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Behavior-genetic analysis of *Phormia regina*: Conditioning, reliable individual differences, and selection

(classical conditioning/Dipteran learning/replication study/test reliability/proboscis reflex)

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ABSTRACT **Using proboscis extension (unconditioned response) to sucrose (unconditioned stimulus), individual blowflies (*Phormia regina*) were classically conditioned to saline and to water (conditioned stimuli) with sensitization controls, thus providing unique, independently replicated evidence both of learning in Diptera and of reliably measured individual differences. Directional and stabilizing selection have bred high and low performance lines markedly different from an unselected control line as a step in the analysis of behavior-genetic correlates. This replicates and extends previous selection analysis with improved conditioning technique. Also, some unwarranted claims of learning in Diptera are discussed.**

Many investigators have long sought a species appropriate for behavior-genetic analysis, i.e., one suitable for separate and simultaneous analyses of the behavioral systems responsive to experience, by the methods of psychology and ethology, and of the relevant biological systems, by the methods of genetics, physiology, and biochemistry. The kind of behavior that has been particularly refractory to such analysis is learning or conditioning, especially in an "appropriate" species. There seems to have been a consensus on the advantages of *Drosophila melanogaster* as the species of choice (1, 2), but there is a history of failures throughout the Diptera to substantiate claims of successful conditioning (3). The recent claims by two groups (4–7) still await independent replication and detailed analysis. One of these is presently being studied in our laboratory using the original apparatus kindly provided by H. Ch. Spatz. A more recent study (8) criticizes the inadequacies of the earlier work and reports conditioning of color vision discrimination, but as a property of populations not of individuals.

Even though the Benzer group (4) discussed habituation and cited the studies by Manning (9) and by Yeatman and Hirsch (3), their claim of successful conditioning is invalidated by their own control observations, which fail to support, and in fact contradict, their interpretation. The analyses in those earlier studies remain valid (ref. 3, pp. 454–455): "Manning (1967) has made the valuable suggestion . . . (which is) the test necessary to distinguish between the alternative interpretations of habituation and conditioning. Such a test would involve running for a second trial those flies choosing the odour on the first trial. A conditioning interpretation predicts that those flies initially choosing an odour would choose it again on the second trial. If only habituation . . . were involved, the choice on the second trial might be random." In none of the recent reports did *Drosophila*, which had met criteria on a test trial, perform any better on a retest than did those that had failed to meet criteria on the test. Despite the statement on p. 708 of ref. 4, ". . . it is . . . an individual rather than a collective property of the flies," there was *no* individual learning. On the other hand, it was correctly reported on p. 711 of ref. 4 that "There is no evidence for an 'intelligent' subset." The behavior observed was unstable and their evidence was unequivocal: individual differences in behavior were unreliable (4).

The discussions of *Drosophila* (ref. 2, p. 1112) advocate using "an inbred strain and isolat(ing) mutants" as a better method than starting "with a genetically heterogeneous pool . . . (and doing) progressive selection." Also, it is stated (ref. 4, p. 708) that "Many flies of identical genotype are readily produced, so that behavioral measurements can be made on populations rather than individuals, yielding instant statistics." However, it appears likely that the reported observations might never have been made on "flies of identical genotype." First, the Canton Special strain has not been maintained by any of the usual rigorous *Drosophila* inbreeding regimens, which involve mating individuals of known close family relationship. Canton Special is a laboratory-maintained, free-mating, wild-type strain and, as Sewall Wright pointed out long ago (ref. 10, p. 174), "matings more remote than between first cousins are of virtually no significance as inbreeding." Furthermore, Clayton and Paietta (11) have shown that *D. melanogaster*, maintained in the laboratory over 47 years—the Oregon-R line—can have more trait relevant (additive) genetic heterogeneity than a conspecific wild population from a natural habitat. Second, irrespective of inbreeding status, certainly the use of a "potent mutagen" (ref. 2, p. 1112) will produce mutations throughout the genome and thus introduce additional genetic heterogeneity (heterozygosity). Therefore, until unambiguous evidence on the genetic status of the Canton Special strain is forthcoming, we should not assume its flies to be "of identical genotype." On the other hand, Médioni and Vaysse (12) have sibmated the Basel-06 strain for 70 generations and waited until generation 40 of inbreeding to begin their study of conditioned behavior.

