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1 **Esterase Activity is Affected by Genetics, Age, Insecticide Exposure, and Viral**
2 **Infection in the Honey Bee, *Apis mellifera***
3
4 Frank D. Rinkevich[1*], Joseph W. Margotta[2], Michael Simone-Finstrom[1], Lilia I. de
5 Guzman[1], and Kristen B. Healy[2]
6
7 [1]USDA-ARS Honey Bee Breeding, Genetics, and Physiology Laboratory, Baton
8 Rouge, LA USA
9
10 [2]Department of Entomology, Life Sciences Annex, Agricultural Center, Louisiana
11 State University, Baton Rouge, LA USA
12
13 *Corresponding Author
14 Email frank.rinkevich@ars.usda.gov

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15 # Abstract

16 Non-target impacts of insecticide treatments are a major public and
17 environmental concern, particularly in contemporary beekeeping. Therefore, it is
18 important to understand the physiological mechanisms contributing to
19 insecticide sensitivity in honey bees. In the present studies, we sought to evaluate
20 the role of esterases as the source of variation in insecticide sensitivity. To address
21 this question, the following objectives were completed: 1) Evaluated esterase
22 activity among honey bee stocks, 2) Assessed the correlation of esterase activity
23 with changes in insecticide sensitivity with honey bee age, 3) Established if
24 esterases can be used as a biomarker of insecticide exposure, and 4) Examined
25 the effects of Varroa mite infestation and viral infection on esterase activity.

26 Results indicated that honey bees have a dynamic esterase capacity that
27 is influenced by genetic stock and age. However, there was no consistent
28 connection of esterase activity with insecticide sensitivity across genetic stocks or
29 with age, suggests other factors are more critical for determining insecticide
30 sensitivity. The trend of increased esterase activity with age in honey bees
31 suggests this physiological transition is consistent with enhanced metabolic rate
32 with age. The esterase inhibition with naled but not phenothrin or clothianidin
33 indicates that reduced esterase activity levels may only be reliable for sublethal
34 doses of organophosphate insecticides. The observation that viral infection, but
35 not Varroa mite infestation, reduced esterase activity shows viruses have
36 extensive physiological impacts. Taken together, these data suggest that honey

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37 bee esterase activity toward these model substrates may not correlate well with
38 insecticide sensitivity. Future studies include identification of esterase substrates
39 and inhibitors that are better surrogates of insecticide detoxification in honey
40 bees as well as investigation on the usefulness of esterase activity as a biomarker
41 of pesticide exposure, and viral infection.

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42 # Introduction

43 The history of honey bee kills upon contact with insecticides has been
44 documented since the advent of modern insecticides [1]. Beekeeper surveys
45 report that pesticide exposure significantly increases annual colony losses [2].
46 Considering that a number of insecticides used in agriculture and vector control
47 exhibit high toxicity to honey bees and that honey bees regularly encounter
48 numerous of pesticides within the colony [3], potential synergistic interactions
49 among these pesticides [4] may contribute to poor colony health.

50 Insects possess an array of metabolic mechanisms such as esterases,
51 cytochrome P450s, and glutathione-S-transferases to detoxify pesticides, plant
52 allelochemicals, and other xenobiotics [5]. Esterases are a type of hydrolase that
53 metabolizes compounds by cleaving the ester bonds of a substrate resulting in
54 separate acid and alcohol products [6]. Quantitative increases [7] as well as
55 qualitative changes in esterase activity [8] may lead to reduction in insecticide
56 sensitivity. In honey bees, esterase expression and activity are upregulated in
57 response to exposure to *p*-coumaric acid [9], coumaphos [10], thiamethoxam
58 [11], deltamethrin, fipronil, and spinosad [12]. Esterase inhibitors significantly
59 increase sensitivity to phenothrin [13], tau-fluvalinate, cyfluthrin [14],
60 fenpyroximate, and thymol [15], suggesting that esterase-mediated
61 detoxification significantly influences pesticide sensitivity. Therefore,
62 understanding the factors that affect honey bee esterase activity may yield
63 insight into differences in insecticide sensitivity.

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64 A myriad of factors such as age, diet, and genetics may affect insecticide
65 sensitivity [13, 16, 17], but little research has been done on the underlying
66 physiological mechanisms. Therefore, we decided to investigate a number of
67 common factors that previous work suggests may affect honey bee physiological
68 processes with a particular focus on esterase activity.

69 The current study aimed to tease apart several factors that influence
70 insecticide sensitivity and esterase activity in honey bees.

71 1) Esterase comparison among honey bee stocks. Earlier studies
72 demonstrated that insecticide susceptibility varies among Italian, Russian, and
73 Carniolan stocks of honey bees, and esterase inhibition has been shown to
74 increase sensitivity to phenothrin [13]. This led us to hypothesize that esterases may
75 contribute to variation in insecticide sensitivity among honey bee stocks and
76 across age.

77 2) Changes in esterase activity with age. Because of changes in pesticide
78 sensitivity occurring with increased age [13], we assessed esterase activity in
79 worker bees of different ages to compare if changes in esterase activity
80 correlated with changes in insecticide sensitivity.

81 3) Esterase inhibition by insecticides. Numerous sublethal effects of
82 pesticides have been demonstrated [18-20], and esterase activity has been
83 proposed as a biomarker of high levels of pesticide exposure [11, 12]. Therefore
84 we assayed the changes of esterase activity upon exposure to experimentally-

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85 determined sublethal levels of the insecticides naled, phenothrin, and
86 clothianidin.
87 4) Impacts of Varroa mite infestation and viruses on esterase activity. All
88 honey bee colonies in the US are infested with the ectoparasitic mite, *Varroa*
89 *destructor* (hereto referred to as the Varroa mite). Varroa mites and the
90 associated viruses they transmit are among the most significant factors relating to
91 colony failure [21, 22]. These factors were both tested because mite infestation
92 and viral infection alone and in combination have multifactorial effects on honey
93 bee physiology and response to insecticide activity [20, 23, 24]. Therefore, the
94 effects of Varroa mite infestation and viral infection on esterase activity were also
95 investigated. Results are discussed in terms of how progression through the honey
96 bee's life history and the impact of biotic factors influence esterase capacity. We
97 further suggest the notion of developing esterases as a potential biomarker of
98 insecticide exposure and viral infection.

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99 # Materials and Methods

100 ## Esterase comparison among honey bee stocks

101 ### Esterase Assays

102 The model substrates for esterase activity (1-naphthyl acetate (1NA), *para*-
103 nitrophenyl acetate (PNPA)), Fast Blue B, sodium dodecylsulfate, and Bradford
104 Reagent were obtained from Sigma (St. Louis, MO). 1NA and PNPA were used
105 because they are model substrates that are representative of general esterase
106 and cholinesterase activity, respectively [25].

107 Esterase activity was performed according to established protocols
108 modified for a 96-well plate [26]. Bee abdomens were homogenized with a
109 disposable pestle in 1 ml of 100 mM sodium phosphate buffer (pH 7.4) in a
110 microcentrifuge tube. Samples were spun for 10 m at 4°C at 10,000g. The
111 supernatant was diluted 1:10 in 100 mM sodium phosphate buffer (pH 7.4) for use
112 in esterase and Bradford assays.

