Effects of Successive Generations of Ether Treatment on Penetrance and Expression of the *Bithorax* Phenocopy in *Drosophila melanogaster*

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ABSTRACT   Ether treatment of embryos in successive generations results in the progressive increase in penetrance of the *bithorax* phenocopy in both a massbred and an inbred line of *Drosophila melanogaster* without artificial selection for the phenocopy. The involvement of cumulative cytoplasmic modifications is suggested by the results of reciprocal crosses between the treated massbred line and its foundation stock, and by the lingering effects observed when ether treatment is withheld in later generations.

Regression analyses showed that the effect of successive generations of ether treatment on penetrance is cumulative and direct, and not mediated by possible effects on viability.

Ether treatment carried out on embryos at four consecutive age intervals in the 14th generation suggests that the effects of long-term etherisation are as follows: an increased tendency to phenocopy at all ages tested; an extension of the critical period during which the phenocopy can be induced; and an increased resistance to the lethal effects of ether in older embryos.

The remarkable consistency in every aspect of the response in the two different lines—one containing at least 20 times as much genetic variation as the other—points to the existence of systematic organismic properties which do not depend on specific alleles in specific genes.

The possible evolutionary significance of our results is discussed.

Phenocopies mimicking the action of mutant genes are readily produced by environmental agents such as heat shocks and noxious chemicals (Goldschmidt, '45; Landauer, '58; Sang and McDonald, '54). Selection for penetrance and/or expression in successive generations often results in genetic assimilation—the appearance of the phenocopy in the absence of the external stimulus (Waddington, '57; Bateman, '59a,b). The phenomenon is ususally explained in terms of subthreshold alleles—exposed by the environmental stimulus—the penetrance and expression of which become further enhanced by selection for additional polygenic modifiers (Bateman, '59b; Maynard-Smith, '66; Milkman, '70; Stern, '58).

Polygenic modifiers of major mutant genes have been identified. For example, modifiers for *veinlet* have been found in homozygous lines selected for increased expression; more-over, selection for the modifiers alone produced at least part of the mutant phenotype in the absence of the major gene (Thompson and Thoday, '75). The effect of the major gene mutant is thus formally equivalent to that of the environmental stimulus in exposing preexisting alternative pathways in the "epigenetic landscape" (Waddington, '57).

When various genetically assimilated lines were analysed (Waddington, '56; Bateman, '59a,b) many genes spread over all chromosomes were usually found to be involved, suggesting that modifiers were indeed selected. In some lines, however, a single major gene or several major genes were also impli-

cated. Waddington ('75) left it an open question as to whether genetic assimilation results from new mutations arising during the experiment or is merely the endpoint of the selection for modifier genes whereby a new developmental trajectory is progressively canalised (or a combination of both).

Whatever the detailed mechanisms involved, the modifier genes models presuppose that the starting population contains variation in modifier genes, and that canalisation (and genetic assimilation) would only take place where there is selection for the phenocopy, either naturally or artificially. The specific prediction which could be made is that canalisation would not take place in a genetically uniform population and where there is no selection for the phenocopy. Thus, Bateman ('59a,b) reported no progress in the penetrance of the *dumpy* and *posteriorcrossveinless* phenocopies in inbred lines after a small number of generations of heat treatment and selection. But neither was there any progress in the massbred line until the high mortality of the phenocopied flies was reduced by shortening the period of heat treatment. No parallel checks on the mortality of phenocopies were performed in the inbred lines. In one inbred line selected for the *posteriorcrossveinless* phenocopy for nine generations, progress was recorded in the last three generations. But that experiment was terminated with the remark: "The fact that a parallel trend was also shown by the unselected line indicated, however, that this apparent response was in fact merely secular" (Bateman,'59b). This happened to be the only instance in which an unselected control was maintained.

One complicating factor in investigating phenocopies, which may be relatively unimportant when the genetic mutants themselves are being studied, is the effect of the environmental stimulus on the organisms. In particular, the possibility cannot be ruled out that cumulative cytoplasmic or maternal influences could lead to an increase in the phenocopy response in successive generations. (This would explain Bateman's ('59b) results cited above.) Such a mechanism operating independently of, or in addition to natural selection, could have important consequences for the rate of phenotypic evolution (Ho and Saunders, '79). The attendant nuclear-cytoplasmic interactions in turn may exert a directive influence on genotypic evolution.

Environmentally induced modifications of the cytoplasm which are transmitted across generations are by no means unknown. Many of these were studied extensively in *Paramecium* by Jollos ('21), Sonneborn ('70), and Beale ('58), in *Amoeba* by Danielli ('58), and in *Aspergillus* by Jinks ('57; '58) and others. In all the above systems, considerable interactions between cytoplasm and nuclear genes were found, which often resulted in large phenotypic effects. Among higher organisms, Harrison ('28) induced heritable changes in the pigmentation of *Pieris* pupae by means of orange light. Fuji ('78) reported the transmission of serum calcium disorders to the offspring of parathyroidectomized female rats up to the F_4 generation. The inheritance of phenotypic changes in flax plants treated with various fertilizer, first investigated by Durrant ('62) and more recently by Cullis ('77, '81), probably also involves some form of cytoplasmic modifications and cytoplasmic-nuclear interactions.

