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Exposure to a sublethal dose of imidacloprid induces cellular and physiological changes in *Periplaneta americana*: Involvement of $\alpha 2$ nicotinic acetylcholine subunit in imidacloprid sensitivity

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1 **Title page**

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4

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22 **Author Contribution Statement**

23 Valerie Raymond and Delphine Goven conceived and designed research. Alexandre Bantz conducted all
24 experiments. Myriam Siegwart and Sandrine Maugin contributed to enzymatic bioassay. Alexandre Bantz,
25 Delphine Goven, Myriam Siegwart and Sandrine Maugin analyzed data and Valerie Raymond coordinated the
26 study. Valerie Raymond, Delphine Goven and Alexandre Bantz wrote the manuscript. Myriam Siegwart and
27 Sandrine Maugin helped to draft the enzymatic bioassay part of the manuscript. All authors read and approved
28 for publication the manuscript.

29 **Abstract**

30 Neonicotinoids are the most important class of insecticides used as pest management tools during several
31 decades. Exposition of insect to sublethal dose of insecticide induces physiological and cellular changes that
32 could contribute to the adaptation of the insects in order to loss their sensitivity to insecticides. The aim of our
33 study is to demonstrate that a subchronic exposure to a sublethal dose of a neonicotinoid imidacloprid is
34 sufficient to induce molecular changes leading to a loss of imidacloprid sensitivity. We report that in the
35 cockroach, *Periplaneta americana*, subchronic exposure to a sublethal dose of imidacloprid induced weak
36 changes in detoxification enzyme activity and a significant decrease of the nicotinic acetylcholine $\alpha 2$ mRNA.
37 This molecular effect is correlated to a decrease of imidacloprid sensitivity of cockroaches. Using RNA
38 interference, we shown the key role of nicotinic acetylcholine $\alpha 2$ subunit in imidacloprid sensitivity. Thus,
39 quantitative changes in insecticide targets lead to decreased sensitivity to insecticides. This parameter needs to
40 be considered in order to develop sustainable insect resistance management strategies.

41

42 **Keywords:** insect; nicotinic acetylcholine receptor; insecticide sensitivity; acclimation; RNA interference;
43 imidacloprid

44

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46

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48 **1. Introduction**

49 Agriculture has to face a great challenge which is the increased needs for food of a growing population and the
50 protection of the environment. Since few years, control and management of pest insects have become one of the
51 most important global priorities. Indeed, crop protection against pest insects is one of the major component to
52 keep and improve crop yields. Chemical pesticides are among the most widely used tools for pest control
53 (Casida and Durkin, 2013) and appear important to preserve the quality of agricultural production at a moderate
54 cost. However, widespread use of pesticides during several decades leads to the emergence of resistance
55 resulting in a decrease in their efficacy (Bass and Jones, 2018). Indeed, **with** decades of use of pesticides in order
56 to protect and enhance crop yields, it appears that an **increasing** number of insect species can cope with higher
57 dose of pesticide. The selective pressure induced by the use of insecticide compounds promotes the expansion of
58 insects developing **particular** mechanisms that allow them to resist effectively against treatments. These
59 mechanisms have been well studied. Insecticide resistance resides in physiological, cellular and molecular
60 changes that can affect directly insecticide targets, metabolism or behavior. Among the well-known mechanisms,
61 mutations of the insecticide targets, increased activity of detoxification enzymes, thickening of the cuticle or
62 behavioral avoidance are considered as critical phenomena in the development of resistance (Li et al., 2007;
63 Ffrench-Constant, 2013; Bass et al., 2015; Ffrench-Constant et al., 2016; Zalucki and Furlong, 2017;
64 Balabanidou et al., 2018; Pavlidi et al., 2018). However, these last years, scientists have brought their attention
65 on the effect of sublethal doses of insecticides that could contribute to the development of insect resistance
66 (Guedes et al., 2017; Bantz et al., 2018).

67 Following a field application of insecticides, a spatial and temporal dispersal of compounds could occur. In fact,
68 insecticides degrade over time and can volatilize with wind. Therefore, the concentration of products initially
69 used to kill pests decreases until it becomes sublethal (Vryzas, 2018). As it has been shown for neonicotinoids
70 (Bonmatin et al., 2015), decline of environmental pesticide concentration could occur over a long period ranging
71 from a few months to several years. In this case, organisms may be exposed to low doses of insecticides that do
72 not induce lethal effect. Nevertheless, exposure to a sublethal dose of insecticides could also induce different
73 effects on insect physiology favoring the development of resistant or adapted insects (Bantz et al., 2018). It has
74 been demonstrated that exposure to a sublethal dose of insecticide can stimulate biological processes in order to
75 increase insect survival and reproduction (Lalouette et al., 2016; Rix et al., 2016; Zhao et al., 2018). However,
76 adverse effects of sublethal doses on honeybees and bumblebees have also been reported in the literature
77 (Desneux et al., 2007; Feltham et al., 2014; Catae et al., 2018; Tison et al., 2019). Thus, increase **in** our

78 knowledge on the impact of sublethal doses of insecticides is necessary to improve pest control. In our study, we
79 focused on the effect of sublethal dose of imidacloprid which is the first neonicotinoid insecticide launched in
80 1991. Neonicotinoids are one of the most important chemical classes of insecticides (Ihara and Matsuda, 2018;
81 Matsuda et al., 2020). Their success is mainly due to their efficacy against a range of insect pests by foliar, soil
82 and seed treatment applications. They act mainly as selective agonists on insect nicotinic acetylcholine receptors
83 (nAChRs) (Matsuda et al., 2020). Efficacy of neonicotinoids on insect nAChRs depends on many cellular and
84 molecular factors such as subunit composition or phosphorylation/dephosphorylation process (Raymond et al.,
85 2017). Insect resistances to these insecticides were attributed to modifications of the detoxification enzyme
86 activity or target-site mutations but also quantitative changes in nAChR subunits (Bass et al., 2015; Zhang et al.,
87 2015; Matsuda et al., 2020). A previous study demonstrated that the nicotine-elicited currents recorded from
88 cockroach *Periplaneta americana* neurosecretory cells called dorsal unpaired median (DUM) neurons can be
89 modified after a subchronic exposure to a sublethal dose of imidacloprid (Benzidane et al., 2017). DUM neurons
90 which are commonly used as biological models to study the mode of action of insecticides (Pelhate et al., 1990;
91 Stankiewicz et al., 2012) are known to express two distinct α -bungarotoxin-insensitive nAChR subtypes named
92 nAChR1 and nAChR2 (Courjaret and Lapied, 2001; Bodereau-Dubois et al., 2012; Sun et al., 2017). These
93 nAChRs can be distinguished according to their pharmacological profiles and to their subunit composition.
94 Indeed, only nAChR1 is sensitive to imidacloprid (Courjaret and Lapied, 2001) and it has been shown that $\alpha 3$,
95 $\alpha 8$ and $\beta 1$ subunits were involved in nAChR1 subtypes whereas $\alpha 1$, $\alpha 2$ and $\beta 1$ subunits participated to nAChR2
96 subtypes (Sun et al., 2017). Using patch-clamp recordings, it was demonstrated that subchronic exposure of
97 cockroaches to a sublethal dose of imidacloprid decreased the sensitivity of the DUM neurons to imidacloprid
98 without modifying the sensitivity of these cells to nicotine suggesting that i) the sensitivity of nAChR1 to
99 imidacloprid and the nAChR1 activity were diminished and ii) the functionality of nAChR2 was increased
100 (Benzidane et al., 2017). As no change in expression level of subunits forming nAChR1 was observed in
101 quantitative PCR experiments, this decrease of nAChR1 sensitivity to imidacloprid was linked to the increase of
102 basal calcium level that could activate calcium-dependent phosphorylation/dephosphorylation pathways.
103 Interestingly, it has been demonstrated that the function of nAChR2 which is insensitive to imidacloprid was
104 increased and that the expression of the $\alpha 1$ and $\alpha 2$ subunits involved in the subunit composition of nAChR2 was
105 significantly modified (Benzidane et al., 2017). We suggest that cellular mechanisms elicited by a sublethal dose
106 of imidacloprid and observed on DUM neurons could lead to a decrease sensitivity of whole cockroach
107 organisms to this insecticide when the cockroaches are exposed to the same imidacloprid treatment. The purpose

