ISOZYME STUDIES OF TWO *MELANOCONION* MOSQUITOES, *CULEX OCOSSA* AND *CX. PANOCOSSA*.

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ABSTRACT. Isozyme profiles of mosquitoes from laboratory colonies of *Culex ocossa* and *Cx. panocossa* for 18 enzymes were made and compared. It is possible to differentiate the larvae, pupae and adults from these colonies by their distinct acid phosphatase (ACP), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (adults only), phosphoglucoisomerase (GPI) and zanthine dehydrogenase (XDH) isozyme patterns. The isozymes produced by other enzymes could not be used for specific identification, due to certain similarities in their isozyme migrations. Study of 100 field collected and taxonomically identified males, 86 *Cx. ocossa* and 14 *Cx. panocossa*, showed that each male of a particular species' field sample had an isozyme profile for ACP, GAPDH, GPI and XDH that was identical to the profile of the appropriate in colony species.

The isozyme profile of these 4 enzymes was used to specifically identify *Cx. ocossa* and *Cx. panocossa* collected from a mixed population of the 2 species collected in the Juan Mina area of central Panama. Adults attracted to man bait and larvae (later reared to adults) were collected at 8 sites. These specimens were specifically identified by isozyme analysis, and both larval and adult collections were shown to consist of 80% *Cx. ocossa* and 20% *Cx. panocossa*. Neither species had a greater attraction to man bait than would be expected from their species' densities.

INTRODUCTION

The name *Culex (Melanoconion) aikenii* (Aiken and Rowland, 1906) was considered to be a *nomen dubium* and the names *ocossa* Dyar and *panocossa* Dyar and Knab were resurrected from synonymy with *aikenii* (from Dyar 1928) and applied to the two species that were recognized under that name (Belkin 1970).

At Gorgas Memorial Laboratory (GML), it was demonstrated that *Cx. aikenii* (in the sense of Dyar 1928) was a natural vector of Venezuelan equine encephalitis virus (VEE) (Galindo and Grayson 1971) and that *Cx. ocossa* and *Cx. panocossa* could be experimentally infected with the virus (L. Kramer, unpublished).

These two species, which have been in colony at GML for over 7 years (Adames and Galindo 1972), at present can be identified with certainty only by characters on the male terminalia. On the Atlantic watershed, the Panama Canal roughly marks the boundary of their distributions, *Cx. ocossa* to the east and then south and *Cx. panocossa* to the west and then north. On the Pacific side the species are allopatric, *ocossa* east of the province of Chiriqui and *panocossa* in western Chiriqui. In one Atlantic area near the Panama Canal, Juan Mina, these species are sympatric. Precipitin tests of blood engorged females collected in the Juan Mina area and identified as "*Cx. aikenii*" and *Cx. ocossa/panocossa* showed the presence of human blood (Tempelis and Galindo 1975 and Christensen, personal communication). If a method were available to identify adult females of these species, the Juan Mina population could be used to obtain information on their host preference, that is, whether 1 of the 2 species has a greater attraction to man, and therefore would present a greater threat as a VEE vector in the area.

Studies on specific identification of mosquitoes by electrophoresis have demonstrated that biochemical characters can be used in association with other

taxonomic characters to identify specimens of morphologically similar species. Miles (1978) tested over 20 enzymes on mosquitoes in the *Anopheles gambiae* group of species, and reported that certain of the species could be identified by species-specific unique alleles for certain enzymes. In a study by Saul et al. (1977a) the authors reported an electrophoretic method of identifying adults of 2 species of *Aedes* which are similar in appearance and are sympatric throughout most of their range, one most probably a vector of La Crosse encephalitis the other not. In another paper by Saul et al. (1977b) certain species of *Culex* were identified by electrophoresis. Studies by Mahon et al. (1976) and Bullini and Colluzi (1973) note certain unique allozymes which can be used to identify species of the *An. gambiae* complex. The work by Bullini and Coluzzi includes a review of many other electrophoretic studies which have been done on mosquitoes. Trebatoski and Haynes (1969) compared the enzymes of 12 species in 3 genera of mosquitoes, and obtained species-specific patterns for all 12 species with certain enzymes. In addition to these studies on species identification in mosquitoes, studies have been reported on specific identification of *Drosophila* by electrophoresis (Ayala and Powell 1972 and Ayala et al. 1972).

In an attempt to identify adult females of *Cx. ocossa* and *Cx. panocossa* their isozyme patterns from cellulose acetate electrophoresis were studied.

