PARTHENOGENESIS AND GENETIC VARIABILITY. I. LINKAGE AND INBREEDING ESTIMATIONS IN THE FROG, *RANA PIPIENS*[1,2]

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THE scarcity of genetically defined variants has been onerous in the case of amphibians and other organisms used in experimental embryology, the major interest of the authors. Few mutants have been described in these organisms and fewer yet have been mapped (LINDSLEY, FANKHAUSER and HUMPHREY 1956; HUMPHREY 1959).

Genetic studies of amphibians have dealt primarily with *Ambystoma mexicanum*, *Xenopus laevis*, and *Rana pipiens*. These studies concern such characteristics as alteration of pigmentation (MOORE 1942; VOLPE 1956; HUMPHREY 1967), early developmental lethality, and various morphological abnormalities (HUMPHREY 1959, 1960, 1962, 1966; UEHLINGER 1969). Linkage was established in only one of the above studies when HUMPHREY (1959) demonstrated a tight linkage between fluid imbalance (*f*) and a gene causing abnormalities of the gills (*g*) of *A. mexicanum*.

An earlier linkage study in amphibians (LINDSLEY, FANKHAUSER and HUMPHREY 1956) utilized the accessibility of meiotic events to experimental manipulation. By altering the course of the second meiotic division in female dioecious organisms, second-division segregants are obtained which yield data that permit determination of gene–kinetochore (*G–K*) distances of any mutant gene (ANDERSON 1925; BEADLE and EMERSON 1935; PAPAZIAN 1952; BARRATT *et al.* 1954). Using such information LINDSLEY *et al.* (1956) attempted to measure *G–K* distances for three genetic factors in *A. mexicanum*: *d* causing a white phenotype, *f* causing fluid imbalance, and the sex factor. In this study, eggs which retained their second polar bodies after cold exposure were fertilized with sperm from males homozygous for the recessive genes (*d* or *f*) and produced triploid progeny. All three of these factors segregated independently of their kinetochores, and *G–K* distances could not be measured.

Gynogenetic reproduction, employing the inhibtion of meiosis II and yielding diploid rather than triploid progeny, can be used to map *G–K* distances as well as

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to develop "inbred" strains*. This mode of reproduction is known in several natural populations of invertebrates and vertebrates (SUOMALAINEN 1950; WHITE 1948, 1970; OLSEN 1962; CARSON 1967) and has been produced experimentally in vertebrates when the appropriate meiotic events are altered (TYLER 1955; BEATTY 1967; GRAHAM 1970; TARKOWSKI, WITKOWSKA and NOWICKA (1970).

Thus it seems possible that the genetic analysis of amphibians could take advantage of this mode of reproduction and of techniques and facilities now available for the laboratory maintenance of amphibians (NACE 1968; NACE and RICHARDS 1969). In this light, the purposes of this investigation are three-fold: (1) to describe linkage studies performed upon *R. pipiens* using gynogenetic and biparental "crosses"; (2) to determine *G–K* distances for factors which might be linked; and (3) to present estimations of the degree of "inbreeding" established by the gynogenetic mode of reproduction. These data resulted from a program to establish "inbred" strains of *R. pipiens* (NACE 1968). The accompanying paper (ASHER 1970) extending the theory developed here presents deterministic models describing gynogenetic and other parthenogenetically reproducing populations.

This study reviews a tool for uncovering and mapping the multitude of genetic variants which exist in *R. pipiens*, and provides a mathematical basis for estimating rates of "inbreeding" in gynogenetically produced strains of amphibians. It is anticipated that this information will be applied to the acquisition of a more precise understanding of many developmental events.

MATERIALS AND METHODS

Animals: R. Pipiens used as parents were obtained from dealers in Vermont and Wisconsin. The frogs and their progeny were maintained and raised under conditions in which both disease and nutrition were monitored (NACE 1968).

Genes studied: Three well characterized genes were studied: (1) *K*, kandiyohi, (2) *B*, burnsi, and (3) *m*, melanoid. The dominant gene *K* alters the pigmentation pattern of the background on which large dorsal spots are arranged (VOLPE 1955, 1956). The phenotypic expression of this gene ranges from numerous pinpoint spots to mottling or scalloping of the dorsal background pattern. The dominant gene *B* alters the number of major dorsal spots (MOORE 1942; VOLPE 1960, 1961). These dorsal spots may be reduced in number or may be totally absent. The third gene, *m*, is a recessive mutant which, when homozygous, alters the differentiation of iridophores and xanthophores to such an extent that the frogs can be described as "transparent" (RICHARDS, TARTOF and NACE 1969).

Data collected: In addition to the mode of reproduction, two sets of data are considered in this paper: (1) the phenotype of each individual, and (2) the age at death measured from foreleg eruption, i.e., larval Stage XX (TAYLOR and KOLLROS 1946) of each frog involved in this investigation during the period 1966 to 1969.

Since kandiyohi and burnsi phenotypes may be difficult to score at metamorphosis, the initial phenotypes scored were reviewed after several weeks and reclassified in a few cases. The melanoid phenotype is easily distinguished during larval stages (RICHARDS, TARTOF and NACE 1969) and could be scored prior to metamorphosis.

Biparental progeny: The biparental crosses were made by artificial insemination using eggs and sperm obtained by standard procedures (RUGH 1962; NACE 1968). Crosses of wild-type males

* The term inbreeding does not truly apply to the parasexual process described here. No term exists which describes a system of reproduction leading to increased homozygosity independent of the mechanism of reproduction. Rather than formulate a new term, we will use the term "inbreeding" in quotes when it relates specifically to parthenogenetic animals.

to K and B females and crosses involving the very rare KB double heterozygote (MERRELL 1965; MERRELL and RODELL 1968) and homozygotes (*KK* and *BB*) were not made. The double heterozygotes and homozygotes for *K* and *B*, and the heterozygous $+m$, produced in the Amphibian Facility, were not mature at the time this analysis was undertaken.

