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ADAPTATION OF *DROSOPHILA WILLISTONI* EXPERIMENTAL POPULATIONS TO EXTREME *p*H MEDIUM

II. DEVELOPMENT OF INCIPIENT REPRODUCTIVE ISOLATION

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Summary

Experimental populations of *Drosophila willistoni* adapted to *low*, *median* and *high* *p*H substrate (*p*H 2, *p*H 5 and *p*H 10) were tested by the multiple choice technique, and a significant sexual preference was observed for homogamic insemination, which was greater among populations derived from the centre of the species distribution than the ones collected near the margin of its distribution range. It was also observed that some of the F_1 and F_2 hybrids, between flies adapted to different foods, were reproductively inferior on the parental substrate.

The greater divergence of the central populations may be due to their significantly greater chromosomal and genic polymorphism than the marginal populations.

1. Introduction

Indirect and direct selective processes may contribute to the initiation of reproductive isolation between spatially isolated populations (Littlejohn, 1969). Incipient isolation usually starts with minor changes in courtship and hybrid male sterility, as Winge (1971) demonstrated in *Drosophila willistoni quechua* (Ayala, 1973). The complexity of natural environments and the lack of detailed ecological information on the factors responsible for race and species divergence in *Drosophila* make it desirable to use an experimental approach to establish the agents and the time necessary to start and develop incipient reproductive isolation in allopatric or sympatric conditions. Knight, Robertson and Waddington (1956), Thoday and Gibson (1962, 1970), Gibson and Thoday (1963), Ehrman (1964), Del Solar (1966), Scharloo (1967), Chabora (1968), and others have contributed significantly to this field.

In this experiment we report the detection of incipient isolation between separated experimental populations of *D. willistoni* adapted for many generations to three types of food having different *p*H levels.

2. Material and methods

A detailed description of the experimental populations is given by Oliveira and Cordeiro (1980) and a brief outline of methods will suffice. Samples of the *D. willistoni* natural populations were collected in riparian

woods at the National Park near Brasilia, D.F., and in semi-isolated woods of the grassland region of Rio Grande do Sul state in the " Eldorado " locality, Guaíba County. The F_1 generation from each sample was distributed between three population cages for Brasilia and three for Eldorado. The cages had 12 food containers each. In the next generation each cage contributed to three others with the same kind of food, giving a total of nine Brasilia and nine Eldorado cages. In successive generations the number of containers with extreme *p*H food was gradually increased in the respective population cages so that ultimately there were, for each locality, three cages with only *low p*H food (*p*H 2-3), three with only *high p*H food (9-10) while three remained unchanged with the standard food (*p*H 5), here named *median p*H. The symbols to represent the cages are: B = Brasilia and E = Eldorado, followed by: l = *low*, m = *median*, and h = *high*, for the three levels of *p*H. The populations were numbered in order: Bl_1, Bl_2, $Bl/_3$ Bm_1, Bm_2, Bm_3, Bh_1, Bh_2, Bh_3, El_1, etc.

Sexual isolation was studied by the multiple choice technique. The flies were sexed and aged for 3 days. The vials used for maturation of these flies were examined afterwards to control mistakes. We found after several trials that it is adequate for this test to place together 3-day-old males and females for only 4 hours at 25°C. The testing was terminated by immobilising the flies in an 8°C thermostable chamber until the females could be dissected and their ventral receptacle and spermathecae examined under a microscope for mobile spermatozoa. For the calculation of Levene's (1949) coefficient of isolation ($K_{1,2}$), population 1 males are placed with population 1 and population 2 females and the number of intra and inter-population inseminations are counted. The inverse crosses are tested using No. 2 males with Nos. 2 and 1 females. The formulae used are:

$$K_{1,2} = \frac{\ln q_{1,1} - \ln q_{1,2}}{\ln q_{1,1} + \ln q_{1,2}} \qquad K_{2,1} = \frac{\ln q_{2,2} - \ln q_{2,1}}{\ln q_{2,2} + \ln q_{2,1}}$$

Where $1\text{-}q_{ij}$ is the frequency of insemination of females of type j by males of type i. Standard errors of these values can be calculated according to Levene (1949).