Selection, however, is a powerful method for analyzing populations and their attributes. It operates through the phenotype (presently, behavior and its components) and articulates the genetic architecture. Response to selection provides immediate evidence for behavior-genetic correlates.

Building upon the careful analyses of responses and conditions in the blowfly *Phormia regina* by Dethier *et al.* (13, 14) and working with individual animals of this species, Nelson (15) has demonstrated classical conditioning and reliable measurement of individual differences in response to the training procedures. Furthermore, her claim for the Diptera has withstood the scrutiny of both independent replication and detailed analysis in a selective breeding study validating genetic cor-

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Abbreviations: CES, central excitatory state; CS, conditioned stimulus.

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relates for the foundation population distribution of individual differences (16).

Nelson showed (15) that an unconditioned proboscis extension response, elicited by a 0.5 M sucrose stimulus applied to the labellar lobes, can be conditioned to a neutral stimulus of either distilled water or 1 M saline applied to the front tarsi if control is included for sensitization, known as the central excitatory state (CES). The conditioning procedure with CES control consists of 15 trials presenting in succession three stimuli, the first two being neutral (CS_1 and CS_2) and the third unconditioned, with temporal contiguity but not overlap between CS_1 and CS_2 (each presented for 4 sec) and partial overlap between CS_2 and unconditioned stimulus (presented during the fourth and last sec of CS_2) and a 10-min intertrial interval. Two neutral stimuli, rather than one, permit CS_1 to drain off responses resulting from CES produced by the unconditioned stimulus and CS_2 to measure the strength of the conditioned association. The 10-min intertrial interval permits the CES to wane. Flies, deprived of food and water for 3 days from eclosion, were mounted by depressing their partially opened wings into tackiwax-coated paraffin cylinders on wooden applicator stocks. Over the 15 reinforced trials, the proportion of flies responding with proboscis extension increased markedly to CS_2 but not as much to CS_1. The first seven trials were considered training and the last eight were used as the test, in which individual differences were measured by three categories of conditioned response score: good (6, 7, or 8), fair (3, 4, or 5), and poor (0, 1, or 2). In addition to the foregoing, Nelson performed six control experiments. The flies observed in these studies were captured and held in the experimenter's hand during transfer from living cages to experimental mounting. They were never anesthetized. The observations presented here confirm and extend previous work and report the initial steps in both genetic and phenotypic component analysis.

MATERIALS AND METHODS

The Nelson paradigm (15) was used with the following modifications. Each fly was introduced into a disposable plastic micropipet tip from which the end had been cut off, leaving a hole just large enough for the fly's head and forelegs to protrude. The fly was eased forward into position (head and forelegs protruding) with the soft bristles of a paintbrush (Fig. 1). This arrangement was mounted on a wooden rod, and 10 such mountings were placed in a holding rack and suspended horizontally about 36 cm above the surface of a table at which the experimenter was seated. This improved restraining technique has the advantage of preserving the wings (used later in mating) and appears to be less stressful. Two test tubes of 100-mm depth and 13-mm diameter containing CS_1 and CS_2 (distilled H_2O and 1.0 M NaCl), respectively, were taped together and raised in the experimenter's left hand so that the fly's front tarsi were immersed in CS_1 for 4 sec and then immediately in CS_2 for the next 4 sec. During the fourth and last sec of CS_2 presentation, the unconditioned stimulus (a drop of 0.5 M sucrose) was touched to the labellum with a capillary tube by the experimenter's right hand. Whereas in previous work a fly was moved to and from the stimuli and the resting position, now an immobile fly can be stimulated, thus eliminating several irrelevant sources of excitation. In one 2.5-hr session, 10 flies were trained and tested over 15 trials with a 10-min intertrial interval. The 4-sec stimulus intervals were timed with a metronome and the 10-min intertrial intervals with a stopwatch. Observations were made in controlled environment chambers at 25° and 50% relative humidity under overhead fluorescent lighting, usually early afternoon near the peak of the activity cycle.

