

1 **Title**

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

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27 **Keywords**

28 Insecticide resistance, Cytochromes P450, Glutathione-S-transferase,  
29 GAL4/UAS, *Anopheles*, Functional analysis

30 **ABSTRACT**

31 Resistance in *Anopheles gambiae* to members of all four major classes (pyrethroids, carbamates,  
32 organochlorines and organophosphates) of public health insecticides limits effective control of  
33 malaria transmission in Africa. Increased expression of detoxifying enzymes has been associated  
34 with resistance, but direct functional validation in *An. gambiae* has been lacking. Here we perform  
35 transgenic analysis using the GAL4/UAS system to examine insecticide resistance phenotypes  
36 conferred by increased expression of the three genes - *Cyp6m2*, *Cyp6p3* and *Gste2* - most often  
37 found upregulated in resistant *An. gambiae*. We report the first evidence in *An. gambiae* that  
38 organophosphate and organochlorine resistance is conferred by overexpression of GSTE2 in a  
39 broad tissue profile. Pyrethroid and carbamate resistance is bestowed by similar *Cyp6p3*  
40 overexpression, and *Cyp6m2* confers only pyrethroid resistance when overexpressed in the same  
41 tissues. Conversely, such *Cyp6m2* overexpression increases susceptibility to the organophosphate  
42 malathion, presumably due to conversion to a more toxic metabolite. No resistant phenotypes are  
43 conferred when either *Cyp6* gene overexpression is restricted to the midgut or oenocytes,  
44 answering long standing questions related to the importance of these tissues in resistance to  
45 contact insecticides. Validation of genes conferring resistance provides markers to guide control  
46 strategies, and the observed negative cross-resistance due to *Cyp6m2* gives credence to proposed  
47 dual insecticide strategies to overcome pyrethroid resistance. These transgenic *An. gambiae*  
48 resistant lines are being used to test potential liabilities in new active compounds early in  
49 development.

## 50 **SIGNIFICANCE STATEMENT**

51 Insecticide resistance in *Anopheles gambiae* mosquitoes can derail malaria control programs, and  
52 to overcome it we need to discover the underlying molecular basis. Here, for the first time, we  
53 characterise three genes most often associated with insecticide resistance directly by their  
54 overproduction in genetically modified *An. gambiae*. We show that overexpression of each gene  
55 confers resistance to representatives of at least one insecticide class and, taken together, the three  
56 genes provide cross-resistance to all four major insecticide classes currently used in public health.  
57 These data validate the candidate genes as markers to monitor the spread of resistance in  
58 mosquito populations. The modified mosquitoes produced are also valuable tools to pre-screen  
59 new insecticides for potential liabilities to existing resistance mechanisms.

## 60 INTRODUCTION

61 From the year 2000 until recently, the number of worldwide malaria cases had steadily fallen  
62 mainly due to the widespread rollout of insecticide treated bed nets in endemic areas (1, 2), which  
63 offer protection against bites from *Plasmodium* infected *Anopheles* mosquitoes. There is growing  
64 evidence suggesting that the stalling in malaria control can be at least partially attributed to the  
65 increasing levels of insecticide resistance in *Anopheles* vectors (3). Resistance in dominant  
66 African *Anopheles* vectors has been recorded to all major insecticide classes currently used in  
67 public health (pyrethroids, organochlorines, carbamates and organophosphates) (4). Therefore,  
68 understanding the mechanisms by which mosquitoes evolve resistance is critical for the design of  
69 mitigation strategies and in the evaluation of new classes of insecticides.

70 Research into the molecular mechanisms that give rise to resistance in mosquitoes have  
71 identified target site modifications and increased metabolic detoxification as the two main  
72 evolutionary adaptations (5), that often co-exist in *An. gambiae*. Families of detoxification enzymes,  
73 including cytochromes P450 (CYP) and glutathione-S-transferases (GST), can provide phase I  
74 metabolism of insecticides and phase II conjugation reactions that alter the toxicity of compounds  
75 and increase polarity, enhancing excretion (6, 7).

76 To identify and characterise the role of the causative resistance genes from these detox families,  
77 a sequential process of transcriptomic, proteomic and *in vivo* functional analysis is often applied  
78 (8). Candidate genes with upregulated transcription or strong signatures of selection in resistant  
79 mosquitoes are typically expressed in bacteria to provide evidence of insecticide depletion and/or  
80 metabolism *in vitro* (9–19). Further studies have used the *Drosophila* transgenic model to  
81 determine whether expression of single *Anopheles* genes confers increased tolerance to  
82 insecticides (13–18, 20).

83 This workflow has implicated a role in resistance of two cytochrome P450 genes, *Cyp6m2* and  
84 *Cyp6p3*, and a Glutathione S Transferase gene, *Gste2*, that are consistently upregulated in  
85 resistant field populations found across Africa (21). However, there are often discrepancies in  
86 results from recombinant protein activity and transgenic *Drosophila* analyses. For example, while  
87 expression studies of *Cyp6m2* and *Cyp6p3* in *E. coli* (10, 11) and *Drosophila* (15) suggest that  
88 both gene products can detoxify pyrethroids, the two systems produce conflicting results in  
89 respect to carbamate (15) and organochlorine insecticide detoxification (12, 15, 19). Moreover,  
90 the involvement of *An. gambiae* and *An. funestus* *Gste2* orthologues in resistance to pyrethroid  
91 insecticides has produced contradictory results when explored in *Drosophila* (16, 20).

92 Clearly, functional validation of *Anopheles* genes directly in the mosquito would provide the  
93 benchmark approach to address these questions, however to date transgenic tools to perform  
94 such analysis have been limited. To this end, we have developed the GAL4/UAS expression

95 system in *An. gambiae* (22–24) which allows genes to be overexpressed in a susceptible  
96 mosquito background and for resultant resistance phenotypes to be examined using the standard  
97 insecticide assays that have been developed for comparative analysis in mosquitoes by WHO  
98 (25).

99 *In vivo* functional analysis in *Anopheles* can also help discover the mosquito tissues that are  
100 specifically involved in insecticide metabolism. Our previous research indicated high P450 activity  
101 in the midgut and oenocytes, since the essential P450 co-enzyme CPR is highly expressed in  
102 these tissues, and RNAi knockdown of *Cpr* increased mosquito sensitivity to a pyrethroid  
103 insecticide (26). Moreover, *Cyp6m2* has been reported as enriched in the *An. gambiae* midgut  
104 (11) and *Cyp6p3* was found upregulated in midguts from pyrethroid resistant populations (27).

105 Here we have used the GAL4/UAS system to overexpress *Cyp6m2* or *Cyp6p3* genes in multiple  
106 tissues or specifically in the midgut or oenocytes of a susceptible *An. gambiae* strain and assayed  
107 the modified mosquitoes against representatives of each insecticide class available for public  
108 health use. In doing so, we determined the resistance profile generated for each gene and  
109 compared these results to those obtained in *Drosophila* and *in vitro*. We then analysed the other  
110 major candidate, *Gste2*, to examine its role in conferring DDT resistance and also extending its  
111 testing to other classes of insecticides in which its role has yet to be tested *in vivo*.

112 In this work, we report the first use of the GAL4/UAS system in *Anopheles* as a benchmark to  
113 determine whether single candidate genes and/or expression in individual tissues are able to  
114 confer WHO-defined levels of resistance to the four public health classes of insecticides,  
115 including for the first time organophosphates. Crucially we find that, when assayed in *An.*  
116 *gambiae*, overexpression of *Cyp6m2*, *Cyp6p3* or *Gste2* produce cross-resistance phenotypes  
117 that encompass members of all four classes of insecticides currently used for malaria control.