The coefficient of joint isolation of two populations is given by the arithmetic mean: $K_{1,2} = (K_{1,2}+K_{2,1})/2$ and the coefficient of excess insemination: $m_{1,2} = (K_{1,2}-K_{2,1})/2$. The joint coefficient measures the true reproductive isolation between the two populations under the artificial conditions of the experiment, while the coefficient of excess insemination can serve as a measure of the extent to which the gene flow between the two populations is in one direction only.

Another measurement of reproductive isolation tested in this work is defined as " hybrid inferiority " under the parental medium conditions. For this test, 5-day-old virgin females and males were used. All possible crosses among Brasilia populations were made. For each combination two replicate crossings were started with 10 pairs of flies which were transferred every 2nd day to a new culture bottle, giving two series of six bottles with the appropriate *p*H substrate. The eclosed progeny flies were counted and again two sets of 10 pairs were mated and transferred as above to make two replicate series of six vials for each combination. The emerging flies were counted as a measure of their productivity.

3. Results

The multiple choice sexual isolation tests were performed after 26, 32, 52, and 69 generations of selection for the three different *p*H foods in the Brasilia populations as summarised in table 1. It can be observed that according to the Levene (1949) index there is a significantly greater frequency of homogamic insemination in the following crosses: males *high* × *low* + *high* females; males *median* × *median* + *low* females; and males *low* × *median* + *low* females. However in the 52nd generation the crossing of males *high* × *low* + *high* females, and males *median* × *median* + *low* females shows no significant results. It is possible that the relatively smaller sample tested, and a sporozoan infection observed in this generation could have interfered with the

Table 1

Sexual isolation between D. willistoni *populations from Brasilia selected for different* p*H levels* (*M* = *Intermediate; H* = *high and L* = *low* p*H medium; n* = *number of examined flies;* % *inseminated flies*)

			Homogamic		Heterogamic				
Generation	Male	Females	n	%	n	%	Isol. coef.	Joint isol. coef.	Insem. excess
26	M	M+H	148	29·05	165	27·27	0·038 ± 0·11	—	—
32	M	M+H	73	15·08	72	26·39	−0·304 ± 0·171	−0·030 ± 0·108	0·274 ± 0·108
	H	M+H	53	35·85	81	51·85	−0·244 ± 0·131		
52	M	M+H	97	48·45	99	50·50	−0·029 ± 0·103	−0·003 ± 0·084	−0·026 ± 0·084
	H	M+H	58	60·34	46	58·70	0·023 ± 0·132		
69	M	M+H	269	65·43	270	54·44	0·150 ± 0·206	0·081 ± 0·105	0·069 ± 0·105
	H	M+H	247	60·42	244	59·43	0·012 ± 0·060		
26	M	M+L	126	27·78	125	13·60	0·380 ± 0·13*	—	—
32	M	M+L	91	37·36	96	20·83	0·333 ± 0·125*	0·294 ± 0·081*	0·039 ± 0·081
	L	M+L	102	52·94	97	36·08	0·255 ± 0·102*		
52	M	M+L	72	63·89	69	55·07	0·120 ± 0·111	0·221 ± 0·068*	−0·101 ± 0·068
	L	M+L	130	66·92	134	43·28	0·322 ± 0·077*		
69	M	M+L	293	69·62	289	50·86	0·254 ± 0·050	0·181 ± 0·036*	0·072 ± 0·036*
	L	M+L	293	65·53	294	57·48	0·109 ± 0·054*		
26	H	L+H	131	36·64	112	25·00	0·226 ± 0·11*	0·222 ± 0·072*	0·004 ± 0·072
	L	L+H	114	52·63	139	38·13	0·218 ± 0·09*		
32	H	L+H	96	61·46	93	40·86	0·289 ± 0·098*	0·391 ± 0·075*	0·102 ± 0·075
	L	L+H	89	47·19	72	19·44	0·493 ± 0·116		
52†	H	L+H	77	61·04	74	52·70	0·114 ± 0·110	0·166 ± 0·142	−0·052 ± 0·142
	L	L+H	18	61·11	11	45·45	0·217 ± 0·262		
69	H	L+H	275	72·00	274	59·49	0·169 ± 0·052*	0·032 ± 0·037	0·137 ± 0·037*
	L	L+H	300	52·33	301	59·80	−0·105 ± 0·056		

