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Expression of metallothionein and α-tubulin in heavy metal-tolerant *Anopheles gambiae* sensu stricto (Diptera: Culicidae)

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ABSTRACT

Anopheles mosquitoes have been shown to adapt to heavy metals in their natural habitats. In this study we explored the possibility of using *Anopheles gambiae* sensu stricto as bio-reporters for environmental heavy metal pollution through expressions of their metal-responsive metallothionein and α-tubulin genes. The study was undertaken with third instar larvae after selection by cadmium, copper, or lead at LC_{30} through five successive generations. Expression levels were determined in the 5th generation by semi-quantitative RT-PCR on the experimental and control populations. The data were analyzed using one-way ANOVA. The highest metallothionein ($F_{3,11}=4.574$, $P=0.038$) and α-tubulin ($F_{3,11}=12.961$, $P=0.002$) responses were observed in cadmium-tolerant treatments. There was significantly higher expression of metallothionein in cadmium or copper treatments relative to the control ($P=0.012$), and in cadmium than in lead treatments ($P=0.044$). Expressions of α-tubulin were significantly higher in cadmium than in control treatments ($P=0.008$). These results demonstrate the capacity of *An. gambiae* s.s. to develop tolerance to increased levels of heavy metal challenge. The results also confirm the potential of heavy metal-responsive genes in mosquitoes as possible bio-indicators of heavy metal environmental pollution. How the tolerance and expressions relate to *An. gambiae* s.s. fitness and vectorial capacity in the environment remains to be elucidated.

1. Introduction

There are currently seven recognized sibling species in the *Anopheles gambiae* complex, including *An. gambiae s.s.* and *An. arabiensis*, the most prolific and widespread African malaria vectors in sub-Saharan Africa. Both species are highly anthropophilic and their larvae typically thrive in structurally simple, sunlit, temporary water collections devoid of vegetation (Gillies and de Meillon, 1968). Although malaria is generally associated with rural areas in Africa, studies have documented frequent occurrence of *Anopheles* larvae in urban environments (Robert et al., 2003; Hay et al., 2005; Donnelly et al., 2005). An important question is how these mosquitoes respond to different characteristics encountered in these environments. Absence of favorable mosquito larval habitats in urban environments has been associated with reduction in mosquito species diversity (Chinery, 1984, 1995; Trape and Zoulani, 1987; Coene, 1993; Coluzzi, 1993). The species that succeed in these environments encounter a variety of larval habitats, some of which may be highly polluted with domestic and industrial sewage (Sattler et al., 2005; Awolola et al., 2007; Djouaka et al., 2007), and heavy metals (Mireji et al., 2008). Understanding the effects of environmental pollutants and responses of *Anopheles* mosquitoes is important for elucidating any adaptive changes that may take place and how these affect their population dynamics and vectorial capacity.

Tolerance to heavy metals in insects is associated with transcription of genes encoding for defense proteins such as metallothioneins, glutathione (Chin and Templeton, 1993; Coogan et al., 1994), α-tubulin and mucin (Rayms-Keller et al., 2000; Mattingly et al., 2001). Cysteine-rich, metal-binding metallothionein proteins have been implicated in regulating intracellular availability of some heavy metals (Klaassen et al., 1999). Recently,

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α-tubulin proteins, which are a component of the microtubules, have been linked to heavy metal tolerance (Mattingly et al., 2001). Expression levels of metallothionein or α-tubulin genes in aquatic organisms, such as *Anopheles* larvae, are useful indicators of evolving tolerance in the insect (Ayala and Coluzzi, 2005) and bio-reporters of environmental pollution by heavy metals (Reed et al., 2003).

In our previous study, we found evidence of global proteomic expressions in *An. gambiae* s.s. exposed to heavy metals (Mireji et al., 2006). This study explores the use of metallothionein and α-tubulin genes in *An. gambiae* s.s. as indicators of tolerance to some environmental heavy metal (copper, cadmium, or lead) pollutants.

2. Materials and methods

2.1. Heavy metals

Cadmium, copper and lead in the form of cadmium chloride ($CdCl_2$, 99.99%), copper II nitrate hydrate ($Cu(NO_3)_2 \cdot 2.5H_2O$, >99%), and lead II nitrate ($Pb(NO_3)_2$, 99.5%) were obtained from Fisher Scientific, Fair Lawn, NJ, USA, Sigma-Aldrich, Laborchemikalien, GMBH, Germany and Prolabo, Fontenay, France, respectively.

2.2. Test insects

An. gambiae s.s. (Mbita strain) larvae collected from water bodies at Mbita Point, Suba District, western Kenya (Seyoum et al., 2002), were maintained in an insectary at the International Center of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya, at a density of 500 larvae per 3 liters of distilled water. To simulate natural conditions, the average temperature in the insectary was 29±2 °C (day) and 24±2 °C (night), with relative humidity (RH) ranging from 57±4% (day) to 72±5% (night). Larvae were fed Tetramin fish food daily. Adult mosquitoes were kept in standard 30×30×30 cm^3 cages in an insectary at 27±2 °C, 65–70% RH and a photoperiod of 12:12 h (L–D); 6% glucose solution was offered *ad libitum*. Three- to four-day-old females were starved for 12 h, after which they were allowed to feed regularly on an anaesthetized mouse for approximately 10-min at 18:00 h. Engorged females were left in the cages until gravid.

Overall maintenance of the colony followed standard operating procedure for rearing *Anopheles* mosquitoes (Ford and Green, 1972). Approval for feeding mosquitoes on mice was obtained from the Kenya National Ethical Review Board (protocol number KEMRI/RES/7/3/1); the protocol was reviewed and approved by the KEMRI Animal Care and Use Committee (ACUC).

