

1 **Zoophytophagous mites can trigger plant-genotype specific defensive responses**  
2 **affecting potential prey beyond predation: the case of *Euseius stipulatus* and**  
3 ***Tetranychus urticae* in citrus**

4

5 **Running title: zoophytophagous phytoseiid mites can trigger plant defensive**  
6 **responses**

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23 **ABSTRACT**

24 **BACKGROUND:** Zoophytophagous predators can trigger plant defense affecting prey  
25 populations beyond predation. *Euseius stipulatus* is a presumed zoophytophagous  
26 phytoseiid common in citrus. The response of citrus to one of its potential prey, *T.*  
27 *urticae*, is genotype dependent, with *Citrus reshni* and *C. aurantium* exhibiting extreme  
28 susceptibility and resistance, respectively. Volatile blends produced upon infestation  
29 affected the behavior of these two mites. We wondered whether *E. stipulatus* could  
30 trigger similar responses.

31 **RESULTS:** *E. stipulatus* triggered genotype-dependent defense responses in citrus.  
32 While *C. aurantium* upregulated the JA, SA and flavonoids defensive pathways, *C.*  
33 *reshni* upregulated JA only. Likewise, different volatile blends were induced. These  
34 blends were exploited by *E. stipulatus* to select less defended plants (i.e., those where  
35 higher pest densities are expected) and, interestingly, did not prevent *T. urticae* from  
36 choosing *E. stipulatus*-infested plants. To the best of our knowledge this is the first time  
37 that this type of responses is described for a zoophytophagous phytoseiid.

38 **CONCLUSION:** The observed responses could affect herbivore populations through  
39 plant-mediated effects. Although further research is needed to fully characterize them  
40 and include other arthropods in the system, these results open opportunities for more  
41 sustainable and effective pest control methods (i.e., combining semiochemicals and  
42 biological control).

43

44 **KEY-WORDS:** spider mites, phytoseiids, direct and indirect defense, HIPV,  
45 semiochemical, biological control.

## 46 1 INTRODUCTION

47 Omnivores are consumers that feed on resources at more than one trophic level.<sup>1</sup> In the  
48 case of arthropods, Coll and Guershon<sup>2</sup> called true omnivores those species that feed on  
49 both plants and prey in nature. This category contains many terrestrial arthropods  
50 including plant feeding predators, which are also known as zoophytophagous  
51 predators.<sup>3</sup> Among these species, predatory bugs (Hemiptera: Heteroptera), especially  
52 Miridae, have recently received attention because of their increasing interest as  
53 biological control agents in augmentative releases against important agricultural pests.<sup>4</sup>  
54 <sup>12</sup> These omnivores have been proven to affect the performance of herbivores not only  
55 directly by predation but also through induced plant defense. Zoophytophagy, though, is  
56 not restricted to Heteroptera. Phytoseiidae mites (Acari: Mesostigmata) constitute  
57 another important group of omnivorous biological control agents.<sup>13-14</sup> Several studies  
58 have shown that some phytoseiid species can feed directly on the plant.<sup>15-17</sup> Cheliceral  
59 traits typical of phytoseiid plant feeders have been observed in five genera including the  
60 genus *Euseius* De Leon, where this feeding habit could be widespread.<sup>17,18</sup> Leaf-feeding,  
61 though, may be plant specific. In a study where leaf feeding on plants labeled with  
62 radioactive phosphoric acid by the omnivorous predators *Euseius hibisci* (Chant), *E.*  
63 *fructicolus* (Gonzales and Schuster), and *E. stipulatus* (Athias-Henriot) was evaluated,  
64 only *E. hibisci* proved to feed from avocado leaves, its natural host, whereas none of  
65 them showed evidence of feeding from lemon foliage.<sup>19</sup> The genus *Euseius* is one of the  
66 most common genera in citrus worldwide.<sup>20-21</sup> Indeed, *E. stipulatus* is the most abundant  
67 phytoseiid species in citrus orchards in the Mediterranean basin.<sup>22</sup> In Spain, this  
68 prevalence occurs both in the canopy and in the cover crops associated with citrus,  
69 irrespective of the species/cultivar and management practices used in the orchard.<sup>23-25</sup>  
70 This mite species is considered key in the natural regulation of the populations of two

71 important tetranychid herbivorous pest species in this agroecosystem, the two-spotted  
72 spider mite *Tetranychus urticae* Koch and the citrus red mite *Panonychus citri*  
73 McGregor.<sup>26-27</sup> According to Adar et al.<sup>17</sup> phytoseiid direct leaf feeding could be cultivar  
74 specific, and this could explain the results of Porres et al.<sup>19</sup> with *E. stipulatus* on lemon  
75 leaves. The occurrence of such a behavior in this species would most probably imply  
76 the induction of defense mechanisms in the plant, which could trigger further effects on  
77 conspecifics and other co-occurring species, including potential prey. Therefore, we  
78 decided to focus our attention on the system constituted by citrus, *T. urticae* and *E.*  
79 *stipulatus*.

