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Evaluation of Genetic Diversity in Wild Chironomid Populations Using RAPD and Hemoglobin Protein as Molecular Biomarkers

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By

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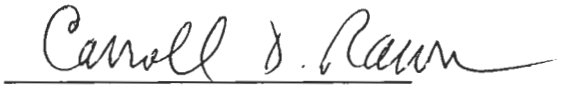

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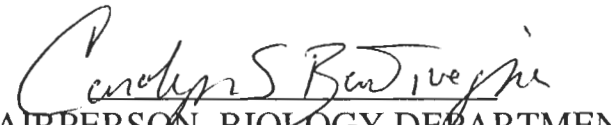

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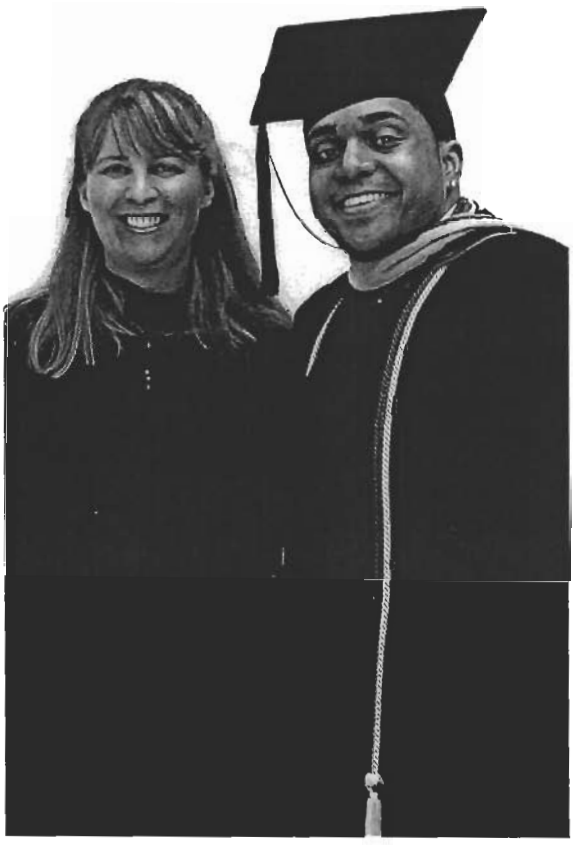

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Abstract

Loss of genetic diversity is detrimental to the survival of many species populations in natural environments that are altered by human activities. There are a variety of molecular techniques available today for determining interspecies and intraspecies genetic diversity. Randomly amplified polymorphic DNA (RAPD) assesses genetic diversity at the individual level based on the differences and similarities of polymorphic bands in the DNA fingerprint. Diversity may also be assessed at the protein level using hemoglobin, which is a multigene family. This study compared RAPD with SDS PAGE of hemoglobin protein as biomarkers of genetic diversity. Chironomids, larvae of midge fly, were obtained from study sites in Kearny Marsh located in the New Jersey Meadowlands. Field sites known to contain heavy metal contamination were capped or uncapped with AquaBlok, an aggregated, clay-based technology. Chironomids were collected from Hester-Dendys placed in capped or uncapped sites for one month in May, August and November. Genetic diversity of species populations was evaluated by multivariate statistics. Results indicate that diversity was more influenced by the presence of AquaBlok than collection date. RAPD results indicated increased levels of diversity on the AquaBlok plots. This suggests that capping may have caused higher levels of competition between species as well as a healthier environment. Hemoglobin profiles showed increased diversity for *C. riparius* on the uncapped plot, suggesting that expression levels of the protein were more variable as a result of environmental stressors for this species. There was a strong negative correlation between genetic diversity and heavy metal contamination using RAPD, indicating lower levels of toxicants were

associated with higher levels of DNA diversity. Furthermore, there was a strong positive correlation between genetic diversity and heavy metal contamination using hemoglobin, indicating higher levels of toxicants were associated with higher levels of protein diversity. This suggested individuals increased levels of hemoglobin expression as a direct result of environmental stressors. Both SDS-PAGE and RAPD proved to be simple, effective methods to study genetic diversity in chironomids.

Introduction

Molecular techniques such as polymerase chain reaction (PCR) have been used in molecular systematics to establish phylogenetic relationships based on similarities and differences at the genetic level. Phylogenetics has also been used to determine intraspecies and interspecies genetic diversity, which is an increasingly useful tool for evaluating environmental health. Impaired populations are alleged to display less genetic diversity while healthy populations are anticipated to show much more variability. Two measures of genetic diversity were investigated in this project: randomly amplified polymorphic DNA (RAPD) which determines diversity at the genomic level and SDS-PAGE of the hemoglobin protein, which determines diversity at the protein level. Hemoglobin was chosen because the test organism, larvae of midge fly (Chironomidae), contains multiple forms of the hemoglobin gene (Tichy, 1975). These forms may be differentially expressed under environmental stress. Placement of AquaBlok (clay-based capping material) in Kearny Marsh afforded the opportunity to investigate how changing environmental conditions might affect genetic diversity.

Kearny Marsh in the New Jersey Meadowlands was chosen as the site for the field study. This marsh has been chronically contaminated by landfills, leachate and run-off, which has resulted in the introduction of metallic contaminants (including As, Cd, Cr, Cu, Pb, Hg, and Zn). Secondly, non-vegetated (i.e. open water) areas have increased and as a result there is a decreased amount of habitat available to wildlife. Finally, Kearny Marsh is a freshwater habitat, making it unusual since only approximately 16% of the entire Meadowlands is freshwater and an optimal field of study (Bentivegna *et al.*, 2004).

AquaBlok has been employed in this study in order to assess how differences in plot from which an individual is collected affects genetic diversity. AquaBlok (AquaBlok, Ltd., Toledo, Ohio) is a patented, composite-aggregate technology comprised of a solid core, an outer layer of clay material, and polymers. Initially, AquaBlok comes in the form of small pebbles, but when placed in water over the sediment it hydrates and forms a barrier between the sediment and water column, which helps to decrease the bioavailability of contaminants (Hull *et al.*, 1998). This would aid in the increase of wetland habitat for wildlife by isolating underlying sediment-borne toxicity and providing a relatively uncontaminated substrate for colonization. For this investigation, wild chironomids were collected from three plots located on the marsh. Two plots contained AquaBlok; one did not contain any AquaBlok and served as control.

Pilot studies on the effectiveness of this clay-based aggregate have been employed on the Eagle River Flats, an estuarine salt marsh ecosystem contaminated with white phosphorus, located near Anchorage, Alaska. This environment proved lethal to the mallard population inhabiting the marsh due to the high levels of environmental toxins found in the sediment. AquaBlok was placed down over the sediment with hopes of reducing bioavailability of the contaminants. Results indicate mallard death and white phosphorus exposure totals greatly decreased after treatment with the substrate (Hull *et al.*, 1998).

Chironomids were chosen for analysis because they are commonly used in ecotoxicological research as ecological indicators with established biological endpoints such as lethality (ASTM, 1992) and growth (USEPA, 1994). Chironomids are

considered sentinel organisms because larvae remain in one location, allowing easy exposure to potentially contaminated sediment, and are indicative of conditions there. Their usefulness in population studies further extends to the fact that they are found in abundance and have a relatively short generation time, namely one generation each month. They are easily collected and are relevant because they are essential components of the food web in the marsh.

The RAPD technique has been used on a wide range of organisms, from insects and fish to various species of plants, to assess genetic diversity. The presence or absence of RAPD bands is indicative of variation in an individual's entire genome (Williams *et al.*, 1990) and allows for the detection of genetic differences (Baral and Bosland, 2002; Brown and Myers, 2002). DNA polymorphisms were extensively utilized as an instrument of assessing genetic diversity in aquatic organisms, such as carp (Yan *et al.*, 2005). Previous studies concluded that genotype-environment interactions do play a key role in an organism's response to environmental stressors as in the case of *Daphnia magna* to sodium bromide and 3,4-dichloroaniline (Soares *et al.*, 1992) and to cadmium (Ward and Robinson, 2005). *Daphnia* adapted to the stressors such that increased genetic variability was associated with better survival after several generations. Furthermore, RAPD was employed in a study that characterized horn flies' resistance to pyrethroids, an environmental control factor for hornflies (Guerrero *et al.*, 1997). RAPD results demonstrated variability amongst susceptible and resistant flies using a resistance-associated marker, named HF-77. Results indicated that the HF-77 was not found in susceptible flies, but was present in approximately 16% of resistant individuals. This

study is a prime example on the effectiveness of RAPD as a biomarker of genetic variability.

Respiratory proteins, such as hemoglobin, are essential for oxygen transport as well as survival for many invertebrates. Some insects, such as the horse botfly, that possess intracellular hemoglobin have specialized storage functions for oxygen (Burmester and Hankeln, 2007). Hemoglobin of this species has the ability to store oxygen until it is released when needed under hypoxic conditions. Other insects such as the fruit fly demonstrate the down regulation of a specific hemoglobin gene, *glob1*, when faced with low oxygen level conditions (Burmester and Hankeln, 2007). This investigation utilized chironomids, which possess extracellular hemoglobin within their hemolymph as a response to hypoxic conditions within the environment (Burmester and Hankeln, 2007). The extracellular hemoglobin enhances the oxygen capacitance of the hemolymph, which in turn enables oxygen transport in the larvae (Burmester and Hankeln, 2007). Polymorphic profiles due to the expression of hemoglobin are essential markers of the organism's response to environmental conditions. Hemoglobin polymorphic profiles as well as morphological distinctions in head capsule (Epler, 2001) helped to classify the species of each individual collected in this study. The use of hemoglobin profiles to identify species is a novel approach developed during this current project.

The present investigation employed the use of AquaBlok in order to study how genetic diversity is affected by varying environmental conditions. It is hypothesized that AquaBlok should establish a healthier environment for all organisms living on plots

containing this clay aggregate. A healthier environment should promote an increase in genetic diversity as well as a higher level of competition. Finally, samples were collected over several dates to determine the effect of season on genetic diversity. Seasonal changes in water conditions may alter hemoglobin expression and affect levels of diversity.

Methods and Materials

Site Selection and Sample Collection

Chironomids were collected from plots in the Kearny Marsh which is located in the New Jersey Meadowlands (**Figure 1**). Plots were treated as follows: plot 1- AquaBlok (AB), plot 4- AB, plot 9- no AB (control). Chironomids were collected from Hester-Dendys placed above the AquaBlok and sediment, but submerged in water for 1 month in triplicate (A,B,C) from plots 1 and 4 or in duplicate (A, B) from plot 9. Plots were sampled in May, August, and November 2006. These are approved by the EPA for benthic macro invertebrate organism collection found in rivers, streams, lakes, and tidal flats. They are hardboard plates which are divided by special nylon spacers that help to attract insect larva and other organisms. The plates have smooth surfaces on each side, are fastened together with a long eye bolt, and can easily be disassembled for specimen examination. Hester-Dendys were cleaned off with brushes and chironomids were separated from other organisms in order to be prepped for hemoglobin analysis and DNA isolation.

Heavy Metal Analysis

Sediment/AquaBlok samples were collected pre- (July 2005) and post- (November 2005) capping using sediment coring devices. Heavy metal content was measured at the Meadowlands Environmental Research Institute in Lyndhurst, N. J. The procedure was as follows. Samples were oven-dried and approximately 1-2 g dry weight of each was mineralized in a 10 ml trace metal grade HNO_3 in Teflon bombs in a microwave digester. The solution was boiled off to near dryness then diluted to 10 ml

with 1% HNO_3. Cd, Cr, Cu, Fe, Pb and Zn were analyzed by either flame or by graphite furnace atomic absorption spectrophotometry. Hg was analyzed using cold-vapor AA in a Bacharach MAS-50D mercury analyzer.

Preparation of Hemolymph Samples

Approximately twenty-five to thirty chironomids were used for hemoglobin analysis from the samples collected from Hester-Dendy replicates. Hemolymph was extracted from each larva by decapitation and bleeding out onto a microscope slide. Approximately 2 µL of hemolymph was drawn up and pipeted into a 1.5 mL centrifuge tube containing 14 µL LDS (Lithium Dodecyl Sulfide) sample buffer (Invitrogen, Carlsbad, California), 2 µL 8M urea (Qiagen, Valencia, California), and 2 µL of 10X 2-mercaptoethanol (Sigma Chemical Co., Saint Louis, Missouri). The head capsules of each sample were stored in 70% ethanol (Pharmco-Aaper, Brookfield, Connecticut) and used for species identification (Epler, 2001), see Appendices 1 through 3. Bodies were placed in 700 µL of DNAzol (Invitrogen, Carlsbad, California) and stored at 4°C until DNA was extracted.

SDS PAGE

Hemolymph proteins were separated on 15 % SDS polyacrylamide gels (Biorad, Hercules, California). The hemolymph samples were all denatured for ten minutes on a heating block at 68°C. Molecular weight standard solution was prepared with 10 µL of LDS sample buffer (Invitrogen, Carlsbad, California) and 5 µL of See Blue Molecular Weight Standard (Invitrogen, Carlsbad, California). Three µL of hemolymph were separated in a 15% SDS polyacrylamide gel. Each gel was run for approximately one to

one and a half hours at 30 mA. When the gel was finished running, it was removed and rinsed three times for five minutes in 200 mL of dH_2O. Each gel was then rinsed in a prefixed solution of 50% methanol (Pharmco-Aaper, Brookfield, Connecticut) and 7% glacial acetic acid (Pharmco, Brookfield, Connecticut) for fifteen minutes. The gel was then washed twice with 200 mL dH_2O for fifteen minutes each. Each gel was then stained with 20 mL of Gel Code Blue Stain (Pierce, Rockford, Illinois) for thirty minutes and then rinsed three times using 200 mL dH_2O for five minutes each. Each gel was then dried on blotting paper using a gel dryer for approximately forty-five minutes at 80°C.

DNA Isolation and Optical Density

After hemoglobin removal, chironomid bodies were placed into 700 µL of DNAzol (Invitrogen, Carlsbad, California) and stored there until DNA extraction. DNA extraction was performed using the same DNAzol in which they were stored in accordance with the manufacturer's protocol. Each chironomid body was placed into a glass hand-held homogenizer filled with 700 µL DNAzol. The body was then ground up using ten to fifteen strokes or until a cloudy mist formed. The homogenate was then transferred to a clean 1.5 mL tube and centrifuged for ten minutes at 3,000 rpm. From there, 350 µL of cold absolute ethanol (Pharmco-Aaper, Brookfield, Connecticut) was combined with each supernatant forming a cloudy precipitate and each tube was inverted six or more times. The samples were centrifuged at 12,000 rpm for two minutes after which the supernatant was discarded. The pellets were washed with 75% ethanol (Pharmco-Aaper, Brookfield, Connecticut) by inverting the tube six or more times in order to free the pellet from the bottom. The samples were then centrifuged at 12,000

rpm for two minutes after which the supernatant was once again discarded. The previous wash step was repeated. The pellets were centrifuged once more at 12,000 rpm for two minutes in order to collect any residual supernatant, which was pipeted out with a 10 μL pipet. Finally, 50 μL of 8mM NaOH (Sigma-Aldrich, Saint Louis, Missouri) was added to each sample and the pellet was pipeted up and down in order for it to dissolve back into solution. Samples were stored at 4°C over night and then 1.2 μL of 1M HEPES buffer (Sigma, Saint Louis, Missouri) was added to each sample in order to neutralize the NaOH before an optical density was taken. Optical Density was measured on all the samples and calculated at the following wavelengths: 260nm, 280nm, and 320nm. DNA concentration was determined by multiplying the 260nm value with the DNA standard value, 50 μg/ml. Once the values were known, the amount needed for each sample to be used in RAPD was calculated.

RAPD

Two primers were used for RAPD PCR: SB2 (XXIDT, Coralsville, Iowa): 5'-GGA CTC CAC G-3' and SB6 (XXIDT, Coralsville, Iowa): 5'-TCA CGA TGC A-3'. PureTaq Ready-To-Go PCR Beads (Amersham Biosciences, Piscataway, New Jersey) were used in which each reaction contained 100ng genomic DNA and 0.2 μM primer brought to 25 μL volume using dH_2O. The PCR cycle was as follows: 95°C 5 min; then 95°C 1 min for denaturation, 36°C 1 min for primer annealing, 72°C 2 min for extension for 37 cycles; then 72°C 3 min; and then 4°C for storage. Each reaction (10 μL) was run in duplicate on a 1.5% agarose (Sigma, Saint Louis, Missouri) gel at 100V for approximately forty-five minutes. The gels were then visualized using a Gel-Doc-it

system (UVP Inc., Upland, California) and a picture was taken. The picture was used to score the bands.

RAPD Optimization

Initial attempts at RAPD resulted in faint or no bands; therefore, optimization of the protocol was necessary. Two different methods were attempted, one where the DNA concentration was varied and the other where the primer concentration was varied. In each case, the DNA and primer concentrations were run at 0.2 μM, 0.4 μM, and 1.0 μM. A gel was run with all cases included and the brightest and most prominent bands were observed using a primer concentration of 1.0 μM. The RAPD protocol was then optimized to 100 ng genomic DNA and 1.0 μM primer brought to 25 μL volume using dH_2O.

