

Technical Report No. 52

ENVIRONMENT-ENZYME POLYMORPHISM RELATIONSHIPS

IN TWO HAWAIIAN DROSOPHILA SPECIES

W. W. M. Steiner

Department of Genetics and Development
University of Illinois
Urbana, Illinois 61801

ISLAND ECOSYSTEMS IRP

U. S. International Biological Program

January 1975

ABSTRACT

Seven polymorphic enzyme-gene loci in Drosophila mimica and five such loci in D. engyochracea were investigated in a time-dependent study. Heterozygosity levels were stressed and relationships determined between heterozygosity and different weather variables and between allele frequencies and different weather parameters. Coordinated shifts in heterozygosity level were noted for different loci in each species. In D. mimica, significant correlations between rainfall and specific allele frequencies at the IDH-1, PGM-1 and EST-2 loci were found. Such a correlation was also found for D. engyochracea at the PGM-1 locus. These findings are discussed with respect to the metabolic roles of these enzymes and their value for predicting associated changes in the genetic architecture of the gene pools they reside in. These activities are consistent with Darwinian theory and not with theories which invoke neutrality.

TABLE OF CONTENTS

	Page
ABSTRACT	i
LIST OF TABLES	ii
INTRODUCTION	1
MATERIALS AND METHODS	2
RESULTS	4
DISCUSSION	14
ACKNOWLEDGEMENTS	26
REFERENCES	27

LIST OF TABLES

TABLE		Page
1	Genetic events observed at polymorphic loci in <u>D. mimica</u> and <u>D. engyochracea</u> (Steiner and Carson 1974)	3
2	Weather data from Kipuka Ki used for correlation to some genetic parameters	5
3	Correlations between observed locus heterozygosity (\bar{h}) estimates given by sex for <u>D. mimica</u>	6
4	Correlations between observed locus heterozygosity (\bar{h}) in <u>D. mimica</u> and 4 weather variables	8
5	Correlations between primary alleles at 7 polymorphic loci in <u>D. mimica</u> and 4 weather variables	9
6	Correlations between observed locus heterozygosity estimates in <u>D. engyochracea</u>	11
7	Correlations between observed locus heterozygosity (\bar{h}) and population heterozygosity (\bar{H}) in Kipuka Ki populations of <u>D. engyochracea</u> to four weather variables	12
8	Correlations between primary alleles at five polymorphic enzyme loci in <u>D. engyochracea</u> and 4 weather variables	13
9	Frequency of the number of heterozygotes occurring at 3 enzyme loci in <u>D. mimica</u> compared with three specific weather parameters in Kipuka Ki (calculated from Steiner and Carson 1974)	23

INTRODUCTION

Steiner and Carson (1974) report extensive genetic variation in two populations of two sympatrically occurring species of endemic Hawaiian Drosophila, D. mimica and D. engyochracea. In the first species, 47.6% of the loci analyzed by electrophoretic techniques were polymorphic, while heterozygosity levels were 19.4% (Kipuka Ki population) and 18.9% (Kipuka Puaulu population). In D. engyochracea, 30% of the 20 gene loci sampled were polymorphic. Heterozygosity levels were 12.8% (Kipuka Ki) and 11.6% (Kipuka Puaulu). An in-depth analysis of the variability revealed that genetic changes occurred in some loci. These were either directional or cyclical in nature. The data for these polymorphic loci in both species are summarized in Table 1.

The foremost question raised by this study, as in others of this nature (Lecam et al. 1972, Selander and Kaufman 1973, Selander and Johnson 1973), is how is this variability maintained? In other words, do the genetic events observed at these loci demonstrate some adaptive function or response? The question is of some evolutionary importance in these representatives of the Hawaiian Drosophila. The endemic Drosophilid fauna of the Hawaiian archipelago have recently undergone extensive speciation (Carson et al. 1970). We are generally interested in the role genic variability may have played in these speciation phenomena. Specifically, we want to know whether or not this variability is adaptive in the environments occupied by D. mimica and D. engyochracea. To answer the latter question, a correlation analysis was undertaken to investigate the relationships existing between the heterozygosity levels and between specific allele frequencies at polymorphic gene loci and environmental parameters occurring in Kipuka Ki. The results of this analysis are presented here and support a hypothesis of selective maintenance at these polymorphic loci.

MATERIALS AND METHODS

The samples, methods, and the experimental design are the same as described in Steiner and Carson (1974). The loci examined are listed in Table 1. For clarification it is pointed out that only polymorphic enzyme loci are examined in this study and that the most commonly occurring allele at a locus is designated at 4. Electrophoretically faster migrating alleles are designated 1-3 (depending on mobility) and slower alleles are designated 5-7.

The weather data were collected on a continual basis by the United States International Biology Program, Integrated Island Research Project, Hawaiian subprogram. The data covering the sampling period of this study are found in Bridges and Carey (1973, 1974). The data are from a meteorological station in Kipuka Ki with the exception of the determination of the number of times it rained in the month preceding collection. This information was obtained from a station approximately 13 km from the collecting area.

Only four environmental parameters were used. These were the amount of rain falling in the first two weeks prior to collection (RAIN 1), the amount of rain falling in the third and fourth week prior to collection (RAIN 2), the mean temperature of the month preceding collection (TEMP), and the average amount of rain that fell in the month preceding collection (RAINFALL). By simplifying the data in this way, the interrelationships between the environmental parameters themselves were reduced.

The reforming of the weather data into a new set was an attempt to recognize those meteorological parameters which would most likely be important in guiding genetic changes observed during the 18-20 month course of this study (6 collections for each species). By grouping rain into two categories, an "immediate" and a "long-term" effect due to moisture could be studied. Moisture is perhaps the most important environmental parameter experienced by Hawaiian Drosophila (Carson et al 1970). Because temperature regimes

TABLE 1. Genetic events observed at polymorphic loci in D. mimica and D. engyochracea (Steiner and Carson, 1974). Data is for the Kipuka Ki populations only.

