

# **Sublethal doses of pyriproxyfen stimulate reproduction and aggressive behavior in a non-target parasitoid wasp**

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1 **Abstract:**

2 Insecticides are commonly used to control populations of pests and disease vectors. However, they  
3 can have multiple unintended effects on non-target species. Assessing their impacts on the physiology  
4 and behavior of beneficial insects, such as biological control agents, is thus necessary to gain insight  
5 into the diversity and nature of such side effects. Here, we investigated the effect of sublethal doses  
6 of the endocrine disrupting insecticide pyriproxyfen, a juvenile hormone agonist, on females of the  
7 solitary ectoparasitoid *Eupelmus vuilleti* (Hymenoptera: Eupelmidae). These parasitoid wasps can be  
8 used as biological control agents to control the cowpea weevil, *Callosobruchus maculatus*  
9 (Coleoptera: Bruchidae), that infests cowpea seeds, *Vigna unguiculata* (Fabacea). To do so, in  
10 addition to classical measures on female fecundity and survival, we focused on female behaviors that  
11 can have important consequences on female fitness and host exploitation. First, we showed that  
12 pyriproxyfen stimulated egg production without affecting female survival. Second, we observed that  
13 low doses of this insecticide had no effect on females' exploration and host discrimination ability but  
14 stimulated their aggressiveness when fighting for host access. Although a negative impact on other  
15 life-history traits cannot be ruled out at this point, these results showed low doses of pyriproxyfen  
16 can have unintended positive effects on ectoparasitoid *E. vuilleti* females, by enhancing at least  
17 temporarily their reproduction and host access in a situation of competition. Our work thus highlights  
18 the importance of studying the diversity of possible unintended sublethal effects of pesticides on  
19 beneficial insects.

20

21 **Keywords:** endocrine disruptor, juvenile hormone agonist, fecundity, behavioral strategies, non-  
22 intentional effects, *Eupelmus vuilleti*

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26 **1. Introduction**

27 It is now well established that beneficial insects play an important role in agro-ecosystem  
28 functioning. Agricultural practices, including pesticide use, must therefore take these insects into  
29 account to be effective. Beneficial arthropods indeed provide many ecosystem services: in addition  
30 to ensuring crop pollination, they also permit natural control of crop pests and maintenance of soil  
31 fertility (Haan et al., 2021). They thus have a recognized economic importance and monetary value.  
32 While 5-8% of global agricultural production, representing an annual market value of \$235-577  
33 billions, is directly dependent on pollination (IPBES, 2016), natural pest control by insects is  
34 estimated to be worth \$4.5 billions per year in the United States of America alone (Losey and  
35 Vaughan, 2006). These natural enemies are mainly predators or parasitoids. For example, the  
36 parasitoid wasp *Lariophagus distinguendus* is used as a biological agent to control the weevil  
37 *Sitophilus granarius*, a major grain pest in temperate climates (Niedermayer et al., 2016).  
38 Nevertheless, while the effects of pesticides are widely investigated in honeybees, due to their  
39 important economic role in crop pollination (as reviewed in Barascou et al., 2021), there are still few  
40 studies focusing on the effects of pesticides on parasitoids (Abbes et al., 2015; Desneux et al., 2004;  
41 Tappert et al., 2017). However, pesticides can have indirect effects on these beneficial arthropods and  
42 decrease their effectiveness in controlling pest populations (Cloyd, 2012). To build integrated pest  
43 management (IPM) programs, it is now necessary to select pesticides having the least possible impact  
44 on pests' natural enemies.

45 Even if research on pesticides' effects on non-target species has increased since the 1950s  
46 (Sánchez-Bayo, 2011; Theiling and Croft, 1988), their sub-lethal effects (i.e. effects measured at  
47 doses that do not induce a significant increase in mortality, Desneux et al., 2007) remain to be  
48 explored in-depth. Such effects can be observed at the physiological level, like the reduction of queen  
49 bees' and drones' fertility, but also at the behavioral level (Barascou et al., 2021). Behavioral changes  
50 can further modulate how animals respond to abiotic and biotic environmental changes and impact  
51 their fitness (Jacquin et al., 2020; Sievers et al., 2019). Analysis of behavioral responses is

52 increasingly used to measure the impacts of environmental stresses such as pesticide exposure  
53 because behavioral alterations tend to be more sensitive than fitness indicators (Montiglio and  
54 Royauté, 2014 for a review). In insects, several studies are reporting sublethal effects of insecticides  
55 on the behavior of non-target species that may be beneficials. For instance, deltamethrin, used  
56 worldwide in many crops but also for public health (Davies et al., 2012; Liao et al., 2018) has a  
57 specific effect on maternal egg care behavior of earwigs, which are recognized as a crop protection  
58 agent against pests in orchards. (Meunier et al., 2020; Mauduit et al., 2021).

59         Hormones mediate both physiology and behavior of animals. By affecting their sensory  
60 systems, central integrators and/or peripheral effectors, hormones allow individuals to adjust their  
61 behaviors to the situation in response to specific stimuli (Nelson, 2010). Juvenile hormone (JH) is  
62 one of the main hormones in insects. In addition to regulating molting processes in immature  
63 individuals, its main known function in adults is to control female reproduction. At the physiological  
64 level, JH has a gonadotropic effect: increased JH levels stimulate the production of vitelline protein  
65 precursors, vitellogenins, by the fat body and their uptake by developing oocytes (Roy et al., 2018).  
66 Thus, high JH titers are generally positively associated with female fertility, such as in the facultative  
67 eusocial bee *Megalopta genalis* (Smith et al., 2013), or in primitively eusocial bumblebees and  
68 halictine bees (Tibbetts and Crocker, 2014). At the behavioral level, JH is known to play various roles  
69 in reproductive behavior, from stimulating sexual pheromone production to regulating female  
70 receptivity (Ringo, 2002). Aggressive behaviors during reproduction can also be mediated by JH. In  
71 odonates, for instance, territory defense is more intense in males with high JH levels (Córdoba-  
72 Aguilar and Munguía-Steyer, 2015). Similarly, in eusocial Hymenoptera, aggressive and dominance  
73 behaviors are more frequent in females presenting high JH circulating levels (Tibbetts et al., 2020,  
74 2011). Juvenile hormone also mediates competitive behaviors for access to reproduction sites in a  
75 parasitoid wasp: JH-supplemented *Eupelmus vuilleti* females are more aggressive when fighting for  
76 hosts against intraspecific competitors than control females supplemented with a solvent (Mathiron  
77 et al., 2019). Given the important role of these behaviors in driving female reproduction success in

78 such beneficial insects used to control pest populations (here, the cowpea weevil), it is now essential  
79 to investigate the effects of exposure to endocrine disrupting pesticides on such behaviors in non-  
80 target insects.

