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Supplementary Information for

Functional genetic validation of key genes conferring insecticide resistance in the major African malaria vector, *Anopheles gambiae*

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Supplementary method

***Cyp6* expression analysis using the ΔΔCt method**

Threshold cycle (Ct) values were corrected for primer efficiency applying the formula *Corrected Ct = Ct (Logx / Log2)*, where x = 2 if efficiency is >100% and x = 1+Efficiency if efficiency is <100%.

Ct values obtained for target genes were normalised against those of the housekeeping genes RPS7 and Ubiquitin using the formula *ΔCt = Ct target – Ct housekeeping.*

Mean ΔCt values of GAL4/+ mosquitoes were then compared to those of GAL4/UAS mosquitoes using the formula *ΔΔCt = ΔCt GAL4/+ – ΔCt GAL4/UAS*. Fold change (FC, x) expression between the two populations was calculated with the formula *FC = (2^ GAL4/UAS ΔΔCt) / (2^ GAL4/+ ΔΔCt)*.

Statistical differences in fold change expression between GAL4/UAS and GAL4/+ were determined using t test (unpaired, two-tails, assuming equal variance) in GraphPad Prism version 8.2.1 (GraphPad Software, La Jolla California USA, www.graphpad.com).

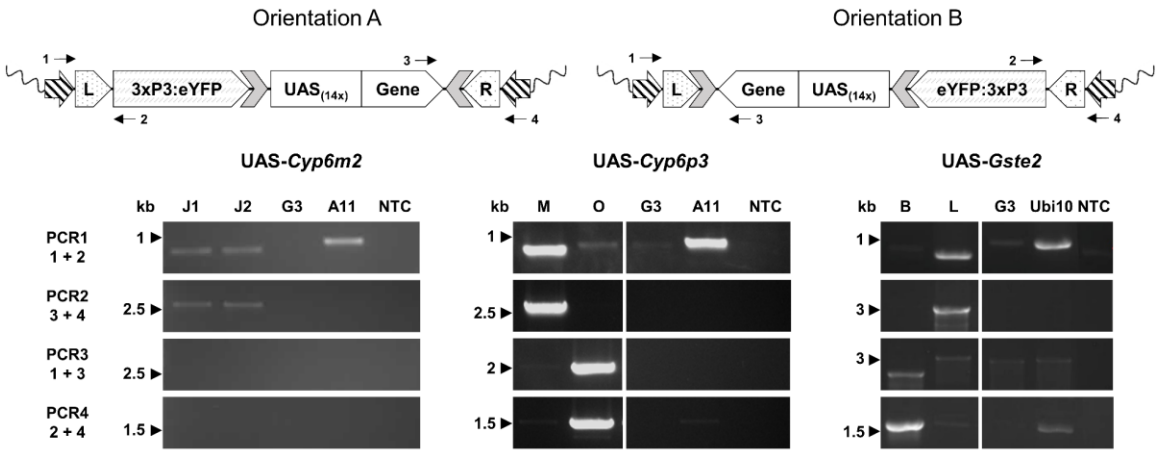

Figure S1. Orientation of insertion of the UAS transgenic cassette in representative individuals of responder lines. Two orientations of insertion, A and B, are possible after recombination of the *attP* sites in the docking mosquito genome and the attB sites in the UAS plasmids. Striped arrows: piggyBac transposon arms; 3xP3: eye and nerve-cord specific promoter; eYFP: enhanced yellow fluorescent protein; grey arrows: Gypsy insulators; $UAS_{(14x)}$: upstream activating sequence (14 repeats). Each PCR (1-4) gives a distinct amplification fragment that is diagnostic for the orientation of the insertion. →: primer annealing site. 1: PiggyBacR-R2, 2: Red-seq4R, 3: M2intFW or P3intFW or GSTe2_v1/v2, 4: ITRL1R. L, R: attL and attR hybrid sites created after recombination. J1, J2: individuals from the UAS-*Cyp6m2* line; M, O: representative individuals from the UAS-*Cyp6p3* line; B, L: representative individuals from the UAS-*Gste2* line; G3: wild type; A11: docking line control; Ubi10: docking line control; NTC: negative control (water as template).

