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1 No evidence of an immune adjustment in response to a parasitoid

2 threat in *Lobesia botrana* larvae

3
4 Fanny VOGELWEITH¹, Yannick MORET², Denis THIERY³, Lionel DELBAC³, and Jérôme
5 MOREAU²

6
7 (1) Johannes-Gutenberg University of Mainz, Institute of Organismic and Molecular Evolution,
8 Behavioral Ecology and Social Evolution Group, Johannes-von-Müller-Weg 6, 55128 Mainz,
9 Germany

10 (2) Université de Bourgogne, Equipe Ecologie-Evolution, UMR 6282 Biogéosciences, 6 Bd
11 Gabriel, 21000 Dijon, France

12 (3) Institut National de la Recherche Agronomique (INRA), UMR 1065 Santé et Agroécologie
13 du Vignoble (SAVE) Institut des Sciences de la Vigne et du Vin (ISVV), 71 avenue Edouard
14 Bourlaux, 33883 Villenave d'Ornon Cedex, France

15
16 **Corresponding author:** Fanny Vogelweith, fanny.vogelweith@gmail.com

17 Johannes Gutenberg-Universität Mainz

18 Johannes-von-Müller-Weg 6

19 55128 Mainz, Germany

20 Tel: +49 6131 39-27853

21 Fax: +49 6131 39-27850

22

23

24 **Abstract**

25 Immune function is a key determinant of an organism's fitness, and natural insect
26 populations are highly variable for this trait, mainly due to environmental heterogeneity and
27 pathogen diversity. We previously reported a positive correlation between infection prevalence
28 by parasitoids and host immunity in natural populations of the vineyard pest *Lobesia botrana*.
29 Here, we tested whether this correlation reflects a plastic adjustment of host immunity in
30 response to the local presence of parasites. To this end, we measured immunity of non-
31 parasitized *L. botrana* larvae exposed, respectively, to one of the two most common species of
32 parasitoids in vineyards, over 6 days. Larvae were able to sense the parasitoid through visual,
33 chemical, or mechanical cues, but contact larvae-parasitoid were excluded. Contrary to our
34 hypothesis, we found that *L. botrana* larvae did not increase their immune defenses in the
35 presence of parasitoids, despite their ability to sense a potential threat. Our results therefore
36 suggest that the positive correlation between infection prevalence by parasitoids and *L. botrana*
37 immunity among natural populations may result from micro-evolutionary changes resulting
38 from long-term local selection pressures imposed by parasitoids in wild populations rather than
39 plastic adjustments of immunity.

40

41 **Key-words:** *Campoplex capitator*, insect immunity, grapevine moth, *Phytomyptera nigrina*,
42 plasticity, prophylaxis.

43

44 **1. Introduction**

45 Animals live in dynamic environments and face variation in resource availability,
46 climate and risk of infection over their whole lifespan. Thus, to survive and reproduce
47 successfully, these organisms must allocate resources among competing physiological systems,
48 such as immunity and growth (van der Most et al., 2011), to maximize fitness in changing
49 environments. Immunity is one of the major physiological mechanisms regulating host survival
50 (Lochmiller and Deerenberg, 2000). In insects, an important part of this defense relies on non-
51 specific and constitutive mechanisms that involve the coordinated action of hemocytes and the
52 phenoloxidase (PO) system (Siva-Jothy et al., 2005). Hemocytes are immune cells circulating
53 in the hemolymph involved in the recognition and encapsulation of pathogens (Lavine and
54 Strand, 2002). Conversely, PO mostly mediates the melanization of foreign objects and operates
55 through the activation of the prophenoloxidase (PPO) cascade, its inactive precursor typically
56 stored in the hemolymph and the hemocytes (Cerenius and Söderhäll, 2004).