108 of our study is to determine 1) if an exposure of the cockroaches to a sublethal dose of imidacloprid modifies
109 their sensitivity to this insecticide and 2) which main cellular and molecular mechanism is involved in the
110 adaptation of the cockroaches to this exposure in order to develop new strategies for pest management. In our
111 study, we demonstrate that a subchronic exposure to a sublethal dose of imidacloprid affects the sensitivity of the
112 cockroaches to this insecticide and this effect is maintained over time. Using enzymatic bioassay, we
113 demonstrate that the main enzymes well-known to be involved in detoxification of imidacloprid (mixed-function
114 oxidases, Glutathione S-Transferase and carboxylesterases) do not play a key role in the loss of sensitivity to
115 imidacloprid of cockroaches. Thus, we made a focus on the $\alpha 2$ nAChR subunit for which it has been shown that
116 the subchronic exposure of the cockroach to a sublethal dose of imidacloprid decreased its expression
117 (Benzidane et al., 2017) and we show that if we only decrease the expression of this subunit, we obtain a
118 decrease on the sensitivity of the cockroaches to imidacloprid similar to that observed after a subchronic
119 exposure to a sublethal dose of imidacloprid. Thus, the $\alpha 2$ nAChR subunit plays a crucial role in the adaptation
120 of the cockroaches to imidacloprid treatment. To take into account these results which contribute to better
121 understand how insect can derive benefit from an exposure to a sublethal dose of insecticide could be important
122 to develop news tools for pest management.

123

124 **2. Material and methods**

125 *2.1. Insects*

126 Experiments were performed on adult male cockroaches *Periplaneta americana*. They were reared under
127 standard conditions in our laboratory at 29°C with a photo-cycle of 12 h light / 12 h dark.

128

129 *2.2. Insecticide exposure*

130 Imidacloprid (Sigma-Aldrich, Saint Quentin Fallavier, France) stock solution (100 mg/ml) and subsequent
131 dilutions were prepared as previously described by Benzidane et al. (2017). To determine lethal doses 10, 50 and
132 90 (LD₁₀, LD₅₀ and LD₉₀ respectively), cockroaches were treated by ingestion of 10 μ l of sucrose solution (10%
133 w/v) containing the different doses of imidacloprid ranging from 0.01 μ g to 20 μ g/cockroach and mortality rate
134 was estimated 48 h after exposure. To study the effect of subchronic exposure to a sublethal dose, cockroaches
135 were daily and orally exposed *ad libitum* 30 days to imidacloprid sublethal dose (0.025 μ g/cockroach) used in
136 Benzidane et al. (2017). Control experiments were performed under the same experimental conditions without
137 imidacloprid. Mortality rate was then evaluated after acute intoxication using three different doses (LD₁₀, LD₅₀

138 and LD₉₀) at the 30th day and also after a resting period of 30 days without imidacloprid exposure. For all
139 experiments at the 30th and the 60th day, 2 groups of cockroaches named respectively control and exposed
140 cockroaches were used.

141

142 2.3. Curve fitting and data analysis

143 Using GraphPad Prism version 8.0.2 (GraphPad Software, La Jolla, CA, USA), mortality curves were plotted as
144 a nonlinear regression and fitted according to the Hill equation, $Y = m + (X^{nH}) \frac{(M-m)}{X^{nH} + LD_{50}^{nH}}$, where Y is the
145 percentage of mortality observed at the dose value X , m and M are respectively the minimum and maximum
146 values of the fitting curve, nH is the Hill coefficient and LD_{50} is the dose of imidacloprid leading to 50% of
147 mortality. Results were expressed as means \pm S.E.M..

148

149 2.4. Enzymatic bioassay

150 Detoxification metabolism of *P. americana* cockroaches has been assessed as previously described (Siegwart et
151 al., 2017) by measuring enzymatic activities of mixed-function oxidases (MFO), Glutathione S-Transferase
152 (GST) and carboxylesterases (CE), three of large detoxification enzyme families commonly associated with
153 pesticide metabolic resistance. Briefly, dissections were performed in a climatic chamber at 11°C to limit insect
154 movement and reduce biochemical reactions. Living cockroaches were immobilized ventral side up on ice. After
155 removing the ventral cuticle, the content of the abdominal cavity was extracted and homogenized in 800 μ l of
156 Hepes buffer (50 mM, pH 7) on ice. The homogenate was partitioned into two samples: one sample was used
157 extemporaneously for the assay of MFO activity and the other was centrifuged for 15 min at 10 000 g. Its
158 supernatant was stored at -80°C before being used as a source of proteins for the measurement of GST and CE
159 activities. The total protein content of each sample was measured using the Bradford protocol with bovine serum
160 albumin to build the standard curve (Bradford, 1976). The absorbance was measured using a microplate reader
161 (Infinite 200, Tecan, Männedorf, Switzerland).

162

163 2.4.1. Mixed-function oxidases (MFO)

164 Enzymatic activities of mixed-function oxidases have been assessed with the classical ECOD method adapted
165 for *in vivo* assay as it has been previously described (Siegwart et al., 2017). 400 μ l of 7-ethoxycoumarin (0.4
166 mM) was added to the homogenate of cockroach abdominal content and keep on ice. Samples were incubated
167 for 4 h at 30°C, the reaction was stopped by adding 800 μ l of 1.5 M glycine buffer (pH 10.3) and centrifuged at

168 11 000 g for 5 min at 4°C. Then, 200 µl of supernatant were individually placed in wells of black microplates
169 (96-wells, Corning Costar, New York, U.S). Three wells per microplates containing HEPES buffer instead of the
170 homogenate content were used as blank. Fluorescence of 7-hydroxycoumarin (HC) was quantified with 380nm
171 excitation and 465nm emission filters. Protein dosages were made on each sample after fluorescence
172 measurement. A 10-fold dilution was operated before the dosage using the Bradford method (Bradford, 1976).
173 The MFO activity was expressed as pg of 7-HC/mg of total protein/min by using a standard curve of 7-HC (0.5-
174 4.5 nmoles/well) in order to convert fluorescence in 7-HC quantity.