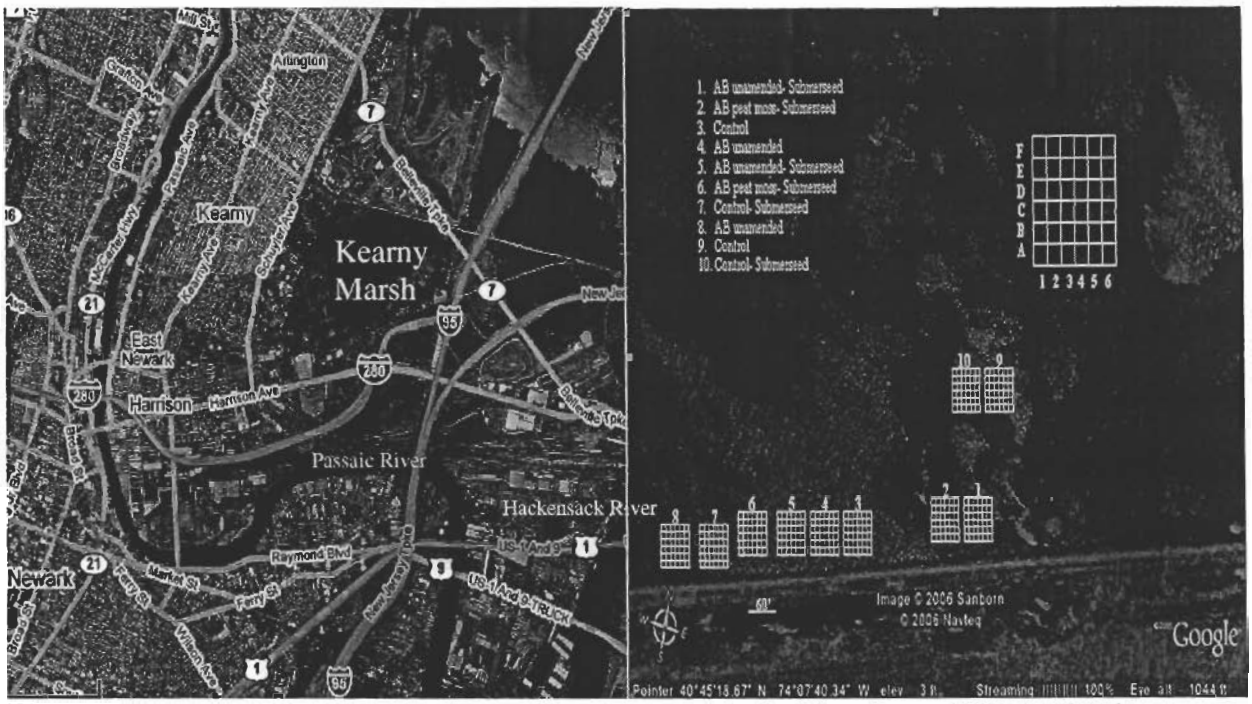

Figure 1: Kearny Marsh. Left: Kearney Marsh located in the New Jersey Meadowlands near major highways and between the Passaic and Hackensack Rivers. Right: Sampling Sites at Kearny Marsh, NJ: Plots 1, 4, and 9.

Data Analysis

Hemoglobin bands were scored visually and analyzed using statistical package for the social sciences (SPSS) version 12.0.0 on an eight band scoring system. Bands were scored 0=absent, 1=light band, and 2=dark band. Divergence between individuals was based on multivariate data analysis that compared bands of one individual to bands of each other individual of the same species in its treatment group. Treatment groups consisted of plot, collection date or plot on a particular collection date. The method used was hierarchal clustal analysis using furthest neighbor and interval squared Euclidean distance to generate a proximity matrix.

Every scorable RAPD band was assigned as a variable in SPSS (version 12.0.0) and each individual a case. A binary system (1=present, 0=absent) scored whether or not the individual did or did not have each band. Divergence between individuals was based on multivariate data analysis as described above for hemoglobin (**Appendix 4**).

Microsoft Excel was used to calculate average distance of each individual compared to the other individuals within a given group, after which the group average and standard deviation was calculated. Individual averages were grouped by treatment and species in order to run one way analysis of variance to test for significance between treatments by the factors that were tested: influence of plot, influence of collection date, and influence of plot and date. Finally, Pearson's Correlation was used to compare an individual's average for hemoglobin to that of RAPD for each primer to determine if there was any association between the two biomarkers.

Results

The study site was analyzed for pre- and post-capping heavy metal concentrations. It was necessary to determine levels of contaminations prior to and after the placement of AquaBlok in order to show if heavy metal contamination was actually reduced by the use of AquaBlok and thereby established a healthier environment for the chironomids. AquaBlok was placed on plots 1 and 4, while plot 9 had no AquaBlok so it could be used as a control. Cadmium, chromium, copper, mercury, nickel, lead, and zinc were among the contaminants found in the Kearny marsh. Results (**Table 1**) showed a general decrease in heavy metal concentration after capping. Values that are highlighted in yellow indicate concentrations that were above the Lowest Effects Limit (LEL) and values highlighted in red indicate those that were above the Severe Effects Limit (SEL). The LEL and SEL are EPA approved values that were established by the Ontario Aquatic Sediment Criterion. The values signify concentrations at which a particular metal is detrimental or even lethal to organisms. After capping, cadmium was above the LEL on all plots; chromium was above the LEL on plots 1B and plot 9 (uncapped); and copper was above the LEL on plots 1A, 4B, and 9A. Mercury was above the LEL on plot 1B; mercury was above the LEL on plots 1B, 9B, and all of plot 4; nickel was above the LEL on plots 1A, 4A, and 4B; lead was above the LEL on plots 1A, 4A, and 4B; and zinc was above the LEL on plots 4C and all of plot 9. Copper was above the SEL on plots 1B, 9B, and 9C; mercury was above the SEL on plots 1A, 4B, 4C, and all of plot 9; nickel was above the SEL on plots 1A, 1C, 9A, and 9C; and lead was above the SEL on plot 1B and all of plot 9. Cadmium and mercury were the two contaminants that stood out as major

problems even after capping because cadmium was found above the LEL on all the plots and mercury was above the SEL on a majority of the plots.

Experiments were run in order to establish a working RAPD protocol that produced significant results. However, RAPD is known for being unreliable with poor reproducibility because of its sensitivity to contamination and sensitivity to changes in critical PCR conditions, namely, Taq polymerase concentration and quality, primer concentration, DNA concentration and quality, number of amplification cycles and even the quality of PCR water used (Munthali *et al.*, 1992). Fortunately, most of the PCR components used in this study were standardized due to the use of PureTaq Ready-To-Go PCR Beads (Amersham Biosciences, Piscataway, New Jersey). This was essential in stabilizing PCR conditions and improving reproducibility for each reaction.

Initial experiments run using the existing RAPD protocol produced gels with faint or no bands (not pictured). An optimization experiment (**Figure 2**) determined the best results were obtained using 100 ng of genomic DNA and 1.0 μM primer.

This established RAPD protocol was used to assess genetic diversity on the successfully isolated samples from all three collection dates from the three significant plots. RAPD was run on each sample set using three different primers individually: SB2, SB3, and SB6. However, SB3 did not produce consistent or reproducible bands; therefore, this primer set was abandoned (**Figure 3**). On the other hand, SB2 and SB6 primer sets both produced significant results that were reproducible (**Figures 4 and 5**); therefore, the results were able to be scored and subjected to statistical analyses.

Once the gels were visualized, bands were scored present or absent and then were analyzed using several different statistical tests. However, it was first essential to identify each sample in order to separate groups by species. Each individual was classified using hemoglobin profiles by running SDS-PAGE on hemolymph isolated from each species (**Figure 6**) and comparing them to head capsule morphology, an established method in identifying chironomids (Epler, 2001). Profiles were constructed using an eight band system scored on a 0 to 2 scale where 0 = absent, 1 = faint band, 2 = prominent band. The *Glyptotendipes lobiferus* profile was demonstrated by a prominent 1^{st} band as well as prominent 4^{th} and 5^{th} bands. A *Chironomus. riparius* profile exhibits no distinct top bands; however, there are prominent 4^{th} and 6^{th} bands. Finally, Hybrid profiles exhibit bands featured both for *G. lobiferus* and *C. riparius*, namely a prominent 1^{st} band, prominent triplet of the 4^{th}, 5^{th}, and 6^{th} band, and finally two faint 7^{th} and 8^{th} bands. Each species head capsule (see **Appendices 1 through 3**) was compared to the corresponding hemoglobin profile in order to classify individuals. Morphological features of a *G. lobiferus* head capsule (**Appendix 1**) include rounded teeth with shallow grooves between them. *G. lobiferus* have a single large tooth in the center as well as a rounded mandible. *C. riparius* head capsules (**Appendix 2**) demonstrate very pointed teeth with deep grooves between teeth. More specifically C. riparius possess a triplet of teeth in the center and sharp, straight mandible. Finally, Hybrid head capsules (**Appendix 3**) demonstrate features of both *C. riparius* and *G. lobiferus*. Hybrids have rounded teeth with shallow grooves, but possess the triplet of teeth in the center. Finally, they possess a sharp, rounded mandible. A total species count was calculated using the

profiles and head capsules for each collection date and plot (**Table 2**). Individuals collected in September 2005 and May 2006 were only *G. lobiferus*. Individuals collected in August 2006 were predominantly *G. lobiferus*, however; there were several *C. riparius* found on plots 1 and 9. There was also a single case of a Hybrid collected from plots 1 and 9 each in August. Individuals collected in November 2006 were predominantly Hybrids on plots 1 and 9. *G. lobiferus* were found in fewer numbers on these plots during this month, however, *G. lobiferus* were still found in greater number on plot 4 during this month. *C. riparius* were also scarce as only two individuals were found on plot 9 in November. Hybrids became the predominant species collected in November on plots 1 and 9. Hemoglobin gels were scored and also subjected to the same statistical tests as those used with RAPD (**Figure 7**).

Table 1: Heavy Metal Concentrations Pre- and Post-Capping with AquaBlok. Heavy metal concentrations (mg/kg) were determined using an EPA approved analysis method. Each heavy metal concentration was calculated prior to and after AquaBlok capping. Values in black indicate heavy metal contamination levels prior to capping with AquaBlok, whereas, blue values indicated heavy metal contamination levels after capping with AquaBlok. LEL = Lowest Effects Limit based on Ontario Aquatic Sediment Criterion. SEL = Severe Effects Limit based on Ontario Aquatic Sediment Criterion. Values highlighted in yellow indicate concentrations above the LEL, values highlighted in red indicate concentrations above the SEL.

Heavy Metal *Plot/Triplicate*		**Cd** (mg/kg)	**Cr** (mg/kg)	**Cu** (mg/kg)	**Hg** (mg/kg)	**Ni** (mg/kg)	**Pb** (mg/kg)	**Zn** (mg/kg)
1 A	Pre-Cap	2.35	41.1			41.6		530
	Post-Cap	3.13	5.55	23.3			32.2	72.4
1 B	Pre-Cap	3.79	82.6			54.6		346
	Post-Cap	4.66	49.9		0.27	60.9		415
1 C	Pre-Cap	3.44						725
	Post-Cap	2.72	0.52	2.91	0.04		4.58	41.9
4 A	Pre-Cap	6.46						
	Post-Cap	3.69	5.35	10.8	0.10	36.5	64.2	71.9
4 B	Pre-Cap	5.87	99.2					
	Post-Cap	3.81	6.66	25.9		70.6	36.4	86.9
4 C	Pre-Cap	2.20	48.9		0.68	42.1		273
	Post-Cap	2.82	5.15	8.65		41.8	26.8	132
9 A	Pre-Cap	7.68	82.2					
	Post-Cap	3.88	104	284				429
9 B	Pre-Cap	1.79	16.6	56.1	0.95	18.4		185
	Post-Cap	2.66	58.3			38.5		467
9 C	Pre-Cap	2.95	33.3		0.10	42.6		416
	Post-Cap	4.73	94.6					520
	LEL	0.6	26	16	0.20	16	31	120
	SEL	10.0	110	110	2.00	75	250	820

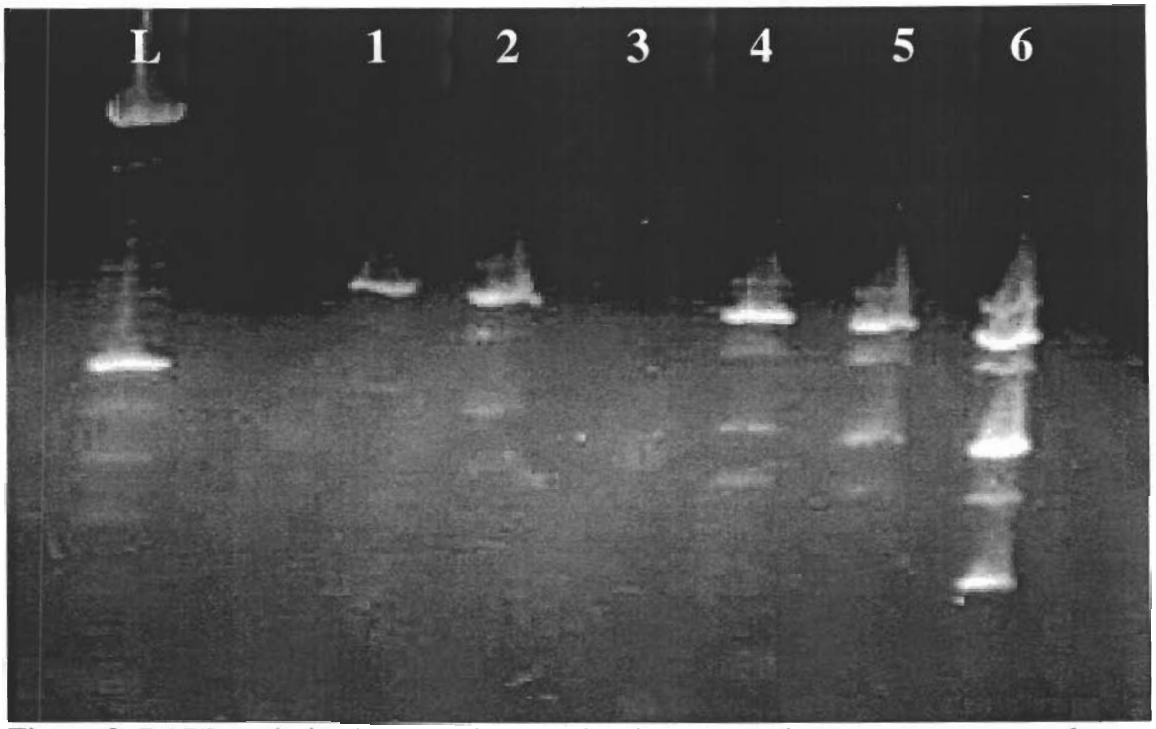

Figure 2: RAPD optimization experiment gel. The two varying components, namely DNA and primer concentration (SB2) were tested at their initial concentrations as well as a two-fold and five-fold increase of each. (L) 100 bp. (1) 100 ng Genomic DNA + 0.2 μM Primer. (2) 200 ng Genomic DNA + 0.2 μM Primer. (3) 500 ng Genomic DNA + 0.2 μM Primer. (4) 100 ng Genomic DNA + 0.2 μM Primer. (5) 100 ng Genomic DNA + 0.4 μM Primer. (6) 100 ng Genomic DNA + 1.0 μM Primer. Conditions for used in PCR sample 6 were used for the rest of the experiments.

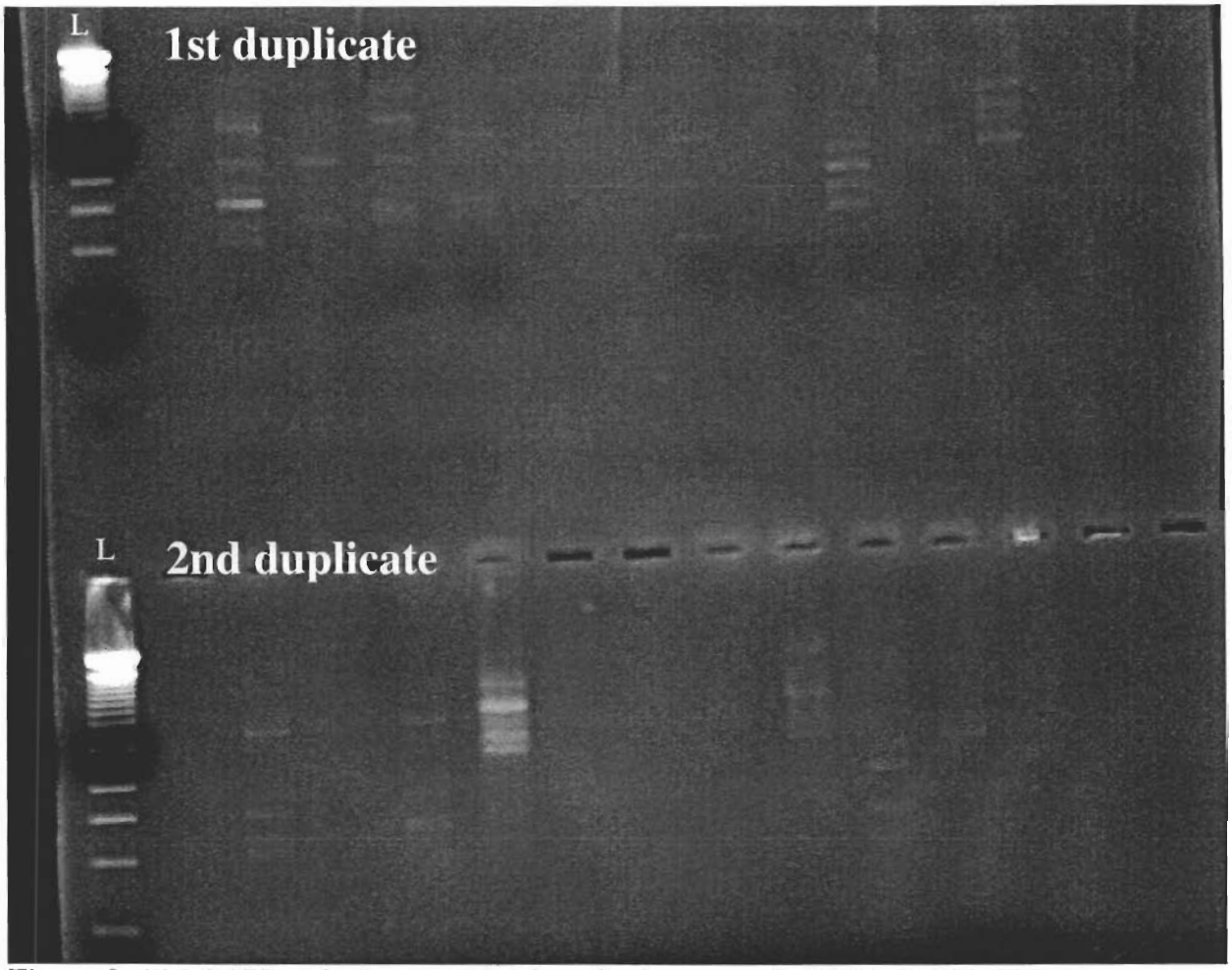

Figure 3: SB3 RAPD gel. An example of a gel using genomic DNA run with SB3 primer. Top and bottom lanes are duplicates: the same DNA in two different PCR reactions. Many bands did not show up and of those that did, many were faint and not reproduced. (L) 100 bp.