57 Insect immunity is associated with inherent costs because it requires energy to build up,
58 maintain and use (Armitage et al., 2003), and thus reduces an individual's ability to invest into
59 other physiological systems. Moreover, the activation of insect immunity produces chemical
60 substances that are harmful for the producer and induce cumulative damage in its body
61 (González-Santoyo and Córdoba-Aguilar, 2012; Nappi and Ottaviani, 2000). For this reason,
62 individuals are expected to flexibly adjust their investment into the immune system to find an
63 optimized balance between their ability to fight off pathogens on the one hand, and saving
64 energy as well as limiting the accumulation of toxic immune components in their body on the
65 other hand. Organisms can indeed be exposed to different threats such as parasites and
66 parasitoids, which can vary among and between populations, as reported in *Drosophila*
67 *melanogaster* (Kraaijeveld and Alphen, 1995; Tinsley et al., 2006; Corby-Harris and
68 Promislow, 2008), *Gammarus pulex* (Cornet et al., 2009) and *Lobesia botrana* (Moreau et al.,

69 2010; Vogelweith et al., 2013a). These different threats induce substantial variation in the
70 investment in immunity within and among wild populations (Corby-Harris and Promislow,
71 2008; Cornet et al., 2009; Vogelweith et al., 2013a). For example, recent investigations among
72 natural populations of the grapevine moth (*L. botrana*) revealed that levels of immune defenses
73 in larvae were positively correlated to parasitoid infection prevalence (Vogelweith et al.,
74 2013a). At a geographical scale, populations with high levels of immune defenses in non-
75 parasitized larvae (high hemocyte concentrations, PPO enzyme activities and antimicrobial
76 peptides) were also those exhibiting the highest rates of infection by parasitoids (Vogelweith et
77 al., 2013a).

78 Although this confirms that parasitoid pressure strongly affects host immune investment
79 (Bryan-Walker et al., 2007; Corby-Harris and Promislow, 2008), the exact cause of such a
80 positive relationship remains unknown. Two non-mutually exclusive selective pressures could
81 shape and drive such a variation in immune traits. First, parasites can mediate a local selection
82 response in favor of (constitutively expressed) high levels of immune defense among hosts,
83 which would result in positive covariation between parasite prevalence and immune defense
84 (Lindström et al., 2004; Kalbe and Kurtz, 2006; Tschirren and Richner, 2006). Alternatively,
85 such a positive correlation could arise if hosts plastically adjust their immune response to the
86 (locally varying) risk of infection, since the ability to sense the current risk of parasitism through
87 visual, chemical, or mechanical cues has been reported in some insect species (Peacor, 2003;
88 Fievet et al., 2008). For example, when the risk of parasitism increases, insects show
89 prophylactic investment in their immune defense even in the absence of parasites (Barnes and
90 Siva-Jothy, 2000; Wilson and Reeson, 1998). The velvetbean caterpillar (*Anticarsia*
91 *gemmatalis*) increased investment in primary defense barriers against parasites, such as the
92 midgut, in response to increased conspecific density and an increased risk of infection (Silva et
93 al., 2016). Such a plastic modulation of immune defenses can be rapid as reported in adult

94 bumble-bee workers (*Bombus terrestris*) (Ruiz-González et al., 2009). A plastic investment in
95 immunity is then likely to result in positive covariation between parasite prevalence and
96 immune defense. To understand how parasitoids can modulate insect immunity, it is important
97 to know which of the two above detailed selective pressures shapes investment into insect
98 immunity.

99 To our knowledge, no study experimentally investigated how insect can plastically
100 modulate their immune defenses in response to parasitoid pressure. Here, we test the hypothesis
101 whether the presence of parasitoids in the local environment induces a plastic increase of the
102 investment in immune defense. To this end, we exposed *L. botrana* larvae to either parasitoids
103 (excluded physical contacts), or no parasitoid for a period of six days and measured immune
104 parameters (hemocyte concentration and PPO activities system) on the last day of exposure. If
105 *L. botrana* larvae indeed flexibly adjust their immunity to the parasitoid threat, we expected an
106 increase of all immune parameters in the presence of parasitoids. In this scenario, the larvae of
107 *L. botrana* would assess cues related to the presence of parasitoids and adjust their investment
108 in immunity to match any increased threat of infection. *L. botrana* appears to be a good
109 candidate because (1) a variation of larval immune parameters with the parasitoid pressure has
110 already been reported among wild populations (Vogelweith et al., 2013a) and (2) the ability of
111 *L. botrana* larvae to sense the current in the risk of parasitism has been previously demonstrated
112 (Vogelweith et al., 2013b).

