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Widespread occurrence of copy number variants and fixation of pyrethroid target site resistance in *Anopheles gambiae* (*s.l.*) from southern Côte d'Ivoire

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ABSTRACT

Resistance to pyrethroid and organophosphate insecticides in the malaria vector *Anopheles gambiae* (*s.l.*) is conferred by a variety of genetic mutations, including single nucleotide polymorphisms (SNPs) and copy number variants (CNVs). Knowledge of the distribution of these mutations in mosquito populations is a prerequisite for establishing better strategies for their management. In this study, a total of 755 *Anopheles gambiae* (*s.l.*) from southern Côte d'Ivoire were exposed to deltamethrin or pirimiphos-methyl insecticides and were screened to assess the distribution of SNPs and CNVs known or believed to confer resistance to one or other of the insecticide classes. Most individuals from the *An. gambiae* (*s.l.*) complex were identified by molecular tests as *Anopheles coluzzii*. Survival to deltamethrin (from 94% to 97%) was higher than to pirimiphos-methyl (from 10% to 49%). In *An. gambiae* (*s.s.*), the SNP in the Voltage Gated Sodium Channel (*Vgsc*) at the 995F locus (*Vgsc*-995F) was fixed, while other target site mutations were rare or absent (*Vgsc*-402L: 0%; *Vgsc*-1570Y: 0%, Acetylcholinesterase *Acel*-280S: 14%). In *An. coluzzii*, *Vgsc*-995F was the target site SNP found at highest frequency (65%) followed by other target site mutations (*Vgsc*-402L: 36%; *Vgsc*-1570Y: 0.33%; *Acel*-280S: 45%). The *Vgsc*-995S SNP was not present. The presence of the *Ace1*-280S SNP was found to be significantly linked to the presence of the *Ace1-CNV*, *Ace1*_AgDup. Significant association was found between the presence of the *Ace1*_AgDup and pirimiphos-methyl resistance in *An. gambiae* (*s.s.*) but not in *An. coluzzii*. The deletion *Ace1*_Del97 was found in one specimen of *An. gambiae* (*s.s.*). Four CNVs in the *Cyp6aa/Cyp6p* gene cluster, which contains genes of known importance for resistance, were detected in *An. coluzzii*, the most frequent being Dup 7 (42%) and Dup 14 (26%). While none of these individual CNV alleles were significantly associated with resistance, copy number in the *Cyp6aa* gene region in general was associated with increased resistance to deltamethrin. Elevated expression of *Cyp6p3* was nearly associated with deltamethrin resistance, although there was no association of resistance with copy number. Use of alternative insecticides and control methods to arrest resistance spread in *An. coluzzii* populations is merited.

1. Introduction

Malaria is a serious vector-borne disease caused by a protozoan parasites *Plasmodium* spp. and transmitted to humans by *Anopheles* mosquitoes (Harbach, 2007; Fontenille, 2008; WHO, 2021). Between 2010 and 2016, a significant decline in malaria incidence and mortality was reported (Bhatt et al., 2015; WHO, 2018a). However, more recently (from 2019 to 2020), malaria cases have increased, with 14 million more people contracting malaria and 69,000 more malaria deaths than in 2019. It was estimated that about 2/3 of these additional deaths in 2020 were related to disruptions in malaria service delivery during the COVID-19 pandemic (WHO, 2021).

Africa remains the continent most affected by malaria, with 95% of the 241 million malaria cases recorded worldwide in 2020 (WHO, 2021). In Côte d'Ivoire, over 6 million cases were reported in 2019 (WHO, 2020). Among the 40 species of *Anopheles* considered to be principal vectors of malaria (Hay et al., 2010; Tawe et al., 2017), species from the *An. gambiae* (*s.l.*) complex (*An. gambiae* (*s.s.*), *An. coluzzii*, and *An.*

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arabiensis) and *An. funestus* have been identified as the main vectors in Côte d'Ivoire (Dossou-Yovo et al., 1995; Adja et al., 2015; Tawe et al., 2017; WHO, 2018b, 2020; Kgoroebutswe et al., 2020). Insecticide-based control methods such as long-lasting insecticidal nets (LLINs), and/or indoor residual spraying (IRS) are used by more than 50% of the population (WHO, 2020). A recent study conducted in rural localities of Agboville, Côte d'Ivoire, revealed that pyrethroids were the most intensely used insecticide both on crops and domestically (Kouamé et al., 2022).

This reliance on insecticide-based methods of control makes the spread of insecticide resistance in *Anopheles* mosquitoes a concerning problem (Edi et al., 2014a; Fodjo et al., 2018; Hancock et al., 2020), requiring effective management of insecticide use, informed by an understanding of the local resistance profile. Target site mutations and metabolic resistance are the main mechanisms involved in mosquito resistance to insecticides (Müller et al., 2008; Ranson et al., 2011; Chouaïbou et al., 2019). Target site resistance involves mutations in the protein targeted by the insecticide. For example, single nucleotide polymorphisms (SNPs) in the voltage-gated sodium channel (*Vgsc*) and acetylcholinesterase (*Ace1*) genes have been found to increase resistance to pyrethroids/DDT and organophosphates/carbamates, respectively (Martinez-Torres et al., 1998; Weill et al., 2004; Ochomo et al., 2015; Baffour-Awuah et al., 2016). These mutations have since been detected in many African populations of *Anopheles gambiae* (*s.l.*) (Bass et al., 2007; Tia et al., 2017; Lynd et al., 2018; Koukpo et al., 2019; Etang et al., 2022). The first and most widely acknowledged resistance mutation in *Vgsc* was *Vgsc*-995F (previously known as *Vgsc*-1014F), a mutation which has since been shown to have multiple independent origins and to have spread throughout much of sub-Saharan Africa (The *Anopheles gambiae* 1000 Genomes Consortium, 2017). More recently, other mutations in *Vgsc* have been discovered (Clarkson et al., 2021), including many that occur only in conjunction with *Vgsc*-995F (such as *Vgsc*-1570Y, previously known as *Vgsc*-1575Y) and one whose presence is in complete negative linkage disequilibrium (i.e. mutually exclusive) with *Vgsc*-995F (*Vgsc*-402L). These additional *Vgsc* SNPs have received limited attention so far (Clarkson et al., 2021; Williams et al., 2022; Lucas et al., 2023). In *Ace1*, the SNP *Ace1*-280S (previously known as *Ace1*-119S) and a copy number variant (CNV) that increases the number of copies of *Ace1* in the genome, combine to provide resistance to organophosphates (Weetman et al., 2015; Grau-Bové et al., 2021).