175

176 2.4.2. *Glutathione S-transferase (GST)*

177 GST activity was determined in transparent microplates (96-wells, Sterilin[®], Newport, UK) using 2,4-dinitro-
178 chlorobenzene (CDNB) as substrate (Nauen and Stumpf, 2002). The reaction mixture in one well was made of 2
179 µl of enzymatic extract, 198 µl of a solution containing: 10 µl of 50 mM glutathione (GSH), 185 µl of HEPES
180 buffer (50 mM, pH 7.0) and 3 µl of 50 mM CDNB. Three wells per microplate filled with 2 µl of HEPES buffer
181 (50 mM, pH 7.0) instead of enzyme extract were used as blank. Absorbance was measured after 2 min of
182 incubation at 25°C in kinetic mode every 30 s at 340 nm. We used the molar extinction coefficient (9.6 mM⁻¹.cm⁻¹)
183 of CDNB-glutathione to convert absorbance in µmol of CDNB-glutathione according to Siegwart et al.
184 (2017) and GST activity was expressed as µmol/min/mg of total proteins.

185

186 2.4.3. *Carboxylesterases (CE)*

187 The same protein extracts were used as for GST activity measure. Total non-specific CE activity was measured
188 with α-naphthyl acetate (α-NA) as substrate (Reyes et al., 2007). The reaction mixture was 1 µl of protein extract
189 added to 194 µl of 30 µM α-NA in HEPES buffer (50 mM, pH 7.0) in each microplate well. After 20 min of
190 incubation at 22°C in darkness, the reaction was stopped and colored by adding 55 µl of 0.2% Fast Garnet GBC
191 diluted in 2.5% sodium dodecyl sulphate solution. Absorbance was recorded at 590 nm, after incubation for 20
192 min in darkness at room temperature. The standard curve with α-Naphtol (0-18 nmol/well) was generated to
193 express activity in nmol/min/mg of total proteins in accordance with Siegwart et al. (2017).

194

195 2.5. *Double-stranded RNA synthesis and ingestion*

196 The double-stranded RNA (dsRNA) were produced with MEGAscript T7 kit (Ambion, Grand Island, NY, USA)
197 according to the manufacturer's instructions. Briefly, the dsRNA template (100 pb) targeting the α2 subunit

198 sequence (accession number JQ585635.1) was amplified by PCR with the forward primer 5'-
199 TAATACGACTCACTATAGGGATGGACGGCGACCCTCCGTG-3' and the reverse primer 5'-
200 TAATACGACTCACTATAGGGGAACTAATAACACCATAATA-3', each containing the T7 RNA polymerase
201 promoter sequence (5'-TAATACGACTCACTATAGGG-3'), from the recombinant PCR-Blunt plasmid
202 including the $\alpha 2$ subunit sequence previously constructed in our laboratory. In parallel, the dsRNA template of
203 the bacterial LacZ sequence (100 pb) obtained after a PCR amplification using primers containing the T7
204 promoter sequence (forward primer: 5'-TAATACGACTCACTATAGGGATGACCATGATTACGCCAAG-3'
205 and reverse primer: 5'-TAATACGACTCACTATAGGGTGGCGGCCGTTACTAGTGGA-3') was used as
206 control. Then, PCR products were purified with NucleoSpin[®] Gel and PCR Clean-up kit (Macherey Nagel,
207 Düren, Germany) and quantified using the GeneQuant[™] 100 spectrophotometer (Thermo Fisher Scientific,
208 Illkirch, France). The transcription reaction was performed overnight at 37°C on 100 ng of PCR product in a
209 reaction mix (20 μ l) containing 2 μ l of NTPs, 2 μ l of T7 RNA polymerase and 2 μ l of T7 10X Buffer. A DNase
210 treatment is carried out and transcription products were then purified with NucleoSpin[®] miRNA kit (Macherey
211 Nagel, Düren, Germany). Finally, dsRNAs were denatured at 95°C during 5 min followed by a rehybridization
212 step of 1 h 30 min at room temperature and the quality of the produced dsRNA were examined by agarose gel
213 electrophoresis.

214 For dsRNA ingestion experiments, cockroaches received dsRNA lipoplexes. The dsRNA lipoplex solution was
215 prepared according to the protocol described by Lin et al. (Lin et al., 2017). Thus, 0.5 μ g of dsRNA (0.2 μ g/ μ l)
216 were mixed with 1 μ l of Escort IV[®] Transfection Reagent (Merck, Darmstadt, Germany) and 6.5 μ l of 5%
217 glucose solution and incubated 30 min at room temperature. Each cockroach was fed once with 10 μ l of dsRNA
218 lipoplex solution.

219

220 2.6. qPCR experiments

221 To evaluate imidacloprid subchronic exposure effect on the expression level of nAChR $\alpha 2$ subunit and to
222 validate dsRNA targeting $\alpha 2$ subunit, quantitative PCR (qPCR) experiments were performed on the terminal
223 abdominal ganglia (TAG) of cockroaches. Thus, total RNAs were extracted from TAG removed from nerve cord
224 of control and treated cockroaches using Nucleospin[®] RNA kit (Macherey Nagel, Düren, Germany) according to
225 the manufacturer's protocol. After a sodium acetate/ethanol precipitation, 500 ng of purified RNA were reverse
226 transcribed using RevertAid H Minus First Strand cDNA Synthesis[®] Kit (Thermo Fisher Scientific, Waltham,
227 MA, USA). Relative cockroach mRNA subunit expression was quantified by qPCR on CFX Connect[™] Real-

228 Time PCR detection system (Biorad, Hercules, CA, USA) and normalized to the expression level of the
229 housekeeping gene actin used in Benzidane et al. (2017). Primer sets were designed based on the *Periplaneta*
230 *americana* $\alpha 2$ subunit sequence (accession number JQ585635.1) and housekeeping gene actin (accession number
231 AY116670.1) published on GenBank database available at NCBI (<https://www.ncbi.nlm.nih.gov/genbank/>).
232 Each reaction of qPCR was carried out in 20 μ l containing 5 μ l of a 20-fold dilution of cDNA, 1 μ M of each
233 primer set ($\alpha 2$: Forward 5'-TAACAACCTCCATCTCATGCC-3', Reverse 5'-GATCTTGCCACTCATGTTCC-
234 3'; actin: Forward 5'-GACTACTGGTATTGTGCTGG-3', Reverse 5'-AAAGCTGTAACCACGCTCAG-3')
235 and 10 μ l of 2X Takyon No Rox SYBR[®] Master Mix Blue dTTP (Eurogentec, Seraing, Belgique). The
236 optimized qPCR program consisted in initial step at 95°C for 5 min followed by 40 cycles of denaturation step of
237 15 s at 95°C and a hybridization step of 1 min at 60°C. The efficiency of each qPCR amplification for gene-
238 specific primers remained between 98% and 102% and all qPCR experiments were performed in duplicate.
239 Relative mRNA expression levels of $\alpha 2$ nAChR subunit were determined according to the $2^{-\Delta\Delta C_t}$ method (Pfaffl,
240 2001; Livak and Schmittgen, 2001).

