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1 **Molecular bases of P450-mediated resistance to the neonicotinoid**
2 **insecticide imidacloprid in the mosquito *Ae. aegypti***

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25

26 **Abstract**

27 Resistance to chemical insecticides including pyrethroids, the main insecticide class used
28 against mosquitoes, led to a regain of interest for neonicotinoids. In this context, the present
29 study aims at characterizing the molecular basis of neonicotinoid resistance in the mosquito
30 *Aedes aegypti*. Resistance mechanisms were studied by combining transcriptomic and
31 genomic data obtained from a laboratory strain selected at the larval stage for 30 generations
32 with imidacloprid (Imida-R line). After thirty generations of selection, larvae of Imida-R line
33 showed a 8-fold increased resistance to imidacloprid and a significant cross-tolerance to the
34 pyrethroids permethrin and deltamethrin. Cross-resistance to pyrethroids was only observed
35 in adults when larvae were previously exposed to imidacloprid suggesting a low but inducible
36 expression of resistance alleles at the adult stage. Resistance of the Imida-R line was
37 associated with a slower larval development time in females. Multiple detoxification enzymes
38 were over-transcribed in larvae in association with resistance including the P450s *CYP6BB2*,
39 *CYP9M9* and *CYP6M11* previously associated with pyrethroid resistance. Some of them
40 together with their redox partner NADPH P450 reductase were also affected by non-
41 synonymous mutations associated with resistance. Combining genomic and transcriptomic
42 data allowed identifying promoter variations associated with the up-regulation of *CYP6BB2* in
43 the resistant line. Overall, these data confirm the key role of P450s in neonicotinoid
44 resistance in *Ae. aegypti* and their potential to confer cross-resistance to pyrethroids, raising
45 concerns about the use of neonicotinoids for resistance management in this mosquito
46 species.

47 **Keywords**

48 Mosquito, resistance, Imidacloprid, Cytochrome P450, nextgen sequencing

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55 **1. Introduction**

56 Arboviruses threaten public health in many tropical and subtropical countries. One of
57 the most important vector of human arboviruses is the mosquito *Aedes aegypti*, which can
58 transmit yellow fever, chikungunya (CHIKV), dengue (DENV), and Zika (ZIKV) viruses
59 (Crawford et al. 2017). The health and economic impact of arboviral diseases has increased
60 dramatically over the last 50 years (Vorou 2016). Despite decades of efforts, control
61 strategies have failed to protect human from arboviruses due to an increase of global trade
62 and ineffective vector control strategies (Franz et al. 2015). Indeed, in absence of treatments
63 or vaccines for most arboviral diseases, arbovirus prevention mainly relies on intensive
64 vector control which itself often relies on insecticide treatments (Smith et al. 2016). However,
65 the usage of the same insecticides for decades led to the emergence and spread of
66 resistance to most chemical insecticides including pyrethroids, the latest insecticides used for
67 vector control (Ranson et al. 2010). Resistance to insecticides is widespread in the dengue
68 vector *Aedes aegypti* (Moyes et al. 2017) with high resistance levels frequently observed in
69 natural populations often affecting the efficacy of vector control (Dusfour et al. 2011; Dusfour
70 et al. 2019).

71 Resistance of mosquitoes to chemical insecticides has been associated to several
72 physiological adaptations among which target-site insensitivity and increased insecticide
73 metabolism are of key importance (Hemingway et al. 2004). Non-synonymous mutations
74 decreasing insecticide target sensitivity are well-characterized in mosquitoes. For example,
75 mutations affecting the voltage-gated sodium channel (knock down resistance mutations,
76 known as *kdr* mutations) have been well described in *Ae. aegypti* and are often present at
77 high frequencies in pyrethroid-resistant populations (Black and Vontas 2007; Moyes et al.
78 2017; Smith et al. 2016). Metabolic resistance is linked to an increased activity of
79 detoxification enzymes including cytochrome P450 monooxygenases (P450s or CYPs for
80 genes), carboxyl/cholinesterases (CCEs), glutathione S-transferases (GSTs) and UDP-
81 glycosyl-transferases (UDPGTs) (David et al. 2013; Li et al. 2007) which metabolize the

82 insecticide. This increased detoxifying activity is frequently caused by an over-expression of
83 detoxification genes through transcriptional regulation or gene duplications though the
84 selection of structural variants enhancing insecticide metabolism may also be involved
85 (Faucon et al. 2015; Faucon et al. 2017).

86 A classical way to manage resistance is by alternating insecticides from different
87 families. However, the use of the same chemical families for decades together with the
88 limited portfolio of insecticides authorized in public health frequently impair such
89 management strategy (Dusfour et al. 2019). Enlarging the portfolio of insecticides available
90 for vector control by repurposing insecticides developed for crop protection is a possible way
91 to improve resistance management. In this frame, several studies pointed out the potential of
92 neonicotinoids for mosquito control (Darriet and Chandre 2013; Paul et al. 2006; Pridgeon et
93 al. 2008) and vector control products containing neonicotinoids are currently being developed
94 by the industry (Agossa et al. 2018; Fuseini et al. 2019). As other neonicotinoids,
95 imidacloprid is an insecticide targeting nicotinic acetylcholine receptors (nAChR) in insect
96 nervous systems (Tomizawa and Casida 2005). This insecticide has been extensively used
97 in agriculture against various crops pests (Elbert et al. 2008; Mullins 1993). Neonicotinoids
98 such as imidacloprid alone or in combination with other insecticides have been shown to be
99 efficient against mosquitoes resistant to pyrethroids suggesting that they might represent a
100 new tool for managing resistance (Darriet and Chandre 2013; Paul et al. 2006). However, the
101 implementation of neonicotinoids in vector control may eventually lead to the selection of
102 novel or existing resistance alleles in mosquitoes. Several studies demonstrated the capacity
103 of various agricultural pests to become resistant to neonicotinoids. In most cases, resistance
104 was caused by target site insensitivity (Crossthwaite et al., 2014) or an overproduction of
105 P450s (Feng et al. 2010; Karunker et al. 2008; Puinean et al. 2010). In addition, multiple
106 P450s from various insects were shown to be capable of metabolizing imidacloprid, including
107 *Drosophila melanogaster* CYP6G1, *Bemisia tabaci* CYP6CM1vQ, *Nilaparvata lugens*
108 CYP6AY1 and CYP6ER1, and *Laodelphax striatellus* CYP6AY3v2 and CYP417A2v2 (Bao et

109 al. 2016; Jouben et al. 2008; Karunker et al. 2009; Miah et al. 2017; Wang et al. 2017).
110 Despite the strong interest for neonicotinoids in vector control, resistance mechanisms of
111 mosquitoes to these insecticides remain poorly investigated (Corbel et al. 2004; Darriet and
112 Chandre 2013; Uragayala et al. 2015). A recent study pointed out the higher neonicotinoid
113 resistance of *Anopheles coluzzi* populations from an intense agriculture area although
114 resistance mechanisms were not investigated (Mouhamadou et al. 2019). In *Ae. aegypti*, the
115 selection of a laboratory strain with imidacloprid at the larval stage for 8 generations lead to a
116 ~5-fold increased resistance phenotype mostly expressed at the larval stage (Riaz et al
117 2013). Biochemical and gene expression studies suggested the key role of P450 over-
118 transcription in resistance with multiple *CYP* genes strongly over-transcribed in the resistant
119 strain (Riaz et al 2013, David et al 2014). Among them, substrate docking simulations
120 identified *CYP6BB2* as a potential imidacloprid metabolizer (Riaz et al 2013). However, the
121 low resistance level obtained after only 8 generations of selection and the lack of genomic
122 data did not allow to fully characterize the molecular bases of the resistance phenotype.

