THE EFFECT OF TEMPERATURE ON CROSSVEIN FORMATION IN CROSSVEINLESS-LIKE STRAINS OF *DROSOPHILA MELANOGASTER*[1]

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GOLDSCHMIDT (1935) first demonstrated responses to temperature shock when he showed that heat treatment of normal flies resulted in individuals whose phenotypes resembled those observed in known mutants including crossveinless (*cv*). Such individuals were termed phenocopies. MILKMAN (1962, 1964, 1966) and MOHLER (1965a) have demonstrated that some of the selected crossveinless-like (cvl) lines contain genes which show increased susceptibility to heat shock while others do not, and that heterozygotes from crosses of susceptible cvl lines with a wild type are also more sensitive to heat shock (MILKMAN 1961; MOHLER 1965a). These observations suggest that the temperature shock is acting upon the same functional system as some of the genes whose action it copies.

Phenocopy experiments with wild-type Drosophila have demonstrated a crossvein limiting response, recognized by the production of crossvein defects (MILKMAN 1961, 1962, 1963). However, phenocopy experiments with cvl strains have shown, in addition to a crossvein limiting response, crossvein restoring responses (MOHLER 1965a; MILKMAN 1966). The sensitive periods of these two opposing responses are well in advance of the visible morphological effects of crossvein formation (MOHLER and SWEDBERG 1964). Such findings suggested that temperature shock might be employed as an experimental means of discriminating among the various components involved in normal crossvein development. For MILKMAN (1961, 1962, 1963) suggested that the phenocopy response to high temperature in wild-type Drosophila could be interpreted as due to a series of changes in the tertiary structure of a protein necessary for the formation of complete crossveins. Thus, a series of studies designed to identify and characterize the responses to temperature shock was started.

In this paper the results of an investigation on the types and nature of the responses to temperature shock in a cvl strain of *Drosophila melanogaster* are reported. It will be shown that there are at least five responses to temperature shock: two involved in crossvein restoration, two crossvein limiting responses, and a change in the quantitative differences between the two sexes following temperature shock.

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MATERIALS AND METHODS

The cvl line used was cvl-5-hi, produced by MOHLER (1965b) by direct selection. The phenotypic difference between the two cvl-5-hi strains used in this study and the original wild-type source depends upon a major gene at 48.1 on chromosome 3 (THOMPSON 1967) and upon polygenic modifiers on the X and chromosome 2. The two cvl-5-hi strains have the following chromosome constitution: one strain contains unselected X and second chromosomes from the original Orinda (Ona) source, and the selected third chromosome (Ona X, Ona II, 5-hi III); the other strain has all three major chromosomes from the selected source (5-hi X, 5-hi II, 5-hi III). These two coisogenic strains were constructed according to the procedure outlined by MOHLER (1965b) involving the use of dominantly marked balancer chromosomes. Most of the experiments were carried out on the Ona X, Ona II, 5-hi III strain which has an intermediate level of crossvein defects between that of wild type and *cv*.

Cultures of these strains were maintained in stock bottles on a cornmeal-agar-molasses-Brewer's yeast-propionic acid medium. Experimental cultures were handled in the following manner: mass matings were placed in half pint bottles and subcultured every 3 to 4 days. The eggs and larvae developed at 25.0 ± 0.5° C in constant temperature incubators on the standard medium supplemented with a thick suspension of live yeast on the 4th or 5th day of incubation.

White prepupae were collected and placed on the walls of shell vials and aged in a Precision water bath at 23.0 ± 0.1°C. Whiteness of the prepupae indicates that it was collected within one hour after the onset of puparium formation, the time of collection is therefore taken as time zero in pupal development. High temperature treatments were made in constant temperature water baths, holding the pupae either in vials or teabags; warmup time in vials is approximately 2 minutes and in teabags 2 seconds or less (MILKMAN 1963). After the pupae were subjected to temperature shocks they were returned to the 23° water bath and remained there for 24 hours. They were then placed in a constant temperature incubator at 25.0 ± 0.5°C to complete development.

Following eclosion the adult flies were rated as to the extent of missing crossvein by using a system which arbitrarily divides the posterior crossvein into fifths, so that a rating of zero indicates the entire crossvein is present in both wings, while a rating of ten indicates total lack of crossvein in both wings. The flies were also scored with respect to the present showing interruption and the site of interruption. MOHLER's (1965a) statistical convention has been adopted: r_{10} = mean rating among all flies; r'_{10} = mean rating among flies showing crossvein defects; n = number of flies; P = penetrance; and N = number of wings.