241

242 2.7. Statistical analysis

243 For acute intoxication of imidacloprid at LD₁₀, LD₅₀ and LD₉₀, enzymatic activities and qPCR experiments,
244 statistical analyzes were performed with nonparametric Mann-Whitney test ($p < 0.05$) using GraphPad Prism
245 version 8.0.2 (GraphPad Software, La Jolla, CA, USA). In these cases, statistical analyzes were considered as
246 significant for * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

247

248 3. Results

249 3.1. Mortality rate after an acute intoxication to imidacloprid

250 Cockroaches were treated individually by ingestion of sucrose solution containing the different doses of
251 imidacloprid and the mortality rate was evaluated at 48 h ($n = 30$ to 90 cockroaches for each dose). Imidacloprid
252 induced dose-dependent mortality at doses ranging from 0.05 μ g to 20 μ g per cockroach (Fig. 1). According to
253 the mortality curve, we determined the lethal dose 10 (LD₁₀), lethal dose 50 (LD₅₀) and lethal dose 90 (LD₉₀)
254 equal respectively to 0.5 μ g, 2.5 μ g and 10 μ g per cockroach. The aim of our study is to determine the effect of
255 an exposure to a sublethal dose of imidacloprid. In accordance with the work of Benzidane et al. (2017) and our
256 mortality curve, the dose of 0.025 μ g was chosen as the sublethal dose for the subchronic exposure experiments.
257 In our experiments, no mortality was observed on cockroaches exposed during 30 days at this dose.

258

259 *3.2. Effect of subchronic exposure to a sublethal dose of imidacloprid on the sensitivity of the cockroaches to this*
260 *insecticide*

261 First of all, the impact of an exposure to the sublethal dose of imidacloprid during 30 days was assessed on the
262 mortality of the cockroaches. Exposed and control cockroaches were treated with an acute dose of imidacloprid
263 corresponding to LD₁₀, LD₅₀ or LD₉₀ and the mortality rate was determined 48 h after their intoxication (Fig. 2a).
264 When the mortality rate of the exposed group was compared to that of the control group, a significant decrease
265 of this rate was observed for all the tested doses ($p < 0.05$). No acute toxicity was observed anymore at LD₁₀ on
266 the exposed cockroaches and a decreased mortality rate by 59% at the LD₅₀ and 31% at the LD₉₀ compared to the
267 control group was registered. The mortality rate was at LD₅₀ of $22.5 \pm 2.5\%$ instead of $55 \pm 5\%$ for the control
268 group and at LD₉₀ of $60 \pm 4.1\%$ instead of $87.5 \pm 4.8\%$ for the control group ($n = 4$ experiments with 10
269 cockroaches for each dose). These results indicate that cockroaches exposed to a sublethal dose of imidacloprid
270 during 30 days present a decrease sensitivity to imidacloprid. To determine if this effect is maintained overtime,
271 the same experiments were performed on cockroaches exposed to imidacloprid during 30 days and then kept
272 another 30 days without imidacloprid. In this set of experiments, we demonstrated that the mortality rate of
273 exposed cockroaches was still decreased compared to the control group (Fig. 2b). Indeed, mortality rates of
274 exposed cockroaches were significantly decreased by 68%, 55% and 33% at LD₁₀, LD₅₀ and LD₉₀ respectively
275 (from $12.3 \pm 3.5\%$ for the control group to $4.75 \pm 2.75\%$ for the exposed group at LD₁₀, $p < 0.01$; from $52.2 \pm$
276 2.3% to $25.2 \pm 3.2\%$ at LD₅₀, $p < 0.05$ and from $92.9 \pm 1.9\%$ to $74.8 \pm 4.9\%$ at LD₉₀, $p < 0.01$; $n = 4$ to 8
277 experiments with 10 to 15 cockroaches for each dose). Thus, after an exposure during 30 days to a sublethal dose
278 of imidacloprid, cockroaches become less sensitive to imidacloprid and this phenomenon persist at least 30 days
279 even if cockroaches were not exposed anymore to the insecticide. To go further, we investigated the cellular and
280 molecular changes induced by this exposure to imidacloprid leading to a decreased sensitivity of the cockroaches
281 to this insecticide. As a decreased sensitivity to insecticides can occur through different mechanisms and since
282 changes to detoxification pathways as well as molecular targets of the insecticides are two of the most common
283 ways, we focused our studies on detoxification enzymes (MFO, GST and CE) and nAChRs.

284

285 *3.3. Effect of subchronic exposure to a sublethal dose of imidacloprid on the activity of detoxification enzymes*

286 Although MFO are considered to be one of major detoxifying enzymes conferring resistance to imidacloprid in
287 several insects (Li et al., 2007; Bass et al., 2011a; Li et al., 2016; Romero and Anderson, 2016), surprisingly, no

288 change of their activity was observed in exposed cockroaches at 30 days (Fig. 3a) and 60 days (Fig. 3b)
289 compared to control cockroaches under our experimental conditions. Moreover, the GST activity of exposed
290 cockroaches was only 1.11 and 1.28 times higher than control cockroaches at 30 days (Fig. 4a) and 60 days (Fig.
291 4b) respectively, even if these differences were significant ($p < 0.01$ and $p < 0.0001$, respectively). Indeed, just
292 after intoxication control group had a mean of GST activity of 483.5 ± 10.3 $\mu\text{mol}/\text{min}/\text{mg}$ of total proteins ($n =$
293 71) against 538.2 ± 14.1 $\mu\text{mol}/\text{min}/\text{mg}$ of total proteins ($n = 62$) for exposed cockroaches. At 60 days, control
294 group had a mean of GST activity of 337 ± 7.8 $\mu\text{mol}/\text{min}/\text{mg}$ of total proteins ($n = 63$) against 432.8 ± 12.5
295 $\mu\text{mol}/\text{min}/\text{mg}$ of total proteins ($n = 59$) for exposed cockroaches. In a same way as MFO and GST, CE activity
296 has been measured (Fig. 5). At 30 days, no statistical difference between control and exposed cockroaches was
297 observed (Fig. 5a). However, at 60 days, a slight significant difference was observed between the 2 groups. CE
298 activity of exposed cockroaches was increased of 1.1-fold compared to control cockroaches with a mean of CE
299 activity of 36.7 and 31.5 nmol of α -Naphthol/min/mg prot respectively ($p < 0.0001$) (Fig. 5b). By contrast to GST,
300 CE were not involved in both sublethal imidacloprid intoxication phase and resting period but only in the resting
301 period. Taken together, these results suggest that detoxifying enzymes may not be the main mechanism linked to
302 the loss of sensitivity of cockroaches to imidacloprid.

