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The Effects of Cadmium on the Hemoglobin Genes of Chironomus Riparius

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THE EFFECTS OF CADMIUM ON THE HEMOGLOBIN GENES OF CHIRONOMUS RIPARIUS

BY

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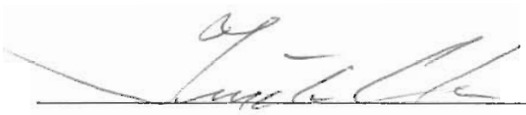
Approved by

Mentor



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Abstract

Effects of cadmium (Cd) on specific hemoglobin (Hb) genes and hemoglobin proteins were investigated in *Chironomus riparius*, an aquatic midge fly larvae. Based on previous studies, the low molecular weight, Hb proteins have shown a decrease in intensity when exposed to Cd. In this study, chironomids were exposed to increasing concentrations of Cd at different time points to study a change in mRNA expression as a possible mechanism by which the proteins were being affected. The concentrations of Cd used were 0 μM, 0.3 μM, 3 μM, and 30 μM and the time points were 0 h, 12 h, 24 h, and 72 h. Genes of interest included Hb III and Hb IV, which produce protein monomers and Hb VII and Hb IX, which produce protein dimers. Three toxicity tests were run where mRNA expression and protein levels were measured to determine if mRNA levels are directly responsible for the low molecular weight protein levels after exposure to Cd.

The objectives of this experiment were to examine a possible relationship between decreasing low MW protein bands and a decrease in mRNA expression. Another objective was to determine if there were any similarities in expression between genes on the same chromosome. Differences in expression between monomeric and dimeric proteins were also investigated.

After analyzing the data, it was clear that mRNA expression varied between the three experiments. It appeared that Cd had no consistent concentration or time related effects on mRNA expression of the Hb genes studied. However, the protein levels did decreased significantly at higher concentrations of Cd showing that Cd was having an effect on low molecular weight Hb proteins. Effects on high molecular weight proteins were not significant.

Correlations between mRNA and Hb protein showed that there was no repeatable relationship between gene expression and low MW protein levels for any of the genes analyzed. It was therefore concluded that the response of Hb protein to Cd was not related to changes in mRNA expression.

Based on the expression of Hb VII and Hb IX, there was evidence that dimeric proteins may be regulated in a similar manner. When Hb IX expression was normalized by dividing it by Hb VII expression, most results showed no significant differences in the expression of IX during Cd exposures. This meant that the two genes were responding to Cd in the same way. This can be explained by the two genes both being located on Chromosome II and in the same gene cluster. When comparing Hb III and Hb IV after normalization to Hb VII, it appeared that the two genes were not regulated the same despite both genes being on Chromosome III. Hb III showed less variation between both the toxicity tests and each concentration. There was much more variation between toxicity tests and Cd concentrations for Hb IV. This might have been explained by the genes being located on opposite DNA strains. In conclusion, there was evidence that the dimeric proteins, Hb VII and IX, may be regulated in a similar manner. Hb III and Hb IV, however, are monomeric proteins. There was no consistent relationship between Hb III and Hb IV; and therefore, it did not appear that monomeric proteins are regulated in the same manner as dimeric proteins.

Understanding the relationships between polymeric Hb proteins and mechanisms of action of environmental pollutants has contributed to our understanding of Hb as a biomarker in chironomids.

Introduction

Chironomids are aquatic larvae of non-biting midge flies. These chironomid species live in different aquatic environments across the globe. Chironomids live in the sediment in these aquatic environments. They survive in a multitude of environmental factors, such a high salinity and a wide range of environmental stressors. Studying chironomids is extremely important since they can help assess the health of their ecosystem. They are a major food source for both larger invertebrates as well as small vertebrate species. They form the base of the food web in their ecosystem (Ha, 2008). Without the chironomids feeding the larger organisms of the environment, the ecosystem can collapse.

Chironomids are a popular animal model for studying ecological response to pollutants and contamination (Govinda, 2000; Groenendijk, 2002). Chironomids are an EPA-approved test organism for studying environmental toxicity (USEPA, 1996). They can survive where many other larval insects cannot. Unlike other larval insects, chironomids have behavioral and physiological adaptations that allow them to survive. They can live in the profundal zone in lake sediments where both hypoxic (oxygen concentrations are less than 3 mg/L) and anoxic conditions (Pinder, 1995; Lee, 2006).

Chironomids express at least 16 different hemoglobin (Hb) proteins and 12 globin polypeptides (Gruhl 2000). The large quantity of hemoglobin proteins gives the chironomid its characteristic red color. The hemoglobin can exist either as a monomer, which would contain one amino acid chain, or as a dimer, which would contain two amino acid chains. Two genera, specifically *Chironomus ramosus* and *Chironomus thummi thummi* (also known as *Chironomus*

riparius), express hemoglobin proteins in both of these forms (Das, 1996).These hemoglobin proteins make up at least 80% of the total hemolymph proteins expressed in the organisms (Laufer et al, 1982). These proteins are expressed at specific stages of development and only in certain tissues (Schmidt, 1988). These genes are essential for the survival of the chironomids in suboxic environments. Since the genes are necessary, they are valuable as potential indicators of environmental stress and for studying genetic mechanisms.

Based on the cyano-methemoglobin method to determine total hemoglobin content in the hemolymph of cells, it has been found that at least 60% of the hemolymph contained proteins, and out of that protein, 92% of the proteins are hemoglobin (Choi, 2004; Ha, 2004). Since there is a high protein content, it is easy to detect hemoglobin on gels by staining with simple stains such as commaisee blue stain (Jacobs and Bentivegna, 2005).

The evolution of the many copies of hemoglobin genes is theorized to have started by duplication and variation of a single ancestral gene (Kleinschmidt, 1981). During duplication, this ancestral gene was replicated so each daughter cell received two identical copies of the ancestral gene. Over time, as the cells continued to divide, small mutations in the nucleotide sequence started to appear. This led to two genes that started out identical later having sequence differences. These sequence differences eventually led to functional differences in the organism (Hardison, 1999).

Environmental contamination can have influences on the chironomids at both cellular and molecular levels in the organism (Timmermans, 1992; Michailova, 2004; Lee 2006). Levels of low molecular weight hemoglobin proteins decrease after exposure to certain types of contamination, such as heavy metal contamination. This phenomenon of a decrease in low

molecular weight hemoglobin proteins has been seen in both environmental conditions as well as laboratory conditions (Oh, 2009).

The decrease in protein levels can be caused by different mechanisms inside each organism. In this study, mRNA expression was chosen as a potential mechanism for the decrease in protein levels. mRNA expression was chosen because of the well characterized relationship between protein levels and mRNA expression in eukaryotic cells. To make proteins, an mRNA transcript of the gene has to be made, so this is a logical starting point to examine the possible mechanisms of lowering levels of certain hemoglobin proteins. Studies have shown that mRNA expression can be used to observe changes caused by environmental contamination. Previous work has shown that levels of Hb mRNA and oxygen consumption can be decreased when exposed to atrazine, an organic pollutant (Anderson, 2008).This shows a direct relationship between the mRNA and effects on protein concentration. Another study shows that cadmium can modulate gene expression of heat-shock chaperone proteins, such as HSP40 and HSP90 (Planello, 2010).

For this study, chironomids were exposed to cadmium. Cadmium was chosen for a few reasons. Cadmium is a common heavy metal pollutant in aquatic environments. Cadmium comes from a multitude of sources, such as landfills, urban development, and exhaust from vehicles. Cadmium has already been shown to have an effect on the low molecular weight hemoglobin proteins on *C. riparius* (Oh, 2009).When these chironomids were exposed to cadmium, there was a decrease in hemoglobin proteins compared to animals that were not exposed to the heavy metal. Cadmium has also been shown to have effects on RNA and protein expression in other eukaryotic organisms. In rat liver, RNA polymerase activity decreases as the

concentration of cadmium increases (Hidalgo, 1976). Cadmium also has inhibitory effects on multiple intermediate steps of RNA synthesis in murine lymphocytes (Gallagher 1981).

RNA has been studied in chironomids that have been exposed to cadmium as well. Cadmium interacts with ribosomal genes and can result in drastic functional changes of the nucleolus. A depletion of the ribosomal genes is a long-term effect of cadmium exposure and leads to cell damage (Planello 2006). Exposure to cadmium also can increase the expression of non-coding RNA (ncRNA) and has been used to biomonitor genetic activity during exposure to contaminants (Martinez-Guitarte, 2009). These two studies show that it is possible to use chironomid mRNA expression as a biomarker for environmental toxicology studies.

Cadmium binds readily to the sediment in contaminated environments. Despite this binding, chironomids can survive in these contaminated sediments where they are most active. The chironomids can survive this exposure to cadmium due to the higher levels of tolerance to cadmium compared to other heavy metals and environmental toxins (Ha, 2008). The LC 10, LC50, and LC90 at 24 hours of exposure to cadmium are 93.05 mg/L (507μM), 169.5 mg/L (924μM), and 308.08 mg/L (1.68mM), respectively (Ha, 2008).

Previous studies have used benthic macroinvertebrates to study physiological effects to heavy metals and other contaminants. The research in this study was looking at molecular changes, not morphological changes or mortality. The correlation between cadmium exposure and mRNA synthesis and expression has not been defined. This study focused on measuring the levels of hemoglobin proteins at different concentrations of cadmium for different exposure times. If Cd decreased mRNA expression of hemoglobin genes at concentrations similar to those

associated with changes in hemoglobin proteins, then this could be one mechanism by which Cd could cause toxicity in environmentally exposed chironomids.

In this study, the heavy metal being used was cadmium. Multiple concentrations and time points were examined to see what their effects were. Concentrations of 0μM, 0.3μM, 3μM, and 30μM of cadmium were studied as well as 0 h, 12 h, 24 h, and 72 h exposure times. Previous studies with 72 h of exposure and a cadmium concentration of 30μM showed the same disappearance of low molecular weight hemoglobin proteins as chironomids living in contaminated field sites with similar, combined concentrations of Cd and other toxic metals. The 30μM concentration, however, was not high enough to kill the chironomids. This makes it an ideal concentration to study.

Four unique hemoglobin genes were studied: Hb III, Hb IV, Hb VII, and Hb IX. These four hemoglobin genes were studied for a variety of reasons. All four of these genes have been previously sequenced and those sequences can be easily found on the National Center for Biotechnology Information (NCBI) website. Hb III and Hb IV are both monomeric proteins, and Hb VII and Hb IX are both dimeric proteins. This is important because these different structures may lead to different functions in the animal. Also, the genes are located on different chromosomes. Hb III and Hb IV are located on Chromosome III whereas Hb VII and Hb IX are located on Chromosome II. Since these hemoglobin genes are located on different chromosomes, they may be differentially regulated. It is known that Hb VII and Hb IX are located extremely close to each other on Chromosome II, so it would be expected that they are regulated similarly to each other and differently than Hb III and Hb IV.