123 In this context, the present study aims at further characterizing the resistance
124 mechanisms having been selected in this resistant strain by combining transcriptomic and
125 genomic data obtained from individuals selected for 30 generations with imidacloprid. An
126 additional line was also created by releasing the selection pressure from generation 11 in
127 order to improve the power of resistance allele detection and investigate fitness costs
128 associated with resistance. A combination of RNA-seq and pool DNA-seq targeting the
129 exons and promoters of 750 genes potentially involved in resistance was used in an attempt
130 to better characterize the genetic bases of this P450-mediated resistance phenotype.

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135 **2. Material and methods**

136 *2.1 Ethical statement*

137 Adult mosquitoes were fed on mice. Mice were maintained in an animal house agreed
138 by French Ministry of animal welfare (n° B 38 421 10 001) and used in accordance to EU laws
139 and the relevant ethic committee recommendations (ComEth Grenoble - C2EA - 12).

140

141 *2.2. Insecticide selection*

142 Mosquitoes were reared in standard insectary conditions (26°C, 14h/10h light/dark
143 photoperiod, 80% relative humidity). Larvae and adults were fed with hay pellets and filter
144 papers impregnated with honey respectively. The susceptible laboratory strain Bora-Bora,
145 (French Polynesia), was used as a parental strain for insecticide selection. The Imida-R
146 resistant line was obtained by selecting Bora-Bora individuals with imidacloprid (Sigma-
147 Aldrich, Germany) for 30 successive generations. Selection was performed following WHO
148 guidelines for larvicide bioassays (WHO, 2016) by exposing 3rd-4th-stage larvae for 24h to
149 imidacloprid. The dose of imidacloprid (500 to 1500 µg/L) was adjusted at each generation to
150 reach a mortality rate of 70-80%. Surviving larvae were transferred in tap water until
151 emergence. At each generation, a negative control was performed with the solvent used for
152 imidacloprid solubilization (acetone) in order to control for mortality not induced by the
153 insecticide. After reproduction females were blood fed to generate eggs for the next
154 generation. Each generation was initiated with more than 7000 individuals for limiting
155 bottleneck effect. In order to investigate fitness costs associated with resistance, a second
156 line (Imida-S) was created by releasing the selection pressure from G₁₁ to G₃₀.

157

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160 *2.3. Larval bioassays with imidacloprid*

161 Dynamics of larval resistance in each line was studied through comparative
162 bioassays with imidacloprid in the Imida-R and the Imida-S lines as compared to the
163 susceptible strain Bora-Bora at generations 6, 9, 11, 12, 14, 16, 19, 25 and 30. At each
164 generation bioassays were performed on 4th-stage larvae not previously exposed to
165 insecticide. For each line, three replicates of 25 larvae were maintained in 50 mL insecticide
166 solution for 24h before recording mortality. Six different insecticide concentrations ranging
167 from 150 to 3500 µg/L killing from 5% to 95% individuals were used for each line. LC₅₀ and
168 their 95% confident intervals (CI₉₅) were estimated for each line at each generation with a
169 log-probit model using XL-Stat (Addinsoft, Paris, France). Resistance ratios (RR₅₀) between
170 each line and the susceptible strain Bora-Bora were computed at each generation based on
171 their respective LC₅₀ values.

172

173 *2.4 Larval and adult bioassays with pyrethroids*

174 Cross-resistance of the Imida-R line to the pyrethroid insecticides permethrin and
175 deltamethrin was investigated in larvae and adult female at the end of the selection process
176 using single-dose bioassays. All mentioned concentrations were nominal concentrations.
177 Comparative bioassays were performed on 4th-stage larvae of the Imida-R line and the
178 susceptible Bora-Bora strain not previously exposed to insecticide. For each line, five
179 replicates of 25 larvae were maintained in 50 mL insecticide solution for 24h before recording
180 mortality. A single-dose of permethrin (1 µg/l) and deltamethrin (0.125 µg/L) were used.
181 Comparative bioassays were also conducted on four days-old non-blood fed adult females of
182 the Imida-R line and the susceptible Bora-Bora strain using WHO test tubes with 0.75%
183 permethrin and 0.05% deltamethrin papers. Bioassays were performed with 5 replicates of
184 25 females according to WHO procedure except that exposure times were reduced to 6 min
185 and 5 min for permethrin and deltamethrin respectively (WHO, 2016). Mortality rates were

186 recorded after a 24h recovery time. Two comparisons were tested. First, the susceptibility of
187 Bora-Bora females and G₃₀ Imida-R females not exposed to any insecticide were compared.
188 Then, the susceptibility of Bora-Bora females and G₃₀ Imida-R females obtained from 4th
189 stage larvae surviving to an imidacloprid exposure of 1500 µg/L for 24h (~70% larval
190 mortality) were compared.

191

192 *2.5 Individual development rates*

193 Development rates up to imago emergence were compared between the Imida-R,
194 imida-S and Bora-Bora lines in order to investigate for fitness costs potentially associated
195 with resistance as defined from (Agossa et al. 2018; Prud'homme et al. 2017; Prud'homme et
196 al. 2018). From 1h post-hatching to imago emergence, 100 G₃₀ individuals (3 replicates)
197 were reared in glass dishes (1 mosquito per mL). Feeding was performed daily with 0.2 to 0.4
198 mg/individual of crushed hay pellets at days 0 to 10 as described in Prud'homme et al. (2017;
199 2018). The time-to-emergence was defined as the number of hours separating hatching and
200 adult emergence. The distributions of time-to-emergence obtained from males and females
201 of each line were compared using a Kaplan-Meyer's test.

202

203 *2.6 RNA and DNA extractions*

204 For RNA-seq, three pools of thirty 4th stage larvae from each line (Imida-R, Imida-S
205 and Bora-Bora) not exposed to insecticide were used for RNA extractions using the
206 RNAqueous-4PCR kit (Life technologies) according to manufacturer's instructions Genomic
207 DNA contaminants were removed using DNase. For targeted DNA-seq, mosquitoes (two
208 pools of 65 individuals) were collected and used for genomic DNA extraction as described in
209 Faucon et al. 2015

210

211 2.7 RNA sequencing

212 Three RNA-seq libraries were prepared per line using the TruSeq Stranded mRNA
213 sample Prep kit (Illumina) following manufacturer's instructions. After quality check, libraries
214 were sequenced (paired reads) in multiplex on an Illumina GAIIx sequencer by Hybrigenics-
215 Helixio (Clermont-Ferrand, France). After unplexing and removing adaptors FastQC
216 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>) was used on each sample to
217 assess reads quality. Reads were then filtered based on their length, pairing and quality
218 using Trimmomatic (Bolger et al. 2014). Trimmed reads (only paired reads) were then
219 mapped to *Ae. aegypti* genome (AaegL5 assembly) using Strand NGS V2.2 (Strand Life
220 Sciences). Reads were then filtered based on the sequence and mapping quality and read
221 coverage was quantified for each sample and normalized as described in Faucon et al.
222 (2017).

223 Transcription ratios between each line were computed across all biological replicates
224 and associated P values were obtained using an ANOVA test with Benjamini-Hochberg
225 correction for multiple comparison and a post-Hoc Tukey HSD test. Genes satisfying the four
226 following conditions in either direction were considered differentially expressed in association
227 with resistance (DE genes): $FC[Imida-R / Bora-Bora] \geq 1.5$ and $P \text{ value} \leq 0.005$ (*differential*
228 *expression in Imida-R versus Bora-Bora*) AND $FC[Imida-S / Bora-Bora] \geq 1.1$ and $P \text{ value} \leq$
229 0.005 (*moderate differential expression in Imida-S versus Bora-Bora*) AND $FC[Imida-R /$
230 $Imida-S] \geq 1.0$ and $P \text{ value} \leq 0.005$ (*higher differential expression in Imida-R as compared to*
231 *Imida-S*) AND $\{FC[Imida-R / Bora-Bora] / FC[imida-S / Bora-Bora]\} > 0$ (*same direction in*
232 *Imida-R and imida-S*).

