THE GENETIC BASIS OF NATURAL VARIATION. VI. SELECTION OF A CROSSVEINLESS STRAIN OF DROSOPHILA BY PHENOCOPYING AT HIGH TEMPERATURE[1]

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As a class, crossveinless polygenes in Drosophila are common, numerous, and diverse. Crossvein defects result from many rare combinations of alleles with individually small effects, and these various combinations can be distinguished by a number of characteristic properties, including genetic and environmental interactions and morphological details (MILKMAN 1962a, 1964; TIMOFEEFF-RESSOVSKY 1934). Similarly, there are three distinct temperature ranges in which posterior crossveins can be produced *during a single sensitive period* in *Drosophila melanogaster*, and this kind of phenomenon is not restricted to this species (MILKMAN 1962b).

WADDINGTON (1953) showed that phenocopy sensitivity at 40.5°C can indicate a genetic predisposition for a corresponding trait and that selection for a given trait may efficiently be begun with selection for its phenocopy. MILKMAN (1961) showed that a strain so selected has dominant alleles enhancing response to the treatment used, while strains selected directly often do not (unpublished data). MOHLER (1964), however, has a directly selected crossveinless strain which coincidentally does have dominant alleles imposing crossveinless phenocopy sensitivity.

In view of the differences between the induction of posterior crossvein defects at 36.5°C and at 40.5°C (MILKMAN 1962b); and because sterility is not a problem in the 36.5° technique but is at 40.5°; and because this form of indirect selection may often be more efficient than direct selection, an attempt was made to select a true-breeding polygenic crossveinless strain from a Syracuse stock, beginning by selection for the production of posterior crossvein defects at 36.5°C. Such a strain was rapidly produced.

Two other questions were posed: (1) Can this technique be used to measure genetic variability? (2) Does selection for increased cve phenocopy sensitivity at 36.5° bring about increased sensitivity to other temperatures? The first question was approached by the use of irradiation (other approaches will be discussed later), and the second by direct tests during selection.

MATERIALS AND METHODS

A stock was assembled from 34 lines of Syracuse flies. Of 40 original Syracuse lines, each

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derived from a single wild-inseminated *D. melanogaster* female, each of six had given rise to a crossveinless (cve) polygenic strain in response to direct selection. Of the remaining 34, two had not responded to selection, and the other 32 had not met the minimal criterion (10 cve flies in 1000 F_2 flies examined) for an attempt to be made. These remaining 34 lines contributed to a mixed foundation stock.

The flies were raised under standard conditions. Animals were collected at the time of puparium formation, aged 25 hours at 23°, and treated at 36.5°C for 3 hr 20 min in a Precision water bath (MILKMAN 1962b). The treated flies were rated on a scale of 12 (normal = 0; posterior crossveins completely absent = 12) and selected. The resulting generation was allowed to breed without treatment. Accordingly, a generation of treatment and selection alternated with a generation of maximal increase in population. The treatment duration was reduced progressively as warranted. Test treatments at other temperatures were used as described. Direct selection was begun in each generation after the sixth, using untreated cve siblings of the treated flies and of course selecting in every subsequent generation, not alternate generations.

In the irradiation experiment, four lines were set up from a highly inbred "isogenic" Oregon-R stock. Each line was begun with 50 males and 50 virgin females. With the sexes kept separate, these flies were placed in empty shell vials, 10 or 20 flies per vial. The control line received no irradiation, and the three treated lines received 8, 80 and 800r respectively. After X-irradiation the flies were mass-mated in each line; and mass-mated F_2's were collected at the time of puparium formation and subjected to the kind of treatment and selection described above. It should be emphasized that each irradiated line was irradiated only at the start of the experiment and never subsequently.

