

# The Midgut Epithelium of Aquatic Arthropods: A Critical Target Organ in Environmental Toxicology

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The midgut epithelium of aquatic arthropods is emerging as an important and toxicologically relevant organ system for monitoring environmental pollution. The peritrophic matrix of aquatic arthropods, which is secreted by the midgut epithelium cells, is perturbed by copper or cadmium. Molecular biological studies have identified and characterized two midgut genes induced by heavy metals in the midgut epithelium. Many other metal-responsive genes (MRGs) await characterization. One of the MRGs codes for an intestinal mucin, which is critical for protecting the midgut from toxins and pathogens. Another codes for a tubulin gene, which is critical for structure and function of the midgut epithelial cells. Perturbation of expression of either gene could condition aquatic arthropod survivorship. Induction of these MRGs is a more sensitive and rapid indicator of heavy-metal pollution than biological assays. Characterization of genes induced by pollutants could provide mechanistic understanding of fundamental cellular responses to pollutants and insight into determinants of aquatic arthropod population genetic structure and survivorship in nature. **Key words:** aquatic arthropods, heavy metals, metal-responsive genes, midgut epithelium, mucin, tubulin. *Environ Health Perspect* 110(suppl 6):911–914 (2002). <http://ehpnet1.niehs.nih.gov/docs/2002/suppl-6/911-914beaty/abstract.html>

Heavy-metal pollution of streams from contemporary and historic mining operations poses significant environmental problems in the Rocky Mountain West. In Colorado it is estimated that heavy metals from over 5,000 abandoned waste dumps or tailing pipes impact over 2,600 km of streams (1). These hazardous waste sites expose aquatic organisms and other wildlife to multiple heavy metals as well as to other pollutants, which can exert impacts on aquatic ecosystems at different levels of organization (1–10). Theoretically, organisms such as aquatic arthropods that experience long-term exposure to the mixtures would be most impacted by such heavy-metal pollution. Interspecific differences in aquatic arthropod populations, including reduced diversity and shifts in benthic community composition at sites located downstream from mining sites, occur in response to heavy-metal pollution (1,4,11). In addition, we previously demonstrated that the population genetic structure of *Baetis tricaudatus* was also perturbed in the Arkansas River in Colorado, presumably in response to impediments to gene flow in the population caused by mortality arising from exposure to pollution from heavy-metal mixtures from mining sites (2). In the latter study, intraspecific differences in population structure were demonstrated using a mitochondrial 16S ribosomal DNA gene of *B. tricaudatus* as a measure of genetic diversity in the species in the Arkansas River. Reduced variability in genetic polymorphisms in regions of metal exposure suggests differences in heavy-metal responsiveness in the population. Unfortunately, the use of such a selectively neutral gene provided no insight into the

molecular mechanisms that conditioned arthropod survivorship. Delineating potential metal-responsive genes (MRGs) or other genes that impact gene flow and condition the population genetic structure of aquatic arthropods could provide a mechanistic understanding of fundamental responses to ecotoxicological insults and could be exploited to develop new molecular bioreporter systems (2,5,12–16). RNA or protein products of a given MRG could be exploited as specific biomarkers of the presence or absence of heavy metals (2,5,12–16).

Insects exposed to heavy metals at sublethal doses exhibit pathological effects on many biological activities (1–3,5,7,11,17). Concentrations and toxic effects of metals depend on the bioavailability of the metal and the size, age, sex, and developmental stage of the arthropod (2,6,8,10,11,18). Resistance to heavy metals varies with different genotypes and strains within a species (2,5–7). However, little information was available concerning the molecular genetic responses of aquatic arthropods to pollutants. We conducted a series of studies to define anatomic and molecular determinants of certain aquatic Diptera susceptibility and resistance to heavy metals.

