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Sub-lethal metal stress response of larvae of *Aedes aegypti*

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Abstract

Aedes aegypti (Diptera: Culicidae) has adapted to urban environments; the urbanisation process provides suitable habitats for this disease vector subsequently increasing the probability of the transmission of pathogens in high-density environments. Urban environments provide metal stressed larval habitats. However, little is known about the physiological cost of metal stress or how this might affect the performance of this mosquito species. This study aims to characterise the sub-lethal physiological consequences of metal stress in *Aedes aegypti*. Various parameters of mosquito physiology under larval metal stress are assessed including larval metallothionein expression and the effects of larval metal stress on adult performance and their progeny. Results show that environmentally relevant larval metal stress compromises larval and adult development and performance, and results in larval metal tolerance along with an increase in lipid consumption. These performance costs are coupled to a dramatic increase in metallothionein expression in the midgut. Metal stress results in lowered adult body mass and neutral storage lipids at emergence, starvation tolerance, fecundity and starvation tolerance of offspring compared to non-metal stressed individuals. Ironically, larval metal stress results in increased adult longevity. Together, these findings indicate that even low levels of environmentally relevant larval metal stress have considerable physiological consequences for this important disease vector.

Keywords

Aedes aegypti; metal stress; metallothionein; performance; storage lipids

Introduction

An increase in urban populations in developing countries has been accompanied by increased industrial activities, an indiscriminate use of pesticides, a lack of environmental regulation and an increase in poorly planned cities, all resulting in environmental pollution (Montgomery, 2008). The presence of *Aedes aegypti* (Linnaeus in Hasselquist, 1762) (Diptera: Culicidae) larvae has been confirmed in polluted habitats such as drains containing domestic wastewater and man-made aquatic habitats (Barrera *et al.*, 2008), habitats previously not associated with this species of mosquito. These observations suggest that pollutant tolerant strains of *A. aegypti*, the primary vector of dengue and yellow fevers, are developing. Indeed, domestic and feral forms of *A. aegypti* have been recognized in Africa (Crovello & Hacker, 1972).

The urbanisation process provides suitable larval breeding sites in the form of septic tanks, discarded metal cans, cisterns, drains, sewers, tires and dumpsters for this mosquito subsequently increasing the probability of the transmission of diseases in high-density urban environments (Frankie & Ehler, 1978). Metallic habitats are of particular interest as they provide a source of metal contamination directly to the breeding site. In addition, there are non-point sources of metal pollution (run off, fall out, indiscriminate use of metal containing herbicides). Together, these sources of metal contamination contribute to the accumulation of metals in these breeding sites. For these reasons, *A. aegypti* larvae are subject to physiological stress as a result of metals in the environment. Metal tolerance physiology in vector mosquitoes has been overlooked and needs to be considered as an adaptation this disease vector is undergoing (Posthuma & Van Straalen, 1993; Poupardin *et al.*, 2008).

Adaptations to novel environments provide distinct advantages; however, performance costs are associated with them. These costs, in turn, might affect the disease transmission dynamics of a vector *i.e.*, vector capacity (Alto & Lounibos, 2012). Anthropogenic changes to the environment such as urbanisation and pollution can result in physiological changes in disease vectors with potential epidemiological consequences.

Little is known about the physiological cost of metal pollution or how this might affect the performance of mosquito species (Rayms-Keller *et al.*, 1998; Sarkar *et al.*, 2004; Mireji *et al.*, 2010). Of the many metals that larvae of *A. aegypti* are exposed to in an urban environment, copper (Cu^{2+}) is of particular interest. It is used indiscriminately as an algicide, fungicide and root treatment agent and in the manufacture of artificial containers (Barrera *et al.*, 2008; Mireji *et al.*, 2008) and can be used to model metal toxicity. It is an essential trace element with an important role in several cellular processes such as respiration, oxidative stress defence and immune function and is found in a number of important metabolic enzymes such as cytochrome *c* oxidase, Cu-Zn-superoxide dismutase 1 (SOD1), tyrosinase, lysyl oxidase and multicopper oxidase (Puig & Theile, 2002). *Aedes aegypti* mosquitoes need to regulate this toxic agent that is also essential to basal metabolism. An investigation of Cu^{2+} tolerance physiology can also serve as a model for mercury, silver, zinc and cadmium, as each of these share similar detoxification pathways *via* metallothionein binding. Metallothioneins are small, ubiquitous, cysteine rich proteins with a high affinity for transition metals and have an important role in the homeostasis of essential metals, the detoxification of nonessential metals and the oxidative stress response (Amiard, *et al.*, 2006). Thus, an understanding of *A. aegypti* metal toxicity and tolerance is pertinent.