We decided to reinvestigate the *bithorax* phenocopy, following Waddington's ('56) original procedure as closely as possible, but without artificial selection in both a massbred line and inbred lines of the same genetic background. An increase in response in successive generations would mean an increase in penetrance or expression, or both. If our conjecture is correct, this should occur in the inbred as well as the massbred line without artifical selection. Natural selection *against* the phenocopy is not ruled out. Waddington ('56) found evidence of this in his experiments; we also found that bithorax flies are less viable and less fertile than normal flies (Ho et al, unpublished). Any increase in penetrance or expression would have to occur in spite of natural selection, thus making the test of our hypothesis more stringent.

MATERIALS AND METHODS

The massbred line (*Mass*), initiated by wild-caught flies from Massachusetts, USA, and reared in the laboratory for about 5 years, was kindly supplied by Dr. B. Charlesworth. Two inbred lines (*H* and *C*) were derived from the massbred line by single-pair brother-sister mating for 25 generations. Samples of 50–100 flies in each line were analysed for isoenzyme variation by starch gel electrophoresis (Harris and Hopkinson, '76). *Mass* was polymorphic for alcohol dehydrogenase (ADH), esterase (E), glycerophosphate dehydrogenase (GPD), phosphoglucomutase (PGM), and isocitrate dehydrogenase (ICD); and monomorphic for 6-phosphogluconate dehydrogenase (6GD), phosphoglucose-isomerase (GPI), and malate dehydrogenase (MDH). Line *C* was

homozygous for all the above enzymes, whereas *H* was polymorphic for GPD, indicating the presence of residual genetic heterogeneity. All the lines were reared on a malt-molasses-and-cornmeal-based recipe and maintained at 25°C on a 12-h light/dark cycle.

For ether treatment, six to nine batches of eggs were collected in food vials for periods of 1 h over 2–3 days from flies 3–7 days old which had been subjected to a prelaying period of 16 h with or without an additional period of 1 h. Ether treatment was performed between 2.5 and 3.5 h after deposition (Waddington, '56) by inverting the vials for 25 min over a 3–4 cm depth of ether in a sealed dessicator. There was considerable variation between batches. Much of the variation stemmed from the age distribution of the eggs around the sensitive period of phenocopy induction (see Results), and was difficult to control. The other sources of variation came from factors affecting the intensity of treatment such as the size of the etherising chamber, the amount of ether, length of exposure, etc. For these reasons, when comparing different lines, etherisation was carried out as far as possible together in the same dessicator, or appropriately randomised over several dessicators. Otherwise, the conditions of etherising were reproduced as accurately as possible for the lines compared.

Mortality rates were monitored by counting samples of eggs in each batch. In later generations, when mortality due to ether treatment increased by more than 5%, the time of treatment was reduced to between 20 and 23 min. There was no effect of ether treatment on the total length of the life cycle. The timing of emergence was remarkably constant and predictable in all generations of treatment. The *penetrance* (percentage of bithorax flies among emerged adults) and the grade of expression were determined either by complete ascertainment under a dissecting microscope, or by examining representative vials from each batch of treated eggs. Phenocopies were graded I to VII according to the size of the total area transformed, which varied continuously as reported by Gloor ('47). Phenocopy spots were present on both dorsal and ventral surfaces. *Expressivity* is given by the sum of the percentage (of all phenocopies) in each grade multiplied by the grade. The sexes were scored separately.

RESULTS
Penetrance in successive generations

Penetrance tended to increase in successive generations in all three lines (Fig. 1). The initial frequencies were typically low (1.5–3%, though under optimal conditions, penetrance could be as high as 10% in the foun-
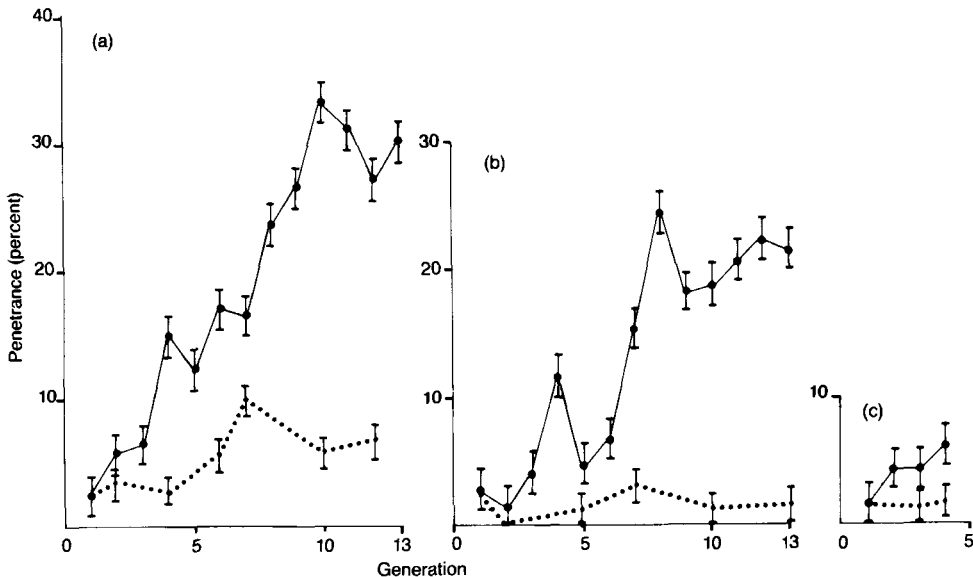