MATERIALS AND METHODS

The laboratory colonies of *Cx. ocossa* and *Cx. panocossa* were maintained under almost identical conditions in 2 different buildings at GML. The *Cx. ocossa* colony was derived from material collected near Tocumen airport, about 15 km NE of Panama City, and the *Cx. panocossa* colony was established from specimens collected at Juan Mina, on Gatun Lake, about 20 km NW of Panama City. The larvae were fed dry yeast (one tablet per approximately 200 larvae per pan per 7 days). A single water lettuce leaf (*Pistia stratiotes* Linn.) was placed in each pan, and the water was constantly aerated. Pupae were collected and allowed to emerge in cages about 61 cm cube. Adults were fed on hamsters, and subsequent oviposition occurred in a pan containing *Pistia* leaves. Eggs were allowed to hatch and the larvae divided into groups of about 200.

Field collections of both adults and larvae were made at 8 sites in the Juan Mina area between September and December, 1978. The larvae were collected among the *Pistia* with dippers, and the adults were collected as they landed on the collector. Only a few adult specimens contained blood; therefore, only man bait attraction of these 2 species can be tested. The adults were immediately placed in a liquid nitrogen container, and both adults and larvae were transported back to GML. The adults, all females, were identified as *Cx. ocossa/panocossa* and then held at −70°C until their isozyme patterns could be tested. The larvae were identified as *Cx. ocossa/panocossa* and were reared in the laboratory. The adult females from the larval collections were identified as *Cx. ocossa/panocossa* and were stored at −70°C. One hundred of the males which were obtained from larval collections were specifically identified by characters on their terminalia. Males were identified to species by examination of the style under the highest magnification of the stereoscopic microscope.

ELECTROPHORESIS

A total of 18 enzymes were tested on the laboratory colony mosquitoes. Many of the enzyme procedures are similar to those described by Shaw and Prasad (1970) and Ayala (1975). These procedures were adapted for cellulose acetate electrophoresis and mosquito tissue (Kreutzer et al. 1977, Kreutzer 1979, Kreutzer and Christensen 1979 and 1980). The procedures for 5 additional enzymes adapted for mosquito tissue and cellulose acetate electrophoresis are pre-

sented in Table 1. The electrophoresis equipment was from Helena Laboratories, Beaumont, Texas.

Over 200 specimens of each species were run for each enzyme; however in many cases data for more than 1 enzyme were obtained from single mosquitoes. Each mosquito from the Juan Mina collections was tested for ACP, GAPDH, GPI, PGM and XDH, and specific identification of these specimens was made from the electrophoresis data.

In this study individual whole adult mosquitoes (occasionally larvae or pupae) were used to obtain isozyme information. It is possiblke to obtain isozyme data for certain enzymes (PGI and PGM) from a single leg of an adult.

Table 1. Conditions for electrophoresis and components for developing zymograms.

Enzyme	Cell buffer	Reaction buffer	Developing components[1]
FUM[2]*	1[3]	A	500 mg Fumaric acid (K salt); 15 mg β-nicotinamide adenine dinucleotide (DPN); 15 mg MTT tetrazolium (MTT); 10 mg Phenazine methosulphate (PMS); 200 units Malic dehydrogenase (Porcine Heart).
α-GDH	2[3]	B	50 mg α-glycerophosphate (Na_2 salt); 15 mg MTT; 15 mg β-DPN; 10 mg PMS.
GAPDH	2[3]	B	80 mg Fructose-1,6-diphosphate (Na_4 salt); 15 mg MTT; 15 mg β-DPN; 10 mg PMS; 100 mg Sodium arsenate (Na salt); 35 units aldolase (Rabbit muscle).
ICD	2[3]	C	50 mg DL-Isocitric acid (Na_3 salt); 100 mg $MnCl_2$; 15 mg MTT; 15 mg β-nicotinamide adenine dinucleotide phosphate (TPN) (Na salt); 10 mg PMS.
XDH	2[3]	C	100 mg Hypoxanthine; 15 mg MTT; 15 mg β-DPN; 10 mg PMS.

OTHER ENZYMES[4]

Cell Buffers:

1. Add 0.2 M NaH_2PO_4 to 0.2M Na_2HPO_4 to reach pH 7.0.
2. 0.1 M Tris/0.1 TM Maleic acid/0.1 M EDTA (Na_2)/0.1 M $MgCl_2$; adjust to pH 7.4 with HaOH.