In contrast, metabolic resistance occurs through changes in either the sequence or expression of a complex array of enzymes and detoxification pathways that remove the insecticide from the system. Cytochromes P450 (often from the Cyp6 and Cyp9 families) have been reported to be the primary family associated with metabolic resistance to most insecticides (David et al., 2013). For example, over-expression of *Cyp6m2, Cyp6p3, Cyp9k1* and *Cyp6p4* has been reported to increase metabolic resistance to pyrethroids and organophosphates (David et al., 2013; Vontas et al., 2020).

Mutations leading to metabolic resistance are considerably harder to identify than for target site resistance, because of the variety of routes by which changes in expression can be generated. One type of mutation that has recently been implicated is copy number variants (CNVs). These occur when a section of the genome is deleted or duplicated (Fig. 1). Many studies have shown the importance of CNVs in insecticide resistance both in metabolic resistance (Weetman et al., 2018) and in target site modification (Weetman et al., 2015; Assogba et al., 2016; Martins et al., 2017). Recent work on whole-genome sequencing data revealed the widespread presence of at least 44 CNVs in *An. coluzzii* and *An. gambiae* (*s.s.*), in regions of the genome previously implicated in metabolic resistance, with many of these CNVs showing signals of positive selection (Lucas et al., 2019a). This strongly hinted at the possibility that these CNVs are rapidly increasing in frequency as a selective response to insecticide use. However, due to the difficulty of execution, there is only limited evidence (Njoroge et al., 2022) about the contribution of CNVs to resistance in many mosquito populations.

Resistance of *An. gambiae* (*s.l.*) to several insecticides has been reported in Côte d'Ivoire, as has the presence of resistance associated mutations (*Vgsc*-995F, *Vgsc*-402L, *Vgsc*-1570Y and *Ace1*-280S) and some metabolic resistance through elevated expression of detoxification genes (N'Guessan et al., 2003; Nauen, 2007; Edi et al., 2014b, 2017; Chouaïbou et al., 2017; Tia et al., 2017; Clarkson et al., 2021). For better management, it is important to have up-to-date and locally-relevant data. We hypothesized that the presence of high selection pressure in agricultural areas would lead to high levels of resistance, particularly towards pyrethroid insecticides, which were the most used (Kouamé et al., 2022).

Assessing the susceptibility of malaria vectors and the resistance mechanisms linked to the observed resistance is necessary to design tailored policies for malaria control and eradication. In this study, the susceptibility of *An. gambiae* (*s.l.*) individuals from rural localities in southern Côte d'Ivoire was assessed towards two insecticides: deltamethrin and pirimiphos-methyl (PM), belonging to the pyrethroid and the organophosphate chemical class, respectively. The choice of these insecticides was based on previous studies, where they were reported to be the main insecticides used by farmers both on their crops against agricultural pests and in their houses against mosquitoes (Kouamé et al., 2022). Deltamethrin was the most widely used pyrethroid, while PM was reported to also be widely used for mosquito management. In addition to the detection of the SNPs in individual mosquitoes exposed to each insecticide, four CNVs in the *Cyp6aa/Cyp6p* region and two known resistance-associated CNVs in the *Ace1* region (Assogba et al., 2016; Lucas et al., 2019a), were screened (Fig. 1).

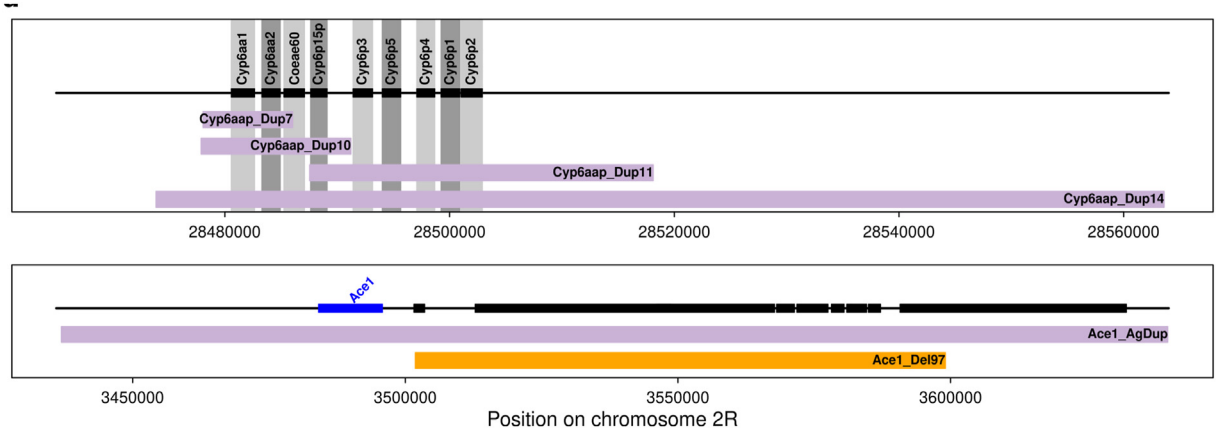

Fig. 1. Genomic range of the CNVs screened in the *Cyp6aa/Cyp6p* and *Ace1* regions. CNVs are shown as rectangular purple bars and the deletion inside the *Ace1*_AgDup CNV is shown as an orange bar. Genes are shown as bead plots above the CNVs.

2. Materials and methods

2.1. Study site and mosquito collection

This study was carried out in four rural localities in the department of Agboville, southern Côte d'Ivoire: Boa Vincent (5°53'41.1"N, 4°35'32.136"E), Ango (5°51'50.364"N, 4°22'54.624"E), Gbalékro (5°56'57.408"N, 4°16'41.34"E) and Ouanguié (5°51'59.112"N, 4°13'42.888"E) (Fig. 2). These localities received LLINs from the National Malaria Control Programme (NMCP) in 2017. The hydrographic network of this region is dominated by the Agneby stream (N'Guessan et al., 2009).