Fig. 1. Penetrance in successive generations of ether treatment in (a) *Mass*, (b) *H*, and (c) *C*. Solid lines represent the progress of the experimental flies; broken lines represent the respective foundation stocks tested at intervals with the experimental. Vertical bars represent standard errors.

dation stock of *Mass.* Line *C* showed considerable inbreeding depression: Its range of mortality on ether treatment was between 60 and 75%, as compared to 40 and 65% in *H* and 35 and 60% in *Mass* (Table 2). Line *C* died out after the fourth generation, at which time its penetrance of 6% was significantly higher than that of the first generation and its foundation stock. There was no difference in penetrance between the sexes.

Expression in successive generations

The distributions among seven grades of expression in females and males in successive generations are depicted in Figure 2. The distributions seem to be discordant between the sexes in the early generations and to converge from generations 4 or 5. Chi-squared tests did not give significant results for any generation, however, indicating the absence of gross difference in expression between the sexes.

Expressivity increased to a maximum around generations 3 or 4, then decreased and stabilised around 280 to 300 between generations 5 and 8 (Table 2). When unemerged pupae were dissected, a disproportionately large number turned out to be bithorax and of the highest grades in *Mass* as in all other lines. This confirms the selection against bithorax flies, especially of high grades first noted by Waddington ('56), and may explain the decrease in expressivity in later generations. At generations 9 and 10 ether treatment was done between 2¼ and 3¼ h. This resulted in an immediate increase in expressivity, suggesting that expression may be governed primarily by the time of ether treatment in relation to the timing of the critical period of phenocopy induction (see below).

Similar data were collected for the *H* line (summarised in Table 2). Again, no statistically significant difference in expression between the sexes could be demonstrated. Expressivity in the *H* line (as well as penetrance) was lower in every generation compared to the *Mass*, even though the same early increase to peak expression around generations 3 or 4 was evident.

Reciprocal crosses between the long-term-treated Mass *line and and its foundation stock*

After six generations of ether treatment, reciprocal crosses were set up between virgins of the treated *Mass* line and its control
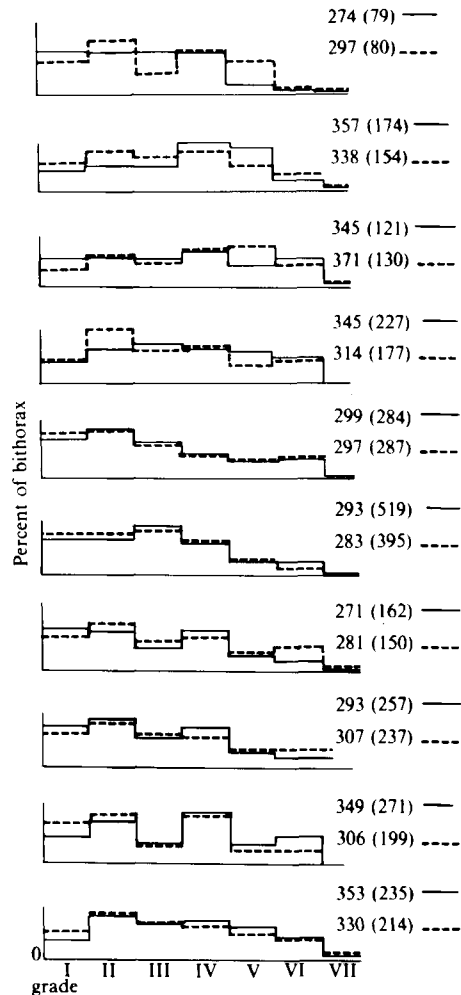

Fig. 2. Distribution of grades of expression in successive generations of ether treatment in *Mass.* From top to bottom, generations 1 to 10. Solid lines represent females, and broken lines, males.

or foundation stock. The *Mass* line was chosen because its total population was much larger than *H* (which could ill-afford to be effectively split into two subpopulations, virgins for the cross and nonvirgins to continue the line), and because the test for cytoplasmic effects does not depend on isogenicity—a criterion not satisfied by *H* in any case.

The eggs were etherised as usual and the adult offspring scored and allowed to mate and lay eggs for the next generation of treatment. The results are summarised in Table 1. The F_1 reciprocal crosses differed signifi-

TABLE 1. *Penetrance and expressivity in progeny of crosses between the long-term-treated* Mass *line and its control foundation stock*

Cross	Penetrance[1]	Viability[2]	Expressivity	
			Females[3]	Males[3]
C♀ × C♂	9.96 (2,248)**	62.51 (1,578)	274 (79)	197 (80)
T♀ × T♂	16.53 (6,226)**	61.88 (2,534)	271 (162)	281 (150)
C♀ × T♂	11.73 (307)*	54.91 (448)	217 (23)	264 (14)
T♀ × C♂	15.92 (1,143)*	59.72 (1,738)	277 (82)	246 (73)
(C♀ × C♂)[2]	19.82 (1,807)**	38.58 (1,366)	357 (174)	338 (144)
(T♀ × T♂)	23.76 (2,841)**	43.97 (1,867)	293 (257)	307 (237)
(C♀ × T♂)[2]	10.93 (183)**	45.00 (293)	310 (10)	282 (11)
(T♀ × C♂)[2]	26.79 (984)**	42.07 (1,084)	317 (98)	268 (98)

*0.050 > P > 0.025.
**0.025 > P > 0.010.
***0.010 > P > 0.005.
****0.005 > P > 0.001.
*****P < 0.001.
[1]Percentage of emerged adults; number scored in parentheses.
[2]Percentage of eggs counted; number in parentheses.
[3]See text; number scored in parentheses.