113

114 **2. Material and methods**

115 *2.1. Model insect*

116 The European grapevine moth *L. botrana* (Lepidoptera: Tortricidae) is currently the major
117 grape pest in Europe and has also spread to American viticulture (Gilligan et al., 2011). This
118 grapevine moth is a polyphagous insect that completes 2-5 generations a year (depending on

119 latitude) on different grape cultivars, where the larvae can do considerable damage (Thiéry,
120 2008). The larvae used for this experiment come from inbred stock maintained diapause-free at
121 the French National Institute for Agricultural Research (INRA) (Villenave d'Ornon, France)
122 for more than 10 years. This strain is based on a great number of caged adults (several thousand
123 per week) to which wild adults are periodically added. This laboratory strain has conserved
124 some plasticity because considerable variation is found in immune parameters between larvae
125 (Muller et al., 2015). In addition, a very similar pattern in terms of basal immunity, response to
126 an immune challenge and parasitoid escape behavior have been found between inbred stock
127 and wild populations sampled in French vineyards (Vogelweith et al., 2014). We used larvae
128 from the laboratory to ensure that they were not previously exposed to parasitoids because this
129 could have affected their immunity. Larvae were fed with an ad libitum amount of a semi-
130 artificial diet (Vogelweith et al., 2015), and maintained in boxes (18 × 11.5 × 7 cm) under
131 standard laboratory conditions (22 ± 1°C, 70 ± 10% r.h., and a L16:D8 photoperiod) at a density
132 of approximately 100 individuals per 300 ml of diet.

133

134 2.2. Parasitoid populations

135 To mimic imminent parasitoid attack on the larvae, we used the two most common parasitoids
136 of *L. botrana* larvae: *Campoplex capitator* (Hymenoptera: Ichneumonidae) and *Phytomyptera*
137 *nigrina* (Diptera: Tachinidae) (Moreau et al., 2010). These parasitoids are known to be larval-
138 parasitoid only and parasitize larvae of *L. botrana* from the 2nd to the 4th instar in vineyards in
139 the south of France where they occur in sympatry (Thiéry, 2008). The parasitism rate of these
140 two parasitoids strongly depends on the year and the vineyard considered (Moreau et al., 2010;
141 Vogelweith et al., 2013a). For instance, in 2012, we observed a parasitism rate of 10% by *C.*
142 *capitator* and 5% by *P. nigra* while in 2011, we found a parasitism rate of 90% by *C. capitator*
143 in the same vineyard (Vogelweith, unpublished data). Both parasitoid species were obtained from

144 parasitized *L. botrana* larvae collected on Grenache in a vineyard close to Perpignan (southern
145 France) in May 2012 using the procedure by Vogelweith et al. (2013a). In brief, the larvae were
146 collected at the end of their development in grapes and kept in large polyethylene boxes (60 ×
147 40 × 21.4 cm) in the laboratory where they were checked daily for pupation. Pupae were placed
148 individually in glass tubes (70 × 9 mm diameter) closed with cotton plugs and stored under
149 standard laboratory conditions (22 ± 1°C, 70 ± 10% r.h., and L16:D8 photoperiod). The tubes
150 were checked daily for the emergence of either the moth or the parasitoid. All the parasitoids
151 were of the same age and were used two days after their emergence in the experiment.