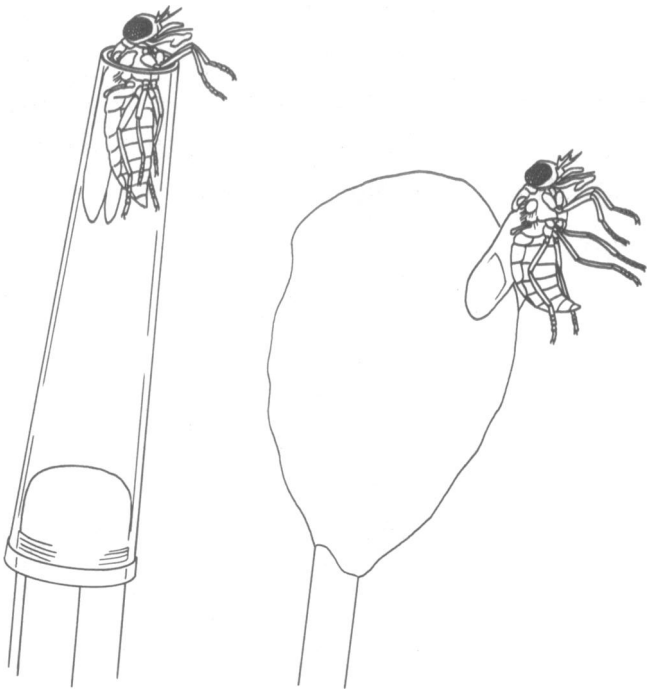

FIG. 1. Comparison of fly-restraining techniques. (*Right*) Wings pressed into tackiwax (Nelson's study, ref. 15). (*Left*) Micropipet-tip body sheath (present study).

The conditioned response was proboscis extension during the first 3 sec of CS_2. Responses to CS_1 afford some (undoubtedly not complete) control for CES effects, and those to the unconditioned stimulus represent unconditioned responses. During the test, any individual failing to emit at least five responses to the unconditioned stimulus was discarded. As in previous studies, when the tarsi were immersed in water, any fly that responded with full proboscis extension and labellar lobes wide open was allowed to drink in order to compensate for possible dehydration during the experiment.

Bidirectional and Stabilizing Selection. By the new method, a sample ($n = 53$) from the same free-mating wild-type foundation population as in the previous study (16) was trained, tested, and classified into a distribution of reliably measured individual differences, from which bidirectional selective breeding was started. As before, "good" flies (eight pairs) with the highest scores were isolated to found the "bright" line, but now "poor" flies (eight pairs), isolated to found the "dull" line, were chosen to exclude individuals scoring zero, i.e., stabilizing selection was used for the dull line, because flies might never respond for a variety of reasons unrelated to innate conditionability (pathology, trauma, etc.). All females were virgin. A control line was started with eight pairs from an untested sample and continued in that way for six generations, since when it has been maintained with 20 pairs. Over the generations, sample sizes ranged from a minimum of 29 to a maximum of 79.

Enough flies were tested every selected generation (with exceptions noted below) to find eight pairs qualifying as "good" in the bright and "poor" in the dull to breed bright and dull lines, respectively. Of course, selection intensity decreased with the progress of selection and the concomitant increase in the proportion qualified for breeding. Every third generation, the control was also tested. The order of presentation of the two stimuli was reversed on alternate generations of selection, being $CS_1(H_2O)$ and $CS_2(NaCl)$ for even-numbered and $CS_1(NaCl)$