TABLE 2. *Viability, penetrance, and expressivity in successive generations of ether treatment*[1]

G	V	P	A	E$_{(♀♀)}$	E$_{(♂♂)}$	
1	64.98 (414)	2.48 (1,046)	1.61	274 (79)	297 (80)	Mass
	53.09 (162)	2.86 (734)	1.52	259 (38)	264 (44)	H
2	59.46 (698)	5.85 (1,616)	3.48	357 (257)	338 (237)	Mass
	54.31 (569)	1.43 (558)	0.78	280 (30)	296 (31)	H
3	53.22 (1,227)	6.50 (2,568)	3.46	345 (163)	371 (114)	Mass
	50.72 (414)	4.00 (575)	2.03	284 (55)	314 (59)	H
4	51.55 (1,837)	14.97 (5,158)	7.72	345 (177)	314 (177)	Mass
	52.20 (542)	11.73 (563)	6.12	306 (35)	295 (37)	H
5	56.69 (2,891)	12.73 (8,319)	7.23	299 (284)	297 (287)	Mass
	48.14 (1,208)	4.78 (607)	2.30	253 (17)	284 (19)	H
6	54.34 (1,406)	17.08 (4,034)	9.28	293 (519)	283 (395)	Mass
	55.46 (2,023)	6.80 (1,398)	3.77	304 (42)	282 (45)	H
7	61.88 (2,534)	16.53 (6,226)	10.23	271 (162)	281 (150)	Mass
	46.78 (1,353)	15.24 (1,562)	7.13	237 (89)	292 (83)	H
8	43.97 (1,867)	23.76 (2,841)	10.44	293 (257)	307 (237)	Mass
	35.87 (683)	24.24 (660)	8.69	275 (63)	261 (54)	H
9	54.17 (864)	26.75 (2,994)	14.49	349 (271)	306 (199)	Mass
	53.97 (1,297)	18.83 (1,384)	10.16	298 (84)	283 (65)	H
10	42.52 (762)	33.33 (1,413)	14.17	353 (235)	330 (214)	Mass
	40.90 (2,093)	18.93 (1,949)	7.74	320 (140)	307 (121)	H
11	41.08 (964)	31.18 (917)	12.81	380 (166)	322 (144)	Mass
	39.18 (1,031)	20.54 (935)	8.05	309 (151)	317 (90)	H
12	48.71 (1,006)	27.39 (1,278)	13.34	262 (145)	263 (133)	Mass
	60.78 (663)	23.83 (389)	14.48	286 (139)	281 (113)	H
13	59.73 (822)	30.05 (1,471)	17.95	271 (119)	272 (117)	Mass
	55.68 (925)	21.25 (1,473)	11.83	226 (247)	213 (213)	H

[1]G, V, P, A, and E represent generation, viability, penetrance, absolute penetrance, and expressivity, respectively; see text.

cantly in penetrance, each resembling the line from which the *maternal* parent was drawn. In the F₂ of the crosses, the difference in penetrance became even more exaggerated. While the same resemblance to the maternal parental line was maintained in the cross involving treated females, that involving control females showed little or no increase in penetrance over its F₁, falling considerably below the second generation of treatment in the control line. The reasons for this are unclear. One possibility is interaction between cytoplasm from control females and nuclear genes from treated males which have been subject to segregation and recombination. This assumes that genetic changes

may have occurred as the result of the six previous generations of ether treatment.

The relationship among generations of treatment, viability, penetrance, and expressivity

The detailed data for 13 generations of ether treatment in *Mass* and *H* are given in Table 2. Regression analyses were performed after arcsine transformation of the frequency data (Sokal and Rohlf, '81). Penetrances were expressed as absolute frequencies (as percentage of embryos treated, rather than as percentage of adults emerged). As the total numbers of embryos treated were not known, the absolute penetrance (hereafter referred to as A) was estimated to be the product of viability (V) and penetrance (P). This removes confounding effects of viability on penetrance. For instance, if absolute penetrance were unaffected by viability, then a spurious negative correlation between them would result from the mere fact that as viability decreases, penetrance expressed as percentage of *surviving* adults is bound to increase. The results of our analyses are summarised in Table 3.

Correlations among variables within and between lines. Significant correlations exist between generations of treatment (G) and absolute penetrance (A) and between expressiv-

ity (E) of females and males within both lines (rows 2 and 7). Between lines, correlations of viabilities, absolute penetrances, and expressivities are all significant (rows 8–10).