Larvae and pupae of *Anopheles* mosquitoes were collected from their natural breeding sites (pools and puddles around the village and associated rice and yam farms) using the dipping method, from September to October 2019. Samples were taken to the insectary, where they were sorted according to their provenance and development stage. Larvae were kept in water and fed with fish food (Tetramin ®) under standard conditions (temperature of 25–28 °C and relative humidity of 65–75%), while pupae were placed directly into cages for adult emergence. Upon emergence, adult mosquitoes were fed on 10% sugar solution soaked in cotton. Species were identified using identification keys (Gillies & Coetzee, 1987), and 3–5 day-old *An. gambiae* (*s.l.*) females were used for molecular analyses and bioassays.

2.2. WHO tube assay method

Susceptibility to two insecticides, deltamethrin (0.05%) and PM (0.25%), was assessed according to WHO recommendations (WHO, 2016) using tube assays. According to the protocol, 80–100 3–5 day-old adult non-blood-fed females were placed into four tubes (20–25 per tube) containing insecticide impregnated filter papers. For the negative control, 40–50 females were taken into two tubes with Wheaton paper without any insecticide. After 1 h of exposure, mosquitoes were transferred to holding tubes for observation and were fed on 10% sugar solution for 24 h. Mortality was recorded after this period.

2.3. Species identification

DNA from mosquitoes was extracted using a modified cetyl trimethyl ammonium bromide (CTAB 2%) whole mosquito extraction method (Chabi et al., 2019). After the purification of the DNA pellet with 70% ethanol, a final centrifugation was performed for 5 min at 12,000 *rpm* at room temperature. The tubes were drained and dried overnight. Then 25 µl of DNAse-free water was added into each tube and incubated at 55 °C in a water bath for 5 min. After DNA extraction, the SINE PCR (Santolamazza et al., 2008) and the SYBR green-based real-time PCR (Chabi et al., 2019) were used to identify species. Full PCR details are provided in the Supplementary file 1: Methods.

2.4. Target site mutations

To detect mutations at the *Vgsc*-995 codon, we used a slightly modified version of a previously developed locked nucleic acid (LNA) assay (Lynd et al., 2018), which is able to detect the wild type, *Vgsc*-995F and *Vgsc*-995S alleles in a single reaction. Briefly, the mix solution of 10 µl contained 5 µl of PCR master mix (IDT PrimeTime), 3.3 µl of dH_2O, 0.2 µl of each of the two primers VGSC-2F and VGSC-1R, 0.1 µl of each of the three probes (Serine: Cy5; Phenylalanine: Fam; Leucine: Hex), and 1 µl of the DNA template. The reaction conditions were as previously published (Lynd et al., 2018).

The detection of *Vgsc*-402L resistance mutation was also performed using an LNA assay (Williams et al., 2022), while TaqMan assays were used to detect the *Vgsc*-1570Y mutation (Jones et al., 2012) and the *Ace1*-280S target site substitution (Bass et al., 2010).

2.5. Copy number assignment using PCR

Samples were screened for four CNVs described in genomic regions of known importance for metabolic resistance and known to be found in West Africa (*Cyp6aap*_Dup7, *Cyp6aap*_Dup10, *Cyp6aap*_Dup11 and *Cyp6aap*_Dup14 (Lucas et al., 2019a)) using custom PCR primers (see Supplementary Table S1 for primer sequences and Supplementary file 1: Methods, for evidence of primer efficacy) and two CNVs in the *Ace1* target site for PM (*Ace1*_AgDup and *Ace1*_Del97) using previously published primers (Assogba et al., 2016). The CNVs names, preceded by "*Cyp6aap*_X" were rewritten as "Dup X" throughout the paper.

PCRs for tandem duplication CNVs can only detect presence/absence of the CNV (heterozygotes and homozygote mutants cannot be distinguished). In contract, the CNV Dup7 is a tandem inversion, making it possible to design a PCR assay that can distinguish heterozygotes from mutant homozygotes. The details about the mix solution and the PCR reactions are provided in Supplementary file 1: Methods.

As *Ace1* is the target site for PM, the *Ace1*_AgDup and *Ace1*_Del97

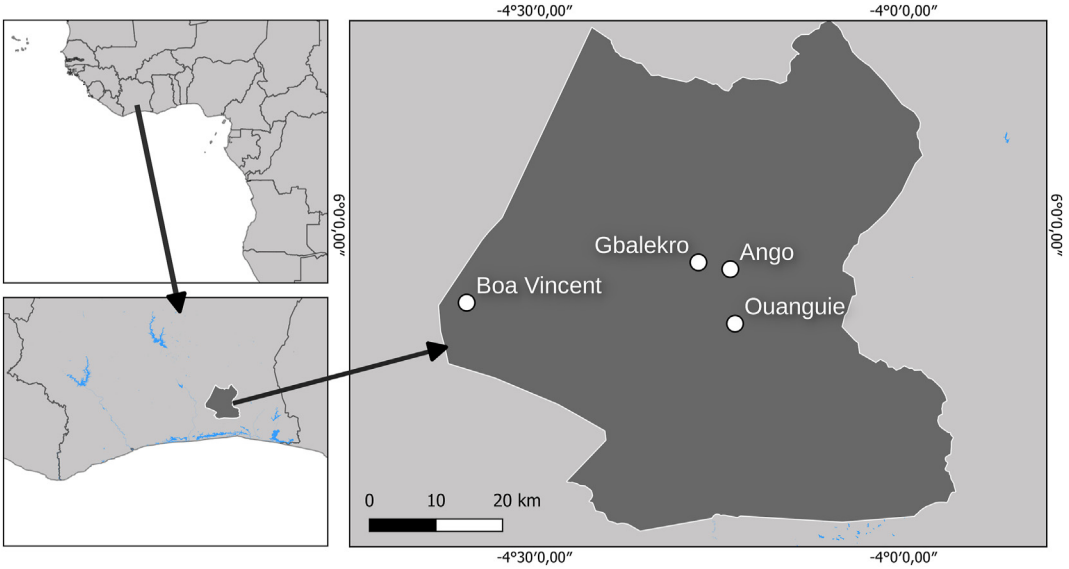

Fig. 2. Study sites in the department of Agboville.