In addition to the different locations, Hb III and Hb IV both have two copies: Hb III.1 and III.2 and Hb IV.1 and IV.2. Hb III.1 and IV.1 run in series on the sense strand, while Hb III.2 and VI.2 run in series on the opposite strand. For each gene, only one strand was optimized for analysis. Hb III.2 and Hb IV.1 were the genes that were able to be analyzed by PCR. By running in opposite directions, this could allow for differential regulation.

Studying these different hemoglobin genes will provide a clearer picture as to what is going on in chironomids after cadmium exposure. By studying different time points and different cadmium concentrations, we will find out not only what effects cadmium has on the expression of hemoglobin genes but also if the responses are concentration dependent or time dependent. This may help explain why there is a decrease in levels of low molecular weight hemoglobin proteins when chironomids are exposed to cadmium.

Methods and Materials

The species that was studied in this experiment was *Chironomus riparius*. There was a lab population kept in a separate room with ambient room temperature (68-72 °F) and 16 h light/8 h dark. The organisms were kept in two 2.5 gallon fish tanks with a layer of acid washed sand covering the bottom of each tank. The sand was washed with 10% HCl, rinsed with deionized water, and baked dry. This cleaned the sand and got rid of any contaminants in the sand.

The water in each tank had a hardness of about 200 ppm and was constantly aerated to maintain dissolved oxygen levels. The water was a mix of filtered tap water(CDPRM1206/CDFC01204, Millipore Co, Bedford, MA), which had a hardness of about 300 ppm, and deionized water (Milli-Q model system, ≥18 Ω), which had a hardness of 0 ppm. The water was changed every two weeks to remove contaminants and waste. The chironomids were fed a suspension of ground fish food (Tetra Cichlid Sticks, Tetra Sales, Blacksburg, VA) once every week.

I. Toxicity Tests

To find out the effects of cadmium, a toxicity test was set up. The first step was to acid wash the plastic carboys, 1 L polycarbonate test vessels and play-sand using 10% HCl. Carboys and test vessels were washed with the 10% HCl and soaked overnight. The next day, they were washed thoroughly with reverse osmosis/deionized water (ddH_2O) until the pH returned to > 6.0. For the play-sand, it was soaked for 3 hours and then washed copiously with ddH_2O. After the pH reached > 6.0, it was placed in an oven overnight to dry at 80 °C.

Each treatment and control water was set up in a separate, acid-washed carboy. Enough test water was added to a carboy so as to provide 250 ml per replicate initially and to allow for approximately 80% daily water change per replicate during the exposure period. A mixture of 50% ddH_2O water and 50% filtered water was used to make up the test water. The ddH_2O was prepared using a Milli-Q model system, ≥18 Ω. The filtered water was made by passing tap water through particle and carbon filters (CDPRM1206/CDFC01204, Millipore Co, Bedford, MA). This filtered water typically had a hardness of > 300 mg/L (total hardness). A mixture of the two types of water was used to ensure that the water hardness was around 200ppm. To check the hardness of the water, a LaMotte Hardness kit (Carolina, Burlington, NC) was used. The cadmium was added to the test water in the carboys. A stock solution of 1.65g/L cadmium chloride was made. For 30 µM, 10 mL of the stock solution was added. For 3 µM, 1 mL of stock solution was added and for the 0.3 µM concentration, 100 µL of the stock solution was added.

An air pump was turned on and a hose was put into each carboy to allow aeration. The solutions in the carboys were aerated for 24 h prior to adding the water to test vessels and initiating the experiments.

Test vessels contained 60g of acid washed sand and 250mL of the aerated test solutions. Each concentration and time point was run in duplicate. Twenty 3^{rd}-4^{th} instar chironomids (*Chironomus riparius*) were added to each test vessel. Chironomids were fed daily1mL of 0.04 g/mL chironomid food. Chironomid food was made by adding ground Cichlid fish food to the filtered water. At 24 h and 48 h, approximately 80% of the test solution in each test vessel was replaced in order to insure sufficient levels of dissolved oxygen.

At the end of each time point, chironomids were collected from the containers and split into two groups. One group was reserved for the RNA isolation. For each sample, 5 chironomids were added to a 1.5mL centrifuge tube with 1mL RNA*later*® (Ambion, Austin, Texas) and stored at -20°C until ready for the RNA isolation. The second group was reserved for protein extraction. The proteins were extracted immediately after collection.

II. Hemoglobin Analysis

A. Protein Extraction

To extract proteins, each hemoglobin sample was added to a buffer consisting of14µL NuPAGE® LDS 4X LDS Sample Buffer (Invitrogen, Carlsbad, California), 2µL mercaptoethanol (Invitrogen, Carlsbad, California), and 2µL 8M Urea. The hemoglobin was collected by blotting a chironomid dry, decapitating it and allowing the hemolymph to drain on to a microscope slide. Approximately 2µL was collected and added to 18µL of the buffer above for a final volume of 20 µL. Samples were stored at -20° until it was time to run and analyze them through a SDS polyacrylamide gel.

B. Polyacrylamide Gel Electrophoresis

Hemoglobin proteins were analyzed using 16.5% Tris-HCI Precast Gels (Bio-Rad, Hercules, California) and the chamber was filled with1X Tris buffer electrophoresis buffer (Bio-Rad, Hercules, California). The Tris buffer was made up of 2.5 mM Tris, 19.2 mM glycine, and 0.01% SDS and the pH of the buffer was 8.3. Each well of the gel was loaded with 5µL of sample or ladder. The ladder was SeeBluePre-Stained ladder (Invitrogen, Carlsbad, CA). The power source was set at 100V.

Following electrophoresis, each gel was stained using the following procedure. It was washed 3-4 times in 200mL of distilled water for 5 minutes per wash with shaking. Then50mL of gel fixing solution (50% methanol, 7% acetic acid) was added for 15 minutes. After 15 minutes, the gel prefix solution was removed and water was added to wash again. It was washed twice for 15 minutes each. The water was removed, 20mL Gel Code Blue stain(Bio-Rad, Hercules, CA) was added and gels were shaken for 30 minutes. After the staining, 100mL of boiling distilled water was added to destain the gel. This was done twice for 30 minutes each. The water was removed and 50mL gel drying solution (20% ethanol, 4% glycerol) was added for 5 minutes. The gel was then placed on a piece of gel blotting paper and dried on a Savant gel drier for 1 h at 68°C

C. Hemoglobin Quantification and Statistical Analysis

To quantify the hemoglobin protein bands, the gels were scanned using a photo scanner and saved as a .tif file. They were analyzed using the program ImageQuant 5.1. This is a program that measures the intensity of the pixels of each hemoglobin protein band. The hemoglobin bands were grouped into two categories: high molecular weight proteins (all pixels in a box encompassing 17 to 11 kDa bands) and low molecular weight (all pixels in a box encompassing <11 kb- 3 kDa bands) proteins. These two groups were normalized by dividing pixel intensity of the protein bands by pixel intensity of the 49 kDa band on the ladder, which was run on each gel. This normalization accounted for differences in gel staining and photography.

To further analyze the protein bands, statistics were run to determine if the changes in treatment were statistically different. To run the statistical tests, SPSS, a statistics computer

program, was used. A one-way ANOVA test was run to compare the control (0 μM Cd) and the different treatments using either the high molecular weight or low molecular weight protein bands. The resulting F-stat determined if the treatment had a significant effect. To expand the statistical tests, a Tukey post-hoc test was run as well. The Tukey post-hoc test compared all treatments against each other to determine if there were significant differences between Cd concentrations and/or times of exposure, $p \leq 0.05$.

III. Hemoglobin mRNA Expression

A. Isolation of Total RNA

Approximately 5 to 6 chironomids were used per sample for RNA isolation. The chironomids were put into a sterile glass homogenizer. A volume of 250 μL of TriReagent (Ambion, Austin, Texas) was added to the homogenizer, and the chironomids were homogenized until there was no tissue visible. Another 250 μL of TriReagent was added and homogenized into the sample. This was repeated until there was a final volume of 1 mL. The homogenate was transferred into a pre-labeled 1.5 mL centrifuge tube. The sample was incubated at room temperature for 5 minutes. Then 200 μL of chloroform was added and the sample was vortexed for 15 seconds and incubated at room temperature for 3 minutes. The sample was centrifuged at 14,000 rpm for 15 minutes at 4°C. After 15 minutes, the top chloroform layer was transferred to a clean 1.5 mL centrifuge tube. A volume of 500 μL of 100% isoproponol was added to the sample and it was vortexed for 15 seconds. After vortexing, the sample was incubated at room temperature for 15 minutes. After the incubation, the sample was centrifuged at 14,000 rpm for 10 minutes at 4°C. The supernatant was then taken out and discarded, leaving only the pellet. A volume of 500 μL of cold 70% ethanol was added to the pellet and vortexed for 15 seconds. The sample was centrifuged at 14,000 rpm for 10 minutes at 4°C. The supernatant was removed.

The ethanol step was repeated again to ensure complete washing. As much ethanol as possible was removed and the sample was placed under the hood, cap opened, to continue to dry. When the pellet turned white, 100 µL of RNase and DNase free water (Invitrogen, Carlsbad, CA) was added and mixed. To completely dissolve the pellet, it was incubated at 60°C for 10 minutes. The RNA concentration was determined using a Beckman/Coulter DU 730 spectrophotometer. Samples were stored at -70°C.

B. DNase Reaction

DNA contamination was removed from RNA samples using the Turbo DNA-free kit in accordance to the manufacturer's protocol (Ambion, Austin, TX). This was done using 5 µg of isolated RNA, 2 µL of DNase, 2 µL of 10x buffer, and enough DNase/RNase-free water to make a final volume of 20 µL. The reaction was heated at 37°C for 30 minutes. After the incubation, 2 µL of inactivation solution was added and mixed. The sample was incubated at room temperature for 1.5 minutes. It was centrifuged at 13,000 rpm for 1.5 minutes. The supernatant was transferred to a new tube, and the RNA concentration was determined using a spectrophotometer.

C. Reverse Transcriptase Reaction

The Reverse Transcriptase reaction was performed to take the total RNA that was isolated from the treated chironomids and transform it to complementary DNA (cDNA). This was done for a few reasons. cDNA is much easier to work with than RNA because it is harder to contaminate than RNA. It is also more stable and easier to duplicate using Polymerase Chain Reaction (PCR).

