RESEARCH ARTICLE

Transcriptional responses of *Anopheles gambiae s.s* mosquito larvae to chronic exposure of cadmium heavy metal [version 1; referees: awaiting peer review]

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Abstract

Background: *Anopheles gambiae* larvae traditionally thrive in non-polluted environments. We previously documented the presence of the larvae in heavy metal polluted urban aquatic environments and the associated biological cost. The goal of this study was to unravel the molecular dynamics involved in the adaptation of the mosquitoes to the heavy metals.
Methods: Total RNA was extracted from third instar larvae of both cadmium treated populations and untreated control populations. The RNA concentrations were normalized and complementary DNAs were prepared. Then annealing control primer (ACP) technology was applied to establish transcriptional responses in *An. gambiae* larvae following several generational (n=90) chronic exposures to cadmium. Differentially expressed genes were determined by their differential banding patterns on an agarose gel. Gel extraction and purification was then carried out on the DEGs and these were later cloned and sequenced to establish the specific transcripts.
Results: We identified 14 differentially expressed transcripts in response to the cadmium exposure in the larvae. Most (11) of the transcripts were up-regulated in response to the cadmium exposure and were putatively functionally associated with metabolism, transport and protein synthesis processes. The transcripts included ATP-binding cassette transporter, eupolytin, ribosomal RNA, translation initiation factor, THO complex, lysosomal alpha-mannosidase, sodium-independent sulfate anion transporter and myotubularin related protein 2. The down-regulated transcripts were functionally associated with signal transduction and proteolytic activity and included Protein G12, adenylate cyclase and endoplasmic reticulum metallopeptidase.
Conclusions: Our findings shed light on pathways functionally associated with the adaptation to heavy metals that can be targeted in integrated vector control programs, and potential *An. gambiae* larvae biomarkers for assessment of environmental stress or contamination.

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Introduction

Heavy metal pollution has become a global environmental problem and severely threatens biological diversity and human health. Our studies on adaptation to heavy metals have documented presence of the mosquitoes in polluted habitats (Mireji *et al.,* 2008) with growing evidence that this adaptation comes at a biological cost to the mosquito (Mireji *et al.,* 2010b). Similar biological costs to adaptations have also been observed elsewhere in *Culex pipiens* L responses to cadmium, copper, lead and mercury (El-Sheikh *et al.,* 2010). To date, molecular dynamics underpinning heavy metal tolerance in insects have been tied to transcripts and genes associated functionally with immunity (Sorvari *et al.,* 2007) and defense and repair mechanisms such as glutathione transferases and heat shock proteins (Liao & Freedman, 1998; Kim *et al.,* 2000; Stohs *et al.,* 2001). We have previously putatively implicated metallothioneins, alpha-tubulin and cytochrome p450 genes associated with defense, repair and pyrethroid metabolism mechanisms in insects with heavy metal tolerance, using single gene assessment approaches with *Anopheles gambiae* mosquito larvae (Mireji *et al.,* 2010b; Mireji *et al.,* 2006; Musasia *et al.,* 2013). Here, we have emulated *ab initio* relatively higher throughput annealing control primer (ACP) transcriptional profiling, to identify:

1) Pathways functionally associated with heavy metal adaptation observed in the field and their associated biological costs (Mireji *et al.,* 2008; Mireji *et al.,* 2010b); and

2) Potential *An. gambiae* larvae biomarkers that can be applied for assessment of environmental stress or contamination.

Methods

Sample insects

Anopheles gambiae s.s mosquitoes that had been collected from the Mbita field station (00.025'S, 34.013'E), Homa Bay County in Kenya were used for the study. The colony was kept in the Animal Rearing and Quarantine Unit (ARQU) at the International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya. Larval stages of *Anopheles gambiae s.s.* were selected for tolerance to cadmium heavy metal through chronic exposures of Maximun Acceptable Toxicant Concentration (MATC) that had been empirically determined (Mireji *et al.,* 2010a). Cadmium metal tolerant strains and control (untreated) strains of the mosquito were raised separately and in triplicates. All subsequent generations of the mosquito were subjected to chronic exposures of cadmium metal as described in Mireji *et al.,* (2010a). Standard Operating Procedure (SOP) for the rearing of *Anopheles* mosquitoes was followed for colony maintenance (Ford & Green, 1972). Cadmium used in our study was applied as Cadmium Chloride ($CdCl_2$) 99.99% pure (Fisher Scientific LLC, Fair Lawn, NJ, U.S.A).

RNA isolation

Total RNA was extracted from the third instar larvae of experimental and control *An. gambiae* populations using Trizol (Invitrogen). Quantification of the extracted RNA was done using the micro-spectrophotometer Genequant pro (Amersham Pharmacia Ltd., Bucks, UK). In addition, DNaseI digestion was carried out to remove any residual DNA that could present in the extracted RNA. Total RNA that was isolated and stored at -80°C.

