Molecular drivers of insecticide resistance in the Sahelo-Sudanian populations of a major malaria vector

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Abstract

Information on common markers of metabolic resistance in malaria vectors from countries/regions sharing similar eco-climatic characteristics can facilitate coordination of malaria control. Here, we characterized populations of the major malaria vector *Anopheles coluzzii* from Sahelian transect/region, spanning four sub-Saharan African countries: Nigeria, Niger, Chad and Cameroon. Genome-wide transcriptional analysis identified major genes previously implicated in pyrethroid and/or cross resistance to other insecticides, overexpressed across the Sahel, including CYP450s, glutathione S-transferases, carboxylesterases, and cuticular proteins. Several, well-known variants/markers of insecticide resistance were found in high frequencies - including in the voltage-gated sodium channel (V402L, I940T, L995F, I1527T and N1570Y), the *acetylcholinesterase*-1 gene (G280S) and the *CYP4J5*-L43F, which is fixed. High frequencies of the phenotypically important chromosomal inversions, 2La, 2Rb and 2Rc were observed (~80% for 2La and 2Rb). The 2La alternative arrangement is fixed across the Sahel. Low frequencies of these inversions (<10%) were observed in the fully insecticide susceptible laboratory colony of *An. coluzzii* (Ngoussou). Several of the most commonly overexpressed metabolic resistance genes sit in these three inversions. Two commonly overexpressed genes, *GSTe2* and *CYP6Z2* were functionally validated. Transgenic *Drosophila melanogaster* expressing *GSTe2* exhibited extremely high DDT and permethrin resistance (mortalities < 10% in 24h). Serial deletion of the 5' intergenic region, to identify putative nucleotide(s) associated with *GSTe2* overexpression, revealed that simultaneous insertion of adenine nucleotide and a transition (T->C), between Fork-head box L1 and c-EST putative binding sites are responsible for the high overexpression of *GSTe2* in the resistant mosquitoes, across the Sahel. Transgenic flies expressing *CYP6Z2* exhibited

marginal resistance towards 3-phenoxybenzylalcohol (a primary product of pyrethroid hydrolysis by carboxylesterases) and a type II pyrethroid, α-cypermethrin. However, significantly higher mortalities were obtained in *CYP6Z2* transgenic flies compared with controls, on exposure to the neonicotinoid, clothianidin. This suggests a possible bioactivation of clothianidin into a toxic intermediate, which if true make it an ideal insecticide against populations of *An. coluzzii* overexpressing this P450. These findings will facilitate regional collaborations within the Sahel region, and refine implementation strategies through re-focusing interventions, improving evidence-based, cross-border policy towards local and regional malaria pre-elimination.

Keywords: *Anopheles coluzzii*, Sahel, insecticides, pyrethroid, DDT, metabolic, resistance, genes, inversions.

Background

Since the year 2000 the massive scale-up in vector control interventions and treatment with antimalarial drugs had cut malaria incidence by ~40 % across Africa (1). Bolstered by this progress the World Health Organization (WHO) has been pushing to eliminate malaria, as proposed in the Global Technical Strategy (GTS) 2016-2030 (2). Unfortunately, the GTS, an ambitious framework with targets to reduce global malaria burden by 90 % in 15 years was dealt an immediate blow by a rebound in malaria transmission, with increased cases between 2016 and 2019 (3, 4). This stark warning of the risk posed to control and elimination efforts was a reflection of the lack of progress in the primary regions of interest in sub-Saharan Africa, which constitute 96 % of the 627,000 malaria-related deaths in 2020 alone (4, 5). Indeed, as the WHO widens the net of malaria elimination (E-2025) its important to acknowledge that no meaningful progress will be made without progress in sub-Saharan Africa, where none of the six countries having the highest global burden of malaria (e.g. Nigeria alone contributing ~27 % of all cases) is on the path to elimination (6).

The recent escalation of insecticide resistance in the major malaria vectors (7-9) makes the development of molecular tools to anticipate emergence and predict spread of resistance across the African continent imperative, in order to achieve malaria control and elimination. For decades no simple molecular assays were available to track metabolic resistance and assess its impact on malaria control and transmission, until the recent discovery of two DNA markers in the *cis*-regulatory region of two cytochrome P450s, *CYP6P9a* (10) and *CYP6P9b* (11) in the major malaria vector *Anopheles funestus*; findings which allowed the design of simple PCR assays to detect and track metabolic resistance in the field. Unfortunately, these markers explain resistance only in southern Africa for *An. funestus*, since genetic basis of pyrethroid

resistance, and cross-resistance with other insecticides is complex in other African regions with different fronts, driven by distinct genes in the presence of barriers to gene flow (10, 12, 13). No major metabolic resistance markers for P450s and/or GSTs (functionally validated and characterised for epidemiological impact in the natural populations) exist for the major malaria vectors of the *Anopheles gambiae* Complex, though it is the omnipresent vector species with widespread presence across Africa (14). This is hindering early detection and tracking of molecular drivers of resistance in this species, slowing down evidence-based control measures and resistance management.

Major genomic regions associated with metabolic resistance to pyrethroids in *An. gambiae* sensu lato include the *CYP6* P450 clusters on the 2R and 3R chromosomes, and a *GST* epsilon (*GSTe*) cluster on chromosome 3R (15), with some key resistance-associated genes functionally validated. These genes include *CYP6P3* shown to confer cross resistance to pyrethroids and organophosphates (16, 17), *CYP6M2* conferring cross resistance to pyrethroids (18, 19) and DDT (19), as well as *GSTe2* shown to confer resistance to DDT (20). For *GSTe2*, in addition to the I114T marker (20), a recent study has found a novel mutation (*Gste2*-119V) associated with resistance (21) using a high-throughput genotypic panel for markers. Functional validation and fild data is required establish the empirical evidence of the role of this mutation in resistance. What has been missing in the case of *Anopheles gambiae/coluzzii* is reliable molecular markers of resistance for major metabolic gene families, e.g. P450s (with field and laboratory validated data) to aid creation of DNA-based diagnostic assays which will allow (i) easy tracking of resistance in the field (e.g. the case of 119F-*GSTe2* mutation in *An. funestus* (22), and (ii) determination of the operational impact of the resistance markers in the field, as

recently done for 119F-*GSTe2* (23) and *CYP6P9a*_R/*CYP6P9b*_R markers (10, 11) in *An. funestus*.

The *An. gambiae s.l.*, especially populations in the semi-arid steppe, exhibit high frequency of paracentric chromosomal inversions [one of the most effective instruments for speciation and local adaptations (Ayala et al., 2014, Dobzhansky, 1971, Kirkpatrick, 2010)], maintained in spatially and temporally heterogenous environment, and which segregate along climatic gradients of increasing aridity (24)]. The 2La inversions are associated with resistance to desiccation in adults (Fouet et al., 2012, Gray et al., 2009) and thermal stress in larvae (Cassone et al., 2011). It was also shown that inversion 2La assorts with insecticide resistance, e.g., dieldrin plus fipronil (Brooke et al., 2000), and is associated with thermotolerance and permethrin resistance in the Sahelain *An. coluzzii* (Ibrahim et al., 2021).

To support malaria pre-elimination effort in sub-Saharan Africa we targeted the Sahelo-Sudanian region, which represent northern-most limit of malaria endemicity in sub-Saharan Africa, and where malaria is highly seasonal [offering excellent target for pre-elimination effort through sustained seasonal vector control and seasonal malaria chemoprevention (25)]. Focusing on the predominant malaria vector, *An. coluzzii* from Sudan/Sahel transects of four countries, Nigeria, Niger, Chad and Cameroon (7, 26-28) we identified the major metabolic resistance genes mediating pyrethroid resistance and cross-resistance in this region, established the genetic variants which explained the resistance. We also functionally validated the roles of two major candidate genes in the resistance (*CYP6Z2* and *GSTe2*), as well as single nucleotide polymorphism in the 5′ regulatory elements of *GSTe2*, responsible for its overexpression in the resistant population.

Materials and Methods

Study Site and Mosquito Sampling

Blood fed female *An. coluzzii* mosquitoes, resting indoor were collected at one locality each in Sudan/Sahel (Additional Figure S1): Hadiyau (HAD: 12°21′38″N, 9°59′15″E), a Sudan/sub-Sahel village in northern Nigeria; Takatsaba (TAK: 13°44'01.8"N 7°59'05.2"E), a Sahel village in southern Niger; Simatou (SIMAT: 10°50'40.7"N 14°56'40.9"E), a Sudan/sub-Sahel village in Maga Department, far north of Cameroon; and Massakory (CHAD: 12° 6′ N, 15° 02′ E), a Sahel town in Chad Republic. Details of sampling approaches and resistance profiles of mosquitoes collected from Nigeria, Niger, and Chad are available in previously published articles (7, 26, 28). As in the above countries the Simatou F_1 females were also highly pyrethroid resistant, with a mortality of only 3.7% from WHO tube bioassays using 0.05% deltamethrin, and no mortality at all with 0.75% permethrin (data not published).

Genome-wide transcriptional analysis for common insecticide resistance genes in the Sahel/sub-Sahel regions

RNA extraction, library preparation and sequencing

The RNA was extracted using the Arcturus PicoPure RNA isolation Kit (Applied Biosystems, CA, USA) from three pools of 8 F_1 *An. coluzzii* females (2-4 day old) alive after exposure to deltamethrin (resistant, R), unexposed (control, C), and also from unexposed females of the fully susceptible laboratory colony of *An. coluzzii*, Ngoussou (susceptible, S) (29). RNA isolation was carried out following the manufacturer's protocol with *Dnase* I-treatment to

remove contaminating DNA. The quantity and quality of RNA was measured using a NanoDrop spectrophotometer (ThermoFisher, MA, USA) and Bioanalyzer (Agilent, CA, USA).

Library preparation, sequencing and data quality control were carried out by the Centre for Genomic Research (CGR), University of Liverpool, UK. RNA samples were subjected to poly(A) mRNA enrichment and libraries prepared from the poly(A) mRNA-enriched materials (dual-indexed, strand-specific RNAseq libraries were prepared using the NEBNext polyA selection and Ultra Directional RNA library preparation kits). Libraries were sequenced on a single lane of an Illumina HiSeq 4000 (paired-end, 2x150 bp sequencing, generating data from >280 M clusters per lane). Basecalling and de-multiplexing of indexed reads were performed by CASAVA version 1.8.2 (Illumina). De-multiplexed fastq files were trimmed to remove Illumina adapter sequences using Cutadapt version 1.2.1 (30). Option -O 3 was used, so that the 3' end of any reads which matched the adapter sequence for 3 bp or more were trimmed. Reads were further trimmed to remove low quality bases using Sickle version 1.200 (31) with a minimum window quality score of 20. Reads shorter than 20 bp after trimming were removed. If both reads from a pair passed this filter, each was included in either the R1 (forward reads) or R2 (reverse reads) file. If only one of a read pair passed this filter, it is included in the R0 (unpaired) file. Statistics were generated using fastq-stats from EAUtils (32). Summary of total number of reads for each sample and distribution of trimmed read length for forward (R1) and reverse (R2) reads and reads unpaired after trimming (R0) are provided in Additional Figure S2.

194 ***Data analysis and estimation of transcript abundance by tag counting and differential gene***
195 ***expression***

196 Paired data for each replicate per country was aligned to the *An. gambiae* reference transcript
197 ome AgamP4.10 downloaded from VectorBase (https://vectorbase.org/) in salmon (0.11.4),
198 using 'validate mappings', 'seqBias', 'gcBias' and 'rangeFactorizationBins 4' flags. Read
199 mapping results (pre-alignment and post-alignment descriptive statistics (flagstat output
200 files) showing sequencing depth and coverage are given in Additional Tables 1 and -2,
201 respectively. Salmon results were converted into a gene expression matrix using the
202 Bioconductor package 'tximport' for input to DESeq2 1.26.0 (33). Differential gene expression
203 was tested for the three possible combinations of Exposed (R, deltamethrin resistant),
204 Unexposed (C, control) and Susceptible replicates (S). For results interpretation log_2-fold
205 change thresholds of 1 was imposed, with false discovery rate adjusted p-values of 0.05
206 applied to accept significance. Principal components analysis implemented in DESeq2 was
207 used to examine relationships between respective replicates and treatments. This was carried
208 out based on the 500 most variable genes, with data transformation (normalisation/scaling) as
209 implemented in VST (DESeq2). For visualisation of expression results, volcano plots were
210 created using the Enhanced Volcano package (34) using the top most overexpressed genes
211 from lists which were prepared with log_2FC cut off of 1 and p value of 0.01. Heatmaps were
212 generated (35) using the list of the top 50 most overexpressed metabolic resistance genes with
213 log_2FC cut off of 1 and p value of 0.01.

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Gene Ontology Enrichment and Mapping/Functional annotations

The enrichment analysis for GO terms was carried out using a topGO package (36) against *An. gambiae* (AgamP4.10), with data reannotated using EggNOG v5.0 (http://eggnog5.embl.de/#/app/home). Gene lists used in topGO are from the $log_2FC = 0$, $p < 0.05$ analysis (the default in DESeq2). For the data from each site (country) six set of results were generated (R, C and S contrasts) for genes either up- or down-regulated in each contrast, for molecular function (MF) and biological process (BP) ontologies. For analysis the GO terms lists for MF were used as input into the Revigo (37) for interpretation and visualisation, querying the Whole Uniprot database, and SimRel setting for semantic similarity measurements.

Quantitative PCR measurement of expression profiles of metabolic resistance genes

The level of expression of 12 resistance-associated genes was validated by qRT-PCR using the primers provided in Additional Table 3. These include the *GSTe2* (AGAP009194), *GSTZ1* (AGAP002898), *CYP6Z2* (AGAP008218), *CYP6Z3* (AGAP008217), *CYP4C27* (AGAP009246), *CYP4G16* (AGAP001076), *CYP4G17* (AGAP000877), *CYP6P3* (AGAP002865), *CYP6M2* (AGAP008212), *CYP9K1* (AGAP000818), a *UGT-B19* (AGAP007920) and *COEBE3C* (AGAP005372). The qRT-PCR was carried out using three technical replicates each of cDNA extracted from 1 μg of total RNA of three biological replicates each from the Resistant (R), Control (C) and Ngoussou (S). Protocol followed was as established in previous studies (38), with relative expression level and fold change (FC) of each target gene in R and C relative to

S calculated according to the $2^{-\Delta\Delta CT}$ method incorporating the PCR efficiency (39), after normalization with the housekeeping genes ribosomal protein S7, *RPS7* (AGAP010592) and glycerol-3-phosphate dehydrogenase, *GPDH* (AGAP007593). Significant differences were calculated using ANOVA with Dunnett's post hoc test.