Correlation in expressivity between the sexes confirms the earlier observations that there is no essential sex difference in expression and that their response to successive generations of treatment are also similar. The between-lines correlations of V, A, and E are to be expected on the basis of a general similarity of response in each generation to uncontrolled and unknown variation in the conditions of treatment (and rearing). These similarities are perhaps not surprising given the identity of "genetic background" between the two lines.

The effect of generations of treatment on viability and absolute penetrance. Absolute penetrance is very highly and significantly correlated with generations of treatment in both *Mass* and *H* (row 2, Table 3) whereas neither viability (row 1) nor expressivity (row 4) is so correlated. This in itself suggests that the effect of G on A is cumulative and direct. Some interaction among G, V, and A is apparent, however. In *Mass*, though not in *H*, V is negatively correlated with both G and A (rows 1 and 3). Even though the correlation is not statistically significant in either case on account of the small number of observa-

TABLE 3. *Correlation among generations of treatment, viability, absolute penetrance, and expressivity*[1]

A. Correlation	r_{xy}	$t_{[11]}$	$F_{[1,11]}$	
1. GV	−0.50	−1.92	3.69	*Mass*
	−0.11	−0.32	0.15	*H*
2. GA	0.96	11.36*****	128.99*****	*Mass*
	0.91	7.04*****	49.60*****	*H*
3. VA	−0.42	−1.53	2.35	*Mass*
	0.05	0.16	0.03	*H*
4. GE$_{(♀♀)}$	−0.13	−0.42	0.18	*Mass*
	0.03	0.09	0.01	*H*
5. VE$_{(♀♀)}$	−0.47	−1.75	3.07	*Mass*
	−0.18	−0.62	0.38	*H*
6. AE$_{(♀♀)}$	−0.09	−0.28	0.81	*Mass*
	0.01	0.02	0.00	*H*
7. E$_{(♀♀)}$E$_{(♂♂)}$	0.80	4.24****	17.95****	*Mass*
	0.66	2.92**	8.55**	*H*
8. V$_{(Mass)}$V$_{(H)}$	0.58	2.37*	5.61*	
9. A$_{(Mass)}$A$_{(H)}$	0.87	5.99*****	35.90*****	
10. V$_{(Mass)}$V$_{(H)}$	0.69	3.15***	9.89***	
B. Partial correlation	$r_{xy.z}$	$t_{[10]}$		
11. GV.A	−0.38	−0.97		*Mass*
	−0.39	−1.37		*H*
12. GA.V	0.95	10.07*****		*Mass*
	0.92	7.34*****		*H*
13. VA.G	0.25	0.81		*Mass*
	0.38	1.29		*H*

[1]Probability of *t* and *F* values as in Table 1; symbols as in Table 2.

tions involved, the possibility cannot be ruled out that changes in V could have contributed to the increase in A with successive generations of ether treatment. For example, the strength of ether treatment may have been unconsciously increased in successive generations—hence the negative correlation between V and G. The negative correlation between V and A in turn suggests that as the strength of treatment increased, mortality and penetrance would also increase. Such an explanation is unlikely to account for much of the total cumulative increase in A, however, as the latter is far greater than the penetrance of controls included in some later generations of treatment (Fig. 1). The absence of correlation between G and V and between V and A in *H* is also evidence against the above interpretation, unless of course, *H*, being inbred, behaves differently from *Mass*. Further analyses indicate that the responses of *Mass* and *H* are in fact remarkably similar.

Despite moderately large differences between *Mass* and *H* in the total correlations between G and V and between V and A (rows 1 and 3) the partial correlations are very similar (rows 11 and 13). This means that once the confounding effect of variation in the third factor is removed, the correlation between any pair is the same in the two lines—suggesting the presence of common processes involved in the response to ether treatment. Note that the partial and total correlations between G and A are almost identical (compare row 2 with row 12), reinforcing our interpretation of a direct and cumulative effect of generations of treatment on penetrance in both lines.

Relationship between viability and absolute penetrance. The partial correlation between V and A (row 13, Table 3) is positive whereas that between G and V is negative in both lines. Although neither is by itself statistically significant, the fact that the correlations are so similar, both in extent and in direction in two different lines, suggests that the phenomenon may be real and hence worthy of comment. Viability decreases with generation. Given that this is not due to an unconscious increase in the strength of ether treatment in successive generations, then one must assume that generations of ether treatment decrease viability in a cumulative way to some extent. Paradoxically, the partial correlation between V and A is *positive*, suggesting that viability as such contributes to the increase in penetrance. If so, its effect

over the generations must be largely counteracted by the decrease in viability with generations of treatment. The positive partial correlation between V and A is easily explained: Phenocopies, especially those showing extreme expression, are known to be less viable than normal flies. Hence, they would be less tolerant to adverse environmental conditions. When conditions are such that general mortality is high, more bithorax than normal flies will succumb, so that the resulting absolute penetrance will be low. Conversely, under favourable conditions, the survival of bithorax flies will improve, thereby increasing absolute penetrance. This interpretation is consistent with the observation that penetrance and expressivity are both higher among unemerged pupae than in emerged flies. There seems to be little room for doubt that the phenocopy is selected against, and is not directly associated with higher tolerance to ether.