Membrane Buffers:

One part appropriate cell buffer to 14 parts distilled water.

Reaction Buffer:

A. Add 0.05M NaH_2PO_4 tp 0.05M Na_2HPO_4 to reach pH 7.0.
B. 0.03 M Tris; adjust to pH 8.0 with 50% HCl.
C. 0.03 M Tris; adjust to pH 7.5 with 50% HCl.

1. To make 60 ml of stain. All chemicals Sigma.
2. This system requires cooling during electrophoresis.
3. These systems are run for 15 minutes at 180 volts.
4. EST* (Kreutzer 1979): ACP—15 minutes at 170 volts, GPT, PFK, G6PDH, GOT—15 minutes at 200 volts, HK—15 minutes at 200 volts, LDH—15 minutes at 175 volts, MDH—15 minutes at 180 volts, ME—15 minutes at 160 volts, PGD, GPI, PGM—20 minutes at 200 volts, conditions for electrophoresis and components for developing zymograms of these enzymes (Kreutzer and Christensen 1980).

* Fumarase (FUM), α-glycerophosphate dehydrogenase (α-GDH), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Isocitrate dehydrogenase (ICD), Xanthine dehydrogenase (XDH), Esterase (non specific) (EST), Acid phosphatase (ACP), Alanine aminotransferase (GPT), 6-Phosphofructokinase (PFK), Glucose-6-phosphate dehydrogenase (G6PDH), Glutamate oxaloacetate transaminase (Aspartate aminotransferase (GOT), Hexokinase (HK), Lactic dehydrogenase (LDH), Malic dehydrogenase (MDH), Malic enzyme (ME), 6-phosphogluconic dehydrogenase (PGD), Phosphoglucoisomerase (GPI), Phosphoglucomutase (PGM).

RESULTS AND DISCUSSION

Of the 18 enzymes tested on colony specimens of *Cx. ocossa* and *Cx. panocossa* 7 produced bands with species-specific migrations, Table 2. Figure 1 is a photograph of zymograms for certain enzymes. The PFK bands can be used but with a certain amount of ambiguity, because the electromorph, .91, which is fixed in the *Cx. panocossa* colony was recovered at a low frequency (<.01) from the *Cx. ocossa* colony. Data from additional natural populations might be combined with the PFK data presented here, table 2, on the .91 electromorph which overlaps at a low frequency in both species to measure the degree of genetic divergence between the species at that locus. It is possible that the PFK gene may show a variable degree of evolution in natural populations of each species and thus provide information on the genetic structure of these and possibly other closely related *Melanoconion* species. The ICD band, .93, from one presumed locus is fixed in *Cx. panocossa* and is also present in the *Cx. ocossa* at a high frequency (>.99). The bands produced by the enzymes ACP, GAPDH, GPI, and XDH, the presumed second locus of ICD and one presumed locus of MDH are species-specific and can be used to specifically identify specimens from either colony.

Unpublished studies on field samples of *Cx. ocossa* have shown that frequencies for some enzymes are neither the same in each sample nor are they the same as the frequencies in the laboratory colonies. Allozyme frequency differences among different geographical populations have also been noted by Bullini and Coluzzi (1973), Miles (1978) and others. In order to determine whether the electromorphs of ACP, GAPDH, GPI and XDH which are species-specific in the laboratory colony sample (Table 2) are similarly specific in the Juan Mina area, 100 randomly chosen adult males from the Juan Mina larval sample were identified by their terminalia, and their individual profile for these enzymes was obtained. The enzyme profile of each identified field mosquito correlated with the profile of colony mos-

Table 2. Enzymes and relative migrations (Rf) of electromorphs which can be used to specifically identify mosquitoes from the GML Laboratory colonies of *Culex ocossa* and *Cx. panocossa*, and data from 100 males collected in the Juan Mina area of Central Panama (near the Panama Canal).

