Insect Biochemistry and Molecular Biology 139 (2021) 103655

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

A whole transcriptomic approach provides novel insights into the molecular basis of organophosphate and pyrethroid resistance in *Anopheles arabiensis* from Ethiopia

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ARTICLE INFO

Keywords:
Anopheles arabiensis
Ethiopia
Insecticide resistance
RNA-Seq
Pyrethroid
Organophosphate

ABSTRACT

The development of insecticide resistance in malaria vectors is of increasing concern in Ethiopia because of its potential implications for vector control failure. To better elucidate the specificity of resistance mechanisms and to facilitate the design of control strategies that minimize the likelihood of selecting for cross-resistance, a whole transcriptomic approach was used to explore gene expression patterns in a multi-insecticide resistant population of *Anopheles arabiensis* from Oromia Region, Ethiopia. This field population was resistant to the diagnostic doses of malathion (average mortality of 71.9%) and permethrin (77.4%), with pools of survivors and unexposed individuals analyzed using Illumina RNA-sequencing, alongside insecticide susceptible reference strains. This population also demonstrated deltamethrin resistance but complete susceptibility to alpha-cypermethrin, bendiocarb and propoxur, providing a phenotypic basis for detecting insecticide-specific resistance mechanisms. Transcriptomic data revealed overexpression of genes including cytochrome P450s, glutathione-s-transferases and carboxylesterases (including CYP4C36, CYP6AA1, CYP6M2, CYP6M3, CYP6P4, CYP9K1, CYP9L1, GSTD3, GSTE2, GSTE3, GSTE4, GSTE5, GSTE7 and two carboxylesterases) that were shared between malathion and permethrin survivors. We also identified nineteen highly overexpressed cuticular-associated proteins (including CYP4G16, CYP4G17 and chitinase) and eighteen salivary gland proteins (including D7r4 short form salivary protein), which may be contributing to a non-specific resistance phenotype by either enhancing the cuticular barrier or promoting binding and sequestration of insecticides, respectively. These findings provide novel insights into the molecular basis of insecticide resistance in this lesser well-characterized major malaria vector species.

1. Introduction

Globally, malaria mortality has fallen since 2010, largely due to the scale-up of diagnosis, treatment and insecticide-based vector control interventions. However, since 2016, the rates of decline have stalled in the World Health Organization regions of Africa, Southeast Asia and the Western Pacific and even reversed in the Eastern Mediterranean and the Americas (World Health Organization, 2020). Concurrently, insecticide resistance among major malaria vector species has become widespread, affecting approximately 90% of countries with ongoing malaria transmission (World Health Organization, 2020) and threatening vector control efforts worldwide.

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https://doi.org/10.1016/j.ibmb.2021.103655
Received 21 July 2021; Received in revised form 15 September 2021; Accepted 20 September 2021
Available online 23 September 2021

In Ethiopia, insecticide resistance in the principal malaria vector species *Anopheles arabiensis* has been a public health concern for decades. Indoor residual spraying (IRS) using DDT was first implemented in 1959, and insecticide-treated net (ITN) distribution was initiated in 1997 and scaled up since 2005 (President's Malaria Initiative, 2019). Following the detection of DDT resistance in 2009, DDT was replaced with deltamethrin for IRS, initially alongside bendiocarb from 2011 until 2013, after which bendiocarb and propoxur were sprayed in different geographical areas. In 2015, pirimiphos-methyl was introduced and is now used alongside propoxur across the country (Messenger et al., 2017). In parallel, more than 80 million pyrethroid-treated long-lasting insecticidal nets (LLINs) have been distributed in Ethiopia since 2008 (President's Malaria Initiative, 2019). This heterogeneous use of different chemicals has resulted in highly focal, dynamic resistance patterns across Ethiopia, broadly reflecting longitudinal shifts in the national insecticide policy (Messenger et al., 2017; Alemayehu et al., 2017). Populations of *An. arabiensis* are now largely resistant to DDT and deltamethrin, with reduced susceptibility to malathion, pirimiphos-methyl, propoxur and bendiocarb reported in some locations (Messenger et al., 2017; Alemayehu et al., 2017). The presence of the L1014F-*kdr* allele was first reported from areas surrounding the Gilgel-Gibe hydroelectric dam in southwestern Ethiopia in 2010 (Yewhalaw et al., 2010). In these populations, L1014F-*kdr* was practically fixed and this target site mutation is now commonly detected elsewhere in Ethiopia at varying frequencies (Messenger et al., 2017). Elevated levels of glutathione-S-transferases (GSTs) have also been observed in some *An. arabiensis* populations from Oromia and Benishangul-Gumuz regions (Alemayehu et al., 2017). To date, other target site mutations, including L1014S-*kdr*, N1575Y and G119S-*Ace-1*, have not been detected in Ethiopia (Messenger et al., 2017; Alemayehu et al., 2017).

In Oromia region, *An. arabiensis* has demonstrated resistance to insecticides belonging to four of the chemical classes historically used for adult vector control (pyrethroids, carbamates, organophosphates and organochlorines) (Messenger et al., 2017; Alemayehu et al., 2017). In this area, restoration of susceptibility following pre-exposure to the synergist piperonyl butoxide (PBO) (Messenger et al., 2017; Birhanu et al., 2019), coupled with a lack of association between phenotypic resistance and L1014F-*kdr* frequency and the complete absence of other target-site mutations (L1014S-*kdr*, N1575Y and G119S-*Ace-1*), suggest that metabolic mechanisms may play an important role in resistance (Messenger et al., 2017; Alemayehu et al., 2017).

In African *Anopheles,* several cytochrome P450 monooxygenases (CYP450s), carboxylesterases (COEs) and GSTs, have been functionally associated with pyrethroid resistance (Müller et al., 2008a; Stevenson et al., 2011; Chiu et al., 2008; Ibrahim et al., 2016a; Riveron et al., 2014). In addition to detoxification enzymes, other gene families, including α-crystallins, hexamerins and ATP synthases (Ingham et al., 2018), Maf-S, Dm and Met transcription factors (Ingham et al., 2017, 2018), D7r2 and D7r4 salivary gland proteins (Isaacs et al., 2018), a sensory appendage protein, SAP2 (Ingham et al., 2019) and cuticular proteins (Balabanidou et al., 2016a) have been associated with insecticide resistance. While over-expression of a number of these proteins is conserved across countries and sub-species of the *An. gambiae* s.l. complex (Ingham et al., 2018), there is still a considerable paucity of data regarding the molecular basis of resistance in *An. arabiensis,* especially in Ethiopia (Messenger et al., 2017; Alemayehu et al., 2017; Simma et al., 2019). Currently, only CYP6P4 and GSTD3 have been directly linked to local deltamethrin and DDT resistance (Simma et al., 2019).

In Ethiopia, nationwide insecticide resistance management strategies rely on the tactical deployment of IRS and LLINs with differing active ingredients. For such strategies to succeed, there needs to be a clear understanding of the specificity of resistance mechanisms to individual insecticides and the likelihood of selecting for cross-resistance mechanisms. To improve our understanding of these factors in *An. arabiensis*, we undertook a whole transcriptomic approach to characterize gene expression patterns in a multi-insecticide resistant field population of *An. arabiensis* from south-west Ethiopia.

2. Materials and methods

2.1. Study area and mosquito collections

Adult mosquitoes were collected from Asendabo, Oromia region, Ethiopia (7°40′31″ N, 36°52′56″ E), where organophosphate and pyrethroid resistance had been previously reported in *An. arabiensis* populations (Messenger et al., 2017). Mosquitoes were sampled at the end of the long rainy season, between 3rd September-10th October 2017, following IRS with bendiocarb by the National Malaria Control Program (NMCP) in this area in June 2017.

Upon obtaining householder consent, indoor-resting, blood-fed female *Anopheles* mosquitoes were collected from the walls of 12 houses (situated approximately <5 km apart) between 4:00 and 6:00 a.m. using handheld aspirators. Mosquitoes were held in paper cups with access to 10% sucrose and transported to the Tropical and Infectious Diseases Research Center (TIDRC) in Sekoru, Oromia region (7°54′50″ N, 37°25′23.6″ E). F_1 progeny were obtained from field-collected mosquitoes using forced-oviposition (Morgan et al., 2010). Blood-fed, field--collected mosquitoes, morphologically identified as *An. gambiae* s.l. (Gillies and Coetzee, 1987), were maintained for 4–5 days until fully gravid and checked daily for survival. Each fully gravid female was transferred to a 1.5 ml microcentrifuge tube containing damp cotton wool and allowed to lay eggs. Eggs from 246 adult *An. gambiae* s.l. were transported to the U.S. Centers for Disease Control and Prevention (CDC), Atlanta, USA, and pooled for rearing in the CDC insectary.

An. arabiensis from the insecticide susceptible Dongola reference strain (originating from Sudan, obtained from the Malaria Research and Reference Reagent Resource Center, MR4) and the Sekoru insecticide susceptible laboratory strain (originating from Ethiopia, obtained from the Vector Biology and Control Research Unit, TIDRC, Jimma University) (Balkew et al., 2010), were also reared in the CDC insectaries. All adult mosquitoes were maintained under standard insectary conditions (27±2 °C, 80% relative humidity, light:dark cycles of 14:10 h) with access to 10% sucrose solution *ad libitum*. F_1 adult females of each strain were randomly mixed in cages for subsequent bioassays.

2.2. Insecticide resistance bioassays

CDC bottle bioassays for malathion (organophosphate) and permethrin (pyrethroid) were conducted according to published guidelines (Centers for Disease Control and Prevention, 2012). Stock solutions of the diagnostic dose required to kill 100% of susceptible mosquitoes (malathion: 50μg/bottle and permethrin: 21.5μg/bottle), were prepared by diluting technical grade insecticide in 50 ml of acetone. Each Wheaton 250 ml glass bottle along with its cap was coated with 1 ml of the stock solution by rolling and inverting the bottles. In each test, a control bottle was coated with 1 ml of acetone. Bottles were left to dry in the dark for 3 h and were washed thoroughly and re-coated before every test. Following a 2-h acclimatization period in paper cups with access to 10% sucrose, approximately, 20–25 unfed, 3 day-old adult female *An. gambiae* s.l. were introduced into each bottle using a mouth aspirator and knock-down/mortality was recorded after 30 min of exposure. Additionally, a susceptible reference *An. arabiensis* strain (Dongola or Sekoru) was assayed in parallel. Bioassays were conducted between 15:00 and 17:00 each day to avoid any bias in RNA transcript expression related to circadian rhythm. Multiple replicates were performed per insecticide to obtain sufficient phenotyped material for RNA-sequencing analysis. A mosquito was defined as 'alive' at the diagnostic time if it was capable of standing and flying in a coordinated manner; surviving mosquitoes (defined as resistant) and non-exposed mosquitoes (from acetone-treated bottles) were stored separately at −80 °C. Additionally, non-exposed, unfed, 3 day-old adult female *An.*

arabiensis from the Sekoru and Dongola susceptible laboratory strains were also preserved for analysis at −80 °C.