CNVs were screened only in samples exposed to PM ($n = 182$). For the detection of the *Cyp6aap* CNVs, 46 samples exposed to deltamethrin were initially screened to identify CNV alleles that were common in the population. CNVs which were found in at least 9 (20%) samples were then screened in all samples exposed to both deltamethrin and PM. CNVs Dup10 and Dup11 were rare among the 46 initial samples screened and were therefore not analyzed further. CNVs Dup7 and Dup14 were found in more than 20% of the first 46 samples. They were therefore carried forward for further analysis ($n = 169$ exposed to deltamethrin +182 exposed to PM).

2.6. Copy number assignment with gDNA using qPCR

Assessment of copy number in *Cyp6aa2* and *Cyp9k1* was performed using qPCR on genomic DNA (gDNA). *Cyp6aa2* was used instead of *Cyp6aa1* because all currently known *Cyp6aa1* CNVs in Côte d'Ivoire include *Cyp6aa2*, and primers were more easily developed than for *Cyp6aa1*.

Copy number of *Cyp6aa2* and *Cyp9k1* in 36 individuals of *An. coluzzii* (profile to deltamethrin: 25 resistant and 11 susceptible) was calculated using Ribosomal Protein S7 (AGAP010592) and *Cyp4g16* (AGAP001076) as assumed single-copy standards for comparison. Primers are shown in Supplementary Table S1. The qPCR reaction is detailed in the Supplementary file 1: Methods.

2.7. Gene expression using RT-qPCR

For gene expression, RNA was extracted from 20 pools of 4 deltamethrin-resistant female *An. coluzzii* and 6 pools of 4 unexposed control female *An. coluzzii*, using the PicoPure RNA isolation kit (Arcturus, Applied Biosystems, USA). Then, first-strand complementary DNA (cDNA) was synthesized from extracted RNA using SuperScript III (Invitrogen, USA). The details of the reaction are shown in Supplementary file 1: Methods.

Gene expression of *Cyp6aa1*, *Cyp9k1* and *Cyp6p3* was calculated relative to two housekeeping genes: elongation factor (HKEF AGAP005128) and Ribosomal Protein S7 (RPS7 AGAP010592). Primers details are provided in Supplementary Table S1.

Reactions contained 10 µl of SYBR 2× mastermix, 1.2 µl of forward primer (5 µM), 1.2 µl of reverse primer (5 µM), 6.6 µl of nuclease-free water and 1 µl of genomic DNA. Cycling was conducted on an AriaMx system with the following cycling conditions: an initializing step at 95 °C for 3 min; followed by an amplification phase of 40 cycles of 95 °C for 10 s and 60 °C for 10 s; with a final melt curve at 95 °C for 1 min, at 55 °C for 30 s and at 95 °C for 30 s, respectively.

2.8. Data analysis

The mortality rate of the mosquitoes exposed to each insecticide was estimated as the proportion of exposed mosquitoes dead after 24 h. According to WHO recommendations, when the mortality percentage is in the range of 98–100%, this indicates susceptibility of the mosquitoes. A mortality rate of 90–97% suggests probable resistance of mosquitoes which have to be proved by other tests and a mortality rate under 90% indicates resistance to the insecticide tested (WHO, 2016).

The samples used for molecular analysis were a subset of the full complement of samples used for bioassays. Since the subsets were not strictly proportional to the number of dead/alive samples from the exposures, values of species-level allele and genotype frequencies needed to be corrected to account for the resulting bias, by calculating a weighted average of the frequencies within each phenotypic group (with weights calculated according to the proportion of the total sample represented by each phenotype). Both raw and corrected frequencies are presented in the results, which never differed by more than one percentage point.

Fisher's test was used to assess associations between phenotypes (dependent variable) and the presence of each mutation/CNV. Binomial test was used to assess associations between (i) the presence of both *Ace1* CNV and *Ace1* mutation; (ii) association between the Dup 14 and the Dup 7. Analysis of qPCR data (both copy number and gene expression) was performed on ΔCt values. Bartlett's homogeneity test of variance was performed to ensure equal variance between phenotypes. Then, general linear modelling was used to assess significant ΔCt differences between phenotypes (included as the independent variable). The copy number of the two genes *Cyp6aa2* and *Cyp9k1*, and the three genes *Cyp6aa1*, *Cyp9k1* and *Cyp6p3*, were included as continuous variables in the models for gDNA qPCR and RT-qPCR respectively. The *P*-value of each term was obtained by comparing models with and without the term using the *anova* function in R. Least significant terms were sequentially removed from the model until only significant terms remained.

All statistical analyses were conducted in the software package R version 4.1.3. (https://www.r-project.org).

3. Results

3.1. Species composition of the Anopheles gambiae (s.l.) complex

Morphologically identified mosquitoes belonging to the *An. gambiae* (*s.l.*) complex ($n = 362$) were chosen from the four sites to conduct species identification. Both *An. gambiae* (*s.s.*) and *An. coluzzii* were found in all the localities (Supplementary Table S2); *An. coluzzii* was the most abundant species in all the sites, representing 91% of all samples successfully identified to species. Twelve samples were not successfully identified and were excluded from the analysis. No *An. arabiensis* specimens were identified.

3.2. Insecticide resistance

A total of 391 females of *An. gambiae* (*s.l.*) mosquitoes, were exposed to 0.05% deltamethrin and 364 were exposed to 0.25% PM. No mortality was observed in control tubes in either test. Based on the WHO criteria, mosquitoes from all four sites were found to be resistant to deltamethrin (Table 1). For PM, three of the four collections were characterised as resistant with only the collection from the village of Boa Vincent scored as probably resistant. Mortality in mosquitoes exposed to deltamethrin was lower (from 3% to 6%) compared to PM (51–91%).

