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Pyrethroid tolerance is associated with elevated expression of antioxidants and agricultural practice in *Anopheles arabiensis* sampled from an area of cotton fields in Northern Cameroon

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Abstract

Spraying of agricultural crops with insecticides can select for resistance in nontarget insects and this may compromise the use of insecticides for the control of vector-borne diseases. The tolerance of the malaria vector, *Anopheles arabiensis* to deltamethrin was determined in a field population from a cotton-growing region of Northern Cameroon both prior to and midway through the 4-month period of insecticide application to the cotton crop. A 1.6-fold increase in the median knockdown time was observed. To determine whether this increased tolerance was associated with constitutively elevated levels of genes commonly associated with insecticide resistance, RNA was extracted from F_1 progeny from family lines of field-caught mosquitoes and hybridized to the *Anopheles gambiae* detox chip. The experimental design avoided the confounding effects of colonization, and this study is the first to measure gene expression in the progeny of gravid, wild-caught mosquitoes. Several genes with antioxidant roles, including superoxide dismutases, a glutathione S-transferase and a thioredoxin-dependent peroxidase, and a cytochrome P450 showed elevated expression in mosquito families collected during the insecticide-spraying programme. These genes may constitute an important general defence mechanism against insecticides. Intriguingly, the levels of expression of these genes were strongly correlated suggesting a common regulatory mechanism.

Keywords: cytochrome P450, detox chip, insecticide resistance, malaria, metabolic detoxification, oxidative stress

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Introduction

The mainstays of vector-based malaria control programmes are insecticide-treated materials and indoor residual spraying (WHO 2000). Currently, the only commercially available class of insecticides with the efficacy and low mammalian toxicity requirements needed for impregnation of insecticide-treated materials are pyrethroids, and these insecticides are commonly used as residual sprays too. As a result, the emergence of pyrethroid resistance threatens to compromise success of insecticide-based malaria control (Elissa *et al*. 1993; Vulule *et al*. 1994; Chandre *et al*. 1999; Hargreaves *et al*. 2000; Awolola *et al*. 2002; Stump *et al*. 2004; N'Guessan *et al*. 2007).

It is often suggested that the extensive use of insecticides in agriculture contributes to the emergence of insecticide resistance in mosquitoes (Lines 1988). For the sub-Saharan malaria vector *Anopheles gambiae*, this hypothesis is supported by the observation of a spatial and temporal

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correlation between the use of insecticides in agriculture and the frequency of a knockdown resistance (*kdr*) allele in field populations (Diabate *et al*. 2002). The *kdr* alleles are characterized by single nonsynonymous point mutations in the voltage-gated sodium channel protein, which result in reduced sensitivity to DDT (dichlorodiphenyltrichloroethane) and pyrethroids (Martinez-Torres *et al*. 1998; Ranson *et al*. 2000). The impact of target site insensitivity on the efficacy of insecticide-treated nets (ITN) has been the subject of several somewhat contradictory studies (Kolaczinski *et al*. 2000; Hougard *et al*. 2003; Henry *et al*. 2005; N'Guessan *et al*. 2007). An important caveat underlying differences among these studies relates to neglect of the role of metabolic mechanisms in determining resistance phenotype. We set out to address this deficit by using a microarray-based approach to detect correlations between phenotypic resistance to pyrethroids and expression of 254 genes belonging to gene families commonly associated with resistance, in mosquitoes from an area of extensive agricultural use of insecticides. We selected Pitoa, a site in Northern Cameroon, where a broad spectrum of insecticides is intensively used in cotton field cultivation. The primary malaria vector in this region is *Anopheles arabiensis*, a member of the *Anopheles gambiae* species complex which comprises at least seven cryptic sibling species (Hunt *et al*. 1998). Adult *A. arabiensis* females bite and rest indoors or outdoors and, in contrast to *A. gambiae sensu strictu* (s.s.), which is also a major malaria vector in Africa, bite both humans and domestic animals such as cattle (White 1974). Larvae are often found in water bodies in the vicinity of agricultural farm land; hence, both adult and larval stages potentially come into contact with the insecticides.

The current study aims to identify candidate genes conferring metabolic-based resistance in the malaria vector *A. arabiensis* in a natural field setting. To assess the impact of selection, rather than an immediate response to the exposure to insecticides, both phenotype and gene expression is measured in the progeny of gravid, wild-caught females. Using a microarray, we determined transcription profiles of a suite of potentially insecticide-detoxifying genes and correlate this with the observed phenotype before and after selection by insecticides. A previous survey of the susceptibility status of *A. gambiae sensu lato* (s.l.) in Cameroon found increased tolerance to the pyrethroid deltamethrin but no evidence of the presence of either of the two known L1014F (Martinez-Torres *et al*. 1998) or L1014S (Ranson *et al*. 2000) *kdr* mutations at this location, making it an ideal location for studying metabolic resistance as the *kdr* target site mutation would be absent (Etang *et al*. 2003). Mosquitoes were collected prior to and midway through a 4-month period of insecticide application to the cotton crop in 2005. Median time to knockdown was used as an indicator of the level of pyrethroid tolerance in each individual F_1 family. RNA was extracted from the remainder of the progeny and hybridized to the *A. gambiae* detox chip (David *et al*. 2005), a custom made array that contains unique probes for the genes encoding the major detoxification and redox metabolism genes.