Gynogenetic progeny: Gynogenetic diploid progeny of *R. pipiens* were produced using ultraviolet-irradiated sperm for activation of the eggs and temperature shock for inhibition of the second meiotic anaphase (NACE 1968). These procedures are modifications of those used by BRIGGS (1947) to produce triploid *R. pipiens* and by KAWAMURA, NISHIOKA and their co-workers (1939, 1967, 1970) to produce gynogenetic diploids and tetraploids of several species of Japanese Ranidae following parthenogenetic activation.

The sperm used for activation in this study were obtained either from *R. clamitans* or from *R. pipiens* heterozygous for either *K* or *B*. These sperm, suspended in 10% Holtfreter's solution, were irradiated for 5 min with a 15 watt Westinghouse Sterilelamp (G15T8) at a distance of 84 cm. This empirically determined exposure did not destroy the capacity of sperm to activate the eggs as revealed by the high percentage of eggs undergoing cleavage. Inactivation of the genetic material of the sperm was indicated by the development of activated eggs beyond the blastula stage. Recall that an arrested blastula is the invariable result of fertilizing *R. pipiens* eggs with normal *R. clamitans* sperm (MOORE 1955). The genetic inactivation of the sperm was also indicated by the absence of either the kandiyohi or burnsi mutant among the offspring when their irradiated sperm were used.

Eggs activated by irradiated sperm normally develop as haploids and usually die in early larval stages (PORTER 1939). Temperature shock at 37°C for 4 min, 20 min after activation at 18°C, however, produces gynogenetic diploids by inhibiting the second meiotic anaphase. The efficacy of this treatment was variable; in our experience with over 65 gynogenetic reproductions of *R. pipiens*, the percentage of viable embryos produced ranged from zero to 34.

THEORETICAL CONSIDERATIONS

Linkage: Figure 1 illustrates the anticipated behavior of chromosomes during gynogenetic diploid reproduction. Three *expectations* follow from this representation and from considerations of linkage: (1) in the absence of recombination, homozygous progeny are produced by a female heterozygous for any locus; (2) the frequencies of both homozygous genotypes (*AA* and *aa*) are equal; and (3) the frequencies of heterozygotes depend upon the distance of the locus from its kinetochore.

We present below, in closed form to facilitate computations, a mapping function derived by BARRATT *et al.* (1954), and based upon previous work by HALDANE (1919), RIZET and ENGELMANN (1949), and PAPAZIAN (1951). It is described by a set of parametric equations, with parameter t (uncorrected map distance), which represents the relationship between the probability of recombination (y) and corrected map distance (x):

$$y = 2/3 \frac{(e^{3kt} - 1)\ (e^{2t} - 1)}{e^{(2+k)t}\ (e^{2kt} - 1)} \tag{1}$$

$$x = \frac{(1 - e^{-2t})}{(1 - e^{-2kt})}\ kt \tag{2}$$

where

y = probability of recombination
x = corrected map distance
k = coefficient of coincidence
t = uncorrected map distance

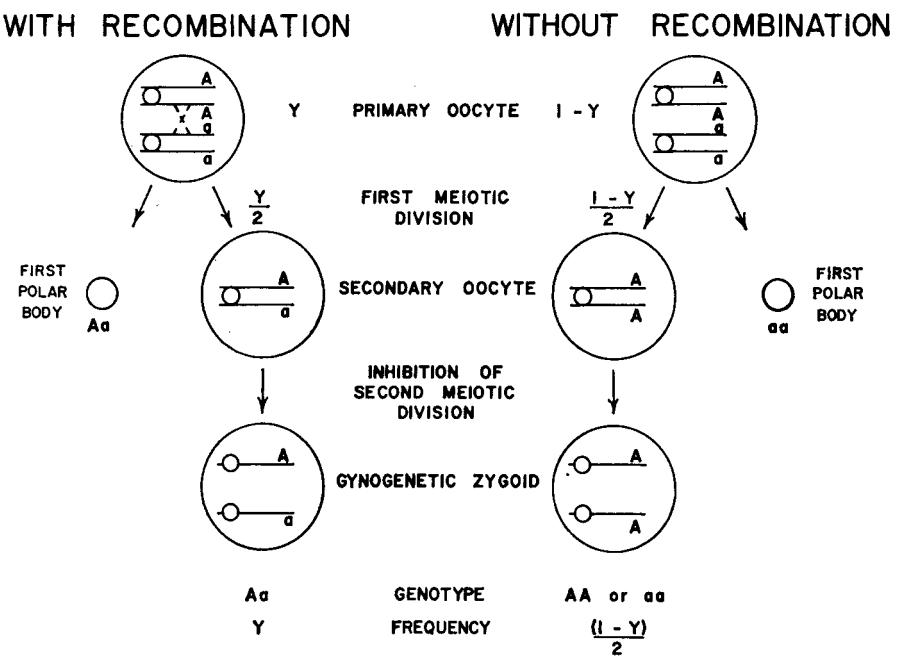

FIGURE 1.—Anticipated chromosomal behavior during gynogenetic diploid reproduction in *Rana pipiens*. A single pair of homologous chromosomes carrying the alleles *A* and *a* is shown with and without the occurrence of recombination between the gene and its kinetochore. *Y* represents the frequency of effective recombination which, in the case of this type of parthenogenesis, is equivalent to the frequency of heterozygotes or second-division segregants. $1 - Y$ is the frequency of no recombination which corresponds to the total frequency of homozygotes.

This mapping function was used by BARRATT *et al.* (1954) to map *G–K* distances in Neurospora. The frequency of tetratypes (second-division segregants) in Neurospora is equivalent to the frequency of heterozygotes in gynogenetic "crosses"; hence, this map function can be used to calculate *G–K* distances in any parthenogenetic organism in which second-division segregants can be recognized.