303

304 *3.4. Key role of $\alpha 2$ nAChR subunit in the sensitivity of the cockroach to imidacloprid*

305 Nicotinic acetylcholine receptors are targets of imidacloprid and some nicotinic subunit expression can be
306 altered after an exposure to a sublethal dose of this insecticide (Benzidane et al., 2017). Indeed, in this previous
307 study (Benzidane et al., 2017), using the same exposure protocol, it was demonstrated that $\alpha 2$ subunit expression
308 was decreased. To determine if the decrease of the sensitivity to imidacloprid that we observed at 30 days and 60
309 days was linked to the $\alpha 2$ nAChR subunit, qPCR experiments were performed on the terminal abdominal ganglia
310 of exposed cockroaches during 30 days and of cockroaches kept another 30 days without imidacloprid treatment
311 (Fig. 6). After subchronic exposure, the expression of $\alpha 2$ mRNA was significantly decreased by approximately
312 63% compared to the control group ($p < 0.01$, $n = 5$ to 6 experiments with 6 samples per experiment) (Fig. 6a).
313 Interestingly, at 60 days, 30 days after the end of imidacloprid exposure, expression of $\alpha 2$ mRNA was still
314 decreased by 31% ($p < 0.05$, $n = 5$ to 9 experiments with 6 samples per experiment) (Fig. 6b).
315 To investigate further this putative involvement of $\alpha 2$ nAChR subunit in imidacloprid sensitivity, we have
316 conducted RNAi experiments targeting this subunit. The first step was to confirm that $\alpha 2$ dsRNAs were able to
317 decrease $\alpha 2$ mRNA expression on non-exposed cockroaches to imidacloprid. Control dsRNA have been

318 designed from bacterial gene *LacZ* which was not express in cockroaches. dsRNAs were encapsulated with a
319 cationic liposome carrier to form dsRNA lipoplexes. 96 h after a unique ingestion of dsRNA lipoplexes
320 containing either *LacZ* dsRNA (control group) or $\alpha 2$ dsRNA, expression level of $\alpha 2$ mRNA was measured in the
321 terminal abdominal ganglia of cockroaches. Ingestion of $\alpha 2$ dsRNA lipoplexes decreased significantly by 24%
322 its mRNA expression compared to the control group ($p < 0.05$, $n = 7$ to 8 experiments with 6 samples per
323 experiment) (Fig. 7a) **without modifying the expression of other nicotinic acetylcholine subunits (data not**
324 **shown)**. Our results shown that subchronic exposure to a sublethal dose of imidacloprid decreased the expression
325 of $\alpha 2$ subunit mRNA and reduced the sensitivity of cockroaches to this insecticide. Thus, to determine if the $\alpha 2$
326 subunit played a key role in the imidacloprid sensitivity, we performed an acute intoxication of control and $\alpha 2$
327 dsRNA treated cockroaches using 2 different doses of imidacloprid (LD_{50} and LD_{90}) (Fig. 6b). The percentage of
328 mortality of non-exposed cockroaches treated with dsRNA targeting $\alpha 2$ nAChR subunit was of $22 \pm 2.1\%$
329 instead of $48.8 \pm 5.6\%$ for the control group at LD_{50} and of $60 \pm 5.5\%$ instead of $86 \pm 4\%$ for the control group at
330 LD_{90} . These results demonstrated that disruption of $\alpha 2$ nAChR subunit induced a decrease of imidacloprid
331 sensitivity ($p < 0.05$, $n = 5$ experiments with 10 to 12 cockroaches per experiment). Thus, ingestion of lipoplex
332 containing dsRNA targeting $\alpha 2$ nAChR subunit mimicked the effect of subchronic exposition to a sublethal dose
333 of imidacloprid.

334

335 **4. Discussion**

336 In this study, we demonstrated that a chronic exposure to a sublethal dose of imidacloprid is sufficient to
337 decrease the sensitivity of the cockroaches *Periplaneta americana* to imidacloprid. Moreover, this effect is
338 maintained over time even if the cockroaches were no longer fed with imidacloprid. Thus, for the first time, we
339 shown that the cockroaches are able to adapt to their toxic environment in order to cope with a subsequent
340 stressor such as a future exposure to the same insecticide. As it is hypothesized that exposure to low doses of
341 insecticides could increase mutations that confer insecticide resistance (Guedes et al., 2017), it is crucial to
342 understand physiological mechanisms induced by an exposure to a sublethal dose of insecticide in order to
343 develop sustainable insect resistance management strategies. In the literature, most studies relate the resistance
344 mechanisms induced by intensive use of insecticide (Ffrench-Constant, 2013; Ffrench-Constant et al., 2016;
345 Zalucki and Furlong, 2017; Balabanidou et al., 2018; Pavlidi et al., 2018) but few studies are devoted to
346 mechanisms elicited by a sublethal dose of insecticides (Bantz et al., 2018).

347 In insects, resistance to insecticides is multifactorial but in most cases, enhanced metabolic detoxification and
348 target alteration are responsible for this phenomenon (Ffrench-Constant, 2013; Crosssthaite et al., 2014; Bass et
349 al., 2015; Matsuda et al., 2020). In our work, we evaluated at first the activity of mixed-function oxidases
350 (MFO), carboxylesterases (CE) and Glutathione S-transferase (GST) in exposed cockroaches. Enzymatic assays
351 were designed to assess these three major classes of detoxification enzymes studied to characterize resistant
352 insects (Hemingway et al., 2004; Daborn et al., 2012; Romero and Anderson, 2016; Bass and Jones, 2018;
353 Pavlidi et al., 2018; Navarro-Roldán et al., 2020). In our study, we demonstrate that after an exposure to the
354 sublethal dose of imidacloprid during 30 days, only the activity of GST slightly increased, no modification of
355 MFO and CE activities was found in our conditions. Nevertheless, after 30 days of rest, the activity of both
356 detoxification enzymes (GST and CE) slightly increased by a factor of approximately 1.2 whereas the activity of
357 MFO is not changed. If we compare our results with those obtained on resistant insects, we cannot affirm that
358 these metabolic changes are the main mechanism inducing the adaptation of the exposed cockroaches to
359 imidacloprid. Indeed, several studies made on neonicotinoid resistant insects reported that the activity of these
360 enzymes was enhanced at least 2.5 times (Wang et al., 2002; Feng et al., 2010; Yang et al., 2016; Ma et al.,
361 2017). Moreover, we found that the activity of both enzymes was mainly modified 30 days after the imidacloprid
362 exposure, in other words, in absence of imidacloprid. According to these results, we suggest that the weak
363 modifications of GST and CE activity that we observed on exposed cockroaches to a sublethal dose of
364 imidacloprid could participate in the adaptation of the cockroaches but they can't be considered as the main
365 mechanism leading to this phenomenon.