GeneFishing™ Reverse Transcription

The total RNA extracted from experimental and control *An. gambiae* populations were normalized to same concentrations and directly used for the synthesis of first strand complementary DNA (cDNA) using reverse transcriptase (Hwang *et al.,* 2003). Reverse transcription was carried out in a final reaction volume of 20µl containing 2µg of the purified mRNA at 42°C for 1.5 hours. The components of the reaction were: 4µl of 5X reaction buffer (Promega, Madison, WI, U.S.A), 2µl of 10µmol cDNA synthesis dT-ACP 1 primer (5'- CGTGAATGCTGCGA CTACGATIIIII$(T)_{18}$-3'), 5µl dNTPS- 2mM each, 0.5µl RNase inhibitor(40U/µl, Promega) and 1µl Moloney murine leukemia virus reverse transcriptase (200U/µl, Promega). The synthesized first strand cDNA was diluted by adding 80µl ultra-purified water. Storage was at -20°C awaiting PCR procedure.

ACP based- GeneFishing™ PCR

Annealing control primer based PCR using the GeneFishing TM DEG kit from Seegene, Seoul, South Korea (Kim *et al.,* 2004), was used to determine differentially expressed genes in the heavy metal treated group and the control population.

Synthesis of the second strand cDNA and PCR was carried out in a single tube. The second strand was synthesized in one cycle of first stage PCR at 50°C, in a final reaction volume of 20µl. The components in the reaction tubes included 3–5µl of diluted first strand cDNA, 1µl 10Mm dT-ACP2 reverse primer (5'-CTGTG AATGCTGCGACTACGATIIIII$(T)_{15}$-3'), 10µl 2x master mix (Seegene, Seoul, South Korea) and 1µl 10µM arbitrary ACP (forward primer).

PCR procedures for the synthesis of the second strand were completed in one cycle, at 94°C for 1 min then 50°C for 3min and 72°C for 1 min.

The second stage of the PCR protocol consisted of 40 cycles at 94°C for 40s, 65°C for 40s, 72°C for 40s and the final extension for 10 min at 72°C. 2% agarose gel electrophoresis with ethidium bromide staining was used for separation of the PCR products.

Gel extraction

Differentially expressed bands in the control and cadmium exposed population subjected to the same primer set were excised from the agarose gel using a scapel under Ultra Violet illumination. The gel slices were then purified using the QIAquick® gel extraction kit (QIAGEN, Inc., Valencia, CA), following the instructions from the manufacturer.

Cloning

Gel-purified PCR products were directly cloned into a pGEMT Easy vector (Invitrogen, Carlsbad, CA, USA), using JM109 competent cells. Colonies were grown at 37°C for 18 hours on Luria broth agar plates, containing ampicillin, X-gal and IPTG for blue/white colony screening. Cloned plasmids were then purified using the GeneJET™ Miniprep kit (Fermentus, Thermo Fisher Scientific Inc.), as per the instructions from the manufacturer.

Sequencing

Sequencing was done with ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using M13 primers. The sequences were edited using VecScreen and BioEdit software. Edited sequences were analyzed by searching for similarities in VectorBase against the *Anopheles gambiae* PEST strain transcripts sequences, AgamP4.6 geneset using the BLASTn search program (Altschul *et al.,* 1990)

Results

We successfully implemented the ACP system to identify differentially expressed genes (DEGs) in larvae chronically exposed to cadmium, as previously demonstrated in blastocyst experiments (Cui *et al.,* 2005; Hwang *et al.,* 2004; Hwang *et al.,* 2005). Our differential banding patterns of the cDNA representation of DEGs is summarized in Figure 1. Fourteen DEGs were identified after chronic exposure of *An. gambiae* larvae to cadmium heavy metal (Table 1). Most (11) of the differentially expressed genes were induced in cadmium exposed relative to the cadmium un-exposed controls. Our BLAST (REF) results revealed that the cadmium induced transcripts were clustered into metabolism (AGAP008584-RA, AGAP001249-RA and AGAP009563-RA), transport (AGAP012302-RA and AGAP002638-RA) and protein synthesis (AGAP028915-RA, AGAP004750-RA, AGAP028391-RA, AGAP003870-RA, AGAP028907-RA, AGAP028818-RA and AGAP028899-RA) processes.

Three of the DEGs identified were suppressed in the cadmium exposed larvae and these included AGAP006187-RA, AGAP002262-RA and AGAP003078-RA.

Dataset 1. Dataset 1: Sequence data obtained after sequence analysis using the BioEdit software

http://dx.doi.org/10.5256/f1000research.13062.d187045

The sequences were subsequently taken through a BLAST search. The results of the sequence analysis are shown on the manuscript.