80 In previous studies, our group demonstrated that the responses of citrus to damage from  
81 *T. urticae* was genotype dependent.<sup>28-31</sup> Sour orange, *Citrus aurantium* L. (Sapindales:  
82 Rutaceae), showed reduced leaf damage symptoms, supported lower mite populations  
83 and reduced oviposition rates compared with Cleopatra mandarin, *Citrus reshni* Hort. ex  
84 Tan., and these effects were transmitted from the roots to the grafted cultivar.  
85 Hormonal, metabolomic and gene expression analyses of the main defense pathways  
86 indicated a relevant role of the oxylipin and the flavonoid pathways. Furthermore, when  
87 *T. urticae* and *E. stipulatus* had to choose between infested sour orange and Cleopatra  
88 mandarin, they preferred poorly defended Cleopatra mandarin plants<sup>30-31</sup>. This result  
89 was observed irrespective of the infestation status of the plant (i.e., uninfested and  
90 infested plants) for *T. urticae*, whereas *E. stipulatus* preferred sour orange when both  
91 genotypes were uninfested.<sup>29</sup> These results were attributed to the different volatile  
92 blends (including Herbivore Plant Induced Volatiles, HIPVs, for infested plants)  
93 produced. Because the HIPVs produced by sour orange can induce resistance in  
94 Cleopatra mandarin,<sup>28</sup> the effect of induction on mite choice was further studied. *T.*  
95 *urticae* still preferred less defended uninfested Cleopatra plants, whereas *E. stipulatus*

96 chose better protected but prey-free induced mandarin plants.<sup>29</sup> As the blends produced  
97 by infested sour orange, and induced Cleopatra mandarin proved attractive to  
98 phytoseiids but not to the herbivore,<sup>31</sup> they may pave the way for developing new more  
99 sustainable tools to manage these species. Should *E. stipulatus* directly feed on the  
100 plant, similar responses are expected. In this study, we have characterized the response  
101 of the two citrus genotypes mentioned earlier to *E. stipulatus* infestation, as well as the  
102 behavior of *T. urticae* and *E. stipulatus*, when offered uninfested and *E. stipulatus*-  
103 infested plants. Our initial hypothesis is that because of the presumed direct feeding of  
104 *E. stipulatus* in citrus, the observed responses will be genotype dependent and similar to  
105 those already observed upon *T. urticae* infestation. In short, plants with relatively  
106 stronger activation of direct defense pathways against *T. urticae* (i.e., oxylipins,  
107 flavonoids) upon *E. stipulatus* feeding should be avoided by the zoophytophagous  
108 predator. Keep in mind that these plants would offer higher levels of potentially toxic  
109 secondary metabolites relative to less defended hosts and, therefore, would sustain  
110 lower prey densities.<sup>32</sup> The same rationale would apply to the herbivore. However, in  
111 both cases, to decrease predation/cannibalism risk, an over-ruling of predator odors over  
112 HIPVs could result in a preference for uninfested versus *E. stipulatus*-infested plants.

113

## 114 **2 MATERIALS AND METHODS**

### 115 **2.1 Plant material**

116 Sour orange (*C. aurantii*) and Cleopatra mandarin (*C. reshni*), the two citrus rootstocks  
117 exhibiting extreme responses to *T. urticae*<sup>30, 32</sup> were used. Three-month-old plants of  
118 both species (with about 10 fully developed leaves) were maintained in a climatic  
119 chamber at  $60 \pm 10\%$  relative humidity (RH) and under a 16:8 h L:D (light:dark)

120 photoperiod combined with a day/night thermal regime of  $25 \pm 2^\circ$  and  $20 \pm 2^\circ\text{C}$ ,  
121 respectively. These plants were grown on vermiculite and peat (1:3; v:v) in 320-ml pots.  
122 No insecticides or acaricides were used and fertilization consisted of a modified  
123 Hoagland's solution applied every 3 days<sup>33</sup> (Bañuls et al., 1997). Lemon fruits obtained  
124 from a pesticide-free experimental orchard at UJI Campus were used to maintain *T.*  
125 *urticae* stock colonies. Finally, bean plants (*Phaseolus vulgaris* L. cv. Buenos Aires  
126 roja) grown at UJI greenhouse in pesticide-free conditions were used to maintain *E.*  
127 *stipulatus* colonies.

