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Abstract

There are few events in nature that are more predictable than the emergence of periodical cicadas. The insects emerge from the ground after 13 or 17 years (depending on brood and species) of development. Karlin et al., (1991) biochemically examined over 750 *Magicicada tredecassini* belonging to Brood XIX which emerged during the spring of 1985. In this study they found evidence for rapid deterioration of heterozygosity for two esterase loci, Gi-3-pdh and Gpi, and suggested that this deterioration may be related to differential mating classes. To test this hypothesis, we re-sampled from this same brood at the same location during fall (1993) and winter (1994), nine years into the 13 year development of this brood. The current biochemical data suggest no significant deviations from Hardy-Weinberg expectations for either Est-3, Gl-3-pdh or Pgm-1, but in several cases Est-1 or Est-2 displayed significant departures. Our failure to find excess heterozygosity in the nymphal sample is interpreted to support weakly the size-mediated mating system hypothesis.

Introduction

There are three morphologically identifiable species of periodical cicadas (genus *Magicicada*) each of which has 13- and 17-year life cycle forms. These are considered as six separate species (Alexander and Moore, 1962), although the recent findings for the Decim pair calls this treatment into question (Martin and Simon, 1988, 1990; Simon et al., 1993).

Periodical cicada year classes termed "broods", have been analogized with incipient species because adults of each year class are temporally reproductively isolated from all other year classes (Simon, 1988). Members of a year-class are synchronized. They emerge from the ground within a two week period with most of the individuals emerging during days two and three (Karban, 1982). The emergence occurs regularly, every 13 or 17 years depending on particular location (Marlatt, 1907; Alexander and Moore, 1962; Lloyd and Dybas, 1966; Lloyd and White, 1976). Thus, for the 17-year cicadas potentially 17 different year classes could exist (broods I-XVII), and for 13-year cicadas there could be 13 different reproductively isolated broods (XVIII-XXX) (Marlatt, 1898). Currently, only 12 17-year broods and three 13-year broods are known to exist (Simon, 1988). In this study we focused our attention on one species, *Magicicada tredecassini*, from Brood XIX in northwestern Arkansas.

In a previous study, Karlin et al. (1991) examined almost 500 individuals captured as they emerged from the ground and another 500 individuals captured three weeks later in chorus centers for several biochemical traits. They interpreted the data to suggest that heterozygosity for two esterase loci (Est-1 and Est-3), glyceraldehyde-3-phosphate dehydrogenase (Gi-3pdh), and glucose phosphate isomerase (Gpi) decreased markedly between sample periods. They proposed that the observed decline in heterozygosity could be a result of a phenotypic mating system linked to individual size as proposed by Karban (1982). If the larger (or smaller) males are more (or less) heterozygous, one class may mate earlier and hence be removed preferentially from the chorus population sample. Observations in support for this hypothesis include finding the larval population in Hardy-Weinberg equilibrium for the genes in question. Although finding Hardy-Weinberg equilibrium is a weak demonstration of support, if the genes are not in equilibrium frequencies, we can strongly reject the hypothesis.

One alternative hypothesis would implicate a constant decline in heterozygosity over the life cycle of the periodical cicadas. This hypothesis leads us to predict a heterozygote excess for these genes in the larval population allowing for the decline we observed in the adult population. Thus, we can use heterozygote excess as detected by deviations from Hardy-Weinberg expectations to support this hypothesis.

Materials and Methods

Nine-year-old nymphs of 13-year periodical cicadas (*Magicicada tredecassini*) were collected at a single locality, near Durham, Washington Co., Arkansas, in October, 1993 and February, 1994. The main study site used by Karlin et al. (1991) was revisited for this study.

Oak trees (*Quercus* sp.), hickory trees (*Carya* sp.) and black locust trees (*Robinia pseudoacacia*) showing obvious evidence of cicada galleries (scaring on the outer branches) were selected. We carefully dug 10 - 20 cm. into the ground approximately 1 - 2 m from the tree base and searched from nymphs. Once an individual was located, careful excavation followed to locate additional individuals. To insure genetic diversity, we sampled 5 - 15 individuals from each of 10 trees. Individuals collected from a tree were placed together into a plastic bag filled with dirt. The bag was refrigerated on ice until returned to the laboratory at University of Arkansas - Little Rock. Once in the lab, the bags were frozen at -70°C until used.

On the morning of electrophoresis, individual cicadas were partially thawed and their total length measured with vernier calipers. Measurements were recorded to the nearest 0.1 mm. To prepare proteins for electrophoresis, the thorax was macerated in an equal volume of double distilled water. The resultant slurry was absorbed onto two filter paper wicks (Whatman #3, 5 x 11 mm) for inoculation into starch slabs. Horizontal starch gel electrophoresis and histochemical staining followed the procedures outlined in Karlin et al. (1991) and Selander et al. (1971). The gel buffers and stains were as reported by Karlin et al. (1991).