Detection of Signatures of Selective Sweep

To detect signature of select sweeps in the major metabolic resistance genes of interest, a RNAseq population genetics pipeline, the Snakemake workflow (https://zenodo.org/record/6078337) was utilised. The workflow aligns RNA-Seq reads to the reference genome, and calls genomic variants with *Freebayes*, at a user-provided level of ploidy, in our case 16 (8 diploid pooled mosquitoes). F_{st} (40) per gene between population pairs and Tajima's D per gene within population were estimated. This was performed against all SNPs passing quality and missingness filters. Population branch statistic (PBS) scans may also be performed with the Snakemake, conditional on the presence of three suitable populations (41). It is also possible to run Hudson's F_{st} and PBS scans, taking the average for each protein-coding gene, as opposed to in windows. The population genetics statistical analyses were calculated in scikit-allel v1.2.1 (42).

Establishment of allele frequencies of variants in genes of interest

After genome alignment, RNA-Seq-Pop utilises samtools (43) to query specific positions of the genome, calculating raw allele frequencies at those sites with a custom R script.

Detection of chromosomal inversion polymorphisms and metabolic genes within its breakpoint

A modified version of the Python 3 program, compkaryo (44) was used to karyoptype the major, *An. coluzzii/gambiae* phenotypically important inversion polymorphisms in chromosome 2, and calculate its frequencies, *in silico*, using the previously identified tag SNPs significantly associated with inversions. This allows to predict with high confidence genotypes of the six common polymorphic inversions on chromosome 2 in individually sequenced genomes of the field *An. coluzzii,* as well as in the Ngoussou. Compkaryo uses the Ag1000 database (The *Anopheles gambiae* 1000 Genomes Consortium 2017) (15) by leveraging a subset of cytologically karyotyped specimens to develop a computational approach for karyotyping applicable to whole genome sequence. Modifications in the Snakemake pipeline allows for variable ploidy (useful in the case of replicates from our pooled RNAsequencing samples) here.

Functional validation of the commonly overexpressed resistance-associated genes

Comparative analysis of coding sequences of major resistance genes

To establish presence of allelic variants which could impact catalytic activities, full length coding sequences (cDNAs) of the two resistance-associated genes, *GSTe2* and *CYP6Z2* were amplified and sequenced from alive mosquitoes in the four Sahel countries, as well as from Ngoussou. This was done using total RNA extracted from 5 individual pools of 8 F_1 *An. coluzzii* females (2-4 day old) alive after exposure to deltamethrin (resistant, R for *CYP6Z2*) or DDT (R for *GSTe2*). Protocol for RNA extraction was as described in previous section, above.

Amplification was done using Phusion HotStart II Taq Polymerase (ThermoFisher SCIENTIFIC, MA, USA) and the Full primers listed in Additional Table 4. The PCR mix comprised 5x Phusion HF Buffer (containing 1.5 mM $MgCl_2$), 85.7 μM deoxynucleotides (dNTPs), 0.34 μM each of forward and reverse primers, 0.015 U of Phusion HotStart II DNA Polymerase (Fermentas, MA, USA), 10.71 μL of ddH_20 and 1 μL cDNA. Thermocycling conditions were 1 cycle at 95 °C for 5 min; followed by 35 cycles each of 94 °C for 20 s, 60 °C for 30 s, 72 °C for 2 min (1 min for *GSTe2*); and finally, one cycle at 72 °C for 5 min. PCR products were cleaned with a QIAquick® PCR Purification Kit (QIAGEN, Hilden, Germany) and ligated into the pJET1.2/blunt cloning vector using the CloneJET PCR Cloning Kit (ThermoFisher SCIENTIFIC, MA, USA). These were then cloned into *E. coli DH5α*, plasmids miniprepped with the QIAprep® Spin Miniprep Kit (QIAGEN) and sequenced on both strands using pJET1.2 primers.

Polymorphisms were detected through examination and manual editing of sequence traces using BioEdit version 7.2.3.0 (45) and nucleotide differences in sequences aligned using CLC Sequence Viewer 7.0 (http://www.clcbio.com/). Different haplotypes were compared by constructing a phylogenetic maximum likelihood tree using MEGA X (46). Genetic parameters of polymorphism including number of haplotypes (h) and its diversity (H_d), number of polymorphic sites (S) and nucleotide diversity (π) were computed using DnaSP v6.12.03 (47).

304 ***Characterization of the 5′ regulatory regions of GSTe2 and CYP6Z2***

305 *Amplification, cloning and sequence characterisation of 5′ regulatory element*

306 To investigate presence of genetic variants in the regulatory elements, which could be
307 responsible for overexpression of *GSTe2*, 351 bp intergenic regions (spanning the 43 bp 3′ UTR
308 of *GSTe1*, 248 bp flanking sequence and 60 bp 5′UTR of *GSTe2*) preceding the start codon were
309 amplified from 10 each of DDT-alive and -dead females from the 4 Sahel countries, as well as
310 from the Ngoussou females (primers provided in Additional Table 4). For *CYP6Z2*, a 1078 bp
311 intergenic region was retrieved from the VectorBase and used for amplification of the putative
312 5′-regulatory elements. Primers spanning 38 bp 3′ UTR of *CYP6Z1*, a 937 bp flanking sequence
313 and 103 bp 5′UTR of *CYP6Z2*, preceding the start codon of *CYP6Z2* were used to amplify
314 fragments from 10 each of deltamethrin-alive and -dead females from Nigeria and Niger, as
315 well as from the Ngoussou females. Amplification was carried out using HotStart II
316 Polymerase (ThermoFisher SCIENTIFIC, MA, USA) with similar thermocycling conditions as
317 outlined above for coding region of *GSTe2* and *CYP6Z2*, respectively. Purification of PCR
318 amplicons, cloning into pJET1.2 vector, sequencing and polymorphism analysis were done as
319 outlined above.

320 The 351 bp 5′-UTR fragments of *GSTe2* and 1078 bp fragment of the *CYP6Z2* were analysed
321 with the Gene Promoter Miner (http://gpminer.mbc.nctu.edu.tw/) and MatInspector (48) to
322 identify putative promoter elements, predict transcription start (TSS) and potential
323 transcription factor binding sites.

324

Cloning of GSTe2 and CYP6Z2 5' regulatory elements in PGL3-Basic vector and dual luciferase reporter assay

Following analysis of the above sequences the 351 bp intergenic fragments of *GSTe2* were amplified from the most predominant sequences of DDT-alive, DDT-dead and Ngoussou. Same was done for for *CYP6Z2* amplifying 1087 bp fragment from deltamethrin-alive and -dead, and Ngoussou. Primers bearing *kpn*I and *BgL*II sites (Additional Table 4) allowed incorporation into pGL3-Basic reporter vector containing luciferase gene from the firefly *Photinus pyralis* (Promega, Wisconsin, USA). Amplification was carried out using Phusion HotStart II Polymerase, with conditions as above, followed by purification of PCR amplicons, and cloning into pJET1.2 vector. Positive colonies (sequencing primers for pGL3-Basic provided in Additional Table 4) were miniprepped; the minipreps digested with the above restriction enzymes, gel-purified and ligated upstream of luciferase gene in pGL3-Basic vector already linearized with the same restriction enzymes. Positive colonies were cloned and miniprepped and concentrations of the recombinant plasmids adjusted to 200 ng/µL before co-transfection. Protocols for maintenance of the *An. gambiae* cell line 4a-3B (MRA-919, https://www.beiresources.org/), co-transfection of 200 ng of either *GSTe2* or *CYP6Z2* promoter constructs, LRIM promoter in pGL3-Basic vector (49), or promoter-less pGL3-Basic control, with 1 ng/µL of internal control, sea pansy *Renilla reniformis* luciferase containing the *Drosophila* Actin *5C* promoter in pRL-null (49), are provided in Additional File 1 (additional text for Methods and Results). Protocol for cell lysis and measurement of the activities of the firefly luciferase (Dual-Luciferase Reporter Assay kit, Promega), with normalisation using the Renilla luciferase activity, were as outlined in the Promega Quick Protocol, and provided in detail in Additional File 1.

348 Assay background was also measured using lysate from non-transfected control cells. Results
349 were compared using a two-tailed Chi-Square test of independence using GraphPad Prism
350 7.02 (GraphPad Inc., La Jolla, CA, USA).

351 *Generation of GSTe2 promoter deletion constructs and assays*

352 The intergenic region separating *GSTe1* from *GSTe2* was progressively delineated. Partial
353 fragments of the 5′ regions (encompassing the 3′-UTR of *GSTe1*, the *GSTe1*/*GSTe2* flanking
354 region and the 5′-UTR of the *GSTe2*) were created by sequential deletion using forward
355 primers (provided in Additional Table 4, with numbers in primer names referring to distances
356 from the AUG start codon of *GSTe2*). Reverse primers were those initially used for
357 amplification of the full 351 bp nucleotide fragments. These fragments include (i) 308
358 nucleotides fragment generated from deletion of the 43 bp 3′-UTR of the *GSTe1* (-308 primers)
359 obliterating cellular-Myb (c-Myb) transcriptional factor binding site; (ii) 291 nucleotide
360 fragments generated from deletion of *GSTe1* 3′-UTR plus 38 nucleotides of the flanking region
361 (-270 primers), which removed in addition δ elongation factor 1 (δEF1) binding site; (iii) 262
362 nucleotide fragments produced from deletion of *GSTe1* 3′-UTR plus 46 nucleotides of the
363 flanking region (-262 primers), which obliterated in addition Forkhead box L1 (FOX-L1)
364 putative binding site; as well as (iv) 232 nucleotide fragments produced from deletion of
365 *GSTe1* 3′-UTR plus 75 nucleotides of the flanking region (-232 primers), located 11 nucleotides
366 from the c-EST/grainy head transcription factor binding site. Also, to assess the importance of
367 the *GSTe2* 5′UTR, 43 nucleotides upstream the AUG codon were deleted (from the 60
368 nucleotide 5′-UTR of both the Sahel-alive and Ngoussou), leaving the putative transcription
369 start site motif untouched. This was done using the original forward primer for the 351

nucleotides intergenic region, with a newly designed primer, GSTe2_minus_5'-UTR-R (Additional Table 4).

Characterisation of major resistance genes using transgenic analysis

Cloning and microinjection of GSTe2 and CYP6Z2 in Drosophila melanogaster

Transgenic flies expressing recombinant GSTe2 and CYP6Z2 were generated using GAL4/UAS system and used in contact bioassays, to confirm if over-expression of these genes alone can confer resistance to insecticides. Transgenic flies were created as outlined in a previous publication (50). Details of amplification of full-length *GSTe2* and *CYP6Z2* from the predominant alleles of sequences of cDNA, cloning into pUASattB vector, microinjection into germ-line *D. melanogaster* lines, crossing of the UAS-lines with the GAL4-Actin driver strain are outlined in Additional File 1.

To confirm overexpression of *GSTe2* and *CYP6Z2* in the transgenic flies three replicates each of 6 females (both experimental and control group) were used for qRT-PCR using a previously established protocol (38). Total RNA and cDNA were extracted as described above, and the relative expression levels of transgenes were assessed, with normalization using the *RPL11* housekeeping gene. The qtrg primers used for the two genes and the *RPL11* primers are provided in Additional Table 4.

Insecticide Susceptibility Contact Bioassay

For insecticide bioassays, 3- to 4-day old experimental and control F_1 females were exposed to 0.15 % deltamethrin, 2 % permethrin, 0.05 % α-cypermethrin, 4 % DDT and 2 % clothianidin-impregnated papers prepared in acetone and Dow Corning 556 Silicone Fluid (BHD/Merck,

Hesse, Germany). Flies overexpressing *CYP6Z2* were also exposed to the primary product of hydrolysis of pyrethroids: 4 % and 20 % (5x) 3-phenoxybenzaldehyde (PBAld) and 3-phenoxybenzylalcohol (PBAlc). Transgenic flies expressing *GSTe2* were exposed to 2 % permethrin, 0.15 % deltamethrin, 0.05 % α-cypermethrin and 4 % DDT only. Impregnated papers were rolled and introduced into 45 cc plastic vials to cover the entire wall and the vials plugged with cotton soaked in 10 % sucrose (38). 20–25 flies were placed in each vial, and the mortality plus knockdown scored at 1 h, 3 h, 6 h, 12 h and 24 h of exposure to the insecticides. For each insecticide, assays were performed in 6 replicates and Student's t-test used to compare the mortality plus knockdown between the experimental groups and the control.

Results

Genome-wide transcriptional profile of the Sahelian *An. coluzzii* populations

A three-way pairwise comparison was conducted for the data from each country: resistant *vs* susceptible (R-S), resistant *vs* unexposed control (R-C) and unexposed control *vs* susceptible (C-S). This captures background variations due to geographical differences in the resistant vs susceptible (R-S) comparison, accounts for genes overexpressed due to induction (R-C comparison), as well as genes that are constitutively overexpressed (C-S comparison). A total of 1384 genes were significantly differentially expressed (FDR-adjusted $p < 0.05$ and $\log_2$ fold change threshold of $1/FC \geq 2$) in R-S comparison in Nigeria (1077 upregulated and 307 down-regulated); 1185 genes were differentially expressed in C-S (1002 upregulated and 183 downregulated); and 295 genes in R-C (129 upregulated and 166 downregulated). Of these, 52 genes were commonly differentially expressed in all 3 comparisons (Figure S3, panel a),

including the upregulated genes, *COEAE80* (AGAP006700), *CYP4H18* (AGAP028019), *CYP4H17* (AGAP008358) and cuticular proteins, *CPLCX3* (AGAP006149), *CPR59* (AGAP006829), *CPR76* (AGAP009874) and *CPR75* (AGAP009871). The Additional Figure S3, panels a-d depicts the differentially expressed genes for the four countries. For Niger, 881 genes were differentially expressed in R-S comparison (619 upregulated and 262 down-regulated) (Additional Figure S3b); 1256 genes were differentially expressed in C-S (986 upregulated and 270 downregulated); and 196 genes in R-C (81 upregulated and 115 downregulated). Of these, 22 genes were commonly differentially expressed in all 3 comparisons [including the upregulated *aminopeptidase N1* (AGAP012757), *CYP6Z2* (AGAP008218), *chymotrypsin-3* (AGAP006711) and *chymotrypsin-2* (AGAP006710), and an acid trehalase (AGAP008547)]. For Chad, 1392 genes were differentially expressed in R-S comparison (975 upregulated and 417 down-regulated) (Additonal Figure S3c); 1284 in C-S (105 upregulated and 269 downregulated); and 526 genes in R-C (270 upregulated and 256 downregulated). Of these, 97 genes were commonly differentially expressed in all 3 comparisons [including *CYP4C27* (AGAP009246), *SULTD1* (AGAP012672), *aminopeptidase N1*, and diverse cuticular proteins, e.g. chitinase (*Cht24*, AGAP006191, *CPFL1* (AGAP010902), *CPCFC1* (AGAP007980), *CPLCX3*, *CPR24* (AGAP005999), *CPR106* (AGAP006095) and *CPR130* (AGAP000047). Finally, for Cameroon, 376 genes were differentially expressed in R-S comparison (204 upregulated and 172 down-regulated) (Additional Figure S3d); 932 in C-S (778 upregulated and 154 downregulated); and 116 genes in R-C [only 9 upregulated and 107 downregulated (probably due to a single, low quality replicate in the raw data from Cameroon (Additional Figure S2). Not surprising, only 7 genes were commonly differentially expressed in all 3 comparisons. These include the highly upregulated gene, *GSTe2* (AGAP009194) and *chymotrypsin-1* (AGAP006709).