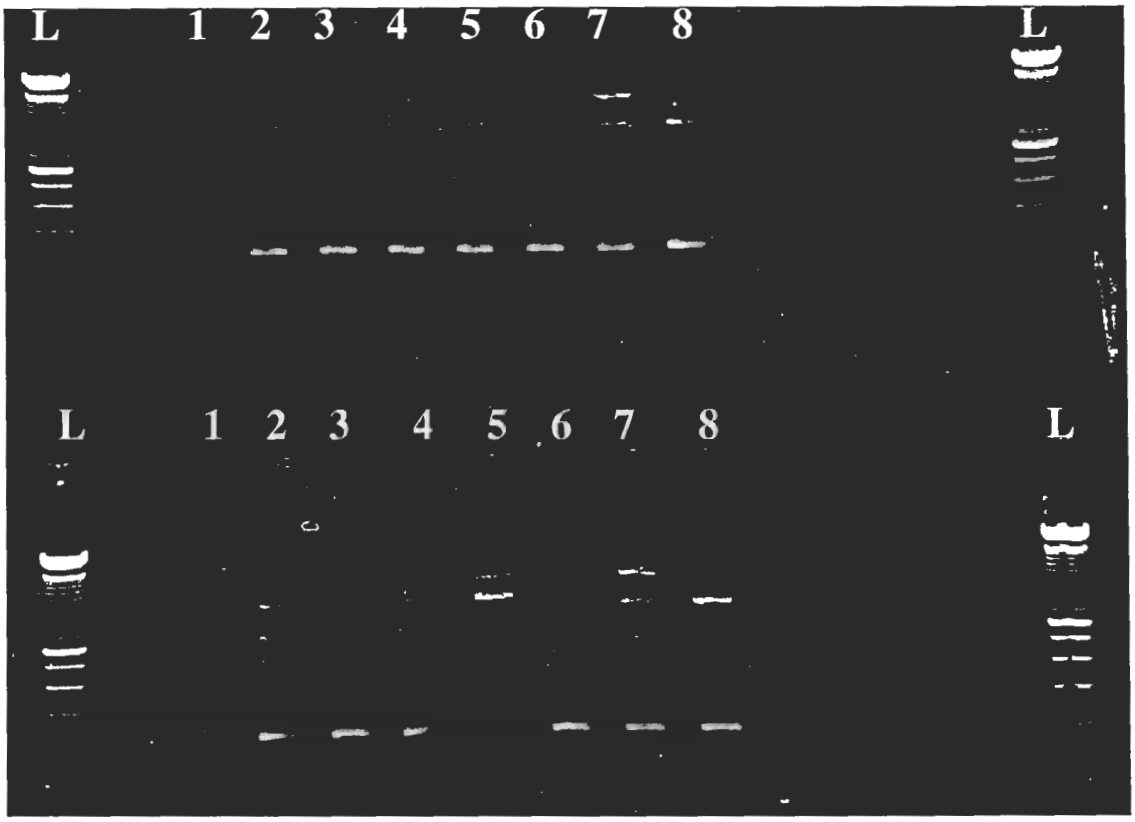

Figure 4: August Plot 9 RAPD gel with SB2. An example of a gel using samples isolated from Plot 4 run with SB2 primer. Top row: 1st set of duplicate reactions. Bottom row: 2nd set of duplicates. (L) 100 bp ladder (1) Sample A3 (2) Sample A4 (3) Sample A8 (4) Sample A10 (5) Sample B2 (6) Sample B3 (7) Sample B5 (8) Sample B6

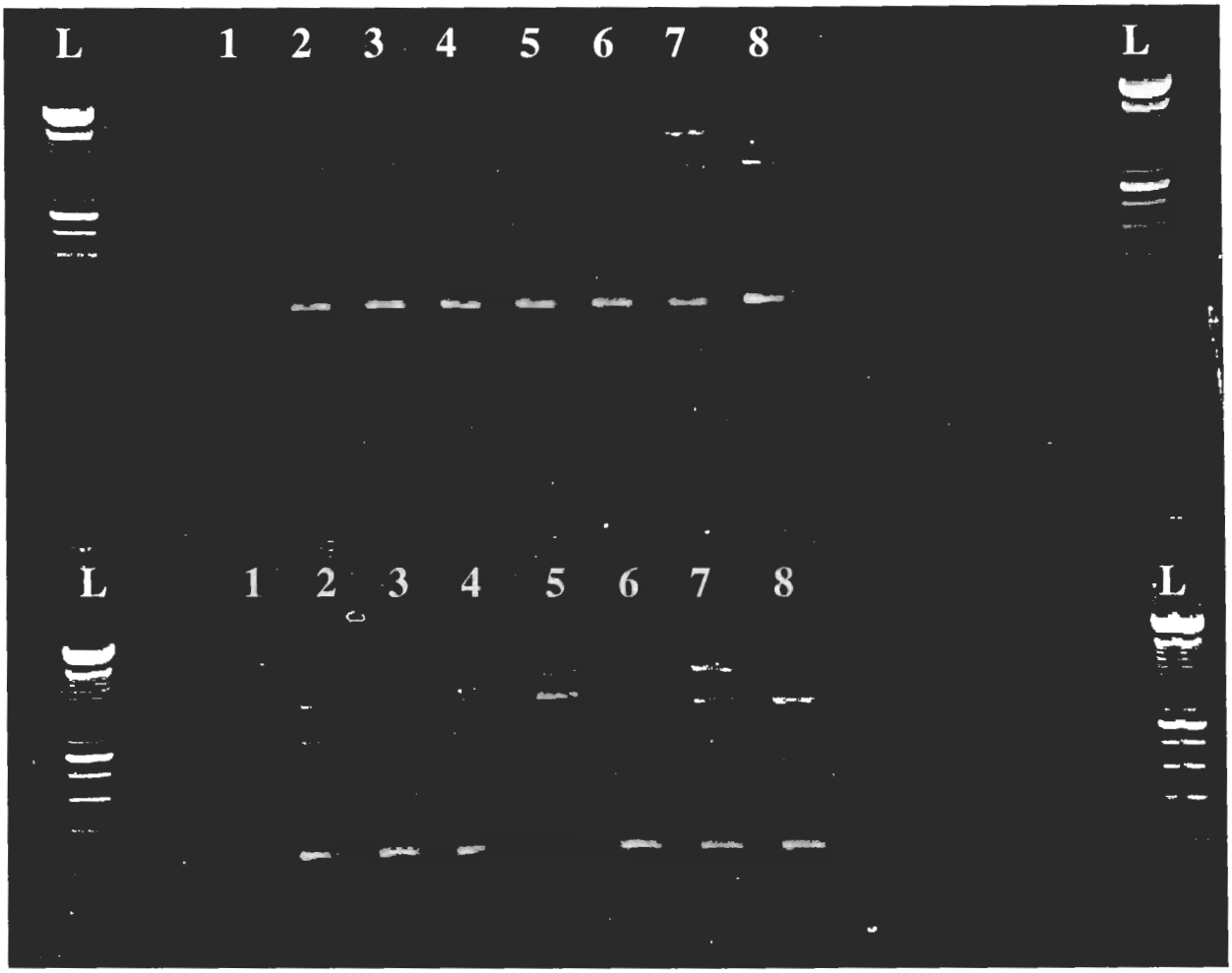

Figure 5: August Plot 9 RAPD gel with SB6. An example of a gel using samples isolated from Plot 4 run with SB6 primer. Top row: 1st set of duplicate reactions. Bottom row: 2nd set of duplicates. (L) 100 bp ladder (1) Sample A3 (2) Sample A4 (3) Sample A8 (4) Sample A10 (5) Sample B2 (6) Sample B3 (7) Sample B5 (8) Sample B6

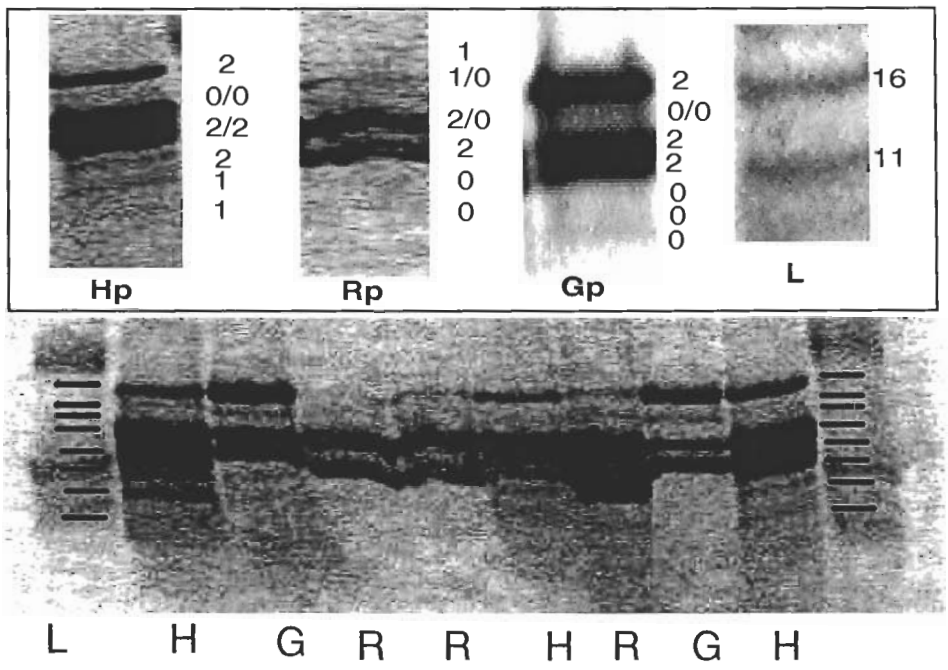

Figure 6: Hemoglobin Profiles. SDS-PAGE was run on hemolymph isolated from species found in the three plots from each collection date and used to establish hemoglobin profiles for each species using an eight-band system. Numbers next to each other represent band above/band below. Numbers represent band intensity: (0) absent band (1) light band (2) intense band. Top: Profiles for each species (Hp = Hybrid Profile, Rp = *C. riparius* Profile, Gp = *G. lobiferus* Profile) and L = molecular weight standard in kDa. Bottom: Gel including all species to assess differences amongst the species' hemoglobin profiles. (L) Molecular weight standard (H) Hybrid profile (G) *G. lobiferus* profile (R) *C. riparius* profile

Table 2: Species Count by collection date and plot. A tally of each species per plot and month and each species percentage of the total number of individuals found on the plot on the specific collection date.

	Species	**Sept 2005**	**May 2006**	**August 2006**	**November 2006**
AB-SS (Plot1)	Glypto	25 (100%)	23 (100%)	23 (79%)	7 (35%)
	Riparius	0	0	5 (17%)	0
	Hybrid	0	0	1 (4%)	13 (65%)
AB (Plot 4)	Glypto	25 (100%)	25 (100%)	31 (100%)	25 (83%)
	Riparius	0	0	0	0
	Hybrid	0	0	0	5 (17%)
Control (Plot 9)	Glypto	25 (100%)	25 (100%)	14 (70%)	1 (5%)
	Riparius	0	0	5 (25%)	2 (10%)
	Hybrid	0	0	1 (5%)	17 (85%)

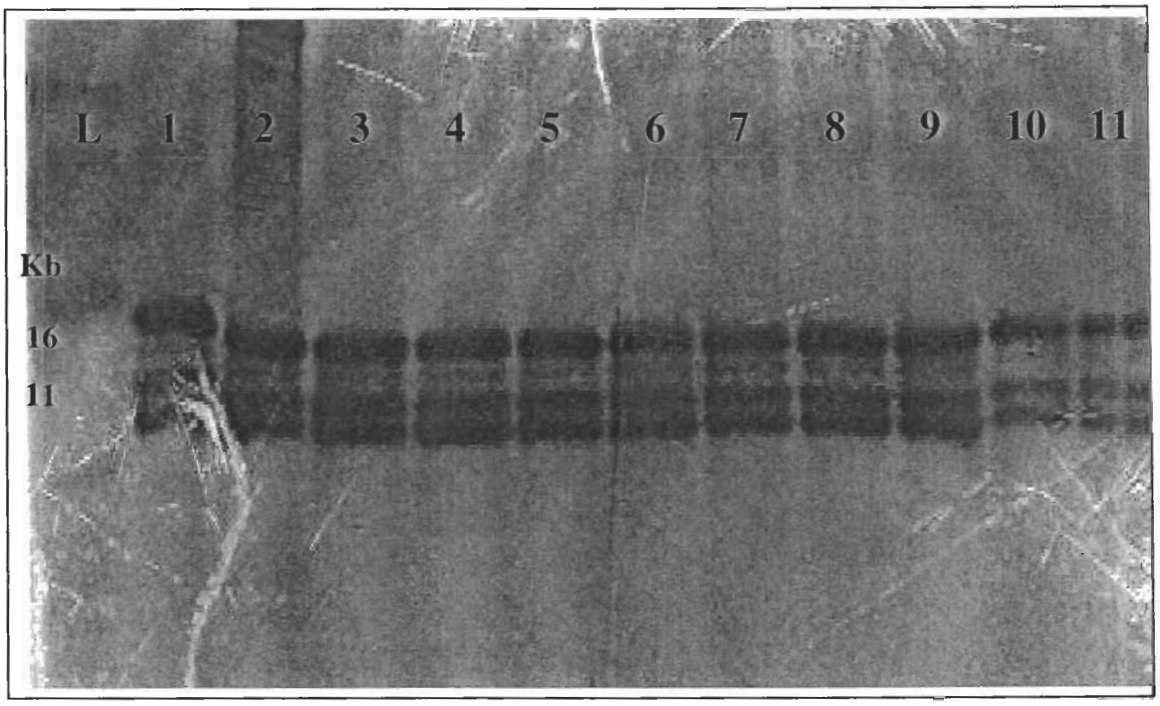

Figure 7: August Plot 4 hemoglobin profile. SDS-PAGE gel of the hemolymph isolated from *G. lobiferus* collected in August from plot 4. (L) Molecular weight standard (1) Sample B1 (2) Sample B2 (3) Sample B3 (4) Sample B4 (5) Sample B5 (6) Sample B6 (7) Sample B7 (8) Sample B8 (9) Sample B9 (10) Sample B10 (11) Sample B11

Effect of Plot on Diversity

Individual average distance was calculated by Microsoft Excel using proximity matrices generated by SPSS (version 12.0.0). The first factor tested was to determine if plot had an influence on genetic diversity. This was done by grouping similar species together regardless of collection date for both RAPD and hemoglobin (**Tables 3 and 4**). Graphs were then generated by plotting the average diversity of a species against the plot from which it was collected (**Figures 8, 9, and 10**).

Results for RAPD indicated a trend of decreasing diversity for *G. lobiferus* across plots for both primer sets (**Table 3 and Figures 8 and 9**). Plot 1 had the highest average diversity but was not statistically different from plot 4. Plot 9 had the lowest diversity and was statistically different from both plots 1 and 4. *C. riparius* was only analyzed in plot 9, which reduced the effectiveness of this study for this species. The diversity of *C. riparius* was statistically similar to *G. lobiferus* and Hybrid on plot 9. Hybrid data resembled that of *G. lobiferus* as average diversity generally decreased from plot 1 through plot 9 except for the SB2 primer set. Hybrids showed a slight increase in diversity on plot 9 when the SB2 primer was used. Using SB6, diversity was statistically lower in plots 4 and 9 compared to plot 1. Plot 1 had the highest average diversity overall indicating that it is the healthiest plot for chironomids. However, both plots 1 and 4 had AquaBlok. This suggested that another factor was contributing to the diversity in plot 1 compared to plot 4. Plot 1 was located adjacent to plot 9, so migration of species might have increased competition and added to diversity.