Additional resistance intensity bioassays were undertaken with F_1 field mosquitoes to characterize susceptibility levels to carbamates (bendiocarb and propoxur) and pyrethroids (alpha-cypermethrin, deltamethrin and permethrin), following exposure to 1, 2, 5 and 10 times the diagnostic doses. Bioassay data were interpreted according to the WHO criteria: mortality of 98% or higher indicates susceptibility, mortality of 90–97% is suggestive of resistance, and mortality of less than 90% indicates resistance (World Health Organization, 2013). Mortality in untreated control bottles was less than 5% in all resistance intensity bioassays. Mean percent mosquito mortality was calculated across all replicates for a given insecticide.

2.3. Molecular species identification

Prior to pooling specimens for RNA extraction, 4–6 legs from each mosquito tested in bioassays were removed and genomic DNA was extracted using the Extracta™ DNA Prep for PCR-Tissue kit (QuantaBio, USA), according to the manufacturer's protocol. Molecular identification of *An. gambiae* s.l was carried out using species-specific PCR with primers for *An. gambiae* s.s., *An. arabiensis* and *An. quadriannulatus* (Wilkins et al., 2006): AR-3T (5′-GTGTTAAGTGTCCTTCTCCGTC-3'; specific for *An. arabiensis*), GA-3T (5′-GCTTACTGGTTTGGTCGGCATGT-3; specific for *An. gambiae s.s.*), QD-3T (5′-GCATGTCCACCAACGTAAATCC-3'; specific for *An. quadriannulatus*) and IMP-UN (5′-GCTGCGAGTTGTAGAGATGCG-3'; common for all species). Each 25 μl reaction volume contained 20–40 ng of DNA, 5X Green GoTaq® Reaction Buffer (Promega), 25 mM $MgCl_2$, 2 mM of each dNTP, 1U GoTaq® DNA polymerase and 25 pmol/μl of primers AR-3T, GA-3T, QD-3T and IMP-UN. PCR cycling conditions were: 95 °C for 5 min, followed by 30 amplification cycles (95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s) and a final elongation step at 72 °C for 5 min. Amplified PCR products were visualized on 1.5% agarose gels, stained with GelRed™ (Biotium, USA). Positive control DNA from *An. arabiensis* Sekoru, *An. gambiae* s.s. Kisumu and *An. quadriannulatus* Sangwe strains and no-template negative controls were included with all reaction runs. PCR products of 387bp, 463bp or 636bp were indicative of *An. arabiensis*, *An. gambiae* s.s. or *An. quadriannulatus*, respectively.

2.4. Target site mutation detection

The presence of the G119S *Ace-1* mutation was determined using PCR restriction fragment length polymorphism analysis (Weill et al., 2004). Amplifications were performed in 25 μl reactions containing 20–40 ng of DNA, 5X Green GoTaq® Reaction Buffer (Promega), 2.5 mM of each dNTP, 1U GoTaq® DNA polymerase, 25 pmol/μl of primers MOUSTDIR1 (5′-CCGGGNGCSACYATGTGGAA-3′) and MOUSTREV1 (5′-ACGATMACGTTCTCYTCCGA-3′). PCR cycling conditions were 95 °C for 5 min, followed by 35 amplification cycles (95 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min) and a final elongation step at 72 °C for 5 min. PCR products were initially visualized on 2% agarose gels, stained with GelRed™ (Biotium, USA) before incubation with *Alu*I restriction enzyme (New England Biolabs, USA) at 37 °C for 16 h, followed by 65 °C for 20 min. DNA fragments were visualized on 2% agarose gels, stained with GelRed™ (Biotium, USA). DNA from *An. arabiensis* Sekoru was used as a negative control alongside a no-template control. DNA from *An. coluzzii* AKDR was used as a positive control. Undigested PCR products of 194bp indicated the susceptible allele (wild type) and 120bp and 74bp digested fragments indicated the presence of the resistant allele. The presence of all three bands indicated the sample was a heterozygote.

West African *kdr* (L1014S) and East African *kdr* (L1014F) alleles were detected using protocols for allele-specific PCR (AS-PCR) (Martinez-Torres et al., 1998; Ranson et al., 2000). Primers IPCF (5′-GATAATGTGGATAGATTCCCCGACCATG-3′), AltRev (5′-TGCCGTTGGTGCAGACAAGGATG -3′), WT-R (5′-GGTCCATGTTAATTTGCATTACTTACGAATA -3′) and East-F (5′-CTTGGCCACTGTAGTGATAGGAAAATC-3′) were used to detect the L1014S allele (AS-PCR East), whereas primers IPCF, AltRev, WT-R and West-F (5′-CTTGGCCACTGTAGTGATAGGAAATGTT-3′) were used to detect the L1014F allele (AS-PCR West). Each 25 μl reaction volume contained 20–40 ng of DNA, 5X Green GoTaq® Reaction Buffer (Promega), 25 mM $MgCl_2$, 2 mM of each dNTP, 1U GoTaq® DNA polymerase, 2.5 pmol/μl of primers IPCF and AltRev and either 5 pmol/μl of primer WT-R and 2.5 pmol/μl of primer East-F to detect the L1014S allele (AS-PCR East), or 25 pmol/μl of primer WT-R and 8.8 pmol/μl of primer West-F to detect the L1014F allele (AS-PCR West). PCR cycling conditions were 95 °C for 5 min, followed by 35 amplification cycles (95 °C for 30 s, 57 °C for East or 59 °C for West for 30 s, 72 °C for 30 s) and a final elongation step at 72 °C for 5 min. Amplified PCR products were visualized on 2% agarose gels, stained with GelRed™ (Biotium, USA). DNA from *An. gambiae* Kisumu was used as a negative control alongside a no-template control. DNA from *An. coluzzii* AKDR and *An. gambiae* s.s. RSP-ST were used as positive controls for L1014F and L1014S, respectively. Successful amplification was indicated by a PCR product of 314 bp; additional bands of 214bp and 156bp identified susceptible (wild type) and resistant alleles, respectively. Pearson's Chi squared tests were used to evaluate deviations from Hardy-Weinberg equilibrium at the population-level.

2.5. RNA extraction and cDNA library preparation

Total RNA was isolated from three pools containing five mosquitoes each from the following groups: mosquitoes phenotyped as resistant following a malathion or permethrin bioassay, non-insecticide exposed mosquitoes and susceptible *An. arabiensis* colony mosquitoes from Dongola and Sekoru strains. RNA was extracted using the Arcturus® PicoPure® RNA isolation kit (Life Technologies, USA) and quantified using the Agilent RNA ScreenTape 4200 assay, according to the manufacturers' protocols. Two micrograms of starting material were treated with Baseline-ZERO™ DNase (Lucigen, USA) and ribosomal RNA was removed using the Ribo-Zero™ Magnetic Core Kit and Ribo-Zero™ rRNA Removal kit (Illumina, USA), according to the manufacturers' protocols. Individual RNA-Seq libraries were prepared from each pool of extracted RNA using the ScriptSeq™ v2 RNA-Seq library preparation kit (Illumina, USA), using 12 cycles of PCR amplification, according to the manufacturer's protocol. Libraries were purified using Agencourt AMPure XP beads (Beckman Coulter, USA) and assessed for quantity and size distribution using the Agilent DNA ScreenTape D5000 assay.

2.6. RNA-sequencing, quality control and read mapping

Two experiments, each comprising nine RNA-Seq libraries, were sequenced as 2 × 125bp paired-end reads, on the Illumina HiSeq platform at the CDC. The first experiment (henceforth "malathion experiment") contained three biological replicates each of malathion bioassay survivors, non-exposed mosquitoes and the susceptible Dongola strain. The second experiment (henceforth "permethrin experiment") contained three biological replicates each of permethrin bioassay survivors, non-exposed mosquitoes and the susceptible Sekoru strain. Each experiment was sequenced on two HiSeq lanes to give an estimate of technical variation.

De-multiplexed paired end sequencing reads for each sample were

evaluated for quality using FastQC v0.11.5 (Andrews, 2016). Concatenated files for R1 and R2 reads were used for downstream analysis. Initially concatenated files for each sample were trimmed and filtered using fastp v0.21.0 (Chen et al., 2018) to remove adapter and low-quality reads according to the following criteria: minimum base quality score = 20, minimum length required = 25, polyG and poly tail trimming = True. Trimmed and filtered read pairs (R1/R2) were aligned against the reference genome, *An. arabiensis* Dongola (genome assembly version = AaraD1.11, GeneBank assembly identifier = GCA_000349185.1; GeneBank WGS Project = APCN01), directly downloaded from VectorBase (release 48) (Giraldo-Calderón et al., 2015), using 'subjunc' v2.0.1, part of the subread aligner v2.0.1 (Liao et al., 2013), with default parameters. The resulting alignment was filtered to remove reads with low mapping quality ($q < 10$) and sorted successively using Samtools v1.10 (Li et al., 2009). Descriptive statistics for the malathion and permethrin read libraries and sequencing alignments are shown in Table S1.

Tags (a read pair or single, unpaired read) mapped to the sense orientation of the annotated An. arabiensis *Dongola* genes (gene set of AaraD1.11 in gff downloaded from release 48 from Vector Base), were quantified using FeatureCounts, as part of the subread-aligner package v2.0.1 (Liao et al., 2013). The tag count with FeatureCount was carried out using the following criteria: 1) count only read pairs that have both ends aligned; 2) count fragment instead of reads; 3) minimum number of overlaps required = 1; 4) feature_type = exon; 5) attribute type = gene_id; and 6) strandness = sense. The FeatureCount analysis generated a tag count matrix table which was inputted to edgeR (Robinson et al., 2010) for differential expression analysis. Metrics describing the transcriptome alignments for the malathion and permethrin experiments are shown in Table S2.

2.7. Differential transcription analysis and GO enrichment analysis

To remove the effect of noise and lowly expressed genes, for each pairwise comparison, genes with a total tag count less than 50 across all libraries (control *vs* treatment) were filtered out before further analysis. Only genes with a total tag count equal to or higher than 50 were considered. The function calcNormFactors (part of the edgeR package (Robinson et al., 2010)), using the TMM (Trimmed Mean M-values) method, was used to normalize tag count among samples, by finding a set of scaling factors for the library sizes that minimized the log-fold changes between samples for most genes. The tag count was not normalized for gene length and GC content, as these values do not vary from sample to sample, so this would be expected to have little effect on DEGs. The DEGs between control (unexposed) and resistant (exposed) mosquitoes were selected after multiple testing using the decideTests function, part of the limma package (Ritchie et al., 2015). A critical value absolute fold-change = 2 and FDR (False Discovery Rate) ≤ 0.01 was used. Different pairwise comparisons were conducted: 1) between resistant field mosquitoes (treatment) and unexposed field mosquitoes (control): CON-M *vs* MAL-R and CON–P *vs* PERM-R; 2) between a susceptible laboratory strain and exposed field mosquitoes: DON *vs* MAL-R, SEK *vs* PERM-R and DON *vs* PERM-R; 3) between the two susceptible laboratory strain: DON *vs* SEK; and 4) between field mosquitoes exposed to different insecticides: MAL-R vs PERM-R.