To perform the Reverse Transcriptase reaction, 1 µg of DNased RNA was added to a 0.2 µL PCR tube. The total volume of RNA solution that was added to each PCR tube was 10 µL, so the RNA concentration was 1 µg/10 µL, or 0.1 µg/1 µl. To the tube, 2 µL of random primers, 2 µL of buffer, 0.8 µL dNTPs, 1 µL of reverse transcriptase, 1 µL of RNase inhibitor, and water to make a total volume of 20 µL was added (Applied Biosystems, Foster City, CA). After all of the reagents were added, the sample was placed in a PCR machine. The run was set up as follows:

- 25°C for 10 minutes
- 37°C for 120 minutes
- 85°C for 5 minutes
- 4°C until taken out of the machine

The sample was stored at -20°C until it was needed.

D. Polymerase Chain Reaction

To perform the Polymerase Chain Reaction, GeneAmp® Fast PCR Master Mix (2X) was used (Applied Biosystems, Foster City, California). The Fast PCR Mix contained all of the dNTPs, PCR buffer, and polymerase enzymes. Into each DNase and RNase free 0.2µL PCR tube, 12.5µL of the Fast PCR Master Mix was added. To the mix, 8.5µL DNase/RNase free water, 2µL of cDNA, and 1µL of both forward and reverse primers was added. The primers for each gene varied as well as the annealing temperatures. All primers were ordered from Integrated DNA Technologies (Coralville, IA). The primer sequence information is in the following table. It includes gene name, chromosome on which it is located, sequences of forward (F) and reverse (R) primers, and annealing temperature used in PCR. In addition to the

samples, a negative control was run as well. The negative control contained all of the components of the samples except there was no cDNA. This was to ensure that the both the primers and water were not contaminated.

Gene	Chromosome	Primer sequence	Annealing Temp
Hb III.2	III	F 5'CCAAACATTGAAGCCGATGTCAAT3' R 5'AAAGTGTCAAGAGTTGCACCC3'	56°C
Hb IV.1	III	F 5'CTGATCCCTTGATTGAGTCG3' R 5'CCTCATTCTTGCCTTGTGCT3'	50°C
Hb VII	II	F 5' CCAGACATTCAAGCCCGTTTC R 5' GAGATTCCACGTGCCTTGTG	57°C
Hb IX	II	F 5'TGTTGCTCTTGTTGGAAACG R5'ATCAGCGGTAGCATCATTCC	54°C

The PCR runs for each gene had the same cycle settings with only the annealing temperatures differing. The setup for each run was:

Initial Denaturation: 94°C for 3 minutes

Denaturation: 94°C for 30 seconds

Annealing: Varying temperature for each gene for 30 seconds

Extension: 72°C for 30 seconds

Final Extension: 72°C for 3 minutes

Storage: 4° until taken out of the machine

E. No Reverse Transcriptase Verification

Before doing Agarose Gel Electrophoresis on the cDNA, a No Reverse Transcriptase (No RT) reaction was run. The purpose of the No RT reaction was to confirm that there was no DNA contamination. To do this, a normal Reverse Transcriptase reaction was performed except the Reverse Transcriptase enzyme was not added. Any DNA in the sample was from contamination and not from converting the mRNA to cDNA.

The No RT and RT reactions were run at the same time to ensure that all conditions were exactly the same. After the reaction was completed, samples were prepared by PCR as if the samples contained cDNA. The No RT samples were tested by running the samples through an agarose gel. If the wells showed no PCR bands, then there was no contamination of the samples. If any band was present, then there was contamination in the samples and the Reverse Transcriptase was repeated.

F. Agarose Gel Electrophoresis

In order to view the results of the PCR, agarose gel electrophoresis was done. The running buffer and gel buffer was 1X TAE at a pH of 8.3. It was made from combining 20mL UltraPure™ DNA Typing Grade® 50X TAE Buffer (Invitrogen, Carlsbad, CA) and 980mL distilled water. To the diluted TAE buffer, 40μL10 mg/mL Ethidium Bromide was added to the running buffer to allow visualization of the DNA in the gel.

PCR samples were separated on 1.5 % agarose (USB, Cleveland, OH) gels. Samples contained 10μL of the PCR reaction, 9μL of distilled water and 1μL of loading dye (Invitrogen, Carlsbad, CA) making a total volume of 20μL. A 100 bp DNA ladder (Invitrogen, Carlsbad, CA) was also loaded on to each gel. The power source was set to 120V and was allowed to run for 30 minutes.

G. Quantification and Statistical Analysis of mRNA Expression

When the gel was finished running, the image was captured using a Gel-Doc camera system and a UV light. After the picture was taken, it was saved as a .tif file. That .tif file was loaded into ImageQuant 5.1 for analysis. This is the same program that was used to quantify the high and low molecular weight protein bands (see above).

To quantify each sample, they had to be adjusted to a standard. The standard in each gel was the DNA ladder. The positive control band was used because it had to have a strong, consistent, band intensity. The positive control was a DNA product from Hb VII. This band had a similar intensity in each agarose gel, so it was used to compare across different gels. This adjustment accounted for differences in photography and Ethidium bromide staining between gels. Each DNA band resulting from PCR was adjusted by dividing its pixel intensity by that of the 500 bp band in the ladder.

The next step in analyzing the gels was to normalized the gene expression data and thereby reduce technical error. It also allowed differences in gene regulation to be assessed. Normalization was done by taking the adjusted pixel density (see above paragraph)for each gene, Hb III, Hb IV, and Hb IX and dividing by it by that for Hb VII from the same sample and multiplying by 100 to get a percent expression. This normalized the expression of Hbs III, IV and IX to another gene in the same mRNA preparation. If the normalized expression was over 100, Hb VII was expressed less than the compared gene, and if it is lower than 100, Hb VII was expressed more.

The samples were run through the statistical program SPSS to determine if the differences or similarities in band intensities were of statistical significance. A one-way ANOVA followed by Tukey post-hoc test was run as described above for hemoglobin protein analyzes.

H. Cloning of cDNA bands from Hb 3.2, 4.1, 7 and 9

In order to clone the cDNA products generated by reverse transcription PCR, the bands were extracted from the agarose gels. The Gel Extraction kit used was the QIAEX II Gel Extraction Kit (Qiagen, Valencia, California). To start the gel extraction, the band was excised from the agarose gel by cutting out the band with a scalpel. The gel slice was placed in a 1.5mL centrifuge tube. QX1 solution, 300μL, was added to the gel slice. The QIAEXII beads were resuspended by vortexing for 30 seconds, and 10μL was added to the sample. It was incubated at 50°C for 10 minutes. The samples were centrifuged for 30 seconds at 13,000rpm. The supernatant was removed and 500μL QX1 solution was added and vortexed. It was centrifuged for 30 seconds at 13,000rpm and the supernatant was removed. Buffer PE, 500μL, was added and vortexed. The sample was centrifuged for 30 seconds at 13,000 rpm and the supernatant was removed. This step was performed twice to ensure complete washing. After both washes, the samples were left to air dry. Once dry, 100μL DNase/RNase free water was added and was vortexed. The samples incubated at room temperature for 5 minutes and then centrifuged. The supernatant was collected and placed in a separate tube. To perform the cloning, the samples from the Gel Extraction were used. The kit used for the cloning was the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA). Chemically competent E. *coli* cells were used for this experiment. Fresh PCR product, 4μL, was combined with 1μL Salt Solution from the kit. TOPO® vector,

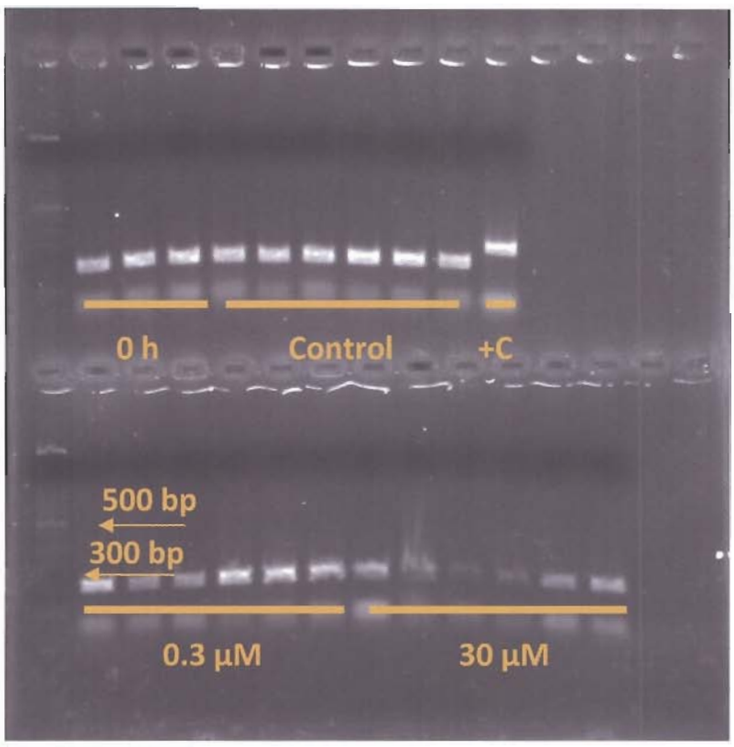
1μL was added to make a total volume of 6μL. The mixture was mixed and incubated at room temperature for 5 minutes. After 5 minutes, the sample was placed on ice.

One vial of One Shot® cells was thawed on ice for each transformation. TOPO® Cloning reaction from last step, 2μL, was added to a vial of cells and mixed gently. The vial was then incubated on ice for 30 minutes. The cells were then heat-shocked for 30 seconds at 42°C without shaking. The cells were then immediately transferred to the ice. Room temperature S.O.C. media, 250μL, was added to the cells. The cells were incubated at 37°C and shaken at 200 rpm for 1 h.

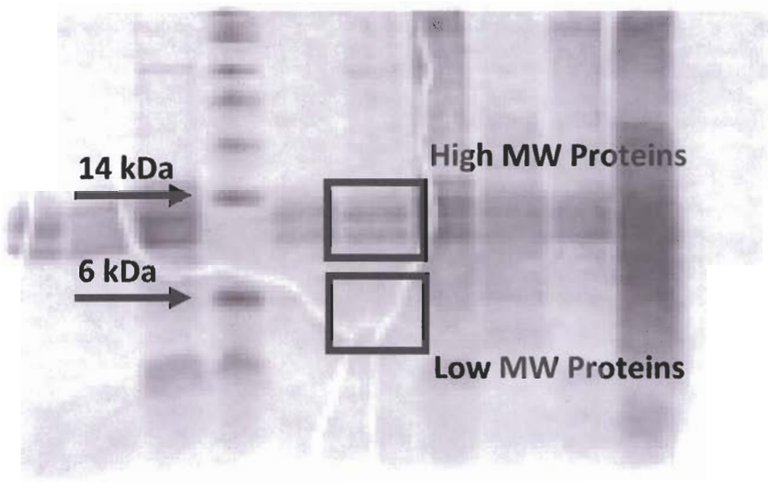
While the cells incubated, the LB plates were made. For every 100mL of media made, 100μL of 100X kanamycin sulfate was added (Invitrogen, Carlsbad, CA). After the plates were poured and solidified, 40μL X-Gal in DMF was spread on the plates. They were pre-warmed for 30 minutes. After the cells incubated, two volumes were plated. One set of plates had 20μL room temperature S.O.C. and 15μL of cells. The other set of plates contained 20μL room temperature S.O.C. and 25μL of cells. After plating, the cells were incubated overnight at 37°C.