## 118 RESULTS

### 119 *Mosquito lines generated for UAS-regulated expression of Cyp6m2 and Cyp6p3*

120 YFP marked UAS-*Cyp6m2* and -*Cyp6p3* lines were created by site directed recombination  
121 mediated cassette exchange (RMCE) into a docking (CFP:2x*attP*) line A11 (24) to produce  
122 mosquitoes carrying transgene insertions in the same genomic site. By mitigating for genomic  
123 position effects, this allows more reliable comparison of the effects of *Cyp6m2* and *Cyp6p3*  
124 overexpression on resistance.

125 A summary of the screening and crossing strategy used to create the UAS responder lines is  
126 illustrated in Table 1. RMCE results in canonical cassette exchange in two potential orientations,  
127 however integration of the whole donor transgene can also occur into either *attP* site. Fluorescent  
128 marker screening of F<sub>1</sub> progenies from F<sub>0</sub> pooled mosquitoes revealed that cassette exchange  
129 and integration events occurred in all experiments as shown by the recovery of individuals  
130 carrying single (YFP: exchange) or double (CFP/YFP: integration) markers (Table 1).

131 Molecular analysis revealed one exchange orientation (A) in transgenic UAS-m2 individuals and  
132 both orientations for UAS-p3 transformation as indicated by diagnostic PCR (Fig. S1). Overall, we  
133 found at least two events for UAS-m2 transformation, having equal efficiencies of 2% for  
134 cassette-exchange and integration (1/49 F<sub>0</sub> founders); while for the UAS-p3 transformation, at  
135 least nine transformation events (six cassette exchanges, three in each orientation (A and B), and  
136 three transgene integrations) were detected, with a minimum cassette-exchange efficiency of 5%  
137 (6/124 F<sub>0</sub>) and integration efficiency of 2% (3/124 F<sub>0</sub>). For comparative functional analysis,  
138 representative *Cyp6* lines in orientation A were maintained and crossed with alternative GAL4  
139 driver lines.

### 140 *CYP6M2 or CYP6P3 overexpression in multiple tissues causes distinct profiles of* 141 *resistance to pyrethroids and bendiocarb*

142 We previously described the production of a GAL4 driver line, Ubi-A10, directing widespread  
143 tissue expression (23). To quantify the overexpression achieved with this driver, we performed  
144 RT-qPCR in the progeny of Ubi-A10 driver and UAS-*Cyp6* crosses. This revealed significant  
145 2447x ( $P=0.005$ ) and 513x ( $P<0.001$ ) increases of *Cyp6m2* and *Cyp6p3* transcript abundance in  
146 adult females compared to native expression in respective controls (Fig. 1A). Western analysis  
147 also readily detected CYP6M2 in the adult female progeny of the Ubi-A10/UAS-m2 crosses, but

148 was beyond the level of detection in sibling controls (Ubi-A10/+ and +/UAS-m2) (Fig. 1B). No  
149 suitable antiserum was available for analysis of CYP6P3.

150 WHO discriminating dose assays were then performed to assess the susceptibility of mosquitoes  
151 overexpressing *Cyp6m2* or *Cyp6p3* compared to their Ubi-A10/+ siblings. WHO tube bioassays  
152 are used to screen for the emergence of resistance in field populations and involve exposing  
153 mosquitoes to fixed concentration of insecticides (twice the LC<sub>99</sub> for a susceptible strain) for 60  
154 minutes, followed by a twenty-four-hour recovery period before recording mortality (25). The  
155 parental strains used here are susceptible (>90% mortality) to all the insecticides tested,  
156 therefore a decrease in mortality in test assays can be directly attributable to the overexpression  
157 of the specific candidate gene.

158 Mosquitoes overexpressing either *Cyp6* gene under the Ubi-A10 driver showed resistance to  
159 permethrin (*Cyp6m2* 28% mortality,  $P<0.001$ ; *Cyp6p3* 43% mortality,  $P<0.001$ ) and deltamethrin  
160 (*Cyp6m2* 88%,  $P=0.04$ ; *Cyp6p3* 52%,  $P=0.004$ ) compared to controls (Fig. 1C). A significant  
161 difference in mortality was observed between mosquitoes overexpressing the two different *Cyp6*  
162 genes for deltamethrin assays ( $P=0.003$ ), while no significant difference was observed for  
163 permethrin ( $P=0.15$ ). However, only *Cyp6p3* overexpressing mosquitoes showed resistance to  
164 bendiocarb (13% mortality  $P<0.001$ ) (Fig. 1C). No resistance to DDT was observed with either  
165 gene in conjunction with the Ubi-A10 driver (Fig. 1C).

### 166 *CYP6M2 or CYP6P3 multi-tissue overexpression increases susceptibility to* 167 *malathion*

168 Malathion is an organophosphate pro-insecticide that is activated to a more toxic compound *in*  
169 *vivo* through P450-based oxidative reactions (28). Preliminary analysis at a standard WHO  
170 diagnostic dose and 60-minute exposure killed all test and control mosquitoes, however during  
171 exposure it was clear that Ubi-A10-directed *Cyp6* overexpression induced more rapid knock-  
172 down compared to controls suggesting malathion activation by these P450s. We therefore  
173 examined the relative sensitivity of mosquitoes overexpressing *Cyp6m2* or *Cyp6p3* when  
174 exposed to the same diagnostic dose of this organophosphate for a shorter time (25 minutes)  
175 (Fig. 2). Under these conditions, mosquitoes overexpressing *Cyp6m2* under the control of the  
176 Ubi-A10 driver showed significantly higher mortality rates compared to controls (95% vs 15%,  
177  $P<0.001$ ) and Ubi-A10/UAS-p3 mosquitoes (95% vs 34%  $P=0.002$ ). Although, the latter also  
178 showed a trend of increased mortality compared to Ubi-A10/+ controls (34% vs 8%  $P=0.05$ ).

179 *Overexpression of GSTE2 in multiple tissues causes resistance to diagnostic*  
180 *doses of DDT and Fenitrothion*

181 To extend the analysis to the role of GSTE2 in insecticide resistance in *An. gambiae*, we utilised  
182 the previously described Ubi-A10 GAL4 line (23) as a docking line for the first time. Integration of  
183 the UAS cassette into a single docking site in this case would provide Ubi-A10GAL4 and UAS-  
184 *Gste2* at the same locus (Ubi-A10GAL4:UAS-e2) and should natively overexpress *Gste2* without  
185 the need for crossing separate lines. Alternatively, cassette exchange would generate a regular  
186 UAS-*Gste2* responder line. After embryonic injections and screening, three exchange events, two  
187 in orientation A and one in orientation B (Fig. S1), and three integration events were  
188 independently recovered with an overall transformation efficiency of 9% (6/65 F<sub>0</sub>), exchange  
189 efficiency of 5% (3/65 F<sub>0</sub>), and integration efficiency of 5% (3/65 F<sub>0</sub>) (Table 1).

190 To obtain comparable data for *Gste2* and the *Cyp6* genes, we focused our analysis on the  
191 progeny from crosses between UAS-e2 and Ubi-A10GAL4 mosquitoes. When exposed to  
192 diagnostic doses of DDT, GSTE2 overexpressing mosquitoes showed a significantly lower  
193 mortality (7%,  $P < 0.001$ ) compared to controls, while no significant difference in resistance was  
194 found when exposed to diagnostic doses of permethrin, deltamethrin, malathion or bendiocarb  
195 (Fig. 3). A trend of increased tolerance was observed in mosquitoes overexpressing *Gste2*  
196 against malathion (Fig. 3), and further analysis with the related organophosphate fenitrothion  
197 indicated high resistance in Ubi-A10/UAS-e2 mosquitoes, showing 8% ( $P < 0.001$ ) mortality (Fig.  
198 3).