Locus	<u>D. mimica</u>			<u>D. engyochracea</u>		
	Event	No. of Alleles ^a	h	Event	No. of Alleles ^a	h
Acid Phosphatase-1 (ACPH)	Cyclical change	3	.473	---	---	---
Alkaline Phosphatase-3 (APH-3)	random	6	.745	---	---	---
Asterase-2 (EST-2)	directional change ^b	5	.651	random	3	.652
Asterase-3 (EST-3)	---	---	---	random ^b	4	.604
Hexokinase-3 (HK-3)	directional change ^b	3	.340	---	---	---
Isocitrate Dehydrogenase (ICDH-1)	directional change	2	.216	---	---	---
Leucine Aminopeptidase-1 (LAP-1)	random	5	.666	---	---	---
Leucine Aminopeptidase-2 (LAP-2)	---	---	---	random	3	.501
Methanol Dehydrogenase-1 (ODH-1)	---	---	---	random	2	.078
Phosphoglucomutase-1 (PGM-1)	directional change	3	.203	directional change	3	.175

occurring at more than 1% in frequency

lack of heterozygotes at this locus

locus heterozygosity level

do not appear to fluctuate widely on a daily basis, it seemed logical to group the data by month. It should be pointed out that Kipuka Ki normally exists in a drier state than Kipuka Puauulu (Smathers, 1968). In addition, records indicate that the period of sampling of this study was drier than usual. These points, however, do not affect the final analysis but may aid in making final interpretations where the genetic data is concerned. The reduced weather data set is found in Table 2. The correlation analysis was done, using a program written by Dr. M. P. Mi and his staff (Department of Genetics, University of Hawaii), on the University of Hawaii IBM 360 computer.

RESULTS

Table 3 lists the results of the correlation analysis for the heterozygosity values at each locus in D. mimica. For the most part, observed shifts in frequency occurring across collections at some loci appear to be correlated to frequency shifts at other loci. The observations are consistent across the sexes but must be viewed with some reserve since the numbers analyzed are not large and since linkage relationships are unknown in the Hawaiian Drosophila. The correlation coefficients listed are all significant ($P < 0.01$).

There are essentially two patterns observable in these correlations. In the first, APH-3, HK-3, LAP-1 and PGM-1 are all highly and positively correlated. These same loci are negatively correlated with EST-2. In the second pattern, IDH-1 and EST-2 are positively correlated while both are negatively correlated to ACPH-1. In the same pattern, APH-3, HK-3 and LAP-1 are positively correlated to ACPH-1 with no significant correlation existing for PGM-1 and ACPH-1. The only correlation not fitting these patterns is that positive correlation existing between IDH-1 and PGM-1, and it is extremely low.

TABLE 2. Weather data from Kipuka Ki used for correlation to some genetic parameters.

D. engyochracea collections:

<u>SOURCE</u>	<u>Oct. 72</u>	<u>March 73</u>	<u>June</u>	<u>Sept.</u>	<u>Dec.</u>	<u>March 74</u>
RAIN 1 (mm)	019	044	066	004	224	036
RAIN 2 (mm)	005	006	020	052	136	036
TEMP. (°C)	14	11	14	14	14	11
RAINFALL	004	---	009	005	006	011

D. mimica collections:

<u>SOURCE</u>	<u>July 72</u>	<u>Jan. 73</u>	<u>March</u>	<u>June</u>	<u>Sept.</u>	<u>Dec.</u>
RAIN 1 (mm)	009	006	051	003	020	023
RAIN 2 (mm)	008	026	045	006	035	007
TEMP. (°C)	18	15	10	14	14	13
RAINFALL	003	005	---	004	004	017

TABLE 3. Correlations between observed locus heterozygosity estimates given by sex for *D. mimica*. Coefficients are all significant with $P < 0.01$.

Sex	Variable	\bar{h}						
		EST-2	PGM-1	ACPH-1	APH-3	HK-3	IDH-1	LAP-1
Females	EST-2 \bar{h}^a							
	PGM-1 \bar{h}	-0.74	--					
	ACPH-1 \bar{h}	-0.78	--	--				
	APH-3 \bar{h}	-0.88	0.97	0.39	--			
	HK-3 \bar{h}	-0.78	1.00	0.21	0.98	--		
	IDH-1 \bar{h}	0.52	0.19	-0.94	--	--		
	LAP-1 \bar{h}	-0.92	0.95	0.47	1.00	0.96	--	
	\bar{H}^b	-0.66	0.99	--	0.94	0.99	0.30	0.90
(N = 202)								
Males	EST-2 \bar{h}							
	PGM-1 \bar{h}	-0.54						
	ACPH-1 \bar{h}	-0.79	--					
	PAH-3 \bar{h}	-0.80	0.94	0.28				
	HK-3 \bar{h}	-0.68	0.76	--	0.85			
	IDH-1 \bar{h}	0.50	0.23	-0.90	--	0.29		
	LAP-1 \bar{h}	-0.84	0.91	0.38	0.99	0.80	--	
	\bar{H}	-0.52	0.93	--	0.90	0.92	0.43	0.84
(N = 163)								

^a \bar{h} is the observed locus heterozygosity

^b \bar{H} is the population heterozygosity

These two patterns suggest that some basic underlying mechanism may be involved in their maintenance. Examination of Table 4 provides insight as to what this mechanism may be. Examination of the correlation coefficients occurring between the level of locus heterozygosity and the four weather variables reveals two correlations which are significant ($P < 0.05$). The first is a negative correlation of PGM-1 heterozygosity to RAIN 2. The second is a positive correlation of LAP-1 heterozygosity to RAIN 2. Thus low levels of variability at the former locus and high levels at the latter locus are found associated with high rainfall. The results in Table 4 indicate that enzyme loci associated with these loci may show associated effects. The results of Table 3 suggest that such effects exist.

In a more detailed analysis, correlations were drawn between alleles occurring at 5% in frequency or higher (primary alleles) at each polymorphic locus with the weather parameters. The coefficients are listed in Table 5.

Four alleles at three loci show significant correlations, each locus with different weather variables. These are allele PGM-1⁴ with RAIN 2, IDH-1⁴ and IDH-1⁵ with RAIN 1 and EST-2³ with RAINFALL. The positive correlation of PGM-1⁴ with RAIN 2 suggests this is the basis for the observation of the high homozygosity during high rainfall periods associated with this locus. At the IDH-1 locus, IDH-1⁴ correlates positively also, while IDH-1⁵ has a significant negative correlation. Although Table 4 indicates a negative correlation exists between IDH-1 heterozygosity and RAIN 2, it is not significant as is that for PGM-1.

At the esterase locus EST-2³, a fast migrating allele which has an average frequency of 22.7% (Steiner and Carson, 1974), has a positive correlation with the number of rainfall events occurring in the month prior to collection. This would indicate that heterozygosity should be higher at this locus during periods of higher rainfall. Correlations of weather parameters with primary alleles at other loci were not significant.