To analyze the clones, only white colonies were chosen. White colonies should have contained the vector with inserted cDNA gene product. For each gene, two types of PCR reactions were set up. The first reaction contained the regular PCR setup, except the primers were different. The gene-specific forward primer was used with the vector primer, M13R. The second reaction was a normal PCR setup with the gene-specific reverse primer and the vector primer, M13F. After the PCR was run, the samples were run through a1.5% agarose gel. If bands were present, they were extracted using gel extraction (see above). Results were validated by amplifying the extracted PCR sample using both gene-specific primers. After the gel was run

and the bands were present, the samples were sent to McLab (San Francisco, CA) or GeneWiz (Plainfield, NJ) for sequencing.



Picture 1. Agarose Gel of cDNA of Hb IV from Toxicity Test 1. The picture shows an example of an agarose gel of Hb IV. The leftmost lane on the top and bottom of the gel is the 100 bp ladder. The rightmost lanes of the top of the gel are the positive control (+C) and the negative control.



Picture 2. Protein Gel of 72 h 30μM samples from Toxicity Test 1. This is an example of a protein gel from 9 individuals that were exposed to 30μM Cadmium for 72 h. Lane 4 is the ladder that was used to measure the size of the protein bands. It is clear that in this example that the low molecular weight proteins disappeared while the high molecular weight proteins are still strong.

Results

Toxicity Test 1

The goal of the toxicity test was to find out what effects Cd has on the different hemoglobin genes of chironomids. In Toxicity Test 1, the time points that were examined were 0 and 72 h. The Cd concentrations were 0μM, 0.3μM, and 30μM. All genes, Hb III, Hb IV, Hb VII, and Hb IX, were analyzed. All four genes showed a concentration dependent decrease in mRNA expression at 72 h after Cd exposure compared to 0μM Cd at 72 h (Table 1). Hb IV was the only gene to show a statistically significant decrease compared to both controls- 0 μM Cd at 72 h and 0 h. The expressions of Hb VII and IX increased significantly at 72 h for 0 μM Cd compared to 0 h. In fact, Hb VII showed a similar pattern to Hb IX. This would be expected since the two genes are located closely on Chromosome II. Similarly, Hb III and IV showed increased expression at 72 h without Cd compared to 0 h; however, it was not significant. These results indicated that the testing conditions simulated Hb expression but the addition of Cd reduced it in this toxicity test.

The mRNA expressions of Hb III, IV and IX were normalized by dividing by the expression of Hb VII from the same cDNA preparation. This controlled for differences in sample preparation much like normalizing by house-keeping genes such as β-actin. However, it also allowed comparisons of dimer gene expression (Hb VII and Hb IX) with monomer gene expression (Hb III and Hb VI). These comparisons might have indicated whether genes on different chromosomes were regulated differently. After dividing Hb III with Hb VII, it was clear that Hb III was expressed more than Hb VII (Figure 1). In the test with the least Hb III expression, it was 120% more expressed than Hb VII. Hb IV showed decreased levels of expression relative to Hb VII overall in Toxicity Test 1, with the lowest level of expression

occurring at 30 μM Cd. This was consistent with the raw data (Table 1); however, there was no significant difference in Hb IV expression relative to that of Hb VII with increasing Cd concentrations. This meant that Hb IV was not more sensitive to Cd than Hb VII. Hb IX was expressed at levels similar to Hb VII at 72 h, between 80-100%, for all concentrations, Hb IX also showed a similar pattern of expression compared to Hb VII. Interesting, Hb III, IV and IX all had significantly higher levels of expression than HB VII at 0 h compared to 72 h for all treatments. The reason for this was unknown.

When looking at the proteins, it was clear that there was a relationship between the expression of mRNA and levels of low molecular weight protein. At 0 h, control, and 0.3μM Cd, there was no decrease in the levels of low molecular weight proteins (Table 2). At 30μM Cd, however, there was a clear decrease in the levels of low molecular weight proteins (Figure 2). This decrease in the low molecular weight proteins was statistically significant at 30μM compared to the other concentrations. The high molecular weight protein bands stayed consistent at all four concentrations.

Treatment	HbIII	HbIV	HbVII	HbIX
0 µM	217±4[a]	153±16[c]	128±2[e]	136±3[g]
0.0 µM	214±4[a]	170±11[c]	181±2[f]	160±12[h]
0.3 µM	191±26[ab]	134±25[c]	132±17[e]	123±15[g]
30 µM	188±10[b]	100±22[d]	124±16[e]	127±11[g]

Data represent average ± SD, n = 5-6.

Values that share a common letter were not statistically different, $p > 0.05$.

Table 1. Effect of Cd on Hb mRNA expression at 72 h- Toxicity Test1 Data are presented as pixel densities of bands on scanned gels divided by 1000. They are the combined average ± SD of amplified cDNA samples from two treatment replicates, n= 5-6. Treatments that share a letter were not statistically significant, $p > 0.05$. SD= standard deviation. 0 h = mRNA expression the day the experiment was initiated.

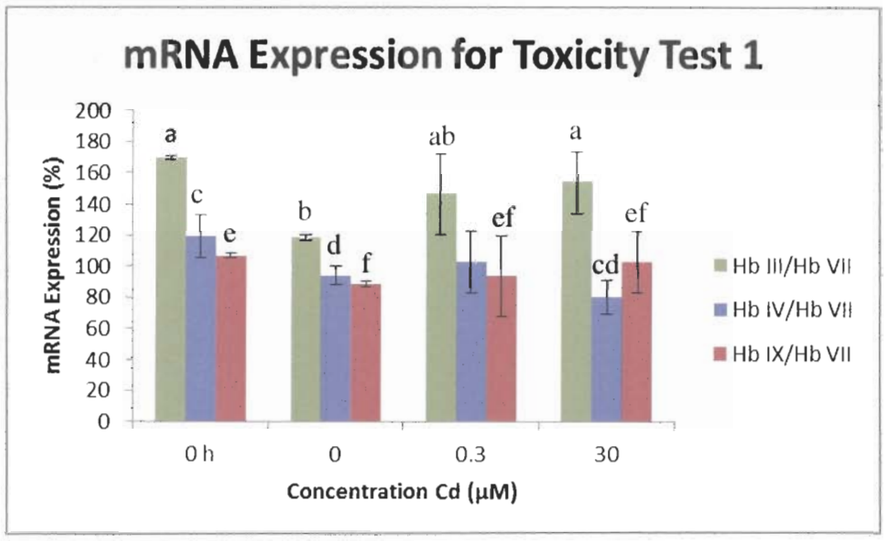


Figure 1. Percent Control for Toxicity Test 1 This figure shows Hb III, Hb IV, and Hb IX divided by Hb VII and multiplied by 100 to get a percent expression compared to Hb VII. Treatments that share the same letter are statistically similar ($p>0.05$). n=3 for 0 h, n=5 for 0 µM, 0.3 µM and 30 µM. 0 h = mRNA expression the day the experiment was initiated.

	0 h	0.0 µM	0.3 µM	30 µM
High MW Protein	48.6±32.5[a]	61.9±20.8[ab]	74.5±8.8[b]	48.8±18.8[a]
Low MW Protein	31.9±21.3[a]	40.5±8.0[a]	33.4±7.5[a]	9.0±7.0[b]

Data represent average ± SD, n = 9-10.

Values that share a common letter were not statistically different, $p > 0.05$.

Table 2. Effect of Cd on High and Low Molecular Weight Protein bands at 72 h- Toxicity Test 1 Data are presented as pixel densities of bands on scanned gels divided by 1000. They are the combined average ± SD of hemoglobin samples from four treatment replicates, n= 5 individual chironomids. Replicates were made of five chironomids each from two different test vessels. Treatments that share a letter were not statistically significant, $p > 0.05$. SD= standard deviation. Day 0 = protein expression the day the experiment was initiated.

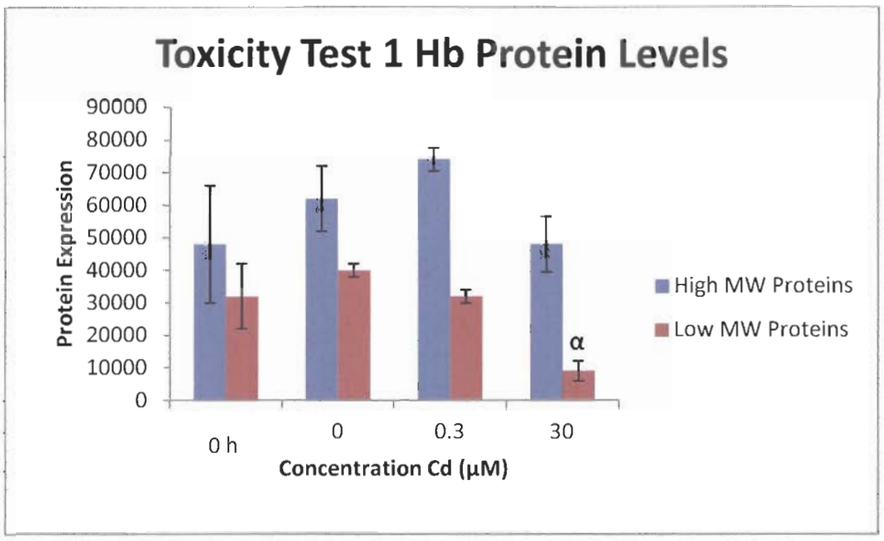


Figure 2. High and Low Molecular Weight Protein bands- Toxicity Test 1 Data are presented as averages of each treatment, n = 10. Error bars show the Standard Deviation of the samples. Samples with an alpha symbol (α) are statistically different than all other samples, $p>0.05$.