113 For 1NA endpoint assays, 20 ul of homogenate was added to a well of a
114 96-well plate (model 9017, Corning Life Sciences, Corning NY) in duplicate. Each
115 well received 200 ul of 0.3 mM 1NA (final concentration, dissolved in 100 mM
116 sodium phosphate buffer (pH 7.4)). Plates were held at room temperature (RT) for
117 15 min. Fifty ul of staining solution (0.15 g Fast Blue B dissolved in 15 ml distilled
118 water and 35 ml of 5% (w/v) sodium dodecylsulfate), and color was allowed to
119 develop for 5 m at room temperature. Plates were read at 570 nm in a

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120 Spectramax 190 with SoftMax Pro 7.0 software (Molecular Devices, Sunnyvale,
121 CA). Standard curves were run in parallel each day with 2-fold serial dilutions of
122 1-naphthol.

123 The PNPA kinetic assay [27] was performed with 20 ul of enzyme
124 homogenate added to a 96 well plate in duplicate. Control wells received 20 ul
125 of 100 mM sodium phosphate buffer (pH 7.4). Just prior to the PNPA assay, 0.1 ml
126 of 100 mM PNPA (dissolved in acetonitrile) was added to 9.9 ml of 50 mM sodium
127 phosphate buffer (pH 7.4) and vortexed 5 s. Each well received 200 ul of the
128 diluted PNPA solution (1 mM PNPA final concentration), and the changes in
129 absorbance were immediately read every 10 s for 2 m in a Spectramax 190 at 405
130 nm. PNPA activity was calculated by subtracting the average control activity
131 from the experimental samples.

132 Protein concentration was determined by the Bradford method [28] by
133 placing 10 ul of supernatant into a 96 well plate in duplicate. Each well received
134 200 ul of diluted Bradford Reagent (BioRad, Hercules CA), incubated at room
135 temperature for 5 m, and then absorbance was read in a Spectramax 190 at 595
136 nm. A standard curve was generated using serial 2-fold dilutions of bovine serum
137 albumin. Esterase activity towards 1NA and PNPA was standardized by protein
138 content.

139

140 Honey bee colonies and collections

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141 Italian, Carniolan, and Russian queens were purchased from commercial
142 breeders. Colonies were established at the USDA-ARS Honey Bee Breeding,
143 Genetics, and Physiology Lab in Baton Rouge, LA. All colonies were maintained
144 using standard management practices with no miticide applications, antibiotic
145 treatments, or supplemental feeding.

146 Frames of emerging adult worker bees were removed from colonies and
147 held at 33±1°C, 70±5% RH in continuous darkness overnight. Newly emerged adult
148 bees were sorted into groups of 20 in 475 ml wax paper cups and supplied with
149 cotton balls soaked with 50% sucrose solution (w/v). These bees were held at the
150 environmental conditions listed above until 3-days of age and then frozen at -
151 80°C until further use in esterase assays described above. A total of 30 bees (5
152 individuals from 6 colonies) for each of the 3 honey bee stocks were used in
153 esterase assays. Esterase activity levels between stocks were compared with
154 Wilcoxon-Rank Sum test with post-hoc multiple comparisons test (α=0.05) using
155 JMP (SAS, Cary, NC).

156

157 Changes in esterase activity with age

158 Colonies with normal demographics

159 Brood frames were removed from 6 colonies of Italian honey bees each
160 consisting of 2 deep boxes with brood frames and 1 medium honey super with
161 >30,000 worker bees. Adults were allowed to emerge overnight from brood
162 frames, marked with a dot of enamel paint on the notum, and returned to their

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163 respective colonies. Marked bees were collected either from inside the hive or
164 returning from a flight every 3 to 5 days up to 31-days of age. At least 5 bees were
165 collected each sampling date from each colony. Samples were frozen at -80°C
166 until used in esterase assays described above. The correlation of age and
167 esterase activity was compared with Spearman's Rank Correlation using JMP.
168

169 Correlation of esterase activity with changes in insecticide sensitivity 170 with age

171 Newly emerged adult bees were marked with enamel paint and returned
172 to source colonies as described above. A total of 10 source colonies were used.
173 Bees were collected at 3-, 14-, 21-, and 28-days of age in groups of 10 into wax
174 paper cups covered with nylon tulle secured with a rubber band. Topical
175 bioassays with phenothrin (98.4% purity, ChemService, West Chester PA) and
176 naled (99.0% purity) were performed as previously described [13]. Bees were
177 anaesthetized with CO_2 for <30s and a 1 ul drop of insecticide was applied to the
178 notum with a Hamilton repeating syringe. Control bees were treated with
179 acetone. Treated bees were provided a cotton ball soaked with 50% sucrose
180 solution and held in an incubator under the environmental conditions listed
181 above. At least 1 rep at each dose was used from each colony on each
182 collection day. A subsample of 8 bees was collected at each collection date and
183 stored at -80°C until used in esterase assays described above. The LC_{50} was
184 calculated using Probit analysis with Abbott's correction for control mortality [29]

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185 in Minitab (State College, PA) and expressed in units of ug insecticide/bee. The
186 correlation of esterase activity with age and insecticide sensitivity was compared
187 with Spearman's Rank Correlation using JMP.
188

189 Insecticide inhibition of esterase activity

190 Clothianidin bioassay

191 Clothianidin is a neonicotinoid insecticide that is widely used as a seed
192 treatment for corn and soy beans. It is frequently found in honey bee colonies
193 and may have detrimental impacts on honey bees [30, 31]. The LC_{50} for
194 clothianidin (99.5% purity, ChemService, West Chester PA) was determined by a
195 feeding bioassay according to previously published methods [13]. Newly
196 emerged adult Italian honey bees from 3 colonies were sorted into groups of 20
197 in 475 ml wax paper cups and held at 33±1°C with 70±5% RH in continuous
198 darkness until 3-days of age. Bees were fed 50% sucrose solution (w/v) containing
199 concentrations of clothianidin that produced >1% and <99% mortality *ad libitum*
200 through a perforated microcentrifuge tube. Mortality was recorded after 24 h. The
201 LC_{50} was calculated using Probit analysis with Abbott's correction for control
202 mortality [29] in Minitab (State College, PA) and expressed in units of ng
203 clothianidin/ml.
204

205 *In vivo* esterase inhibition

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206 Newly emerged adult Italian honey bees from 6 colonies were collected
207 and aged to 3-days of age as described above. An experimentally-determined
208 maximum-sublethal treatment of phenothrin (0.1 ug/bee, topical [13]), naled
209 (0.066 ug/bee, topical [13]), or clothianidin (2.15 ng/ml, feeding, from above
210 experiment) was administered to these bees. To determine the dose-
211 dependence of esterase inhibition, sublethal doses of naled (i.e., 0.05, 0.033, and
212 0.025 ug/bee) were applied to 3-day old bees in a separate experiment. Control
213 bees were treated with 1 ul of acetone for control topical bioassays or 50%
214 sucrose solution with 0.001% acetone for feeding bioassays. Bees were collected
215 at 24 hours after treatment and frozen at -80°C until use in esterase assays
216 described above. Esterase activity data from insecticide exposed bees were
217 compared with Wilcoxon-Rank Sum Test with post-hoc multiple comparisons test
218 (α=0.05) using JMP. The correlation of naled doses and esterase activity was
219 compared with Spearman's Rank Correlation using JMP.
220

221 Impacts of Varroa mite and virus on esterase activity

222 Varroa mite infestation

223 Frames of emerging adults were removed from 4 colonies of Italian bees
224 that showed no symptoms of viral infection. Emerging adults and the associated
225 brood cells from which they emerged were examined for Varroa mite infestation
226 by a single foundress [32]. Varroa mite infested and uninfested adults were
227 collected into separate wax paper cups provisioned with a cotton ball saturated