Materials and methods

Field collections and bioassays

Study site. All collections were carried out in the village of Pitoa, Northern Cameroon (9°21′N, 13°31′E). The study site lies within the Sudanese climate zone with an annual rainfall of 700–1000 mm. Malaria transmission is seasonal and occurs during the rainy season between May and October. As the production of cotton is an important income source for the region, insecticides are intensively used to protect cultivation from agronomical pests. According to the 'Société de développement du coton du Cameroun' (Sodecoton), cotton fields are sprayed fortnightly between July and October. The first two spraying interventions consist of carbamates, followed by a cocktail of pyrethroids and organophosphates.

Mosquito collections. Mosquitoes were collected, either as larvae from natural breeding sites located within the cotton fields or as blood-fed females using aspirators inside human dwellings approximately 300 m distant from the cotton fields. The collections were carried out prior to (July) and midway through (late August/early September) the 4-month period (July–October) of insecticide application to the cotton crop. Specimens were morphologically identified as members of the *Anopheles gambiae* species complex (Gillies & De Meillon 1968; Gillies & Coetzee 1987). A recent study showed two members of the *A. gambiae* species complex are found here with 85% belonging to *Anopheles arabiensis* and 15% to *A. gambiae* s.s. (Chouaibou *et al*. 2006).

World Health Organization test tube assays. Susceptibility of *A. gambiae* s.l. to 0.05% deltamethrin was assessed using World Health Organization (WHO) test kits according to standardized procedures (WHO 1998). During the 1 h exposure, time to knockdown was continuously recorded. Data were fitted by a log(exposure time)-logit logistic regression model (Pampel 2000) using the statistical software package R (http://www.r-project.org) to predict the exposure time which would knock down 50% (KDT_{50}) of the mosquito population.

Net assays. Gravid females collected from human dwellings were kept individually in plastic cups covered with netting and provided with sugar solution. Eggs collected on wet filter paper, placed at the bottom of the cups, were transferred to plastic bowls, and each family reared separately.

Larvae were provided with ground TetraMin Baby Fish Food. For species identification, DNA was extracted from dead mothers following Livak's protocol (Livak 1984) and diagnosed by the polymerase chain reaction (PCR) from Scott *et al*. (1993). One-day post-eclosure, adult females were either subject to a net bioassay or frozen down for microarray and quantitative reverse transcription PCR analysis (see below). From each family, we aimed to expose 11 females to deltamethrin-impregnated (25 mg/m^2) netting in a modified wire-ball knockdown assay (WHO 2006). Instead of a ball, the netting was wrapped around a frame (10 × 10 × 10 cm). The mosquitoes were introduced and the time to first knockdown recorded.

Knockdown resistance (kdr) *screen.* To control for the presence of *kdr* alleles in families analysed for gene expression, six female siblings from each family were screened for the presence of both the L1014F (Martinez-Torres *et al*. 1998) and L1014S (Ranson *et al*. 2000) *kdr* mutation by a hot oligonucleotide ligation assay (HOLA) (Lynd *et al*. 2005) from DNA extracted using Livak's method (Livak 1984).

Microarray experiments

Target preparation and microarray hybridizations. Adult mosquitoes were collected the morning after emergence and immediately killed on dry ice. Frozen mosquitoes were transferred to RNA*later* Ice (Ambion) and kept below –20 °C until further processing. Total RNA was extracted using the PicoPure RNA Isolation Kit (Arcturus) from batches of 10 female siblings per family. Prior to amplification extracted RNA was treated with DNase (RNase-Free DNase Set, QIAGEN). RNA quantity was then measured using a Nanodrop spectrophotometer (Nanodrop Technologies). Five microgram of total RNA from each batch were then reverse transcribed and amplified in one single round using the RiboAmp RNA Amplification Kit (Arcturus). Target samples for hybridization to the microarray were prepared from 8 μg of amplified sample RNA and 4 μg RNA control spike mix (Lucidea Universal Score Card, Amersham Biosciences). Prior to fluorescent-labelling, antisense RNA quantity and quality were again assessed by a spectrophotometer (Nanodrop) and agarose gel electrophoresis. Fluorescent nucleotides, Cyanine 3-dUTP (Cy3) or Cyanine 5-dUTP (Cy5) (GeneBeam, Enzo), respectively, were incorporated into first-strand cDNA by reverse transcriptase using random hexamer primers (Life Technologies) and Superscript III reverse transcriptase (Invitrogen). After degradation of the aRNA template strand using 1 μL 1 M NaOH, 20 mM EDTA and incubating for 5 min at 70 °C, cDNA targets were pooled together and purified using the CyScribe GFX Purification Kit (Amersham Biosciences). Dye incorporation efficiency was measured using a spectrophotometer (Nanodrop). After adding 5 μg of poly dA oligo (Amersham Biosciences) to prevent nonspecific hybridization, the labelled cDNA targets were vacuum-dried and resuspended in 15 μL formamide-based hybridization buffer (Corning). Labelled targets were hybridized to an updated version of the detox chip (David *et al*. 2005) for 16 h at 42 °C. The detox chip is a custom-made microarray chip which in its current version contains fragments of 254 *A. gambiae* s.s. genes from families generally associated with metabolic-based detoxification. These genes include 103 cytochrome P450s, 31 esterases, 35 glutathione S-transferases and 85 additional genes such as peroxidases, reductases, superoxide dismutases, ATP-binding cassette transporters, tissue-specific genes and housekeeping genes. Detailed annotation and description of the spotted probes can be found at ArrayExpress, Accession no. A-MEXP-862. After incubation, slides were washed according to the manufacturer's instructions and intensity images were scanned by a Genepix Personal 4100a microarray scanner (Axon Instruments) at a resolution of 10 μm.