To calculate the *G–K* distances for *K*, *B*, and *m*, the proportion of heterozygotes which is equivalent to the probability of effective recombination (y) was first computed from a consideration of "expectation 2" when dealing with a dominant gene (frequencies of *AA* are expected to be equal to the frequencies of *aa*):

$$y = \text{Proportion of dominants } (AA \text{ and } Aa) \text{ minus recessives } (aa) \qquad (3)$$

Corrected map distances were then determined from this value by using a graph (Figure 2) or an extensive table (not presented) generated by solving the equations. The standard errors for these map distances were then computed by first determining the standard deviation of the proportion y and then reading these values from Figure 2. Only two values of k (1.0 and 0.2) were used in the present calculations. They were chosen from data derived from several organisms and represent estimations of chromosome interference ($1 - k$) values which have proven to be reasonably predictive, and thus delimit the most likely extremes

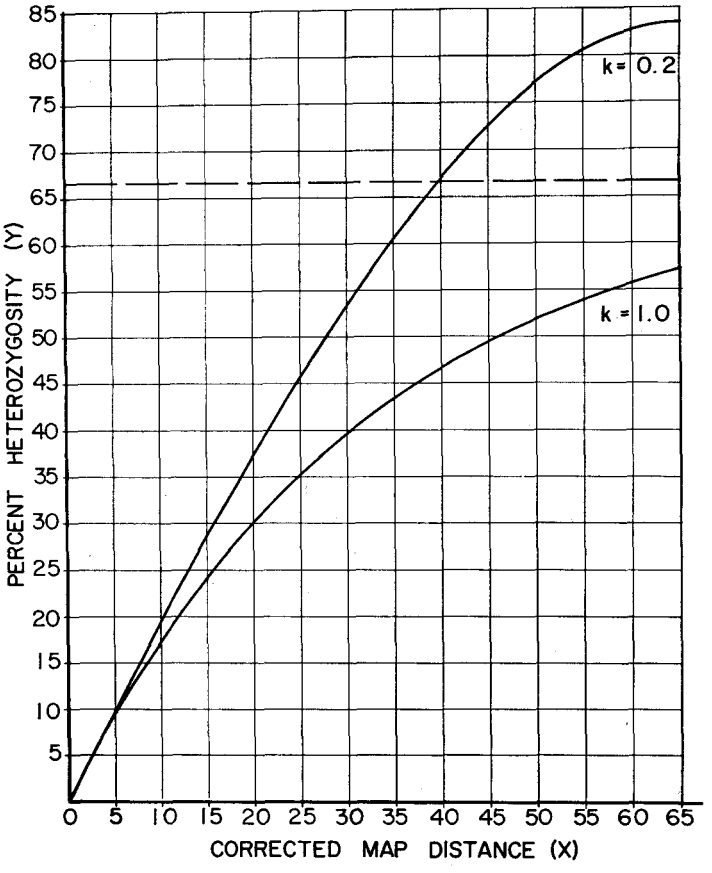

FIGURE 2.—Graphic representation of the mapping function (from BARRATT *et al.* 1954) used a to determine corrected gene–kinetochore distances (X) from the percent of heterozygotes or second-division segregants (Y) recovered from gynogenetic diploid reproduction using females heterozygous for various genes. The dashed line at 66.6% indicates the asymptote approached as the gene segregates independently of its kinetochore.

(ANDERSON and RHOADES 1931; BEADLE and EMERSON 1935; PAPAZIAN 1951; BARRATT *et al.* 1954).

"*Inbreeding*": The extent of inbreeding can be represented either by: (1) the coefficient of inbreeding (WRIGHT 1921; FALCONER 1960); or by (2) the fixation index (WRIGHT 1951). When selection and other modifying factors are negligible, both methods yield the same numerical estimations (JAIN and WORKMAN 1967). We have chosen to use the fixation index (F) in the present paper to estimate the increase in homozygosity since it can be used to describe inbreeding with and without selection. For gynogenetically reproducing populations, the following equations (paralleling those derived by WORKMAN and JAIN (1966) for a selfing population) represent the genotypic and gene frequencies at a given generation (n) assuming no selection against homozygotes and no lethality:

$$p_{n+1}^2 = p_n^2 + (1-\gamma)(1-F_n)p_nq_n$$
$$q_{n+1}^2 = q_n^2 + (1-\gamma)(1-F_n)p_nq_n \qquad (4)$$
$$R_{n+1} = 1 - p_{n+1}^2 - q_{n+1}^2$$

where

n = number of generations
R = frequency of observed heterozygosity (Aa)
p = frequency of A
q = frequency of $a = 1 - p$
p^2 = frequency of AA
q^2 = frequency of aa
$p_n = p_n^2 + \frac{1}{2}(R_n)$
$q_n = 1 - p_n$
$F_n = 1 - R_n/(2p_nq_n)$

Values of the fixation index (F_{n+1}) were computed for genes at different distances (x) from the kinetochore by solving the mapping function (equation (1)) for γ, and using this value in the recurrent equations (4) to obtain p, q, and R for substitution in the equation for the fixation index.

Given the size of each chromosomal arm in map units (computed from data presented by DiBerardino 1962; Green 1966; Strickberger 1968), we may obtain an estimation of the average heterozygosity per generation for the entire genome using the following relationship:

$$\bar{y} = \frac{\sum_{i=1}^{26} \int_0^{c_i} \gamma^n dx}{\sum_{i=1}^{26} c_i} \qquad (5)$$

where

$\bar{y}$ = average heterozygosity at generation n
c_i = length of each of 26 chromosomal arms
n = the generation of inbreeding used as a power of γ

Since $\bar{y}$ represents the expected proportion of heterozygous loci in the entire genome of a single individual at generation n, $(1 - \bar{y})$ represents the expected proportion of homozygous loci or the proportion of individuals in the population expected to be entirely homozygous.