TABLE 4. Correlations between observed locus heterozygosity (\bar{h}) in D. mimica and 4 weather variables.

<u>Variable</u>	<u>RAIN 1</u>	<u>RAIN 2</u>	<u>TEMP.</u>	<u>RAINFALL</u>
ACPH \bar{h}	0.72	0.31	0.22	0.32
APH-3 \bar{h}	0.24	0.72	0.06	-0.54
LAP-1 \bar{h}	0.29	0.98*	-0.12	-0.37
EST-2 \bar{h}	0.04	-0.72	-0.30	0.27
HK-3 \bar{h}	-0.18	0.40	0.83	-0.73
IDH-1 \bar{h}	-0.49	-0.45	0.48	-0.57
PGM-1 \bar{h}	-0.44	-0.95*	0.10	0.26

a See text or Table 1 for further description of variables.

* $P < 0.05$

TABLE 5. Correlations between primary alleles at 7 polymorphic loci in D. mimica and 4 weather variables.

<u>Variables</u> ^a	<u>RAIN 1</u>	<u>RAIN 2</u>	<u>TEMP</u>	<u>RAINFALL</u>
ACPH-1 ³	0.52	0.00	0.47	0.28
ACPH-1 ⁴	-0.65	-0.18	-0.34	-0.30
APH-3 ³	0.03	0.11	-0.81	0.48
APH-3 ⁴	-0.32	-0.52	-0.13	-0.20
APH-3 ⁵	0.21	0.17	0.30	0.29
APH-3 ⁶	0.57	-0.24	0.37	0.45
LAP-1 ³	0.64	0.15	0.40	0.21
LAP-1 ⁴	0.36	-0.56	0.12	0.73
LAP-1 ⁵	-0.48	0.58	-0.21	-0.64
EST-2 ³	0.66	-0.35	-0.69	0.98*
EST-2 ⁴	-0.14	0.77	0.31	-0.43
EST-2 ⁵	-0.84	-0.06	0.78	-0.89
HK-3 ⁴	0.06	-0.46	-0.79	0.66
HK-3 ⁵	0.25	0.94	0.16	-0.55
IDH-1 ⁴	0.96*	0.17	-0.60	0.81
IDH-1 ⁵	-0.97*	-0.15	0.54	-0.80
PGM-1 ³	-0.27	-0.77	-0.24	0.53
PGM-1 ⁴	0.40	0.95*	-0.04	-0.31

a See text or Table 1 for definition of variables.

* P < 0.05

Table 6 lists the observed significant correlation coefficients between the heterozygosity levels at five polymorphic loci in D. engyochracea. One basic pattern exists to explain the relationships involved. First, EST-3, LAP-2 and ODH-1 are negatively correlated to PGM-1. EST-3 and ODH-1 are positively correlated to LAP-2, and although LAP-2 and EST-3 are also positively correlated, a negative correlation exists between the heterozygosity levels for ODH-1 and EST-3. To support this pattern, EST-2 correlated negatively with LAP-2 and ODH-1 and positively with PGM-1.

The correlation of the heterozygosity values for each locus for each collection with the weather parameters revealed one significant, negative relationship (table 7).

Again PGM-1 heterozygosity appears to have some kind of relationship to RAIN 2. The negative correlation indicates that high rainfall is associated with low heterozygosity values for this locus. Although not significant, a high negative correlation for this locus extends into the period covered by RAIN 1. A high but nonsignificant positive correlation coefficient is given for LAP-2 heterozygosity and RAIN 2 as well; LAP-2 and its associated loci (TABLE 6) demonstrate an opposing fluctuation in heterozygosity to PGM-1 and EST-2.

Lower levels of heterozygosity at the PGM-1 locus during high rainfall in RAIN 2 predicts that we should find the most common allele occurring at increased frequency during that same period. Table 8 reveals that a high positive and significant coefficient does indeed exist between PGM-1⁴ and RAIN 2. The locus appears to be strongly interactive with amounts of rain falling in the first two weeks prior to collection with some high interaction observed for the PGM-1⁵ allele with RAIN 2 also. None of the other loci show high levels of correlation between their alleles and the various weather parameters, including LAP-2. This locus has other rare alleles (less than 5% frequency) which may confound effects observed at this locus

TABLE 6. Correlations between observed locus heterozygosity estimates in D. engyochracea. Coefficients are all significant with $P < 0.05$.

<u>Sex</u>	<u>Variable</u>	<u>EST-3</u>	<u>LAP-2</u>	<u>ODH-1</u>	<u>EST-2</u>	<u>PGM-1</u>
FEMALES	EST-3 \bar{h}					
	LAP-2 \bar{h}	0.58				
	ODH-1 \bar{h}	-0.15	0.35			
	EST-2 \bar{h}	---	-0.54	-0.97		
	PGM-1 \bar{h}	-0.86	-0.94	-0.27	0.49	
	\bar{H}	0.60	---	0.37	-0.46	-0.49
	(N = 294)					
MALES	EST-3 \bar{h}					
	LAP-2 \bar{h}	0.51				
	ODH-1 \bar{h}	-0.52	---			
	EST-2 \bar{h}	0.25	-0.43	-0.96		
	PGM-1 \bar{h}	-0.86	-0.86	---	---	
	\bar{H}	0.67	---	---	---	-0.46
	(N = 167)					

TABLE 7. Correlations between observed locus heterozygosity (\bar{h}) and population heterozygosity (\bar{H}) in Kipuka Ki populations of D. *engyochracea* to four weather variables.

<u>Variable</u> ^a	<u>RAIN 1</u>	<u>RAIN 2</u>	<u>TEMP</u>	<u>RAINFALL</u>
PGM-1 \bar{h}	-0.92	-0.97*	0.09	0.03
ODH-1 \bar{h}	0.39	0.32	0.09	0.50
LAP-2 \bar{h}	0.78	0.94	0.22	-0.01
EST-3 \bar{h}	0.65	0.66	-0.41	-0.03
EST-2 \bar{h}	0.16	0.45	-0.28	0.55
\bar{H}	0.36	0.52	-0.51	0.74

a See text or table 1 for description of variables.

* $P < 0.05$

TABLE 8. Correlations between primary alleles at five polymorphic enzyme loci in D. engyochracea and 4 weather variables.