All data analysed together (Additional Figure S3e) revealed no single gene differentially expressed in common; possibly due to the low quality with the Chad unexposed (C) data. Analysis of data from Nigeria, Niger and Chad, revealed a single gene (AGAP000046, transporter major facilitator superfamily) differentially expressed across all countries (Additional Figure S3f). Niger and Chad shared *hexamerin* (AGAP010658), *aminopeptidase N1*, and unknown protein, AGAP0290967; Nigeria and Niger share only a single gene, AGAP003248; while seven genes were common to Nigeria and Chad, including *CPLCX3*.

Principal component analysis for the top 500 most variable genes in all experimental arms revealed data from field samples (R and C) from all four countries clustering closer in PC1 and PC2 axes, away from the data from the susceptible females, Ngoussou (Additional Figure S4).

Analysis of the common differentially expressed genes across the Sahel

The most differentially expressed genes were presented in Figure 1, a volcano plot of fold change *vs* significance levels for Nigeria and Niger, and Figure 2, for Chad and Cameroon. Exhaustive lits of these genes of interest is provided in Additional File 2. Comparisons of genes commonly, upregulated and/or downregulated in R-S/R-C/C-S, from the four countries revealed similar transcriptomic profiles between R and C compared with the S. The most commonly and consistently overexpressed genes across the Sahel (taking account mean expressions) are the chymotrypsins, 3, -2 and -1 (*CHYM3*/AGAP006711 and *CHYM2*/AGAP006710 and *CHYM1*/AGAP006709) (Figures 1 and 2, Additional File 2), the glutathione S-transferase, *GSTe2* (AGAP009194), an aquaporin, *AQP3* (AGAP010326), *CYP6Z2* (AGAP008218), *CYP6Z3* (AGAP008217), *CYP4C27* (AGAP009246), a chitinase, *Cht24* (AGAP006191), a thioester-containing protein-1, *TEP-1* (AGAP010815),), a trehalose 6-

461 phosphate synthase/phosphatase, *TPS*1/2 (AGAP008227), a lipase (AGAP002353), and
462 AGAP012818 (V-type protein ATPase subunit A). More on these genes is provided in the
463 sections, below.

464 Other genes commonly overexpressed in data from two or three countries include
465 AGAP005501 (dehydrogenase/reductase SDR family 11) upregulated in Nigeria and Niger (R-
466 S and C-S comparisons), and in R-S for Chad and Cameroon; AGAP008091 (CLIP-domain
467 serine protease, *CLIPE1*), upregulated in all countries in R-S and C-S comparisons; a chitinase,
468 *Cht5-5*, AGAP013260) upregulated in Nigeria, Nigeria and Chad R-S and C-S, with both
469 induced upregulated in R-C in Chad, as heme peroxidase (*HPX15*), upregulated in R-S and C-
470 S comparisons in Nigeria, Chad and Cameroon, as well as malate dehydrogenase
471 (AGAP000184), upregulated in Nigeria, Chad and Cameroon, R-S and C-S.

472 However, the most overexpressed genes in Nigeria are a cubilin, a histone (H2B), carbonic
473 anhydrase I, and a cuticular protein, *CPR131* (Additional File 2), while for Cameroon its H2B,
474 Serpin 9 inhibitory serine protease inhibitor, galectin 4 and a Protease m1 zinc
475 metalloprotease.

476 Several genes were significantly downregulated across the four countries, particularly the
477 cytochrome P450s and the ATP-binding cassette transporters. The most consistently down-
478 regulated P450s were *CYP6AK1* (AGAP010961), downregulated in R-S and C-S comparisons
479 in Nigeria, Niger and Cameroon, while down-regulated in all comparisons in Chad; *CYP4H24*
480 (AGAP013490), downregulated in R-S and C-S comparisons for Nigeria and Cameroon, and
481 downregulated in all comparisons in Niger and Chad; *CYP6M4* (AGAP008214),
482 downregulated in R-S and C-S comparisons in Nigeria, Niger and Cameroon, while

downregulated in all comparisons in Chad; and finally *CYP6P5* (AGAP002866), downregulated in R-S and C-S comparisons in Niger and Chad, but only in C-S in Nigeria.

Analysis of commonly overexpressed metabolic resistance genes across the Sahel

Special attention was given to the known metabolic resistance genes, implicated in insecticide resistance in *Anopheles* and/or other insects, including, CYP450s, GSTs, carboxylesterases, cuticular proteins, chemosensory proteins/SAPs, uridine diphosphoglucuronosyltransferases, etc, in addition to other important genes, e.g., the immune proteins. Analysis of the data from the list of genes significantly, differentially expressed (FDR-adjusted $p < 0.05$ and $\log_2 FC = 1/FC \geq 2$) revealed the major, commonly upregulated metabolic resistance genes in the four Sahel countries. Top 50 genes from these lists (Additional File 3) from each country are displayed as heatmaps of fold changes in Additional Figure S5a, -b, -c and -d). The most common genes linked with resistance and/or other physiologically important phenotypes are tabulated in Table 1, showing fold changes of 55 genes (R-S and C-S comparisons), common to all countries, or three or two countries at least. The most commonly and consistently overexpressed genes across the Sahel (taking account mean expressions) are the chymotrypsins, -1, -2 and -3 (*CHYM1*/AGAP006709, *CHYM3*/AGAP006711 and *CHYM2*/AGAP006710) (Table 1, Additional Figure S5, Additional File 2, Results section), with fold change for *CHYM1* in R-S and C-S comparisons of 31.62 and 54.41 for Nigeria, 12.31 and 41.59 for Niger, and 18.00 and 18.08 for Chad, and 5.69 and 11.77 for Cameroon; for *CHYM3*, 32.45 and 58.43 for Nigeria R-S and C-S, respectively, 7.30 and 43.27 for Niger, 13.40 and 7.24 for Chad, and 2.59 and 8.15 for Cameroon. Of *Anopheles* metabolic resistance genes, *GSTe2* (AGAP009194) was the most consistently upregulated gene across the Sahel, FC = 9.61 and 7.16, respectively for Nigeria R-S and C-S comparisons; 6.55 and 11.32 for Niger; 9.09 and 10.02

for Chad, 9.68 and 20.67 for Cameroon. Other common GSTs were *GSTZ1*, upregulated in three countries (Nigeria, Niger and Chad), *GSTe4*, *GSTU1*, *GSTD1-4* and *GSTD3*.

Other most consistently and highly upregulated genes include a lipase, AGAP002353: FC of 26.73 and 20.80 for Nigeria R-S and C-S comparisons, 16.53 and 29.76 for Niger, 14.95 and 9.95 for Chad, 14.58 and 14.53 for Cameroon; a chitinase, *Cht24* (AGAP006191), FC = 21.12 and 38.55 for Nigeria R-S and C-S, 40.08 and 67.18 for Niger, 91.42 and 5.71 for Chad, 44.26 and 40.53 for Cameroon); a V-type protein ATPase subunit a (AGAP012818, FC = 22.12 and 14.61 for Nigeria, 31.05 and 25.43 for Nigeri, 6.00 and 4.31 for Chad, 44.92 and 43.4 for Cameroon), and an aquaporin, *AQP3* (AGAP010326, FC = 14.81 and 11.03 for Nigeria, 12.09 and 21.94 for Niger, 14.86 and 1.21 for Chad, 14.17 and 17.3 for Cameroon).

The most overrepresented gene family, and most consistently upregulated across the Sahel were the cytochrome P450s, with *CYP6Z2*, *CYP6Z3*, and *CYP4C27* taking the front seat, in addition to the two P450s linked to cuticular resistance, *CYP4G16* (upregulated in Nigeria and Chad) and *CYP4G17* (upregulated in Nigeria, Niger and Chad). The three P450s, *CYP6Z2, CYP6Z3 and CYP4C27* are consistently overexpressed: FC for *CYP6Z2* is 6.55 and 2.10 for Nigeria R-S and C-S comparisons, 6.73 and 2.32 for Niger, 13.96 and 1.48 for Chad, 11.84 and 8.39 for Cameroon; FC for *CYP6Z3* is 4.73 and 1.82 for Nigeria, 6.09 and 1.42 for Niger, 3.51 and 1.11 for Chad, 7.11 and 3.79 for Cameroon; and FC for *CYP4C27* is 7.36 and 4.6 for Nigeria, 4.03 and 5.64 for Niger, 6.04 and 2.62 for Chad, 2.01 and 2.63 for Cameroon. The two well-known insecticide resistance genes *CYP6P3* and *CYP6M2* did not future prominently across the Sahel of these countries.

The second most commonly overrepresented gene families belong to cuticular proteins, including cuticular proteins Rebers and Riddiford (RR), several cuticular proteins of low

529 complexity (CPLC) and chitin-binding cuticular proteins (CPAP), with the commonly
530 upregulated ones across all four countries being *CPR75* (AGAP009871) and *CPR76*
531 (AGAP009874).

532 Two carboxylesterases picture prominently across the Sahel – the beta esterase, *COEBE3C*
533 (AGAP005372) upregulated in all four countries, while *COEJHE5E* was common to Nigeria,
534 Niger and Chad. For Phase II metabolism enzymes, several uridine-diphospho-
535 glucuronosyltransferases, UGTs were upregulated, including the *UGT-B19*, which is
536 upregulated in Nigeria and Niger (Table 1), and unknown UGT (AGAP006222) upregulated
537 across all the countries (Additional File 2).

538 Other genes in the top 55 metabolic genes include the malate dehydrogenase, *TPS* (*TPS1/2*),
539 *AQP2*, thioester-containing proteins, *TEP1*, *-12* and *-14*, a cyanogenic-beta-glucosidase
540 (AGAP006422) and three chemosensory proteins, *CSP5*, sensory appendage proteins, -1 and -
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549 **Table 1: Common detoxification and metabolic genes differentially upregulated in Sahel**
550 ***An. coluzzii* (FDR-adjusted p < 0.05).**

	NIGERIA		NIGER		CHAD		CAMEROON		
Gene	**R-S**	**C-S**	**R-S**	**C-S**	**R-S**	**C-S**	**R-S**	**C-S**	**Gene Description**
AGAP006400	2.2	4.2	1.7	4.5	-	-	2.1	2.8	alkaline phosphatase 2
AGAP028491	4.3	2.8	1.4	2.7	2.9	2.6	-	-	aquaporin, AQP2
AGAP010326	14.8	11.0	12.1	21.9	14.9	1.2	14.2	17.3	Aquaporin, AQP3
AGAP005372	9.5	12.2	3.3	4.3	6.6	3.6	1.4	2.6	carboxylesterase, COEBE3C
AGAP005837	4.7	5.0	1.4	3.3	1.9	2.6			carboxylesterase, COEJHE5E
AGAP004904	3.9	2.2	-	-	2.3	1.7	-	-	catalase, CAT1
AGAP029127	4.1	3.2	-	-	1.7	2.2	-	-	chemosensory protein 5, CSP5
AGAP006191	21.1	38.6	40.1	67.2	91.4	5.7	44.3	40.5	chitinase, Cht24
AGAP013260	4.4	11.5	1.3	3.9	3.1	0.8	-	-	chitinase, Cht5-5
AGAP006898	8.9	5.5	-	-	2.2	3.9	-	-	chitinase, Cht6
AGAP006709	31.6	54.4	12.3	41.6	18.0	18.1	5.7	11.8	chymotrypsin-1, CHYM1
AGAP006710	9.9	19.4	2.1	8.8	4.2	3.9	-	-	chymotrypsin-2, CHYM2
AGAP006711	32.5	58.4	7.3	43.3	13.4	7.2	2.6	8.2	chymotrypsin-3, CHYM3
AGAP000987	4.3	2.7	-	-	1.3	2.6	-	-	cuticular protein, CPAP3-A1b
AGAP000988	5.3	1.6	-	-	0.7	5.4	-	-	cuticular protein, CPAP3-A1c
AGAP000986	7.3	4.6	-	-	1.7	3.5	-	-	cuticular protein, CPAP3-D
AGAP006149	3.8	0.8	-	-	0.7	5.8	-	-	cuticular protein, CPLCX3
AGAP010123	33.3	54.9	2.4	13.2	-	-	-	-	cuticular protein, CPR131
AGAP005456	5.2	1.8	-	-	1.2	2.2	1.1	2.1	cuticular protein, CPR15
AGAP006009	-	-	2.9	1.3	-	-	3.9	1.6	cuticular protein, CPR30
AGAP009871	8.7	2.7	1.1	2.6	2.2	4.2	1.7	2.6	cuticular protein, CPR75
AGAP009874	10.6	2.2	1.1	2.6	3.7	5.6	1.1	2.0	cuticular protein, CPR76
AGAP009879	7.4	2.6	-	-	1.9	4.6	-	-	cuticular protein, CPR81
AGAP006422	7.5	9.4	-	-	8.4	7.3	2.1	2.0	cyanogenic beta-glucosidase
AGAP002417	5.5	4.2	0.9	1.2	1.9	1.9	-	-	cytochrome P450, CYP4AR1
AGAP009246	7.4	4.6	4.0	5.6	6.0	2.6	2.0	2.6	cytochrome P450, CYP4C27
AGAP012957	5.0	2.8	-	-	2.9	2.5	2.0	2.6	cytochrome P450, CYP4D17
AGAP001076	5.8	4.7	1.5	1.8	3.8	2.4	0.9	1.5	cytochrome P450, CYP4G16
AGAP000877	3.8	2.0	2.3	1.9	3.8	2.1	1.2	2.1	cytochrome P450, CYP4G17
AGAP008358	-	-	2.9	4.1	3.4	3.3	3.6	2.0	cytochrome P450, CYP4H17
AGAP007480	5.4	3.9	1.3	2.7	2.5	2.0	-	-	cytochrome P450, CYP6AH1
AGAP002865	1.11	0.5	2.3	1.9	0.7	12.8	2.1	2.3	cytochrome P450, CYP6P3
AGAP008207	3.6	1.9	-	-	2.1	1.6	1.7	2.4	cytochrome P450, CYP6Y2
AGAP008218	6.6	2.1	6.7	2.3	14.0	1.5	11.8	8.4	cytochrome P450, CYP6Z2
AGAP008217	4.7	1.8	6.1	1.4	3.5	1.1	7.1	3.8	cytochrome P450, CYP6Z3
AGAP000818	3.0	1.2	0.8	0.5	0.9	1.6	3.4	2.2	cytochrome P450, CYP9K1
AGAP010400	3.6	2.6	1.6	2.8	3.3	1.4	-	-	flavin-containing monooxygenase
AGAP007920	5.7	2.4	2.8	1.8	-	-	-	-	glucuronosyltransferases, UGT-B19
AGAP004164	2.2	1.9	1.7	2.3	1.4	1.3	-	-	glutathione S-transferase, GSTD1-4
AGAP009194	9.6	7.2	6.6	11.3	9.1	10.0	9.7	20.7	glutathione S-transferase, GSTe2
AGAP009193	3.3	2.3	1.1	2.1	1.5	1.6	-	-	glutathione S-transferase, GSTe4
AGAP000947	4.4	2.3	1.2	2.2	1.9	3.2	-	-	glutathione S-transferase, GSTU1
AGAP002898	5.5	3.5	1.7	2.6	2.8	2.4	-	-	glutathione S-transferase, GSTZ1
AGAP002198	-	-	2.0	5.8	4.8	6.6	-	-	glycine-N-methyltransferase
AGAP013327	-	-	1.2	7.3	7.9	20.1	5.1	14.8	heme peroxidase, HXP15
AGAP002353	26.7	20.8	16.5	29.8	15.0	10	14.6	14.5	lipase
AGAP000184	5.2	6.3	-	-	4.9	4.3	1.2	1.9	malate dehydrogenase
AGAP008051	3.7	4.0	1.1	1.3	1.7	1.7	-	-	sensory appendage protein 1, SAP1
AGAP008052	5.5	10.8	1.0	1.5	3.6	1.1	-	-	sensory appendage protein 2, SAP2
AGAP010815	5.7	14.4	4.1	8.6	7.8	3.8	1.7	3.1	thioester-containing protein, TEP1
AGAP008368	3.3	6.1	1.4	3.9	3.4	2.0	-	-	thioester-containing protein, TEP14
AGAP008654	4.6	17.4	2.1	10.0	-	-	-	-	thioester-containing protein, TEP12
AGAP008227	4.1	4.0	4.0	2.0	4.6	1.5	2.6	4.1	trehalose 6-phosphate synthase/phosphatase
AGAP012818	22.1	14.6	31.1	25.4	6.0	4.3	44.9	43.4	V-type proton ATPase, subunit a