RESULTS

Types of responses: Heat shocks of 20 minutes duration, a subthreshold treatment in wild type (MILKMAN 1961), produced effects in the Ona X, Ona II, 5-hi III strain at three specific pupal ages (Figure 1, Table 1). (1) 20-minute heat shocks at 14 hours of pupal development restored the wild-type phenotype. (2) 20-minute heat shocks at 22 hours produced a more extreme cvl phenotype. (3) 20-minute heat shocks from 28 hours through 34 hours in pupal development produced a reduction in mean rating. This last difference between control and treated samples is due to a reduction in penetrance (Table 1).

Since the Ona X, Ona II, 5-hi III strain is known to be retarded in stages of wing vein development (MOHLER and SWEDBERG 1964) by at least 2 hours when compared with wild type and other cvl strains, another cvl-5-hi strain (5-hi X, 5-hi II, 5-hi III) which is not retarded was used to demonstrate the repeatability of the observed responses. The 5-hi X, 5-hi II, 5-hi III strain, which contains modifiers for increased penetrance and expressivity from the cvl-5-hi line (con-

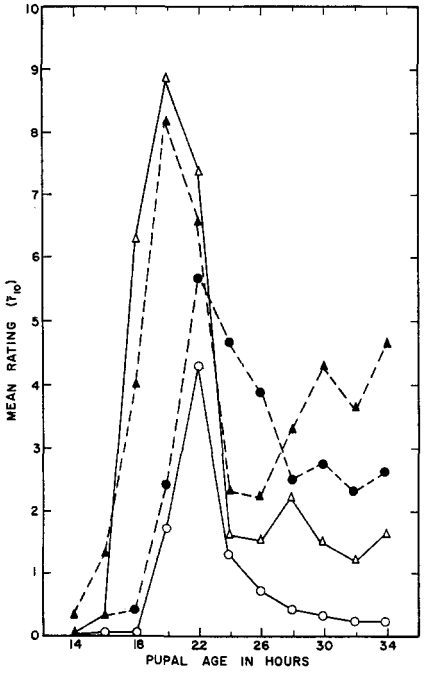

FIGURE 1.—Age response of Ona X, Ona II, 5-hi III and 5-hi X, 5-hi II, 5-hi III to 20 minute temperature shocks (40.5°C). Dotted lines females; solid lines males; circles Ona X, Ona II, 5-hi III, and triangles 5-hi X, 5-hi II, 5-hi III.

trol $\bar{r}_{10}$ for females is 4.8 and for males is 2.3), produced similar responses to 20-minute heat shocks as did the Ona X, Ona II, 5-hi III strain, with the exception that the peak period for the crossvein limiting response occurred at 20 hours rather than 22 hours (Figure 1).

MOHLER (1965a) reported for the crossveinless-like strain cvl-6b differential

TABLE 1

Age response of Ona X, Ona II, 5-hi III to 20-minute heat shock

Pupal age (hours)	Females			Males		
	n*	P†	r'_{10}‡	n	P	r'_{10}
14	49	2.4	2.0	22	0	0
16	141	16.3	1.8	84	0	0
18	139	26.6	1.5	123	0.8	2.0
20	140	52.9	4.5	105	37.1	4.6
22	130	89.2	6.4	116	81.9	5.2
24	144	82.6	5.7	103	30.1	4.4
26	140	77.1	5.1	99	19.2	3.6
28	187	62.6	4.0	124	13.7	3.1
30	192	74.5	3.6	141	13.5	2.4
32	202	65.4	3.5	150	9.3	2.3
34	150	75.3	3.5	122	7.4	2.2
Control	842	88.4	3.4	695	21.1	2.0

* n=Number of individuals per sample.
† P=Penetrance.
‡ r'_{10}=Mean rating among those flies showing interruptions.

responses, following temperature shock, of the two sections of the posterior crossvein adjacent to the fourth and fifth longitudinal veins respectively. Such regional specificity of response to heat shock was also observed in Ona X, Ona II, 5-hi III, where most of the interruptions occurred at the two longitudinal veins, with few center interruptions occurring at any developmental age (Table 2).