3.3. Deltamethrin target site mutations

A total of 168 females of *An. gambiae* (*s.l.*) exposed to deltamethrin were screened for the presence of mutations in *Vgsc*. *Vgsc*-995F was found in both *An. coluzzii* and *An. gambiae*, while *Vgsc*-402L was found only in *An. coluzzii*. The *Vgsc*-1570Y SNP was found in only one *An. coluzzii* specimen from Boa Vincent. Supplementary Table S3 shows the distribution of each mutation within species and villages. The *Vgsc*-995S SNP was not observed in any sample in this study.

Specimens homozygous for the *Vgsc*-995F allele were almost always wild-type for *Vgsc*-402L and *vice versa* except for two samples that are likely to be genotyping errors (Table 2). When the mutation *Vgsc*-995F was found under the heterozygous form, the *Vgsc*-402L mutation was also present in the heterozygous form. Concerning the *Vgsc*-402L mutation,

Table 1
Mortality rate (and number of specimens exposed (*n*, *N*)) of *An. gambiae* (*s.l.*) to insecticides.

Locality	Deltamethrin ($N = 391$)		Pirimiphos-methyl ($N = 364$)	
	Mortality rate (*n*)	Status	Mortality rate (*n*)	Status
Boa Vincent	6% (97)	R	91% (98)	PR
Ango	4% (100)	R	51% (96)	R
Gbalékro	3% (97)	R	70% (76)	R
Ouanguié	3% (97)	R	83% (94)	R

Abbreviations: R, resistant; PR, probable resistant, need more investigations.

Table 2
Genotypes at the mutually exclusive *Vgsc*-995F and *Vgsc*-402L loci in *An. gambiae* (*s.l.*), shown by resistance status to deltamethrin (alive/dead after exposure).

		995F/402L	995F	995F + 402L	402L
An. coluzzii	Alive	1	53	70	16
	Dead	1	7	4	0
An. gambiae (*s.s.*)	Alive	0	11	0	0
	Dead	0	5	0	0

Notes: 995F/402L: homozygote for both Vgsc-995F and Vgsc-402L (this genotype is likely to be the result of a genotyping error since haplotypes containing both the Vgsc-995F and Vgsc-402L mutations have never previously been found). 995F: homozygote for Vgsc-995F and wild-type at the Vgsc-402 locus. 995F + 402L: heterozygote, with one copy of Vgsc-995F and one copy of Vgsc-402L. 402L: homozygote for Vgsc-402L and wild-type at the Vgsc-995 locus.

two separate SNPs (G to C and G to T) are known to produce the mutant amino acid L at this locus. At the amino acid level, three genotypes were identified (LL, LV, VV). A single individual of *An. coluzzii* found to carry the T allele for *Vgsc*-402L was among these samples.

In *An. coluzzii*, the heterozygous genotype 995F + 402L and the resistant *Vgsc*-995F genotypes were both found at high frequencies of 49% and 40% respectively (Table 2), both before and after correction for frequency of dead and alive samples in the original exposures. The overall allele frequency of *Vgsc*-402L was 35% (36% after correction). No significant association was found with resistance to deltamethrin (Fisher's test, $P = 0.0865$, $n = 152$, Supplementary Table S3). In *An. gambiae* (*s.s.*), all specimens screened were found to carry the homozygous resistant genotype *Vgsc*-995F (Table 2).

3.4. Pirimiphos-methyl (PM) target site mutations

We found a high frequency (85%, 84% after frequency correction) of the heterozygous *Ace1*-280S genotype in *An. coluzzii*. In *An. gambiae* (*s.s.*), the susceptible genotype was predominant (79%, 80% after correction), with only a single sample (7% before and after correction) found with the homozygous resistant genotype (Table 3).

In *An. coluzzii*, the homozygous *Ace1*-280S genotype was found only in the localities of Ango and Boa Vincent. There was a significant association between resistance to PM and genotypes for *An. coluzzii* (Fisher's test, $P < 0.001$, $n = 168$, Table 3). In alive samples, only the heterozygous (94%) and the homozygous resistant (6%) genotypes were observed (Supplementary Table S3). In *An. gambiae* (*s.s.*), survival to PM was associated with *Ace1*-280S genotype (Fisher's test, $P = 0.03297$, $n = 14$, Table 3). Only two *An. gambiae* (*s.s.*) samples survived exposure to PM; both were found to carry the heterozygous and the homozygous resistant genotypes respectively (Table 3).

The frequency of the *Ace1*_AgDup CNV was at 82% (before and after correction) in *An. coluzzii* and 14% (13% after correction) in *An. gambiae* (*s.s.*). The presence of the *Ace1*_AgDup was significantly associated with the *Ace1*-280S mutation (Binomial test, $P < 0.0001$, $n = 182$, Supplementary Table S4). There was a significant association between the presence of this CNV and ability to survive to PM in *An. gambiae* (*s.s.*) (Fisher's test, $P = 0.01099$, $n = 14$, Supplementary Table S4) but not in *An. coluzzii* (Fisher's test, $P = 0.8273$, $n = 168$, Supplementary Table S4). Only one specimen belonging to *An. gambiae* (*s.s.*) was found to carry the *Ace1*_Del97 deletion.

3.5. Metabolic resistance: CNV alleles in Cyp6aa/Cyp6p region

The CNVs Dup7, Dup10 and Dup11 were found only in *An. coluzzii* (Supplementary Table S4). In *An. gambiae* (*s.s.*), a single specimen was found to carry the Dup 14. Dup10 and Dup11 were found in 3 and 6 of the original 46 samples screened and were not investigated further. Dup7 was found in 42% of *An. coluzzii* (before and after correction), while Dup14 was found in 26% (27% after correction).

No significant association was found between Dup7 genotypes and resistance to deltamethrin (Fisher's test, $P = 0.13$, $n = 133$, Supplementary Table S4) or PM (Fisher's test, $P = 0.62$, $n = 134$, Supplementary Table S4). These three genotypes were found in all localities except in Ouanguié where no CNV allele was identified.

