GEOGRAPHIC VARIATION, POPULATION STRUCTURE, AND BALANCING SELECTION IN RED RIVER PUPFISH, *CYPRINODON RUBROFLUVIATILIS*

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PREFACE

This dissertation contains two chapters intended for separate publication. The first chapter has been published in the *Journal of Fish Biology* (45:291-302) and is cited in Chapter 2 as Ashbaugh et al. 1994. Chapter 2 is formatted for publication in *Copeia*.

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CHAPTER I

GENIC DIVERSITY IN RED RIVER PUPFISH *CYPRINODON RUBROFLUVIATILIS* (ATHERINIFORMES: CYPRINODONTIDAE) AND ITS IMPLICATIONS FOR THE CONSERVATION GENETICS OF THE SPECIES

I. INTRODUCTION

Knowledge of the genetic structure of natural populations is essential for effective management in conservation biology (Frankel, 1974). Such information is especially important for stream fishes because of the opportunities for restricted gene flow and, therefore, heightened levels of population subdivision (Meffe, 1986; Allendorf and Leary, 1988). In this paper, we provide a protein electrophoretic analysis of genetic variation in the Red River pupfish, *Cyprinodon rubrofluviatilis* Fowler (Atheriniformes: Cyprinodontidae). Natural populations of the species are restricted to saline waters in upper reaches of the Red and Brazos river systems in west Texas and Oklahoma. Populations in two other basins of the region, the Colorado and South Canadian river drainages, presumably represent rather recent anthropogenic introductions (Echelle

et al., 1987; Echelle and Echelle, 1992).

At present, *C. rubrofluviatilis* is abundant and widespread within its rather restricted historic range. However, changes in status can occur very rapidly, as illustrated by developments following the recent introduction of sheepshead minnow (*Cyprinodon variegatus* Lacepede) into the range of Pecos pupfish (*Cyprinodon pecosensis* Echelle and Echelle) in west Texas. In apparently less than five years, panmictic admixtures of the two species developed over several hundred river-kilometers, approximately half the original geographic range of the native species (Echelle and Connor, 1989). Similar effects apparently ensued over a smaller area when *C. variegatus* was introduced into the range of Leon Springs pupfish (*Cyprinodon bovinus* Baird and Girard), another species endemic to western Texas (Hubbs, 1979).

Anthropogenic introductions of both *C. rubrofluviatilis* and *C. variegatus* into "foreign" waters in western Texas and Oklahoma have occurred several times since the 1960s, possibly as an incidental effect of sportfishing activities (Stevenson and Buchanan, 1973; Echelle et al., 1977; Hubbs, 1979; Echelle and Connor, 1989; J. Pigg, pers. comm.). The patterns of variation described in this paper should be useful in detecting the genetic results of such activities and in making future decisions regarding the management of

genic diversity in *C. rubrofluviatilis*.

Previous genetic information on *C. rubrofluviatilis* is limited to a protein electrophoretic survey of three small samples (N = 9-12) included in a phylogenetic analysis of several pupfishes in drainages associated with the western Gulf of Mexico (Echelle and Echelle, 1992). The results indicated a relatively high level of genetic distinctness between the populations of *C. rubrofluviatilis* in the Red and Brazos rivers. Our purposes in the present study were to describe the geographic distribution of genic diversity within *C. rubrofluviatilis* and to provide insight into the history of the introduced populations.

II. MATERIALS AND METHODS

Seine collections of Red River pupfish were made between June and September of 1991 at the 17 sites shown in Fig. 1. Voucher specimens from 15 of the 17 collection sites were preserved in formalin and deposited in the Oklahoma State University Collection of Vertebrates (catalogue numbers = OSUS 24177-24191). The two remaining collections (sites 1 and 2; Fig. 1) were taken in August 1991 from the South Canadian River just downstream from Lake Meredith, Hutchison County, Texas (Site 1) and from near Canadian, Hemphill County, Texas (Site 2). The two largest tributaries on the south side of the upper Red River drainage (Pease and Wichita rivers) may contain extant

populations of Red River pupfish, but we were unsuccessful in obtaining specimens from those areas. Fish were frozen on dry ice, transported to the laboratory, and stored at -60°C. For each specimen, liver, epaxial muscle, and a mixture of eye and brain were homogenized separately in deionized water to obtain water-soluble protein extracts. Standard methods of horizontal starch gel electrophoresis (Murphy et al., 1991) were used to resolve products encoded by 26 enzyme and general protein loci (Table I). To minimize expense, all loci were examined in an initial survey of six specimens from each site. Those loci exhibiting at least two alleles were then surveyed in additional specimens from each site. Except for a few loci that posed scoring difficulties due to weak resolution of bands in some individuals (e.g., *ADH**), samples of at least 20 specimens were obtained per polymorphic locus per sample.

Statistical analyses were performed with the BIOSYS-1 program (Swofford and Selander, 1981). Percent polymorphism (P) was computed as the proportion of loci in which the common allele occurred at a frequency less than 0.95. Average heterozygosity per individual ($\overline{H}$) was estimated from allele frequencies. The fixation index (F_{IS}) and an exact significance test with Levene's correction for small sample sizes were used to examine genotypic frequencies for agreement with Hardy-Weinberg expectations. The

standardized variance (F_{ST}) and heterogeneity chi-square test were used to assess levels of population subdivision. F_{ST} was expressed as the arithmetic mean over all polymorphic loci. Hierarchical analyses were performed to estimate the apportionment of total genic diversity among and within samples, streams, drainages, and the entire species. The designated hierarchy of sites was as follows (site numbers as in Fig. 1): ((1,2))((3,4)(5,6)(7,8)(9,10,11)(12))((13,14)(15))((16)(17)). Total genic diversity (H_T) was computed from the BIOSYS printout as the average of the "total limiting variance" across all 26 loci examined. Rogers' (1972) genetic distance (D) was computed for all samples. The resulting matrix of distances was summarized in a dendrogram constructed by the unweighted pair group method of analysis with arithmetic averaging (UPGMA).

III. RESULTS

Allele frequencies at polymorphic loci are given in Table II. More than one allele was found at 15 of the 26 loci surveyed. Three of a total of 130 tests for goodness of fit to Hardy-Weinberg expectations were significant (alpha level = 0.05): *G3PDH** from site 4 (P = 0.009), *LDH-B** from site 8 (P = 0.002), and *ADH** from site 12 (P = 0.027). This was less than the number of significant tests expected due to sampling error alone (6.5 = 130 X 0.05). The mean

fixation index across all polymorphic loci (F_{IS} = 0.040) also revealed no general tendency toward deviation from Hardy-Weinberg expectations.

Genetic variability was lower ($\overline{H}$ = 0.000-0.017, P = 0.0-0.115) in the Brazos River samples than in samples from the other three drainages: Colorado ($\overline{H}$ = 0.091, P = 0.231), Red ($\overline{H}$ = 0.090, P = 0.254), and South Canadian ($\overline{H}$ = 0.088, P = 0.231). The sample from the Double Mountain Fork of the Brazos River (site 15, Fig. 1) was monomorphic, at all loci examined, for the common allele present in the two samples from the Salt Fork of the Brazos River (sites 13 and 14). The less common alleles in the Salt Fork samples were rare (frequency = 0.03-0.08).

The major observations from the UPGMA dendrogram (Fig. 2) are as follows: 1) There were two groups of tightly clustered samples, one comprising the three Brazos River samples (D = 0.008-0.009) and the other comprising the samples from the Red River and the presumably introduced populations in the South Canadian and Colorado rivers (D = 0.012-0.059); 2) These two clusters were separated by a relatively large genetic distance (D = 0.240); 3) Within the two clusters, there was a pronounced tendency for clustering to reflect the geographic sampling pattern, the only major exceptions being the two samples from the Colorado River drainage: one (site 16) clustered with a subcluster

comprising the three samples (sites 9-11) from the Prairie Dog Town Fork of the Red River; the other (site 17) was the most divergent member of the large cluster comprising all samples except those from the Brazos River drainage. Otherwise, the hierarchical structure of the dendrogram closely corresponds with the hierarchical structure of drainages.

The Red and Brazos river populations were effectively fixed for different alleles at four loci (*sAH**, *LDH-B**, *MPI**, and *PGM-1**), the only overlap being the rare occurrence, in Red River populations, of two alleles typical of Brazos River populations: *MPI*b* and *PGM-1*b* occurred in two(frequency = 0.025) and four (0.025-0.050) of the 10 samples from the Red River, respectively. With two exceptions, all alleles detected in the presumably introduced populations in the South Canadian and Colorado river drainages were those typical of Red River populations. The exceptions were low frequencies (0.000-0.050), in the two Colorado River samples, of the *sAH** and *MPI** alleles typical of Brazos River populations.

Genetic heterogeneity over all 17 samples indicated a high degree of population subdivision (F_{ST} = 0.476). Exclusion of the Brazos samples from the data set resulted in much reduced heterogeneity (F_{ST} = 0.089). Heterogeneity among the 10 samples from the Red River drainage was

relatively low (F_{ST} = 0.056). All drainages except the Brazos exhibited statistically significant among-sample heterogeneity in allele frequencies at individual polymorphic loci and over all loci. Significant heterogeneity (P = 0.000-0.021) occurred at seven of the 14 polymorphic loci in samples from the Red River drainage (*GPI-A**, *LDH-B**, *G3PDH**, *CK-C**, *sMEP-1**, *GPI-B**, *mAH**). Significant heterogeneities occurred at a smaller number of loci in the South Canadian and Colorado river samples: *GPI-A** in both drainages (P = 0.005-0.026) and *sAH** (P = 0.011) in the Colorado.

The hierarchical analysis indicated that, on the average, 53.4% of the total genic diversity ($\overline{H}_T$ = 0.141) is explained by within-sample diversity. An additional 43.4% is attributable to allele frequency differences among populations in different drainages. Removal of the Brazos River samples from the data set resulted in a reduced $\overline{H}_T$ value (0.096), a much greater within-sample percentage of total diversity (93.0%), and a correspondingly reduced between-drainage percentage (1.6%). Removal of all samples except those from the Red River drainage resulted in a still higher within-sample percentage of $\overline{H}_T$ (96.1%; $\overline{H}_T$ = 0.092). The percentage of $\overline{H}_T$ due to allele frequency differences between streams within drainages was 2.7%, 4.7%, and 3.7%, respectively, in the three analyses just described, and the

corresponding percentages due to differences between sites within the same stream were 0.5%, 0.7%, and 0.2%.

IV. DISCUSSION

The populations of *C. rubrofluviatilis* in the Red and Brazos rivers represent two genetically discrete entities (herein, the Red and Brazos river "forms"), with no sharing of alleles at 8% (2) of the gene loci examined and effectively no sharing of alleles at two additional loci. This suggests that the two forms have had a long history of isolation. Indeed, a phylogenetic analysis of allozyme variation suggested that they may represent cryptic species with independent origins (Echelle and Echelle, 1992). Our study supports the finding from previously limited sampling (Echelle and Echelle, 1992) that the common *LDH-B** allele (*LDH-B*c*) in the Red River form (frequency = 0.95-1.00) is absent in the Brazos River form. This allele has otherwise been detected only in *C. tularosa* (frequency = 1.00), a species from southcentral New Mexico (Echelle and Echelle, 1992, 1993). *LDH-B*c* is distributed as a derived allele (synapomorphy) that would link *C. tularosa* and the Red River form of *C. rubrofluviatilis* to a common ancestor not shared with the Brazos River form (Echelle and Echelle, 1992). We cannot, however, discount the possibility that the Brazos River form was once polymorphic at *LDH-B** and subsequently

lost *LDH-B*c*, perhaps as a result of a population bottleneck(s). Such bottlenecks would explain the paucity of genetic variation in the Brazos River form ($\overline{H}$ = 0.00-0.01).

The genic diversity reported here for *C. rubrofluviatilis* ($\overline{H}_T$ = 0.14) is slightly greater than the maximum value (0.13) reported by Gyllensten (1985) and Echelle (1991) in reviews of previous allozyme studies of genetic variation in fishes (considering only those species assayed from multiple localities and for 15 or more gene loci). Those reviews reported mean $\overline{H}_T$-values of 0.06, 0.04, and 0.04 from studies of marine, anadromous, and non-migratory freshwater species, respectively (Gyllensten, 1985), and 0.04 from studies of threatened fishes of western North America (Echelle, 1991). The high level of genic diversity in *C. rubrofluviatilis* is due primarily to 1) the consistently high within-sample heterozygosity in the Red River form ($\overline{H}$ = 0.076-0.101), which is well above the average for fishes in general (0.051, Nevo et al., 1984), and 2) the marked differences between the Red and Brazos river forms.

The hierarchical analysis indicated that the average local population of the Red River form of *C. rubrofluviatilis* contained 96% of the genic diversity present in this form. However, there was evidence of

significant, although rather minor, population subdivision. Samples from the same stream within the Red River drainage were invariably more similar to each other than to samples from other streams within the drainage. The only significant physical barrier to gene flow is the Lake Altus dam on the North Fork of the Red River, which would isolate populations at sites 3 and 4 from the remainder of the populations. Isolation by distance, together with reduced population densities in connecting waters downstream, may explain the small amount of divergence among populations in different tributaries of the Red River. The species becomes less abundant in downstream areas near the confluences of these streams, possibly as a result of interactions with the more complex fish communities in the less saline waters in those areas (Echelle et al., 1972).

The introduced populations of *C. rubrofluviatilis* in the South Canadian and Colorado river drainages appear to have originated from one or more Red River populations. All alleles detected in samples from the South Canadian River were observed in Red River populations. With two exceptions, the same was true for the Colorado River samples. The exceptions involved rare occurrences (frequency = 0.05) of the *sAH** and *MPI** alleles characteristic of the Brazos River form. This may represent low-level introgression of alleles from the Brazos River form, possibly as a result of introductions subsequent to

establishment of a relatively large introduced population of the Red River form.

The history of the introductions of *C. rubrofluviatilis* in the South Canadian and Colorado river drainages is not well understood. In Texas, the species appears to have been absent from both drainages in the early 1950s (C. Hubbs, pers. comm.). However, it was detected in the upper Colorado River drainage in the 1960s (Williams, 1969) and is now locally abundant, primarily in saline waters of the area. The first record of the species in the South Canadian River drainage was a collection of a single specimen from a site in Roberts County, Texas in 1973 (Echelle et al., 1977). It now is common in the Texas Panhandle portion of the South Canadian River eastward from Lake Meredith (pers. obs.) and exists as a sparse population downstream as far as Cleveland County in central Oklahoma (G. R. Luttrell, pers. comm.). In addition, J. Pigg (pers. comm.) recently collected one specimen from a site in the Cimarron River in north-central Oklahoma, suggesting the possibility that yet another introduced population has been established.