Path analysis. To make more explicit the presumed causal relationships between generations of treatment, viability, and penetrance, path analysis (Sokal and Rohlf, '81) was performed on the data, assuming the path diagram in Figure 3. The path coefficients are standardized regression coefficients. The coefficients of paths G → A and V → A are obtained by multiple regression of A on G and V; that of G → V is obtained by simple regression of V on G. The coefficients of unknown causal paths, U_a → A and U_v → V, are the residual variation unexplained by each of the above regressions, respectively.

Once again it can be seen that generations of treatment have a major and direct effect on absolute penetrance. The increment in determination of A due to V, over and above that due to G, is not significant in either *Mass* or *H* (the F values are 0.6513 and 1.673,
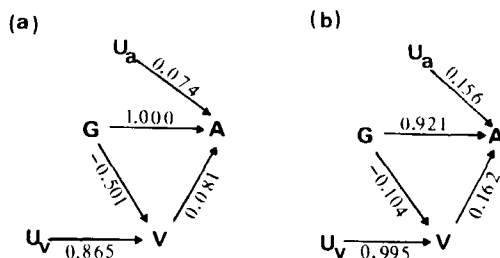

Fig. 3. Path diagram for *Mass* (a) and *H* (b). See text.

respectively, on 1,10 degrees of freedom). While G affects A directly and cumulatively so that only a small proportion of the effect is assigned to unknown causes (U_a), its action on V is indirect and complex. The large residual variance which is unexplained by the regression of V on G means that the major causes of variation in V remain unknown (hence the large path coefficients for U_v).

Critical period of phenocopy induction: Penetrance and expression

At generation 14 an experiment was carried out to compare the critical periods of phenocopy induction in the treated *Mass* and *H* with their respective foundation stocks. Eggs were collected at half-hourly intervals, and all the vials were randomised and treated together for 20 min.

Table 4 gives the viabilities, penetrances, and expressivities for *Mass* and *H* compared with their respective foundation stock at four consecutive age intervals. There appears to be no obvious change in the timing of the critical period of phenocopy induction in the long-term-treated lines compared to the controls. The most sensitive interval remains at 2–2.5 h (the preblastoderm stage (Bownes, '75; Ho, et al., '82)). However, the drop in penetrance between the peak interval and the next (2.5–3 h) appears to be *proportionately* less in the treated lines. In *H* penetrance remains abnormally high at the old-

est age interval, suggesting an extension of the critical period of phenocopy induction to older ages. This is more clearly seen when absolute penetrances are plotted (Fig. 4). The difference in magnitude between treated and controls remains, and the trend toward an extension of the critical period is particularly evident in *H*.

Expressivity is a function of age in all lines; the peak of expression corresponds approximately to the peak of penetrance. Expressivity is generally higher in the treated lines than in the controls at all intervals, although the difference in mean is not significant, except in one case (Table 4). This is mainly because our scoring system is not sufficiently finely graded. The distributions of the grades of expression are shown in Figure 5. Note that in the oldest interval of *H*, high-grade phenocopies (IV or above)—characteristic of earlier intervals—are present in moderate numbers. This is consistent with the extension of the critical period suggested by the penetrance data. We have since repeated similar experiments using a number of different *Drosophila* stocks under nearly identical conditions of ether treatment and rearing, and have never yet come across phenocopies above grade IV in the oldest age intervals (Ho et al., '82).

A somewhat unexpected finding was the highly significant increase in viability in both treated lines for the intervals 2.5–3 h

TABLE 4. Viability, penetrance, and expressivity as the result of etherization at four consecutive age intervals[1]

Interval		V	P	E	
		A. *Mass* lines			
I	(1½–2h)	16.18 (717)	37.50 (256)*****	267 ± 16 (94)**	Control
		15.53 (425)	60.58 (104)*****	327 ± 24 (45)**	Treated
II	(2–2½h)	30.02 (976)	40.98 (471)*****	283 ± 13 (133)	Control
		26.57 (335)	63.45 (238)*****	315 ± 14 (128)	Treated
III	(2½–3h)	41.66 (1,325)*****	11.19 (777)****	203 ± 12 (76)	Control
		53.10 (532)*****	20.29 (473)****	212 ± 14 (74)	Treated
IV	(3–3½h)	56.31 (1,471)*****	0.82 (971)	164 ± 15 (11)	Control
		73.81 (443)*****	1.93 (621)	193 ± 19 (14)	Treated
		B. *H* lines			
I	(1½–2h)	20.00 (80)	32.00 (50)***	244 ± 32 (16)	Control
		21.82 (536)	53.85 (195)***	289 ± 16 (109)	Treated
II	(2–2½h)	35.71 (84)	39.29 (112)**	249 ± 20 (41)	Control
		40.35 (342)	52.22 (383)**	270 ± 10 (200)	Treated
III	(2½–3h)	40.57 (106)*****	10.27 (292)*****	196 ± 20 (27)	Control
		59.90 (763)*****	24.57 (578)*****	250 ± 12 (159)	Treated
IV	(3–3½h)	50.68 (146)**	1.90 (211)*****	150 ± 18 (8)	Control
		61.41 (425)**	14.84 (468)*****	221 ± 20 (53)	Treated