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228 with 50% sucrose solution. These bees were held in an incubator at 33±1°C with
229 70±5% RH in continuous darkness until 3-days of age, then stored at -80°C until
230 used in esterase assays as described above. Esterase activity from Varroa mite
231 infested bees was compared with Wilcoxon-Rank Sum Test using JMP.
232

233 **Virus injection**

234 Solutions of *Deformed wing virus* (DWV), *Chronic bee paralysis virus* (CBPV),
235 and *Black queen cell virus* (BQCV) were semi-purified by grinding 10 symptomatic
236 adult bees (DWV, CBPV) or larvae (BQCV) in phosphate buffered saline (PBS) and
237 centrifuged in a 15 mL tube at 4,700g for 20 m at 4°C. The supernatant was then
238 filtered through a 0.2 um syringe filter and maintained at until 4°C use within a
239 week following standard protocols [33]. Viral titers were determined using
240 standard curves generated from plasmid standards containing the sequence
241 listed above (generated by GeneArt, Invitrogen). Linearized plasmid standards
242 containing 10^5 to 10^{12} copies per reaction were used as templates to assess primer
243 efficiency and quantify the amount of virus following standard practices [34-36].
244 Linear standard equations were generated using the $\log_{10}$ of the initial plasmid
245 copy number. The genomic region encoding a capsid protein for each of the
246 viruses was as follows: DWV Forward— GAG ATT GAA GCG CAT GAA CA and
247 Reverse— TGA ATT CAG TGT CGC CCA TA (AY292384.1, [37]); CBPV Forward—
248 CGC AAG TAC GCC TTG ATA AAG AAC and Reverse—ACT ACT AGA AAC TCG

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249 TCG CTT CG (EU122229.1, [38]); BQCV Forward—TTT AGA GCG AAT TCG GAA
250 ACA and Reverse— GGC GTA CCG ATA AAG ATG GA (HQ655494.1, [37]).
251 Newly emerged adult bees (<1 h) from 3 colonies were collected into a 20
252 ml scintillation vial and chilled on ice. Bees were injected between the dorsal
253 abdominal tergites with 3 ul of semi-purified virus (DWV @ 10^7 copies/ul; CBPV @
254 10^4 copies/ul; BQCV @10^7 copies/ul) using a Hamilton syringe fitted with a 30G
255 needle at an infusion rate of 1ul/sec using a Micro4TM Microsyringe Pump
256 Controller adapted from standard methods [33, 39]. Two sets of control bees were
257 either uninjected or injected with PBS. Adults were collected in to a wax paper
258 cup provisioned with a cotton ball with 50% sucrose solution and held in an
259 incubator at 33+1°C with 70+5% RH in continuous darkness until 3-days of age.
260 Survivors were collected and stored at -80°C until used in esterase assays as
261 described above. Esterase activity in virus-injected bees was compared via One-
262 Way ANOVA with Tukey's HSD post-hoc test using JMP.

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263 # Results

264 ## Esterase activity varies among honey bee stocks

265 Carniolan and Italian honey bees exhibited significantly higher esterase
266 activity towards 1NA (χ^2=10.4, df=2, p<0.01, Fig 1) compared to Russian honey
267 bees. Italian honey bees showed significantly higher esterase activity towards
268 PNPA compared to both Carniolan and Russian honey bees, and Carniolan
269 honey bees had significantly higher activity compared to Russian honey bees
270 (χ^2=17.5, df=2, p<0.01, Fig 1).

271
272 **Fig 1. Esterase activity towards 1NA and PNPA from Carniolan, Italian, and**
273 **Russian honey bees.** The bar with an * indicate significant differences between
274 stocks. Data are the average ± SEM.
275

276 ## Changes in esterase activity with age

277 ### Esterase activity positively correlates with honey bee age

278 Esterase activity towards both 1NA and PNPA was significantly correlated
279 with age in honey bees in colonies with normal demographics (1NA ρ=0.86, df=13
280 p<0.01, Fig 2A; PNPA ρ=0.91, df=13, p<0.01, Fig 2B).

281
282 **Fig 2. Changes in esterase activity with age.** Esterase activity towards 1NA (A) and
283 PNPA (B) significantly increased with age. Data are the average ± SEM.
284

285 ### Changes in insecticide sensitivity with honey bee age do not correlate
286 ### with esterase activity

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287 Sensitivity to phenothrin did not significantly decrease with age in honey
288 bees from colonies with normal demographics (ρ=0.80, df=3, p=0.20; Fig 3A), but
289 older bees (i.e. 21- and 28-day old bees) were significantly less sensitive to
290 phenothrin than younger bees (i.e. 3- and 14-day old bees). Naled sensitivity
291 significantly increased with age in honey bees from colonies with normal
292 demographics (ρ=-1.00, df=3, p<0.01; Fig 3B). Phenothrin sensitivity did not
293 correlate with esterase activity towards 1NA (ρ=0.00, df=3, p=1.0; Fig 4A) or PNPA
294 (ρ=0.80, df=3, p=0.20; Fig 4C). There was a significant negative correlation of
295 naled sensitivity with esterase activity towards PNPA (ρ=-1.00, df=3, p=<0.01 Fig
296 4D), but not 1NA (ρ=-0.40, df=3, p=0.60 Fig 4B)
297

298 **Fig 3. Changes in insecticide sensitivity with age in honey bees from colonies**
299 **with normal demographics.** Sensitivity to phenothrin decreased with age (A),
300 while sensitivity to naled increased with age (B). Letters indicate significant
301 differences in insecticide sensitivity at different ages. Data are the average $\pm$
302 95% CI.
303
304

305 **Fig 4. Correlation of esterase activity and insecticide sensitivity.** Esterase activity
306 towards 1NA was not correlated with sensitivity to phenothrin (A) or naled (B).
307 There was no correlation of esterase activity towards PNPA and phenothrin
308 sensitivity (C), but it was significantly correlated with naled sensitivity (D). Data
309 are shown as the LD_{50} $\pm$ 95%CI.
310

311 *In vivo* esterase inhibition by insecticides

312 Determination of maximum sublethal clothianidin concentration

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313 The LC_{50} for clothianidin was 132.6 ng/ml (Table 1). The ratio of the LC_{90} to
314 LC_{10} was 92-fold. Based upon these results, the maximum sublethal clothianidin
315 concentration was calculated to be 2.1 ng/ml and verified by bioassays.

316 **Table 1. Clothianidin toxicity to Italian honey bees.** The LC values are expressed in
317 ng clothianidin/ml sucrose solution. Values in parenthesis represent the 95%
318 confidence interval and standard error for the LC values and slope, respectively.
319

Compound	n	LC_{10}	LC_{50}	LC_{90}	Slope
Clothianidin	1052	13.8 (6.9-22.5)	132.6 (103.9-160.3)	1276.2 (986.4-1813.2)	1.3+(0.1)

320

321 Sublethal insecticide exposure in vivo esterase inhibition varies with 322 insecticide class

323 Both 1NA and PNPA activities were significantly inhibited by application of
324 sublethal dose of naled (1NA Z=-3.03, $p<0.01$; PNPA Z=-6.05, $p<0.01$, Fig 5).
325 Exposure to sublethal treatments of phenothrin or clothianidin did not significantly
326 affect 1NA or PNPA activity 24 hours post treatment (Fig 5). Further application of
327 lower sublethal doses of naled resulted in dose-dependent inhibition of 1NA (ρ=-
328 0.82, df=7, p=0.02) and PNPA activity (ρ=-0.75, df=7, p=0.05, Fig 6). Inhibition of
329 PNPA activity was greater than inhibition of 1NA activity at 0.05 ug/bee (χ^2=12.5,
330 df=1, $p<0.01$) and 0.066 ug/bee (χ^2=13.6, df=1, $p<0.01$; Fig 6).