The annotation set of the AraD1.11 reference genome included 13,307 protein-coding genes and 378 additional non-coding genes (Table S3) (https://legacy.vectorbase.org/organisms/dongola/aaradd111). However, Gene Ontology (GO) description of only 9074 of these genes was provided in VectorBase (Giraldo-Calderón et al., 2015) (cellular component: 4784; molecular function: 7261; biological processes: 5316). To increase the annotation efficiency, the predicted protein gene set fasta file of AraD1.11 was downloaded from VectorBase (release 48) (Giraldo-Calderón et al., 2015) and was used for functional annotation using Blast2GO (Conesa and Götz, 2008). A Blastp search of the protein fasta file was conducted against the *Insecta* category of the non-redundant protein NCBI database, with a maximum e-value cut-off of 1e−3. Additionally, the RefSeq protein IDs corresponding to the best blast hits of each query sequence were mapped to the GO database as curated and updated in the last release of Blast2GO database (November 2020). The resulting non-annotated genes from the Blast2GO analysis were mapped to the *An. gambiae* proteome (AgamP4.13) using a Blastp search with a maximum e-value cut-off of 1e−10 for ortholog inference. The best alignments (based on e-value and alignment score) were considered as orthologous genes, were ID mapped to the GO annotation of AgamP4.13 using the panda's python library (McKinney, 2011). The newly annotated genes were concatenated with the Blast2GO annotation, which was used as the background for the functional enrichment analysis of the DEGs. From this analysis, 10,456 (78.6%) of 13,307 protein coding genes were GO annotated.

GO term enrichment analysis of up- and down-regulated genes was carried out using Goatools (Klopfenstein et al., 2018) based on the go-basic database (release 2021-02-01). The list of 10,456 annotated genes of *An. arabiensis* with their associated GO terms was used as the background reference set. The *P* values used to evaluate significantly enriched GO terms were calculated based on Fisher's exact test and corrected by Benjamini-Hochberg multiple test correction method. Finally, we used a FDR adjusted *P*-value <0.05 to tag statistically significant overrepresented GO terms associated with the list of DEGs.

2.8. qRT-PCR validation of RNA-seq data

A subset of eleven differentially transcribed genes was selected for quantitative real-time reverse transcription PCR validation (qRT-PCR). One microgram of RNA from three replicates of malathion resistant or permethrin resistant, non-exposed and Dongola strain mosquitoes were used to synthesize cDNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) with oligo-dT20 (New England Biolabs, USA), according to the manufacturer's instructions. Primer sequences and efficiencies are detailed in Table S4. Standard curves of Ct values for each gene were generated using a five-fold serial dilution of cDNA to assess PCR efficiency. Reactions were performed using either a QuantStudio 6 Flex Real-Time PCR system (Applied Biosystems, USA) with PowerUp SYBR Green Master Mix (Applied Biosystems, USA) or a Stratagene Mx3005P Real-Time PCR system (Agilent Technologies) with LightCycler® 480 SYBR Green I Master Mix (Roche, UK). cDNA from each sample was used as a template in a three-step reaction: 50 °C for 2 min, denaturation at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C and a final step of 15 s at 95 °C, 1 min at 60 °C, and 15 s at 95 °C. The relative expression level and Fold Change (FC) of each target gene from resistant field samples, relative to the susceptible laboratory strain (Dongola), were calculated using the $2^{-\Delta\Delta CT}$ method (Rao et al., 2013), incorporating PCR efficiency. Two housekeeping genes, ribosomal protein S7 (RpS7: AARA000046) and ubiquitin (AARA016296), were used for normalisation.

2.9. Sequence polymorphism analysis

The RNA-Seq reads of all resistant groups and susceptible strains were mined for the prevalence of non-synonymous Single Nucleotide Polymorphisms (SNPs) involved in *Anopheles* spp resistance to either DDT, organophosphate or pyrethroid insecticides. The primary target of the analysis was the *para* Voltage-Gated Sodium Channel (*VGSC*) gene (AARA017729), for which the presence of 21 recently reported non-synonymous SNPs (A1125V, A1746S, A1934V, D466H, E1597G, F1920S, I1527T, I1868T, I1940T, K1603T, L995F, L995S, M490I, N1575Y, P1874L, P1874S, T791M, V1254I, V1853I, V402L, and V1853I) were investigated (Clarkson et al., 2021). Additionally, non-synonymous variants G119S in the acetylcholinesterase (*Ace-1*) gene (AARA001814), L119F and I114T in GSTe2 (AARA008732) (Mitchell et al., 2014; Lucas et al., 2019), were also investigated. Prevalence of the target site mutations in the RNA-Seq datasets was

determined as follows. The coding sequences (CDS) corresponding to VGSC, Ace-1, and GSTe2 from AaraD1.11 were downloaded from VectorBase (Giraldo-Calderón et al., 2015) and were aligned separately with their respective homologous gene retrieved from the AgamP4.4 gene set, using Clustalw Omega (Sievers et al., 2011). Next, the sequence (~30–40 nucleotides) flanking the codon and the site of interest from each gene in *An. arabiensis* was identified and extracted from the alignment as described here (Lol et al., 2019). The resulting flanking sequence was BLASTn (Chen et al., 2015) searched against the AaraD1.11 reference genome (release 48 in Vectorbase) (Giraldo-Calderón et al., 2015), which gave the exact chromosomal numerical position of the nucleotide. Finally, the sorted bam files, which were previously used as the input featureCount for DEG analysis were separately uploaded to Integrative Genomics Viewer (IGV) (Thorvaldsdótti et al., 2013) and zoomed to the position to the flanking sequence. The allele frequency in the population was calculated as the percentage of RNA-Seq reads spanning the codon with the SNP of interest.

3. Results

3.1. Phenotypic insecticide resistance

Indoor resting F_0 adult *An. gambiae* s.l. were collected from houses in Asendabo, Oromia region, Ethiopia from July-September 2017 and F_1 progeny were generated by forced-oviposition (Morgan et al., 2010). Susceptibility to the diagnostic doses (1X) of malathion (organophosphate) and permethrin (pyrethroid) was determined for 273 F_1 *An. gambiae* s.l. mosquitoes, using U.S. Centers for Disease Control and Prevention (CDC) bottle bioassays (Centers for Disease Control and Prevention, 2012). These mosquitoes were subsequently confirmed via species-specific PCR as *An. arabiensis* (Wilkins et al., 2006). The average mortality to malathion was 71.9% [95% CI: 65.3–78.5] and to permethrin was 77.4% [95% CI: 44.0–100.0%]. Resistance intensity assays, using an additional 1183 PCR-confirmed F_1 *An. arabiensis*, were conducted with alpha-cypermethrin (1X), bendiocarb (1X), propoxur (1X), deltamethrin (1X, 2X, 5X and 10X) and permethrin (1X, 2X, 5X and 10X) (20). Complete (100%) mortality was observed to the diagnostic doses of alpha-cypermethrin, bendiocarb and propoxur, while moderate to intense resistance was detected to deltamethrin and permethrin, with small proportions of mosquitos capable of surviving five to ten times the diagnostic concentrations (Fig. 1).

3.2. Target site mutations

Phenotyped individuals were screened for known insecticide resistance target site mutations. The G119S-*Ace-1* mutation was not detected in any mosquitoes from the malathion bioassays (n = 173). The L1014F-*kdr* mutation was identified in 52% (30/58) of *An. arabiensis* exposed to the diagnostic dose of permethrin, with allele frequencies of 0.65 in surviving mosquitoes and 0.26 in dead mosquitoes. A greater proportion of *An. arabiensis* surviving permethrin bioassays were homozygous for L1014F-*kdr* (46%; 6/13) compared to those that died (9%; 4/45), and 38.5% of survivors (5/13) and 33% of dead individuals (15/45) were heterozygous. The L1014S-*kdr* allele was not detected in any sample tested.

3.3. RNA sequencing quality control and mapping metrics

Malathion or permethrin bioassay survivors, field mosquitoes which were not exposed to insecticide, and two *An. arabiensis* susceptible reference strains (originally from Sudan or Ethiopia – Dongola or Sekoru, respectively) were submitted for transcriptomic analysis. For the malathion experiment, Illumina RNA-sequencing generated more than 620 million raw reads across three biological replicates, sequenced in technical duplicate with an average of 68.9 (±5.1) million reads per group. (Table S1). After filtering and quality trimming, an average of 67.6 (±5.0) million reads were retained per group (98.15%) for subsequent analysis. An average of 51 (±7.8) million quality filtered reads per group (75.40%) were mapped to the whole *An. arabiensis* Dongola AaraD1.11 reference genome, with around 59% of the counted fragments mapped to all exonic features of the gene set (Table S1). The permethrin experiment generated more than 569 million reads across three biological replicates, sequenced in technical duplicate with an average of 63.3 (±10.9) million reads per group (Table S1). Quality control filtering retained an average of 61.4 (±10.7) million reads per population (97.02%), with an average of 42.6 (±14.3) million total filtered reads aligned to the reference genome (69.48%) and around 64% of the counted fragments successfully assigned to exons of the gene set (Table S1). Full results for the analyses of the malathion and permethrin experiments are presented in Table S5, and results of gene ontology (GO) enrichment analysis for sets of differentially expressed genes (DEGs) are shown in Table S6.

