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Molecular Characterization of Hemoglobin Protein in Larvae of 4th Instar Chironomidae for Evaluating Environmental Quality

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MOLECULAR CHARACTERIZATION OF HEMOGLOBIN PROTEIN IN LARVAE OF 4TH INSTAR CHIRONOMIDAE FOR EVALUATING ENVIRONMENTAL QUALITY

By

JUN T. OH

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Molecular Bioscience from the Department of Biological Sciences of Seton Hall University

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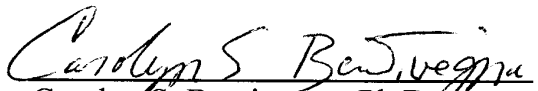

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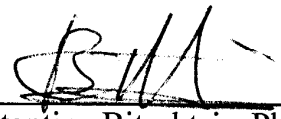

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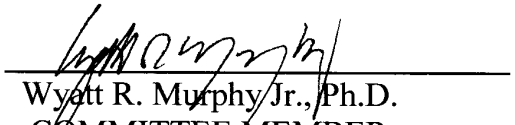

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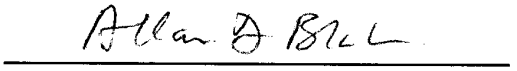

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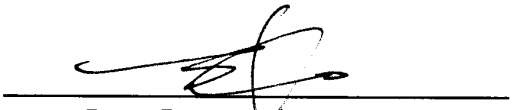

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Abstract

Studying environmental quality is a challenging task. It is a complicated exercise since the environment is constantly influenced by numerous variables such as climate change, anthropogenic activities, and unexpected natural disasters. Traditionally, exposure to chemical pollutants depended on chemical and physical analysis of environmental media. Unfortunately, this approach has not taken into consideration bioavailability of the chemical(s) of interest to exposed organisms and/or modification of the chemical (bioactivation/detoxification) by the organism. Benthic macroinvertebrates (BMIs) have been chosen as bioindicators for numerous environmental biomonitoring programs geared towards the assessment of aquatic ecosystems. Biomonitoring requires a more subtle measure – a biomarker – which should be dependable, reliable, and specific for assessing various ecological issues and human health risks. A wide array of biomarkers has focused on sublethal changes at the cellular and molecular levels. Good cellular and molecular biomarkers can respond quickly to low concentrations of contaminants with some specificity for particular types of contaminants. It is important that the mechanism by which they respond to contaminants be understood. This improves their reproducibility and provides an understanding of their limitations. In this study, various molecular responses exhibited by hemoglobin (Hb) protein from hemolymph of larvae of Chironomidae, or chironomids were characterized, in order to develop a biomarker for evaluating environmental quality. Hb proteins in the hemolymph of wild chironomids were separated by SDS-PAGE and compared to head capsule morphology. Results showed unique profiles for different genera and particular bands that identified

species. However, some species had multiple profiles. The source of Hb polymorphisms observed among wild species was investigated by determining the effect of proteases – chymotrypsin, trypsin, and pepsin – and an environmental stressor, cadmium (Cd), on Hb profiles. Results showed that individual and/or combination of proteases could account for intraspecies Hb protein profiles. However, 3.0 μM Cd generated its own distinct profile with upper bands similar to early 4^{th} instar larvae and a loss of lower bands. Cd's mechanism of action was investigated by measuring endpoints associated with Hb biosynthesis pathways. Endpoints included porphobilinogen (PBG) synthase activity and expressions of genes: hemoglobin IV and VII (Hb IV & VII), ubiquitin (Ub), and metallothionein (MT). Results showed that the effect of Cd on Hb protein profiles could be explained by inhibition of PBG synthase, up-regulation of Ub and down-regulation of Hb IV and VII. Overall, Hb protein profiles could be used to identify different genera and some species of wild chironomids. Hb profile polymorphisms could be explained by larval stage of development, levels of protease activity and modulation of Hb biosynthesis pathways.

Introduction

The adverse effect of environmental stressors is a major concern in the field of ecotoxicology – an interdisciplinary branch between ecology and toxicology. Ecotoxicology studies the effect of toxic molecules, either in the form of artificial origin (organic pesticides, pharmaceutical drugs, and endocrine disruptors) or natural agents (heavy metals and polycyclic aromatic hydrocarbons (PAHs), on biological systems (Holmstrup et al., 2010; Pauwels et al., 2013). The challenge in ecotoxicological research is to develop methods which could accurately identify, detect and characterize the biological impact of contaminant exposure for ecological risk assessment (Pauwels et al., 2013).

Traditionally, the detection of toxic chemical compounds in an environment of interest was conducted through chemical analysis of the water and sediment samples. However, relying solely on the chemical analysis of environmental samples does not provide any indication of deleterious effects of contaminants on biological systems (Brenner et al., 2014; Cajaraville et al., 2000). Therefore, a supplemental bioassay approach to the traditional monitoring techniques is necessary, biomonitoring. Biomonitoring involves using the response of organisms to assess the degree of contamination or "health" of an ecosystem. The response can be based on biochemical, physiological, morphological, or ecological measurements (Hare, 1992). However, to achieve an accurate measurement of the responses manifested by the organisms require a selection of indicator taxa. Among many candidate taxa for biomonitoring of aquatic environments, various species of periphyton, benthic macroinvertebrates (BMI), and fish

have received much attention (Li et al., 2010). The United States Environmental Protection Agency (USEPA) has established bioassessment protocols for select indicator organisms as well approaches for monitoring changes in water quality, population numbers, community composition, or ecosystem functioning with empirically defined reference conditions (Barbour et al., 1999). More subtle approaches could also be conducted – such as observing morphological abnormalities of antennal and mouthpart deformities found on head capsules of chironomids (Diptera: Chironomidae) caused by various anthropogenic stressors in aquatic systems (Madden et al., 1992; Martinez et al., 2003; Swansburg et al., 2002; Warwick,1985).

Biomarkers can be incorporated into biomonitoring in order to assess possible impacts of stress on indicator organisms more precisely. Biomarkers are defined as measurements in body fluids, cells, or tissues that can indicate biochemical or cellular modifications resulting from the presence of toxicants or stress. This definition was later modified to take into account characteristics of organisms, populations, or communities, including behavior, in which measureable responses reflect changes to the environment. The concept of the biomarker approach for assessing adverse effects or stress is based on the hypothesis that the effects of stress are typically manifested, first at lower levels of biological organization, before disturbances are realized at the population, community, or ecosystem levels. Thus, the biomarkers measured at the molecular or cellular level have been proposed as sensitive 'early warning' signals of specific detrimental biological endpoints that may occur at later response levels (Cajaraville et al., 2000; Brenner et al., 2013; Hauser-Davis 2012; Nogueira et al., 2010).

Among many biomarkers used in biomonitoring programs, the following have received special attention: metallothionein induction, acetylcholinesterase inhibition, cytochrome P450 system induction, imposex, lysosomal enlargement and lysosomal membrane destabilization, and peroxisome proliferation. These biomarkers can be used to evaluate exposure to and effect of different contaminants. For example, metallothionein for heavy metals and acetylcholinesterase inhibition and cytochrome P450 induction for organic xenobiotics and organometallic compounds (Cajaraville et al., 2000). However, even well-established biomarkers could face challenges and limitations, such as variability, costs for analyses, and laboratory errors which would potentially lead to misinterpretation of data and never achieving their full potential (Mayeux, 2004). Therefore, a clear understanding of mechanism of action of the biomarker of choice is necessary for making an accurate ecological risk assessment.

The goal of this study is to characterize hemoglobin (Hb) proteins found in hemolymph of larvae of Chironomidae at the molecular level as a potential biomarker for evaluating environmental quality. The larvae of Chironomidae, commonly called chironomids or bloodworms, are usually a major component of the BMI community. They are abundant and distributed globally, live in almost any aquatic habitat, tolerate a wide range of salinities and form an integral part of the diet for both vertebrate and invertebrate organisms – placing them in an important position in the aquatic foodweb. Therefore, chironomids have been frequently used for assessment of both acute and sublethal toxicity of contaminated sediments and water (Epler, 2001). Chironomids are holometabolous insects that pass through four distinct stages in their life cycle. They

spend the greatest part of their life cycle in larval form (Chetelat et al., 2008; Ebrahimnezhad & Fakhri, 2005). Worldwide, there may be more than 10,000 species of chironomids (Armitage et al., 1995). Their distribution is closely related to dissolved oxygen, organic matter, temperature, and different degrees of water depth – different species of larvae can live in/on sediments and vegetation (Das & Handique 1996; Epler, 2001; Ebrahimnezhad & Fakhri, 2005). Methods for culturing and testing are established by the USEPA, which has a standard protocol, Ecological Effects Test Guideline, for testing toxic sediments using chironomids (USEPA, 1996).

One of the striking traits of chironomids is that they have Hb – an oxygen molecule carrier. Hb proteins in invertebrates are complex and have different overall architectures. In chironomids, Hb proteins are synthesized by the insect's fat body and secreted directly into hemolymph starting in the second instar (Bergtrom et al., 1976). Concentrations of Hb proteins gradually increase as the larvae reach later instars (Bergtrom et al., 1976; Schin et al., 1974). Due to high concentrations of Hb in hemolymph (Tichy, 1975), large amounts are easily obtained from individuals for research purposes (Bentivegna et al., 2009). The abundance and presence of Hb in chironomids is physiologically relevant. They allow the larvae to sustain aerobic metabolism under adverse environment conditions such as polluted and hypoxic sediments (Lee et al., 2006; Saffarini et al., 1985). Hb proteins in the genus, *Chironomus*, show a high degree of polymorphism in addition to a high affinity for oxygen (Osmulski & Leyko, 1986). Hb polymorphism is stage-, species-, and tissue-specific, where different species have different numbers of Hbs. For example, *Chironomus thummi* has as

many as 10 and *Chironomus tentans* has up to 14 Hb proteins (Bergtrom et al., 1976; Schmidt et al., 1988; Vafopoulou-Mandalos & Laufer, 1982). The architecture of the Hb molecule includes one (monomer) or two subunits (dimer) per molecule instead of the four typical of vertebrates (Das & Handique, 1996; Osmulski & Leyko., 1986; Wollmer et al., 1972). The heterogeneity characterizing chironomid Hbs may be adaptive to exogenous and endogenous factors. This is demonstrated by the markedly higher contribution of dimeric Hbs in hemolymph of summer larvae compared to spring larvae of *Chironomus thummi thummi* (Osmulski & Leyko, 1986).

Our laboratory has been studying chironomid Hb as a biomarker for environmental contaminants. Previous studies indicated that Hb polymorphisms might be useful for molecular taxonomy of wild chironomids and detection of environmental contaminants in the field (Bentivegna et al., 2009; Oh, 2009). The data presented in those studies conveyed two vital pieces of information.

First, each genus of chironomids appeared to have one or more characteristic Hb protein profiles when matched with corresponding head capsule analysis. Head capsule morphology of chironomid larvae has been a principle means for taxonomically identifying larval species (Cranston, 2000; Epler, 2001). Determining the species of chironomids is important since there is substantial variation among species in their response to environmental change; species-level identification provides the most relevant data when using population and community level parameters to evaluate a study site (Carew et al., 2003). However, there are many challenges faced when relying on head capsule morphology alone for taxonomic identification. For example, it can require a

high level of taxonomic skill and large-scale routines often lead to misidentifications even when identification is restricted to genera, families or orders (Carew et al., 2003; Epler 2001; Pfenninger et al., 2007). Therefore, Hb protein profiles detected by SDS-PAGE might provide a simplified but accurate method for taxonomic identification of wild chironomids that would facilitate their use in field work.

Second, the Hb protein profiles, regardless of species, were modulated by cadmium toxicity – showing changes in the patterns of different molecular weight bands. Cadmium (Cd) was chosen for laboratory studies as it is a representative toxic heavy metal commonly found in urbanized waterways and because its concentrations exceeded EPA sediment standards at Kearny Marsh, NJ where some of our field work with chironomids had been conducted (Bentivegna et al., 2004). Acute Cd toxicity tests showed that there was a loss of both small and large molecular weight proteins, when exposed to high concentration of Cd. A key finding of the study was that Hb protein profiles among genera could exhibit different profiles, but display similar sensitivity to Cd where there was a consistent concentration response observed on SDS-PAGE (Bentivegna et al., 2009). This finding suggested that there could be a link between Hb proteins in the wild population and Cd exposure.

Overall, our previous findings led us to believe that the polymorphisms observed among wild population was likely due to a unique genetic history, causing changes in the overall structure of Hb protein synthesis over multiple generations. It appeared that each individual species could have evolved its own way of processing the Hb protein synthesis. In the case of modulation of Hb protein profiles upon exposure to Cd, the changes in the

profiles could be exerted by adverse effects of Cd on Hb protein synthesis. This suggested that Hb profiles had potential as a biomarker for heavy metal toxicity and evaluating environmental health. Overall, it appeared that a more clear understanding of the mechanisms associated with chironomid Hb polymorphisms was needed in order to validate its usefulness for species identification and as a biomarker of environmental stressors.

In this study, comprehensive research on Hb proteins was carried out to explore the essence of Hb proteins with the intention of developing it as a true multi-functional biomarker for fieldwork research. We begin by describing how Hb proteins could contribute to the development of a novel means of identification of wild chironomid species collected from two locations in Maine and New Jersey, USA. Their Hb proteins in the hemolymph were separated by SDS-PAGE and Hb protein profiles were generated. The technique described here supports and supplements the current standard method of taxonomic identification using larval head capsule morphology or even other methods such as PCR-based approaches. This study further investigates the potential role of endogenous proteolytic enzymes – chymotrypsin, trypsin, and pepsin – on Hb protein and how activity of individual and/or combination of proteases could produce distinct Hb protein profiles of the wild population. It appeared that there is a possible connection between Hb protein polymorphism and the enzymatic activity. Lastly, molecular mechanisms examining the response of Cd during heme biosynthesis pathway is delineated. More specifically, the response of key genes and an enzyme involved in the pathway are described upon exposure to Cd.

Materials and methods

Description of sampling sites

Chironomids were collected from four different sampling sites, comprised of one river and three wetlands, all located on the east coast of the United States during summers of 2007, 2008, and 2012. Two sites were from the state of New Jersey and the other two sites were from the state of Maine. Kearny Marsh (KM) (latitude: 40.756035, longitude: -74.125314) of the New Jersey Meadowlands is located between the Passaic and Hackensack Rivers and is surrounded by major highways such as Interstate 95. High levels of toxic heavy metals have been found in this marsh (Bentivegna et al. 2004). Rahway River (RR) (latitude: 40.750402, longitude: -74.259387) is located in a residential area in Essex County of New Jersey. Chironomids were collected from the east branch which runs through the town of South Orange. North East Creek (NEC) (latitude: 44.417107, longitude: -68.31316) and Bass Harbor (BH) (latitude: 44.24071, longitude: -68.346591) are located in Mount Desert Island, Maine, with close proximity to sea water. Both sites are near Acadia National Park, which is considered to be a relatively pristine area when compared to the New Jersey watershed (USEPA, 2010ab).

Chironomid collection

Chironomids in Maine were collected using a Hester-Dendy sampler (Wildco, Yulee, FL) or by hand picking them from rocks and aquatic vegetation. Chironomids in KM were collected using Hester-Dendy, while those in the RR were collected using Surber samplers (Wildco). The live larvae were placed into a Ziploc bag containing site

water and transported back to the laboratory in a cooler. One to four hours transpired between chironomid collection and Hb protein collection (see below). In addition to the wild larvae, investigations included a laboratory strain of *Chironomus riparius* (Environmental Consulting & Testing, Superior, WI) that was cultured and maintained under controlled conditions in our laboratory. The laboratory population was used for life stage and Cd toxicity studies (see below). The larvae used in these studies were reared from a single egg mass deposited by adult flies.

Water quality

The following water quality parameters were measured at KM, NEC, and BH: temperature (°C), pH, dissolved oxygen (mg/L), salinity (ppt), and redox potential (mV). The parameters were measured using a YSI meter, model 556 (YSI Environmental, Yellow Springs, OH). Water quality parameters at RR sites included temperature (°C), pH, dissolved oxygen (DO mg/L), and hardness (mg/L). Hardness and dissolved oxygen were measured using LaMott testing kits (Carolina Science and Math, Burlington, NC). The pH was measured using a Corning pH M240 (Corning Science Products, Corning, NY).

Species identification

Head capsules and their corresponding body were collected for hemoglobin analysis and species identification. The process involved decapitation of live larvae such that hemolymph was drained and collected as described below. After collection of

hemolymph, the head capsule and remaining body of a particular chironomid were stored in 70% ethanol until mounting. Permanent mounting was performed according to Epler (2001). All mounted head capsule samples were keyed to the lowest possible taxonomic unit based on larval body morphology and head capsule morphology keys (Epler, 2001). Representative mounts of head capsules were sent to Epler for identification.