Hemoglobin results (**Table 4 and Figure 10**) showed similar levels of diversity across all plots for *G. lobiferus* and Hybrid. *C. riparius* diversity was statistically similar on plot 9; however, there was a drastic increase in diversity of *C. riparius* from plot 1 to plot 9. This may have indicated that expression levels of hemoglobin were more variable as a result of environmental stressors for this species on plot 9. No *C. riparius* species were found on plot 4.

Table 3: Plot influence on average diversity over all collection dates for RAPD using both primer sets. Average diversity and standard deviation were calculated using Microsoft Excel after furthest neighbor binary squared Euclidean distance analysis was run on each individual within a plot grouped by species. No DNA from *C. riparius* species were successfully isolated from plot 1 and there were no larvae found on plot 4. Shared letters represent statistically similar groups for each primer set. ND = No Data.

Plot Influence on Average Diversity over All Collection Dates RAPD

Plot		*G. lobiferus* Grp Avg (SD)	*C. riparius* Grp Avg (SD)	Hybrid Grp Avg (SD)
1	**SB2**	5.33 (0.61) a	ND	5.28 (0.63) ac
	SB6	6.06 (0.99) ac	ND	6.06 (1.13) ab
4	**SB2**	4.74 (0.78) a	ND	4.00 (1.00) abc
	SB6	4.60 (0.81) bd	ND	4.00 (0.87) cde
9	**SB2**	3.59 (1.21) bc	4.57 (0.95) ab	4.35 (0.66) bc
	SB6	3.50 (0.93) e	3.60 (1.29) de	3.87 (0.43) e
Furthest Neighbor Binary Squared Euclidean Distance				$p \leq 0.05$

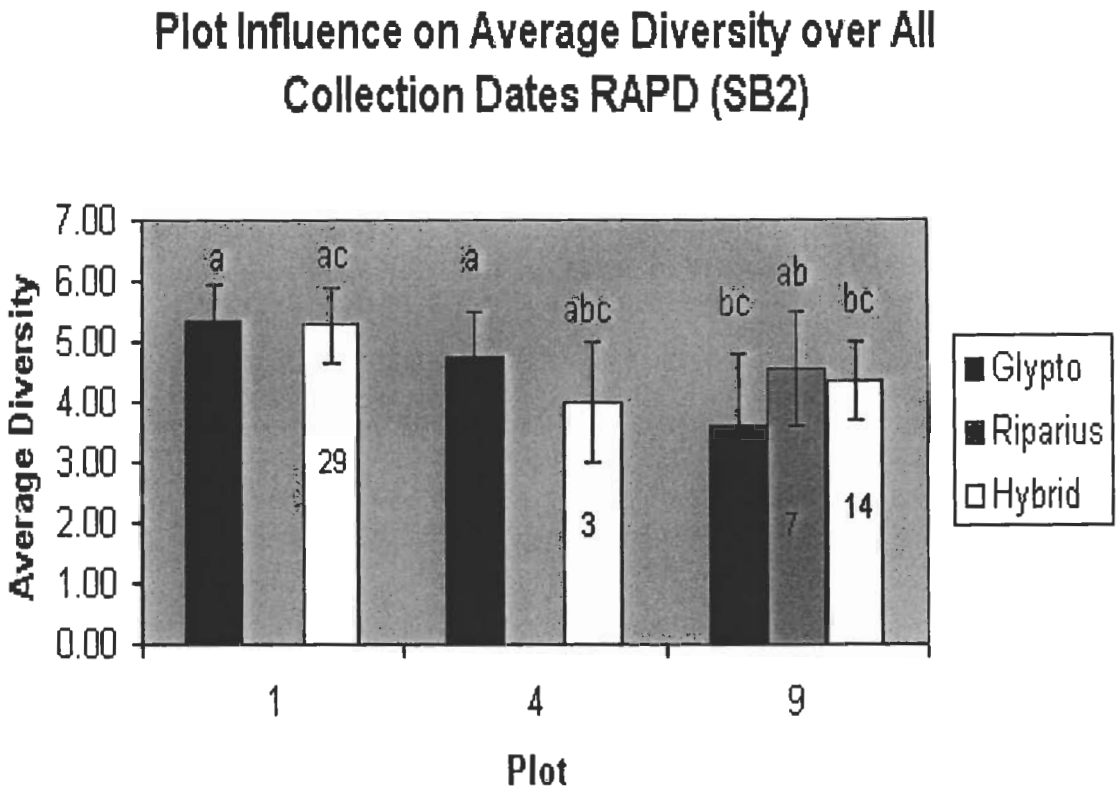

Figure 8: Plot influence on average diversity over all collection dates for RAPD using SB2. RAPD diversity values (average ± SD) were obtained from proximity matrices. Diversities of individuals from each plot were averaged. No DNA from *C. riparius* species were successfully isolated from plot 1 and there were no larvae found on plot 4. Numbers within the bars represent the number of successfully isolated individuals. Shared letters above the bars represent statistically similar groups. Plots 1 and 4 were similar for *G. lobiferus* and Hybrid, respectively, but significantly different from Plot 9 for *G. lobiferus* (one way ANOVA, Tukey's post hoc, $p \leq 0.05$).

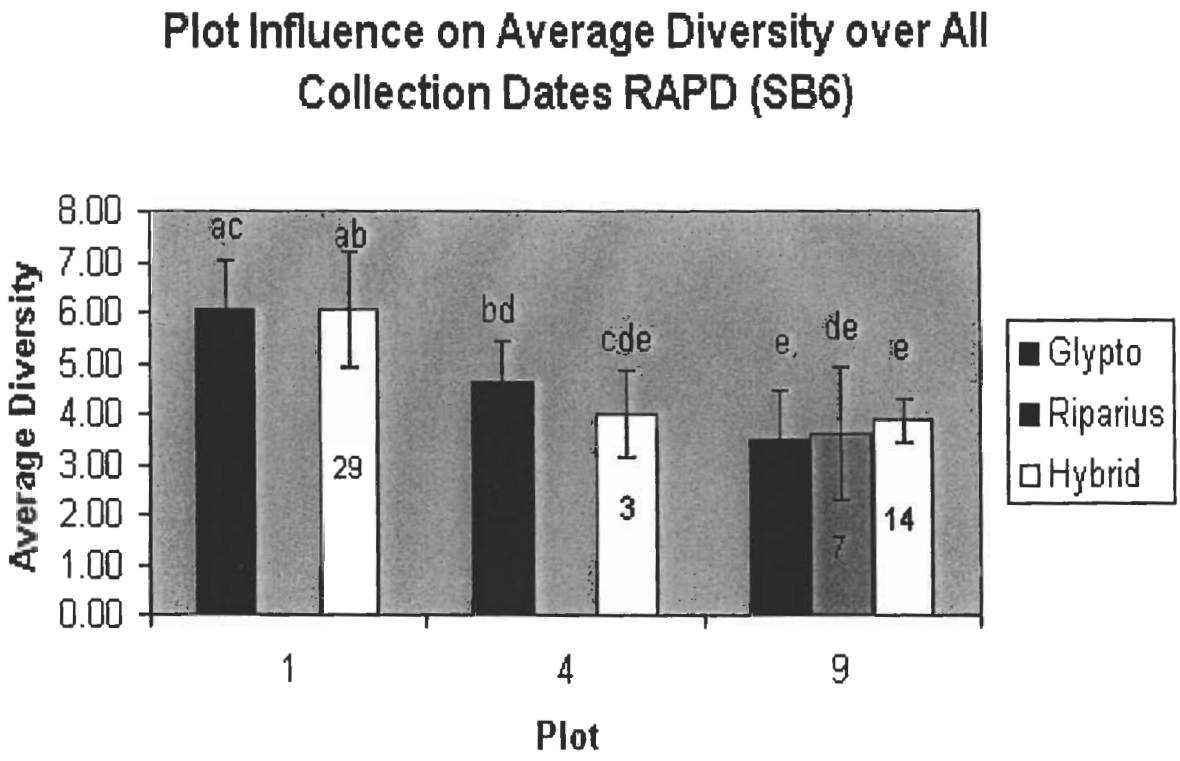

Figure 9: Plot influence on average diversity over all collection dates for RAPD using SB6. RAPD diversity values (average ± SD) were obtained from proximity matrices. Diversities of individuals from each plot were averaged. No DNA from *C. riparius* species were successfully isolated from plot 1 and there were no larvae found on plot 4. Numbers within the bars represent the number of successfully isolated individuals. Shared letters above the bars represent statistically similar groups. All plots were different from each other for all species except; however, plots 4 and 9 were similar for Hybrid (one way ANOVA, Tukey's post hoc, $p \leq 0.05$).

Table 4: Plot influence on average diversity over all collection dates for hemoglobin. Average diversity and standard deviation were calculated using Microsoft Excel after furthest neighbor binary squared Euclidean distance analysis was run on each individual within a plot grouped by species. No *C. riparius* larvae were found on plot 4. Shared letters represent statistically similar groups. ND = No Data.

Plot Influence on Average Diversity over All Collection Dates Hemoglobin

Plot	*G. lobiferus* Grp Avg (SD)	*C. riparius* Grp Avg (SD)	Hybrid Grp Avg (SD)
1	0.74 (0.75) a	1.20 (0.27) ab	0.64 (1.20) ab
4	0.92 (1.06) a	ND	0.80 (0.67) ab
9	0.82 (0.73) a	2.20 (0.56) b	1.12 (0.29) ab
Furthest Neighbor Binary Squared Euclidean Distance			$p \leq 0.05$

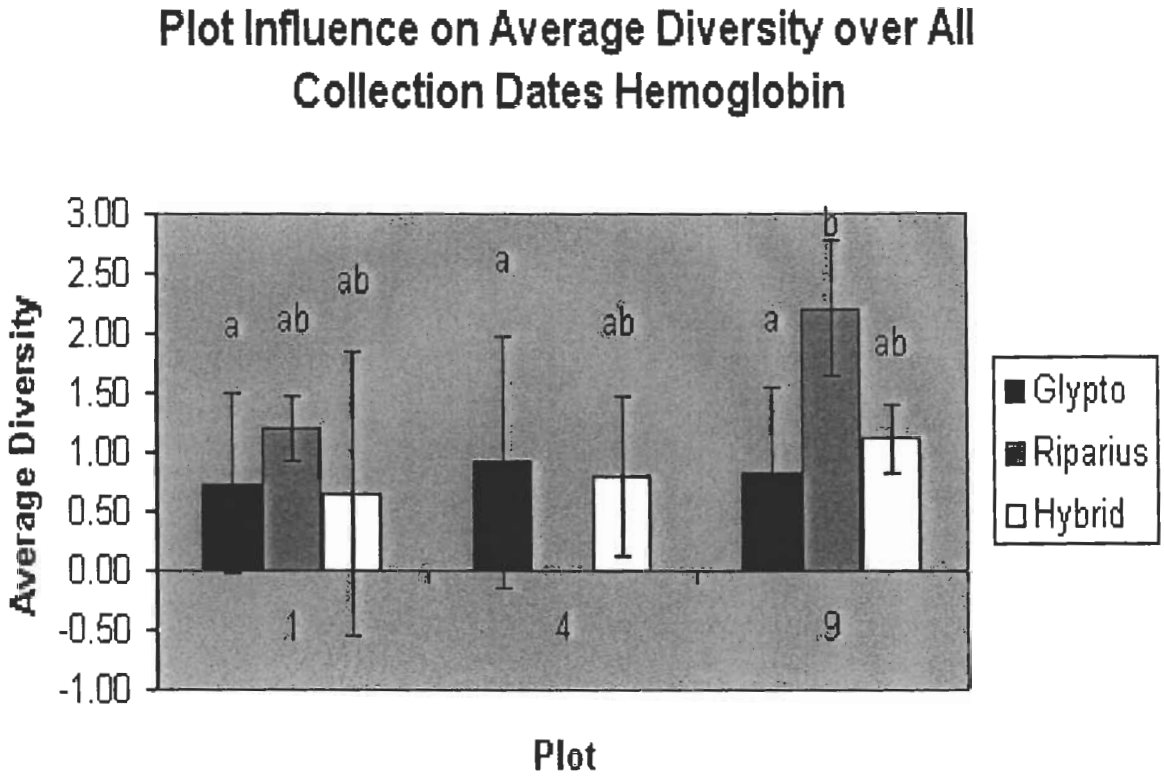

Figure 10: Plot influence on average diversity over all collection dates for hemoglobin. Hemoglobin diversity values (average ± SD) were obtained from proximity matrices. Diversities of individuals from each plot were averaged. No *C. riparius* larvae were found on plot 4. Numbers of individuals from which hemolymph was extracted from is listed in the species count table. Shared letters above the bars represent statistically similar groups. All plots were statistically similar for *G. lobiferus* and Hybrid. *C. riparius* at plot 9 were statistically different from all *G. lobiferus*, but similar to Hybrid (one way ANOVA, Tukey's post hoc, $p \leq 0.05$).

Effect of Collection Date on Diversity

The second factor tested was to determine if collection date had an influence on genetic diversity. This was done by grouping similar species together regardless of plot for both RAPD and hemoglobin (**Tables 5 and 6**). Graphs were then generated by plotting the average diversity of a species against the collection date during which it was collected (**Figures 11, 12, and 13**).

RAPD (**Table 5 and Figures 11 and 12**) results demonstrated similar levels of diversity for *G. lobiferus* between May and August; however there was a decrease in diversity in November for both primer sets. This decreased level of diversity could be a result of lower levels of this species found during this month. Collection date influence cannot be determined for the other two species because they were only found on a single collection date. However, when Hybrid was compared to *G. lobiferus* in November, *G. lobiferus* had lower diversity. Increased DNA diversity in Hybrid could have been due to interbreeding of *C. riparius* and *G. lobiferus*. Collection date was not a good factor to compare species using RAPD because only one species was found during this sampling period.

Hemoglobin results (**Table 6 and Figure 13**) showed low levels of diversity for *G. lobiferus* during May and November, but greatly increased levels in August. This could have indicated increased expression levels of the protein as a result of environmental stressors for this species during August. The same could be said for *C. riparius* in August and Hybrids in November as exhibited by high levels of diversity

during those months and because they are statistically similar to *G. lobiferus* in August as well.

Table 5: Date influence on average diversity over all plots with RAPD using both primer sets. Average diversity and standard deviation were calculated using Microsoft Excel after furthest neighbor binary squared Euclidean distance analysis was run on each individual collected on a certain date grouped by species. No *C. riparius* larvae were found in May and not enough were found in November to run a statistical analysis. No Hybrid larvae were found in May or August. Shared letters represent statistically similar groups for each primer set. ND = No Data.

Date Influence on Average Diversity over all Plots RAPD

Month		*G. lobiferus* Grp Avg (SD)	*C. riparius* Grp Avg (SD)	Hybrid Grp Avg (SD)
May	**SB2**	4.95 (0.57) a	ND	ND
	SB6	4.68 (0.96) a	ND	ND
Aug	**SB2**	4.77 (0.92) a	4.95 (1.16) a	ND
	SB6	4.77 (0.92) a	4.95 (1.16) ab	ND
Nov	**SB2**	2.85 (1.44) b	ND	4.60 (0.59) a
	SB6	3.65 (1.80) b	ND	5.79 (1.10) a

Furthest Neighbor Binary Squared Euclidean Distance $p \leq 0.05$

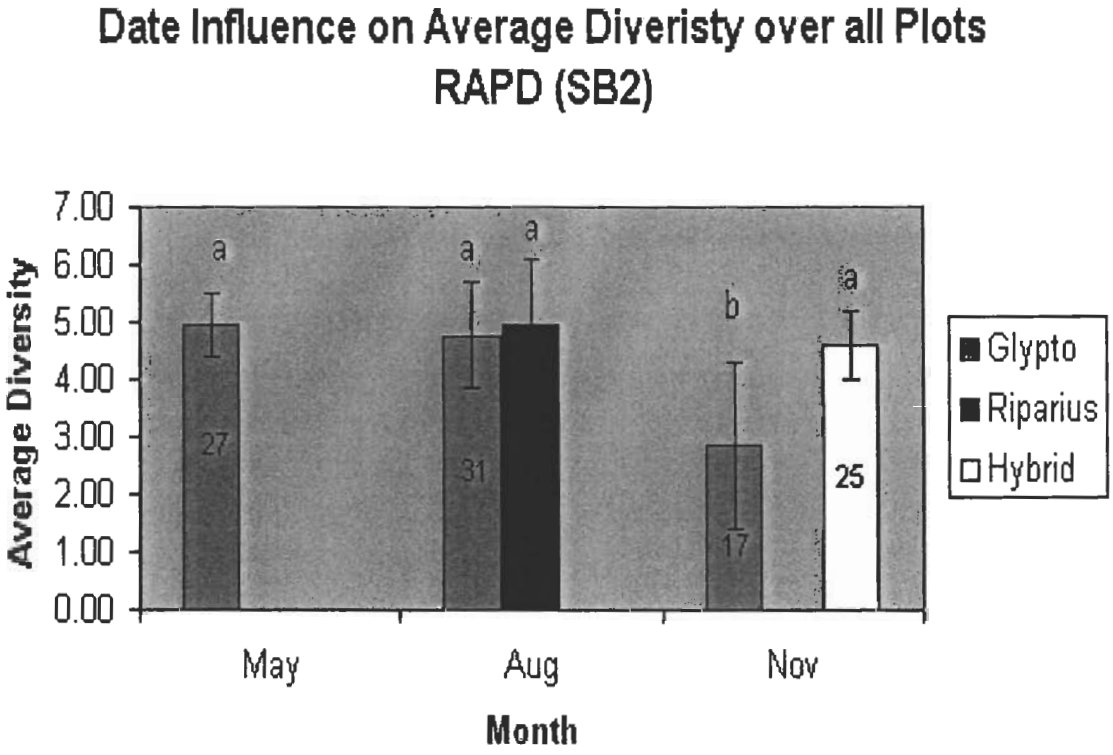

Figure 11: Date influence on average diversity over all plots for RAPD using SB2. RAPD diversity values (average ± SD) were obtained from proximity matrices. Diversities of individuals from each plot were averaged. No *C. riparius* larvae were found in May and not enough were found in November to run a statistical analysis. No Hybrids were found in May or August. Numbers within the bars represent the number of individuals from which DNA was successfully isolated. Shared letters above the bars represent statistically similar groups. May and August were statistically similar, but significantly different from November for *G. lobiferus*. *C. riparius* for August and Hybrid for November were similar to May and August for *G. lobiferus* (one way ANOVA, Tukey's post hoc, $p \leq 0.05$).