## *Aedes aegypti*: Anatomic Basis of Heavy-Metal Toxicity to Larvae

Initially, studies were conducted using *Ae. aegypti* mosquitoes as a model arthropod system to define the anatomic basis of heavy-metal toxicity (17). The mortality rate of third instar larvae exposed to certain heavy metals (mercury, cadmium, and copper) was metal- and dose-dependent. Interestingly, exposure to

heavy metals compromised the integrity of the peritrophic matrix (PM) of larval *Ae. aegypti* (Figure 1); a significant proportion of the metal-exposed larvae failed to produce a dis-sectible PM (17). The compromising of the PM was also metal and dose dependent, and the concentrations of heavy metals used were well within those found in contaminated aquatic ecosystems and aquifers (17). The integrity of the PM is critical to protect the arthropod midgut from toxins, microbial infections, digestive enzymes, and physical trauma (19–21). Thus, heavy metals could compromise the integrity of the PM, and thereby condition survivorship in arthropod populations, that is, predispose their ability to overcome a potentially lethal event. However, these studies provided no information concerning the molecular basis of the arthropod response to heavy metals. Thus, molecular biological studies were conducted with *Ae. aegypti* and *Chironomus tentans* to identify specific MRGs in the midgut epithelium.

## *Ae. aegypti*: Molecular Characterization of a Metal-Responsive *Ae. aegypti* Intestinal Mucin cDNA

Because midgut epithelial cells secrete the components of the PM, we used a molecular biological approach to identify MRGs and other genes in the arthropod midguts. Although the metallothionein genes were considered to be likely candidates as biomarkers for heavy-metal pollution (15,22,23), other metal-responsive genes were also candidates to be induced (8,9,13,14,24–26).

To identify MRGs in the mosquito gut, we screened an *Ae. aegypti* larval cDNA library with radioactively labeled cDNA probes made from polyA + RNA isolated from metal-exposed or unexposed (control) larvae. Several plaque-purified clones bearing cDNA whose corresponding RNAs were

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more abundant in larvae exposed to heavy metals were recovered. One of these metal-responsive cDNAs was characterized. Northern blot analysis indicated that the gene coded for a midgut-specific transcript of about 1.0 kilobase. The complete nucleotide sequence of the 1,034 base pair (bp) cDNA was determined. The cDNA had a single 825 bp open-reading frame coding for a 275 amino acid protein, and the predicted translated product of the gene has three cysteine-rich domains (CRDs), and a proline-threonine-serine-rich box (PTSB) flanked by CRD1 and CRD2. A comparison of the predicted translated CRD sequences of the cDNA against other known protein sequences revealed significant similarity to invertebrate mucin-, peritrophin-, chitinase-binding, and chitinase genes (10) and with vertebrate and invertebrate mucin genes (21,27–32). Thus, we designated the gene *Ae. aegypti* intestinal mucin 1 (*AEIMUC1*).

In larvae, the expression of the *AEIMUC1* gene was restricted to the midgut, and its relative abundance was increased in larvae exposed to heavy metals (Figure 2). In adults, *AEIMUC1* was also expressed in the adult female midgut, where its abundance was increased by exposure to iron chloride, by dengue virus infection administered in an artificial blood meal (33), or by the ingestion of a blood meal (Figure 3). *AEIMUC1* expression was not detected in embryo or pupal stages or in adult males (data not shown).

Insect intestinal mucinlike genes have been isolated from larvae of the cabbage patch moth *Trichoplusia ni* (21,32) and the mosquito *Anopheles gambiae* (30). *AEIMUC1* exhibits

similarity to these and other insect midgut proteins (10). The overall organization of the CRDs and the PTSB of our *AEIMUC1* most closely resembles that of the moth larval intestinal mucin. Both the mucus layer and the PM have been proposed to play an important role in protecting the arthropod digestive epithelium against toxins, pathogens, and abrasive foreign particles. The fact that *AEIMUC1* is induced by virus infection tends to support this hypothesis. Mucins are well known for their ability to bind pathogens such as viruses and bacteria, thereby inhibiting infection (10,21,27–31). To overcome the mucin blockade, numerous pathogens have developed specific mucin-degrading enzymes (29,34–36). Certain baculoviruses actually code for a mucin-digesting enzyme (enhancin), that enhances baculovirus infections in moths (32). The role of mucins and heavy metals in conditioning survivorship in aquatic arthropods remains to be determined. Certainly, concentrations of heavy metals that qualitatively or quantitatively alter mucin expression or compromise the integrity of the PM could reduce resistance of aquatic arthropods to microbial and toxicological insults. As noted previously, the integrity of the PM in *Ae. aegypti* is compromised by exposure to heavy metals (17).