RESULTS

Viability: Before the frequency of heterozygosity can be used for the computations of linkage distance, we must be certain that the alleles in question do not cause changes in viability which modify the frequency of their appearance. With the exception of one type of cross, the biparental crosses (Table 1) that serve as controls of the gynogenetic "crosses" confirm that K and B behave as simple Mendelian dominant genes which have no effect upon viability. The exception concerns the cross B female × K male involving 11 different females (6, 6a, and 6b in Table 1) from which four equally frequent types of progeny were expected. The chi-square (Snedecor and Cochran 1967) for this cross indicates a signifi-

TABLE 1

Summary of biparental progeny from parents heterozygous for the genes K *(kandiyohi) and* B *(burnsi) with analyses of fit to expected ratios*

Cross	Females Used	Phenotype Female	Phenotype Male	Observed Progeny +	B	K	KB	Expected ratio	Chi-square
1	1	+	B	58	52	...	...	1:1	.33
2	2	B	B	12	42	...	...	1:3	.22
3	10	+	K	94	...	88	...	1:1	.20
4	3	K	K	21	...	59	...	1:3	.05
5	7	K	B	48	42	49	47	1:1:1:1	.60
6	11†	B	K	61	65	35	42	1:1:1:1	12.46**
6a		B	K	126	...	77	...	1:1	11.83*
6b		B	K	96	107	...	...	1:1	.60

† The homogeneity test for progeny from these 11 females gave a $\chi^2_{18} = 26.28$ with $.05 < P < .10$. $\chi^2_{18} = 28.87$ at $P = .05$.

* Progeny from cross 6 have been grouped according to phenotypes K and + (a), or B and + (b). Note the deficiency in K progeny.

** Indicates a highly significant difference with $\chi^2_3 = 11.3$ at $P = .01$.

cant deficiency of kandiyohi progeny. The chi-square of the homogeneity test for uniformity of progeny frequencies among the females of this cross ($\chi^2_{18} = 26.28$) indicates that these genotypic frequencies are uniform. No biparental data are available at present which would reveal the influence of *m* upon zygotic viability. If the gene *m* is similar to the speckle mutant described by BROWDER (1968), we would expect no difference in viability caused by *m*.

From these crosses, there appears to be a deficiency in kandiyohi progeny derived from eggs produced by burnsi females fertilized by sperm from kandiyohi males. Since this interaction depends on the male, it cannot influence the frequency of progeny from gynogenetic "crosses," and hence should not influence the linkage measurements.

Linked lethality: A second problem in obtaining gene frequency values to be used in determining linkage relationships arises from the possibility that the gene in question could be linked to a recessive lethal gene. Such a lethal gene would alter the genotypic ratios according to the phase (*cis* or *trans*) and strength of linkage. If the lethal and the recessive gene being mapped are linked in the *cis* position, the homozygous recessive class will be deficient. As the distance between the two genes increases, the lethal gene would have a smaller differential effect upon the phenotypic ratios.

In order to test for the possible influence of lethality, we need only see whether ratios of progeny from individual females are homogeneous. If these ratios were not homogeneous, the test would suggest the possible influence of linked lethal

TABLE 2

Contingency analysis to determine whether ratios of gynogenetic progeny from individual females heterozygous for K *(kandiyohi) are homogeneous*

	Female	Observed K	d^2/e*	Observed +	d^2/e	Total
	1	2	.37	3	.57	5
	2	7	.13	5	.21	12
	3	23	.04	13	.06	36
	4	5	.04	4	.07	9
	5	47	.17	35	.27	82
	6	9	.39	3	.61	12
	7†	9	2.26	0	3.52	9
	8	22	.18	11	.28	33
	9	24	.38	21	.58	45
Totals	9	148		95		243
	Proportion of total	.61		.39		1.0

* $\Sigma(d^2/e) = \chi^2 = 10.1$ ($\chi^2_8 = 15.5$ at $P = .05$).

† Largest contributor to chi-square. This female shows a deficiency in wild-type progeny. As she produced normal biparental ratios for the segregation of *K* and +, she may be carrying a closely linked lethal. The total χ^2, however, is not significantly affected.

TABLE 3

Contingency analysis to determine whether ratios of gynogenetic progeny from individual females heterozygous for B *(burnsi) are homogeneous*

	Female	Observed B	d^2/e*	Observed +	d^2/e	Total
	1	32	.19	9	1.02	41
	2	14	.02	2	.11	16
	3	18	.02	4	.08	22
	4	14	.01	3	.04	17
	5	46	.003	9	.01	55
	6	7	.01	1	.05	8
	7	3	.35	2	1.85	5
	8	12	.10	1	.53	13
	9	122	.06	19	.45	141
Totals	9	268		50		318
	Proportion of total	.84		.16		1.0

* $\Sigma(d^2/e) = \chi^2 = 4.9$ ($\chi^2_8 = 15.5$ at $P = .05$).

TABLE 4

Contingency analysis to determine whether ratios of gynogenetic progeny from individual females heterozygous for m *(melanoid) are homogeneous*

Female	Observed +	d^2/e*	Observed *m*	d^2/e	Total
1	28	.01	7	.02	35
2	199	.001	46	.003	245
Totals					
2	277		53		280
Proportion of total	.81		.19		1.0

* $\Sigma(d^2/e) = \chi^2 = .03 \quad (\chi^2_1 = 3.8 \text{ at } P = .05)$.

genes. These tests are examined in Tables 2, 3, and 4 in which the gynogenetic progeny from females heterozygous for the genes *K*, *B*, and *m*, respectively, are tabulated.

Chi-square analyses of the data indicate that these females produced offspring showing homogeneous ratios. Consequently, as it is unlikely that all females should have the same lethal gene linked in the same relationship, we conclude that linked lethals have not differentially influenced the phenotypes of the gynogenetic progeny produced, and thus should not affect the linkage estimates made from these data.