An interesting observation upon the sex differences can be made from observing Figure 1 and Table 1. The crossvein phenotypes produced by heat shock in the two sexes are quantitatively similar through 22 hours but differ markedly subsequent to this period.

In order to determine whether the major gene which has been identified with the cvl-5-hi strains behaves in the same fashion as MOHLER's cvl-6b (1965a) and MILKMAN's cve (1961), heterozygotes were produced between Ona X, Ona II, 5-hi III and the Orinda wild type (Ona X, Ona II, Ona III). Unlike cvl-6b and cve, cvl-5-hi does not confer increased sensitivity to heat shock when heterozygous, and no response was obtained with 20-minute heat shocks (40.5°C). Whatever the function of the major gene may be, it is relevant to the response to heat shock, for heat shock can destroy its effect at certain times in development (see Figure 1 and Table 1). It may be that the responses observed with the cvl-5-hi strains are occurring in wild type but are obscured there by the threshold effect. With this in mind, further characterization of the separate responses was next undertaken.

Characterization of the separate responses: Pupae treated with 40.5°C at 22 hours of pupal development show three responses dependent upon the duration of the treatment. (1) Short durations, from one minute up to 5 minutes, cause restoration of the crossvein. (2) Durations of up to 20 minutes produce more ex-

TABLE 2

Changes in specificity during age response of Ona X, Ona II, 5-hi III to 20-minute heat shocks

	Females			Males		
Pupal age (hours)	N*	% L4†	% L5‡	N	% L4	% L5
14	98	1.0	0.0	44	0.0	0.0
16	282	0.0	0.8	168	0.0	0.0
18	278	0.7	1.0	246	0.4	2.8
20	280	25.4	27.5	210	17.6	18.6
22	260	68.8	58.9	232	50.9	47.0
24	288	57.3	44.1	206	14.6	17.5
26	280	48.9	24.6	198	9.1	6.1
28	374	35.3	16.6	248	6.1	3.6
30	384	43.0	15.1	282	4.6	2.8
32	404	41.3	8.9	300	2.0	3.3
34	300	51.7	7.7	244	0.8	3.7
Control	1684	20.6	68.3	1390	0.9	12.1

* N = Number of wings per sample
† % L4 = Percentage of wings with an interruption at the fourth longitudinal vein.
‡ % L5 = Percentage of wings with an interruption at the fifth longitudinal vein.

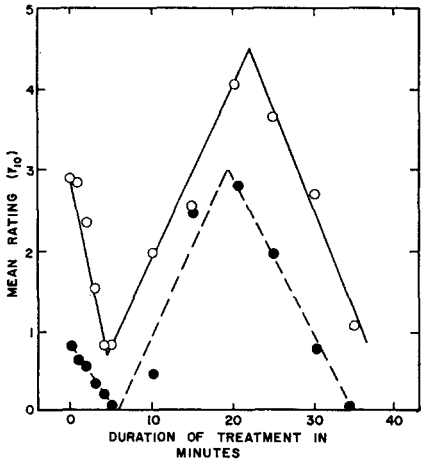

FIGURE 2.—Dosage response of Ona X, Ona II, 5-hi III at 22 hours of pupal age (40.5°C). Open circles females, solid circles males.

treme crossveinlessness. (3) Durations longer than 20 minutes have a crossvein restoring response. For the detailed information see Figure 2.

It is well known that changes in conformation of a protein can be induced with high temperature, and that such conformational changes have characteristically high energies of activation. MILKMAN (1961, 1962, 1963) concluded from temperature dose studies that the phenocopy induction in wild-type Drosophila was due to a series of changes in the tertiary structure of a protein. In order to test whether the various responses demonstrated in this study might also be due to conformational changes in a protein (or proteins), temperature coefficients and the corresponding energies of activation were determined for the responses.

Temperatures at one-degree intervals between 38.5° and 43.5°C (six in all) were compared as to their effect on the crossvein limiting response and the crossvein restoring responses, and it was found that all temperatures at 22 hours gave qualitatively the same result as that shown in Figure 2.

First, a temperature coefficient for the crossvein limiting response at 22 hours of pupal development was calculated by determining the least squares estimates of the regression lines for the 38.5° and the 42.5° treatments, and measuring the ratio between treatment durations giving identical rating values. This calculation gave a temperature coefficient of 1.9 per degree rise in temperature (Q_1), which corresponds to an activation energy of about 126,000 calories per mole (calculation based on the Arrhenius equation, cited in WEST 1963). This high activation energy is characteristic of conformational change of a protein due to heat (EYRING and STEARN 1939) and the changes in phenotype can be interpreted as due to conformational changes in a protein underlying the phenotype. In Figure 3 the data are plotted with response as a linear function of time by converting the treatments of the various temperatures to equivalent times at 40.5°C. The fit tests the validity of the temperature coefficient of 1.9.