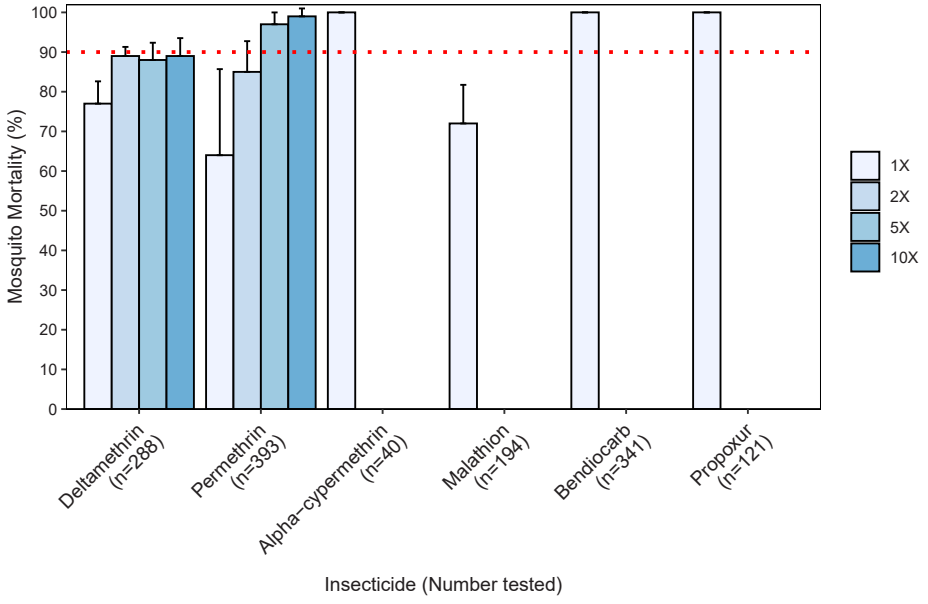

Fig. 1. Bottle bioassay results for pyrethroid (deltamethrin, permethrin and alpha-cypermethrin), organophosphate (malathion) and carbamate (bendiocarb and propoxur) insecticides among *An. arabiensis* from Asendabo, Ethiopia. Bars show the mean mortality after 30 min of insecticide exposure across bottle replicates with 95% confidence intervals. The red dashed line indicates the threshold of 90% mortality, below which a population is considered resistant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. Differentially expressed genes associated with malathion resistance

Differential expression analysis was performed on transcripts retained after quality control and removal of genes with low read counts. Aligned reads were mapped to the *An. arabiensis* genes dataset (AaraD1.11) to quantify levels of gene expression, with between 52 and 69% of alignments successfully assigned to the exonic regions of the reference genome (Table S2). Three pairwise comparisons were conducted for malathion: resistant *vs* susceptible (R–S; MAL-R *vs* DON), resistant *vs* unexposed control (R–C; MAL-R *vs* CON-M) and unexposed control *vs* susceptible (C–S; CON-M *vs* DON). The R–C comparison allowed us to account for induction of transcription during insecticide exposure; genes were filtered by analysing their expression profiles in the susceptible Dongola strain, with the assumption that constitutive resistance genes will be significantly differentially expressed between both bioassay survivors and the non-exposed field mosquitoes, when compared to the susceptible strain.

At the most conservative level (*P*-values adjusted for multiple testing based on a false discovery rate (FDR) < 0.01 and fold change (FC) > 2), a total of 1212 (12.2%; 872 upregulated and 340 downregulated) genes were significantly differentially expressed in mosquitoes that survived malathion exposure and 598 (6.0%; 398 upregulated and 200 downregulated) were significantly differentially expressed in non-insecticide exposed field mosquitoes as compared to the susceptible strain (Fig. 2A; Table 1). A total of 170 (1.8%; 137 upregulated and 33 downregulated) genes were significantly differentially expressed in mosquitoes that survived malathion exposure compared to their non-insecticide exposed counterparts (Fig. 2A; Table 1).

Of the genes that were differentially expressed in all treatment groups (n = 9), 2 were upregulated while 7 were downregulated in one or more conditions (Fig. 2A). Five of these genes had retrievable annotations, all of which were molecular functions or cellular components (for R–C/R–S/C–S comparisons: AARA017080 = peptide methionine sulfoxide reductase, FCs = 2.57, 0.43 and 0.18; AARA016556 = sulfotransferase, FCs = 2.23, 23.88 and 9.92; AARA007045 = protease M1 zinc metalloprotease, FCs = 0.40, 0.18 and 0.44; AARA002630 = transient receptor potential protein, FCs = 0.21, 0.49 and 2.37; and AARA002503 = ion binding protein, FCs = 0.37, 0.04 and 0.17,

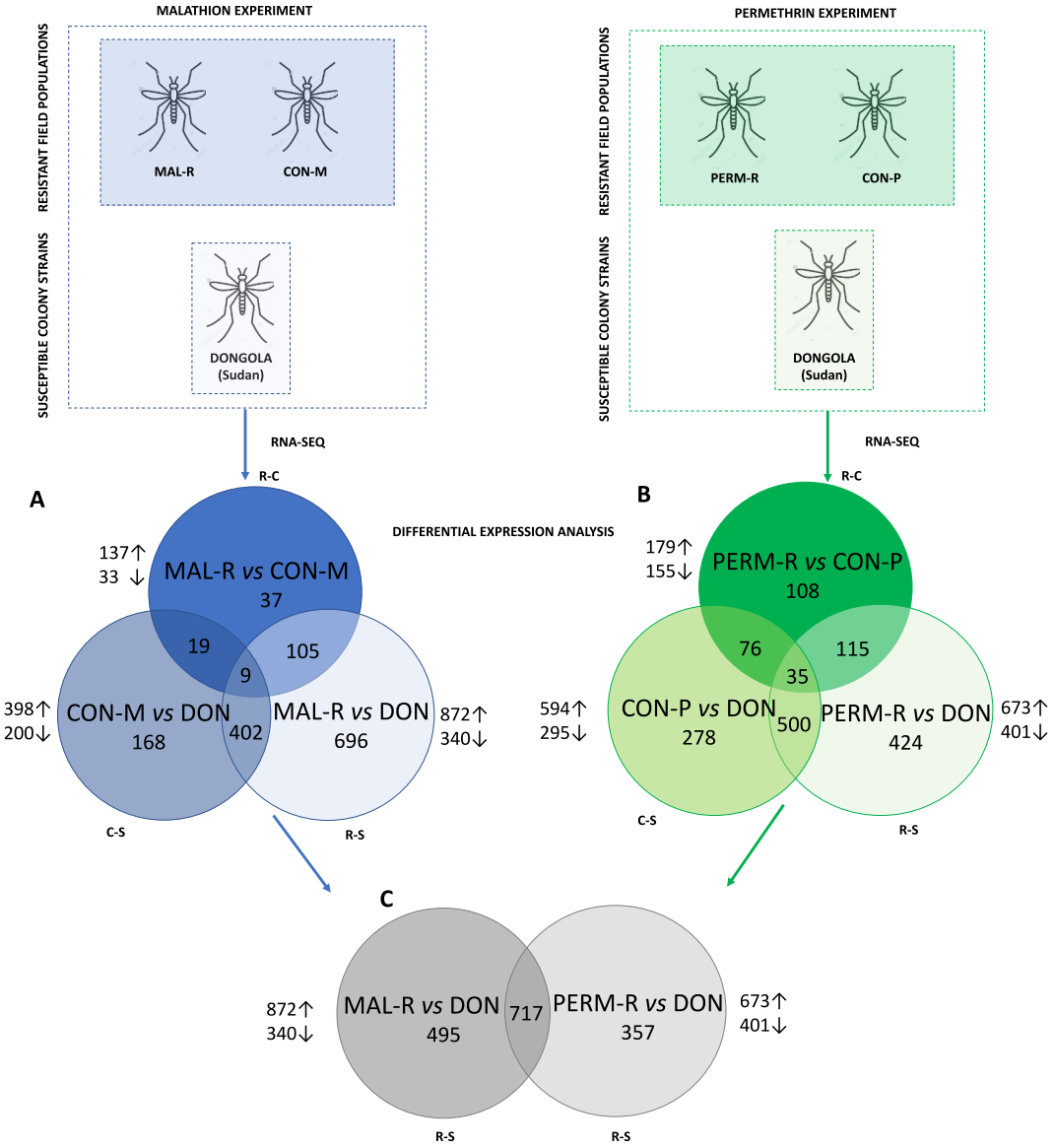

Fig. 2. Experimental design and differentially expressed genes among resistant (R), susceptible (S) and unexposed (C) mosquito populations in malathion (A) and permethrin (B) experiments and in both (C). Each Venn diagram section shows the number of differentially expressed genes meeting each set of conditions (*P*-values were adjusted for multiple testing based on FDR<0.01 and FC > 2). For a list of all DEGs for each comparison see Table S5.

Table 1
Summary of differential gene expression analyses for malathion and permethrin experiments.

Condition	# of genes tested	DE genes (adjP<0.05)		DE genes (adjP<0.01)		DE genes(|FC|>2 & adjP<0.05)		DE genes(|FC|>2 & adjP<0.01)	
		Up	**Down**	**Up**	**Down**	**Up**	**Down**	**Up**	**Down**
MAL-R *vs* CON-M	9609	455	163	209	59	203	61	137	33
MAL-R *vs* DON	9959	1998	1586	1557	1027	893	364	872	340
CON-M *vs* DON	9906	661	972	392	616	229	456	398	200
PERM-R *vs* CON–P	9669	595	424	351	246	214	192	179	155
PERM-R *vs* SEK	9293	2471	2640	1955	2005	1083	1156	1057	1126
CON–P *vs* SEK	9752	2790	2745	2259	2259	1193	2790	1153	1159
MAL-R *vs* PERM-R	9551	139	173	68	94	60	102	45	77
DON *vs* SEK	9961	3074	3148	2579	2619	1564	1447	1557	1414
PERM-R *vs* DON	9885	1565	1256	1086	754	714	450	673	401
CON–P *vs* DON	9999	1354	1046	981	645	632	343	594	295

DON = Dongola susceptible colony; MAL-R = alive after malathion exposure; PERM-R = alive after permethrin exposure; SEK=Sekoru susceptible colony; CON-M = field population not exposed to insecticide in malathion experiment. CON–P = field population not exposed to insecticide in permethrin experiment.
DE = differentially expressed; FC = fold change; adjP = *P*-value adjusted for multiple testing (22).

respectively).

A total of 402 genes were differentially expressed commonly in the R–S and C–S groups (Fig. 2A). Among the top 10 over-expressed genes with retrievable annotations were enzymes with structural, cellular or immune functions, including chitinase (AARA007329: FCs = 50.04 and 10.80 for R–S/C–S comparisons, respectively), D7r4 short form salivary protein (AARA016237: FCs = 33.29 and 31.34), cytoplasmic actin (AARA015772: FC = 29.53 and 7.33), cuticular protein CPLCG (AARA011115: FCs = 26.80 and 20.12), alkaline phosphatase (AARA002132: FCs = 26.33 and 11.83), sulfotransferase (AARA016556: FCs = 23.88 and 9.92), serine protease (AARA009441: FCs = 23.73 and 24.43), polyubiquitin (AARA016579: FCs = 21.67 and 31.07), ADP/ATP carrier protein (AARA017958: FCs = 21.15 and 5.23) and deoxyribonuclease (AARA000505: FCs = 17.0 and 12.15). A total of 19 genes were differentially expressed commonly in the R–C and C–S groups (Fig. 2A). Among the top over-expressed genes with retrievable annotations were notably two odorant binding proteins (for R–C/C–S comparisons, respectively: AARA007908: FCs = 5.17 and 0.17; AARA004722: FCs = 3.24 and 0.19).