Dataset 2. Dataset 2: Sample of the colony PCR experiment

http://dx.doi.org/10.5256/f1000research.13062.d187046

The gel photo of a colony PCR of 20 samples that was carried out after blue/white colony screening using M13 primers.

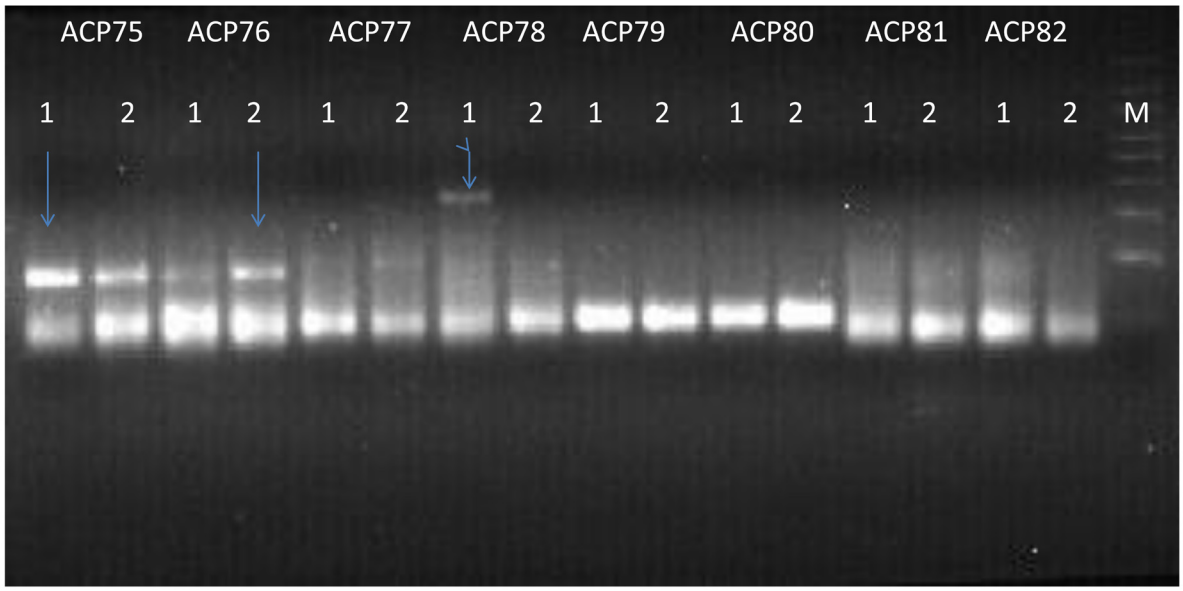

Figure 1. Differential cDNA banding patterns in cadmium treated and control population of mosquito larvae. The arrows indicate the DEGs observed using ACP 75, ACP 76 and ACP 78 primer set. Number 1 represents Cadmium population while 2 represents control population. M= 50bp molecular marker. High intensity of a band represents an up-regulation of a particular gene in cadmium or control population.

Table 1. Blastn results from VectorBase. Sequence data obtained was blasted against *Anopheles gambiae* PEST strain transcript sequences, AgamP4.6 geneset in May 2017.

Gene	Gene name	Description of gene product	E-Value	% ID	Expression pattern
AGAP002638-RA	ABCH1	ATP-binding cassette transporter (ABC transporter) family H member 1	3	77.5	Up
AGAP001249-RA		Eupolytin	3e-31	98.7	Up
AGAP028915-RA	SSU_rRNA_eukaryotic	Eukaryotic small subunit ribosomal RNA	8e-79	98.2	Up
AGAP004750-RA		Translation initiation factor 4G	6.4	87	Up
AGAP028915-RA	SSU_rRNA_eukaryotic	Eukaryotic small subunit ribosomal RNA	8e-78	99.4	Up
AGAP006187-RA		Protein G12	6.8	100	Down
AGAP003078-RA		Endoplasmic reticulum metallopeptidase 1	1.5	80.6	Down
AGAP028391-RA	lsu rRNA		3e-103	100	Up
AGAP028915-RA	SSU_rRNA_eukaryotic	Eukaryotic small subunit ribosomal RNA	4e-49	96.6	Up
AGAP028915-RA	SSU_rRNA_eukaryotic	Eukaryotic small subunit ribosomal RNA	5e-81	98.8	Up
AGAP003870-RA	Thoc7	THO complex subunit 7	6.4	87	Up
AGAP008584-RA		Lysosomal alpha-mannosidase	3.4	90.5	Up
AGAP010252-RA	RpL23	60S ribosomal protein L23	4e-12	100	Up
AGAP028907-RA	SSU_rRNA_eukaryotic	Eukaryotic small subunit ribosomal RNA	3e-06	91.2	Up
AGAP028818-RA	5_8S_rRNA	5.8S ribosomal RNA	3e-37	98.9	Up
AGAP028899-RA	SSU_rRNA_eukaryotic	Eukaryotic small subunit ribosomal RNA	2e-08	100	Up
AGAP009563-RA		Myotubularin related protein 2	0.74	91.3	Up
AGAP002262-RA		Adenylate cyclase 8	9.6	100	Down
AGAP012302-RA		Sodium-independent sulfate anion transporter	0.36	88.9	Up