This study aims to characterise *A. aegypti*'s physiological responses to sub-lethal concentrations of Cu^{2+} . Various parameters of mosquito physiology are assessed under larval metal stress, including larval metallothionein expression and the effects of larval metal stress on adults and their progeny (adult body mass at emergence, longevity, starvation resistance, fecundity and maternal effects).

Materials and methods

Exposure to metal stress

Aedes aegypti (Rockefeller strain) pharate 1st instars (eggs less than a week old) were hatched in deoxygenated water. Upon emergence, three replicate groups of 200 larvae were reared to adulthood in 23 × 40 × 15cm polypropylene plastic pans containing 1L of reconstituted soft water (RSW) for the treatment referred to as “reared clean”. For the metal stressed treatment, three replicate groups of 200 1st instar larvae were reared with copper (Cu²⁺) in RSW. Cu₂ concentration varied (0.75 – 1.25 ppm) depending on the specific nature of each experiment. Although metal concentrations in Aedine larval habitats have not been assessed, this concentration range has been shown to be ecologically relevant for Culicine and Anopheline mosquitoes (Sarkar *et al.*, 2004; Mireji, *et al.*, 2008). These authors reported that environmental metal concentrations in urban habitats are well above this range. Therefore, the physiological responses observed in this study are not toxic effects from unrealistic laboratory exposures, but rather model realistic organismal responses to environmental stress.

Larval diet consisted of 750 µl of 10% liver powder suspension (MP Biomedicals Inc., Aurora, OH) added to the pan every day except for the second day. Pupae were collected daily throughout the pupation period and transferred to an appropriate container for adult emergence. Adult mosquitoes were offered a cotton pad soaked in 3% sucrose solution. Four-day old female mosquitoes were fed porcine blood equilibrated to 37 °C and ATP added to the blood meal to a final concentration of 1 mM immediately before use. After blood feeding, females were housed individually (*n* = 15) so that eggs oviposited on paper could be evaluated. For maternal effects experiment, the eggs were hatched as mentioned above. Insectary conditions were 28 °C and 80% relative humidity under an LD 16: 8 h photoperiod. All assays were performed under insectary conditions ~12:00 pm.

Micrographs of representative individuals of 24 h old larvae and three day old adult females for each treatment were produced with a Leica MZ6 stereo dissecting microscope equipped with a Leica DC500 digital camera and LAS 2.8.1 software at 40 × magnification. All images were processed with Photoshop C2 software from Adobe to remove background glare and reflection, the actual larval and tissue component of the images were never altered.

Metal tolerance

Increased tolerance to metal stress was demonstrated by rearing larvae under the above mentioned conditions except with 0.75 ppm Cu²⁺ (to minimize physiological stress and mortality) or in RSW without Cu²⁺. When larvae from each treatment reached early 3rd instar stage they were subjected to an acute toxicity assay in 15 cm diameter glass petri dishes containing 100 ml of RSW or test water in a time response manner by exposing them to the LC₅₀ concentration determined by the dose response curve (2 ppm Cu²⁺) until 100% mortality had occurred (*n* = 20, in duplicate). Median LT₅₀ values were determined by generating a survivorship curve and performing a log rank test (Mantel-Cox).