199 Preliminary analysis of Ubi-A10GAL4:UAS-e2 (integration) mosquitoes indicated the expected  
200 increase in GSTE2 protein in whole body extracts compared with Ubi-A10 controls (Fig. S2A) and  
201 a resistance phenotype against DDT in the F<sub>1</sub> generation of transformed male and female  
202 mosquitoes (Fig. S2B).

203 *Oenocyte or midgut specific overexpression of CYP6M2 or CYP6P3 does not*  
204 *confer resistance to insecticides*

205 To examine the role of oenocytes and midgut tissues in P450-based metabolism of insecticides  
206 we utilised previously published GAL4 driver lines to regulate tissue specific expression. The  
207 specificity of these GAL4 drivers has been established following crosses with UAS regulated  
208 fluorescent gene reporter lines (22, 24), but to examine the relative increase in tissue-specific  
209 *Cyp6* gene expression, we performed RT-qPCR and western blot analysis in progeny from  
210 alternative driver and *Cyp6* responder crosses.



211 Using the midgut driver (GAL4-mid), *Cyp6m2* and *Cyp6p3* transcripts were 2730x ( $P=0.002$ ) and  
212 659x ( $P=0.011$ ) more abundant in midguts dissected from GAL4/UAS mosquitoes compared to  
213 controls (Fig. 4A). A low level of overexpression was detected in the remaining carcass of  
214 GAL4/UAS mosquitoes compared to that of controls (*Cyp6m2*: 77x,  $P=0.038$ ; *Cyp6p3*: 7x,  
215  $P=0.08$ ). In GAL4-*oeno* crosses, *Cyp6m2* and *Cyp6p3* were specifically upregulated in transgenic  
216 dissected abdomens (66x,  $P=0.013$  for *Cyp6m2*; 153x,  $P<0.001$  for *Cyp6p3*) where oenocytes  
217 are located (Fig. 4B). Background overexpression was also found in the remaining carcass of  
218 GAL4/UAS-m2 and -p3 adults compared to controls (26x,  $P<0.001$ ; 2x,  $P<0.001$  respectively). In  
219 western blot analysis, CYP6M2 antiserum again only detected the target protein in GAL4/UAS  
220 mosquitoes. CYP6M2 was found exclusively in dissected midguts (and whole mosquitoes) from  
221 the progeny of GAL4-mid crosses, but was not observed in GAL4/UAS carcasses or extracts from  
222 controls (Fig. 4C). Similarly, in GAL4-*oeno* crosses CYP6M2 signal was only detected in whole  
223 adult female extracts and in dissected abdomen integument, but not in the remaining carcass or  
224 control extracts (Fig. 4D).

225 Adult females overexpressing *Cyp6m2* in the midgut (Fig. 4E) or in the oenocytes (Fig. 4F)  
226 showed complete susceptibility to permethrin, deltamethrin, DDT, and bendiocarb. Similar results  
227 were obtained with *Cyp6p3* (Fig. 4E and F), however potential resistance (95% mortality,  
228  $P=0.013$ ) was suggested in oenocyte specific *Cyp6p3* overexpressing mosquitoes when exposed  
229 to permethrin (Fig. 4F). Further analysis was performed to detect subtle differences in  
230 susceptibility by repeating the assays with reduced exposure time (Fig. S3). However, no  
231 significant decrease ( $P<0.01$ ) was found in the mortality rates of mosquitoes overexpressing  
232 *Cyp6m2* or *Cyp6p3* in the midgut or oenocytes compared to their respective controls when  
233 exposed for 20 minutes to the same diagnostic doses of the four insecticides (Fig. S3).

234 Finally, the 25-minute reduced exposure bioassay for malathion showed no significant difference  
235 in the mortality of mosquitoes overexpressing *Cyp6m2* or *Cyp6p3* in midgut or oenocytes  
236 compared to controls (Fig. S4).

## 237 DISCUSSION

238 *In vivo* functional analysis is critical to provide evidence of causative links between candidate  
239 genes and their proposed phenotypes. Here we demonstrate the utility of new GAL4/UAS-based  
240 tools to characterise gene function directly in *An. gambiae* by reporting the first use of the system  
241 to validate the ability of single candidate genes to confer WHO-defined resistance to different  
242 classes of insecticides. Overall, the transgenic analysis in *An. gambiae* is more in accordance  
243 with data generated from recombinant protein studies of insecticide metabolism rather than those  
244 obtained from *Drosophila* survival assays (Table 2).

245 In *Anopheles*, multi-tissue overexpression of *Cyp6m2* and *Cyp6p3* demonstrated that resistance  
246 to permethrin and deltamethrin (type I and II pyrethroids respectively) can be conferred by the  
247 sole overexpression of either *Cyp6* gene. *Cyp6p3* expression also conferred resistance to  
248 bendiocarb (carbamate); while the overexpression of either *Cyp6* gene did not alter DDT  
249 (organochlorine) sensitivity. These phenotypes correlate with the profile of metabolism or  
250 substrate depletion of the respective insecticides for the two recombinant P450 enzymes (Table  
251 2). More variable results have been observed using *Drosophila* as an *in vivo* model, with  
252 overexpression of *Cyp6m2* surprisingly generating increased tolerance to bendiocarb compared  
253 with *Cyp6p3*, despite *in vitro* analysis not detecting activity against bendiocarb for *Cyp6m2* (15,  
254 19). DDT tolerance was also observed in *Cyp6m2* overexpressing fruitflies, but data for *Cyp6p3*  
255 could not be generated (15) (Table 2). In this study, DDT resistance was monitored by dose  
256 response assays over a 24 hr exposure time, whilst bendiocarb resistance was not observed  
257 when measured through such dose response assays but was reported following 24 hr exposure  
258 to a diagnostic dose. In the latter case, the controls used to compare *Cyp6m2* and *Cyp6p3*  
259 overexpression showed very different levels of sensitivity to bendiocarb, which appeared to  
260 contribute to the differences in resistance levels observed; whilst there was no data for the  
261 respective *Cyp6p3* controls in the DDT analysis for comparison. It may thus be a difference in  
262 genetic background that gives rise to the discrepant results observed in *Drosophila*. However, it  
263 should also be noted that the different methods of insecticide bioassay may not yield directly  
264 comparable results to the diagnostic WHO level of resistance in mosquitoes used in this study  
265 and extensively used to assess the emergence of resistance in endemic countries. Our data in  
266 mosquitoes unequivocally indicate though that the expression of single *Cyp6* genes can confer  
267 resistance to different pyrethroids, and that *Cyp6p3* overexpression confers cross resistance to  
268 prominent representatives of at least two classes of public health insecticides.

269 In contrast to our *Cyp6* studies, increased *An. gambiae* *Gste2* (*AgGste2*) expression generates  
270 clear DDT resistance, while resistance to bendiocarb and pyrethroids was not observed. These  
271 phenotypes again validate predictions from the DDT activity observed *in vitro* for recombinant

272 AgGSTE2 (9, 13) as well as the increased DDT tolerance (13) and lack of pyrethroid tolerance  
273 (20) observed when overexpressed in *Drosophila*. The corresponding *in vitro* data for AgGSTE2  
274 activity against bendiocarb and pyrethroids have not been reported, and this is the first time that  
275 bendiocarb resistance has been examined *in vivo* following *Gste2* overexpression.

