UC Irvine

UC Irvine Previously Published Works

Title

Continuous variation: Possible role of regulatory genes

Permalink

<https://escholarship.org/uc/item/7tr2c4g3>

Journal

Genetica, 52-53(1)

ISSN

0016-6707

Authors

Ayala, FJ
McDonald, JF

Publication Date

1984-12-01

DOI

10.1007/BF00121808

Copyright Information

This work is made available under the terms of a Creative Commons Attribution License, available at <https://creativecommons.org/licenses/by/4.0/>

Peer reviewed

CONTINUOUS VARIATION: POSSIBLE ROLE OF REGULATORY GENES*

F.J. AYALA & J.F. MCDONALD

Department of Genetics, University of California, Davis, California 95616, U.S.A.

It is proposed that there may be considerable overlap between regulatory genes as defined by molecular geneticists and minor genes, or polygenes, as understood in quantitative and evolutionary genetics. Variation in metric traits and in fitness may largely be due to variation in regulatory genes modifying the activity of only one or very few major genes.

Evidence in favor of the hypothesis is derived from the study of alcohol tolerance in *Drosophila melanogaster*. Flies selected for higher alcohol tolerance exhibit higher levels of alcohol dehydrogenase (ADH), an enzyme encoded by the *Adh* gene, located at position 50.1 on the second chromosome. The increase in ADH activity is not due to changes at the *Adh* locus itself, but to changes in regulatory genes that determine the amount of ADH enzyme in the flies.

The study of a natural population demonstrates that genes regulating the levels of ADH activity are located on the third chromosome (although additional regulatory genes may exist on other chromosomes). Moreover, there is extensive natural variation in such regulatory genes. These genes do not act by means of post-transcriptional or post-translational modifications affecting the catalytic efficiency of the gene product (ADH), but rather by controlling the amount of ADH enzyme, which in turn determines the levels of ADH activity of given *Adh* genotypes. Although the precise mechanism of gene regulation is not known, the results are consistent with regulation by means of macromolecules specifically binding at control sites adjacent to the structural gene locus.

Indirect evidence as well as theoretical arguments suggest that gene regulation may play a critical role in evolution. Tolerance of environmental alcohol is an important adaptation in *D. melanogaster*. Therefore, the results provide direct evidence of the importance of regulatory genes in eucaryotic evolution. Alcohol

tolerance is also a metric trait. Other continuously varying traits, including economically important ones of interest in plant and animal breeding, may, like alcohol tolerance, be largely determined by variation in regulatory genes modifying the activity of major genes.

Structural and regulatory genes versus major and minor genes

Genes are classified differently in different branches of genetics or by geneticists with different interests. In particular, molecular geneticists distinguish between *structural* genes and *regulatory* genes. Structural genes are those whose RNA templates are translated into proteins (polypeptides). A broad definition of regulatory genes is the following: 'Regulatory genes are those that control the activity of other genes' (Dobzhansky et al., 1977, p. 24; see also MacIntyre & O'Brien, 1976). It should be noted that, as defined, structural and regulatory genes are not mutually exclusive classes, nor do they include all kinds of genes. Some genes are both structural and regulatory; for example, those coding for 'effector' molecules that regulate the transcription of other genes by binding to their operators or promoter sites. Moreover there are genes which are neither structural nor regulatory, such as the genes coding for ribosomal RNA and transfer RNA.

Population geneticists, concerned with the study of evolutionary processes, or quantitative geneticists, concerned with continuous variation, distinguish be-

* Paper based on a presentation by F.J. Ayala at the Symposium on 'Problems in Evolutionary and Population Genetics' of the XIV International Congress of Genetics in Moscow on August 23, 1978.

tween Mendelian traits and metric traits (Falconer, 1960). A Mendelian trait is determined by a single *major* gene whose alleles have large enough effects to be distinguishable as qualitatively different. A metric trait is jointly determined by several *minor* genes, or *polygenes*, whose interacting effects result in phenotypic variation without natural discontinuities. These traits are called metric or quantitative because their study depends on measurement rather than on simple identification of qualitatively distinct phenotypes. Blue versus brown eye-color and the ABO blood groups are examples of Mendelian traits, while human height or the milk production of cows are examples of metric traits. Metric traits are important to evolutionists since fitness and fitness components, such as fertility, longevity, length of development, and so on, are metric traits.

The distinction between major and minor genes is not absolute because the effects of major genes may be somewhat modified by minor genes; also the same gene may be major with respect to one trait but minor with respect to another. Moreover variation due to nongenetic causes may blur the distinction between Mendelian and metric traits. Nevertheless, this distinction is often valid and has important operational consequences: Mendelian traits are studied by counting the individuals in each class while metric traits are studied by measuring the trait and breaking the continuity of the trait by means of arbitrary class boundaries.

In this paper, we suggest as a working hypothesis that there may often be a profound relationship between the regulatory genes of the molecular geneticist and the minor genes of the quantitative geneticist. Or, to state it differently, that, underlying metric traits, there may often be only one major gene locus — or very few — whose expression is under the control of regulatory genes. In many instances, the hypothesis may amount to no more than the claim that minor genes may be considered, from another point of view, as regulatory genes. (It is not implied, of course, that all regulatory genes are 'minor genes', as these are understood by quantitative geneticists.) But even this is of more than terminological interest — methods of investigating continuously varying traits as well as ways of genetic thinking may be at stake.

Our case will be made here by showing the validity of the hypothesis in one single instance: alcohol tolerance in *Drosophila melanogaster*. This is a continu-

ously varying trait and responds to selection like a typical character studied by quantitative geneticists (Ward, 1975; David & Bocquet, 1977). The trait also is of evolutionary interest because *D. melanogaster* flies encounter environments with variable concentrations of alcohol which affect their fitness (Brown, 1934; McKenzie and Parsons, 1972, 1975; Briscoe et al., 1975).