Experimental design. Target RNA from each family was cohybridized with a common reference target. The common reference consisted of RNA pooled from a total of 150 individuals from the Kisumu laboratory strain. The common reference design was chosen to allow for comparing the families collected during the spraying programme with the families collected before the spraying programme. The indirect design made it also possible to relate resistance phenotype and expression levels family by family and to include an additional data set obtained from field-collected material in Kenya in the analysis. Each set of microarray experiments comparing the family with the common reference consisted of two technical replicates; amplified RNA from each sample was split into two aliquots which were cohybridized to two arrays while the Cy3 and Cy5 labels were swapped between hybridizations.

Microarray data analysis

Preprocessing. After visual inspection of each array, spot and background intensities were calculated from the scanned array images using GENEPIX Pro 5.1 software (Axon Instruments). Raw intensities were then analysed with LIMMA 2.4 software package (Smyth 2005) running in R. R is available from the CRAN repository http://www.r-project.org. LIMMA is part of the BIOCONDUCTOR project at http://www.bioconductor.org (Gentleman *et al*. 2004). Any spot meeting one of the following criteria was excluded from the analysis: (i) median intensity values for both channels below 300, (ii) signal-to-noise ratio below 3, and (iii) median intensity in one or both channels at saturation. For each spot, background intensities were subtracted from the total spot intensities using the method 'normexp' with an offset of 50. Background-corrected intensities were

Table 1 Oligonucleotide primer sequences used in the quantitative RT-PCR analysis

Gene	Accession no.	Primer	Sequence (5′– 3′)	Transcript length
GSTS1-2	GenBank: AF513639	Left	TTGTTATCGCGTGCAATGTC	552 bp
		Right	CGCTGATGATTGGGAGAACT	
SOD3B	GenBank: AY745233	Left	TACAATCCGGACGGGAACGA	301 bp
		Right	TCAGATGCAATCGTTCGGCA	
S7 ribosomal	GenBank: L20837	Left	GCACGTCGTGTTCATTGCCG	418 bp
		Right	GAACGTAACGTCACGGCCAGTCA	

transformed to intensity log-ratios which were first normalized within each array using the intensity-dependant global 'loess' function. In a second normalization step, intensity log-ratios were scaled to equalize the median absolute value across all arrays to account for technical biases between arrays (Smyth & Speed 2003).

Differential expression. For the comparison between the two family groups, collected either before or during the spraying programme, differences in relative expression levels were assessed by fitting linear models for each individual probe on the array using a common value for the between-replicate correlation (Smyth *et al*. 2005). To account for technical replicates (dye-swaps), measurements from the same RNA pool (families) were first averaged and then compared between two groups, collected either during or before the spraying programme. The contrast, i.e. the linear combination of the model's coefficients, mean relative expression levels during spraying programme minus mean relative expression levels before spraying programme, was then tested for significance using a moderate *t*-statistics (Smyth 2004). To account for multiple testing, *P* values were adjusted adopting the approach of Benjamini & Hochberg (1995) to control for the false discovery rate as described in (Smyth 2005).

Cluster analysis. In order to obtain a sufficiently large data set for a robust cluster analysis data from 12 families of *A. gambiae* s.s., the closely related sister species to *A. arabiensis* (Besansky *et al*. 2003), were combined with the *A. arabiensis* families collected in Pitoa. The *A. gambiae* s.s. families were collected from the Asembo Bay area, Nyanza Province in West Kenya, between April and July 2005. Field collections and microarray experiments followed identical protocols as for the Pitoa samples. However, the bioassays performed on the F_1 progeny differed in that mosquitoes were exposed to nets treated with α-cypermethrin instead of deltamethrin. In addition, the Asembo families showed the presence of the L1014S *kdr* mutation with a frequency of 35%. A relationship between resistance phenotype and either *kdr* status or gene expression could not be established from the Asembo data set, and in the present study, these families were solely included in the cluster analyses for measuring co-expression between loci covered by the detox chip. The cluster analysis was also run for the Pitoa and Asembo expression data separately to confirm the presence of a similar signature in each population thereby justifying the combination of the two data sets. To capture genes that are co-expressed, the standard Pearson correlation coefficient was computed first as a measure of similarity between any pair of genes. The resulting distance matrix then provided the basis to run the hierarchical ordered partitioning and collapsing hybrid (HOPACH) algorithm in R for building a hierarchical tree of clusters (van der Laan & Pollard 2003). For each gene in each cluster, the proportion of bootstrap data sets, with 1000 bootstrap resamplings where the gene is in the cluster, was computed as an estimate of membership of the gene. Data were visualized using the MAPLETREE 2.3 open source software available at http://mapletree.sourceforge.net.

Quantitative reverse transcription PCR

An aliquot of the RNA extracted for microarray experiments served as template for making cDNA by reverse transcription using an oligo $d(T)_{14}$ primer and Superscript III reverse transcriptase (Promega). The number of copies of *GSTS1-2* and *SOD3B* was calculated by measuring the incorporation of the fluorescent dye SYBR Green I (QIAGEN) into double-stranded PCR product using the primers in Table 1 and comparing this value to a calibration curve produced from the amplification of the same gene fragment from the plasmids of known concentration ranging from 1 ng/μL to 1 fg/μL. Primers were designed based on the *A. gambiae* s.s. genome available in Ensembl (Birney *et al*. 2006) and their specificity confirmed by sequencing genomic DNA from 10 *A. arabiensis* specimens from the families investigated in this study. The PCR conditions were 95 °C for 15 min, followed by 37 cycles of 94 °C for 20 s, 54 °C for 25 s and 72 °C for 30 s with the fluorescence read at 82 °C. Each family was measured in two runs and three repeats per run. The copy number of the ribosomal gene S7 [Accession no. AY380336] (Salazar *et al*. 1993) was used to normalize for variation in total cDNA concentration.