	Laboratory Colonies								
	ocossa		*panocossa*		*ocossa* (86 specimens)		*panocossa* (14 specimens)		Number
Enzyme	Rf[1]	Frequency	Rf	Frequency	Rf	Frequency	Rf	Frequency	of Loci
ACP	1.00	1.00	1.12	1.00	1.00	1.00	1.12	1.00	1*
PFK	1.00	>.99	.91	1.00	Not Determined		Not Determined		1*
	.91	<.01							
GAPDH	1.00	1.00	.72	>.99	1.00	1.00	.72	1.00	1*
			.61	<.01					
ICD	1.00	<.01	.93	1.00	Not Determined		Not Determined		2*
	.93	>.99							
	.33	1.00	.44	1.00					
MDH	1.00	1.00	1.00	1.00	Not Determined		Not Determined		2*
	.26	1.00	.13	1.00					
GPI	1.00	1.00	1.06	1.00	1.00	1.00	1.06	1.00	1*
XDH	1.00	1.00	.77	1.00	1.00	1.00	.77	1.00	1–2*

$$^{1}\text{Rf} = \frac{\text{distance of band migration}}{\text{most anodal band in GML colony } ocossa}$$

* Assumed for these species. ACP = Iqbal, et al. (1973); GAPDH—Ved Brat and Whitt (1974); XDH—Narang and Kitzmiller (1972); ICD and MDH—Steiner and Joslyn (1979).

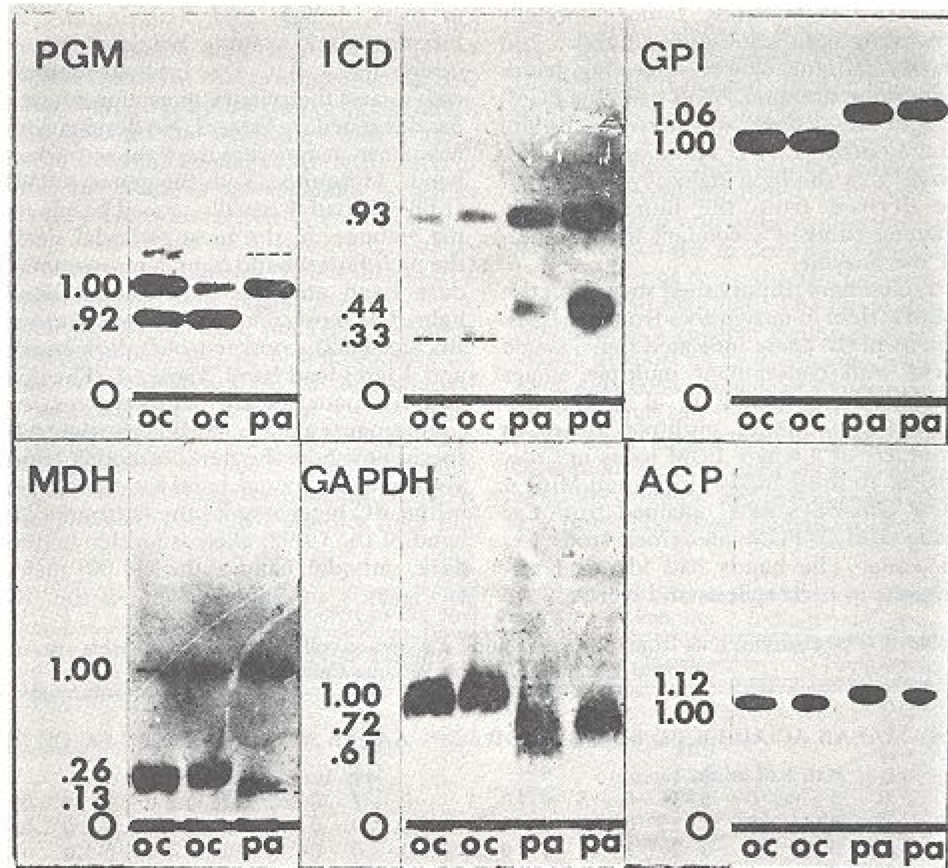

Fig. 1. Zymograms for certain enzymes. O = origin. The numbers at the left of each zymogram are Rf values; the most anodally migrating band for each enzyme from *Cx. ocossa* has arbitrarily been assigned a value of 1.00, and all other electromorphs or isozymes have proportional Rf values. oc = *Cx. ocossa* pa = *Cx. panocossa*. Anode is at the top of each zymogram.

quitoes. Each of the 86 *Cx. ocossa* males had the 1.00 electromorph for ACP, GAPDH, GPI and XDH, and each of the 14 *Cx. panocossa* males had the 1.12 ACP band, the .72 GAPDH band, the 1.06 GPI band and the 77 XDH band, Table 2. No other bands were obtained for these enzymes among these 100 males. These data suggest that the Juan Mina sample and the laboratory colony samples had certain electromorph similarities for these enzymes, and that the field collected mosquitoes identified as *Cx. ocossa/panocossa* could be specifically identified from their electromorph profile for the enzymes ACP, GAPDH, GPI and XDH.

