THE GENETIC BASIS OF NATURAL VARIATION. IX. COMPARATIVE STUDIES ON *cve* PHENOCOPY SENSITIVITY IN *DROSOPHILA*¹

ROGER D. MILKMAN

Department of Zoology, Syracuse University, Syracuse, New York

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SINCE WADDINGTON's (1953) association of crossveinless phenocopy sensitivity and spontaneous crossvein defects, these two aspects of crossvein-making ability have often been studied together. It is true that many crossveinless (*cve*) polygenes, so named because they contribute to spontaneous crossvein defects, do not enhance susceptibility to the induction of such defects by heat shock. Others do, however, and these are common in natural populations. Accordingly, it is often useful to study natural variation in crossvein-making ability by measuring phenocopy sensitivity of all the members of a population, rather than by looking for the fairly rare spontaneous crossvein defects, a simpler and more frequently used procedure.

Phenocopy sensitivity studies are of interest in several ways. WADDINGTON (1953) used sensitivity to heat shock as a selective criterion in a procedure which ultimately led to the production of a true-breeding polygenic *cve* strain. More recently (MILKMAN 1962a, 1964; MOHLER 1965b), direct selection has been shown to produce similar results rapidly and conveniently in certain strains; but where relevant genetic variation is low, only phenocopy selection may possibly provide the efficiency necessary to assemble whatever *cve* genes are present. The remarkable variety of responses depending upon temperature during a single short period of development provides a basis for detailed comparison of different strains. How uniform are the developmental labilities among various strains of *D. melanogaster*?

MOHLER (1965a) and THOMPSON (1966) have shown that under certain circumstances heat shock can promote rather than hinder posterior crossvein formation. The generality of this phenomenon is worth investigating. Finally, because of the rarity of spontaneously crossveinless flies, any attempt to *increase* crossvein-making ability must employ selection for phenocopy resistance. WADDINGTON's (1953) original attempt was not successful, and subsequent phenocopy selection experiments (MILKMAN, unpublished) have also failed to increase resistance.

The present paper summarizes a variety of experiments bearing on these aspects of phenocopy sensitivity and upon their consequences.

MATERIALS AND METHODS

Flies were raised at 23° or 25°C under standard conditions. They were treated in shell vials in constant temperature baths according to methods described previously (MILKMAN 1961,

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1962b). Individuals were rated as to number of 12ths missing from their posterior crossveins (0 = normal, 12 = complete absence of both posterior crossveins) regardless of location of the gap. r is the average rating for all flies in a sample, r' the average rating for the cve flies of a sample. The origin and maintenance of "Scow" strains are described by SCOWCROFT (1966). The strains were extracted from a selection program for increased and decreased number of scutellar microchaetae. Homozygous $se\ h^1$ males were mated to randomly chosen $+/se\ h^1$ females (se , sepia eye, 3-26.0; h^1 , hairy wing, 3-26.5). In each generation the $se\ h^1$ males were selected on the basis of individual male performance within the best 30% of families (based on mean male performance). The stocks have been maintained in mass culture, homozygous for se and h^1 , since June, 1965, by SCOWCROFT. My stocks, similarly maintained, are derived from these and have been in my laboratory since October, 1965. Further details are presented with the individual experiments.

Strains BM-1 and Bex-1 were sent me by DR. JACK BENNETT of Northern Illinois University; they were subsequently selected for increased cve expression. All other cve strains have been described before (MILKMAN 1964, 1965b; MOHLER 1965b).

In the figures from 2 on, each pair of points (male and female) represents at least 40 treated pupae.

RESULTS

Selection for phenocopy sensitivity: To determine whether selection for phenocopy sensitivity provided higher resolution than direct selection, three Syracuse and two South Lancaster strains were chosen, each of which contained no cve flies among 1000 F_2 flies derived from a single wild-inseminated female (of 49 Syracuse and South Lancaster strains, only six contained no cve individuals in 1000 F_2 flies examined) (MILKMAN 1964). It was decided to use treatments at 36.5°C, since death and sterility do not accompany phenocopying at this temperature (MILKMAN 1965a); they do to a bothersome extent at higher treatment temperatures. Although very little variation has been encountered in the time of peak sensitivity (25 hours at 23° after puparium formation), other ages were tried in some cases. The standard exposure time used, 200 minutes, has been found effective before (MILKMAN 1965a). Other durations were used in addition. For comparison, the responses to 200 minutes at 36.5° of Oregon-R and of a mixed wild strain derived from all but the most crossveinless of the Syracuse strains were used. For Oregon-R, about 20% of the males and 60% of the females showed posterior crossvein defects; in the mixed strain about 15% of the males and 35% of the females responded in this way.