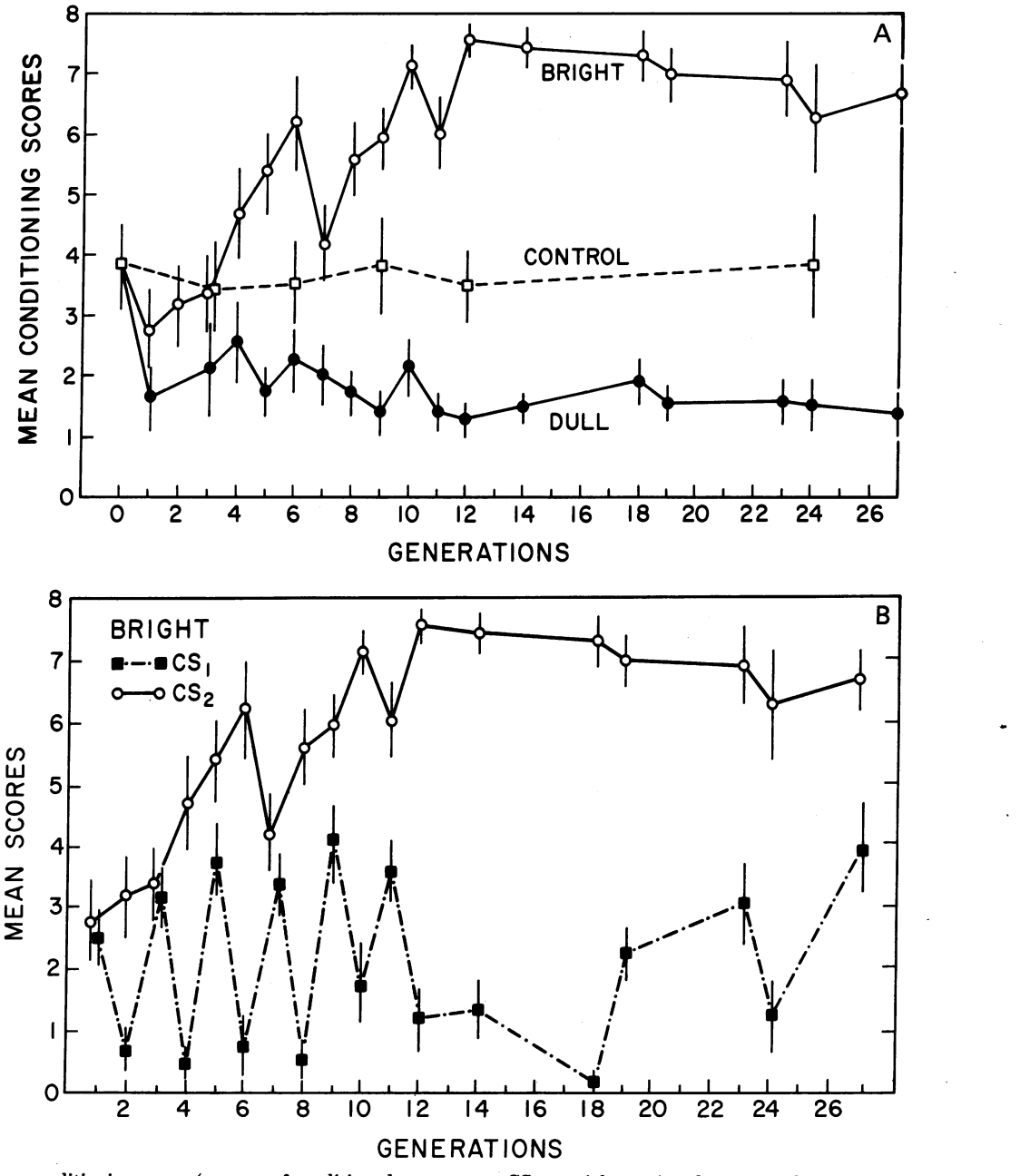

FIG. 2. (*A*) Mean conditioning scores (average of conditioned responses to CS_2 on trials 8–15) and 95% confidence intervals for bright, dull, and control lines over 27 generations of selection. (Some confidence intervals have been slightly displaced for clarity.) (*B*) Comparison, for bright line over generations, of mean response scores to CS_1 and to CS_2 on conditioning trials 8–15. Sequence of conditioned stimuli was reversed on alternate generations: CS_1 (H_2O) and CS_2 (NaCl) for even-numbered generations, and CS_1 (NaCl) and CS_2 (H_2O) for odd.

and CS_2(H_2O) for odd-numbered generations. Selection was continuous over the first 12 generations (except that generation 2 of the dull line was unselected), then intermittent over the next 15, being relaxed for both lines at generations 13, 15, 16, 17, 20, 21, 22, 25, and 26.