Preparation of hemolymph samples and SDS-PAGE

Hemolymph was extracted from each larva by decapitation and bleeding out onto a microscope slide. Immediately, approximately 2 µL of hemolymph was drawn up and transferred into a 1.5 mL centrifuge tube containing 14 µL of lithium dodecyl sulfide (LDS) sample buffer (Invitrogen, Carlsbad, CA), 2 µL of 8M of urea (Qiagen, Valencia, CA) and 2 µL of 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO). The samples were stored at -20 °C until use. SDS-PAGE involved separating a 5 µL aliquot of hemolymph protein mixture on 16.5% Tris-Tricine gels (BioRad, Hercules, CA) under denaturing conditions using 1X Tris/Tricine/SDS electrophoresis buffer (Biorad). All hemolymph samples in LDS sample buffer were boiled prior to loading. The protein concentration of the hemolymph samples used to generate Hb protein profile indices (see below) and protease digestions (see below) was not measured prior to SDS-PAGE separation. However, the protein concentrations of all other hemolymph samples including *C. riparius* instar Hb proteins (see below) and 96 hour acute Cd toxicity test (see below) were first analyzed using Bicinchoninic Acid Kit, following the manufacturer's protocol (Sigma) prior to SDS-PAGE separation. This ensured that

approximately 200 and 1500 µg of total protein per sample was loaded and separated by SDS-PAGE for instar Hb proteins and 96 hour acute Cd toxicity test, respectively. SeeBlue® Plus2 Protein Standard (Invitrogen) was used as a molecular weight ladder. The gel was run at 100 V followed by gel washing, fixing, staining, and drying steps. Gels were washed in ddH2O and then fixed in a fixing solution containing 50 % methanol (Pharmco-Aaper, Brookfield, CT), 7 % glacial acetic acid (Pharmco-Aaper), and 43% ddH_20. After fixation, the gel was washed with ddH_2O and stained with Gel Code Blue (Pierce, Rockford, IL). The gel was de-stained by boiled ddH_2O.The gel was dried on blotting paper using a gel dryer. All gels were then scanned and imaged (see below).

Liquid chromatography-mass spectrophotometry (LC-MS) proteomic analysis

To verify that the proteins contained in particular SDS-PAGE bands were indeed hemoglobin, four prominent bands ranging from 5 to 12.5 kDa were excised and tested using LC-MS proteomic analysis. This was done using standard protocols at the Biological Mass Spectrometry Facility of the Center for Advanced Biotechnology and Medicine of Robert Wood Johnson Medical School and Rutgers, the State University of New Jersey. The LC-MS data were searched against a subset of Uniprot database with entries containing the keyword “Chironomidae” and CRAP.fasta (www.theGPM.org) and using a local version of the Global Proteome Machine (GPM cyclone, Beavis Informatics Ltd, Winnipeg, Canada).

Hemoglobin protein band pixel quantification

The gel was scanned using a Gel Doc-It Imaging Transilluminator System (UVP, Upland, CA). The picture was loaded onto VisionWorksLS program (UVP) for quantification analysis of the bands. In order to measure each band in a single lane, a 20 band system was developed using different molecular weights ranging from 4 kDa to 17 kDa. Pixel intensity of each band within the 20 band system was initially quantified as a raw score and then further normalized by subtracting the background pixel intensity – a blank space in each lane. The relative intensity ratio for each band was determined by dividing the normalized pixel by the highest pixel found in that lane. Only the bands with relative intensity of 30% or greater were considered in this study as bands of interest.

Hemoglobin protein molecular weight determination

For each hemolymph sample separated on SDS-PAGE gels, molecular weights were determined for all Hb bands in a single lane (Hemes 1998). Relative mobility (Rf) for each protein was determined using the formula: Rf = distance of protein migration/distance of dye migration. A standard curve was generated for each gel by plotting Rf versus log scale of the molecular weights (log MW) of protein standards. Finally, Rf value of each Hb protein in a single lane was used to estimate its molecular weight by interpolation to the standard curve. The ladder for SDS-PAGE contained the protein standards. Information on their molecular weights was provided by the manufacture's protocol (Invitrogen).

Hemoglobin protein profiles

Hb protein profiles consisted of one or more bands from a particular larva. They were associated with their corresponding head capsules, which were identified to the lowest possible taxonomic level using head capsule morphology. Hb protein profiles of all taxa were compared for uniqueness. Amino acid sequencing has shown that major Hb proteins have molecular masses in the range of 16 and 17.5 kDa; therefore, bands below 7 kDa were thought to be Hb protein degradation products, and were excluded from all comparisons.

Bright field imaging of chironomid head capsules

Each mounted head capsule of a chironomid was viewed using a 10X objective lens on a Zeiss Axioskop microscope (Carl Zeiss Ltd, Cambridge, United Kingdom). Images were captured using a Leica DFC 300 FX digital camera (Leica Camera Inc, Allendale, NJ). The entire head capsule could not be imaged in a single field of view using this objective, so two overlapping areas were collected (the superior and inferior portions of the head capsule) and a panoramic montage was created using the Photomerge function in Photoshop CS5 (Adobe Systems Incorporated, San Jose, CA) to generate a complete image of a head capsule. Additionally, for each area, 15 to 20 images from successive focal depths were collected and three dimensional montages for each portion of the head capsule were generated using the Do Stack function in CombineZP URL (http://www.hadleyweb.pwp.blueyonder.co.uk/).

Comparison of stage-specific Hb protein profile

Chironomids from a single egg mass were collected every three days starting at approximately 6 days after hatching and continuing through pupation and emergence of the adult. At each time point, three hemoglobin samples were generated. Each sample consisted of the combined hemolymph of five individuals. All individuals collected at the first time point (6 days) had visible red pigment on the thoracic segments including the head capsule, when viewed under the dissecting microscope (Parco Scientific Company, Westland, MI). Head capsules were collected from the same individuals used for hemolymph analyses. The widths of the head capsules were measured in order to determine larval instar using methods previously described (Watts & Pascoe, 2000). Hemolymph from pupae and adult flies were collected the same way as from larvae and was separated by SDS-PAGE as described above.

Hemoglobin protease digestion

Hemolymph collected from 10 laboratory strain *C. riparius* was artificially digested with chymotrypsin, trypsin, and pepsin (Sigma). The concentrations of proteases were as follow: 0.1 mg/mL of chymotrypsin, 1.0 mg/mL of trypsin, and 1.0 mg/mL of pepsin. For chymotrypsin and trypsin digestion, 15 µL of hemolymph was digested with 5 µL of protease and incubated in 30 µL of assay buffer 1 containing 20 mM Tris-HCl and 10 mM $CaCl_2$ with pH=8.5 at 37 °C. For pepsin digestion, assay buffer 2 containing 0.084N HCl and 35 mM NaCl with pH=2 (Thomas et al., 2004) was used. 10 µL of digested hemolymph was collected at 5, 30, and 60 minutes, transferred to a fresh tube

containing 10 µL of LDS buffer, and immediately stored at -20 °C. To verify the actual protease activity, 5 µL of specific inhibitors, 2 mg/mL of aprotinin (Sigma) for chymotrypsin and trypsin, and 2 mg/mL of pepstatin A (Sigma) for pepsin, were used. The digested hemolymph samples were separated via SDS-PAGE to generate Hb protein profiles (see above).

96 hour acute cadmium toxicity test

Laboratory cultured larvae of *C. riparius* were reared to third or early 4th instar from the same fertilized egg masses. From the total population, 30 larvae were randomly picked and used for each concentration – 0, 0.3, and 3.0 µM Cd (Sigma) for 96 hours. This corresponded to 0, 55, and 550 µg/L, respectively. Acid-washed sand was provided as a substrate and larvae were fed daily. The test water was obtained by making reconstituted water (USEPA, 2006) from the ½ deionized water (Millipore Milli-Q, Billerica, MA) and ½ filtered tap water with reagent grade chemicals: $NaHCO_3$, $CaSO_4$, $MgSO_2$, and KCl (Sigma). The test water (250 ml) was added to 1 L polypropylene containers, and $CdCl_2$ was spiked into it from a stock concentration. The test water was aerated, changed every 24 hours, and a new spike was added. At 96 hour, the average hardness of the reconstituted water was between 160 to 180 ppm, the average pH was between 7.2 to 7.4, the average temperature was between 20 to 21 °C, and the dissolved oxygen was between 7 to 9 ppm. Chironomids were exposed to the following concentrations of Cd: 1) control; nominally 0 µM, actual value measured in-container: 0.0215 ± 0.0334 µM, 2) 0.3; nominally 0.3 µM, actual value measured in-container:

0.0681 ± 0.043 µM, and 3) 3.0; nominally 3.0 µM, actual value measured in container: 0.64 ± 0.0639 µM. All water samples were prepared by following the EPA/600/4-91/010 under Method 200.7 and analyzed by inductively coupled plasma mass spectrometry (ICP-MS) at Environmental and Occupational Health Sciences Institute, Rutgers, the State University of New Jersey. Chironomids were collected for Hb protein (see above), porphobilinogen synthase assay (see below), and gene expression study (see below) at 12, 24, 48, 72, and 96 hours. Three independent experiments (indicated by trial numbers I, II, and III) were conducted. Each independent experiment included an analysis of Hb protein profile, porphobilinogen synthase activity, and quantitative polymerase chain reaction of the genes of interest.

Porphobilinogen (PBG) synthase Assay

Three larvae were collected from each test vessel and plotted dry using tissue wipers (VWR, Radnor, PA) and placed into 500 µL of chilled 100 mM potassium phosphate buffer (pH 6.5) and completely homogenized using a glass 2 mL tissue grinder (Fisher Scientific, Waltham, MA). The homogenate was transferred into a 1.5 mL microcentrifuge tube (VWR, Radnor, PA) and 400 µL was transferred to a clean tube, for the PBG synthase assay. This left 100 µL of homogenate for protein quantification using the Bicinchoninic Acid assay (see below). To the 400 µL of homogenate, 10 µL of dithiothreitol (DTT) (Sigma) was added and the sample was vortexed. The sample was then incubated at 37 ºC for 10 min using an IncuBlock heat block (Denville Scientific, South Plainfield, NJ). Following incubation, 100 µL of 100 mM of the substrate, amino-

levulinic acid (ALA) (Frontier Scientific, Logan, UT), was added to the sample. This solution was incubated for 60 min at 37 ºC in order to generate the product, porphobilinogen (PBG). Next, the amounts of PBG generated were determined using a modified Ehrlich's assay. This involved adding 500 µL of 10% trichloroacetic acid (TCA) (Sigma) containing 100 mM $HgCl_2$ (Alfa Aesar, Ward Hill, MA) to each sample and vortexing. The samples were then centrifuged using a Biofuge 13 centrifuge (Heraeus, Hanau, Germany) for 5 min at 10,000 RPM. Next, 500 µL of modified Ehrlich's reagent [2% dimethylaminobenzaldehyde (DMAB, Mallinckrodt, Phillipsburg, NJ) in 5N HCl] was added to 500 µL of each supernatant. Samples were inverted several times and incubated at room temperature for 10 min. After 10 min, the absorbance was taken at OD555nm using Spectramax M5 plate-reader (Molecular Devices LLC, Sunnyvale, CA). All values were normalized to the total protein mass which was measured by Bicinchoninic Acid (BCA) solution (Sigma). The same homogenate (100 µL) prepared for the PBG synthase assay was used to determine the total protein mass. The final units were reported as µmol/µg protein.

RNA isolation and quantitative polymerase chain reaction (qPCR)

Total RNAs were extracted by homogenizing three larvae in TRIzol (Invitrogen). From the total RNAs, a cDNA library was constructed using oligo-dT primers (Applied Biosystems, Foster City, CA). The cDNA was used as a template to amplify genes of interest with PCR. Genes targeted for quantification included β-actin (GenBank: AB070370), hemoglobin protein IV (Hb IV, GenBank: X00920), hemoglobin protein VII

(Hb VII, GenBank: U01342), ubiquitin (Ub, see below) and metallothionein (MT, GenBank: HQ260607). StepOnePlus System 96-well PCR instrument (Applied Biosystems) carried out each qPCR run. Sequences of the primer sets are provided in Table 6. For each qPCR run, the ΔΔCt method was chosen to calculate relative gene expression, and all data were normalized against β-actin, as a housekeeping gene (Livak 2001). Samples were analyzed in triplicate for each gene and compared to corresponding 12 hour control. All Melt curves were generated for each qPCR run to validate a single product formation. The product size was verified by agarose gel electrophoresis.

Table 1. Primers used to amplify specific genes.

Gene	Primer sequence	Product size (bp)	Tm (°C)	GenBank Accession No.
β-actin	5'GTCGCGATTTGACTGACTACTT3' 5'GTCCAATGCAACATAGCACAAC3'	122	62	AB070370
Hb IV	5'CTCGACTCAATCAAGGGATCAG3' 5'GTTTGGAAGGTCTCCGATGAT3'	90	62	X00920
Hb VII	5'GAAATCCTTGCTGCTGTCTTTG3' 5'CGAATGCACCAGTATCCTTGA3'	103	62	U01342
Ub	5'ATCAGACAATGTACGACCATCTT3' 5'GTTGAGCCATCAGACACCATT3'	126	62	N/A
MT	5'GGGCTGCAAATGTTGTTCACA3'	130	55	HQ260607

Cloning and sequencing of Ubiquitin

The sequence for Ub was not available in NCBI, and it was necessary to generate degenerative primer sets for Ub gene in order to develop gene specific primers for qPCR. Degenerative primers were generated based on the available known Ub sequence homology among six closely related species: *Aedes aegypti* (GenBank: XM_001664216), *Anopheles funestus* (GenBank: DQ910360), *Anopheles stephensi* (GenBank: AJ415521), *Culex quinquefasciatus* (GenBank: XM_001864341), *Drosophila melanogaster* (GenBank: M22428), and *Papilio xuthus* (GenBank: AK401158). Using degenerative primers, PCR was performed and multiple bands of the anticipated size were observed on an agarose gel stained with ethidium bromide. The bands were extracted from the agarose gel using QIAEX II Gel extraction Kit (Qiagen). The cloning was done using TOPO TA Cloning kit (Invitrogen). The purified PCR product was ligated into TOPO® vector and transformed into Transform One Shot® TOP10, chemically competent *E.coli* cells. The cells were spread on LB-plate containing 50 µg/mL Kanamycin sulfate (Invitrogen), 200 mg/mL IPTG (isopropyl-beta-D-thiogalactopyranoside) (Invitrogen), and 20 mg/mL X-gal (Invitrogen). Plasmid DNA was isolated and amplified with the gene-specific forward primer and M13R vector primer. The PCR product was separated by agarose gel and the band was extracted from the agarose gel using gel extraction kit. The purified plasmid PCR product was confirmed by commercial sequencing (GeneWiz, Plainfield, NJ). A gene specific primer set for Ub was generated using the confirmed nucleotide sequence information.

Statistical analyses

The results are reported as the mean ± SD and repeated thstatistical tests were performed using GraphPad Prism5 (GraphPad Software, La Jolla, CA). Statistically significances were established at $p<0.05$ for (*), $p<0.01$ for (**) and $p<0.001$ for (***) and determined by One-way ANOVA followed by Dunnett's multiple comparison test or Bonferroni's Multiple Comparison Test.

Results

Water quality of the sampling sites

Water quality parameters of the four sites at the time of chironomid collection are shown in Table 2. KM stood out as having water temperatures approximately 8 °C warmer than the others and supersaturated DO. The redox of KM was slightly higher as well but had a wide range, 21.9 – -153 mg/L. These factors indicated a eutrophic environment at KM. The pH averages at KM, RR, NEC, and BH were 8.29, 7.10, 6.39, and 6.33, respectively. Salinity averaged higher at BH and KM, 2.90 and 1.71 ppt, respectively, and lower at NEC, 0.65 ppt. RR was a freshwater site and hardness averaged 180 ppm.

Table 2. Range of water quality parameters at the four chironomid collection sites.

Site	Temperature (°C)	pH	DO (mg/L)	Salinity (ppt)	Hardness (ppm)	Redox (mV)
KM (NJ)	26.70 – 30.30	7.49 – 8.59	7.32 – 11.90	1.73 – 1.74	NA	21.9 – -153
RR (NJ)	21.0 – 22.0	6.80 – 7.40	2.20 – 9.40	NA	120 – 240	NA
NEC (ME)	19.40 – 21.0	5.92 – 6.86	5.57 – 6.60	0.45 – 0.84	NA	-106 – -114
BH (ME)	18.4 – 21.7	6.16 – 6.49	6.02 – 6.28	2.10 – 3.70	NA	-45 – -142

DO, Dissolved oxygen; Redox, Reduction and oxidation potential; NA, Not available;
KM (NJ), Kearny Marsh, New Jersey; RR (NJ), Rahway River, New Jersey; NEC (ME), North East Creek, Maine; BH (ME), Bass Harbor, Maine
The table was adopted from Oh et al., 2014.

Chironomid head capsule identification

Sixty-six chironomids were analyzed for head capsule morphology (Table 3). This included one group – *Thienemannimyia* and four genera – *Chironomus*, *Cricotopus*, *Dicrotendipes* and *Glyptotendipes*. Within the four genera, there were four described species and three unidentifiable species based on head capsule morphology. Those not identified to species were denoted as "sp." or named after the region in which the chironomid was found – "sp. ME1" for an unknown species collected in Maine.

Table 3. Chironomid taxonomy based on head capsule morphology. Chironomids were collected from four different sampling sites during 2007 to 2012. n = total number of chironomids analyzed for a particular species. Subfamily of each species is also provided.

Taxonomy Identification	Site	N	Collection Date	Subfamily
Chironomus riparius Meigen	KM, RR	14	Jul-07, Aug-12	Chironominae
Chironomus sp. ME1	BH	9	Aug-08	Chironominae
Cricotopus bicinctus Meigen	RR	3	Sep-12	Orthocladiinae
Dicrotendipes modestus (Say)	RR,NEC,BH	10	Jul-07, Aug-08	Chironominae
Glyptotendipes paripes Edwards	KM	14	Jul-07	Chironominae
Glyptotendipes sp.	KM	14	Jul-07	Chironominae
Thienemannimyia group sp.	RR	2	Sep-12	Tanypodinae

Site abbreviations as in Table 2
The table was adopted from Oh et al., 2014.