Of the five strains studied, three (X, P-12 and SL-3) showed little or no response to a 200-minute treatment at 36.5°, administered 25 hours (at 23°) after puparium formation. In all three strains, other ages from 18 to 27 hours were tried to no avail. Since phenocopy selection requires fairly large numbers of flies, even when phenocopy induction and selection take place only in alternate generations, it was impractical to attempt in these three cases.

For strain P-12, other temperatures were also tried (Table 1). In general, response was low, but at 40.5° the production of posterior crossvein defects was comparable to that in Oregon-R. This observation is one of many leading to the conclusion (MILKMAN 1961, 1962b, 1966b) that several quite distinct temperature effects on crossvein formation exist, and that they are separable according to temperature range. Additional discordant differences in phenocopy sensitivity

TABLE 1

cve phenocopying of strain P-12 at various temperatures

Temperature (°C)	Duration (min)	Males			Females		
		+	<i>cve</i>	<i>r</i>	+	<i>cve</i>	<i>r</i>
36.0	180	24	0	0	28	7	0.4
36.0	240	27	3	0.2	20	9	0.6
36.0	300	30	3	0.1	23	11	1.1
36.0	420	18	1	0.1	23	13	0.9
37.0	180	142	10	0.1	129	15	0.2
37.5	240*	2	0	0	5	5	1.9
38.0	210	25	8	0.3	35	1	0.0
38.0	240	27	5	0.2	15	3	0.2
38.0	270	36	5	0.3	20	2	0.1
38.5	180	14	2	0.1	24	0	0
38.5	210*	1	4	1.6	5	1	0.3
40.5	25	281	34	0.2	321	40	0.2
40.5	30	107	80	1.4	66	115	2.6
40.5	35*	0	14	7.0	0	17	7.2

* Low survival.

at various temperatures will be presented in the next section of this paper. An interesting sidelight is the demonstration in strain P-12 of an unusual quantitative relationship between temperature and death. One would expect that with an increase in temperature the duration tolerated would decrease systematically. Similarly, a lethal exposure period at one high temperature would certainly be expected to be lethal at a higher temperature. Yet Oregon-R males have been shown to survive $4\frac{1}{2}$ hours at 38.0° but not at 37.5° (MILKMAN 1962b)! Now a similar observation has been made for both sexes of P-12. The survival of various temperatures and durations is summarized in Figure 1. The comparison between responses to $4\frac{1}{2}$ hours at 37.5° and to $4\frac{1}{2}$ hours at 38.0° was made twice, with similar results. Actually, the discrete temperature ranges for crossvein defect induction (MILKMAN 1962b) predict the possibility of this observation on lethality: one merely extends the relationship to *vital* structures or processes.

The other two strains, W and SL-7, showed somewhat greater responses to phenocopy treatment. Response to phenocopy selection was small and limited in

FIGURE 1.—Survival of 25-hour P-12 pupae at various temperatures. Note anomalous positions of 38.0° and 37.5° (arrows). In general each point represents at least 60 treated pupae; 150 were treated at 37.5° for $4\frac{1}{2}$ hours and 150 at 38.0° for $4\frac{1}{2}$ hours.

the case of strain W. Strain SL-7 showed more promise; selection was terminated after a few generations, however, because of the relation between effort and results. Treatment and selection were carried out in generations alternating with those for re-expansion of the stocks (MILKMAN 1965a), and durations were reduced periodically. In both cases, response was much lower than that obtained (MILKMAN 1965a) from the mixed strain described above. Incidence of cve flies among the untreated siblings remained low and sporadic. To summarize the results of attempts to assemble cve combinations by phenocopy selection in strains with no spontaneous cve flies, the attempts either failed outright or showed very little promise of success.

With respect to the usefulness of this technique, it may be pointed out once more that the five strains used were among the six which had contained no cve flies in 1000 F_2 flies counted, as opposed to 43 others which contained one or more. Since only five of the latter strains yielded true-breeding cve strains in response to direct selection, some of the 38 intermediate strains may yet provide an application for the phenocopy selection method. On the other hand, unsuccessful attempts at direct selection in these strains, together with inferences drawn from some tentative models constructed for cve, suggest that the lower the incidence of the cve phenotype, the larger is the proportion of cve due to nongenetic variation. If this is so, we can expect rather little response to phenocopy selection in the intermediate strains as well.