81 Insect Growth Regulators (IGR) are insecticides with selective toxicity on biochemical  
82 pathways or processes essential for insect growth and development. They consequently affect the  
83 development of targeted insects by disrupting their molting process, leading to their death, but also  
84 to malformations and reduction in fertility in imagoes. Adults are also subjected to sublethal effects,  
85 such as a reduced fertility in females or the developmental inhibition of specific imago organs  
86 (Desneux et al., 2007). When they interfere with the action of arthropods' hormones (e.g. ecdysone,  
87 juvenile hormone), they can be considered as endocrine disrupting insecticides (Sánchez-Bayo,  
88 2012). Among IGRs, the pyriproxyfen has been synthesized to act as a JH agonist, by competing with  
89 JH molecules on receptors (Sullivan and Goh, 2008). Since the early 1990s, it is commonly used in  
90 agriculture to control insect pests but also for veterinary purposes, as an antiparasitic for domestic  
91 animals. It is also increasingly used in a sanitary context, to prevent *Aedes aegypti* mosquito larvae  
92 from becoming adults and transmitting viruses (Hustedt et al., 2020). Yet, numerous studies have  
93 shown that pyriproxyfen disrupts the development, physiology, and behavior of a variety of taxa,  
94 from aquatic species (e.g., fish, amphibian tadpoles, crustaceans, daphneids; see Devillers, 2020;  
95 Moura and Souza-Santos, 2020) to terrestrial species, in particular non-target insects (Devillers and  
96 Devillers, 2020). For instance, exposure of honeybee larvae to sublethal doses of pyriproxyfen leads  
97 to the greater avoidance of young bees by other colony members, and accepted bees exhibit less social  
98 behavior and more self-directed behavior than untreated bees. In particular, they spend more time  
99 grooming themselves and being immobile (Fourrier et al., 2015). Since the effect of pyriproxyfen on  
100 fitness is highly species-dependent, it is not possible to generalize what is observed in one species to  
101 others (Cloyd, 2012).

102 In the present study, we investigated the potential effects of pyriproxyfen on a non-target  
103 beneficial insect, the parasitoid wasp *Eupelmus vuilleti* Crawford (Hymenoptera: Eupelmidae).

104 Females of this species parasitize and feed on larvae and pupae of the cowpea weevil, *Callosobruchus*  
105 *maculatus* (Fab) (Coleoptera: Bruchidae), which infest cowpea seeds, *Vigna unguiculata* (L.) Walp  
106 (Fabaceae) (Terrasse and Rojas-Rousse, 1986), both in the field and in storage areas. These parasitoid  
107 wasps can be used as biological control agents to control this pest. Nevertheless, IGRs such as  
108 methoprene, a juvenile hormone analog, are often used to reduce *C. maculatus* populations in grain  
109 stocks (Abo-Elghar et al., 2004). The use of pyriproxyfen is also effective for a long period of time:  
110 it reduces *C. maculatus* oviposition rate, egg hatching rate during the month post-treatment, and  
111 suppresses adult emergence up to 8 months after treatment (Abo-Elghar et al., 2004). Moreover, the  
112 tri-trophic system (*E. vuilleti*, *C. maculatus* and the cowpea seeds) is located in the western part of  
113 the Afro-tropical ecozone (i.e Burkina Faso, Mali, Nigeria, Benin and Togo; Gibson and Fusu, 2016).  
114 Its range overlaps that of *Aedes aegypti* (Kamal et al., 2018; Thabet et al., 2020), against which  
115 pyriproxyfen is used (Hustedt et al., 2020). As a result, *E. vuilleti* females are likely exposed to  
116 pyriproxyfen in the field and in grain stocks. Although not targeted by this insecticide, occasional  
117 exposure, even at reduced doses, may affect their behaviors and reproductive success, hence their  
118 efficiency as biological control agents. Here, we, therefore, measured the effects of sub-lethal doses  
119 of pyriproxyfen on *E. vuilleti* females' reproduction (egg maturation and host exploitation) and  
120 competitive behaviors for host access.

121

## 122 **2. Material and methods**

### 123 **2.1 Laboratory breeding**

124 *Eupelmus vuilleti* and *C. maculatus* individuals were collected in 2007 in Togo, West Africa.  
125 Parasitoids were then mass-reared in a climatic chamber, maintained at 28°C, with a photoperiod of  
126 12:12LD following the method described by Jaloux et al., (2004). Their hosts were also maintained  
127 in the laboratory, on cowpea seeds of the Blackeye California variety, under controlled conditions  
128 (12:12 LD, 30°C : 25°C, 30% humidity).

129 *Eupelmus vuilleti* females used in experiments were isolated upon emergence to prevent any  
130 experience of oviposition on a host of unknown quality and competition between females before  
131 assays. They were then individually placed in a Petri dish (diameter: 9.2 cm, height: 1.4 cm) and  
132 provided with a cotton ball soaked with water for hydration. One male was added during 24h for  
133 mating and oogenesis stimulation (Terrasse and Rojas-Rousse, 1986), except in the survival  
134 experience as explained below. Females were also provided with hosts that were replaced daily until  
135 behavioral assays were carried out. In experiments measuring discrimination ability and exploratory  
136 activity of females, hosts were provided as one seed containing 2 to 3 larvae of *C. maculatus*. For the  
137 other trials, they were presented as individual fourth-instar *C. maculatus* larvae dissected from seeds  
138 and located in a transparent gelatin capsule (length: 18mm, diameter: 5mm). This mimics the cowpea  
139 seed and allows better experimental control of host larval stage and it also facilitates the observation  
140 of oviposition by the parasitoid (the egg can be seen through the gelatin capsule during its deposition  
141 on the host; Gauthier and Monge, 1999), without altering female behavior (Gauthier and Monge,  
142 1999; Jaloux et al., 2004), despite a slight reduction in the number of eggs laid (Damiens et al., 2001).  
143 All behavioral experiments were carried out in a climate room with a LD photoperiod of 12:12,  
144 maintained at 28°C and constant 70% relative humidity.