366 The second part of our work was focused on the molecular target of the imidacloprid which are the nAChRs
367 (Matsuda et al., 2020). In a previous study, it has been reported that an exposure to a sublethal dose of
368 imidacloprid decreased the expression of the $\alpha 2$ nAChR subunit (Benzidane et al., 2017). In our experiments, we
369 also shown that the quantity of nicotinic $\alpha 2$ mRNA was diminished after 30-day exposure to a sublethal dose of
370 imidacloprid but we demonstrated as well as that a decrease of nicotinic $\alpha 2$ mRNA was maintained after 30 days
371 without imidacloprid exposure. If we analyze these results with our toxicological data, we suggest that the $\alpha 2$
372 nAChR subunit could play a key role in the sensitivity of the cockroaches to imidacloprid. To confirm our
373 hypothesis, we used RNAi targeting $\alpha 2$ nAChR subunit. Using toxicological test, we determined that non-
374 exposed cockroaches treated with dsRNA $\alpha 2$ are less sensitive to acute imidacloprid intoxication than control
375 cockroaches. Indeed, at the dose inducing 50% of the mortality of the control cockroaches, only around 20% of
376 the treated dsRNA $\alpha 2$ cockroaches died. Moreover, a decrease in the mortality rate was also observed at the LD₉₀

377 value. These results are similar to those obtained after a subchronic exposure to a sublethal dose of imidacloprid.
378 Thus, we can conclude that $\alpha 2$ nAChR subunit play a major role in the sensitivity change of the cockroaches
379 with respect to imidacloprid. According to the literature, decrease of imidacloprid sensitivity correlated to
380 nAChRs is often linked in insects to target-site mutations (Bass et al., 2015; Kim et al., 2015; Matsuda et al.,
381 2020). Sequencing of nAChR subunit encoding genes from insect resistant to imidacloprid led to the
382 identification of different point-mutations. In the green peach aphid *Myzus persicae* and in the cotton aphid
383 *Aphis gossypii*, a substitution of an arginine to threonine was observed at the 81 position in the loop D of the $\beta 1$
384 nAChR subunit (Bass et al., 2011b; Hirata et al., 2015). The loop D is known to play a role in the binding of
385 neonicotinoids (Matsuda et al., 2020). In the brown planthopper *Nilaparvata lugens*, a single point-mutation at a
386 conserved position (Y151S) in two α nAChR subunits, N1 α 1 and N1 α 3 was attributed to imidacloprid resistance
387 (Liu et al., 2005, 2006). However, more recently, Zhang and colleagues (2015) demonstrated that the reduction
388 of $\alpha 8$ nAChR subunit expression affected the sensitivity of *N. lugens* to imidacloprid. Thus, quantitative changes
389 in insecticide targets could also lead to decreased sensitivity to insecticides. However, in the case of *N. lugens*,
390 $\alpha 8$ subunit belongs to nAChRs having a high-affinity binding site for imidacloprid (Li et al., 2010). In
391 cockroaches *Periplaneta americana*, the $\alpha 2$ nAChR subunits are combined with $\alpha 1$ and $\beta 1$ subunits to form the
392 nAChR2 subtypes (Sun et al., 2017) and the nAChR2 is insensitive to imidacloprid (Benzidane et al., 2017;
393 Courjaret and Lapied, 2001; Sun et al., 2017). However, in a previous study, it has been shown that DUM
394 neurons coming from cockroaches exposed to the same sublethal dose of imidacloprid during 30 days and
395 showing a decrease of $\alpha 2$ mRNA expression are less sensitive to application of imidacloprid but are able to
396 optimize functional properties of nAChR2 (Benzidane et al., 2017). The authors suggested that intracellular
397 signaling calcium-dependent pathways could regulate nAChR2 function. Indeed, different cellular and molecular
398 factors including calcium-dependent signaling pathways modulate the sensitivity of nAChR2 to neonicotinoid
399 insecticides (Bodereau-Dubois et al., 2012; Calas-List et al., 2013; Mannai et al., 2016). However, it wasn't
400 demonstrated if the nAChR2 subtypes from exposed cockroaches are made of the same nAChR subunits as that
401 those from non-exposed cockroaches. Further studies are needed to determine how a decrease of $\alpha 2$ subunit
402 expression could contribute to optimize the function of nAChR2 subtypes to maintain physiological functions.

403

404 **5. Conclusions**

405 Our study demonstrated for the first time that an exposure to a sublethal dose of imidacloprid induce molecular
406 changes allowing cockroaches to adapt to this treatment. This work which brings knowledge on the role of $\alpha 2$

407 nAChR subunit in imidacloprid sensitivity could help to understand how insects deal with exposure to sublethal
408 dose of imidacloprid in order to circumvent its effect and to develop new strategies to improve pest control.
409 Thus, the understanding of physiological mechanisms induced by an exposure to a sublethal dose of insecticide
410 is one of the important step to develop sustainable insect resistance management strategies.

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629 **Figure legends**

630

631 **Fig. 1.** Mortality rate of adult male cockroaches evaluated 48 h after acute intoxication using imidacloprid doses
632 ranging from 0.01 µg to 20 µg per cockroach. Between 30 to 90 cockroaches were used for each tested dose. **The**
633 **highest dose, which did not induce any mortality was estimated to be 0.025 µg/cockroach and was chosen as the**
634 **sublethal dose for the subchronic exposure experiments.** LD₁₀, LD₅₀ and LD₉₀ represent the dose inducing 10%,
635 50% and 90% of mortality respectively. Data are means ± S.E.M..

636

637 **Fig. 2. (a)** Histograms showing mortality rates observed at 48 h in control and exposed cockroaches to sublethal
638 dose of imidacloprid (**0.025 µg/cockroach/day**) during 30 days. Control mortality rates are expressed as means ±
639 S.E.M. (n = 4 experiments with 10 cockroaches per experiment; * *p*<0,05, Mann-Whitney Test). **(b)** Histograms
640 showing mortality rates observed at 48 h in control and exposed cockroaches to a sublethal dose of imidacloprid
641 during 30 days followed by 30 resting days without insecticide (n = 4 to 8 experiments with 10 to 15
642 cockroaches per experiment; * *p*<0.05; ** *p*<0.01, Mann-Whitney Test).

643

644 **Fig. 3. (a)** Effect of 30-day exposure to a sublethal dose of imidacloprid on mixed-function oxidase activity.
645 Box-and-whisker plots indicate the median, the 25th and the 75th percentiles (box edges) and the range (whiskers)
646 (n = 60 cockroaches for the control experiment group and n = 59 for the exposed cockroach group, ns : non-
647 significant with Mann-Withney test). **(b)** Mixed-function oxidase activity on the 60th day **corresponding to the**
648 **day after 30 days imidacloprid exposure followed by 30 days without insecticide.** Control group included 60
649 cockroaches and the exposed group 56 cockroaches (ns : non-significant with Mann-Whitney test). MFO activity
650 is expressed as pg of 7-HC/mg of total protein/min (pg/mg prot /min).

651

652 **Fig. 4. (a)** Effect of 30-day exposure to a sublethal dose of imidacloprid on Glutathione S-Transferase (GST)
653 activity. Box-and-whisker plots indicate the median, the 25th and the 75th percentiles (box edges) and the range
654 (whiskers) (n = 71 cockroaches for the control experiment and n = 62 for the exposed cockroach group, **
655 *p*<0.01, Mann-Withney test). **(b)** GST activity on the 60th day **corresponding to the day after 30 days**
656 **imidacloprid exposure followed by 30 days without insecticide.** Control group included 63 cockroaches and the
657 exposed group 59 cockroaches (**** *p*<0.0001 with Mann-Whitney test). GST activity is expressed as
658 µmol/min/mg of total proteins (µmol/min/mg prot).