Significant differential expression of some members of the detoxification gene families associated with metabolic resistance were observed among R–S and C–S comparisons (Table 2; Fig. 3A). These included nine CYP450s (CYP9K1, CYP9J5, CYP6AA1, CYP4C36, CYP6AA1, CYP9L1, CYP6M2, CYP6M3 and CYP6P4), six GSTs (GSTE2, GSTE3, GSTE4, GSTE5, GSTE7 and GSTD3) and two COEs (AARA016305 and AARA016468). With the exception of GSTD3 and GSTE3, the FCs of all of these detoxification enzymes increased in response to malathion exposure (Table 2). Two additional CYP450s were also upregulated between R–C conditions (CYP4G16, FC = 3.40; and CYP4G17, FC = 2.03) (Supplementary Fig. S1; Table 2).

Significant differential expression of eighteen mosquito salivary gland proteins were identified among R–S and C–S comparisons (Table 2; Fig. 3A), most notably D7r4 short form salivary protein (FCs = 33.29 and 31.34 for R–S/C–S, respectively), TRIO salivary gland protein (FCs = 4.26 and 7.16), AARA009957 (FCs = 10.48 and 7.57) and salivary gland protein 7 (FCs = 5.87 and 6.12). Among these salivary gland proteins, twelve were downregulated following malathion exposure (Table 2); one salivary gland protein was significantly overexpressed between R–C conditions (AARA008387, FC = 2.04). Furthermore, fifteen proteins associated with cuticular function were significantly overexpressed in the R–S condition, including chitinase (AARA007329) (FCs = 50.04 and 10.80 for R–S/C–S, respectively), cuticular protein CPLCG family (AARA011115) (FCs = 26.80 and 20.12), cuticular protein RR-2 family (AARA001131) (FCs = 14.39 and 22.16) and cuticular protein RR-1 family (AARA003903) (FCs = 10.06 and 6.09). The majority of these were upregulated after insecticide treatment (Table 2), with an additional cuticular protein RR-2 family member, significantly overexpressed between R–C conditions (AARA017766, FC = 2.45) (Supplementary Fig. S1).

In malathion resistant mosquitoes, several ontologies were enriched in genes overexpressed relative to susceptible mosquitoes (Table S5). In particular, many of these ontologies were associated with metabolic processes, including "cellular metabolic process" (GO:0044237), "catalytic activity" (GO:0003824) and "generation of precursor metabolites and energy" (GO:0006091). Between R–C conditions, additional metabolic ontologies were upregulated, including "generation of precursor metabolites and energy" (GO:0006091) and "cellular metabolic process" (GO:0044237), potentially associated with increased physiological stress in response to insecticide exposure (Adedeji et al., 2020).

3.5. Differentially expressed genes associated with permethrin resistance

Differential transcription analysis for the permethrin experiment was performed relative to both DON and Sekoru (SEK) susceptible laboratory strains; the latter analysis was performed with the assumption that this more geographically proximate colony from Ethiopia would be a better biologically comparator than DON. However, greater variation in gene expression was observed, with 2183 (23.5%; 1057 upregulated and 1126 downregulated) and 2312 (23.7%; 1153 upregulated and 1159 downregulated) genes significantly differentially expressed between SEK and mosquitoes that survived permethrin exposure and non-exposed field mosquitoes, respectively (Supplementary Fig. S2; Table 1). A multi-dimensional scaling plot revealed significant variation between SEK and all other mosquito populations (Supplementary Fig. S3); downstream analyses for the permethrin experiment were therefore performed relative to DON.

Consistent with the malathion experiment, three pairwise comparisons were conducted for permethrin: resistant *vs* susceptible (R–S; PERM-R *vs* DON), resistant *vs* unexposed control (R–C; PERM-R *vs* CON–P) and unexposed control *vs* susceptible (C–S; CON–P *vs* DON). Among mosquitoes that survived permethrin exposure and non-exposed field mosquitoes, 1074 (10.9%; 673 upregulated and 401 downregulated) and 889 (8.9%; 594 upregulated and 295 downregulated) genes were significantly differentially expressed (at *P*-values adjusted for multiple testing based on a FDR<0.01 and FC > 2), respectively, when compared to the susceptible Dongola strain (Fig. 2B; Table 1). A total of 334 (3.5%; 179 upregulated and 155 downregulated) genes were significantly differentially expressed in permethrin survivors as compared to their non-exposed counterparts (Fig. 2B; Table 1).

Of the genes that were differentially expressed in all treatment groups (n = 35), 3 were upregulated while 32 were downregulated in one or more conditions (Fig. 2B). Eleven had retrievable annotations, which were mostly molecular functions or biological processes (for R–C/R–S/C–S comparisons: AARA015710 = CLIP-domain serine protease, FCs = 2.21, 4.35 and 1.97; AARA015772 = cytoplasmic actin, FCs = 4.24, 51.86 and 12.20; AARA016057 = ATP binding cassette

Table 2
Significantly differentially expressed genes of interest in comparisons of resistant *vs* susceptible (R–S) and control *vs* susceptible (C–S) groups in the malathion and permethrin experiments (FDR<0.05 and FC > 2).

Gene Category	Gene ID	Gene Description[a]	FC MAL-R *vs* DON (R–S)	FC CON-M *vs* DON (C–S)	FC PERM-R *vs* DON (R–S)	FC CON–P *vs* DON (C–S)
Detoxification Enzymes	AARA003630	CYP325C2	NP	NP	4.04	3.49
	AARA008772	CYP4C36	3.22	2.46	3.99	3.54
	AARA011787	CYP4G16	3.40	NS	2.44	NS
	AARA002563	CYP4G17	2.03	NS	FC < 2	NS
	AARA004676	CYP6AA1	2.54	2.45	FC < 2	2.44
	AARA015644	CYP6M2	4.10	2.85	3.26	2.43
	AARA015642	CYP6M3	2.20	FC < 2	2.82	2.19
	AARA015787	CYP6P3	2.29	2.09	NS	3.62
	AARA015789	CYP6P4	8.64	6.72	4.02	8.06
	AARA003376	CYP9J5	2.34	2.11	NS	2.03
	AARA002507	CYP9K1	3.33	2.68	2.55	2.17
	AARA015621	CYP9L1	2.33	2.47	2.15	2.41
	AARA015764	GSTD3	3.48	4.21	3.32	5.36
	AARA015765	GSTD10	NP	NP	28.25	NP
	AARA008732	GSTE2	3.09	3.03	2.46	2.38
	AARA015728	GSTE3	3.90	4.30	3.04	3.44
	AARA015648	GSTE4	6.01	3.23	3.59	3.27
	AARA015649	GSTE5	3.01	2.11	2.88	2.24
	AARA008734	GSTE7	6.73	NP	5.31	5.22
	AARA016305	Carboxylesterase (AGAP028695)	3.56	4.04	NP	3.07
	AARA016468	Carboxylesterase (AGAP028426)	7.18	4.34	4.56	4.62
	AARA004790	Carboxylesterase (AGAP013509)	3.74	NP	4.87	2.61
	AARA001582	Carboxylesterase (AGAP001101)	3.96	FC < 2	2.14	2.89
Salivary Gland Proteins	AARA001829	TRIO salivary gland protein (AGAP001374)	4.26	7.16	2.32	4.63
	AARA008387	Salivary gland protein (AGAP006506)	7.16	2.79	NS	NS
	AARA009957	Salivary gland protein (AGAP003841)	10.48	7.57	NP	6.34
	AARA010442	Salivary gland protein 1-like (AGAP000607)	3.15	5.00	NS	4.07
	AARA014717	Salivary gland protein (AGAP006507)	3.66	3.11	NS	NS
	AARA016088	Salivary gland protein 7-like (AGAP008215)	5.87	6.12	4.80	11.35
	AARA016089	Salivary gland protein (AGAP008216)	2.76	3.88	NS	2.82
	AARA016177	Salivary gland protein 2-like (AGAP006504)	2.89	2.60	FC < 2	2.19
	AARA016208	Salivary gland protein (AGAP013423)	2.09	2.22	NS	NS
	AARA016220	Salivary gland protein (AGAP000612)	2.07	2.60	NS	2.50
	AARA016221	Salivary gland protein 1-like (AGAP000611)	3.09	4.28	2.25	4.73
	AARA016222	Salivary gland protein 1-like (AGAP000610)	2.18	2.45	FC < 2	2.44
	AARA016223	Salivary gland protein 1-like (AGAP000609)	3.08	3.91	2.47	3.27
	AARA016236	D7 short form salivary protein (AGAP008282)	3.53	3.73	FC < 2	6.14
	AARA016237	D7 short form salivary protein (D7r4) (AGAP008281)	33.29	31.34	20.84	43.89
	AARA016239	D7 short form salivary protein (AGAP008283)	2.10	2.50	FC < 2	2.50
	AARA016540	D7 long form salivary protein (AGAP008279)	2.32	2.49	FC < 2	2.51
	AARA011280	D7 long form salivary protein (AARA011280)	3.19	NP	2.46	2.37
Cuticular Proteins	AARA001131	Cuticular protein RR-2 family (AGAP000047)	14.39	22.16	NS	37.08
	AARA002197	Chitin synthase (AGAP001748)	3.05	4.01	NS	6.04
	AARA002622	Cuticular protein RR-1 family (AGAP000344)	4.17	4.12	2.44	6.66
	AARA003897	Cuticular protein RR-1 family (AGAP009879)	5.53	4.44	2.60	6.00
	AARA003899	Cuticular protein RR-1 family (AGAP009877)	3.01	2.40	NS	2.50
	AARA003903	Cuticular protein RR-1 family (AGAP009871)	10.06	6.09	19.34	15.68
	AARA004016	Cuticular protein CPLCP11 (AGAP009758)	6.47	6.84	NS	13.05
	AARA007329	Chitinase (AGAP006191)	50.04	10.80	93.30	16.76
	AARA009226	Chitinase (AGAP006898)	2.86	2.22	2.18	2.93
	AARA011115	Cuticular protein CPLCG family (AGAP008449)	26.80	20.12	NS	34.49
	AARA011120	Cuticular protein CPLCG family (AGAP008444)	6.51	2.99	3.91	6.79
	AARA002342		2.82	NS	3.08	2.84

(*continued on next page*)

Table 2 (*continued*)

Gene Category	Gene ID	Gene Description[a]	FC MAL-R *vs* DON (R–S)	FC CON-M *vs* DON (C–S)	FC PERM-R *vs* DON (R–S)	FC CON–P *vs* DON (C–S)
		Cuticular protein RR-2 family 16 (AGAP005459)				
	AARA016553	Cuticular protein (AGAP000987)	2.10	3.42	3.77	4.28
	AARA016552	Cuticular protein (AGAP000988)	6.49	6.88	6.53	11.34
	AARA016147	Cuticular protein RR-1 family (AGAP006001)	5.31	NS	4.66	4.90
	AARA007248	Cuticular protein RR-2 family (AGAP006283)	NS	4.79	2.19	5.86
	AARA001390	Cuticular protein (AGAP003308)	4.51	3.85	NS	5.60
	AARA002344	Cuticular protein RR-2 family 15 (AGAP005456)	2.17	NS	NS	2.33
	AARA002509	Cuticular protein RR-2 family (AGAP000820)	8.05	13.88	NS	22.50

CON-M = field population not exposed to insecticide; CON–P = field population not exposed to insecticide; DON = Dongola susceptible colony; MAL-R = alive after malathion exposure; PERM-R = alive after permethrin exposure; FC = fold change; FC < 2 = indicates a significantly differentially expressed gene with fold change less than 2; NP = not present in dataset due to low sequencing coverage; NS = not significant.