Protein composition analysis by LC-MS

LC-MS was used to verify protein composition of the bands separated on SDS-PAGE. Bands at 12.5, 11, 7, and 5.5 kDa were chosen from a hemolymph sample of *C. riparius* (laboratory strain) for LC-MS analysis. The major proteins identified for one of the four bands (12.5 kDa) analyzed is shown in Table 4. Information provided includes the protein name, molecular mass (kDa), the amino acid length, and the corresponding accession numbers in the NCBI database. According to the base peak ion chromatogram, isomers of Globin *Chironomus thummi thummi* (CTT) Hemoglobin-VIIB (CTT-VIIB) and Globin CTT-II were the dominant proteins in the 14.5 kDa band and accounted for the majority of its intensity. CTT is the former name of *C. riparius*, the species used for this analysis. Similar protein species of Hbs were also observed in the 11, 7, and 5.5 kDa bands (data not shown). The LC-MS analysis indicated that the Hb proteins detected in these four bands might be breakdown products of the major Hb proteins, as the expected sizes of those Hb proteins range from 16.8 to 17.4 kDa, but no bands were found to be in this range on SDS-PAGE for *C. riaprius*.

Table 4. LC-MS data of the most abundantly identified proteins in Hb band 5 separated by SDS-PAGE. CTT represents *Chironomus thummi thummi*, which now is called *Chironomus riparius*. AA represents the total number of amino acids in the sequence.

Protein Name	Molecular Mass (kDa)	Length (AA)	Accession number in the NCBI database
Globin CTT-VIIB-4	16.8	161	P84296
Globin CTT-VIIB-6	16.9	161	P12549
Globin CTT-VIIB-8	17	161	Q23763
Globin CTT-II beta	17.4	160	P02222

The table was adopted from Oh et al., 2014.

Comparison of head capsule and corresponding Hb protein profile index

The head capsule of wild *Chironomus riparius* and its associated Hb proteins generated by SDS-PAGE were compared (Fig. 1). An important landmark of *C. riparius* head capsule was three dark inner teeth on the mandible (Fig. 1a). According to the head capsule morphology, we determined that all individuals analyzed appeared to be the same species, regardless of the sampling sites, KM (n=2) and RR (n=4). *C. riparius* had three varying Hb protein profiles – P1, P2 and P3 (Fig. 1b). P1 was obtained from a chironomid collected in RR whereas P2 and P3 were from KM. All three profiles shared bands at 14.5, 12.5 and 11 kDa. Both P1 and P2 had an extra band at 11.5 kDa, and P3 showed an absence of bands at 15.5 and 11.5 kDa. P1 did not have bands below 11 kDa, whereas both P2 and P3 did.

An unidentifiable species of wild *Chironomus* was collected only at BH and was denoted as *Chironomus* sp. ME1. The head capsule of this species had two dark inner teeth on the mandible and a significantly downsized outer tooth on the mentum (Fig. 2a) – traits which are atypical of *C. riparius*. There was only one Hb protein profile (P4) found in all individuals of *Chironomus* sp. ME1, n=9 (Fig. 2b). P4 had three bands at 15.5, 14.5, and 11.5 kDa.

Cricotopus bicinctus was collected at RR (n=3). The most prominent feature on the head capsule was the jagged edges of the molar margin of the mandible (Fig. 3a). Interestingly, *C. bicinctus* displayed a Hb protein profile (P5) containing only one band at 17 kDa (Fig. 3b). P5 was found in all three individuals of this species.

Dicrotendipes modestus was found at NEC (n=3), BH (n=1), and RR (n=6). The main distinguishable characteristic of this species' head capsule was the striations on the ventromental plate: there was a mean of 32 strial ridges (Fig. 4a). The Hb protein profile consisted of three distinct bands in the range of 17.5, 15, and 13.5 kDa (Fig. 4b). Although *D. modestus* was found in three different locations, the single Hb protein profile (P6) and head capsule morphology were consistent among all individuals collected.

Glyptotendipes paripes was only found at KM (n= 14). Taxonomy of the genus *Glyptotendipes* has been under revision by Michael Heyn; however, the *Glyptotendipes paripes* has been exceptionally well characterized and is, unlike many other *Glyptotendipes* species, usually identifiable in all life stages. Two common features of *G. paripes* head capsule were the smooth anterior margin of the ventromental plate and the darkened internal area posterior to the mandible (Fig. 5a). *G. paripes* had two similar Hb protein profiles, P7 and P8, n= 11 and 3, respectively (Fig. 5b). Both profiles shared two intense bands, one at 17 kDa and the other at 13 kDa. However, P8 had an extra band at 16 kDa that was not visible in P7. In addition, P8 lacked bands in the range of 11 to 8 kDa.

There was an unidentifiable species of *Glyptotendipes* found in KM along with *G. paripes* (Fig. 6a). Other than the 13 teeth on the mentum – a well characterized landmark of the *Glyptotendipes* genus – there was a lack of characteristic traits, preventing species-level identification using head capsule morphology. The smooth anterior margin of the ventromental plate and the darkened internal area posterior to the mandible of *G. paripes*

were not visible on this species (Fig. 5a). The head capsule of *Glyptotendipes* sp. and its associated Hb proteins were compared (Fig. 6). This unidentified species of *Glyptotendipes* had three varying Hb protein profiles (Fig. 6b). All three profiles, P9, P10 and P11 were obtained from chironomids collected in KM, n = 4, 8, and 2, respectively. All three profiles shared four bands at 17, 16, 14, and 13 kDa with varying intensity. P9 and P11 did not have bands in the range of 11 to 8 kDa, whereas P10 had multiple bands in that range. P11 had an extra band at 15 kDa that was absent in both P9 and P10.

The last species studied was a member of the *Thienemannimyia* group, a complex of several closely related genera of the subfamily Tanypodinae. Larvae of this group are difficult or impossible to identify to genus without an associated pupa or adult male. The individuals collected in this study at RR (n=2) could not be identified to the genus or species-level. The head capsule of *Thienemannimyia* group sp. and its associated Hb proteins were compared (Fig. 7). The head capsule morphology of *Thienemannimyia* group sp., which is typical for the subfamily Tanypodinae, was strikingly different from the other genera and species found in this investigation (Fig. 7a). There was only one corresponding Hb protein profile, P12, found in the two individuals collected (Fig. 7b).

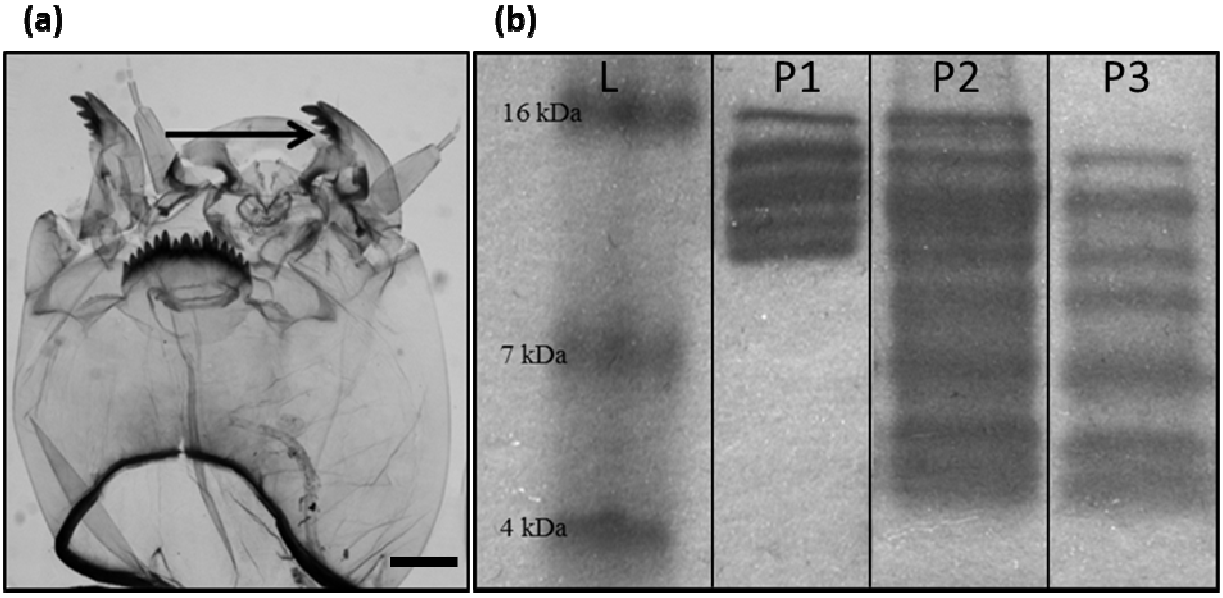

Fig. 1 Comparison of head capsule with Hb proteins of *Chironomus riparius*. Presented are (a) head capsule at 10X magnification and (b) molecular weight ladder (L) and Hb protein profiles 1 – 3 (P1, P2, and P3, respectively). *C. riparius* has a mandible with three dark inner teeth indicated by the solid arrow (a). One species was associated with one head capsule morphology (a) and three Hb profiles (b). P1 was found in all chironomids collected in Rahway River, NJ, n=4. P2 and P3 were in chironomids collected in Kearny Marsh, NJ, n= 5 for each profile. The figure was adopted from Oh et al., 2014. Scale bar = 100 µm.

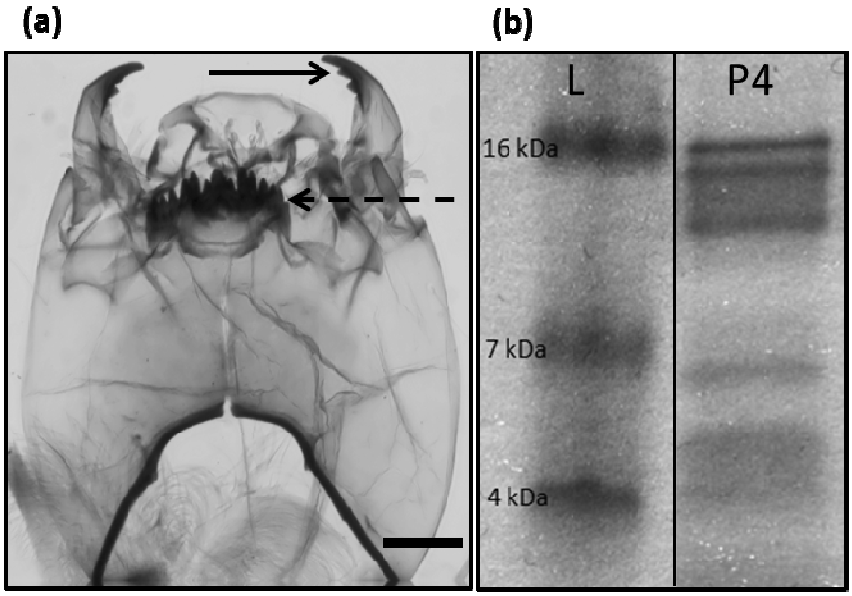

Fig. 2 Comparison of head capsule with Hb proteins of *Chironomus* sp. ME1. Presented are (a) head capsule at 10X magnification and (b) molecular weight ladder (L) and Hb protein profile 4 (P4). Distinctions between *C. riparius* and this unknown species are the two dark inner teeth of the mandible indicated by the solid arrow and a reduced outer tooth on the mentum indicated by the dotted arrow (a). One species associated with one head capsule morphology (a) and one Hb profile (b). This single Hb protein profile was found in all individuals of this unknown species collected at Bass Harbor, ME, n=9. The figure was adopted from Oh et al., 2014. Scale bar = 100 µm.

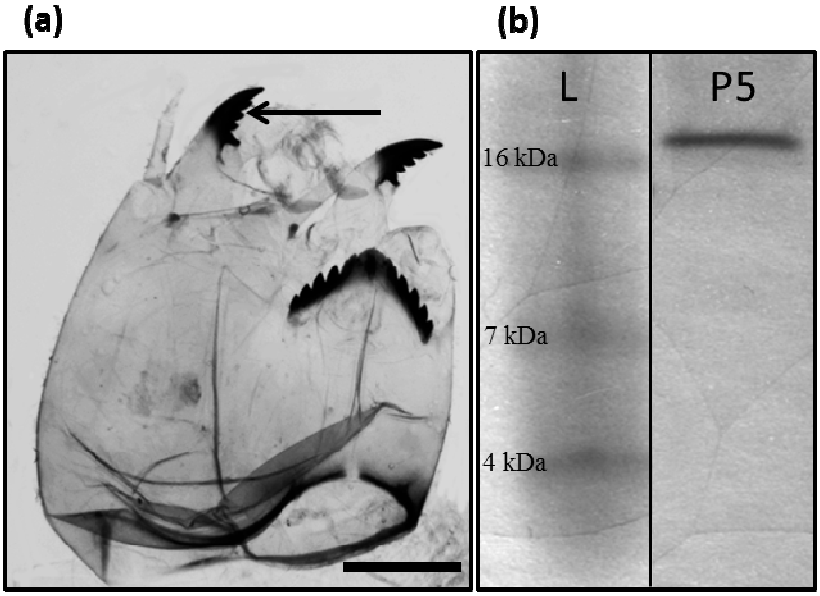

Fig. 3 Comparison of head capsule with Hb proteins of *Cricotopus bicinctus*. Presented are (a) head capsule at 10X magnification and (b) molecular weight ladder (L) and Hb protein profile 5 (P5). The solid arrow (a) indicates the inner teeth of the mandible. One species was associated with one head capsule morphology (a) and one Hb profile (b). This single Hb protein profile was found in all individuals of this species, which was only found at Rahway River, NJ, n=3. The figure was adopted from Oh et al., 2014. Scale bar = 100 µm.

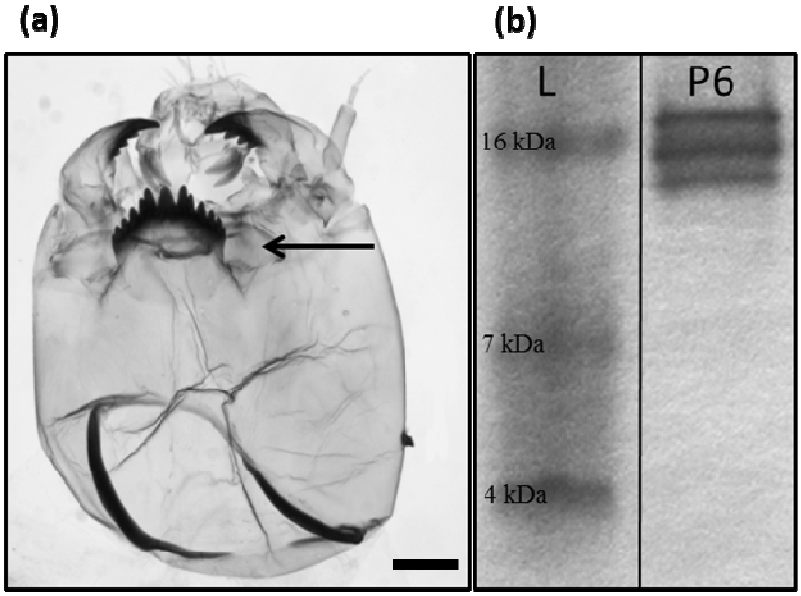

Fig. 4 Comparison of head capsule with Hb proteins of *Dicrotendipes modestus*. Presented are (a) head capsule at 10X magnification and (b) molecular weight ladder (L) and Hb protein profile 6 (P6). The solid arrow (a) indicates the ventromental plate. One species was associated with one head capsule morphology (a) and one Hb profile (b) in all individuals collected. *D. modestus* was found at three of the four sites investigated – North East Creek, ME (n=3), Bass Harbor, ME (n=1) and Rahway River, NJ (n=6). The figure was adopted from Oh et al., 2014. Scale bar = 100 µm.

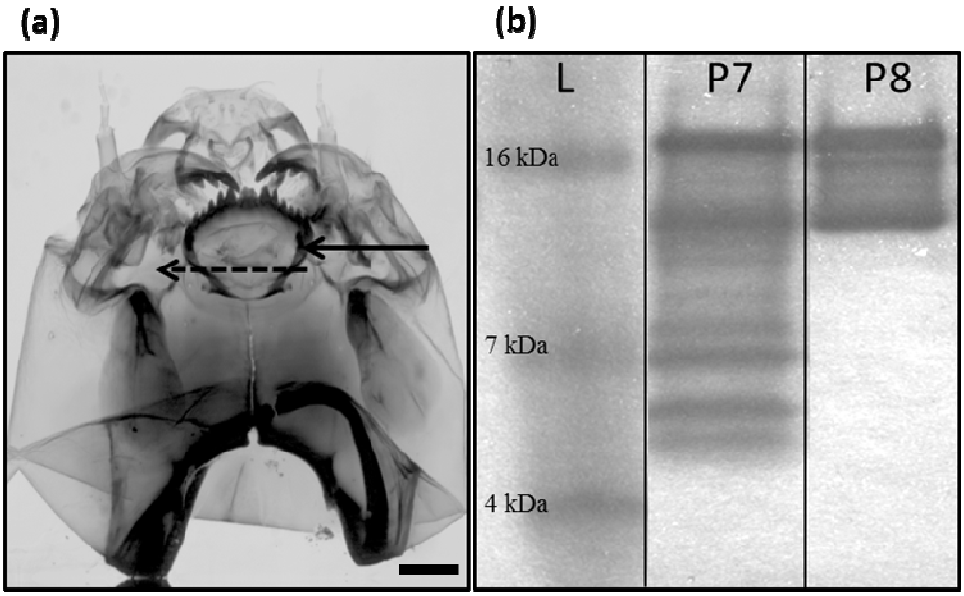

Fig. 5 Comparison of head capsule with Hb proteins of *Glyptotendipes paripes*. Presented are (a) head capsule at 10X magnification and (b) molecular weight ladder (L) and Hb protein profiles 7 and 8 (P7 and P8, respectively). Common traits of *G. paripes* include the smooth anterior margin of the ventromental plate indicated by the dotted arrow and the darkened internal area posterior to the mandible indicated by the solid arrow. One species was associated with one head capsule morphology (a) and two Hb protein profiles (b). Both profiles were found in chironomids collected at Kearny Marsh, NJ, n=11 for P7 and n=3 for P8. The figure was adopted from Oh et al., 2014. Scale bar = 100 µm.