Dose response curve

Early 3rd instar larvae reared under the above mentioned conditions in RSW without Cu²⁺ were exposed to Cu²⁺ (0, 1.2, 1.6, 1.9, 2.4, 3.2, and 4 ppm) (from CuSO₄) ($n=25$, in triplicate) for 24 h. Lowest observed effect concentration (LOEC) and LC₅₀ values were determined by log transforming the Cu²⁺ concentration and fitting a sigmoidal nonlinear curve.

Performance assays

To determine adult body mass newly emerged adult females from each treatment (larvae reared in RSW only or in 1 ppm Cu²⁺) were collected, dried at 95 °C for 24 h and analysed gravimetrically with a Cahn 29 electrobalance to within 0.01 of a milligram ($n=50-69$).

For the adult starvation tolerance assay three replicate groups of 15 newly emerged adult females from each treatment were assayed in one pint mosquito cages and provided deionized water *ad libitum*. The longevity assay was performed in a similar manner except 3% sucrose was provided. For the filial larvae starvation tolerance assay (maternal effects) three replicate groups of 25 newly emerged 1st instars generated from mosquitoes reared under the above mentioned protocol were assayed in 15 cm diameter glass petri dishes containing 100 mL of RSW in the absence of food. The number of individuals found not responsive to mechanical stimulation every 24 h in each assay were counted as “dead” and removed. A Kaplan-Meier survivorship analysis was then performed.

Reagents and copper measurement

Reconstituted soft water was used in all bioassays. RSW was prepared by adding reagent-grade NaHCO₃ (48 mg), CaSO₄ 2H₂O (30 mg), MgSO₄ (30 mg) and KCl (2 mg) to 1 L of Nanopure water (pH~7.4). The absence of contaminating metals was confirmed by inductively coupled plasma mass spectrometric analysis (ICP-MS). All reagents were purchased from Fisher Scientific, Pittsburgh, Pennsylvania. Test solutions were prepared by adding reagent grade copper sulphate pentahydrate to RSW. Nominal copper (Cu²⁺) concentrations were confirmed ICP-MS using an HP 4500 plus IPC-MS instrument (Hewlett-Packard Co., Wilmington, Delaware), equipped with a Babington-type nebulizer and an ASX-500 autosampler (Cetac Technologies Inc., Omaha, Nebraska).

Quantification of total neutral lipids

To determine if metal tolerance is a lipid dependant process larvae were reared as described in the first section of Materials and methods in RSW only. Early 3rd instar larvae were then exposed to 1.25 ppm Cu²⁺ in RSW under insectary conditions in the absence of food for 5 d. A slightly higher concentration of Cu²⁺ was used for this experiment because 3rd instar larvae are less sensitive to metal stress than 1st instars. During that 5 d period the larvae moulted to the 4th instar stage. Negative control larvae were held in clean RSW. Neutral lipids were quantified using a triglyceride quantification kit (Biovision, Mountain View, California; cat# K622-100). Three 4th instar larvae were assayed per biological replicate and assays performed in triplicate. To stain neutral lipids, larvae were fixed in 4% formaldehyde overnight, washed with cold PBS, stained with Oil Red-O for 30 min., washed with cold

PBS and imaged with a Leica Model MZ6 stereomicroscope at 40× magnification (adapted from Nishiura *et al.*, 2007).

Quantification of follicles per ovary

A sub-sample of adults was analysed for follicle quantification. 60–72 h old adult females were anesthetized by chilling for 10 min at 4° C prior to dissection. Ovaries from these mosquitoes ($n = 15$) were removed, rinsed in Aedes physiological saline (APS) (MgCl₂ 0.6 mM; KCl 4.0 mM NaHCO₃ 1.8 mM; NaCl 150.0 mM; HEPES 25.0 mM; CaCl₂ 1.7 mM) and stained with 0.5% neutral red solution in acetate buffer at pH 5.2 (Sigma–Aldrich, St. Louis, MO) for 5 s to more easily visualize follicles (Clifton & Noriega, 2011). The ovaries were rinsed a second time in APS and placed under a coverslip. Photographs were taken of the previtellogenic ovaries using a DM 5500 B Leica fluorescence microscope, a Leica DFC 310 FX mounted camera and Leica LAS imaging software. Ovaries were later scored using Leica LAS imaging software for total follicle count. A mean follicle value was then calculated to represent previtellogenic follicle output for the cohort.