We shall show that *D. melanogaster* flies selected for increased alcohol tolerance differ from their controls in regulatory genes that determine the amount of alcohol dehydrogenase, an enzyme encoded by a structural gene locus on the second chromosome; while no differences can be detected at the structural locus itself. We shall moreover show that natural populations of *D. melanogaster* possess considerable variation with respect to regulatory genes located on the third chromosome; and that these third-chromosome regulatory genes control the amount of alcohol dehydrogenase enzyme in the flies.

Fig. 1. Selection for alcohol tolerance in an experimental population of *Drosophila melanogaster*. Selection was practiced by exposing 100 females and 100 males to a given concentration of alcohol, which was changed from generation to generation so that only about 20 percent of the flies would survive after three days of exposure. The concentration of alcohol used for the selection (●) is shown together with the LC 50 (the concentration needed to kill 50 percent of the flies) after two days of treatment (x). (Data from David & Bocquet, 1977.)

Effects of selection for alcohol tolerance

D. melanogaster flies were collected by Professor Jean David of the Université Claude Bernard (Lyon, France) in Colmar, France. A strain started with these flies was divided into two populations. One population, Selected (S), was subject to intense selection for ethanol tolerance for 28 generations; the other population was maintained as a Control (C). Selection was practiced by exposing the flies to a certain concentration of alcohol that was adjusted from generation to generation so that about 80 percent of the flies would die during three days of exposure; the surviving flies were used to produce the following generation.

A gradual increase in alcohol tolerance took place in the selected population. This can be seen in Figure 1 in two ways. First, by the increasing concentration of alcohol needed to kill approximately 80 percent of the flies in the three days. Second, by an evaluation of the LC 50 (i.e., the alcohol concentration needed to kill 50 percent of the flies) in two days. These two measures of alcohol tolerance happen to be fairly similar.

After the 28th generation of selection, flies from the Selected and the Control populations were transferred to our laboratory at the University of California at Davis, where the rest of the experiments described in this paper were conducted. The first experiment as a test of alcohol tolerance by measuring adult survivorship in the presence of ethanol. Adult flies, five days after emergence from the puparium, were placed at room temperature in vials containing 2.5 ×

Fig. 2. Survival of adult *Drosophila melanogaster* flies from a Selected (●) and a Control (○) population in six different concentrations of ethanol.

5 cm filter-paper strips saturated with 1 ml of a water solution consisting of 3% sucrose and variable percentages of ethanol. The vials, each with 10 flies of the same sex and a filter-paper strip, were immediately sealed with Parafilm. The number of surviving flies was monitored at various intervals for a total of 100 hours. Two replicate vials were prepared for each sex, strain, and ethanol concentration.

The results are shown in Figure 2. With alcohol concentrations of 8 and 10%, there are no conspicuous differences between the Selected and the Control populations. However, when the ethanol concentration is 12% or higher, the Selected flies exhibit higher tolerance than the Control flies. When the ethanol concentration is 14%, 16%, or 18%, more than 50% of the Selected flies survive after 100 hours of exposure, while all or nearly all Control flies have died.

The question arises, what is the physiological basis of the difference in alcohol tolerance between the Selected and the Control populations? Several investigators (e.g., McDonald & Avise, 1976) have shown that *Drosophila* flies with higher levels of alcohol dehydrogenase (ADH) activity are also more tolerant of environmental alcohol. If this is a causal relationship one might expect that more tolerant flies would also have higher levels of ADH activity. Levels of ADH activity were measured in the Selected and in the Control populations using the methods of Avise &

Fig. 3. Levels of alcohol dehydrogenase (ADH) activity during ontogeny in a Selected (●) and a Control (○) population of *Drosophila melanogaster*. L, larvae; P, pupae; A, adults.

McDonald (1976; see also McDonald et al., 1977). Measurements were made using crude homogenates of third-instar larvae, early pupae, late pupae (eyes and wing pads visible), and 6-10 day-old adults. The results are shown in Figure 3. Clearly, selection for ethanol tolerance has increased the levels of ADH activity in the Selected population. It is worth noticing that the response has affected all life stages although the selection was done on adults only. This result complements the observations of Ward (1975), who obtained significant increases in ADH activity at all life stages in a strain selected for higher ADH tolerance at the larval stage.

The Selected population has adapted to high ethanol concentrations by increasing the activity levels of ADH, the critical enzyme involved in ethanol metabolism. In order to ascertain whether the increased ADH activity was a specific response or rather was part of a generalized response, activity levels were measured in four other enzymes — α -glycerophosphate dehydrogenase (α GPDH), isocitrate dehydrogenase (IDH), phosphoglucose isomerase (PGI) and aldehyde oxidase (AO). The methods of Avise & McDonald (1976) were used for the first three enzymes. AO was assayed using acetaldehyde as substrate and coupling its oxidation with dichloroindophenol. As in the case of ADH, these four assays were made using crude homogenates of third-instar larvae, early pupae, late pupae, and adults. As shown in Figure 4 the Selected and Control populations exhibit virtually identical patterns of enzyme activity for all four enzymes. The increase in ADH activity appears to be a specific response by which the Selected population has achieved higher ethanol tolerance.

Genetic and molecular basis of the selection response

At the physiological level, the Selected population has achieved higher ethanol tolerance by means of higher ADH activity. What is the genetic basis of this increase in ADH activity? One possibility is that the Selected and the Control populations differ at the structural gene coding for ADH and therefore that the enzyme itself is different in the two populations. Higher ADH activity might be accomplished by changes in the enzyme structure increasing its catalytic efficiency. Another possibility, not mutually exclusive with the previous one, is that the selection has

affected genes other than the structural locus coding for ADH; for example, by changing regulatory loci that increase the amount of ADH present in the Selected flies.