331

332 **Fig 5. Effect of sublethal treatments of insecticides on esterase activity.** Capital
333 and lower case letters indicate significant differences in 1NA and PNPA activity,
334 respectively

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335 **Fig 6. Relative inhibition of esterase activity towards 1NA and PNPA to sublethal**
336 **doses of naled.** Capital and lower case letters indicate significant differences in
337 1NA and PNPA activity, respectively. The asterisk indicate significant differences
338 between substrates at respective naled doses.
339

340 Viruses but not Varroa affect esterase activity

341 Varroa mite infestation does not affect esterase activity

342 Esterase activity towards 1NA (χ^2=0.30, df=1, p=0.58, Fig 7) or PNPA (χ^2=0.28,
343 df=1, p=0.59, Fig 7) was not affected by Varroa mite infestation, as honey bee
344 that pupated with a single Varroa mite feeding on them exhibited no differences
345 in esterase activity compared to bees that developed without Varroa infestation.
346

347 **Fig 7. Varroa mite infestation does not affect esterase activity towards 1NA or**
348 **PNPA.** Data are shown as average ± SEM.
349

350 Viral infection reduces esterase activity

351 Esterase activity towards 1NA significantly decreased in bees injected with
352 BQCV, CBPV, and DWV relative to both uninjected and PBS-injected controls
353 (F=18.8, df=12, p<0.01, Fig 8). PNPA activity was significantly reduced in bees
354 injected with BQCV and CBPV compared to both uninjected and PBS-injected
355 controls. DWV-injected bees had lower PNPA activity compared to uninjected
356 controls (F=19.7, df=12, p<0.01, Fig 8).

357 **Fig 8**. **Viruses reduce esterase activity.** Honey bees injected with BQCV (n=12),
358 CVPV (n=16) or DWV (n=16) have significantly reduced esterase activity relative
359 to uninjected (n=16) or PBS-injected controls (n=12). Capital and lower case
360 letters indicate significant differences in esterase activity towards 1NA and PNPA,
361 respectively. Data are the average ± SEM.

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362 # Discussion

363 Our research shows plasticity in honey bee esterase activity due to a wide
364 variety of life-history traits and external pressures. These physiological differences
365 may underlie the wide range in the reports of honey bee pesticide sensitivity [16,
366 17] as well as emphasizing the need for detailed descriptions of the insecticide
367 bioassay conditions in order to produce data that is comparable among
368 researchers. The results we report on factors affecting esterase activity in honey
369 bees are concepts that are easily applicable to other physiological systems such
370 as immune function, nutrition utilization, and development where experimental
371 conditions may dramatically affect the results. Honey bee colonies are complex
372 and dynamic systems that are highly adaptable to changes in foraging resources,
373 pathogen infection, parasite infestation, and pesticide exposure in particular.
374 Understanding the physiological basis of how honey bees mediate these stresses
375 allows for improved colony management strategies to promote honey bee
376 colony health.
377

378 ## Esterase activity does not correlate with insecticide sensitivity

379 We utilized 1NA and PNPA because they are model substrates that are
380 indicative of general esterase and choline esterase activity, respectively [25, 40].
381 However, esterase activity towards the model substrates 1NA and PNPA may not
382 reliable surrogates of esterase activity towards most insecticides in honey bee. It

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383 is possible that the activity of esterases capable of detoxifying organophosphates
384 (OPs) cannot be assessed using the model substrates we employed. For example,
385 a mutation in the E3 esterase of OP resistant strains of the sheep blowfly confers
386 hydrolase activity towards the OP chlorfenvinphos while losing the ability to
387 metabolize the model substrates 1NA and PNPA [8]. The difficulties of connecting
388 insecticide sensitivity and esterase activity towards model substrates has been
389 especially noted in OP resistant mosquitoes [41-43]. Thus, identification of honey
390 bee-specific esterase substrates (including the insecticide itself) and inhibitors are
391 urgently needed to accurately assess the metabolic contribution of esterases
392 toward insecticide sensitivity.

393 The high esterase activity in Italian honey bees was an unexpected result
394 since this stock of honey bees was the most sensitive to many insecticides and
395 esterase inhibition produced the lowest level of synergism in phenothrin bioassays
396 [13]. Comparison of esterase activity generated here with previously reported
397 LD_{50}/LC_{50} values among honey bee stocks [13] suggest variable roles for esterases
398 in insecticide sensitivity. Although data points were limited in our previous study
399 (and thus unable to be statistically analyzed appropriately), there was a positive
400 association of esterase activity towards 1NA and PNPA with the LD_{50} of malathion
401 among honey bee stocks. This is consistent with esterase-mediated detoxification
402 of malathion [44]. There was no correlation with esterase activity towards 1NA and
403 PNPA with the LD_{50} values of naled, etofenprox, resmethrin, or imidacloprid [13],
404 suggesting other factors besides esterases are more important for explaining

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405 differential sensitivity to these insecticides among these honey bee stocks [45, 46].
406 Interestingly, esterase activity negatively correlated with the LD_{50}s of phenothrin
407 and thiamethoxam [13], suggesting that esterases may bioactivate these
408 compounds to more toxic metabolites. However, the role of esterases in
409 bioactivation of these compounds would be unusual as esterases are likely
410 responsible for phenothrin detoxification [47], and P450s are responsible for
411 bioactivation of thiamethoxam [48].

412 Comparison of the levels of esterase inhibition suggests a secondary role of
413 esterases in determining phenothrin sensitivity. Italian honey bees had the highest
414 levels of esterase activity towards PNPA but the lowest of level of synergism in
415 phenothrin bioassays when the maximum sublethal dose of coumaphos was used
416 to inhibit esterase activity [13]. However, this assumes that coumaphos provided
417 similar levels of esterase inhibition among honey bee stocks and that coumaphos
418 inhibits the esterases that are involved in phenothrin detoxification. Future studies
419 on esterase inhibition with different inhibitors will help determine the types of
420 esterase that contribute to activity towards these substrates and if there is any
421 differences in the effectiveness of these inhibitors among honey bee stocks.

422 A third line of evidence that suggests a diminished role of esterases in
423 insecticide detoxification is shown in the current study with the lack of correlation
424 of esterase activity with phenothrin sensitivity and the negative correlation with
425 naled sensitivity as bees aged. While esterases are important for phenothrin
426 sensitivity in 3-day old bees [13, 14], the lack of correlation of esterase activity and

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427 phenothrin sensitivity with age suggests that other factors (i.e. P450s [14, 49]) may
428 underlie the changes in phenothrin sensitivity with age. The negative association
429 of esterase activity with naled sensitivity suggests esterase activity bioactivated
430 naled. Bioactivation of OPs is typically accomplished via P450-mediated
431 conversion of a thiophosphate to the active oxon species [50]. However, naled
432 does not possess a thiophosphate. Therefore, bioactivation of naled by esterases
433 is very unlikely due to its chemical structure. These results suggest that other factors
434 besides esterases are important for determining the increased naled sensitivity
435 with age in honey bees. Taken together, findings from current and previous work
436 suggest that esterases activity as measured by metabolism of model substrates
437 may play a secondary role in determining pesticide sensitivity [14].
438

439 Esterase activity increases with honey bee age

440 Our results show that esterase activity increases with age in honey bees. This
441 is consistent with the increase in cytochrome P450 and glutathione-S-transferase
442 activities with age documented in honey bees [49, 51]. Sensitivity decreases to
443 the pyrethroid, phenothrin, with age, while sensitivity increases to the OP, naled
444 (Fig 3; [13]). Both P450s and esterases are involved in determining phenothrin
445 sensitivity [13]. However, the lack of correlation of esterase activity with phenothrin
446 sensitivity suggests that other factors, such as P450s, may be more important than
447 esterases for causing the changes in phenothrin sensitivity with age. The increase

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448 in naled sensitivity with age is contradicted by the increase in esterase activity
449 with age.