<u>Variable</u> ^a	<u>RAIN 1</u>	<u>RAIN 2</u>	<u>TEMP</u>	<u>RAINFALL</u>
PGM-1 ⁴	0.91	0.97*	-0.07	0.00
PGM-1 ⁵	-0.81	-0.91	-0.42	0.50
EST-2 ³	-0.06	-0.38	0.22	-0.46
EST-2 ⁴	-0.08	-0.11	-0.79	0.21
EST-2 ⁵	0.08	0.39	-0.08	0.42
EST-3 ³	-0.33	-0.21	0.32	-0.76
EST-3 ⁴	-0.47	-0.50	0.52	0.01
EST-3 ⁵	0.50	0.43	-0.80	0.57
LAP-2 ⁴	-0.34	-0.66	-0.21	-0.02
LAP-2 ⁵	-0.84	-0.57	-0.14	0.23
ALDOX-1 ³	0.39	0.34	0.33	---
ALDOX-1 ⁴	-0.38	-0.14	-0.74	---

* P < 0.05

a ODH locus not included.

but which present a sampling problem. Because Chi-square contingency tests between samples proved negative and the stability of the alleles at ODH-1 over time was evident (Steiner and Carson, 1974), no correlation to weather data was attempted for this locus.

DISCUSSION

In a recent review Lewontin (1973) discusses the development of models in recent years which have attempted to describe the dynamics of multiple-locus systems. A number of these models demonstrated that there exists more than one possible point of stable equilibrium. These points are dependent on the intensity of linkage and the degree of interaction between the loci which determine fitness. If the genes are tightly linked and interacting, the loci are not independent at equilibrium but are correlated. Thus there will exist excesses of coupling or repulsion gametes. For intermediate values of recombination, alternative points of equilibrium exist in which loci may be correlated or uncorrelated. Genes found correlated in one population may be uncorrelated in another, although selection may be much the same in both cases.

Developments such as these have ignored any effect due to environment. This is because of a lack of knowledge of how genetic systems react to specific parameters, many of which may be hard to partition from other factors. New approaches are required to understand how biochemical genetic variation is maintained in natural populations. An attempt must be made to understand not only the behavior of linked systems with each other but with respect to environment as well. Lewontin (1973) points out that more emphasis must be placed on the development of the theory of multiple-locus systems in temporarily fluctuating environments.

In this study, an attempt is made to bridge the gap between knowledge concerning genetic variation and its possible adaptive function in natural

populations. Examination of two isolated populations of Drosophila mimica and Drosophila engyochracea on the Island of Hawaii reveal high levels of genetic variability in both species (Steiner and Carson, 1974). D. mimica shows higher variability. In addition, both demonstrate heterogeneity in allele frequencies over time. Changes at polymorphic gene systems detected in this study appear to be of three kinds: a) long-term and directional, b) short-term and cyclical and c) those which manifest no consistent pattern.

For the frequency of several gene alleles in D. mimica, significant correlations to specific weather variables are observed. These are the amount of rainfall in the 2 weeks immediately preceding collection (RAIN 1) for IDH-1, the amount of rainfall in the third and fourth weeks prior to collection (RAIN 2) for PGM-1 and the number of times it rained in the month preceding collection (RAINFALL) for EST-2. In D. engyochracea, a similar correlation to RAIN 2 is observed for changes at the PGM-1 locus. These are the same loci which tend to show either directional or cyclical changes in frequency over time.

Relationship to an environmental weather pattern may be expected for a generalist enzyme which acts on external metabolites. The quality, type or quantity of the metabolites may in turn be dependent on some component of weather. Esterase is such a generalist enzyme, although it probably plays a role internally as, for example, in lipid mobilization. The metabolic enzymes PGM and IDH, which act on known internally-occurring metabolites produced in specific energy-producing pathways, are also associated with specific environmental components. It is difficult to explain how this type of enzyme can be affected directly by any environmental variable. A more likely explanation would be that the polymorphic loci are linked to genes susceptible to the environmental parameter in question. It is equally likely that shifts in the balance of environmental forces might spark related shifts in the energy requirement of the organisms which comprise the sampled

gene pool. Both hypotheses could even explain why significant positive correlations exist between the heterozygosity levels at both loci.

The fact that the PGM-1 locus in both species is significantly correlated to total rainfall in the third and fourth weeks prior to collection has special significance for the above hypotheses. It indicates that the phenomenon is not artificial and that common components of the environment can act across species at a single locus in the same locality.

Each of the components, temperature or rainfall, may act individually although joint effects cannot be ruled out. For example, insects normally have an external coating of wax which serves a water-proofing function. Such waxes are often composed of numerous constituents including n-series paraffins, esters of fatty acids or primary alcohols, and acids and alcohols in the free state. Chain lengths of the paraffins and fatty acids vary from 12-34 carbon atoms while primary alcohols from 20-36 atoms long have been identified.

The melting point of natural insect waxes covers a wide range. Studies on the properties and permeability of these suggest that the innermost layer is in the form of an oriented monolayer (Gilmour, 1965). This may be formed by the attraction of polar groups to the hydrated layers of the cuticle. Rising temperature supposedly causes a sudden increase in permeability by disrupting the monolayer at a transition temperature characteristic for the species. Such transition temperatures of different species can be correlated to some extent with the conditions of their environments, being highest in those insects living in hot, dry environments. The monolayer may be an important factor in the control of permeability, as is the nature and physical architecture of the wax layer. In shifting temperature regimes, it is possible that D. mimica and D. engyochracea may become more susceptible to temperature independent of moisture.

It is important to note that water production in the Krebs citric acid cycle is of some importance. Enzymes associated with this cycle include PGM

and IDH. On the other hand, an enzyme possibly associated with lipid or cuticular wax formation may very well take the form of an esterase, since in the above compounds many ester bonds are used in the layering process. Perhaps it is not surprising to find that in each of the above enzymes, the most common allele increases in frequency as rainfall or rainfall events increase.

Studies indicate that many polymorphic loci may show relationships with environmental components. Of these, EST and PGM are particularly often associated with some component of the environment. Esterase-environment associations are reported for almost every organism studied. Cases where these enzymes have been related to environmental parameters have been reported for Uta stansburiana (McKinney et al, 1972), Drosophila melanogaster (Johnson and Schaffer, 1973), Drosophila obscura (Lakovaara and Saura, 1971), Drosophila pavani (Kojima et al, 1972) and Drosophila paulistorum (Richmond, 1972). Esterases are particularly important for the ant Pogonomyrmex barbatus (Johnson et al, 1969). For the most part, these studies are related to latitude or temperature and relationships to moisture are inferential. We have, for example, already seen that amount of rainfall in the Hawaiian environment is inversely related to temperature. In the other studies only in P. barbatus is a strong moisture relationship detected after applying analytical techniques to the data.