Indirect selection: It has often been found, intentionally or otherwise, that selection for certain phenotypes leads to progressive changes in apparently unrelated phenotypes (LERNER 1958). Such results may be due to closely linked genes, to an unrecognized relation at one of many levels between the two phenotypes, or perhaps to a general lessening of developmental stability (or "buffering") (WADDINGTON 1957). A case in point is the discovery by SCOWCROFT (1966) of certain changes in wing vein making ability in a *se h'* strain. A subsequent detailed investigation in this area has led to findings of interest with respect to the mechanisms suggested above for indirect effects of selection. Three strains were studied: Hiscow, a strain selected for high scutellar microchaeta number and showing great numbers of hairs on the wings; Loscow, a strain selected for low scutellar hair number and showing few hairs on the wings; and Unscow, an unselected strain. All three strains had been in mass culture for about 6 months prior to the start of this experiment. SCOWCROFT had originally reported (personal communication) that the Unscow strain appeared to be particularly well buffered with respect to wing vein development. The evidence was the suppression of the effects of a variety of wing-venation mutant genes (radius incompletus, plexate, cubitus interruptus and crossveinless) which SCOWCROFT incorporated individually into this strain. "In all cases in which the mutant impaired vein making ability, the presence of hairy¹ restored (fully or partially) that portion of the vein removed" (SCOWCROFT, personal communication). One might then ask whether the modifiers of the effect of *h'* on microchaetae modify its effect on venation similarly. If this were true, Hiscow would be even more resistant to the induction of venation defects by genetic and by environmental factors.

FIGURE 2.—Crossveinless phenocopy response in the SCOWCROFT strains at 36.5°. At least 40 flies of each strain were treated at each duration.

FIGURE 3.—Phenocopy response in the SCOWCROFT strains at 37.5°. Points above the maximal value of 12 are cases of death of the entire sample.

Accordingly, it was of interest to see how the strains compared in their phenocopy sensitivity, and a variety of temperatures were used, including temperatures in three discrete ranges known to produce crossveinless phenocopies and in a fourth range which usually does not. The results are summarized in Figures 2, 3, 4 and 5. Unexpectedly, Hiscow is not only the most sensitive of the strains, but it is the most sensitive strain I have ever examined, excepting of course strains se-

FIGURE 4.—Phenocopy response in the SCOWCROFT strains at 38.5°. Hiscow and Loscow did not survive 2½ hours.

FIGURE 5.—Phenocopy response in the SCOWCROFT strains at 40.5°. Note complete lack of response in Unscow.

lected for phenocopy sensitivity. This extreme sensitivity is seen at 36.5° , 38.5° , and at 40.5° . However, Hiscow shows only a moderate response to 37.5° . Loscow responds negligibly to 36.5° , extremely to 37.5° , moderately to 38.5° , and rather strongly to 40.5° . Unscow responds negligibly to 36.5° , even more extremely than Loscow to 37.5° , and yet not at all to 38.5° and 40.5° . It is the first strain tested whose posterior crossveins do not respond to 40.5° ; this finding fits well with SCOWCROFT's genetic observations.

One simple basis for some of these differences might be differences in developmental rates in general, or in the period of maximum sensitivity in particular. Such underlying differences would have to be great indeed to account for the observed results at the lower temperatures, which show relatively long periods of high sensitivity in several strains. But at 40.5° , such differences might provide a plausible explanation. Accordingly, age response studies were made, and they are summarized in Figure 6. No differences are found between Hiscow and Loscow; Unscow fails to respond at any age tested. Moreover, since the 40.5° sensitivity period lasts for several hours, it can be concluded that the simple shift in sensitive period necessary to explain these observations would have to be too great to jibe with the known developmental schedule of *Drosophila* and with the observation that the three strains actually appear to have comparable developmental rates. In this regard, however, the very real and difficult problem raised by HARKER's (1965a, b) recent work should be mentioned. She has found striking variations in developmental rates of *D. melanogaster* pupae which depend on their history of exposure to light. The degree of this variation, together with the demonstration of genetically determined variations in the animals' developmental

FIGURE 6.—Phenocopy response at 40.5° versus age in the SCOWCROFT strains. Maximal response in each case is at 25 hours.