* Homogamic insemination significantly greater than the heterogamic.
† Sporozoan infection.

outcome of this experiment. Nevertheless, the crosses: males *low* × *median* + *low* females shows significant homogamic sexual preference.

The same kind of multiple choice tests was performed for the Eldorado experimental populations after 34, 52, and 69 generations of selection. The results are summarised in table 2. The most consistent incipient isolation by sexual preference was exhibited by the males *median* × *median* + *low* females where the homogamic inseminations were more frequent than the heterogamic. In the Eldorado crossings of males *low* × *low* + *high* females, males *median* × *median* + *high* females, and males *low* × *median* + *low* females, significant homogamic preferences were observed only in one of the generations. There was not a continuity of response as in Brasilia's populations.

TABLE 2

Sexual isolation between D. willistoni *populations from Eldorado selected for different* pH *levels (M = intermediate; H = high and L = low* pH *medium; n = number of examined flies; % inseminated flies*

Generation	Male	Females	Homogamic n	Homogamic %	Heterogamic n	Heterogamic %	Isol. coef.	Joint isol. coef.	Insem. excess
34	M	M+H	103	53·40	102	52·94	0·006 ± 0·098	0·082 ± 0·067	−0·075 ± 0·067
	H	M+H	106	58·49	112	47·32	0·156 ± 0·093		
52	M	M+H	39	79·49	38	68·42	0·158 ± 0·140	0·085 ± 0·096	0·073 ± 0·096
	H	M+H	49	71·43	44	70·45	0·013 ± 0·131		
69	M	M+H	278	67·27	280	48·21	0·263 ± 0·071*	0·111 ± 0·044*	0·152 ± 0·044*
	H	M+H	299	66·55	292	69·52	−0·041 ± 0·053		
34	M	M+L	117	52·99	125	33·60	0·297 ± 0·091*	0·238 ± 0·068*	0·059 ± 0·068
	L	M+L	129	41·86	124	31·45	0·179 ± 0·102		
52	M	M+L	69	84·05	69	59·42	0·342 ± 0·097*	0·079 ± 0·063	0·421 ± 0·063*
	L	M+L	74	86·49	78	48·72	0·500 ± 0·082*		
69	M	M+L	290	63·10	290	49·65	0·185 ± 0·053*	0·098 ± 0·037*	0·097 ± 0·037*
	L	M+L	294	63·94	294	64·63	−0·009 ± 0·053		
34	H	L+H	97	50·51	96	39·58	0·165 ± 0·106	0·260 ± 0·074*	−0·095 ± 0·074
	L	L+H	83	57·83	89	33·71	0·355 ± 0·104		
52	H	L+H	44	75·00	47	68·08	0·096 ± 0·131	0·137 ± 0·089	0·040 ± 0·089
	L	L+H	50	86·00	48	75·00	0·173 ± 0·123		
69	H	L+H	282	64·89	285	62·10	0·011 ± 0·056	−0·024 ± 0·039	0·013 ± 0·039
	L	L+H	285	62·10	282	66·31	−0·037 ± 0·054		

* Monogamic insemination significantly greater than the heterogamic.