Toxicity Test 2

Toxicity Test 2 was run with the same concentrations as Toxicity Test 1, 0 µM, 0.3 µM, and 30 µM, but with new time points. The time points run in this study were 12 h, 24 h, and 72 h. In this test, Hb IV again showed a response to Cd, though it was different from Toxicity Test 1 (Table 3). Hb IV expression appeared to show time-dependent effects. It was increased by 30 µM Cd treatments at 24 h (404 ± 49) and 72 h (366 ± 52) compared to 0 µM Cd controls at 12, 24 and 72 h (226±47, 295 ± 71 and 268 ±37, respectively); however, the differences were not statistically different. The 0.3 µM Cd treatments supported the trend such that expression for 0.3 µM Cd at 72 h was significantly greater than at 12 h and 24 h. However, was no clear concentration related effects at any time points. The time related increases in Hb IV expression in Toxicity Test 2 differed from Toxicity Test 1 where results showed decreases in Hb IV with Cd exposure.

mRNA expressions of Hb III, VII and IX were also different between Toxicity Test 1 and 2. At all three time points in Toxicity Test 2, Hb III and XI were very consistent, and there were no concentration or time dependent changes. Hb VII showed no concentration dependent responses at 12 h and 72 h. However at 24 h, expression was decreased by 0.3 and 30 µM Cd compared to 0 µM Cd. The reason for this effect only at 24 h was unknown. Overall, the changes for Hb VII expression in raw data were not time or concentration dependent in Toxicity Test 2. These results differed from Toxicity Test 1 where Hb III, VII and IX were suppressed at 72 h by 30 µM Cd compared to 0 µM Cd.

Normalization of mRNA expression to Hb VII showed similar results for Hb IX but different ones for Hb III and IV when comparing Toxicity Tests 1 and 2. For Hb III, there was

less mRNA expression compared to Hb VII (Figure 3) as opposed to more (Figure 2). There was a consistent percentage of expression of about 80% Hb III compared to Hb VII. Hb III showed no concentration dependent effects of Cd exposure as it did in Toxicity Test 1.

For Hb IV, some treatments expressed less mRNA relative to Hb VII and some expressed more. This was unlike Toxicity Test where there was a lower expression of Hb IV compared to Hb VII for all treatments. There was an increase of Hb IV expression at 72 h for the 0.3 μM and 30 μM concentrations. There was also an increase at 24 h for 30 μM Cd. This was consistent with the raw data (Table 3) and suggested a concentration and time related effect of Cd on Hb IV. There was a statistical difference for 0.3 μM at 72 h compared to both 12 h and 24 h. There was also a statistical different for 30 μMat 24 h compared to 12 h, but not between the 12 h and 72 h samples.

For Hb IX, results of normalized data for Toxicity Tests 1 and 2 were similar. Again, Hb IX showed was less expression relative to Hb VII. However, this occurred at 0 h as well as at all other time points. As in Toxicity Test 1, there were no concentration dependent effects of Cd at 72 h for Hb IX. Its expression was similar to Hb VII. There was one exception: the 24 h control was significantly suppressed compared to other data points. The reason for this was unknown.

Overall the major differences between Toxicity Tests 2 and 1 was that Hb IV appeared to be expressed at relatively higher levels than Hb VII when exposed to Cd in Toxicity Test 2, while Hb III appeared to be expressed at relatively constant levels in Toxicity Test 2 and was not affected by Cd.

When looking at the proteins, it was evident that the low molecular weight protein bands were affected by 72 h Cd exposure in Toxicity Test 2 (Table 4) as they were in Toxicity Test 1

(Table 2). For 0.3 μM and 30 μM, the low molecular weight protein bands decreased when compared to the Control at 72 h (Figure 4). The decrease in protein levels for 30 μM showed a similar pattern as in Toxicity Test 1 (Figure 2). However, in Toxicity Test 2, the decrease in protein levels at both the 0.3 μM and 30 μM Cd indicated either an increase in organism sensitivity or a lack of concentration dependence. The high molecular weight proteins stayed consistent across concentrations as found in Toxicity Test 1.

Treatment	HbIII	HbIV	HbVII	HbIX
0 h	253±11[a]	320±22[a]	270±45[a]	289±12[a]
12 h0.0 µM	251±8[a]	226±47[ab]	320±20[ab]	222±39[b]
12 h0.3 µM	244±11[a]	257±18[a]	314±30[ab]	228±15[b]
12 h30 µM	276±46[a]	222±27[ab]	348±11[b]	225±34[b]
24 h 0.0 µM	278±51[ab]	295±71[ac]	368±25[b]	192±25[b]
24 h 0.3 µM	218±11[b]	186±13[b]	257±7[a]	227±14[b]
24 h 30 µM	237±4[a]	404±49[c]	289±47[a]	247±19[b]
72 h 0.0 µM	251±21[ab]	268±37[a]	302±21[a]	242±30[b]
72 h 0.3 µM	271±9[a]	416±68[c]	333±30[ab]	226±51[b]
72 h 30 µM	257±16[a]	366±52[ac]	328±47[ab]	227±32[b]

Data represent average ± SD, n = 5-6.

Values that share a common letter were not statistically different, $p > 0.05$.

Table 3. Effect of Cd on Hb mRNA expression at 12 h, 24 h, and 72 h- Toxicity Test 2 Data are presented as pixel densities of bands on scanned gels divided by 1000. They are the combined average ± SD of amplified cDNA samples from two treatment replicates, n= 5-6. Treatments that share a letter were not statistically significant, $p > 0.05$. SD= standard deviation. Day 0 = mRNA expression the day the experiment was initiated.

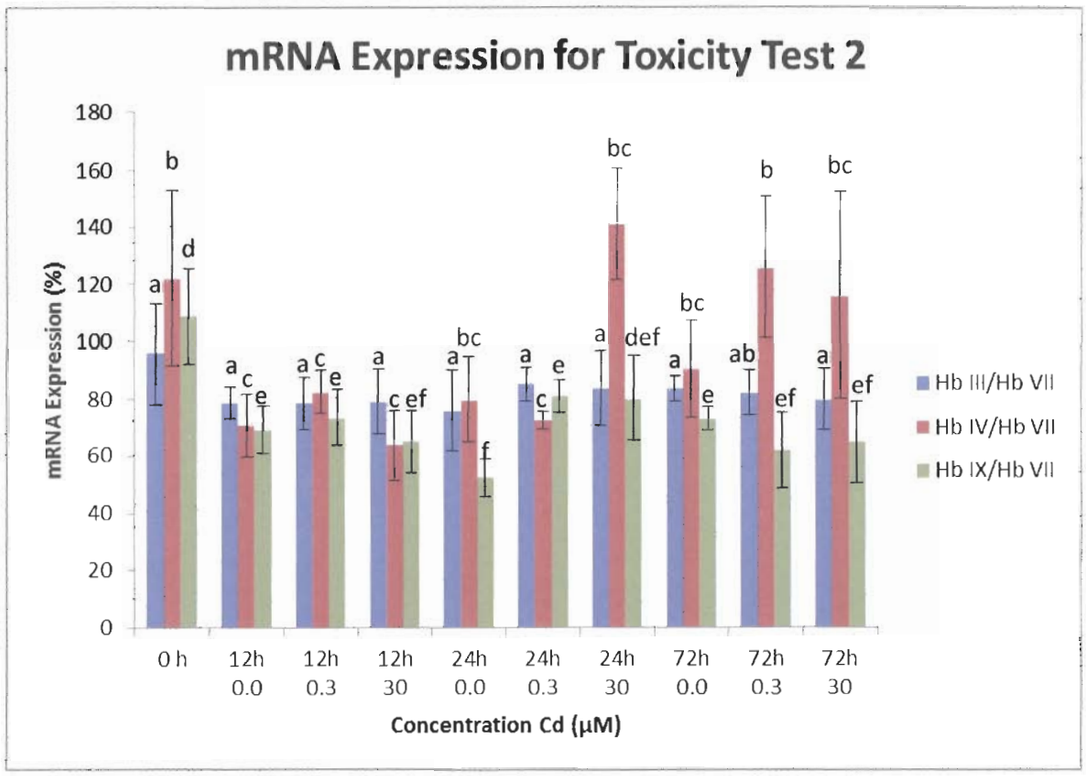


Figure 3. Percent Control for Toxicity Test 2 This figure shows Hb III, Hb IV, and Hb IX divided by Hb VII and multiplied by 100 to get a percent expression compared to Hb VII. Treatments that share the same letter are statistically similar ($p>0.05$). n=3 for 0 h, n=6 for 0 µM, 0.3 µM and 30 µM. 0 h = mRNA expression the day the experiment was initiated.

	Control	0.3 µM	30 µM
High MW Protein	30.5±4.5[a]	26.2±4.2[a]	28.2±2.6[a]
Low MW Protein	26.9±3.3[b]	23.7±3.0[c]	23.5±3.4[c]

Data represent average ± SD, n = 9-10.

Values that share a common letter were not statistically different, $p > 0.05$.

Table 4. Effect of Cd on High and Low Molecular Weight Protein bands at 72 h- Toxicity Test 2 Data are presented as pixel densities of bands on scanned gels divided by 1000. They are the combined average ± SD of hemoglobin samples from two treatment replicates, n= 10totalchironomids. Treatments that share a letter were not statistically significant, $p > 0.05$. 0 h = protein expression the day the experiment was initiated.

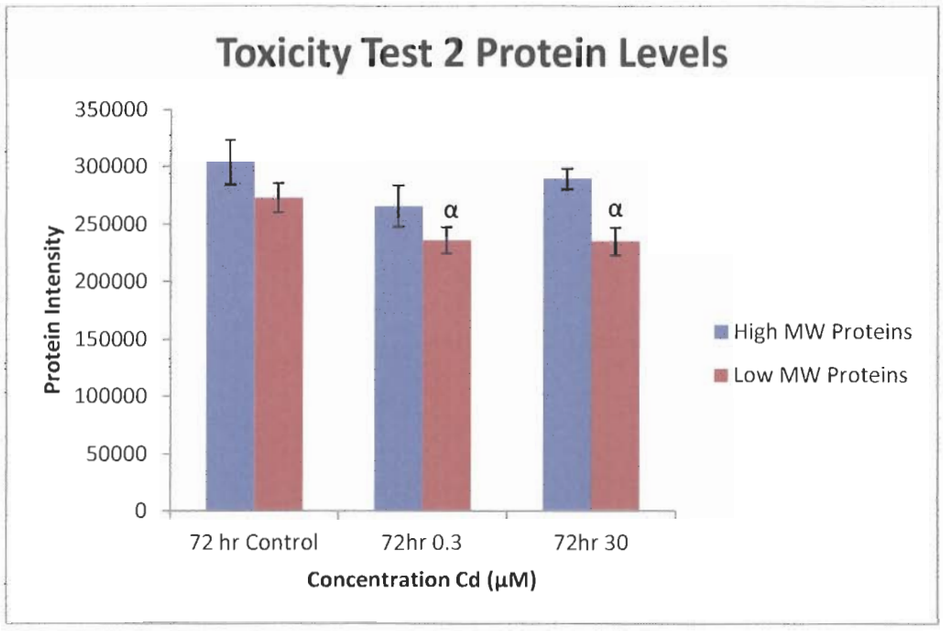


Figure 4. High and Low Molecular Weight Protein bands- Toxicity Test 2 Data are presented as averages of each treatment. The error bars show the Standard Deviation of the samples, n=10. Samples with an alpha symbol (α) are statistically different than control, p>0.05.