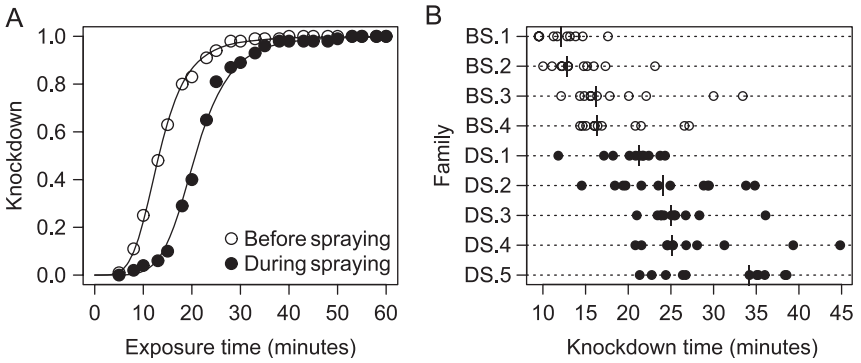

Fig. 1 Mosquitoes collected during the spraying programme showed extended knockdown times (KDT). (A) Proportion of *Anopheles gambiae* s.l. individuals knocked down as a function of exposure time to 0.05% deltamethrin according to WHO standards (WHO 1998) before and during the 2005 insecticide spraying programme in Pitoa, Northern Cameroon. Response curves are fitted to data using logit analysis (Pampel 2000). Mosquitoes collected during the spraying programme showed extended KDT; KDT_{50} increased by over 7 min from 13 min 12 s prior to 20 min 43 s during the spraying programme. (B) Bioassay data recorded for the *A. arabiensis* families analysed in the gene expression studies. Assays were performed on F_1 progeny from wild-caught, gravid mosquitoes. For each family, KDTs from 11 females exposed to deltamethrin-treated bed nets were recorded. (Exceptions are family BS.1 and DS.6 with 10 and 12 individuals, respectively.) Open circles, specimens collected before spraying programme (BS); filled circles, specimens collected during spraying programme (DS). Vertical bars show family-specific median KDT.

Results

Gene expression of 254 genes putatively involved in metabolic resistance and insecticide susceptibility was measured in F_1 progeny of nine families. Five families were collected before and five during the spraying programme. Due to poor quality of the extracted RNA from one of the five families collected before the sprays, only four families from this collection could be used in the analysis.

Levels of pyrethroid susceptibility before and during the cotton spraying programme

WHO susceptibility tests performed on adult *Anopheles gambiae* s.l. mosquitoes, reared from field-caught larvae, showed a decrease in mortality between tests performed before and during the spraying programme. As the WHO susceptibility tests were performed on adults emerging from larvae collected in the cotton fields, specimens could not be identified a priori at the species level using molecular markers. Subsequent molecular species identification on a random sample of 40 individuals per time point revealed 81% of the specimens belonging to *Anopheles arabiensis* and 19% being *A. gambiae* s.s. Mortality after a 1 h exposure to 0.05% deltamethrin as counted 24 h post-exposure decreased by 10% from 0.93 (six out of 80 tested individuals) before to 0.83 (14 out of 83 tested individuals) during the spraying programme (χ^2 test, two-sided, $P = 0.023$, $N = 163$). In the same series of experiments, the median time to knockdown also increased between the two sampling points (Fig. 1A). Using logistic regression analysis, the estimated time to knock down half of the mosquito population (KDT_{50}) is 13 min 12 s before and 20 min 43 s during the spraying programme, a 1.6-fold increase. While the above tests were performed on a mixture of *A. arabiensis* and *A. gambiae* s.s., a similar, significant 1.7-fold increase in time to knockdown (from 14 min 38 s to 25 min 5 s; Wilcoxon rank sum test, two-sided, $P = 0.016$, $N = 9$) was also observed in laboratory-reared F_1 progeny of wild-caught gravid *A. arabiensis* females exposed to deltamethrin-treated netting (Fig. 1B).

Association between gene expression and insecticide resistance

Knockdown resistance (*kdr*) to DDT with cross-resistance to pyrethroid insecticides may be associated with a modification of the voltage-gated sodium channel protein by substitutions of the leucine residue 1014 by either phenylalanine (Martinez-Torres *et al*. 1998) or serine (Ranson *et al*. 2000). To determine whether the families subject to gene expression analysis contained either of the *kdr* mutations, six randomly chosen sisters from each of the nine families were screened for the presence of L1041F and L1014S *kdr* alleles using the HOLA technique (Lynd *et al*. 2005). Neither of the *kdr* mutations was detected. Although we cannot discount the presence of alternative, as yet undescribed target site mutations, this result is in accordance with a previous study (Etang *et al*. 2003) which has identified the presence of decreased susceptibility in *A. gambiae* s.l. from Pitoa to the pyrethroid deltamethrin but not to DDT with all survivors lacking a *kdr* mutation. As both of these insecticide classes share the same target site, substitutions in this protein would be expected to affect tolerance to both insecticide classes.