233 Polymorphism data obtained from RNA-seq were analyzed using Strand NGS V2.2
234 (Strand Life Sciences). SNPs were called against the reference genome from filtered reads
235 using the parameters described in Faucon et al. (2017). SNPs for which allele frequency
236 variations satisfied the following conditions in either direction were considered associated

237 with resistance (Diff SNPs): $f[\text{Imida-R}] - f[\text{BoraBora}] > 40\%$ (*high frequency variation between*
238 *Imida-R and Bora-Bora*), AND $f[\text{Imida-S}] - f[\text{Bora-Bora}] > 20\%$ (*moderate frequency variation*
239 *between Imida-S and Bora-Bora*) AND $f[\text{Imida-R}] / f[\text{Imida-S}] > 0$ (*frequency variations versus*
240 *Bora-Bora in the same direction for Imida-R and Imida-S*). Genic effects were computed to
241 identify non-synonymous differential SNPs (NS Diff SNPs).

242

243 *2.8 Pool targeted DNA sequencing*

244 Gene capture and sequencing were performed by Hybrigenics-Helixio (Clermont-
245 Ferrand, France). The capture library consisted in 51,073 overlapping RNA probes of 120 bp
246 (baits) targeting 3458 exons belonging to 789 genes and their upstream promoter regions
247 (up to 1 kb upstream ATG) as described in Faucon et al. (2015). These genes, chosen
248 according to their potential role in insecticide resistance. Capture was performed according
249 to the 'SureSelect Target Enrichment System for Illumina Paired-end Sequencing Library
250 version 1.5' protocol with 3 μg gDNA. Captured DNA fragments were amplified, purified,
251 multiplexed and paired-end sequenced on a Genome Analyzer IIx (Illumina) producing 76 bp
252 reads. Sequencing deep was adjusted to reach an average coverage of targeted regions
253 $>60\text{X}$ for each sample. Reads quality was checked using FastQC and reads were then
254 filtered based on their length, pairing and quality using Trimmomatic (Bolger et al. 2014) as
255 described in Faucon et al. (2015). Only paired reads were retained for mapping. Reads were
256 mapped to *Ae. Aegypti* genome (AaegL5 assembly) using BWA (Li and Durbin 2009) with
257 default parameters implemented into a Galaxy pipeline (<http://galaxyproject.org>). Mapped
258 reads were loaded into Strand NGS V2.2 (Strand Life Sciences) and further filtered
259 according to their mapping quality (alignment score ≥ 95). Only primary hits were conserved
260 In case of multiple mapped reads.

261 Copy number variations affecting each targeted gene were estimated based on
262 coverage data estimated from filtered reads as described in Faucon et al. (2015). Detection

263 of Copy Number Variations (CNV) was based on coverage fold change (FC) between
264 samples. Genes satisfying the following conditions were considered affected by a positive
265 CNV associated with resistance: FC[Imida-R / Bora-Bora] \geq 1.5-fold (*corresponding to a*
266 *duplication in \geq 50% individuals*) AND FC[Imida-S / Bora-Bora] $>$ 1.05-fold (*corresponding to*
267 *a duplication in \geq 5% individuals*) AND FC[Imida-R / Imida-S] \geq 1-fold (*more copies in Imida-*
268 *R as compared to Imida-S*).

269 Polymorphisms (SNPs, MNPs and indels) were called against the reference genome
270 by using Strand NGS V2.2 (Strand Life Sciences) and for each variation, allele frequencies
271 were computed for each line based on the number of reads supporting each allele. Only
272 substitutions showing $>$ 5% allele frequency variation across lines were retained. SNPs for
273 which frequency variations satisfied the same conditions as for RNA-seq polymorphisms
274 analysis (see above) were considered associated with resistance (Diff SNPs). Genic effects
275 were computed as described above

276 For promoter regions, the correlation between the frequency of SNPs located
277 upstream detoxification genes over-transcribed in association with resistance and their
278 transcription levels were tested across all conditions using Pearson's tests. Then, the effect
279 of variations positively correlated with transcription levels was examined *in silico* by
280 comparing transcription factor binding sites (TFBS) at each locus between the reference and
281 the variant alleles. TFBS were screened with Promo V3.0 ([http://alggen.lsi.upc.es/cgi-](http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3)
282 [bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3](http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3)) using Transfac V8.3 database restricted
283 to animals and factors of at least six bp as described in Faucon et al. (2017). DNA patterns
284 showing \leq 5% matrix dissimilarity with known TFBS were retained and compared between
285 reference and variant haplotypes. Differential TFBS were then manually curated and those
286 likely having a major impact on gene regulation were identified based on i) their differential
287 occurrence between reference and variant alleles, ii) their known enhancer effect on
288 detoxification genes, iii) their expected effect in regards of the positive/negative correlation
289 between the frequency of the variant allele and transcription level and iv) their position

290 relative to core promoter elements and the putative transcription starting site. Putative
291 transcription starting sites were manually identified based on the presence of core promoter
292 elements previously identified in arthropods.

293

294 **3. Results**

295 *3.1 Dynamics of resistance through the selection process*

296 Monitoring larval resistance to imidacloprid along the selection process confirmed the
297 gradual increase of resistance in the selected Imida-R line with a RR_{50} reaching 9.5-fold at
298 generation 19 and then stabilizing around 8-fold as compared to the susceptible parental line
299 Bora-Bora (Fig. 1). The release of imidacloprid selection from generation 11 (Imida-S line)
300 led to the gradual decrease of resistance with a RR_{50} decreasing to 3.2-fold at generation 30.

301 *3.2 Larval and adult cross-resistance to pyrethroids*

302 Comparative bioassays revealed a significant cross-resistance of the Imida-R line to
303 the two pyrethroids permethrin and deltamethrin at the larval stage as compared to the
304 parental Bora-Bora strain (Fig. 2A). No significant constitutive cross resistance was observed
305 in adult females (Fig. 2B) when obtained from unexposed larvae. However, when the adult
306 females of the imida-R line used for bioassays were obtained from larvae surviving
307 imidacloprid exposure, a significant increased resistance to both pyrethroids was observed
308 for the Imida-R line as compared to the parental Bora-Bora strain suggesting that the
309 expression of resistance alleles can be induced by the insecticide (Fig. 2C).

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314 *3.3 Fitness costs associated with resistance*

315 Comparison of larval development rates between the three lines revealed a delay of
316 emergence for Imida-R females as compared to females of the susceptible parental Bora-
317 Bora strain ($X^2=139$, $df=1$, $p\text{-value}\approx 0$) while no significant delay was observed for Imida-
318 S females (Fig. 3A). No significant differences were observed in males between the Imida-R
319 or the imida-S lines and the parental Bora-Bora strain (Fig. 3B).

320

321 *3.4 Transcription level variations associated with imidacloprid resistance*

322 The 6,145 annotated transcripts showing a transcription level > 0.5 RPKM were
323 manually associated to 13 functional categories (Fig. 4A). Twenty annotated genes were
324 differentially transcribed in association with resistance with a higher transcription ratio
325 observed in the Imida-R line than in the Imida-S line (DE genes, see methods). These DE
326 genes encompassed 14 genes over-transcribed and 6 genes under-transcribed. Functional
327 categories enrichment analyses performed on the differentially transcribed genes indicated a
328 strong enrichment of cytochrome P450s (P450s) and glycosyl-transferases (UGT) among
329 over-transcribed genes (Fig. 4A). Four P450s belonging to *CYP6* (*CYP6BB2* and *CYP6M11*),
330 *CYP9* (*CYP9M9*) and *CYP4* (*CYP4H28*) families and two *UGTs* (*AAEL002688* and
331 *AAEL001816*) were found over-transcribed in association with imidacloprid resistance.
332 Among them the three P450 genes *CYP6BB2*, *CYP9M9* and *CYP4H28* showed the highest
333 transcription ratios in the resistant line Imida-R (3.2-fold, 2.96-fold and 2.89-fold respectively)
334 and lower transcription ratios in the Imida-S line obtained by releasing the selection pressure.
335 Other differentially transcribed genes belong to various functional categories that were not
336 enriched nor commonly associated with known insecticide resistance mechanisms (Fig. 4B).