[a] = gene description based on orthology to *An. gambiae* PEST.

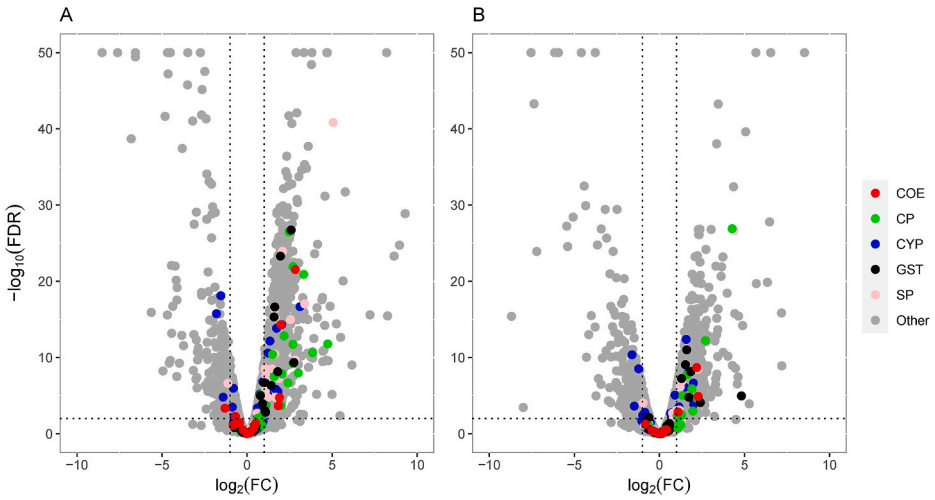

Fig. 3. Volcano plots of gene expression for MAL-R vs DON (A) and PERM-R vs DON (B). The X-axis shows the $\log_2$ fold-change (negative and positive values are down and up-regulated respectively relative to the laboratory strain). Y-axis shows -$\log_{10}$ of the adjusted P-value (-$\log_{10}$FDR values greater than 50 were displayed as 50). Detoxification gene families are indicated in red (COE: carboxylesterases), blue (CYP: cytochrome P450s) and black (GST: glutathione-S-transferases). Cuticular proteins are indicated in green (CP) and salivary gland proteins are indicated in pink (SP). In each plot, genes overexpressed in the population are >0 on the x-axis. Vertical dotted lines indicate 2-fold expression differences and the horizontal line indicates a *P*-value of 0.01. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

transporter, FCs = 0.41, 2.39 and 5.82; AARA016221 = salivary gland protein 1-like, FCs = 9.47, 2.25 and 4.73; AARA002374 = MIP18 family protein CG7949, FCs = 2.38, 4.01 and 1.67; AARA003468 = peptide methionine sulfoxide reductase, FCs = 3.63, 0.42 and 0.11; AARA003599 = TRPL translocation defect protein 14 isoform, FCs = 2.20, 3.47 and 1.57; AARA009096 = diacylglycerol kinase 1 isoform, FCs = 0.41, 0.22 and 0.53; AARA016129 = sorbitol dehydrogenase, FCs = 0.04, 0.35 and 7.99; AARA017544 = serine protease 7-like, FCs = 2.58, 4.70 and 1.82; and AARA018460 = lysosomal alpha-mannosidase, FCs = 0.42, 4.24 and 10.04, respectively).

A total of 500 genes were differentially expressed commonly in the R–S and C–S groups (Fig. 2B). The top 10 over-expressed genes with retrievable annotations were similar to the malathion experiment, including chitinase (AARA007329: FCs = 93.30 and 16.76 for R–S/C–S comparisons, respectively), D7r4 short form salivary protein (AARA016237: FCs = 20.84 and 43.89), cytoplasmic actin (AARA015772: FCs = 51.86 and 12.20), alkaline phosphatase (AARA002132: FCs = 29.70 and 13.74), sulfotransferase (AARA016556: FCs = 33.61 and 16.61), polyubiquitin (AARA016579: FCs = 21.57 and 67.65) and ADP/ATP carrier protein (AARA017958: FCs = 25.15 and 10.50). Cuticular protein RR-1 (AARA003903: FCs = 19.34 and 15.68) and hexamerin (AARA016988: FCs = 15.78 and 7.50) were also highly upregulated.

Consistent with the malathion experiment, key metabolic enzymes were significantly differentially expressed between R–S and C–S comparisons (Table 2; Fig. 3B), including eight CYP450s (CYP6M2, CYP4C36, CYP6AA1, CYP9K1, CYP6M3, CYP6P4, CYP325C2 and CYP9L1), six GSTs (GSTE2, GSTE3, GSTE4, GSTE5, GSTE7 and GSTD3) and three COEs (AARA016468, AARA001582 and AARA004790). Six of these detoxification genes were downregulated following permethrin exposure, including CYP6AA1, CYP9L1, GSTD3, GSTE3 and two COEs (AARA016468 and AARA001582).

One additional CYP450 was also significantly overexpressed between R–C conditions (CYP6Z3, FC = 2.02). A further GST (GSTD10) was highly overexpressed in both R–S and R–C conditions (FCs = 28.25 and 5.94, respectively), but was not present at sufficient sequence coverage in the C–S comparison. In addition, six mosquito salivary gland proteins were identified among R–S and C–S comparisons (Table 2), most notably D7r4 short form salivary protein (FCs = 20.84 and 43.89 for R–S/C–S, respectively), and salivary gland protein 7 (FCs = 4.80 and 11.35), which in contrast to the malathion experiment, were both downregulated in response to permethrin exposure. A further eleven proteins associated with cuticular function displayed differential expression patterns (Table 2; Fig. 3B), including chitinase (AARA007329; FCs = 93.30 and 16.76, for R–S/C–S, respectively), cuticular protein RR-1 family (FCs = 19.34 and 15.68) and cuticular protein (FCs = 6.53 and 11.34, for R–S/C–S). An additional chitinase was significantly overexpressed between R–C conditions (AARA007329, FC = 5.56) (Supplementary Fig. S1).

Similar to the malathion experiment, ontologies enriched in the permethrin experiment also included terms related to “metabolic process” (GO:0008152), “generation of precursor metabolites and energy”

(GO:0006091), "oxidoreductase activity" (GO:0016491) and "carbohydrate metabolic process" (GO:0005975).

3.6. Differentially expressed genes associated with multi-insecticide resistance

A total of 717 (45.7%; 512 upregulated and 205 downregulated) transcripts were significantly differentially expressed in mosquitoes that survived either malathion or permethrin exposure, compared to the susceptible strain (Table S7). Eight key upregulated metabolic enzymes were shared between both resistant groups (MAL-R *vs* DON and PERM-R *vs* DON), including six CYP450s (CYP6P4, CYP4C36, CYP4G16, CYP6M3 and CYP9K1 and CYP9L1), six GSTs (GSTD3, GSTE2, GSTE3, GSTE4, GSTE5 and GSTE7) and three COEs (AARA004790, AARA016468 and AARA001582) (Table 2; Fig. 4); two additional CYP450s (CYP9M2 and CYP304B1) were both downregulated. Unique detoxification DEGs to the malathion resistant group were CYP9J5 (FCs = 2.34 and 2.11 for R–S/C–S, respectively), CYP6P3 (FCs = 2.29 and 2.09) and one COE (AARA016305: FCs = 3.56 and 4.04 for R–S/C–S, respectively). One detoxification DEG was unique to the permethrin resistant population, CYP325C2 (FCs = 4.04 and 3.49, for R–S/C–S, respectively), but was not present at sufficient sequence coverage in the malathion resistant population.

Among salivary gland DEGs, six were shared between both resistant populations (Table 2; Fig. 4): D7r4 short form salivary protein (AARA016237), D7 long form salivary gland protein (AARA011280), salivary gland protein 1-like members (AARA016223 and AARA016221), TRIO salivary gland protein (AARA001829) and salivary gland protein 7-like members (AARA016088). Twelve additional salivary gland proteins were exclusive to the malathion resistant population and none to the permethrin resistant population (Table 2; Fig. 4).

Among cuticular DEGs, ten were shared between both resistant populations: cuticular protein RR-1 family members (AARA002622, AARA003897, AARA003903 and AARA016147), chitinases (AARA007329 and AARA009226), a cuticular protein CPLCG family member (AARA011120), a cuticular protein RR-2 family 16 member (AARA002342), cuticular proteins (AARA016553 and AARA016552). There were eight and one DEGs which were unique to the malathion and permethrin resistant populations, respectively (Table 2; Fig. 4).

Finally, we mined the RNA-seq data to investigate expression patterns of other recently described resistance mechanisms in *An. gambiae* complex members (Ingham et al., 2018, 2019) (Table 3). We identified orthologues in *An. arabiensis* of four α-crystallins, two hexamerins, ATPase subunit e and SAP2 which were significantly differentially expressed between R–S/C–S conditions.

3.7. Detection of resistance target site mutations

RNA-Seq reads from the malathion and permethrin experiments were screened for target site mutations associated with DDT, pyrethroid, organophosphate or carbamate resistance and known voltage-gated sodium channel (VGSC) mutations in *An. gambiae* s.l. (Tables S8 and S9). Consistent with the target site PCR data generated in this study, we did not detect the presence of either L1014S *kdr* or G119S *Ace-1* mutations in any populations. The L1014F-*kdr* mutation was detected in all groups except DON, with average population allele frequencies of CON-M = 27%; CON–P = 24%; MAL-R = 31%; PERM-R = 79%; and SEK = 55% (Table S8). None of the previously described GSTe2 target site mutations (L119F and I114T) (23,24) were present in our dataset, nor was N1575Y, which is linked to L1014F-*kdr* and found at variable frequencies in parts of West and Central Africa (Jones et al., 2012; Collins et al., 2019; Lynd et al., 2018). Of 20 recently described non-synonymous VGSC mutations from West and Central Africa (Clarkson et al., 2021), we detected the presence of seven (R254K,

