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POPULATION CAGE EXPERIMENTS WITH A VERTEBRATE: THE TEMPORAL DEMOGRAPHY AND CYTONUCLEAR GENETICS OF HYBRIDIZATION IN *GAMBUSIA* FISHES

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Abstract.—The dynamics of mitochondrial and multilocus nuclear genotypic frequencies were monitored for 2 yr in experimental populations established with equal numbers of two poeciliid fishes (*Gambusia affinis* and *Gambusia holbrooki*) that hybridize naturally in the southeastern United States. In replicated "small-pool" populations (experiment I), 1018 sampled individuals at six time periods revealed an initial flush of hybridization, followed by a rapid decline in frequencies of *G. affinis* nuclear and mitochondrial alleles over 64 wk. Decay of gametic and cytonuclear disequilibria differed from expectations under random mating as well as under a model of assortative mating involving empirically estimated mating propensities. In two replicate "large-pond" populations (experiment II), 841 sampled individuals across four reproductive cohorts revealed lower initial frequencies of F_1 hybrids than in experiment I, but again *G. holbrooki* alleles achieved high frequencies over four generations (72 wk). Thus, evolution within experimental *Gambusia* hybrid populations can be extremely rapid, resulting in consistent loss of *G. affinis* nuclear and cytoplasmic alleles. Concordance in results between experiments and across genetic markers suggests strong directional selection favoring *G. holbrooki* genotypes. Results are interpreted in light of previous reports of genotype-specific differences in life-history traits, reproductive ecology, patterns of recruitment, and size-specific mortality, and in the context of patterns of introgression previously studied indirectly from spatial observations on cytonuclear genotypes in natural *Gambusia* populations.

Key words.—Allozymes, disequilibrium, *Gambusia*, genetic drift, hybrid zones, mitochondrial DNA.

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The availability of multilocus data from nuclear and cytoplasmic genomes has fostered a growing appreciation of the evolutionary inferences that may be drawn from molecular genetic characterizations of natural populations. However, because of the usual lack of direct historical information on breeding structure, recruitment, gene flow, selective regimes, and other demographic variables in nature, static descriptions of genotypic distributions have limitations as a sole source of evolutionary inference. One augmentative approach involves analyses in controlled "population cage" settings, where the temporal dynamics of population processes and molecular genetic features can be monitored directly. Laboratory populations of bacteria and fruit flies have proved particularly useful in studies of genetic drift, mutational pressure, and components of selection (Anderson 1989; Buri 1956; Dykhuizen and Hartl 1983; Hall 1983; Hedrick and Murray 1983; Sperlich and Pfriem 1986; Wallace 1948; Wright and Dobzhansky 1946). However, virtually no such experimental genetic research has been conducted on vertebrate populations because of the obvious constraints posed by long generation time, handling and sampling difficulties, and sample sizes.

Here we employ an experimental "population-cage" approach to monitor temporal changes in cytonuclear genotypic composition following population contact and hybridization between two species of live-bearing poeciliid fishes, *Gambusia affinis* and *Gambusia holbrooki*. These species occur in freshwater habitats throughout the southeastern United States, and they offer several advantages for experimental genetic analysis. Individuals are small in size such that large populations can be maintained, and the species have a short generation time [(typically four to six broods per year (Scribner 1992; Leberg 1990)] such that multigeneration dynamics can be monitored. Taxonomically informative genetic markers are available from both the nuclear and mitochondrial genomes (Scribner and Avise

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1993a,b). Interpretations of temporal population-genetic changes within hybrid zones should be facilitated by an extensive literature for *G. affinis* and *G. holbrooki* on species-specific differences in life-history traits (Reznick 1981; Scribner 1993), population dynamics (Scribner 1993), and spatio-temporal genetic variation in nature and in controlled settings (McClenaghan et al. 1985; Leberg 1992). Finally, the mixed-species experimental design employed in the current study should facilitate analyses of multilocus dynamics because differences in factors such as mating preference, fertility, and viability likely will be magnified, making their genetic consequences easier to document over short time periods.

The mosquitofish *G. affinis* and *G. holbrooki* hybridize naturally across several drainages in the southeastern United States, as evidenced by the appearance of interspecific recombinant nuclear and cytonuclear genotypes (Wooten et al. 1988; Wooten and Lydeard 1990; Scribner 1992; Scribner and Avise 1993a,b). Nonetheless, populations within the presumptive hybrid region exhibit significant gametic disequilibria and high fixation indices, likely because of assortative mating and/or differential fitnesses of species-specific versus recombinant genotypes. Furthermore, heterospecific genetic markers have been observed in low frequency outside the central hybrid region, and these could reflect contemporary introgressive penetrance across the zone, the evolutionary "footprints" of past hybridization in a moving hybrid region, and/or the mere retention of ancestral polymorphisms (Scribner 1992; Scribner and Avise 1993a,b). Thus, the static genetic characterizations of natural *Gambusia* populations have raised several possibilities about the hybridization process that warrant further examination in a controlled experimental framework.

In this study of the genetics and demography of experimental hybrid populations, the objectives are to (1) describe temporal changes in nuclear and cytoplasmic genotype frequency within controlled and replicated populations set up as mixtures of two parental species; (2) compare results to expectations of temporal change under models of genetic drift and assortative mating; and (3) relate the experimental results to predictions based on species differences in life-history traits and population dynamics and to prior molecular-genetic observations on the natural *G. affinis–G. holbrooki* hybrid zone.

Materials and Methods

Two large-scale experiments were conducted on the Savannah River Site (SRS) near Aiken, South Carolina. These experiments were designed to monitor cytonuclear changes during a 2-yr period (approximately four generations) following simulated secondary contacts between *Gambusia affinis* and *Gambusia holbrooki*. Genotypes were assayed at five nuclear (allozyme) loci and mitochondrial DNA (mtDNA), all of which exhibit nearly fixed allelic differences between the two species. Founding *G. affinis* and *G. holbrooki* came from allopatric populations (Lake Arthur in south-central Louisiana, and a tributary of the Savannah River in west-central South Carolina, respectively) previously characterized as genetically distinct for the markers employed in the current study (Scribner 1992; Scribner and Avise 1993a). Species specificity of the allozyme markers was verified further by characterization of 100 additional individuals from each locale.

All experimental populations were maintained in isolation (to eliminate the confounding effects of gene flow), and physical environmental conditions were standardized as much as possible across replicates. The two sets of experiments differ in the probabilities of stochastic genetic change because of inherent differences in founding effective population size, population carrying capacity, and sampling regime.

Experiment I—Small Pools

Sampling Design.—Replicate aquatic communities were established using 12 pools each approximately 2.4 m in diameter and 30 cm deep. Pools were filled with water on March 15, 1990, and to each was added 2 kg of dried pond debris and 2.5 m of artificial cover (synthetic vegetation). Pools were left open to allow colonization by invertebrates, and 500 ml of strained water from each of four aquatic environments found on the SRS was added monthly throughout the experiment to maintain a seasonally diverse planktonic prey source. Pools were thus self-sustaining "mesocosms," complete with predator and prey sources, producers, and structural complexity.