145

## 146 2.2 Exposure to pyriproxyfen

147 Because *E. vuilleti* juveniles develop (from the host) inside the cowpea seeds, they are unlikely  
148 to be exposed to sprayed pesticides. We therefore focused on the effects of pyriproxyfen of free-living  
149 adult females, using external application to mimic, in a controlled way, female exposure to pesticide  
150 sprayed on treated areas. Females were thus topically applied with pyriproxyfen (Sigma-Aldrich, Inc.  
151 #34174) diluted in pure acetone using one of four concentrations: 0 (control), 9.6, 96 or 960 µg/mL.  
152 Such doses are consistent with what females can encounter in treated fields, since the Togolese  
153 legislation authorizes the use of pyriproxyfen at 30g/ha, which is equivalent to 300ng/cm<sup>2</sup> (Unité de  
154 coordination nationale REDD+, direction nationale ODEF, 2020). Our two lower doses were within



155 that range, as they consisted of applying 9.6 and 96 ng of active substance to females. To apply the  
156 solution, females were briefly cold anesthetized at -20°C, and received 1 µL of solution on the ventral  
157 side of their thorax, where the presence of numerous spiracles (pores) allow the solution to enter their  
158 body. Afterwards, females were individually placed in a 1.5mL tube for 4 or 24 hours before being  
159 used in experiments.

160

### 161 2.3 Female survival

162 To measure the survival of females in dependence of the solution to which they are exposed,  
163 females were treated with either acetone (control, N = 24) or pyriproxyfen (N = 23 for each of the 3  
164 concentrations) on the day of their emergence. A fifth group of 23 females (i.e. additional control  
165 group) underwent the same procedure as for the other treatments (e.g. handling and cold anesthesia)  
166 but no solution was applied. Females were then individually placed in a Petri dish with a cotton ball  
167 soaked with water (but no male and no host) and checked daily, until their death.

168

### 169 2.4 Gonadotropic effect

170 The gonadotropic effect of pyriproxyfen was tested by dissecting 3-day old females and  
171 counting their mature eggs, 4h or 24h after exposure (N = 20 for each concentration and time tested).  
172 Mature (i.e. ready-to-lay) eggs were identified based on their homogeneous content and their long  
173 and thin pedicel (Bodin et al., 2009). In the 4h treatment, females were treated on day 3, whereas  
174 those in the 24h treatment were treated on day 2 and remained in a tube for 24 hours. These latter  
175 females therefore had the same age at the time of the dissection as those of the 4h treatment but had  
176 access to one fewer host. The egg load of control females (pure acetone treatment) could therefore  
177 not be compared between both time delays because oviposition modifies egg load.

178 In the following behavioral assays, as egg number is known to influence host exploitation  
179 behavior including aggressiveness and contest outcome in *E. vuilleti* (Mathiron et al., 2019, 2018;

180 Mohamad et al., 2010), we chose to investigate the effect of pyriproxyfen on behavior at a time where  
181 egg load was not modified. Since our results showed the number of mature eggs increased 24h after  
182 pyriproxyfen application but not yet 4h after (see Results section), we performed all behavioral  
183 observations four hours after pyriproxyfen application.

184

## 185 2.5 Mobility and exploration

186 The arena used to measure the influence of pyriproxyfen on female mobility and exploration  
187 consisted of nine chambers connected by thin corridors and sandwiched between two glass plates  
188 (Supplementary Material, Figure S1). The central chamber was connected to four others, each linked  
189 to a peripheral zone. A half seed containing a single larva of the beetle *C. maculatus* was placed in  
190 one of the four peripheral chambers. The complete device was placed on an infrared table with a  
191 BASLER BCA 1300 (Germany) camera fixed above (Media Recorder v4.0, Noldus Information  
192 Systems, The Netherlands). Three-day-old females treated with acetone or one of the three  
193 concentrations of pyriproxyfen (N = 27, 26, 27 and 27 respectively) were placed, 4 hours after  
194 exposure, in the central area of the device, initially closed with a ring blocking the different exits.  
195 After a 5-minute acclimation period, the ring was removed, allowing the female to freely move in the  
196 entire arena. Their movement were recorded for 60 minutes. The number of eggs laid was  
197 subsequently counted by opening the seeds and inspecting the hosts' surface. Female egg load was  
198 measured by dissecting females (as previously explained). We then analyzed videos both manually  
199 and using ToxTrac software (version 2.92 (2021/03/30)) to measure the distance covered by females,  
200 their speed or time spent stationary. The time taken to arrive at the seed and the time spent on it were  
201 also noted.

202

203

204

## 205 2.6 Host discrimination

206 To evaluate the influence of pyriproxyfen on females' host discrimination ability, three-day-  
207 old females (N = 20 for each concentration of pyriproxyfen: 0, 9.6, 96 and 960  $\mu\text{g}/\text{mL}$ ) were  
208 individually introduced four hours after treatment, for one hour, into a Petri dish (diameter: 9.2cm,  
209 height: 1.4cm) at equidistance between of two seeds. Each seed contained a single fourth-instar larva  
210 of *C. maculatus*: one had been parasitized 2 hours before the trial by another 3-day-old *E. vuilleti*  
211 female (not used further in experiments) while the other was unparasitized. Using BORIS software  
212 (v 7.10.2 – 2021-01-16), we recorded the occurrence and duration of the following behaviors:  
213 grooming, resting, antennal exploration and oviposition. The location where these behaviors were  
214 displayed was noted: on the unparasitized or parasitized host or on the Petri dish, called 'neutral zone'.  
215 At the end of each experiment, cowpea seeds were opened to count the number of eggs present on  
216 the host and confirm the oviposition observed during experiments.

217

## 218 2.7 Female aggressiveness and conflict resolution

219 To identify females individually during contests, we marked them, on the day before the trial,  
220 with a yellow or red dot of acrylic paint deposited on the dorsal part of their thorax, avoiding touching  
221 the tegula (wing insertion), and after reducing their locomotor activity by cold exposure. Three-day-  
222 old females were then either treated with pure acetone or 9.6  $\mu\text{g}/\text{mL}$  pyriproxyfen and maintained 4h  
223 without host before running behavioral assays, to increase their motivation to lay eggs. It has to be  
224 noted that, due to the complexity of setting up these trials, we tested only one pyriproxyfen  
225 concentration which was the lowest dose of pyriproxyfen having a gonadotropic effect (i.e 9.6 $\mu\text{g}/\text{mL}$ ,  
226 see results section) and compared its effect to control (i.e. females treated with acetone).