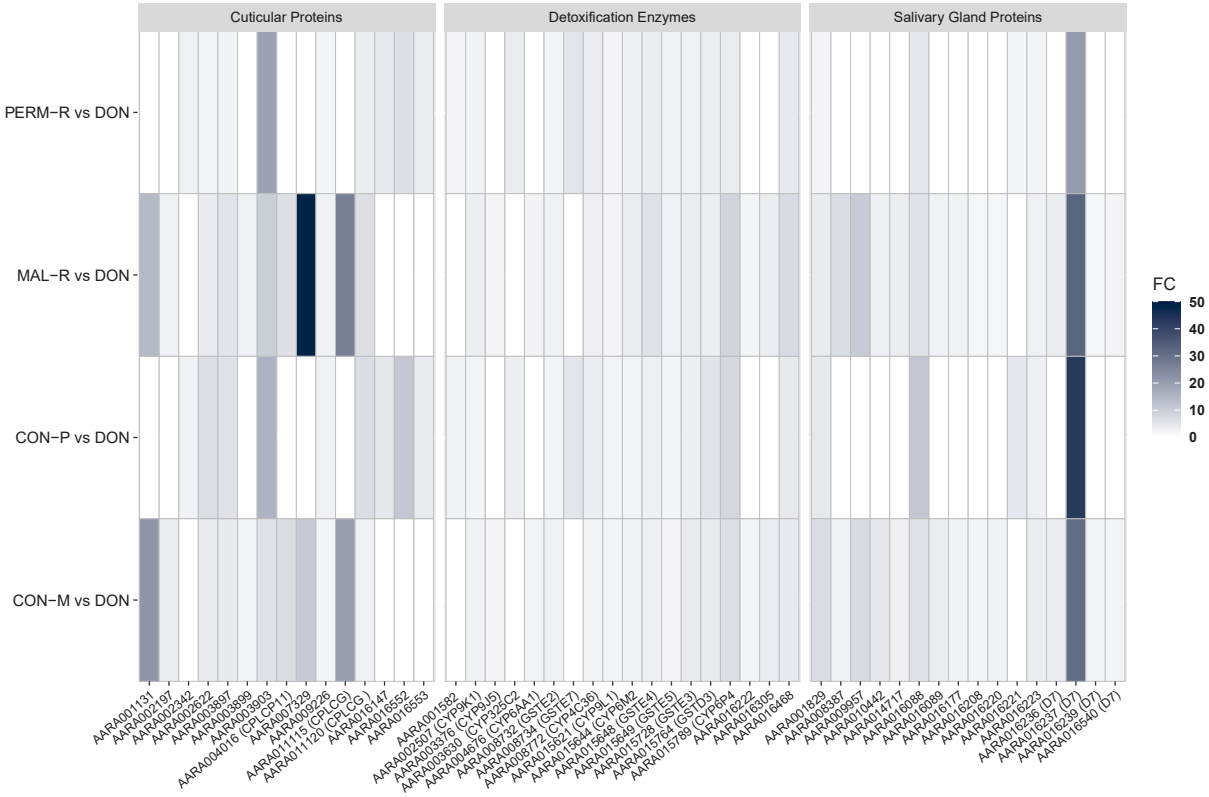

Fig. 4. Heatmaps summarizing expression of cuticular proteins, detoxification enzymes and salivary gland proteins, showing fold-change values relative to the susceptible strain. CON-M = field population not exposed to malathion; CON–P = field population not exposed to permethrin; DON = Dongola susceptible colony; MAL-R = alive after malathion exposure; PERM-R = alive after permethrin exposure; FC = fold change.

Table 3
Significantly differentially expressed genes of interest in comparisons of resistant *vs* susceptible (R–S) and control *vs* susceptible (C–S) groups in the malathion and permethrin experiments (FDR <0.05).

Reference	Gene Category	Gene ID[#]	Gene Description[a]	FC MAL-R *vs* DON (R–S)	FC CON-M *vs* DON (C–S)	FC MAL-R *vs* CON-M (R–C)	FC PERM-R *vs* DON (R–S)	FC CON–P *vs* DON (C–S)	FC PERM-R *vs* CON–P (R–C)
12	α-crystallins	AARA007814	α-crystallin chain B	4.96	5.83	NS	4.21	8.27	NS
		AARA007816	α-crystallin chain A	2.57	2.40	NS	2.00	2.37	NS
		AARA007817	α-crystallin chain A	1.67	1.96	NS	1.48	2.61	0.57
		AARA018370	α-crystallin chain B	1.97	NS	NS	1.67	NP	NP
12	Hexamerin	AARA016988	Hexamerin	NP	12.33	0.09	15.78	7.50	NS
		AARA002101	Hexamerin	NP	11.13	0.13	10.94	5.29	NS
12	ATPase Subunit e	AARA015629	F-type H + -transporting ATPase subunit e	4.58	NS	2.11	4.15	2.97	NS
15	Sensory Appendage Protein	AARA005074 (AGAP008052)	SAP2	0.72	0.53	NS	0.90	0.60	1.49

CON-M = field population not exposed to malathion; CON–P = field population not exposed to permethrin; DON = Dongola susceptible colony; MAL-R = alive after malathion exposure; PERM-R = alive after permethrin exposure; FC = fold change; NP = not present in dataset due to low sequencing coverage; NS = not significant; [#] = gene ID retrieved based on orthology to *An. gambiae*.
[a] = gene description based on orthology to *An. gambiae* PEST.

A1125V, I1868T, P1874L, F1920S, A1934V and I1940T) across the Asendabo field population at very low frequencies (range of 1–7%); 2 of these were also found in SEK (I1868T and I1940T).

3.8. qRT-PCR validation of relative expression levels estimated by RNA-Seq

Quantitative RT-PCR was used to validate the FCs of eleven genes (CYP4G16, CYP4G17, GSTM3, CPR130, GSTE7, CYP6M2, D7r4 short form salivary protein, chitinase, cuticular protein RR-1 family, CYP6M3 and GSTE3), relative to two housekeeping genes (40S ribosomal protein S7; RPS7 and ubiquitin) (Fig. 5). The majority of the qRT-PCR results supported the directionality of the changes in expression levels as estimated by RNA-Seq.

4. Discussion

Using a whole transcriptomic approach, we investigated the molecular basis of resistance to malathion and permethrin in *An. arabiensis* from southwest Ethiopia. Our analyses allowed for comparisons between insecticides, to detect shared expression patterns between different active ingredients and to identify novel diagnostic markers associated with phenotypic resistance. In addition to malathion and permethrin resistance, the field population was also resistant to

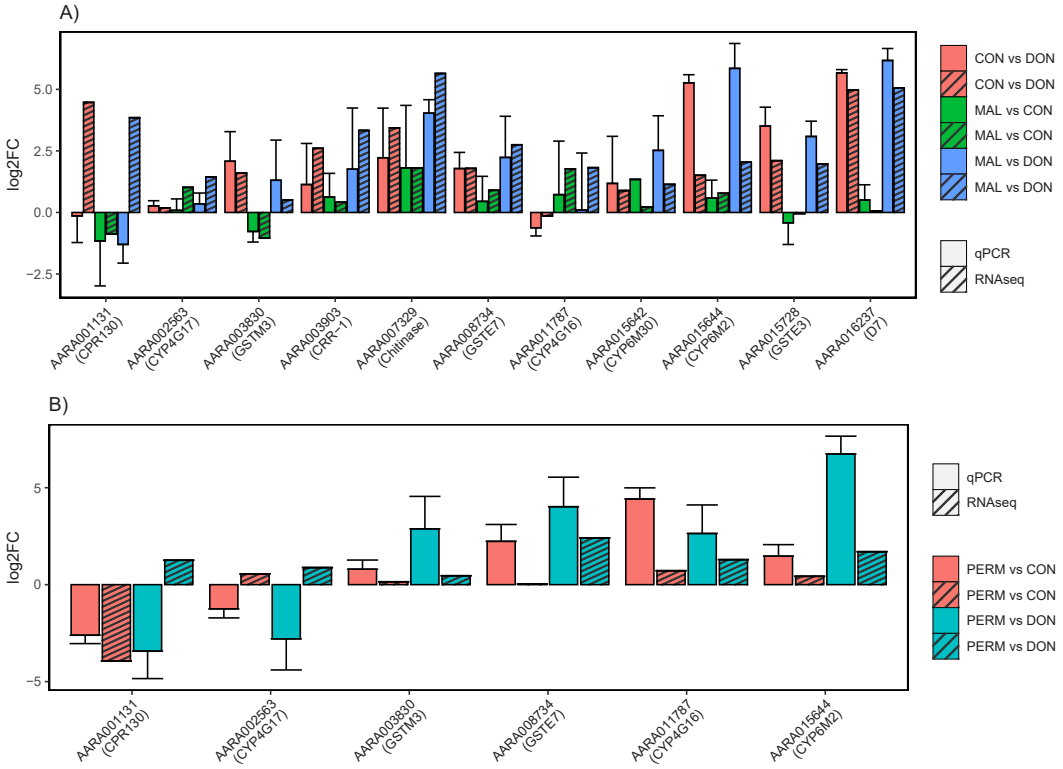

Fig. 5. Comparison of expression levels of DEGs measured by qRT-PCR and RNA-Seq in malathion (A) and permethrin (B) experiments.

deltamethrin but remained susceptible to alpha-cypermethrin, bendiocarb and propoxur. A previous study from the same region reported susceptibility to the putative diagnostic doses of clothianidin (neonicotinoid) and chlorfenapyr (pyrrole) (Dagg et al., 2019). Bioassay results indicated that insecticide-specific mechanisms may be important in this *An. arabiensis* population, as demonstrated by the lack of cross-resistance between active ingredients belonging to the same chemical class (for example permethrin and alpha-cypermethrin). Insecticide resistance profiles in Asendabo aligned with recent nationwide insecticide resistance monitoring results (Messenger et al., 2017). However, bendiocarb and alpha-cypermethrin tolerance appeared dynamic, with reduced local *An. arabiensis* mortality to both chemicals reported during previous years but absent in our study (Messenger et al., 2017).