Three virgin females and three males of *G. affinis* and *G. holbrooki* (total $N = 12$) were placed into each pool on April 15, 1990 (see fig. 1A for design). Each set of replicates subsequently was sampled at regular intervals (weeks 6, 12, and 18

during 1990 and weeks 52, 58, and 64 during 1991). Each 6-wk period corresponds to approximately 0.5 generations (mean generation length of *Gambusia* is about 80 d; Scribner, unpubl. data). Estimates of generation length were determined independently in the laboratory under constant (27°C) temperature regimes. Generation length in *Gambusia* is temperature dependent (Vondracek et al. 1988) and may be shorter at the higher temperatures experienced during certain times of the year (see beyond).

Demographic and Genetic Characterization.—During each sampling period, population size was estimated using serial removals of fish from each replicate pool. Sampling was conducted using a dip net to remove as many fish as possible in each of eight 2-min periods. Total population size was estimated for each replicate based on the decline in fish numbers in these serial samples using the computer program CAPTURE (Otis et al. 1978). For each of the six sampling periods (I–VI), two replicate pool populations (1 and 2) were chosen randomly and destructively sampled for genetic characterization.

Statistical Analyses.—Nuclear and mtDNA genotype frequencies were estimated for each replicate sampled for each of the six sampling periods. Measures of interpopulation divergence in nuclear gene frequency between replicates (a measure of drift) sampled each period were assessed using G_{st} (Nei 1987), the significance of which was tested by χ^2 tests (Workman and Niswander 1970). A corresponding measure for mtDNA (F_{st}) was calculated treating haplotypes as alleles at a single locus. Significance of F_{st} was tested using the V-statistic (DeSalle et al. 1987).

Multilocus nuclear genotypes were used to estimate composite measures of gametic phase disequilibrium (Weir 1979; Weir and Cockerham 1989) for each replicate pool sampled during each period. χ^2 tests (Weir and Cockerham 1989) were employed to evaluate whether the composite disequilibrium measures for each replicate pool differed significantly from zero. Any non-zero disequilibria were assumed to reflect associations between *G. affinis* and *G. holbrooki* alleles resulting from initial stocking conditions or population processes (e.g., assortative mating or genotype-specific differences in fitness), rather than to linkage along a chromosome [because based on genetic maps for other poeciliid fishes, no physical linkage among the five loci is indicated (Morizot and Siciliano 1983)]. Nonetheless, there remain other grounds for caution in interpreting disequilibrium measures because of possible biases stemming from allele frequency differences (Hedrick 1987) across time periods, and because most measures of disequilibrium are computed from genotypic frequencies under an assumption of random gamete union (Hill 1974), which is unlikely to be strictly valid in hybrid zones.

Statistical associations between nuclear and cytoplasmic loci were measured using both genotypic and gametic cytonuclear disequilibrium statistics as defined in Asmussen et al. (1987). In brief, for a diploid population with two alleles at a nuclear locus (*A* and *a*) and two alleles (*M* and *m*) at a mitochondrial locus, genotypic cytonuclear disequilibria are quantified by the departures of genotypic frequencies from expectations under random mating [i.e., D_1 = freq. (*AA/M*) − freq. (*AA*) freq. (*M*)]. Similarly, the gametic disequilibrium summarizes the association between nuclear and mitochondrial alleles [i.e., D = freq. (*A/M*) − freq. (*A*) freq. (*M*)]. Significance levels were evaluated relative to the hypothesis $D_1 = D_2 = D_3 = D = 0$. Changes in disequilibrium values over sampling periods were compared to expected values under models of (1) random mating (recursive equations in Asmussen et al. 1987) and (2) assortative mating (as defined in Arnold et al. 1988), in which female *G. holbrooki* and *G. affinis* parental types were assumed to mate assortatively with probabilities $\alpha = 0.716$ and $\beta = 0.244$, respectively. (Frequencies of homo- and heterospecific matings were determined empirically in the laboratory (Scribner, unpubl. data), and the above mating probabilities were calculated as defined in Arnold et al. 1988.) Expected cytonuclear genotype counts for each whole generation (gen. 1 = week 12; gen. 2 = week 52; gen. 3 = week 64) derived under each model were compared to observed counts using χ^2 analysis. Because each replicate population and time period was independent, an experiment wide goodness-of-fit for each model was generated as the sum of each population-period χ^2. A scaling factor (C) was then used to adjust χ^2 tests (Brier 1980) to account for heterogeneity between replicates, where $C = (\chi^2_a)/(a - 1)$ (the χ^2 test for homogeneity between replicates each generation divided by the degrees of freedom for that test).

Experiment II—Large Ponds

Two large replicate populations were established in concrete-lined ponds [approximately 12 m long, 7 m wide, and 1.5 m deep (fig. 1B)].

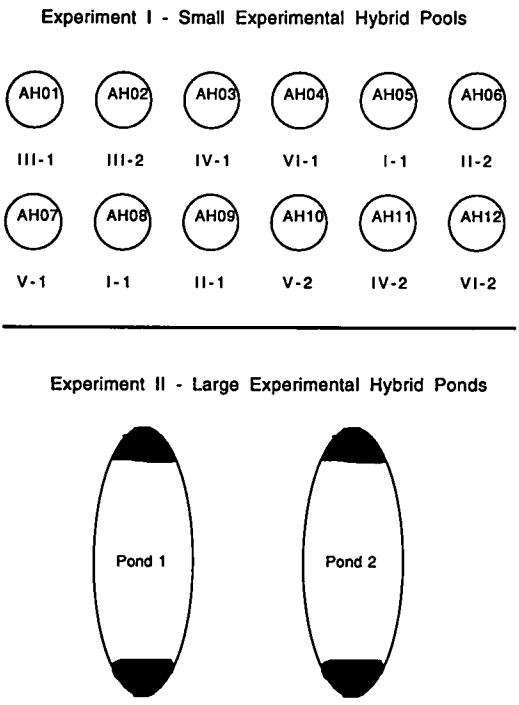

FIG. 1. Summary of experimental pool and pond designs. A. Small pools: roman numerals represent the six sampling periods for the replicate sets of pools 1 and 2. Altogether 12 pools (AH01–AH12) were monitored. B. Large ponds: two replicate ponds were monitored, each with two brooding areas indicated by shading.

Ponds were filled with water on March 15, 1990, and to each was added 6 kg of dried pond debris and 10 m of artificial cover. About 1500 ml of strained water from each of four aquatic environments found on the SRS were added monthly throughout the experiment, thereby creating a self-supporting mesocosm as outlined above. To provide a refuge for progeny from cannibalism and to facilitate sampling, shallow "brooding" areas were established on both ends of each pond (fig. 1B).