227 We set up dyadic encounters between either (i) two control females (i.e. both females were  
228 exposed to pure acetone; N = 45), or (ii) one control female and one female exposed to pyriproxyfen  
229 (N = 36). Each dyad consisted of visually similar-sized wasps of distinct color. No effect of color on

230 contest was found (Logistic analysis, LR  $\chi^2 = 0.374$ , df = 1, P = 0.541). At the beginning of each  
231 observation, both females were simultaneously introduced into an arena consisting of three circular  
232 chambers connected by a narrow corridor (following Petersen and Hardy, 1996), with a *C. maculatus*  
233 fourth instar larva placed in a gelatin capsule in the central chamber. During each encounter, we  
234 recorded which female was (i) first on host, (ii) first to oviposit, and (iii) first to initiate agonistic  
235 interactions with her opponent. We also noted the number of the following behaviors: antennal  
236 contact, pushing (i.e. a female pushed her opponent with her body), chasing the opponent, climbing  
237 to the opponent or kicking the opponent. All these behaviors, apart from antennal contact, were  
238 considered aggressive. Contests lasted a maximum of 1 h or were stopped when one of the females  
239 exited the central chamber for more than 2 min, or when neither female touched the host or had any  
240 agonistic interactions for more than 2 min. A female was designated the winner of the contest when  
241 her opponent left the central chamber for more than 2 min. Similarly, when both females in a dyad  
242 remained in the central chamber for the entire duration of the observation, or when both females  
243 apparently lost interest in the host, the winning female was designated as the one that exhibited  
244 oviposition behavior. Contests were considered unresolved, when the interaction was not interrupted  
245 and both females exhibited oviposition behavior. This was also the case when none of the protagonists  
246 appeared to be interested in the host.

247         After each encounter, females were frozen at -20°C, and the number of eggs laid on the host  
248 by the winner counted. Subsequently, females were weighed using an electronic scale (Sartorius  
249 Quintix 125 D-15, accuracy 0.01 mg) and dissected to count the number of mature eggs in their  
250 abdomens. The initial mature egg load of the winning females was calculated as the number of mature  
251 eggs in their ovaries plus the number of eggs laid on the host.

252

## 253 2.8 Statistical analysis

254         All statistical analyses were performed with R software v4.0.4 and R studio v1.4.1106 with  
255 the packages car, DHARMA, emmeans, and using  $\alpha = 0.05$ . The general approach was to use

256 parametric analyses. However, when this was not possible because the parametric assumptions were  
257 not met, nonparametric tests were used. The distribution and homoscedasticity of each model was  
258 checked using the 'testResiduals' function of the DHARMA package (Hartig, 2021).

259 To estimate differences in female mortality due to pyriproxyfen treatment, we carried out  
260 parametric survival analyses using the 'survreg' function of the Survival package. An initial model  
261 comparison showed that a Weibull distribution had a better fit than an exponential distribution ( $P <$   
262  $0.005$ ).

263 To explore the effect of pyriproxyfen exposure on female egg load, 4h and 24 h after  
264 application, we ran two linear models, followed by Tukey's 2-to-2 multiple comparisons when  
265 appropriate.

266 In the mobility and exploration experiment, we analyzed the effect of pyriproxyfen exposure  
267 on several variables. We investigated the effect of pesticide concentrations on the total distance  
268 covered by females in the arena, the resting time and the time they spent on the seed using Kruskal-  
269 Wallis tests. The effect on the latency before reaching the seed was evaluated using a generalized  
270 linear model with a Gamma error distribution, and the effect on the number of eggs laid on a host  
271 with a Poisson error structure. It has to be noted that 8 of the 114 females were removed from this  
272 later analysis because either the number of eggs laid could not be accurately determined (i.e. the host  
273 was damaged during its extraction from the cowpea seed making potential parasitoid eggs impossible  
274 to locate) or were sterile (i.e. they were no oocytes in their ovaries).

275 To explore the effect of pyriproxyfen on female host discrimination ability, we tested whether  
276 the probability that females laid eggs on unparasitized and parasitized hosts varied with the  
277 concentration of pesticide they were exposed to, using a generalized linear mixed model ('glmer'  
278 function of the lme4 package) with a Binomial error structure, taking the identity of the female as a  
279 random variable. The effect of pyriproxyfen exposure on immobility, grooming and foraging time

280 was evaluated by Kruskal-Wallis tests, and antennal exploration time by a linear regression (with a  
281 normal error structure).

282         Among the 81 dyadic encounters set up to explore the effect of pyriproxyfen exposure on  
283 female aggressiveness and contest resolution, 65 were clearly resolved: only these were considered  
284 in the following analyses. The effect of the treatment on the aggressiveness of the focal females was  
285 evaluated using two generalized linear mixed models with the identity of the fight as a random  
286 variable ('glmer' function). For the first model, the response variable was whether or not females  
287 expressed aggressive behaviors (binomial error structure). The total number of agonistic behaviors  
288 displayed by each female was considered in the second model (Poisson error structure). The relative  
289 difference in egg load between females was calculated as the result of '((egg load of focal female -  
290 egg load of opposing female) / egg load of focal female) x 100)'. Two GLMs were then run with a  
291 binomial distribution of errors (logit link function). The first model explored the influence of  
292 treatment (acetone or pyriproxyfen), relative egg load difference and their interaction on the  
293 probability of the focal female winning the contest. A similar analysis was performed with the relative  
294 difference in mass instead of egg load. The differences in mass and egg load were not entered into  
295 the same model because these two variables are correlated. Indeed, the greater the mass of a female,  
296 the greater her egg load (Linear model,  $F_{1,63} = 10.526$ ,  $P = 0.002$ ). Binomial tests ('binom.test'  
297 function) were also performed to explore whether the probability of the focal female exploiting the  
298 host first was significantly different from the null expectation at 0.5.