FIGURE 7.—Survival of SCOWCROFT strains treated at 37.5° and 38.5° . Note that Unscow is the most sensitive at 37.5° and the least sensitive at 38.5° .

responses to light, make close control vital. In any event, systematic fluctuations in phenocopy response related to time of day have been sought and not found under the conditions prevailing in my laboratory.

Viability differences at 37.5° and 38.5° were also striking. These are summarized in Figure 7. The opposite extremes shown by the unselected strain are noteworthy.

It will be noted that (1) striking variation in response is seen with respect to specific temperatures; (2) some effects are correlated with direction of selection; and (3) selection, irrespective of direction, has in some cases increased and in other cases decreased resistance both to death and to morphogenetic changes. No specific mechanism or adaptive significance can be advanced for these effects in any but the most general and obvious terms. Once again, however, the distinctions among effective temperatures are seen, and the multifarious effects of new combinations of existing alleles are demonstrated.

In view of their sensitivity to crossvein defect production at various high temperatures, the three strains were examined for spontaneous defects. At the temperatures at which they were kept normally (23° or 25°C), no cve flies were seen in routine operations. But at 18° the incidence in two strains is noteworthy, as shown in Table 2, which also records the finding of two cve Hiscow males in a further study of 25° flies. Accordingly, observation of yet another variable reveals distinctions among the strains. It is of interest that the spontaneous cve tendency goes with sensitivity to certain temperatures and not with others. Whether the positive correlations reflect any causal connection is of course unclear; a fairly extensive program of selection experiments might be required for conclusive information.

The three strains were also crossed to several known crossveinless strains, both simple and polygenic. The results are also presented in Table 2. It will be seen that the few F_1 cve flies are largely in the Hiscow crosses, again demonstrating discordance between effects on microchaeta number and posterior crossvein formation. In the F_2 's, no evidence was seen for a general antagonism to crossveinless on the part of any particular Scowcroft strain, since the incidence of cve in the F_2 's does not seem to vary much with the Scow parent used. This type of experiment, however, is not nearly as rigorous or informative as incorporating particular venation mutants into the respective selected strains.

Restoration of crossvein-making ability by heat: MOHLER (1965a) has recently shown that the posterior fifth of the posterior crossvein (note that the crossveins are considered to run in an anterior-posterior direction, and so the posterior region is adjacent to the 5th longitudinal vein) is actually restored while other parts of the crossvein are being destroyed by a particular heat shock. Subsequently, he and THOMPSON (1966) have shown that flies of MOHLER's crossveinless strain cvl-5 can be made normal by the application of a heat shock around the 14th hour after puparium formation. THOMPSON has made another interesting observation on a stock carrying cvl-5 third chromosomes and wild Orinda first and second chromosomes (MOHLER 1965b): while intermediate durations at 40.5° (15, 20, 25 minutes) markedly increase crossvein defects at 22 hours, lower

TABLE 2

Incidence of cve in SCOWCROFT's strains and in crosses (F_2 , 18°C)

cve parent		High		Unselected		Low	
		% cve	r'	% cve	r'	% cve	r'
P-6	♂	58	6.8	38	6.9	12	7.2
	♀	4	6.5	8	8.8	12	5.3
MUS 13½	♂	12	5.8	4	9.0	10	5.6
	♀	6	3.7	4	8.5	16	8.1
E'	♂	58	6.3	52	6.0	60	7.0
	♀	32	3.2	24	3.8	16	3.6
SL-8	♂	34	7.5	46	6.7	38	5.8
	♀	12	2.8	12	5.2	8	3.5
per	♂	16	5.0	26	7.8	10	8.6
	♀	10	4.8	22	5.0	12	7.3
cv	♂	28	11.6	44	11.8	28	11.9
	♀	14	10.0	0	..	6	5.7
self, 18°	♂	11	2.5	0	..	0	..
	♀	6	3.3	0	..	3	4.3
self, 25°	♂	2	3.0	0	..	0	..
	♀	0	..	0	..	0	..

Crosses: N = 50, each sex. Selves: N = 100, each sex. r': average rating, cve flies.

In F_1 's at 25° (N = 20, sex), there were cve flies only in Unscow × E' (1 female), Hiscow × E' (1 male, 7 females), and Hiscow × MUS 13½ (1 female).

FIGURE 8.—Enhancement of posterior cross-vein formation in crossveinless strains treated at 40.5°. Solid lines: 20-minute treatment, dashed lines: as indicated by numbers in parentheses, L: mature third instar larval stage, C: control values. Treatments made at 12, 13, 14, 15, 16, 17, 18, 19, and 20 hours for all strains and additional ages for some.