276 Although DDT tolerance was also observed in *Drosophila* overexpressing the orthologous *An.*  
277 *funestus Gste2 (AfGste2)* (16, 18), conflicting results were reported about activity towards  
278 pyrethroids. For example, recombinant AfGSTE2 depleted permethrin but not deltamethrin *in*  
279 *vitro*, yet *Drosophila* acquired increased tolerance to both insecticides when *AfGste2* was  
280 overexpressed (16, 18). RNAi analysis in deltamethrin resistant *Ae. aegypti* of *AaGste2* has also  
281 indicated a role in pyrethroid resistance (29). It is possible that the variation observed in  
282 resistance profiling are due to intrinsic differences in the activity of GSTE2s derived from the  
283 different mosquito species. In this context, it has been speculated that the predominant pyrethroid  
284 detoxification role of GSTs in some insects is sequestration or protection against oxidative stress  
285 rather than direct metabolism (30). Our results show that even high levels of *AgGste2*  
286 overexpression do not confer WHO diagnostic levels of resistance to this class of insecticides in  
287 isolation. It is possible that *AgGste2* may need to work in concert with other genes, that are not  
288 upregulated in the sensitive genetic background of the *An. gambiae* transgenic lines, to produce a  
289 pyrethroid resistance phenotype. Future work will test this hypothesis by co-expression of other  
290 UAS regulated detoxification genes using the Ubi-A10GAL4:UAS-e2 (integration) line. Although  
291 beyond the scope of this work, this mosquito line expresses GAL4 and GSTE2 and can be  
292 crossed with other UAS lines to provide co-expression with other detoxification enzymes to  
293 examine additive or synergistic interactions.

294 Although GSTs have been associated with organophosphate (OP) metabolism through  
295 biochemical studies (7), we report the first evidence that the expression of a single gene can  
296 provide OP resistance in mosquitoes. The high resistance shown towards fenitrothion by *Gste2*  
297 overexpressing *An. gambiae* is intriguing. It is currently unclear if GSTE2 detoxifies fenitrothion by  
298 sequestration, free radical protection or directly through conjugation/modification. Evidence from  
299 early studies (31) suggest that *Anopheles* GST activity is associated with the conversion of  
300 fenitrothion to the non-toxic metabolite desmethyl fenitrooxon through an oxidised intermediate.  
301 Similar analysis in the *Gste2* overexpressing lines would clarify which of these mechanisms is  
302 involved. Further investigation is also needed on the OP malathion, for which we report  
303 suspected resistance when *Gste2* is overexpressed.

304 We have also demonstrated that *Cyp6* overexpression increases susceptibility to malathion, as  
305 well as conferring permethrin resistance, which may have direct implications on insecticide  
306 management, especially if replicated with other OPs that may be used for *Anopheles* control (32).  
307 Such sensitivity profiles are readily explained by the bio-activation of malathion to its more toxic

308 metabolite malaoxon (33) by a P450-mediated mechanism (28). Here we provide the first direct *in*  
309 *vivo* evidence that CYP6 enzymes can confer negative cross resistance. Furthermore, there  
310 appears to be substrate specificity in the alternative P450-mediated reactions, since we observed  
311 higher mortality when assayed against *Cyp6m2* overexpression compared to *Cyp6p3*. This may  
312 suggest that *Cyp6m2* favours the higher steady state production of the toxic intermediate  
313 compared to *Cyp6p3*.

314 Malathion activation by *Cyp6m2* is also supported by recent evidence provided by Ingham et al  
315 (34) who found that knock down of the transcription factor Maf-S results in increased survival  
316 following malathion exposure. One of the P450s downregulated by Maf-S knockdown was  
317 *Cyp6m2*, whereas *Cyp6p3* transcription was not modified. Taken together, the results provide  
318 experimental evidence to support the use of OPs, and potentially other pro-insecticides activated  
319 by CYP6 enzymes, for *Anopheles* control in areas where pyrethroid resistance is also conferred  
320 by detoxification by the same enzyme/s. One such strategy involves combining the use of  
321 pyrethroid-based bed nets with OP-based residual wall spraying or impregnated hangings (32).  
322 This takes advantage of the additive effect of the two classes of insecticides, while sensitising  
323 *Cyp6*-based pyrethroid resistant mosquitoes to malathion (35). In conjunction with recombinant  
324 enzyme assays, the modified mosquitoes described may thus become valuable tools to assess  
325 the susceptibility of new public health pro-insecticides, for example chlorfenapyr (36), to activation  
326 and detoxification by xenobiotic metabolising P450 genes in *Anopheles*.

327 When validating resistance phenotypes conferred by transgenic overexpression, the spatial  
328 pattern of overexpression can give clues to the identity of key tissues of detoxification. The  
329 expression driven by Ubi-A10 is spread over multiple tissues, which makes it impossible to  
330 pinpoint which tissue/s are particularly important for generating the resistance phenotype. Here,  
331 we directly investigated the involvement of the midgut and oenocytes in conferring P450-  
332 mediated resistance. Critically, we did not observe clear resistance to any insecticide class when  
333 either *Cyp6m2* or *Cyp6p3* were specifically expressed in either of these tissues, despite achieving  
334 highly enriched expression and the knowledge that oenocytes and the midgut express abundant  
335 P450 co-enzyme CPR (26). Furthermore, since our previous expression profiling of the Ubi-A10  
336 driver indicated lack of expression in Malpighian tubules (23) yet resistance to multiple  
337 insecticides was observed with this driver, it would appear that the insecticides tested are not  
338 predominately metabolised in the Malpighian tubules either, and other unidentified tissues may be  
339 critical, alone or in combination, for detoxification. As described earlier, some evidence of tissue  
340 specificity of P450s associated with insecticide resistance has been derived from transcriptomic  
341 analysis of crude dissections of tissues and body segments from pyrethroid resistant and  
342 sensitive strains (27). This study indicated that *Cyp6p3* is more highly expressed in the midgut of  
343 the resistant strain, whereas *Cyp6m2* has a broader upregulation in midgut, Malpighian tubules

344 and the abdomen (integument, fat body and ovaries). The relevance of elevated *Cyp6p3* levels in  
345 the midgut of the examined resistant strain is difficult to reconcile with the lack of a resistance  
346 phenotype when the same gene is overexpressed in this tissue with the GAL4/UAS system.

347 Previous *Drosophila* studies have shown that overexpression using drivers active in multiple  
348 tissues, such as actin5C-GAL4 (14–18) or tubulin-GAL4 (20) are generally needed to modify  
349 resistance. Nevertheless, there are few examples in which tissue-specific drivers have been used  
350 to validate *Cyp6* gene based resistance in *Drosophila*. Yang et al (37) demonstrated the central  
351 role of Malpighian tubules for *DmCyp6g1*-mediated DDT resistance, whilst Zhu et al (38)  
352 demonstrated the importance of neuronal expression to provide deltamethrin resistance in  
353 *Drosophila* expressing *T. castaneum Cyp6bq9*. Even in this latter analysis, however, the neuronal  
354 driver showed leaky expression in other tissues, leading to the possibility that the observed  
355 phenotype results from expression in multiple tissues. Overall, a more detailed analysis with  
356 further tissue-specific drivers, as they become available, is needed to clarify the potential  
357 involvement of specific tissues in the detoxification of insecticides in *An. gambiae*.