These alternatives were investigated by means of a series of tests addressed to ascertain whether the ADH enzyme produced by the two populations is different. If the ADH enzyme is different it would not necessarily follow that the *Adh* locus itself is different, since the enzyme might conceivably be modified after translation. But if the ADH enzyme were identical in the two populations, it would be warranted to conclude that no differences exist at the *Adh* locus. The tests performed are the following (McDonald et al., 1977).

Electrophoresis. Using standard electrophoretic procedures (Ayala et al., 1972) the two populations were surveyed by means of starch and polyacrylamide gel electrophoresis. All individuals surveyed from both populations were found to be homozygous for the *F* (Fast) allele at the *Adh* locus; no differences were detectable between the populations. Electrophoretic identity, however, does not necessarily imply complete enzyme identity, since not all amino acid substitutions are detectable by electrophoresis. Electro-

Fig. 4. Changes in activity of four enzyme systems during ontogeny in a Selected (●) and a Control (○) population of *Drosophila melanogaster*. AO, aldehyde oxidase; α GPDH, α -glycerophosphate dehydrogenase; PGI, phosphoglucose isomerase; IDH, isocitrate dehydrogenase. L, larvae; P, pupae; A, adults.

phoresis detects mostly those amino acid substitutions resulting in net electrical charge differences or in major changes in the size or shape of the molecule. **Product inhibition.** The ability of ADH to convert ethanol into acetaldehyde is inhibited by acetaldehyde. ADH purified according to the methods of Chambers et al. (1979) was assayed in the presence of 0, 1, 2, 5, 7, 10, and 15 mM acetaldehyde. The inhibitory effect of acetaldehyde is effectively identical for the ADH from the Selected and the Control populations as shown in Figure 5; the inhibitory kinetics are consistent with the notion that aldehyde is a competitive hyperbolic inhibitor (Day et al., 1974).

Temperature stability. The thermostability of proteins is affected by both the temperature to which the protein is exposed and the duration of the exposure. The thermal stability of ADH was studied in two ways using for each test 20 μ l of purified ADH. First, the time was kept constant (5 minutes), but the temperature was varied from 22°C to 50°C. Second, the temperature was held constant (45°C) but the time was variable, from 0 to 70 minutes. The tests were made by incubation of ADH in a water bath and then transfer of the samples to ice until assayed for ADH activity.

Fig. 5. Inhibitory effect of various acetaldehyde concentrations on the conversion of NAD to NADH by purified ADH from a Selected (●) and a Control (○) population of *Drosophila melanogaster*.

The results are shown in Figure 6. When incubated for 5 minutes, the ADH activity remained unaffected up to 40°C but rapidly decreased above this temperature. When ADH was incubated at 45°C its activity gradually decreased to zero as the length of exposure increased from 0 to 70 minutes. The significant result for the present purposes is, however, the virtually identical response of ADH from the Selected and from the Control population in both tests.

pH optimum. The effect of pH on the reaction rate of ADH was ascertained using two different buffers: 10 mM of glycylglycine buffer within a pH range from 7.0 to 9.5; and 10 mM boric acid-0.1 M KCl within a pH range from 6.5 to 9.3 adjusted with 0.1 M NaOH. Each assay was made with 10 μ l of purified ADH at room temperature with ethanol as substrate. The peaks of maximum activity occurred at pH 9.2 in glycine buffer and at pH 8.9 in borate buffer, but the responses of the Selected and the Control populations were again virtually identical (Fig. 7).

Substrate specificity. The substrate specificity of

Fig. 6. Thermal stability of ADH purified from a Selected (●) and a Control (○) population of *Drosophila melanogaster*. Left, percent of activity remaining after exposure for 5 minutes to various temperatures. Right, percent of activity remaining after exposure to 45°C for varying lengths of time.

Fig. 7. Activity of purified ADH from a Selected (●) and a Control (○) population of *Drosophila melanogaster* as a function of pH. Left, activity in a glycyl-glycine buffer. Right, activity in a borate buffer.

crude extracts from the Selected and Control populations was examined using solutions of five alcohols: isopropyl, ethyl, allyl, isobutyl, and *n*-butyl. The rationale for this test is as follows. We know that the Selected population exhibits higher ADH activity than the Control population when ethanol is the substrate. But if the two ADH enzymes are different, they may behave differently with other substrates – the relative advantage of the Selected population compared to the Control population might disappear or at least change. Table 1 shows that ADH from the Selected population exhibits higher activity on all five substrates than ADH from the control population. Moreover, the ratio between the Selected and the Control population is virtually identical for the five alcohols tested. (Mean and standard error of this ratio are 1.36 ± 0.01 for protein extracts and 1.12 ± 0.01 for crude extracts.) The homogeneity of the difference between the Selected and the Control populations is consistent with the hypothesis that the ADH enzyme itself has not been changed by the selection, but rather higher ADH activity has been achieved in some other way.

Michaelis constants. Michaelis constants (K_m) are rel-

ative measures of the ability of enzyme to bind to substrate – a lower K_m indicating a higher affinity. Such ability is the rate-limiting step in most enzyme-catalyzed reactions. Michaelis constants were estimated using five NAD and five ethanol concentrations following standard procedures (McDonald et al., 1977). For each population, four separate estimations were made using crude extracts and two using purified ADH. The averages and standard errors of these estimates are given in Table 2 for two primary and two derivative Michaelis constants. No significant differences exist between the Selected and the Control populations. True differences could, of course, be masked by the typically large (Day et al., 1974) standard errors, but the values are not consistently lower in one or the other population. The Selected population has lower K_m in three, and higher K_m in five, of the eight comparisons.