152

153 2.3. Experimental design

154 Fourth instar larvae from the laboratory culture (see above) were exposed in groups of five to
155 one female parasitoid (either *C. capitator* or *P. nigrina*) to assess the effect of parasitoid
156 presence on larval immune parameters. In a previous experiment using then same protocol
157 (Vogelweith et al., 2013b), we did not find any difference between control groups either
158 exposed to a non-parasitoid fly or nothing. For this reason, we used additional groups of
159 unexposed *L. botrana* larvae (empty cup) as sole control. The groups of larvae were placed in
160 plastic boxes (98 × 98 × 49 mm) containing 50 ml of semi-artificial diet (Vogelweith et al.,
161 2015) (Fig. 1). A plastic cup (30 mm diameter and 30 mm height) containing one female of *C.*
162 *capitator* or *P. nigrina* (n = 6 parasitoids and n = 30 larvae, respectively), or no parasitoid (n =
163 5 and n = 20 larvae) was positioned in the middle of each box. The central cup was transparent
164 and pierced with small holes, which allowed for visual, olfactory and vibratory stimulation of
165 *L. botrana* larvae by the parasitoid. The parasitoids were offered a drop of honey as food for
166 the duration of the experiment. The experimental boxes were maintained in standard laboratory
167 conditions (22 ± 1°C, 70 ± 10% r.h., and L16:D8 photoperiod). Six days later, a sample of
168 hemolymph was collected from each larva for measurements of the hemocyte concentration

169 and the activity of the PO-PPO system as described in Vogelweith *et al.* (2011). The larvae that
170 reached metamorphosis (5%) did not provide hemolymph and were equally distributed among
171 treatments. Note that we did not directly expose the larvae to the parasitoid because of the very
172 aggressive behavior of *C. capitator*; since this parasitoid is able to perform more than 10 stings
173 in less than a minute (Vogelweith, unpublished data) inducing consequent injuries to the larvae,
174 possibly affecting immune measurements.

175

176 *2.4. Hemolymph collection and measurement of the three immune parameters*

177 We measured three key immune parameters on 4th instar larvae well known to reflect parasitoid
178 resistance (Prévost and Eslin, 1998; Carton *et al.*, 2008): the concentration of circulating
179 hemocytes, as well as the PO and total-PO activity, assays established to be sensitive enough
180 to detect rapid changes in immunity (Ruiz-González *et al.*, 2009; Vogelweith *et al.*, 2013c).
181 Each larva was chilled on ice for 20 min, and 1 µl of hemolymph was first extracted with a
182 sterile glass capillary (Hirschmann Laborgeräte, Eberstadt, Germany) from a wound inflicted
183 to the posterior part of the ventral side of the abdomen. This extract was immediately diluted in
184 25 µl of cold sodium cacodylate/CaCl₂ buffer (0.01 M sodium cacodylate, 0.005 M CaCl₂; pH
185 6.5) to measure the two immune parameters. A 10 µl sample of this solution was immediately
186 removed for the counting of hemocytes; the remainder was stored at – 27°C for measurement
187 of the enzymatic activity of the PPO system.

188 The concentration of hemocytes was measured immediately after hemolymph
189 extraction. This measurement was done using a Neubauer Improved Haemocytometer under
190 phase contrast microscopy (magnification x 400), as described in Vogelweith *et al.* (2011). The
191 activity of the PPO system was estimated by measuring the enzymatic activity of naturally
192 activated PO enzymes (PO activity), and the activity of the proenzyme together with that of the

193 activated PO (total-PO activity). These measurements were based on a spectrophotometric
194 assay described by Vogelweith et al. (2011, 2013a, 2014).

195

196 *2.5. Body size of larvae*

197 After the collection of hemolymph, larval body size was estimated by measuring the distance
198 between the most distant lateral sides of the head capsule margins (HC width) (Delbac et al.,
199 2010; Vogelweith et al., 2013c) using a Nikon SMZ-10A stereoscopic microscope and a VTO
200 232 video analysis system (Linkam Scientific Instruments).

201

202 *2.6. Statistical analyses*

203 All statistical analyses were conducted using the software R v3.1.2 loaded with the packages
204 *car*, *lme4* and *MASS*. The hypothesis of a plastic adjustment of immune parameters (hemocyte
205 concentration, PO and total-PO activities) to the parasitoid presence was analyzed using three
206 linear mixed models (LMMs), in which the parasitoid presence, parasitoid species and the body
207 size of the larvae were entered as explanatory variables, whereas the ID of experimental box
208 was used as a random effect. Note that to fulfill homoscedasticity and Gaussian distribution,
209 these models were computed using log+1-transformed hemocyte concentration, PO and total-
210 PO activities. We used Pearson's rank correlation coefficient with a confidence interval of 95
211 % (C.I.) for the relationship between immune parameters and larval body size (Extract Model
212 Fitted Values). When the C.I. included 0, the correlation was not significant.