551 ***Gene Ontology Enrichment Analysis***

552 Gene ontology enrichment analysis, for genes significant in comparisons ($log_2FC = 1$, $p < 0.05$)
553 revealed crucial differences in overrepresented GO terms between the up- and -
554 downregulated genes, in data from the four countries. For example, the most over-
555 represented, semantically similar GO terms associated with xenobiotics metabolism is
556 oxidoreductase activity ((highest frequency of 12.9 %, Additional Figure S6a, REVIGO Table
557 View), which cluster together in Nigerian R vs S comparison (for genes upregulated in R).
558 Other over-represented GO terms in this comparison include glutathione S-transferase
559 activity (the most enriched/specific term), peroxidase activity, odorant binding and chitin
560 binding. In contrast, GO terms over-represented in R vs S (down-regulated in R) were mostly
561 involved in neurotransmission, metal ions binding and receptor channelling activities
562 (Additional Figure S6b).

563 For R vs C comparison (upregulated in C) the over-represented GO terms include
564 oxidoreductase (frequency = 12.88 %), glutathione S-transferase activity (the most
565 enriched/specific term), peroxidase activity, glucuronosyltransferase activity, aldehyde
566 oxidase activity, chitin binding, carbohydrate binding activities, etc, (Additional Figure S6c).
567 In contrast the most enriched GO terms, downregulated in C were endopeptidase activities,
568 Toll binding, oxidoreductase (frequency = 1.21 %) and monooxygenase activities (Additional
569 Figure S6d). For the rest of the three countries similar contrasts were also observed, between
570 phenotypes, and detailed in Additional File 1, Results section.

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Quantitative PCR validation of expression profiles of metabolic resistance genes

The relative expression levels of 12 metabolic resistance genes were validated. The qRT-PCR results support the transcriptomic patterns obtained from the RNAseq analysis, with *GSTe2* as the most overexpressed gene, followed by *CYP6Z2* (Figure 3). For example, for R-S and C-S comparisons *GSTe2* had fold changes of 49.25 ± 5.05 and 28.45 ± 4.45, for Nigeria ($p \leq 0.0001$); 37.01 ± 2.22 and 33.34 ± 6.61 for Niger ($p \leq 0.0001$); 29.97 ± 5.39 and 30.16 ± 6.16 for Chad ($p \leq 0.0001$); and 28.30 ± 3.30 ($p \leq 0.0001$) and 17.03 ± 7.03 for Cameroon ($p \leq 0.001$). *CYP6Z2* has R-S and C-S fold changes of 15.93 ± 2.54 ($p \leq 0.001$) and 7.35 ± 1.52 ($p \leq 0.05$), for Nigeria; 24.21 ± 2.17 ($p \leq 0.001$) and 20.83 ± 3.02 for Niger ($p \leq 0.001$); 15.02 ($p \leq 0.001$) ± 2.01 and 6.09 ± 2.08 for Chad ($p \leq 0.05$); and 10.58 ± 3.00 ($p \leq 0.01$) and 3.43 ± 1.43 for Cameroon. Correlation analyses for R-S comparisons for all genes revealed positive and significant association (Additional Figure S7) between the RNA-seq and qRT-PCR data in data from Niger ($R^2 = 0.58$, $p = 0.03$) and Chad ($R^2 = 0.408$, $p = 0.025$), with positive, but non-significance seen in Cameron ($R^2 = 0.58$ $p = 0.08$) and Nigerian ($R^2 = 0.503$, $p = 0.06$).

Detection of Signatures of Selective Sweeps

Signatures of selection were investigated in the major metabolic resistance genes, by estimating Tajima's D per gene within populations, and *F*st per gene between population pairs. These tests of neutrality revealed several genes exhibiting high genetic differentiation, or possibly undergoing expansion. Among the top 100 most overexpressed metabolic genes across the four countries (Additional File 2), 13 genes were possibly undergoing genetic differentiation. These include *TEP1*, and *TEP3*, with average Tajima's D of -1.4 and -1.6

595 respectively, in populations from Chad, and average *F*st values of 0.58 and 0.49 for all
596 countries (Additional File 3, provide the number of SNPs and chromosomal location for each
597 gene), *CYP9K1* (average Tajima's D = -1.00 for Cameroon, and combined *F*st = 0.55), *CPR15*
598 (average Tajima's D = -0.94, and *F*st = 0.17); *CPAP3-A1b* (Tajima's D = -0.70 in Chad, -0.95 in
599 Nigeria, and -1.32 in Cameroon, with combined *F*st of 0.55) and *CPLCX3*. Several genes from
600 the GST family exhibited strong genetic differentiation, with reduced variations prominently
601 in *GSTe2* (< 3 SNPs in Chad and Cameroon samples), with Tajima's D of -1.45 and -1.15 for
602 Nigeria and Niger, and combined *F*st of 0.02. Detailed analysis is provided in Additional File
603 1, and Additional File 3 contain the respective frequencies of these variables.

604

605 **Identificaton of genetic variants associated with insecticide resistance**

606 The RNA-Seq-Pop workflow calculated allele frequencies of variants of interest found in the
607 raw RNA-sequencing read data. Figure 4 displays allele frequencies of each variant in
608 respective treatment replicates used for the RNAseq, as well as Ngoussou. Several mutaions
609 were found within the voltage-gated sodium channel. In addition to the L995F *kdr* mutation,
610 the recently identified V402L and I1527T mutations exist in all the field populations across the
611 Sahel, at frequencies ranging from 29 % to 67 %. We also detected the N1570Y mutation, which
612 shares the same haplotype as L995F. The G280S *acetylcholinesterase*-1 mutation was also
613 observed in low to moderate frequencies in all populations except for Ngoussou and samples
614 from Niger. The L43F pyrethroid resistance marker of *CYP4J5* was also seen, fixed in most
615 populations.

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Detection of chromosomal inversion polymorphisms and metabolic genes within its breakpoints

The frequency of the major inversion polymorphisms in chromosome 2 were calculated, considering ploidy (8 individual female mosquitoes were pooled for RNA extraction, for each replicate). Additional Table 5 provided the frequencies of the respective inversions for the populations from each country, as well as for Ngoussou. High frequency of the 2La, 2Rb and 2Rc were observed, in contrast to the 2Rd, 2Rj and 2Ru which were in lower frequencies [except for the 2Rj in Nigeria (30.38 %) and 2Ru in Cameroon (24.29 %)]. The 2La inversion was found fixed in the field populations (100 % in Nigeria and Cameroon, 99.92 % in Niger and 99.21 % in Chad), in contrast with Ngoussou, with frequency of only 6.26 %. Similar pattern was observed with the 2Rb inversion, with high frequencies in three field populations (79.01 %, 84.85 %, 80.22 % in Nigeria, Niger and Chad), but lower in Cameroon (29.08 %) and Ngoussou (5.35 %). Frequencies of the 2Rc inversion were 88.54 %, 85.52 %, 88.82 %, 46.99 % and 9.89 % for Nigeria, Niger, Chad, Cameroon and Ngoussou respectively. Some genes among the top 100 most overexpressed metabolic resistance genes (above) and which were likely undergoing recent population expansion were located within the 2La, 2Rb and 2Rc inversions (Additional File 4). These include the *hsp83* (AGAP006958) and molecular chaperone hptG (AGAP006961), *CPLCX3* (AGAP006149), *CPLCA1* (AGAP006145,) within the inverted 2La chromosomal arm. Other genes within 2La inversion include a group of chitinase enzymes (*Cht6*, *-8*, *-11* and *-24*), most notably the *Cht24* and *Cht6*, which are among the top 50 most overexpressed genes in all countries. Other genes include: *COEJHE5E*, one of the 14 carboxylesterases in the 2La region, and which was among the top 100 most overexpressed genes across the Sahel; the most overexpressed chymotrypsin genes, *CHYM1*

and *CHYM2*; three ionotropic receptors, *IR136* (AGAP006440), *IR139* (AGAP006691) and *IR142* (AGAP006407); as well as five P450s, *CYP301A1*, *CYP302A1*, *CYP4J5*, *CYP4J9* and *CYP4J10*. The most overrepresented genes within 2La inversions are the CPR cuticular proteins (73 in total), with *CPR21*, *-26*, *-30*, *-59* and *-140* from the list of the top 100 resistance genes from the Sahel.

Carbonic anhydrase I (the most overexpressed metabolic resistance in Nigeria, AGAP013402) sits within 2Rb inversion. Four chitinases (*Cht4*, *Cht5-1*, *Cht5-3*, and *Cht5-5*) are located within 2Rb inversion; *Cht5-1* and *Cht5-5* were among the observed top 50 most overexpressed metabolic genes in the Sahel. Lipase (*AGAP002353*) which is the among the top 6 overexpressed metabolic gene in the four countries sit within the 2Rb inversion. Eight CYP450s reside in the 2Rb inversion, including *CYP4D15*, *CYP4D17* and *CYP4AR1*, which were among the top 100 metabolic resistance genes. Three cuticular proteins, *CPR7*, *-8* and *-9* were also within the 2Rb inversion.

Interesting genes sitting within 2Rc inversion include *GSTZ1*; 5 ionotropic receptor genes, *IR7i* (AGAP013363), *IR7u* (AGAP013285), *IR7t* (AGAP002763), *IR7w* (AGAP013416) and *IR41a* (AGAP002904); a carboxylesterase, *COEAE6O* (AGAP002863); and 11 CYP450s, including *CYP6AA1* (AGAP002862), *CYP6AA2* (AGAP013128), *CYP6P15P* (AGAP002864), *CYP6P3* (AGAP002865), *CYP6P5* (AGAP002866), *CYP6P4* (AGAP002867), *CYP6P1* (AGAP002868), *CYP6P2* (AGAP002869), *CYP6AD1* (AGAP002870) and *CYP6Z4* (AGAP002894)

Investigation of the polymorphism in the coding sequences of *GSTe2* and *CYP6Z2*

Analysis of the polymorphism patterns of full-length cDNA sequences of *GSTe2* (666 bp) and *CYP6Z2* (1479 bp) from the Sahel region of Africa revealed complete homogeneity for *GSTe2*, with no polymorphism detected in the field populations (all sequences were identical to those from Ngoussou and the AGAP009194 reference). This suggests a fixed allele, consistent with the observation from the analyses from the fixation index (Additional File 3). For *CYP6Z2* (1479 bp), homogeneity was observed within each country and Ngoussou, except for Niger, characterised by an unusually high polymorphism (Additional Figure S8a, -b). *CYP6Z2* is polymorphic with 10 haplotypes across the Sahel, with 75 polymorphic sites of which 65 were synonymous, and 12 led to amino acids substitutions. The bulk of the polymorphisms were contributed from larger variations in the Niger and Cameroon (S = 42 and 23 respectively), while highest homogeneity was observed in Chad, with a single haplotype. Haplotype diversity is high (H_d = 0.921), from 10 haplotypes out of only 20 sequences, with the lowest H_d in the Chad sequences, suggesting a directional selection/fixed allele. The haplotypes cluster according to origin on the maximum likelihood phylogenetic tree, except for Niger (Additional Figure S8c).

Investigation of the role of intergenic region elements in overexpression of *GSTe2* and *CYP6Z2*

Investigation of polymorphism in the intergenic region/regulatory elements

To investigate polymorphisms in the regulatory elements of the above genes, the 351 intergenic regions of *GSTe2* (spanning the 43 bp 3'-UTR of *GSTe1*, 248 bp flanking sequence

and 60 bp 5'-UTR of *GSTe2*) preceding the start codon were amplified from 10 each of DDT-alive and -dead females from the 4 Sahel countries, as well as from Ngoussou females, successfully. For *CYP6Z2,* 1078 bp (spanning 38 bp 3' UTR of *CYP6Z1,* 937 bp flanking sequence and 103 bp 5'UTR of *CYP6Z2,* preceding the start codon of *CYP6Z2*) were used to amplify fragments from 10 each of deltamethrin-alive and -dead females from Nigeria, and from Ngoussou females.