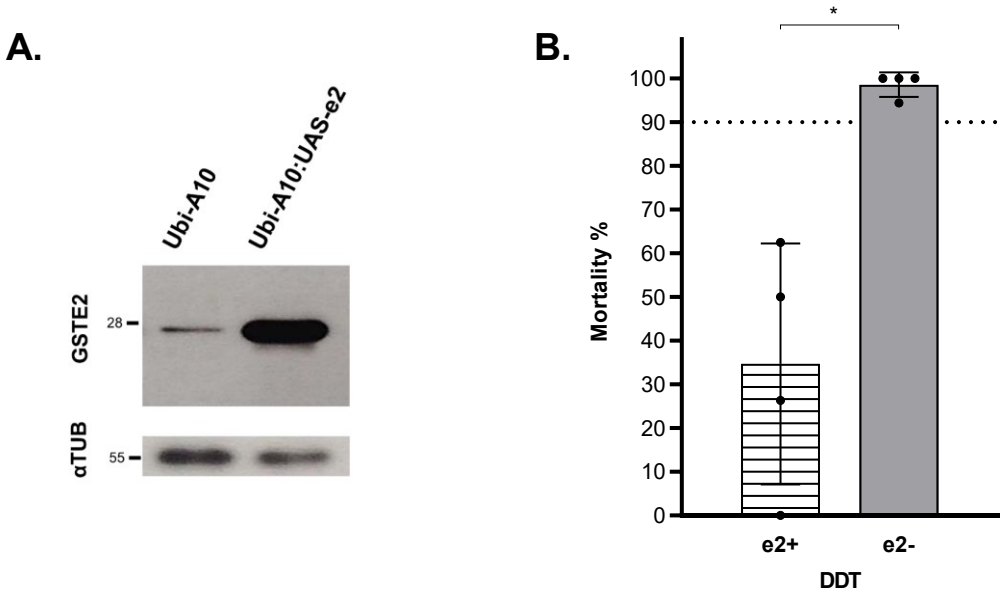

Figure S2. Multi-tissue overexpression of GSTE2 in the integration line affects sensitivity to the organochlorine insecticide DDT. A) Expression of GSTE2 and α-tubulin in adult females of the Ubi-A10:UAS-e2 line with respective Ubi-A10/+ controls. Protein extract from the equivalent of 1/10 of a whole female mosquito was loaded in each lane. **B)** Sensitivity to DDT of Ubi-A10:UAS-e2 females overexpressing *Gste2* (e2+) ubiquitously under the control of the Ubi-A10 driver compared to negative sibling controls (e2-) was measured by WHO tube bioassay. Bars represent SD (N = 4, Table S2). Dotted line marks the WHO 90% mortality threshold for defining resistance. Welch's t test with $P<0.05$ significance cut off.