RESULTS

Table 1 presents the distribution of individual differences for the control and the foundation population in comparison with the distributions from three other studies. Despite differences in methods and populations, the results are evidently quite similar. Fig. 2*A* shows, for the bright and dull lines, the response to selection in comparison with the control over 27 generations. The initial fluctations remain parallel up to generation 5, when divergence starts; the separation of bright and dull from the control becomes statistically significant at generation 8. The marked drop by the bright line at generation 7 is correlated with an unfortunate release of insecticides into the building atmosphere. Fig. 2*B* shows, for the bright line, the comparison between the rising and consistently higher level of response to CS_2 and the fluctuating but lower level of response to CS_1; note that the response to CS_1 is stronger in the odd generations when that stimulus is NaCl than in the even generations when it is H_2O.

Fig. 3 shows cumulated means and variances for the sexes separately. Females have a larger variance than males in both lines and have a lower mean only in the bright line. The success of stabilizing selection can be seen in Fig. 4, which compares

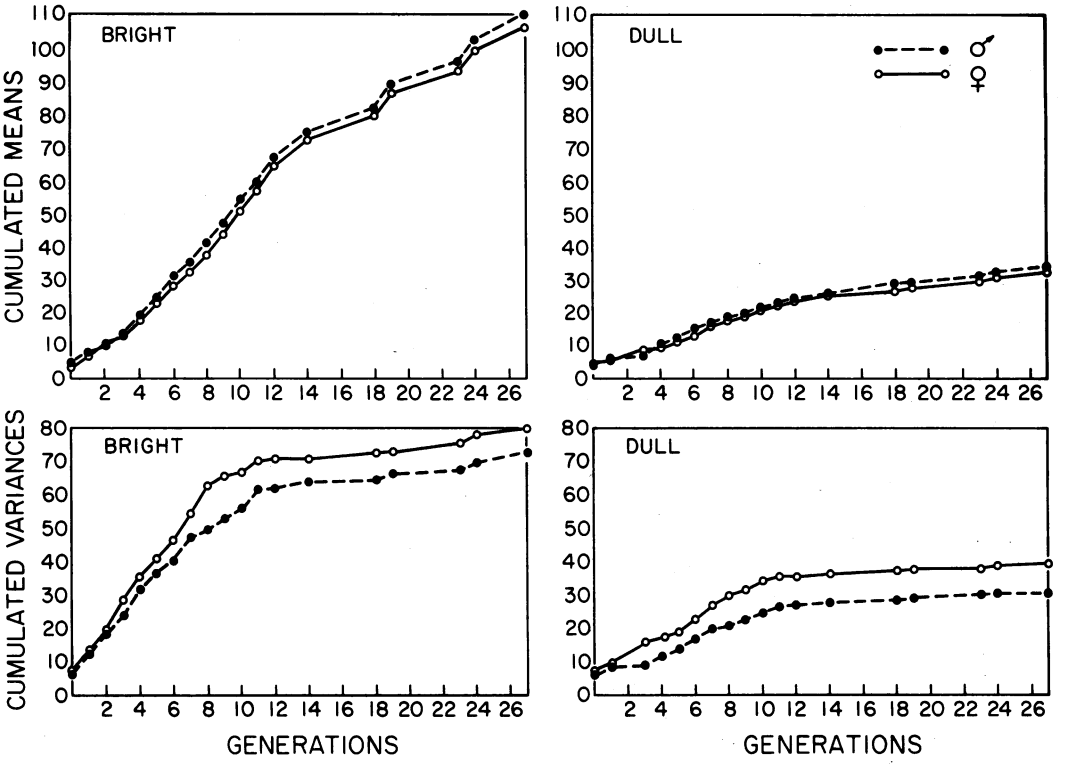

FIG. 3. Cumulated means and variances of conditioned responses to CS_2 from bright and dull lines over generations, showing consistently higher female variance.

the proportion of dull individuals never responding in the present and previous studies.

DISCUSSION

There are several uniquely important features of these observations. They represent the second corroboration of what is now the only independently replicated evidence for conditioning throughout the Diptera. The behavioral changes have been observed in reliably measured and identifiable individuals. The response to selection validates the existence of genetic correlates for the foundation population distribution of individual differences and begins the genetic analysis of *P. regina* conditionability. Also, it should be noted that the two successful *P. regina* selective breeding studies were done with a laboratory strain maintaincd well over 20 years on a breeding regimen no different from that used with the *D. melanogaster* Canton Special strain. Certainly the individuals in these strains were *not* of identical genotype.