337

338

339 *3.5 Polymorphisms associated with imidacloprid resistance*

340 Targeted DNA-seq identified a total of 34,858 variations called against the reference genome
341 with 24,252 (69%) being polymorphic between lines. Among them, 421 (1.2%) were
342 associated with resistance to imidacloprid (Diff SNPs) with 64 (0.18%) affecting the protein
343 sequence of 31 annotated genes (NS Diff SNPs). RNA-seq allowed the calling of 130,672
344 variations against the reference genome with 64,172 (49%) being polymorphic between
345 lines. Among them, 906 (0.69%) were associated with resistance to imidacloprid (Diff SNPs)
346 with 120 (0.09%) affecting the protein sequence of 151 annotated genes (NS Diff SNPs).
347 Functional enrichment analysis (from global RNA-seq polymorphism data) identified a 2-fold
348 significant enrichment of Diff SNP affecting P450 genes (Fig. 5A). Such enrichment reached
349 5-fold when only considering Diff NS SNPs. A significant 2.5-fold enrichment of NS Diff SNP
350 was also observed for other enzymes families not likely involved in detoxification processes.

351 A focus on detoxification enzymes revealed 27 NS Diff SNPs affecting 18 distinct
352 detoxification genes (Fig. 5B). These genes included twelve P450s mainly of the CYP4 and
353 CYP9 families, two CCEs, one UGTs, one ABC transporters, one sulfotransferase and one
354 oxidase/peroxidase. The NADPH P450 reductase AAEL025363 was also affected by one NS
355 Diff SNP. Among all NS Diff SNPs affecting detoxification enzymes two were located in the
356 putative substrate recognition sites (SRS) of the P450s CYP6AH1 and CYP9J15.

357

358 *3.6 Genomic variations associated with the over-transcription of detoxification genes*
359 *associated with imidacloprid resistance*

360

361 Copy number variation (CNV) analysis inferred from pool targeted DNA-seq did not
362 evidence any CNV associated with the over-transcription of detoxification genes associated
363 with imidacloprid resistance (data not shown). The mapping of targeted DNA-seq reads to
364 promoter regions allowed identifying 56 Diff SNPs upstream detoxification genes but none of

365 them were affecting genes over-transcribed in association with resistance. Testing for
366 positive correlations between the transcription levels of the seven detoxification genes
367 associated with resistance and the allele frequency of their promoter variations across all
368 lines identified two variations significantly correlated with the over-transcription of the P450
369 *CYP6BB2* ($r > 0.98$ and $p < 0.05$). In silico analysis of *CYP6BB2* promoter sequence
370 suggested that these two variations are potentially enhancing the binding of transcription
371 factors previously identified as transcriptional enhancers of detoxification genes (Fig. 6). The
372 G>T at position -69 (relative to ATG) enhances the binding of the Adf-1 factor while the
373 A>G at position -104 enhances the binding of the COE-1, Nkx2-1 and C/EBP α / β elements.

374

375 **4. Discussion**

376 In the context of the widespread resistance of mosquitoes to most chemical
377 insecticides used in public health, the particular mode of action of neonicotinoids have
378 suggested their use as a possible alternative to current insecticides (Paul et al. 2006;
379 Pridgeon et al. 2008; Tiawsirisup et al. 2007). Imidacloprid and other neonicotinoids alone or
380 in mixture with deltamethrin have demonstrated their efficiency against mosquitoes including
381 those resisting pyrethroids (Corbel et al. 2004; Darriet and Chandre 2013; Uragayala et al.
382 2015). However, a previous study suggested that P450-mediated resistance to imidacloprid
383 can be selected from standing genetic variation after only few generations of selection at the
384 larval stage in *Ae. aegypti* (Riaz et al. 2013). Because no other neonicotinoid-resistant
385 mosquito strain has been described yet, the present study aims at complementing the data
386 obtained by Riaz et al. (2013) and David et al. (2014) by studying the same *Ae. aegypti*
387 selected line but with up to twenty more generations of imidacloprid selection in order to
388 strengthen the resistance phenotype. An additional line was also created by releasing the
389 selection pressure in order to investigate fitness costs associated with resistance and

390 improve the detection power of resistance allele through an integrated approach combining
391 RNA-seq and DNA-seq targeting more than 750 candidate genes.

392 Larval bioassays confirmed the further increased larval resistance of the Imida-R line
393 to imidacloprid as compared to Riaz et (2013) although a saturation of RR_{50} around 8-fold
394 was observed after 25 generations of selection. Considering the full susceptibility of the
395 parental strain Bora-Bora, the saturation of RR_{50} may reflect the progressive enrichment of
396 low-effect resistance alleles and their association through recombination in the Imida-R line
397 and/or the expression of fitness costs associated with resistance (McKenzie and Batterham
398 1994). Relaxing insecticide selection pressure from G_{11} led to the progressive decrease of
399 resistance in the Imida-S line confirming that resistance alleles were not fixed at G_{11} and are
400 associated with significant fitness costs (Chown and Gaston 1999; Harak et al. 1999;
401 Hostetler et al. 1994). Insecticide resistance has frequently been associated with fitness
402 costs often compensated by an increased energetic metabolism. If such compensation does
403 not take place, the energy reallocation for expressing the resistance phenotype may impair
404 fundamental physiological processes such as development and reproduction (Chown and
405 Gaston 1999; Harak et al. 1999; Hostetler et al. 1994). Indeed, in the Imida-R line, the time to
406 emergence increased by nearly 3 days in females as compared to the susceptible parental
407 line. This phenomenon was no longer observed in the Imida-S line supporting the association
408 of this trait with resistance. These findings agree with previous studies reporting an increased
409 larval development time in insecticide resistant *Ae. aegypti* populations (Diniz et al. 2015;
410 Paris et al. 2011). The sex-dependency of this trait observed might be explained by the
411 higher need for females to accumulate resources for the adult stage (Tabbabi and Daaboub
412 2018). Variation in larval development time is the primary cause of variation of generation
413 time in natural mosquito populations (Moeur and Istock 1980). The duration between
414 hatching to adult reproduction is a crucial component of fitness in expanding populations
415 (Charlesworth 1980). In resistant insects, resistance cost is frequently associated with an
416 increased activity of enzymes involved in cellular catabolism (Araujo et al. 2008). Although

417 such compensation response was observed from gene expression data in the Imida-R line
418 after 10 generations of selection (Riaz et al. 2013), it was less apparent after 30 generations
419 of selection suggesting its progressive replacement by less costly mechanisms. However,
420 the increased development time observed might also be associated with an alteration of
421 hormone homeostasis as P450s such as *CYP18A1* and *CYP305A5* likely associated with
422 steroidogenesis in *Ae. aegypti* were both affected by non-synonymous variations in the
423 Imida-R resistant line (Margam et al. 2006; Prud'homme et al. 2018).