In both malathion and permethrin resistant groups, several CYP450s and GSTs, which have been associated with pyrethroid and DDT resistance in populations of *An. arabiensis*, were overexpressed. Upregulation of CYP6M2, CYP6M3, CYP6P4, CYP9K1 and GSTE4, which were shared between both resistant groups, has been documented in pyrethroid and DDT resistant *An. arabiensis* from central Sudan (Abdalla et al., 2014). In addition, we detected overexpression of CYP4C36, CYP6AA1, CYP9L1, GSTD3, GSTE2, GSTE3, GSTE5, GSTE7 and three carboxylesterases (AARA016468, AARA004790 and AARA001582) in both resistant groups; carboxylesterases have previously been implicated in pyrethroid resistance in *An. funestus* from Malawi (Wondji et al., 2012). Overexpression of CYP6P3 and CYP9J5, which were exclusive to malathion survivors, has also been observed in permethrin-resistant *An. arabiensis* from Sudan (Abdalla et al., 2014) and permethrin and DDT resistant *An. arabiensis* from South Africa (Nardini et al., 2012, 2013). Many of these CYP450s are regularly reported from multi-insecticide resistant populations of *An. gambiae* across sub-Saharan Africa and have been shown to metabolize different combinations of type I and type II pyrethroids, DDT and pyriproxyfen *in vitro* (Müller et al., 2008a; Stevenson et al., 2011; Chiu et al., 2008; Mitchell et al., 2012; Vontas et al., 2018; Yunta et al., 2019; reviewed by Vontas et al., 2020). *In vivo* functional characterization of CYP6M2 and CYP6P3 in *An. gambiae* demonstrated that overexpression enhanced susceptibility to malathion by catalysing the bioactivation of this insecticide to its more toxic metabolite malaoxon by a CYP450-mediated mechanism (Voice et al., 2012); with CYP6M2 increasing malaoxon production to a greater degree compared to CYP6P3 (Adolfi et al., 2019). Our contradicting results may be explained by the relative activity of the transcription factor Maf-S, which when knocked-down has been shown to increase survival to malathion exposure (Ingham et al., 2017) and was not found to be significantly differentially expressed in this study. CYP325C2, which was the only unique CYP450 overexpressed in our permethrin resistant population, has been reported from *An. arabiensis* in Kenya (Bonizzoni et al., 2015) and Cameroon (Müller et al., 2008b) with reduced susceptibility to deltamethrin. Interestingly, CYP325C2 was not identified as a DEG in previous transcriptomic analysis performed among deltamethrin and DDT survivors from Asendabo, which may indicate that it is specific to permethrin resistance in this field population (Müller et al., 2008b). Following permethrin exposure, CYP6Z3 was also significantly upregulated in survivors compared to the unexposed population; overexpression of this enzyme has also been implicated in lambda-cyhalothrin resistance in *An. arabiensis* from Tanzania (Jones et al., 2013).

In Ethiopia, spatial and temporal patterns of insecticide resistance have generally correlated with changes in national malaria vector control policy. Intense pyrethroid resistance is not unexpected given the quantity of conventional LLINs which have been distributed across the region (>80 million since 2008), while the continued presence of malathion resistance is more surprising. Malathion was last used extensively for malaria control from 2003 to 2005 by the NMCP in areas with reported DDT resistance (Abose et al., 1998; Yewhalaw et al., 2011). Between 2005 and 2017, malathion susceptibility was monitored in 127 sentinel sites in Ethiopia, with evidence for possible resistance at 55 sites, confirmed resistance at 36 sites and susceptibility at 36 sites (reviewed by Mekuriaw et al., 2019). In general, resistance instability in the absence of insecticidal pressure, largely attributable to fitness costs, has been well documented among a number of medically-important vector species (Grossman et al., 2018; Shi et al., 2015; Williams et al., 2019); with some notable exceptions, particularly dieldrin resistance (Grau-Bové et al., 2020). Selection experiments using field populations of *An. gambiae* have determined that the rate of resistance decay to full pyrethroid susceptibility from moderate resistance intensity can be as little as 15 generations or approximately 1.3 years in typical African settings (Machani et al., 2020). Our transcriptome data revealed shared overexpression of detoxification enzymes between malathion and permethrin resistant groups, which may be responsible for cross-resistance due to ongoing pyrethroid selection and as a result, maintaining decreased malathion susceptibility.

Another explanation for the continued persistence of malathion resistance in this field population might be that underlying resistance mechanisms impart other physiological benefits to individuals in both the presence and absence of insecticidal exposure. We identified nineteen cuticular proteins and associated enzymes which in some cases were upregulated by more than fifty- or ninety-fold in resistant groups compared to the susceptible strain. These were generally much more highly overexpressed than any of the detoxification enzymes and some of which were observably induced by insecticide exposure (including cuticular protein RR-1 family; AARA003903, chitinase; AARA007329 and cuticular protein CPLCG; AARA011115). Evidence is emerging to strongly support a key role for cuticular thickening as a generalist mechanism of insecticide resistance across *Anopheles* populations, through either enriched deposition of cuticular hydrocarbons or changes to structural components of the procuticle (Wood et al., 2010; Balabanidou et al., 2019). Thicker femur cuticles can delay the penetration rate of contact chemicals (Wood et al., 2010) and/or increase the time available for metabolic processes to inactivate the insecticide before it causes inhibition, thus potentially producing a more intense, non-specific resistance phenotype (Balabanidou et al., 2016b). Following malathion exposure, our field population was characterized by a significant increase in CYP4G16 and CYP4G17 expression; both genes are known to facilitate hydrocarbon production, with the former catalysing epicuticular hydrocarbon biosynthesis (Balabanidou et al., 2016b; Yahouedo et al., 2017). Previous analyses of the Asendabo population also support the potential involvement of cuticular resistance, via increased cuticular hydrocarbon quantities but not procuticle thickness (Balabanidou et al., 2016a). Recent multiplex qRT-PCR assays have been developed with CYP4G16 as a candidate surveillance marker for metabolic resistance in *An. gambiae* which will begin to improve our understanding of its relative involvement in regional cuticular resistance (Mavridis et al., 2019). Among the genes we selected for qPCR validation, chitinase (AARA007329) was very highly overexpressed, induced by exposure to malathion (FCs = 50.04 and 10.80, for R–S/C–S, respectively) and permethrin (FCs = 93.3 and 16.76) and may represent an informative cuticular-associated gene for resistance monitoring in *An. arabiensis* populations. Further investigation is required to determine whether chitinase overexpression is a causative factor in resistance or if it is closely associated with a resistance-conferring variant, as it might be expected to enhance insecticide toxicity by promoting faster cuticle degradation (Merzendorfer and Zimoch, 2003).

In this study, we also detected another putative resistance mechanism in the form of eighteen differentially expressed salivary proteins, particularly the D7 short form salivary protein (the ortholog of D7r4 in *An. gambiae*), which was overexpressed by more than twenty-to thirty-fold following malathion exposure but was notably downregulated following permethrin exposure. Overexpression of D7r4 has been observed in pyrethroid-resistant *An. arabiensis* populations from Sudan, Uganda and Zanzibar (Abdalla et al., 2014; Jones et al., 2013; Wilding et al., 2015) and carbamate- and pyrethroid-resistant *An. funestus* and

An. gambiae (Isaacs et al., 2018; Ibrahim et al., 2016b); this is the first report of D7r4 associated with organophosphate (malathion) resistance. It has been suggested that D7 overexpression is symptomatic of a disruption in the tissue-specificity of these salivary gland proteins, allowing these proteins to interact with insecticides in tissues other than the salivary glands (Isaacs et al., 2018). Furthermore, *in silico* modelling of the protein structure of D7r4 has shown it can accommodate bendiocarb in its central binding pocket, supporting a role for this molecule in binding and sequestering insecticide or insecticide metabolites, rather than by direct detoxification (Isaacs et al., 2018). Similarly, we detected overexpressed candidate α-crystallins, hexamerins and an ATPase subunit which have been proposed to play as yet undefined functions in binding and sequestering insecticides (Ingham et al., 2018). By comparison to *An. gambiae,* our understanding of the molecular basis of resistance in *An. arabiensis* is far more limited; however, our findings highlight several potential shared pathways between these major vector species that warrant further investigation.

In addition to gene expression patterns, we also investigated the prevalence of known resistance target site mutations in our field population. We detected L1014F-*kdr* at moderate to high allele frequencies among permethrin survivors, and also confirmed the absence of L1014S-*kdr*, N1575Y, G119S-*Ace-1* and two GSTe2 mutations (L119F and I114T) (Mitchell et al., 2014; Lucas et al., 2019; Jones et al., 2012), which have yet to be reported in Ethiopia (Messenger et al., 2017; Alemayehu et al., 2017; Simma et al., 2019). Furthermore, from our RNA-Seq data, we detected the presence of seven novel mutations in the VGSC of our pooled *An. arabiensis* populations; one in domain one (in the linker between transmembrane segments four and five; R254K), one in the linker between domains two and three (A1125V) and five in the internal carboxyl tail (I1868T, P1874L, F1920S, A1934V and I1940T). These belong to a group of 14 non-synonymous substitutions in the VGSC recently described in *An. gambiae* and *An. coluzzii,* which have likely evolved in association with L1014F-*kdr* and appear to have been positively selected following decades of DDT/pyrethroid use (Clarkson et al., 2021). In particular, the substitutions located in the C-terminal tail have been proposed to disrupt the confirmation of the DIII-DIV linker subdomain, which is normally bound in close proximity to the DIV S6 helix, inactivating the VGSC (Clarkson et al., 2021). The expected outcome would be altered channel inactivation, but this awaits functional validation.

5. Conclusions

Insecticide-resistant mosquito populations remain a significant challenge to global malaria vector control. While substantial progress has been made unraveling resistance mechanisms in major vector species, such as *An. gambiae* and *An. funestus,* comparatively less is known about *An. arabiensis* populations. Using a whole transcriptomic approach, we investigated the molecular basis of resistance to two public health insecticides in *An. arabiensis* from Ethiopia. Study findings revealed shared detoxification enzymes between organophosphate and pyrethroid-resistant vectors and highly overexpressed cuticular-associated proteins and salivary gland-associated proteins, which may play a role in enhancing vector resistance. The advantages of adopting a transcriptomic approach are evidenced by its universal mechanistic characterization, allowing for the discovery of novel candidate resistance genes, which warrant functional validation to determine their contributions to insecticide resistance, including their potential to confer cross-resistance between different insecticides with the same mode of action.

Ethics approval and consent to participate

The study protocol was reviewed and approved by the institutional review boards (IRBs) of the Institute of Health, Jimma University (THRPGD/843/17) and the U.S. Centers for Disease Control and Prevention, USA (2017–227).

Consent for publication

Not applicable.

Availability of data and material

Sequence data generated by this study is available at Sequence Read Archive (SRA) BioProject PRJNA730212 (accession numbers: SAMN19223816-SAMN19223833). All other relevant data are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the CDC's Advanced Molecular Detection (AMD) program. LAM was supported by an American Society for Microbiology/Centers for Disease Control and Prevention Fellowship. SI is supported by the President's Malaria Initiative (PMI)/CDC.

Author contributions

LAM, LMI, DY, SI and AL designed the study. LAM and DY conducted the field work. LAM and LMI undertook mosquito rearing, phenotyping and preparation of samples for sequencing. Laboratory supervision was provided by AL. DD, LAM and LMI performed the formal data analysis. LAM, LMI, DD and AL drafted the manuscript, which was reviewed by DY and SI.

Acknowledgements

The authors would like to thank all of the entomology fieldworkers of the Tropical and Infectious Diseases Research Center (TIDRC), Jimma University for their dedicated work and the residents of Asendabo for their study participation. We gratefully acknowledge members of the Biotechnology Core Facility Branch, U.S. Centers for Disease Control and Prevention (CDC), Atlanta. The following reagent was obtained through BEI Resources, NIAID, NIH: *Anopheles arabiensis,* Strain DONGOLA, MRA-856, contributed by Mark Q. Benedict. We thank Dustin Miller for providing PCR controls, Gareth Weedall and Steven E. Massey for analytical expertise and Yikun Li for PCR technical advice.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2021.103655.

Disclaimer

The findings and conclusions in this paper are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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