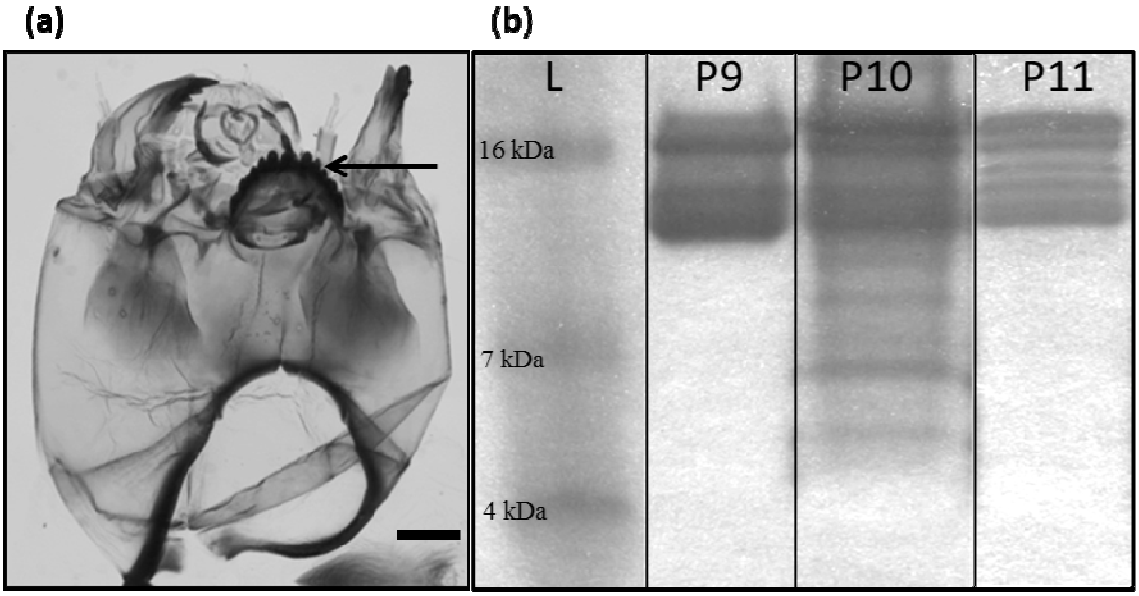

Fig. 6 Comparison of head capsule with Hb proteins of *Glyptotendipes* sp. Presented are (a) head capsule at 10X magnification and (b) molecular weight ladder (L) and Hb protein profiles 9 – 11 (P9, P10, and P11, respectively). The solid arrow (a) indicates the larval mentum. One species was associated with one head capsule morphology (a) and three Hb protein profiles (b). All three profiles were found in chironomids collected at Kearny Marsh, NJ, n=4, 8, and 2, for profiles P9, P10, and P11, respectively. The figure was adopted from Oh et al., 2014. Scale bar = 100 µm.

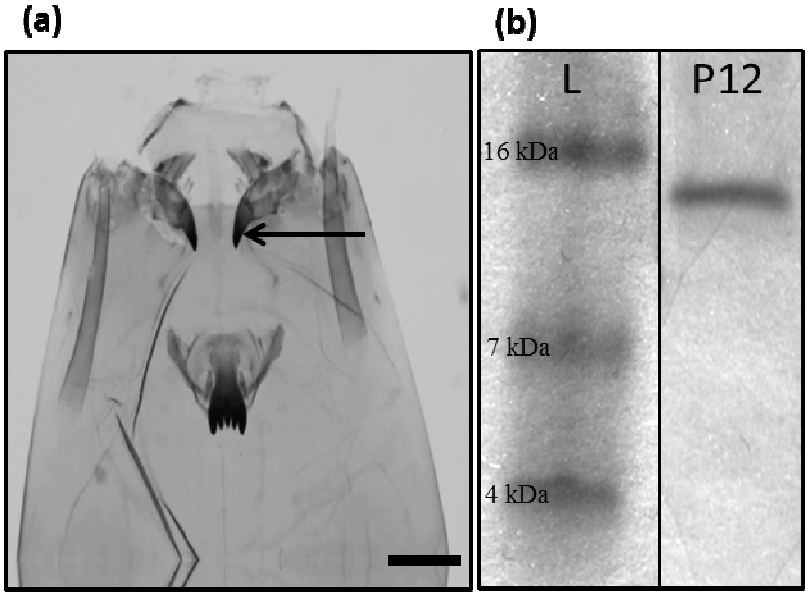

Fig. 7 Comparison of head capsule with Hb proteins of *Thienemannimyia* group sp. Presented are (a) head capsule at 10X magnification and (b) molecular weight ladder (L) and Hb protein profile 12 (P12). The solid arrow (a) indicates a short apical tooth on the mandible. One species was associated with one head capsule morphology (a) and one Hb protein profile (b). The *Thienemannimyia* group sp. individuals were collected at Rahway River, NJ, n=2. The figure was adopted from Oh et al., 2014. Scale bar = 100 µm.

Hb protein profile comparison

Profiles of all species were compared to determine if there was any relationship between species within a genus or among the different genera (Fig. 8). Band numbers 1 to 20 represented all of the Hb bands found in the different profiles. The numbers matched up with molecular weights ranging from 17.5 to 8 kDa. Profiles of the genus *Chironomus* (P1 – P4) shared a band at 14.5 kDa, regardless of species and collection site. Profiles of *C. riparius* (P1 – P3) shared two more bands: 12.5 and 11 kDa. These bands might have been unique to all *C. riparius* individuals or only to *C. riparius* in NJ, where the individuals with these three profiles were collected. *Cricotopus* spp. (P5) had one band at 17 kDa, which was shared by *Glyptotendipes* spp. However, the profile of *C. bicinctus* (P5) only had one band at 17 kDa, whereas species of *Glyptotendipes* had multiple bands below 17 kDa. Thus, results indicated that a band at 17 kDa alone might identify members of the genus *Cricotopus*, but other Orthocladiinae must first be investigated. *Dicrotendipes* spp. (P6) had one consistent profile regardless of collection site. Those bands at 17.5, 15, and 13.5 kDa were not shared by other genera except the one at 15 kDa, which was shared with *Glyptotendipes* sp. (P11). The band at 17.5 kDa was the highest MW band observed and not found in any of the other species. The uniqueness of P6 indicated that bands in this profile could be used to identify *D. modestus*. Profiles of the genus *Glyptotendipes* (P7 – P11) shared two bands at 17 and 13 kDa. Results indicated that these two bands were characteristic of the genus *Glyptotendipes*, since no other genera found had a band at 13 kDa. Another common band in *Glyptotendipes* was at 16 kDa. It was found in all profiles of the unidentified

Glyptotendipes species, P9 – P11, but only 1 of 2 profiles found for *G. paripes*. Distinguishing *Glyptotendipes* at the species-level may be difficult since the profiles of *G. paripes* (P7 and P8) were not considerably different from those of the unknown species. *Thinemannimyia* group sp. (P12) had one band at 14 kDa which was also found in *Glyptotendipes* sp. (P9 – P11).

MW (kDa)	Band #	*Chironomus*				*Cricotopus*	*Dicrotendipes*	*Glyptotendipes*					*Thienemannimyia*
		P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
17.5	1						—						
17	2					—		—	—	—	—	—	
16.5	3												
16	4								—	—	—	—	
15.5	5	—	—		—								
15	6						—					—	
14.5	7	—	—	—	—								
14	8									—	—	—	—
13.5	9						—						
13	10							—	—	—	—	—	
12.5	11	—	—	—									
12	12												
11.5	13	—	—		—								
11	14	—	—	—									
10.5	15							—			—		
10	16												
9.5	17												
9	18							—			—		
8.5	19		—	—									
8	20							—					

Fig. 8 Comparison of all Hb protein profiles. Bands of each profile (P1 – P12) were plotted against molecular weight of Hb protein ranging from 17.5 to 8 kDa. A black line within a column indicates the presence of a band at that particular molecular weight. Band intensity was not considered in this analysis. Profiles are grouped by genus. The figure was adopted from Oh et al., 2014.

Changes in Hb protein profile during a typical life cycle of C. riparius

Although the Hb protein profile index corresponded well with head capsule morphology, there were multiple profiles for some species, particularly in the genera *Chironomus* and *Glyptotendipes*. These multiple profiles could complicate the use of this technique for taxonomic identification. Therefore, it was investigated whether or not developmental stage could account for this observation. To study the influence of developmental stage on Hb profiles, hemolymph was collected from a laboratory cohort of *C. riparius* through most of their life cycle. The hemolymph was collected at second instar (lane 1), 3rd instar (lane 2), 4th instar (lane 3 – 6), pupa (lane 8), and adult (lane 9) (Fig. 9). The instar was based on average head capsule width of larvae contributing to each hemolymph sample (Table 5). Head capsule widths measured in this study showed a range similar to that found by Watts & Pascoe (2000).

Results for the developmental study showed stage-specific changes in synthesis of Hb protein. A band at 12.5 kDa initially appeared at the late 2nd instar (lane 1, Fig. 9) and showed a perpetual synthesis throughout the larval stages that carried into both pupa and adult stages. Interestingly, a band at 12.5 kDa was also observed in P1 – P3 of wild *C. riparius* individuals (Fig. 1), indicating that this band might be unique to *C. riparius* species since it was not observed in the unknown *Chironomus* species (Fig. 2). In addition, larvae at the onset of 4th instar (lane 3) showed a banding pattern similar to P3, in which a band at 15.5 kDa was missing. This finding indicated that the P3 Hb profile found in wild *Chironomus* might have been from an individual collected at the onset of 4th instar. Bands below 7 kDa – which were considered to be degraded Hb protein

product – appeared during the 3rd instar and remained throughout the larval stages. The distinctive profile of *C. riparius* was observed by the mid 4th instar (lanes 4 – 6), which was indistinguishable from those of P1 and P2 found in wild *Chironomus* (Fig. 1). During the transition from larva to pupa (lanes 6 – 8), most of the bands were retained with the exception of bands between 4 and 6 kDa in pupa, a newly synthesized band at 16 kDa in pupa, and the disappearance of the 15.5 kDa band in adult. Synthesis of the 15.5 kDa band completely ceased in adult while the 16 kDa band became more prominent – suggesting that loss and formation of these could be due to metamorphosis.

Table 5. Mean head capsule width (mm) of a laboratory strain *C. riparius* used in a developmental study. Mean head capsule width was calculated from the five larvae whose hemolymph was combined to make a representative Hb sample (Fig. 9).

Lane	Days after hatching	Mean (mm)	Range (mm)	Instar
1	6	0.205	0.2 to 0.21	Second
2	9	0.3375	0.29 to 0.38	Third
3	12	0.6133	0.58 to 0.64	Fourth
4	15	0.5966	0.54 to 0.64	Fourth
5	18	0.643	0.62 to 0.68	Fourth
6	21	0.6475	0.62 to 0.68	Fourth

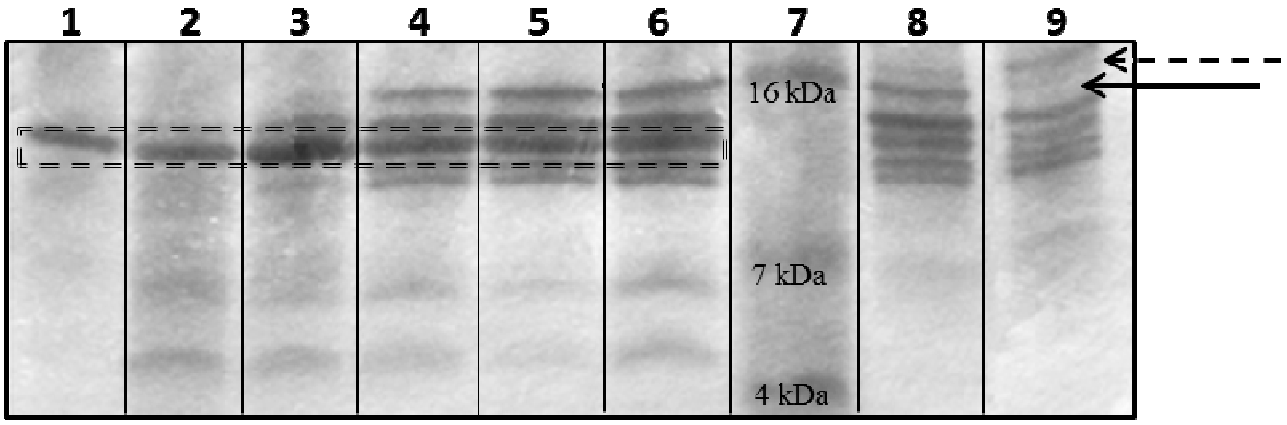

Fig. 9 Changes in Hb protein throughout the life cycle of a laboratory population of *C. riparius*. Dotted box indicates 12.5 kDa band, which was found to be unique to *C. riparius* and observed to be continuously synthesized throughout the life cycle. Each lane presents one of three samples collected at the same time point. Lane 1: 2nd instar, lane 2: 3rd instar, lanes 3, 4, 5, and 6: 4th instar, lane 7: ladder, lane 8: pupa, and lane 9: adult (Table 5). Dotted black arrow indicates newly synthesized band. Black arrow indicates loss of the original 15.5 kDa band, which was present starting at 4th instar. The figure was adopted from Oh et al., 2014.

Atypical Hb protein profiles of wild C. riparius

During several collections of wild chironomids, unusual Hb profiles were found for *C. riparius* (Fig. 10). Even though these profiles had not occurred in the laboratory population, head capsule morphology identified these wild larvae as *C. riparius,* suggesting intra-species differences of Hb proteins in the wild chironomid populations. The most frequently found profile was P1 where at least 17 *C. riparius* showed this distinct profile. P3 was also found to be a popular profile among with n=12. However, P3 had an unusual accumulation of lower bands (<4 kDa). P2 and P4 were considered rare profiles with n=4 and n=2, respectively. At first, these unusual profiles were disregarded, as they were initially thought to be degraded samples. However, as indicated by LC-MS data, every band tested was found to be a digested product of approximately 17 kDa sized Hb proteins family (Table 4). Therefore, it was theorized that the different Hb protein profiles seen in the wild population could be due to posttranslational modifications (PTMs) such as endogenous proteolysis. To better understand the possible relationship between Hb protein polymorphism and the activity of proteases, Hb proteins from the laboratory strain *C. riparius* were artificially digested with three different proteases: chymotrypsin, trypsin, and pepsin.

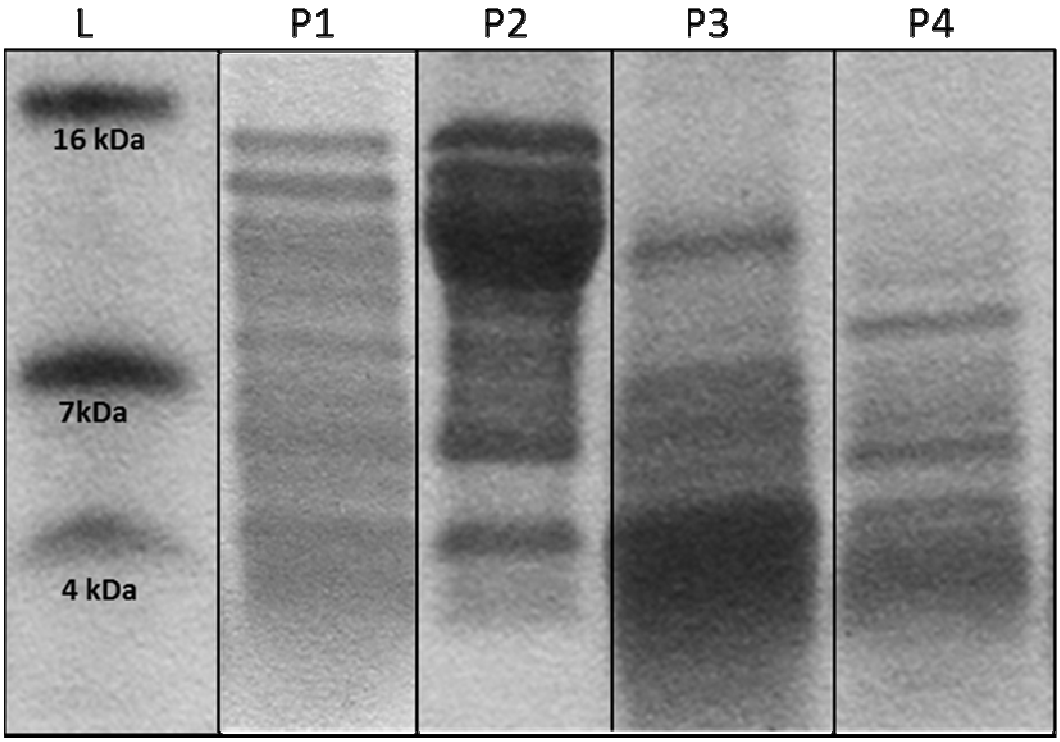

Fig. 10 Common and rare patterns observed in wild *C. riparius* Hb protein profiles. Each lane represents Hb protein separated from hemolymph of one individual collected in KM, matched with its corresponding head capsule. Accumulation of degraded proteins was observed in P3 and P4. These rare profiles and accumulation of low MW proteins were not seen in the laboratory strain *C. riparius* population. P1, n=17 P2, n=4 P3, n=12, P4, n=2

Modulation of Hb protein profile of laboratory strain C. riparius by proteolysis

To test the theory that the polymorphisms were due to natural proteolytic activity, Hb proteins collected from laboratory strain *C. riparius* were artificially digested with chymotrypsin, trypsin, and pepsin for 5, 30, and 60 minutes (Fig. 11). Three independent experiments were carried out for each protease. This test was necessary in order to determine whether or not Hb proteins when digested with proteases could generate similar profiles seen in the wild Chironomus species. Different time points represented the temporal effect of protease activity – the longer the incubation, the higher activity of protease.