A total of 240 adult *G. affinis* and *G. holbrooki* (60 males and 60 females of each species) was stocked into each pond on April 15, 1990. Females were not necessarily virgins, but may have been bred the previous fall (*Gambusia* have the capacity to store sperm; see Constantz 1989). Thus, the first sampled cohort was "cohort 2," produced from breeding within the ponds. In contrast to experiment I, the sampling in experiment II was not destructive of the entire pond, such that replicate populations were monitored directly through time. Cohorts 2 and 4 were produced during 1990 and cohorts 6 and 8 produced during 1991. During each sampling period, offspring of the desired cohort were collected twice daily during a 14-d period from each of the shallow brooding areas. Sampling duration was set to maximize the numbers of females contributing to each sample. Sampling was intended to maximize the number of offspring collected prior to juvenile mortality factors (i.e., cannibalism by adults; see Scribner 1993). All offspring sampled from each brooding area were kept in separate enclosures. A subset (N = 100–120) of each cohort was subsequently retained and raised to sexual maturity for genetic analysis and remaining individuals were returned to each pond.

Demographic and Genetic Characterization.—A total of 841 individuals was characterized for mtDNA and allozyme genotype. Spatial structure within ponds was monitored by comparing genotype frequencies of offspring obtained from each of the two brooding areas. These samples always proved statistically homogeneous and were therefore combined in subsequent analyses. Differences in cohort genotypic composition between replicate ponds were tested by χ^2 tests. After collections of each cohort sample, estimates of population size were made following the procedures outlined above.

Comparison of Designs for Experiments I and II.—Experiment II differs in several important respects from experiment I. First, because many more individuals were stocked initially into each pond (N = 240 per replicate versus 12 per replicate in experiment I), the potential for founder effect and drift should be considerably lower. Second, in contrast to the genetic characterizations of entire populations in experiment I, sampling from the large ponds involved neonates only. Thus, the genetic data from experiment II (particularly cohorts 2 and 4 of the first year) reflect mating influences, fecundities, and early viability effects only (i.e., prior to appreciable mortality caused by adult cannibalism), whereas the data in experiment I also could include juvenile mortality effects. Third, direct demographic data in experiment II are more limited for interpreting genetic results thus precluding some of the kinds of statistical tests administered in experiment I. Fourth, the ponds' larger areas might increase environmental heterogeneity, with various possible biological effects including increased habitat partitioning, greater opportunity for assortative mating [asymmetry in degree of

assortative mating has been documented (Scribner, unpubl. data)], diminished interspecific competition, and/or amelioration of potential density dependent viability or fertility effects.

Molecular Procedures.—Mitochondrial DNA was extracted from each individual (N = 1859 over both experiments) using a rapid-isolation alkaline lysis preparation (Tamura and Aotsuka 1988). The mtDNA was resuspended in 60 μl TE and dialyzed to remove excess salt using the "drop dialysis" procedure (Marusyk and Sergeant 1980). Restriction digests using the species-diagnostic *Hin*dIII (Scribner 1992) were conducted overnight under conditions recommended by the enzyme supplier (Boerhinger Mannheim). Digestion fragments were end labeled with ^{35}S-radionuclides, separated through 1.0% agarose gels, and revealed by autoradiography (Brown 1980; Maniatis et al. 1982).

Cellular debris obtained during low-speed centrifugation was frozen at −70°C and used for allozyme electrophoresis. Five nuclear loci that exhibit large allele frequency differences between the *Gambusia* species (Wooten and Lydeard 1990; Scribner, unpubl. data) were analyzed: adenosine deaminase (E.C. 3.5.4.4; *Ada*), aspartate aminotransferase-1 (E.C. 2.6.1.1; *Aat-1*), malate dehydrogenase-1 (E.C. 1.1.1.37; *Mdh-1*), peptidase-A (E.C. 3.4.11 or 3.4.13; *Pep-A*, leucyl alanine as substrate), and aconitate hydratase-1 (E.C. 4.2.1.3; *Acon-1*). Electrophoretic techniques have been described previously (McClenaghan et al. 1985; Wooten and Lydeard 1990). Mobilities were determined for each allele relative to a reference *G. affinis* electromorph ("100") placed on each gel. Use of five diagnostic loci facilitated the characterization of each individual as either parental *G. holbrooki*, parental *G. affinis*, F_1, *G. holbrooki* backcross, or *G. affinis* backcross. Use of cytoplasmically inherited mtDNA allowed determination of maternal parentage.

Results

Experiment 1

Following stocking, population numbers expanded rapidly over weeks 6–18, peaking at about 160 individuals during 1990 (fig. 2). Numbers declined 45% over the winter and remained relatively unchanged throughout the summer of 1991. Differences in population growth rate between years were caused primarily by differences in population age structure, differences in resource availability, and density-dependent differences in offspring viability and female fecundity (Scribner 1992, 1993).

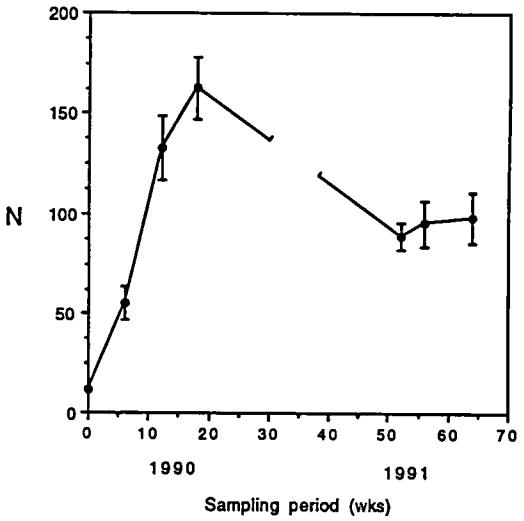

FIG. 2. Estimates of total population size (mean ± SE over replicates) for the small pool populations of *Gambusia affinis* and *Gambusia holbrooki* during the six sampling periods of 1990 and 1991.

Allozyme frequencies changed dramatically over the six periods of population sampling (table 1), and changes were generally consistent across loci. From initial stocking conditions (P_o = 0.50), all allozyme frequencies shifted rapidly and directionally toward fixation for *Gambusia holbrooki* alleles, such that by week 64 the mean frequency across replicates was 0.88 (table 1). Rates of mitochondrial DNA (mtDNA) haplotype change were initially greater than those observed for nuclear alleles. The direction of change was generally in favor of *G. holbrooki* haplotypes, such that a mean frequency of 0.94 across replicates was reached within 64 wk (table 1).

The degree of interreplicate variance in nuclear and mitochondrial frequency was significant during some sampling times (periods 2, 3, 5, and 6 for nuclear loci and periods 1, 4, and 5 for mtDNA) but did not increase consistently through time as would be predicted under random genetic drift. In general, the degree of interreplicate divergence for mtDNA did not consistently exceed that for nuclear loci nor did it increase in a directional manner (table 1).

Temporal genetic changes are further evidenced in the multilocus genotypic structure, as summarized by frequencies of parental, F_1, and backcross nuclear genotypes (fig. 3). At week 6,

TABLE 1. Genotypic frequencies for five diagnostic allozyme loci and the species-specific mitochondrial DNA (mtDNA) haplotypes for *Gambusia* in the two replicate sets of small pool populations† during six sampling periods.