Artificial introductions of another species of pupfish (*C. variegatus*) into saline waters of western Texas have occurred several times since the 1960s (Stevenson and Buchanan, 1973; Hubbs, 1979; Echelle and Connor, 1989). The cause of such introductions is not known for either *C. rubrofluviatilis* or *C. variegatus*, although incidental

transport by bait- and sportfishing activity is a likely possibility. Populations established by such means are likely to show effects of genetic drift due to the small effective population size (N_e) of founding populations. Correspondingly, in our UPGMA analysis of genetic distances, the Canadian River samples and one sample from the Colorado River drainage clustered well outside the group comprising the potential parent populations from the Red River drainage. Nevertheless, the relatively high heterozygosities in these introduced populations suggest that the founding events were followed by a rapid increase in N_e, and subsequently there have been no prolonged or repeated population bottlenecks (Nei et al., 1975; Motro and Thomson, 1982).

Apparently there has been a reduction in allelic diversity that might have occurred in the founders of the introduced populations, even though overall heterozygosity was not notably affected. Genetic drift during severe reductions in N_e causes an immediate loss of rare alleles, which contribute little to measures of overall heterozygosity (Nei et al., 1975; Allendorf, 1986). To search for such losses, we considered the population in each major Red River tributary as a potential parent population and contrasted its allelic composition with that of each introduced population (only those alleles occurring in the potential parent at an average frequency of 0.01 or higher

were considered). The two samples from the South Canadian River were missing a total of 8, 11, 11, and 8 alleles that were present in, respectively, the North, Elm, Salt, and Prairie Dog Town forks of the Red River. The corresponding numbers for the Colorado River drainage were 5, 9, 8, and 5. If the average frequency of these alleles is 0.01 in the introduced populations, then the probability of missing the complete set of alleles as a result of sampling error in two 20-specimen samples ranges from 0.0001 to 0.002 for the South Canadian River populations and from 0.0007 to 0.018 for the population in the Colorado River drainage. The alleles involved are not necessarily absent from the introduced populations, but the average of their frequencies apparently is less than 0.01, and it is possible that losses have occurred.

The major implications of our results for conservation of genetic diversity in *C. rubrofluviatilis* are twofold: 1) The genetic integrity of populations in the Red and Brazos rivers should be given priority over that of the apparently introduced populations in the South Canadian and Colorado river drainages. 2) The Red and Brazos river forms should be managed and maintained as separate entities. The two forms apparently are fixed for different alleles at a large number of protein-encoding loci (12% of those examined). In an allozyme survey of *C. variegatus* and the so-called "inland members of the *C. variegatus* complex", Echelle and

Echelle (1992) found that overall genetic differences between the Red and Brazos river forms of *C. rubrofluviatilis* were greater than, or equivalent to, those between either form and certain other, morphologically well-differentiated, species of the group. For example, Rogers' index of genetic distance was 0.18-0.21 between the Red and Brazos river forms, while it was only 0.11-0.15 between each one and *C. variegatus*. Thus, the Red and the Brazos river forms are worthy of preservation as separate entities, regardless of whether or not they are eventually recognized as different species.

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Table I. Proteins, presumptive loci, tissues, and buffer systems used in this survey.

Protein	Locus	Tissue	Analytical system
Aconitate hydratase (EC 4.2.1.3)	sAH*	Liver	1
	mAH*	Muscle	1
Adenylate kinase (EC 2.7.4.3)	AK*	Eye-brain	2
Alcohol dehydrogenase (EC 1.1.1.1)	ADH*	Liver	1
Aspartate aminotransferase (EC 2.6.1.1)	sAAT-1*	Muscle	2
Creatine kinase (EC 2.7.3.2)	CK-A*	Eye-brain	2
	CK-B*	Eye-brain	2
	CK-C*	Eye-brain	2
Fumarate hydratase (EC 4.2.1.1)	FH*	Eye-brain	2
General protein	PROT-1*	Muscle	2
Glucose-6-phosphate isomerase (EC 5.3.1.9)	GPI-A*	Eye-brain	1
	GPI-B*	Muscle	2
Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	GAPDH-1*	Eye-brain	1
Glycerol-3-phosphate dehydrogenase (EC 1.1.1.8)	G3PDH*	Muscle	1
Isocitrate dehydrogenase (EC 1.1.1.42)	sIDHP-1*	Liver	1
	mIDHP-1*	Muscle	1
L-Lactate dehydrogenase (EC 1.1.1.27)	LDH-A*	Eye-brain	2
	LDH-B*	Eye-brain	2
	LDH-C*	Eye-brain	2
Malate dehydrogenase (EC 1.1.1.37)	sMDH-A*	Eye-brain	3
	mMDH-1*	Eye-brain	3
Malic enzyme (NADP+) (EC 1.1.1.40)	sMEP-1*	Eye-brain	3
Mannose-6-phosphate isomerase (EC 5.3.1.8)	MPI*	Muscle	1
Phosphoglucomutase (EC 5.4.2.2)	PGM-1*	Eye-brain	1
Phosphogluconate dehydrogenase (EC 1.1.1.44)	PGDH*	Eye-brain	1
Superoxide dismutase (EC1.15.1.1)	sSOD-1*	Liver	1

Enzymes and numbers follow the International Union of Biochemistry (1984); locus symbols follow Shaklee et al. (1990).

Analytical systems as follows: (1) after Stein et al. (1985) except adjusting with 10 N NaOH--electrode buffer: 0.1M Tris, 0.03 M citric acid, pH 7.5; gel buffer: 1 vol. electrode buffer + 6 vol. H_2O; (2) after Turner (1983) -- stock solution: 0.9 M Tris, 0.5 M boric acid, 0.1 M disodium EDTA, pH 8.6; electrode buffer: 1 vol. Stock + 6.9 vol. H_2O; gel buffer: 1 vol. stock solution + 24 vol. H_2O; (3) after Shaw and Prasad (1970) -- electrode buffer: 0.69 M Tris, 0.16 M citric acid, pH8.0; gel buffer: 0.02 M Tris, 0.03 citric acid, pH 8.0

Table II. Allele frequencies for 15 polymorphic gene loci and genetic variability ($\bar{H}$ and P) across a total of 26 loci (11 monomorphic) assayed in 17 samples of *Cyprinodon rubrofluviatilis*.

Locus and allele		Drainage and site: South Canadian River		Red River					
		1	2	3	4	5	6	7	8
sAH*	a								
	b	0.050	0.026	0.100	0.100	0.125	0.125	0.083	0.075
	c	0.950	0.974	0.900	0.900	0.875	0.875	0.917	0.925
mAH*	a					0.050	0.125	0.025	
	b	1.000	1.000	1.000	1.000	0.950	0.875	0.975	1.000
ADH*	a								
	b	0.342	0.237	0.350	0.325	0.263	0.368	0.132	0.175
	c	0.658	0.763	0.650	0.675	0.737	0.632	0.868	0.800
	d								0.025
CK-C*	a	0.025						0.125	0.100
	b	0.975	1.000	1.000	0.975	1.000	0.975	0.850	0.900
	c				0.025		0.025	0.025	
GPI-A*	a					0.125		0.025	0.025
	b			0.200	0.225	0.025	0.100	0.050	0.050
	c	0.375	0.125	0.050	0.100	0.275	0.325	0.425	0.325
	d			0.050	0.025	0.025		0.075	0.125
	e	0.350	0.700	0.375	0.325	0.275	0.350	0.400	0.425
	f			0.050	0.050		0.025		
	g	0.275	0.175	0.250	0.250	0.200	0.200	0.025	0.050
	h			0.025	0.025	0.075			
GPI-B*	a	0.050		0.150	0.275	0.100	0.025	0.105	0.125
	b	0.950	1.000	0.800	0.700	0.900	0.975	0.868	0.850
G3PDH*	a								
	b			0.025	0.025				
	c	0.425	0.250	0.800	0.725	0.579	0.650	0.421	0.500
	d	0.575	0.750	0.175	0.250	0.421	0.350	0.579	0.500
	e								
sIDHP-1*	a	0.125	0.026				0.025	0.025	0.050
	b	0.875	0.974	1.000	1.000	1.000	0.975	0.975	0.950
	c								

Table II. Continued

Locus and allele		Drainage and site: South Canadian River 1	South Canadian River 2	Red River 3	Red River 4	Red River 5	Red River 6	Red River 7	Red River 8
LDH-B*	a								
	b								0.100
	c	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.900
LDH-C*	a								
	b	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
mMDH-1*	a					0.025	0.025	0.025	
	b	1.000	1.000	1.000	1.000	0.975	0.975	0.975	1.000
	c								
sMEP-1*	a	0.325	0.405			0.079	0.025	0.050	0.025
	b	0.675	0.571	1.000	1.000	0.921	0.975	0.950	0.975
	c		0.024						
MPI*	a	1.000	1.000	1.000	0.975	0.975	1.000	1.000	1.000
	b				0.025	0.025			
PGM-1*	a								
	b						0.025		
	c	1.000	1.000	1.000	1.000	0.975	0.975	1.000	1.000
	d					0.025			
PGDH*	a	0.975	0.950	1.000	1.000	1.000	1.000	0.950	0.925
	b	0.025	0.050					0.050	0.050
	c								0.025
$\bar{H}$		0.100	0.075	0.081	0.092	0.097	0.094	0.093	0.101
P		0.269	0.192	0.192	0.192	0.269	0.192	0.308	0.346

Table II. Continued.

Locus and allele		Drainage and site								
		Red River				Brazos River			Colorado River	
		9	10	11	12	13	14	15	16	17
sAH*	a					1.000	1.000	1.000	0.050	0.050
	b	0.075	0.125	0.150	0.184				0.250	0.575
	c	0.925	0.875	0.850	0.816				0.700	0.375
mAH*	a					1.000	1.000	1.000		
	b	1.000	1.000	1.000	1.000				1.000	1.000
ADH*	a		0.024							0.075
	b	0.175	0.190	0.125	0.237				0.350	0.425
	c	0.825	0.786	0.875	0.763	0.950	0.950	1.000	0.600	0.500
	d					0.050	0.050		0.050	
CK-C*	a				0.025				0.025	
	b	0.975	1.000	1.000	0.925	1.000	1.000	1.000	0.975	1.000
	c	0.025			0.050					
GPI-A*	a									
	b	0.075	0.150	0.150					0.150	0.183
	c	0.250	00.175	00.200	0.147	1.000	1.000	1.000	0.225	0.567
	d	0.025	0.100	0.075					0.025	
	e	0.625	0.500	0.550	0.676				0.575	0.250
	f									
	g	0.025	0.025	0.025	0.147				0.025	
	h				0.029					
GPI-B*	a		0.025	0.075	0.075					0.050
	b	1.000	0.975	0.925	0.925	1.000	1.000	1.000	1.000	0.950
G3PDH*	a						0.025			
	b			0.025		0.025	0.050			
	c	0.737	0.525	0.625	0.575	0.975	0.925	1.000	0.675	0.600
	d	0.263	0.450	0.350	0.200				9.325	0.400
	e		0.025		0.225					
sIDHP-1*	a									
	b	1.000	0.975	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	c		0.025							

Table II. Continued

Locus and allele		Drainage and site: Red River 9	Red River 10	Red River 11	Red River 12	Brazos River 13	Brazos River 14	Brazos River 15	Colorado River 16	Colorado River 17
LDH-B*	a					1.000	1.000	1.000		
	b		0.025	0.050	0.025					
	c	1.000	0.975	0.950	0.975				1.000	1.000
LDH-C*	a					0.025	0.075			
	b	1.000	1.000	1.000	1.000	0.975	0.925	1.000	1.000	1.000
mMDH-1*	a			0.025						
	b	0.950	0.975	0.950	1.000	1.000	1.000	1.000	1.000	1.000
	c	0.050	0.025	0.025						
sMEP-1*	a	0.125	0.050	0.175	0.075				0.100	0.025
	b	0.800	0.875	0.800	0.925	0.950	0.975	1.000	0.900	0.975
	c	0.075	0.075	0.025		0.050	0.025			
MPI*	a	1.000	1.000	1.000	1.000				1.000	0.950
	b					0.974	1.000	1.000		0.050
	c					0.026				
PGM-1*	a					0.053				
	b	0.025	0.050		0.025	0.947	1.000	1.000		
	c	0.975	0.950	1.000	0.975				1.000	1.000
	d									
PGDH*	a	0.975	0.975	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	b	0.025	0.025							
	c									
$\bar{H}$		0.076	0.092	0.089	0.089	0.017	0.017	0.000	0.088	0.094
P		0.231	0.231	0.308	0.269	0.115	0.115	0.000	0.192	0.231

FIG. 1. Collection sites for *Cyprinodon rubrofluviatilis* in Texas and Oklahoma. Open circles represent possible introduced populations of the species.