Digestion with 0.1 mg/mL of chymotrypsin generated two new bands just above the lower bands with molecular weights ranging approximately 5 to 9 kDa, and it eliminated a top band at 16 kDa (Fig. 11A). At 5 minutes of digestion, one of the doublet bands at 16 kDa began to disappear. Simultaneously, a new band was formed at 7 kDa, and there were increased intensities of both lower bands. This profile at 5 minutes indicated digestion of the top band yielding digested products ranging from 4 to 7 kDa. After 30 minutes of digestion, a strong band at 6 kDa was generated and one at 12.5 kDa began to disappear. By 60 minutes of digestion, the band at 12.5 kDa had all but disappeared while the rest of the profile was similar to that at 30 minutes.

Digestion with 1.0 mg/mL of trypsin showed a pattern similar to that of chymotrypsin where the top band (lower doublet at 16 kDa) was the main target (Fig. 11B). At 5 minutes of digestion, a band at 5 kDa had appeared. This band was also seen after 30 minutes of digestion by chymotrypsin. Trypsin completely digested the top band

by 30 minutes and some of the 12.5 kDa band. However, the band at 7 kDa generated by chymotrypsin was never seen with trypsin indicating different cleavage sites. At 60 minutes, the 12.5 kDa band was completely digested while the rest of the profile was similar to that at 30 minutes.

Pepsin digestion was completely different from chymotrypsin and trypsin (Fig. 11C). At 5 minutes digestion with 1 mg/mL of pepsin, the profile was similar to that of control. However, an accumulation of digested products less than 4 kDa was observed. This finding indicated a wide range of cleavage sites of pepsin, targeting all Hb protein bands, unlike the others, in which the initial target was the top band at 16 kDa. The lower bands ceased to be visible after 30 minutes with more intense accumulation below 4 kDa. By 60 minutes of digestion, most of the upper bands except the 12.5 kDa band had disappeared, indicating a completely dissimilar mode of proteolytic activity exhibited by pepsin.

The profiles generated by individual proteases or a combination of them appeared imitate profiles seen in the wild population. For example, accumulation of the proteins less than 4 kDa, a unique feature of pepsin, was observed most notably in P3 and P4 (Fig. 10), suggesting a possible activity of pepsin-like protease(s) in P3 and P4 individuals. Another example is shown by the chymotrypsin digestion where Hb proteins digested with for 30 minutes (Fig. 11A) exhibited a profile similar to that of the P1observed in the wild *C. riparius* (Fig. 10). When these two profiles were aligned, bands were matched up with one another where several bands were evenly spaced out ranging from 4 to 14 kDa in both profiles. To some degree, trypsin digestion mimicked P4 where some of the top

bands, especially band at 12.5 kDa ceased to be visible. Overall, the polymorphisms observed in the wild chironomids could be due to either a specific protease or combination of various proteases with different target cleavage sites on Hb proteins.

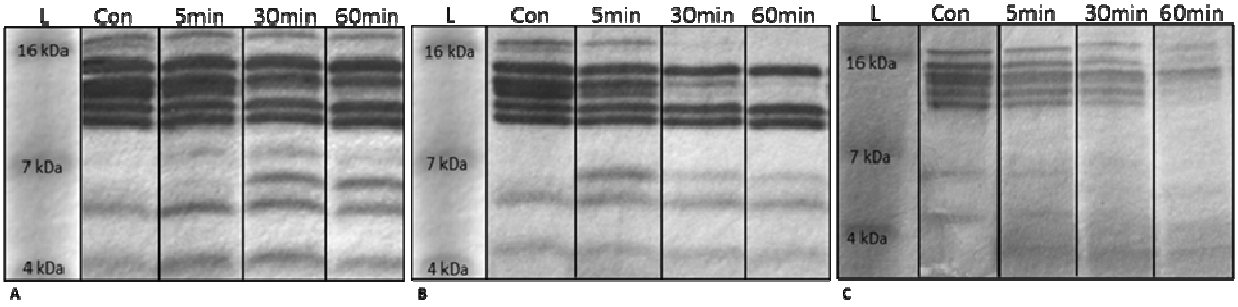

Fig. 11 Hb protein profiles of laboratory strain *C. riparius* digested with A) chymotrypsin, B) trypsin, and C) pepsin for 5, 30, and 60 minutes. Various banding patterns were observed in each protease digestion. Saturation of proteins <4 kDa observed in Hb proteins digested with pepsin. Profiles of Hb protein treated with inhibitors were similar to the control profile (data not shown).

Hb proteins response to Cd exposure

Together with finding from LC-MS and digestion data, it was plausible to believe that the overall Hb protein synthesis involves translation of Hb proteins undergoing proteolysis to generate multiple bands. However, the actual mechanism regulating the Hb protein synthesis resulting in polymorphism was still in question. Some of the wild *C. riparius* showing Hb polymorphisms were collected at KM, which is moderately contaminated with a wide range of toxicants including heavy metals. Therefore, one theory was that the profiles observed in the wild *C. riparius* might have been caused by external stimuli. Among numerous candidates, Cd is one of the heavy metals frequently found at KM. Thus, to test the theory that the polymorphism could be a response to Cd, an acute Cd toxicity test was conducted using laboratory strain *C. riparius* larvae exposed to Cd at concentrations ranging from control, 0.3, and 3.0 µM for 96 hours.

Three independent 96 hour acute Cd toxicity tests were conducted and the representative profiles of those tests are shown in Fig. 12. Changes in Hb protein profiles over 96 hours for both control and 0.3 µM Cd treatment groups were found to be similar in all three trials (Fig. 12AB). This indicated that 0.3 µM was the no observed adverse effect level (NOAEL) for Cd. Both control and 0.3 µM groups showed changes in Hb profiles beginning at 48 hour when there was addition of newly formed upper bands. These additional bands continued to progress throughout 96 hours. On the other hand, 3.0 µM Cd group did not show any significant changes in the profiles over 96 hours of Cd exposure (Fig. 12C). The 3.0 µM Cd group maintained its 12 hour status-quo until 48 hours, and then by 72 hours, the lower bands began to disappear in all three trials. This

loss of bands indicated that at 3.0 µM Cd, synthesis of the low MW weight bands was ceasing. More importantly, the profiles generated by the 3.0 µM Cd group did not mimic profiles found in the wild species (Figs. 1 – 8 and 10), suggesting that the profile generated by exposure to 3.0 µM Cd at 72 hours was a unique profile. Therefore, this unique profile could be used to detect presence of Cd at concentrations of at least 3.0 µM. Overall, it appeared that Cd at 3.0 µM reduced Hb protein synthesis and/or Hb degradation, as addition of bands was not visible by 48 hours, but rather, loss of the lower bands was observed.

More subtle difference in Hb protein profiles was determined by measuring the pixels of the bands visualized on SDS-PAGE (Fig. 13). Data on trial 2 was not available as the SDS-PAGE broke apart when gels were dried. On gels from other trials, an area containing all bands ranging from 4 to 16 kDa was measured.

In the absence of Cd, there was a statistical significant increase in band intensity at 72 hours and it continued to progress onto 96 hours (Fig. 13AB). This increase in band intensity was consistent with the addition of bands observed on SDS-PAGE by 72 hours. In the presence of 3.0 µM Cd, band intensity did not show any statistical changes throughout 48 hours; however, after 72 hours, there was a significant decrease in band intensity indicating a reduction in number of bands and pixels (Fig. 13CD). It appeared that the effect of Cd at the Hb protein level was not instantaneous, where the earliest significant effect was observed at 72 hours; consistent with the loss of lower bands observed on SDS-PAGE. The loss of band intensity coincided with mean body mass such that the 3.0 µM Cd treated group showed a considerable reduction in body mass

compared to control (Table 6). However, the loss of band intensity did not coincided with metamorphosis as the head capsule widths indicated that the larvae were in their 4^{th} instar (Table 6). Overall, Hb band number and pixel intensity increased overtime in the absence of Cd. Concentrations of 3.0 µM Cd had profiles similar to that of 12 hour control until 48 hours, at which point some upper bands failed to appear. After 72 hours, Hb protein intensity was reduced and lower bands disappeared. This change in Hb profile was associated with poor growth but not metamorphosis.

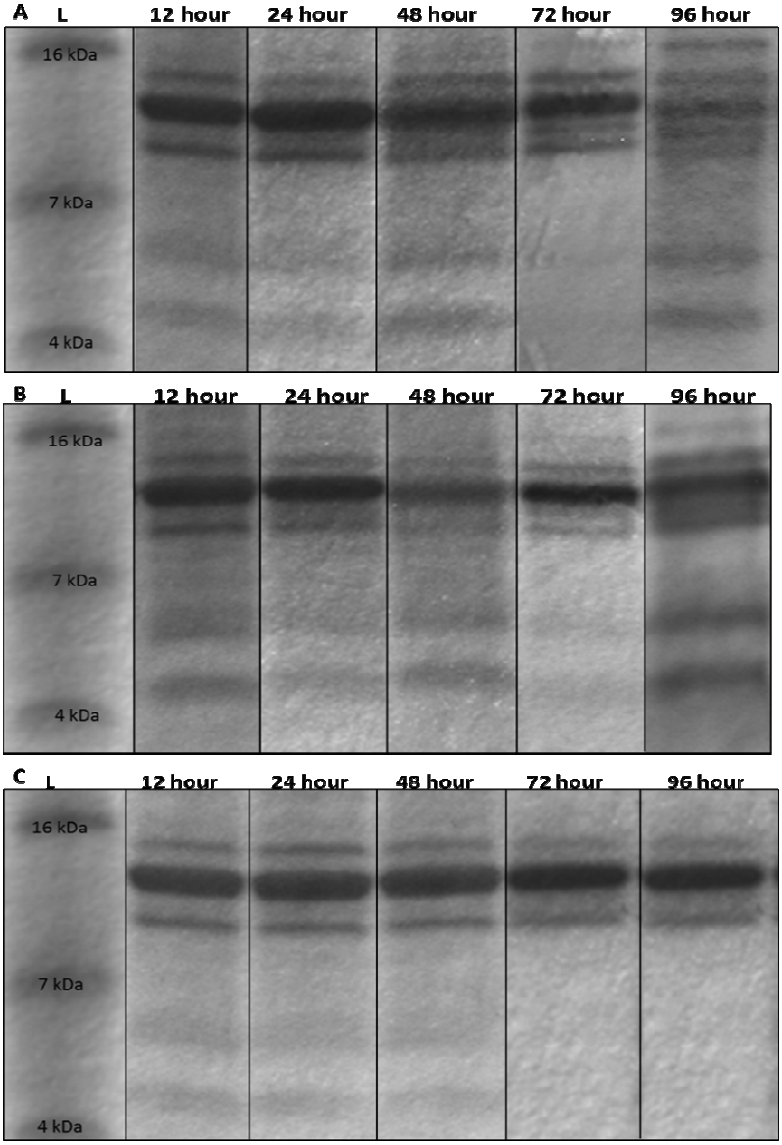

Fig. 12 Changes in Hb protein profiles over 96 hours. Panel A shows Hb protein profiles of the control, B shows 0.3 µM Cd treated groups and C shows Hb protein profiles of 3.0 µM Cd treated group. Control and 0.3 µM showed a similar response to Cd where there was increase in number of bands beginning at 48 hours and progressed onto 96 hours. Profiles of 3.0 µM Cd treated group remained its 12 hour profile until 48 hour and at 72 hour, the low MW proteins have disappeared. n=3 for each hour and concentration where each n consisted of three individual chironomids.

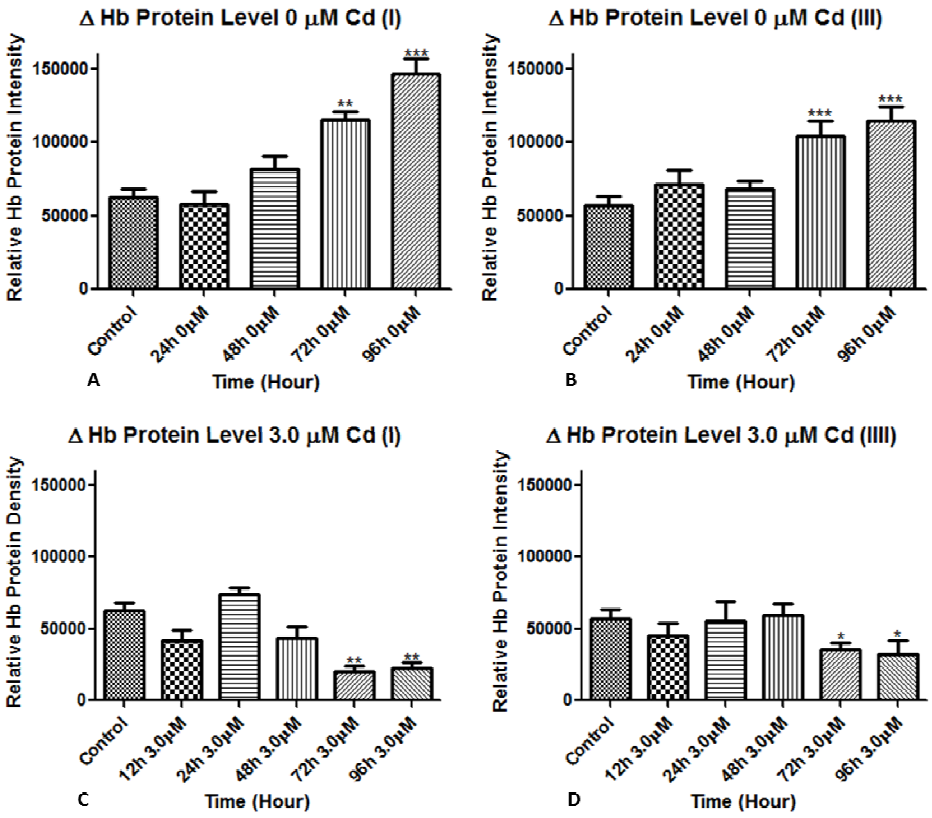

Fig. 13 Total Hb protein level was quantified by densitometry and converted to relative density by subtracting the background. The relative density of the whole region containing all the bands was normalized to signal of 12 hour 0 µM group. Values are depicted as mean ± standard deviation (n=3) where one n is representative of 3 chironomids. Asterisks indicate significant differences between 12 hour 0 µM group and Cd treated groups. Statistically significances were established at $p<0.05$ for (*), $p<0.01$ for (**) and $p<0.001$ for (***) determined by One-way ANOVA followed by Dunnett's multiple comparison test. Data for trial II is not shown.

Table 6. Chironomid body mass and head capsule width after 96 hours with or without Cd. Average body mass of one individual was measured in mg and its standard deviation is shown. Average head capsule width of one individual was measured in mm and its standard deviation is shown. For each concentration of Cd, n=30 for body mass, n=10 for head capsule width measurement.

[Cd]	Mean Body Mass (mg)	Head Capsule Width (mm)
Control	0.70 ± 0.073	0.68 ± 0.020
0.3 µM	0.62 ± 0.114	0.68 ± 0.023
3.0 µM	0.26 ± 0.056	0.63 ± 0.038

Effect of Cd on heme synthesis

According to the 96 hour acute Cd toxicity test, it was clear that 3.0 µM Cd was a concentration high enough to suppress the overall Hb protein synthesis; delaying the progression of normal banding pattern and loss of the lower bands by 72 hours. To better understand the mode of action of Cd on Hb protein synthesis, we analyzed one of the early stages of heme synthesis (Fig. 14), the production of porphobilinogen (PBG) by PBG synthase activity. PBG synthase catalyzes condensation of two molecules of delta-aminolevulinic acid (ALA) to form one molecule of PBG (Gultepe et al., 2009). The purpose of quantifying the amount of PBG present was to identify whether or not Cd had early influences on the multi-step process of heme synthesis pathway – potentially inhibiting the activity of PBG synthase causing decreased level of PBG production and ultimately affecting the final stage, production of heme molecule.

Quantification of PBG synthase activity (PBG) was measured in three independent trials (Fig. 15). In the absence of Cd, it appeared that the activity of PBG over the course of 96 hours was relatively similar to that of the 12 hour control group in trial I (Fig. 15A). Although there was a slight decrease in PBG production at 48 hours, it recovered by 72 hours and maintained its level through 96 hours. In trial I with the presence of Cd, there was a significant decrease in PBG, except at 24 hours (Fig 15D). This indicated Cd toxicity on heme production. In trial II, the response in the absence of Cd was similar to that observed for trial I, accept this time PBG at 96 hours was significantly lower than control (Fig. 15B). However, when Cd was added, a substantial reduction in PBG was observed from 24 to 96 hours (Fig. 15E). Both trials I and II in the

presence of Cd correlated with SDS-PAGE, where there was no addition of new bands and a loss of lower bands. In trial III, PBG was increased in the absence of Cd and showed a relatively similar PBG production in the presence of Cd (Fig. 15CD). The similar PBG production in the presence of Cd could be due to the fact that PBG production of the control group was low when compared to that of the controls in the other two trials. As high as two fold differences between the PBG production of trials II and III was observed, indicating that the activity of PBG synthase in trial III control was substantially low. Overall, it appeared that Cd did inhibit the activity of PBG synthase in the cytosol, possibly reducing the overall production of heme and thereby adversely affecting Hb protein synthesis.