450 Previous work that showed that decrease in phenothrin sensitivity and an
451 increase in naled sensitivity as honey bees aged in single cohort colonies, which
452 are comprised of bees that are of the same chronological age but shift
453 physiologically to conduct the different tasks needed for a functioning hive that
454 would typically be divided across bee ages (e.g. feeding larvae vs. foraging) [13].
455 Those results are consistent with the results reported here for colonies with normal
456 demographics (Fig 3). The similar changes in esterase activity and insecticide
457 sensitivity with age in both types of colonies suggest that altered colony
458 demographics do not affect insecticide sensitivity under our experimental
459 conditions. It also suggests that chronological age is more significant than task
460 (e.g. in-hive worker vs. forager) in determining sensitivity to insecticides, which is
461 significant for toxicological bioassays.

462

463 Esterase inhibition by insecticides

464 Clothianidin sensitivity

465 The LC_{50} value for clothianidin obtained with Italian honey bees was the
466 same as the LC_{50} values for thiamethoxam reported in previous studies [13, 52].
467 Thiamethoxam must be bioactivated by cytochrome P450s [48] into clothianidin
468 [53]. The similar LC_{50} values for thiamethoxam and clothianidin suggest that honey
469 bees have a high metabolic capacity for this particular bioactivation. However,

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470 this phenomenon appears to be a common process as many other insects also
471 possess similar LC_{50} values for thiamethoxam and clothianidin [53-57].
472

473 Esterase inhibition is insecticide-dependent

474 The patterns of esterase inhibition by exposure to insecticides were
475 expected based on their respective target sites. Naled is an OP that inhibits
476 acetylcholinesterase activity, and most honey bee esterase activity is performed
477 by choline esterases [40], therefore, it is not surprising naled inhibits esterase
478 activity towards these model substrates. Since mortality occurs at doses of naled
479 >0.066 ug/bee, it appears that inhibition of 26% and 42% of esterase activity
480 towards 1NA and PNPA, respectively, results in mortality. Esterases significantly
481 influence phenothrin sensitivity [13]. However, at the experimentally determined
482 sublethal dose, phenothrin did not affect esterase activity towards 1NA or PNPA.
483 Esterase activity was not affected by clothianidin exposure. This result is expected
484 because studies on the effects of esterase inhibitors on sensitivity to clothianidin
485 (or thiamethoxam) in honey bees have not been reported and these compounds
486 have no ester bonds. Reports in which thiamethoxam (which is bioactivated *in*
487 *vivo* to clothianidin [53]) has been shown to induce or inhibit esterase activity at
488 concentrations near the LC_{50} [58], or at concentrations lower than the LC_{50} that
489 would still result in low levels of mortality [11]. Our study indicates that esterases
490 are not significantly inhibited *in vivo* at the much lower experimentally-
491 determined sublethal concentrations of clothianidin that we employed here.

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492 While other studies have shown altered esterase activity to insecticide exposure
493 at levels higher than the experimentally-determined sublethal levels we used here
494 [11, 58], it is likely that mortality would be a more definitive and more convenient
495 measure of insecticide exposure.
496

497 Viruses transmitted by Varroa, but not Varroa infestation, 498 impair esterase activity

499 Varroa infestation during the honey bees' development did not alter
500 esterase activity when they emerged as adults. Honey bees that have been
501 infested by Varroa mites have smaller body size [32] and reduced expression of
502 genes involved in metabolic detoxification [59, 60]. Our results, however, show no
503 effect of Varroa mite infestation on esterase activity. This is consistent with the lack
504 of change in insecticide sensitivity with varying Varroa mite infestation at the
505 colony level [61]. Therefore, it appears that single-foundress Varroa infestation on
506 its own may have little impact on pesticide sensitivity. However, the significant
507 reduction in esterase activity by injection of virus shows that Varroa infestation
508 can indirectly affect esterase activity as a disease vector. It is well known that viral
509 infection can affect pesticide sensitivity [20, 24], and our results demonstrate that
510 viruses may have significant effects on detoxification capacity. Future
511 experiments with varying levels of viruses as well as focused investigation on the

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512 expression of genes involved in metabolic detoxification will demonstrate the
513 impacts of viruses on honey bee health.
514

515 Conclusions

516 This study demonstrates that honey bee esterase activity is very dynamic
517 and significantly influenced by honey bee stock, age, insecticide exposure, and
518 viral infection. Our results suggest a diminished or secondary role of esterases in
519 determining insecticide sensitivity and that esterase activity toward model
520 substrates does not accurately represent esterase activity towards insecticides.
521 However, development of low-cost, high throughput assays using the insecticide
522 as the esterase substrate would yield unambiguous results on the importance of
523 esterases in determining insecticide sensitivity. The utility of using esterase activity
524 towards model substrates as biomarkers of insecticide exposure should be
525 pursued further and validated in order to be used as an accurate diagnostic tool.
526 Despite these findings, reducing the quantity of insecticides as well as cautious
527 and accurate application of insecticides near honey bee colonies as well as in
528 foraging areas can reduce the potential negative impacts of insecticides on
529 honey bee colony health. Besides potential effects from insecticides, honey bees
530 are confronted with the significant and realistic problems of Varroa mites [62],
531 introduced pathogens [63], loss of foraging area [64], and reduced queen health
532 [65] and the complex interactions among all of these factors may work in concert
533 to contribute to poor colony performance and productivity.