In the microarray analysis, three transcripts were consistently expressed at levels over threefold in families

Table 2 Top 10 candidate genes differentially expressed between the families collected before and during the 2005 spraying programme in Pitoa, Northern Cameroon. *P* values are corrected for multiple comparisons

Gene	GenBank Accession no.	Function	Location	Fold	*P* value
Over-expressed in families collected during the spraying programme					
CYP4G16	AY062189	Cytochrome P450	X	4.5	5.20×10^{-08}
SOD3B	AY745233	Cu-Zn superoxide dismutase	3L	4.1	4.43×10^{-10}
GSTS1-2	AF513639	Glutathione S-transferase	3L	3.5	1.73×10^{-08}
SOD3A	AY745232	Cu-Zn superoxide dismutase	3L	2.1	2.91×10^{-07}
SOD2	AY524130	Cu-Zn superoxide dismutase	2L	2.0	3.01×10^{-12}
TPX4	AY745235	Thioredoxin-dependent peroxidase	3L	1.9	2.79×10^{-07}
Over-expressed in families collected before the spraying programme					
CYP4H24	AY062206	Cytochrome P450	X	3.2	5.54×10^{-09}
CYP6P3	AF487534	Cytochrome P450	2R	2.6	1.66×10^{-07}
CYP325C2	DQ013849	Cytochrome P450	2R	2.4	1.39×10^{-09}
CYP6AG1	AY745223	Cytochrome P450	2R	1.7	4.31×10^{-08}

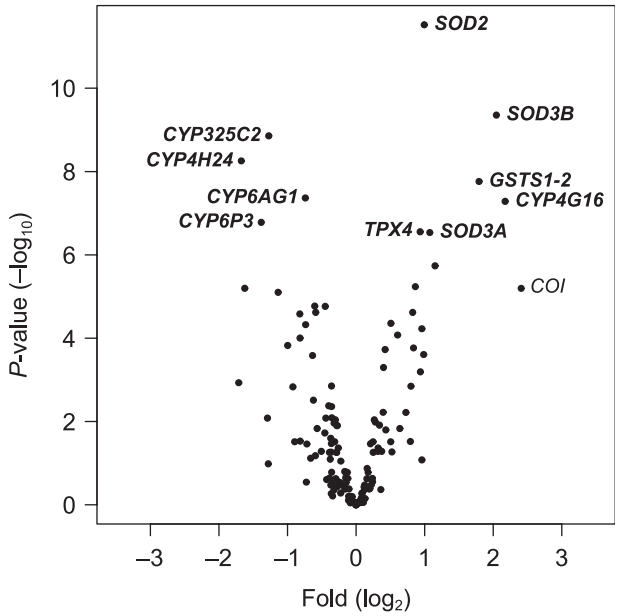

Fig. 2 Microarray analysis of putative metabolic resistance genes showing differences in expression levels between the families collected before and during the insecticide spraying programme. Each dot represents the mean estimates for one unique probe on the detox chip. The names in bold letters show the 10 genes providing best evidence (lowest *P* values) for being differentially expressed between the two family groups (values are given in Table 2).

collected during the insecticide spraying programme, compared to those families selected before spraying began. These transcripts were derived from the cytochrome P450 (P450), *CYP4G16* (4.5-fold), the SOB3B transcript from the superoxide dismutase *SOD3* gene (4.1-fold) and the GSTS1-2 transcript from the glutathione S-transferase *GSTS1* gene (3.5-fold) (Fig. 2 and Table 2). Transcripts from the superoxide dismutase *SOD2* (2-fold), an alternative transcript of the superoxide dismutase *SOD3* (SOD3A, 2.1-fold), and the thioredoxin-dependent peroxidase, *TPX4* (1.9-fold), although showing lower expression levels, were also among candidate genes of interest when probes were ranked and selected according to their *P* values (Fig. 2 and Table 2). Noteworthy, although statistically less significant, cytochrome oxidase I (*COI*) was 5.3-fold over-expressed in the more insecticide-tolerant families (Fig. 2). At the other end of the range, four P450s were expressed at lower levels during insecticide spraying (Fig. 2 and Table 2); *CYP4H24* (3.2-fold), *CYP6P3* (2.6-fold), *CYP325C2* (2.4-fold), and *CYP6AG1* (1.7-fold).

Quantitative reverse transcription PCR (RT-PCR) was used to validate microarray data by measuring mRNA copy numbers for the two transcripts GSTS1-2 and SOD3B in families sampled for microarray analysis. The mRNA copy numbers of *GSTS1-2* and *SOD3B* were normalized against the copy number of the ribosomal gene *S7*. In agreement with the microarray data (correlation between microarray and RT-PCR values; $r_{GSTS1\text{-}2} = 0.94$ and $r_{SOD3B} = 0.89$), RT-PCR measurements showed much higher transcript levels of *GSTS1-2* and *SOD3B* in the families collected during the spraying programme (Fig. 3).