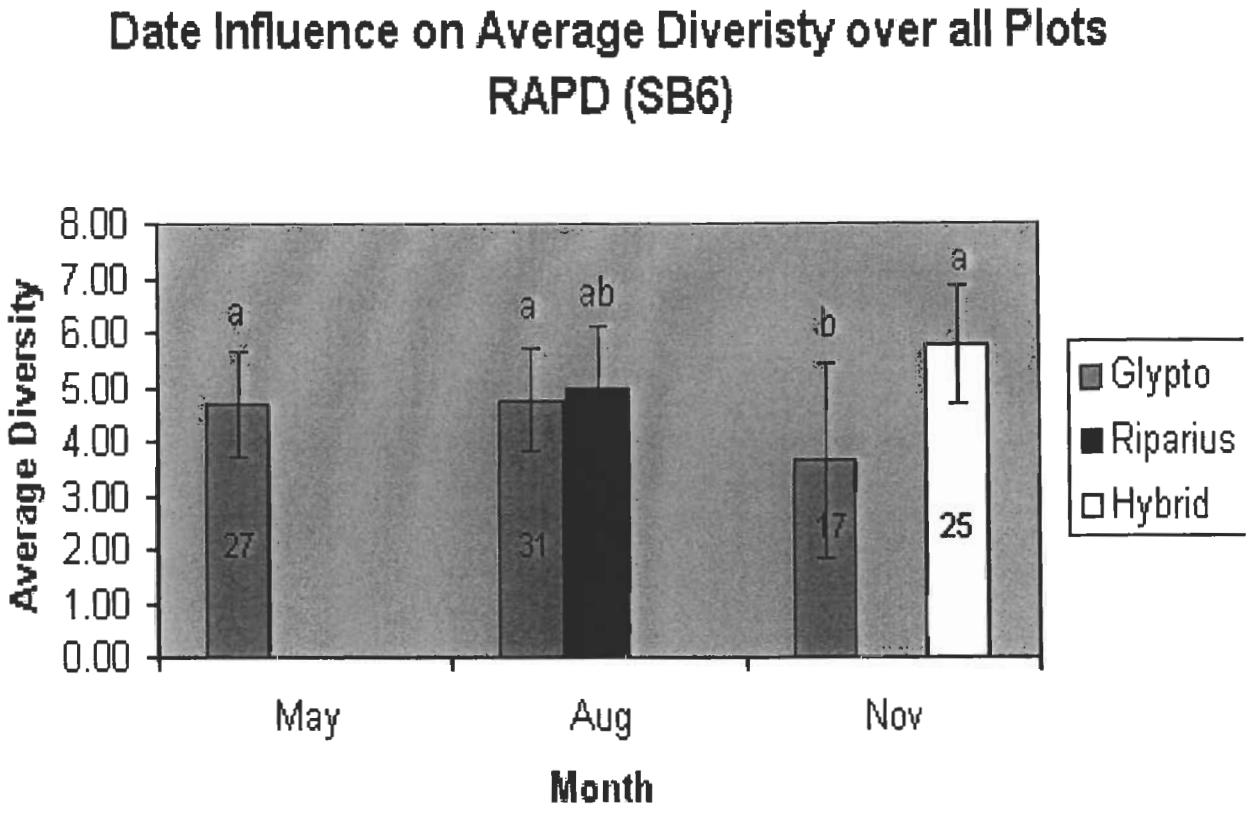

Figure 12: Date influence on average diversity over all plots for RAPD using SB6. RAPD diversity values (average ± SD) were obtained from proximity matrices. Diversities of individuals from each plot were averaged. No *C. riparius* larvae were found in May and not enough were found in November to run statistical analysis. No Hybrids were found in May or August. Numbers within the bars represent the number of successfully isolated individuals. Shared letters above the bars represent statistically similar groups. May and August were statistically similar, but significantly different from November for *G. lobiferus*. *C. riparius* for August and Hybrid for November were similar to May and August for *G. lobiferus*; however, *C. riparius* in August was also similar to *G. lobiferus* in November as well (one way ANOVA, Tukey's post hoc, $p \leq 0.05$).

Table 6: Date influence on average diversity over all plots for hemoglobin. Average diversity and standard deviation were calculated using Microsoft Excel after furthest neighbor binary squared Euclidean distance analysis was run on each individual collected on a certain date grouped by species. No *C. riparius* larvae were found in May and not enough were found in November to run a statistical analysis. No Hybrid larvae were found in May or August. Shared letters represent statistically similar groups. ND = No Data.

Date Influence on Average Diversity over all Plots Hemoglobin

Month	*G. lobiferus* Grp Avg (SD)	*C. riparius* Grp Avg (SD)	Hybrid Grp Avg (SD)
May	0.27 (0.54) a	ND	ND
Aug	1.12 (0.61) b	1.20 (0.45) b	ND
Nov	0.24 (0.67) a	ND	0.84 (0.40) b

Furthest Neighbor Binary Squared Euclidean Distance $p \leq 0.05$

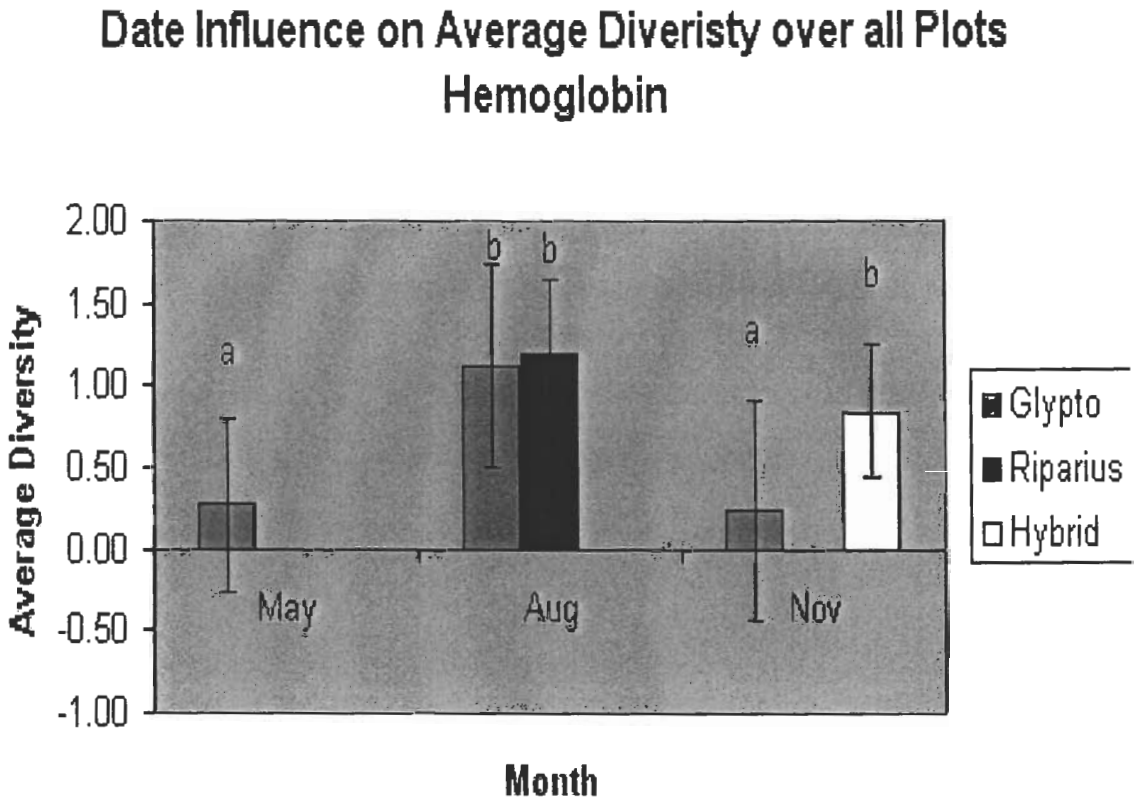

Figure 13: Date influence on average diversity over all plots for hemoglobin. Hemoglobin diversity values (average ± SD) were obtained from proximity matrices. Diversities of individuals from each plot were averaged. No *C. riparius* species were found in May and not enough were found in November to run statistical analysis. No Hybrids were found in May or August. Numbers of individuals from which hemolymph was extracted from is listed in the species count table. Shared letters above the bars represent statistically similar groups. *G. lobiferus* were only similar in May and November and statistically different from August and all other species. *G. lobiferus* in August were statistically similar to *C. riparius* in August and Hybrid in November (one way ANOVA, Tukey's post hoc, $p \leq 0.05$).

Effect of Plot and Collection Date on Diversity

The final factor tested was how plot and collection date together affected a species average diversity. This was done by analyzing individuals collected at the same time, from the same plot, of the same species, and averaging their diversity values (**Tables 7 through 10**). Graphs were generated by plotting the average diversity against the plot from which each species was collected and grouping by collection date for both RAPD and hemoglobin (**Figures 14 through 22**).

RAPD results (**Tables 7 through 9 and Figures 14 through 19**) indicated decreasing diversity over plot; however, it also displays varying activity between months for *G. lobiferus* for both primer sets. There is a general decrease in diversity between months when viewed at each plot. The highest values were seen in May and August at plots 1 and 4, but drastically lower values were observed in November at plots 1 and 4. Plot 9 on the whole had lower values. This indicated plots 1 and 4 could be a healthier, more competitive environment for *G. lobiferus* species. Furthermore, warmer weather could have contributed to the increased diversity values as well. Not enough data was collected to assess this factor for *C. riparius*. Hybrid data was conflicting for the two primer sets, where SB2 showed a gradual increase in diversity over plots, but SB6 showed a gradual decrease in diversity over plots. No conclusions could be drawn about how plot affected Hybrid species. Temporal data could not be assessed since Hybrids were only found in November.

Hemoglobin results (**Table 10 and Figures 20 through 22**) indicated similar values across plots in May and August, respectively, for *G. lobiferus*. There was an

increase in diversity from May to August suggesting there was more stress on the species respiration, thus increasing expression of the protein in August. November data increased at plot 1, but decreased over the rest of the plots. However, this could have been due to the number of species decreasing at these plots. *C. riparius* data was only available for August plots 1 and 9; however, both plots exhibited similar levels of high diversity. This further supported a temporal effect on the expression of the hemoglobin protein. Finally, Hybrid species were only found in November; therefore, seasonal effect could not be established. On the other hand there was an increasing trend in diversity between plots 1 and 9; indicative conditions on plot 9 caused more expression of the hemoglobin protein for this species due to the higher diversity value at this location. The average diversity of plots 1 and 4 and plots 4 and 9 are statistically similar to each other, respectively.

Table 7: Average diversity by plot and collection date RAPD for *G. lobiferus* using both primer sets. Average diversity and standard deviation were calculated using Microsoft Excel after further neighbor binary squared Euclidean distance analysis was run on each individual collected on a certain date and plot. Not enough *G. lobiferus* were collected to be analyzed from plot 9 in November. Shared letters represent statistically similar groups for each primer set. ND = No Data.

Average Diversity by Plot and Collection Date RAPD (*G. lobiferus*)

Plot		May Grp Avg (SD)	August Grp Avg (SD)	November Grp Avg (SD)
	SB 2	4.49 (0.69) a	4.98 (0.64) a	1.78 (0.51) bc
1	**SB6**	4.58 (0.57) a	4.98 (0.64) a	2.67 (0.58) bce
	SB 2	4.51 (0.56) a	2.84 (0.91) b	1.88 (0.50) d
4	**SB6**	3.87 (0.83) ab	2.84 (0.91) c	1.94 (0.81) de
	SB 2	2.27 (0.59) bd	1.00 (0.31) c	ND
9	**SB6**	2.75 (0.76) c	1.00 (0.31) d	ND

Furthest Neighbor Binary Squared Euclidean Distance $p \leq 0.05$

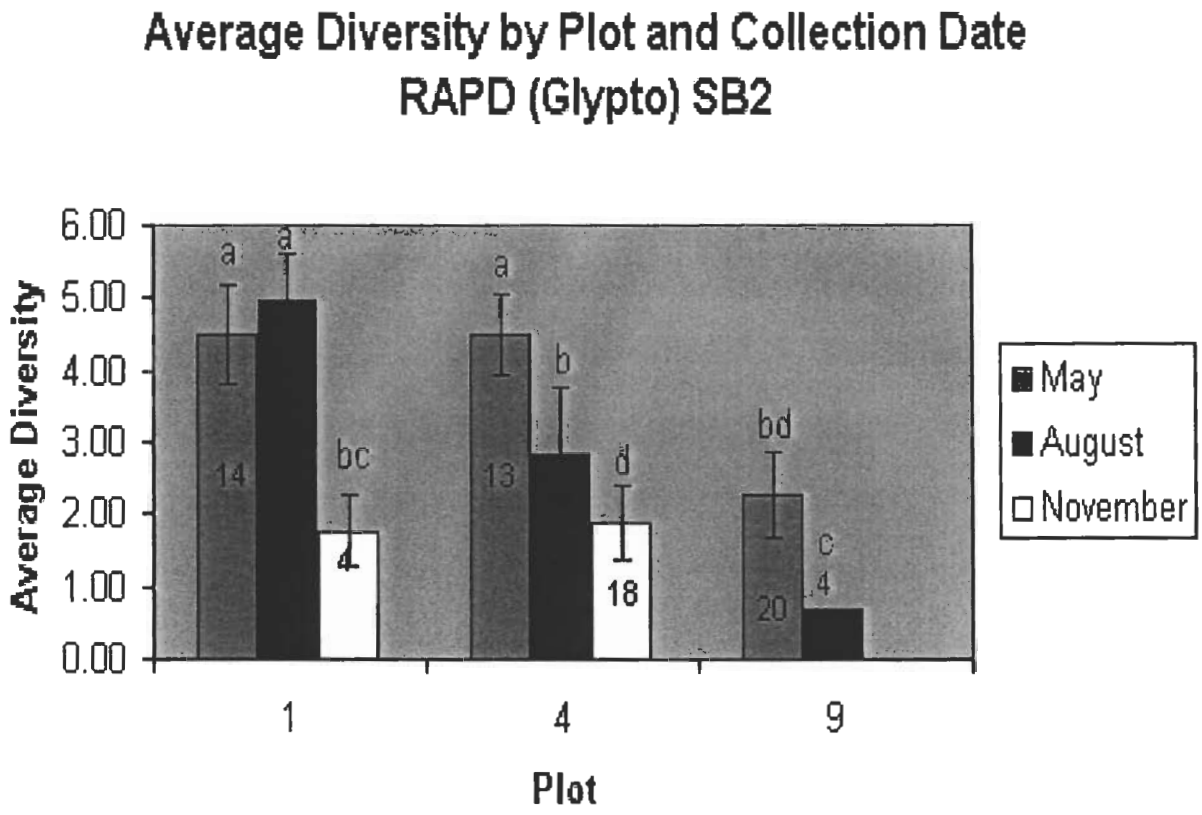

Figure 14: Average diversity by plot and collection date RAPD for *G. lobiferus* using SB2. RAPD diversity values (average ± SD) were obtained from proximity matrices. Diversities of individuals from each plot were averaged. Not enough *G. lobiferus* were collected to be analyzed from plot 9 in November. Numbers within the bars represent the number of successfully isolated individuals. Shared letters above the bars represent statistically similar groups. May and August on plot 1 are statistically similar to each other as well as to May on plot 4. November plot 1 average diversity it similar to that of August plot 4, May plot 9, and August plot 9. Finally, *G. lobiferus* in November plot 4 are statistically similar to those in May on plot 9.