Linkage: The biparental data presented in this study do not help to determine whether the three genes of interest (*K*, *B*, and *m*) are linked. Previous biparental data (ANDERSON and VOLPE 1958; VOLPE 1960) indicate, however, that *K* and *B* are unlinked. The proportion of gynogenetic heterozygotes and the corresponding gene–kinetochore distances computed for each of these genes appear in Table 5. Two genes (*B* and *m*) segregate independently of their kinetochores (a ratio of 1:5 or .17:.83 indicates independent assortment) while *K* is linked at 11.3 or 13.2 map units, depending upon the assumed strength of interference ($1 - k = .8$ and 0). The proportions reported previously (VOLPE and DASGUPTA 1962; NACE and RICHARDS 1969; VOLPE 1970) yield linkage estimates which fall within the confidence intervals of the more accurate values given in Table 5. The paper by

TABLE 5

Gene–kinetochore distances (G–K) *for three unlinked genes* (K, B, *and* m) *derived from gynogenetically produced phenotypic ratios*

Gene	Chi-square for 1:5 ratio	Proportion* heterozygous (y)	Linkage (x)† k=0.2 G–K	Range	k=1.0 G–K	Range
K	88.01**	.22 ± .05	11.3	(8.6–14.1)	13.2	(9.7–17.3)
B	.20	.69 ± .05	41.4	(37.3–47.2)	not mapable	
m	1.03	.62 ± .06	36.3	(32.2–40.8)	not mapable	

* Derived (equation (3)) from values in Tables 2, 3, and 4, and includes the 95% confidence interval.

† Derived from values in Figure 2 and includes the 95% confidence interval.

** Indicates a highly significant difference with $\chi^2_1 = 6.6$ at $P = .01$.

VOLPE (1970) presented linkage data which showed *K* and *B* at 9 and 32 map units from the kinetochore, respectively. When map distances were recalculated using VOLPE's data and assuming the corrections for double exchanges and interference presented here, map distances of 8.2 and 37.7 were obtained. The 95% confidence intervals for the linkage values reported here are 8.6–14.1 and 37.3–47.2 map units (Table 5).

Two females, one heterozygous for *K* and the other heterozygous for *B*, were found to be heterozygous for *m* as well. Their gynogenetic progeny are recorded in Table 6. As *K* is linked to its kinetochore, its expected gynogenetic segregation ratio is .61 kandiyohi to .39 wild type (Table 2). As *B* and *m* segregate independently of their kinetochores, they should independently exhibit the ratio of .83 dominants to .17 recessives (the theoretical value for independent segregation of the gene and kinetochore). If *m* is not linked to either *K* or *B*, the product of these ratios will represent expected ratios for *K* and *m* or *B* and *m* segregants. The chi-square values of Table 6 indicate that the observed ratios do not differ from these predicted ratios. Thus, two conclusions can be drawn from these observations: (1) since there are no deficiencies in the kandiyohi or burnsi classes,

TABLE 6

Chi-square analysis to determine whether the genes K *(kandiyohi), or* B *(burnsi), and* m *(melanoid) segregate independently in gynogenetic progeny from females heterozygous for both* K, *or* B, *and* m

	Phenotype	Expected* Progeny	Observed Progeny	d^2/e†
	K+	18.3	21	.40
	Km	3.7	4	.02
I	++	11.7	8	1.17
	+m	2.3	3	.21
Totals		36.0	36	1.81
	B+	97.9	94	.16
	Bm	19.6	28	3.60
II	++	19.6	14	1.60
	+m	3.9	5	.31
Totals		141.0	141	5.67

* Expected ratios were computed assuming independent segregation of *K*, or *B*, and *m* and assuming (Table 5) *K* linked to its kinetochore (K:+ = .61:.39) and both *B* and *m* independent of their kinetochores (B:+ and +:m = 5:1).

† $\Sigma(d^2/e) = \chi^2 = 1.81$, for I, and 5.67, for II, which are below the critical value of $\chi^2_3 = 7.8$ at P = .05.

m does not interact with K or B to alter viability; and (2) the gene m segregates independently of both K and B.

"*Inbreeding*": The probability that an individual is homozygous for a given gene (or the proportion of individuals in the population homozygous for a given gene) is given by the fixation index (F, equation (4)) in Table 7. We have assumed that $F_0 = 0$ for the base population. Given additional information about the base population, F_0 may take on values other than zero.

On the conservative assumption that the amount of genetic material (DNA) in the genomes of mammals and amphibians is equal (STRICKBERGER 1968), we have estimated the total map size of the genome of *R. pipiens* using map distances accepted for the house mouse. The total map length of the genome of the house mouse has been variously estimated as 847.2 map units for 135 genes (GREEN 1966), and as 1970 units (CARTER 1955). If we accept these figures as minimum and maximum estimates for the mouse and assume an equivalence of mouse and frog, we can, for the sake of discussion, consider the total frog map length to be approximately 1000–2000 units. Visualizing these 1000–2000 map units distributed uniformly among the 26 chromosome arms of the frog according to the physical lengths of each arm (DIBERARDINO 1962), we have computed the proportion of homozygosity in the entire genome. Table 8, showing these indices, should be compared with Table 7, which considers each gene separately.