424 Our RNA-seq approach identified several genes encoding detoxification enzymes
425 belonging to the P450 and UGT families being over-transcribed in association with
426 imidacloprid resistance. Such an increase in detoxification enzyme transcription levels was
427 previously associated with increased P450 activities in Imida-R larvae and a higher P450-
428 dependent imidacloprid metabolism suggesting that, as in other insects, P450s play a key
429 role in imidacloprid resistance (Riaz et al., 2013). Among the four P450s identified, *CYP6BB2*
430 and *CYP9M9* were previously found over-transcribed in the Imida-R line at G₁₁ (David et al.
431 2014; Riaz et al. 2013), supporting their potential role in imidacloprid resistance. *CYP9M9*
432 was also shown to be induced in larvae following exposure to imidacloprid (David et al. 2010)
433 and over-transcribed in some pyrethroid-resistant populations (Smith et al. 2016). The high
434 homology of *CYP6BB2* substrate binding site with *Bemisia tabaci* *CYP6CM1vQ* known to
435 metabolize imidacloprid also supports its role in imidacloprid resistance (Karunker et al.
436 2009). *CYP6BB2* over-transcription through up-regulation and/or gene duplication has also
437 been frequently associated with pyrethroid resistance (Cattel et al. 2020a; Faucon et al.
438 2015; Faucon et al. 2017; Moyes et al. 2017; Smith et al. 2016) and this P450 has been
439 shown capable of metabolizing permethrin (Kasai et al. 2014). Although the ability of
440 *CYP6M11* to metabolize insecticides remains to be demonstrated, this gene was shown to
441 be induced by several xenobiotics including the insecticide permethrin (Poupardin et al.
442 2010) and was also frequently associated with pyrethroid resistance in *Ae. Aegypti* (Dusfour
443 et al. 2015; Goindin et al. 2017; Marcombe et al. 2012; Marcombe et al. 2009; Moyes et al.

444 2017). Altogether, these data support the capability of these P450s to confer resistance to
445 various insecticides and their role in the cross-resistance of the Imida-R line to pyrethroids at
446 the larval stage. In this regard, the absence of constitutive cross-resistance to pyrethroids
447 observed in adults may be explained by the lower expression of some of these genes such
448 as *CYP9M9* in adult females (Poupardin et al. 2010). Such stage-dependent resistance
449 phenotype was also observed for imidacloprid in the Imida-R line by Riaz et al. (2009). Then,
450 the significant cross-resistance phenotype observed in adult females obtained from larvae
451 surviving imidacloprid suggest that the lower constitutive cross-resistance of adults may be
452 compensated by P450 induction following insecticide exposure as shown for both *CYP6M11*
453 and *CYP9M9* (Poupardin et al 2010).

454 Over-transcription can be caused by gene Copy Number Variation (CNV) or
455 transcriptional regulation. In mosquitoes, copy number variations (CNV) has been highlighted
456 as a frequent mechanism leading to the over-expression of detoxification enzymes in
457 response to insecticide selection (Cattel et al. 2020b; Faucon et al. 2015; Faucon et al. 2017;
458 Lucas et al. 2019; Weetman et al. 2018). By combining controlled crosses with targeted
459 DNA-seq, (Cattel et al. 2020a) identified multiple P450s affected by CNV in association with
460 insecticide resistance in a multi-resistant population from French Guiana. Among them, a
461 duplication of a *CYP6* gene cluster including *CYP6BB2* was associated with pyrethroids
462 resistance. In the present study, no CNV affecting *CYP6BB2* or any other detoxification gene
463 was detected in association with resistance as a probable consequence of an absence of the
464 duplicated resistance alleles in the parental line. Such absence of CNV suggests that the up
465 regulation of detoxification genes can be rapidly selected by insecticides from standing
466 genetic variation and that CNV may act as an additive adaptive mechanism further
467 enhancing gene expression. Indeed, though the over-expression of *CYP6BB2* has been
468 associated with a gene duplication in the South American region, this gene was also found
469 over-expressed solely due to up regulation in a resistant line from Singapore (Kasai et al.
470 2014; Smith et al. 2018). Although mutations affecting promoters are frequently linked to up

471 regulation of P450 in insecticide-resistant populations, transcription factors involved have
472 rarely been identified (Feyereisen et al. 2015; Ingham et al. 2017). Here, we identified two
473 substitutions showing an allele frequency correlated with the transcription level of *CYP6BB2*
474 and likely enhancing the binding of transcription factors previously associated with P450 up-
475 regulation. Among them, C/EBPs and Nkx2-1 were shown to induce the over-transcription of
476 P450s in mammals (Kolla et al. 2007; Martinez-Jimenez et al. 2005). In addition, C/EBP-like
477 transcription factors have been found upregulated in DDT-resistant anopheles populations
478 (Kolla et al. 2007; Martinez-Jimenez et al. 2005) suggesting their involvement in insecticide
479 resistance in mosquitoes. Recently, Smith et al. (2018) demonstrated the role of trans-
480 regulation in the overexpression of several P450s including *CYP6BB2*. Undoubtedly,
481 characterizing the resistance spectrum and the variety of genetic events leading to the over-
482 expression of mosquito P450s playing a key role in insecticide resistance such as *CYP6BB2*
483 is of high interest for resistance monitoring and management.

484 Though genes encoding the nicotinic acetylcholine receptors (nAChR) targeted by
485 imidacloprid were included in the genes targeted by DNA-seq, no non-synonymous mutation
486 affecting these genes were found associated with imidacloprid resistance. Indeed, no target-
487 site mutation affecting the nAChR has been described yet in mosquitoes (Bass et al. 2015).
488 Even if such mutation exists in *Ae. aegypti* natural populations, its absence in the susceptible
489 parental line would have precluded its selection in the present study. In turn, multiple
490 detoxification enzymes were affected by non-synonymous mutations associated with
491 resistance including several P450s and their redox partner the NADPH P450 reductase.
492 Although most of these variations are probably not functionally associated with resistance
493 and may have been enriched by genetic drift, this may also reflect the strong selection
494 pressure undertaken by genes involved in P450-mediated metabolism during selection with
495 imidacloprid. Indeed, particular detoxification enzyme variants may be associated with an
496 increased metabolic activity against imidacloprid. Such a variation affecting *GSTE2* and
497 conferring an increased DDT metabolism has been identified in *An. funestus* and is under

498 strong selection pressure in Africa (Riveron et al. 2014). Similarly, variants of the esterase
499 $\alpha E7$ from *Lucilia cuprina* have been associated with increased activity toward
500 organophosphates and deltamethrin (Coppin et al. 2012; Devonshire et al. 2007; Heidari et
501 al. 2005; Newcomb et al. 1997). In our study, the P450 *CYP9M9* whose over-transcription
502 was associated with imidacloprid resistance was also affected by two non-synonymous
503 variations associated with resistance. In addition, one cluster of *CYP* genes including
504 *CYP6AH1*, *CYP4J14*, *CYP4J15* and *CYP4J16* located at ~ 58 Mb on chromosome 2
505 appeared particularly affected by imidacloprid selection with 10 NS Diff SNP associated with
506 resistance. Among them, two variations affecting *CYP6AH1* and *CYP4J15* were located
507 within the SRS of P450s and may affect the metabolism of insecticides (Feyereisen 2005).
508 Interestingly the M362I point mutation affecting *CYP6AH1* previously associated with
509 resistance at G₁₀ (David et al., 2014) was also associated with resistance at G₃₀. Whether
510 these particular mutations have arisen due to drift effects, are highlighting the selection
511 imprint affecting these P450s, or are involved in insecticide metabolism requires additional
512 work. Nevertheless, the strong enrichment of P450s within the genes affected by non-
513 synonymous variations supports the selection of P450 variants involved in acute insecticide
514 degradation in the early steps of the selection process (Faucon et al. 2015; Faucon et al.
515 2017; Kasai et al. 2014; Smith et al. 2018).