RESULTS

The rapidity and extent of phenocopy selection in the mixed strain is shown

TABLE 1

Response of the mixed strain to selection

Minutes at 36.5°C	Generation	Males			Females		
		Percent cve	r′	r	Percent cve	r′	r
200	0	14	2.9	0.4	31	3.5	1.1
	1	18	3.7	0.7	35	4.4	1.5
	2	47	4.4	2.1	84	5.7	4.7
	3	59	5.0	2.9	88	6.1	5.4
	4	74	5.9	4.4	92	6.7	6.2
	5	55	5.4	3.0	90	7.0	6.2
	6	73	5.5	4.0	97	7.7	7.5
	7	92	7.7	7.1	98	8.5	8.3
120	7	57	4.7	2.6	83	6.1	5.0
	8	48	5.2	2.5	76	6.9	5.3
90	8	22	5.0	1.1	58	5.1	2.9
	9	29	4.3	1.3	50	5.3	2.6
60	7	4	6.3	0.2	8	3.2	0.3
	9	3	10.0	0.3	19	4.7	0.9
	10	34	6.6	2.2	43	3.9	1.7
	11	41	7.5	3.1	50	7.2	3.6
	12	62	9.4	5.8	78	7.1	5.6
30	12	59	9.5	5.6	68	9.0	6.1
	13	90	9.7	8.7	80	10.2	8.2
15	13	83	9.7	8.0	83	10.2	8.4

r′ = average defect rating, cve flies. r = average defect rating, all flies.

TABLE 2

Response of all strains to selection for susceptibility to 3 hours, 20 minutes at 36.5°C

Strain	Sex	Generation of selection 0	1	2	3	4
A. *Percent cve*						
Wild mix	M	14	18	47	59	74
	F	31	35	84	88	92
Oregon-R, 0r	M	21	18	37	34	12
	F	58	61	67	75	72
Oregon-R, 8r	M	22	27	46	39	18
	F	67	64	77	83	74
Oregon-R, 80r	M	18	26	40	49	34
	F	68	59	78	70	69
Oregon-R, 800r	M	23	28	47	37	40
	F	56	71	84	70	71
B. *Average defect rating, all flies*						
Wild mix	M	0.4	0.7	2.1	2.9	4.4
	F	1.1	1.5	4.7	5.4	6.2
Oregon-R, 0r	M	0.6	0.5	1.2	1.3	0.4
	F	3.1	3.0	3.5	4.1	4.2
Oregon-R, 8r	M	0.7	1.1	1.7	1.4	0.5
	F	3.7	3.4	4.5	5.0	4.8
Oregon-R, 80r	M	0.6	0.9	1.5	1.8	1.3
	F	3.2	2.8	4.8	4.8	4.0
Oregon-R, 800r	M	1.0	1.1	1.8	1.9	1.7
	F	3.4	4.0	5.3	4.2	4.2
C. *Average defect rating, cve flies*						
Wild mix	M	2.9	3.7	4.4	5.0	5.9
	F	3.5	4.4	5.7	6.1	6.7
Oregon-R, 0r	M	2.9	2.8	3.2	3.8	3.3
	F	5.3	4.9	5.2	5.5	5.8
Oregon-R, 8r	M	3.2	4.1	3.7	3.6	2.8
	F	5.5	5.3	5.8	6.0	6.5
Oregon-R, 80r	M	3.3	3.5	3.7	3.7	3.8
	F	4.7	4.7	6.2	6.9	5.8
Oregon-R, 800r	M	4.4	3.9	3.8	5.1	4.2
	F	6.1	5.6	6.3	6.0	5.9

in Table 1. This may be compared with attempts at similar selection in highly inbred Oregon-R flies, both unirradiated and lightly irradiated (Table 2). Neither the unirradiated controls nor the lightly irradiated strains responded to selection.

Returning to selection in the mixed strain, the increased sensitivity to other temperatures is shown in Table 3. It should be noted that in unselected wild strains, certain of these temperatures (30 to 36°, 37.0°) do not produce posterior crossvein defects in appreciable numbers at any duration, nor are the responses to 40.5° comparable (MILKMAN 1962a,b). After a number of generations of selection, the incidence of cve in the untreated siblings rose (Table 4). Using such cve flies. true-breeding strains have been obtained by direct selection begin-