## 128 **2.2 Spider mite stock colony**

129 The colony of *T. urticae* used in the assays was initiated with specimens collected in  
130 clementine orchards in the region of La Plana (Castelló, Spain) in 2001. Mites were  
131 maintained on lemons kept in a climatic chamber ( $22 \pm 2.5^\circ\text{C}$  and  $75 \pm 5\%$  RH and 16:8  
132 h L:D photoperiod). Colonies consisted of 8–10 lemons, which were replaced weekly in  
133 groups of four. Adult females obtained from these stock colonies were directly used in  
134 Y-tube olfactory choice assays (see below). In this case, females were subjected to a 24-  
135 h starvation period before the assay. Starvation took place in 50 ml plastic vials where  
136 mites in groups of 15 could drink on a 2 cm in diameter water-soaked cotton ball.

## 137 **2.3 *Euseius stipulatus* stock colony**

138 This colony was initiated with specimens collected in clementine orchards in Montcada,  
139 not far from UJI Campus, in 2012. These phytoseiids were maintained in a climatic  
140 chamber at the same environmental conditions as above. The rearing took place on  
141 detached leaf units consisting of a single bean leaflet placed upside down on moistened  
142 cotton, placed on top of a water-saturated foam cube (3–4 cm thick) in an open plastic  
143 box ( $35 \times 20 \times 7 \text{ cm}^3$ ) half-filled with water. Moist cotton was folded over the edges of

144 the leaves to prevent mites from escaping. *Typha* L. spp. (Typhaceae) pollen, was added  
145 every 3 days to feed this phytoseiid mite. Same as before, adult females of this  
146 predatory mite were directly removed from the colony and subjected to a 24-h  
147 starvation period in vials in groups of seven before use in the Y-tube olfactory choice  
148 assays. Furthermore, specimens from this colony were also used to infest citrus plants  
149 for the same assays and for those were plant volatiles were extracted. In this case, a total  
150 of 25 adult females per plant were used. These mites were deposited on different leaves  
151 with a soft-bristle paintbrush. Infested plants remained in a climatic chamber for up to  
152 48 hours at the same environmental conditions as those explained in 'Plant Material'.  
153 Plants were kept separated by both genotype and infestation status to avoid any  
154 exposure to plant volatiles from the other treatments, which could induce undesired  
155 defensive responses<sup>28</sup>.

#### 156 **2.4 Y-tube olfactory choice assays**

157 Olfactory choice assays were conducted using a Y-tube olfactometer according to Bruin  
158 et al.<sup>34</sup> This assay involves the use of a 4-cm-diameter, Y-shaped glass tube with a 13-  
159 cm base and two 13-cm arms containing a Y-shaped 1-mm diameter metal wire of the  
160 same dimensions, which occupies the core of the olfactometer. The two short arms were  
161 directly connected via a plastic pipeline to the outlets of two identical 5-l glass vessels  
162 containing different odor sources. Each vessel was connected to an air pump that  
163 produced a unidirectional airflow of 1.5 l/h from the arms to the base of the tube. The  
164 air was purified with a granular activated charcoal filter (Sigma-Aldrich). The  
165 environmental conditions inside the Y-tube were  $23 \pm 2^\circ\text{C}$  and  $60 \pm 10\%$  RH. Adult  
166 females offered water only during the 24 h starvation period before the assay, were  
167 individually deposited at the beginning of the long arm of the wire using a soft-bristle  
168 paintbrush. Females were allowed to make a choice within 10 min. As soon as a mite

169 reached the end of one of the two short arms of the tube, the mite was removed from the  
170 set-up and discarded. Mites failing to reach either end of the short arms within the  
171 allocated time were scored as 'no choice'. Different 2-choice experiments involving  
172 infested and uninfested plants of both genotypes, as well as *E. stipulatus* alone were  
173 performed. Each combination was evaluated four times at different dates (i.e., four  
174 replicates). Each replicate included 10 responding mites, which meant that up to 13  
175 mites per combination per date were tested as the non-choice rate ranged from 0 to 3.  
176 The glass vessels were switched after five females had been tested to reduce the effects  
177 of spatial influence on choice. In the case of assays with plants, the plants were replaced  
178 after every 10 females had been tested, and the whole system was rinsed with ethanol  
179 (70%), followed by air drying. To exclude any bias from the set-up, before the  
180 beginning of the assays, 10 mites were exposed to clean air in both arms. A random  
181 choice was expected.