Present studies clearly demonstrate that the *AEIMUC1* gene is induced by heavy metals at sublethal concentrations. However, the mechanism by which increased metal concentrations compromise PM formation or stability remains to be determined.

Expression of *AEMUC1* is a molecular biomarker that is more sensitive than classic toxicological end points (e.g., mortality rates)

for detecting exposure to metal pollution. For example, exposure of third instar *Ae. aegypti* larvae to 0.5 ppm cadmium for 24 hr results in virtually no larval mortality, and as much as 5 ppm cadmium is needed to cause significant mortality (10,17). In contrast, *AEIMUC1* RNA was induced in larvae exposed to as little as 0.01 ppm cadmium, which is a dramatic increase over the sensitivity of the mortality bioassay.

### Molecular Characterization of a Metal-Responsive *C. tentans* Alpha-Tubulin cDNA

Because *Ae. aegypti* is a container breeder that does not breed in natural aquatic ecosystems, we chose to identify genes of interest in more environmentally relevant organisms, such as *C. tentans*, that live in natural ecosystems (rivers, lakes, ponds, etc.). We used a molecular approach to characterize MRGs in *C. tentans* larvae (8). We screened a *C. tentans* larval cDNA library with radioactively labeled cDNA probes made from polyA + RNA isolated from metal-exposed or unexposed (control) larvae. Several plaque-purified clones bearing cDNAs whose corresponding RNAs were more abundant in larvae exposed to cadmium (Figure 4) were recovered. One of these metal-responsive cDNAs was characterized. The complete nucleotide sequence of the 1,676 bp cDNA (1,676 bp) was determined, revealing a 1,350 bp single open-reading frame coding for a 450 amino acid protein. The predicted protein is acidic, with an isoelectric point of 4.96 and a molecular weight of approximately 50 kilodaltons (8). The translated protein sequence of the cDNA

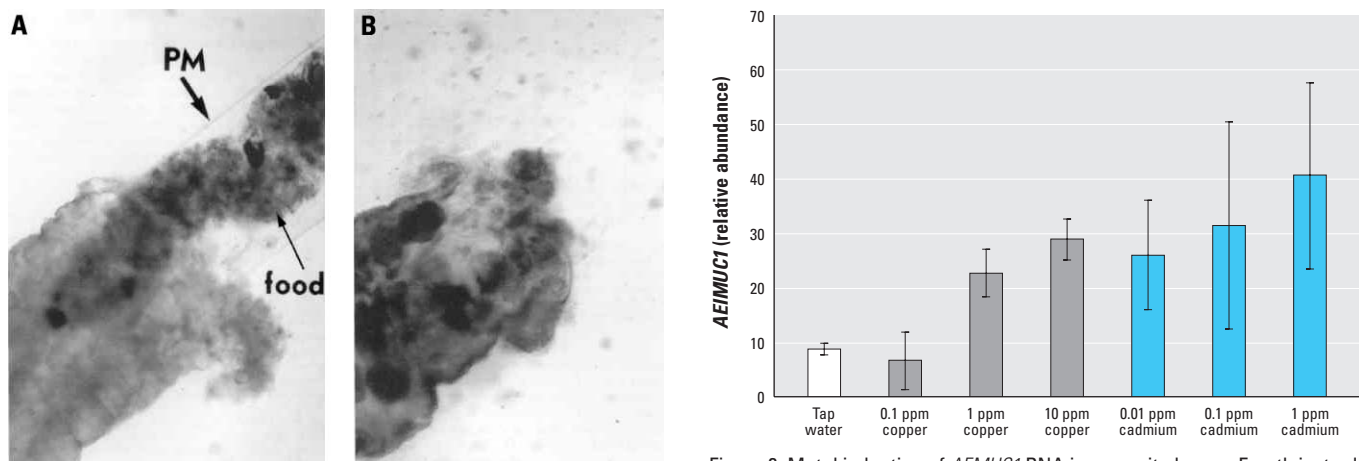


Figure 1. Copper perturbs the PM of *Ae. aegypti* larvae. Removal of the midgut epithelium from larvae grown under standard conditions (17) shows the PM completely surrounding the food bolus (A). In contrast, in larvae exposed to 32 ppm copper for 24 hr, removal of the midgut epithelium causes dispersal of the food bolus, indicating the PM is absent or poorly formed (B).