Electrophoretic data were recorded as inferred individual enzyme genotypes for each polymorphic protein. To determine whether the genotype frequencies were in agreement with Hardy-Weinberg expectations based on gene frequencies, the data were analyzed using BIOSYS-1 (Swofford and Selander, 1981). Chi-square probabilities were modified (Harris, 1985) to account for the number of tests performed and for small sample sizes. To determine gene frequency differences between populations samples we used Nei's genetic distance and identity estimates (Nei, 1978).

Results and Discussion

Of the total of 113 individuals surveyed for esterases, Gi-3-pdh, Gpi, and Pgm-1, we found that glucosephosphate isomerase (Gpi) was not interpretable in any of the nymphs. Similarly, beta-naphtyl proprionate esterase-4 (Est-4) was not consistently interpretable on our gels. Although some individuals were not scorable for some enzymes, we were able to score 112 individuals for Est-1, 108 for Est-2, 112 for Est-3, 113 for Gi-3-pdh, and 77 for Pgm-1. We found significant ($P < 0.05$ adjusted) departures from Hardy-Weinberg expectations for Est-1 and Est-2 when the entire sample was considered (Table 1). In these two cases, the departure resulted from excess homozygosity (Fixation Index = +0.276 and +0.429 for Est-1 and Est-2 respectively) for Est-1^{aa}, Est-1^{bb}, Est-2^{bb} homozygotes (Table 1). When we pooled all rare homozygotes and all rare heterozygotes for Est-1, no significant deviations from equilibrium frequencies were observed. Hence, only Est-2 genotypes were not in equilibrium frequencies after pooling. Because we only found two of the four known genes for Est-2 (Karlin et al., 1991) in the nymphal sample, the absence of those genes could have resulted in the skewed genotype frequencies. We consider these results to indicate strongly no departures from Hardy-Weinberg expectations for the five proteins studied. In terms of the original hypotheses, the absence of excess heterozygosity suggests that heterozygosity does not decline steadily throughout the life cycle of periodical cicadas.

Table 1. Genotype frequencies for 5 polymorphic loci in *Magicicada tredecassini* from Durham, Arkansas, 1994.

Locus	Genotype	Observed Frequency	Expected Frequency[1]	Chi-Square
Est-1	AA	2	0.53	
	AB	12	9.18	
	AC	0	4.66	
	AD	0	1.08	
	BB	45	36.45	
	BC	26	37.45	
	BD	0	8.61	
	CC	15	9.33	
	CD	9	4.38	
	DD	3	0.47	34.38* df=6
Est-2	AA	76	68.4	
	AB	20	35.2	
	BB	12	4.4	18.33* df=1
Est-3	AA	64	58.48	
	AB	34	45.04	
	BB	14	8.48	5.87 ns df=1
Pgm-1	AA	22	20.13	
	AB	35	38.73	
	BB	20	18.14	0.463 ns df=1
GI-3pdh	AA	82	79.80	
	AB	26	30.40	
	BB	5	2.8	1.569 ns df=1

1- Expectations are based on Hardy-Weinberg Equilibrium
*P <0.05

To evaluate gene frequency differences between the nymph population and the adult population sampled in 1985 (Karlin et al., 1991), we used genetic distance and identity estimates (Nei, 1978). Because we were not able to survey all of the enzymes as in Karlin et al., (1991), the adult data set was reduced to only those enzymes surveyed in the nymph population. Both the genetic distance (D = 0.03) and the genetic identity (I = 0.968) suggest that any differences in gene frequencies are due to chance. Although we found no differences in average

individual heterozygosity between the adult and nymph samples, Karlin et al., (1991) observed more alleles in the adult population. This was expected as a result of the smaller sample from the nymph population.

Karlin et al. (1991) suggested that the differences observed between emerging and chorus samples could have resulted from preferential mating patterns linked to individual size. Lloyd and Dybas (1966) found that cicada nymphs grow at different rates. Often at one site individuals of the same age class were in different instar stages. That suggests that one class grows rapidly and "waits" up to four years for the "slower" growing nymphs to catch-up. It is thus possible that nymphs of different sizes emerge differentially, and as predation pressure on adults is severe (see Karban, 1982), genotypic differences between emergent samples and chorus samples may result.

In this study we found two different size-class nymphs. One class, 48 individuals, was comprised of small individuals usually less than 16.5 mm in total length, while the other class was comprised of large nymphs (larger than 16.5 mm, 64 individuals). When we separated the population by size class and recomputed genotype frequencies (Table 2), we found no differences in gene frequencies between the two groups (D= 0.000, I=1.000), nor did we found evidence for genetic heterogeneity (Chi-square = 5.53, df=7, ns) across all proteins. Furthermore, no departures from Hardy-Weinberg expectations were observed for any enzyme in the small nymphs, while only Est-1 and Est-2 demonstrated departures in the large nymphs (Table 2). Once again, in the large nymphs, the departures were due to homozygote excesses for Est-1^{bb}, Est-1^{cc}, Est-2^{aa}, and Est-2^{bb}, and pooling eliminated the homozygote excess for Est-1. Again, these departures are in the opposite direction of what we would expect if there was a continual reduction of heterozygosity, and we interpret them to be consistent with the differential mating hypothesis.