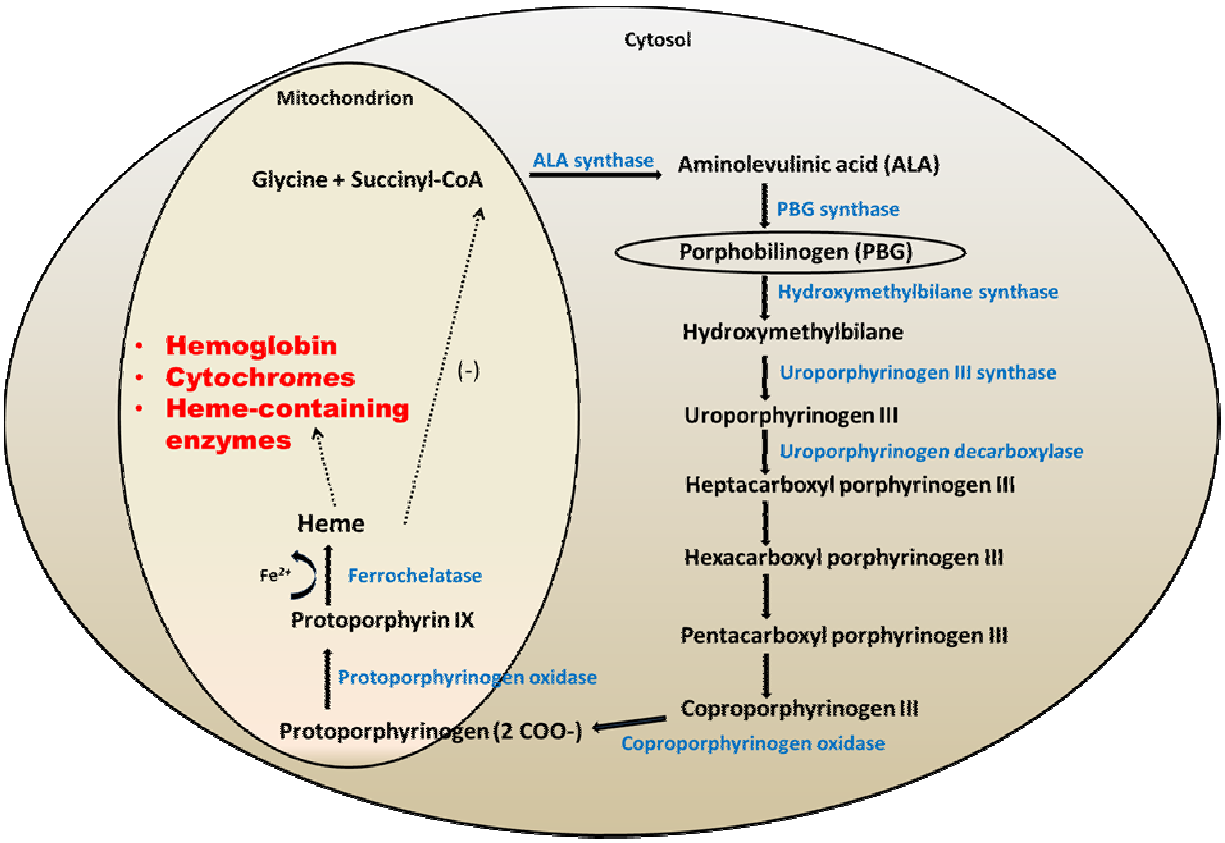

Fig. 14 Heme synthesis pathway. A pathway involving various enzymes catalyzing a heme molecule from glycine and succinyl-CoA, eventually leading to hemoglobin protein production. The level of porphobilinogen (highlighted in oval) was quantified as a measure of Cd toxicity.

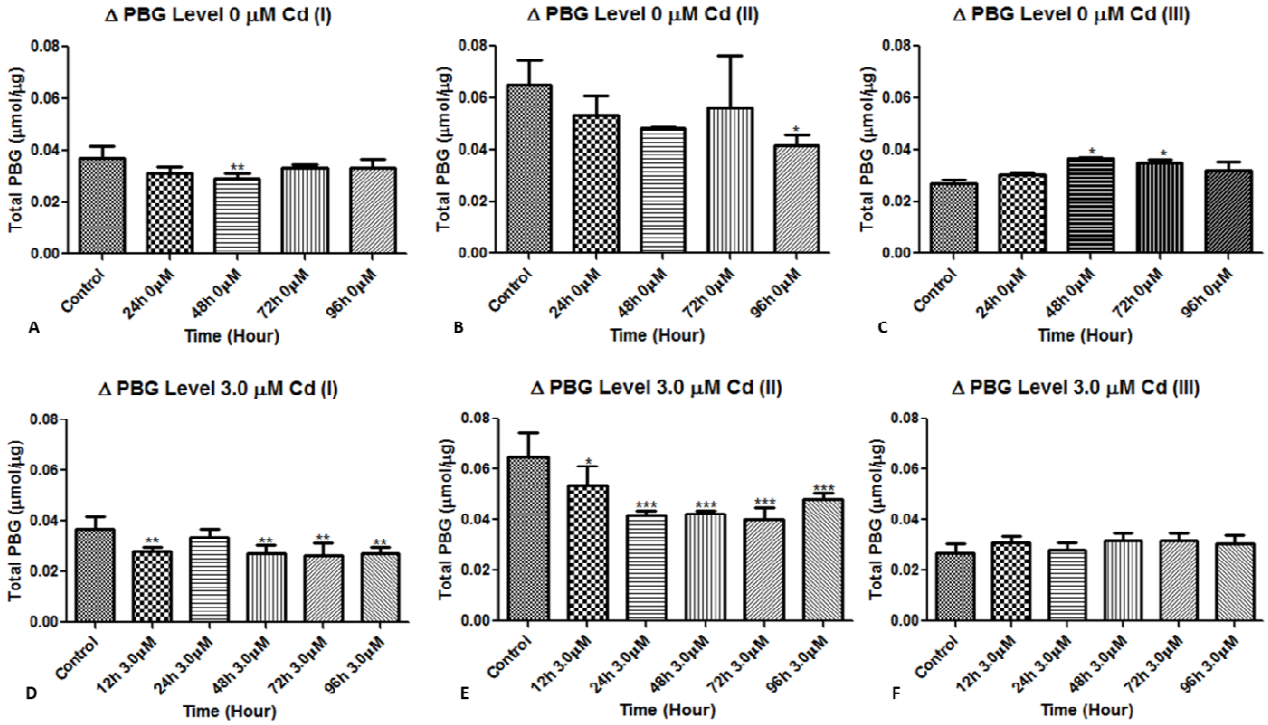

Fig. 15 Quantification of PBG molecules. Activity PBG synthase was analyzed by measuring the amount of PBG generated over 96 hours in both presence (3.0 µM Cd) and absence of Cd. Panel A and B shows the changes in the PBG in trial I, C and D in trial II, and E and F in trial III. All treatment was compared to 12 hour 0 µM group. Values are depicted as mean ± standard deviation (n=3) in each trial, where one n is representative of 3 chironomids. Statistically significances were established at $p<0.05$ for (*), $p<0.01$ for (**) and $p<0.001$ for (***) determined by One-way ANOVA followed by Dunnett's multiple comparison test. Data provided by Monsheimer, 2014 (personal communication).

Effect of Cd on Hb IV and VII genes

To further investigate the mode of action of Cd on Hb proteins, the expression of the genes which code for Hb proteins were analyzed using qPCR in three independent experiments. Among many candidates, Hb IV (monomer) and VII (dimer) genes were chosen, since their products were the most abundant proteins found in all of the bands tested by LC-MS (Table 4). Quantification of the expression of the two Hb genes was necessary to understand if there is a relationship between gene and its corresponding protein seen on SDS-PAGE.

In the absence of Cd, the expression of Hb IV appeared to be up-regulated at 48 hour (Fig. 16ABC). In trial I, the expression was significantly increased at 48 hour but it ceased by 72 hour returning to control levels (Fig. 16A). The expression of Hb IV in trial II also showed a significant increase, almost 6-fold differences when compared to that of the control (Fig. 16B). However, this up-regulation was maintained even after 96 hours reaching almost 10-fold differences by 72 hour. In trial III, the expression of Hb IV showed a significant increase at 48 hour and reaching 15-fold difference by 72 hour (Fig. 16C). Although the expression came back down by 96 hour, it was still relatively higher than that of the control. Overall, in all three trials, it appeared that the up-regulation of Hb IV gene at 48 hour was necessary for addition of new bands observed on SDS-PAGE. This up-regulation was maintained at least until 72 hour indicating that the continued expression of Hb IV was necessary in order to generate additional bands after 48 hours which completed the fourth instar Hb profile.

In the presence of Cd, Cd toxicity was observed as soon as 12 hour, where all three trials showed a significant decrease in Hb IV expression (Fig. 16DEF). Trials I and II showed a significant down-regulation of Hb IV all throughout 96 hours (Fig. 16DE). However, in trial III, Cd exposure of this group showed a substantial recovery of Hb IV gene at 96 hour (Fig. 16F). Although this recovery was not significant when compared to that of the control (Bonferroni's Multiple Comparison Test), it was significantly different from other time points (data not shown). However, this unusual recovery could be due to the fact that the trial III group had a relatively high expression of Hb IV gene as there was no sign of recovery on SDS-PAGE. Overall, the down-regulation of Hb IV upon Cd exposure was correlated with SDS-PAGE where the addition of bands at 48 hour was not observed and that by 72 hour, there was loss of the lower bands. These results could be explained by Cd suppression of Hb IV gene resulting in decreased Hb IV production, as one of the major contributors to the lower band.

The expression of Hb VII was similar to that of Hb IV where there was a significant increase at 48 hour in the absence of Cd in trials II and III (Fig. 17BC). Although there was no significant increase at 48 observed in trial I, the relative expression at 48 hour was the highest among other time points in trial I, consistent with the trend observed in other two trials (Fig. 17A). At 48 hour, there was an up-regulation of Hb VII gene up to 3.5 and 4.5-folds in trials II and III, respectively (Fig. 17BC). By 72 hour, only trials I and III showed a similar downward trend where trial II still showed an up-regulation. The expression of Hb VII appeared to reach a similar level as the control except for trial II. Similar to findings observed in Hb IV, when Cd was absence, the up-

regulation of expression of Hb VII gene at 48 hour correlated with Hb protein synthesis on SDS-PAGE at 48 hour, which showed the appearance of new bands. Overall, in all three trials, it appeared that the up-regulation of Hb VII gene at 48 hour was necessary for addition of new bands observed on SDS-PAGE for control. This up-regulation was maintained at least until 72 hour indicating that the continued expression of Hb VII was necessary in order to generate more bands after 48 hours to generate a complete fourth instar profile.

When Cd was present, Hb VII also showed a rapid response where expression was significantly decreased as early as 12 hour (Fig. 17DEF). Similar to Hb IV, both trials I and II showed a sustained down-regulation (Fig. 17DE). However, as seen in trial III of Hb IV, an up-regulation of Hb VII was observed in trial III at 72 and 96 hour (Fig. 17F). Again, this up-regulation in both 72 and 96 hours was not statistically significant when compared to that of the control; the induction at 96 hour was statically different (Bonferroni's Multiple Comparison Test) from other time points (data not shown). Likewise, this recovery was not seen on SDS-PAGE and possibly due to the fact that this group in trial III had a relatively high expression of Hb VII. Overall, changes in Hb genes due to Cd were shown to be rapidly effected where both Hb IV and VII genes were down-regulated as early as 12 hour. In addition, there was no sign of synthesis of new bands by 96 hours on SDS-PAGE, indicating an irreversible effect of Cd on Hb protein synthesis, reducing production of the precursor Hb proteins.

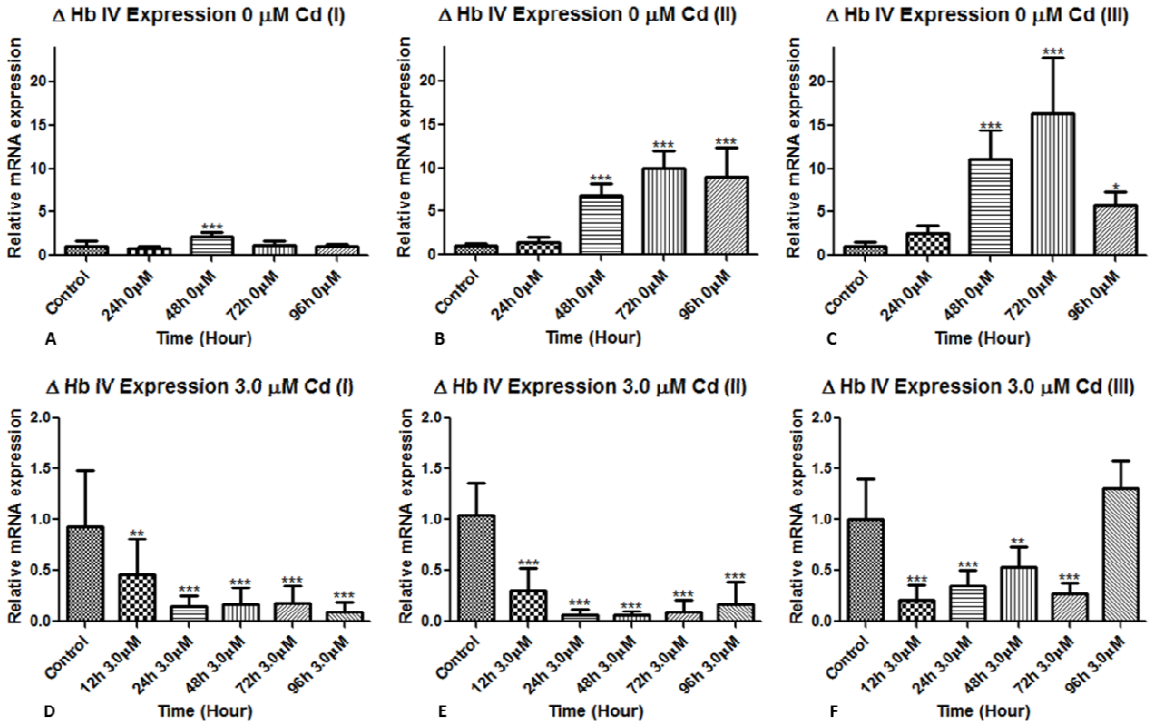

Fig. 16 Changes in the Hb IV gene expression of chironomids treated with 0 and 3.0 µM Cd throughout 96 hours was measured by qPCR. The ΔΔCt method was chosen to calculate relative gene expression, and all data were normalized against β-actin, as a housekeeping gene. Panels A and D represents the relative expression of Hb IV in trial I, B and E in trial II, and C and F in trial III. All treatments were compared to 12 hour 0 µM group. Values are depicted as mean ± standard deviation (n=3) where one n is representative of 3 chironomids. Asterisks indicate significant differences between 12 hour 0 µM group and other time points. Statistically significances were established at $p<0.05$ for (*), $p<0.01$ for (**) and $p<0.001$ for (***) determined by One-way ANOVA followed by Dunnett's multiple comparison test.

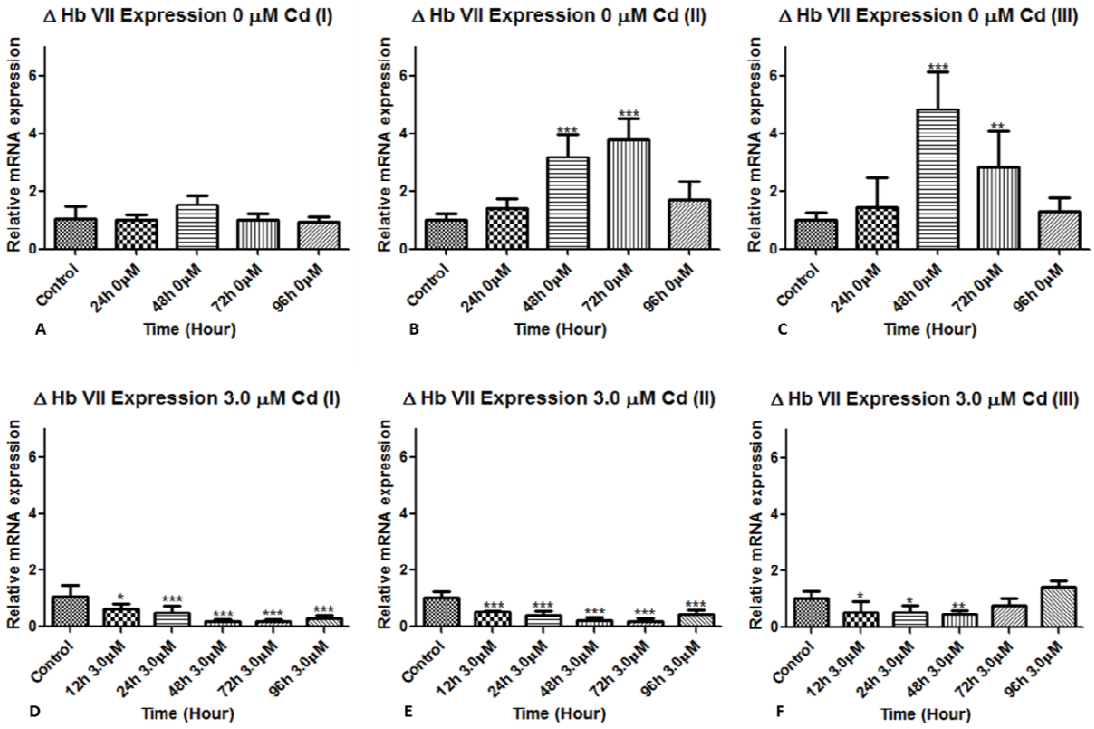

Fig. 17 Changes in the Hb VII gene expression of chironomids treated with 0 and 3.0 µM Cd throughout 96 hours was measured by qPCR. The ΔΔCt method was chosen to calculate relative gene expression, and all data were normalized against β-actin, as a housekeeping gene. Panels A and D represents the relative expression of Hb VII in trial I, B and E in trial II, and C and F in trial III. All treatments were compared to 12 hour 0 µM group. Values are depicted as mean ± standard deviation (n=3) where one n is representative of 3 chironomids. Asterisks indicate significant differences between 12 hour 0 µM group and other time points. Statistically significances were established at $p<0.05$ for (*), $p<0.01$ for (**) and $p<0.001$ for (***) determined by One-way ANOVA followed by Dunnett's multiple comparison test.