Quantitative real-time PCR (qPCR) and metallothionein (AaMtn) induction

To induce AaMtn transcription newly emerged 1st instar larvae hatched from eggs less than a week old were exposed to 1.25 ppm Cu²⁺ in RSW (to maximize physiological stress with minimal mortality) under insectary conditions in the absence of food for 12 h and subsamples collected at 6 and 12 h. To demonstrate the reversible nature of AaMtn transcription metal exposed larvae were transferred back to clean water after 6 h of metal exposure and a subsample collected. Three independent biological replicates for each time point consisting of ~50 larvae derived from three different cohorts of eggs were collected and stored at minus 80°C. To determine if the midgut is a barrier to metal toxicity via AaMtn expression early 4th instar larvae were exposed to 1.25 ppm Cu²⁺ in RSW under insectary conditions in the absence of food for 6 h. Negative control larvae were held in RSW without Cu²⁺. After exposure midgut tissues were separated from the rest of the body (three independent biological replicates of five tissues each) and both midguts and ‘rest of body’ tissues were stored in RNeasy lysis buffer (Qiagen) at minus 80° C. Midgut AaMtn transcription level was analysed and compared to the rest of the body tissue.

Total RNA was isolated and quantified from samples using RNA-binding glass powder as previously described (Noriega & Wells, 1993). Contaminating genomic DNA was removed using the DNA-free™ kit (Ambion, Austin, Texas). The yield and purity of total extracted RNA was evaluated using a NanoDrop (Thermo Scientific). The ratios of A260/A280 were above 1.9. RNA concentrations were standardized based on this quantification. Reverse transcription was carried out using the Verso cDNA Kit (Fisher Scientific). Real time qPCR was performed with the 7300 Real Time PCR System using TaqMan® Gene Expression Assays together with TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, California). The primer probes for housekeeping gene 60S ribosomal protein L32 (rpL32) (Accession number: AAEL003396) and for metallothionein (AaMtn) (Accession number: AAEL008176) were as follows (exon borders in bold):

rpL32 Forward: 5' CCAAGCCAGATTCCTAACATTCAAA 3'

rpL32 Reverse: 5' TGCCCTGTCCGCAACC 3'

rpL32 Probe: 5' CTTGCAATCATTACCACAGCAC 3'

Mtn Forward: 5' GCCAAGCCAGATTCCTAACATTCAA 3'

Mtn Reverse: 5' GCAACCGGAGGTGCACTT 3'

Mtn Probe: 5' AAGTGCTGTGGTAATGATTGC 3'

qPCR reactions were carried in a 20 µl volume according to manufacturer's recommendations for Custom TaqMan® Gene Expression Assays. Reactions were run in triplicate using 1 µl of cDNA per reaction. Standard curves to quantify relative gene copy number were made from serial dilutions of plasmids containing *rpL32* or *AaMtn* gene (300,000, 30,000, 3000, 300 and 30 copies of a plasmid per reaction). Thermo cycling parameters were as follows: polymerase activation at 95°C for 10 minutes was followed by 40 cycles of denaturing 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. Real time data were collected by 7300 System SDS Software and analysed in Microsoft Excel. *AaMtn* transcript levels were normalized with *rpL32* transcript levels in the same sample. Relative *AaMtn* transcript levels are expressed as a number of copies of *AaMtn* transcript per 10,000 copies of *rpL32* transcript. Each qPCR data point is the average of three independent biological replicates.