299

### 300 **3. Results**

#### 301 3.1 Female survival and reproduction

302         Survival did not significantly differ between females manipulated but not exposed to any  
303 solution, females treated with acetone and females treated with one of the different concentrations of  
304 pyriproxyfen ( $\chi^2 = 6.29$ ,  $ddl = 4$ ,  $P = 0.18$ ). In contrast, egg load was significantly higher in females

305 treated with pyriproxyfen, regardless of the concentration received, than in females treated with  
306 acetone, 24h after exposure ( $F_{3,77} = 6.720$ ,  $P < 0.001$ ; Figure 1). Four hours after application, this  
307 effect was, however, not yet visible: female egg load did not significantly differ between all  
308 treatments ( $F_{3,76} = 0.066$ ,  $P = 0.978$ ).

309

### 310 3.2 Mobility and exploration

311 Regardless of the pyriproxyfen concentration received, females' exploration ability to explore  
312 a complex arena and motivation to find an oviposition site did not differ between treatments (Figure  
313 S2). Indeed, the time (Gamma LR- $\chi^2 = 1.787$ ,  $df = 3$ ,  $P = 0.618$ ) and distance (Kruskal-Wallis:  $H =$   
314  $2.229$ ,  $df = 3$ ,  $P = 0.513$ ) walked by females to reach the host –containing seed and the time spent on  
315 that seed ( $H = 2.240$ ,  $df = 3$ ,  $P = 0.524$ ) did not significantly vary across treatments. All females,  
316 regardless of the pyriproxyfen concentration received, spent the same proportion of time resting ( $H$   
317  $= 3.880$ ,  $df = 3$ ,  $P = 0.275$ ) and laid a similar number of eggs (Poisson LR- $\chi^2 = 2.598$ ,  $df = 3$ ,  $P =$   
318  $0.458$ ).

319

### 320 3.3 Host discrimination

321 The effect of pyriproxyfen on female host discrimination ability was assessed in a choice test  
322 between a parasitized and an unparasitized host. The interaction between host value and the different  
323 concentrations of pyriproxyfen tested had no significant effect on whether females laid eggs on  
324 unparasitized or parasitized hosts (LR- $\chi^2 = 6.496$ ,  $df = 3$ ,  $P = 0.090$ ). Females laid eggs on the first  
325 host they encountered, regardless of its quality.

326 Female behavior on hosts before laying eggs was not modified by the treatment (Figure S3).  
327 Indeed, there was no significant difference in the time spent immobile (Kruskal-wallis:  $H = 2.136$ ,  $df$   
328  $= 3$ ,  $P = 0.545$ ), grooming ( $H = 7.268$ ,  $df = 3$ ,  $P = 0.064$ ), or foraging for the seed ( $H = 4.294$ ,  $df = 3$ ,  
329  $P = 0.231$ ), nor any effect on antennal exploration time ( $F_{1,78} = 0.026$ ,  $P = 0.871$ ).

330

### 331 3.4 Aggressiveness and conflict resolution

332           The effect of pyriproxyfen exposure (9.6 µg/mL) on female contest behavior and outcome  
333 during host access was studied. Females exposed to pyriproxyfen were more likely to display  
334 aggressive behavior than those exposed to acetone (logistic analysis, LR- $\chi^2 = 4.146$ , df = 1, P = 0.042).  
335 They also showed a greater number of aggressive behaviors than did control females (Poisson LR- $\chi^2$   
336 = 6.243, df = 1, P = 0.012, Figure 2A). In spite of this aggressiveness, the probability of the focal  
337 female winning was not influenced by pyriproxyfen or acetone treatment, the relative difference in  
338 mature egg load between contestants, or the interaction between these main effects (Table 1). The  
339 difference in reproductive status, i.e. their mature egg load, also had no effect on the probability of  
340 winning when only acetone-acetone dyads were considered (logistic analysis, LR-  $\chi^2 = 0.714$ , df = 1,  
341 P = 0.398). Similarly, the probability that the focal female won did not vary with the relative mass  
342 difference with the opponent, the treatment received or the interaction between both (Table 1). When  
343 considering only acetone-acetone female contests, the effect of mass difference was also not  
344 significant, despite a trend in which heavier females were slightly advantaged (LR  $\chi^2 = 3.615$ , df = 1,  
345 P = 0.057). In contrast, focal females treated with pyriproxyfen were more frequently the first of their  
346 dyad to exhibit host oviposition behavior (binomial test: P = 0.007, Figure 2B), than focal females  
347 exposed to acetone (P = 0.296).

348

### 349 **4. Discussion**

350           This study aimed to evaluate the potential sublethal effects of pyriproxyfen, an insecticide that  
351 regulates growth by mimicking juvenile hormone, in females of *E. vuilleti*, a beneficial parasitoid  
352 wasp that attacks the food stock pest, *C. maculatus*. We found that low doses of pyriproxyfen do not  
353 affect survival but modulate both reproductive physiology and behavior in this parasitoid.  
354 Pyriproxyfen has a gonadotropic effect in *E. vuilleti* females. Additionally, while exploratory and  
355 host discrimination behavior did not seem to be modified after pyriproxyfen exposure, agonistic



356 behaviors were affected. Females exposed to pyriproxyfen were more aggressive during female-  
357 female contests for hosts and were more likely to be the first of the dyad exhibiting oviposition  
358 behavior. Even if many studies focused on the impact of pesticides on egg-laying behavior in insects  
359 (see Barascou et al., 2021 for bees), especially parasitoids (effects of various neurotoxic insecticides:  
360 Delpuech, 2017; Delpuech and Delahaye, 2013; Desneux et al., 2004; Rafalimanana et al., 2002;  
361 Sheng et al., 2021; Wang et al., 2016) due to their economic importance as biological control agents,  
362 very few studies explored the effect of juvenile hormone analogues on adults (Beloti et al., 2015;  
363 Cloyd and Dickinson, 2006) and, to our knowledge, none during intraspecific competition for  
364 oviposition sites. Here, we showed that a pesticide that is an Insect Growth Regulator, IGR, can have  
365 sublethal effects on non-target insects by impacting their fecundity and oviposition behavior under  
366 intraspecific competition.