[1]All symbols as in Table 2; expressivities (females and males are pooled) given with standard errors. For probabilities on the t-test see Table 1.
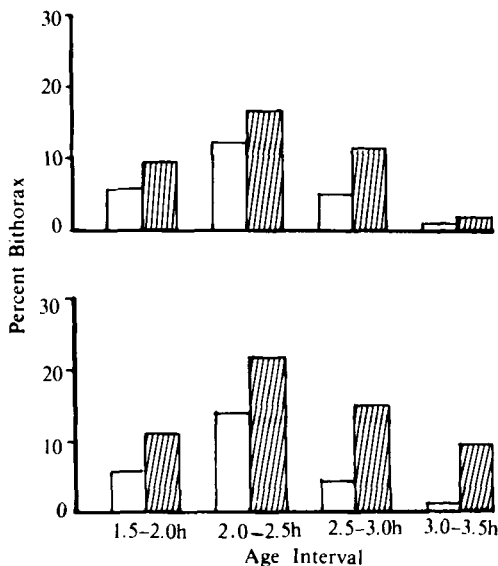
Fig. 4. Absolute penetrance at four age intervals. Top, *Mass* and its foundation stock; bottom *H* and its foundation stock. Controls unshaded.

and 3–3.5 h. The viabilities in successive generations of ether treatment (Table 2) showed no clear evidence of progress as might be expected if selection for ether tolerance was taking place. Instead, viabilities tended to decrease with successive generations until generation 12 or 13. It is possible that an interaction between selection for ether tolerance and cumulative cytoplasmic effects may be involved, which tended at least initially to decrease viability.

We have demonstrated by statistical analyses that the effects of generations of ether treatment on penetrance are largely independent of those on viability. However, it may still be questioned whether the terminal increase in viability may be linked in some way to the apparent extension of the critical period of induction. The most obvious hypothesis is that when treated with ether, the embryo either phenocopies or succumbs. This would almost certainly apply in the early embryonic stages which we know to be much more susceptible to the lethal effects of ether (Table 4, compare viabilities in intervals I and II with those of III and IV; see also Ho et al., '82). Thus, those embryos already sensitive to phenocopy induction will be protected relative to those not yet sensitive. As a result, selection would operate so that after a
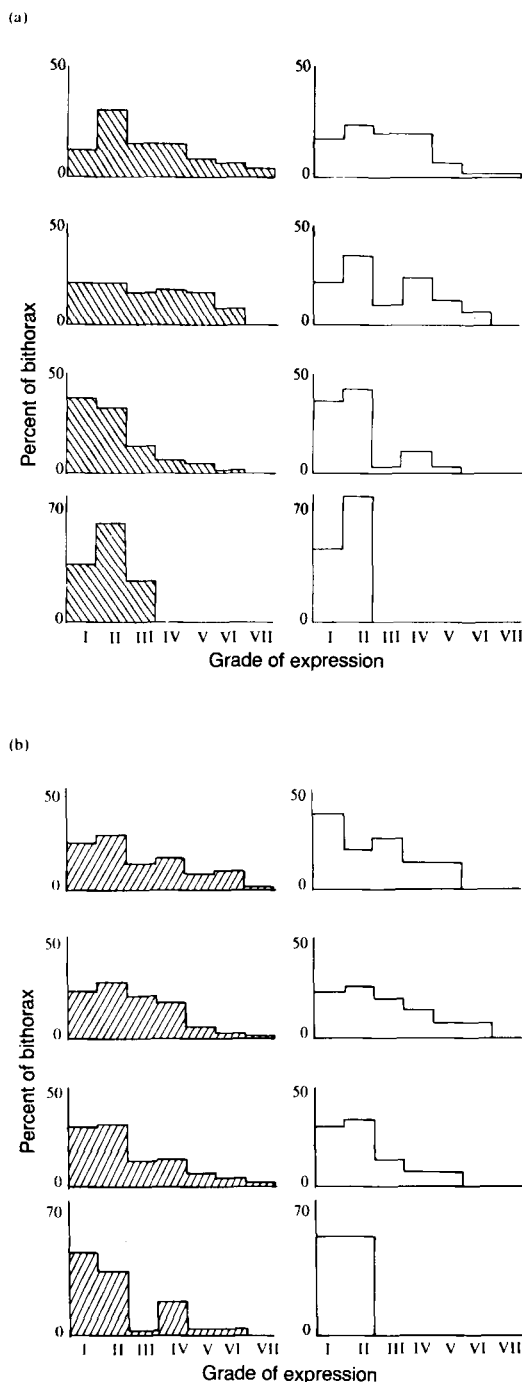
Fig. 5. Distribution of grades of expression at four different age intervals. a. *Mass* and its control. b. *H* and its control. From top to bottom: intervals I–IV (see Table 4). Controls unshaded.

number of generations, the embryos will become prematurely sensitive to ether phenocopy. But it would not lead to the extension of the sensitive period. Another hypothesis which could be entertained is that the pupal to adult viability of the phenocopy may be improved in successive generations due to natural selection against the phenocopy, so that by generation 13 or 14, the increase in viability is almost entirely due to the larger numbers of phenocopies emerging successfully from the pupal stage. This would then account for the increase in penetrance in intervals III and IV, giving the impression that the sensitive period has been prolonged. This hypothesis is unlikely for the following reasons. First, the increase in penetrance in intervals I and II in the treated lines is not accompanied by increased viability, so that phenocopies per se are not more viable. Second, the viabilities of ether-treated embryos increase with age in the controls as in the long-term-treated lines, suggesting that embryos past the sensitive period of phenocopy induction are also protected against the lethal effects of ether. Third, penetrance among unemerged pupae in both control and treated lines are equally high in all intervals (80–100%). We come to the conclusion that whatever the cause of the increase in viability, it is not obviously connected to the increase in penetrance or the apparent extension of the sensitive period of phenocopy induction.