Second, by comparing time to equivalent responses between the various temperatures, a temperature coefficient (Q_1) of 2.1 was calculated for the rapid crossvein restoring response at 22 hours, i.e., the crossvein restoring response

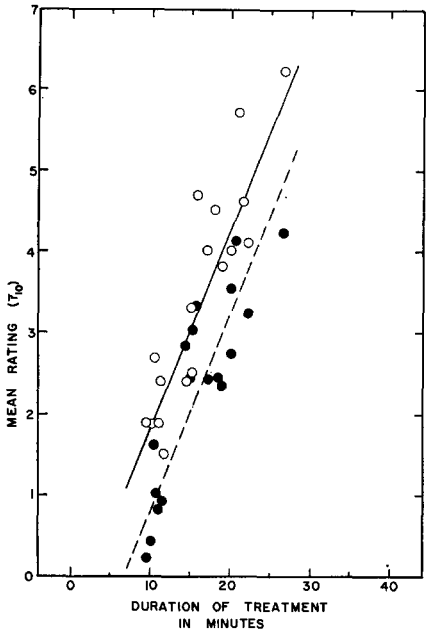

FIGURE 3.—Crossvein limiting response at 22 hours of pupal development in Ona X, Ona II, 5-hi III. All temperatures converted to equivalent times at 40.5°C. $Q_1 = 1.9$. Females open circles, males solid circles.

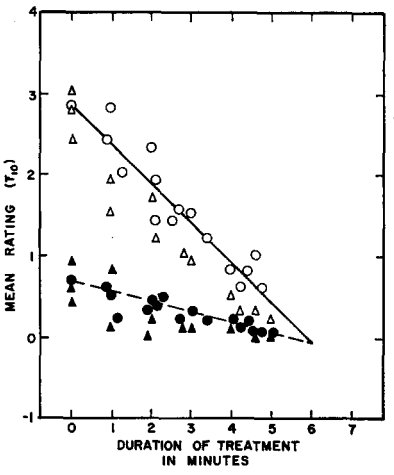

FIGURE 4.—Rapid crossvein restoration with Ona X, Ona II, 5-hi III at 14 and 22 hours of pupal development. All temperature data converted to equivalent times at 40.5°C. $Q_1 = 2.1$. Open triangles females at 14 hours; solid triangles males at 14 hours; open circles females at 22 hours; solid circles males at 22 hours.

following short durations of temperature shock. All the data can be plotted with response as a linear function of time as shown in Figure 4, by converting the treatments of the various temperatures to equivalent times at 40.5°C, again attesting to the reliability of the calculated temperature coefficient. A Q_1 of 2.1 corresponds to an activation energy of about 146,000 calories per mole, which is consistent with conformational change of a protein.

Third, a minimal temperature coefficient (Q_1) of 1.7 was calculated for the crossvein restoring response following long durations of temperature shock at 22 hours. However, this value is reliable only as a minimal estimate, for the calculation is confounded by the fact that very long durations of heat shock cause death, and by the fact that at the lower temperatures (38.5° and 39.5°) the animals have passed out of the most sensitive period before the response is complete, while at the higher temperatures (42.5° and 43.5°) the responses occur so rapidly as to be almost unmeasurable. Nevertheless, a Q_1 of 1.7 appears to be indicative of conformational changes in a protein as the basis of the second restoring response.

Temperature shocks at 14 hours of pupal development show only one response, rapid crossvein restoration. The temperature coefficient calculated for this re-

sponse is identical with that of the rapid restoration at 22 hours, i.e., $Q_1 = 2.1$, and these data fit the same lines as the rapid restoration at 22 hours as shown in Figure 4. Thus, it appears possible that the material affected at 14 hours in development is also affected at 22 hours. Further characterization of the rapid crossvein restoring responses will be considered in greater detail below.