Table 2. Genotype Frequencies for Large (> 16.5 mm) and Small (< 16.5 mm) *Magicicada tredecassini* from Durham, Arkansas, 1994.

		Large Cicadas			Small Cicades		
Locus	Class	Obs. Freq.	Exp. Freq.	Chi-Square	Obs. Freq.	Exp. Freq.	Chi-Square
Est-1	AA	1	0.22		1	0.29	
	AB	6	4.72		6	4.46	
	AC	0	2.33		0	2.35	
	AD	0	0.51		0	0.58	
	BB	28	21.85		17	14.50	
	BC	13	21.85		13	15.62	
	BD	3	4.72		0	3.90	
	CC	10	5.24		5	3.97	
	CD	4	2.33	21.81 (6)	5	2.06	8.69 (6)
	DD	2	0.22	$P<0.05$	1	0.22	ns
Est-2	AA	46	40.75		27	27.66	
	AB	7	17.47	18.94 (1)	13	17.67	2.37 (1)
	BB	7	1.76	$P<0.05$	5	2.66	ns
Est-3	AA	32	29.45		32	29.21	
	AB	23	28.08	1.53 (1)	11	16.58	4.11 (1)
	BB	9	6.45	ns	5	2.21	ns
Pgm-1	AA	10	9.88		12	10.49	
	AB	21	21.24	0.00 (1)	14	17.01	0.62 (1)
	BB	11	10.88	ns	8	6.49	ns
Gi-3 pdh	AA	44	42.57		38	37.16	
	AB	14	16.86	0.901 (1)	12	13.67	0.19 (1)
	BB	3	1.57	ns	2	1.16	ns

Combined Chi-Square of Homogeneity = 5.527 (df=7) ns

Finally, during the course of this investigation, we noticed that several individuals were infected with what appeared to be the fungal pathogen *Massospora cicadina*. We later identified the organism as a pathogenic bacterial agent, tentatively identified as *Bacillus sp.* Approximately half-way through the study, we noticed that the incidence was rather high, and at that point we began to keep records of infected vs. non-infected individuals. In the sub-sample (53 nymphs) in which we recorded pathogen incidence, we found 43% to be infected. We divided those genotypic data by presence of infection and reanalyzed the frequencies. Again we found no heterogeneity (Chi-square = 4.38, df=7, ns) and no differences in gene frequencies (D=0.00, I=1.00) between infected and non-infected individuals. Similarly, the groups did not differ from each other with respect to genotype frequencies (Table 3). We suggest that although this pathogen is not currently known to be a source of mortality, its high incidence suggests that it may have a role in regulating this population. As it produced no apparent genotypic bias in our study we suggest it represents a random factor.

Our observations that Est-2 demonstrated departure from Hardy-Weinberg equilibrium in the nymphs but not in the adults, and our failure to resolve Gpi in the nymphs may suggest specific gene expression in the nymphs. The results lead us to suggest that the genotype frequencies in the nymph population are in close agreement to expectations. Thus, we can not reject our initial hypothesis. We can, however, reject the notion that heterozygosity steadily decreases in this cicada population.

Table 3. Genotype Frequencies for *Magicicada tredecassini* infected with *Bacillus sp.* and those without infection from Durham, Arkansas, 1994.

		Infected Cicadas			non-Infected Cicades		
Locus	Class	Obs. Freq.	Exp. Freq.	Chi-Square	Obs. Freq.	Exp. Freq.	Chi-Square
Est-1	AA	1	0.22		0	0.10	
	AB	3	3.0		4	2.38	
	AC	0	1.55		0	1.19	
	AD	0	0		0	0.21	
	BB	9	7.80		12	9.84	
	BC	6	8.40		6	10.14	
	BD	0	0		0	1.79	
	CC	4	2.02		4	2.38	
	CD	0	0	2.63 (3)	3	0.89	6.83 (6)
	DD	0	0	ns	0	0.05	ns
Est-2	AA	19	16.46		24	21.61	
	AB	1	6.06	12.5 (1)	3	7.78	8.37 (1)
	BB	3	0.46	P<0.05	3	0.61	P<0.05
Est-3	AA	15	14.65		17	16.59	
	AB	6	6.67	0.006 (1)	10	10.81	0.01 (1)
	BB	1	0.65	ns	2	1.59	ns
Pgm-1	AA	3	2.69		1	1.78	
	AB	7	7.62	0.002 (1)	10	8.43	0.19 (1)
	BB	5	4.69	ns	8	8.76	ns
Gi-3 pdh	AA	18	16.46		20	20.63	
	AB	3	6.06	3.43 (1)	9	7.73	0.10 (1)
	BB	2	0.467	ns	0	0.63	ns

Combined Chi-Square of Homogeneity = 4.382 (df=7) ns

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