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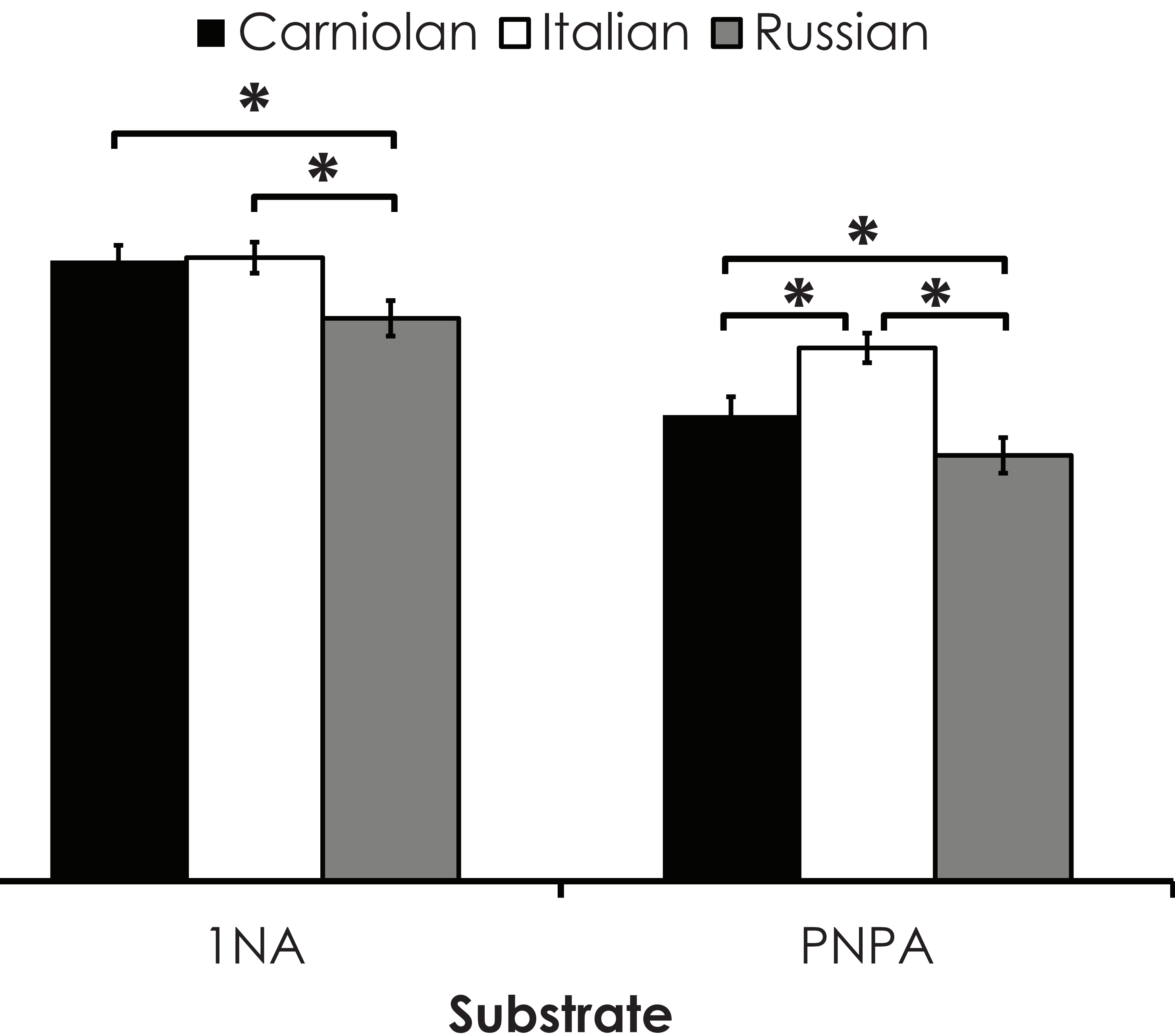

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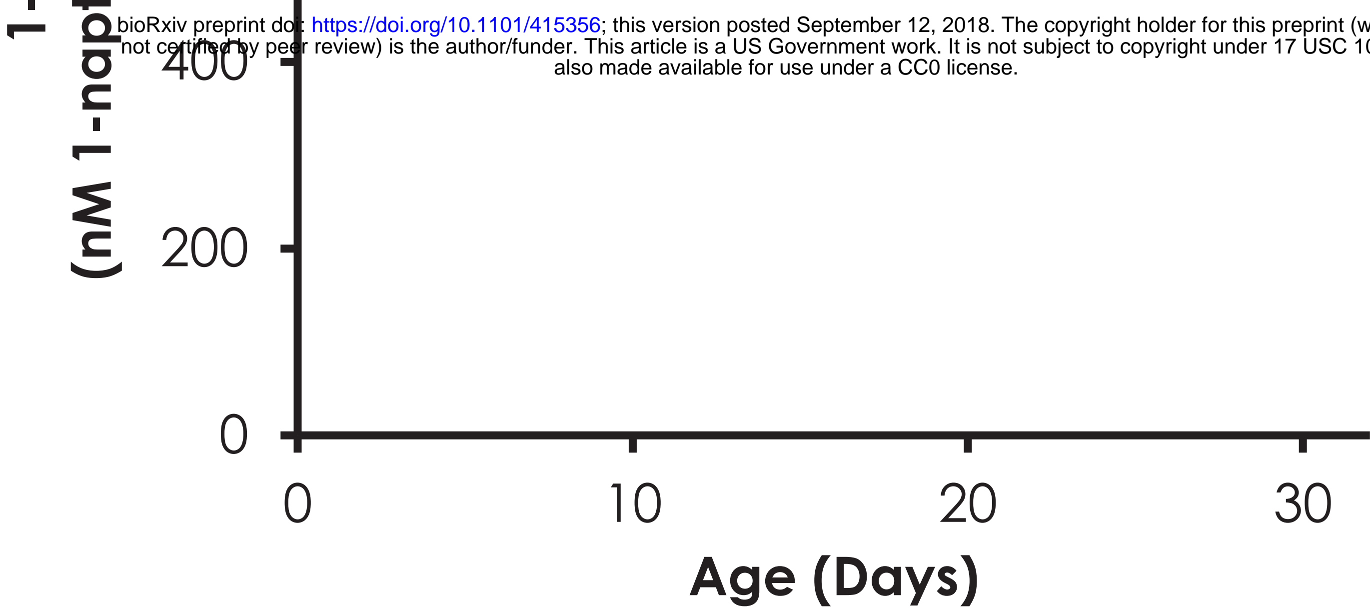

B

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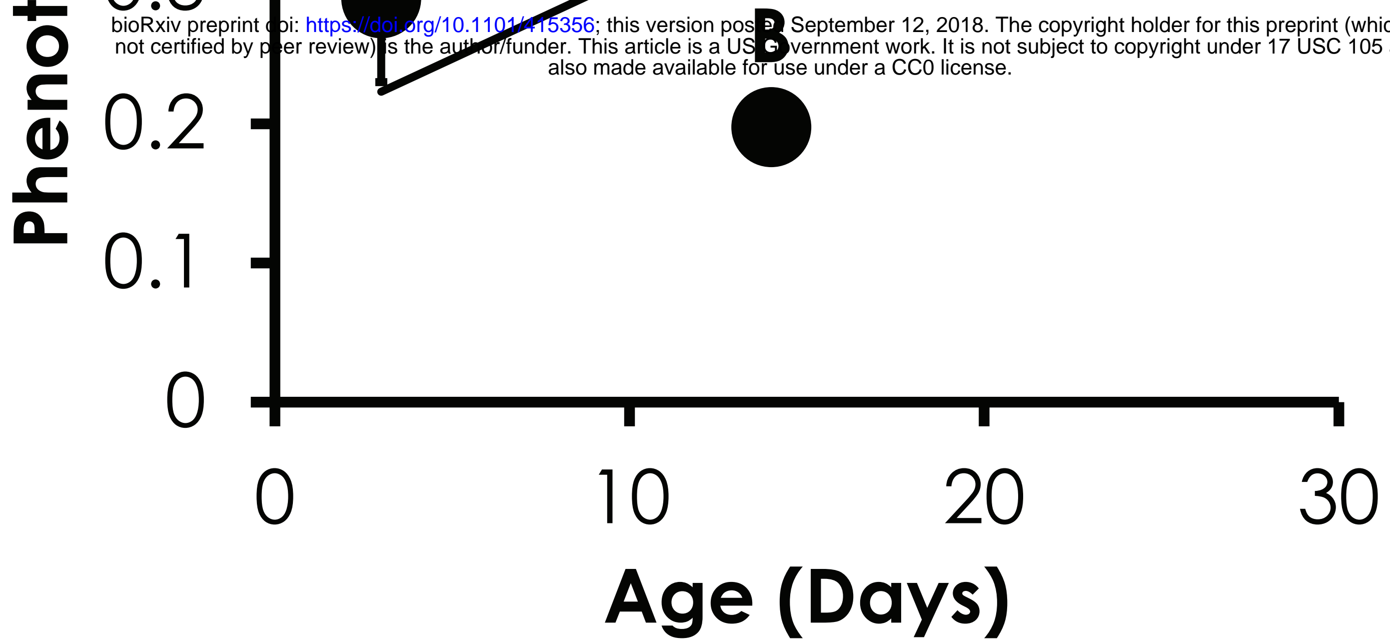