Survivorship: As a consequence of parthenogenetic reproduction, a given proportion of progeny should become homozygous for deleterious genes present in each female. These genes should express themselves through differences in the

TABLE 7

Fixation index (or coefficient of inbreeding) for a gynogenetically reproducing population
$F_0 = 0,\ p = q = .5$
The population is initially at equilibrium prior to reproduction

	Distance from kinetochore (x)											
	10		20		30		40		50		60	
	Coefficient of coincidence (k)											
Generations	1.0	.2	1.0	.2	1.0	.2	1.0	.2	1.0	.2	1.0	.2
1	.827	.806	.699	.628	.604	.467	.534	.335	.482	.230	.444	.174
2	.970	.963	.910	.861	.843	.716	.783	.553	.732	.406	.690	.317
3	.995	.993	.973	.948	.938	.849	.899	.701	.861	.543	.828	.436
4	.999	.999	.992	.981	.976	.919	.953	.800	.928	.648	.904	.534
5			.998	.993	.990	.957	.978	.866	.963	.729	.947	.615
6			.999	.997	.996	.977	.990	.911	.981	.791	.970	.682
7				.999	.998	.988	.995	.940	.990	.839	.983	.737
8					.999	.994	.998	.960	.995	.876	.991	.783
9						.997	.999	.973	.997	.904	.995	.820
10						.998		.982	.999	.926	.997	.852
15								.998		.980		.943
20										.995		.978

TABLE 8

The proportion of the entire genome of Rana pipiens, *with an assumed map length of 1000 or 2000 map units, expected to be homozygous at generation* n

Generations	Map units 1000 k 1.0	.2	2000 k 1.0	.2
1	.697	.596	.573	.438
2	.880	.772	.783	.621
3	.947	.852	.882	.727
4	.975	.896	.933	.797
5	.987	.924	.961	.846
6	.994	.943	.977	.882
7	.997	.956	.986	.908
8	.998	.966	.992	.928
9	.999	.973	.995	.943
10	.999	.979	.997	.956

TABLE 9

Analysis to detect differences in proportions of gynogenetic and biparental progeny dying at various time intervals after tadpoles reach Taylor-Kollros Stage XX

Time in Weeks	Biparentals Deaths in Period	Proportion	Gynogenetic Deaths in Period	Proportion	Total	z
1	67	.229	102	.330	169	2.75**
2	25	.086	22	.071	47	.68
3	14	.048	8	.026	22	1.43
4	8	.027	2	.006	10	2.00*
5	6	.021	7	.023	13	.17
10	7	.024	26	.084	33	3.23**
20	6	.020	13	.042	19	1.54
30	16	.055	22	.071	38	.81
40	23	.079	21	.068	44	.52
50	56	.192	45	.146	101	1.51
60	24	.082	17	.055	41	1.32
70	5	.017	12	.039	17	1.63
Alive after 70	35	.120	12	.039	47	3.70**
Totals	292	1.000	309	1.000	601	

* $z = 1.96$ indicates a significant difference ($P = .05$) between biparental and gynogenetic.
** $z = 2.58$ indicates a highly significant difference ($P = .01$).

proportions of survivors when biparental and gynogenetic progeny are compared. Pairwise z tests (SNEDECOR and COCHRAN 1967) were performed to compare the proportions of individuals dying at various times in their development (Table 9). These comparisons show significant differences in the mortality of biparental and gynogenetic progeny; the greatest differences occurred within the first week and at the fifth through tenth weeks following metamorphosis. It is probable that even greater differences would be detected if comparative data were available on deaths during embryonic and larval development.

Homozygosity of progeny: Finally, the theory currently used to calculate linkage assumes the equivalence of the frequencies of homozygous dominant and recessive progeny. On this assumption, the expected ratio of *KK* to *K-* from Table 2 is .39 to .61 (or .64), and of *BB* to *B-* from Table 3 is .17 to .83 (or .20). Of 196 gynogenetically produced kandiyohi progeny (*KK* and *K+*), three that have been tested gave two heterozygotes and one homozygote. Similarly, 3 of 321 burnsi (*BB* and *B+*) progeny tested gave two heterozygotes and one homozygote. Neither of these samples is large enough to test adequately the assumption that the homozygous classes are equal.

DISCUSSION

Viability and linked lethality: In examining the biparental and gynogenetic data, we have detected no differences in viability caused individually by *K* and *B*. We have assumed for the present that this is also true for *m*. Similarly, among gynogenetic progeny derived from individual females, we have detected no significant differences in ratios that would indicate the presence of linked lethal genes which differentially affect the viability of animals scored in the linkage study. These observations indicate that linkage estimates are not adversely influenced by differences in viability caused either by the gene under consideration, or by a lethal gene linked to it, provided it is on a normal genetic background. Gynogenetically produced progeny, however, probably have widely different homozygous genetic backgrounds and hence there might well be differential survival in favor of the progeny heterozygous for *K*, *B*, and *m*. In this case, linkage estimates would be biased upwards. The homogeneity tests (Tables 2, 3, and 4) indicate that any bias in the genotypic ratios must be uniform.

The mathematics needed to analyze parthenogenetic reproduction with selection for heterozygotes is discussed in detail elsewhere (ASHER 1970). Since this analysis requires an unbiased estimate of the linkage distance to determine whether selection favors the heterozygote, this approach cannot be used to correct the possible biases in linkage measurements. Thus, to eliminate this possible source of bias, the linkage measurements should be made upon a highly "inbred" strain which gives normal ratios from biparental crosses. Since these strains are as yet unavailable, the confirmation of linkage estimates presented here awaits their production.

Further examination of the biparental data has revealed an interaction which results in a deficiency of kandiyohi progeny. Such an interaction has not been

reported in other studies of these same genes (VOLPE 1956, 1960, 1970; ANDERSON and VOLPE 1958). As only cross 6, but neither the reciprocal, cross 5, nor cross 3 (Table 1) shows this interaction, the deficiency may be caused not by a process such as meiotic drive, but by a process occurring at or sometime after fertilization.