Data analysis

Graphical representations and statistical analysis were performed with GraphPad Prism Software (San Diego, California) version 6.0 for Windows. Results were expressed as means and standard error of the mean (SEM). Survivorship (LT₅₀) was determined by non-linear regression curve fit. Significant differences in survivorship (LT₅₀) were determined with a log rank method. Significant differences for all other results were determined with either a one tailed student's *t* test or a one way ANOVA followed by a Tukey's test for multiple comparisons.

Results

Larval metal stress altered larval and adult phenotypes

Aedes aegypti larvae reared under the metal stressed treatment exhibited a visibly altered phenotype than those that had been reared in a clean environment with no metal stress. At 24 h of development 1st instar larvae were distinctly different in size and general overall appearance (Fig. 1A and B). The difference in size is likely the effect of clean reared larvae having moulted to the 2nd instar while metal stressed larvae remain in 1st instar stage longer. Previous work has demonstrated that metal stressed larvae exhibit longer developmental time (Perez & Noriega, 2012). In the adult stage, there were also dramatic visible differences between treatments (Fig. 1C). Most notably, ovaries and Malpighian tubules of three day old females were affected. Overall, metal stressed larvae and their corresponding adult tissues were less robust, pale and translucent.

Aedes aegypti 3rd instars responded to Cu²⁺ in a dose dependent manner in a 24 h acute toxicity assay (Fig. 2). The lowest observed effect concentration (LOEC) was 1.6 ppm and

the lethal concentration of 50% of the sample population (LC_{50}), was 2.06 ppm. *A. aegypti* was found to be sensitive to Cu^{2+} and exhibited a narrow response range (~3ppm), *i.e.*, the difference between the LOEC and the concentration that resulted in 100% mortality.

Larvae reared under the metal stress treatment to the 3rd instar stage exhibited significantly longer survivorship (median LT_{50} , 44 vs. 14 h) in an acute toxicity assay than larvae reared in water without Cu^{2+} (Fig. 3).

Metal stress and neutral storage lipids

There was a nutritional cost associated with metal stress. Larvae held in 1.25 ppm Cu^{2+} for 5 d resulted in a 58% greater reduction in neutral lipid reserves compared to those held in clean water (Fig. 4). Fourth instar larvae stained with Oil Red O visually confirmed this reduction in neutral lipid reserves (Fig. 4 inset).

AaMtn was expressed predominately in the midgut of larvae

AaMtn expression in midguts of larvae held in clean water was ~20 fold higher than *AaMtn* expression in the rest of the body representing the basal metabolic state of *AaMtn* without any metal stress; however, under metal stress (4th instars exposed to 1.25 ppm Cu^{2+} for 6 h) *AaMtn* midgut expression was almost ~40 fold greater than the rest of the body indicating that *AaMtn* is up regulated in the midgut (Fig. 5) (*t* test, $p < 0.01$). Midguts of larvae exposed to metal stress expressed approximately twice the *AaMtn* than midguts of larvae held in clean water. However, *AaMtn* expression in metal stressed body tissues and body tissues exposed to clean water were virtually the same.

AaMtn transcription of 1st instars was highly dynamic

Newly emerged 1st instars exposed to 1.25 ppm Cu^{2+} for 12 h expressed *AaMtn* at extremely high relative levels compared to larvae held in clean water (121 fold increase at 6 h and 12 fold increase at 12 h) (Fig. 6). The reduction in relative increase was mostly a result of the increase in basal *AaMtn* expression of larvae held in water without Cu^{2+} . Basal *AaMtn* expression increased tenfold from time points 6 to 12 h. Measurable differences in *AaMtn* expression were observed within 30 min. (not shown). *AaMtn* transcription was also reversible, *AaMtn* expression dramatically decreased in metal exposed larvae that were transferred back to clean water to levels not significantly different than those held in clean water for the entire 12 hours.

Larval metal stress is reflected in adult phenotype

Adult females reared from metal stressed larvae were 17% smaller (*t* test, $P < 0.0001$), with a 20% decrease in starvation tolerance (log rank, $P < 0.0001$), with 39% less neutral lipids ($P < 0.04$, *t* test), 19% greater longevity (log rank, $p < 0.0001$), 14% fewer follicles ($P < 0.0001$, *t* test) and produced filial 1st instar larvae that also had 19% lowered starvation tolerance (log rank, $P < 0.0001$).