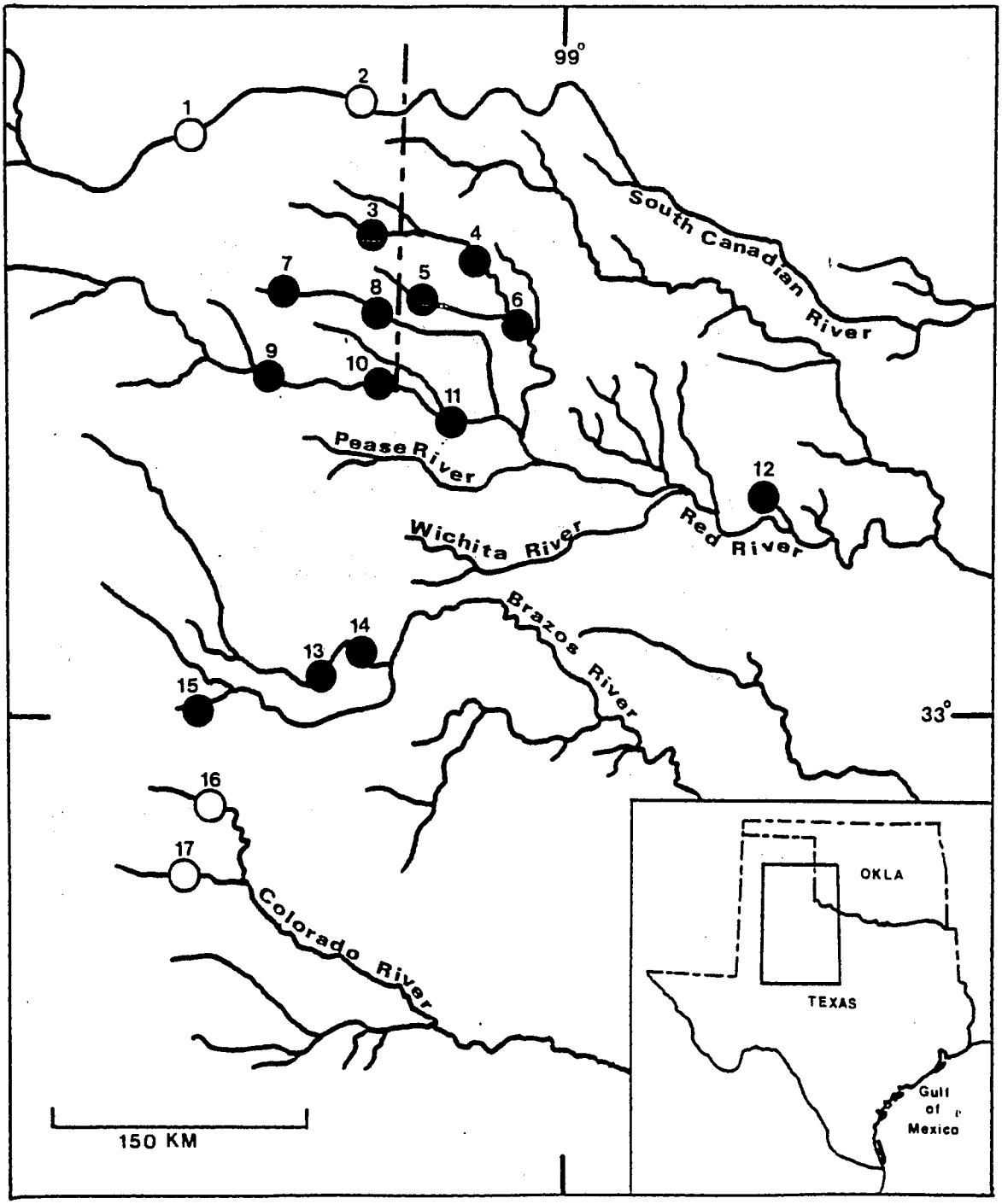
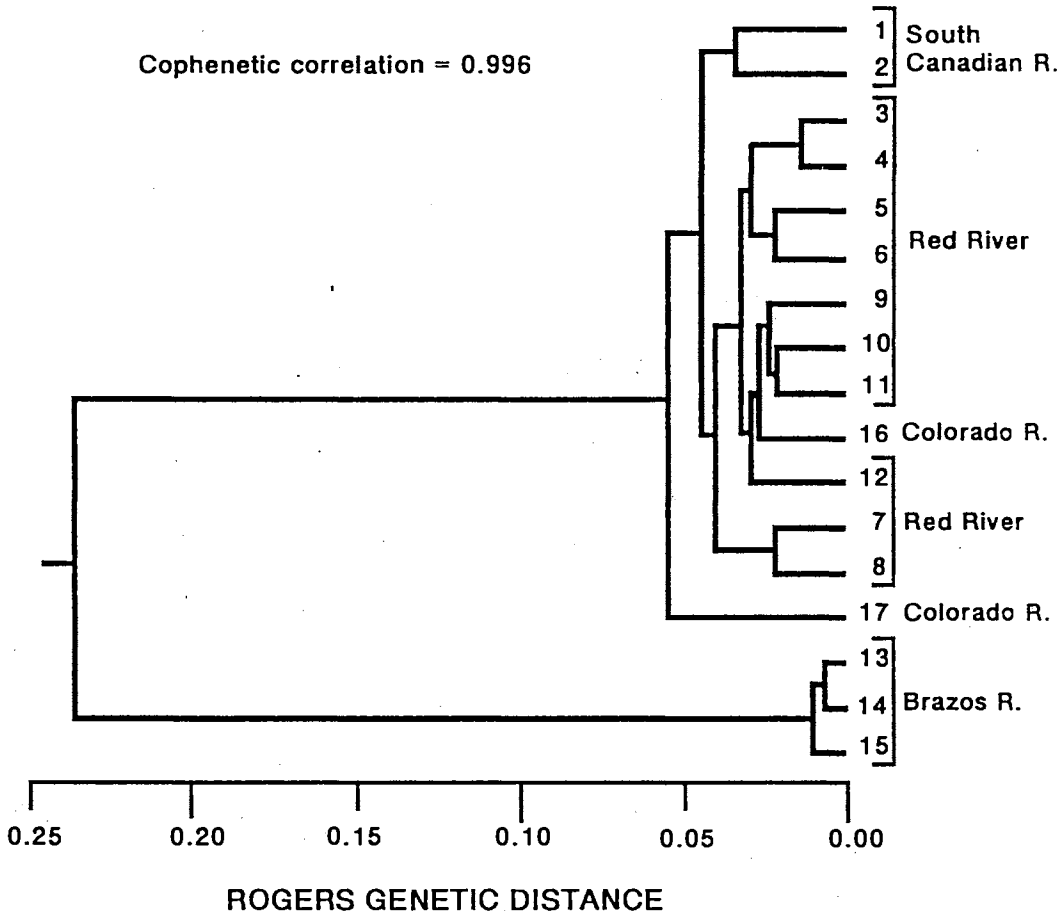

99°
33°
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
South Canadian River
Pease River
Wichita River
Red River
Brazos River
Colorado River
150 KM
OKLA
TEXAS
Gulf of Mexico

FIG. 2. UPGMA phenogram summarizing Rogers' (1972) genetic distances among 17 populations of *Cyprinodon rubrofluviatilis*.


Cophenetic correlation = 0.996
1
2
South Canadian R.
3
4
5
6
Red River
9
10
11
16 Colorado R.
12
7
8
Red River
17 Colorado R.
13
14
15
Brazos R.
0.25
0.20
0.15
0.10
0.05
0.00
ROGERS GENETIC DISTANCE

CHAPTER II

ANALYSIS OF THE POTENTIAL FOR SEASONALLY-BASED BALANCING SELECTION AT TWO ALLOZYME LOCI IN RED RIVER PUPFISH, *CYPRINODON RUBROFLUVIATILIS* (TELEOSTEI: CYPRINODONTIDAE)

One of the most challenging questions facing evolutionists concerns the adaptive significance of polymorphism at protein-coding loci (Koehn et al., 1983). This is the central issue in the so-called neutralist-selectionist debate. Neither view appears sufficient to explain all persistent polymorphisms (Lewontin, 1974), but a number of studies support various forms of balancing selection at some protein-coding loci. The most convincing evidence comes from studies of alcohol dehydrogenase in *Drosophila melanogaster* (Clarke, 1975; Cochrane and Richmond, 1979; Oakeshotte et al., 1981; Gilbert and Richmond, 1982), a lactate dehydrogenase in the fish *Fundulus heteroclitus* (reviewed in Powers et al., 1991), glucose phosphate isomerase in the butterfly genus *Colias* (Watt, 1977; Watt et al., 1983; Watt et al., 1985), and leucine aminopeptidase in the mussel *Mytilus edulis* (reviewed in Koehn and Hilbish, 1987).

In this study, I examine the question of whether seasonally-based balancing selection explains the high

levels of allozymic variation previously observed by Echelle and Echelle (1992) and Ashbaugh et al. (1994) in Red River populations of Red River pupfish (*Cyprinodon rubrofluviatilis* Fowler), a small cyprinodontid native to saline headwaters of the Brazos and Red river drainages in west Texas and southwest Oklahoma (Minckley, 1980). The plains-streams inhabited by *C. rubrofluviatilis* have primarily shallow waters, unstable sand substrates, and extreme temporal heterogeneity in temperature, salinity, and stream discharge (Echelle et al., 1972; Taylor et al., 1993). Thus, plains-streams are excellent natural settings for tests of various balancing selection hypotheses (e.g., Gillespie, 1978; Hedrick, 1978) invoking environmental heterogeneity.

Effects of environmental variation may be more pronounced for small poikilotherms like *C. rubrofluviatilis* because of the absence of homeostatic buffering afforded by large body size and homeothermy (Merritt et al., 1978). Indeed, relatively high heterozygosity may be characteristic of species with life-history traits typical of so-called "r-selected species" (Mitton and Lewis, 1989, 1992; but see Waples, 1991). Heterozygosity in these forms may be important in maximizing reproductive potential. Such species typically occupy unpredictable environments, placing a premium on early maturation (hence, relatively small body size at sexual maturity) and high fecundity. A possible

corollary is that small poikilotherms ("coarse-grained" species; Levins, 1968) in general are more subject to environmentally induced physiological or metabolic effects than larger forms. Thus, in addition to permitting maximization of reproductive potential, heterozygosity may afford greater performance capacity under fluctuating conditions (Selander and Kaufman, 1973).

To assess the role of balancing selection in maintaining protein polymorphism in *C. rubrofluviatilis,* I examined seasonal patterns of variation at a glucose-6-phosphate isomerase locus (*GPI-A**) and a glycerol-3-phosphate dehydrogenase locus (*G3PDH-1**). Enzymes encoded by these loci are central to carbohydrate metabolism and are, therefore, likely targets of selection acting to optimize carbohydrate flux and ATP synthesis (Gillespie, 1991; Powers et al., 1991). Previous studies indicate that alternative genotypes for these enzymes are associated with variables affecting reproductive fitness or general performance (Watt et al., 1985; Oakeshotte et al., 1981). I reasoned that balancing selection would be manifested in patterns of genetic variation not attributable to neutral variation and stochasticity. Such patterns might include consistent seasonal, sexual, or age-class differences in genetic structure, or consistent excesses of heterozygotes.

MATERIALS AND METHODS

Red River pupfish (n = 6,039) were collected by seine in March and August 1992 and 1993 from three sites on the Prairie Dog Town Fork of the Red River in west Texas and Oklahoma (Fig. 3): Highway 70 bridge, 40 km N of Turkey, Hall Co., Texas; Highway 83 bridge, 16 km N of Childress, Childress Co., Texas; Highway 34 bridge, 12 km S of El Dorado, Jackson Co., Oklahoma. Sample times were chosen to provide samples of fish during or immediately following extremes of winter (March) and summer (August). Fish were frozen on dry ice or in liquid nitrogen and subsequently stored at -70°C. A sample of epaxial muscle (individuals > 25 mm TL) or the entire caudal peduncle (individuals < 25 mm TL) was dissected from each individual and ground in an equivalent volume of deionized water. Homogenates were spun at 4,000 X g for 15 seconds and stored at -70°C.

Genotypic variation at *GPI-A** (EC 5.3.1.9) and *G3PDH-1** (EC 1.1.1.8) was screened by horizontal starch-gel electrophoresis. Both loci were resolved in 11.5% starch (StarchArt, Corp.) gels with a Tris-citrate pH 7.5 buffer system (Stein et al., 1985). Electrophoresis proceeded overnight (12-14 hrs) at 25 mA. *GPI-A** gels were stained with a 2% agar overlay containing 0.01 g NAD, 1 ml MTT, 0.1 ml PMS, 0.2 ml $MgCl_2*6H_2O$, 0.08 g D-fructose-6-phosphate, 0.009 ml G6PDH, and 47 ml 0.2 M Tris-HCL (pH 7.0). *G3PDH-1** gels were stained following Murphy et al. (1991). Gels were

incubated at 37°C until bands were scoreable, then fixed in a 5:5:1 solution of ethanol, H_2O, and acetic acid. Alleles were assigned designations indicating percent migration of encoded products relative to that of the most common allele (**100* allele). All allozymes migrated anodally.

Fish were categorized according to collection site, year and season of collection, sex, and size (small fish = TL < 30 mm, large fish = TL ≥ 30 mm). Sex determination was based on presence/absence of dorsal fin pigmentation (adult females and juveniles have a small patch of melanophores near the posterior margin of the dorsal fin), width of anal rays 3-5 (expanded in adult males), and presence/absence of a dark bar on the posterior margin of the caudal fin (present in adult males). Fish < 15 mm TL were classified as juveniles.

I used the BIOSYS-1 program (Swofford and Selander, 1981) to perform chi-square tests of Hardy-Weinberg expectations for three classes of genotypes in each sample: homozygotes for a given allele, heterozygotes for the allele, and all other genotypes. Pooling avoids spurious conclusions resulting from small expected values in chi-square tests (Sokal and Rohlf, 1981). I also used BIOSYS-1 to compute fixation index values (F_{IS}) as measures of deviation from Hardy-Weinberg expectation (positive values = heterozygote deficiencies; negative values = excesses).

I used the FREQ procedure in SAS (SAS Institute Inc.,

1985) to perform contingency chi-square tests for among-sample heterogeneity in single-locus genotypic arrays containing all genotypes with counts of five or more. Subsequently, I tested among-sample heterogeneity in genotype counts based on pooling with respect to common alleles as in Hardy-Weinberg tests described previously. Three such tests were performed, one for each common allele at the two loci (*GPI-A*125*, *GPI-A*100*, and *G3PDH-1*100*).

To test for homogeneity among single-locus estimates of F_{IS} for each seasonal sample and to compare among-sample mean F_{IS} for *GPI-A** and *G3PDH-1**, I used a QBASIC program, FCORR.BAS (Ashbaugh, unpub.; Appendix) to perform tests of homogeneity among correlation coefficients (Sokal and Rohlf (1981).

I used the CATMOD procedure in SAS (SAS Institute Inc., 1985) for loglinear tests (Agresti, 1990) of association between independent variables (site, year, sex, season, and size) and within-locus variation in frequency of individual common alleles, and frequency of homozygotes or heterozygotes for such alleles. The data were structured such that each combination of site, year, sex, season, and size corresponded with two classes of allele frequency (a common allele versus other alleles) and two classes of common allele genotype frequency (homozygotes or heterozygotes for a common allele, and other genotypes). To reduce the complexity of the structural relationships

between explanatory and response variables, I used a stepwise backward-elimination procedure to search a hierarchy of models for the most parsimonious model that adequately fit ($P > 0.05$) the observed data. Subsequently, this model was used to evaluate significance of its component main effects and interactions. In all models, I followed the convention of retaining all main effects and interactions involving only explanatory variables. These can be regarded as fixed "facts of life" (Wrigley, 1985) and their retention is necessary to prevent exaggeration of the pertinent interactions involving explanatory and response variables. In subsequent discussions of loglinear analysis results, the term "main effect" applies to two-way interaction between an explanatory variable and a given response; "interaction" applies to three- or higher-way interaction between two or more explanatory variables and a given response. In tests for balancing selection, genetic response variables associated with significant main effects and/or interactions due to season, sex, and size provide support for balancing selection based on fitness variation between seasons, sexes, or size classes. In contrast, significant higher-order interactions involving site and/or year indicate heterogenous effects between years or among sites and are counter to expectation for balancing selection.

The QBASIC programs LINKED.BAS and DISEQ.BAS (Ashbaugh, unpubl.; Appendix) were used to compute linkage disequilibrium (D) and its theoretical maximum (D_{max}) for each seasonal sample. Because unadjusted D is dependent on allele frequencies (Hedrick, 1987), the standardized linkage disequilibrium, $D' = D / D_{max}$, was computed for each seasonal sample. The LINKED.BAS program estimates D from Hill's (1974) maximum likelihood algorithm (convergence criterion = 10^{-8}). Coupling gametes were defined as those containing a pair of **100* alleles or a pair of alternate alleles (all other less common alleles); repulsion gametes were those with a **100* allele at one locus and an alternate allele at the other locus.

RESULTS

Seven alleles were resolved for *GPI-A** and four for *G3PDH-1** (Table 3). Both loci were dominated by a pair of alleles with mean frequencies as follows: *GPI-A*100*, 0.50; *GPI-A*125*, 0.32; *G3PDH-1*100*, 0.69; and *G3PDH-1*86*, 0.29. In the following discussions, *GPI-A*100*, *GPI-A*125*, and *G3PDH-1*100* are referred to as common alleles for their respective loci. Frequencies of common alleles and observed and expected frequencies of heterozygotes for such alleles are shown in Figs. 4-6. Observed and expected frequencies of common allele homozygotes are shown in Figs. 7-9.