Effects of relaxation of ether treatment at different generations

At generations 4, 9, and 12 in the treated *Mass* line, some flies were raised from untreated eggs in order to start relaxation lines, the penetrances of which were examined after a varying number of generations without ether treatment. The results are shown in Figure 6. Penetrance generally drops off sharply in all cases after one generation of relaxation. In the line started at generation 4, penetrance decreased to control levels after one generation of relaxation. In those started at generations 9 and 12, penetrance did not return to control levels until some generations later. These results are reminiscent of *dauermodifications*[1] (Jollos, '21).

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[1]*Dauermodifications* are a class of environmentally induced modifications which linger on, disappearing gradually over a number of generations after the inducing environmental regime is discontinued.
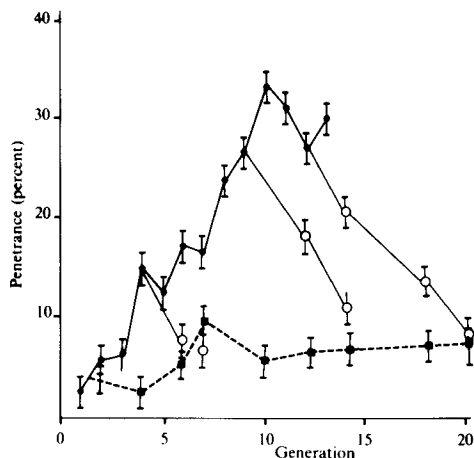

Fig. 6. Relaxation of ether treatment at different generations in *Mass*. Solid circles represent penetrance in the treated *Mass*; unfilled circles represent penetrances in the foundation stock. Vertical bars give standard errors.

DISCUSSION

We have demonstrated an increase in penetrance of the *bithorax* phenocopy in successive generations of ether treatment in one massbred and two inbred lines without artificial selection. A progressive increase in expression was also found in the early generations. Increase in penetrance and expression is usually interpreted in terms of selection for modifier alleles. That such can occur without artificial selection in both inbred and massbred lines suggests that selection for modifier alleles may not be the only explanation. We conjectured that cumulative cytoplasmic effects might be responsible for the increase in the phenocopy response. This was borne out by the results of reciprocal crosses between the long-term-treated line and its foundation stock, as well as by the lingering effects observed when treatment was relaxed in later generations.

A notable feature of our results is the striking consistency in the response of the two different lines—one massbred and the other inbred—in all aspects: penetrance, expression, viability. Even though the inbred line was not isogenic, the amount of genetic variation had been much reduced by the inbreeding regime. (Heterozygosity was estimated to be about 11.5% and 0.5%, respectively, for

Mass and *H*.) In order to explain the results in terms of selection for modifier alleles—conscious or unconscious, direct or indirect—one must assume that almost precisely the same alleles were still present in the inbred as in the massbred line. The only reasonable alternative is to recognize the existence of systemic, organismic properties common to both lines, which do not depend on specific alleles in specific genes. These properties are in part dependent on cytoplasmic constitution, which may in turn be subject to environmental modification.

If cumulative cytoplasmic effects increase sensitivity to ether and extend the sensitive period for phenocopy induction, it is by no means clear what mechanisms are involved. Jinks ('58) included in his classification of cytoplasmic modifications in *Aspergillus* one main category involving "changes in the equilibrium or balance of cytoplasmic elements." "Cytoplasm" includes cortical or membrane components, alterations in which could be propagated independently of the nucleus through a number of cell divisions (Sonneborn, '70). Such changes may underly our observations here. We have investigated the effect of ether on pattern determination and analysed the changes induced in both *Mass* and *H* in greater detail. The results, reported elsewhere (Ho et al., '82), indicate that ether treatment disrupts an early event in pattern determination involving two diffusion-like wavefronts. One of these renders the area swept over sensitive both to ether and to determination by the later wavefront. The effect of chronic ether treatment is to alter the timing and rates of progression of the wavefronts, thus extending the period during which the area is sensitive to ether phenocopy both into earlier and later embryonic ages.

In suggesting the involvement of cytoplasmic effects in the increase in phenocopy response in successive generations of ether treatment, we do not rule out the action of modifier genes. Rather we draw attention to the existence of other mechanisms which can greatly speed up phenotypic changes in evolution in response to environmental challenges. At this stage, we still do not know whether additional mutations in "major genes" are required for genetic assimilation. However, we cannot ignore the possibility that cytoplasmic-nuclear interactions, which accompany all cytoplasmic modifications,

may eventually lead to change in nuclear genes (Meklar, '80; Ho and Saunders, '82).

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