TABLE 3

Response of the mixed strain to various temperatures

Temperature (°C)	Duration (minutes)	Males		Females		N
		r′	r	r′	r	
Generation 10						
40.5	25	5.4	4.0	6.3	3.9	97
	30	7.2	5.8	5.8	4.8	102
	35	9.6	9.2	8.7	8.5	107
	40	11.5	11.5	10.7	10.7	104
37.5	180	6.0	1.6	7.1	2.5	47
	240	8.1	3.9	9.7	1.5	49
	270	6.4	3.9	6.2	3.9	34
untreated	...	6.2	0.1	3.2	0.1	566
Generation 12						
40.5	5	9.5	6.8	9.1	6.9	72
	10	8.0	5.1	7.7	4.5	81
	15	9.3	7.0	7.7	5.0	58
	20	7.6	4.8	6.6	5.3	70
37.0	30	9.1	5.3	8.1	5.8	59
	60	9.4	9.4	8.6	8.6	31
36.5	30	9.5	5.6	9.0	6.1	547
	60	9.4	5.8	7.1	5.6	44
36.0	60	9.5	7.0	9.2	8.1	86
34.0	60	8.4	6.4	7.1	3.9	43
	120	8.3	5.4	9.9	8.5	37
32.0	60	8.3	4.3	9.8	6.1	44
	120	7.6	3.4	7.1	4.2	37
30.0	120	8.8	4.4	9.2	5.4	46
untreated	...	6.9	1.8	7.9	1.8	518
Generation 13						
37.5	30	9.3	6.7	10.2	9.8	55
	60	9.9	9.3	8.8	8.8	52
	90	8.6	6.5	7.6	6.7	55
	120	7.6	6.8	5.0	3.8	13
	180	6.9	6.9	7.6	7.6	15
	210	8.5	7.9	7.4	6.9	46
	240	9.3	8.0	7.2	6.8	29
	270	7.9	6.9	7.5	6.8	28
untreated	...	7.9	4.5	7.7	4.7	548

ning with each of a number of generations. The course of selection is illustrated in Table 5. Comparative analysis of these strains has not begun.

The unirradiated and irradiated Oregon-R strains, as would be expected from their lack of response to phenocopy selection, contained only rare, sporadic cve individuals. The four Oregon-R lines (0, 8, 80, 800r) were each separated into two sublines. One subline was maintained from the time of irradiation, undergoing no selection for susceptibility to 36.5°C. The other subline was derived from flies with a history of four generations of such selection. Untreated flies from each group were examined for posterior crossvein defects, and the results

TABLE 4

Incidence of crossvein defects among untreated flies of the mixed strain

Selection generation	+	cve	Percent cve
0	151	0	0
1	144	0	0
2	123	0	0
3	2433	26	1.1
4	2116	98	4.4
5	1285	8	0.6
6	1396	62	4.3
7	958	43	4.3
8	566	13	2.2
9	1060	6	0.6
10	552	14	2.5
11	355	117	24.8
12	391	127	24.5
13	223	325	59.4
13½*	30	183	85.9

* Progeny of Selection Generation 13. This would ordinarily have been an unselected generation, followed by selection.

are given in Table 6. It would appear that the few cases of cve encountered cannot be ascribed to causes relevant to the present experiment; that is, it is not clear that these individuals reflect genetic variability, and it is highly unlikely that they reflect genetic variability produced by irradiation. Similar sporadic occurrences of cve in Oregon-R are reported by MOHLER (personal communication): one cve female and two cve males in about 13,000 inbred Oregon-R flies examined. Both our strains derive from stocks of P. T. IVES.

DISCUSSION

Both the speed and the extent of selection in the mixed strain are noteworthy, and it is clear that this method promises to be quite useful in assembling *cve* polygenes. Now, before proceeding to specific applications, it would be well to compare the present selection experiments with those involving the direct selection of the progenies of single pairs (MILKMAN 1964). First and foremost, the greater speed and extent of the present selection may simply be due to the greater genetic variability inherent in a strain derived from 34 such pairs. On the other hand, these were the pairs whose progenies contained very few cve flies.