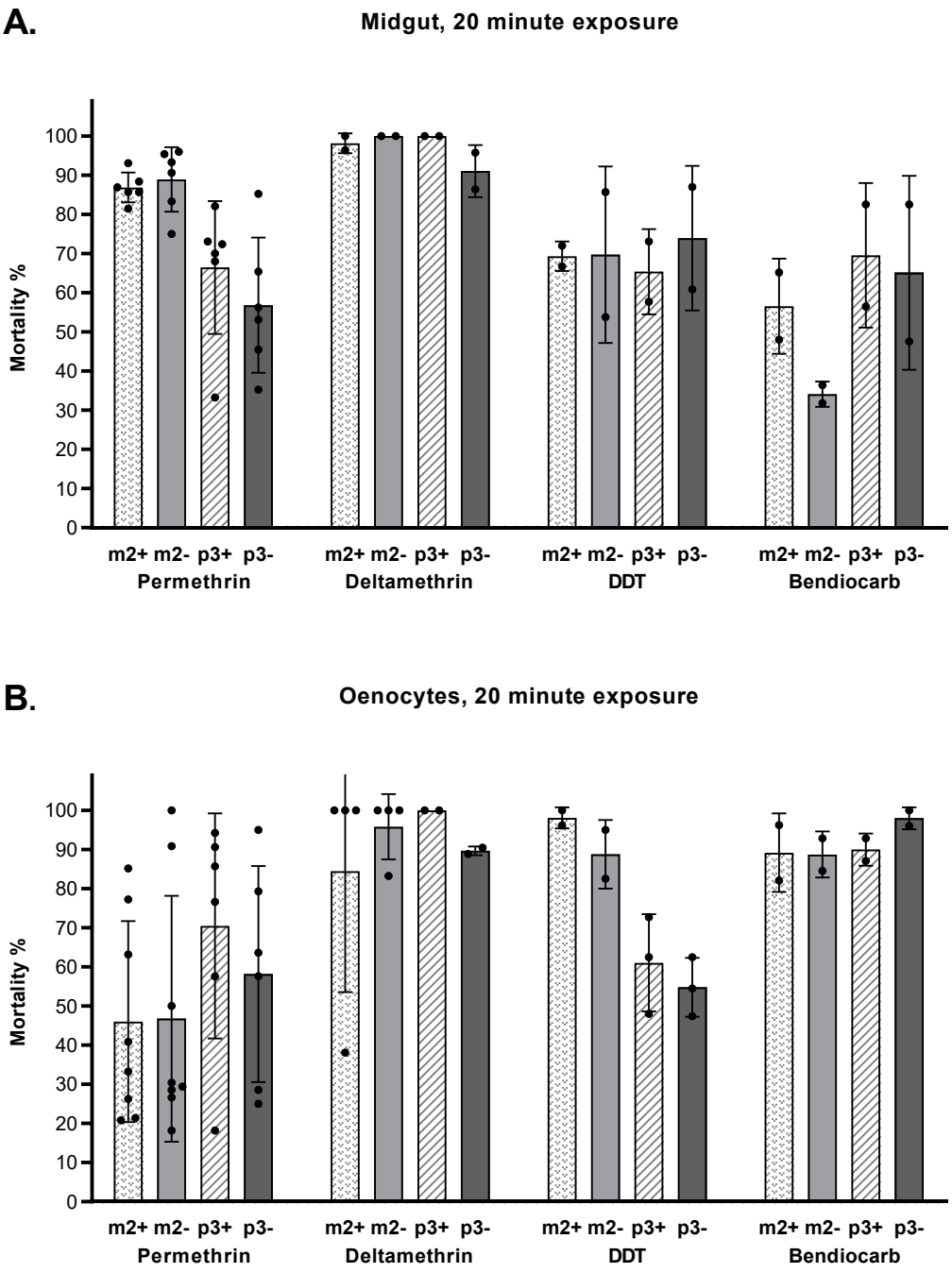

Figure S3. Tissue-specific *Cyp6* gene upregulation does not affect sensitivity to insecticides (reduced exposure). Sensitivity to insecticides of GAL4/UAS (+) females overexpressing *Cyp6m2* or *Cyp6p3* under the control of the midgut (A) or oenocyte specific (B) drivers compared to GAL4/+ controls (-) measured by a modified WHO tube bioassay representing mortality rates after 20 minutes of exposure and 24 h recovery. Bars represent SD (N = 2-8, Table S2). Welch's t test with $P<0.01$ significance cut off.