659

660 **Fig. 5. (a)** Effect of 30-day exposure to a sublethal dose of imidacloprid on carboxylesterase activity. Box-and-
661 whisker plots indicate the median, the 25th and the 75th percentiles (box edges) and the range (whiskers) (n = 71
662 cockroaches for the control experiment and n = 62 for the exposed cockroach group, ns : non-significant with
663 Mann-Withney test). **(b)** Carboxylesterase activity on the 60th day **corresponding to the day after 30 days**
664 **imidacloprid exposure followed by 30 days without insecticide**. Control group included 63 cockroaches and the
665 exposed group 59 cockroaches (**** $p < 0.0001$ with Mann-Whitney test). CE activity is expressed as
666 nmol/min/mg of total proteins (nmol/min/mg prot).

667
668 **Fig. 6.** Comparative histograms illustrating the expression level of $\alpha 2$ nicotinic subunit mRNA after a 30-day
669 exposure to a sublethal dose of imidacloprid **(a)** followed by 30 resting days **(b)**. Quantitative PCR experiments
670 were performed on the terminal abdominal ganglia of control and exposed cockroaches. Results are represented
671 as the mean \pm S.E.M. of 5 to 9 independent experiments with 6 samples per experiment. Data were normalized to
672 the expression of the housekeeping gene actin. * $p < 0.05$, ** $p < 0.01$ (Mann-Whitney test).

673
674 **Fig. 7. (a)** Effect of dsRNA targeting $\alpha 2$ nAChR subunit on the expression of $\alpha 2$ mRNA. Quantitative PCR
675 experiments were performed 96 h after dsRNA ingestion on the terminal abdominal ganglia of cockroaches
676 treated with either LacZ dsRNA (control group) or $\alpha 2$ dsRNA (treated group). Results are represented as the
677 mean \pm S.E.M. of 7 to 8 independent experiments with 6 samples per experiment. Data were normalized to the
678 expression of the housekeeping gene actin. * $p < 0.05$ (Mann-Whitney test). **(b)** Effect of $\alpha 2$ dsRNA ingestion on
679 the mortality rate. The experiments were performed on two groups of non-exposed cockroaches treated with
680 either LacZ dsRNA (control group) or $\alpha 2$ dsRNA (treated group). 96 h after dsRNA ingestion, cockroaches
681 received a single dose of imidacloprid (LD₅₀ or LD₉₀). The mortality rate was then evaluated 48 h after this acute
682 intoxication. Data are means \pm S.E.M. (n = 5 experiments with 10 to 12 cockroaches per experiment; * $p < 0.05$,
683 Mann-Whitney test).