Out of the 90 *GSTe2* 5'-UTR sequences analysed differences were observed between the alive and dead mosquitoes, with a total of 65 sequences from alive and dead females (regardless of country of origin) identical to the 10 sequences of Ngoussou. From the 40 sequences of the dead females, 39 were identical to Ngoussou (regardless of the country). Twelve sequences, all from the alive females (from across the four countries) were similar, with several mutations in putative transcriptional factors binding sites, which may impact overexpression of the *GSTe2.* In short, 8 mutations were shared in common between these 12 sequences of DDT-alive females from across the countries (3 each from Nigeria, Chad and Cameroon) and additional mutations in the Niger samples (4 sequences). These mutations include (i) T->A transition withing the cellular myb-DNA (c-myb) binding domain (Additional Figure S9), (ii) a T->C transversion in the zinc-finger homeodomain, δEF1 (δ elongation factor 1) binding site, (iii) T->A transition in the nuclear matric protein 4 (NMP4), (iv) simultaneous insertion of adenine and a transition T -> C, in positions 113 and 114 respectively, between the Fork-head box L1 and c-EST binding sites, (v) an A -> C transversion in a second NMP4, (vi) a T -> C transition, six nucleotides downstream the nuclear factor κB (NF-κB), (vii) followed by an A -> G transversion 3 nucleotides downstream the NF-κB, (viii) a G -> A transversion, 7 nucleotides upstream the GC box, (ix) a C -> G transition, 2 nucleotides downstream the

arthropod initiator (Inr consensus) sequence, and finally (x) a C -> G transition, within the 5′-UTR of the *GSTe2*, 28 nucleotides downstream the transcriptional start site/49 nucleotides upstream the *GSTe2*, start codon.

Analysis of the 90 sequences revealed a very low polymorphism in the dead mosquitoes (S = 0, for Niger-dead, Chad-dead and Ngoussou), but high polymorphism in the alive (S = 16 for Nigeria-alive, and 13 each for Chad-alive and Cameroon-alive) (Additional Table 6). All sequences produced 17 polymorphic sites, with 6 haplotypes (Additional Figure S10a and -b). High haplotype diversities were obtained from the alive mosquitoes (for example, Nigeria alive, Hd = 0.71, Niger-, Hd = 0.53). Regardless of country of origin, the haplotypes cluster according to phenotype on the maximum likelihood phylogenetic tree, with the alive haplotypes forming a distinct/separate clade (Additional Figure S10c).

With regard to *CYP6Z2* no major differences were observed when comparing the deltamethrin-alive and -dead sequences with the Ngoussou.

Measurement of activities of the 5′ regulatory elements of the GSTe2 and CYP6Z2

Initial promoter analyses were conducted with the 5′- regulatory element sequences of *GSTe2*, for the DDT-alive females (representative sequence with the 8 common polymorphic positions, designated, Sahel-alive), comparing it with the sequence from the DDT-dead/Ngoussou (designated Ngoussou/Sahel-dead). For *CYP6Z2*, a predominant sequence from the Nigeria field sample (with 8 nucleotide insertion) was compared with the Ngoussou sequence.

728 The ability to drive heterologous expression of the firefly luciferase was determined, with the
729 Sahel-alive construct and Ngoussou/Sahel-dead producing increased luciferase activity
730 [~3090× (normalized luciferase activity = 2.58) and 479× (normalized activity = 0.400),
731 respectively] compared with the promoterless pGL3-Basic vector. But the Sahel-alive
732 construct was significantly more active than the Ngoussou/Sahel dead counterpart [promoter
733 activity ~6-fold higher (Tukey HSD Q = 8.11, p = 0.004)]. In contrast, for *CYP6Z2* no significant
734 differences were observed when comparing the field construct (normalized luciferase activity
735 = 5.95 for alive/dead field construct), compared with 5.84 for the Ngoussou construct.

736

737 ***GSTe2 promoter delineation and measurement of activity***

738 Sequential deletion of the intergenic region of the *GSTe2* resulted in progressive reduction in
739 luciferase activity. Deletion of the 43 bp 3'-UTR of the *GSTe1* (-308 from the start codon of
740 *GSTe2*) reduced activity of the Sahel-alive construct by only 18.6 % suggesting that the c-Myb
741 transcriptional factor binding site may not be critical for overexpression (Figure 5). Deletion
742 of an additional 38 nucleotides from the flanking region (-270 fragment, obliterating the δEF1
743 binding site) had comparable impact as above, with activities reducing by 15.3 % only. But
744 removing the *GSTe-1* 3'-UTR and an additional 46 nucleotides from the flanking region (-262
745 fragment, which obliterated in addition the FOX-L1 putative binding site) reduced activity by
746 64.1 % (Q = 6.18, p = 0.01) indicating the importance of this binding site. Deletion of *GSTe1* 3'-
747 UTR plus 75 nucleotides of the flanking region (-232 fragment, located 10 nucleotides from
748 the c-EST binding site) significantly reduced the activity by ~70% (Q = 6.14, p = 0.01),
749 suggesting the importance of the simultaneous insertion of adenine and transition T -> C, in
750 positions 113 and 114 respectively, between the FOX-L1 and c-EST binding sites. Removing

the fragment of the 5'UTR of *GSTe2* (43 nucleotides preceding the AUG codon obliterated the promoter activity, reducing the luciferase expression by 98 % (Q = 9.41, p = 0.002). This is despite presence of all the above binding sites and transcriptional start site in this fragment. Deletion of the 5'-UTR from Ngoussou significantly reduced activities as well (reduction by 88 % compared with the full Ngoussou intergenic region construct, Q = 13.75, p = 0.001). Overall, these findings suggest that the essential binding sites for overexpression of *GSTe2* span the FOX-L1 and c-EST binding sites, with the 5'-UTR essential for activity.

Investigating the role of *GSTe2* and *CYP6Z2* in insecticides resistance using transgenic analysis

A qRT-PCR was conducted using the transgenic flies to first establish overexpression of the above genes. Relative fold changes (FC) of 32.7 ± 4.6 and 25.30 ± 2.8 were obtained in flies overexpressing the *GSTe2* and *CYP6Z2* respectively, compared with control flies (progenies of crosses between the parental line flies with no gene insertion, crossed with GAL4/UAS driver line) (Additional Figure S11).

Contact bioassays carried out using 0.05 % α-cypermethrin revealed a high susceptibility in the transgenic flies expressing *GSTe2* (Act5C-GAL4-UAS-GSTe2) and controls (Figure 6a), with mortalities increasing from 62 % and 70 % respectively in 1 h, to 94 % and 99 % in 24 h. However, significantly reduced mortalities were observed in transgenic flies expressing *CYP6Z2* (Act5C-GAL4-UAS-CYP6Z2) compared to control flies, at 1 h (mortality = 35 % *vs* 70 % in control, $p < 0.001$) and 3 h (mortality = 52 % vs 89 % in the control, $p < 0.001$). High susceptibilities were also seen in all the experimental flies exposed to 0.05 % deltamethrin

(both for *GSTe2* and *CYP6Z2* flies), except for 1 h with flies expressing *CYP6Z2* (mortality = 47 % compared with 58 % in the control flies, $p < 0.010$).

The *GSTe2* transgenic flies were highly resistant to 2 % permethrin, with no mortality at all in 1 h (Figure 6b), and average mortalities of only 8.5 % at 24 h ($p < 0.0001$), compared with 85 % at 1 h for control flies, which increased to 100 % from 3 h.

Initial exposure to 4 % of 3-phenoxybenzaldehyde (PBAld) and 3-phenoxybenzyl alcohol (PBAlc) had no toxic effect on *CYP6Z2* transgenic flies (Figure not shown). However, 5x concentration of these primary products of pyrethroid hydrolysis induced mortalities, albeit low in all flies (Figure 6c). For PBAld, mortalities ranged from 15.5 % and 17.5 % at 1 h, for *CYP6Z2* transgenic flies and control flies respectively, to 30 % and 27.5 % at 24 h. Although low mortalities were observed with PBAlc, but at 1 h and 3 h exposure times the mortalities in the transgenic flies expressing *CYP6Z2* were significantly lower compared with mortalities from the control flies (1 h mortality = 2.5 % *vs* 12.5 %, $p < 0.001$; 3 h mortality = 9.5 % *vs* 17.5 %, $p < 0.001$).

Marginal tolerance towards DDT was observed in the *CYP6Z2* transgenic flies when compared with the control flies, but only at 3 h ($p < 0.001$) and 6 h ($p < 0.01$) (Figure 6d). This is in contrast with the *GSTe2* transgenic flies, with very low mortalities, in ranges of 0 % to 9.52 % for 1 h to 24 h, when compared with control flies (mortality = 34 % in 1 h and 100 % in 6 – 24 h, $p < 0.0001$).

Susceptibility to clothianidin was very high (Figure 6d), but surprisingly the *CYP6Z2* transgenic flies exhibited a contrasting phenotype, with significantly higher mortalities

compared with the control flies, at 1 h (mortality = 67 % vs 42 %, $p < 0.001$), 3 h (mortality = 71 % vs 53 %, $p < 0.001$) and 6 h (mortality = 78 % vs 62 %, $p < 0.01$).

Taken together, these results confirmed that over-expression of *GSTe2* alone is sufficient to confer resistance to type I pyrethroid (permethrin) and DDT, while overexpression of *CYP6Z2* alone, may confer marginal resistance to the α-cypermethrin and PBAlc.

Discussion

Escalating insecticide resistance across Africa (7-9, 51), if not tackled, will probably compromise the malaria control and elimination efforts. Molecular markers of metabolic resistance e.g., (10, 11, 21), will support evidence-based control and resistance management. Identification and validation of resistance markers in malaria vectors across regions of sub-Saharan Africa can promote communication, cooperation and coordination among malaria control/elimination programs, and allow control efforts to be tailored to the vector species involved in transmission across borders (52), and tracking of the evolution and spread of resistance markers across regions (22). To support malaria pre-elimination efforts in Africa, in this study we targeted the Sudan savannah and Sahel (regions of Africa sharing similar eco-climatic conditions, and characterised by high seasonal transmission), which are ideal for control and elimination of malaria using seasonal vector control (25) and chemoprevention.

Anopheles coluzzii is a major malaria vector across the Sudano-Sahelian transects

Contrary to the previous observations that *An. arabiensis* tends to predominate in arid savannas, while *An. gambiae* is the dominant species in humid forest zones (53-55), *An. coluzzii* has repeatedly been identified as the major malaria vector in the Sudan savannah and Sahel

of several, neighbouring countries (including in northern Nigeria, southern Niger, central Chad, and northern Cameroon) (7, 26-28, 56) in recent years, suggesting that this vector has adapted well in drier regions of the Sahel transects, and is probably predominating over *An. arabiensis* and *An. gambiae s.s*. This is not surprising as this species exhibits higher exploitation of breeding sites associated with anthropogenic activities, and behavioural plasticity to avoid predators (57), and is known to survive a long dry season *in situ*/aestivation, which allows it to predominate, becoming the primary force of malaria transmission (58, 59). Also, photoperiod and lower nightly temperature have been shown to significantly increase the longevity of the *An. coluzzii*; mechanisms which allow it to diapause in the dry season and re-establish first in the early rainy season (60).

Common metabolic genes mediate multiple resistance in the Sahelian *An. coluzzii*

Several genes shown to confer metabolic resistance in *Anopheles* mosquitoes and other insects were found constitutively overexpressed and/or induced in this study. *GSTe2* (AGAP009194) is one of the most regularly encountered metabolic genes in resistant populations of the major malaria vectors *An. gambiae*, *An. coluzzii* and *An. funestus* (20-23). *Anopheles gambiae GSTe2* has been validated, using transgenic flies to confer DDT (20) and fenitrothion (61) resistance. It was extensively studied in *An. funestus*, in which it was shown to confer cross-resistance to DDT and permethrin (22), reduce efficacy of the LLINs, PermaNet 2.0 and PermaNet 3.0 (side panels) (23), and even increase the longevity of the resistant populations carrying the 119F mutation (62). These and our findings suggest that the overexpression of this GST alone can confer resistance to three insecticides from three different classes (DDT, permethrin and fenitrothion). The absence of mutations in the cDNA coding sequences of *GSTe2* in *An. coluzzii*

839 from these four countries, suggest that overexpression of this GST is enough to confer
840 resistance.

841 Several cytochrome P450s previously linked with insecticide resistance were found
842 overexpressed across the Sahel. For example, *An. gambiae CYP6Z2* (AGAP008218) known to
843 metabolize carbaryl (63), the insect juvenile hormone analogue insecticide, pyriproxyfen (64)
844 and mitochondrial complex I inhibitors, fenazaquin, pyridaben and tolfenpyrad (65). This
845 P450 also plays a pivotal role in the clearance of pyrethroid insecticides via further catabolism
846 of pyrethroid derivatives (PBAld and PBAlc) obtained by the action of carboxylesterases (66),
847 in line with our findings of this gene conferring marginal tolerance to high concentration of
848 PBAlc, and α-cypermethrin. However, our findings suggest that overexpression of this P450
849 may enhance the efficacy of clothianidin, which will be epidemiologically advantageous in
850 terms of control. Indeed, bioactivation by P450s is known to be a requirement for insecticidal
851 toxicity of several classes of insecticides, e.g., the organophosphates and chlorpenafyr. In
852 contrast to *GSTe2*, the *CYP6Z2* from across Sahel contain three cDNA mutations, which makes
853 it different from Ngoussou coding sequences. These are $K^{211}N$ and $T^{218}S$ mutations, both
854 within the substrate recognitions site 2, and an $A^{282}E$. Other important CYP450s found to be
855 commonly overexpressed across the Sahel include the *CYP4C27* (AGAP009246), *CYP6Z3*
856 (AGAP008217) and *CYP9K1* (AGAP000818), all three shown to be consistently overexpressed
857 in field populations of *An. gambiae/coluzzii* and *An. funestus* across Africa (9, 67, 68). *CYP9K1*
858 has been shown to be epidemiologically important pyrethroid-metabolising P450 linked with
859 metabolism of deltamethrin and pyriproxyfen in *An. gambiae* (69). We also have recently
860 shown that his P450 is involved in pyrethroids in *An. funestus* (70).

Several carboxylesterases were also upregulated/induced across the Sahel, with the *COEBE3C* (AGAP005372) upregulated in all four countries. This beta esterase is enriched in the legs (where xenobiotic detoxification probably occur) of pyrethroid-resistant *An. coluzzii* (71). Other genes consistently overexpressed across the Sahel, include the chymotrypsins (*CHYM3*/AGAP006711 and *CHYM1*/AGAP006709) and a lipase (AGAP002353). Chymotrypsins are known to defend insects against plants' proteinase inhibitors (72); and previous transcriptional studies have shown *CHYM1* and *CHYM3* overexpressed in insecticide resistant populations of *An. gambiae* and *An. coluzzii,* respectively (9, 67). Using *in vitro* and *in vivo* tools, lipases have been linked with deltamethrin resistance in *Culex pipiens* pallens (73).