As to the differences in method, three aspects of the process may be considered: the initial phase: the effect of selecting only in alternate generations; and the final phase. The initial phase of selection probably determines which *cve* genes will be fixed in the strains ultimately obtained. On the genetic constitution of the initial sample depends what is to follow. The present method provides a large number of flies whose average number of *cve* genes is a good deal greater than that of the flies not selected. In contrast, direct selection brings together, in the case of crossvein defects, a very small proportion of the population: 0.5 to 1 percent of a large number of wild flies or 1 to 2 percent in an F_2 from a wild pair (an F_2 containing less would not be used). In

TABLE 5

Response of the mixed strain to direct selection

Indirect selection generation	Direct selection generation	Males					Females				
		+	cve	Percent cve	r'	r	+	cve	Percent cve	r'	r
7	0	468	16	3	6.0	0.2	490	27	5	6.0	0.3
	1	60	26	30	7.7	2.3	83	39	32	7.9	2.5
	2	14	125	90	6.9	6.2	6	119	95	7.6	7.2
	3	0	88	100	7.9	7.9	0	113	100	8.6	8.6
	4	0	88	100	8.2	8.2	1	88	100	9.0	8.9
	5	0	9	100	9.9	9.9	0	26	100	10.8	10.8
8	0	Males and females pooled:					566	13	2.2	5.0	0.1
	1	45	4	8	3.5	0.3	42	7	14	5.3	0.8
	2	65	0	0	0	0	54	2	4	1.5	0.1
9	0	Males and females pooled:					1060	6	0.6	5.3	0
	1	110	21	16	7.3	0.9	111	25	18	5.6	1.0
	2	29	28	49	8.0	3.9	22	40	65	7.6	4.9
	3	(not counted)									
	4	37	18	33	8.1	2.6	23	12	34	5.2	1.8
	5	17	55	76	8.3	6.4	17	66	79	9.5	7.6
	6	0	39	100	8.8	8.8	0	42	100	9.8	9.8
10	0	Males and females pooled:					552	14	2.5	4.5	0.1
	1	35	25	42	7.2	3.0	41	19	32	7.0	2.2
	2	15	27	64	6.2	4.0	18	23	56	6.6	3.7
	3	0	69	100	5.6	5.6	3	68	96	5.7	5.5
	4	0	37	100	7.3	7.3	0	46	100	6.6	6.6
	5	0	32	100	7.8	7.8	0	28	100	7.6	7.6
	6	0	62	100	7.9	7.9	0	59	100	8.2	8.2
11	0	175	62	26	7.8	2.0	180	55	23	8.0	1.9
	1	15	66	82	9.0	7.3	18	74	80	8.9	7.1
	2	0	61	100	9.2	9.2	0	61	100	9.8	9.8
	3	0	44	100	9.8	9.8	0	28	100	10.4	10.4
	4	0	54	100	10.3	10.3	0	59	100	10.6	10.6
	5	0	16	100	11.1	11.1	0	16	100	11.3	11.3
12	0	184	64	26	6.9	1.8	207	63	23	7.9	1.8
	1	23	32	58	7.9	4.6	18	33	65	7.8	5.0
	2	(not counted)									
	3	0	47	100	8.7	8.7	0	48	100	9.9	9.9
13	0	127	172	58	7.9	4.5	96	153	61	7.7	4.7
	1	8	21	72	7.1	5.1	7	24	77	6.2	4.8
	2	(not counted)									
	3	0	46	100	8.8	8.8	0	52	100	9.5	9.5
	4	0	114	100	9.7	9.7	0	120	100	10.0	10.0
13½	0	18	92	84	6.6	5.5	12	91	88	7.4	6.6
	1	7	66	90	8.6	7.8	10	71	88	9.0	7.9
	2	0	31	100	8.8	8.8	0	39	100	9.6	9.6
	3	(not counted)									
	4	(not counted)									
	5	0	114	100	9.5	9.5	0	115	100	10.1	10.1
	6	0	85	100	10.2	10.2	0	123	100	10.2	10.2

TABLE 6

Incidence of cve phenotype among untreated Oregon-R flies at 18°C

Line (Dose, r)	Subline	Number +	Number cve	Sex and rating of cve flies
0	unselected	2,581	3*	♂3 ♀2 ♀2
	selected	2,280	0	
8	unselected	4,467	1	♂5
	selected	2,699	1	♂6
80	unselected	6,177	0	
	selected	2,139	0	
800	unselected	2,932	1	♀4
	selected	2,944	1	♀3

* These three appeared in two batches of 63 and 194 flies, from cultures made two weeks apart with the same parents, counted on the same day. No explanation involving error has proven likely.