	Sampling period‡											
	1990						1991					
	Week 6		Week 12		Week 18		Week 52		Week 58		Week 64	
Locus/allele§	Rep 1 (79)‖	Rep 2 (64)	Rep 1 (113)	Rep 2 (81)	Rep 1 (113)	Rep 2 (109)	Rep 1 (51)	Rep 2 (83)	Rep 1 (88)	Rep 2 (66)	Rep 1 (78)	Rep 2 (93)
Pep-A												
114/114	0.342	0.453	0.726	0.074	0.274	0.554	0.804	0.634	0.874	0.591	0.872	0.710
114/100	0.557	0.484	0.150	0.750	0.567	0.355	0.196	0.338	0.080	0.364	0.103	0.258
100/100	0.101	0.063	0.124	0.176	0.159	0.091	0.000	0.028	0.046	0.045	0.025	0.032
	*	*	*	*					*		*	
Ada												
130/130	0.342	0.453	0.726	0.062	0.274	0.564	0.451	0.610	0.852	0.354	0.846	0.710
130/100	0.557	0.484	0.150	0.750	0.549	0.345	0.549	0.366	0.080	0.554	0.128	0.258
100/100	0.101	0.063	0.124	0.188	0.177	0.091	0.000	0.024	0.068	0.092	0.026	0.032
	*	*	*	*			*		*	*	*	
Mdh-1												
80/80	0.342	0.453	0.726	0.099	0.221	0.536	0.686	0.639	0.776	0.561	0.872	0.710
80/100	0.557	0.484	0.150	0.728	0.646	0.382	0.235	0.313	0.176	0.439	0.103	0.258
100/100	0.101	0.063	0.124	0.173	0.133	0.082	0.079	0.048	0.064	0.000	0.025	0.032
	*	*	*	*	*		*		*		*	
Aat-1												
112/112	0.342	0.453	0.726	0.086	0.319	0.655	0.725	0.651	0.886	0.666	0.949	0.720
112/100	0.557	0.484	0.150	0.750	0.567	0.263	0.275	0.313	0.080	0.334	0.026	0.247
100/100	0.101	0.063	0.124	0.164	0.114	0.082	0.000	0.036	0.034	0.000	0.025	0.033
	*	*	*	*					*	*	*	

TABLE 1. Continued.

Locus/allele§	Sampling period‡											
	1990						1991					
	Week 6		Week 12		Week 18		Week 52		Week 58		Week 64	
	Rep 1 (79)‖	Rep 2 (64)	Rep 1 (113)	Rep 2 (81)	Rep 1 (113)	Rep 2 (109)	Rep 1 (51)	Rep 2 (83)	Rep 1 (88)	Rep 2 (66)	Rep 1 (78)	Rep 2 (93)
Acon-1												
109/109	0.342	0.453	0.726	0.062	0.212	0.564	0.745	0.639	0.875	0.636	0.859	0.720
109/100	0.557	0.484	0.150	0.778	0.612	0.355	0.255	0.325	0.068	0.364	0.115	0.243
100/100	0.101 *	0.063 *	0.124 *	0.161 *	0.176	0.081	0.000	0.036	0.057 *	0.000 *	0.026 *	0.033
Mean allele frequency#	0.621	0.695	0.801	0.442	0.554	0.745	0.834	0.800	0.901	0.767	0.927	0.841
G_{st}††	0.012		0.130‡‡		0.184‡‡		0.007		0.039‡‡		0.018‡‡	
Gambusia holbrooki												
mtDNA frequency	0.667	0.937	0.832	0.815	0.847	0.899	0.941	0.795	0.942	0.815	0.936	0.945
F_{st}§§	0.155‡‡		0.001		0.005		0.042‡‡		0.040‡‡		0.001	

† Each replicate represents one small pool.
‡ Each 6-wk sampling period was assumed to be one-half a generation based on laboratory breeding studies (Scribner 1992).
§ Allelic designations follow Scribner and Avise (1993a,b).
‖ Sample size.
Mean frequency of *G. holbrooki* alleles over five nuclear loci.
†† Measures of divergencce in nuclear gene frequency between replicates. Significance (‡‡) calculated as described by Workman and Niswander (1970).
§§ Measures of divergence in mtDNA haplotype frequency between replicates. Significance (‡‡) calculated as described by DeSalle et al. (1987).
* Significant deviation ($P < 0.05$) of genotypic frequencies from Hardy-Weinberg expectations.