Common metabolic resistance markers probably exacerbate resistance across the Sahel

Several well-known genetic variants implicated in resistance, as well as the recently discovered ones exist in high frequencies in the Sahelian *An. coluzzii*. For example, the pyrethroid resistance *CYP4J5*-L43F marker (74) was found fixed across the Sahel. The G280S/G119S *ace*-1 mutation, found in high frequencies across Sahel confers organophosphate an carbamates resistance (75), and is shown recently to confer resistance to pirimiphos-methyl, in *An. coluzzii*/*gambiae* (76). Several mutations found within the VGSC have recently been described/validated. For example, the resistance mutation, L995F (77) and the V402L/I15227T haplotypes have been observed across Africa (78). Recently, the two mutations (V402L/I15227T) are described to be in tight linkage and mutually exclusive to the classical L995F/S mutations (77). Our results suggest haplotypes carrying the V402L/I1527T

combination plus the L995 replacement do exist in the Sahel *An. coluzzii*. Not only that, it is also in addition to the N1570Y replacement.

In contrast to *An. funestus GSTe2*, where overexpression and 119F mutation combined to confer extreme DDT resistance (22), the absence of amino acid replacements in the *An. coluzzii GSTe2* from the Sahel suggests that overexpression alone is the key mediator of DDT and permethrin resistance. This is supported by the higher activity in the regulatory regions, harbouring an insertion and nucleotide substitutions in the alive mosquitoes. Indeed, some of the mutations we have found within the intergenic region of *GSTe2* are similar to those observed in a previous study (79).

Cuticular resistance mechanism probably playing a key role in Sahelian *An. coluzzii*

Our results suggest cuticular mechanism play a role in pyrethroid resistance in these populations. For example, the findings of *CYP4G16* (AGAP001076) and *CYP4G17* (AGAP000877) overexpressed across the Sahel. The former P450 was previously shown to be involved in epicuticular hydrocarbon biosynthesis associated with resistance (80).

The three major classes of the insect cuticular proteins - the CPR, CPLC and CPAP, were found overrepresented in the top overexpressed genes across the Sahel, with the commonly upregulated ones being *CPR76*, *CPR15* and *CPR30*, a chitin-binding cuticular protein, *CPAP3-A1b*, and cuticular proteins of low complexity, *CPLCX3* and *CPLCA1*. Indeed, *CPAP3-A1b* (AGAP000987) have been shown to be highly overexpressed in deltamethrin-resistant Sahelian population of *An. coluzzii* from Burkina Faso (9) and induced by blood feeding in *An.*

905 *gambiae* (81). The CPR and CPLC cuticular proteins have been described to potentially play a
906 crucial role in insecticide resistance through leg cuticle remodelling/thickening, regulating
907 penetration rate of insecticides in *An. coluzzii* (71). Furthermore, a recent study has found most
908 of the cuticular proteins we have described here, as highly overexpressed in
909 permethrin/malathion resistant populations of Ethiopian *An. arabiensis* (82), e.g., *CPR30*,
910 *CPR75*, *CPR81*, and *CPLCP11*. Out of the several chitinases overexpressed across the Sahel,
911 four were amongst the top 50 most overexpressed metabolic genes. These include the *Cht24*
912 and *Cht6* that have been shown to be overexpressed in the *An. arabiensis* from the above study
913 (82), with the ortholog of *Cht24*, AARA007329 among the top 10 most overexpressed genes in
914 *An. arabiensis*, in line with our observations in *An. coluzzii* across the Sahel. There is an
915 overwhelming need to functionally investigate the role/contribution towards insecticide
916 resistance of these cuticular proteins, chitinases and a chitin synthase (AGAP001748)
917 significantly overexpressed in the field *An. coluzzii* from Nigeria and Chad.

918

919 **Insecticide resistance- and thermotolerance-associated genes sit within**
920 **chromosomal inversions**

921 In this study, the findings of high frequency of 2La, 2Rb and 2Rc inversion polymorphisms in
922 the populations of *An. coluzzii*, compared with the Ngoussou, suggested strong phenotypic
923 adaptations in this species, across the Sahel. Most importantly, in addition to several of the
924 cuticular protein genes associated with resistance (chitinases, chitin synthase, CPR, CPLC and
925 CPAP proteins), several other genes previously implicated in thermotolerance and/or
926 desiccation resistance in *An. gambiae/coluzzii,* and which were highly overexpressed in this
927 study sit within these inversions. For example, the heat shock proteins, *hsp83* (AGAP006958)

928 and *hsp90* hptG (AGAP006961), both of which are known to be heat- and insecticide-stress
929 inducible (83) and were among the core set of hsp genes involved in a common and immediate
930 response to thermal stress in *An. gambiae* populations (84), sit within the 2La inversion. These
931 two genes were among the overexpressed genes in both heat-hardened and permethrin-
932 resistant *An. coluzzii* populations from northern Nigeria (56). Several ionotropic glutamate
933 receptors were found within the 2La inversion breakpoints: *IR136* (AGAP006440), *IR139*
934 (AGAP006691) and *IR142* (AGAP006407). This is not surprising as ionotropic receptors are
935 commonly associated with chemosensation, thermosensation, and hygrosensation (85, 86),
936 characteristics
937 which can confer adaptive advantages in xeric environs. The *IR25a* (AGAP010272) and *IR21*
938 *a* (AGAP008511) are known to mediate both humidity and temperature preference in the fruit
939 fly, *D. melanogaster* (86, 87), in addition to *IR21a* driving heat seeking and heat-stimulated
940 blood feeding in *An. gambiae* (87). These two genes have been shown to be
941 overexpressed/induced in thermotolerant/permethrin-resistant populations of *An. coluzzii*
942 (56).

943

944 Conclusions

945 Information on molecular basis of resistance and/or resistance genes and its markers facilitates
946 evidence-based control measures. In this study we characterised a major malaria vector, *An.*
947 *coluzzii* from the Sahel region of four countries, with findings which could promote evidence-
948 based, cross-border policy towards local and regional malaria control. The study found that
949 across Sahel (where malaria is highly seasonal, reaching its peak in the rainy season), *An.*

coluzzii is a dominant vector. And that a handful of common cross-resistance genes are responsible for multiple insecticide resistance in this species. Findings from this study suggest pleotropic role of some key genes – able to confer insecticide resistance and/or stabilize the insecticide resistance gene, at the same time conferring environmental adaptations, such as the ability to survive thermal stress (thermotolerance), as expected in this Sahelian region. From operational vector control perspective this study provided evidence of the role of key insecticide metabolism gene, *CYP6Z2* in increasing insecticidal potency of clothianidin, which could increase the efficacy of the ingredients in malaria control tools, when targeting field populations overexpressing this key P450.

Declarations

Ethics approval and consent to participate

This study did not use human participants, human data or human tissue. Ethical approval for collection of indoor resting female mosquitoes in Nigeria, Niger, Chad and Cameroon have been provided in the references cited in the Study Site and Mosquito Sampling (Materials and Methods).

Consent for publication

Not Applicable.

Availability of data and materials

The dataset(s) supporting the conclusions of this article are available in the European Nucleotide Archive, with accession PRJEB51644, and secondary accession of ERP136291, for the RNA-seqe raw sequence reads. cDNA sequences of *GSTe2* and *CYP6Z2* were deposited in GenBank (accession numbers: Submission # 2567942 for *GSTe2* and Submission # 2567961 for *CYP6Z2*) and 5'-UTR DNA fragment sequences were deposited in GenBank (accession numbers: Submission # 2568060).

Competing interests

The authors declare that they have no competing interests.

Funding

This research was funded in whole, by the Wellcome Trust [Grant numbers: WT201918/Z/16/Z to SSI and WT217188/Z/19/Z to CSW]. For the purpose of open access, the author has applied a CC BY public copyright licence to any Author Accepted Manuscript version arising from this submission. The Wellcome Trust had no role in the design of this study and collection, analysis, and interpretation of data and in writing of this manuscript.

Authors' contributions

Conceived and designed by SSI and CSW. SSI carried out the molecular analyses, with support from AM, LMJM, EIP and HI. ANF and MMM, participated in field collection of mosquitoes in Nigeria, Chad and Cameroon. SSI carried out data analysis with the support of JH, GDW, SCN for the RNAseq component. SSI wrote the manuscript with inputs from CSW, JH and SCN. All authors contributed to corrections of the final draft and approved final version of the manuscript.

Acknowledgements

Not Applicable

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Figure Titles

Figure 1: A volcano plot of differentially expressed genes showing fold changes and significance in R-S, C-S and R-C comparisons, for Nigeria and Niger populations of *An. coluzzii*.

Figure 2: A volcano plot of differentially expressed genes showing fold changes and significance in R-S, C-S and R-C comparisons, for Chad and Cameroon populations of *An. coluzzii*.

Figure 3: Validation of candidate resistance genes. qRT-PCR of twelve metabolic resistance genes (R-S and C-S comparisons) from Sahelian *An. coluzzii*.

Figure 4: Identification of resistance variants of interest. A heatmap showing frequencies of the resistance variants (haplotypes) in key genes of interest.

Figure 5: Characterization of intergenic region (5′ regulatory element) of *GSTe2*. Results of dual-luciferase reporter assays of the promoter (intergenic region) constructs, showing progressive loss of activity following sequential deletion of the constructs.

Figure 6: Validation of the role of metabolic resistance genes in insecticide resistance. Results of insecticide susceptibility bioassays with transgenic flies expressing *GSTe2* and *CYP6Z2*. **a.** α-cypermethrin and deltamethrin, **b.** permethrin, **c.** 3-phenoxybenzaldehyde and 3-phenoxybenzyl alcohol, **d.** DDT and clothianidin.

Additional Files

Additional File 1: Supplementary text for methods and results.

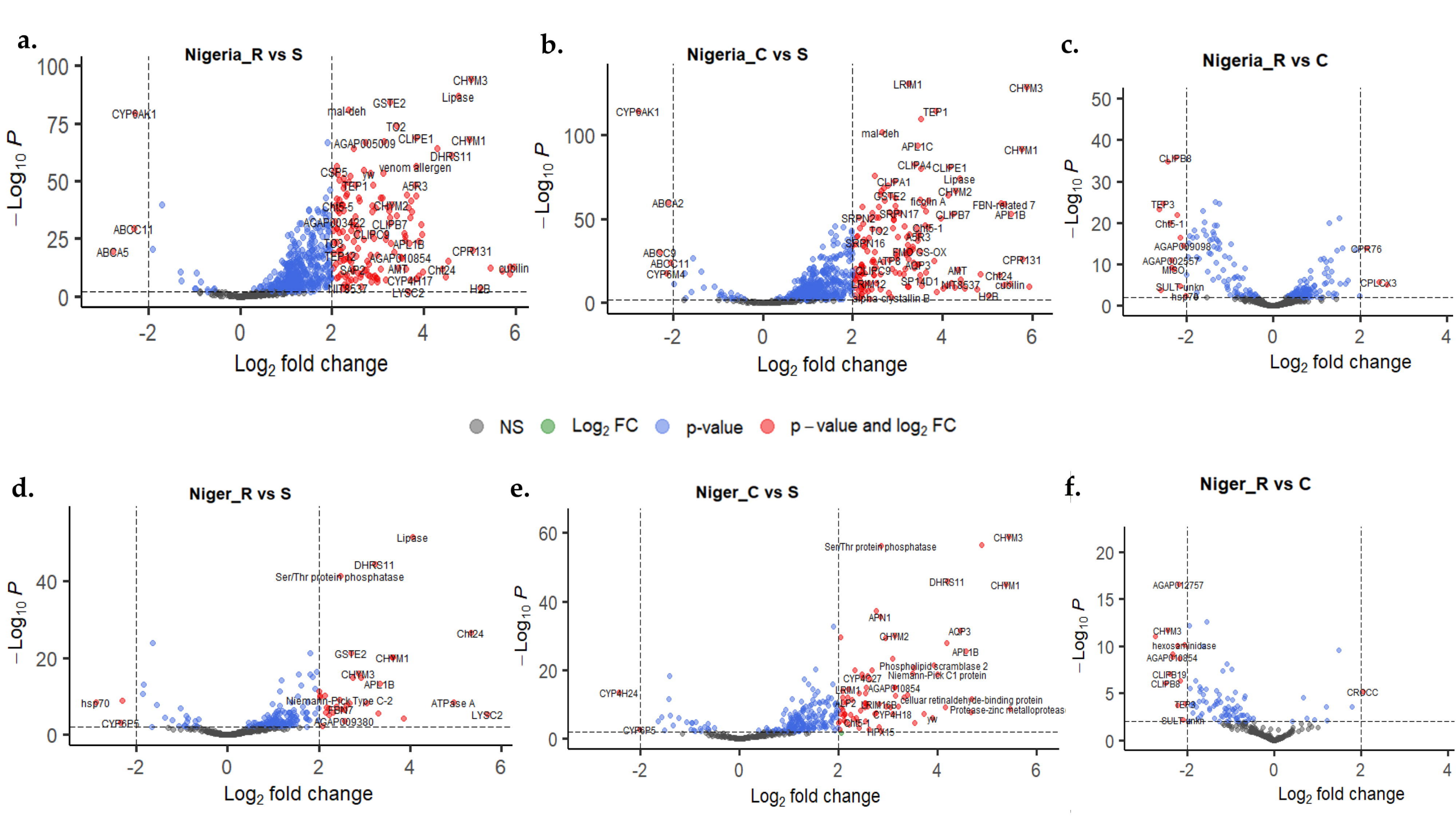
Additional File 2: Common differentially expressed genes across the Sahel.

Additional File 3: Population genetics analyses.

Additional File 4: Genes within 2La, 2Rb and 2Rc inversion polymorphisms.

Additional Tables: Additional tables, S1-S6.