Collections of larvae (reared to adults in the laboratory) and man bait attracted adults were made at 8 sites in the Juan Mina area. Each of the remaining 1153 *Cx. ocossa/panocossa* specimens was tested and had 1 of 2 electromorph profiles. Males and females with the 1.00 ACP, GAPDH, GPI and XDH bands and males and females with the 1.12 ACP, the .72 GAPDH, the 1.06 GPI and the .77 XDH band. The former group was assigned to be *Cx. ocossa* and the latter *Cx. panocossa*. No other bands were obtained for these enzymes. In addition to these 4 enzymes PGM data were collected on each mosquito, Table 3. Each specimen produced

at least 2 PGM bands, 1 more anodally migrating light staining band and a 2nd darkly staining slower migrating band. This same situation, band pairs for PGM, 1 light and 1 dark, was noted by Bullini and Coluzzi (1973). These authors proposed that the light staining band might be derived from the more intensely staining band by secondary modification of the enzyme.

Preliminary unpublished studies on the PGM system in mosquitoes from the GML colony of *Cx. ocossa* indicated that a single locus with codominant multiple alleles was involved. Bullini et al. (1971) reported codominant multiple alleles as products of a single PGM locus in *Culex pipiens*. In the samples from Juan Mina 4 PGM allozymes were obtained from *Cx. ocossa* and 3 PGM allozymes from *Cx. panocossa*. The bands had identical migrations in each species and were always in pairs, 1 light and 1 dark. The allozymes, dark staining bands only, are designated A, B, C, D to facilitate identification, and the relative migration of each band is noted in Table 3. No designations have been assigned to the light secondary bands. Mosquitoes with the genotype AD (1.08/.84) had 4 equally spaced bands on the zymogram, the most cathodal dark, the next fastest band light, a more anodal dark band and the most anodal band light. The most common isozyme pattern, BC (1.00/.92), consisted of 2 dark bands and 1 light lead band, Figure 1. The AD and BC patterns are heterozygotes for codominant alleles. Although the AD specimens show the dark, cathodal, band and the light, anodal, band for each allele, in the BC heterozygote the light anodal, band of the C(.92) allele is hidden by the dark, cathodal, band of the B(1.00) allele. In figure 1 the middle band of the BC

Table 3. PGM isozyme data from *Culex ocossa* and *Cx. panocossa* collected in the Juan Mina area of central Panama (near the Panama Canal).

Site[1]	AA[3]	AB	AC	AD	BB	BC	BD	CC	CD	DD
					TYPES					
Man bait adults (*ocossa*)										
1	0	3	0	0	8	17	0	3	0	0
2	0	0	1	0	15	25	0	22	0	0
3	1	0	2	0	9	20	1	11	0	0
4	0	0	0	0	11	21	0	12	0	0
5	0	0	4	0	19	44	0	29	1	0
6	0	2	0	0	12	16	0	18	0	0
7	0	3	2	0	9	32	0	15	0	0
8	0	2	0	0	15	24	0	18	0	0
Adults from larvae (*ocossa*)										
1	0	3	1	0	16	35	0	14	0	0
2	0	0	0	0	3	9	0	3	0	0
3	0	0	2	0	22	29	0	20	0	0
4	0	0	2	0	22	44	0	35	0	0
5	0	3	1	0	9	27	0	20	0	0
6	0	0	0	0	15	33	0	17	0	0
7	0	0	1	0	22	41	0	26	0	0
8	0	1	1	0	13	47	0	24	0	0

Site[2]	AA	AB	AC	AD	BB	BC	BD	CC	CD	DD
Man bait adults (*panocossa*)										
1	0	1	0	0	12	0	0	0	0	0
2	0	0	0	0	13	1	0	0	0	0
3	0	0	0	0	12	0	0	0	0	0
4	0	0	0	0	7	1	0	0	0	0
5	0	0	0	0	10	2	0	0	0	0
6	0	0	0	0	13	1	0	0	0	0
7	0	0	0	0	12	1	0	0	0	0
8	0	0	0	0	28	1	0	0	0	0
Adults from larvae (*panocossa*)										
1	0	0	0	0	34	0	0	0	0	0
2	0	0	0	0	22	3	0	0	0	0
3	0	0	0	0	6	2	0	0	0	0
4	0	0	0	0	3	1	0	0	0	0
5	0	0	0	0	12	1	0	0	0	0
6	0	0	0	0	25	1	0	0	0	0
7	0	0	0	0	8	2	0	0	0	0
8	0	1	0	0	15	0	0	0	0	0

[1] Each sample at each site of *ocossa* was in Hardy-Weinberg equilibrium.