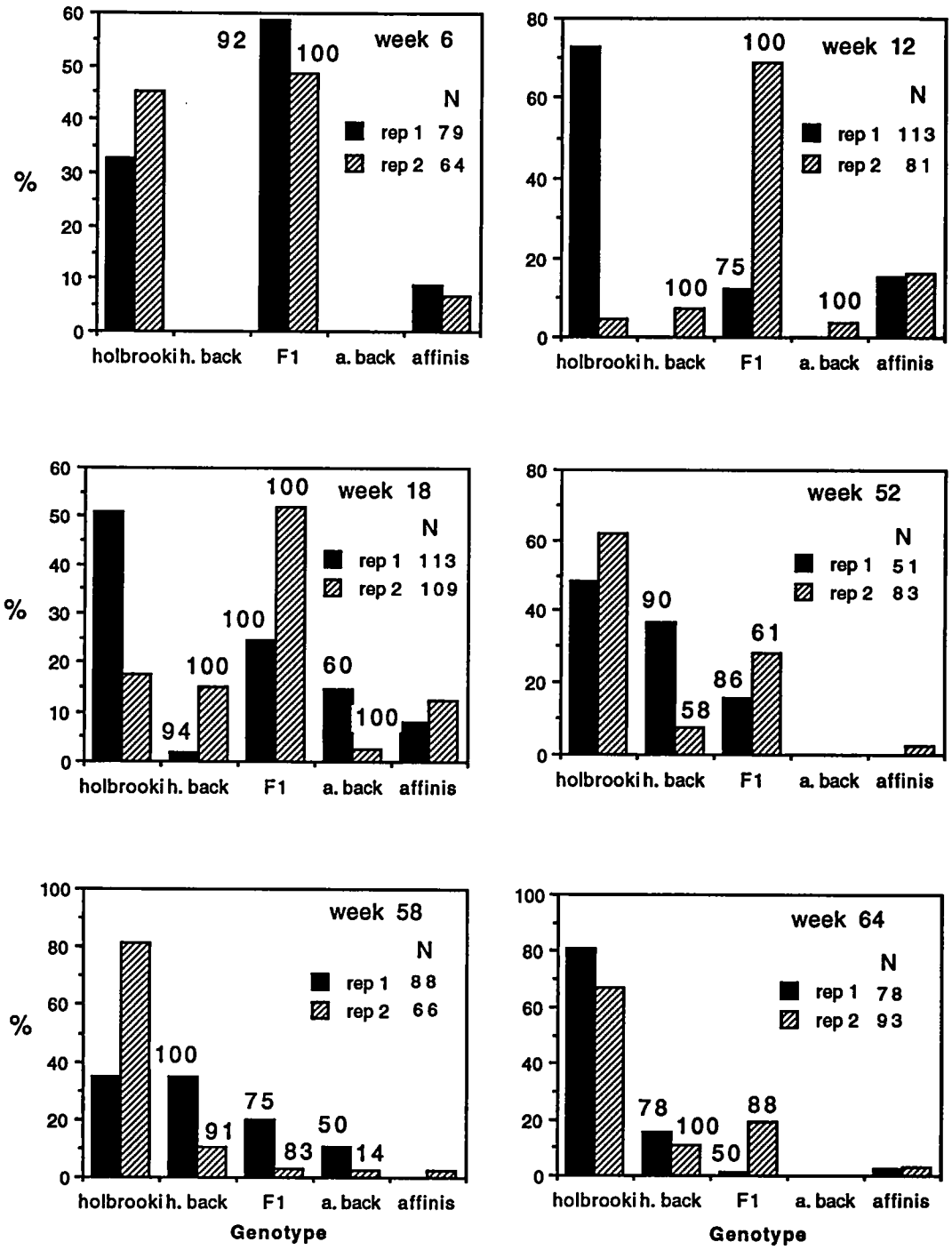

FIG. 3. Histograms showing temporal changes in frequencies of pure parental genotypes, F_1s, and backcrosses, all as indicated by multilocus allozyme composition, for each of two replicate sets of small-pool populations (experiment I). Frequencies of *Gambusia holbrooki* mitochondrial DNA (mtDNA) (×100) in various hybrid classes are indicated by numerical values above the histogram bars.

most individuals were either parental *G. holbrooki* or first generation hybrids with relatively few parental *Gambusia affinis*. At week 12, a large interpopulation G_{st} (table 1) was caused by interreplicate differences in proportions of pure *G. holbrooki* and F_1s (fig. 3). Backcrosses were first observed at this time, whereas parental *G. affinis* were again present only in low frequency. By week 18, the frequency of backcross individuals had increased somewhat, though large interreplicate differences in frequencies of pure *G. holbrooki*, F_1s and backcrosses were observed. After overwintering, populations sampled at week 52 showed further declines in *G. affinis* nuclear alleles and genotypes. From that point forward, most remaining *G. affinis* alleles were present either in F_1 or backcross genotypes. Significant variance among replicates was observed at week 58, but the low frequency of pure *G. affinis* and *G. affinis* backcrosses was consistent throughout the second year (fig. 3).

Most hybrids (84.9% of F_1s and backcrosses) in the pool populations possessed *G. holbrooki* mtDNA (fig. 3), suggesting strong directional selection favoring *G. holbrooki* females or their progeny from interspecific crosses. The low frequency of *G. affinis* mtDNA in hybrid progeny and overall shows that *G. affinis* females were contributing disproportionately fewer offspring to the pool populations than were *G. holbrooki* females.

Significant nuclear and cytonuclear disequilibria (figs. 4, 5) were observed for all pool populations. Nuclear disequilibria declined monotonically from initial stocking ($\Delta_{AB} = 0.250$) to values of 0.068 and 0.129 at week 64 for replicates 1 and 2, respectively (fig. 4). Decay of disequilibria was somewhat less than expected under random mating (fig. 4), a result presumably attributable to the preponderance of pure *G. holbrooki* genotypes in most periods (fig. 3).

Cytonuclear disequilibria (D, D_1, D_2, D_3) also were significantly different from zero (fig. 5). Gametic disequilibrium (D) declined from initial stocking values of 0.250 to values of 0.028 and 0.032 at week 64 in replicates 1 and 2, respectively. The D_1 genotypic disequilibrium values remained consistently positive, indicating an association of *G. holbrooki* mtDNA with *G. holbrooki* nuclear homozygotes. Genotypic disequilibrium values D_2 were positive during the first three sampling periods, indicating excess numbers of heterozygotes possessing *G. holbrooki* mtDNA but became negative in 1991.

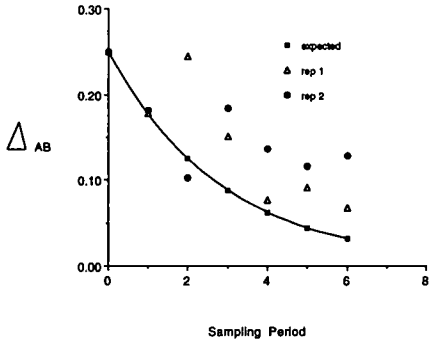

FIG. 4. Temporal estimates of composite gametic disequilibria for nuclear gene comparisons (Δ_{AB}, Weir 1979) for two replicate sets of small-pool populations (experiment I). The solid line represents expected values of Δ_{AB} under random mating.

Values of the genotypic disequilibria D_3 were negative, indicating a tendency for association of *G. affinis* mtDNA with *G. affinis* nuclear homozygotes. Whereas some interreplicate variation in cytonuclear disequilibria was observed, the signs and magnitudes of the statistics were consistent across replicates (fig. 5).

Observed cytonuclear disequilibrium values were compared with those expected under models of random and of assortative mating (fig. 5). Neither model adequately predicts the observed changes ($\chi^2/C = 115.7$, df = 30, $P < 0.01$ for random mating; $\chi^2/C = 95.2$, df = 30, $P < 0.01$ for assortative mating). χ^2 statistics were calculated based on generation times of 80 d and thus may be conservatively biased. Shorter generation times would alter expected genotypic counts but in a manner that would increase the test statistic. Although increased temperatures may in fact decrease generation interval (see above), the timing of sampling and lack of additional specific information precluded the use of estimates other than those empirically estimated in the laboratory (Scribner, unpubl. data).

In contrast to patterns of temporal change in composite nuclear disequilibria, the initial decay of cytonuclear disequilibria was greater than expected under models of assortative or random mating. Lack of fit to these models together with the rapid unidirectional changes in cytonuclear genotypic frequencies suggests that strong directional selection underlies the evolutionary changes in multilocus genotype structure.

Experiment II

Population numbers in the two large ponds exhibited qualitatively similar seasonal changes