The confirmation of this observation based on 203 progeny from 11 females involved in a single type of mating and suggesting an egg–sperm interaction must await additional data. It is of interest, however, at this time to draw attention to other relevant observations which suggest possible relationships between pigmentation and viability. CASLER (1967) mentions that when a single nucleus from parents with *many* dorsal spots was transplated into activated, enucleated eggs from frogs with *few* dorsal spots, no tadpoles reached metamorphosis. The reciprocal transplantation gave "normal" numbers of metamorphosing tadpoles. Nuclear transplantation experiments using *K* or *B* as genetic markers have been performed (MCKINNELL 1962, 1964; SIMPSON and MCKINNELL 1964); however, studies employing both markers simultaneously are lacking. Nevertheless, the CASLER (1967) data suggest that color pattern and viability differences are related in *R. pipiens* and that this relationship depends upon the character of the nuclear and cytoplasmic contributors. The relationship of pigmentation to viability has also been noted in mice. Here the action of the semidominant lethal *Sp* (splotch), which primarily inhibits the production of neural crest derivatives (AUERBACH 1954), demonstrates the association of neural crest functions and viability. As the genes *K* and *B* appear to influence the number and distribution of pigment cells (GILL and NACE 1969) derived from neural crest (HÖRSTADIUS 1950), it is possible that, in view of the donor-host relationship noted by CASLER (1967), the interaction of *K*-bearing sperm with eggs derived from a burnsi (*B*+) mother could reduce the viability of progeny by affecting neural crest derivatives other than pigment cells. Maternal cytoplasmic factors influencing viability have been previously known in amphibians, as in the case of the maternal-effect mutant in *A. mexicanum* (HUMPHREY 1966); but in this example, the effect has not also been correlated with a specific paternal genotype. Thus, the possible significance of the observation reported here is sufficient to warrant bringing it to the attention of investigators in this field.

In addition to the difference in viability associated with a possible interaction of *K* sperm and *B* eggs, we expected gynogenetic progeny, as a consequence of increased homozygosity, to show differences in viability when compared to biparental controls. Table 9 presents the differences observed in such a comparison of postmetamorphic animals. Significant differences were noted in the first, fourth, and fifth through tenth weeks following metamorphic Taylor-Kollros stage XX.

Those frogs dying the week following stage XX were going through metamorphic climax. As metamorphosis involves many physiological and morphological changes (ETKIN 1968) which are undoubtedly under fine genetic control, it is reasonable to expect that the genetic stress of gynogenesis should cause high mortality at this time. Unexplained, however, is the increased mortality among the biparental progeny at the fourth week.

Most interesting is the significant increase in mortality of the gynogenetic over the biparental progeny at 5 through 10 weeks following metamorphosis. This difference might be explained in the following way: *R. pipiens* in the northern latitudes normally hibernates at yearly intervals starting about 10 to 12 weeks after metamorphosis. Hibernation involves complex physiological changes, and if some of these are controlled by an internal clock, it is possible that disruption of this mechanism under constant laboratory conditions could cause reduced viability of these genetically vulnerable animals and lead to increased mortality at this time. The remaining gynogenetic progeny should now be more uniform with respect to their ability to cope with hiberation.

The significance of the selection which occurred at 5 to 10 weeks following metamorphosis is seen at 40 to 70 weeks when there was an increase in the mortality of both groups of animals in comparison with the period from 3 to 30 weeks. During this period of vulnerability (40 to 70 weeks), which again spanned a time of expected hibernation, the deaths of biparental progeny exceeded those of gynogenetic progeny. Although this difference was not considered statistically significant, the trend may indicate physiological differences reflecting the selection which occurred among the gynogenetic animals of an earlier period. The assumption concerning the influence of the internal clock upon frogs under constant laboratory conditions seems to be supported by various unquantified observations which have been made at the Amphibian Facility on the reproductive cycles of animals which have not been given the opportunity to hibernate.

Linkage: Three genetic factors have been mapped in this study: *K*, *B*, and *m*. The map distances appear in Table 5. We have not concerned ourselves with mapping the "sex factor" as LINDSLEY, FANKHAUSER and HUMPHREY (1956) have done in *A. mexicanum* since it is the presumption that among the anurans, females are the homogametic sex (GALLIEN 1965), and thus this factor would not be amenable to this type of analysis, the appearance of some males among gynogenetic progeny notwithstanding (RICHARDS and NACE 1970).

Using the linkage estimates of Table 5, an independent estimate of the total length of the *R. pipiens* genome was obtained by the methods of CARTER (1955). Using the 13 kinetochores as markers and the genes *K*, *B*, and *m* as "new mutants", the size of the frog genome is estimated to be 30.8 ± 7.1 Morgans. This appears to be high when compared to estimates for the house mouse using the same technique. A larger number of mapped loci are needed to increase the accuracy of this estimate.

In order to map various genetic factors accurately, interference must be considered. Positive interference $(1 - k)$ applied uniformly over the entire length of the chromosome can have the effect of increasing the proportion of heterozygotes above the value of .67 expected for independent segregation (Figure 2). Thus, a significant deviation above this value would have given information concerning the magnitude of interference in *R. pipiens*. As none of the ratios exhibits significant increases over the maximum expected value of heterozygosity (.67), no conclusion can be made at this time with regard to interference in this species.

It is apparent from these data that by means of a single gynogenetic reproduction in *R. pipiens*, one can determine: (1) linkage relationships; and (2) map

distances. Simultaneously, it is possible to uncover recessive genes (TOKUNAGA 1949; ROSTAND 1950; RICHARDS, TARTOF and NACE 1969) which could be identified only with a great deal of effort using conventional breeding systems. Several such genes are currently under investigation at the Amphibian Facility (NACE and RICHARDS 1969).

"Inbreeding": Recently, PURDOM (1969) considered gynogenesis as a mode of developing homozygous strains. In addition to retention of the second polar body and regulation during cleavage, he included "retention of the first polar body" as a mode of restoring the diploid condition. Taking retention of the first polar body as the equivalent of inhibition of meiosis I, he suggested that this mode of restoring zygoidy should produce increases in homozygosity equivalent to self-fertilization. Using this assumption, he estimated F from FALCONER's (1960) equations. This led to a large error in the estimates of F for this mode of reproduction (ASHER 1970). In addition, although his equations for estimating homozygosity from second polar body retention were similar to ours (equation 5), they were not recurrent and tended to yield estimates of F which are approximately 15% higher than those shown in Table 10.