Discussion

Metal Tolerance and impact of copper exposure on performance traits

Metal tolerance, the physiological adaptation of an individual over the course of its lifetime to metal stress, is the result of the up regulation of detoxification, sequestration and excretion mechanisms and the down regulation of uptake mechanisms. *Aedes aegypti* is subject to not only the selection pressure of metals in the urban environment, but also suffers the physiological stress associated with it. *Aedes aegypti* are challenged with the need to protect themselves from potentially toxic metals like Cu^{2+} that are at the same time essential to basal metabolism (Balamurugan, 2007). The results of this work have demonstrated that the *A. aegypti* mosquito is sensitive to metal stress and that even a relatively low concentration of metal exposure results in distinctly different larval, adult and F_1 larval phenotypes with different morphological and physiological features. The observed adult phenotypes resulting from larval metal stress result in performance costs similar to those described by other authors (Kosalwat & Knight, 1987b; Mireji *et al.*, 2010). The tissues of the adult mosquitoes reared under the metal stressed environment were less robust in appearance than their clean reared counterparts and the adults and their progeny displayed compromised performance overall. The pale translucent appearance of the tissues is likely the result of diminished lipids reserves (Perez & Noriega, 2012).

Chronic and sub lethal dose effects of metals have been shown in a variety of other dipteran taxa (Kosalwalt & Knight, 1987a; Mireji *et al.*, 2010; Marinkovic *et al.*, 2011). The dose response curve produced under these conditions for Cu^{2+} exhibited a narrow response range of ~3ppm that is similar to the work of Kosalwalt & Knight with midges under similar conditions (Kosalwalt & Knight, 1987b). Rayms-Keller reported a very different LC_{50} for Cu^{2+} in *A. aegypti*, but under very different laboratory conditions (Rayms-Keller *et al.*, 1998). A narrow response range, *i.e.*, a steep dose response curve, suggests that there may be only one mechanism of detoxification (Newman & Unger, 2002); once this mechanism is saturated, Cu^{2+} ions are free to cause oxidative stress and cellular damage resulting in mortality suggesting that there is no redundancy for Cu^{2+} stress management in *A. aegypti*.

Chronic sub-lethal exposure to Cu^{2+} resulted in metal tolerant larvae; this could have occurred in two ways - selection for metal resistant larvae or the induction of metal detoxification mechanisms. Previous work has described ~50% mortality in the metal stress treatment under these same conditions, possibly selecting for surviving individuals that are metal resistant (Perez & Noriega, 2012). The alternative scenario is that the genes responsible for Cu^{2+} metabolism had been up regulated at the time of the acute toxicity assay as a result of Cu^{2+} exposure. Metal detoxification is controlled to a large extent by the zinc finger transcription factor, metal response element-binding transcription factor-1 (MTF-1) (Balamurugan *et al.*, 2004). A major target gene of MTF-1 is metallothionein. In addition, glutathione *S*-transferases confer tolerance to oxidative stress (Leiers *et al.*, 2003) and cytochrome P450-dependent monooxygenases have been shown to be altered by exposure to metals (Poupardin *et al.*, 2008). Cu^{2+} transporter (Ctr1) likely also plays a role by its down regulation (Turski & Thiele, 2007). Transcription for all of these genes could have been altered by exposure to Cu^{2+} to give larvae reared in Cu^{2+} a physiological

advantage in the toxicity assay, *i.e.*, hormesis. Hormesis is defined as a favourable biological response to low exposures of a stressor (reviewed in Sinclair, 2005). Regardless of the mechanism, larvae reared under metal stress have the potential to tolerate higher subsequent metal stress.