B

A
B
C
C

0.14
0.12
0.1
0.08
0.06
0.04
0.02
0

Naled LD_{50} (ug/bee)

0
10
20
30

Age (Days)

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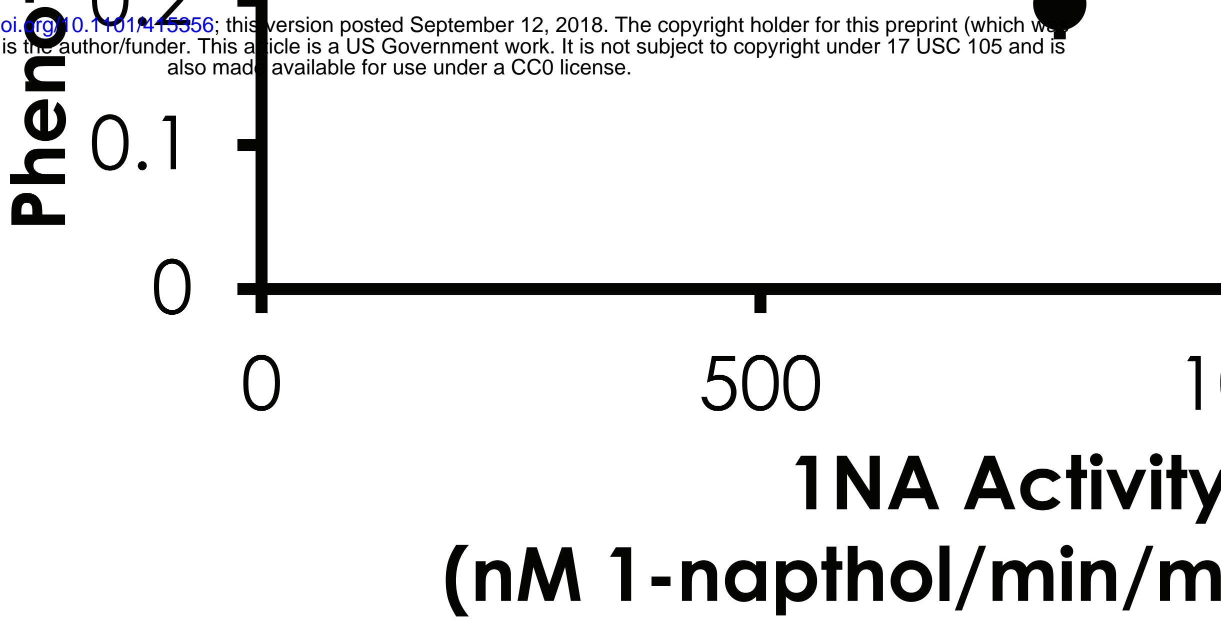

PNPA

C

Phenothrin LD_{50} (ug/bee)

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0

0
200

PNPA Activit

(nM 1-nitrophenol/min/

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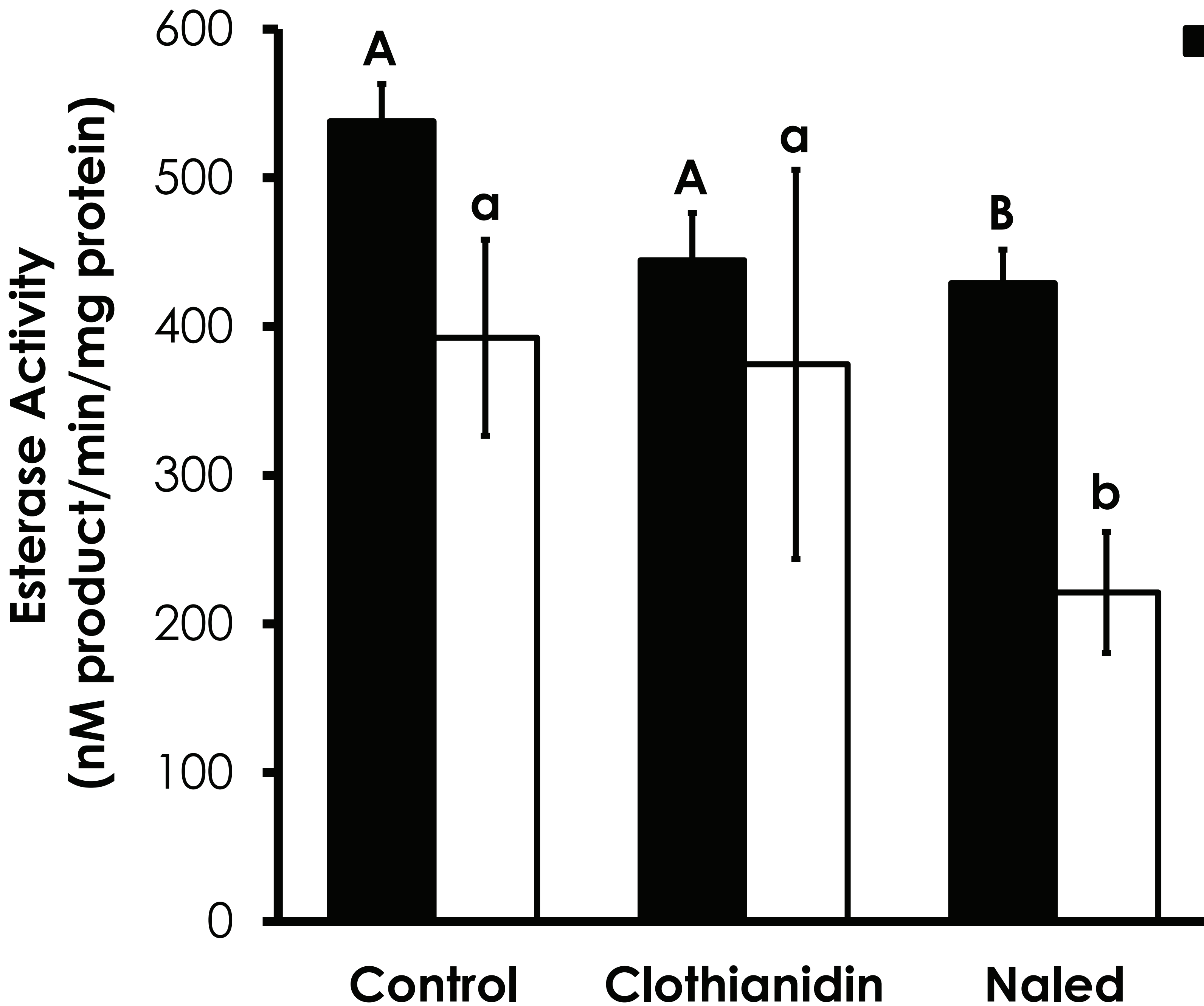