(5 minutes) and higher durations (40 minutes) *both* actually *reduce* the degree of defects in comparison to untreated flies of this stock. It is of interest to investigate the generality of this phenomenon as a further means of distinguishing among crossveinless strains and perhaps to uncover genetic differences which can be made relevant to posterior crossvein formation but which are not ordinarily under the scrutiny of selection when crossveinless strains are being assembled by the usual means (MILKMAN 1962a, 1965a).

The present results on a variety of *cve* strains are summarized in Figure 8. It will be noted that various strains differ as to the time of maximum restoration of crossveins by heat and that unlike *cvl-5* (THOMPSON 1966), none of the strains described here are restored to the wild phenotype in high frequency. On the other hand, the degree of defect to begin with is generally much greater than in the *cvl-5* strain used. One strain, *E'*, shows little or no response, in contrast to all the others tested. The vertical compression of Figure 8 makes some fairly striking differences appear rather modest; but it will be recalled that the difference in ratings of 11 and 6 is the difference between almost no posterior crossveins and crossveins which are 50% complete. It may be noted further that *SL-8* and *cvl-6* have been shown to be quite similar with respect to their *cve* genes (MILKMAN 1966a): they are unusual in having a major gene and modifiers rather than a group of genes contributing about equally; the major genes are indistinguishable as to location (both are at about 1-60) and in allelism testing; and it appears that some of the modifiers are similar, also on the basis of allelism testing (MILKMAN 1966a). These two independently selected strains, from Massachusetts and California, respectively, thus appear to be rather similar with respect to what we call *cve* genes on the basis of their effects on the posterior crossvein under normal conditions. Yet the strains differ clearly in their ability to restore the crossvein under the influence of high temperature early in development. Whether this difference stems from differences in *cve* genes or from differences in genes which are not ordinarily placed in this category (by present criteria) remains to be seen.

DISCUSSION

The observations reported here confirm and extend previous observations on the multiplicity of experimentally accessible effects on posterior crossvein formation. It has previously been demonstrated that a number of different combinations of genes can lead to the common *cve* phenotype (MILKMAN 1962a, 1964, 1965b). The striking diversity among strains in response to various specific temperatures establishes now that numerous accessible developmental pathways exist together before leading to the particular final common path under present consideration, the formation of the posterior crossvein. This conclusion is certainly not unexpected.

Two new approaches to selection for increased crossvein-making ability (or for the increased stability of crossvein formation) have been successful: the testing of individual lines for phenocopy resistance has revealed several which respond unusually little to normally effective treatments at certain temperatures;

selection for superficially unrelated properties has also resulted in strains resistant to certain temperatures. Of course, in line both with the experimental observations and with the above statement on the diversity of variable developmental properties, the term "increased crossvein-making ability" must be qualified as to the conditions under which the difference obtains. We may stabilize any of several developmental components.

The usefulness of the posterior crossvein as a quantitative end point is increased by the demonstration of the diversity of factors affecting it. But the experimental system is protected from confusion only to the extent that one can define the various relevant genetic combinations and effective high temperature treatments (the latter in terms of temperature, duration, and the age and previous thermal history of the pupae).

The unusual order of effect on viability of 37.5° and 38.0° must be emphasized once again because it runs counter to the very reasonable rule that the impact of a high temperature treatment rises with temperature. Although this is usually the case, it has now been demonstrated repeatedly and with more than one strain of flies that there are exceptions. These observations, together with the discontinuity of temperature effects on crossveins and on other structures, remind us that balance is an important consideration in developmental responses to shock. It may be of some interest to recall that CROZIER, PINCUS and ZAHL (1936) once suggested that "suppressed moults" or "metabolic instars" might explain discontinuity over time in the sensitivity of *Drosophila* to one particular kind of environmental stress (alcohol treatment). Since various strains differ so greatly in the nature, degree and time of their responses, this conjecture is no longer tenable.

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SUMMARY

Certain lines of *Drosophila melanogaster* exhibit unusually low response to cve phenocopy treatment at certain temperatures. Selection for a superficially unrelated trait can produce changes in cve phenocopy response; these changes in response to various temperatures often differ strikingly in degree as well as direction. Another case has been found in which killing at 37.5°C is actually faster than at 38.0°C.—The observations of J. D. MOHLER (1965a) and S. THOMPSON (1966) on the enhancement of posterior crossvein formation by certain high temperature treatments have been extended to a number of cve strains.

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