[2] Chi-square tests were not made of the *panocossa* samples because at least 67% of the expected frequencies were less than 5.0 (Cochran 1954).

[3] Rf values for PGM allozymes.
A = 1.08.
B = 1.00.
C = .92.
D = .84.

(1.00/.92) heterozygote is thicker than the most cathodal dark band. This is the usual pattern on zymograms of BC heterozygotes, a dark thin cathodal band, the next anodal band dark and thicker, and the most anodal band light. Each of the *Cx. ocossa* samples, adults or adults reared from larvae, from each of the 8 sites was tested and found in Hardy-Weinberg equilibrium. This indicates that each sample is a representative of the population at that site. *Cx. panocossa* samples could not be tested, because an excessive number, 67%, of the expected frequencies were less than 5.0 (Cochran 1954). All PGM data from the Juan Mina samples were combined, tested and found in Hardy-Weinberg equilibrium, Table 4. *Cx. panocossa* combined samples were not tested, and certain *Cx. ocossa* classes, AA, AD, BD, CD, DD were omitted in the calculations (Cochran 1954). The combined data suggest that in the Juan Mina area there is a single population of *Cx. ocossa*. It is assumed that *Cx. panocossa* is also a single population.

Both larval and adult collections were made at each site to determine whether 1 or the other of these species has a greater attraction to man bait. The larval sample would give a reliable estimate as to the actual proportion of each species in the area. An excess number of 1 species in the adult sample can implicate it as a potentially more aggressive man biter. The combined data for each species collected as adults or larvae are presented in Table 5. These data show that the Juan Mina

Table 4. Tests for Hardy-Weinberg equilibrium of combined data of *Culex ocossa*[1] samples collected in the Juan Mina area of Panama.

	PGM Type[2]	Observed	Expected[2]	Frequencies
Man bait adults	AB	10	7.3	A = .0235
	AC	9	8.5	B = .4540
	BB	98	91.9	C = .5213
	BC	199	213.6	D = .0011
	CC	128	124.1	
				$x^2 = 2.56$ df = 5 classes − 3 alleles = 2 P>.20[3]
Adults from larvae	AB	7	9.1	A = .0135
	AC	8	10.5	B = .4541
	BB	116	114.4	C = .5324
	BC	265	265.8	
	CC	159	154.4	
				$x^2 = 1.24$ df = 5 − 3 = 2 P>.50[3]
Total combined	AB	17	16.4	A = .0185
	AC	17	19.0	B = .4540
	BB	214	206.3	C = .5274
	BC	464	479.4	D = .0005
	CC	287	278.4	
				$x^2 = 1.28$ df = 5 − 3 = 2 P>.50[3]

[1] No tests were made of the *panocossa* samples because 67% of the expected frequencies were less than 5.0 (Cochran 1954).

[2] Classes AA, AD, BD, CD, and DD have not been included because each of their expected frequencies was below 1.0 (Cochran 1954).

[3] Abridged Table IV of Fisher and Yates.

mixed population of larvae consisted of 80% *Cx. ocossa* and 20% of *Cx. panocossa*, and that the adults attracted to man bait consisted of almost identical proportions. Neither species in the Juan Mina population had a greater attraction to man bait than their species' densities would predict.

Table 5. Totals of each *Culex* species identified by ACP, GAPDH, GPI and XDH electromorph analysis in the samples from the Juan Mina Area of Panama.

Site	Man bait adults		Adults from larvae	
	ocossa	*panocossa*	*ocossa*	*panocossa*
1	31	13	63	34
2	63	13	15	40
3	44	12	73	8
4	44	8	103	4
5	97	12	60	13
6	48	14	65	26
7	61	13	90	10
8	59	29	86	17
Totals	447	114	555	137
%	79.7	20.3	80.3	19.7

[1] Certain of the adult males reared from larvae were also identified morphologically by characters on their terminalia .

In this study only adults were used, however most of the enzymes produced identically migrating bands and equal numbers of bands in larvae, pupae and adults; EST and GAPDH did not. It should be possible to measure other field biological parameters of these species. Currently studies are being conducted at GML on field collected blood engorged females which should yield valuable information on the various hosts of these species. Isozyme studies on other *Melanoconion* species are contemplated.

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