Cluster analysis

In order to investigate the coregulation of the putative tolerance genes associated with pyrethroid, we measured correlations between expression levels for each gene pair as a measure of co-expression in 21 *A. gambiae* s.l. (nine *A. arabiensis* and 12 *A. gambiae* s.s.) families. Using the correlation distance metric, we applied the HOPACH clustering algorithm (van der Laan & Pollard 2003) and discovered 24 clusters. When all the 21 families were combined into one data set, all four putatively antioxidant

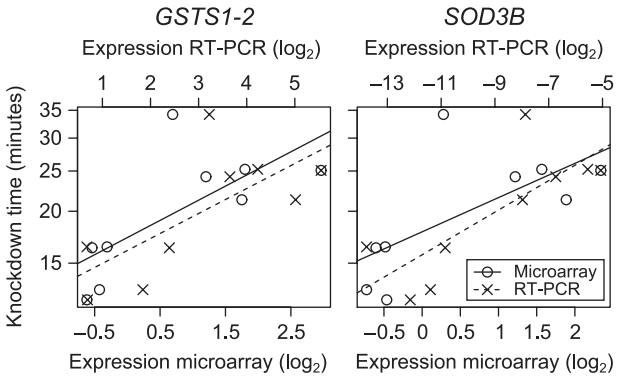

Fig. 3 Time to knockdown against deltamethrin plotted as a function of the expression levels of two transcripts, *GSTS1-2* and *SOD3B*, which were chosen for microarray validation by quantitative RT-PCR. Knockdown times represent median time to knockdown in families included in the microarray analysis (Fig. 1B). Knockdown times are medians of 10–12 female siblings determined by exposure to deltamethrin-treated netting. Both the microarray and the RT-PCR approaches showed a log-log linear relationship between time to knockdown and the mRNA expression levels for *GSTS1-2* and *SOD3B*. The mRNA expression levels as obtained by microarray and RT-PCR are highly correlated ($r_{GSTS1\text{-}2} = 0.94$ and $r_{SOD3B} = 0.89$). Lines represent linear regression models fitted to microarray and RT-PCR data, as follows: *GSTS1-2* (microarray), $\log \mathrm{KDT}_{50} = 2.73 + 0.68 \cdot \log_2 x$, $R^2 = 0.51$, P value = 0.031. *GSTS1-2* (RT-PCR), $\log \mathrm{KDT}_{50} = 2.67 + 0.64 \cdot \log_2 x$, $R^2 = 0.47$, P value = 0.04. *SOD3B* (microarray), $\log \mathrm{KDT}_{50} = 2.74 + 0.58 \cdot \log_2 x$, $R^2 = 0.44$, P value = 0.05. *SOD3B* (RT-PCR), $\log \mathrm{KDT}_{50} = 2.58 + 0.76 \cdot \log_2 x$, $R^2 = 0.62$, P value = 0.012.

genes, *SOD2*, *SOD3* (including the two splicing variants; *SOD3A*, *SOD3B*), *GSTS1* (including the two splicing variants; *GSTS1-1*, *GSTS1-2*), and *TPX4*, as well as the P450 *CYP4G16*, were contained in one single cluster and showed high membership scores for this cluster as determined by 1000 bootstrap resamplings (Fig. 4). If the HOPACH algorithm was run separately for either of the *A. arabiensis* or *A. gambiae* s.s. data set, a similar outcome could be observed. While in the *A. gambiae* s.s. expression data for *SOD2*, *SOD3A*, *SOD3B*, *GSTS1-1*, *GSTS1-2*, *TPX4* and *CYP4G16* fell in one single cluster as for the combined data set, these genes were clustered in two sister cluster in the *A. arabiensis* population. Here, *SOD2*, *SOD3A* and *CYP4G16* were members of the first cluster and the second cluster comprised *GSTS1-1*, *GSTS1-2*, *SOD3B* and *TPX4*.

In summary, mosquitoes collected during the cotton spraying programme showed increased tolerance to the insecticide deltamethrin as compared to mosquitoes sampled before the spraying programme started. A set of constitutively over-expressed genes with antioxidant properties and a single P450, *CYP4G16*, were associated with increased tolerance to deltamethrin in this *A. arabiensis* field population. The antioxidant genes include the superoxide dismutases *SOD2* and *SOD3*, the glutathione S-transferase *GSTS1* and the thioredoxin-dependent peroxidase *TPX4*. The results imply that exposure to insecticides selects for a constitutive enhancement of enzymes involved in antioxidant defence.

Discussion

We investigated insecticide resistance levels and gene expression patterns in the *Anopheles arabiensis* population from Pitoa, Northern Cameroon, before and midway through a large scale insecticide spraying campaign in order to associate differential gene expression with observed phenotypes in a field population. We focused on the mosquitoes' susceptibility towards insecticides to impregnate locally available ITNs. The present study is the first to study gene expression in the progeny of gravid, wild-caught mosquitoes. The use of F_1 family lines, set-up from field-collected, gravid females, allows us to measure constitutive expression levels by splitting genetically related siblings into one group for RNA extraction and one group for bioassays. All other studies to date correlating gene expression with a particular trait in anopheline mosquitoes have been exclusively performed on mosquitoes in laboratory culture in which biases due to inbreeding, low effective population size, and geographic origin confound comparisons and reduce meaningfulness of results to the situation in nature. For insecticide resistance studies, these laboratory colonies are often further selected with a single insecticide to increase the resistance phenotype prior to analysis (Ranson *et al*. 2001; Nikou *et al*. 2003; Ortelli *et al*. 2003; David *et al*. 2005; Müller *et al*. 2007; Vontas *et al*. 2007).