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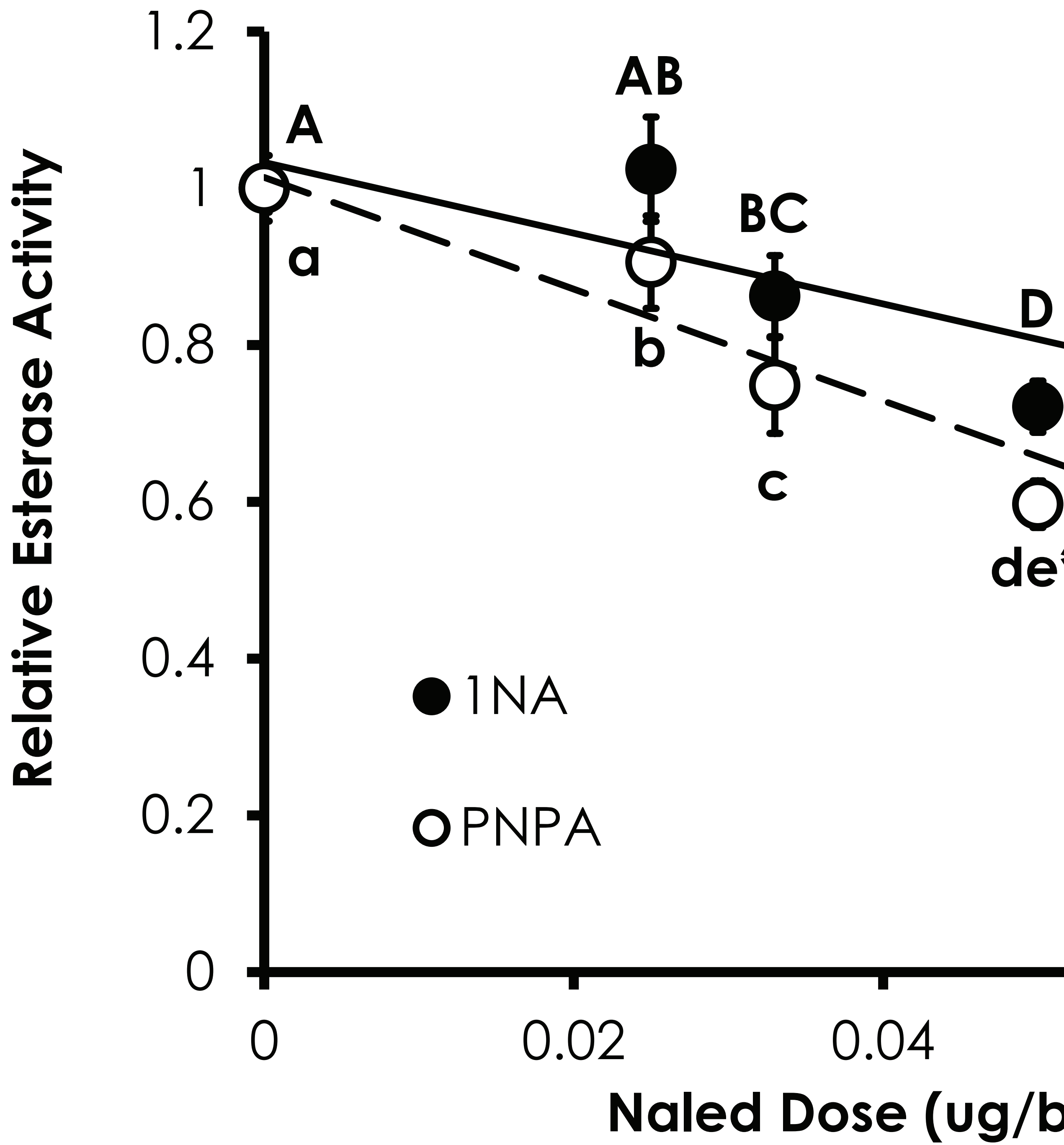

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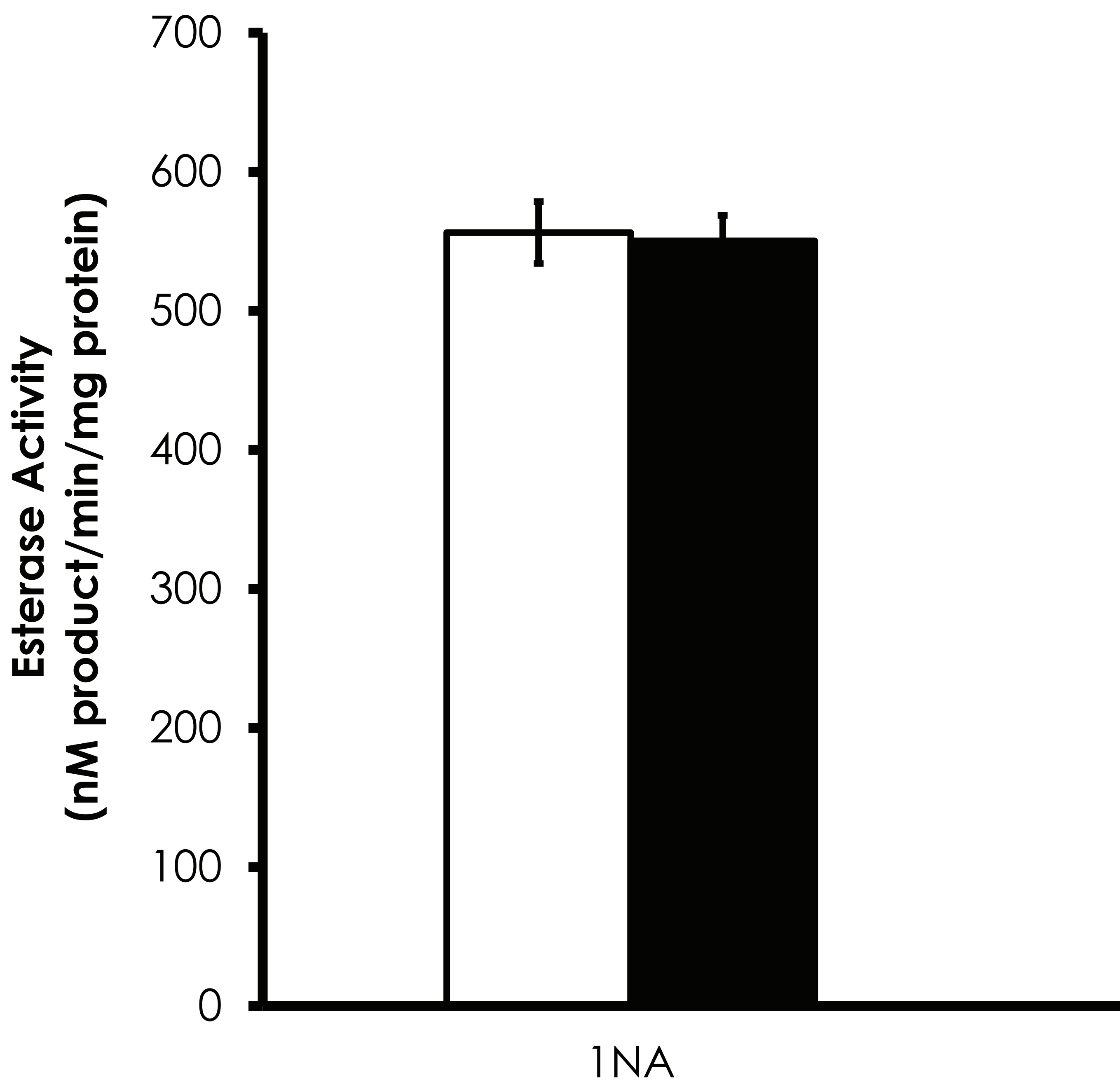

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