Hardy-Weinberg tests.--Pooling genotypes into three classes based on dosage of common alleles (common allele homozygotes and heterozygotes and all other genotypes) indicated no significant deviation from Hardy-Weinberg expectations in the 12 tests for *GPI-A*100* (Table 4). However, seven of the 12 tests were significant for *GPI-A*125* (six deficiencies and one excess of heterozygotes), three of which (all heterozygote deficiencies) remained significant after Bonferroni correction (P_{adj} = 0.004). Although three of the 12 tests for *G3PDH-1*100* indicated significant heterozygote deficiencies, none was significant after the Bonferroni correction (Table 4).

In another assessment of Hardy-Weinberg expectations, I used a sign test (Steel and Torrie, 1980) to search for overall biases toward positive or negative F_{IS} values across all samples for each of the three common alleles. For *GPI-A*100*, there was a non-significant bias toward positive values (7 of 12 values positive; χ^2 = 0.33, *df* = 1, *P* > 0.5). *GPI-A*125* and *G3PDH-1*100*, however, both exhibited significant biases toward positive values (in both, 11 of 12 were positive; χ^2 = 8.3, *df* = 1, *P* = 0.005). Tests of the hypothesis that mean F_{IS} differed significantly from zero (Li and Horwitz, 1953) indicated significance for *GPI-A*125* (χ^2 = 4.91, *df* = 1, P < 0.05) but not for *G3PDH-1*100* (χ^2 =

2.04, $P > 0.05$).

Within-sample Wahlund effects due to sexual or size-class differences.--The possibility for heterozygote deficiency resulting from sex- or size-based Wahlund effect was tested by calculating F_{IS} separately for each sex and size class. If sexes or size classes differ sufficiently to produce a detectable Wahlund effect when lumped as a single sample, the effect (consistent heterozygote deficiency) should disappear when sexes or size classes are analyzed separately. Separate analyses of the sexes revealed biases toward heterozygote deficiency for all three common alleles although significant biases occurred only for *GPI-A*125* in females (11 of 12 positive; $\chi^2 = 8.3$, $df = 1$, $P < 0.005$) and *G3PDH-1*100* in females (10 of 12 positive; $\chi^2 = 5.3$, df = 1, $P < 0.05$). In analyses of size classes, deficiencies outnumbered excesses in all instances except for *GPI-A*100* in small fish, where numbers of excesses and deficiencies were equal; significant biases occurred for *GPI-A*125* in small fish (11 of 12 positive; $\chi^2 = 8.3$, $df = 1$, $P < 0.005$) and for *GPI-A*100* and *G3PDH-1*100* in large fish (10 of 12 positive for both; $\chi^2 = 5.3$, $df = 1$, $P < 0.05$). The consistent, although sometimes non-significant, bias toward heterozygote deficiencies indicates that sex or size-class differences in allele frequencies are inadequate to produce

a Wahlund effect.

Within-sample Wahlund effects due to temporal heterogeneity.--For each site separately, I tested for Wahlund effects resulting from admixtures of temporally differentiated subpopulations by comparing observed numbers of heterozygotes across all season/year samples with numbers expected from allele frequency variation among samples. The expected frequency of heterozygotes under subdivision is given by $2\bar{p}\bar{q}(1 - F)$ where $\bar{p}$ and $\bar{q}$ are mean frequencies of the common allele and all other alleles, respectively, and F is the standardized variance in allele frequencies, F_{ST}, which measures the reduction in heterozygosity due to inbreeding within subpopulations relative to the total population (Hartl and Clark, 1989). Estimates of expected heterozygote frequencies under subdivision were calculated using the WAHL.BAS program (Ashbaugh, Unpubl.; Appendix). Values of F_{ST} used in calculating expected heterozygosities under subdivision measured genetic differentiation among season/year samples within each site separately. For two of the three common alleles of this study, *GPI-A*100* and *G3PDH-1*100*, observed frequencies of heterozygotes across season/year samples did not differ significantly from those predicted for Wahlund effects reflecting temporal differences in allele frequencies (Table 5). For *GPI-A*125*,

however, observed numbers of heterozygotes were significantly less than predicted from this model ($P < 0.001$) at two of the three collection sites. At these sites, Turkey and Childress, observed numbers were 86% and 90% of expected, respectively.

Plots of expected and observed frequencies for the common alleles reveal no consistent seasonal pattern in deviations from Hardy-Weinberg expectations (Figs. 4-9). To further examine the dynamics of deviation of *GPI-A*125* genotypes from Hardy-Weinberg equilibrium, I examined within-site variation in magnitude of deviation-values [(observed - expected)÷ expected] for several *GPI-A** genotypes. Deviation-values for *GPI-A*100* homozygotes and heterozygotes showed no significant within-site variation ($P > 0.05$; test for heterogeneity among correlation coefficients, Sokal and Rohlf, 1981). However, for each of the three sites, deviation-values for *GPI-A*125* homozygotes showed significant heterogeneity among samples ($P < 0.05$ to $P < 0.001$). Deviation in *GPI-A*125* heterozygote frequencies was significantly heterogeneous at El Dorado ($P < 0.001$), but not at the other two sites. This was true both for all *GPI-A*125* heterozygotes and, specifically, for the *GPI-A*125/*100* heterozygote.

Among-sample heterogeneity.--Contingency tests of

heterogeneity after removal of genotypes with low expected counts (≤ 5) revealed a general lack of among-sample differences in genotypic frequency. Although *GPI-A** showed significant heterogeneity (χ^2 = 114.6, *df* = 88, *P* = 0.03), this effect disappeared after removal of the winter 1992 data for El Dorado (χ^2 = 82.9, *df* = 80, *P* = 0.39). A lack of significant heterogeneity also occurred for *G3PDH-1** (χ^2 = 19.3, *df* = 22, *P* = 0.63).

Considering each common allele separately, genotypic frequencies for *GPI-A*100* and *G3PDH-1*100* were homogeneous across samples (χ^2 = 16.4 to 20.6, *df* = 22, *P* > 0.5). Significant heterogeneity was indicated for *GPI-A*125* genotype counts (χ^2 = 38.1, *df* = 22, *P* = 0.02), but again, this was attributable to the winter 1992 sample from El Dorado. Removal of this sample resulted in non-significance (χ^2 = 20.7, *df* = 20, *P* = 0.42).

Season, sex, and size effects.--For *G3PDH-1*100* allele and genotypic frequencies, log-linear models contained interactions indicating confounding influence of site or year or both. Thus, there was no consistent effect due to season, sex, or size. For *GPI-A*125* allele frequency there was a significant effect due to season (χ^2 = 4.14, *df* = 1, *P* = 0.041) such that frequencies of this allele were lower in summer than in winter (Fig. 5).

For multi-allelic loci, a given pair of alleles may not exhibit complementary dynamics, particularly if there are several alleles with frequencies substantially greater than zero. In my study there were three intervals over which allele frequency change could be measured: winter 1992 to summer 1992; summer 1992 to winter 1993; winter 1993 to summer 1993. I calculated frequency changes for *GPI-A*100* and *GPI-A*125* over these three intervals and used Spearman's rank correlation coefficient, r_s, to test their association. The resulting correlation coefficient (r_s = -0.92, $P < 0.001$) indicated significant complementarity in frequencies for these two alleles. Additional tests were performed to measure association between frequency changes for *GPI-A** common alleles and various genotypes containing these alleles. Frequencies of *GPI-A*100* and *GPI-A*125* homozygotes exhibited significant positive associations with changes in frequency for their respective alleles (*GPI-A*100*: $r_s = 0.73$, $P = 0.03$; *GPI-A*125*: $r_s = 0.80$, $P = 0.01$). In contrast, associations between *GPI-A** common alleles and their heterozygotes were not significant ($P > 0.05$).

Linkage disequilibrium.--There was no evidence of linkage disequilibrium after Bonferroni adjustment ($P_{adj} = 0.004$; Table 6). Eight of the 12 *D*-values were negative but this bias toward negative values did not differ significantly

from the equilibrium expectation of equal proportions of positive and negative values ($\chi^2 = 0.11$, $P > 0.05$). Multi-way analysis of variance indicated no significant main effects or two-way interactions involving the factors site, season, and year.

High levels of linkage disequilibrium (e.g., $D' = 0.50$) may go undetected as a consequence of low statistical power owing to insufficient sample size (Zapata and Alvarez, 1992). To augment statistical power, I calculated D and D' for annual samples pooled over seasons at each site. Again, none of the D values was significantly different from zero.

DISCUSSION

Two results from this study suggest selection as an important influence on genetic structure at the *GPI-A** locus. First, one allele, *GPI-A*125*, exhibited marked heterozygote deficiencies relative to other alleles for the two loci examined. This observation is inconsistent with expectations from forces such as localized inbreeding and resultant Wahlund effects because they would produce deficiencies for all heterozygous allele combinations (Hartl and Clark, 1989). The presence of a null allele can also be eliminated as an explanation. Such an allele would cause deficiencies in all types of *GPI-A** heterozygotes; instead, only those for *GPI-A*125* were notably deficient. Such inconsistent patterns among loci and among alleles for the

same locus are potential indicators of selection (Lewontin and Krakauer, 1975).

Second, there was a statistically significant pattern of seasonal variation in frequency of *GPI-A*125*, lower in summer and higher in winter. This observation is consistent with seasonally-based balancing selection and would contribute toward heterozygote deficiencies. However, the magnitude of heterozygote deficiencies for *GPI-A*125* was greater than expected from pooled seasonal samples at two of the three sample localities (Turkey and Childress). This result indicates an added effect of persistent underdominance (heterozygote disadvantage), a result supported by the lack of statistically significant among-sample heterogeneity in magnitude of *GPI-A*125* heterozygote deficiency.

Underdominance as an explanation of heterozygote deficiency has been postulated for six enzyme loci in oysters (Zouros et al., 1980) and for an esterase locus in the fish *Zoarces viviparus* (Christiansen et al., 1977). In both studies, heterozygote deficiencies were most pronounced in early life stages, possibly indicating underdominant zygotic selection. In the oyster study, analysis of a single cohort revealed a trend toward increased multi-locus heterozygosity and reduced heterozygote deficiency in larger individuals. This was interpreted as indicating overdominance expressed as faster growth rate in highly

heterozygous individuals.

To further examine the possibility for underdominance in early life stages in *Cyprinodon rubrofluviatilis* I compared heterozygote deficiencies between presumed juveniles (TL $\leq$ 20 mm) and adults using *t* tests for comparing two correlation coefficients (Sokal and Rohlf, 1981). Due to inadequate samples of juveniles in some samples, such tests were possible for only six of the twelve seasonal collections: Turkey, winter and summer 1992; El Dorado, winter 1992; Childress, winter and summer 1992 and winter 1993. Heterozygote deficiencies were detected in the majority of these samples although biases toward heterozygote deficiency were non-significant in either age class (i.e., deficiencies in four of six samples for juveniles and five of six samples for adults). With one exception, pairwise comparisons between juveniles and adults indicated non-significant differences in heterozygote deficiency between age classes. The lone exception occurred in the El Dorado winter 1992 sample where juveniles had a pronounced excess of *GPI-A*125* heterozygotes (F_{IS} = -0.56) relative to that detected in adults (F_{IS} = -0.07; t = 2.33, df = 1, $P < 0.05$). Thus, there is a lack of compelling evidence for age-dependent variation in underdominant selection against *GPI-A*125* heterozygotes in *C. rubrofluviatilis*. However, these data do not preclude the

possibility that differential mortality of *GPI-A*125* heterozygotes may occur in early life phases not sampled in this study (e.g, zygotes or larvae).

Heterozygote deficiencies are frequently reported in studies of natural populations of plants and animals (Futuyma, 1987), and such deficiencies appear common in fishes (deLigny, 1970). Consistent heterozygote deficiencies at one or more loci have been reported for several fishes in addition to the above mentioned example from *Zoarces*. These include deficiencies at two of four polymorphic loci in *Fundulus zebrinus* (Brown, 1986) and four of twelve in *F. heteroclitus* from New York (Mitton and Koehn, 1975); these species are members of the Fundulidae, a family closely allied with Cyprinodontidae. Like *C. rubrofluviatilis, F. zebrinus* occupies highly variable plains-stream environments; the two species occur syntopically within the range of *C. rubrofluviatilis* and they exhibit a high degree of similiarity in behavior and ecology (Echelle et al., 1972). Other examples of consistent heterozygote deficiencies in plains-stream fishes include one of two polymorphic loci in *Notropis stramineus* from the Kansas River drainage (Koehn et al., 1971) and two of eight in *Cyprinella lutrensis* from northcentral Texas (Rutledge et al., 1990). Additionally, heterozygote deficiencies have been reported for *Poeciliopsis monacha* and

Gambusia holbrooki, two members of the Poeciliidae, another family closely allied with Cyprinodontidae. Heterozygote deficiencies were reported for all four polymorphic loci surveyed in a 1992 study of *P. monacha* from highly variable streams in arid regions of northwestern Mexico (Vrijenhoek et al., 1992). In populations of *G. holbrooki* from Georgia, South Carolina, and North Carolina, heterozygote deficiencies were detected for seven of ten loci exhibiting significant deviation from Hardy-Weinberg equilibrium (Hernandez-Martrich and Smith, 1990).

Underdominant selection was invoked as a possible explanation for the heterozygote deficiencies observed in *F. zebrinus* and *N. stramineus*. In *F. zebrinus*, heterozygote deficiencies at a creatine kinase locus appeared to be the result of increased underdominant selection with age; large fish exhibited more pronounced heterozygote deficiencies (Brown, 1986). In *N. stramineus*, heterozygote deficiencies at an esterase locus were more pronounced in females than in males, thereby indicating possible sex-dependent fitness differences among genotypes.

The loglinear models indicated a consistent seasonal effect only for *GPI-A*125*. However, frequencies of the two common alleles were complementary, *GPI-A*125* being less abundant in summer samples than in winter, while *GPI-A*100* exhibited the reverse trend (Figs. 4 and 5).

The consistent, apparently non-random pattern of seasonal alternation in declines and increases of the two common alleles for *GPI-A** is best explained as resulting from temporal variation in fitness of homozygotes. Documented cases of temporal variation in genetic characteristics of populations that appear consistent with a hypothesis of temporal variation in fitness have been rare relative to purported cases of spatial fitness variation (Hedrick, 1986). This agrees with theory in that conditions for maintenance of polymorphism are much more stringent for temporally-based balancing models than for spatially-based models (Hedrick, 1986).

My results suggest two different modes of selection bearing on the *GPI-A** polymorphism. First, underdominance may explain the consistent relatively pronounced heterozygote deficiencies observed for *GPI-A*125*. Heterozygote deficiency for this allele and the presumed heterozygote disadvantage appear relatively constant among seasons and size classes. Second, temporal variation in selection favoring *GPI-A*100* and *GPI-A*125* homozygotes may explain seasonal changes in frequencies of these alleles.