Overall metal stress resulted in compromised adult performance, *i.e.*, lowered adult body mass and neutral storage lipids at emergence, starvation tolerance, fecundity and starvation tolerance of offspring when compared to their non-metal stressed counterparts. Taken together these are negative performance costs of coping with metal stress. On the other hand, metal stress resulted in a hormetic effect in adults too, *i.e.*, increased longevity. Longevity has been shown to be increased by moderate metal or oxidative stress in several studies with *Drosophila melanogaster* and *Caenorhabditis elegans* (Barsyte *et al.*, 2001; Broughton *et al.*, 2005; Baldal *et al.*, 2006).

AaMtn expression and the midgut

This study has shown the potentially high transcription activity of the *AaMtn* gene to be dynamic, reversible and expressed mostly in the midgut thereby underscoring the role of the midgut as a defensive barrier. *AaMtn* is dramatically up regulated in response to Cu^{2+} in 1st instar *A. aegypti*, a critical life stage where most mortality in Cu^{2+} reared larvae occurs (Perez & Noriega, 2012). The insect midgut is recognized as a barrier to parasites, xenobiotics and pathogens *via* the peritrophic matrix, a protective lining of the midgut (Beatty *et al.*, 2002). Intestinal mucin and alpha tubulin are constituent proteins of the peritrophic matrix and these genes have been shown to respond to metal exposure. This, however, is the first report of metallothionein (Mtn) expression in the midgut of mosquitoes demonstrating a tissue specific physiological role for this protein in a disease vector. This work has demonstrated that *AaMtn* is highly inducible by Cu^{2+} and that *AaMtn* expression in body tissues (without midgut) of both clean reared and metal stressed treatments were virtually the same suggesting that Cu^{2+} did not go beyond the midgut tissue. These results suggest that the midgut is the primary tissue of *AaMtn* expression and acts as a protective barrier for metal stress. These results are in agreement with the findings of Egli *et al.*, who demonstrated in *D. melanogaster* that metallothioneins are expressed in a tissue-specific manner in response to Cu^{2+} , *i.e.*, the midgut (Egli *et al.*, 2006). Taken together, the dynamic and reversible nature of the Mtn gene along with its tissue specificity might explain why it has sometimes been problematic to use as a biomarker for environmental contamination (Roesijadi, 1992; Amird *et al.*, 2006).

Coping with metal stress comes at a cost however. Several studies have shown that metal stress decreases lipid reserves, increases respiration rate or alters the availability of energy yielding substrates (Servia *et al.*, 2006). Under the experimental conditions of this work, larvae exposed to chronic sub-lethal Cu^{2+} utilized more neutral storage lipids than larvae not exposed to Cu^{2+} . These results are in agreement with Servia *et al.* (2006) as well as other investigators and suggest that metal tolerance physiology is a lipid dependant process with neutral storage lipids fuelling *AaMtn* expression.

Implications for vector capacity

It is possible larval metal stress - even at relatively low, ecologically relevant concentrations - might alter adult mosquito performance and susceptibility to viral infection in much the same manner that larval stress increases vector competence as previously described (Alto, *et al.*, 2005a, b, 2008). Briefly, these authors demonstrate repeatedly that larval stress in the form of heat stress, competition, insecticide exposure and nutrient limitation each caused alterations in adult mosquito phenotype and immunity that increased their susceptibility to arboviruses. Larval metal stress is might possibly do the same. Adaptations conferring tolerance to stress, such as metals, are often costly to the organism's fitness and performance (Sakar, *et al.*, 2004; Shirley & Sibly, 1999). Metal resistant strains of mosquitoes exhibit decreased egg viability, increased larval mortality, decreased pupation, emergence and adult survivorship when compared to non-selected strains (Mireji *et al.*, 2010). It has also been shown that insecticide tolerant strains of anopheline and aedine mosquitoes exhibit increased fitness costs (Hemingway & Ranson, 2000). Our results demonstrating the compromised performance of adult female mosquitoes as a consequence of larval metal stress are in agreement with other work indicating that larval stress alters adult performance (Mireji *et al.*, 2010). Three mechanisms have been proposed that might lead to such performance costs: a) detoxification mechanisms might use energy and nutritional resources that are then unavailable for other performance traits such as growth or reproduction (Servia *et al.*, 2006; Sorvari *et al.*, 2007), b) tolerant individuals might be less efficient at metal uptake or utilization resulting in micronutrient deficiency in unpolluted environments and c) oxidative stress may alter gene regulatory mechanisms involving redox signalling (Kitamura & Hiramatsu, 2010).