In the *A. arabiensis* population studied here, mosquitoes became more tolerant during the rainy season coinciding with the use of insecticides in a cotton-growing area. According to Sodecoton, a parastatal organization, which coordinates the cultivation of cotton and pesticide control in Northern Cameroon, cotton fields are sprayed fortnightly between July and October. The first two spraying applications typically consist of carbamates, followed by a cocktail of pyrethroids and organophosphates. Why has increased tolerance occurred? Variation in rates of migration and selection pressure associated with insecticide application (Lenormand *et al*. 1999) might explain the observed shift in pyrethroid tolerance between the two sampling points. The increase in insecticide tolerance is also observed in the progeny of exposed individuals, suggesting the differences in phenotypes are a response to selection, rather than insecticide-induced gene expression. It is also possible that household use of insecticides (e.g. pyrethroid coils, aerosols, ITNs) increases as nuisance biting intensifies during the rainy season and this might also contribute to the observed increase in tolerance.

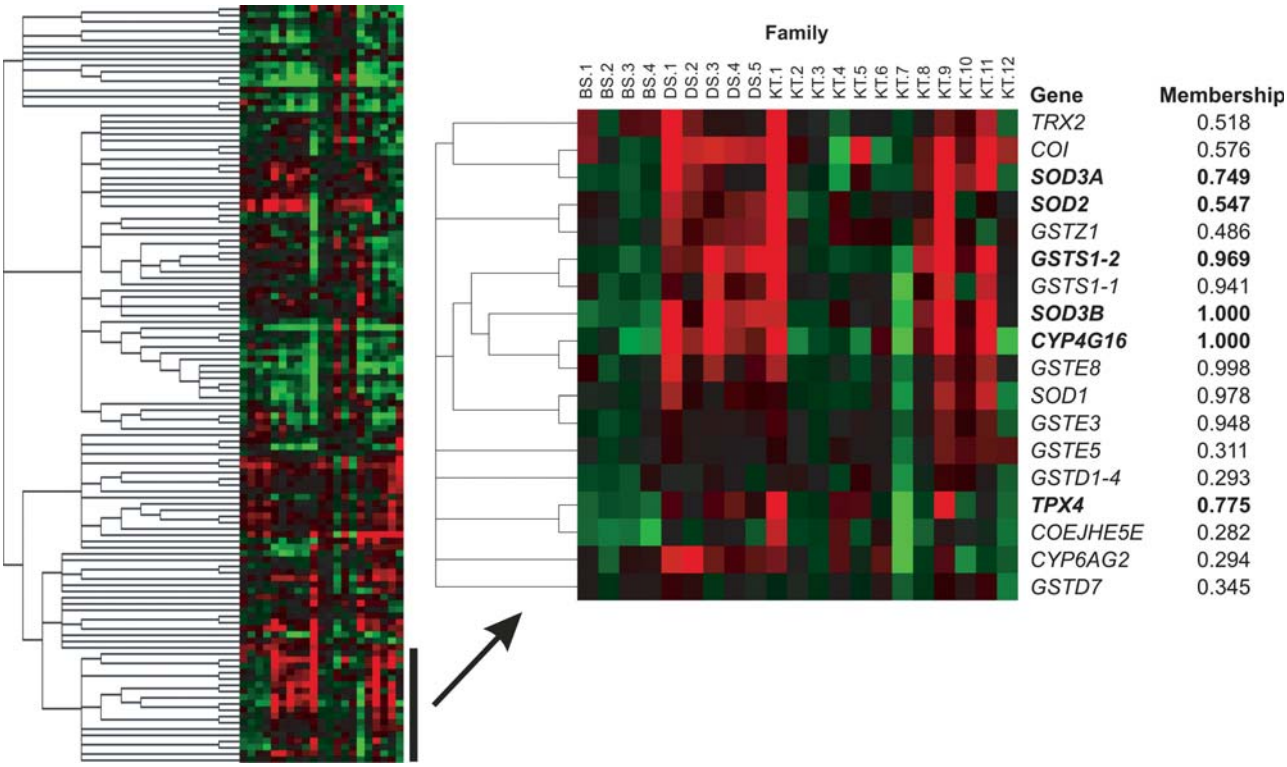

Fig. 4 Cluster analysis results from the HOPACH algorithm (van der Laan & Pollard 2003) with zoom view of a single cluster containing the genes implicated in increased tolerance to pyrethroids (bold letters). Red represents over-expression in families relative to the common reference RNA, and green is the opposite. Numbers give bootstrap estimated memberships in the cluster shown. *CYP4G16*, *SOD3B*, and *GSTS1-2* show particularly high bootstrap reappearance probabilities. The *Anopheles arabiensis* families are either labelled DS or BS for during or before the spraying programme, respectively. KT represents *Anopheles gambiae* s.s. families collected in West Kenya.

In order to measure expression patterns of genes putatively involved in metabolic insecticide resistance in this *A. arabiensis* population, we used the *Anopheles gambiae* detox chip (David *et al*. 2005). The detox chip is a highly specific custom-made microarray to measure expression of genes putatively involved in insecticide detoxification in *A. gambiae* s.s. As previously shown by Vontas *et al*. (2007), this array can to a certain extent also be used for cross-species hybridizations. Here, hybridizations of labelled DNA to the detox chip showed that the percentage of probes detected in the nine *A. arabiensis* families studied ranged between 69% and 93%. For comparison, the detection rate recorded from a series of 12 *A. gambiae* s.s. families using identical protocols ranged from 71 to 91% (data not shown), and hence, the performance of the detox chip appeared to be similar between the two species. *A. arabiensis* and *A. gambiae* s.s. show shared polymorphisms and even full haplotype sharing of mtDNA as well as autosomal sequences (Besansky *et al*. 2003). However, in the absence of a genome sequence for *A. arabiensis*, the exact degree of similarity between the detoxification genes in the two species is not yet known. It is possible that there may have been gene loss or duplications in one of the two species, an event that is not unexpected in multigene families such as the detoxification gene families. Thus, we cannot fully discount the involvement of probe mismatches or missing or duplicated genes as the cause of the observed patterns of gene expression.