In the 106th generation of selection the Brasilia experimental populations were sampled and crossed as indicated, in order to find out whether reproductive isolation in the form of reduced hybrid viability in the parental environments could be detected. The results are presented in table 3. Despite the fact that *high* and *low* populations have developed quite significant differences with regard to their food medium their hybrids are not less fit on the alkaline food. However, on the acid substrate the hybrids are inferior to their parents which are adapted to this food; the productivity of the hybrids at the low *p*H is nevertheless better than that of the high *p*H parents. The hybrids are even more viable in the high *p*H than their low

TABLE 3

Reproductive isolation due to hybrid inferiority between D. willistoni *populations from Brasilia selected for 106 generations for adaptation to extreme* pH *values of the medium*

F_1 ♂×♀	F_1 $\bar{X}$	F_1 t	F_2 ♂×♀	F_2 $\bar{X}$	F_2 t
High *p*H substrate, *p*H 10					
H×H	26·82	—	H×H	53·21	—
L×L	12·86	3·20†	L×L	46·42	2·26*
H×L	29·00	0·47	H×L	68·92	3·01*
Low *p*H substrate, *p*H 2					
L×L	30·25	—	L×L	30·28	—
H×H	3·96	9·70†	H×H	5·88	4·49†
H×L	13·32	6·16†	L×H	14·35	2·63*

Significant at a level of 0·05. † Significant at a level of 0·01.
$\bar{X}$ Eclosed flies from a pair mating (2 days oviposition).

TABLE 4

Reproductive isolation due to hybrid inferiority between D. willistoni *population from Brasilia selected during 122 generations to extreme* p*H medium*

F_1			F_2		
♂×♀	$\bar{X}$	t	♂×♀	$\bar{X}$	t
		Low *p*H substrate			
L×L	16·82	—	L×L	3·71	—
M×M	4·96	5·38*	M×M	2·68	1·30
H×H	3·28	6·28*	H×H	1·64	2·87*
L×M	6·78	4·13†	L×M	1·50	3·16†
M×L	13·96	0·99	M×L	3·18	0·59
L×H	6·07	4·52†	L×H	1·75	2·48*
H×L	11·71	1·88	H×L	4·07	0·40
		Intermediate *p*H substrate			
M×M	37·11	—	M×M	37·89	—
L×L	18·57	4·96†	L×L	16·68	7·34†
H×H	8·86	8·85†	H×H	30·82	2·15*
M×L	29·68	2·39*	M×L	44·21	1·99
L×M	35·89	0·37	L×M	33·25	1·34
M×H	28·86	2·48*	M×H	41·32	1·21
H×M	25·50	3·34†	H×M	35·07	0·89
		High *p*H substrate			
H×H	30·11	—	H×H	35·61	—
M×M	26·96	1·07	M×M	26·25	4·68†
L×L	22·71	2·40*	L×L	19·54	7·51†
H×L	27·71	0·62	H×L	33·36	0·86
L×H	29·75	0·10	L×H	34·57	0·46
H×M	26·93	0·90	H×M	27·96	3·73†
M×H	22·28	2·63*	M×H	34·32	0·70

* Significant at a level of 0·05. † Significant at a level of 0·01.
$\bar{X}$ Eclosed flies from a pair mating (2 days oviposition).
(M = intermediate; L = low; H = high *p*H medium.)

*p*H parents. In the 122nd generation of selection the same tests were carried out and table 4 shows the data obtained for the F_1 and F_2. When the average production per pair mating is compared, most of these results confirm the results for the 106th generation. Again, on the alkaline food the *low/high* hybrids are not significantly inferior to their *high* parents. The opposite was observed for the low *p*H parents which were significantly better on their food than any hybrid except for the *median/low* and *high/low* combinations. However, pooling of *median/low* + *low/median* and *high/low* + *low/high*, the F_1 and F_2 hybrids are significantly inferior to their *low* parents on the acid food. At the median *p*H food (*p*H 5) the hybrids *high/median* and *median/high* are significantly less productive than the flies adapted to this intermediate *p*H level which are not better than the hybrids *low/median* but are better than the *median/low* in this food. If the viabilities of these reciprocal hybrids are pooled the result is significantly inferior to the *median* population in the F_1. In the F_2 generation hybrid inferiority appears only in three out of twelve combinations while in F_1 the proportion is six to twelve. If we pool the reciprocal hybrid crossings this difference increases.