For IDH, an association with altitude has been reported by Kojima et al. (1972) in D. pavani. Although other studies have reported geographical heterogeneity at this locus (Selander et al, 1970), the relationships to moisture in the environment appear to be tenuous. The relationship to elevation in D. pavani is only suggestive at best.

Studies concerning the PGM locus are related to latitude or temperature differences in D. melanogaster (Johnson and Schaffer, 1973) and D. pavani (Kojima et al., 1972). Dobzhansky and Ayala (1973) present the best evidence

that this locus may be related to some environmental parameter. Studying the two *Drosophila* species *D. persimilis* and *D. pseudoobscura*, these workers found an association between gene frequencies and season.

All of the studies reported above, with the exception of those from *P. barbatus*, have attempted to relate gene frequencies to environmental differences by using gross measurements, for example latitude or season. This study is one of very few which defines the environmental parameters which are important in shaping latitudinal or seasonal differences. Thus, it is not surprising, in view of the associations found by previous workers, that relationships between environmental parameters and polymorphism at the PGM-1, IDH-1 and EST-2 loci are found in *D. mimica* and *D. engyochracea*.

That correlations are observed between these loci and environmental moisture is not unexpected. It is obvious that moisture is an important parameter in the Hawaiian environment. Moisture effects on Hawaiian *Drosophila* have been reviewed in Carson et al. (1970).

The fact that heterozygosity levels at different loci are significantly correlated to each other has strong implications for the genetic responses associated with environmental heterogeneity. It suggests that we are observing correlated gene responses in which shifting gene frequencies are reflected in changing heterozygosity patterns.

Adaptation of gene pools to environmental changes and vicissitudes must at some stage require correlated responses of coadapted gene complexes. Interaction between loci may be involved in determining the final outcome of the genetic structure observed in the gene pool of the two species studied. This finding is of special significance to current theory concerning the nature of evolution in the Hawaiian *Drosophila*. The determination of the degree of linkage or of linkage disequilibrium would be of importance in understanding the extent and strength of coadaptation of genic contents involved in the observed responses.

Carson (1971, 1973) and Carson and Sato (1969) have outlined a theory underlying the significance of the finding of correlated changes in gene frequencies and heterozygosities in various species. They suggest that evolution in the Hawaiian Drosophila may to some extent be influenced by stochastic processes. For example, Carson visualizes that species formation may be triggered by major reorganization of the gene pool following a founding event. The newly arisen gene pool would have only a portion of the variability previously carried. It may also experience, after initial founding, a population explosion (flush) with resultant albeit temporary relaxation of natural selection. These stochastic events make necessary a reorganization of the genetic system because of destruction of the former "delicately-balanced, integrated system" of genes.

The Carson theory of speciation requires that some coadaptation be present within the founding gene pool. However, so far no evidence for this crucial point has been obtained. The observed correlated changes in heterozygosity at several gene loci in D. mimica and D. engyochracea is the first evidence that such coadaptation, at least at a very elementary level, exists. It would be of interest to determine if the shifts in heterozygosity are mirrored by similar correlated shifts in specific allele frequencies at each locus. If supported by linkage studies, such an investigation would yield definitive evidence about the nature of integrated and balanced gene systems in Hawaiian Drosophila. Because Kipuka Ki and Kipuka Puauulu have been isolated for a known period of time, their Drosophila populations appear especially promising for study with this regard, particularly if a third, geographically separated population could be found.

Chance genetic drift or even migration might result in the observed patterns that single enzyme loci demonstrate with respect to the environment. This seems unlikely, however, since changes are seen in both populations and the same trends are observed for both species. Preliminary studies of

dispersal in D. mimica indicate that the species is rather sedentary. It is difficult to justify how migration between the only known populations of these species would result in the directional trends observed over time. That adaptation of these gene pools is occurring as a result of Darwinian selection is a necessary conclusion.

Another point favoring this conclusion is the following. Significant numbers of homozygotes occur at the EST-3 locus in D. engyochracea and at the LAP-1 and EST-2 loci in D. mimica (Steiner and Carson, 1974). This suggests that selection against heterozygotes occurs during certain periods of the year. These periods appear to fall primarily in the first 6 months of the year. Thus a locus shows a deviation from the expected number of heterozygotes nine times in the period from January through June versus four times in the period from July through December. Both species are considered in this comparison. Inspection of the heterozygosity correlations reveals high, significant correlations which are negative in nature between EST-2 and LAP-1 and EST-2 and PGM-1 in D. mimica. Significant correlations between LAP-1 heterozygosity and PGM-1 heterozygosity ($P < 0.05$) with RAIN 2 exist as well. The evidence suggests that linkage disequilibrium may be involved in which certain genes, conferring adaptation to wetness or dryness of the environment (or some other related factor), tend to pull other loci out of phase as their recombinants are selected for, thus slightly upsetting the genetic "balance" of the system. Similar phenomena may be occurring in D. engyochracea where EST-3 heterozygosity negatively correlates to PGM-1.

Most population genetic studies in Drosophila have not attempted to analyze population structure at polymorphic enzyme loci from a temporal point of view. In most instances comparisons between adjunct or disjunct populations have served the purpose of describing the genetic architecture of a species. Such studies, however, are limited in their capacity to determine responses within populations to particular environmental features.

Present theories concerning the description and nature of neutrality in gene products have been based on the studies just mentioned. These theories have not been concerned with temporal changes in gene frequencies and have, in fact, ignored the few studies which have been done that are of this nature. They have attempted to define the nature of gene neutrality without considering the effect of either environment or of coadaptation, assuming that the locus under consideration was independent of such factors. At a third level, the biochemical and physiological roles of gene products have been ignored, a point emphasized by Johnson (1974). Hypotheses concerned with neutrality predict that gene frequencies at polymorphic enzyme systems will display random tendencies between populations unless these populations are connected by a few intermittent migrants. A corollary to these predictions is that no associations to environmental parameters should be observed except by chance. Furthermore, enzyme phenotypes should display similar responses with respect to some environmental stress.