The gene expression analysis yielded five up-regulated transcripts in families collected during the insecticide spraying programme, compared to those families selected before spraying began. These transcripts include products from the cytochrome P450 (P450), *CYP4G16*, the superoxide dismutases *SOD2* and *SOD3*, the glutathione S-transferase *GSTS1*, and the thioredoxin-dependent peroxidase, *TPX4*. At the other end of the range, four P450s were expressed at lower levels during insecticide spraying; *CYP4H24*, *CYP6P3*, *CYP325C2* and *CYP6AG1*.

CYP4G16 is a member of the P450 family. Its putative ortholog in *Drosophila melanogaster*, *DmCYP4G15*, whose translated amino acid sequences are 69% identical (Altschul *et al*. 1990) with the *AgCYP4G16*, is predominantly found in the central nervous system, indicating a role in metabolizing endogenous compounds rather than in xenobiotic detoxification (Maibeche-Coisne *et al*. 2000). However, in the German cockroach, *Blattella germanica*, the P450 *CYP4G19*

which shows 64% amino acid identity with the *AgCYP4G16* (Altschul *et al*. 1990) is approximately fivefold over-expressed in adults from a pyrethroid resistant strain as compared to a standard laboratory susceptible strain, and in this species, expression is higher in the abdomen than in the thorax or head (Pridgeon *et al*. 2003). Further studies such as measuring the interaction of the *AgCYP4G16* gene product with pyrethroids are needed to verify that this gene encodes an enzyme that is involved in insecticide metabolism.

It is notable that, with the exception of the P450 discussed above, all of the remaining genes that showed significantly elevated expression during the insecticide spraying season are associated with alleviation of oxidative stress. Remarkably, expression of three of the five up-regulated genes found in the current study, i.e. *SOD3B, GSTS1-2* and *TPX4*, were also found to be elevated in a recently colonized *A. gambiae* s.s. strain from Ghana, highly resistant to the pyrethroid permethrin (Müller *et al*. 2007). Pyrethroids are known to induce oxidative stress and this is associated with the induction of antioxidant activities, as demonstrated by increased superoxide dismutase and catalase activity in rats (Kale *et al*. 1999). Superoxide dismutases protect organisms from the damaging effects of reactive oxygen species by catalysing the redox reaction of superoxide anion to hydrogen peroxide and oxygen (Fridovich 1978). Thioredoxin peroxidases then catalyse the elimination of the reactive hydrogen peroxide. The Sigma GSTs have also been implicated in reducing oxidative stress. For example, *DmGSTS1-1*, shows a protective role against deleterious effects of oxidative stress by glutathione-conjugating activity for 4-hydroxynonenal, an electrophilic aldehyde derived from lipid peroxidation (Singh *et al*. 2001). In the brown plant hopper, *Nilaparvata lugens*, the elevated levels of GSTs present in a pyrethroid-resistant strain attenuate the pyrethroid-induced lipid peroxidation (Vontas *et al*. 2001). Intriguingly, the co-expression of these genes, together with the P450 *CYP4G16*, suggests a common regulatory input; thus, these genes might constitute a functional unit, which warrants further investigation.

Four P450s were over-expressed in the families sampled before the spraying programme; *CYP4H24*, *CYP6P3*, *CYP325C2*, and *CYP6AG1*. To our knowledge, putative functions have not been assigned to any of these four P450 genes. One speculation is that some of these P450s, as previously demonstrated for *CYP2E1* (Gonzalez 2005), might induce oxidative stress, too, and hence suppression of these genes together with the over-expression of antioxidants would protect the insect from oxidative injury. Alternatively, it is plausible that expression of these P450s is reduced as a compensatory response to the elevated levels of oxidative stress induced by over-expression of *CYP4G16*.

Conclusions

The intensified use of insecticides to protect cotton crops coinciding with increased tolerance to pyrethroids supports the hypothesis that agricultural use of insecticides poses selective pressure on the *Anopheles arabiensis* mosquito population in Northern Cameroon. Although the presence of novel *kdr* mutations cannot be ruled out, the absence of known *kdr* mutations supports the hypothesis that the underlying mechanism conferring reduced knockdown to deltamethrin is metabolic. In addition to a single P450, *CYP4G16*, a set of constitutively over-expressed genes with antioxidant properties are implicated in conferring increased tolerance to pyrethroids. These antioxidant genes include the superoxide dismutases *SOD2* and *SOD3*, the glutathione S-transferase *GSTS1* and the thioredoxin-dependent peroxidase *TPX4*. The increased steady-state expression of concerted reductant enzymes could predispose the mosquitoes' resilience to oxidative stress when targeted by pyrethroids or other toxins. Alternatively, increased levels of antioxidants could be a protective mechanism against the reactive oxygen species, which are a by-product of increased P450-catalysed pyrethroid metabolism. Perhaps the first line of defence against pyrethroid insecticides is to select for individuals with a stronger stress-response pathway with selection for increases in insecticide detoxification pathways occurring only after prolonged exposure. Intriguingly, the co-expression of these antioxidant genes together with the P450 *CYP4G16* in two different anopheline species suggests a common regulatory input; thus, these genes might constitute a common functional unit, which warrants further investigation. The current study suggests that a hitherto overlooked group of antioxidant enzymes may hold the key to the development of insecticide tolerance in mosquito field populations.

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