## 358 **Conclusions**

359 This work reports on the first functional analysis of mosquito insecticide resistance genes  
360 conducted in transgenic *An. gambiae*. The mosquitoes generated are resistant, in a solely  
361 metabolism-based manner, to at least one representative insecticide from the major classes used  
362 in public health, and are therefore useful in liability screens of new and repurposed active  
363 compounds, including insecticides, pro-insecticides, synergists and sterilising agents. The lines  
364 can also be used in combination with strains carrying genome edited target sites (e.g. Kdr and  
365 Ace-1R) to examine the additive or synergistic effects of multiple resistance mechanisms.  
366 Similarly, it is possible to use the integration line carrying both Ubi-GAL4 and UAS-*Gste2* to cross  
367 with other UAS-detox genes to analyse metabolic interactions, for example combining phase I  
368 and II metabolism. In addition, the Ubi-A10 driver is active in larval stages (23) and can thus be  
369 used to examine gene function in immature stages.

370 Importantly, for future work, there is growing evidence on the involvement in resistance of genes  
371 that are very difficult to test *in vitro* due to the lack of appropriate assays. These include genes  
372 coding for cuticle components (39), transcription factors (34) and other binding proteins, e.g.  
373 hexamerins and  $\alpha$ -crystallins (21), for which current transgenic tools, including GAL4/UAS, make  
374 *An. gambiae* the most relevant option for functional genetic analysis.

## 375 MATERIALS AND METHODS

### 376 *Plasmid construction*

377 Responder plasmids were designed for the expression of the *An. gambiae* genes *Cyp6m2*  
378 (AGAP008212), *Cyp6p3* (AGAP002865), or *Gste2* (AGAP009194) under the regulation of the  
379 UAS and carried a YFP marker gene regulated by the 3xP3 promoter. The coding sequences of  
380 *Cyp6m2* (1500 bp), derived from the susceptible strain Kisumu, was amplified from  
381 PB13:CYP6M2 (11) using primers M2fw and M2rv (Table S1). The coding sequence of *Cyp6p3*  
382 was obtained by amplifying a 193 bp fragment from Kisumu cDNA using primers P3fw1 and  
383 P3rv1 (Table S1) and a 1362 bp fragment from pCW:17 $\alpha$ -*Cyp6p3* (10) using primers P3fw2 and  
384 P3rv2 (Table S1). P3fw1 and P3rv2 were then used to join the two fragments and obtain the 1530  
385 bp full length *Cyp6p3* coding sequence. The 666 bp *Gste2-114T* coding sequence derived from  
386 the DDT-resistant strain ZAN/U was amplified from the K1B plasmid (13) using primers  
387 *Gste2k1bfor* and *Gste2k1brev* (Table S1). All coding sequences were cloned into the YFP-  
388 marked responder plasmid pSL\*attB:YFP:Gyp:UAS14i:Gyp:attB (24) downstream of the UAS  
389 using EcoRV/XhoI (*Cyp6*) or EcoRI/NcoI (*Gste2*).

### 390 *Creation of UAS responder lines by PhiC31-mediated cassette exchange*

391 For creating responder lines carrying *Cyp6* genes, embryos of the docking line A11 (24), which  
392 carries two inverted *attP* sites and is marked with 3xP3-driven CFP, were microinjected with 350  
393 ng/ $\mu$ l of the responder plasmid and 150 ng/ $\mu$ l of the integrase helper plasmid pKC40 encoding the  
394 phiC31 integrase (40) as described in Pondeville et al (41). The same protocol was followed to  
395 create the *Gste2* responder line using embryos of the docking line Ubi-A10 (23) which carries two  
396 inverted *attP* sites and is marked with 3xP3-driven CFP. Emerging F<sub>0</sub> were pooled into sex  
397 specific founder cages and outcrossed with wild type G3s. F<sub>1</sub> progenies were screened for the  
398 expression of YFP (cassette exchange) and CFP/YFP (cassette integration) in the eyes and  
399 nerve cord. Orientation check to assess the direction of cassette exchange was performed on F<sub>1</sub>  
400 YFP-positive individuals or on the F<sub>2</sub> progeny deriving from single YFP-positive individuals. This  
401 was carried out by PCR using alternative combinations of four primers designed to give a product  
402 only in one of the orientations: PiggyBacR-R2 + Red-seq4R (PCR1) and M2intFW or P3intFW or  
403 *Gste2\_v1* + ITRL1R (PCR2) to detect insertions in orientation A; PiggyBacR-R2 + M2intFW or  
404 P3intFW or *Gste2\_v2* (PCR3) and Red-seq4R + ITRL1R (PCR4) for orientation B. All definitive  
405 responder lines were created from individuals showing orientation of insertion A, which was  
406 chosen for consistency with previous RMCE lines created in this laboratory. Transformation

407 efficiencies were calculated as the number of independent transgenic events (exchanges or  
408 integrations) over the number of surviving F<sub>0</sub> adults.

#### 409 *Driver lines and GAL4 x UAS crosses*

410 Crosses for ubiquitous expression were established between the CFP-marked driver Ubi-A10  
411 (23) and individuals of the responder lines marked with YFP. While to obtain tissue-localised  
412 expression dsRed-marked drivers specific for expression in the midgut (GAL4-mid) (22) or in the  
413 oenocytes (GAL4-oen) (24) were used. Responder lines were kept as a mix of homozygous and  
414 heterozygous individuals so to obtain GAL4/+ progeny to be used as transgenic blank controls.

#### 415 *Cyp6 gene expression analysis*

416 To quantify *Cyp6* gene expression in GAL4/UAS and GAL4/+ individuals, total RNA was  
417 harvested from pools of 2-5-day-old whole adults and their relevant dissected body part (midgut  
418 or abdomen cuticle). The adult tissues remaining after dissection constituted the carcass. Three  
419 biological replicates consisting of 5 mosquitoes (or body parts) each were collected from each  
420 mosquito population. RNA extraction was performed using the TRI Reagent® protocol (Sigma).  
421 To remove genomic DNA contamination, samples were treated with the Turbo DNA-Free kit  
422 (Ambion). RNA was then reverse-transcribed using the SuperScript III First-Strand Synthesis  
423 System (Life Technologies) following the oligo(dT) reaction protocol. RT-qPCR reactions were set  
424 up using 1x Brilliant III Ultra-Fast SYBR® Green qPCR Master Mix (Agilent Technologies) and  
425 primers qM2fw and qM2rv for quantification of *Cyp6m2*, and qP3fw and qP3sub for *Cyp6p3* (15)  
426 (Table S1). The qP3sub primer bears a nucleotide substitution (A11G) to conform its sequence to  
427 that of the G3 strain template. Two housekeeping genes, the ribosomal protein S7 (RPS7)  
428 (AGAP010592) and ribosomal protein L40/Ubiquitin (AGAP007927), were also quantified using  
429 primers qS7fw, qS7rv, qUBfw and qUBrv (42) (Table S1). Transcription data obtained by RT-  
430 qPCR were analysed using the  $\Delta\Delta C_t$  method as described in SI. Gene expression analysis was  
431 not performed to assess upregulation of the *Gste2* transcript.

#### 432 *CYP6 and GSTE2 protein expression analysis*

433 To detect protein expression in GAL4/UAS and GAL4/+ individuals, total protein extracts were  
434 obtained from whole 2-5-day-old female adults and their dissected body parts. Protein extracts

435 equivalent to 1/3 of a mosquito or its body part were analysed to detect CYP6 expression driven  
436 by tissue-specific drivers. With the exception of midgut samples, for which two whole midguts  
437 were analysed. The higher amount of midgut sample was required to visualise signal of the  $\alpha$ -  
438 tubulin loading control. The equivalent of 1/10 of a single female mosquito was used to assess  
439 expression driven by ubiquitous drivers. CYP6s were probed using primary affinity-purified  
440 polyclonal peptide antibodies produced in rabbit against CYP6M2 or CYP6P3 (gifts from Dr M.  
441 Paine), while GSTE2 was probed with anti-GSTE2-28 rabbit primary antibodies (9). Secondary  
442 antibodies were anti-rabbit-HRP IgGs (Bethyl Laboratories). Detection of the loading control  $\alpha$ -  
443 tubulin was performed using primary mouse anti- $\alpha$ tubulin antibodies (Sigma or Fisher Scientific)  
444 and secondary goat anti-mouse-HRP IgG antibodies (Abcam). Signal detection was carried out  
445 using SuperSignal™ West Dura Extended Duration Substrate (Life Technologies).