In summary, a variety of biochemical tests fail to demonstrate any structural differences between the ADH enzyme produced by the Selected and the Control populations; although these tests have, in the past, proven to be sensitive to relatively small differences between ADH molecules in *D. melanogaster*

Table 1

ADH activity in crude extracts of the Selected and Control populations, using different alcohols as substrates. One unit of activity is that which increases 0.001 absorbance units per minute at 340 nm.

Substrate	Units/mg. protein			Units/mg. wet weight		
	S	C	S/C	S	C	S/C
Isopropyl	6400	4800	1.33	184	167	1.10
Ethyl	1180	870	1.36	34.0	30.5	1.11
Allyl	2350	1680	1.40	67.7	58.5	1.16
Isobutyl	2450	1800	1.36	70.6	62.7	1.13
<i>n</i> -Butyl	2200	1630	1.35	63.4	56.7	1.12

Table 2

Michaelis constants (mM) of purified and crude ADH from the Selected and Control populations of *Drosophila melanogaster*

Michaelis constant	Pure ADH		Crude extract	
	Selected	Control	Selected	Control
K_{ethanol}	2.88 ± 0.97	3.04 ± 0.92	11.86 ± 3.68	7.22 ± 1.23
K'_{ethanol}	5.95 ± 2.98	4.51 ± 1.08	9.31 ± 2.45	12.17 ± 2.45
K_{NAD}	0.108 ± 0.070	0.018 ± 0.002	0.075 ± 0.035	0.062 ± 0.012
K'_{NAD}	0.038 ± 0.004	0.025 ± 0.015	0.078 ± 0.044	0.110 ± 0.013

(Vigue & Johnson, 1973; Bernstein et al., 1973; Day et al., 1974; Day & Needham, 1974; Milkman, 1976). Although no amount of analysis short of amino acid sequencing may prove the structural identity of the ADH produced by the Selected and the Control populations, the tests performed strongly suggest that such differences do not exist.

If the differences in ADH activity are not due to

Fig. 8. An agarose gel exhibiting 'Laurell rockets,' which reflect the relative quantity of ADH enzyme present in males and females from a Selected (S) and an Control (C) population of *Drosophila melanogaster*.

structural differences (whether pre- or post-translational) affecting the catalytic activity of the enzyme, then there must be a difference in the number of ADH molecules present in the two populations. This hypothesis was tested by the quantitative immunoelectrophoresis method of Laurell (1966; see McDonald et al., 1977). The method uses antiserum-containing agarose gels. After electrophoresis, the length of the 'Laurell rockets' is linearly related to the number of protein molecules in a sample.

Figure 8 shows a typical gel containing female and male samples from the Control and the Selected populations. The results of four replicate gels are given in Table 3. In both populations, the males have more ADH molecules (as well as greater activity) than the females. The important result is, however, that flies of the Selected population have greater amounts of ADH protein than flies of the Control population. The mean differences in rocket length between the two populations (Selected minus Control) are statistically greater than zero.

Genetically, differences in amount of a given gene product may be explained by duplication of the structural locus and/or by differences in some sort of regulatory gene(s). The present evidence favors a regulatory explanation. Gene duplications at the *Adh* locus have never been detected in natural populations, and have only been identified as very rare events following mutagenesis (Grell, 1969); moreover the specific ADH activity in duplicated strains is roughly proportional to the number of doses of the gene locus

Table 3

Comparison of relative amounts of ADH in the Selected and Control populations by means of 'Laurell rockets'

Sex	Experiment	Rocket length (mm)		
		Selected	Control	Difference (Selected-Control)
Females	1	18.5	14.0	4.5
	2	18.5	16.0	2.5
	3	20.5	16.0	4.5
	4	18.0	15.0	3.0
	Average	18.88 ± 0.55	15.25 ± 0.48	3.63 ± 0.51
Males	1	21.0	17.0	4.0
	2	19.5	17.0	2.5
	3	22.5	22.5	0.0
	4	18.0	12.0	6.0
	Average	20.25 ± 0.97	17.12 ± 1.98	3.13 ± 1.26

(Grell, 1969). On the other hand, genes regulating ADH activity have been identified throughout the *Drosophila* genome and are polymorphic in natural populations (see also Pipkin & Hewitt, 1972; Ward, 1975; McDonald & Ayala, 1978). The hypothesis that regulatory genes controlling the number of ADH molecules are responsible for differences in ADH activity is confirmed by the experiments described below.

In conclusion, we have provided evidence strongly suggesting that differences in a quantitative trait – alcohol tolerance – are predominantly due to differences in ADH activity, which in turn are due to regulatory genes modifying the amount of a gene product (ADH) encoded by a structural gene (*Adh*).

Detection of regulatory genes in natural populations

The evolutionary significance of regulatory genes – as well as the possibility of artificial selection being effective when regulatory genes are involved – depends on the existence of allelic variation with respect to such genes. The effectiveness of the selection for alcohol tolerance, reported in the previous sections of this paper, indicates that variation existed in the Colmar population of *D. melanogaster* with respect to the regulatory genes involved. We propose here a different approach to detect regulatory gene variation in natural populations. This method, moreover, provides a means to ascertain the chromosomal location of such regulatory genes. We shall show for a natural population of *D. melanogaster*: (1) that regulatory gene(s) exist in the third chromosome that affect the *Adh* locus (which is located on the second chromosome); (2) that these regulatory genes exhibit abun-

Fig. 9. Mating schemes used to obtain experimental strains of *Drosophila melanogaster* homozygous or heterozygous for full chromosomes. The superscripts '+1,' '+2,' '+3,' and '+4' represent different wild chromosomes. Flies homozygous for the *Cy* or the *SbSr* chromosomes die before completing metamorphosis and therefore are not shown in the F₃ generation. Only the experimental flies are shown in the F₅ and F₆ generations. The types of flies used for the experiments are labelled (A), (B), and (C).

dant allelic variation; and (3) that the regulatory genes act by changing the amount of ADH protein present in the flies.