Many questions remain unresolved regarding the fitness costs and vectorial capacity of insects that are tolerant to pollutants. The effect of insecticide resistance on vector capacity has been extensively investigated, but not the effect of environmental contaminants. The results of this work indicate that evaluating the effect of larval metal stress on vectorial capacity in a future study could be of value.

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Fig. 1.

Low sub-lethal metal stress compromised larval and adult phenotypes: Larvae reared in clean water (A) are easily distinguishable after 24 h from those reared 1 ppm Cu^{2+} (B). Ovaries (ov) and Malpighian tubules (mt) dissected from 3 d old adults were also compromised as a result of low sub-lethal larval metal exposure (C). Metal stressed larvae and adult tissues were less robust, pale and translucent in appearance (all images-40 $\times$ magnification).

Fig. 2.

Dose response curve for copper: *A. aegypti* 3rd instars responded to Cu²⁺ in a dose dependent manner in a 24h acute toxicity assay (LOEC = 1.6, LC₅₀ = 2.06 ppm), ($n = 25$, assayed in triplicate) (Sigmoidal nonlinear curve fit, $r^2 = 0.95$). Brackets indicate concentrations at which mosquito larvae were reared for subsequent experiments throughout this work.

Fig. 3.

A. aegypti 3rd instars exhibited tolerance: Larvae reared in 0.75 ppm Cu²⁺ to the 3rd instar stage exhibited significantly longer survivorship (median LT₅₀, 44 vs. 14 h) in an acute toxicity assay (2.0 ppm Cu²⁺) than larvae reared in clean water (n=20 performed in duplicate, log rank test, Chi square = 4.65, *P* = 0.03).

Fig. 4.

The nutritional cost of metal stress: Fourth instar larvae held in 1.25 ppm Cu^{2+} for 5 d resulted in a reduction in neutral lipid reserves (NSL) compared to larvae held in clean water ($P = 0.018$, students t test, one tailed) ($n=3$ assayed in triplicate). Representative image of fourth instar larvae stained with Oil Red O; ovals indicate area where fat body is located (inset).

Fig. 5.

AaMtn was expressed predominately in the midgut of larvae: (A) *AaMtn* expression in midguts (mdgt) of 4th instars held in clean water and exposed to 1.25 ppm Cu^{2+} for 6 h was higher than *AaMtn* expression in the rest of the body. (B) Midguts of larvae exposed to metal stress expressed ~2 the *AaMtn* than midguts of larvae held in clean water. *AaMtn* expression in body tissues of metal exposed and clean exposed were the same (t test, one way, $P < 0.01$, $df = 8$) (Three independent biological replicates of five tissues each) (mdgt = midgut; body = rest of the body minus midgut; cln = clean reared; mtl = metal stress).

Fig. 6.

AaMtn transcription is highly dynamic: Newly emerged 1st instars were exposed to 1.25 ppm Cu²⁺ for 12 h or held in clean water. For the rescue effect, larvae that were metal exposed to Cu²⁺ for 6 h were transferred back to clean water and held for 6h. *AaMtn* mRNA is expressed as copy number of *AaMtn* mRNA/ 10,000 copies of rpl32 mRNA. Each q-PCR data point is a mean $\pm$ SEM of three independent biological replicates of ~50 larvae (one way ANOVA with a Tukey's multiple comparison test, $F=7.02$, total df = 19, $P = 0.0022$). Letter designations indicate significant differences for multiple comparison tests ($P < 0.05$).