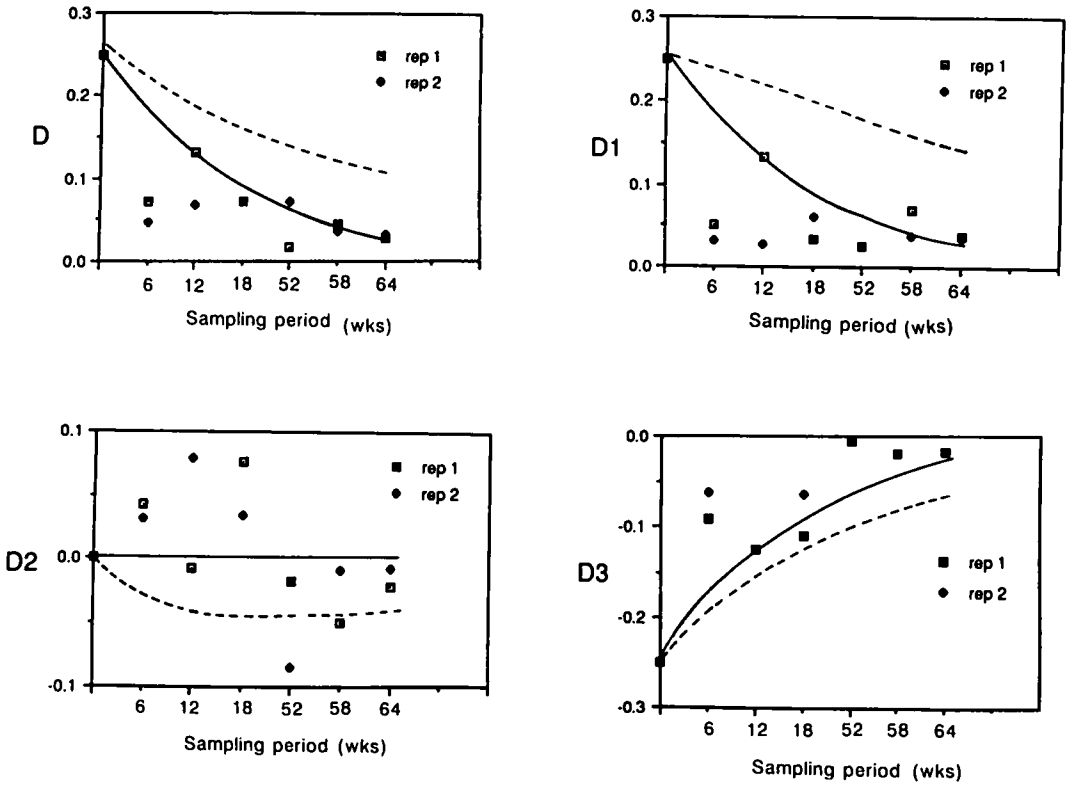

FIG. 5. Temporal estimates of cytonuclear disequilibria (Asmussen et al. 1987) for two replicate sets of small-pool populations (experiment I). Solid and dashed lines represent expected changes in gametic (D) and genotypic (D_1, D_2, and D_3) disequilibria under random and assortative mating (with assumed assortative mating propensities $\alpha = 0.72$ and $\beta = 0.24$, respectively).

(fig. 6) to those of the small pools. Population sizes expanded in the first summer, declined greatly over the winter, and remained relatively constant thereafter.

Changes in allelic and genotypic composition through time in the pond cohorts again reflected a rapid loss of *G. affinis* nuclear and mitochondrial alleles (table 2, fig. 7). At week 6 (cohort 2), individuals were almost exclusively "pure" parental types, with *G. holbrooki* predominating. Prevalence of parental types continued through week 19 (cohort 4), after which pure *G. affinis* declined in frequency. First-generation hybrids in the large ponds never achieved the high frequencies initially observed in the small pools. In contrast to results from experiment I, a greater proportion of hybrids possessed *G. affinis* mtDNA [57.4% versus 15.3% (compare figs. 7 and 3)]. Estimates of multilocus gametic and cytonuclear disequilibrium were not calculated, as only one segment of the populations (neonates) was sampled during each period.

DISCUSSION

In most prior genetic analyses of hybrid zones, researcher have had to draw evolutionary inferences from static or short-term observations in uncontrolled natural settings. Typically, estimates of the relative importance of various factors that can influence hybrid zone dynamics (e.g., strength and mode of selection, dispersal, and mating behavior) have been based on the spatial distribution of genetic markers within and adjacent to areas of species contact (Barton 1979; Mallet et al. 1990; Szymura and Barton 1986, 1991). In contrast, experimental settings allow for considerable control of influencing variables and thus offer special advantages for the study of microevolutionary processes. Experimental designs have been successfully used to monitor, for example, the temporal population dynamics of mitochondrial DNA (mtDNA) genotypes in *Drosophila* (MacRae and Anderson 1988), and population demography and genetic drift in in-

breeding and outbreeding *Gambusia* (Leberg 1990, 1992). However, to our knowledge, this is the first use of a "population-cage" approach to experimentally study the temporal genetic dynamics of vertebrate hybridization.

Rate and Direction of Genetic Change

Changes in Gene Frequency.—The mtDNA and allozyme data from the experimental pools and ponds demonstrate that frequencies of uni- and biparentally inherited genetic characters can change very rapidly following secondary contact between *Gambusia affinis* and *Gambusia holbrooki*. Within approximately 56–72 wk of founding (ca. four generations), experimental populations had undergone a remarkable series of genetic changes, generally as follows. By week 6, F_1 hybrids had appeared and in some cases were abundant, and frequencies of pure *G. affinis* genotypes had declined dramatically to less than one-half their initial frequencies. By weeks 12–19, backcrosses to both *G. affinis* and *G. holbrooki* had made their appearance and were to increase to frequencies as high as 0.30 by the end of the first summer. These genetic changes were accompanied by great expansions in population size. Over the first winter, population numbers

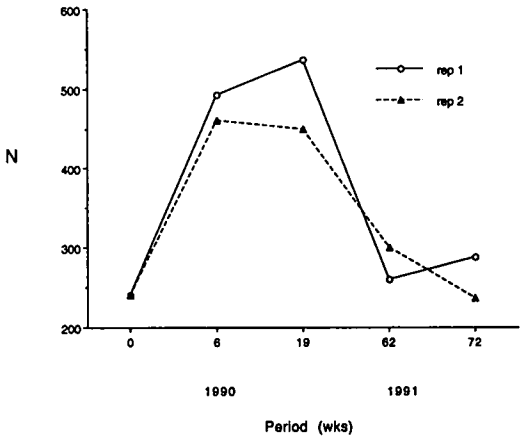

FIG. 6. Estimates of adult population size for two large-pond populations of *Gambusia affinis* and *Gambusia holbrooki* in experiment II.

TABLE 2. Genotypic frequencies for five diagnostic allozyme loci and the species-specific mitochondrial DNA (mtDNA) haplotypes for *Gambusia* juveniles sampled from specific cohorts over two years from each of the two large-pond populations.

	Cohort 2		Cohort 4		Cohort 6		Cohort 8	
Locus/allele	Rep 1 (N = 118)	Rep 2 (N = 110)	Rep 1 (N = 116)	Rep 2 (N = 93)	Rep 1 (N = 102)	Rep 2 (N = 102)	Rep 1 (N = 98)	Rep 2 (N = 102)
Pep-A								
114/114	0.703	0.818	0.595	0.430	0.529	0.539	0.755	0.755
114/100	0.028	0.036	0.147	0.172	0.275	0.176	0.123	0.157
100/100	0.266	0.146	0.258	0.398	0.196	0.285	0.122	0.088
Ada								
130/130	0.703	0.818	0.593	0.440	0.520	0.490	0.735	0.745
130/100	0.028	0.036	0.133	0.187	0.275	0.265	0.153	0.176
100/100	0.266	0.146	0.274	0.373	0.245	0.245	0.112	0.079
Mdh-1								
80/80	0.703	0.818	0.611	0.458	0.500	0.510	0.735	0.682
80/100	0.028	0.036	0.115	0.167	0.314	0.245	0.143	0.186
100/100	0.266	0.146	0.274	0.375	0.186	0.245	0.122	0.132
Aat-1								
112/112	0.703	0.818	0.603	0.484	0.490	0.510	0.755	0.725
112/100	0.208	0.036	0.138	0.187	0.314	0.245	0.153	0.176
100/100	0.266	0.146	0.259	0.329	0.196	0.245	0.092	0.099
Acon-1								
109/109	0.703	0.818	0.621	0.473	0.529	0.490	0.694	0.735
109/100	0.208	0.036	0.121	0.172	0.275	0.245	0.194	0.186
100/100	0.266	0.146	0.258	0.355	0.196	0.265	0.122	0.078
Mean *G. holbrooki* allele frequency	0.716	0.836	0.670	0.545	0.659	0.624	0.811	0.817
Mean *G. holbroki* mtDNA frequency	0.729	0.827	0.678	0.495	0.602	0.559	0.777	0.802