The method takes advantage of the existence of marked balancer stocks of *D. melanogaster* that make it possible to obtain flies homozygous for full chromosomes or heterozygous for desired chromosomes. The mating schemes used in our case are shown in Figure 9. The tester stock combines balanced-lethal-inverted stocks for the second chromosome (the SM5 stock: *Cy/Bl L*²) and for the third chromosome (the TM3 stock: *Sb Sr/e*¹¹) (Lindsley & Grell, 1967). The mating scheme produces in the F₃ generation flies fully homozygous for second and third chromosomes sampled from a natural population (flies labelled A in Figure 9). In the F₅ generation flies can also be obtained that are homozygous for a given second chromosome as well as for different third chromosomes (flies labelled B); this permits ascertaining the influence that different third chromosomes (and the regulatory genes located on them) may have on levels of ADH activity. Finally, in the F₆ generation it is possible to obtain flies homozygous for a given second chromosome but heterozygous for desired third chromosomes (flies labelled C), which in turn makes it

possible to examine dominance-recessivity relations between the third-chromosome regulatory genes.

The second and third chromosomes of *D. melanogaster* are about equally large and jointly account for about 80% of the whole genome of this species. The X chromosome contains about 20% of the genome. And there is a very small dot-like fourth chromosome. In our studies, the X chromosome is always derived from the laboratory stock, while the possible effects of the small fourth chromosome are ignored. The purpose, then, is to ascertain whether regulatory genes affecting the levels of ADH activity exist in the large third chromosome. The *Adh* locus itself has been mapped at position 50.1 on the second chromosome.

For each strain with a desired genetic constitution, enzyme activity is measured using standard spectrophotometric methods in 18 separate samples, nine of males and nine of females. Each three of these come from a separate culture. The experimental design makes it possible to separate the effects on ADH activity due to the *Adh* locus (the second chromosome), to the regulatory genes on the third chromosome, and to environmental effects (different cultures) (McDonald & Ayala, 1978). The activity values

Table 4

Levels of ADH activity in 6 to 10-day-old adult *Drosophila melanogaster* flies from nine strains, each homozygous for a second and third wild chromosome sampled from a natural population. The means and standard errors are based on nine measurements for each sex. One unit of activity is defined as 8×10^{-4} moles of NAD⁺ reduced per ml reaction mixture per mg. live weight.

Strain	Electrophoretic genotype	Chromosome genotype		Mean activity $\pm$ standard error		
		II	III	Males	Females	Overall
1	F/F	1F	1F	19.44 $\pm$ 0.44	17.33 $\pm$ 0.55	18.39 $\pm$ 0.32
2	F/F	2F	2F	27.55 $\pm$ 0.29	23.44 $\pm$ 0.24	25.50 $\pm$ 0.35
3	F/F	3F	3F	25.66 $\pm$ 0.80	21.33 $\pm$ 0.40	23.50 $\pm$ 0.40
4	S/S	1S	1S	9.67 $\pm$ 0.24	8.17 $\pm$ 0.20	8.92 $\pm$ 0.24
5	S/S	2S	2S	9.50 $\pm$ 0.35	8.33 $\pm$ 0.26	8.92 $\pm$ 0.24
6	S/S	3S	3S	6.11 $\pm$ 0.39	5.44 $\pm$ 0.18	5.78 $\pm$ 0.23
7	S/S	4S	4S	11.67 $\pm$ 0.29	11.89 $\pm$ 0.35	11.78 $\pm$ 0.22
8	S/S	5S	5S	7.89 $\pm$ 0.26	5.22 $\pm$ 0.28	6.55 $\pm$ 0.30
9	S/S	6S	6S	11.56 $\pm$ 0.44	9.83 $\pm$ 0.12	10.64 $\pm$ 0.24
Average for F strains*				24.22 $\pm$ 2.45	20.70 $\pm$ 1.79	22.46 $\pm$ 2.12
Average for S strains*				9.40 $\pm$ 0.88	8.15 $\pm$ 1.05	8.77 $\pm$ 0.94

* The standard errors of the averages are calculated from the variance among the corresponding mean values.

Table 5
ADH activity of double homozygous strains with second and third chromosomes from different original strains

Second chromosome	Third chromosome						Activity effects of third chromosomes*
	1F	2F	3F	1S	2S	3S	
1F	18.4 ± 0.3	25.7 ± 0.7	—	—	—	19.9 ± 0.4	2F >> 3S = 6S = 1F
2F	—	25.5 ± 0.3	44.4 ± 0.5	22.5 ± 0.3	—	24.5 ± 0.4	3F >> 2F = 3S > 1S = 5S
3F	—	—	23.5 ± 0.4	15.4 ± 0.2	—	—	3F >> 5S = 1S
1S	—	—	16.0 ± 0.4	8.9 ± 0.2	14.8 ± 0.4	—	3F > 2S >> 6S > 1S
2S	—	10.9 ± 0.4	28.9 ± 0.5	—	8.9 ± 0.2	—	3F >> 2F = 3S
3S	11.0 ± 0.3	7.0 ± 0.2	—	—	—	5.8 ± 0.2	1F >> 2F = 3S = 6S
6S	—	17.8 ± 0.4	24.7 ± 0.5	7.9 ± 0.2	—	—	3F >> 3S >> 2F >> 6S > 1S

* Results of Student-Newman-Keul's multiple comparison tests, where the influence of each third chromosome on ADH activity is ranked and the statistical significance of the difference between each pair of adjacent chromosomes is tested. The meaning of the symbols is: =, not significantly different; >, greater than, with $P < 0.05$; >>, greater than, with $P < 0.01$.

given in the present paper are the means and standard errors for the nine male samples, the nine female samples, or all 18 samples ('Overall').