516 The use of the same insecticides families for decades have led to the emergence and
517 spread of insecticide resistance in mosquitoes, often reducing the efficacy of vector control.
518 One resistance management strategy consists in the introduction of novel active ingredients
519 having a different mode of action and therefore being not likely affected by existing
520 resistance mechanisms. Among insecticides having been proposed for controlling pyrethroid
521 resistance, neonicotinoids were suggested to be of high interest (Darriet and Chandre 2013)
522 since they target nicotinic acetylcholine receptors (nAChR) which constitute a novel target for
523 vector control (Agossa et al. 2018). Though nAChR mutations conferring neonicotinoid
524 resistance have not been identified in mosquitoes, this does not mean that they are not

525 present at low frequency in particular mosquito populations such as those regularly exposed
526 to neonicotinoids used in agriculture. In addition, our study demonstrates that metabolic
527 resistance alleles, likely including P450s, can be rapidly selected upon selection with
528 neonicotinoids and that some of them likely also confer resistance to pyrethroids. Such a
529 P450-mediated cross-resistance between neonicotinoids and pyrethroids has frequently
530 been reported in agricultural pests (Bass et al. 2015; Le Goff et al. 2003; Ma et al. 2019; Wei
531 et al. 2017) and clearly raises concerns about the use of neonicotinoids alone for managing
532 pyrethroid resistance in mosquitoes. Such hypothesis is supported by recent studies pointing
533 out the presence of neonicotinoid resistance alleles in *Anopheles* populations located in
534 intensive agriculture areas (Fouet et al. 2020; Mouhamadou et al. 2019). In this perspective,
535 using neonicotinoids in combination with other insecticides to target mosquitoes should be
536 envisaged, especially in areas where metabolic resistance alleles to pyrethroids are
537 circulating.

538

539 **5. Conclusion**

540 The present study confirms that metabolic resistance alleles can be selected from
541 standing genetic variation after a few generations of selection with imidacloprid in the
542 mosquito *Ae. Aegypti*. As in other insects, P450-mediated insecticide metabolism appears to
543 play a key role in neonicotinoid resistance in mosquitoes and can led to cross-resistance with
544 pyrethroids. Though further work is required to fully validate the functional role of the P450s
545 identified, their previous involvement in insecticide resistance and their homology with known
546 neonicotinoid-metabolizing P450s identified in other insects support their role in
547 neonicotinoid resistance. Whether these P450s will be selected by neonicotinoids in the field
548 cannot be fully ruled out yet, though they represent a good value for studying neonicotinoid
549 resistance in agricultural areas and monitoring resistance dynamics following the
550 implementation of neonicotinoids for vector control.

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556

557 **CRedit author statement**

558 Conceptualization: JPD, SR

559 Methodology: MGZ, TG, SMP, MAR, JPD, SR

560 Investigation: MGZ, TG, SMP, MAR, JPD, SR

561 Data Curation: JPD, SR

562 Writing - Original Draft: JPD, SR

563 Writing - Review & Editing: JPD, SR

564 Supervision: JPD, SR

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567 **Data Availability Statement:**

568 The RNA-seq sequence data from this study have been deposited at the European
569 Nucleotide Archive (ENA; <http://www.ebi.ac.uk/ena>) under the accession number
570 PRJEB42696. DNA-seq data from this study have been deposited at the European
571 Nucleotide Archive under accession number PRJEB42685.

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849 **Figure captions**

850 **Fig. 1.** Evolution of larval imidacloprid resistance through the selection process. Resistance
851 levels are indicated as RR50 based on LC50 estimated from dose-response bioassays. The
852 imida-R line is shown in black while the Imida-S line for which selection pressure was release
853 from generation 11 is shown in grey. For each line best fitting curves are shown.

854

855 **Fig. 2.** Cross-resistance of the Imida-R line to pyrethroids. A. Comparative larval bioassays
856 between the Imida-R line and the susceptible parental Bora-Bora strain. B. Comparative
857 adult bioassays between the Imida-R line and the susceptible parental Bora-Bora strain with
858 individuals obtained from larvae not exposed to imidacloprid. C. Comparative adult bioassays
859 between the Imida-R line and the susceptible parental Bora-Bora strain with individuals
860 obtained from larvae surviving to 1,5 mg/L imidacloprid. Histograms represent mean
861 mortality rates \pm Wald's confident interval across 5 replicates (Fisher exact test: *** $p < 0.001$,
862 * $p < 0.05$).

863

864 **Fig. 3.** Comparison of development rates between lines. A. Distribution of individual time-to-
865 emergence in females. B. Distribution of individual time-to-emergence in males. Each dot
866 represent the mean of three biological replicates \pm SEM. *** $p < 0.001$ as compared to the
867 susceptible parental line (Kaplan-Meyer test).

868

869 **Fig. 4.** Transcription level variations associated with imidacloprid resistance. A. Distribution
870 of predicted gene functions associated with differentially transcribed genes. The number of
871 genes significantly over- and under-transcribed are indicated between brackets. Gene
872 functions significantly enriched compared to all detected genes are indicated (Fisher exact
873 test: * $P < 0.05$, *** $P < 0.001$). B. Transcription ratios of differentially transcribed genes in the
874 Imida-R (black) and Imida-S (grey) lines as compared to the parental susceptible strain.

875

876 **Fig. 5.** Polymorphisms associated with imidacloprid resistance. A. Distribution of predicted
877 gene functions affected by Diff SNPs and NS Diff SNPs detected by RNA-seq. The number
878 of variations are indicated between brackets. Functions significantly enriched as compared to
879 all detected variations are indicated (Fisher exact test: ***P < 0.001). B. NS Diff SNPs
880 affecting detoxification enzymes. Frequency variations in the Imida-R line (black) and in the
881 Imida-S line (grey) as compared to the parental susceptible line are indicated by bar charts.
882 “#”marks indicate NS Diff SNPs identified by DNA-seq while no mark indicate those identified
883 by RNA-seq . For each variation, the allele represented in the histogram is indicated in blue.
884 Boxed amino acid changes indicate variations likely affecting substrate binding site.

885

886 **Fig. 6.** Promoter variations associated with the over-transcription of CYP6BB2 in the
887 resistant line. Only variations positively correlated with transcription level and potentially
888 enhancing the binding of transcription enhancers previously associated with the regulation of
889 detoxification genes are shown. Transcription levels and allele frequencies are shown for
890 each condition (Imida-R line: black; Imida-S line: grey; Susceptible parental line: white).
891 Transcription factor names and transcription initiation site (Inr) are indicated.

