two conditions were carried out by 2×2 contingency chi-squares. The asterisks indicate significant differences under the two conditions at the 5% level. The lines above the bars correspond to binomial errors.

The analysis now shows how selection for high pupation in *D melanogaster* did not modify digging, whereas it was increased when selection was for low pupation sites. This species difference in the selection response suggests that both species have been subjected to different selection pressures on digging behaviour in the wild.

In summary, artificial selection for high pupation sites decreased the digger trait in *D simulans* but not in *D melanogaster*. In this species, however, selection for low pupation height was accompanied by an increase in digging. A common fact is that in both species the high pupating lines show less digging than the low pupating ones, a result that agrees with data of Godoy-Herrera in *D melanogaster* (1978) and in *D pavani* and *D gaucha* (1986).

Another species difference appears when comparing, by contingency chi-squares, the percentages of digging achieved in food placed under or above the animal. In *D simulans*, the number of diggers decreased in the two low lines when food was above the animal ($\chi^2 = 6.99$; df $= 1$; $P = 0.008$ for SL1; $\chi^2 = 4.07$; $P = 0.04$ for SL2; see fig 4). This means that larvae are geopositive, as digging is favoured if food is placed under them. In *D melanogaster*, the differences affect the two high pupating lines ($\chi^2 = 4.22$; df $= 1$; $P = 0.04$ for MH1; $\chi^2 = 7.28$; df $= 1$; $P = 0.007$ for MH2), in which food inversion increases the number of diggers. Here this is interpreted as larvae being geonegative. The remaining lines have the same behaviour in normal and inverted food, which argues that their larvae are neutral with respect to gravity.

Interestingly, a characteristic trait of *D simulans* populations is the tendency of their larvae to pupate in the food, which suggests that their larvae are geopositive. In

clear contrast, populations of *D melanogaster* pupate much higher, which suggests they are geonegative or neutral. It seems that in the selection experiments that gave rise to the lines examined, selection for high pupation sites in *D simulans* eliminates the geopositive tendency present in the base population and in the low lines, whereas selection for low pupation sites in *D melanogaster* was accompanied by selection of geopositive larvae.

DISCUSSION

The study of behavioural traits, because of their singularity, requires researchers to apply careful control of environmental variables and to perform well-designed experiments that allow objective measurements (Ehrman and Parsons, 1976). Another question to consider could be the complexity of a trait. Most animal behaviours, before and/or during their manifestation, are influenced by a battery of stimuli that are simultaneously perceived and processed by the organism. When examining a given behaviour, the question is whether the experimenter is measuring only one well-defined behaviour or whether the response of the animal is the result of a complex mixture of several other simpler behaviours. An example is offered by *Drosophila* geotaxis. After 181 generations of selection for negative geotaxis in the laboratory, Murphey and Hall (1969) found that the flies had also been selected for resistance to starvation and/or desiccation in the maze, reduced locomotor activity and low claustrophobia levels. Thus an apparently simple behaviour influences other undetected behaviours that affect the final measure.

The above question is of great importance if one is concerned with the genetic basis of a complex behavioural trait. If the trait is based on other simpler behaviours or taxes, its genetic basis could be difficult to determine since any analysis would in fact be the analysis of several traits possibly inherited to different extents (Hay, 1985). On the other hand, if different laboratories use different apparatus or achieve different control of the environmental variables affecting the underlying simpler behaviours, different genetic architecture could be found by different investigators. In studying phototaxis in *Drosophila*, Rockwell and Seiger (1973) conclude that there is no a priori reason to suppose that one particular design is better to measure 'true' wild phototaxia. That is to say, different environments could reveal different genetic systems.

In the present work we have found several correlated responses to selection for pupation height. Henderson (1989) suggested that differences between high and low selection lines for traits other than the originally selected could be attributed to genetic drift instead of to a genetical relationship with the selected trait. As previously indicated, our selection lines were not exposed to severe drift, and their inbreeding coefficient values have been estimated to be under 10%. On the other hand, our hypotheses for a causal relationship between pupation height and the traits examined in the present work are reasonable, and for some of them we have found strong experimental support in the sense indicated by Henderson (1989), that is, predictions are confirmed by the differences exhibited by the high and low lines in both *D simulans* and *D melanogaster* species. For instance, an increase in locomotion was detected in four of the eight selected lines, and these were precisely the high pupation lines.

Therefore, we can conclude that larval pupation height is a complex behaviour influenced by, at least, humidity, locomotion, digging behaviour, and gravity. Thus the larvae with higher humidity sensitivity pupate higher, as well as those having greater locomotion. It is also evident that selection has modified the digging behaviour of some lines through changes in gravity perception by the larvae; thus, *D melanogaster* larvae showing high pupation are geonegative, whereas low pupating larvae of *D simulans* are geopositive. Owing to the mixed influence of these factors, it is not too surprising that selection lines showing the same pupation height phenotype had been selected for different levels of locomotion, gravity, etc. In this way, different genotypes attained through modifications of very different genetic systems produce similar pupation height phenotypes. This conclusion stresses the need for caution when interpreting the results of genetic analyses performed on complex behavioural traits whose underlying basic mechanisms are unknown. It is clear from our results that depending on the larval behaviour more directly involved in the observed pupation height in the laboratory, the genetic analyses would reveal different genes.

Finally, it is common to infer, from the results of a genetic analysis, the type of natural selection that has acted in the past on the population (Broadhurst and Jinks, 1974). In this sense, general conclusions drawn from a single population about the nature of differences in a complex trait, such as pupation height, can be misleading if natural populations have variation for different behavioural components, and each author is measuring different things.

ACKNOWLEDGMENT

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