Additional Figures: Additional figures, S1-S11.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b.
Nigeria_C vs S
c.
Nigeria_R vs C
d.
Niger_R vs S
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f.
Niger_R vs C
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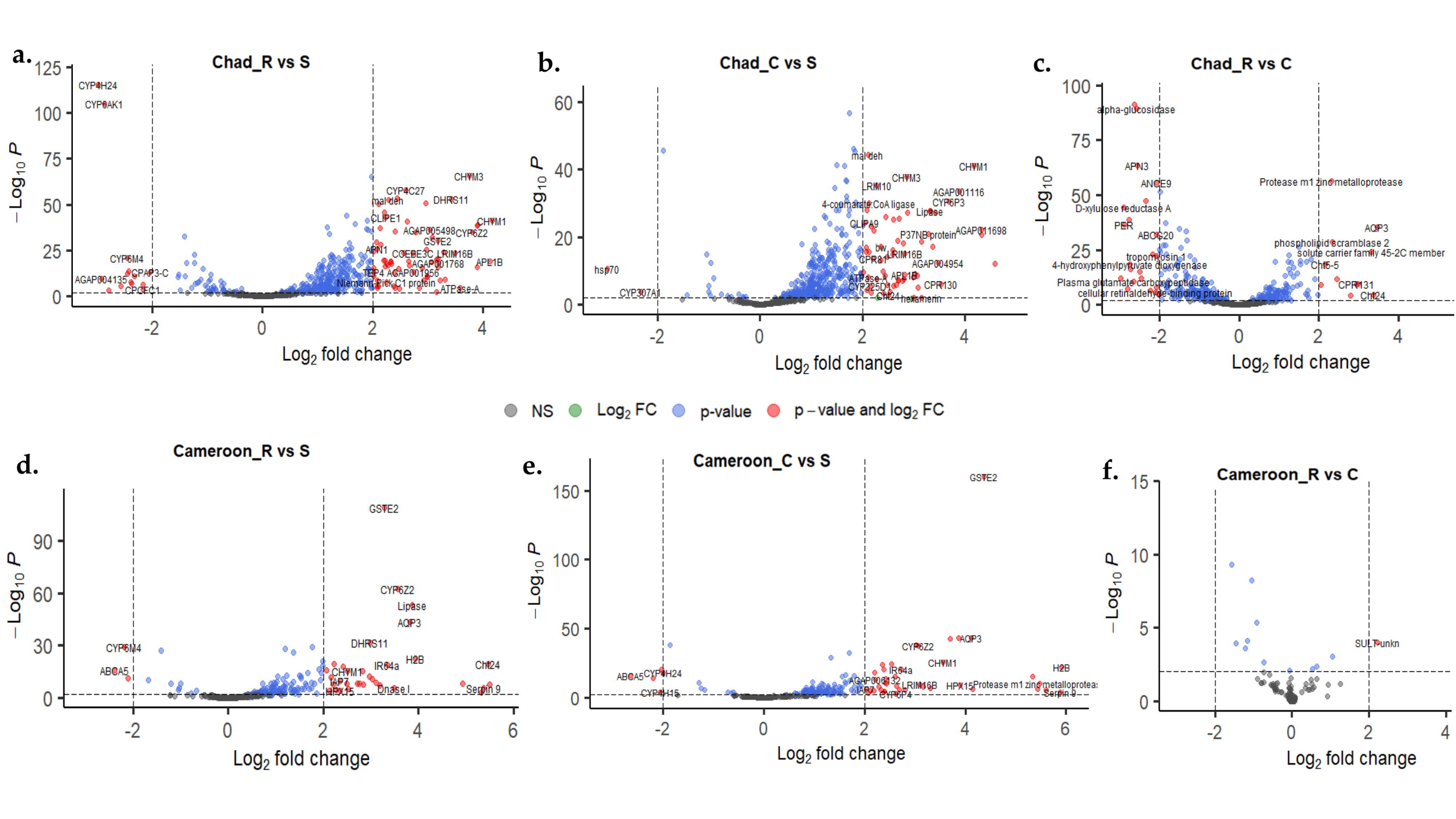
NS
Log2 FC
p-value
p – value and log2 FC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c.
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NS
Log2 FC
p-value
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f.
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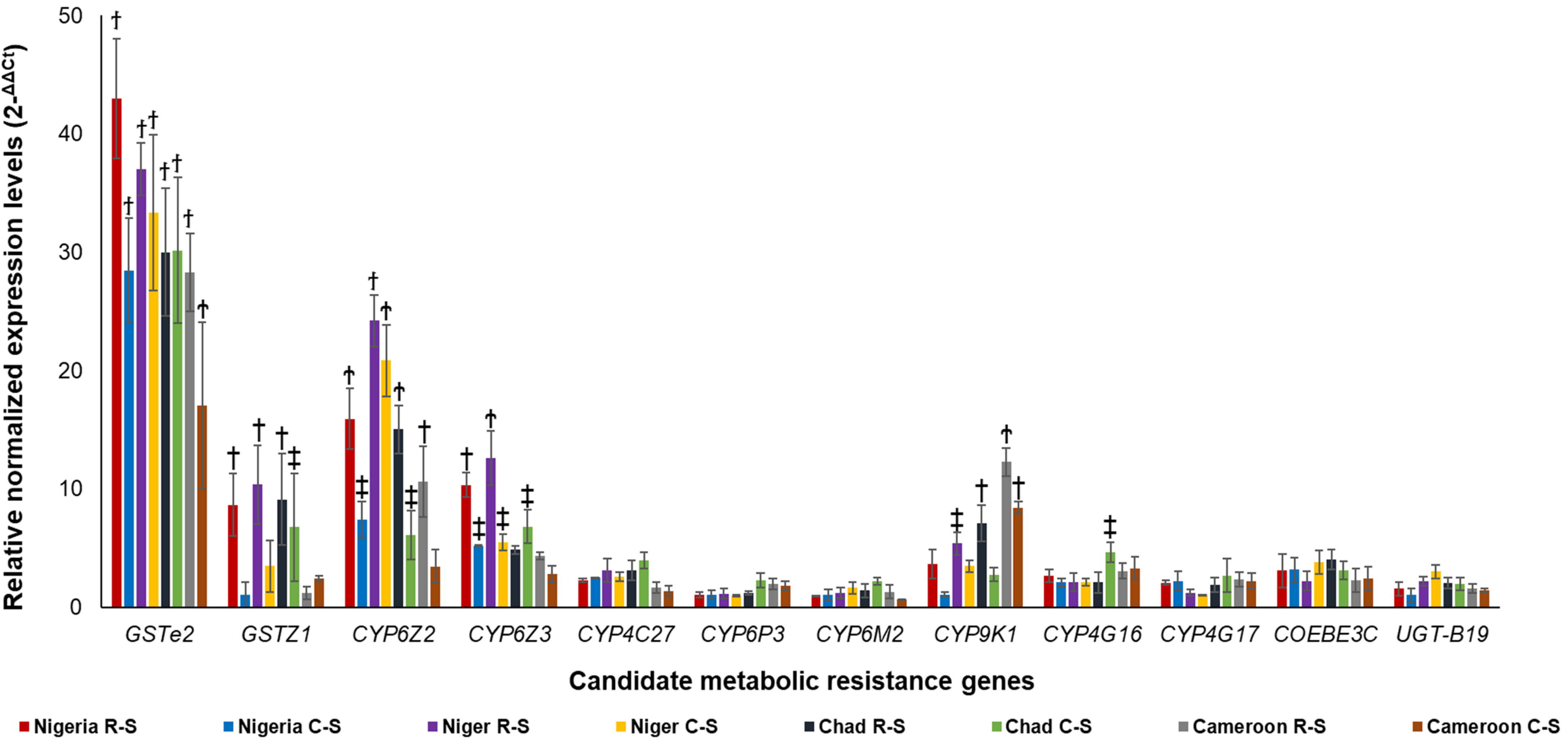
−Log10 P
Log2 fold change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40
30
20
10
0
GSTe2
GSTZ1
CYP6Z2
CYP6Z3
CYP4C27
CYP6P3
CYP6M2
CYP9K1
CYP4G16
CYP4G17
COEBE3C
UGT-B19
Candidate metabolic resistance genes
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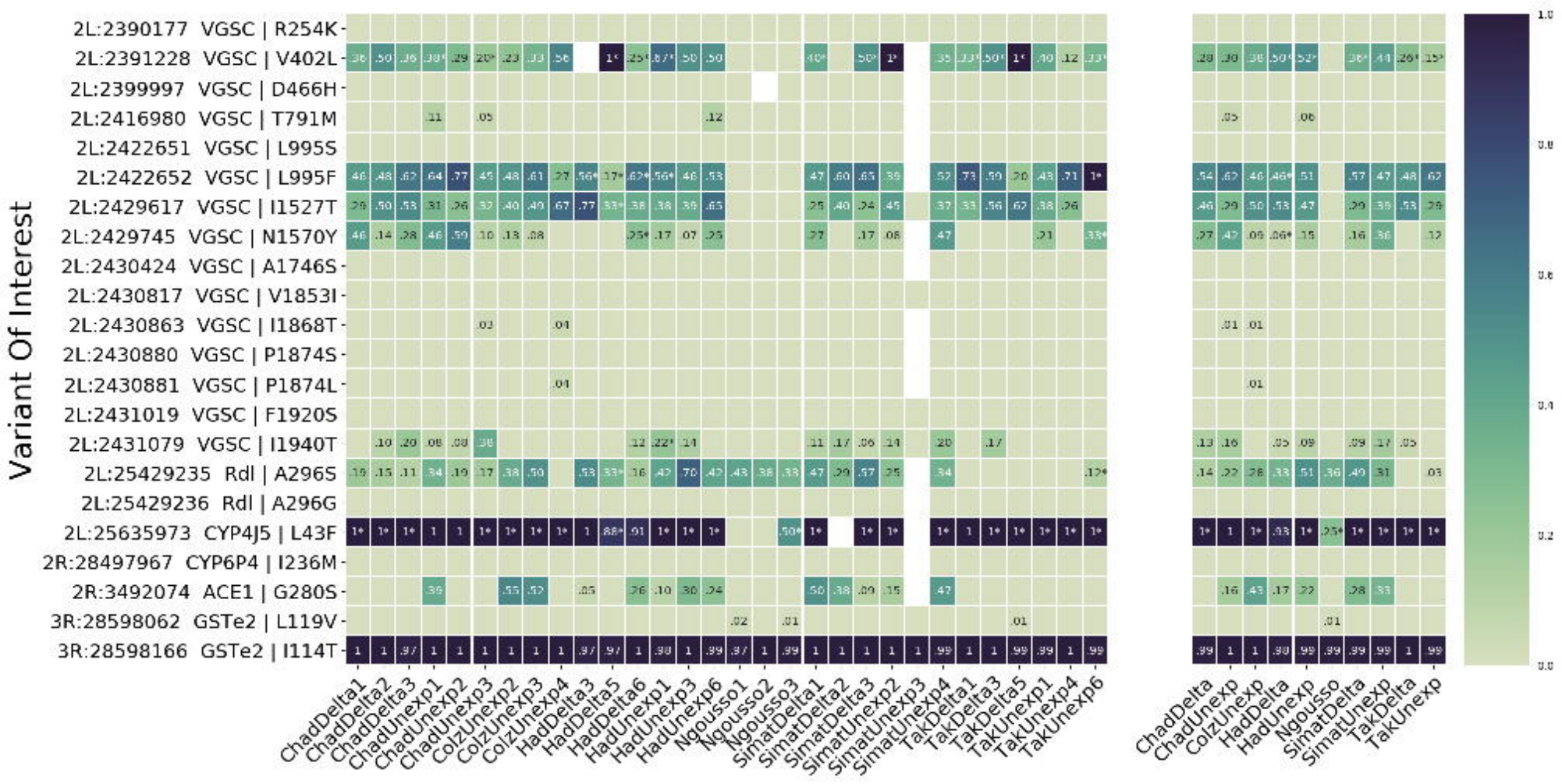
Niger R-S
Niger C-S
Chad R-S
Chad C-S
Cameroon R-S
Cameroon C-S