practice, this adds up to relatively few flies. Moreover, the expected effect of such high selection pressure does not materialize. Often, for example, the inbred progeny of two such cve flies, or of several, or of one crossed with a normal fly, include no cve individuals for several generations. Thus the noise level attributable to nongenetic variation is considerable. Again, if two *cve* genes have antagonistic effects (MILKMAN 1964), then both may be excluded from a small initial sample, although either alone might have acted additively with the others. In short, there is reason to believe that direct selection of cve flies is likely to be inefficient in the gathering together of genes whose subsequent rise in frequency determines the further course of selection. Note that phenocopy selection permits the taking of any proportion of flies within a wide range (based on degree of response) and the resultant gathering of genes with a positive effect in some of many, rather than few, combinations.

As for the limitation of selection to alternate generations, suffice it to say that it does not prevent rapid progress.

In the final phase, the present method shifts to direct selection in order to apply the more stringent standard required to fix the *cve* genes. Now (and perhaps in the phenocopy selection phase as well), genes acting additively with other *cve* genes, but not with heat shock, are also increased in frequency. The speed of the response and the attainment of 100 percent penetrance and high expressivity attest to the strength of the genetic component underlying the phenotypic variability observed after 7 to 13 generations of phenocopy selection. Generation 8 is an exception. Otherwise, a clear contrast is seen in comparing progress from similar penetrances in flies with a history of completely direct selection (MILKMAN 1964). So, in spite of the fact that only some of the genes which act adversely on posterior crossvein formation also do so additively with higher temperatures, the phenocopy selection phase may be more efficient than direct selection in gathering in the relevant genes. This in turn sets the stage for greater progress later on.

The role of phenocopying in selection illustrates the idea held by many geneticists that the environment serves evolution in two ways: by acting as a testing ground for the various phenotypes in the population; and also as an important agent in the production of this phenotypic variability, in collaboration with genetic variability. The process by which genes with individually small effects in subthreshold numbers are thus brought to light has been called "genetic recruitment" (MILKMAN 1961). Need-

less to say, this process does not require environmental variation of the degree represented by an abrupt three-hour exposure to 36.5°C. Moreover, it may be argued that variations of this degree are in fact encountered: that there is a great deal of difference between a dry 29°C and a humid 17°C; and that populations tend to be fringed with lethal circumstances.

With respect to the irradiated flies, these experiments were the first steps in an attempt to measure the production by irradiation of genetic variability akin to that found in nature. Clearly, these first experiments have served only to block out an area that would have been considered most unpromising on the basis of previous information (CLAYTON and ROBERTSON 1955; SCOSSIROLI and SCOSSIROLI 1959). These investigators have studied the production of polygenic variability by much higher doses of irradiation, and their results, together with those of other workers cited by them, are in general agreement with the following statement made by CLAYTON and ROBERTSON (1955) with specific reference to abdominal bristles: "Under irradiation new variation can be detected, but that utilizable by direct selection is small." The authors just cited made no attempt to estimate induced mutation rates, but OKA, HAYASHI and SHIOJIRI (1958) have estimated mutation rates around 2×10^{-4} per locus per 1000r for two sets of polygenes in rice. The nature of the assumptions on which these latter estimates are based makes them very tentative indeed; but taken at face value, these rates are rather similar to those for the production of sex-linked lethals in Drosophila. On the other hand, MUKAI (1964) estimates the spontaneous mutation rate for polygenes affecting viability to be 20 times that for recessive lethals, and he presents the case for a comparable relationship in radiation-induced mutations. This case is based in considerable part on a similarity between the doubling dose for polygenes and that for major genes. In addition to the work cited by MUKAI, that of YAMADA and KITIGAWA (1961) results in estimates of 18r, 29r and 58r for the doubling dose with respect to various bristles in *D. melanogaster*. These are of the same order estimated for major genes in the same species. Clearly, then, if the spontaneous rate for polygenes is 20 times that of major genes, and if the doubling dose is the same, it follows that the radiation-induced mutation rate for polygenes must be 20 times higher also. But all these estimates are based on phenotypic data alone: essentially on the relation between deviations and variances.