The results of this and the previous study (Steiner and Carson, 1974) are particularly important in these regards. First, long-term genetic stability is found present at the APH-3 and ACPH-1 loci in D. mimica. A cyclical trend exists in the data for ACPH-1 which appears to be seasonally dependent. These observations indicate that balancing selection is in operation. This possibly overlies some type of frequency-dependent selection which might be operating at the ACPH-1 locus. Occurring as they do over time in the same population, they are inconsistent with non-Darwinian theories.

Secondly, associations between weather variables, particularly temperature and moisture, are found in allele frequencies at the HK-3, IDH-1 and PGM-1 loci in D. mimica and the PGM-1 locus in D. engyocharcea. In addition, heterozygosity levels at the LAP-1 and PGM-1 loci in D. mimica are correlated to rainfall. These loci along with EST-2 show either directional changes or cyclical trends over an 18 month sample period. These observations are also inconsistent with non-Darwinianism.

Finally, correlated gene responses appear to be present; this suggests epistatic or linkage effects. These are consistent across sexes and even species suggesting that linkage may be involved. This correlation is observed in the heterozygosity levels at polymorphic enzyme systems in D. mimica and D. engyochracea. They argue for correlated gene responses to the environment, which favors the selectionist point of view.

Because of the above pieces of evidence, the writer argues that neutrality of a gene is relative to the relationships and conditions that that particular gene is experiencing. Such neutrality, if it exists, must be dependent on time where the conditions determining its neutrality are likewise dependent.

The two sympatric gene pools occurring in each Kipuka appear to be dynamic in their action and respond readily within their limits to environmental fluctuation. Whether the response is due to selection for an overall optimal genotype to meet all vicissitudes of the environment or to selection for a series of equally-responsive genotypes is not clear; certainly the latter can not be ruled out where a series of multiple-allele loci are involved.

Because of the above two considerations, it is suggested that future linkage studies of the enzymes involved in this investigation would prove particularly valuable. Relationships of these to the chromosome variability reported to exist in D. mimica should also prove informative.

It seems relevant to inquire as to the predictive value that the enzyme allele-weather correlations may contain. For example, the correlations drawn at the EST-2, IDH-1 and PGM-1 loci in D. mimica would suggest that levels of heterozygotes at these loci occurring in nature might vary according to fluctuations in the environment of the weather parameter in question. Table 9 depicts the occurrence of just this situation and is drawn from the collection data presented in Steiner and Carson (1974). It is easy to see that the expected differences in heterozygosity level are met. At the EST-2 locus,

TABLE 9. Frequency of the number of heterozygotes occurring at 3 enzyme loci in D. mimica compared with three specific weather parameters in Kipuka Ki (calculated from Steiner and Carson, 1974).

	Jul	Jan	Mar	Jun	Sept	Dec
RAIN 1 (mm)	9	6	51	3	20	23
RAIN 2 (mm)	8	26	45	6	35	7
RAINFALL	3	5	--	4	4	17
Frequency, EST-2 heterozygotes	.506	.408	.518	.455	.418	.567
Frequency, IDH-1 heterozygotes	.242	.162	.278	.283	.125	.136
Frequency, PGM-1 heterozygotes	.239	.148	.042	.289	.042	.401

4 of the 5 collections show increased or decreased numbers of heterozygotes occurring in the collections when the number of rainfall events is correspondingly high or low. Although the RAINFALL data point is missing for the March collection, the amount of rain falling in RAIN 1 and RAIN 2 indicates that there may have been high incidences of rainfall during that period. Correspondingly the number of heterozygotes occurring in the collection is high. Only the July collection does not meet the expected.

At both the IDH-1 and PGM-1 loci, high levels of variability tend to correlate with low amounts of rainfall and vice versa, with only two collections (Jan, Mar) for IDH-1 not fitting the pattern. Again this is consistent with predictions which might be made from correlations between allele frequency and amounts of rainfall for these two loci. The apparently weather-dependent heterozygosity frequencies are also consistent with Darwinian theory, and not completely random as Neutralist theory might predict. If each expected correlation is treated as an independent whole unit, then a chi-square test of significance reveals that the hypothesis of randomness can be rejected ($\chi^2=7.88$, $df=1$, $P < .005$ corrected with Yates correction factor for small samples). The heterozygosity levels are indeed following a pattern consistent with our predictions.

In this paper it is demonstrated that weather and especially moisture are correlated to genetic events in two endemic, sympatrically occurring, isolated populations of Hawaiian Drosophila. These correlations are generally long-term with the possible exception of the IDH-1 locus. It is important to note that the life cycle in D. mimica is 2-3 weeks while in D. engyochracea it is 3-4 weeks (Steiner, personal observations). It indicates that selection may be associated mostly with developmental stages in both species. D. mimica usually breeds in rotting Sapindus saponaria fruits, while D. engyochracea breeds in rotting bark on this tree. Both species spend pupal stages in the ground. It is these stages that may be

the most susceptible to dry environments, and these stages at which selection associated with moisture is most likely to act in shaping the architecture of the gene pool of the next generation. Although it is not definite that moisture is the selective parameter involved here, it is the single most variable parameter in many Hawaiian ecosystems. As such, it is a force which an adapting gene pool must come to terms with at both the genetic and the physiological level. If the ability to resist desiccation is of any importance as a quantitative, physiological yet adaptive trait, it will be so in such an environment. This hypothesis and related genetic parameters are investigated in following reports.

ACKNOWLEDGEMENTS

I wish to thank the staffs of the Hawaii IBP and the Hawaiian Drosophila Project for their help in this project. Peter Galloway and Lee Annest assisted on several field trips. Gwen Arakaki and Alan Ohta provided technical assistance in the lab. Discussions with Drs. J. N. Ahearn, K. Y. Kaneshiro, F. C. do Val and W. E. Johnson proved invaluable at various stages of the project. Dr. W. E. Johnson provided serious comment on portions of this manuscript.

In addition I would like to thank my wife, Judith, and my daughters Shawna and Angela for their continuing support and understanding throughout. This paper was submitted in partial fulfillment for a Doctor of Philosophy degree in Genetics awarded to W. W. M. Steiner by the Department of Genetics, University of Hawaii, Honolulu. Research was supported by NSF grants GB-23230, GM-27586, and GB-29288.