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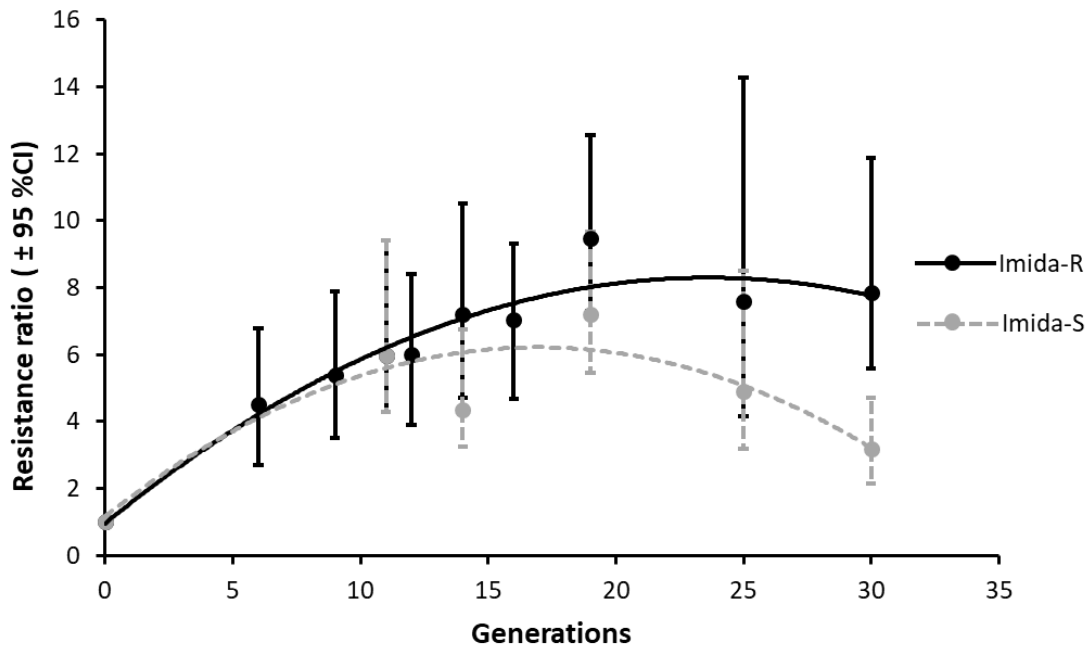
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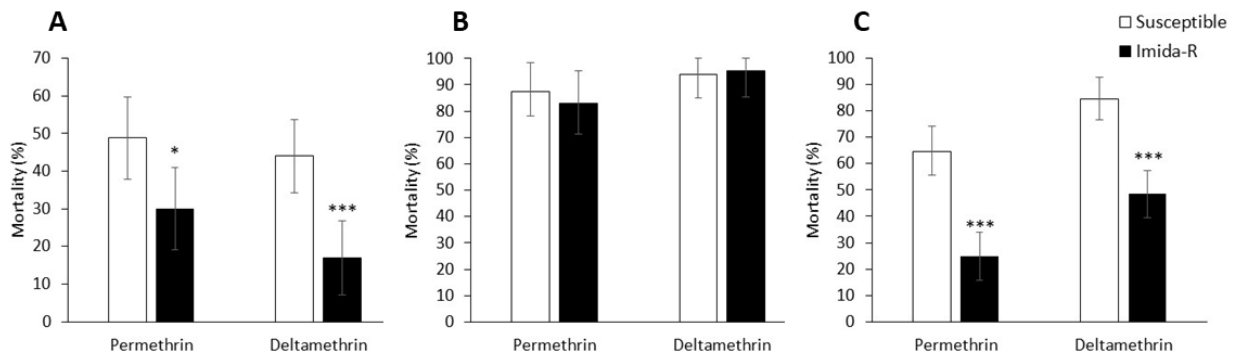
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Fig. 1.



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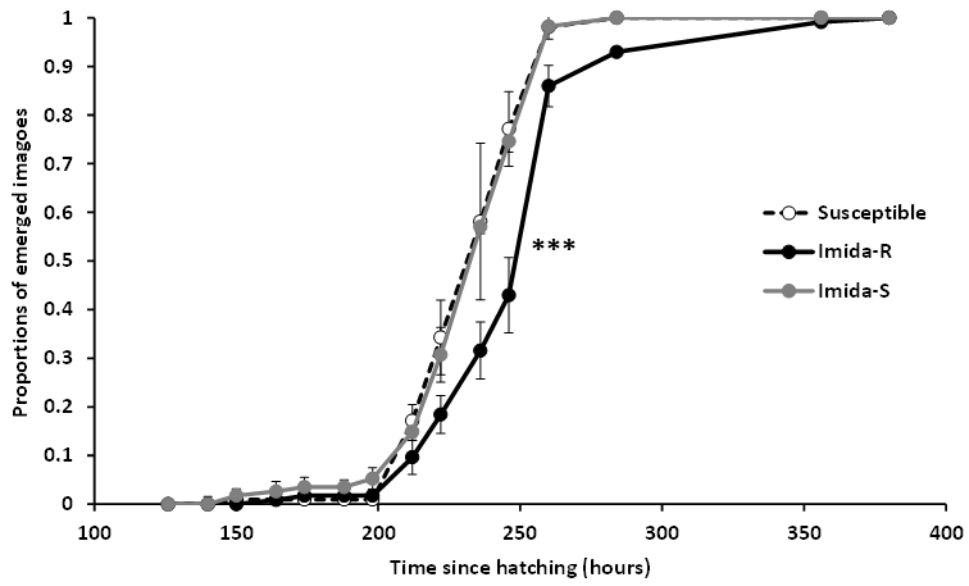
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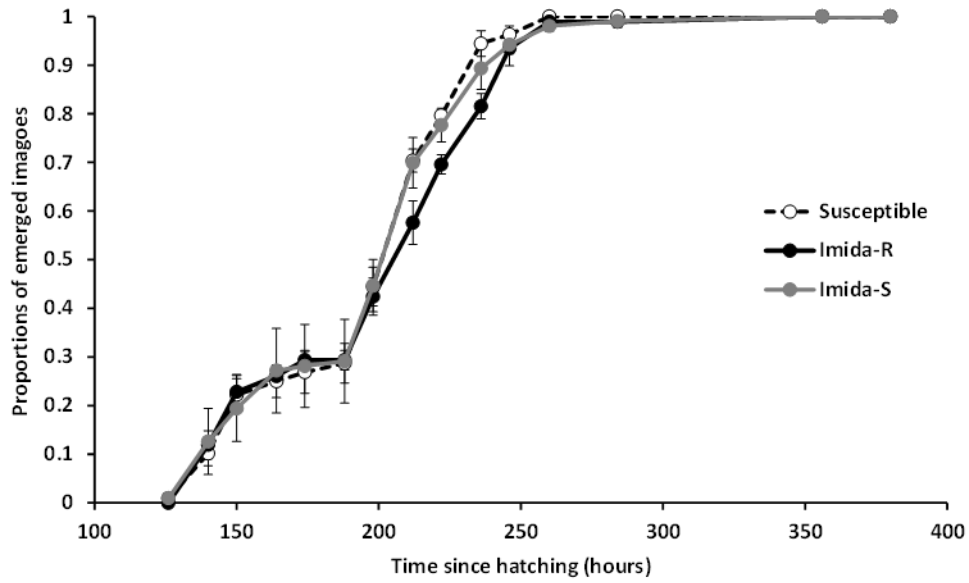
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Fig. 3.

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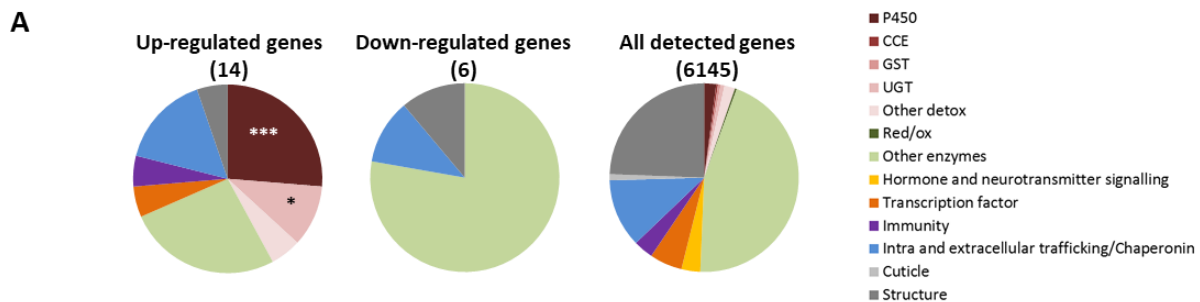