#### 446 *Assessment of susceptibility to insecticides*

447 Susceptibility to insecticides was assessed in mosquitoes overexpressing *Cyp6* genes using the  
448 WHO tube bioassay (25). Pools of 20-25 GAL4/UAS and GAL4/+ adult female mosquitoes were  
449 exposed 2-5 days post-emergence to standard discriminating doses of insecticides – 0.75%  
450 permethrin, 0.05% deltamethrin, 0.1% bendiocarb, 4% DDT – for 60 minutes and mortality rates  
451 assessed after a 24 hour recovery period. For mosquitoes expressing *Cyp6* genes in the midgut  
452 or oenocytes a modified version of the standard WHO test was also performed reducing the  
453 exposure time to 20 minutes (26). For assessing susceptibility to 5% malathion in mosquitoes  
454 overexpressing *Cyp6* genes, the exposure time was decreased to 25 minutes. Mosquitoes  
455 overexpressing *Gste2* were additionally tested for 1% fenitrothion using the recommended 2 h  
456 exposure time. 1-4 biological replicates were performed for each insecticide tested. A total of 2-8  
457 technical replicate tubes were tested for each population. Welch's t-test was performed to  
458 determine statistical differences between mortality rates in GAL4/UAS and GAL4/+. Details on  
459 statistical analysis and replicate numbers of bioassay experiments are reported in Table S2.



460 **ACKNOWLEDGMENTS**

461 We gratefully acknowledge the LSTM and the MRC (MR/P016197/1) for sponsoring PhD  
462 studentships to AA and BP respectively, and to the Innovative Vector Control Consortium for  
463 follow on funding to AA. Thanks also go to Dave Weetman for very useful comments on the  
464 manuscript.

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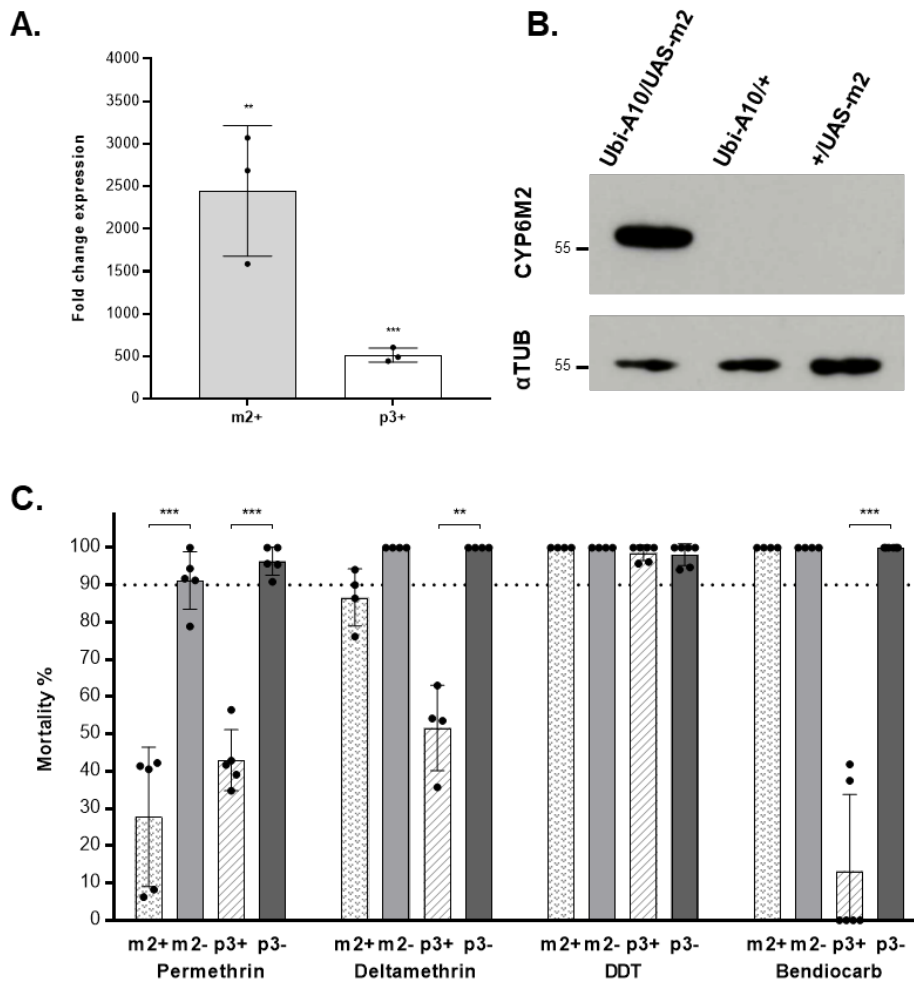
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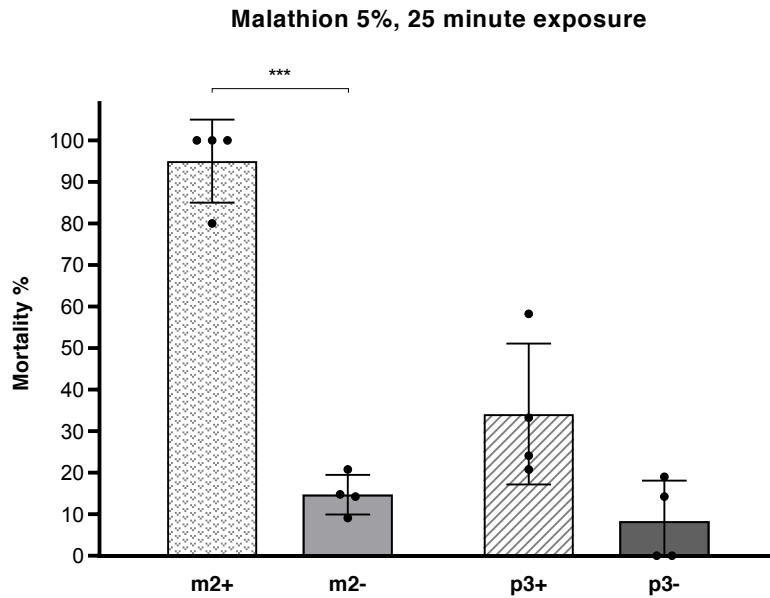
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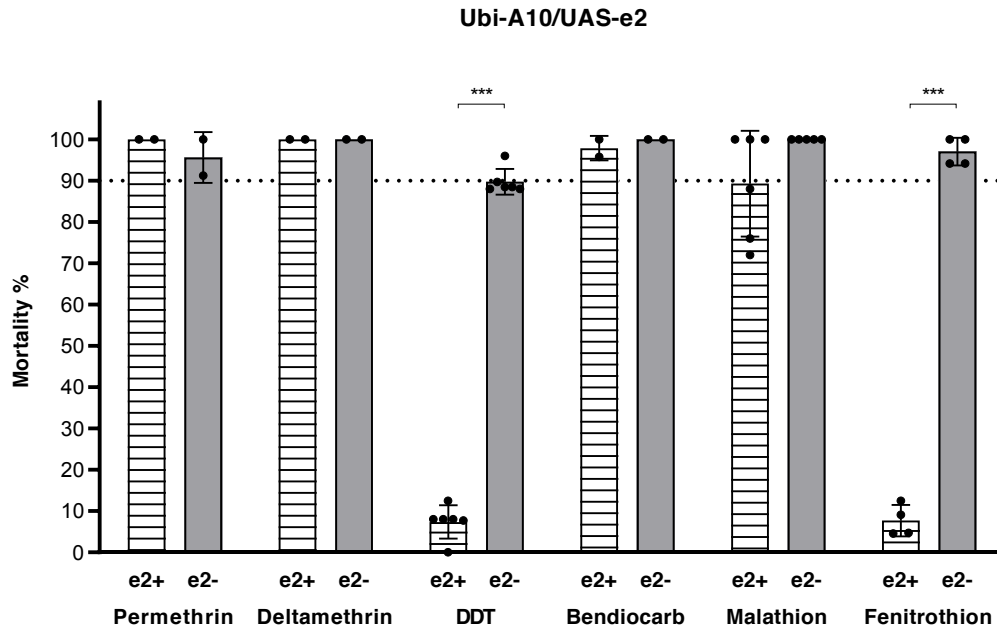
583 **FIGURES AND TABLES**