What does the foregoing suggest regarding seasonally based selection as a model for maintenance of protein polymorphism in *C. rubrofluviatilis*? The data for *GPI-A** do not allow prediction regarding the ultimate effect of the

two modes of selection. The equilibrium frequencies of the various alleles are predicated on relative fitnesses of gentoypes averaged over time. However, the consistent heterozygote deficiencies observed for *GPI-A*125* suggest greater mean fitnesses for homozygotes. This effect promotes instability of polymorphism, and therefore fixation, if one of homozygotes has a mean fitness greater than that of the alternate homozygote (Hedrick, 1983). Thus, the *GPI-A** polymorphism in *C. rubrofluviatilis* may be transient, the current state of polymorphism in the populations analyzed in this study reflecting non-equilibrial allele frequencies. However, seasonal variation in fitness of the homozygotes may retard the rate of allele frequency change toward fixation relative to an underdominance model where fitnesses of homozygotes are constant.

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Table 3. Allele frequencies and sample sizes (n) at the *GPI-A** and *G3PDH** loci in collections of *Cyprinodon rubrofluviatilis* from three collection localities on the Prairie Dog Town Fork of the Red River in Oklahoma and Texas. Collection designations indicate the season (W = winter, S = summer) and year (92 = 1992, 93 =1993) of the collections.

	Turkey				Childress				El Dorado			
Locus	W92	S92	W93	S93	W92	S92	W93	S93	W92	S92	W93	S93
*GPI-A**												
(n)	472	558	467	324	448	434	519	577	547	565	505	503
137	0.001	0.004	0.001	0.000	0.003	0.001	0.002	0.003	0.004	0.000	0.000	0.001
129	0.055	0.067	0.078	0.079	0.056	0.063	0.051	0.058	0.061	0.058	0.066	0.064
125	0.323	0.306	0.321	0.282	0.339	0.303	0.343	0.334	0.340	0.331	0.332	0.319
111	0.063	0.070	0.043	0.046	0.029	0.044	0.037	0.037	0.048	0.040	0.044	0.042
100	0.489	0.491	0.483	0.523	0.498	0.524	0.510	0.509	0.488	0.497	0.494	0.511
93	0.003	0.016	0.011	0.015	0.018	0.008	0.000	0.007	0.011	0.008	0.009	0.013

Table 3. (Continued)

	Turkey				Childress				El Dorado			
Locus	W92	S92	W93	S93	W92	S92	W93	S93	W92	S92	W93	S93
*GPI-A**												
(n)	472	558	467	324	448	434	519	577	547	565	505	503
80	0.066	0.046	0.059	0.054	0.057	0.056	0.058	0.052	0.049	0.065	0.056	0.050
*G3PDH**												
(n)	469	558	467	322	445	433	519	588	537	561	502	505
104	0.003	0.003	0.015	0.014	0.003	0.006	0.013	0.010	0.017	0.015	0.017	0.020
100	0.678	0.682	0.672	0.693	0.700	0.708	0.690	0.689	0.713	0.710	0.679	0.684
86	0.319	0.311	0.308	0.287	0.294	0.286	0.293	0.297	0.267	0.273	0.296	0.291
78	0.000	0.004	0.004	0.006	0.002	0.000	0.004	0.004	0.003	0.002	0.008	0.005

Table 4. Results of chi-square tests for Hardy-Weinberg equilibrium for the *GPI-A** and *G3PDH** loci in seasonal samples of *C. rubrofluviatilis* from three sampling localities on the Prairie Dog Town Fork of the Red River. Sample designations indicate site (TUR = Turkey, CHI = Childress, ELD = El Dorado), season (W = winter, S = summer), and year (92 = 1992, 93 = 1993). Chi-square estimates were based on genotype frequencies pooled on the basis of dosage of common *GPI-A** and *G3PDH** alleles: common allele homozygotes, common allele heterozygotes, and other genotypes. Signs of F_{IS} values indicate deficiencies (+ F_{IS}) or excesses (- F_{IS}) of heterozygotes. One degree of freedom is associated with each test.

Sample	Common allele	Sign of F_{IS}	χ^2	P
TURW92	*GPI-A*100*	-	0.143	0.705
	*GPI-A*125*	+	5.098	0.024
	*G3PDH-1*100*	+	0.512	0.474
TURS92	*GPI-A*100*	-	0.593	0.441
	*GPI-A*125*	+	0.101	0.750
	*G3PDH-1*100*	+	6.980	0.008
TURW93	*GPI-A*100*	+	1.283	0.257
	*GPI-A*125*	+	2.755	0.097
	*G3PDH-1*100*	+	1.522	0.217
TURS93	*GPI-A*100*	+	0.087	0.768
	*GPI-A*125*	+	0.002	0.965

Table 4. (Continued)

Sample	Common allele	Sign of F_{IS}	χ^2	P
TURS93	*G3PDH-1*100*	-	2.026	0.155
ELDW92	*GPI-A*100*	-	0.278	0.598
	*GPI-A*125*	-	4.330	0.037
	*G3PDH-1*100*	+	5.227	0.022
ELDS92	*GPI-A*100*	+	2.421	0.120
	*GPI-A*125*	+	9.366	0.002
	*G3PDH-1*100*	+	0.036	0.849
ELDW93	*GPI-A*100*	+	2.712	0.100
	*GPI-A*125*	+	9.779	0.002
	*G3PDH-1*100*	+	1.700	0.192
ELDS93	*GPI-A*100*	+	0.152	0.697
	*GPI-A*125*	+	0.916	0.338
	*G3PDH-1*100*	+	0.112	0.738
CHIW92	*GPI-A*100*	NA	0.000	1.000
	*GPI-A*125*	+	1.836	0.175
	*G3PDH*100*	+	4.082	0.043
CHIS92	*GPI-A*100*	+	3.518	0.061
	*GPI-A*125*	+	7.634	0.006
	*G3PDH-1*100*	+	0.500	0.479

Table 4. (Continued)

Sample	Common allele	Sign of F_{IS}	χ^2	P
CHIW93	*GPI-A*100*	+	0.150	0.699
	*GPI-A*125*	+	8.483	0.004
	*G3PDH-1*100*	+	0.393	0.531
CHIS93	*GPI-A*100*	-	0.046	0.829
	*GPI-A*125*	+	5.725	0.017
	*G3PDH-1*100*	+	1.353	0.245

Table 5. Chi-square tests comparing observed (H_{OBS}) and expected (H_{EXP}) heterozygote counts for common alleles in samples from Turkey (Texas), Childress (Texas), and El Dorado (Oklahoma). Expected counts estimate effects of pooling differentiated samples . One degree of freedom is associated with each test.

Allele	H_{OBS}	H_{EXP}	χ^2
Turkey			
*GPI-A*100*	908	910	0.003
*GPI-A*125*	726	846	17.080***
*G3PDH-1*100*	755	789	1.420
Childress			
*GPI-A*100*	967	988	0.460
*GPI-A*125*	782	873	9.560***
*G3PDH-1*100*	798	839	1.990
El Dorado			
*GPI-A*100*	1024	1059	1.169
*GPI-A*125*	887	937	2.700
*G3PDH-1*100*	848	889	1.850

*** $P < 0.001$

Table 6. Estimates of gametic disequilibrium between the *G3PDH** and *GPI-A** loci in seasonal samples of *Cyprinodon rubrofluviatilis*. The coefficient *D'* is the standardized measure of *D* referred to its theoretical maximum value, D_{max}, for a given set of allele frequencies. Sample designations indicate site (Turkey = TUR, Childress = CHI, El Dorado = ELD), season (Winter = W, Summer = S), and year (1992 = 92, 1993 = 93). Probability values for significant *D* values are indicated by asterisks: * $P < 0.05$, ** $P < 0.01$. Bonferroni correction resulted in no significant *D* values.

Sample	*D*	D_{max}	*D'*
TURW92	0.0015	0.1863	0.0079
TURS92	0.0137	0.1881	0.0727
TURW93	-0.0198	0.2150	-0.0921
TURS93	-0.0296	0.2074	-0.1425
CHIW92	-0.0259*	0.2086	-0.1251
CHIS92	0.0051	0.1808	0.0281
CHIW93	-0.0015	0.2085	-0.0071
CHIS93	-0.0250*	0.2105	-0.1189
ELDW92	-0.0332**	0.1983	-0.1673
ELDS92	-0.0049	0.2016	-0.0244
ELDW93	-0.0041	0.2116	-0.0192
ELDS93	0.0081	0.1825	0.0447

Fig. 3. Collection localities on the Prairie Dog Town Fork of the Red River in Oklahoma and Texas.

Fig. 3. Collection localities on the Prairie Dog Town Fork of the Red River in Oklahoma and Texas.

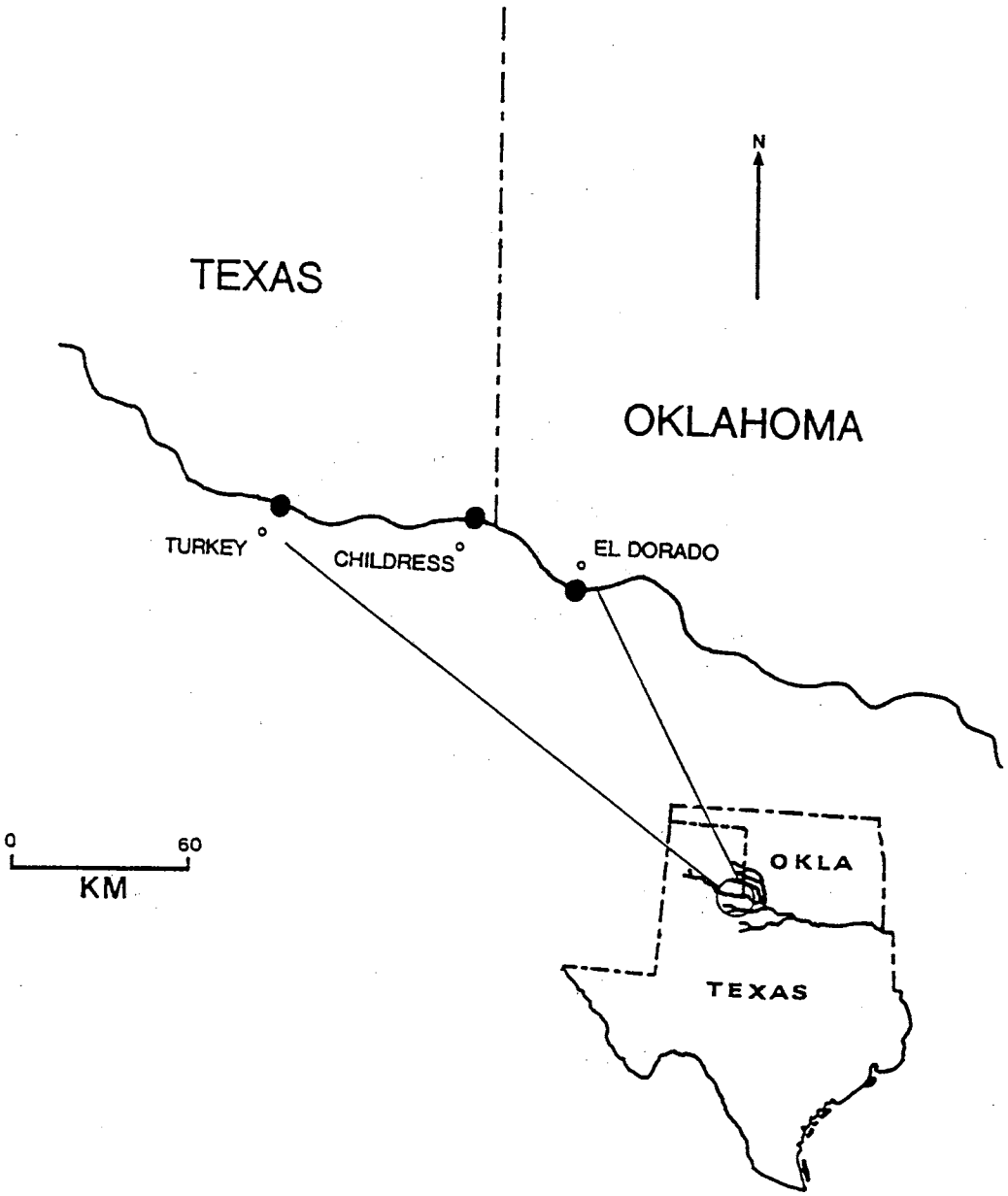


Fig. 4. *GPI-A*100* **allele and heterozygote frequencies in samples of** *Cyprinodon rubrofluviatilis* **from three collection localities on the Prairie Dog Town Fork of the Red River.**

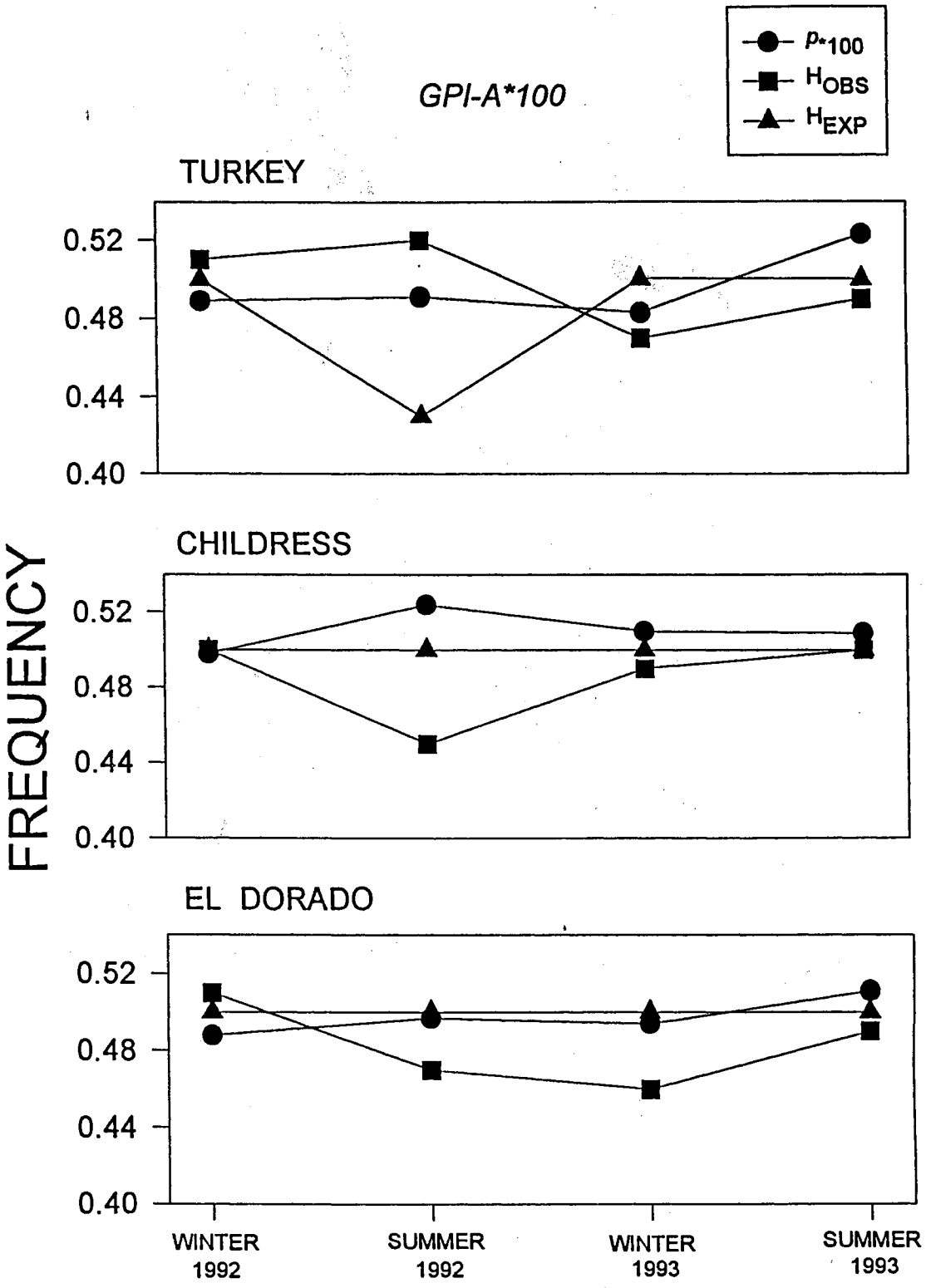

GPI-A*100
p*100
H OBS
H EXP
TURKEY
CHILDRESS
EL DORADO
FREQUENCY
0.52
0.48
0.44
0.40
WINTER 1992
SUMMER 1992
WINTER 1993
SUMMER 1993