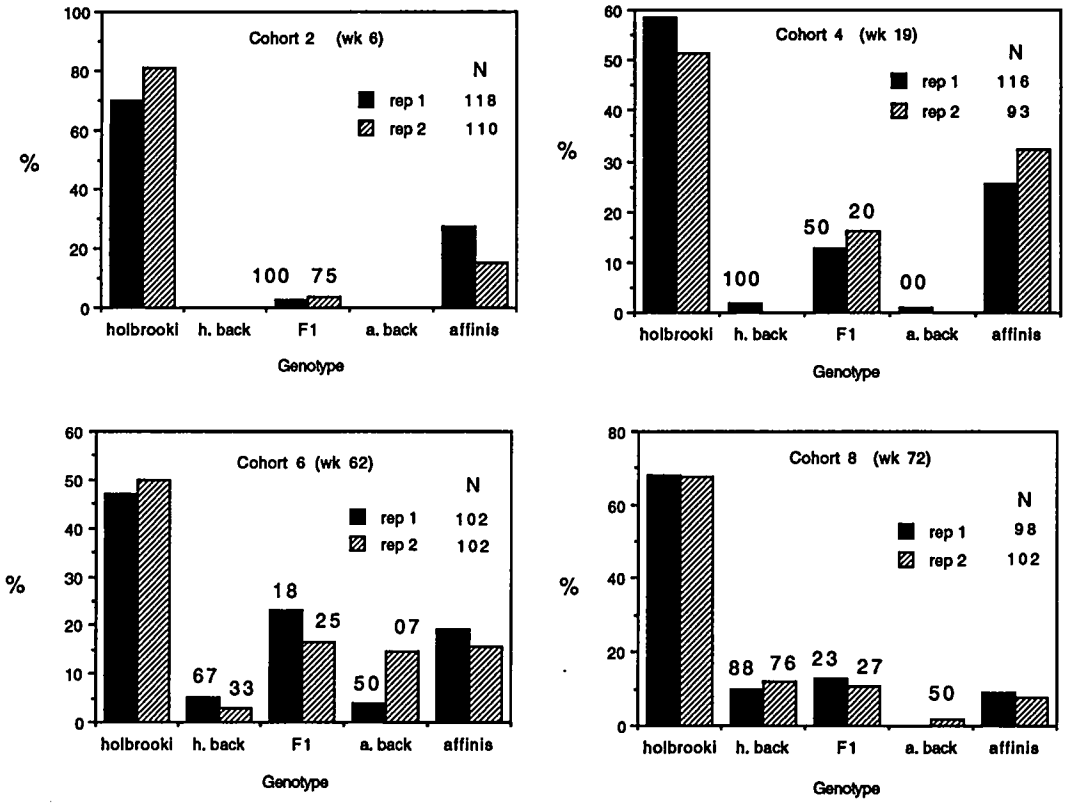

FIG. 7. Histograms showing temporal changes in frequencies of pure parental types, F_1s, and backcrosses, all as indicated by multilocus allozyme composition, for each of two large pond populations (experiment II). Frequencies of *Gambusia holbrooki* mitochondrial DNA (mtDNA) (× 100) in various hybrid classes are indicated by numerical values above the histogram bars.

fell dramatically. Evidently, differences in genotype-specific survival (Scribner 1993) resulted in a further decline in the frequency of pure *G. affinis* genotypes and sharp increases in pure *G. holbrooki* forms. During the second summer, populations stabilized in size, but the decline of pure *G. affinis* genotypes continued. By the end of the second summer, *G. holbrooki* mtDNA and allozyme alleles greatly predominated (mean population frequencies ranged from 0.78–0.94 among experiments), and most of the few remaining *G. affinis* alleles were present in interspecific F_1 hybrids or in recombinant genotypes.

Measures of Gene Association.—Strong associations among genes, chromosomes, and morphological characters are observed in many hybrid zones (Barton and Hewitt 1985; Nagylaki 1976). Disequilibria at any time result from the history and balance of forces that generate and decay genotypic associations. At founding, populations in overlap zones between potentially hybridizing species normally exhibit high nuclear and cytonuclear disequilibria caused by the preexisting multilocus associations that developed in allopatry. Hybridization and introgression will tend to decay these disequilibria at rates influenced by mating propensities and selection for or against recombinant genotypes, whereas continued immigration of pure parental genotypes into the hybrid zone will tend to counter disequilibria decay. Measures of genetic disequilibria have been used to estimate diffusion rates into hybrid zones (Szymura and Barton 1986) as well as to make inferences about the nature of selection and mating preferences (Asmussen et al. 1989; Lewontin 1974; Birley and Haley 1987; Mallet et al. 1990).

In this study, nuclear gametic disequilibria decayed from initial stocking conditions more slowly than expected under random mating, caused largely by a preponderance of pure *G. holbrooki* genotypes in later time periods that has

the net effect of diminishing effective recombination. Taken alone, this observation might indicate some degree of assortative mating or selection favoring *G. holbrooki*. In contrast, cytonuclear disequilibria initially declined more rapidly than expected under random or positive assortative mating. Studies of female mate choice (Scribner unpubl. data) suggest that female *G. holbrooki* mate assortatively with males of their own species, whereas female *G. affinis* mate randomly with regard to genotype. This behavioral asymmetry suggests that F_1 hybrids should show a preponderance of *G. affinis* mtDNA, yet 88% of all hybrids in experiment I carried *G. holbrooki* mitochondria. This conundrum raises the likelihood that rates of change in allele frequency and decay of disequilibria in the pool populations include selection pressures other than mating preferences per se.

Evolutionary Processes Governing Genetic Change

Genetic Drift.—Few empirical studies have monitored temporal changes in both nuclear and cytoplasmic loci (but see Chapman 1989). In spatial genetic surveys conducted at a single point in time, mtDNA differentiation among conspecifics usually exceeds that registered in nuclear (typically allozyme) assays (Avise and Saunders 1984; DeSalle et al. 1987; Moritz et al. 1987). This observation may be attributable to a lower effective population size for maternally inherited mtDNA, but differences in sensitivities of restriction site versus electromorph assays, differences in rates of accumulation of de novo mutations, and differences in levels of male versus female dispersal probably also play important roles in some cases. In the present study, all microevolutionary changes involved shifts in frequencies of preexisting nuclear and mitochondrial polymorphisms in closed populations. Thus, under genetic drift, any consistent differences in the rates of such changes presumably would be attributable to the disparity in effective population size between nuclear and mitochondrial genes.