584 **Fig. 1. Multi-tissue *Cyp6* gene upregulation affects sensitivity to two pyrethroids and a**  
 585 **carbamate insecticide. A)** Relative transcription levels of *Cyp6m2* (m2+) and *Cyp6p3* (p3+) in  
 586 adult females where expression is driven by the Ubi-A10 driver compared to GAL4/+ controls.  
 587 Bars represent SD (N = 3). Unpaired t test, \*  $P < 0.05$ . \*\*  $P < 0.01$ . \*\*\*  $P < 0.001$ . **B)** Expression of  
 588 CYP6M2 and  $\alpha$ -tubulin in adult females from Ubi-A10 x UAS-m2 crosses with respective Ubi-  
 589 A10/+ and +/UAS-m2 controls. Protein extract from the equivalent of 1/10 of a whole female  
 590 mosquito was loaded in each lane. **C)** Sensitivity to insecticides of GAL4/UAS (+) females  
 591 overexpressing *Cyp6m2* or *Cyp6p3* ubiquitously under the control of the Ubi-A10 driver compared  
 592 to GAL4/+ controls (-) measured by WHO tube bioassay. Bars represent SD (N = 4-6, Table S2).  
 593 Dotted line marks the WHO 90% mortality threshold for defining resistance. Welch's t test with  
 594  $P < 0.01$  significance cut off, \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

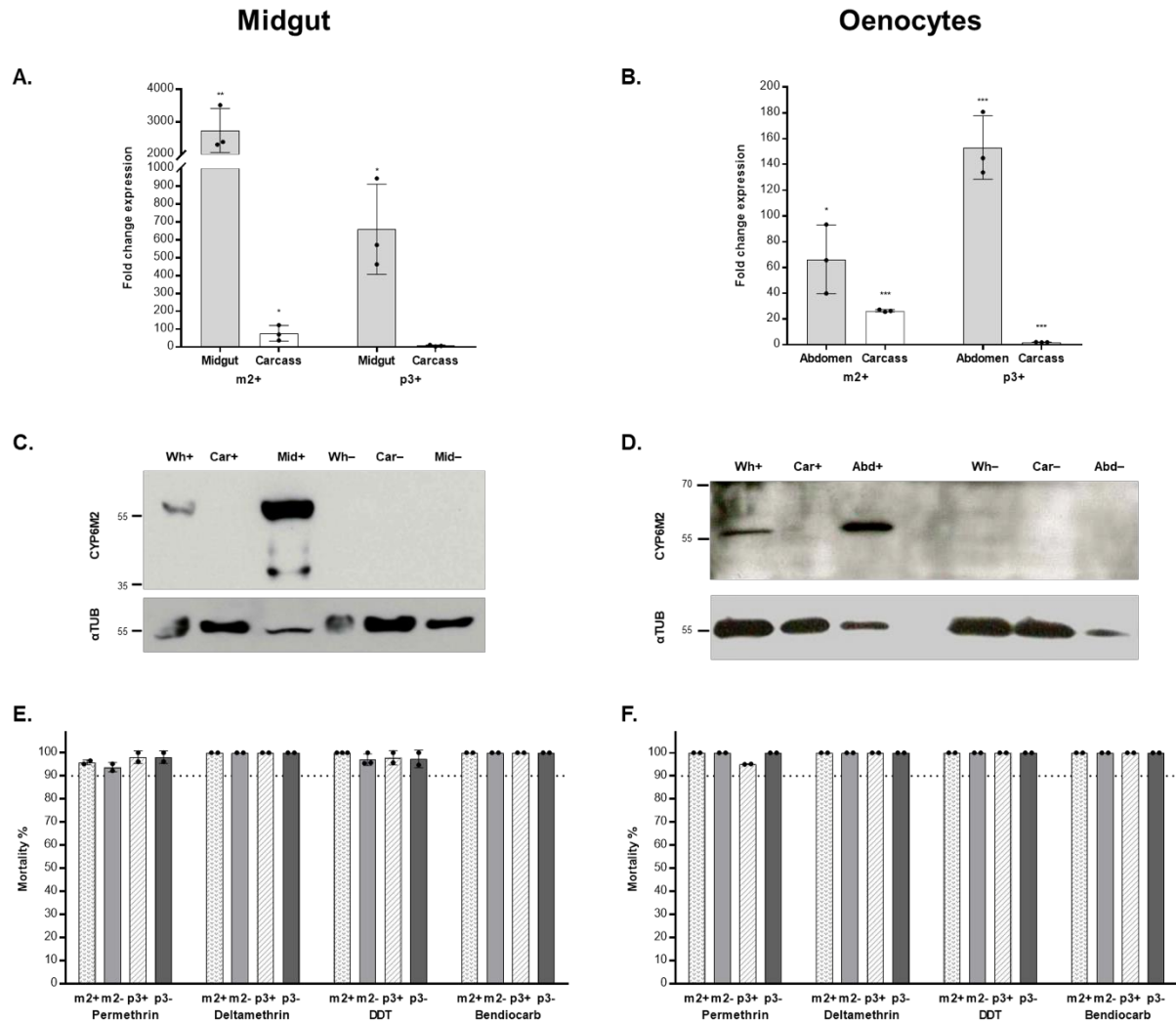


595 **Fig. 2. Multi-tissue *Cyp6* gene upregulation increases sensitivity to the organophosphate**  
596 **insecticide malathion (reduced exposure).** Sensitivity to malathion of females overexpressing  
597 *Cyp6m2* (m2+) or *Cyp6p3* (p3+) ubiquitously under the control of the Ubi-A10 driver compared to  
598 respective GAL4/+ controls (m2-, p3-) measure by a modified WHO tube bioassay representing  
599 mortality rates after 25 minutes of exposure and 24 h recovery. Bars represent SD (N = 4, Table  
600 S2). Welch's t test with  $P < 0.01$  significance cut off, \*\*\*  $P < 0.001$ .



601 **Fig. 3. Multi-tissue overexpression of GSTE2 affects sensitivity to an organochlorine and**  
602 **an organophosphate insecticide.** Sensitivity to insecticides of adult female mosquitoes  
603 overexpressing *Gste2* (e2+) ubiquitously under the control of the Ubi-A10 driver compared to Ubi-  
604 A10 controls (e2-) measured by WHO tube bioassay. Bars represent SD (N = 2-6, Table S2).  
605 Dotted line marks the WHO 90% mortality threshold for defining resistance. Welch's t test with  
606  $P < 0.01$  significance cut off, \*\*\*  $P < 0.001$ .