Temperature shocks (40.5°C) at 28 hours in pupal development induced two responses. As expected, penetrance was reduced by treatment periods up to 20 minutes in length (Figure 1, Table 1). Longer treatments resulted in an unexpected crossvein limiting response (Figure 5). This second response at 28 hours, which requires a certain threshold of temperature shock (20 minutes), is strikingly similar to that found at 25 hours with Oregon-R (see MILKMAN 1962), and MOHLER (1967) is showing, with doses longer than 20 minutes, that Ona X, Ona II, 5-hi III produces a two peak age response curve as does wild type, though the second sensitive period (28 through 32 hours) is delayed by about 4 hours. Thus, it appears that Ona X, Ona II, 5-hi III responds to temperature shock in the same fashion as does wild type except for being delayed by approximately 3 to 4 hours.

Further characterization of the rapid restoration response: Since the rapid crossvein restoring response was observed in both 14 hour and 22 hour pupae, an attempt was made to determine the full extent of the period during which a similar effect could be produced. Pupae ranging in age from 6 to 30 hours were tested. Rapid crossvein restoration was found at almost all ages (Table 3).

The identical temperature coefficient calculated for the rapid crossvein restoration responses at 14 and 22 hours suggested that the same process is active in crossvein formation at both ages. This was tested by the use of split treatments in which pupae were subjected to 5-minute heat shock (40.5°C) at either 14 or 22 hours followed by prolonged treatment (40.5°) at 22½ hours. From Figure 1, it can be seen that the response at 22½ hours should not be greatly different from that at 22 hours, so that it is reasonable to compare treatments at 22 hours with those at 22½ hours. If the same process is being affected at both 14 and 22 hours, pretreatments at these two developmental ages would be expected to add in similar fashion to prolonged treatment at 22½ hours.

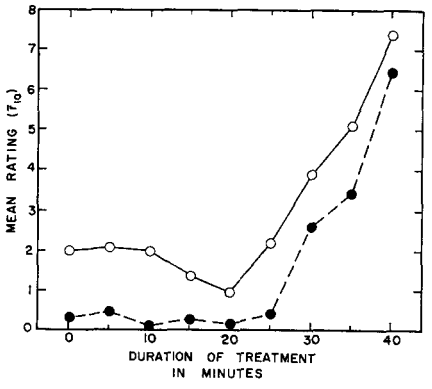

FIGURE 5.—Dosage response of Ona X, Ona II, 5-hi III at 28 hours of pupal age (40.5°C). Open circles females, solid circles males.

TABLE 3

Age response of Ona X, Ona II, 5-hi III to 5-minute heat shock

Pupal age in Hours	Females				Males			
	n	P	$\bar{r}_{10}$*	r'_{10}	n	P	$\bar{r}_{10}$	r'_{10}
6	27	63.0	1.6	2.6	13	61.5	1.2	1.9
8	42	35.7	0.7	2.0	32	15.6	0.4	2.4
10	25	28.0	0.9	3.1	17	5.9	0.1	2.0
12	28	25.0	0.6	2.4	17	0.0	0.0	0.0
14	26	3.9	0.0	1.0	19	5.3	0.1	1.0
16	26	15.4	0.3	1.8	26	0.0	0.0	0.0
18	37	24.3	0.4	1.6	22	0.0	0.0	0.0
20	30	26.7	0.4	1.6	22	0.0	0.0	0.0
22	31	25.8	0.5	1.8	27	3.7	0.0	1.0
24	28	28.6	0.6	2.0	27	0.0	0.0	0.0
26	29	41.4	0.6	1.4	28	3.6	0.0	1.0
28	18	55.6	1.7	3.0	38	7.9	0.1	1.7
30	28	75.0	2.2	3.0	27	14.8	0.3	1.8
Control	141	80.1	2.1	2.6	118	17.8	0.2	1.3

* $\bar{r}_{10}$=Mean rating among all flies.

Pupae subjected to 5-minute pretreatments at 14 hours followed by treatment at 22½ hours showed no increase in crossvein defects over the level observed with 5-minute treatment at 14 hours as shown in Figure 4 (Table 4). MILKMAN (1962, 1963) reported similar responses in wild type to split treatments and termed this phenomenon "protection." However, in contrast to the split treatments at 14 hours, when 22 hour pupae (Ona X, Ona II, 5-hi III) were subjected

TABLE 4

Effect of 5-minute pretreatment on Ona X, Ona II, 5-hi III, followed by increasing durations of treatment at 22½ hours