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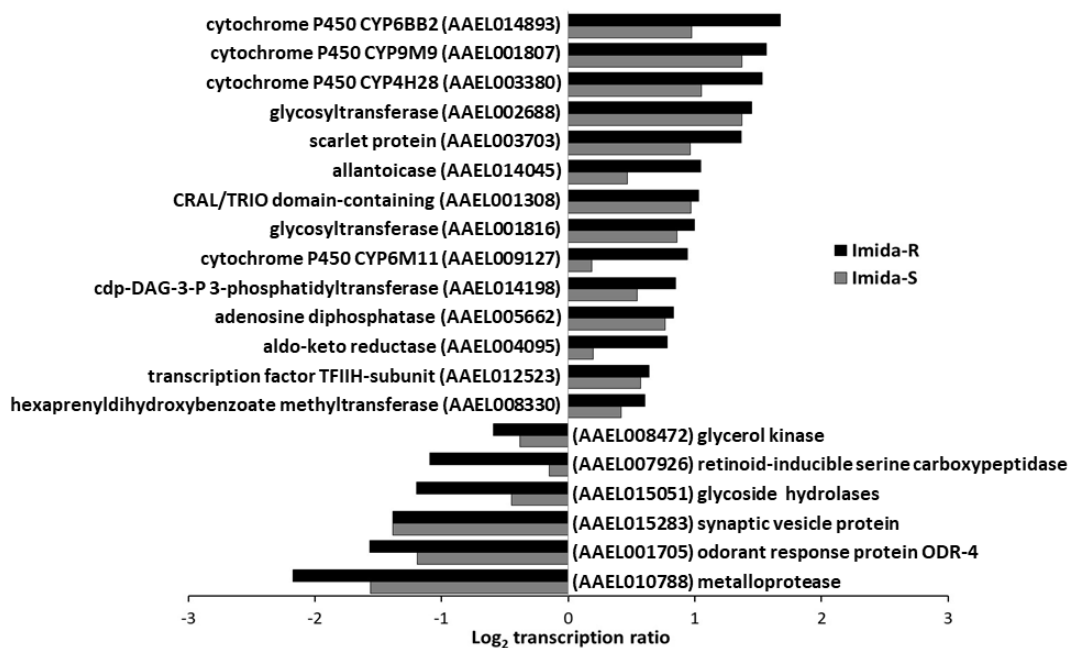


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Fig. 4.



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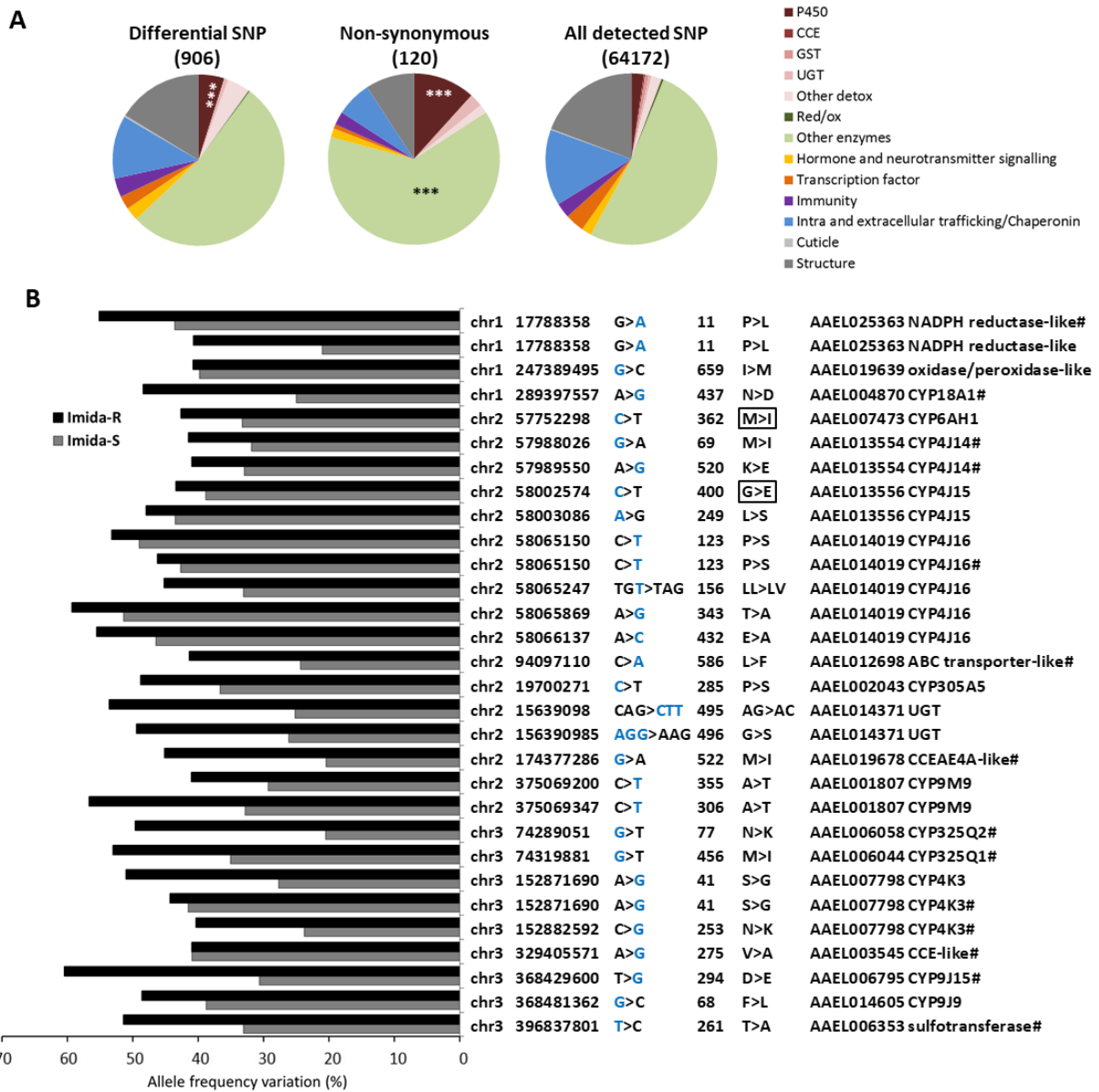
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Fig. 5.



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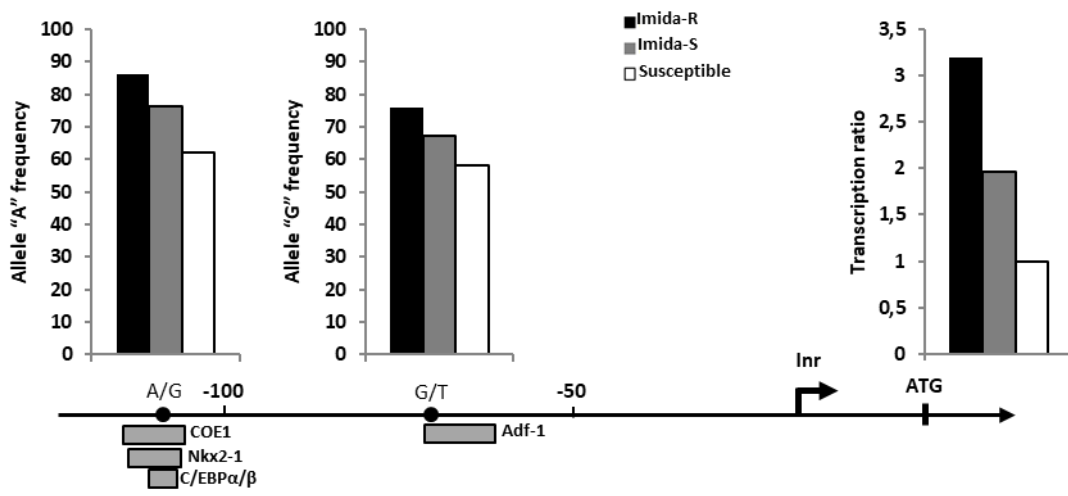
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Fig. 6.



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