607 **Fig. 4. *Cyp6* gene upregulation in the mosquito midgut or oenocytes does not affect**  
 608 **sensitivity to insecticides. A-B)** Relative transcription levels of *Cyp6m2* (*m2+*) and *Cyp6p3*  
 609 (*p3+*) in dissected midguts (A) and abdomens (B) of GAL4/UAS female mosquitoes compared to  
 610 the equivalent body parts in GAL4/+ controls. Carcass is whole body without the relevant  
 611 dissected part. Bars represent SD (N = 3). Unpaired t test, \*  $P < 0.05$ . \*\*  $P < 0.01$ . \*\*\*  $P < 0.001$ . **C-D)**  
 612 Expression of CYP6M2 and  $\alpha$ -tubulin in females from the GAL4-mid x UAS-m2 (C) and GAL4-  
 613 oeno x UAS-m2 (D) crosses. Wh: protein extract from 1/3 of a single whole female; Car: protein  
 614 extract from 1/3 of a single female carcass (whole body without midgut); Mid: two dissected  
 615 midguts; Abd: abdomen cuticle; +: GAL4/UAS-m2; -: GAL4/+. **E-F)** Sensitivity to insecticides of  
 616 GAL4/UAS females overexpressing (+) *Cyp6m2* or *Cyp6p3* in the midgut (E) or in the oenocytes  
 617 (F) compared to GAL4/+ controls (-) measured by WHO tube bioassay. Bars represent SD (N =  
 618 2-3, Table S2). Dotted line marks the WHO 90% mortality threshold for defining resistance.  
 619 Welch's t test with  $P < 0.01$  significance cut off.

620 **Table 1.** Summary of the screening and crossing strategy adopted to create and establish the  
 621 UAS responder lines by RMCE.

| Docking line<br>(No. Embryos)          | F <sub>0</sub> pools<br>(No. and sex) | F <sub>0</sub><br>isofemale | F <sub>1</sub> transgenics |           | Orientation of cassette<br>exchange**                                    |
|--|---------------------------------------|-----------------------------|----------------------------|-----------|--|
|  |                                       |                             | YFP+                       | YFP+/CFP+ |  |
| <b>A11_UAS-<br/>Cyp6m2</b><br>(347)    | M2-1<br>(24 ♀)                        | G                           | 0                          | 2         | N/A  |
|  |                                       | J                           | 2♂                         | 0         | 2 F <sub>1</sub> ♂ - A   |
|  | M2-2<br>(25 ♂)                        | N/A                         | 0                          | 0         | N/A  |
| <b>A11_UAS-<br/>Cyp6p3</b><br>(460)    | P3-1<br>(28 ♀)                        | N/A                         | 7♀, 4♂                     | 1         | 5 F <sub>1</sub> ♀- A x2, B x3   |
|  | P3-2<br>(27 ♀)                        | N/A                         | 2♀, 8♂                     | 2         | 2 F <sub>1</sub> ♀- A, B   |
|  | P3-3<br>(13 ♀)                        | N/A                         | 0                          | 0         | N/A  |
|  | P3-4<br>(56 ♂)                        | N/A                         | 10♀, 13♂                   | 4         | 3 F <sub>1</sub> ♀- A, B x2  |
| <b>Ubi-A10_<br/>UAS-Gste2</b><br>(208) | E2-1<br>(10 ♂)                        | N/A                         | 0                          | 0         | N/A  |
|  | E2-2<br>(12 ♀)                        | N/A                         | 0                          | 0         | N/A  |
|  | E2-3<br>(19 ♂)                        | N/A                         | 2♂                         | 36♀, 44 ♂ | 2 F <sub>1</sub> ♂- A  |
|  | E2-4<br>(24 ♀)                        | A                           | 3♀, 3♂                     | (7)*      | F <sub>2</sub> progeny of 1 F <sub>1</sub> ♂- B                          |
|  |                                       | E                           | 4♀, 3 ♂                    | 2♀, 2♂    | 1 F <sub>1</sub> ♀- A<br>F <sub>2</sub> progeny of 1 F <sub>1</sub> ♂- A |

622 \*did not survive to adulthood.

623 \*\*As cassette exchange may occur in two different orientations with respect to the chromosome,  
 624 designated A or B, orientation check was performed on F<sub>1</sub> YFP-positive individuals or on the F<sub>2</sub>  
 625 progeny deriving from single YFP-positive individuals.

626 **Table 2.** *In vitro* (metabolism and/or depletion) and *in vivo* (*An. gambiae* and *D. melanogaster*)  
 627 functional validation of *An. gambiae* *Cyp6m2*, *Cyp6p3*, and *Gste2* genes.

| Class            | Insecticide  | Gene          | <i>In vitro</i>    | <i>An. gambiae</i><br>(this study) | <i>Drosophila</i> |
|------------------|--------------|---------------|--------------------|------------------------------------|-------------------|
| Pyrethroids      | Permethrin   | <i>Cyp6m2</i> | ✓ ‡ (11), § (19)   | ✓                                  | ✓ (15)            |
|                  |              | <i>Cyp6p3</i> | ✓ ‡ (10), § (19)   | ✓                                  | ✓ (15)            |
|                  |              | <i>Gste2</i>  | N/A                | ✗                                  | ✗ (20)            |
|                  | Deltamethrin | <i>Cyp6m2</i> | ✓ ‡ (11), § (19)   | ✓                                  | ✓ (15)            |
|                  |              | <i>Cyp6p3</i> | ✓ ‡ (10), § (19)   | ✓                                  | ✓ (15)            |
|                  |              | <i>Gste2</i>  | N/A                | ✗                                  | N/A               |
| Organochlorines  | DDT          | <i>Cyp6m2</i> | ✗ § (19) ✓ ‡* (12) | ✗                                  | ✓ (15)            |
|                  |              | <i>Cyp6p3</i> | ✗ § (19)           | ✗                                  | N/A               |
|                  |              | <i>Gste2</i>  | ✓ ‡ (9, 13)        | ✓                                  | ✓ (13, 20)        |
| Carbamates       | Bendiocarb   | <i>Cyp6m2</i> | ✗ § (15, 19)       | ✗                                  | ✓ (15)            |
|                  |              | <i>Cyp6p3</i> | ✓ § (15, 19)       | ✓                                  | ✓ (15)            |
|                  |              | <i>Gste2</i>  | N/A                | ✗                                  | N/A               |
| Organophosphates | Malathion    | <i>Cyp6m2</i> | ✓ ‡ (28), § (19)   | ✓                                  | N/A               |
|                  |              | <i>Cyp6p3</i> | ✓ § (19)           | ✓                                  | N/A               |
|                  |              | <i>Gste2</i>  | N/A                | ✗                                  | N/A               |
|                  | Fenitrothion | <i>Cyp6m2</i> | ✓ § (19)           | N/A                                | N/A               |
|                  |              | <i>Cyp6p3</i> | ✓ § (19)           | N/A                                | N/A               |
|                  |              | <i>Gste2</i>  | N/A                | ✓                                  | N/A               |

628 Presence (✓) or absence (✗) of *in vitro* activity or *in vivo* WHO-defined insecticide  
 629 resistance (*An. gambiae*) or increased insecticide tolerance (*Drosophila*).

630 ‡ metabolism; § depletion

631 \*in presence of added cholate