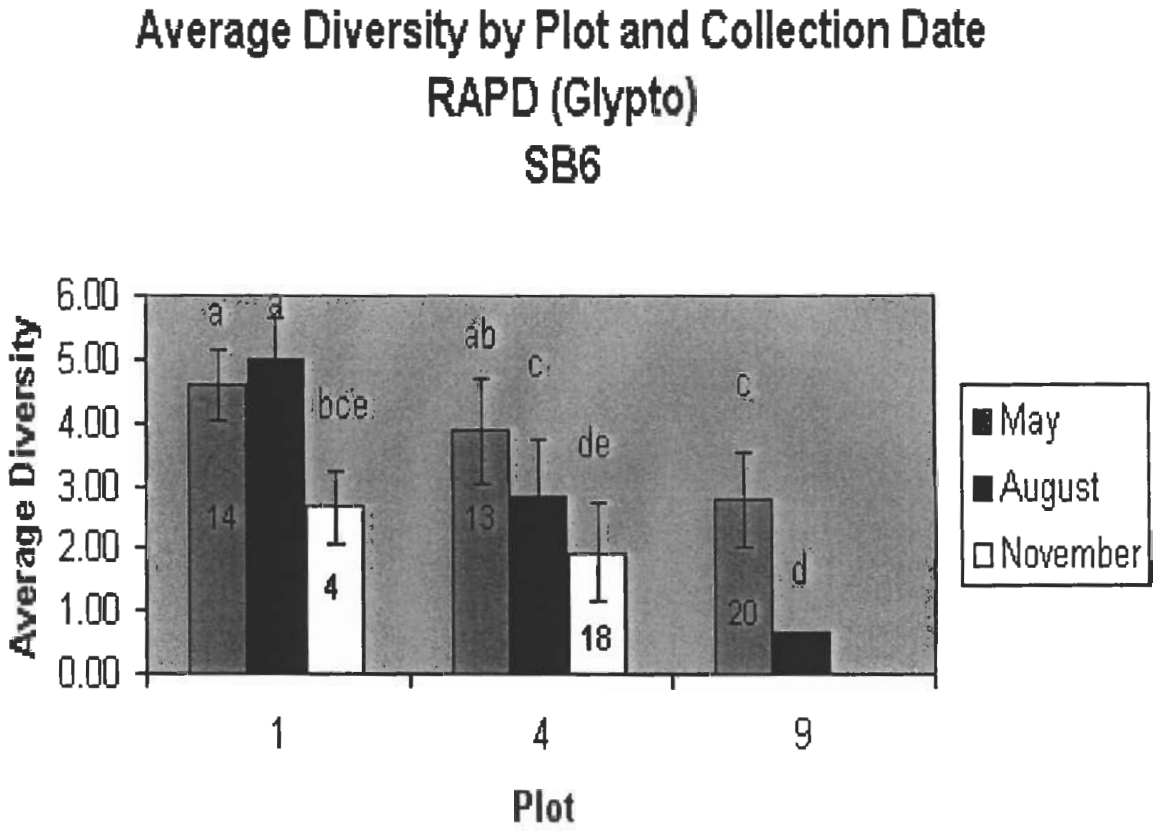

Figure 15: Average diversity by plot and collection date RAPD for *G. lobiferus* using SB6. RAPD diversity values (average ± SD) were obtained from proximity matrices. Diversities of individuals from each plot were averaged. Not enough *G. lobiferus* were collected to be analyzed from plot 9 in November. Numbers within the bars represent the number of successfully isolated individuals. Shared letters above the bars represent statistically similar groups. May and August on plot 1 are statistically similar to each other as well as to May on plot 4. November plot 1 average diversity it similar to that of August plot 4, May plot 9, and November plot 4. Finally, *G. lobiferus* in November plot 4 are statistically similar to those in August on plot 9.

Table 8: Average diversity by plot and collection date RAPD for *C. riparius* using both primer sets. Average diversity and standard deviation were calculated using Microsoft Excel after furthest neighbor binary squared Euclidean distance analysis was run on each individual collected on a certain date and plot. Not enough *C. riparius* larvae were successfully isolated to be analyzed from plot 1 in August and plot 9 in November. No *C. riparius* larvae were collected in May, plot 4 in August and November, and plot 1 in November. ND = No Data.

Average Diversity by Plot and Collection Date RAPD (*C. riparius*)

Plot		May Grp Avg (SD)	August Grp Avg (SD)	November Grp Avg (SD)
	SB 2	ND	ND	ND
1	**SB6**	ND	ND	ND
	SB 2	ND	ND	ND
4	**SB6**	ND	ND	ND
	SB 2	ND	3.60 (1.29)	ND
9	**SB6**	ND	3.60 (1.29)	ND
Furthest Neighbor Binary Squared Euclidean Distance				$p \leq 0.05$

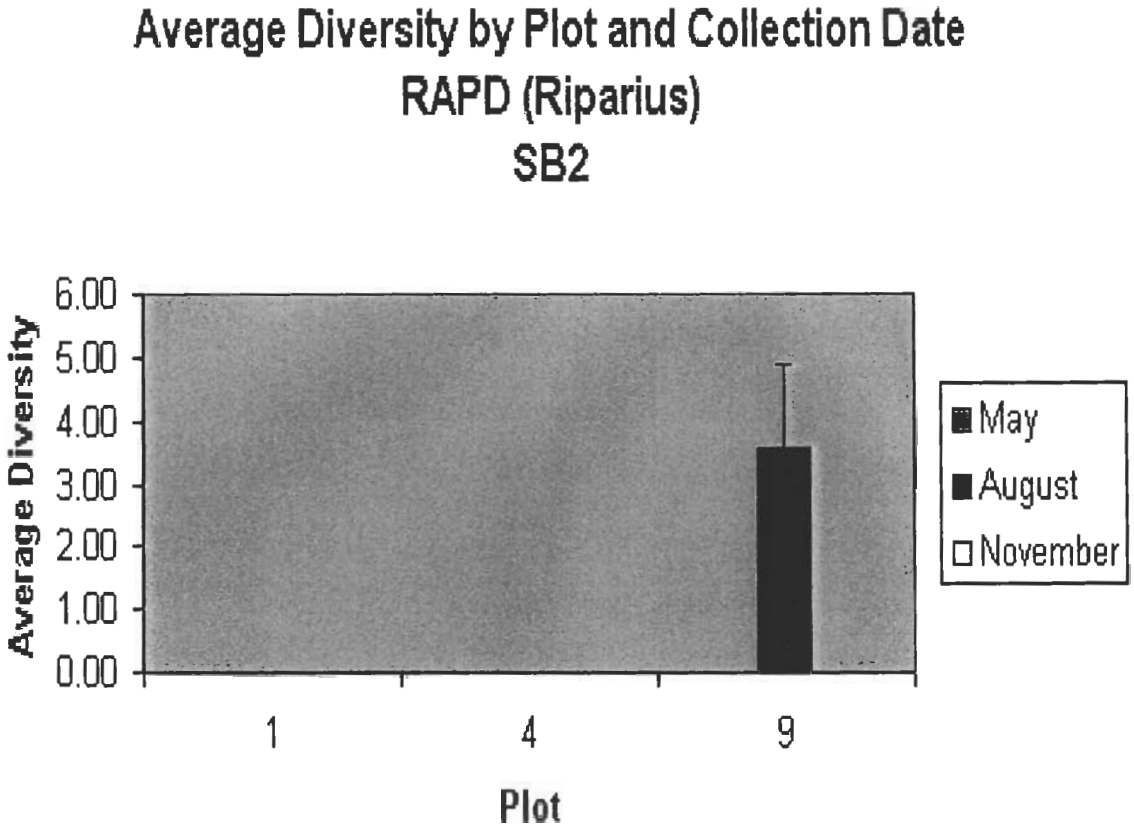

Figure 16: Average diversity by plot and collection date RAPD for *C. riparius* using SB2. RAPD diversity values (average ± SD) were obtained from proximity matrices. Diversities of individuals from each plot were averaged. Not enough *C. riparius* larvae were successfully isolated to be analyzed from plot 1 in August and plot 9 in November. No *C. riparius* larvae were collected in May, plot 4 in August and November, and plot 1 in November. Numbers within the bars represent the number of successfully isolated individuals.

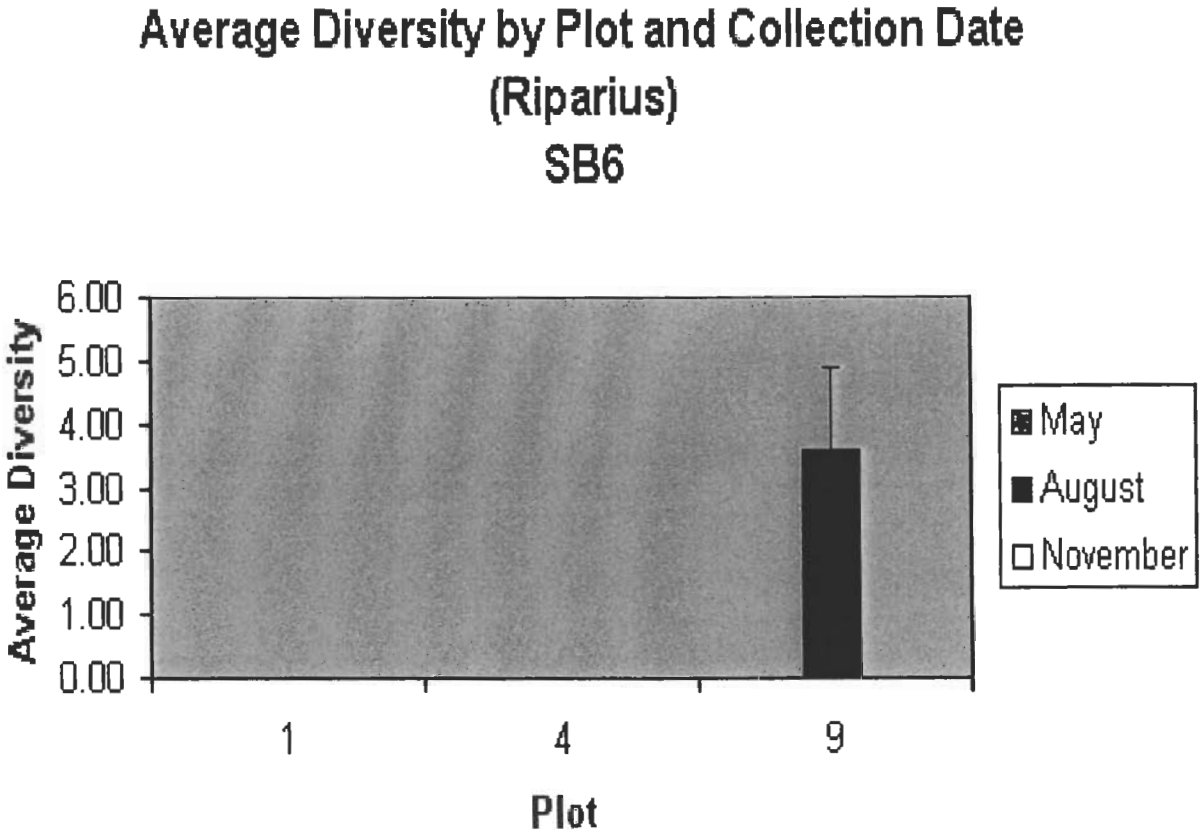

Figure 17: Average diversity by plot and collection date RAPD for *C. riparius* using SB6. RAPD diversity values (average ± SD) were obtained from proximity matrices. Diversities of individuals from each plot were averaged. Not enough *C. riparius* larvae were successfully isolated to be analyzed from plot 1 in August and plot 9 in November. No *C. riparius* larvae were collected in May, plot 4 in August and November, and plot 1 in November. Numbers within the bars represent the number of successfully isolated individuals.

Table 9: Average diversity by plot and collection date RAPD for Hybrids using both primer sets. Average diversity and standard deviation were calculated using Microsoft Excel after furthest neighbor binary squared Euclidean distance analysis was run on each individual collected on a certain date and plot. No Hybrid larvae were collected in May or August. Shared letters represent statistically similar groups for each primer set. ND = No Data.

Average Diversity by Plot and Collection Date RAPD (Hybrid)

Plot		May Grp Avg (SD)	August Grp Avg (SD)	November Grp Avg (SD)
	SB 2	ND	ND	2.68 (0.72) a
1	**SB6**	ND	ND	5.79 (1.10) a
	SB 2	ND	ND	4.00 (1.00) b
4	**SB6**	ND	ND	4.00 (0.87) b
	SB 2	ND	ND	4.35 (0.66) b
9	**SB6**	ND	ND	3.87 (0.43) b

Furthest Neighbor Binary Squared Euclidean Distance $p \leq 0.05$

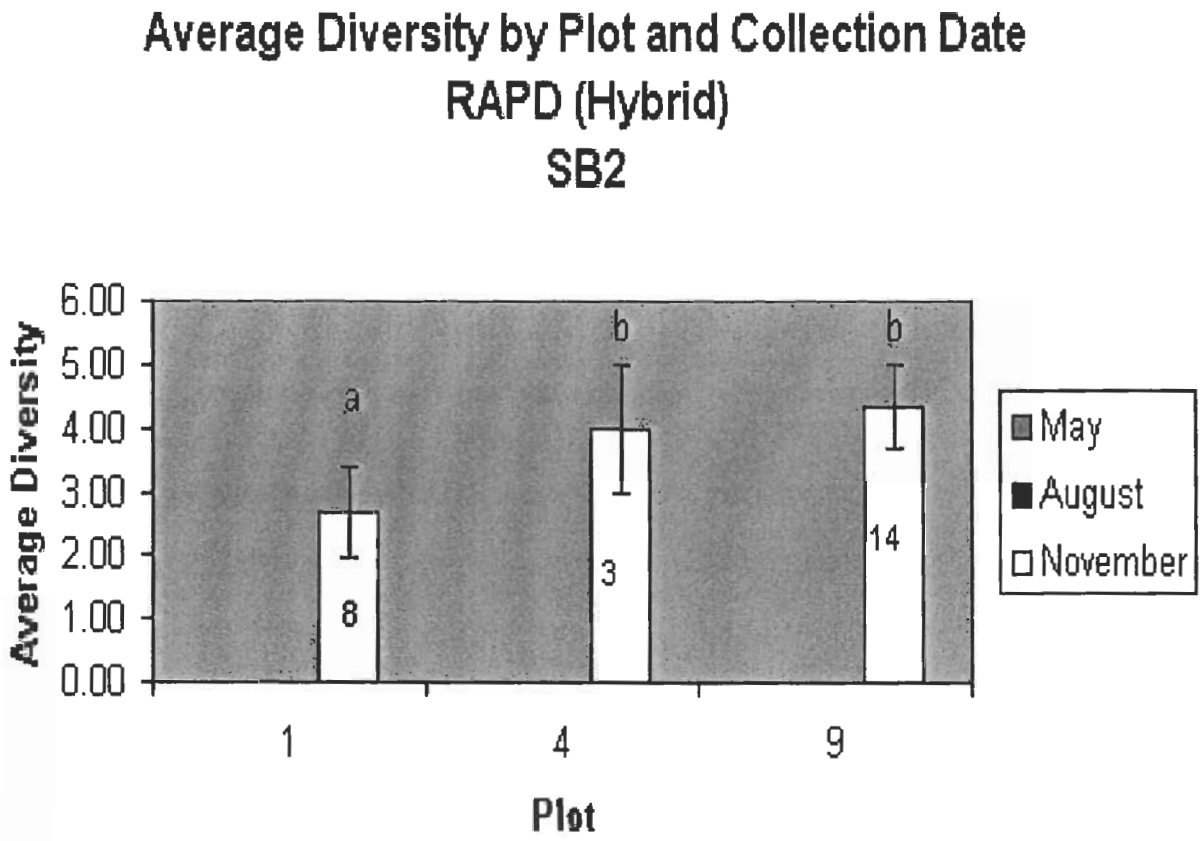

Figure 18: Average diversity by plot and collection date RAPD for Hybrid using SB2. RAPD diversity values (average ± SD) were obtained from proximity matrices. Diversities of individuals from each plot were averaged. No Hybrid larvae were collected in May or August. Numbers within the bars represent the number of successfully isolated individuals. Shared letters above the bars represent statistically similar groups. Hybrids collected in November on plot 4 and 9 are statistically similar, but both are significantly different from those collected in November on plot 1.

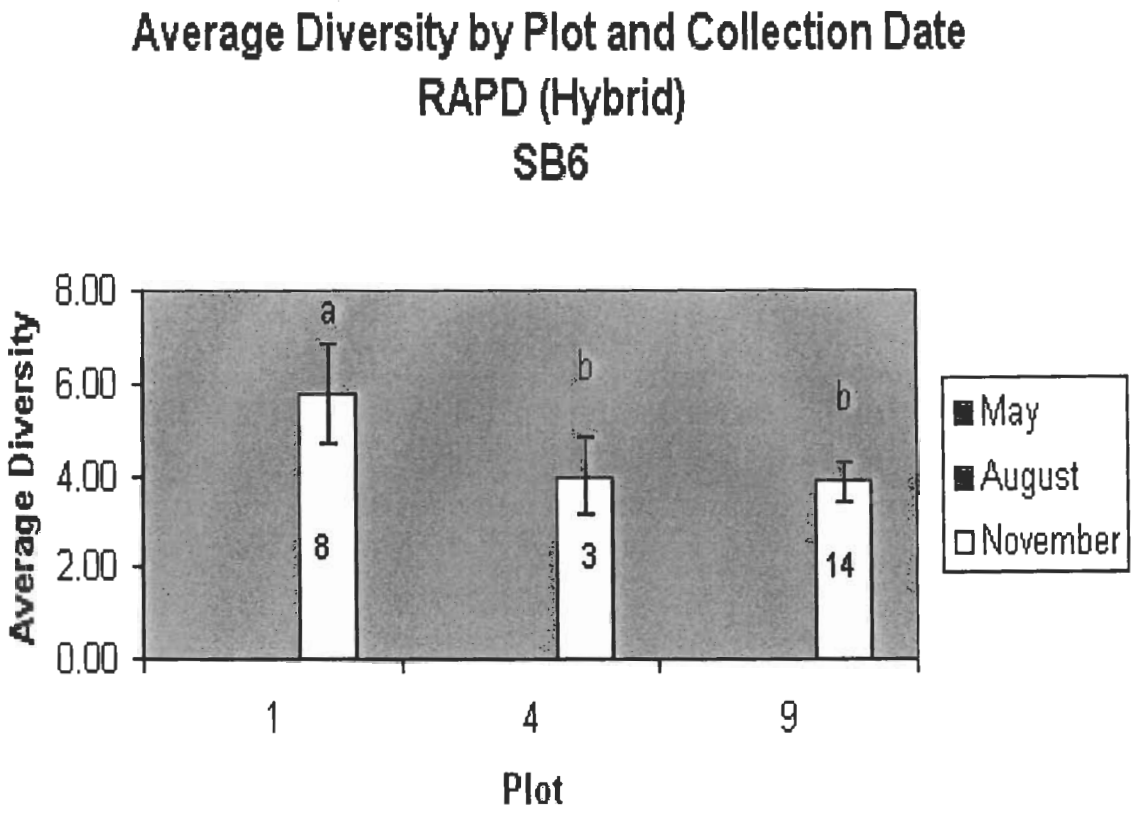

Figure 19: Average diversity by plot and collection date RAPD for Hybrid using SB6. RAPD diversity values (average ± SD) were obtained from proximity matrices. Diversities of individuals from each plot were averaged. No Hybrid larvae were collected in May or August. Numbers within the bars represent the number of successfully isolated individuals. Shared letters above the bars represent statistically similar groups. Hybrids collected in November on plot 4 and 9 are statistically similar, but both are significantly different from those collected in November on plot 1.