4. Discussion

Experimental populations from Brasilia show very significant, consistent incipient sexual isolation in three different multiple choice tests (males *median* × *median* + *low* females, *low* × *low* + *median* females, and males *high* × *low* + *high* females). The only consistently significant result shown by the Eldorado populations was produced by the crossings of males *median* × *median* + *low* females. These results might be related to the fact that the Central Brazilian region (Brasilia) populations have higher frequencies of genic and chromosomal polymorphism than the southern marginal populations of Rio Grande do Sul (Eldorado) according to Pavan *et al.* (1951), Cordeiro *et al.* (1958), Da Cunha and Dobzhansky (1954). The other three cases of significant results are scattered throughout different combinations (table 2). In *D. melanogaster* strains isolated for many generations, Koref-Santibañez and Waddington (1958) showed occasional preferences for homogamic matings in male choice experiments in four of six strains, two of them showing also female homogamic preference. These results, like those observed in the Eldorado populations, suggest that incipient sexual isolation may appear as a chance event among spatially isolated populations. However the more consistent results of Brasilia populations are best compared with those of the Mather and Harrison (1949) strains selected for abdominal chaeta number. Sexual isolation may be a frequent result concomitant with genetic divergence.

Low *p*H populations definitely produced more significant results. No isolation was detected between median and high *p*H populations in Brasilia or Eldorado, except for one case in the latter populations. The comparison of the reproductive performance of F_1 hybrids and their parents in the respective culture media suggests that low *p*H food was more effective as a selective agent than high *p*H food.

The first known test of sexual isolation in *D. willistoni* populations was performed by Dobzhansky and Mayr (1944) for flies from Quirigua, Guatemala and four other locations in Brasil. Incipient sexual homogamic preference was shown only by Brazilian flies, while Guatemalian males preferred Brazilian females. Recently Dobzhansky (1975) showed that *D. willistoni quechua* shows a barely significant sexual isolation index, calculated by the method also used by us. Despite minor differences in the technique of multiple choice testing, our results are comparable with those of Dobzhansky (1975). It is surprising that selection for extreme environments could be so fast and so effective in producing ethologic isolation between flies of the same species and location, at a level observed among naturally occurring subspecies. In *Zea mays* L., Patterniani (1969) developed intraspecific sexual isolation through selection in only six generations.

Unfortunately we did not test hybrid male sterility, which was found to be the most important isolation factor among *D. willistoni* geographic races (De Souza *et al.*, 1961, De Toledo, 1971, and Winge, 1971). The distinction of *D. willistoni* into three geographic races was proposed by Winge, after extensive studies, as: the northern (Central America, West Indies, except Trinidad, and southern Florida), southern (Brasil, Argentina, Colombia and Trinidad), and the transitional race (Peru and Equador) which corresponds to the subspecies named *D. willistoni quechua* by Ayala (1973). These flies are morphologically indistinguishable, yet the high degree of probability

of correct diagnosis of the subspecies on a single individual, with the aid of isoenzyme patterns, ranges from 0·99998 to 0·974 for each of the five loci Ayala and Tracey (1973) used to describe the subspecies. The five loci give the joint probability of $3{\cdot}4 \times 10^{-14}$ for the incorrect diagnosis of the subspecies of a single individual. According to Reguly and Cordeiro (1978) our *high* and *low* populations differ in the allelic frequency of two out of four loci studied, and we have indications that other enzymatic systems show allozymic variations between populations. Allozymes appear to be good markers of genetic divergence among *Drosophila* species as demonstrated by Ayala and Powell (1972) for the *willistoni* sibling group of species.

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