Duration of treatment*	Pretreatment at 14 hours				Pretreatment at 22 hours			
	Females		Males		Females		Males	
	n	$\bar{r}_{10}$	n	$\bar{r}_{10}$	n	$\bar{r}_{10}$	n	$\bar{r}_{10}$
10	36	0.4	28	0.0	40	4.0	31	3.5
15	36	0.6	19	0.0	45	4.5	26	3.7
20	42	0.7	30	0.0	39	5.5	34	4.6
25	41	0.5	33	0.1	34	6.2	31	5.4
30	47	0.6	31	0.0	42	6.1	27	4.5
35	36	0.4	19	0.0	40	5.0	29	4.3
40	..	..	..	..	36	3.4	18	1.2
50	..	..	..	..	18	2.7	18	0.1
60	..	..	..	..	16	0.0	19	0.0
Control†	26	0.0	17	0.0	31	0.5	27	0.0

* Duration of treatment at 22½ hours.
† Control data are taken from Table 3, and are the values obtained with 5 minutes (40.5°C) treatment at 14 and 22 hours.

to 5-minute pretreatment followed by treatment at 22½ hours the combined effect of the two treatments was like the continuous treatment at 22 hours shown in Figure 2 (Table 4). Further, in both of these experiments pretreatment protects against death, for the combined treatment of 40 minutes (5 minutes pretreatment + 35 minutes treatment) is lethal in a continuous treatment.

Although the two split treatments gave different results, it cannot be unequivocally stated that the two rapid crossvein restoring responses at 14 and 22 hours are different on the basis of these experiments, because MILKMAN (1963) has shown that the interval between treatments is important in determining whether additivity or "protection" occurs with split treatments in wild type. It may be that a 25-minute interval is not long enough to determine "protection" at 22 hours.

DISCUSSION

This study has recognized a number of responses to temperature shock in a cvl strain; at least two crossvein restoring responses and two crossvein limiting responses. There is yet another interesting point which has been revealed in this study. that is, the change in the sex differences after heat shock. This change occurs in age response studies up through 22 hours in pupal development, and in dose response with durations as low as 5 minutes and up through 20 minutes at 40.5°C.

High temperature coefficients and the corresponding high energies of activation are characteristic of conformational changes in protein due to heat treatment. Since no other material is known in biological systems which has such a high temperature coefficient, the responses have been interpreted as having their basis in conformational change in protein. The calculation of very high temperature cofficients and activation energies does not constitute absolute proof that the responses are due to conformational change in protein following heat treatment, but the interpretation seems reasonable, and was used by MILKMAN (1962) in interpreting the various effects produced by temperature shock in wild-type Drosophila.

MILKMAN's hypothesis does not, however, explain all of the data now available about posterior crossvein formation, e.g., the recognition of opposing responses to heat shock in MOHLER's study with cvl-6b (1965a) and again in this study. It appears that the responses demonstrated here occur prior to those described by MILKMAN (1961, 1962, 1963) for the following reasons: (1) studies by MOHLER and SWEDBERG (1964) have shown that Ona X, Ona II, 5-hi III is retarded in stages of wing vein development when compared with other cvl strains and with wild type; (2) the crossvein limiting response at 28 hours is like that of Oregon-R at 25 hours in development, and MOHLER (1967) is showing that with doses longer than 20 minutes Ona X, Ona II, 5-hi III responds as does wild type, but the sensitive periods are delayed by about 3 to 4 hours. If the responses of this study are prior to those already described, then they are especially interesting because the affected processes underlying these responses may be involved in setting the stage for what occurs later in development.

I am grateful to DR. JAMES DAWSON MOHLER who directed and guided me during the course of this investigation. The advice and criticism of DR. ROGER D. MILKMAN are also much appreciated.

SUMMARY

Development of the posterior crossvein in crossveinless-like (cvl) strains can be influenced by high temperature shocks at specific times in pupal development. Different developmental responses to heat shock were detected in three sensitive periods; crossvein restoration at 14 hours, crossvein limitation at 22 hours, and a reduction in cvl penetrance at 28 hours. Characterization of these responses revealed that at 22 hours there also exists a rapid crossvein restoration which appears to be identical with the response at 14 hours, and another restoration following prolonged treatment at 22 hours. At 28 hours an additional crossvein limiting response was observed. Temperature coefficients have been calculated for the responses at 14 and 22 hours; these coefficients indicated that the basis of the various responses is in conformational changes of protein.

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