213

214 **3. Results**

215 The presence of parasitoid and parasitoid species had no effect on hemocyte concentration (F_2
216 = 0.48; $p = 0.786$), PO ($F_2 = 0.85$; $p = 0.651$) and total-PO activities ($F_2 = 2.90$; $p = 0.235$) (Fig.
217 2). However, all immune parameters decreased with larval body size (Hemocyte concentration:

218 $F_1 = 7.08$, $p = 0.008$, and $\rho = -0.96$, C.I. 95% = [-0.98; -0.95]; PO activity: $F_1 = 8.59$, $p =$
219 0.003 , and $\rho = -0.91$, C.I. 95% = [-0.94; -0.86]; Total-PO activity: $F_1 = 5.27$, $p = 0.021$, and
220 $\rho = -0.73$, C.I. 95% = [-0.82; -0.61]).

221

222 **4. Discussion**

223 This study aimed to explain the positive relationship between immune parameters and
224 parasitoid prevalence previously observed in populations of *L. botrana* larvae inhabiting
225 different French vineyards (Vogelweith et al., 2013a). In particular, we tested the hypothesis
226 that the presence of parasitoids in the local environment can induce a plastic increase of the
227 investment of *L. botrana* larvae into their immune defense. Our results show that the
228 presence/absence of a parasitoid or the species of the parasitoid present in the environment did
229 not affect immunity of *L. botrana* larvae, although our assays previously allowed us to measure
230 reliable changes in immunity due to environmental variation and our protocol maximized the
231 parasitoid perception by the larvae (Vogelweith et al., 2013b) compared to what presumably
232 occurs under natural conditions. However, our results confirm the negative relationships
233 between larval body size and immunity previously found and discussed in this species
234 (Vogelweith et al., 2013a). To our knowledge, our study is the first to test variation of
235 investment into the larval immune system in response to the presence of parasitoids.

236 Many animals are able to anticipate potential exposure to parasitoids and/or predators
237 by sensing cues such as kairomones, inducing the alteration of several of their traits (e.g.,
238 avoidance behavior, morphological changes, and changes in oviposition site) to reduce the cost
239 of infection/predation (Dicke and Grostal, 2001; Sloggett and Weisser, 2002). For example,
240 Sloggett and Weisser (2002) have shown that the pea aphid (*Acyrtosiphon pisum*) produces
241 winged offspring that leave the host plant to flee colonies infected by parasitoids. Our
242 experimental setting prevented any contact of the moth larvae with the parasitoids, and therefore

243 excluded any direct effect of parasitoid infection, but allowed the larvae to assess different
244 (acoustics, chemicals and visual) cues. We are confident that our experimental setup allowed
245 the detection of the parasitoids by the larvae because, using the same protocol, we previously
246 found that larvae of *L. botrana* accelerated their development in the presence of these
247 parasitoids (Vogelweith et al., 2013b). Such a plastic alteration of the larval growth rate leading
248 to earlier metamorphosis is believed to be adaptive, as it reduces the temporal exposure of the
249 larvae to the parasitoid. Altogether, these results suggest that developmental acceleration
250 appears to be the better option to escape parasitoid infection as compare to enhancing immunity.