Several lines of evidence demonstrate conclusively that these genetic changes did not result solely from random drift. First, the variances in allele frequency across loci and across replicates did not increase consistently over time (table 1), and furthermore, interreplicate divergences revealed no tendency for a greater differentiation in mtDNA than in the nuclear markers. Second, the consistent pattern and direction of change in allele frequencies for mtDNA and for all five allozyme loci across all experiments clearly implicate nonrandom evolutionary forces. Invariably, changes in genetic composition were in the direction of increase for *G. holbrooki* alleles and genotypes. Third, under genetic drift alone no loss of experimentwide gene diversity would be expected but rather a reapportionment of existing diversity into between-replicate differences. However, levels of mtDNA and nuclear genetic diversity declined over time further reflecting consistent loss of *G. affinis* alleles.

Selection.—One striking feature of both experiments was the rapid increase in frequency of *G. holbrooki* mtDNA. This trend appears consistent with results from other laboratory breeding experiments conducted concurrently with this study (Scribner 1993), in which genotype-specific differences in life-history traits and differences in population ecology of "mixed" and parental populations were observed. These breeding studies revealed that homo- and heterospecific offspring from *G. holbrooki* mothers exhibited significantly larger size at birth, faster growth rates, and greater size and younger age at sexual maturity than did their counterparts from *G. affinis* mothers (see also Reznick 1981). These life-history features could place offspring of *G. holbrooki* mothers at a selective advantage, for example by lowering the risk to mortality factors including cannibalism (which increases in density-dependent fashion and may be a major factor contributing to the outcome of interspecific contacts). Such advantages to *G. holbrooki* maternal lineages might be accentuated in the high-density, resource-scarce environmental conditions prevalent through most sampling periods in both the pool and pond experiments.

In experiment II, the raw genotypic data (fig. 7) indicate a somewhat lower incidence of successful hybridization and a lower proportion (43%) of hybrids containing *G. holbrooki* mtDNA. One possibility is that the larger spatial scale of experiment II afforded greater opportunities for mating preferences to be expressed. In addition, neonates were sampled from refugial areas before significant predation, and this might have influenced the genetic results. In contrast, in experiment I, the sampling of entire replicate populations must have reflected differences in juvenile mortality rates in addition to the effects of selection based on other genotypic-specific life-history differences.

Comparisons of Experimental and Field Results

In a previous study, Scribner and Avise (1993a) found that some populations of *Gambusia* in the southeastern United States carry apparent nuclear and cytonuclear recombinants between *G. affinis* and *G. holbrooki* genomes. For example, nuclear markers characteristic of *G. holbrooki* were observed in low frequency in *G. affinis* populations somewhat in advance of a mitochondrially defined contact zone between these species, and nuclear alleles normally characteristic of *G. affinis* were found in low frequency in "*G. holbrooki*" populations. One explanation for the observed discordance in spatial distribution of mtDNA and nuclear markers is differential introgression of female- versus biparentally inherited genes resulting from either sex-biased gene flow or lack of reciprocity in hybrid fertility or viability (i.e., asymmetry in direction of selection). Differential introgression could also characterize different nuclear loci, either through varying selection pressures across loci or chance sampling effects (Hunt and Selander 1973). The presence of *G. affinis* nuclear alleles in *G. holbrooki* populations could have resulted from independent mutations to shared electromorphs, retention of ancestral polymorphisms that predate the population separations, or the evolutionary "footprints" of a moving hybrid zone.

The "population cage" experiments demonstrate unequivocally that *G. affinis* and *G. holbrooki* can and do hybridize when placed into population contact. Furthermore, recombinant genotypes are produced via backcrossing, and it is not difficult to imagine that such introgressive hybridization could provide a means for occasional movements of nuclear or cytoplasmic alleles from one species to the other in nature. However, a major feature of the experimental pools and ponds was the rapid shift in allele frequency toward pure *G. holbrooki,* with little indication of a genetic stabilization at intermediate genotypic frequencies. Thus, by hard criteria, the present experimental results cannot eliminate alternatives to introgression scenarios in accounting for some of the genotypic patterns observed in nature.

Conclusions

Results of the "population cage" experiments reported here are one component of our multifaceted research program on the *G. affinis–G. holbrooki* complex. Prior work has included descriptive genetic surveys of natural populations, and laboratory observations and breeding experiments designed to illuminate reproductive features and other life-history characteristics of potential relevance to the hybridization process. Overall, perhaps the most important take-home message from this research effort is that species-specific population demographies and life histories can influence the dynamics of hybrid zones to a greater degree than generally has been appreciated. In the controlled experimental settings employed, *G. holbrooki* appears to possess several demographic advantages over *G. affinis,* including a higher population carrying capacity, higher rate of recruitment, and lower overwinter mortality. Furthermore, the offspring of *G. holbrooki* mothers exhibit significantly larger size at birth, faster growth rate, and greater size and younger age at sexual maturity than do their counterparts from *G. affinis* mothers, and these differences likely translate into fitness advantages including a diminished susceptibility to cannibalism. Such reproductive and population-demographic differences between *G. affinis* and *G. holbrooki* no doubt have contributed importantly to the dramatic and consistent temporal shifts in cytonuclear genetic composition observed over 2 yr in the experimental pool and pond populations.

Competitive processes are known to play an important role in molding the structure of freshwater fish communities. Interactions within and between species can influence individual growth rates, size distributions, and age- or size-specific mortality and reproductive rates (Evans et al. 1987), which are often magnified in situations of increasing density or resource limitation. Conversely, differences in phenotypic, life-history, or behavior traits also can affect competitive interactions among species and result in changes in the composition of mixed species assemblages (Roughgarden 1977). Our research on the temporal dynamics of hybridization in *Gambusia* species indicates that such population demographic and life-history differences can also dramatically affect the rate and direction of evolutionary genetic change within regions of secondary contact between hybridizing taxa.

Nonetheless, caution is required in extrapolating our experimental results to field situations, because only short time scales were monitored, and potential complicating factors (such as continuing gene flow from pure parental sources and greater heterogeneity in the physical environ-

ment) were intentionally restrained. Many hybrid zones in nature probably are maintained by a balance between dispersal and selection, the very factors that were eliminated or partially controlled, respectively, in the current experiments.

Furthermore, selection may operate differently in contrasting environmental regimes by favoring alternative alleles or genotypic combinations. Previous studies of intra- and interspecific competition in fish communities have stressed the importance of environmental factors in determining the intensity and outcome of competition (Werner and Gilliam 1984). Studies of introgressive hybridization in *Gambusia* (Hubbs 1959; Hubbs and Delco 1962; Hubbs and Peden 1969) also have emphasized the importance of ecological factors in maintaining species integrity in sympatry. For example, Scribner and Avise (1993a) showed that in Atlantic and Gulf coastal drainages within the natural *G. affinis–G. holbrooki* contact region, *G. affinis* predominates in the headwaters whereas *G. holbrooki* generally predominates in the coastal plains. By exhibiting stochastic temporal variation, environments may affect the outcome of interspecific contacts by influencing the degree of resource limitation, which in turn can influence any processes associated with density dependence and niche divergence. Thus, in conclusion, a deeper understanding of the genetic outcomes of species contacts and hybridization in *Gambusia* (or other taxa) will require that the temporal processes be monitored over a broad range of environmental conditions.

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