367         Regarding the effect of pyriproxyfen on reproductive physiology, we showed that, 24 hours  
368 after exposure, *E. vuilleti* females had on average 2 to 3 more mature eggs than control females (i.e.  
369 exposed to solvent), regardless of the concentration applied. Four hours after application, this effect  
370 was, however, not yet visible. These results confirm pyriproxyfen mimics JH's action on reproduction  
371 in *E. vuilleti*. Juvenile hormone, known to be one of the main gonadotropic hormones in insects by  
372 stimulating vitellogenesis (Roy et al., 2018), has indeed been demonstrated to enhance egg maturation  
373 in *E. vuilleti* (Mathiron et al., 2019): injection of JH in females stimulates the maturity of additional  
374 eggs. This gonadotropic effect was observed 24h after exposure but not yet after 4h (Mathiron et al.,  
375 2019). Altogether, these results suggest the complete maturation of new eggs following hormonal (JH  
376 or pyriproxyfen) signal takes between 4h and 24h in this species. Furthermore, the duration of  
377 pyriproxyfen effect on egg maturation remains to be investigated: it can result in a transient increase  
378 that does not necessarily modify females' lifetime egg production, or a longer-lasting effect  
379 enhancing females' reproductive potential. Whether eggs produced following exposure to this  
380 insecticide develop normally should also be analyzed. IGRs can indeed impact egg viability in adult  
381 insects as observed in pests, such as *Platynota idaeusalis* and *Anthonomus grandis grandis* (Myers

382 and Hull, 2003; Rolim et al., 2019). Similarly, exposure of female ladybugs *Tenuisvalvae notata* to  
383 pyriproxyfen resulted in a temporary sterility (Barbosa et al., 2018): treated females laid a similar  
384 number of eggs, but these eggs laid during treatment, and up to 4-5 days after the end of exposure,  
385 were not viable and did not result in the development of any offspring.

386 Under field conditions, the consequences on female fitness, hence parasitoid population size,  
387 will also depend on the availability of hosts when females show an enhanced number of ready-to-lay  
388 eggs. If parasitoid population size increases, the use of low doses of pyriproxyfen would have dual  
389 effects on the control of cowpea pests, both by negatively affecting pest survival, and by favoring  
390 parasitism rate. On the contrary, if parasitoid population size is negatively impacted by egg quality  
391 or asynchrony between mature eggs and available hosts, a negative indirect effect on pest regulation  
392 is expected. The potential consequences of low doses of pyriproxyfen on offspring production in *E.*  
393 *vuilleti* remains to be further investigated to understand its effect on pest control efficiency.

394 In addition to affecting the insects' physiology, pyriproxyfen could also alter their behavior.  
395 Previous experiments carried out in the eusocial bee *Apis mellifera* showed that, despite not being a  
396 demonstrated juvenile hormone-controlled behavior, workers' mobility was impacted by exposure to  
397 pyriproxyfen. Their flight ability was reduced and they spent more time immobile (Fourrier et al.,  
398 2015; Gomes et al., 2020). This pair of studies are among the very few exploring the influence of  
399 pyriproxyfen on this type of behavior in insects. Our video tracking data did not indicate similar  
400 effects in female *E. vuilleti*. This means either these behaviors are not JH-dependent in *E. vuilleti* or,  
401 regardless of the underlying mechanisms regulating them, exploration and mobility were not  
402 disrupted by this insecticide at the doses applied. The ability to discriminate host quality, in particular  
403 its parasitism status, is common in parasitoid wasps (Godfray, 1994) and is often based on the  
404 perception and recognition of chemical cues. After locating their hosts using long-range cues or  
405 signals, females detect and recognize chemical information about host quality through the activation  
406 of sensory neurons of sensillae mainly located on their antennae and ovipositor, and then adjust their  
407 oviposition decisions accordingly (Cury et al., 2019). Neurotoxic pesticides can disrupt the insect

408 olfactory system (Delpuech and Leger, 2011), and smell disruption is a possible effect of endocrine  
409 disruptors. In *Spodoptera littoralis*, bis(2-ethylhexyl)phthalate alters male detection of female sex  
410 pheromone by down-regulating the transcription level of calmodulin and ecdysteroid nuclear  
411 receptors in their antennae (Aviles et al., 2020). We therefore anticipated endocrine disruptors may  
412 alter parasitoid females' discrimination ability in species where host quality assessment is based on  
413 chemical cues perceived during antennal examination, like in *E. vuilleti* (Jaloux, 2004). In contrast to  
414 previous work (Darrouzet et al. 2007), we did not observe host discrimination ability in our  
415 experiment. This surprising difference may be due to the time elapsed since the first oviposition,  
416 which was shorter in our set-up. Nevertheless, our results did not show any effect of pyriproxyfen in  
417 modulating oviposition strategies on parasitized and unparasitized hosts in this parasitoid wasp, at the  
418 tested concentrations: parasitized host acceptance rates did not differ between treated and control  
419 females.

420         Despite the absence of sublethal effects of pyriproxyfen on exploration and host  
421 discrimination behavior in *E. vuilleti*, we observed that female exposure to a low dose of this  
422 endocrine disruptor increased the occurrence and number of aggressive behaviors. Interestingly, other  
423 insecticides that are not JH analogues have already been shown to increase the aggression of insects.  
424 For instance, females of the beetle *Phaedon cochleariae* exposed to pyrethroid lambda-cyhalothrin  
425 show more aggressive behavior than untreated females (Müller et al., 2019). Our result is also in  
426 agreement with what is known about the hormonal regulation of aggressiveness in many species.  
427 Indeed, high levels of JH in adults are often associated with high levels of aggressiveness and elevated  
428 dominance rank (Tibbetts et al., 2020). Furthermore, a similar stimulatory effect of aggression after  
429 exposure to another JH analogue, fenoxycarb, has already been demonstrated in *Schistocerca*  
430 *gregaria* and *Locusta migratoria* (Wiesel et al., 1996). This phenomenon was also found in the  
431 necrophagous beetles *Nicrophorus orbicollis* and *N. tomentosus* when exposed to methoprene (Scott,  
432 2006). In the absence of a resource to fight over, exposure to methoprene indeed increased  
433 individuals' aggressiveness: when a treated female competed with a untreated conspecific, injuries

434 were more frequent than when neither of the opponents was treated (Scott, 2006). Altogether, these  
435 studies, including this current study, support the hypothesis that juvenile hormone-like growth  
436 regulator insecticides can influence the behavior of non-target insects, especially in competitive  
437 situations.