REFERENCES

- Bridges, K. W. and G. V. Carey. 1973. The climate of the IBP sites on Mauna Loa, Hawaii. US/IBP Island Ecosystems IRP Tech. Rep. #22. 141 p.
- Bridges, K. W. and G. V. Carey. 1974. Climate data for the IBP sites on Mauna Loa, Hawaii (supplement). US/IBP Island Ecosystems IRP Tech. Rep. #38. 97 p.
- Carson, H. L. 1973. Ancient chromosomal polymorphisms in Hawaiian Drosophila. Nature 241:200-202.
- Carson, H. L., D. E. Hardy, H. T. Spieth and W. S. Stone. 1970. The evolutionary biology of the Hawaiian Drosophilidae in Essays in Evolution and Genetics in Honor of Theodosius Dobzhansky. M. Hecht and W. Steere (eds.). Appleton-Century-Croft, N. Y. pp. 437-534.
- Carson, H. L. and J. E. Sato. 1969. Microevolution within three species of Hawaiian Drosophila. Evolution 23:493-501.
- Dobzhansky, Th. and F. J. Ayala. 1973. Temporal frequency changes of enzyme and chromosomal polymorphisms in natural populations of Drosophila. Proc. Nat. Acad. Sci. (U. S.) 70:680-683.
- Gilmour, D. 1965. The metabolism of Insects. Univ. Rev. in Biology. W. H. Freeman and Co., S. F.
- Johnson, F. M. and H. E. Schaffer. 1973. Isozyme variability in species of the genus Drosophila, VII. Genotype-environment relationships in populations of D. melanogaster from the Eastern United States. Bioch. Genet. 10:149-163.
- Johnson, F. M., H. E. Schaffer, J. E. Gillaspay and E. S. Rockwood. 1969. Isozyme genotype-environment relationships in natural populations of the Harvester Ant, Pogonomyrmex barbatus, from Texas. Bioch. Genet. 3:429-450.
- Johnson, G. B. 1974. Enzyme polymorphism and metabolism. Science 184:28-37.
- Kojima, K., P. Smouse, S. Y. Yang, P. S. Nair and D. Brncic. 1972. Isozyme frequency patterns in Drosophila pavani associated with geographical and seasonal variables. Genetics 72:721-731.
- Lakovaara, S. and A. Saura. 1971. Genetic variation in natural populations of Drosophila obscura. Genetics 69:377-384.
- Lecam, L., J. Neyman and E. L. Scott (eds.). 1972. Darwinian, Neo-Darwinian and non-Darwinian Evolution, Proceedings Sixth Berkeley Symposium on Mathematics, Statistics and Probability, Vol. V. Univ. of Calif. Press, Berkeley.
- Lewontin, R. C. 1973. Population genetics. Ann. Rev. Genetics 7:1-17.

- McKinney, C. O., R. K. Selander, W. E. Johnson and S. Y. Yang. 1972. Genetic variation in the side-blotched lizard (Uta stansburiana). Studies in Genetics VII. Univ. Tex. Publ. 7213:307-318.
- Richmond, R. C. 1972. Enzyme variability in the Drosophila willistoni group, III. Amounts of variability in the superspecies D. paulistorum. Genetics 70:87-112.
- Selander, R. K. and D. W. Kaufman. 1973. Genetic variability and strategies of adaptation in animals. Proc. Nat. Acad. Sci. (U. S.) 70:1875-1877.
- Selander, R. K., S. Y. Yang, R. C. Lewontin and W. E. Johnson. 1970. Genetic variation in the Horseshoe Crab (Limulus polyphemus), a phylogenetic "relic". Evolution 24:402-414.
- Selander, R. K. and W. E. Johnson. 1973. Genetic variation among vertebrate species. Ann. Rev. Ecol. and System. 4:75-91.
- Smathers, G. A. 1968. A report on the micro-climate in two Hawaiian Kipukas. Report to the Dept. of Entomology, Univ. of Hi., Honolulu.
- Steiner, W. W. M. and H. L. Carson. 1974. Genetic structure and variability in two species of endemic Hawaiian Drosophila. US/IBP Island Ecosystems IRP Tech. Rep. #50. 66 p.

TECHNICAL REPORTS OF THE US/IBP ISLAND ECOSYSTEMS IRP

(Integrated Research Program)

- *No. 1 Hawaii Terrestrial Biology Subprogram. First Progress Report and Second-Year Budget. D. Mueller-Dombois, ed. December 1970. 144 p.
- *No. 2 Island Ecosystems Stability and Evolution Subprogram. Second Progress Report and Third-Year Budget. D. Mueller-Dombois, ed. January 1972. 290 p.
- *No. 3 The influence of feral goats on koa (Acacia koa Gray) reproduction in Hawaii Volcanoes National Park. G. Spatz and D. Mueller-Dombois. February 1972. 16 p.
- *No. 4 A non-adapted vegetation interferes with soil water removal in a tropical rain forest area in Hawaii. D. Mueller-Dombois. March 1972. 25 p.
- *No. 5 Seasonal occurrence and host-lists of Hawaiian Cerambycidae. J. L. Gressitt and C. J. Davis. April 1972. 34 p.
- *No. 6 Seed dispersal methods in Hawaiian Metrosideros. Carolyn Corn. August 1972. 19 p.
- *No. 7 Ecological studies of Ctenosciara hawaiiensis (Hardy) (Diptera: Sciaridae). W. A. Steffan. August 1972. 7 p.
- *No. 8 Birds of Hawaii Volcanoes National Park. A. J. Berger. August 1972. 49 p.
- *No. 9 Bioenergetics of Hawaiian honeycreepers: the Amakihi (Loxops virens) and the Anianiau (L. parva). R. E. MacMillen. August 1972. 14 p.
- *No. 10 Invasion and recovery of vegetation after a volcanic eruption in Hawaii. G. A. Smathers and D. Mueller-Dombois. September 1972. 172 p.
- *No. 11 Birds in the Kilauea Forest Reserve, a progress report. A. J. Berger. September 1972. 22 p.
- No. 12 Ecogeographical variations of chromosomal polymorphism in Hawaiian populations of Drosophila immigrans. Y. K. Paik and K. C. Sung. February 1973. 25 p.
- *No. 13 The influence of feral goats on the lowland vegetation in Hawaii Volcanoes National Park. D. Mueller-Dombois and G. Spatz. October 1972. 46 p.
- *No. 14 The influence of SO₂ fuming on the vegetation surrounding the Kahe Power Plant on Oahu, Hawaii. D. Mueller-Dombois and G. Spatz. October 1972. 12 p.
- No. 15 Succession patterns after pig digging in grassland communities on Mauna Loa, Hawaii. G. Spatz and D. Mueller-Dombois. November 1972. 44 p.
- No. 16 Ecological studies on Hawaiian lava tubes. F. G. Howarth. December 1972. 20 p.
- No. 17 Some findings on vegetative and sexual reproduction of koa. Günter O. Spatz. February 1973. 45 p.
- No. 18 Altitudinal ecotypes in Hawaiian Metrosideros. Carolyn Corn and William Hiesey. February 1973. 19 p.
- No. 19 Some aspects of island ecosystems analysis. Dieter Mueller-Dombois. February 1973. 26 p.
- No. 20 Flightless Dolichopodidae (Diptera) in Hawaii. D. Elmo Hardy and Mercedes D. Delfinado. February 1973. 8 p.