Fig. 5. *GPI-A*125* **allele and heterozygote frequencies in samples of** *Cyprinodon rubrofluviatilis* **from three collection localities on the Prairie Dog Town Fork of the Red River.**

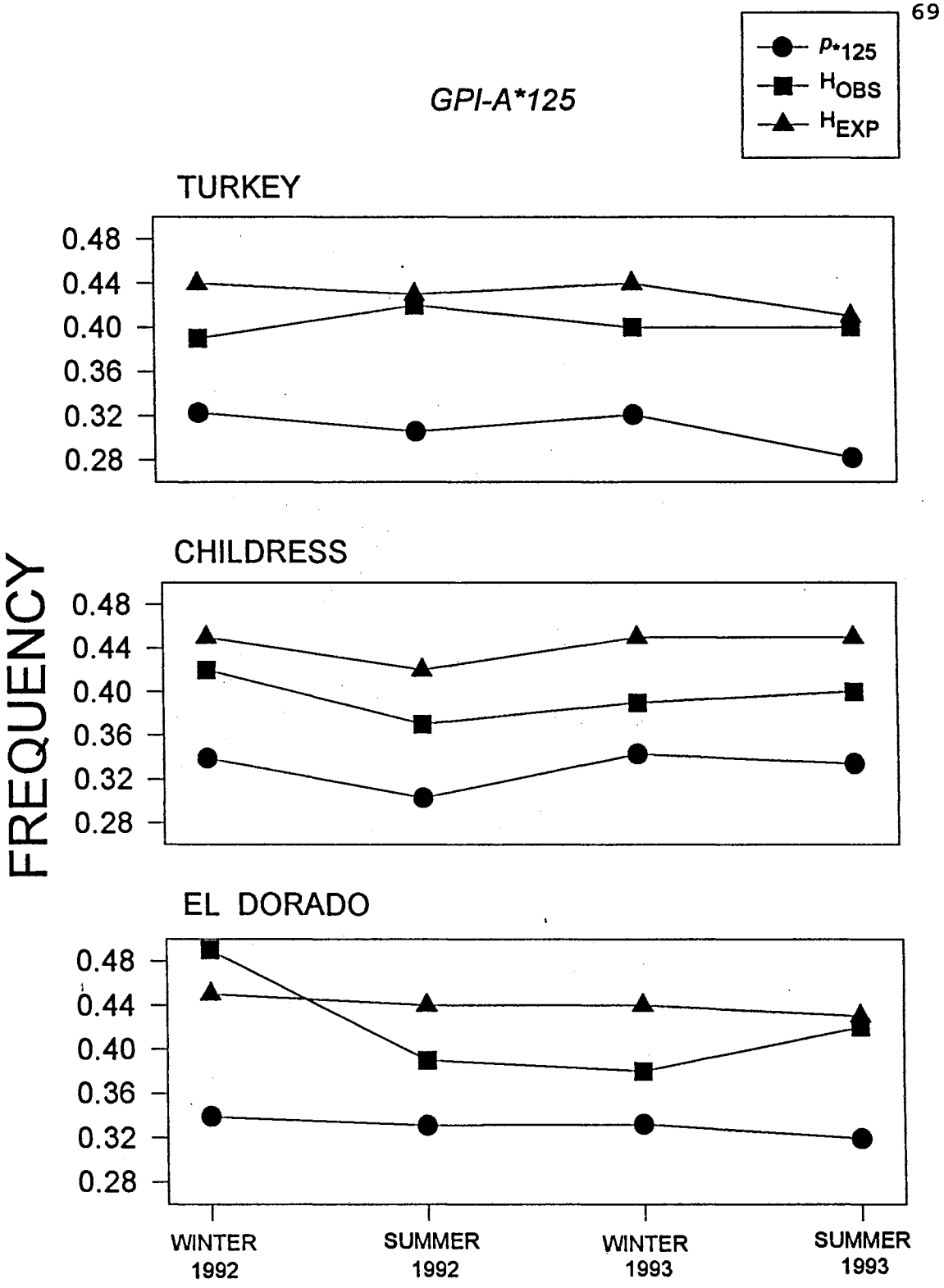

GPI-A*125
P*125
HOBS
HEXP
TURKEY
CHILDRESS
EL DORADO
FREQUENCY
0.48
0.44
0.40
0.36
0.32
0.28
WINTER 1992
SUMMER 1992
WINTER 1993
SUMMER 1993

Fig. 6. *G3PDH-1*100* **allele and heterozygote frequencies in samples of** *Cyprinodon rubrofluviatilis* **from three collection localities on the Prairie Dog Town Fork of the Red River.**

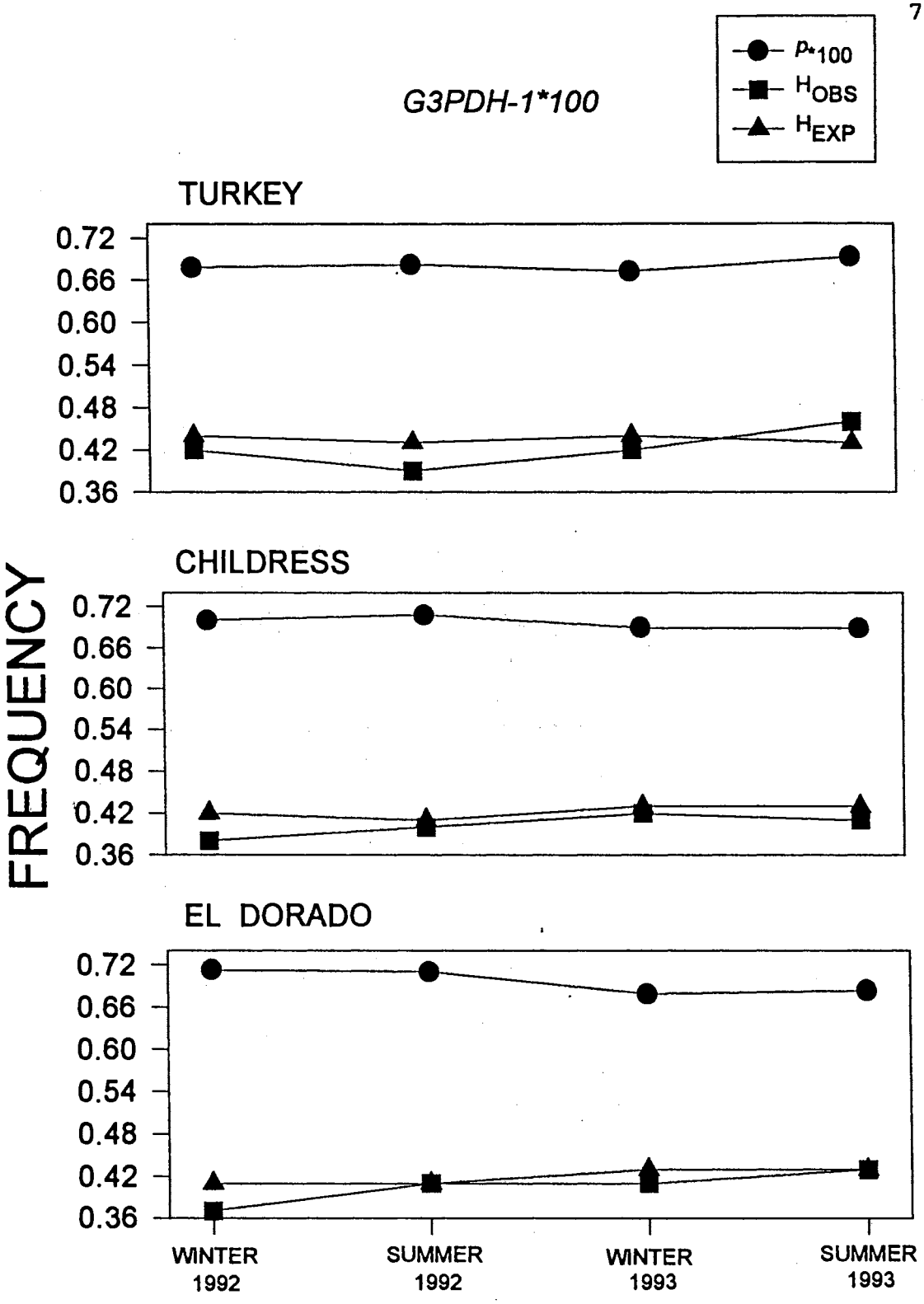

G3PDH-1*100
p*100
H OBS
H EXP
TURKEY
CHILDRESS
EL DORADO
FREQUENCY
0.72
0.66
0.60
0.54
0.48
0.42
0.36
WINTER 1992
SUMMER 1992
WINTER 1993
SUMMER 1993

Fig. 7. ***GPI-A*100*** **allele and homozygote frequencies in samples of** *Cyprinodon rubrofluviatilis* **from three collection localities on the Prairie Dog Town Fork of the Red River.**

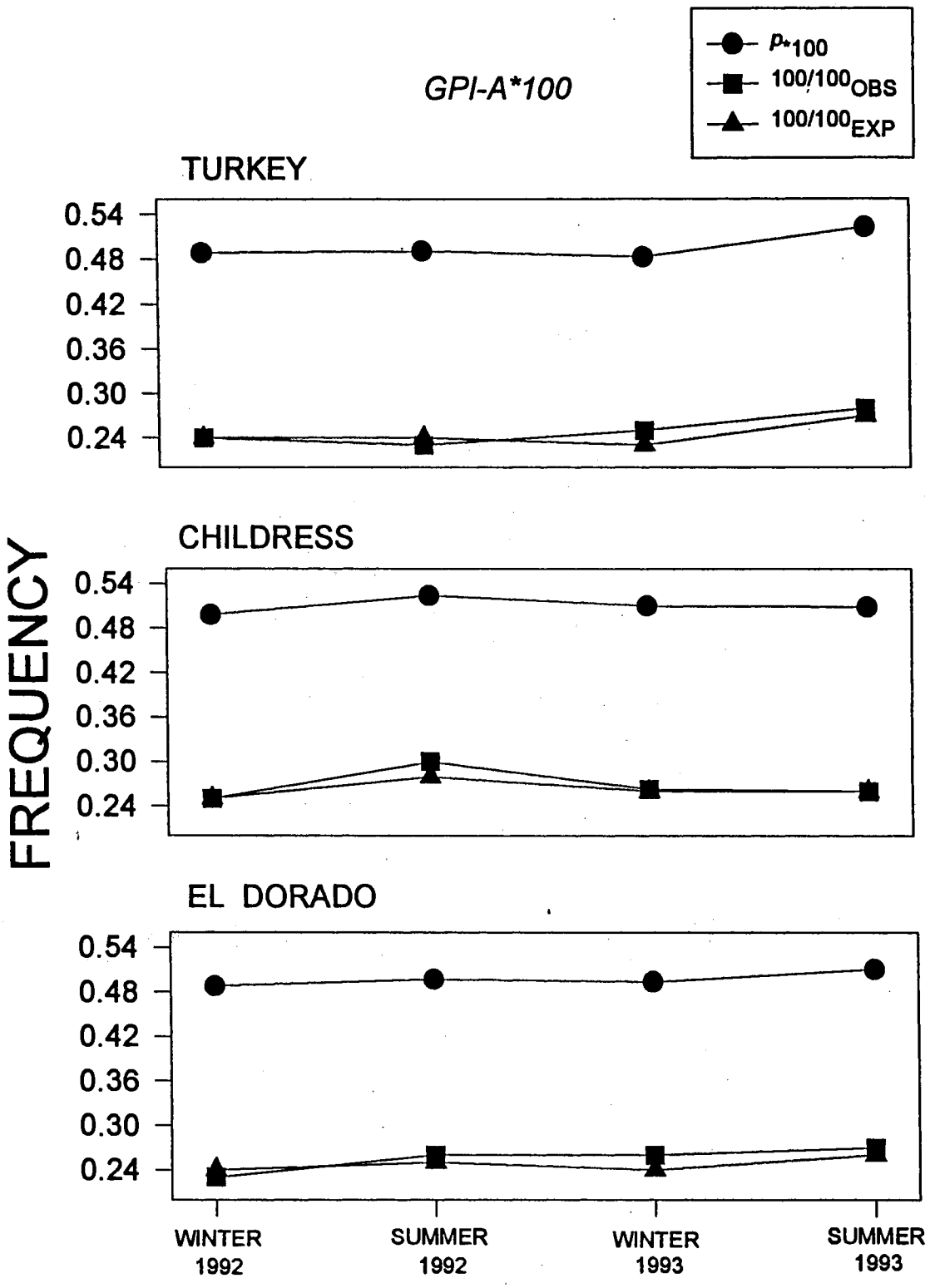

GPI-A*100
p*100
100/100OBS
100/100EXP
TURKEY
CHILDRESS
EL DORADO
FREQUENCY
0.54
0.48
0.42
0.36
0.30
0.24
WINTER 1992
SUMMER 1992
WINTER 1993
SUMMER 1993

Fig. 8. *GPI-A*125* **allele and homozygote frequencies in samples of** *Cyprinodon rubrofluviatilis* **from three collection localities on the Prairie Dog Town Fork of the Red River.**