In the recent reports of conditioning in *Drosophila* (4–8) there was no evidence for individual differences or stability over time. The authors reported finding no "intelligent subset." It was precisely to the question of stability in *Phormia* of the in-

Table 1. Conditioning score proportions

	CS_1 (H_2O), CS_2 (NaCl)			CS_1 (NaCl), CS_2 (H_2O)			
	Good	Fair	Poor	Good	Fair	Poor	*n*
Nelson (ref. 15)	0.39	0.24	0.37	0.31	0.18	0.51	200
Hirsch–McCauley (ref. 16)	0.26	0.30	0.44	0.26	0.26	0.48	102
Jackson*							
DT	0.32	0.26	0.41				99
E	0.23	0.31	0.44				89
C	0.03	0.41	0.55				29
Present study	0.27	0.32	0.41	0.24	0.34	0.42	219

Comparison of distributions of individual differences. The first two studies used the Nelson tackiwax mounting (15) but different populations. The second and fourth studies used the same population but different mounting and stimulus arrangements. The third and fourth used the same mounting and stimulating arrangements, but the third tested three populations—the Dethier population (DT) used by Nelson, the Illinois Entomology population (E) used by Hirsch and McCauley and in the present study, and the derivative control line (C) from this study. *n*, number of subjects.

* Jackson, D. (1976) Master's Dissertation, University of Illinois, Urbana-Champaign, IL.

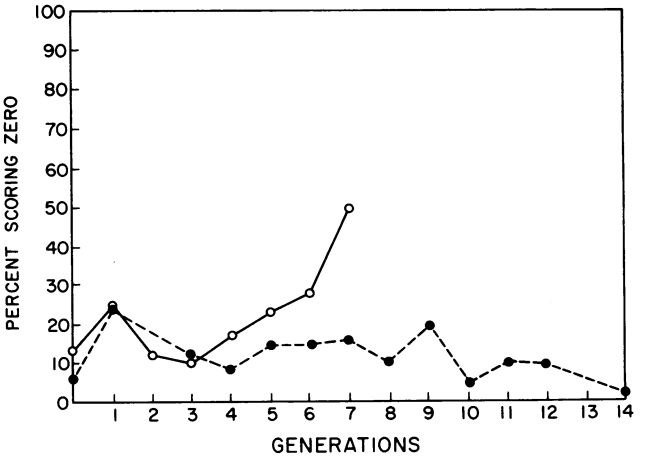

FIG. 4. Response to divergent and stabilizing selection by dull lines in previous (O—O, ref. 15) and present (●--●) studies, showing importance of including or excluding zero score in selection criterion.

dividual differences in conditioning that D. Jackson (1976, Master's Dissertation, University of Illinois, Urbana-Champaign, IL) has addressed himself. Though he takes strong exception to the use of the designation "intelligent subset," he has shown not only that individual differences can be measured reliably, but that, when stored at 1.8°, the behavior of the individuals is stable over time. Flies that on one day achieved high conditioning scores and flies that achieved low conditioning scores maintained their relative differences in performance when retested at the same time 24 hr later. Furthermore, we have obtained excellent evidence for extinction ($n = 59$), extinction followed by spontaneous recovery ($n = 20$), and extinction followed by reconditioning and extinction again ($n = 10$) in selected generations 23 and 24 of the bright line.

Even the simplest conditioned behavior is complex, consisting of relationships between unconditioned stimuli and responses and between conditioned stimuli and responses. In the present instance, at the very least, we are dealing with relationships between unconditioned stimuli and responses, CS_2 and conditioned responses, unconditioned stimuli and CES, and intertrial intervals and CES, in addition to individual differences in each. Only detailed behavior-genetic analyses might unravel the strands of this complexity. The distinguishable phenotypic components of this complex, i.e., lines with different degrees of expression of CES, must be bred by selection from the foundation population. Their analysis can elucidate the role of CES in conditioning. The expression of CES in the bright and dull lines can then be studied in a complementary behavioral analysis. So, Nelson's cryptic suggestion (ref. 15, p. 368) that her "classical conditioning . . . is essentially an extension of the CES phenomenon" thus becomes amenable to detailed experimental behavior-genetic analysis. Is conditioning in *Phormia* "merely" CES or are there separate "states," with the habit developing in a context of optimum excitation?

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