* out of print

- No. 21 Third Progress Report and Budget Proposal for FY 74 and FY 75. D. Mueller-Dombois and K. Bridges, eds. March 1973. 153 p.
- No. 22 Supplement 1. The climate of the IBP sites on Mauna Loa, Hawaii. Kent W. Bridges and G. Virginia Carey. April 1973. 141 p.
- No. 23 The bioecology of Psylla uncatoides in the Hawaii Volcanoes National Park and the Acacia koaia Sanctuary. John R. Leeper and J. W. Beardsley. April 1973. 13 p.
- No. 24 Phenology and growth of Hawaiian plants, a preliminary report. Charles H. Lamoureux. June 1973. 62 p.
- No. 25 Laboratory studies of Hawaiian Sciaridae (Diptera). Wallace A. Steffan. June 1973. 17 p.
- No. 26 Natural area system development for the Pacific region, a concept and symposium. Dieter Mueller-Dombois. June 1973. 55 p.
- No. 27 The growth and phenology of Metrosideros in Hawaii. John R. Porter. August 1973. 62 p.
- *No. 28 EZPLOT: A computer program which allows easy use of a line plotter. Kent W. Bridges. August 1973. 39 p.
- No. 29 A reproductive biology and natural history of the Japanese white-eye (Zosterops japonica japonica) in urban Oahu. Sandra J. Guest. September 1973. 95 p.
- No. 30 Techniques for electrophoresis of Hawaiian Drosophila. W. W. M. Steiner and W. E. Johnson. November 1973. 21 p.
- No. 31 A mathematical approach to defining spatially recurring species groups in a montane rain forest on Mauna Loa, Hawaii. Jean E. Maka. December 1973. 112 p.
- *No. 32 The interception of fog and cloud water on windward Mauna Loa, Hawaii. James O. Juvik and Douglas J. Perreira. December 1973. 11 p.
- No. 33 Interactions between Hawaiian honeycreepers and Metrosideros collina on the island of Hawaii. F. Lynn Carpenter and Richard E. MacMillen. December 1973. 23 p.
- No. 34 Floristic and structural development of native dry forest stands at Mokuleia, N.W. Oahu. Nengah Wirawan. January 1974. 49 p.
- No. 35 Genecological studies of Hawaiian ferns: reproductive biology of pioneer and non-pioneer species on the island of Hawaii. Robert M. Lloyd. February 1974. 29 p.
- No. 36 Fourth Progress Report and Budget Proposal for FY 1975. D. Mueller-Dombois and K. Bridges, eds. March 1974. 44 p.
- No. 37 A survey of internal parasites of birds on the western slopes of Diamond Head, Oahu, Hawaii 1972-1973. H. Eddie Smith and Sandra J. Guest. April 1974. 18 p.
- No. 38 Climate data for the IBP sites on Mauna Loa, Hawaii. Kent W. Bridges and G. Virginia Carey. May 1974. 97 p.
- No. 39 Effects of microclimatic changes on oogenesis of Drosophila mimica. Michael P. Kambysellis. May 1974. 58 p.
- No. 40 The cavernicolous fauna of Hawaiian lava tubes, Part VI. Mesoveliidae or water treaders (Heteroptera). Wayne C. Gagné and Francis G. Howarth. May 1974. 22 p.

* out of print

- No. 41 Shade adaptation of the Hawaiian tree-fern (Cibotium glaucum (Sm.) H. & A.). D. J. C. Friend. June 1974. 39 p.
- No. 42 The roles of fungi in Hawaiian Island ecosystems. I. Fungal communities associated with leaf surfaces of three endemic vascular plants in Kilauea Forest Reserve and Hawaii Volcanoes National Park, Hawaii. Gladys E. Baker, Paul H. Dunn and William A. Sakai. July 1974. 46 p.
- No. 43 The cavernicolous fauna of Hawaiian lava tubes, Part VII. Emesinae or thread-legged bugs (Heteroptera: Reduviidae). Wayne C. Gagné and Francis G. Howarth. July 1974. 18 p.
- No. 44 Stand structure of a montane rain forest on Mauna Loa, Hawaii. Ranjit G. Cooray. August 1974. 98 p.
- No. 45 Genetic variability in the Kilauea Forest population of Drosophila silvestris. E. M. Craddock and W. E. Johnson. September 1974. 39 p.
- No. 46 Linnet breeding biology on Hawaii. Charles van Riper III. October 1974. 19 p.
- No. 47 The nesting biology of the House Finch, Carpodacus mexicanus frontalis (Say), in Honolulu, Hawaii. Lawrence T. Hirai. November 1974. 105 p.
- No. 48 A vegetational description of the IBP small mammal trapline transects - Mauna Loa Transect. James D. Jacobi. November 1974. 19 p.
- No. 49 Vegetation types: a consideration of available methods and their suitability for various purposes. Dieter Mueller-Dombois and Heinz Ellenberg. November 1974. 47 p.
- No. 50 Genetic structure and variability in two species of endemic Hawaiian Drosophila. William W. M. Steiner and Hampton L. Carson. December 1974. 66 p.
- No. 51 Composition and phenology of the dry forest of Mauna Kea, Hawaii, as related to the annual cycle of the Amakihi (Loxops virens) and Palila (Psittirostra bailleui). Charles van Riper III. January 1975. 37 p.
- No. 52 Environment-enzyme polymorphism relationships in two Hawaiian Drosophila species. W. W. M. Steiner. January 1975. 28 p.