251 Overall, our results allow us to reject the hypothesis of a plastic change in immunity to
252 explain the positive relationship between larval immunity and parasitism pressure in *L. botrana*.
253 This suggests that the immunity of *L. botrana* larvae might be shaped by – and adapted to –
254 local conditions in response to the local selective pressure imposed by parasites, as
255 demonstrated in others species (Bryan-Walker et al., 2007; Kortet et al., 2007). Because
256 immunity is costly to express (Kraaijeveld and Godfray, 1997), natural selection should favor
257 optimized investments into immune defenses matching the local prevalence and severity of
258 parasitic infections (Kalbe and Kurtz, 2006). Consequently, on a micro-evolutionary scale,
259 natural selection should favor reduced levels of immune defense in populations subjected to
260 low parasite pressure (Kalbe and Kurtz, 2006; Lindström et al., 2004), whereas on macro-
261 evolutionary scale, immunity should protect species from parasitism has already been found in
262 16 species of Lepidoptera (Smilanich et al., 2009). Further studies should investigate whether
263 local parasitism pressure leads to population-specific micro-evolutionary changes in the
264 immune defense of *L. botrana* larvae. The monitoring and measurements of both larval
265 immunity and parasitism in different vineyards over several years could help to investigate this
266 second hypothesis.

267

268 **Conflict of interest**

269 The authors declare no competing financial interests.

270

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276

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387 **Figure legends**

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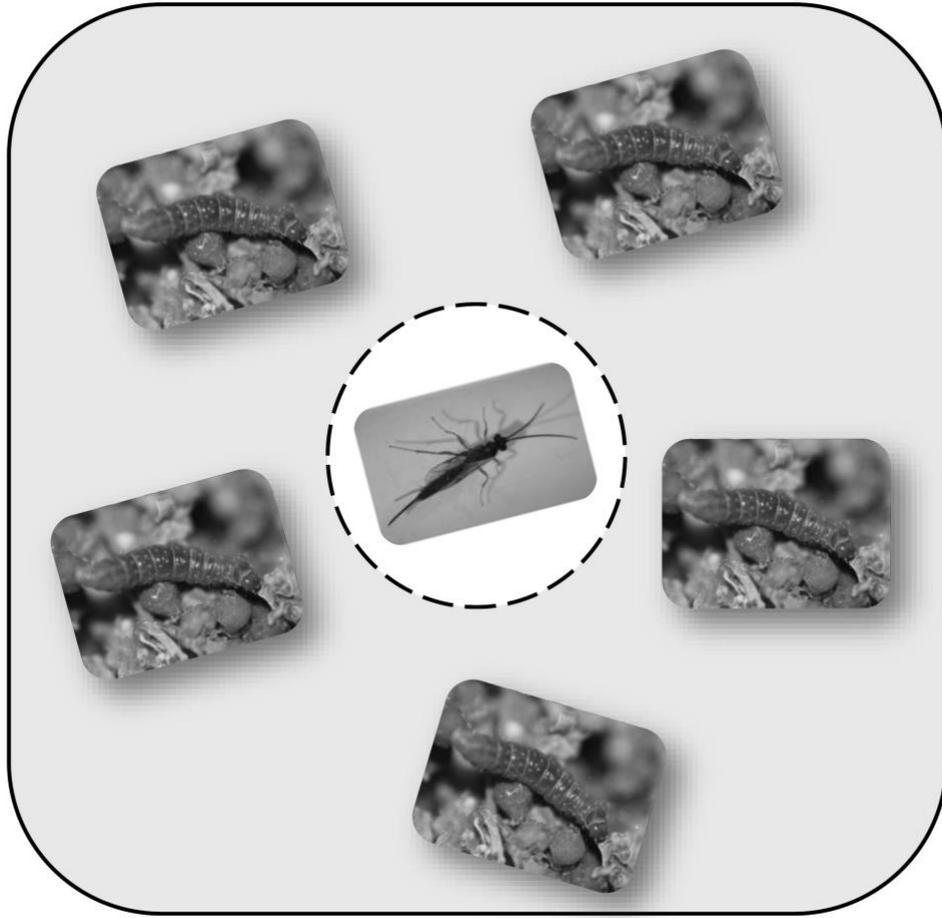
389 **Figure 1.** Diagram of the experimental design. The large square represents the plastic box with
390 the diet (grey) and the larvae. The dotted circle represents the plastic cup with *C. capitator*, *P.*
391 *nigrina* or no parasitoid inside.