It would appear, then, that there is little concrete information on the mutation rate for the production of "small" genetic differences. The ratio of induced lethals to induced polygenes (mutant alleles whose impact on the phenotype is individually very small) is still uncertain. Obviously, questions of this sort must be answered before one can begin to speak comprehensively about the genetic effects of radiation, since the major natural component of genetic variability is likely to be polygenic.

At any rate, the posterior crossvein seems to be a good theater in which to further this investigation. Various techniques of selection, interactions with simple recessives, and heat phenocopying have all been used effectively to amplify the response to genetic variability of the sensitive developmental process leading to crossvein formation (MILKMAN 1960b, 1961, 1962a, 1964). The demonstration that *cve* genes from various sources interact positively in many cases (MILKMAN 1964) increases the likelihood of success in any attempt to select a team of polygenes from a random array of newly induced mutations. Finally, a fairly extensive vocabulary exists with which to describe and compare individual *cve* polygenes. For these reasons, it was considered desirable to try to detect genetic variation after irradiation, beginning with a dose low enough to include the possibility that mutations with very small phenotypic effects are

produced much more often than are lethals. As it turned out, selection after these low doses was unsuccessful. And so the present system will make a meaningful contribution only when a minimal level of irradiation is found which will introduce into a highly inbred line sufficient genetic variability to cause a response to cve selection. It is hoped that the present technique can lead to direct gene-by-gene analysis, without which we can conclude little about the induction of mutations with small phenotypic effects.

We turn now to two additional aspects of genetic variability which may be investigated by the present method. There is good reason to believe, building for example on the findings of TIMOFEEFF-RESSOVSKY (1934) and of DUBININ (1948), that almost any wild inseminated female is capable of giving rise to a true-breeding polygenic *cve* strain. An attempt is currently underway to see if this is so. If it is, an attempt to measure the relevant genetic variability in such individual flies will be made by successive backcrosses to an isogenic line (suggested by BRUCE WALLACE: personal communication). Naturally, since susceptibility to phenocopying is affected by common polygenes, one would expect to find occasional resistant lines after inbreeding wild flies. Strains X and SL-3, each derived from a wild Syracuse female's F_2 which contained no cve flies in 1000 examined (MILKMAN 1964), are such lines, exhibiting essentially no cve phenocopy response to 36.5°C treatments at various ages and durations. Selection against cve phenocopy response has proven unsuccessful at higher temperatures (WADDINGTON 1953; MILKMAN 1960a) and has not yet been tried at 36.5°C.

It is interesting to consider the potential genetic variability in a single pair of flies, where no gene frequency is below 25 percent. With inbreeding, a far higher proportion of the possible combinations of these genes will be attained than in a population with many additional alleles at lower frequencies, although the latter will of course have a lot more different combinations possible. If evolution proceeds by a method which does not waste genetic variability, most possible combinations should actually appear. As things stand now, we know that individuals have a large share of the population's potential genetic variability. Perhaps this share will look even larger after further investigation of the potential genetic variability of individual pairs of flies.

The discontinuities and dissimilarities in the production of posterior crossvein defects at high temperatures are at present susceptible only to the most general explanations. One thinks in terms of balance. It is likely that selection for phenocopy sensitivity at 36.5° so lowers the threshold of interference with a particular process that the balanced relationship no longer holds, and that this particular process is now accessible to derangement over a broad temperature range. This view is supported by the data in Table 3, particularly the data for response to 40.5°C in Generation 12. Here response does not rise with dose. If anything, it falls after a certain duration has been exceeded. This is like the dose-response relationship observed at 36.5° in wild-type Oregon-R flies, where response first rises, then falls with increasing duration. But it is not like the more usual kind of relationship seen in the 37.5° range and in the 40.5° range, where response continues to rise with dose. Beyond this very general outline, nothing more can be said on the basis of the existing observations, except to point out that the increased susceptibility to other temperatures is only with respect to the production of posterior crossvein defects and not to the production of any other morphological changes or to killing.

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SUMMARY

Selection for crossvein defects via phenocopy sensitivity at 36.5°C is a useful method. Selection increases sensitivity to other temperatures as well. This sensitivity is specific, relating to crossveins, but not to survival. Attempts to use this method as a detector of genetic variability are described, using unirradiated and X-irradiated inbred flies. At the very low doses of irradiation used, any genetic variability produced was insufficient to cause a response to selection.

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