Toxicity Test 3

In Toxicity Test 3, the experimental conditions were modified again. The 12 h time point was not run, leaving only the 24 h and 72 h time points. In addition to the 0 h, 0 μM, 0.3 μM, and 30 μM Cd concentrations, chironomids were exposed to 3 μM Cd. For Hb III, there was a slight statistical increase for 0 μM Cd at 24 h compared to 0 h (Table 5). The 30 μM Cd treatment showed a statistical decrease in expression compared to 0 μM and 3.0 μM Cd at 24 h. However, it was statistically similar to 0.3 μM, and its effects did not appear concentration dependent. At 72 h, there were no significant differences between treatments. These results were similar to those for Toxicity Test 2 but not Toxicity Test 1.

The results for Hb IV, at 24 h, showed a statistical decrease in expression at 3 μM compared to 0 h and 0 μM Cd (Table 5). It was also significantly different from both the 0.3 μM and 30 μM concentrations indicating no concentration related effects. At 72 h, all treatments were statistically similar. These results for Hb IV differed from that of Toxicity Tests 1 and 2 where expression was suppressed or enhanced by Cd, respectively.

Hb VII did not show a statistical increase in expression at 24 h for 0 μM Cd compared to 0 h and 3 μM Cd. There was, however, a statistical difference between 30 μM Cd at 24 h. There was no statistical difference between 3 μM and 30 μM. Overall, treatment effects at 24 h were not concentration dependent. It was interesting that Hb VII also showed effects of Cd at 24 h in Toxicity Test 2; however in this test, expression was increased by Cd. At 72 h, like the previous genes, there were no statistical differences between any of the concentrations. These results for Hb VII were similar to those for Toxicity Test 2 but differed from those of Toxicity Test 1 at 72 h.

Hb IX showed no statistically significant differences between any of the treatments at 24 h. There was a change at 72 h, however. The 3 μM treatment showed a very slight statistical decrease in cDNA expression compared to 0 μM and 0.3 μM Cd, but was statistically similar to 30μM. The 0 μM, 0.3 μM, and 30 μM Cd treatments were not significantly different from one another. Overall, there did not appear to be any concentration dependent effects of Cd at 72 h for Hb IX. These results were similar to those for Toxicity Test 2 but different from those of Toxicity Test 1.

When the mRNA expression of Hb III was normalized by dividing by Hb VII, the only significant difference in expression was an increase at 24 h for 0 μM Cd compared to 0 h (Figure 5). There were no Cd related effects for Hb III. Hb IV showed no Cd related effects when normalized by Hb VII. There was a significant increase for 0 μM Cd at 24 h compared to 0 h as with Hb III. This might have suggested some co-regulation of Hb III and IV. Hb IX also showed no Cd related effects when normalized by Hb VII. There was a significant increase in Hb IX for 0 μM Cd at 72 h compared to 0 h. Overall, none of the genes showed differences in expression due to Cd exposure when normalized by Hb VII in Toxicity Test 3. This finding was the same for Hb III and IX in Toxicity Tests 1 and 2 but different for Hb IV, which appeared to respond to Cd in the first two tests.

The protein banding pattern after exposure to Cd was consistent with previous toxicity tests (Figure 6 and Table 6). The high molecular weight protein bands showed no statistical differences regardless of Cd concentrations. The low molecular weight protein levels followed the same pattern as Toxicity Test 1. The 0 h, 0 μM, 0.3 μM, and 3μM concentrations were not statistically different from each other, but the 30 μM concentration showed a significant decrease in protein levels. The decrease at 30 μM Cd was the same result as the previous two toxicity

tests (Figures 2 and 4). This showed consistency between the three experiments for effects of Cd on Hb protein.

Treatment	HbIII	HbIV	HbVII	HbIX
0 h	352±2 [a]	301±10 [a]	373±51 [a]	275±6 [a]
24 h 0.0 µM	370±3 [b]	321±13 [a]	293±12 [b]	280±17 [a]
24 h 0.3 µM	258±22 [d]	296±39 [ab]	320±13 [ab]	278±39 [a]
24 h 3.0 µM	300±48 [acd]	253±33 [b]	320±12 [ab]	285±12 [a]
24 h 30 µM	345±12 [a]	289±16 [ab]	300±9 [ab]	284±15 [a]
72 h 0.0 µM	318±5 [c]	286±12 [ab]	269±37 [b]	273±11 [a]
72 h 0.3 µM	318±4 [c]	290±13 [ab]	304±21 [ab]	270±7 [a]
72 h 3.0 µM	295±8 [c]	282±24 [ab]	269±19 [b]	252±9 [b]
72 h 30 µM	299±23 [c]	295±29 [ab]	286±25 [b]	261±12 [ab]

Data represent average ± SD, n = 5-6.

Values that share a common letter were not statistically different, $p > 0.05$.

Table 5. Effect of Cd on Hb mRNA expression at 24 h and 72 h- Toxicity Test 3 Data are presented as pixel densities of bands on scanned gels divided by 1000. They are the combined average ± SD of amplified cDNA samples from two treatment replicates, n= 5-6. Treatments that share a letter were not statistically significant, $p > 0.05$. SD= standard deviation. 0 h = mRNA expression the day the experiment was initiated.

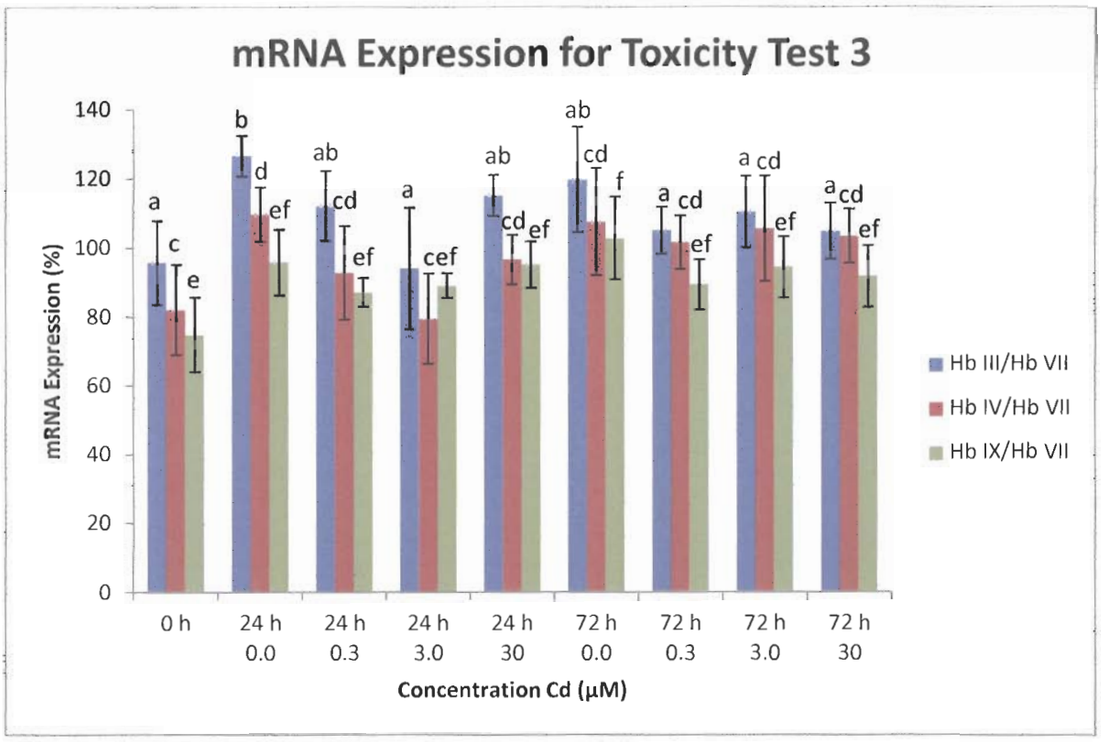


Figure 5. Percent Control for Toxicity Test 3 This figure shows Hb III, Hb IV, and Hb **IX** divided by Hb VII and multiplied by 100 to get a percent expression compared to Hb VII. Treatments that share the same letter are statistically similar ($p>0.05$). n=3 for 0 h, n=5 for 0 µM, 0.3 µM, 3 µM and 30 µM. **0 h** = mRNA expression the day the experiment was initiated.

	Control	0.3µM	3.0 µM	30 µM
High MW Protein	34.2±1.5[a]	32.3±4.4[a]	32.0±4.0[a]	34.0±1.7[a]
Low MW Protein	28.0±2.0[b]	29.0±1.4[b]	28.3±2.4[b]	20.0±2.5[c]

Data represent average ± SD, n = 9-10.

Values that share a common letter were not statistically different, $p > 0.05$.

Table 6. Effect of Cd on High and Low Molecular Weight Protein bands at 72 h- Toxicity Test 3 Data are presented as pixel densities of bands on scanned gels divided by 1000. They are the combined average ± SD of hemoglobin samples from two treatment replicates, n= 10. Treatments that share a letter were not statistically significant, $p > 0.05$. SD= standard deviation. 0 h = protein expression the day the experiment was initiated.

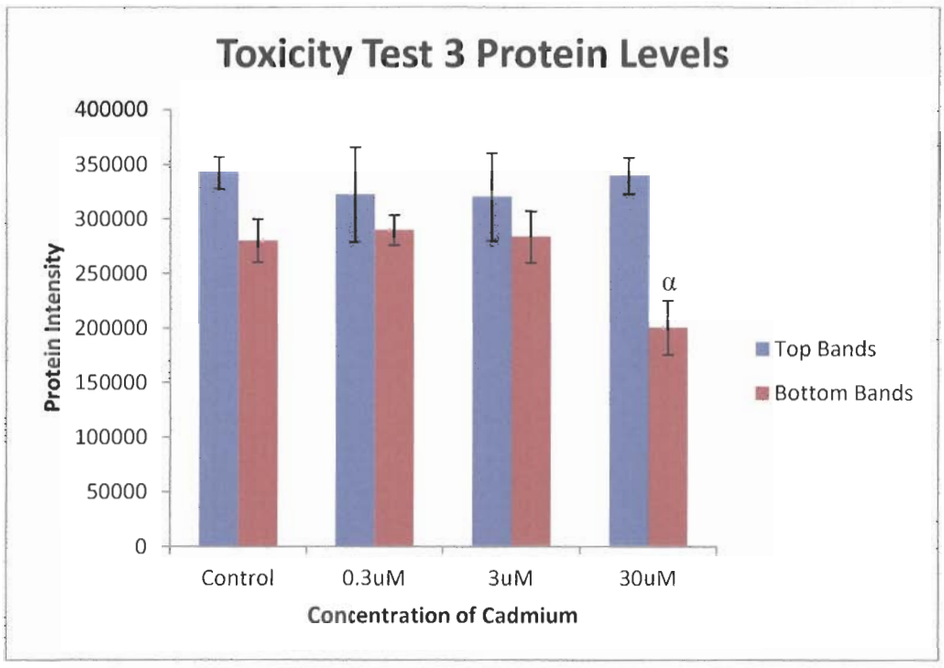


Figure 6. High and Low Molecular Weight Protein Bands- Toxicity Test 3 Data are presented as averages of each treatment, n = 10. The error bars show the Standard Deviation of the samples. Samples with an alpha symbol (α) are statistically different than control, p>0.05.