Variant Of Interest
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2L:2422651 VGSC | L995S
2L:2422652 VGSC | L995F
2L:2429617 VGSC | I1527T
2L:2429745 VGSC | N1570Y
2L:2430424 VGSC | A1746S
2L:2430817 VGSC | V1853I
2L:2430863 VGSC | I1868T
2L:2430880 VGSC | P1874S
2L:2430881 VGSC | P1874L
2L:2431019 VGSC | F1920S
2L:2431079 VGSC | I1940T
2L:25429235 Rdl | A296S
2L:25429236 Rdl | A296G
2L:25635973 CYP4J5 | L43F
2R:28497967 CYP6P4 | I236M
2R:3492074 ACE1 | G280S
3R:28598062 GSTe2 | L119V
3R:28598166 GSTe2 | I114T
ChadDelta1
ChadDelta2
ChadDelta3
ChadUnexp1
ChadUnexp2
ChadUnexp3
ColzUnexp2
ColzUnexp3
ColzUnexp4
HadDelta3
HadDelta5
HadDelta6
HadUnexp1
HadUnexp3
HadUnexp6
Ngousso1
Ngousso2
Ngousso3
SimatDelta1
SimatDelta2
SimatDelta3
SimatUnexp2
SimatUnexp3
SimatUnexp4
TakDelta1
TakDelta3
TakDelta5
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TakUnexp4
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ChadUnexp
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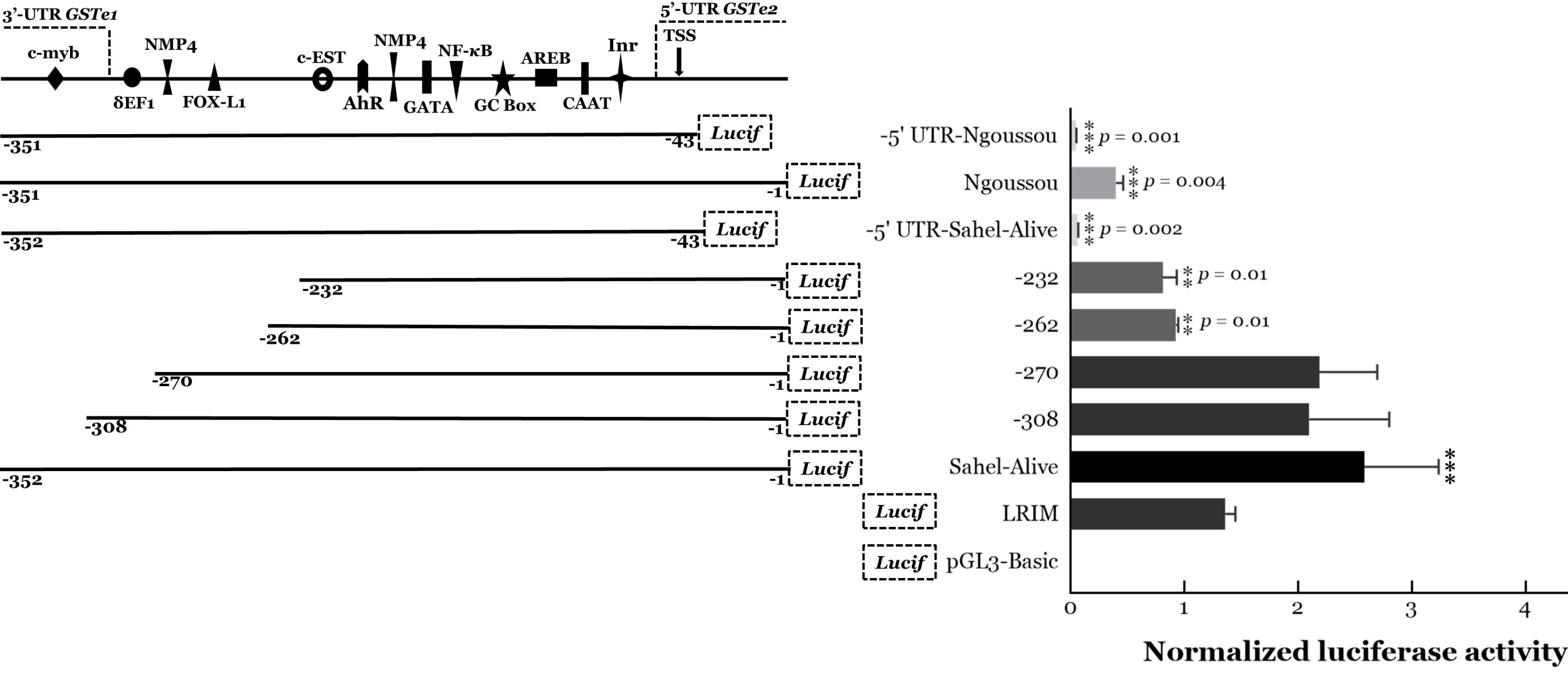
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AREB
CAAT
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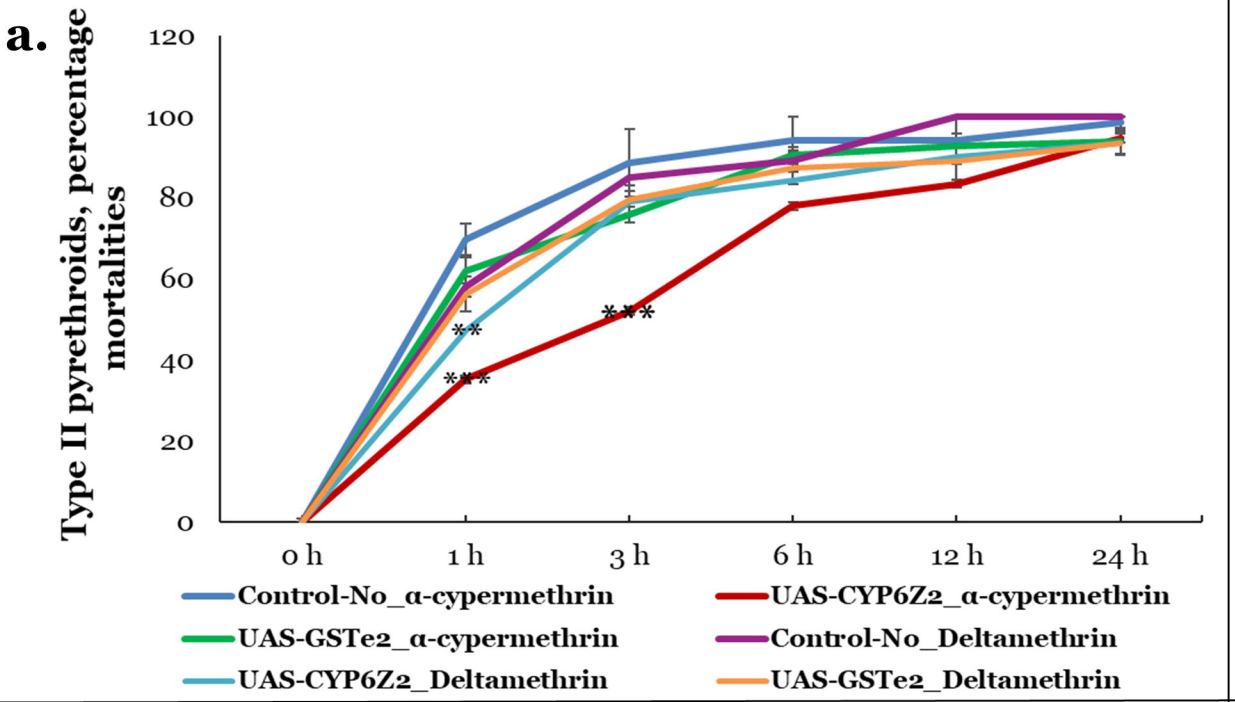
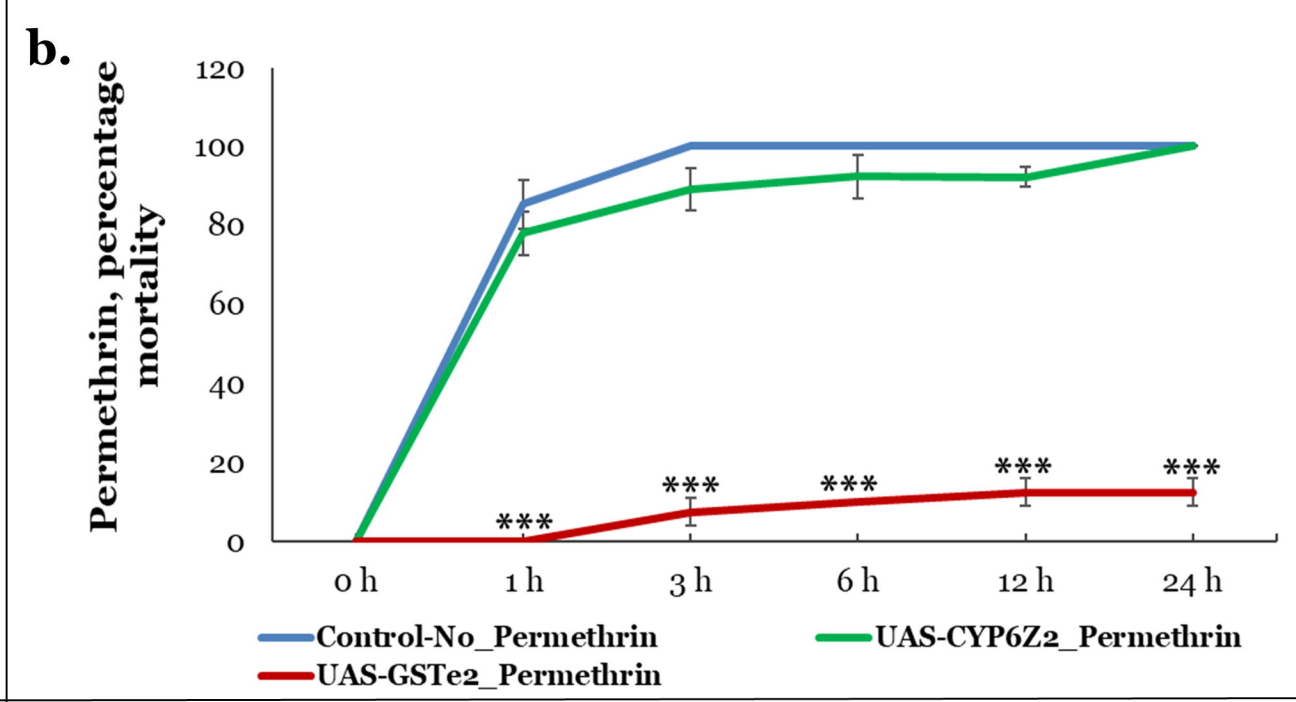
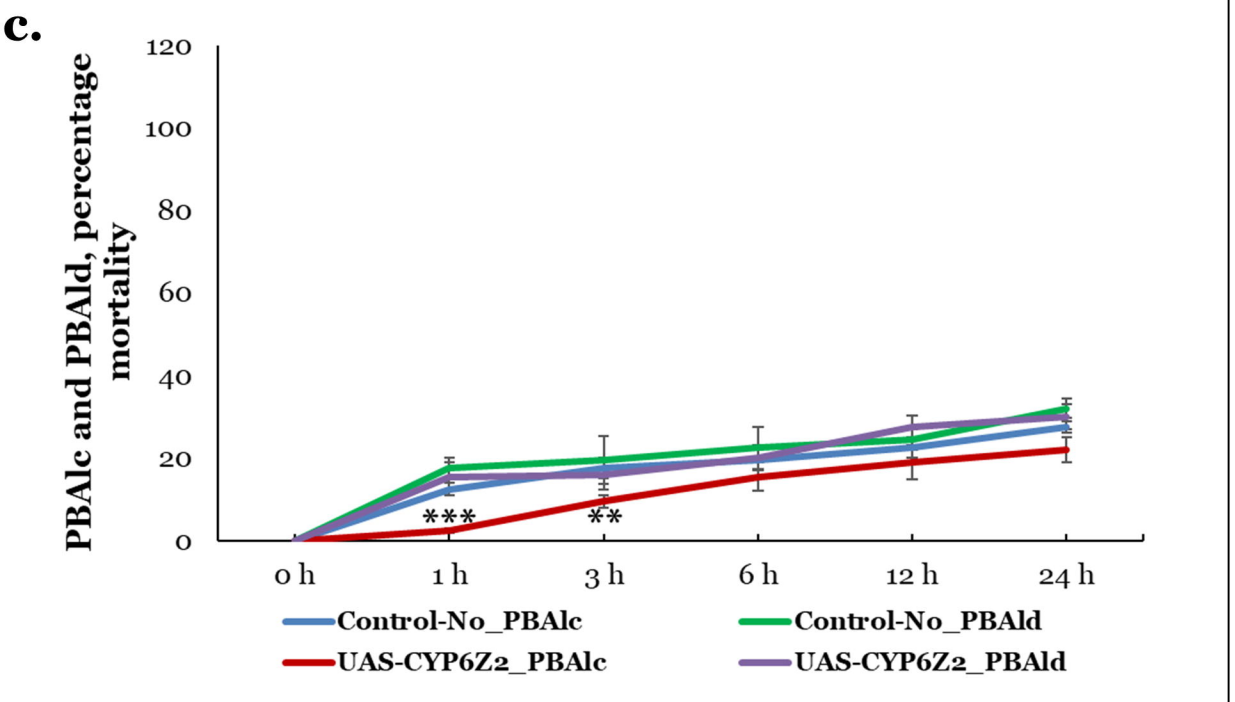
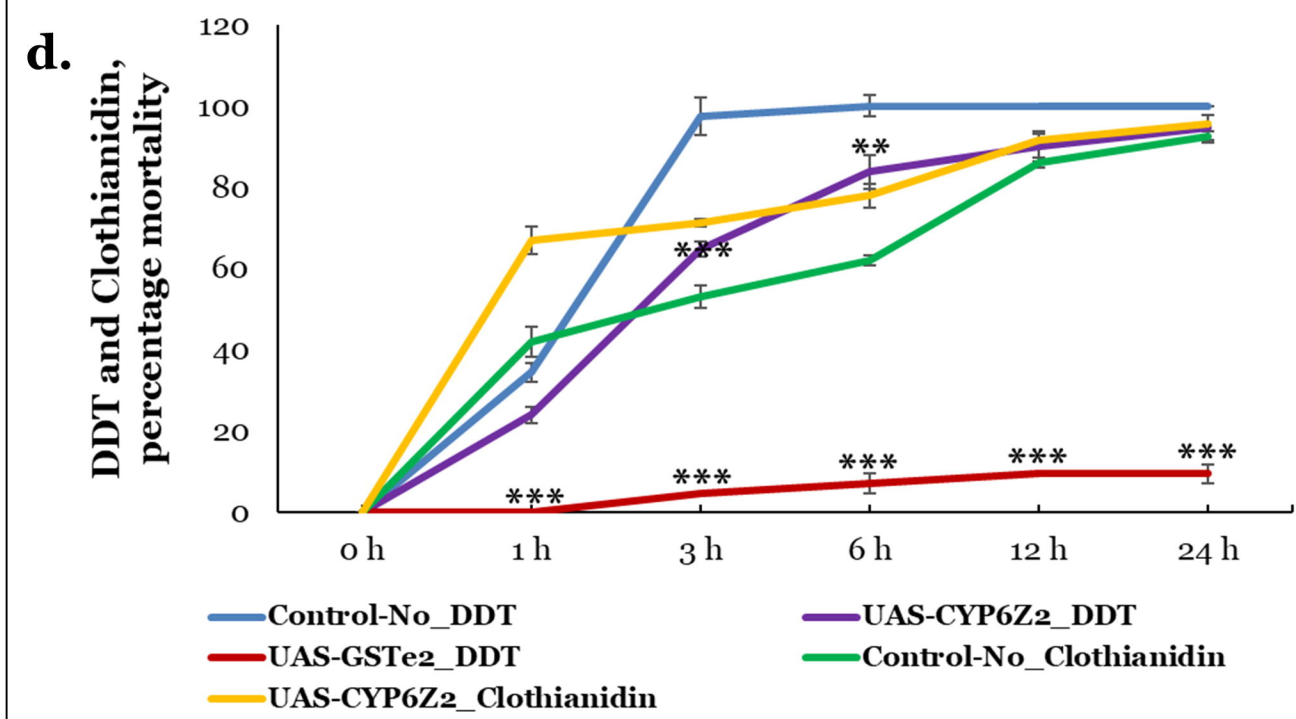
Lucif
-352
-1
Lucif
Lucif
Lucif
-5' UTR-Ngoussou
p = 0.001
Ngoussou
p = 0.004
-5' UTR-Sahel-Alive
p = 0.002
-232
p = 0.01
-262
p = 0.01
-270
-308
Sahel-Alive
LRIM
pGL3-Basic
0
1
2
3
4
Normalized luciferase activity


a.
Type II pyrethroids, percentage mortalities
120
100
80
60
40
20
0
0 h
1 h
3 h
6 h
12 h
24 h
**
***
***
Control-No_α-cypermethrin
UAS-CYP6Z2_α-cypermethrin
UAS-GSTe2_α-cypermethrin
Control-No_Deltamethrin
UAS-CYP6Z2_Deltamethrin
UAS-GSTe2_Deltamethrin


b.
Permethrin, percentage mortality
120
100
80
60
40
20
0
0 h
1 h
3 h
6 h
12 h
24 h
***
***
***
***
***
Control-No_Permethrin
UAS-CYP6Z2_Permethrin
UAS-GSTe2_Permethrin


c.
PBAlc and PBAld, percentage mortality
120
100
80
60
40
20
0
0 h
1 h
3 h
6 h
12 h
24 h
***
**
Control-No_PBAlc
Control-No_PBAld
UAS-CYP6Z2_PBAlc
UAS-CYP6Z2_PBAld


d.
DDT and Clothianidin, percentage mortality
120
100
80
60
40
20
0
0 h
1 h
3 h
6 h
12 h
24 h
***
***
**
***
***
***
Control-No_DDT
UAS-CYP6Z2_DDT
UAS-GSTe2_DDT
Control-No_Clothianidin
UAS-CYP6Z2_Clothianidin