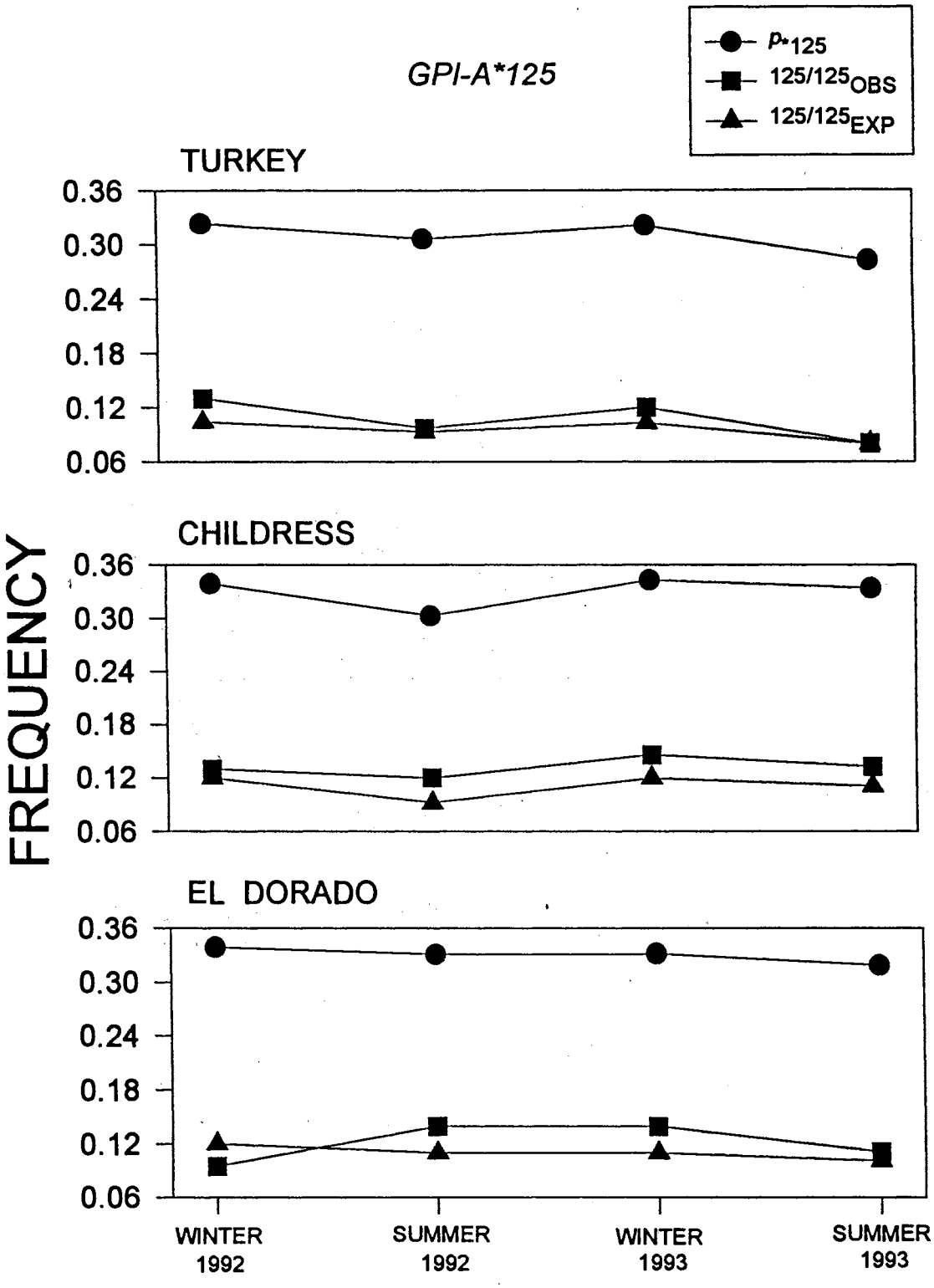
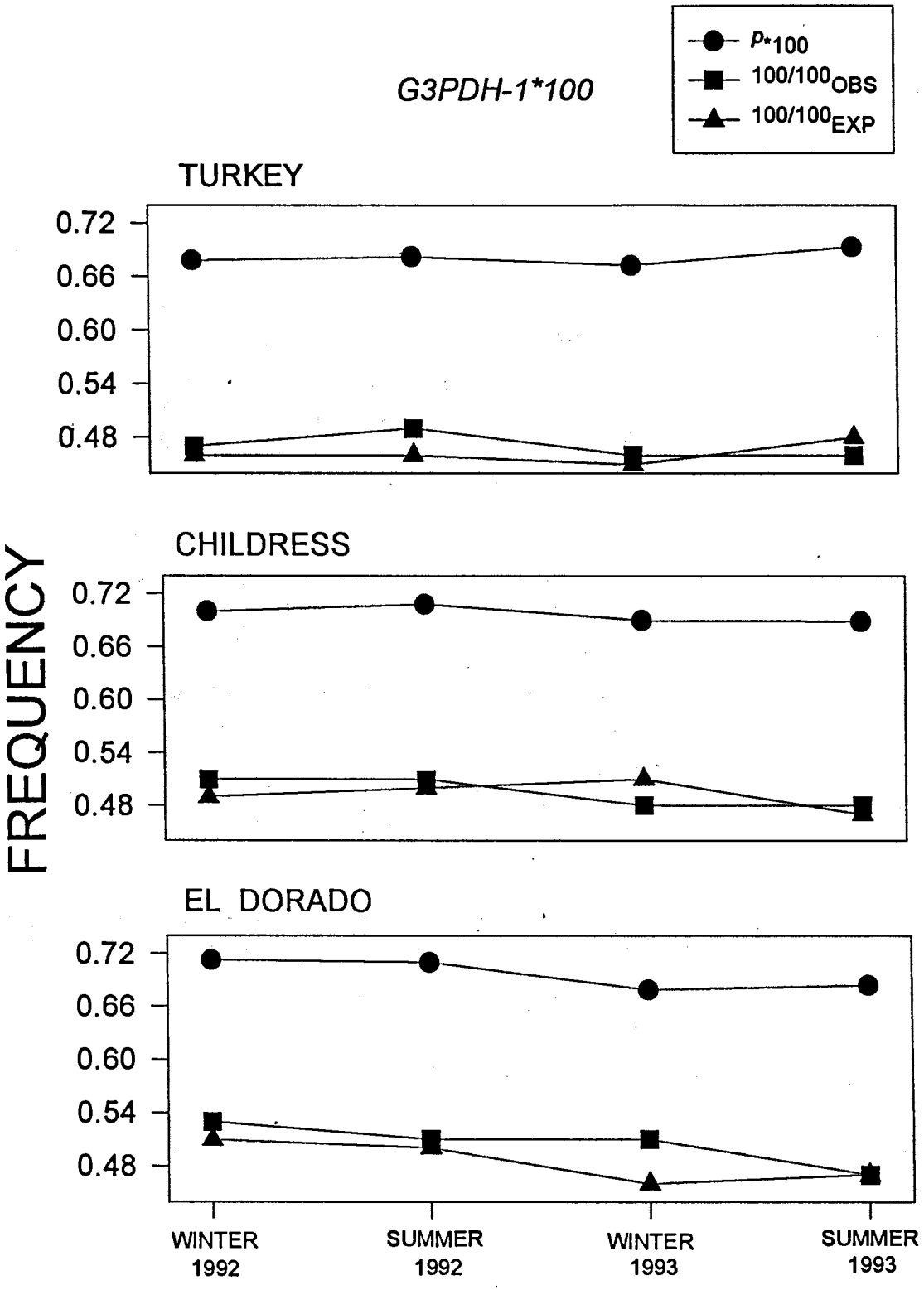

GPI-A*125
p*125
125/125OBS
125/125EXP
TURKEY
CHILDRESS
EL DORADO
FREQUENCY
0.36
0.30
0.24
0.18
0.12
0.06
WINTER 1992
SUMMER 1992
WINTER 1993
SUMMER 1993

Fig. 9. *G3PDH-1*100* **allele and homozygote frequencies in samples of** *Cyprinodon rubrofluviatilis* **from three collection localities on the Prairie Dog Town Fork of the Red River.**


G3PDH-1*100
p*100
100/100OBS
100/100EXP
TURKEY
CHILDRESS
EL DORADO
FREQUENCY
0.72
0.66
0.60
0.54
0.48
WINTER 1992
SUMMER 1992
WINTER 1993
SUMMER 1993

APPENDIX

LISTINGS OF COMPUTER PROGRAMS USED IN THIS STUDY

```
    CLS
    PRINT "FCORR.BAS--A QBASIC PROGRAM BY NICK ASHBAUGH"
    PRINT
    PRINT "    AS POINTED OUT BY VRIJENHOEK ET AL. (1992. EVOLUTION 46:1642-1657),"
    PRINT "F-STATISTICS CAN BE VIEWED AS CORRELATION COEFFICIENTS.  F(IS), FOR"
    PRINT "EXAMPLE, MEASURES THE PROBABILITY THAT TWO ALLELES AT A LOCUS IN A"
    PRINT "DIPLOID INDIVIDUAL ARE IDENTICAL BY DESCENT.  INBREEDING AMONG"
    PRINT "RELATIVES OR CERTAIN KINDS OF POPULATION STRUCTURE CAN GIVE RISE TO"
    PRINT "POSITIVE F(IS) VALUES.  DEPENDING ON THE NATURE OF THE FORCES BEARING ON"
    PRINT "ALLELE OR GENOTYPE FREQUENCIES IN POPULATIONS, ONE CAN PREDICT EITHER"
    PRINT "COMPARATIVELY HETEROGENEOUS OR UNIFORM DISTRIBUTIONS OF F(IS) VALUES"
    PRINT "FOR SEPARATE LOCI.  IF GENETIC DRIFT AND GENE FLOW ARE PREDOMINANT"
    PRINT "FORCES INFLUENCING GENETIC STRUCTURE AND VARIATION IN POPULATIONS,"
    PRINT "F(IS) VALUES SHOULD VARY LITTLE AMONG LOCI.  NATURAL SELECTION, HOWEVER,"
    PRINT "TENDS TO AFFECT ALLELE FREQUENCIES SUCH THAT F(IS) VALUES VARY"
    PRINT "AMONG LOCI; I.E., SELECTION AFFECTS EACH LOCUS IN A DIFFERENT WAY."
    PRINT "THIS PROGRAM PROVIDES A TEST OF HOMOGENEITY AMONG F(IS) VALUES USING"
    PRINT "A METHOD OUTLINED IN SOKAL AND ROHLF'S 1981 EDITION OF BIOMETRY "
    PRINT "(PP. 588-589)."
    PRINT
    INPUT "DO YOU WISH TO USE THE PROGRAM? (Y/N) ", Q$
    IF Q$ = "Y" OR Q$ = "y" THEN GOTO START ELSE GOTO TERM
START:
    '
    CLS
    '
28  PRINT "NUMBER OF LOCI OR POPULATIONS (IF TESTING HETEROGENEITY AMONG"
    INPUT "MEAN F(IS) VALUES) TO BE COMPARED"; L
    IF L = 2 THEN
      GOTO SPEC:
    ELSEIF L < 2 THEN
      GOTO 28
```

```
    ELSE GOTO 35
35  PRINT
    END IF
    DIM LS(100), FIS(100), N(100), NMIN(100), Z(100), ZZ(100), ZW(100), ZZW(100)
    NSUM = 0
    NMSUM = 0
    ZWSUM = 0
    ZZWSUM = 0
    FOR G = 1 TO L
      PRINT "NAME OF LOCUS OR POPULATION NUMBER "; G;
      INPUT LS(G)
      PRINT
      PRINT "F(IS) FOR "; LS(G);
      INPUT FIS(G)
      PRINT
      INPUT "NUMBER OF INDIVIDUALS SAMPLED FOR THE CURRENT F(IS)? ", N(G)
      PRINT
      NMIN(G) = N(G) - 3
      NSUM = NSUM + N(G)
      NMSUM = NMSUM + NMIN(G)
      Z(G) = .5 * LOG((1 + FIS(G)) / (1 - FIS(G)))
      ZZ(G) = Z(G) ^ 2
      ZW(G) = NMIN(G) * Z(G)
      ZZW(G) = NMIN(G) * ZZ(G)
      ZWSUM = ZWSUM + ZW(G)
      ZZWSUM = ZZWSUM + ZZW(G)
    CLS
    NEXT G
    PRINT "******TEST OF THE HYPOTHESIS OF HETEROGENEITY AMONG F(IS) VALUES******"
    PRINT
    PRINT
    ZWAVG = ZWSUM / NMSUM
    CT = ZWAVG * ZWSUM
    CHISQ = ZZWSUM - CT
    DF = L - 1
    PRINT "OBSERVED CHI-SQUARE = "; CHISQ
```

```
    PRINT
    PRINT "DF = "; DF
    COMF = ((2.718282 ^ ZWAVG) - (2.718282 ^ -ZWAVG)) / ((2.718282 ^ ZWAVG) + (2.718282 ^ -ZWAVG))
    PRINT
    PRINT
    PRINT "COMMON F(IS) VALUE (SEE SOKAL AND ROHLF, P. 590): "; COMF
    PRINT
    PRINT "_____"
    PRINT
    INPUT "DO YOU WISH TO END YOUR SESSION? (Y/N) ", W$
    IF W$ = "Y" OR W$ = "y" THEN GOTO TERM ELSE GOTO START
SPEC:
    CLS
    FOR G = 1 TO L
      PRINT "NAME OF LOCUS OR POPULATION NUMBER "; G;
      INPUT L$(G)
      PRINT
      INPUT "NUMBER OF INDIVIDUALS SAMPLED FOR THE CURRENT F(IS) VALUE? ", N(G)
      PRINT
      PRINT "F(IS) FOR "; L$(G);
      INPUT FIS(G)
      NMIN(G) = N(G) - 3
      Z(G) = .5 * LOG((1 + FIS(G)) / (1 - FIS(G)))
      CLS
    NEXT G
    PRINT "******TEST OF THE HYPOTHESIS OF HETEROGENEITY AMONG F(IS) VALUES******"
    T = ABS(Z(1) - Z(2)) / (((1 / NMIN(1)) + (1 / NMIN(2))) ^ .5)
    PRINT
    PRINT
    PRINT "OBSERVED T-VALUE = "; T
    PRINT
    IF T < 1.96 THEN
      PRINT "THERE IS NO STATISTICAL EVIDENCE THAT THE F(IS) VALUES ARE"
      PRINT "SIGNIFICANTLY DIFFERENT AT THE P = .05 LEVEL."
    ELSEIF T = 1.96 THEN
      PRINT "THE OBSERVED T-VALUE HAS AN OBSERVED SIGNIFICANCE LEVEL OF .05."
```

```
    PRINT "THE TWO F(IS) VALUES ARE SIGNIFICANTLY DIFFERENT."
  ELSE PRINT "THE OBSERVED T-VALUE HAS AN OBSERVED SIGNIFICANCE LEVEL OF < .05."
    PRINT "THE TWO F(IS) VALUES ARE SIGNIFICANTLY DIFFERENT."
  END IF
  PRINT
  PRINT "_____"
  INPUT "DO YOU WISH TO END YOUR SESSION? (Y/N) ", W$
  IF W$ = "Y" OR W$ = "y" THEN GOTO TERM ELSE GOTO START
TERM:
  '
  CLS
  '
  END
```