Natural populations of *D. melanogaster* are polymorphic at the *Adh* locus for two electrophoretically distinguishable alleles, *F* ('Fast') and *S* ('Slow'). We started our study by obtaining strains homozygous for nine second and nine third chromosomes derived from nine different wild males collected in Napa County, California. The mating scheme given in Figure 9 shows that these original homozygous strains (A in the figure) carry one second and one third chromosome which happened to be associated with each other in a wild fly. Three of these original strains carried the *F* allele at the *Adh* locus, the other six carried the *S* allele. The wild chromosomes, seconds as well as thirds, are symbolized as 1F, 2F, 3F, 1S, 2S, ... 6S; where 1F, for example, represents one second chromosome carrying the *F* allele and also the third chromosome extracted simultaneously with that second chromosome.

The mean levels of ADH activity in the nine original strains are given in Table 4. There is considerable variation in ADH activity among the strains. Much of the variation can be attributed to the electrophoretic genotype, since strains homozygous for

the *F* allele have significantly higher activity (average, 22.46 ± 2.12) than strains homozygous for the *S* allele (average, 8.77 ± 0.94). But there is significant variation in ADH activity even among strains with the same electrophoretic genotype. (Analysis of variance shows that the heterogeneity among strains with a given electrophoretic genotype is statistically significant, at the 0.05 level for the *F* strains and at the 0.005 level of significance for the *S* strains.)

The results shown in Table 4 are not necessarily inconsistent with the possibility that the variation in ADH activity is all due to the *Adh* locus. This is so because identity of electrophoretic genotype does not necessarily imply identity at the structural gene locus coding for ADH, since not all differences in the primary structure of proteins are detected by electrophoresis. Therefore, we have two possibilities: first, that the variance in ADH activity is in part due to gene loci other than the structural *Adh* locus; and second, that all the variation is due to differences in the structural locus, *Adh*, coding for ADH. A decision between these two alternatives can be made by examining sets of strains in which the genotype in all strains of a set is identical at the *Adh* locus, but not everywhere else in the genome, i.e., by substituting parts of the genome without changing the *Adh* locus.

Fig. 10. Mean ADH activity compared between *Drosophila melanogaster* flies heterozygous for two wild third chromosomes (flies of type C in Figure 9) and flies homozygous for each one of the two third chromosomes (flies of types A and B in Figure 9). The arrows indicate the expected no-dominance values. The mean activity is always significantly higher (using Standard t-tests) than the expected co-dominance value, ** = $P < 0.01$, *** = $P < 0.001$.

In order to determine whether any of the variation in ADH activity could be due to genes on the third chromosome (the large autosome not carrying the *Adh* structural locus), we constructed the strains homozygous for a given second chromosome but for different third chromosomes (strains of the type labelled B in Figure 9). We obtained 21 strains containing various combinations of second and third chromosomes. The mean levels of ADH activity observed in these 21 strains (and, for comparison, in the original homozygous strains) are given in Table 5.

Two conclusions can be drawn from the data: (1) there are one or more gene loci in the third chromosome of *D. melanogaster* that affect the levels of ADH activity; and (2) there is variability segregating in the population at the gene or genes in the third chromosome that modify the levels of ADH activity. The structural locus coding for ADH is in the second chromosome; yet replacing the third chromosomes in flies with identical second chromosomes results in significant changes in ADH activity. This happens with every second chromosome tested. For example, the mean activity of a strain homozygous for the 1S second chromosome is 8.9 ± 0.2 , 10.4 ± 0.2 , 14.8 ± 0.4 , and 16.0 ± 0.4 when it is homozygous for the third chromosomes 1S, 6S, 2S, and 3F, respectively. Because different third chromosomes have different effects on ADH activity when combined with any given second chromosome, it follows that there is allelic variation at the third chromosome gene or genes that modify ADH activity.

The results shown in Table 5 indicate moreover the existence of gene(s) in the second chromosome that interact with the third chromosome genes that modify the levels of ADH activity. As an example, consider the third chromosomes 1F and 2F – when combined with the second chromosome 1F, the 2F third chromosome results in higher activity than the 1F third chromosome; but when combined with the second chromosome 3S, the 2F third chromosome results in lower activity than the 1F third chromosome. Another example is provided by comparing the third chromosomes 2F and 3S – the first one results in higher activity when combined with the second chromosome 1F, but both have virtually identical effects when combined with either the 2F or the 3S second chromosomes. It is not possible to decide from the data whether this variable interaction is due to the ADH locus itself or to other gene(s) on the

second chromosomes. However, if this interaction is due to the *Adh* locus, it must be the case that variation exists at this locus between alleles electrophoretically identical, because the 2F and 3S third chromosomes have different effects when combined with the 1F and 2F second chromosomes, although both of these have the *F* allele at the *Adh* locus.

The results so far described involve strains fully homozygous for the second and third chromosomes. The dominance-recessivity relationships of the third-chromosome regulatory effects can be investigated using strains heterozygous for two third chromosomes of known effects (*i.e.*, flies of the type labelled C in Figure 9). The mean activity levels of nine heterozygous strains are represented in Figure 10. In every case the mean ADH activity is significantly higher in the heterozygous flies than the average of the strains homozygous for the corresponding two third chromosomes. This indicates that higher-activity genes are dominant over lower-activity genes, although the dominance is not always complete. In five of the nine cases, the dominance is only partial since the flies homozygous for the higher-activity chromosome exhibit significantly higher ADH activity than the heterozygous flies; in the other four cases (the first, fourth, sixth, and eighth cases, starting from the left in Figure 10), the dominance of the higher-activity chromosomes appears to be complete.