Figure 2. Metal induction of *AEIMUC1* RNA in mosquito larvae. Fourth instar larvae were exposed to the metals at the respective concentrations for 24 hr. Total RNA isolated from larvae was fractionated in an agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized with a radioactively labeled DNA probe made from *AEIMUC1* cDNA as described (10). The relative abundance of *AEIMUC1* RNA was measured and plotted as a function of the different experimental conditions (10). The relative abundance of *AEIMUC1* RNA was increased 4.6-, 3.6-, and 3-fold by 1, 0.1, 0.01 ppm cadmium, respectively ( $p > 0.001$ ); 10 or 1.0 ppm copper increased *AEIMUC1* RNA by 3.3- and 2.6-fold, respectively ( $p > 0.001$ ) (10). Error bars are standard errors of the mean (SEM).

revealed significant similarity with vertebrate and invertebrate alpha-tubulins (37–39). Thus, we designated the gene, which was induced in the midguts of *C. tentans* larvae exposed to cadmium (Figure 4), *C. tentans* tubulin-1 (*CTTUB1*).

Indirect immunofluorescent analysis of metal-exposed *C. tentans* midguts revealed a dramatic change in *CTTUB1* localization in midgut epithelial cells, suggesting that microtubule structure and function were perturbed as a consequence of heavy-metal exposure. It is possible that microtubule

assembly was prevented by nitrotyrosination of unpolymerized alpha-tubulin, but further experimentation will be required to elucidate the molecular basis of the phenomenon (40). Clearly, heavy metals that impact fundamental cellular processes could have dramatic impacts on aquatic arthropod survivorship (37). Certainly, concentrations of heavy metals that qualitatively or quantitatively alter alpha-tubulin or alpha-tubulin gene expression could reduce or increase the resistance of aquatic arthropods to microbial and toxicological insults (8).

As with *AEMUC1* (10), *CTTUB1* expression at the RNA or protein level is a more sensitive biomarker of metal exposure than traditional bioassays (8). For *C. tentans* larvae exposed for 6 days, the LD<sub>50</sub> for copper and cadmium are 2.5 and 5 ppm, respectively (11). In contrast, as little as 0.1 ppm cadmium or copper induces *CTTUB1* in a 1-day exposure. Thus, a molecular-based system provides both a temporal and a sensitivity advantage over the biological assay.

## Summary

The *Ae. aegypti* *AEMUC1* and *C. tentans* *CTTUB1* are excellent examples of MRGs of aquatic arthropods that can be readily identified using molecular biological techniques. The importance and need of more sensitive and quantifiable biological markers in ecotoxicological testing has been stressed previously (2,12,13). Identification and characterization of more MRGs in the arthropod midgut may lead to additional, sensitive, molecular-based, *in vitro* approaches for environmental bioreporting (14). In addition, understanding the molecular response of arthropods to heavy metals will provide mechanistic understanding of the factors that condition aquatic arthropod survivorship and population genetic structure in response to pollutants in nature. Significant advances are being made in developing and use of new molecular, biochemical, and genetic markers in recent years, and the use of population genetic approaches in ecotoxicology is increasing dramatically (41–44). These approaches provide important new tools for environmental biomonitoring and risk assessment.