LINKED.BAS

```
CLS
PRINT "LINKED.BAS PROGRAM -- A QBASIC PROGRAM BY NICK ASHBAUGH"
PRINT
PRINT
PRINT "   THIS PROGRAM CALCULATES THE LINKAGE DISEQUILIBRIUM"
PRINT "PARAMETER, D, FOR PAIRS OF DIALLELIC LOCI AT WHICH"
PRINT "ALLELES ARE CODOMINANTLY EXPRESSED.  IT ALSO PROVIDES"
PRINT "A CHI-SQUARED TEST OF THE NULL HYPOTHESIS OF D = 0."
PRINT "D IS ESTIMATED USING THE MAXIMUM LIKELIHOOD ALGORITHM"
PRINT "OF HILL (SEE HEREDITY. 1974. 33:229-239).  THE CONVERGENCE"
PRINT "CRITERION IN THIS PROGRAM WAS ARBITRARILY SET AT 10E-8."
PRINT "PLEASE SEE HILL'S PAPER OR HARTL AND CLARK'S PRINCIPLES OF"
PRINT "POPULATION GENETICS (1988; PP. 55-57) FOR ADDITIONAL DETAILS."
PRINT
PRINT "DO YOU WISH TO USE THE PROGRAM (Y/N)";
INPUT QUERY$
IF QUERY$ = "Y" OR QUERY$ = "y" THEN GOTO START ELSE GOTO FINE
START:
CLS
T = 1000
REDIM NEWP(T + 1)
REDIM NEWQ(T + 1)
REDIM NEWR(T + 1)
PRINT "ENTER OBSERVED PHENOTYPE COUNTS:"
PRINT
PRINT
INPUT "A1A1/B1B1"; N11
PRINT
INPUT "A1A1/B1B2"; N12
PRINT
INPUT "A1A1/B2B2"; N13
PRINT
```

```
INPUT "A1A2/B1B1"; N21
PRINT
INPUT "A1A2/B1B2"; N22
PRINT
INPUT "A1A2/B2B2"; N23
PRINT
INPUT "A2A2/B1B1"; N31
PRINT
INPUT "A2A2/B1B2"; N32
PRINT
INPUT "A2A2/B2B2"; N33
CLS
NTOT = 2 * (N11 + N12 + N13 + N21 + N22 + N23 + N31 + N32 + N33)
X11 = (2 * N11) + N12 + N21
X12 = (2 * N13) + N12 + N23
X21 = (2 * N31) + N21 + N32
X22 = (2 * N33) + N23 + N32
PEST = (X11 + X12 + N22) / NTOT
QEST = (X11 + X21 + N22) / NTOT
PROD = PEST * QEST
FOR G = 0 TO T
NEWR(0) = ((1 / (2 * NTOT)) * (X11 - X12 - X21 + X22) + .5 - ((1 - PEST) * (1 - QEST)))
NEWP(G + 1) = ((N22 * NEWR(G)) * (1 - PEST - QEST + NEWR(G))) / ((NEWR(G) * (1 - PEST - QEST + NEWR(G))) + ((PEST -
NEWR(G)) * (QEST - NEWR(G))))
NEWQ(G) = X11 + NEWP(G + 1)
NEWR(G + 1) = NEWQ(G) / NTOT
IF ABS(NEWR(G + 1) - NEWR(G)) <= .00000001# THEN GOTO CALC
NEXT G
CALC:
DISEQ = NEWR(G + 1) - PROD
PRINT "LINKAGE DISEQUILIBRIUM PARAMETER = "; DISEQ
N = .5 * NTOT
VAR = (PEST * (1 - PEST) * QEST * (1 - QEST)) / N
SD = VAR ^ .5
PRINT "STANDARD DEVIATION OF D = "; SD
K = (N * DISEQ ^ 2) / (PEST * (1 - PEST) * QEST * (1 - QEST))
```

```
PRINT
PRINT
PRINT
PRINT "TESTING THE NULL HYPOTHESIS OF NO LINKAGE DISEQUILIBRIUM (D = 0)"
PRINT "----------"
PRINT
PRINT "CHI-SQUARE VALUE = "; K
PRINT
PRINT "THERE IS A SINGLE DEGREE OF FREEDOM ASSOCIATED WITH THE"
PRINT "CHI-SQUARE VALUE USED IN THIS TEST."
PRINT "THE CRITICAL CHI-SQUARE VALUE AT THE .05 PROBABILITY LEVEL"
PRINT "AND ONE DEGREE OF FREEDOM IS 3.841."
PRINT
IF K > 10.828 THEN
  PRINT "PROBABILITY OF OBSERVED CHI-SQUARE IS < .001"
  ELSEIF K = 10.828 THEN
    PRINT "PROBABILITY OF OBSERVED CHI-SQUARE = .001"
  ELSEIF K > 6.635 THEN
    PRINT "PROBABILITY OF OBSERVED CHI-SQUARE IS < .01"
  ELSEIF K = 6.635 THEN
    PRINT "PROBABILITY OF OBSERVED CHI-SQUARE = .01"
  ELSEIF K > 3.841 THEN
    PRINT "PROBABILITY OF OBSERVED CHI-SQUARE IS < .05"
  ELSEIF K = 3.841 THEN
    PRINT "PROBABILITY OF OBSERVED CHI-SQUARE = .05"
ELSE
    PRINT "PROBABILITY OF OBSERVED CHI-SQUARE IS > .05"
END IF
PRINT
IF K < 3.841 THEN
  PRINT "THERE IS NO STATISTICAL EVIDENCE FOR LINKAGE DISEQUILIBRIUM BETWEEN"
  PRINT "THE LOCI UNDER STUDY."
END IF
IF K >= 3.841 THEN
  PRINT "THE RESULTS OF THE CHI-SQUARE TEST INDICATE THAT THERE IS LINKAGE"
  PRINT "DISEQUILIBRIUM BETWEEN THE LOCI UNDER STUDY."
```

```
END IF
PRINT
PRINT
PRINT
PRINT "DO YOU WANT TO CALCULATE ANOTHER D (Y / N)";
INPUT Q$
IF Q$ = "Y" OR Q$ = "y" THEN GOTO START ELSE GOTO FINE
FINE:
CLS
END
```

```
CLS
INPUT "ENTER NO. OF SAMPLES FOR WHICH YOU'D LIKE DMAX VALUES ", N
PRINT
PRINT
FOR POP = 1 TO N
PRINT
PRINT
PRINT "DISEQ. PARAMETER FOR POP "; POP; ": "
INPUT DISEQ
PRINT
PRINT "ENTER GENOTYPE COUNTS"
PRINT "————————————"
PRINT
PRINT "A1A1: ";
INPUT N1
PRINT "A1A2: ";
INPUT N2
PRINT "A2A2: ";
INPUT N3
PRINT "B1B1: ";
INPUT N4
PRINT "B1B2: ";
INPUT N5
PRINT "B2B2: ";
INPUT N6
P1 = ((2 * N1) + N2) / (2 * (N1 + N2 + N3))
Q1 = 1 - P1
P2 = ((2 * N4) + N5) / (2 * (N4 + N5 + N6))
Q2 = 1 - P2
D1 = P1 * Q2
D2 = P2 * Q1
D3 = P1 * Q1
D4 = P2 * Q2
IF DISEQ > 0 THEN DPR1 = DISEQ / D1
```

```
IF DISEQ > 0 THEN DPR2 = DISEQ / D2
IF DISEQ < 0 THEN DPR1 = DISEQ / D3
IF DISEQ < 0 THEN DPR2 = DISEQ / D4
LPRINT "DMAX FOR SAMPLE "; POP
LPRINT "----------"
LPRINT
LPRINT "UNADJUSTED DISEQUILIBRIUM PARAMETER "; DISEQ
IF DISEQ > 0 THEN LPRINT "DMAX1 = "; D1
IF DISEQ < 0 THEN LPRINT "DMAX1 = "; D3
LPRINT "DADJ1 = "; DPR1
LPRINT
IF DISEQ > 0 THEN LPRINT "DMAX2 = "; D2
IF DISEQ < 0 THEN LPRINT "DMAX2 = "; D4
LPRINT "DADJ2 = "; DPR2
LPRINT
LPRINT
NEXT POP
'
CLS
'
END
```

```
    CLS
    PRINT "WAHL.BAS -- A QBASIC PROGRAM BY NICK ASHBAUGH"
    PRINT
    PRINT "    OFTEN IN CASES WHERE A POPULATION IS FRAGMENTED INTO MORE"
    PRINT "OR LESS ISOLATED DEMES THERE IS INTER-DEMIC VARIATION IN ALLELE"
    PRINT "FREQUENCIES SUCH THAT POOLING SAMPLES FROM THESE SEPARATE DEMES"
    PRINT "RESULTS IN A DEFICIENCY OF HETEROZYGOUS GENOTYPES RELATIVE TO EXPECTATIONS"
    PRINT "FOLLOWING FROM THE HARDY-WEINBERG THEOREM.  THIS IS REFERRED TO AS"
    PRINT "THE WAHLUND EFFECT.  AN ALTERNATE SITUATION IS ONE WHERE EACH SEPARATE"
    PRINT "DEME IS CHARACTERIZED BY DEFICIENT FREQUENCIES OF HETEROZYGOTES."
    PRINT "CONSISTENT DEFICIENCIES AMONG DEMES MAY BE THE RESULT OF SEVERAL"
    PRINT "POSSIBLE CAUSES INCLUDING SELECTIVE INFERIORITY OF HETEROZYGOTES,"
    PRINT "SEX DIFFERENCES IN SELECTION, ETC. (SEE KOEHN ET AL., 1971"
    PRINT "AM. NATURALIST 105:51-69 FOR AN OVERVIEW OF SOME OF THESE FACTORS)."
    PRINT
    PRINT "  THE KERNEL OF THIS PROGRAM CONSISTS OF TWO CALCULATIONS: 1.) HARDY-WEINBERG"
    PRINT "EXPECTED FREQUENCY OF HET'S. GIVEN POPULATION SUBDIVISION (A FUNCTION OF THE"
    PRINT "INBREEDING COEFFICIENT) AND 2.) DEVIATION OF OBSERVED FREQUENCY OF HET'S."
    PRINT "RELATIVE TO THE EXPECTED FREQUENCY UNDER POPULATION SUBDIVISION."
    PRINT "SEE LI'S 1955 POPULATION GENETICS TEXT OR KOEHN ET AL. 1971 (P. 57) FOR MORE"
    PRINT "DETAILS."
    PRINT
    PRINT
    INPUT "DO YOU WANT TO CONTINUE THIS SESSION? (Y/N) ", Q$
    IF Q$ = "Y" OR Q$ = "y" THEN GOTO START ELSE GOTO TERM
START:
    DIM POP$(1000), N1(1000), N2(1000), N3(1000), P(1000), Q(1000)
    DIM W(1000), WP(1000), WPP(1000)
    CLS
    PRINT
    INPUT "ENTER NUMBER OF POPULATIONS SAMPLED: ", N
    PRINT
    INPUT "ENTER TOTAL NUMBER OF INDIVIDUALS SAMPLED: ", NTOT
    PRINT
```

```
INPUT "ENTER NAME OF LOCUS UNDER STUDY: ", LOCUS$
PRINT "_____"
PRINT
PSUM = 0
WPSUM = 0
WPPSUM = 0
N2SUM = 0
FOR G = 1 TO N
PRINT
PRINT "ENTER NAME OF POPULATION NO. "; G;
INPUT POP$(G)
PRINT
CLS
PRINT "GENOTYPE COUNTS FOR "; LOCUS$; " IN THE "; POP$(G); " SAMPLE:"
PRINT
INPUT "A1A1: ", N1(G)
PRINT
INPUT "A1A2: ", N2(G)
PRINT
INPUT "A2A2: ", N3(G)
P(G) = (2 * N1(G) + N2(G)) / (2 * (N1(G) + N2(G) + N3(G)))
Q(G) = 1 - P(G)
W(G) = (N1(G) + N2(G) + N3(G)) / NTOT
WP(G) = W(G) * P(G)
WPP(G) = W(G) * (P(G) ^ 2)
WPSUM = WPSUM + WP(G)
WPPSUM = WPPSUM + WPP(G)
PSUM = PSUM + P(G)
N2SUM = N2SUM + N2(G)
CLS
NEXT G
PAV = PSUM / N
QAV = 1 - PAV
PQAV = PAV * QAV
VARP = WPPSUM - (WPSUM ^ 2)
F = VARP / PQAV
```

```
HET = 2 * PQAV * NTOT
HETF = 2 * F * PQAV * NTOT
HETEX = HET - HETF
HETDEV = N2SUM - HETEX
'
CLS
'
PRINT "********HETEROZYGOTE COUNTS**************"
PRINT
PRINT
PRINT "OBSERVED NO. OF HETEROZYGOTES: "; N2SUM
PRINT
PRINT "HARDY-WEINBERG EXPECTED NO. OF HET'S.: "; HET
PRINT
PRINT "H-W EXP. WITH SUBDIVISION: "; HETEX
PRINT
PRINT "DEVIATION OF OBSERVED HETEROZYGOTE NUMBER FROM THAT"
PRINT "UNDER WAHLUND EFFECT (POOLED ISOLATES): "; HETDEV
PRINT "_____"
PRINT
PRINT "DEFICIENCIES OWING TO WAHLUND EFFECT ARE INDICATED BY"
PRINT "OBSERVED HETEROZYGOSITIES SIMILAR TO THOSE EXPECTED WITH POPULATION"
PRINT "SUBDIVISION.  OTHERWISE, THE DEFICIENCIES ARE NOT A CONSEQUENCE"
PRINT "OF POOLING DIFFERENTIATED POPULATIONS; I.E., EACH POPULATION"
PRINT "EXHIBITS A CONSISTENT PATTERN OF HETEROZYGOTE DEFICIENCY."
PRINT "SUCH SITUATIONS HERE ARE INDICATED BY STRONGLY NEGATIVE DEVIATIONS"
PRINT "OF OBSERVED HETEROZYGOSITIES RELATIVE TO THOSE EXPECTED UNDER POPULATION"
PRINT "SUBDIVISION."
PRINT
INPUT "DO YOU WISH TO CONTINUE THIS SESSION? (Y/N) ", C$
IF C$ = "Y" OR C$ = "y" THEN GOTO START ELSE GOTO TERM
TERM:
CLS
END
```

VITA

Nicholas A. Ashbaugh

Candidate for the Degree of

Doctor of Philosophy

Dissertation: GEOGRAPHIC VARIATION, POPULATION STRUCTURE, AND BALANCING SELECTION IN RED RIVER PUPFISH, *CYPRINODON RUBROFLUVIATILIS*

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