438         Additionally, *E. vuilleti* females treated with pyriproxyfen were more likely to be the first of  
439 their dyad exhibiting host exploration compared to control females (exposed to solvent). This could  
440 reflect a greater motivation among pyriproxyfen treated females to lay eggs. Indeed, JH is known to  
441 stimulate egg maturation within 24h (Mathiron et al., 2019), which in turn increases the value that  
442 females place in the host (i.e. higher subjective value, Mohamad et al., 2010), hence their motivation  
443 to access hosts. Juvenile hormone would thus be the direct or indirect signal modulating motivation  
444 to access the host. Exposure to pyriproxyfen would induce a similar signal and lead to an increase in  
445 female motivation to take possession of hosts. It would then be interesting to analyze how long this  
446 effect lasts. Nevertheless, this increased probability of being first on host (and being first to exhibit  
447 oviposition behavior) did not translate into a higher probability of being first to lay an egg (i.e. a fully  
448 realized win of a contest for host resources). The oviposition sequence of females was frequently  
449 interrupted by aggressive behavior of their opponent, leading to their departure from the host or even  
450 from the central chamber, while the opponent fully parasitized the host. As a result, females exposed  
451 to the insecticide were not more likely than control females to win the overall conflict. These  
452 surprising results are consistent with previous work showing that, while *E. vuilleti* agonistic behaviors  
453 are modulated by JH (Mathiron et al., 2019), contest outcomes are mediated by another hormone,  
454 ecdysone (Mathiron et al., 2020). Thus, whereas *in silico* analyses revealed a possible interaction of  
455 pyriproxyfen with USP-EcR, the receptor dimer known to mediate the effects of ecdysteroids in bees  
456 (Spaggiari et al., 2021), it seems that in *E. vuilleti* there is no cross communication between  
457 pyriproxyfen and ecdysteroid activity.

458

459

## 460 5. Conclusion

461 Our results revealed exposure to sublethal doses of pyriproxyfen enhances, at least  
462 temporarily, egg maturation, aggressiveness and motivation to access host during competition in *E.*  
463 *vuilleti* females. If these effects lead to a higher fitness in these parasitoids, they could improve the  
464 biological control of the cowpea seed pest, *C. maculatus*. Similarly, positive effects of sublethal  
465 concentrations of other insecticides on natural enemies of pests have been identified (Müller, 2018;  
466 Stothard and Hardy, unpublished). For instance, the frequency of flight and walking, as well as the  
467 duration and distance of these movements in ladybugs *Harmonia axyridis* exposed to beta-  
468 cypermethrin is increased (Xiao et al., 2017). Female fecundity is also enhanced (Xiao et al., 2016).  
469 As flight performance is important for predation but also for dispersal, such changes in a beneficial  
470 insect may have important consequences in its biocontrol efficiency. In addition, pesticides can cause  
471 transgenerational effects and impact populations over multiple generations even though they are no  
472 longer present in the environment (Müller, 2018). However, because a same insecticide can have  
473 effects on both pests and beneficial organisms, care must be taken to ensure that the dose used is more  
474 beneficial to the latter species than to the former. Furthermore, positive sublethal effects are rare and  
475 in most cases insecticides have harmful effects on non-target insects (Desneux et al., 2007). Finally,  
476 the influence of pesticides on interactions between sympatric species, whether interspecific or  
477 between prey and predators, must be investigated to ensure the maintenance of ecosystem dynamics  
478 in IPM programs.

479 Under the experimental conditions of this study, pyriproxyfen modulated upward *E. vuilleti*  
480 females' fecundity and aggressiveness during conflict for host access. This highlights that  
481 pyriproxyfen may have, though relatively specific, unintended effects on non-target species,  
482 including parasitoids used to control cowpea pests. At this point, it remains difficult to anticipate the  
483 consequences at the population level of these positive effects on reproduction and behavior and  
484 effects on other life-history traits cannot yet be ruled out. This study raises the importance of studying  
485 the diversity of unintentional sublethal effects of pesticides that may affect non-target species,

486 including arthropods, to improve knowledge of their potential risks to species used in biological  
487 control.

488

#### 489 **Competing interest statement**

490 The authors have no competing interests to declare.

491

#### 492 **Ethical Note**

493 No animal ethics approval was required. Nevertheless, we handled all individuals with care, and we  
494 kept handling time to an absolute minimum. Acrylic paint used for marking does not cause injury to  
495 wasps. Dissected wasps were freeze-killed prior to decapitation.

496

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500

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738

739 Figure legends

740

741 **Figure 1:** Average number of mature eggs (mean +/- SEM) in *E. vuilleti* females 24h after being  
742 treated with pyriproxyfen at four different concentrations. n indicates the sample size per treatment.  
743 \* indicates a significant difference ( $P < 0.05$ ) in mature eggs between the four treatments. Two  
744 different letters indicate a significant difference at  $\alpha < 0.05$ .

745

746 **Figure 2:** Female behavior during conflicts for access to a host depending on whether *E. vuilleti*  
747 females were treated with acetone or pyriproxyfen at  $9.6\mu\text{g/mL}$  four hours before. A. Number of  
748 aggressive behaviors (mean +/- SEM) displayed by a focal female, treated with pyriproxyfen or  
749 acetone, towards its opponent, a non-treated female. B. Proportion of focal females exploiting the  
750 host first. Proportions were compared to chance (i.e. 50%). Figures in the bars indicate the number  
751 of females observed in each situation. \* and \*\* indicate a significant difference at  $\alpha < 0.05$  and  $\alpha <$   
752  $0.001$ , respectively. ns: indicates a non-significant difference.