Molecular basis of the differences in ADH activity

The experiments just reported demonstrate the existence of regulatory genes on the third chromosome that modify the levels of ADH activity. We may now raise the question of what is the molecular mechanism by which the regulatory genes on the third chromosome effect levels of ADH activity. The molecular basis of genetic regulation in higher organisms is far from adequately known. Experimental evidence and some theoretical models suggest that interchromosomal control may not be an uncommon phenomenon (McClintock, 1965; Britten & Davidson, 1969; Ward, 1975; Stein & Stein, 1976; Nelsen et al., 1976). The mechanisms of such nonadjacent control are believed to involve the synthesis of macromolecules, whether RNA or protein. The control may be exercised either through direct modification of the target gene product (*i.e.*, through post-translational or post-

transcriptional action) or through regulation of the rate of transcription of the structural gene via specific binding at control sites.

These alternatives can be tested. If the third chromosome genes modify ADH activity exclusively by some form of post-translational modification of the protein (or of post-transcriptional changes in the messenger RNA) affecting the catalytic efficiency of the enzyme, the amount of ADH protein should not be different in strains with different third chromosomes. Accordingly, we have used the Laurell-rocket method to measure the relative amounts of ADH protein in different strains.

Figure 11 gives the results of one test. Both strains used have the 2F second chromosome, but one has the 2F third chromosome while the other has the 3F third chromosome. As shown in Table 5, the mean ADH activity of the 2F(II)-2F(III) strain is 25.5 ± 0.3 , or 57% of 44.4 ± 0.5 , the mean activity of the 2F(II)-3F(III) strain. Rocket length was measured in crude homogenates of both strains, and in two serial dilutions (one-half and one-quarter) of the 2F(II)-3F(III) strain.

Fig. 11. Relative quantity of ADH enzyme as estimated by the length of 'Laurell rockets' in two strains of *Drosophila melanogaster*. The two serial dilutions of strain 2F(II)-3F(III) demonstrate the linear relationship between amount of ADH enzyme and rocket length. The amount of ADH enzyme in strain 2F(II)-2F(III) is approximately two-thirds the amount of enzyme in strain 2F(II)-3F(III).

The results given in Figure 11 (the two points on extreme right) show that the lower-activity strain has indeed fewer ADH molecules than the higher-activity strain. This figure additionally demonstrates the linear relationship between the number of ADH molecules and the length of Laurell rockets: the length of these in the diluted samples is approximately one-quarter and one-half of their length in the undiluted sample.

Differences in the amount of ADH protein can be due to modification of the rate of synthesis of the polypeptide, or of the rate of degradation, or to both. (These rates might be modified at the level of the protein itself or through changes in the half-life of the mRNA or hnRNA.) Changes in the rate of synthesis would be consistent with the hypothesis that the regulation of ADH activity occurs by specific binding of macromolecules at control sites adjacent to the *Adh* locus.

Regulatory genes, adaptation, and continuous variation

We have demonstrated in *Drosophila melanogaster* the existence of ample variation in regulatory genes located on the third chromosome that modify the levels of activity of ADH, an enzyme encoded by a structural gene located on the second chromosome. Moreover, increased ADH activity in a population selected for alcohol tolerance was achieved by changes in regulatory genes rather than in the structural gene coding for ADH; the efficient response to selection suggests the ample occurrence in natural populations of regulatory genes modifying levels of ADH activity. Evidence gathered by other authors (Ward & Hebert, 1972; Pipkin & Hewitt, 1972; Ward, 1975) further indicates that variation in regulatory genes modifying ADH activity levels is widespread in natural populations.

The regulatory genes act by changing the number of ADH molecules in the flies. This excludes for the present case the hypothesis of gene regulation by means of post-transcriptional and post-translational modifications affecting the catalytic efficiency of the gene product (ADH); and is consistent with the hypothesis of gene regulation by means of macromolecules specifically binding at control sites adjacent to the structural genes.

Levels of ADH activity are adaptively important for *D. melanogaster* flies, which live in environments with varying alcohol concentrations. The importance of regulatory genes in bringing about adaptive changes is well documented in procaryotes (Lerner et al, 1964; Lin et al., 1976). Indirect evidence (e.g., Valentine & Campbell, 1975; Wilson, 1976), as well as theoretical arguments (Britten & Davidson, 1969, 1971), suggests that gene regulation may have played a critical role in the eucaryotes as well. We have provided direct evidence showing that such is in fact the case in a particular situation – the adaptation of *D. melanogaster* to environmental alcohol. Fitness may, indeed, be affected by regulatory genes.

Fitness is a metric trait, exhibiting continuous variation. Particular adaptations – involving morphology, physiology, or behavior – may also be largely modified by regulatory genes, which may thus play an important role in adaptive evolution. We propose that the results presented here may have relevance to metric traits in general: continuous variation may largely reflect allelic variation in regulatory genes. If so, the understanding of gene regulation may be of fundamental importance in animal and plant breeding and not only in molecular genetics and in evolution. Traits such as the milk production of cows, the number and size of chicken eggs, the protein content of corn, and the yield of tomato plants, may be largely under the control of regulatory genes.