A strong association was observed between the presence of both Dup7 and Dup14 CNVs in our sample (Binomial test, $P < 0.0001$). Most of the samples in which Dup 14 was identified (62%), were heterozygous for Dup7. Dup14 was never found in samples that were homozygous for Dup7. No significant association was found between other CNVs spanning the same region.

3.6. Copy number in Cyp6aa2 and Cyp9k1

Cyp6aa2 copy number was found to be significantly associated with resistance to deltamethrin in *An. coluzzii* (generalised linear model, $P = 0.01263$, $n = 36$) (Fig. 3), with copy number of *Cyp6aa2* being 1.4-fold higher in alive samples than in dead ones. However, no association was found between deltamethrin phenotype and *Cyp9k1* copy number ($P = 0.0989$, $n = 36$).

3.7. Gene expression of Cyp9k1, Cyp6aa1 and Cyp6p3

Among the three genes, only *Cyp6p3* was nearly significantly expressed between groups (generalised linear model, $P = 0.0549$, $n = 22$); with expression in alive samples higher than in control ones (Fig. 4). For the other genes, *Cyp9k1* ($P = 0.3342$, $n = 22$) and *Cyp6aa1* ($P = 0.2272$, $n = 22$), there was likewise a higher expression of these genes in alive samples than in control ones, although the difference was not significant.

4. Discussion

Malaria vector resistance to several chemical classes of insecticides represents a major threat to the effectiveness of vector control programmes. Among the genetic mechanisms known to confer resistance to mosquitoes, SNPs and more recently CNVs have been identified to play an important role in the evolution of insecticide resistance (Jones et al., 2012; Stica et al., 2019; Clarkson et al., 2021; Njoroge et al., 2022). In this study, we investigated the levels of resistance to deltamethrin and PM, and their association with genetic markers, in southern Côte d'Ivoire.

4.1. Target site resistance

We observed resistance to both insecticides, particularly to deltamethrin (97–100%), which could be due to the intensive use of pyrethroids in this region (Kouamé et al., 2022). The low levels of mortality to

Table 3
Ace1-280S genotypes frequencies sorted by locality and *An. gambiae* (*s.l.*) species exposed to pirimiphos-methyl.

Status	Locality				*An. coluzzii* ($n = 168$)		*An. gambiae* (*s.s.*) ($n = 14$)	
	Ango ($n = 43$)	Boa Vincent ($n = 44$)	Gbalékro ($n = 46$)	Ouanguié ($n = 49$)	Alive	Dead	Alive	Dead
SS	0	12	9	11	0	21	0	11
RS	38	31	37	38	46	96	1	1
RR	5	1	0	0	3	2	1	0

Abbreviations: *n*, number of samples; SS, homozygous susceptible genotype; RS, heterozygous genotype; RR, homozygous resistant genotype.

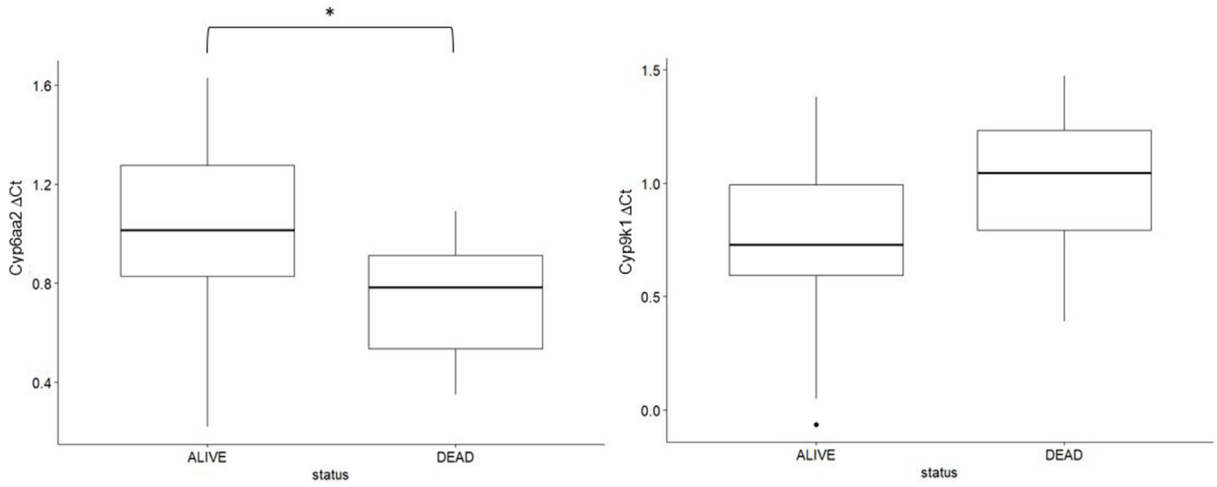

Fig. 3. Copy number of *Cyp6aa2* and *Cyp9k1* as a function of status after exposure to deltamethrin. $^*P < 0.05$.

deltamethrin meant that we had few susceptible samples for our phenotypic association analyses, and the resulting low statistical power should therefore be taken into consideration when interpreting our results.

Many studies have shown the important role of the *Vgsc*-995F SNP, previously known as *Vgsc*-1014F, in *An. gambiae* (*s.l.*) resistance to pyrethroids in West Africa (Baffour-Awuah et al., 2016; Djegbe et al., 2017; Fodjo et al., 2018; Chouaïbou et al., 2019). However, less attention has been paid to the more recently discovered valine to leucine mutation *Vgsc*-402L, another variant of the *Vgsc* located at segment 6 in the domain I (Clarkson et al., 2021). The strong negative linkage between *Vgsc*-995F and the *Vgsc*-402L SNP observed in our study confirms the complete negative linkage disequilibrium reported between these two alleles in studies so far (Clarkson et al., 2021; Williams et al., 2022). The distribution of these SNPs among *An. gambiae* (*s.l.*) aligns with the findings of Clarkson and colleagues that showed that *An. gambiae* (*s.s.*) predominantly carry *Vgsc*-995F, while *An. coluzzii* carry a mix of *Vgsc*-995F and *Vgsc*-402L with very few wild types (Clarkson et al., 2021). The difference in genotype frequencies between species in *An. gambiae* (*s.l.*) re-affirms the importance of molecular tools for disaggregating morphologically identical species for phenotypic and genetic analysis.