Figure 1

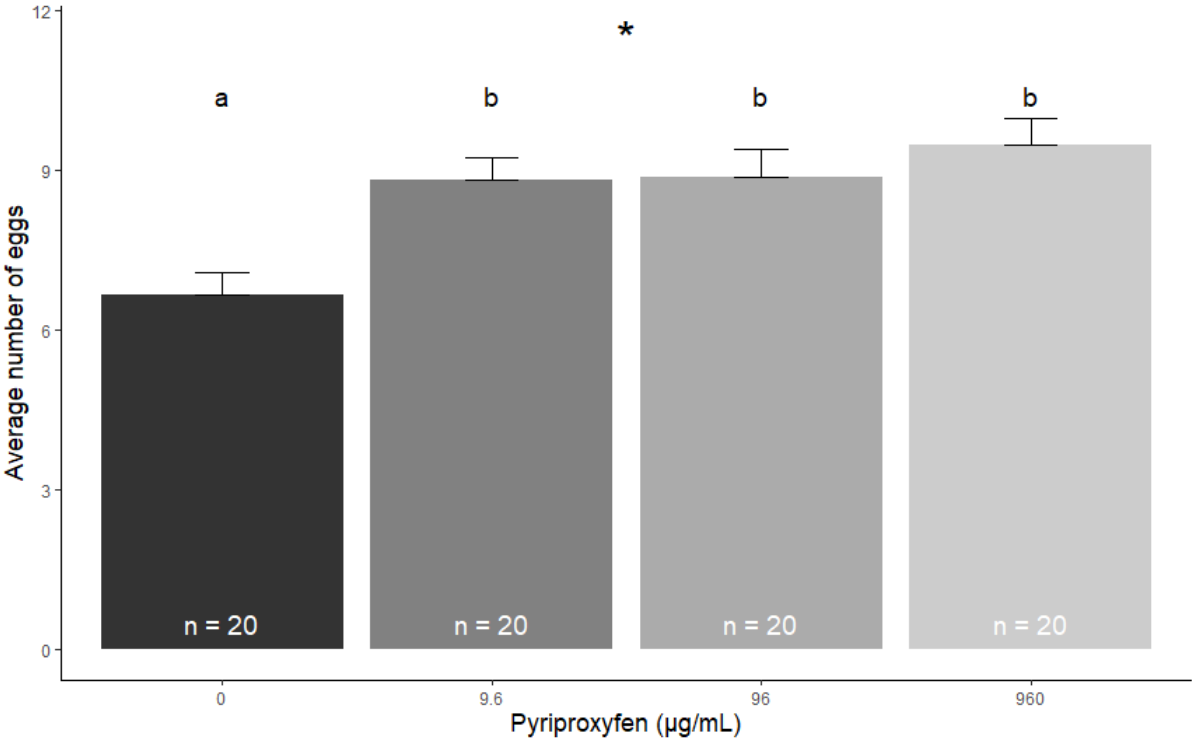
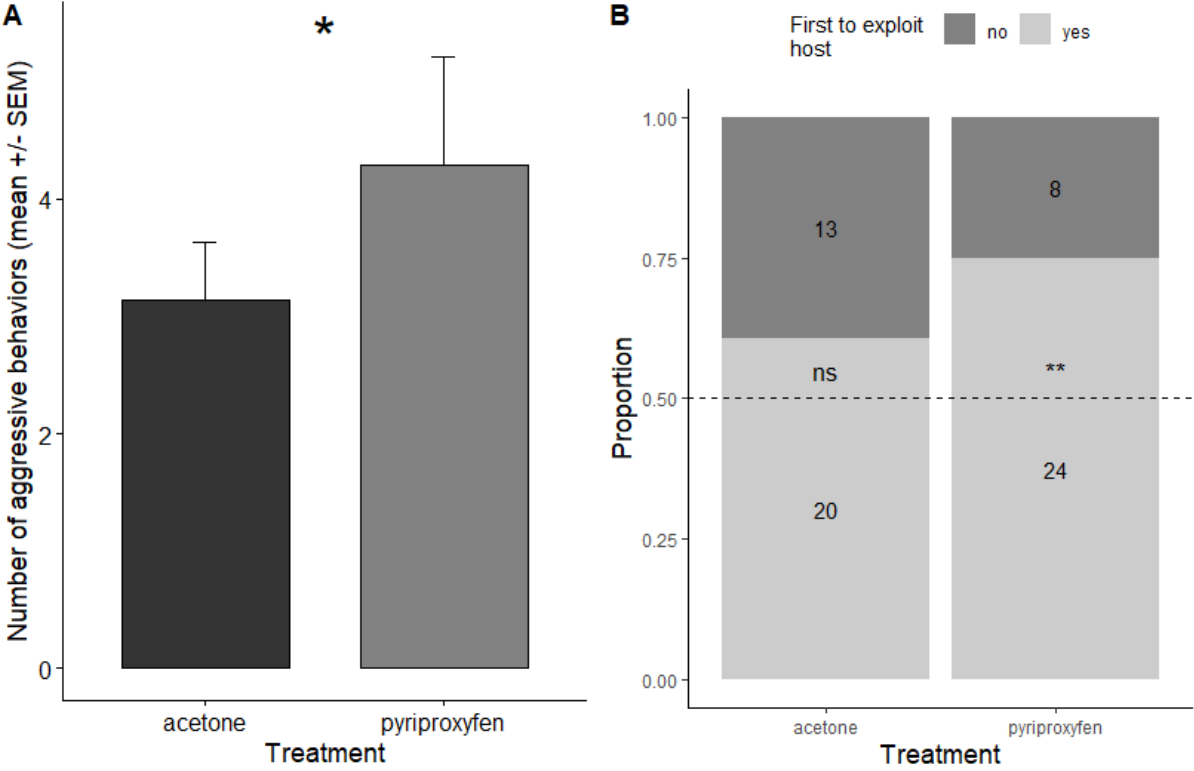


Figure 2



**Table 1:** Effect of relative egg load or mass difference and treatment on the probability of the focal female winning.

<b>Factors affecting victory</b>	<b>Df</b>	<b>LR <math>\chi^2</math></b>	<b>P</b>
Model 1 : Relative egg load difference			
Relative egg load difference	1	0.101	0.751
Focal female treatment	1	1.514	0.219
Relative egg load difference x Focal female treatment	1	0.727	0.394
Model 2 : Relative mass difference			
Relative mass difference	1	2.470	0.116
Focal female treatment	1	2.680	0.102
Relative mass difference x Focal female treatment	1	1.300	0.254

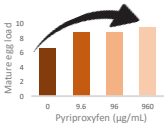
**Side effects of a pesticide on a non-target species:  
Does pyriproxyfen, a juvenile hormone agonist, impact physiology and behavior in a beneficial insect?**

External application of  
pyriproxyfen

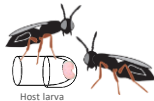


♀ parasitoid of crop pest

**Increase in egg production**



**Increase in female aggressiveness**



**➔ Sublethal doses of this pesticide has unintended effects on a beneficial insect, what might subsequently disrupt its ecosystem services**