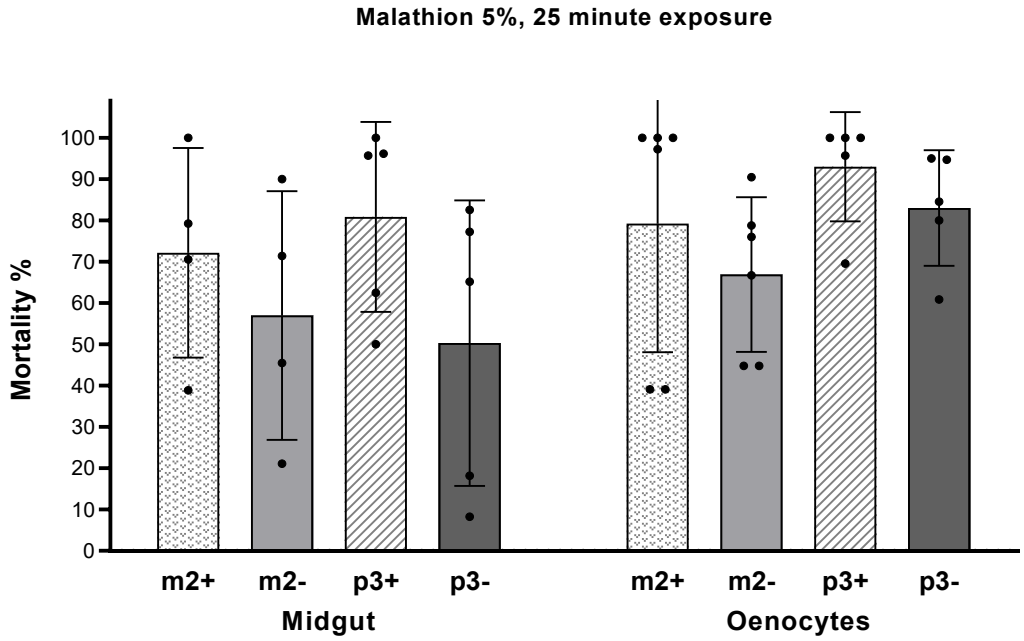

Figure S4. Tissue-specific *Cyp6* gene upregulation does not affect sensitivity to the organophosphate insecticide malathion (reduced exposure). Sensitivity to malathion (reduced exposure) of females overexpressing *Cyp6m2* (m2+) or *Cyp6p3* (p3+) tissue-specifically (midgut, oenocytes) compared to respective controls (m2-, p3-) measure by modified WHO tube bioassay representing mortality rates after 25 minutes of exposure and 24 h recovery. Bars represent SD (N = 4-6, Table S2). Welch's t test with $P<0.01$ significance cut off.

Table S1. Primers used in this study

Primer	Sequence 5'-3'
M2fw	TTCTGATATCAAAAATGTTTAGCTTGTTGGATTTCA
M2rv	TTCTCTCGAGCTAAATCTTATCCACCTTCAACCAC
P3fw1	TTCTGAATTCAACGATGGAGCTAATTAACGCGGTGCTGG
P3rv1	GGTACAGCTCCTGATGGATGTCGGC
P3fw2	GCCGACATCCATCAGGAGCTGTACC
P3rv2	TTCTCTCGAGCTACAACTTTTCCACCTTCAAG
Gste2k1bfor	GGGGGAATTCGAAAATGTCCAACCTTGTACTGTACAC
Gste2k1brev	GGGGCTCGAGTTAAGCCTTAGCATTCTCCTCCTT
PiggyBacR-R2	TTTGCCTTTCGCCTTATTTTAGA
Red-seq4R	CGAGGGTTCGAAATCGATAA
M2intFW	CGTATAGGGCTGGCGTATCT
P3intFW	GCTGAGAAAGTTCCGCTTCT
GSTe2_v1	TGTAAATTCGGCCCTGCACT
GSTe2_v2	GTGTAAATTCGGCCCTGCAC
ITRL1R	TGACGAGCTTGTTGGTGAGGATTCT
qM2fw	TACGATGACAACAAGGGCAAG
qM2rv	GCGATCGTGGAAGTACTGG
qP3fw	TGTGATTGACGAAACCCTTCGGAAG
qP3sub	ATAGTCCACAGATGGTACGCGGG
qS7fw	AGAACCAGCAGACCACCATC
qS7rv	GCTGCAAACTTCGGCTATTC
qUBfw	CGACTCCGTGGTGGTATCAT
qUBrv	GCACTTGCGGCAAATCATCT