The *Vgsc*-402L allele in our study population was at a higher frequency (36% in *An. coluzzii*) than in previous studies on specimens from Côte d'Ivoire (8.5%; Clarkson et al., 2021). Samples screened by Clarkson et al. (2021) were collected in rice fields from Tiassalé in 2012 (The *Anopheles gambiae* 1000 Genomes Consortium, 2020), and the difference from the results of our study could therefore be due to differences between collection sites, or be the result of an increase in frequency of this mutation over time. Such an increase with time in a field population from West Africa has been found in Burkina Faso, where frequency of *Vgsc*-402L in *An. coluzzii* increased from 18% to 37% from 2016 to 2019 (Williams et al., 2022). Unlike some previous studies that have reported the presence of the mutation from leucine to serine (*Vgsc*-995S) in central Côte d'Ivoire, at low frequencies (Chouaïbou et al., 2017; Fodjo et al., 2018) this SNP was not detected in our work. Our results confirm that *Vgsc*-995F remains the main mutation found in *An. gambiae* (*s.s.*) in our study area.

The absence of genetic variation at the *Vgsc*-995 and *Vgsc*-402 loci in *An. gambiae* in our study population precluded the analysis of phenotypic association with *Vgsc* variants in this species. In *An. coluzzii*, while the wild-type allele was absent, we were able to compare the *Vgsc*-995F and *Vgsc*-402L mutations against each-other. Although we found no significant difference in resistance status, the *P*-value was close to significance (smaller than 0.1) despite the relatively low power of the test, and the direction of difference (higher resistance for *Vgsc*-402L compared to *Vgsc*-995F) was the same as observed in another recent study of *An. coluzzii* from Benin (Lucas et al., 2023). Combined with the high frequency of *Vgsc*-402L which we observed compared to previous data from Côte d'Ivoire (Clarkson et al., 2021), our study lends further weight to the emerging picture that *Vgsc*-402L is gradually replacing *Vgsc*-995F as a more highly resistant allele with lower fitness costs (Williams et al., 2022; Lucas et al., 2023). Currently, the *Vgsc*-402L and *Vgsc*-995F alleles appear to be in perfect negative linkage disequilibrium, but it remains unknown whether a combination of the two mutations on the same haplotype would provide yet higher levels of resistance. This question could be addressed through genetic modification, for example by CRISPR/Cas9 (Grigoraki et al., 2021).

For mosquitoes exposed to PM, resistance was strongly related to the presence of the *Ace1*-280S mutation, as reported elsewhere (Alou et al., 2010; Elanga-Ndille et al., 2019). *Ace1*-280S frequency was higher in *An. coluzzii* than in *An. gambiae* (*s.s.*), as found in samples from Madina in Ghana (Grau-Bové et al., 2021), but in contrast to samples from Aboisso (southern Côte d'Ivoire), where frequencies of 23.64% and 96.34% were found in *An. coluzzii* and in *An. gambiae* (*s.s.*), respectively (Grau-Bové et al., 2021). Unlike some previous studies in Côte d'Ivoire where the resistant alleles were found only in heterozygous form (Edi et al., 2012; Assogba et al., 2016; Keïta et al., 2020), our study revealed the presence of homozygous resistant genotypes in both *An. coluzzii* and *An. gambiae* (*s.s.*) in the localities of Boa Vincent and Ango. Homozygous resistant genotypes at *Ace1*-280S have previously been found in Ghana, Benin and Togo (Assogba et al., 2016; Lucas et al., 2019b; Grau-Bové et al., 2021). The continued presence of the homologous CNV allele (containing no wild-type copies of the *Ace1*-280S locus) despite its documented fitness costs (Djogbénou et al., 2010; Assogba et al., 2016) invites further investigation.

4.2. CNV and gene expression

Like the *Ace1*-280S SNP, the Ace1_AgDup CNV was found to be widespread among our samples, which is to be expected as the SNP is nearly always found in conjunction with the CNV (Assogba et al., 2016; Grau-Bové et al., 2021). Interestingly, we found no significant association between PM resistance and the presence of *Ace1*_AgDup in *An. coluzzii*, despite the high frequency of this CNV in resistant samples. Previous results have demonstrated a strong correlation between the CNV and resistance (Grau-Bové et al., 2021), but in particular showed that number of copies was more important than presence/absence of the CNV. Since the PCR that we used, developed in a previous study, does not include a control primer to confirm the success of the PCR reaction, it is prone to false negatives, where PCR failure is interpreted as absence of *Ace1*-AgDup. The difference in outcome between our association test for *Ace1*-280S and *Ace1*-AgDup was largely driven by 8 resistant samples