Correlation between mRNA and Protein expression

Cd caused the most modulation of the Hb IV gene, so a correlation was run using the Hb VII normalized, concentration-response data such that mRNA expression of Hb IV/Hb VII was compared with levels of low molecular weight Hb protein bands. The low molecular weight bands were chosen because there was a concentration-dependent decrease in their levels at 72 h, which was statistically significant for 30 μM Cd in all experiments.

Toxicity Test 1 showed a very strong correlation between normalized Hb IV and the low molecular weight proteins (Figure 7). The R^2 value for this experiment was 0.9157. This showed that there was a clear relationship between the decrease in Hb IV mRNA expression and the low MW proteins. Toxicity Test 2 did not have a strong correlation. The R^2 value for this experiment was 0.3848. This indicated that the relationship between Hb IV and the low molecular weight proteins was not as strong as Toxicity Test 1.The relationship between Hb IV and the low molecular weight proteins for Toxicity Test 3 was nonexistent. The R^2 value for the correlation was 0.0337. This proved that there was no repeatable relationship between the normalized mRNA expression of Hb IV and Hb protein levels despite the fact that Cd did significantly reduce levels of low molecular weight Hb protein. This experiment disproved the hypothesis that changes in Hb mRNA expression are causing the reduced levels of low molecular weight protein.

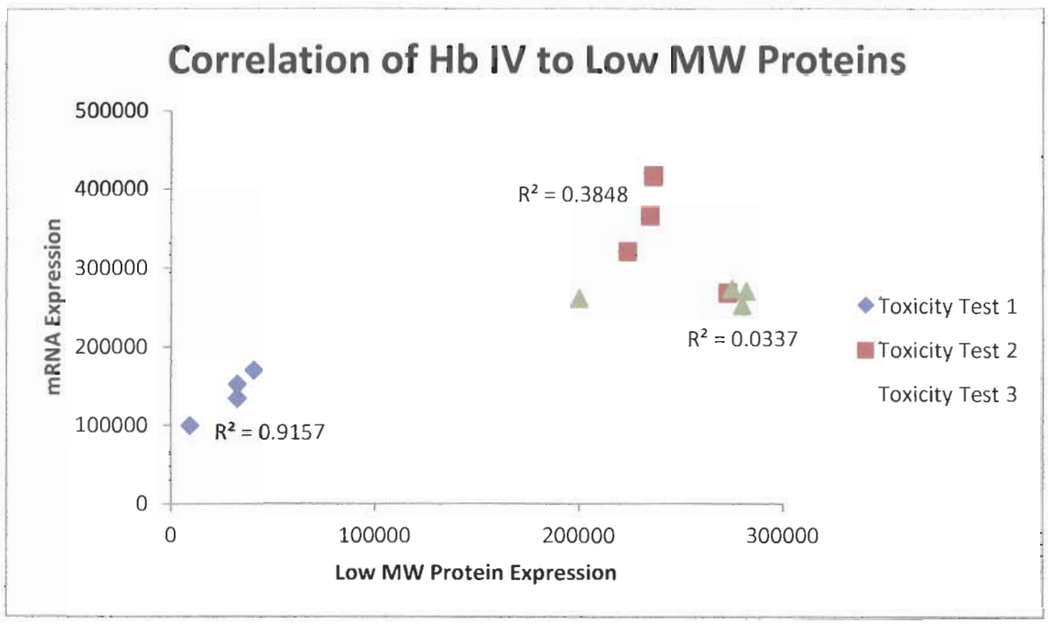


Figure 7. Correlation of Hb IV/Hb VII and Low MW Protein Intensity This graph showed the correlation between the average ratio of Hb IV/Hb VII for each Cd concentration at 72 h. The R^2 value was calculated by SPSS, n=4 for each experiment.

Cloning Data

The cloning data confirmed that the cDNAs measured were from the genes of interest. The accession number from GenBank for the gene segment of Hb III was Y10622.1. For the gene, the percent identity was 100% and all 164 bases that were sequenced matched up with the gene in the NCBI database (Figure 8). The e-value for the comparison between the experimental Hb III and the known sequence was 1e-79, which showed that there was an extremely low chance that the correct alignment was due to random chance. The e-value proved that the alignment was due to the experimental gene being correct.

The accession number for GenBank's sequence of Hb IV is X00920.1.The percent identity for the alignment between the experimental Hb IV gene and the one from the database was 100% (Figure 9). All 143 of the base pairs matched up perfectly with the gene sequence from GenBank. The e-value for this alignment was 3e-66, which, like Hb III, showed that there was an extremely small chance that there alignment occurred due to random chance.

The alignment for Hb VII showed a strong similarity between the GenBank and experimental sequences (Accession number AF001292) (Figure 10). The percent identity for the alignment was 98%: 85 out of 86 bases correctly aligned between the two sequences. The e-value was 6e-34, which, although it was not as strong as Hb III and Hb IV, showed that there was a very good chance that the alignment did not occur due to random chance.

For Hb IX, the accession number generated by GenBank is AF001292. Hb IX showed a better alignment than Hb VII. Out of 130 total base pairs, 129 aligned perfectly with the GenBank sequence for a percent identity of 99% (Figure 11). There was very little evidence that the alignment would have been due to random chance with an e-value for the alignment of 3e-58.

All four of the genes showed a high percent identity and a low e-value. The combination of those two pieces of data confirmed that all of the cDNAs amplified by PCR were from the Hb genes under study.

```
Y10622.1|C. thummi thummi globin gene cluster E
Score = 297 bits (328),  Expect = 2e-77
Identities = 164/164 (100%), Gaps = 0/164 (0%)
Strand=Plus/Plus

Query  1     CCAAACATTGAAGCCGATGTCAATACATTCGTTGCCTCACACAAGCCTCGTGGAGTTACA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3201  CCAAACATTGAAGCCGATGTCAATACATTCGTTGCCTCACACAAGCCTCGTGGAGTTACA  3260

Query  61    CATGATCAATTAAACAACTTCCGTGCTGGATTCGTCAGCTACATGAAGGCTCACACTGAC  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3261  CATGATCAATTAAACAACTTCCGTGCTGGATTCGTCAGCTACATGAAGGCTCACACTGAC  3320

Query  121   TTCGCTGGAGCTGAAGCAGCCTGGGGTGCAACTCTTGACACTTT  164
             |||||||||||||||||||||||||||||||||||||||||||
Sbjct  3321  TTCGCTGGAGCTGAAGCAGCCTGGGGTGCAACTCTTGACACTTT  3364
```

Figure 8. Sequence alignment for Hb III This figure shows the alignment of the cloned sequence (Query) compared to the known database sequence from the NCBI database (Sbject) for Hb III. The database sequence was from *Chironomus thummi thummi*'s globin gene cluster E, which is where Hb III is located. There is a 100% match between the known sequence and the E-value is 2e-77, which shows that there is a very good chance that this result did not happen by chance. This alignment shows that the cloned sequence is not only extremely similar to the database sequence but there is a very high probability that it is not a false positive. *C. thummi* is the former name of *C. riparius*, the species used in this study.

```
X00920.1Chironomus thummi thummi globin gene for globin IV

Score =  259 bits (286),  Expect = 3e-66
Identities = 143/143 (100%), Gaps = 0/143 (0%)
Strand=Plus/Plus

Query  1    AATCAGCACAGTCCAATCATCATTTGCTGGAGTTAAGGGAGATGCTGTTGGTATCCTCTA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  365  AATCAGCACAGTCCAATCATCATTTGCTGGAGTTAAGGGAGATGCTGTTGGTATCCTCTA  424

Query  61   TGCCGTTTTCAAAGCTGATCCATCAATCCAAGCCAAATTCACACAATTCGCTGGAAAGGA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  425  TGCCGTTTTCAAAGCTGATCCATCAATCCAAGCCAAATTCACACAATTCGCTGGAAAGGA  484

Query  121  CCTCGACTCAATCAAGGGATCAG  143
            |||||||||||||||||||||||
Sbjct  485  CCTCGACTCAATCAAGGGATCAG  507
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Figure 9. Sequence alignment for Hb IV This figure shows the alignment of the cloned sequence (Query) compared to the known database sequence from the NCBI database (Sbject) for Hb IV. The database sequence was from *Chironomus thummi thummi*'s globin gene for Hb IV, which is where Hb IV is located and where the primers are based off of. There is a 100% match between the known sequence and the cloned sequence and the E-value is 3e-66. This alignment confirms that the gene that has been modified is in fact Hb III and that there is a very strong possibility that this alignment did not happen by chance. *C. thummi* is the former name of *C. riparius*, the species used in this study.

AF001292 Chironomus thummi thummi globin VIIA.1 (ctt-7A.1), globin 9.1(ctt-9.1), globin II-beta (ctt-2beta), non-functional globin
XIII (ctt-13RT), globin XII (ctt-12) and globin XI (ctt-11)
genes, complete cds

Score = 150 bits (166), Expect = 6e-34 Identities = 85/86 (98%), Gaps = 0/86 (0%)
Strand=Plus/Plus

```
Query  1    CCAGACATTCAAGCCCGTTTCCCACAATTCGCCGGAAAGGACGTTGCTTCAATCAAGGAT  60
            |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  771  CCAGACATTCAAGCCCGTTTCCCACAATTCGCTGGAAAGGACGTTGCTTCAATCAAGGAT  830