2.3. Selection for heavy metal tolerance

An. gambiae s.s. third instar larvae were selected for heavy metal tolerance in the F_1–F_5 generations by exposing them to lethal concentrations (LC_{30}) of cadmium, copper, or lead resulting in 30% mortality. The usual practice of selecting for 50% mortality (LC_{50}) resulted in adult survival rates that were too low for analysis. In each of 3 replicates for each metal treatment, 300 larvae were selected from each generation and exposed to the test treatment for 24 h in 1500 ml of water in polypropylene cylindrical pans (radius 10.5 cm and height 24.1 cm). The larvae were not fed during the exposure period. The susceptibility of *An. gambiae* s.s. to each metal in successive generations was monitored by determining the LC_{50} values, as indicated below. A control colony not exposed to metals was reared simultaneously in a separate room and handled in the same manner through all manipulations. Twenty-five LC_{30} survivors from the 5th-generation selection were sampled from each treatment and replicate, and were frozen at −70 °C for subsequent metallothionein and α-tubulin expression analyses.

2.4. Acute toxicity tests

Twenty-four-hour toxicity range tests of cadmium, copper, or lead were conducted on each generation using third instar *An. gambiae* s.s. larvae. Three replicates ($n=25$ larvae per replicate) were exposed to five lead, cadmium, or copper concentrations within established toxicity-response ranges (Finney, 1971), in 400 ml of distilled water in the polypropylene cylindrical pans. The concentrations were validated by direct quantitative determination of cadmium, copper or lead separately in each exposure concentration and replicate using a Buck Scientific 210VGP flame atomic absorption spectrophotometer (Buck Scientific, East Norwalk, Connecticut, USA). Quality control was achieved using certified reference sediment material for cadmium, copper, and lead (IAEA 433) from the International Atomic Energy Agency (Wyse et al., 2004). Larval mortalities were evaluated 24 h post-exposure; lethal concentrations and; linear regressions were conducted using probit analysis (Finney, 1971). Changes in slope between generations indicated development of tolerance (Brown and Pal, 1971). The LC_{30} was used for selection, while LC_{50} was used to assess changes in tolerance.

2.5. RNA and DNA extraction

Total RNA was isolated from each of the frozen (−70 °C) larval samples using the guanidine isothiocyanate-based protocol of the Total RNA Isolation System® (Promega, Madison, WI). Integrity of extracted RNA was validated by electrophoresis in 1.0% agarose (Sigma-Aldrich Chemie, Gmbh) RNA denaturing gel in 1.4% sodium phosphate with 1 μg/ml ethidium bromide staining for visualization. The yield and quality of RNA was determined spectroscopically (Sambrook et al., 1989). The genomic DNA was extracted from a pool of 25 third instar *An. gambiae* larvae by conventional phenol-chloroform DNA extraction method with RNAase (Sambrook et al., 1989). The RNA and DNA extracted were used for cDNA synthesis and for detection of gDNA contamination in each transcription product, respectively.

2.6. Reverse transcriptions

Reverse transcriptions were conducted using TaqMan Reverse transcriptase reagents (Applied Biosystems, Foster City, CA). Oligo $d(T)_{16}$ was used as primer in the first step of cDNA synthesis in the reaction mix. The mix consisted of 1× TaqMan RT buffer, 5.5 mM $MgCl_2$, 1.5 μg total RNA, 2.5 μM oligo-dT, 0.4 U/μl RNAse inhibitor, 500 μM dNTPs, 125 U/μl Multiscribe Reverse Transcriptase and H_20 in a total volume of 10 μl. This was incubated at 25 °C for 10 min to maximize primer-RNA template binding, reverse-transcribed at 48 °C for 30 min, and the reverse trancriptase inactivated at 95 °C for 5 min. The cDNA generated was stored at −20 °C.

3. PCR

3.1. Primer selection

3.1.1. Metallothionein

An. gambiae metallothionein gene (Genebank accession no. AAX86006) was obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) database using bioinformatic searches. In order to discriminate against possible genomic DNA in the cDNA, metallothionein primers were manually designed from the genomic sequence such that the 5′ and the 3′ primer pair spanned different exons.

3.2. α-Tubulin

A functional homolog of previously characterized heavy metal-responsive *Chironomus tentans* α-tubulin gene (Genebank accession no. AF272829) (Mattingly et al., 2001) in *An. gambiae* was identified by BLAST analysis (Altschul et al., 1997) of the gene against the *An. gambiae* genome (Holt et al., 2002). The resultant BLAST results were screened for α-tubulin conserved domain through InterProScan (Mulder et al., 2003) analysis, following which the homolog (Genebank accession no. XM_309723) was isolated. Primers for amplification of this homolog were designed *in silico* using primer3 software (Rozen and Skaletsky, 2000).

3.3. Ribosomal protein S7

An. gambiae Ribosomal Protein S7 (RP S7) gene was selected as an internal neutral/loading control (Salazar et al., 1993). The DNA sequence of the gene (Genebank accession no. AAA03087) was obtained from the genomic database maintained by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The primers were designed using similar procedure to those used for α-tubulin primers described above. The following primers were obtained: metallothionein 5′-ATGCCCTGCAAGTGCTGTGG-3′ and 5′-GAGCCGTACAACTCCATCCTG-3′, α-tubulin 5′-GGCGATCATCATCTACGTTGC-3′ and 5′-GCAGCCGCAGCAACCGCCGGAC-3′, and