Discussion

We identified ATP-binding cassette transporters belonging to the superfamily of membrane proteins that are present in all living organisms (Dean & Annilo, 2005). They are normally associated with movement of substrates such as amino acids, peptides, sugars, metals, inorganic ions, lipids, lipopolysaccharides and xenobiotics across biological membranes (Dawson & Locher, 2006; Hollenstein *et al.,* 2007a). The ABC transporters have been shown to affect development, metabolism and insecticide resistance in insects (Borycz *et al.,* 2008; Dow & Davies, 2006; Ricardo & Lehmann, 2009; Vache *et al.,* 2007). The silencing of the ABCH1 gene has been shown to result in the death of larvae and pupae (Guo *et al.,* 2015). Therefore, induction of the ABC transporters may suggest that they are involved in cadmium transport through membranes to reduce toxicity of cadmium metal to the larvae in their environment.

The induction of the eupolytin gene may have a role in the activation of defense molecules. In a study involving the ground beetle *Eupolyphaga sinensis*, eupolytin-1 gene encoding a protease was shown to be involved in the activation of plasminogen and hydrolysis of fibrinogen (Yang *et al.,* 2011).

Ribosomal genes are involved in protein synthesis and upregulation of the various arrays of ribosomal RNAs in this study, which suggests their role in enhancing the survival of *An. gambiae* in the heavy metal polluted environment by the transcription and translation of genes which are important in the adaptation of the larvae to xenobiotics.

The nuclear structure referred to as THO complex is usually conserved in all kingdoms, and it has an important role in the packing of pre-mRNA molecules into RNA-protein assemblies which are termed mRNPs (Köhler & Hurt, 2007). A study carried out recently has shown that the THO complex is required for efficient expression of some genes, ensuring genetic stability thereby preventing transcription-associated recombination (Gewartowski *et al.,* 2012). The expression of the THO complex is suggestive of its role in expressing defense genes that enhance survival of larvae in a Cadmium polluted environment.

Suppression of AGAP006187-RA, AGAP002262-RA and AGAP003078-RA transcripts that included G- Proteins couple receptors to adenylyl cyclase stimulation indicated increasing levels of cAMP and a cascade of events that constitute the signal transduction pathway that drive cellular responses. Metallopeptidases are a ubiquitous and diverse group of enzymes containing both endopeptidases and exopeptidases. Although they vary widely at the sequence, structural, and functional levels, they all require a metal ion for catalytic activity (Rawlings & Salvesen, 2013).

The suppression of these important genes involved in signal transduction and proteolytic activity would account for the larval mortality rates that are usually observed in larvae raised in the cadmium heavy metal environment.

Our findings shed light on the adaptation of the *An. gambiae* mosquito to heavy metals by differentially expressing particular genes in response to a toxicant impact. A study to determine differentially expressed genes in cadmium-exposed *sebastes schlegeli* unraveled genes related to pathogenesis, extrinsic stresses, and catalytic metabolites (Woo & Yum, 2014). Previous studies have indicated that metallothionein and α-tubulin genes that are present in insects can be used as potential biomarkers (Hare, 1992; Klerks & Weis, 1987; Mattingly *et al.,* 2001; Roesijadi, 1994). Metallothionein was assessed through *C. quinquefasciatus* mosquito larvae for Copper, Cadmium and Zinc aquatic environmental levels (Sarkar *et al.,* 2004). Therefore, the genes identified might be useful in the development of potential biomarkers that can be used to assess the level of environmental pollution of heavy metal origin in *An. gambiae* mosquitoes.

Conclusions

We were able to identify genes that are differentially expressed due to chronic exposure of *An. gambiae* larvae to cadmium metal using the ACP-based PCR method. However, application of more sensitive techniques like those used in proteomics would generate more data.

Data availability

Dataset 1: Sequence data obtained after sequence analysis using the BioEdit software. The sequences were subsequently taken through a BLAST search. The results of the sequence analysis are shown on the manuscript. DOI, 10.5256/f1000research.13062.d187045 (Muturi *et al.,* 2017a).

Dataset 2: Sample of the colony PCR experiment. The gel photo of a colony PCR of 20 samples that was carried out after blue/white colony screening using M13 primers. DOI, 10.5256/f1000research.13062.d187046 (Muturi *et al.,* 2017b).

Competing interests

No competing interests were disclosed.

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Data Source

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