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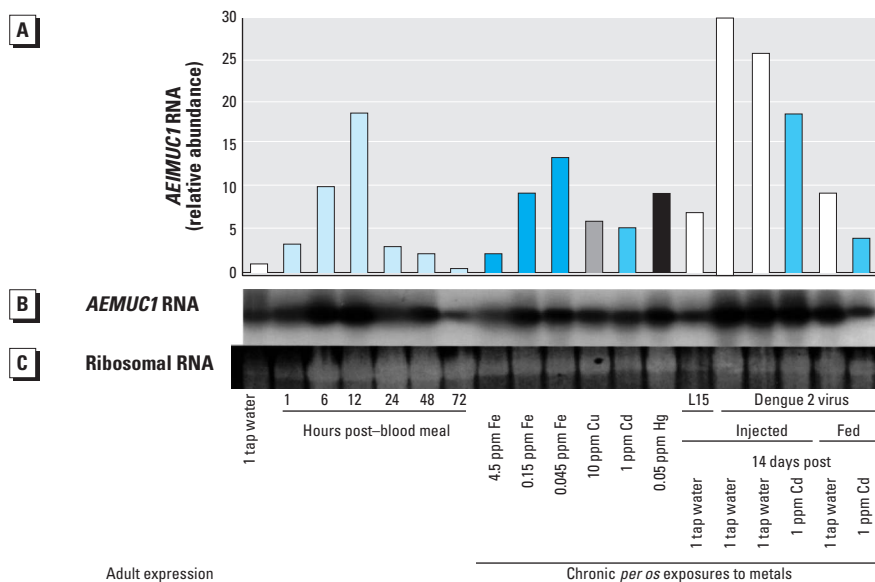


Figure 3. Heavy-metal, blood meal, and virus induction of *AEMUC1* RNA in adult mosquitoes. Total RNA, 10  $\mu$ g/lane, isolated from female adult mosquitoes was fractionated in an agarose–formaldehyde gel, transferred to a nylon membrane, and hybridized with a radioactively labeled DNA probe made from *AEMUC1* cDNA (B) as described (10). Ribosomal RNA control for each is stained with ethidium bromide (C). The relative abundance of *AEMUC1* RNA as a function of different treatments is presented in A. For virus studies, mosquitoes were infected with approximately 100 tissue culture infective dose<sub>50</sub> dengue 2 virus and maintained for 14 days before processing.

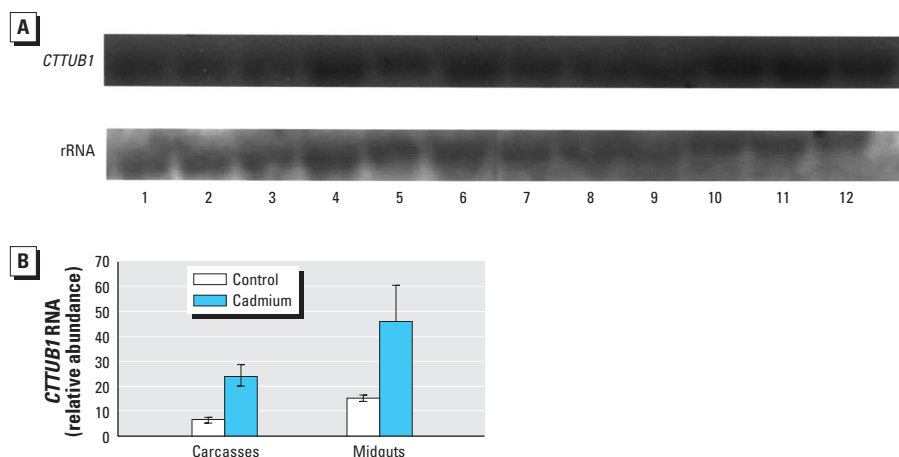


Figure 4. Tissue-specific induction of *CTTUB1* RNA. Total RNA, 10  $\mu$ g/lane, isolated from dissected midguts or carcasses (containing no midgut) of larvae exposed to 0.1 ppm cadmium for 24 hr, was fractionated in a denaturing agarose–formaldehyde gel. RNA was transferred to a nylon membrane and hybridized with a biotinylated probe made from *CTTUB1* cDNA (B). RNA-biotinylated probe complex was visualized using NorthSouth psoralen–biotin Northern blot detection kit by Pierce. Ribosomal RNA was visualized by staining with ethidium bromide. Figure reproduced from Mattingly et al. (8) with permission from Elsevier.

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