Effect of Cd on Ub gene

To further investigate the effect of Cd on Hb proteins, changes in Ub expression was analyzed by qPCR in order to determine whether or not Ub played a role in posttranslational modification of the Hb proteins – more specifically the effect of Cd on digestion of Hb proteins. The Ub gene had not been sequenced for *C. riparius*. Therefore, a fragment of Ub's nucleotide sequence was needed for making gene specific primers. Generating gene specific primers of Ub involved alignment of known ubiquitin sequences of other species made available in NCBI. From the alignment, degenerative primers based on the consensus sequence were found. Standard PCR amplification with these degenerative primer sets provided several potential gene products detected as bands on ethidium bromide stained agarose gels. The most intense band was excised, and the DNA from that band was purified as a template for molecular cloning. The sequence information from that particular band is shown in Fig. 18A as the query sequence. Since there was no genomic nucleotide sequence information available for chironomid Ub, one of the most closely related species, *Aedes aegypti*, was chosen as a subject match. The alignment showed 86% identity confirming that the cloned band was a partial sequence of Ub. From the initially acquired sequence, a gene specific primer set was generated containing a forward primer of 5'ATCAGACAATGTACGACCATCTT3'and a reverse primer of 5'ACATAGGAAGTTGAGCCATCAG3'. This primer set amplified a 135 base-pair partial region of Ub in *C. riparius* and was used to amplify partial coding sequence of ubiquitin (Fig. 18B)

In the absence of Cd, there were no significant changes in the expression Ub in all three trials, indicating the expression Ub was not time-dependent as both Hb genes were found to be (Fig. 19). Although there was a slight reduction in expression of Ub gene at 24 hour and 72 hour in trial I and II, respectively, the overall expression was not significantly different from that of the control indicating a relatively stable cellular activities involving degradation of damaged proteins (Fig. 19 AB).

However, in the presence of Cd, other than trial I, both trials II and III showed a significant increase at 24 hour, suggesting a relatively early ubiquitin labelling or ubiquitination of damaged proteins, potentially caused by Cd (Fig. 17EF). However, the up-regulation was short in trial II. Only in trial III was the expression of Ub prolonged until 72 hour after which it returned to control levels. The prolonged expression Ub in trial III suggested that there could be more damaged proteins caused by Cd than in the other two trials. In general, Cd did appear to cause a change in the Ub expression, suggesting a surge of ubiquitin protein at early time points. Together suppression of Hb proteins production and Ub marking damaged Hb proteins for degradation could explain the lack of new upper appearance and loss of the lower bands in Cd presence.

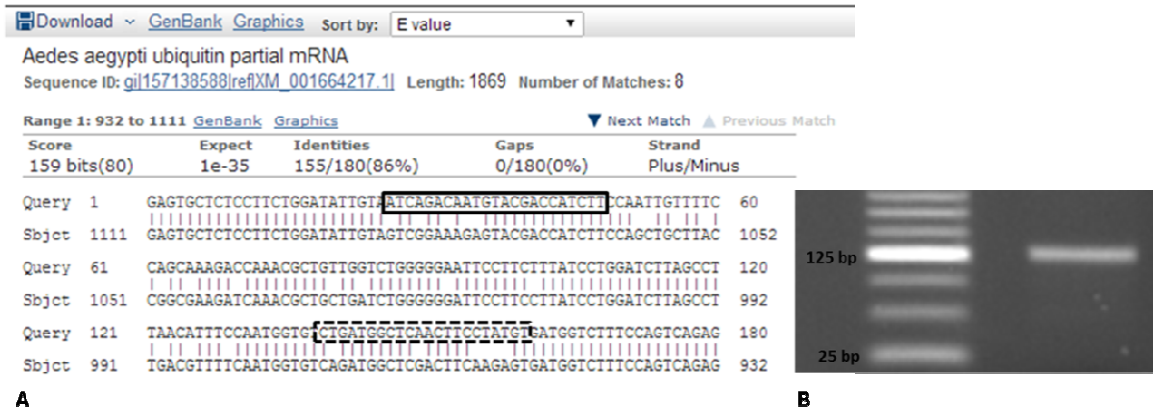

Fig. 18 Cloned and sequenced nucleotide information of ubiquitin (Ub) gene found in *C. riparius*. Since ubiquitin gene of *C. riparius* is currently unavailable, the query sequence (cloned) was matched up with the subject sequence of one of the closely related species, *Aedes aegypti* (annotated gene sequence available in NCBI, accession number: XM_001664217) with 86% identity, confirming ubiquitin gene of *C. riparius* (Panel A). Primer set containing forward primer 5'ATCAGACAATGTACGACCATCTT3' (indicated by the black box) and reverse primer 5'ACATAGGAAGTTGAGCCATCAG3' (indicated by the dotted box) was generated in order to amplify a partial region (135 base-pairs) of Ub using PCR (Panel B).

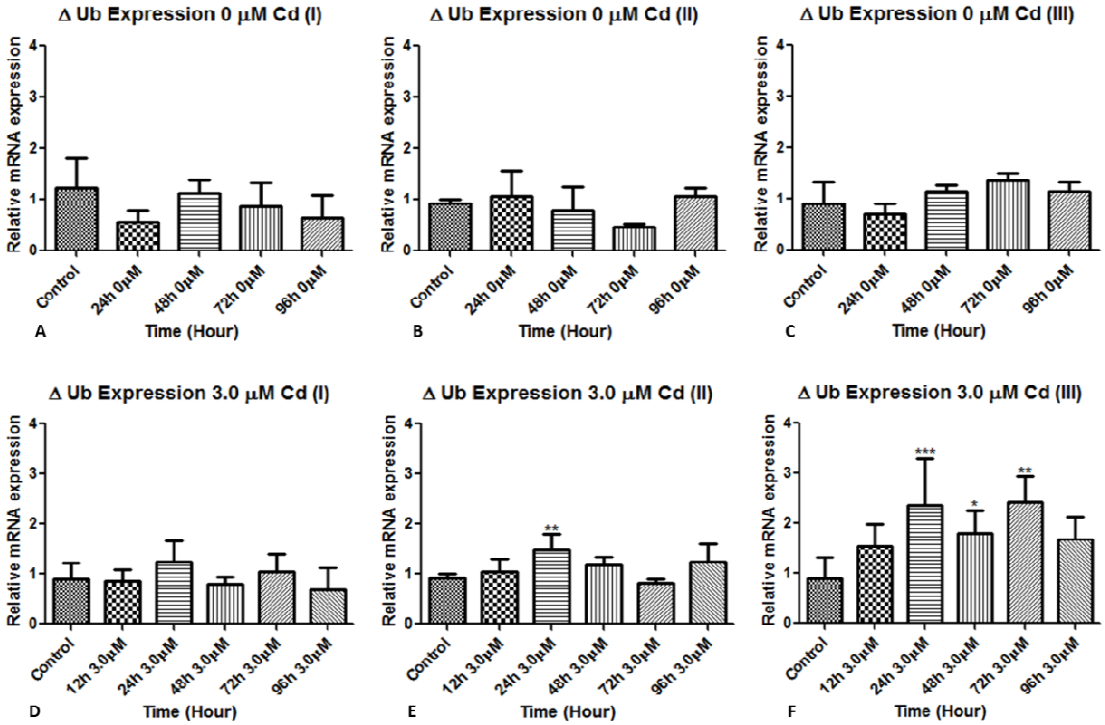

Fig. 19 Changes in the Ub gene expression of chironomids treated with 0 and 3.0 µM Cd throughout 96 hours was measured by qPCR. The ΔΔCt method was chosen to calculate relative gene expression, and all data were normalized against β-actin, as a housekeeping gene. Panels A and D represents the relative expression of Ub in trial I, B and E in trial II, and C and F in trial III. All treatments were to 12 hour 0 µM group. Values are depicted as mean ± standard deviation (n=3) where one n is representative of 3 chironomids. Asterisks indicate significant differences between 12 hour 0 µM group and other time points. Statistically significances were established at $p<0.05$ for (*), $p<0.01$ for (**) and $p<0.001$ for (***) determined by One-way ANOVA followed by Dunnett's multiple comparison test.

Markers of Cd toxicity: MT

Cd is known to induce generation of reactive oxygen species (ROS), including the superoxide radical, hydroxyl radical, and hydrogen peroxide causing cellular damages such as lipid peroxidation, protein denaturation, and DNA damage. As a protective mechanism, metal binding proteins, such as metallothionein (MT), are the first responder for detoxification and cysteine metabolism of heavy metals (Fang et al., 2010; Jeppe et al., 2014). Therefore, changes in the normal expression of MT could be used as an indication of presence of Cd. Results showed that MT was over-expressed even in the absence of Cd (Fig. 20 BC). Other than trial I, both trials II and III showed a significant increase in MT expression, suggesting that MT is as highly expressed as Hb genes in chironomids. In trial I, there was no statistical difference in expression from 12 to 96 hours. In trials II and III, an upward trend of MT expression was observed.

In the presence of Cd, the results varied among trials (Fig. 20DEF). The expression of MT in trial I had no statistical changes, although it had a downward trend suggesting suppression by Cd (Fig. 20D). In trial II, there was a significant increase at 24 hour; however, this induction soon came down to a level similar to that of the control (Fig. 20E). Interestingly, the expression of MT was up-regulated at 72 hour and maintained throughout 96 hours in trial III (Fig. 20F). There was not a single consistent trend among the three trials, indicating chironomids must have other means of Cd detoxification. Overall, the expression of MT appeared to be inconsistent and did not contribute to the changes in the Hb protein profiles observed on SDS-PAGE.

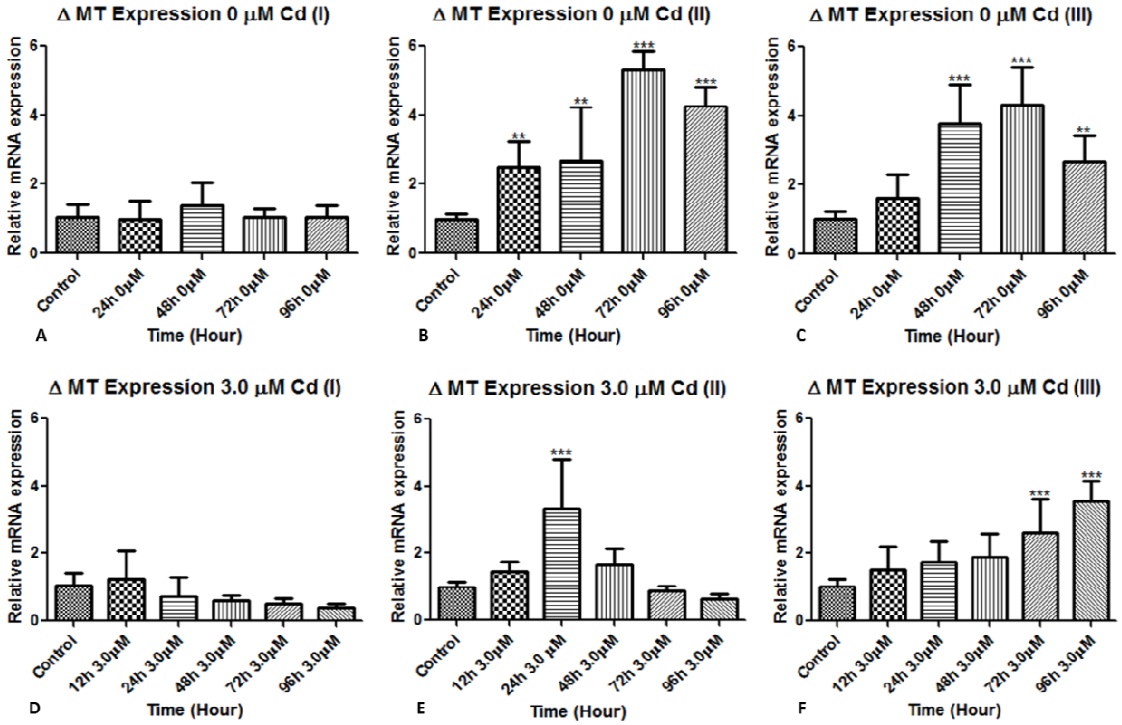

Fig. 20 Changes in the MT gene expression of chironomids treated with 0 and 3.0 µM Cd throughout 96 hours was measured by qPCR. The ΔΔCt method was chosen to calculate relative gene expression, and all data were normalized against β-actin, as a housekeeping gene. Panels A and D represents the relative expression of MT in trial I, B and E in trial II, and C and F in trial III. All treatments were compared to 12h 0 µM group. Values are depicted as mean ± standard deviation (n=3) where one n is representative of 3 chironomids. Asterisks indicate significant differences between 12h 0 µM group and other time points. Statistically significances were established at $p<0.05$ for (*), $p<0.01$ for (**) and $p<0.001$ for (***) determined by One-way ANOVA followed by Dunnett's multiple comparison test.

Discussion

With a dependable biomarker, evaluating the environmental health of a particular area by deducing the actual cause of the change in the environment becomes possible. Species identification is the first step in evaluating the quality and it becomes even more crucial when changes in communities are used to study biodiversity and to biomonitor species-level responses to anthropogenic stressors. Chironomids are important members of the BMI community and an easier method for their identification would facilitate their use in field studies. The approach of this study was to compare a new technique of identification, SDS-PAGE of Hb proteins, with a well-established one, larval head capsule morphology. Identification of chironomids using SDS-PAGE was validated by showing that a unique combination of Hb protein profiles was associated with just one head capsule morphology and by proving LC-MS that the bands from SDS-PAGE gels were actually composed of a group of a wide range of digested Hb proteins, Hb VII being the major contributor. The use of Hb protein polymorphism visualized on SDS-PAGE enhanced the ability to distinguish one individual from the other. For example, the consistent presence of particular band(s) for each species studied indicated that Hb protein profiles could be used to study taxonomic relationships. Nonetheless, the artificial digestion of Hb proteins coupled with LC-MS data indicated that the major Hb proteins undergo natural proteolysis – generating unique Hb protein profiles which accounted for polymorphism. Depending on the type and rate at which protease act on its substrate, heterogeneous profiles were observed in the wild population and that these profiles were not generated upon exposure to one toxicant per se, but possibly due to a myriad of

environmental cues. However, detection of Cd could be done in the laboratory setting; separating Hb proteins collected from chironomids by SDS-PAGE. The exposed chironomids showed a distinctive modulation in their Hb protein profiles, which were not seen in the wild species. Both heme and hemoglobin synthesis were significantly suppressed by Cd toxicity, where the activity of PBG synthase was inhibited and the two major Hb protein genes IV and VII, were also down-regulated. Ubiquitin also appeared to be responding to Cd where its expression was up-regulated upon Cd exposure; together with the suppression of the overall hemoglobin synthesis including PBG synthase inhibition, a unique profile showing early 4^{th} instar bands along with the loss of the lower bands was generated. Thus, this particular profile generated upon exposure to 3.0 µM Cd could be an indicator of Cd toxicity. However, utilizing this technique for Cd detection required an additional tool, head capsule width measurement; to further validate the developmental stage of the chironomids being tested. This extra step was required in order to avoid any confusion which could arise when comparing Hb protein profiles of an early instar, i.e. 3^{rd} instar chironomid versus 4^{th} instar chironomid exposed to Cd.

In this study, Hb protein profiles enhanced the ability to identify chironomid species. For example, individuals of *Cricotopus bicinctus*, *Thienemannimyia* group sp. and *Dicrotendipes modestus* showed distinctive Hb profiles with no variation. However, *Cricotopus bicinctus* and *Thienemannimyia* group sp. were only found at one site, RR, and only two to three individuals of each species were analyzed. Studying more individuals from more locations may increase the number of Hb profiles for these two species. On the other hand, *D. modestus* found at three different locations had three

distinct bands – making a consistent, unique profile that was not found in any other species.