392

393 **Figure 2.** Immune response of *L. botrana* larvae to the presence of a parasitoid. (a) Hemocyte
394 concentration ($\times 10^3$ hemocytes/ μl), (b) PO and (c) total-PO activities ($\times 10^3 V_{\text{max}}$ value) in the
395 hemolymph of *L. botrana* larvae after six days in the absence (white boxplot) or in the presence
396 of the parasitoids (grey boxplot): *C. capitator* (*C.c.*) or *P. nigrina* (*P.n.*). The edges of the
397 rectangles represent the first and third quartiles, the central features are the medians, the dashed
398 lines are the maxima and minima, and the black circles are the arithmetic means. Numbers
399 below the x-axis represent the number of larvae measured in the experiment.

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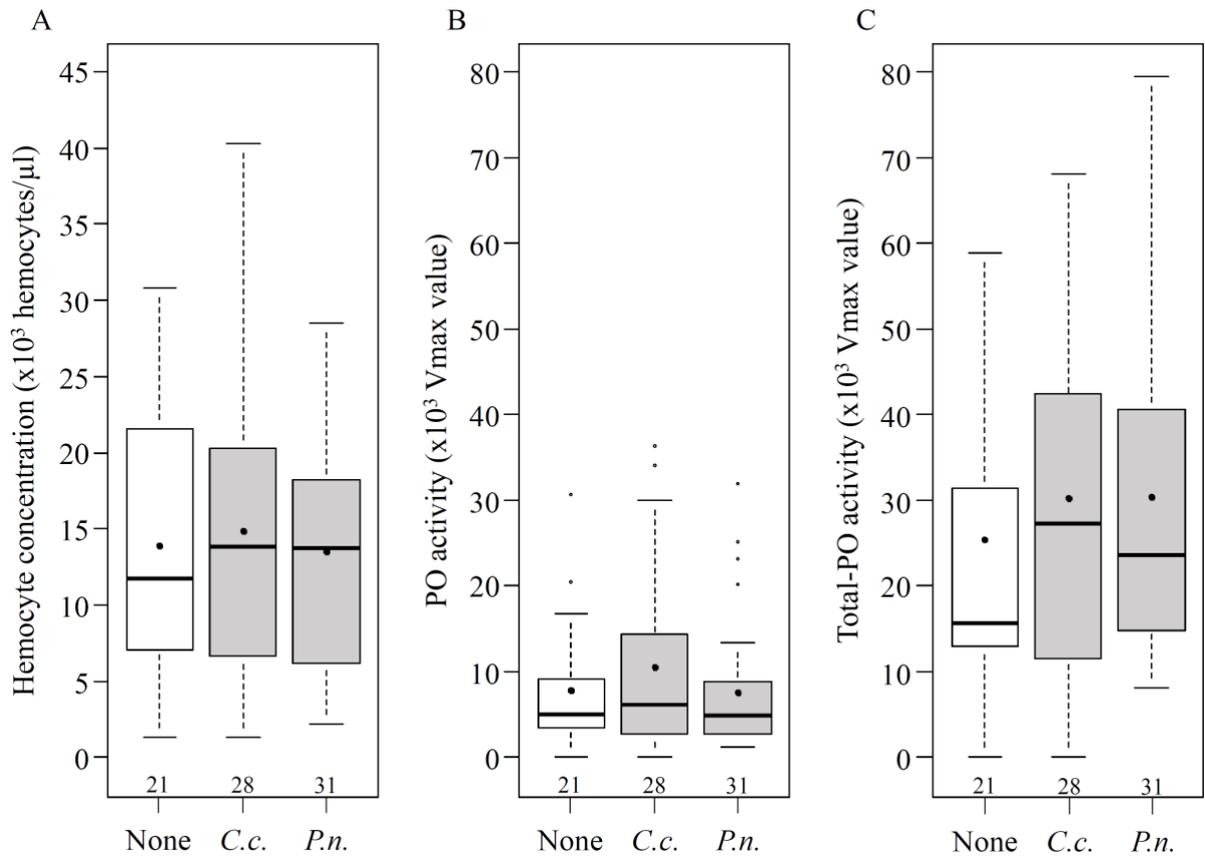
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Figure 1
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Figure 2
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