Table 10: Average diversity by plot and collection date Hemoglobin in all species. Average diversity and standard deviation were calculated using Microsoft Excel after furthest neighbor binary squared Euclidean distance analysis was run on each individual collected on a certain date and plot. All *G. lobiferus* individuals were similar on plot 4 in November and not enough were collected in November on plot 9 to run statistical analysis. No *C. riparius* larvae were collected in May, August on plot 4, and November on plots 1 and 4. Not enough *C. riparius* larvae were successfully collected to run statistical analysis on from November on plot 9. No Hybrid larvae were collected in May or August on plot 4. Not enough Hybrid larvae were collected in August plots 1 and 9 to run statistical analysis on. Shared letters represent statistically similar groups for each species. ND = No Data.

Average Diversity by Plot and Collection Date Hemoglobin

Plot		May Grp Avg (SD)	August Grp Avg (SD)	November Grp Avg (SD)
1	***G. lobiferus***	0.17 (0.40) a	0.76 (0.59) bcde	1.14 (1.26) e
	C. riparius	ND	1.20 (0.27) a	ND
	Hybrid	ND	ND	0.56 (0.36) a
4	***G. lobiferus***	0.28 (0.27) acd	0.85 (0.68) e	0.00 (0.00) a
	C. riparius	ND	ND	ND
	Hybrid	ND	ND	0.80 (0.67) ab
9	***G. lobiferus***	0.32 (0.77) ab	0.79 (0.58) bde	ND
	C. riparius	ND	1.05 (0.31) a	ND
	Hybrid	ND	ND	1.03 (0.19) b

Furthest Neighbor Binary Squared Euclidean Distance $p \leq 0.05$

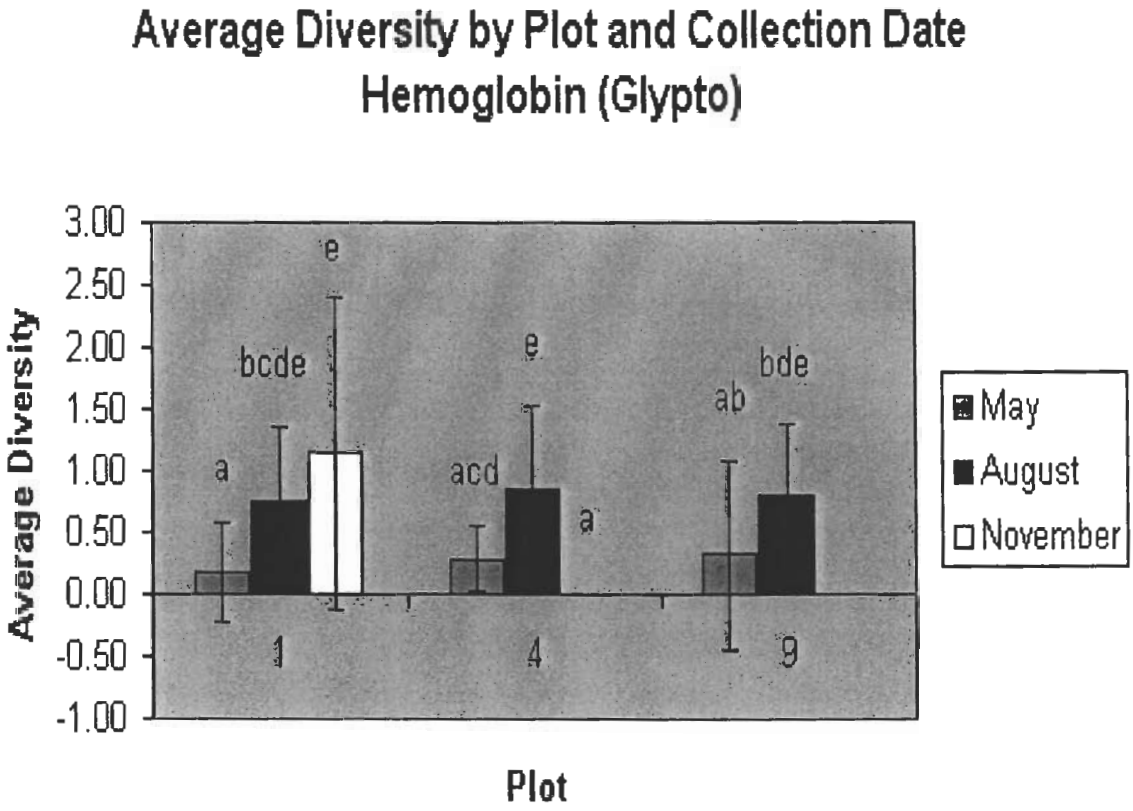

Figure 20: Average diversity by plot and collection date Hemoglobin for *G. lobiferus*. Hemoglobin diversity values (average ± SD) were obtained from proximity matrices. Diversities of individuals from each plot were averaged. Not enough *G. lobiferus* larvae were collected in November on plot 9 to run statistical analysis. Numbers of individuals from which hemolymph was extracted from is listed in the species count table. Shared letters above the bars represent statistically similar groups. There was no average diversity value for November on plot 4 because all individuals were genetically identical. All May values were statistically similar to each other as well as to November plot 4 individuals. Individuals from August plot 1 were similar to those on November plot 1, May plots 4 and 9, August plots 4 and 9, and November plot 1.

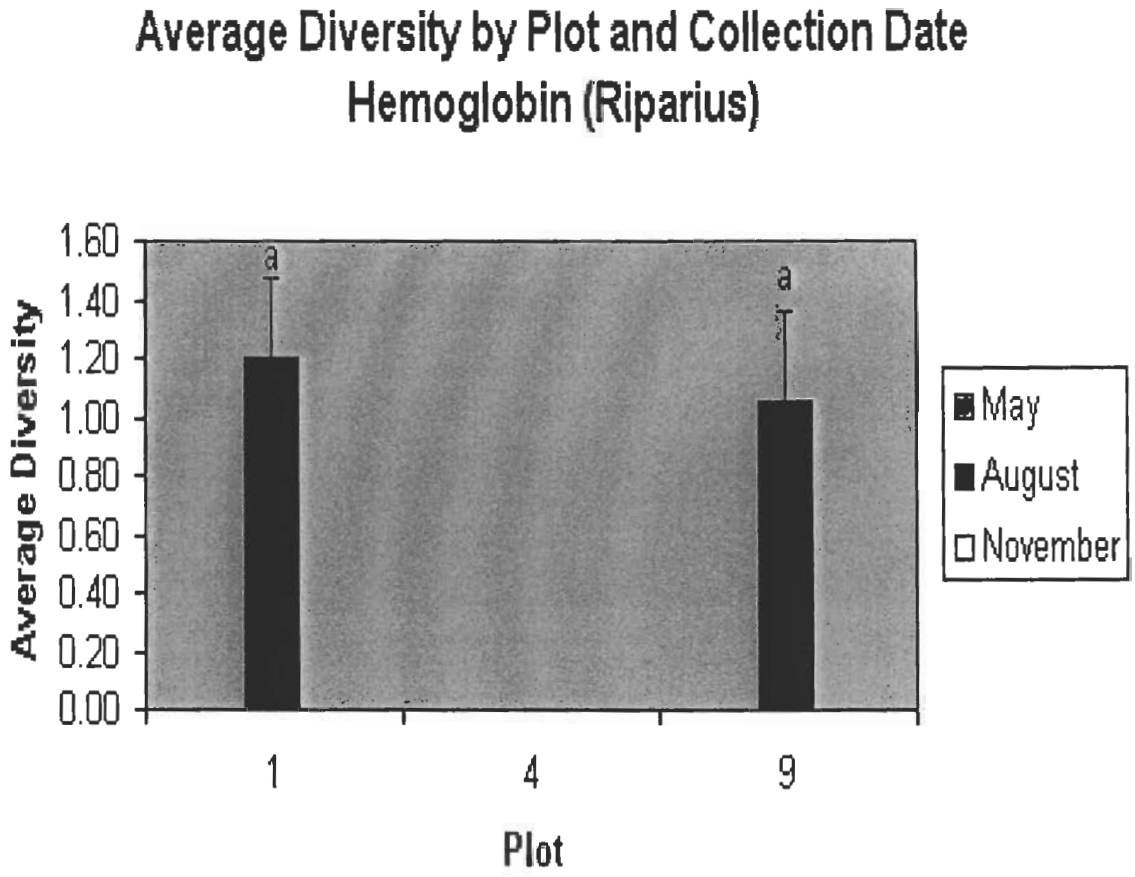

Figure 21: Average diversity by plot and collection date Hemoglobin for *C. riparius*. Hemoglobin diversity values (average ± SD) were obtained from proximity matrices. Diversities of individuals from each plot were averaged. No *C. riparius* larvae were collected in May, August on plot 4, and November on plots 1 and 4. Not enough *C. riparius* larvae were successfully collected to run statistical analysis on from November on plot 9. Numbers of individuals from which hemolymph was extracted from is listed in the species count table. Shared letters above the bars represent statistically similar groups. Individuals from August plot 1 were statistically similar to those from August on plot 9.

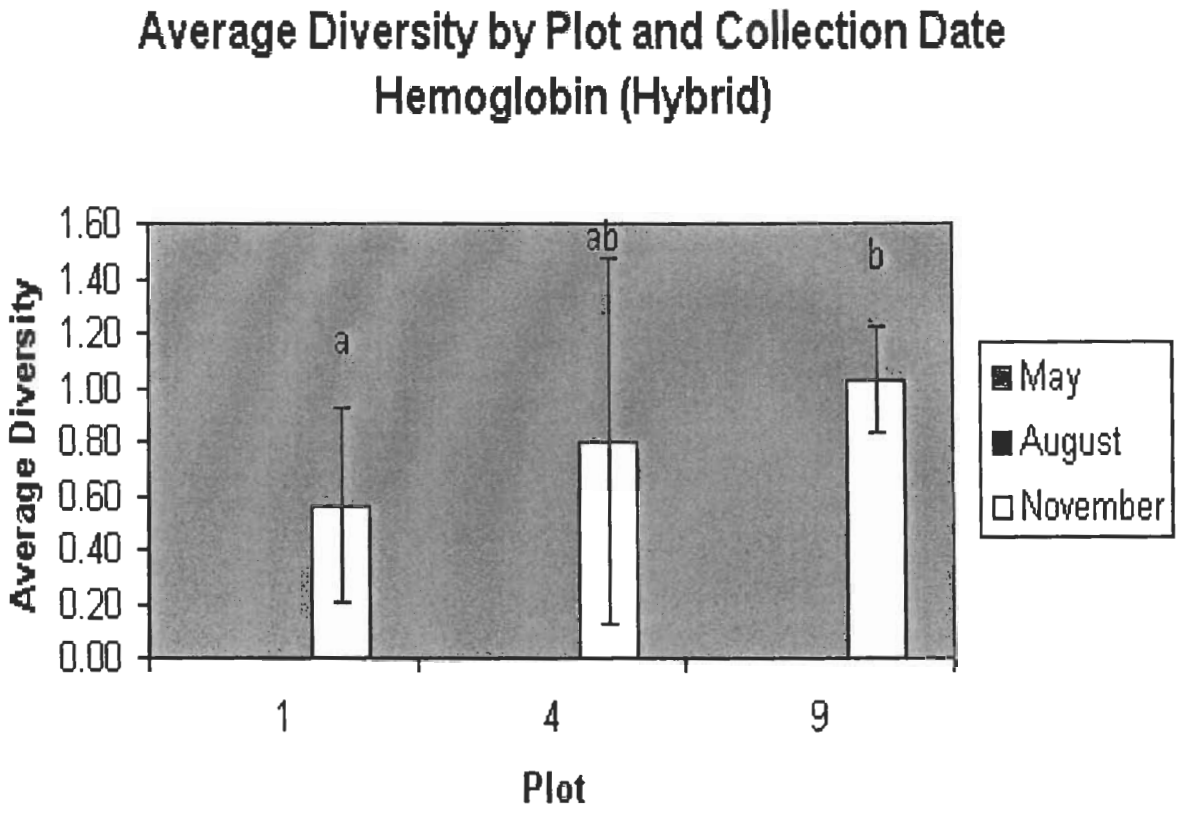

Figure 22: Average diversity by plot and collection date Hemoglobin for Hybrid. Hemoglobin diversity values (average ± SD) were obtained from proximity matrices. Diversities of individuals from each plot were averaged. No Hybrid larvae were collected in May or August on plot 4. Not enough Hybrid larvae were collected in August plots 1 and 9 to run statistical analysis on. Numbers of individuals from which hemolymph was extracted from is listed in the species count table. Shared letters above the bars represent statistically similar groups. Individuals collected in November from plot 1 were statistically similar to those collected in November from plot 4, but significantly different from those from November plot 9. Individuals from November plot 4 were statistically similar to those collected in November from plot 9.

Correlation of Data

Data was correlated using Pearson's correlation method and defined significant if $p \leq 0.05$. The data correlated include: RAPD to hemoglobin, RAPD to heavy metals, and hemoglobin to heavy metals (**Table 11**).

RAPD did not correlate with hemoglobin data except for *G. lobiferus* in August. RAPD and hemoglobin data for *C. riparius* did not correlate at all and lacked data for May and November collection dates. RAPD date correlated with hemoglobin data for Hybrids in November; however, only for the SB2 primer. The SB6 primer for Hybrid in November did not correlate RAPD with hemoglobin; furthermore, there was a lack of data since none were collected in May or August.

RAPD data correlated well with heavy metals data with an overall negative correlation for *G. lobiferus* (**Figure 23**). This indicated a general trend where an increased level of heavy metals contamination resulted in a lower average genetic diversity. Conversely, as the total concentration of heavy metals decreased, genetic diversity increased. No correlation was able to be made for *C. riparius* for all collection dates and Hybrid for May and August collection dates.

Hemoglobin data correlated well with heavy metals in *G. lobiferus* for August and November. There was a strong positive correlation, which indicated at higher levels of contamination there were higher diversity values. Conversely, at low concentrations of heavy metals, there were low values of average diversity. Results suggested the possibility of an increase in expression of the hemoglobin protein due to the stress of contaminants or other stressors, thus resulting in higher diversity values.

Table 11: Pearson's Correlation Values for RAPD, Hemoglobin and Heavy Metal Data. Correlation values (r^2) for data comparing RAPD to hemoglobin, RAPD to metals, and hemoglobin to metals. A box marked with 'X' represents a correlation that was not calculated due to lack of data. Correlations were considered significant if they had a p value less than or equal to 0.05.