Two genera, *Chironomus* and *Glyptotendipes*, had more than one Hb profile associated with each head capsule. This complicated the use of their profiles for species identification. *C. riparius* and both species of *Glyptotendipes* were found in KM. Due to shared bands, a unique profile for each genus was not found; although, they did have characteristic bands such as 12.5 kDa in *C. riparius* (Fig. 1 P1 – P3) and 17 and 13 kDa in both *Glyptotendipes* species (Fig. 5 P7 – P8 and Fig. 6 P9 – P11). Since chironomids have been shown to secrete stage-specific hemoglobins (Vafopoulou-Mandalos & Laufer 1982; Vafopoulou-Mandalos & Laufer 1984), a developmental study was undertaken to determine if one or more of the Hb bands in the different profiles could be used to consistently identify the species (Fig. 9). The study was performed using a laboratory population of *C. riparius*. Results showed one consistent band at 12.5 kDa that was first observed at second instar and continued to be observed throughout the whole life cycle. This indicated that despite differences in stage-specific profiles the presence of this band could be used to identify *C. riparius*. This was supported by the absence of this band in the unknown *Chironomus* species (Fig. 2) as well as the rest of the other wild chironomids collected (Fig. 8).

Results from the developmental study appeared to account for the variable profiles seen in wild *C. riparius* (Fig. 1 P1 – 3). The Hb protein profile for P3 was missing the 15.5 kDa band which was found in both P1 and P2, indicating that the P3 individual might have been transitioning between the third and fourth instar (compare

lanes 3 and 4 in Fig. 9). This finding suggested that while head capsule width could be used to identify larval instars, Hb protein profile might be another tool once it is fully characterized for a particular species. Findings also indicated that it would be beneficial to analyze multiple instars of a particular species to determine which band or combination of bands are consistently present and therefore representative of the species.

Interestingly, some Hb protein profiles were found to be rare and unable to explain the extreme cases of polymorphism observed (Fig. 10). These profiles were initially believed to be Hb protein profiles from degraded samples, as the findings from the developmental study could not explain the mechanism behind these unusual profiles. However, the results of LC-MS analysis showed that proteins found in the band with molecular weight of 12.5 kDa were partial products or fragments of mainly Hb genes VII and II (Table 4). This finding indicated that there is a presence of endogenous proteases which are responsible for generating a unique individual profile for each species. Since the full length protein products of these Hb genes have a molecular weight of approximately 17 kDa, the Hb proteins in the band at 12.5 kDa and others must have been altered so as to reduce their molecular weights. Research has shown that proteins are degraded by a number of natural processes (Jensen, 2004; Mann & Jensen, 2003). It appeared that upon Hb protein translation; it is in the inactive state, where it requires further processing to become active. A previous study has shown that Hb IV gene is an intron-less globin gene and has characteristics of secretory proteins – a region coding for an amino-terminal signal peptide also containing start codon (Antoine & Niessing, 1984). Interestingly, the amino acid sequence of mature Hb IV starts 14 codons after the start

codon, 13 of which are hydrophobic, indicating a signal sequence. It appeared that the hydrophobic leader peptide is probably responsible for the vectorial transport of Hb IV across the endoplasmatic reticulum. This is consistent with earlier studies suggesting Hb proteins are synthesized as preglobins in the larval fat body and subsequently secreted into the hemolymph as free molecules (Antoine & Niessing, 1984). Therefore, the appearance of digested products of major Hb proteins in across all bands tested including both high and low molecular weight, could be explained by the fact that Hb proteins could be undergoing proteolysis at least in the hydrophobic leader sequence reducing its actual molecular weight.

Fig. 11 shows *C. riparius* Hb proteins artificially digested by three different proteases – chymotrypsin, trypsin, and pepsin. This generated profiles that were different from the untreated Hb proteins. Although generating the exact profiles seen in the wild population was not possible, the types and rates of protease activities acting upon Hb protein could have played a critical role in producing profiles similar to those seen in the wild population. Chymotrypin digestion appeared to be responsible for making bands between 5 to 10 kDa (Fig. 11); its profile looked to be similar to that of one of the common profiles seen in wild *C. riparius* (Fig. 10 P1). Trypsin digestion at 60 minutes (Fig. 11) generated a profile similar to that of the one of the rare profiles seen in the wild *C. riparius* (Fig. 10 P4). An interesting digestion pattern was shown by pepsin where accumulation of the low molecular weight proteins was observed below 4 kDa (Fig. 11). This type of digestion correlated with the profiles of the wild species where P3 and P4 in Fig. 10 also showed an intense accumulation below 4 kDa. Evidence of digestion of Hb

proteins was shown in *C. pallidivitatus*, where Schin et al., (1974) suggested an existence of a degradative mechanism controlling the concentration of hemoglobin, and was measured by quantifying formation of bile pigment. These pigments were found in considerable quantity within the meconium at the time of adult emergence. Secretion of serine peptidases including chymotrypsin and trypsin has been implicated in variety of cellular regulations including immune response by *Anopheles sp*. and hydrolysis of nutrients from food by *Aedes aegypti*. Despite the high similarity of serine peptidases among mosquito species, each enzyme has a unique set of accessory catalytic residues that are thought to be important for determining substrate specificity (Saboia-Vahia et al., 2013). However, the activity of these peptidases could be influenced by environmental stressors such as di-(2-ethylhexyl)-phthalate (DEHP) exposure (Park & Kwak, 2008). Taken together, in the wild, individual and/or a combination of proteases and their activity governed by varieties of external cues could be responsible for generating profiles seen in the wild species, suggesting differences in both individual and population levels of Hb proteins digestion.

Many studies have shown that environmental stress can influence chironomids at both the molecular and cellular level (Ha & Choi 2008; Lee *et al*. 2006; Nair *et al*. 2011). Findings in this study suggested that water chemistry might have contributed to hemoglobin polymorphism detected by multiple Hb protein profiles for some species. For example, two major differences between *C. riparius* at RR and KM were the absence of the 8.5 kDa band in RR chironomids as well as the presence of bands below 7 kDa in KM chironomids. These differences might be attributed to salinity given that RR is freshwater

while KM is oligohaline. This idea was supported by the presence of bands below 7 kDa in *C*. ME1, which was collected at another oligohaline site. The modifications in Hb protein seen at oligohaline sites might reflect a role in salt tolerance. Research has suggested that *C. salinarius* uses their hemolymph for osmotic regulation (Cartier *et al*. 2011). *C. salinarius* appeared to absorb and eliminate excess salt using their Hb proteins or were able to adjust their intracellular Hb protein levels according to the external environment. Interestingly, the three polymorphic species found in this study – *Chironomus, Glyptotendipes* and *Dicrotendipes* – were all found at oligohaline sites. In addition, the two taxa that showed a lack of polymorphism, *Cricotopus bicinctus* and *Thienemannimyia* group sp., were found only at freshwater sites. This limited evidence suggested that polymorphism in Hb proteins may be an important feature of chironomid adaptation to environmental parameters.

Due to a high concentration of Cd found in KM, it was first assumed that Cd could be influencing the endogenous proteases activity – causing polymorphisms within the same species. However, 96 hour acute Cd toxicity tests showed that on SDS-PAGE, Hb protein profiles of 3.0 µM Cd exposed group were not only unique and different from the control Hb protein profile, but also the profile was not similar to those found in the wild species (Fig. 12). In the absence of Cd, there was an increase in number of bands in a profile and addition of bands was observed up to 96 hours indicating a surge of natural Hb protein synthesis. This increase in Hb protein production was consistent with a previous study where Hb concentrations were at maximum during the late fourth instar (Schin et al., 1974). In the presence of Cd, early onset of 4^{th} instar Hb protein profile was

maintained until 48 hours, an additional 24 hour delay, when compared to that of the control group. There was a loss of lower bands by 72 hours, indicating repression of natural Hb protein synthesis and perhaps, digestion of those major Hb proteins. When profiles were compared with the head capsule width and overall body mass, Cd toxicity clearly caused considerable inhibition on larvae growth. In previous study, growth of *C. tentans* larvae exposed to uranium was shown to be decreased. However, larvae were able to recover their growth similar to that of the control level, once transferred to clean water (Muscatello & Liber, 2010). This recovery observed in larvae of *C. tentans* could suggest that larvae exposed to Cd in our study could potentially reallocated energy into Cd detoxification, thereby inhibiting growth. Both active and passive transport mechanisms have been proposed to explain the uptake of metal from solution into aquatic invertebrates. The active transport required the expenditure of energy and can be protein carrier-mediated (e.g., copper, zinc), via ion pump (e.g., cadmium, calcium), and/or by endocytosis (e.g., iron, lead) (Muscatello & Liber, 2010). Evidence of entry of Cd into *C. riparius* was shown by Leonard et al., (2009) where Cd was transported into anterior midgut and out into the hemolymph where it was excreted through Malpighian tubules. However, Cd did not affect the metamorphosis as the larvae exposed to 3.0 µM Cd were considered to be in 4^{th} instar indicating that metamorphosis could be a separate mechanism, which might not be influenced by Cd detoxification. SDS-PAGE data indicated that Cd alone was not a factor in generating profiles seen in wild species or artificial protease digestions, but Cd toxicity did generate a unique profile which could be used as a biomarker to identify adverse effects of Cd. It is important to note that 3.0 µM

Cd was not an environmentally-relevant concentration, meaning that wild species are experiencing lower concentration but for longer duration. It is well documented that chironomids of the early stages of larva (2^{nd} instar) showed approximately 8 fold higher sensitivity (24 hour LC50) towards Cd than that of the 4^{th} instar (Williams *et al.* 1986). Therefore, a chronic low concentration Cd toxicity test would be required to verify whether or not modulation of bands by high Cd concentrations could also be seen in low Cd concentration chronic (a full life cycle) exposures. The result from this future test would validate the use of Hb protein profiles as a biomarker for Cd exposure.

Cd toxicity was prevalent at the heme synthesis level where the changes in Hb proteins observed on SDS-PAGE were consistent with Cd's toxic effect on PBG synthase activity. Upon exposure to 3.0 µM Cd, the level of PBG appeared to be decreased, indicating suppression of PBG synthase activity (Fig. 15). The activity of PBG synthase was decreased as early as 12 hours, indicating a susceptibility to Cd. However, a mechanism illustrating Cd toxicity on heme synthesis was unclear and appeared to be similar to other well documented metal toxicity such as lead (Pb). Pb has been shown to inhibit hemoglobin production by interfering with PBG synthase and ferrochelatase activity, the latter catalyzes the insertion of iron into protoporphyrin IX (Scinicariello et al., 2007). Pb appeared to displace the necessary metal cofactor for PBG synthase, Zn, which then inhibited heme synthesis. Pb had a high affinity for sulfhydryl (SH) groups, binding to SH group of PBG synthase and inhibiting its activity (Hodgson, 2004). However, detoxification of Cd has been known to involve formation of complexes with high cysteine proteins such as metallothionein (see below) and induction of antioxidant

enzymes such as families of superoxide dismutases and glutathione due Cd generation of reactive oxygen species (ROS) (Hensbergen et al., 2001, Fang et al., 2010, Liu et al., 2009, Park et al., 2012). Therefore, whether Cd has a direct influence on PBG synthase or indirect one, for example, on ROS generation and genotoxicity, how Cd might have produced the unique Hb profile seen during 3.0 μM exposure should be further investigated in order to understand its mechanism of action.

Suppression of Hb genes IV and VII expressions was observed as early as 12 hours in the presence of Cd (Figs. 16 and 17). As major Hb proteins, their early response indicated that these Hb genes were highly susceptible to Cd toxicity, where the responses manifested were immediate and consistent with the changes shown in the SDS-PAGE. Significantly decreased expressions of these selected Hb genes suggested that there was a clear link between the Hb gene expression and the Hb protein synthesis shown on SDS-PAGE. The adverse effect of Cd on Hb genes observed in this study was validated by another study; where Cd was shown to down-regulated 12 hemolymph proteins (Choi & Ha, 2009). The up-regulation of the Hb genes matched up with the addition of bands at 48 hour and continued until 96 hour. It appeared that the profile displayed by 72 hour in the absence of Cd represents a profile of fully mature larvae, and in order to fully mature as 4^{th} instar larvae, the 48 hour induction of genes of the major Hb proteins is necessary. Similarly, without the continuous expressions of the major Hb proteins, the larvae would maintain their early 4^{th} instar profile along with loss of the lower bands, indicating no further Hb proteins to be digested. Although there appeared to be no direct relationship, it seemed that the down-regulation of Hb protein genes in response to Cd could be

indirectly generating ROS through unbound heme, or free heme. When free heme is not a component of hemoproteins, due to its lipophilicity, it has been found toxic to cells; promoting lipid peroxidation and production of ROS, resulting in membrane injury and cell apoptosis (Khan & Quigley, 2011). In addition, oxygen radicals were shown to stimulate ubiquitin-dependent degradation of newly synthesized proteins (Medicherla & Goldberg, 2008). Thus, the polymorphism observed in response to Cd could due to: 1) the proteins that make up the early 4^{th} instar profile are insensitive to oxidative stress and do not undergo degradation, 2) newly synthesized Hb proteins are sensitive and rapidly undergo degradation process, unable to generate lower bands.

To further investigate the effect of Cd on Hb proteins, changes in Ub expression was analyzed by qPCR in order to determine whether or not Ub played a role in PTM of the Hb proteins – more specifically the effect of Cd on digestion of Hb proteins. The theory was that Cd could be accelerating the rate of digestion, as Cd could be binding to Hb proteins replacing Fe^{2+}. Leonard et al., 2009 showed that Cd is transported into the anterior midgut cells from the lumen and out of the cells into the hemolymph. Since the production of Hb protein is in the fat body (Bergtrom *et al*. 1976), it was likely that Cd could alter proper folding of Hb protein by binding to it, since fat body is located in close proximity to the midgut and Cd could enter the fat body. Thus, Ub was chosen to be studied since its function is related to process degradation of the damaged proteins.

Ub is a 76-residue protein found in nearly every eukaryote. Its DNA sequence is also highly conserved. Ub is involved in a complex process known as the ubiquitin-proteasome pathway, where it functions as marking damaged proteins for proteolytic

degradation (Krauss, 2008). It is possible that low concentrations of toxicants abnormally stimulate ubiquitinating enzymes resulting in increased degradation of target proteins such as Hb. Over stimulation of ubiquitinating enzymes might be linked to the unique Hb profile observed in Cd exposed larvae. The abnormal lack in upper band development and loss of lower bands caused by Cd could be due to Ub posttranslational modification. More specifically, Ub might have initiated marking of damaged Hb proteins for protein turnover. Through series of enzymatic processes involving other Ub-related proteins, the marked proteins were then delivered to proteasomes where actual degradation of the marked proteins occurred. Cd has been known for its competition with transport with calcium (Leonard et al., 2009). Therefore, as a non-essential metal, Cd is believed to cause damages and/or generate mis-folded proteins. The idea was that if Cd alters the Hb conformation by binding to it or damages it in anyway, the Hb proteins would be subjected to Ub for degradation.

Ubiquitin appeared to be responding to Cd where there was an induction of the expression as early as 24 hours (Fig. 18). This induction of Ub suggested a potential activation of a biological process known as the ubiquitin-proteasome pathway, where Ub functions as marking damaged proteins for proteolytic degradation. The idea is that once Cd was transported through the midgut and entered the hemolymph, it bound to heme or globin molecules. A recent proteomic study showed that 14 proteins disappeared and six proteins seemed to be newly expressed upon exposure to Cd in larvae of *C. riparius* (Lee et al., 2006). Of the six proteins, one of them was ubiquitin-actin fusion protein. This mechanism is consistent with the observed induction of Ub at 24 hour. However,

ubiquitin-proteasome system is known to have complex regulation involving numerous enzymes for each step; activation, conjugation, and ligation of ubiquitin molecule. In addition, there are deubiquitylating enzymes (DUBs) counteracting the ubiquitylation of a particular protein (Petroski, 2008). Therefore, deducing the actual relationship between Cd exposure and ubiquitin requires further investigation.

The response of MT to Cd was not what was expected (Fig. 20). In the absence of Cd, it appeared that a high expression of MT was necessary for growth and maturation over 96 hours as observed in control over. However, there was an inconsistent response of MT in the presence of Cd. The response of MT in the presence of Cd found in this study was not consistent with others. The expression of MT mRNA was induced significantly in *C. riparius* after exposure to three different concentrations of Cd (Park & Kwak, 2012). However, the highest Cd concentration used in that study was 20 µg/L, which is equivalent to 0.1 µM, 30 time less than 3.0 µM used in our study. It appeared that MT responds to low concentration of Cd but not high concentrations. The inconsistency in MT response in our study suggested that at high concentration, chironomids are relying on other means of detoxification of Cd. One possibility was the sequestration of Cd within the gut (Leonard et al, 2009). However, in order to test whether chironomids were selecting detoxification routes through midgut, quantification of Cd within the meconium of larvae at 96 hour is necessary to ensure that Cd has been cleared from hemolymph.

This study contributes to the development of a novel means of identification of wild chironomid species using Hb protein profiles detected by SDS-PAGE. The

technique described here supports and supplements the current standard method of taxonomic identification using larval head capsule morphology or even other methods such as PCR-based approaches described above. Identification using Hb profiles requires less taxonomic expertise, could be used to identify larvae with deformed head capsules, and most importantly, could handle large numbers of samples. The consistent presence of particular bands for each species studied indicated that Hb profiles could be used to study taxonomic relationships and determine instars in chironomids. The regulation of overall Hb protein synthesis responsible for the polymorphisms observed in wild and laboratory species is likely a complex biological activity involving numerous enzymes, which could be influenced by a wide range of unknown external cues. This study provided a glimpse of how Hb protein synthesis functions normally. It involved synthesis of different sized bands which appeared during 4^{th} star. Experiments showed that the appearance of bands could be impacted by an environmental stressor such as Cd. However, the profile produced did not explain the polymorphisms found in wild populations of C. riparius. Profiles were explained by protease digestion. Overall, this study adds proteolysis as a novel constituent of Hb protein as a biomarker, that should now be considered as a useful tool in biomonitoring of the environmental quality

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