Query  61   ACTGGTGCATTCGCCACACACGCCGG  86
            |||||||||||||||||||||||||
Sbjct  831  ACTGGTGCATTCGCCACACACGCCGG  856
```

Figure 10. Sequence alignment for Hb VII This figure shows the alignment of the cloned sequence (Query) compared to the known sequence from the NCBI database (Sbject) for Hb VII. The database sequence was from *Chironomus thummi thummi*'s globin VIIIA.1 gene, which is where Hb VII is located and where primers were designed for Hb VII. There is a 98% match between the known sequence and the cloned sequence and the E-value is 6e-34. Combining both the percent similarity and the E-value, this alignment confirms that the gene that has been studied is Hb VII. *C. thummi* is the former name of *C. riparius*, the species used in this study.

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AF001292 Chironomus thummi thummi globin VIIA.1 (ctt-7A.1), globin 9.1
(ctt-9.1), globin II-beta (ctt-2beta), non-functional globin
XIII (ctt-13RT), globin XII (ctt-12) and globin XI (ctt-11)
genes, complete cds

Score =  233 bits (126),  Expect = 3e-58
Identities = 129/130 (99%), Gaps = 1/130 (0%)
Strand=Plus/Plus

Query  110   TTGTTGCTCTTGTTGGAAACGAATCAAATGCCCCAGCCATGGCTACACTCATCAATGAAT  169
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2141  TTGTTGCTCTTGTTGGAAACGAATCAAATGCCCCAGCCATGGCTACACTCATCAATGAAT  2200

Query  170   TATCAACAAGCCATCACAACCGTGGAATCACAAAGGGACAATTCAATGAATTCCGT-CAT  228
              |||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct  2201  TATCAACAAGCCATCACAACCGTGGAATCACAAAGGGACAATTCAATGAATTCCGTTCAT  2260

Query  229   CACTCGTCTC  238
              ||||||||||
Sbjct  2261  CACTCGTCTC  2270
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Figure 11. Sequence alignment for Hb IX This figure shows the alignment of the cloned sequence (Query) compared to the known sequence from the NCBI database (Sbject) for Hb IX. The database sequence was from *Chironomus thummi thummi*'s globin VIIIA.1 gene cluster. Hb IX is located on the same gene cluster as Hb VII. There is a 99% match between the known sequence and the cloned sequence and the E-value is 3e-58. These two results confirm that the sequence that was cloned was the same sequence that was found in the database. The E-value proves that there is an extremely small chance that this close alignment is random. *C. thummi* is the former name of *C. riparius*, the species used in this study.

Discussion and Conclusions

In this study, changes of hemoglobin (Hb) low molecular weight protein levels were caused by exposing chironomids to increasing concentrations of Cd. The main objective was to relate the changing low molecular weight Hb proteins to changes in mRNA expression for multiple Hb genes. Based on previous studies, the disappearance of low molecular weight protein levels was proven to be a consistent response. The mechanism for this change was unknown, so mRNA expression was studied as a possible explanation. Since proteins are translated from mRNA, the mRNA was collected and transcribed into cDNA and studied. Understanding the mechanism(s) by which heavy metals affect Hb proteins will support the development of Hb in chironomid as a biomarker for environmental pollutants.

Hb has been used as a successful environmental biomarker. It has been used in a variety of species. One example was rainbow trout. In that study, musk ketones, commonly found in personal care products, were studied to see what effects they have on total hemoglobin protein in rainbow trout (Mottaleb, 2007). The fish were exposed to 0.010, 0.030, 0.10, 0.30 mg musk ketone/gram of fish. The Hb in this experiment was affected. 2-amino-MK (AMK) was found to be in higher concentrations in Hb extracts, especially at the 0.10 mg/g dosage. Another example was the effect of cadmium exposure on the Hb genes of the larval sheepshead minnow, *Cyprinodon variegatus* (Dangre, 2010). The Cd concentration used in this experiment was approximately1.6 μM and exposure was for 1, 3, 5, and 7 days. Hb in this experiment showed an increase in gene expression after exposure to hypoxic conditions. Cd did not seem to have an effect on Hb expression, however. This result was similar to what occurred in the present study in chironomid.

Since *Chironomus riparius* thrive in toxic environments, it was important to study how they can survive heavy metal contaminants such as Cd. The concentrations used for Cd exposure, 0.3 μM, 3 μM, and 30 μM, were concentrations that have been shown to reduce the level of low molecular weight Hb protein in our experiments. These concentrations were higher than some of the polluted wetlands where chironomids have been found. Based on previous studies, toxic heavy metals, like Cd, were at concentrations of about 0.8 μM in New Jersey marshland (Bentivegna, personal communication). Since chironomids have been shown to be resistant to heavy metals, higher Cd concentrations had to be used to induce molecular changes. It is possible that exposure of earlier instars, 1^{st} -3^{rd}, for more than 96 h would have resulted in effective concentrations similar to those found in the marsh.

Previous work has shown that Cd can, in fact, cause toxic effects in chironomids. Increasing concentration of Cd caused physiological changes such as a decrease in biomass and, at the highest concentrations, a lack of viability (Heinis, 1990). Concentrations used in this study ranged from 0.546 μM to 5.46 μM. The accumulation of Cd can also cause degradation of posterior midgut cells in chironomid. Cadmium exposure occurred for 96 hours at 10, 100, and 250 μg (Seidman, 1986). The concentrations used in this study were not high enough to fatally harm the chironomids (survival rate of over 70%).

There have been reports that environmental pollutants can affect Hb genes of various chironomid species. Some species studied included *C. riparius, C. tentans* and *Glyptotendipes pallens.* Hemoglobin genes appeared to be a target of environmental pollutants in *C. riparius* (Ha, 2008). There was a decrease in hemoglobin gene expression after exposure to the pollutants, such as nonylphenol, bisphenol A diglycidyl ether, and benzo[a]pyrene, proving that hemoglobin can be used as a potential biomarker. It was not clear if high or low molecular

weight proteins were affected in Ha's studies. The Hb protein findings of Ha (2008) supported the results in our studies. In all three of the toxicity tests presented here, there was a clear decrease in low molecular weight protein levels at 30 μM Cd. This decrease in protein expression was statistically significant. The high molecular weight proteins appeared to be unaffected by Cd and showed no statistically significant changes. These findings build on Ha's work by showing different sensitivities of Hb proteins to Cd.

It has been shown that chironomid hemoglobin genes can be modulated by certain environmental pollutants. Lee (2005) proved that hemoglobin expression increased when exposed to alkyl phenolic compounds and decreased when exposed to pesticides. In this experiment, *C. tentans* ORF (A-E) and *C. tentans* DNA Hb IX gene fragments were studied. The exposure time was 24 h and the hardness was not reported. The results in Lee's study were different than ours. In our study, it seemed that Cd did not cause a consistent concentration or time dependent response. In Toxicity Test 1, there was a decrease in Hb IV expression at 30 μM (Table 1). Hb IX and Hb III did not show a concentration dependent response. This decrease in Hb IV expression correlated very strongly with low molecular weight protein expression (Figure 7). Toxicity Test 2 showed that there was a statistical increase in expression of Hb IV at 30 μM Cd at 24 h (Table 3). For Hb III and Hb IX, there were not any major statistical changes for any of the concentrations. The correlation between the low molecular weight proteins and Hb IV was very weak since the protein expression decreased like Toxicity Test 1 (Figure 7). Toxicity Test 3 showed no statistical changes for Hb III, Hb IV, and Hb IX (Table 5). The correlation between low molecular weight Hb proteins and Hb IV was, again, very weak (Figure 7). Even though the genes' responses to Cd were variable, the low molecular weight proteins were affected consistently at 30 μM Cd. This proved that there was an effect cause by Cd to these

proteins but that it was not due to changes in Hb gene expression. There have not been any other studies that have looked at effects of Cd on any *C. riparius* genes or compared the response of different Hb genes.

Regulation of the dimers Hb VII and Hb IX were evaluated during this study. In previous studies, Hb VII and Hb IX were found to be located on the same chromosome (Weber, 1984). This was also found when researching the published sequences of the particular Hb genes used in this study (Kao, 1994).According to the sequencing information, Hb VII and IX were found in series on Chromosome II in *C. riparius*. Because the two genes were located in the same area, it was anticipated that they would be regulated similarly. When looking at the data, it appeared that there was some evidence that they were expressed similarly. In Toxicity Tests 1 and 3, it appeared that the levels of expression of the two genes were the same (Figures 1 and 3). There was no statistical difference between the two genes. In Toxicity Test 2, however, there was a statistical decrease of Hb IX compared to Hb VII.

Normalization of Hb III and IV by Hb VII allowed the gene regulation of monomeric Hbs to be compared to a dimeric one, Hb VII. Both Hb III and Hb IV have been located on Chromosome III.(Hankeln 1998, Antoine 1984) Hb IV was located on the opposite strand of Hb III: Hb IV ran 5' to 3' whereas Hb III ran 3' to 5'. In Toxicity Test 1, Hb III showed increased expression relative to Hb VII at 72 h with 30 µM Cd. This increase was statistically significant between 0 µM and 30 µM Cd indicating a concentration related response. Hb IV did not show a significant increase in mRNA expression at 30 µM Cd compared to 0 µM Cd (Figure 1); therefore, it was apparently not under the same regulation as Hb III. In Toxicity Test 2, Hb III showed no differences relative to Hb VII; however, Hb IV increased in a time and concentration dependent manner at 24 and 72 h (Figure 3). This indicated again that Hb III and IV were

regulated independently of each other. In Toxicity Test 3, the only differences from Hb VII occurred at 24 h (Figure 5). Hb III and IV showed a similar and significant decline at 3.0 μM suggesting that they were under similar regulation. Taken together this data showed no consistent evidence that Hb III and IV were co-regulated even though they were on the same chromosome and both produced monomeric Hb proteins. The fact that they were located on opposing DNA strands may explain this. The data for dimers, Hb VII and IX, did indicate co-regulation. This may be because they were located on the same chromosome in series.

After analyzing all three toxicity tests, it was clear that mRNA expressions vary between each test, especially Hb IV at 72 h. At that time point, Hb IV mRNA expression decreases in Toxicity Test 1, increases in Toxicity Test 2, and was constant in Toxicity Test 3. While these mRNA expressions vary between each test, the low molecular weight protein levels followed a consistent pattern. At 30 μM, the protein levels decreased in every test. In conclusion, since the Hb protein levels decreased consistently and the mRNA was not consistent, the research demonstrated that the mRNA expressions of the genes studied did not have a direct effect on the levels of low molecular weight proteins.

Some future studies can be performed based on the data from the three Toxicity Tests. One future study would look at other mechanisms that could be responsible for the decrease in low molecular weight proteins. A possible mechanism could be a decrease in heme synthesis, which would reduce hemoglobin protein levels. Another future study would be to determine how, or if, gene regulation differs on different chromosomes. Regulation of monomeric and dimeric proteins and how they differ can also be explored.

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