Pearson's Correlation Values for RAPD, Hemoglobin and Heavy Metal Data					
Month	Biomarker	Primer	*G. lobiferus*	*C. riparius*	Hybrid
May	*Hb vs. RAPD*	**SB2**	-0.189	X	X
		SB6	-0.162	X	X
	Hb vs. Metals		0.163	X	X
	RAPD vs. Metals	**SB2**	-0.870*	X	X
		SB6	-0.646*	X	X
August	*Hb vs. RAPD*	**SB2**	-0.656*	-0.688	X
		SB6	-0.632*	-0.688	X
	Hb vs. Metals		0.909*	X	X
	RAPD vs. Metals	**SB2**	-0.387*	X	X
		SB6	-0.387*	X	X
November	*Hb vs. RAPD*	**SB2**	0.276	X	0.537*
		SB6	0.580	X	-0.086
	Hb vs. Metals		0.686*	X	0.294
	RAPD vs. Metals	**SB2**	0.626*	X	0.583*
		SB6	0.871*	X	-0.560*
* = p value ≤ 0.05					

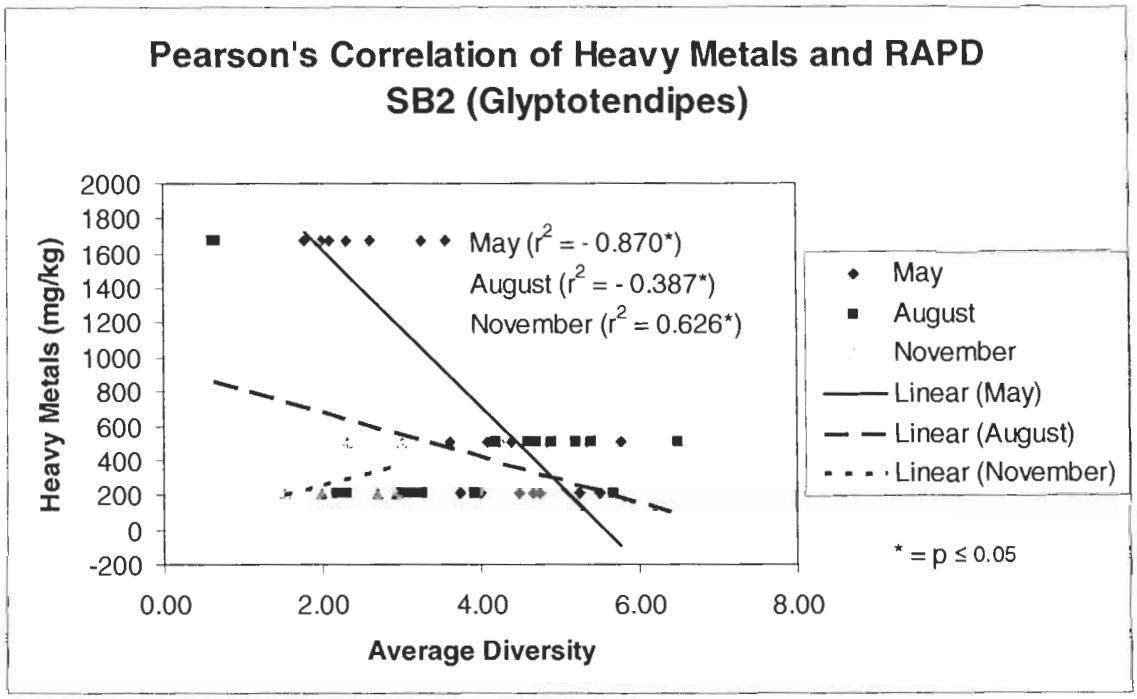

Figure 23: Pearson's correlation of heavy metals and RAPD using SB2 primer for *G. lobiferus*. RAPD data correlated with heavy metals data demonstrating an overall negative correlation for *G. lobiferus* species. Results indicated a general trend where an increased level of heavy metals contamination caused a lower average genetic diversity. There were multiple, overlapping data points with low diversity values for August that appear as one or two points on the graph. No correlations could be made for *C. riparius* on any collection dates, nor Hybrid for May and August collection dates. Correlations were significant for p values that were less than or equal to 0.05.

Discussion

This investigation attempted to develop two biomarkers in chironomids. It used the placement of AquaBlok to generate a treatment (capped) and control (uncapped) in an otherwise similar ecosystem. It was discovered that the capped sites reduced heavy metal contamination, which in turn should have produced a healthier environment for the capped plots (**Table 1**). This healthier environment could have led to species migration, which resulted in increased competition and corresponding levels of diversity. *G. lobiferus* was the predominant species on most plots. Over time, however, *C. riparius* were recruited and there was an emergence of a new variety, which appeared to be a fusion of the two species called Hybrid.

RAPD was able to detect differences in genetic diversity between plots (**Figures 8 and 9**). Plots 1 and 4 were both treated (capped) with AquaBlok, whereas plot 9 was the control plot which remained uncapped. Results for RAPD indicated a trend of decreasing diversity for *G. lobiferus* across plots. Plot 1 had the highest average diversity but was not statistically different from plot 4. Plot 9 had the lowest diversity and was statistically different from both plots 1 and 4. Plot 1 had the highest average diversity overall indicating that it was the healthiest plot as seen by the low heavy metal concentrations (**Table 1**). AquaBlok established a healthier environment as demonstrated by the higher levels of diversity on plots 1 and 4. This result is similar to what was seen at the Eagle River Flats near Anchorage, Alaska where AquaBlok was utilized to lower white phosphorus concentrations in order to create a healthier habitat for the wildlife (Hull *et al.*, 1998). The increased diversity at plot 1 compared to plot 4 was interesting as both

had AquaBlok. Emigration of species from plot 9 to plot 1 and increased competition may have accounted for higher levels of diversity in plot 1. Increased competition has been known to affect species biodiversity and ecosystem interactions. Models have been constructed to illustrate how plant biodiversity is affected by species competition and resource availability (Tillman *et al.*, 1997). The investigators' models demonstrated how increased competition and resource consumption directly increased plant biodiversity and greater variation among species traits.

RAPD results demonstrated similar levels of diversity for *G. lobiferus* between May and August; however there was a decrease in diversity in November for both primer sets (**Figures 11 and 12**). This decreased level of diversity could be a result of fewer individuals found during this month. Collection date influence could not be determined for the other two species because they were only found on a single collection date.

Hemoglobin results showed similar levels of diversity across all plots for *G. lobiferus* and Hybrid (**Figure 10**). However, *C. riparius* diversity showed a drastic increase in plot 9 compared to plot 1. The hemoglobin variability was likely due to changes in expression, as RAPD indicated that genetic diversity of *C. riparius* was similar to that of *G. lobiferus* on plot 9 (**Figure 9**). This could be due to the fact that RAPD assesses the entire genome of an individual, whereas hemoglobin considers expression of the protein.

Hemoglobin results showed low levels of diversity for *G. lobiferus* during May and November, but greatly increased levels in August (**Figure 13**). This could be indicative of increased expression levels of the protein as a result of environmental

stressors for this species during August. The increased diversity during this month and on the uncapped plot is due to the increased number of bands seen in the individuals' profiles as a direct result of up regulation of the hemoglobin protein. Studies have shown that season does have an effect on metabolic parameters in *Aegla platensis* (Oliveira *et al.*, 2007). Hemolymph was extracted from this freshwater crustacean, analyzed for metabolic parameters, and was seen to fluctuate seasonally. Investigators concluded that storage of these energetic substrates are needed seasonally for gamete production in the summer, and incubation and egg laying during fall and winter (Oliveira *et al.*, 2007). Chironomids possess hemolymph with similar storage capabilities that allows oxygen to be released under hypoxic conditions (Burmester and Hankeln, 2007). Since chironomids possess hemolymph with similar functions as those of hemolymph of crustaceans, the differences in diversity over collection dates could be indicative of seasonal fluctuations due to metabolic parameters.

Analysis of heavy metal contamination was done on substrates from each of three plots. After capping total metal concentrations were averaged, Plot 1 (AB) had a moderate average (SD), 503 mg/kg (541). Plot 4 (AB) had the lowest average, 217 mg/kg (23), whereas plot 9 (CN-uncapped) had the highest average, 1675 mg/kg (102). It was hypothesized that environmental stressors, such as heavy metals, would decrease genetic diversity among chironomids. Therefore, capping the sediment with AquaBlok was expected to reduce heavy metal exposure and result in higher genetic diversity. RAPD in *G. lobiferus* suggested that high genetic diversity was associated with low heavy metal concentrations in capping material (**Figure 23**). This is demonstrated by

significant correlation values after RAPD and hemoglobin were individually correlated to heavy metals and to each other (**Table 11**). When RAPD was correlated to metals in May and August for *G. lobiferus* there was a significant negative correlation for both primers: May – r2 = -0.870 (SB2), r^2 = -0.646 (SB6) and August – r^2 = -0.387 (SB2 and SB6).

When considering all factors, the effect of plot was best discriminated by RAPD. This is reflected in studies where genotype-environment interactions play a key role in an organism's response to environmental stressors. Investigators demonstrated in *Daphnia magna* that genetic diversity increases over several generations in response to sodium bromide and 3, 4-dichloroaniline (Soares *et al.*, 1992) and to cadmium (Ward and Robinson, 2005). Collection date was more influential when investigating hemoglobin. RAPD represented the entire genome of an individual: both a healthy environment and species competition were associated with high diversity. Hemoglobin variability appeared to be associated with changes in expression levels. Expression seems to be affected by seasonal differences rather than contaminant levels. Overall the data indicated that environmental conditions and species competition can have a profound impact on the individual's genetic make up and survival.

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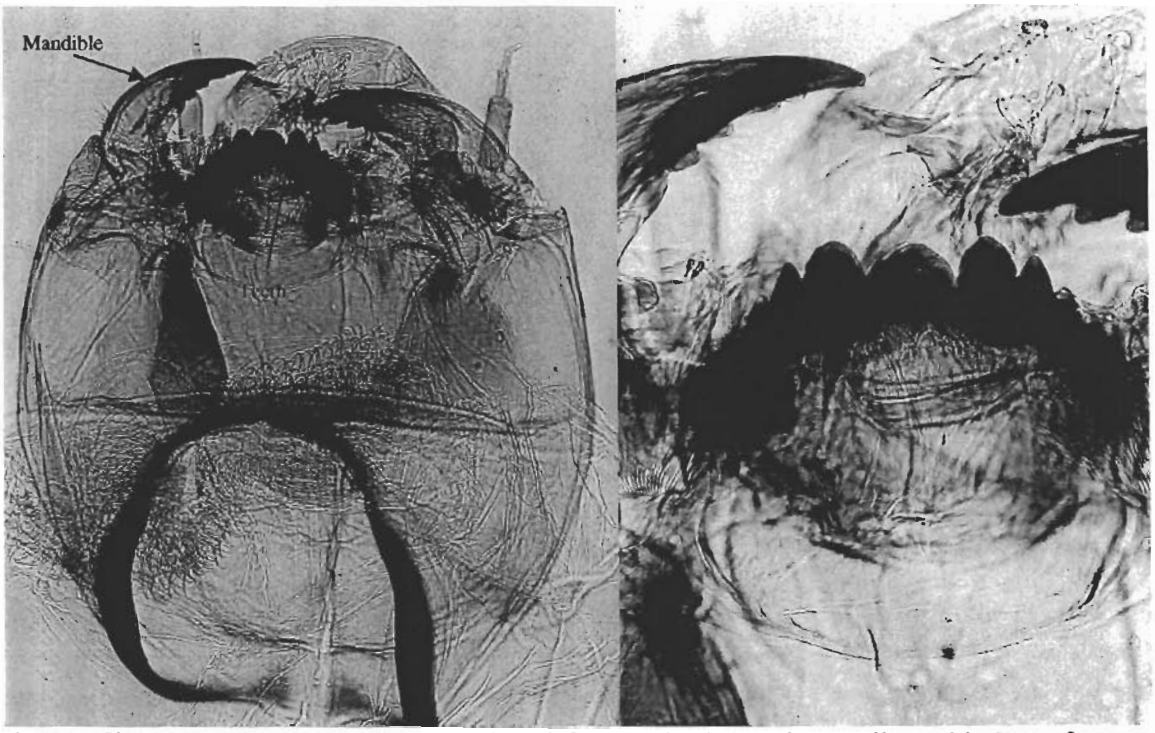
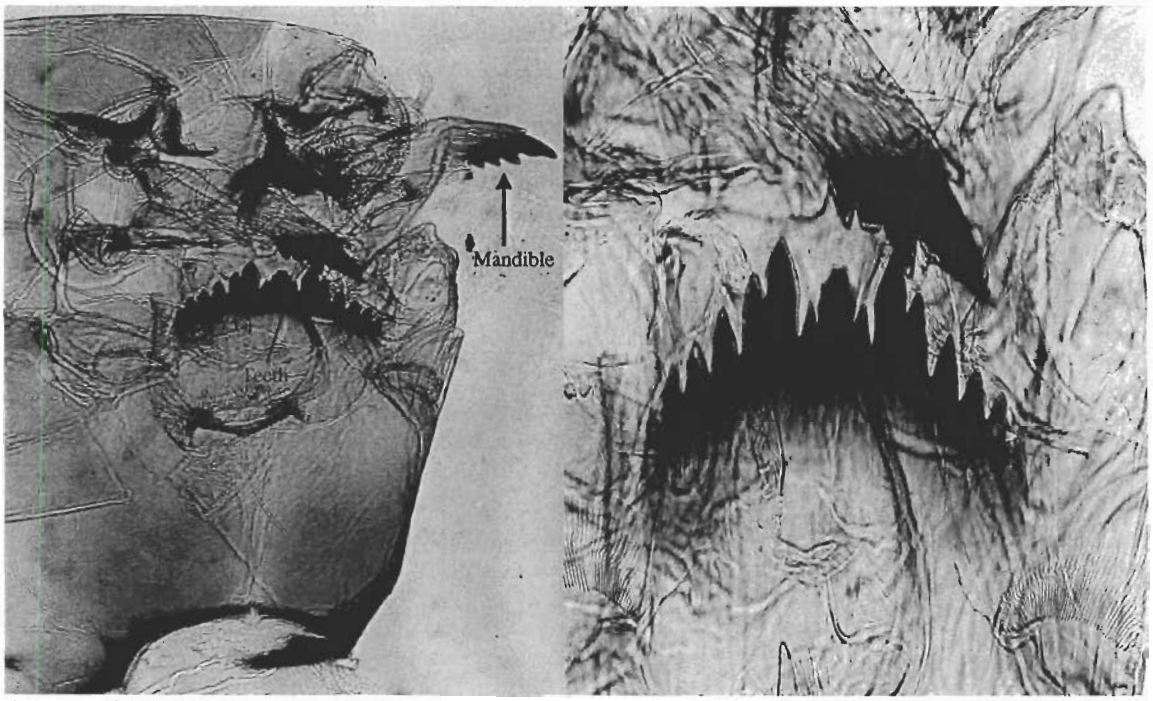
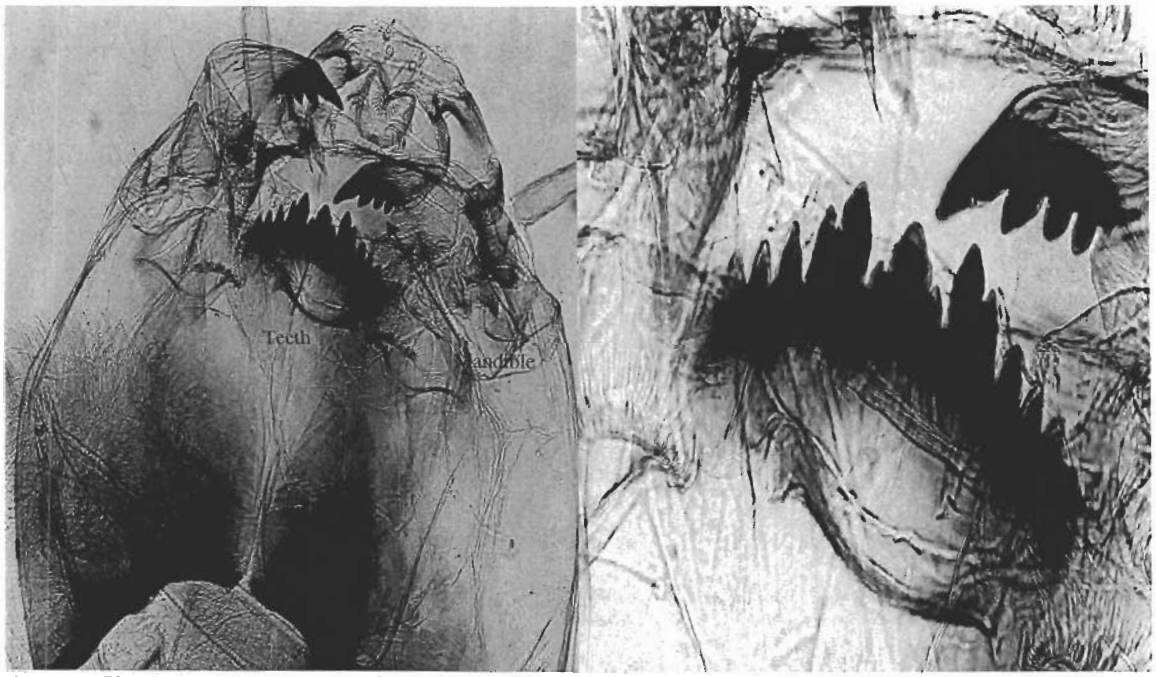
Appendix



Appendix 1: Head capsule pictures of *G. lobiferus* individual #7 collected in May from plot 1. Left: 10X capture of head capsule using Olympus photomicroscope. Right: 40X capture of head capsule using Olympus photomicroscope.



Appendix 2: Head capsule pictures of *C. riparius* individual #6 collected in August from plot 1. Left: 10X capture of head capsule using Olympus photomicroscope. Right: 40X capture of head capsule using Olympus photomicroscope.



Appendix 3: Head capsule pictures of Hybrid individual #1 collected in November from plot 9. Left: 10X capture of head capsule using Olympus photomicroscope. Right: 40X capture of head capsule using Olympus photomicroscope.

Proximity Matrix								
Case	Binary Squared Euclidean Distance							
	2:E21 A2	3:E21 A3	4:E21 A4	5:E21 A5	6:E21 A6	7:E21 B6	8:E21 C2	
2:E21 A2		1	3	1	5	7	6	3.83
3:E21 A3	1		2	2	6	6	7	4.00
4:E21 A4	3	2		4	6	4	7	4.33
5:E21 A5	1	2	4		6	6	5	4.00
6:E21 A6	5	6	6	6		6	7	6.00
7:E21 B6	7	6	4	6	6		7	6.00
8:E21 C2	6	7	7	5	7	7		6.50
This is a dissimilarity matrix							grp avg	4.95
							sd	1.16

Appendix 4: Proximity matrices are like multiplication tables showing the Squared Euclidean value between *C. riparius* individuals from August shown here. A low value (0.000) indicated no variability of genetic information and high homology in the fingerprint. A high value (8.000) indicated high diversity and high variability. The average of each pairing was considered the representative distance of a particular individual from another individual.