References

- Avise, J.C. & J.F. McDonald (1976). Enzyme changes during development of holo- and hemi-metabolic insects. *J. comp. Biochem. Physiol.* 53B: 393-397.
- Ayala, F.J., J.R. Powell, M.L. Tracey, C.A. Mourão & S. Pérez-Salas (1972). Enzyme variability in the *Drosophila willistoni* group. IV. Genic variation in natural populations of *Drosophila willistoni*. *Genetics* 70: 113-139.
- Bernstein, S.C., L.H. Throckmorton & J.L. Hubby (1973). Still more genetic variability in natural populations. *Proc. natn. Acad. Sci. U.S.A.* 70: 3928-3931.
- Briscoe, D.A., A. Robertson & J. Malpica (1975). Dominance at the Adh locus in response of adult *Drosophila melanogaster* to environmental alcohol. *Nature* 255: 148-149.
- Britten, R. & E. Davidson (1969). Gene regulation for higher cells: a theory. *Science* 165: 349-357.
- Britten, R. & E. Davidson (1971). Repetitive and non-repetitive DNA sequences and a speculation on the origins of evolutionary novelty. *Q. Rev. Biol.* 46: 111-138.
- Brown, M.J. (1934). Alcohol-tolerance of *Drosophila*: an instance of adaptation to an environment lethal to most organisms. *J. Hered.* 25: 244-246.
- David, J. & C. Bocquet (1977). Genetic tolerance to ethanol in *Drosophila melanogaster*: Increase by selection and analysis of correlated responses. *Genetica* 47: 43-48.
- Day, T.H., P. Hillier & B. Clarke (1974). The relative quantities and catalytic activities of enzymes produced by alleles at the alcohol dehydrogenase locus in *Drosophila melanogaster*. *Biochem. Genetics* 11: 155-165.
- Day, T.H. & L. Needham (1974). Properties of alcohol dehydrogenase isozymes in a strain of *Drosophila melanogaster* homozygous for the Adh-slow allele. *Biochem. Genet.* 11: 167-175.
- Dobzhansky, Th., F.J. Ayala, G.L. Stebbins & J.W. Valentine (1977). *Evolution*. W.H. Freeman, San Francisco, xiv + 572 pages.
- Falconer, D.S. (1960). *Introduction to Quantitative Genetics*, Oliver and Boyd, Edinburgh and London, vi + 365 pages.
- Grell, E.H. (1969). Induction of duplications of genes which specify enzymes in *Drosophila melanogaster*. *Genetics* 61s: 23 (abstract).
- Laurell, C. (1966). Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. *Analyt. Biochem.* 15: 45-52.
- Lerner, S.A., T.T. Wu & E.C.C. Lin (1964). Evolution of a catabolic pathway in bacteria. *Science* 146: 1313-1315.
- Lin, E.C.C., A.J. Hacking & J. Aguilar (1976). Experimental models of acquisitive evolution. *Bioscience* 26: 548-555.
- Lindsley, D. & E. Grell (1967). Genetic variations of *Drosophila melanogaster*. *Publs Carnegie Inst.* 627.
- MacIntyre, R. & S. O'Brien (1976). Interacting gene-enzyme systems in *Drosophila*. *A. Rev. Genet.* 10: 281-318.
- McClintock, B. (1965). The control of gene action in maize. *Brookhaven Symp. Biol.* 18: 162-181.
- McDonald, J.F. & J.C. Avise (1976). Evidence for the adaptive significance of enzyme activity levels: Interspecific variation in α -GPDH and ADH in *Drosophila*. *Biochem. Genet.* 14: 347-355.
- McDonald, F.J. & F.J. Ayala (1978). Genetic and biochemical basis of enzyme activity variation in natural populations. I. Alcohol dehydrogenase in *Drosophila melanogaster*. *Genetics* 89: 371-388.
- McDonald, J.F., G.K. Chambers, J. David & F.J. Ayala (1977). Adaptive response due to changes in gene regulation: A study with *Drosophila*. *Proc. natn. Acad. Sci. U.S.A.* 74: 4562-4566.
- McKenzie, J.A. & P.A. Parsons (1972). Alcohol tolerance: an ecological parameter in the relative success of *Drosophila melanogaster* and *Drosophila simulans*. *Oecologia* 10: 373-388.
- McKenzie, J.A. & P.A. Parsons (1975). Microdifferentiation in a natural population of *Drosophila melanogaster* to alcohol in the environment. *Genetics* 77: 385-394.
- Milkman, R. (1976). Further evidence of thermostability variation within electrophoretic mobility classes of enzymes. *Biochem. Genet.* 14: 383-387.
- Nelsen, R., J. Lehman & R. Metzberg (1976). Regulation

- of phosphate metabolism in *Neurospora crassa*: Identification of the structural gene for repressible acid phosphatase. *Genetics* 84: 183-192.
- Pipkin, S. & N. Hewitt (1972). Variation of alcohol dehydrogenase levels in *Drosophila* species hybrids. *J. Hered.* 63: 267-270.
- Stein, G. & J. Stein (1976). Chromosomal proteins: their role in the regulation of gene expression. *Bioscience* 26: 488-498.
- Valentine, J.W. & C.A. Campbell (1975). Genetic regulation and the fossil record. *Am. Scient.* 63: 673-680.
- Vigue, C. & F. Johnson (1973). Isozyme variability in species of the genus *Drosophila*. VI. Frequency-property-environment relationships of allelic alcohol dehydrogenases in *D. melanogaster*. *Biochem. Genet.* 9: 213-227.
- Ward, R. (1975). Alcohol dehydrogenase in *Drosophila melanogaster*: a quantitative character. *Genet. Res.* 26: 81-93.
- Ward, R. & P. Hebert (1972). Variability of alcohol dehydrogenase activity in a natural population of *Drosophila melanogaster*. *Nature New Biol.* 236: 243-244.
- Wilson, A.C. (1976). Gene regulation in evolution. In: *Molecular Evolution*, F.J. Ayala (ed.), Sinauer Associates, Massachusetts.