Table S2. Bioassay experiments

	Cross	Insecticide	Standard exposure					Reduced exposure				
			Experiment (Biol. reps)	Tot. tech. reps	*P*	t	df	Experiment (Biol. reps)	Tot. tech. reps	*P*	t	df
UBIQUITOUS	Ubi-A10 x UAS-m2	Permethrin 0.75%	2	5	0.0007	7	5.3	NS	NS	NS	NS	NS
		Deltamethrin 0.05%	2	4	0.04	3.5	3	NS	NS	NS	NS	NS
		DDT 4%	1	4	N/A	N/A	N/A	NS	NS	NS	NS	NS
		Bendiocarb 0.1%	1	4	N/A	N/A	N/A	NS	NS	NS	NS	NS
		Malathion 5%	NS	NS	NS	NS	NS	2	4	<0.0001	14.5	4.3
	Ubi-A10 x UAS-p3	Permethrin 0.75%	3	5	<0.0001	13.3	5.6	NS	NS	NS	NS	NS
		Deltamethrin 0.05%	1	4	0.004	8.4	3	NS	NS	NS	NS	NS
		DDT 4%	2	6	0.7	0.3	9.1	NS	NS	NS	NS	NS
		Bendiocarb 0.1%	2	6	0.0001	10.3	5	NS	NS	NS	NS	NS
		Malathion 5%	NS	NS	NS	NS	NS	2	4	0.05	2.6	4.8
	Ubi-A10 x UAS-e2	Permethrin 0.75%	1	2	0.5	1	1	NS	NS	NS	NS	NS
		Deltamethrin 0.05%	1	2	N/A	N/A	N/A	NS	NS	NS	NS	NS
		DDT 4%	3	6	<0.0001	39.6	9.4	NS	NS	NS	NS	NS
		Bendiocarb 0.1%	1	2	0.5	1	1	NS	NS	NS	NS	NS
		Malathion 5%	3	6	0.1	2.	5	NS	NS	NS	NS	NS
		Fenitrothion 1%	2	4	<0.0001	35.2	5.9	NS	NS	NS	NS	NS
	Ubi-A10:UAS-e2	DDT 4%	2	4	0.0184	4.6	3	NS	NS	NS	NS	NS
MIDGUT	GAL4-mid x UAS-m2	Permethrin 0.75%	1	2	0.35	1.3	1.4	3	6	0.6	0.6	7.1
		Deltamethrin 0.05%	1	2	N/A	N/A	N/A	1	2	0.5	1	1
		DDT 4%	2	3	0.18	2	2	1	2	0.98	0.02	1.1
		Bendiocarb 0.1%	1	2	N/A	N/A	N/A	1	2	0.2	2.5	1.1
		Malathion 5%	NS	NS	NS	NS	NS	2	4	0.5	0.8	5.8
	GAL4-mid x UAS-p3	Permethrin 0.75%	1	2	>0.99	0	2	3	6	0.35	0.98	10
		Deltamethrin 0.05%	1	2	N/A	N/A	N/A	1	2	0.3	1.9	1
		DDT 4%	1	2	0.9	0.1	1.9	1	2	0.6	0.6	1.6
		Bendiocarb 0.1%	1	2	N/A	N/A	N/A	1	2	0.9	0.2	1.8
		Malathion 5%	NS	NS	NS	NS	NS	3	5	0.1	1.6	7

OENOCYTES	GAL4-oeno x UAS-m2	Permethrin 0.75%	1	2	N/A	N/A	N/A	4	8	0.96	0.05	13.5
		Deltamethrin 0.05%	1	2	N/A	N/A	N/A	2	4	0.5	0.7	3.4
		DDT 4%	1	2	N/A	N/A	N/A	1	2	0.4	1.4	1.2
		Bendiocarb 0.1%	1	2	N/A	N/A	N/A	1	2	0.96	0.05	1.6
		Malathion 5%	NS	NS	NS	NS	NS	3	6	0.4	0.8	8.2
	GAL4-oeno x UAS-p3	Permethrin 0.75%	1	2	0.013	49	1	3	6	0.5	0.8	10
		Deltamethrin 0.05%	1	2	N/A	N/A	N/A	1	2	0.05	12.9	1
		DDT 4%	1	2	N/A	N/A	N/A	2	3	0.5	0.7	3.3
		Bendiocarb 0.1%	1	2	N/A	N/A	N/A	1	2	0.2	2.3	1.8
		Malathion 5%	NS	NS	NS	NS	NS	3	5	0.3	1.2	8

N/A: mortality is 100% in all replicates; NS: not screened.

Each experiment included mosquitoes deriving from independent crosses or from subsequent gonotrophic cycles of the same cross, therefore they were considered as biological replicates. 1-4 experiments were performed for each insecticide tested. Generally, two technical replicate tubes containing 20-25 females were tested for each population in each experiment. Standard exposure was 60 minutes for all insecticides except fenitrothion for which recommended exposure is 2 hours. Reduced exposure was 20 minutes for permethrin, deltamethrin, DDT and bendiocarb, and 25 minutes for malathion. Recovery time is 24 hours for all experiments.