When, using our recurrent equations, gynogenesis is compared to more common inbreeding systems such as full-sibling matings, backcrossing to an inbred strain, or selfing (Table 10), it is apparent that gynogenesis (using inhibition of meiosis II or second polar body retention) should be far more effective in producing homozygosity than is any other system previously described (LI 1955; FALCONER 1960). With respect to genes within 46 map units from the kinetochore, this system of reproduction produces homozygosity faster than selfing (Table 10). Both of these conclusions were stated previously by WHITE (1948, footnote, page 283) in a brief reference to some unpublished calculations made by HALDANE.

TABLE 10

Comparison of fixation indices (or inbreeding coefficients) for gynogenesis and other closed systems of inbreeding

Generations	Full-sib* mating	Backcrossing* to inbred strain or selfing	Gynogenesis† 10 map units k 1.0	k .2	46 map units k 1.0	k .2	Entire genome‡ k 1.0	k .2
1	.250	.500	.827	.806	.501	.266	.697	.596
2	.375	.750	.970	.963	.751	.461	.880	.772
3	.500	.875	.995	.993	.876	.604	.947	.852
4	.594	.938	.999	.999	.938	.709	.975	.896
5	.672	.969			.969	.786	.987	.924
6	.734	.984			.985	.843	.994	.943
7	.785	.992			.992	.885	.997	.956
8	.826	.996			.996	.915	.998	.966
9	.859	.998			.998	.938	.999	.973
10	.886	.999			.999	.954		.979

* From FALCONER (1960 *op. cit.* p. 91).
† Data in part from Table 7.
‡ Assumes a total map length of 1000 map units (see Table 8).

To put these data into a practical context, we must consider the time between generations as well as the amount of homozygosity gained in each generation. At the present time, the minimum time between generations for *R. pipiens* under laboratory conditions is approximately one year (NACE 1968). Previous experience of one of us (JHA) with the house mouse indicates that approximately three full-sibling or backcross generations can be produced in a year. Mice thus reproduce three times more rapidly than frogs. However, depending upon *G–K* distance and interference, frogs can attain in 3 generations, or 3 years, the same degree of homozygosity ($F = .99$ to $.60$) as 9 generations of full-sibling matings in the house mouse ($F = .86$). Considering the entire genome, these three gynogenetic generations give an average homozygosity for all genes of .95 to .73, depending upon interference and total map length (Table 8). Thus, in terms of developing "inbred" strains of experimental vertebrates, gynogenetic reproduction of amphibians appears to be an extremely effective system for the production of homozygosity. Strains of amphibians similar to the congenic strains available in the mouse are now being developed by appropriate selection.

The gynogenetic mode of reproduction, with zygoidy restored by inhibition of the second meiotic division, offers several advantages with regard to studies of natural populations. First, as the degree of homozygosity is proportional to the distance of a gene from its kinetochore, one can compare frequencies of genes in natural populations having different *G–K* distances. Knowing the map location of a given gene, one can determine whether this gene and others linked to it exhibit predictable and verifiable fixation indices for each generation. Alterations might indicate selective processes acting differentially upon the genome. Second, as some species, e.g., the insects *Lecanium hesperidum*, *L. hemisphaericum*, *Pristiphora pallipes*, and *Diprion polytomum*, reproduce entirely by this method (SUOMALAINEN 1950), these populations are conceptually more easily handled than a population which employs a mixed system such as inbreeding and outcrossing. Finally, given the size of the genome, one is able to compute fixation indices for its entirety. This could be an advantage when comparing the extent of polymorphism in different populations.

Thus, this system provides some important advantages over other inbreeding systems with respect to studies of population genetics. The accompanying paper (ASHER 1970) will investigate the genetic structure of naturally reproducing parthenogenetic populations.

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SUMMARY

Gynogenesis can be used in four ways: (1) to detect linkage relationships; (2) to construct linkage maps; (3) to produce inbred strains; and (4) to produce information concerning natural populations.—Leopard frogs and their progeny were maintained and raised in the Amphibian Facility of The University of

Michigan and were used to map three genes: *K* (kandiyohi), *B* (burnsi), and *m* (melanoid). The information collected from gynogenetic and biparental control progeny included the date of metamorphosis, phenotype, and age attained if death occurred.—Linkage and inbreeding estimates were based upon a mapping function.—The data indicated that: (1) the genes *K* and *B* do not individually lower viability; (2) phenotypic ratios are not differentially altered by any linked lethals; and (3) burnsi mothers produce deficiencies in kandiyohi progeny when fertilized by sperm from kandiyohi males.—The gynogenetic data indicated that the gene *m* segregates independently of either *K* or *B*. The gene–kinetochore distances and the 95% confidence interval for each gene were as follows: *K* at 11.3 (8.6–14.1) map units, *B* at 41.4 (37.3–47.2), and *m* at 36.3 (32.2–40.8). These values were based upon a coefficient of coincidence (k) of 0.2. For $k = 1.0$, *K* was at 13.2 (9.7–17.3) map units, and the other genes (*B* and *m*) segregated independently of their kinetochores.—When postmetamorphic mortality of gynogenetic progeny was compared to that of their biparental controls, increased mortality was found in the first, fourth, and fifth through tenth weeks following metamorphosis.—Fixation indices (or inbreeding coefficients), computed for gynogenesis as an "inbreeding" system, indicated that three generations of gynogenesis in frogs is the equivalent of nine generations of full-sibling matings in mice; this end point is reached by both in approximately 3 years. Depending upon linkage and interference, gynogenesis can be a more effective "inbreeding" system than self-fertilization.

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