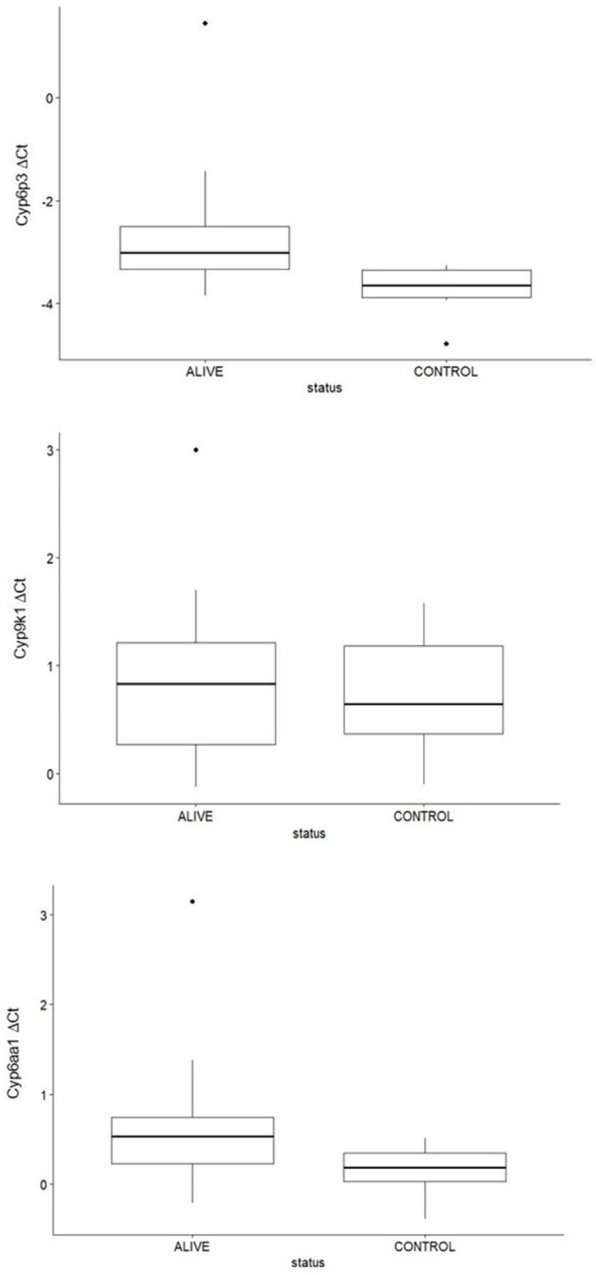

Fig. 4. Comparison of *Cyp6p3*, *Cyp9k1* and *Cyp6aa1* expressions between individuals surviving deltamethrin exposure (alive) and unexposed (control).

that were positive for *Ace1*-280S but negative for *Ace1*-AgDup, and which may have been incorrectly classified.

In a study conducted by Assogba et al. (2018) in Togo, Benin and Côte d'Ivoire, *An. gambiae* (*s.l.*) samples were collected from 2012 to 2017 and screened for the detection of *Ace1*_Del97, which is a secondary deletion of all co-amplified genes within the *Ace1*_AgDup, except *Ace1*. *Ace1*_Del97 was found to be widespread in these three countries (Assogba et al., 2018). However, in our study, only one specimen was found to carry this CNV. The reason for this low frequency of *Ace1*_Del97 could be the low frequency of *An. gambiae* (*s.s.*) mosquitoes in our sample, which would be consistent with the previous observations which found this deletion to occur more frequency in *An. gambiae* (*s.s.*) than *An. coluzzii* (Assogba et al., 2018).

In the *Cyp6aa/p* cluster, no association was found between any individual CNV allele and resistance to deltamethrin or to PM. However, copy number of *Cyp6aa2* (a gene that is spanned by the CNV alleles Dup7, Dup10 and Dup14 (Lucas et al., 2019a)) was higher in samples resistant to deltamethrin compared to susceptible samples. Although the gene whose copy number was investigated was *Cyp6aa2*, all known CNVs in the region that cover this gene also cover *Cyp6aa1*, and thus the relative importance of these two genes cannot be distinguished. In East Africa, a single resistance-conferring CNV allele in *Cyp6aa1* has spread to high frequencies in *An. gambiae* (*s.s.*) across a large geographical range, providing resistance to deltamethrin (Njoroge et al., 2022). In contrast, in West Africa, CNVs in the *Cyp6aa* region are rare in *An. gambiae* (*s.s.*), while several different CNV alleles together reach high frequencies in *An. coluzzii* (Lucas et al., 2019a, 2023). Our results show that while no single CNV allele can be identified as the cause of metabolic resistance, as is the case in East Africa, increased copy number brought about by the CNVs in general provides resistance to deltamethrin, adding to the mounting body of evidence that CNVs in the *Cyp6aa* cluster of genes provide deltamethrin resistance.

The tight link between Dup7 and Dup14 could be explained if the two CNVs sometimes co-exist on the same haplotype background, which is further supported by the high frequency of Dup7 heterozygotes in samples carrying Dup14. It therefore appears that Dup7 is often found within the Dup14 CNV.

RT-qPCR confirmed that expression of *Cyp6p3* plays an important role in survival to deltamethrin as described elsewhere (Edi et al., 2014b; Matowo et al., 2022). In contrast, we found no association between the expression of *Cyp6aa1* or *Cyp9k1* with resistance to deltamethrin, despite the significant association between copy number in the *Cyp6aa* region and resistance. The direction of difference was however in the expected direction (higher expression was found in the resistant compared to control samples), and gene expression studies of resistance carry the difficulty that resistant samples cannot be compared directly to susceptible samples, since the latter are killed as a result of the experiment. Resistant samples must therefore be compared to unexposed controls, which also include some resistant samples. We therefore cannot exclude the possibility that a larger sample set would have revealed a significant difference in *Cyp6aa1* expression between resistant samples and the controls.

5. Conclusions

This study shows high and moderate resistance levels of *An. gambiae* (*s.l.*) species from Côte d'Ivoire, to deltamethrin and PM, respectively. In *An. coluzzii* samples, resistance to PM was found to be associated with the 280S SNP in *Ace1*, while resistance to deltamethrin, in the context of ubiquitous target site resistance, was associated with copy number in the *Cyp6aa* gene cluster. Updating malaria vector resistance screening by considering new variants like the *Vgsc* 402L and CNVs in each species composing the *An. gambiae* (*s.l.*) complex is necessary to adopt better strategies for monitoring insecticide resistance.

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Ethical approval

Not applicable.

CRediT authorship contribution statement

Ruth M.A. Kouamé: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Visualization, Writing - original draft,

Writing - review & editing. Amy Lynd: Methodology, Formal analysis, Investigation, Resources, Validation. Jackson K.I. Kouamé: Methodology, Investigation. Laura Vavassori: Methodology, Writing – review & editing. Kouabénan Abo: Supervision, Writing – review & editing. Martin J. Donnelly: Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. Constant Edi: Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Supervision, Project administration. Eric Lucas: Conceptualization, Methodology, Investigation, Software, Formal analysis, Data curation, Writing – review & editing, Resources, Visualization, Validation, Project administration, Supervision.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data supporting the conclusions of this article are included within the article and its supplementary files. Raw data are available upon request to the corresponding author.

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Appendix A. Supplementary data

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