Fig.1

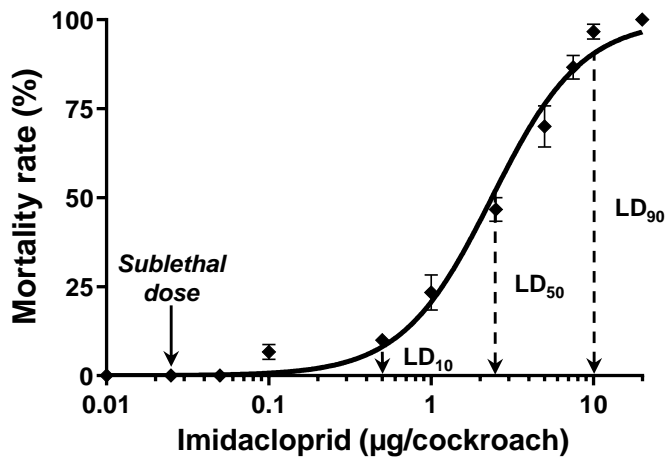


Fig.2

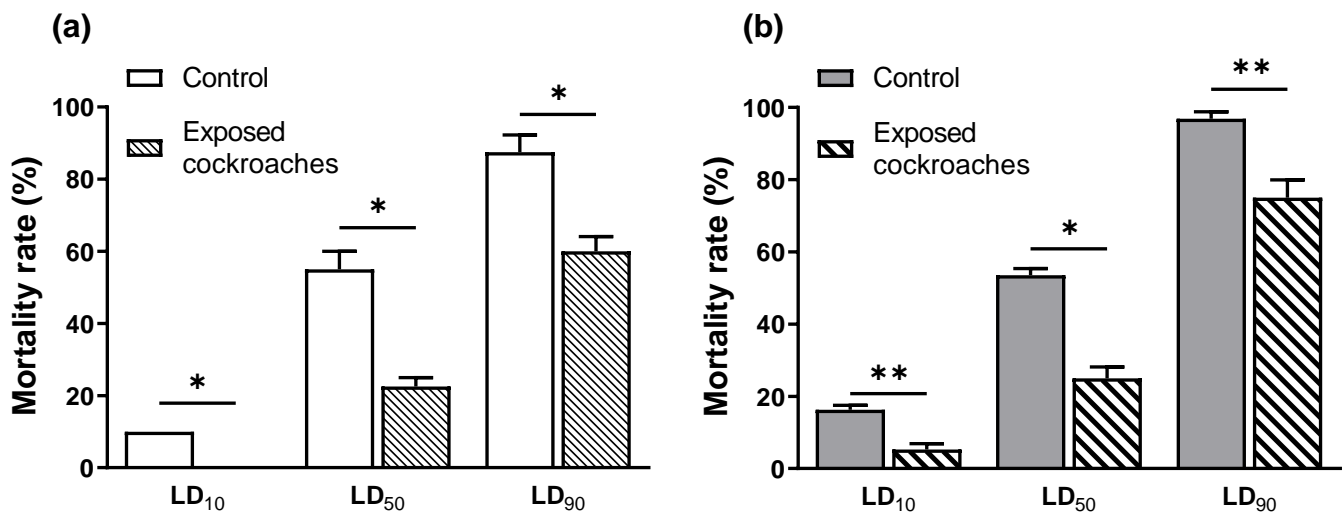


Fig.3

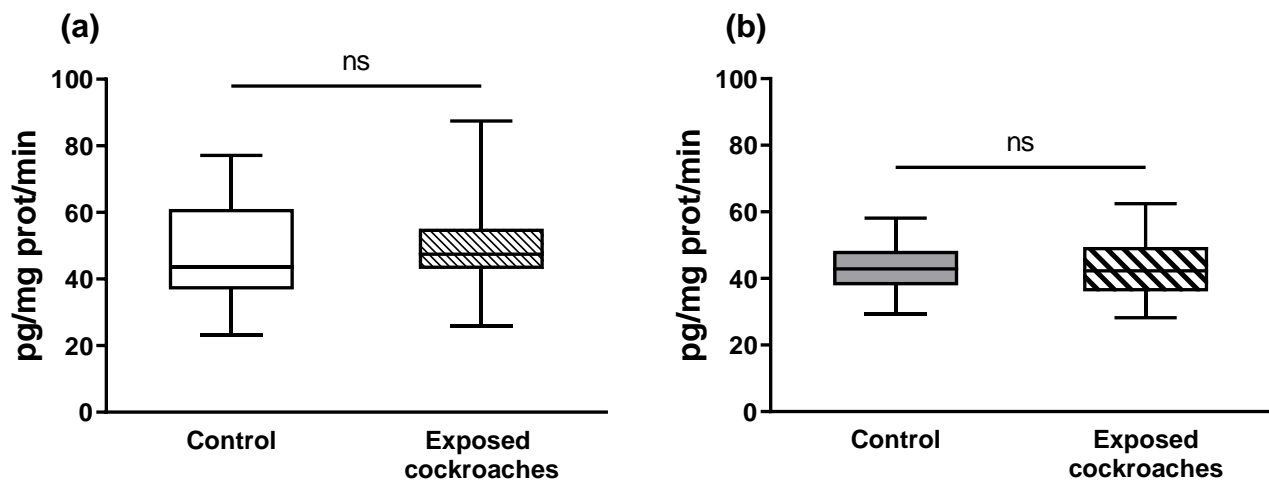


Fig.4

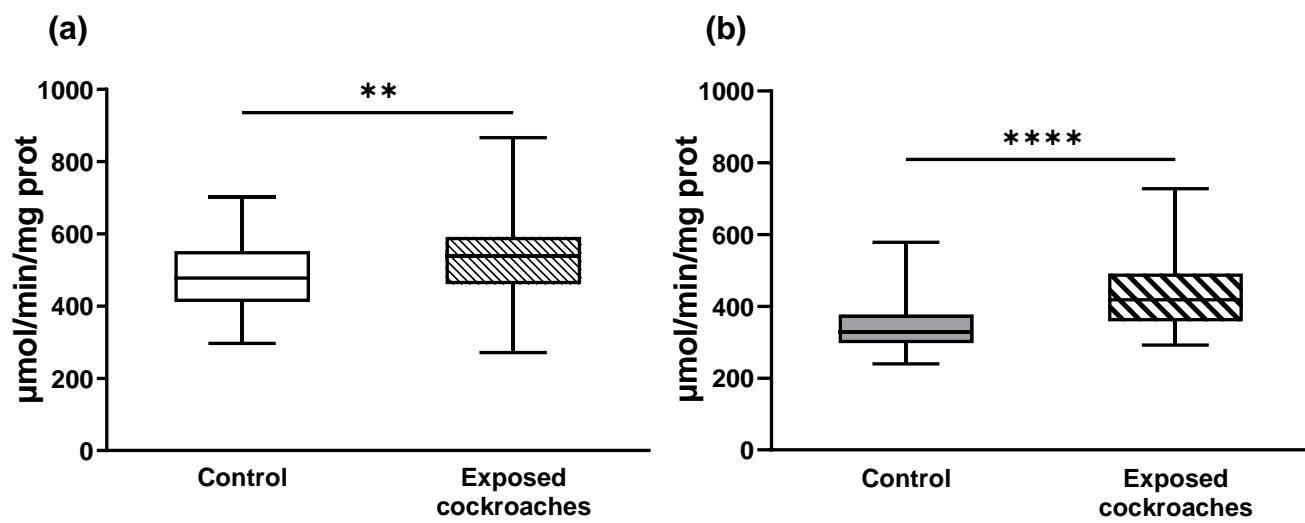


Fig.5

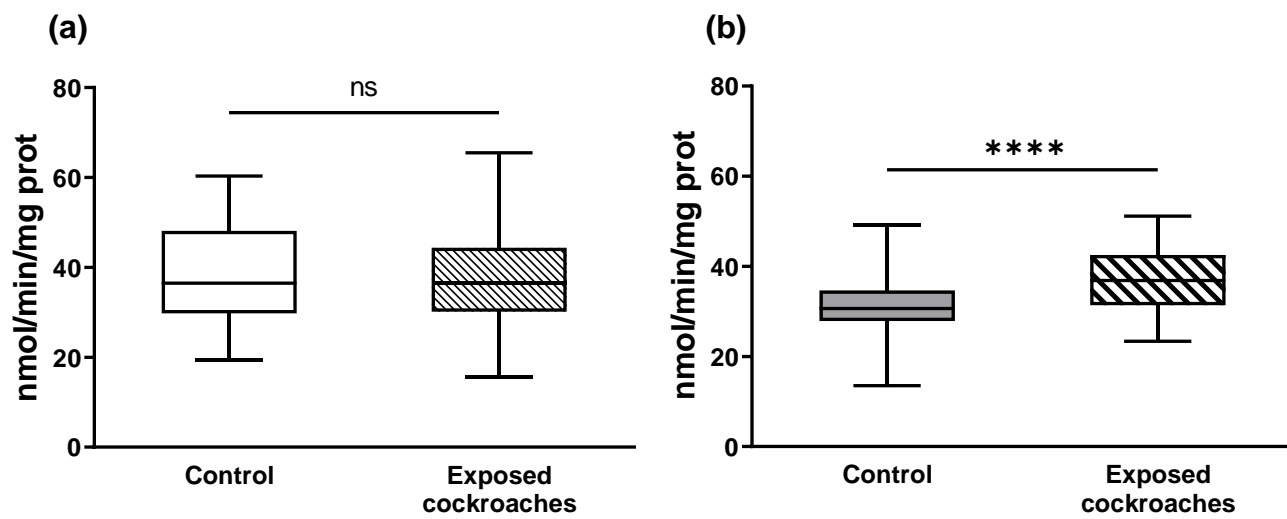


Fig.6

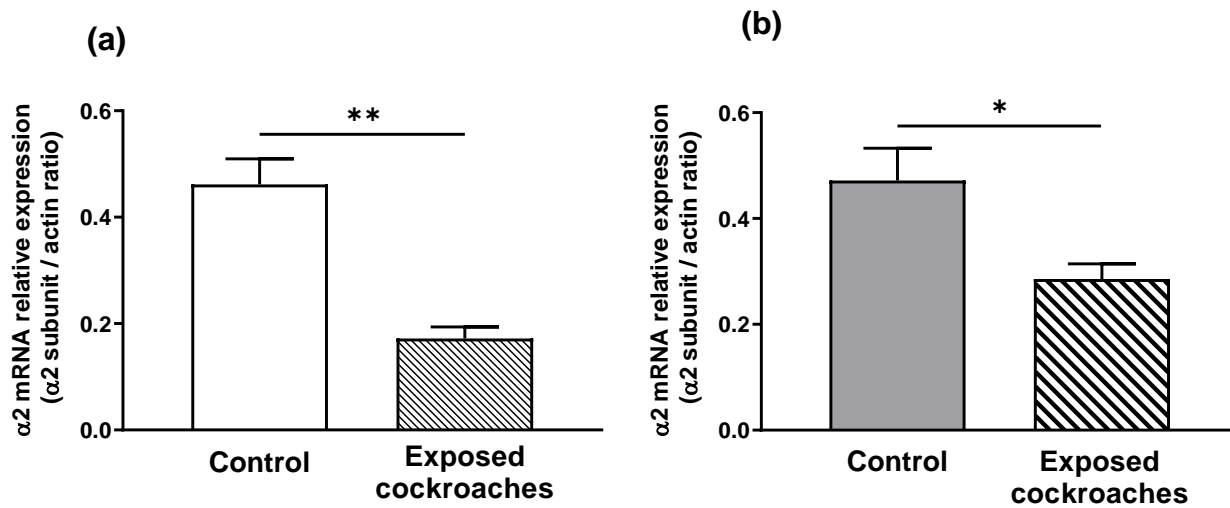


Fig.7