## 182 **2.5 Quantitative real-time reverse transcription-polymerase chain reaction (qRT- 183 PCR) analysis**

184 Three assays including 3 plants per treatment each were carried out. For each assay, six  
185 sour orange and six Cleopatra mandarin plants were used. For each genotype three  
186 plants were infested with *E. stipulatus* as previously explained, whereas the other three  
187 were remained uninfested and were used as controls. 48 hours after infestation at the  
188 same temperature and RH conditions as before, leaves were cut and immediately  
189 introduced into 50 ml Falcon vials, which were immersed in liquid nitrogen and stored  
190 at -80° C until extraction. Leaves from the same treatment were pulled together in the  
191 same vial. RNA was extracted using a Plant RNA protocol with trizol. For qRT-PCR  
192 experiments, 1 µg of total RNA was digested with 0.7 µg of DNase (RNase-free DNase  
193 I) in 0.7 µl of DNase buffer and Milli-Q water up to 4.9 µl and incubated for 30 min at



194 37°C. After incubation, 0.7 µl of EDTA was added and incubated again at 65°C for 10  
195 min to inactivate DNase (Thermofisher Scientific Inc.). The RT reaction was performed  
196 by adding 7 µl of DNase reaction, 2 µl of PrimeScript buffer and 0.5 µl of PrimeScript  
197 RT and Oligo-dT respectively (PrimeScript RT Reagent Kit, Takara Bio Inc.). The  
198 reaction mixture was incubated at 37°C for 15 min. Complementary DNA from the RT  
199 reaction, 10X diluted, was used for qPCR. Forward and reverse primers (0.3 µM) were  
200 added to 5 µl of Maxima SYBR Green qPCR Master Mix, 1 µl of cDNA and 3 µl Milli-  
201 Q sterile water (Maxima SYBR Green/ROX qPCR, Thermofisher Scientific Inc.). qPCR  
202 was carried out using the Smart Cycler II (Cepheid, Sunnyvale, CA, USA) sequence  
203 detector with standard PCR conditions. qRT-PCR analysis was replicated three times.  
204 The expression of lipoxygenase 2 (*LOX2*; accession Cit.16756.1.S1\_s\_at; forward  
205 primer: 5'→3' GAACCATATTGCCACTTTTCG; reverse primer 5'→3':  
206 CGTCATCAATGACTTGACCA) pathogenesis-related protein 5 (*PR5*; accession  
207 BAI63297.1; forward primer: 5'→3' CATCAAGCTTCACAGTGCTTAG; reverse  
208 primer 5'→3': CCACAACGTACAGACTGATGAC) and Chalcone synthase (*CHS*;  
209 accession CF417078; forward primer: 5'→3': AGACGATCCTCCCTGACTCT; reverse  
210 primer 5'→3': CTCCACTTGGTCCAGAATTG) genes was determined.<sup>32</sup> Relative  
211 expression was compared with the housekeeping gene glyceraldehyde 3-phosphate  
212 dehydrogenase (*GAPDH*; accession Cit.122.1; forward primer: 5'→3':  
213 GGAAGGTCAAGATCGGAATCAA; reverse primer 5'→3':  
214 CGTCCCTCTGCAAGATGACTCT).

215

## 216 **2.6 Collection of headspace volatiles in plants occupied by *E. stipulatus***

217 Volatiles from the two citrus genotypes, including uninfested and *E. stipulatus*-infested  
218 plants, were collected using a headspace collection system similar to that described by  
219 Bruinsma et al.<sup>35</sup> 5-l glass vessels (Duran, Mainz, Germany) ventilated with carbon-  
220 filtered pressure-air at 1.5 l h<sup>-1</sup> were used. Pasteur pipettes with 300 mg of Porapak  
221 (Sigma-Aldrich, Barcelona, Spain) were used as a volatile retention filter. These filters  
222 were in the air outlet hole at the top of the glass vessel. Plants were individually  
223 introduced into these glass vessels. The system (glass vessels and Porapak filters) was  
224 cleaned with acetone and dried in an oven 1 hour prior to the assay. Volatiles collection  
225 took place in a climatic chamber at 60 ± 10% RH and under a 16:8 h L:D photoperiod  
226 combined with a day/night thermal regime of 25 ± 2° and 20 ± 2°C, respectively. When  
227 necessary, plants were infested with 25 *E. stipulatus* adult females, (as explained above)  
228 which could feed directly on the plant, cannibalize conspecifics, or try to escape. In this  
229 case, the volatiles were collected during the first 24 hours of infestation as maintaining  
230 the plants under these conditions for longer (i.e., 48 hours as in the previous assays)  
231 resulted in deposition of water droplets in the interior of the vessel. These droplets may  
232 affect the efficiency of the collection. Furthermore, previous studies showed that gene  
233 expression and hormone concentration in infested citrus plants did not significantly  
234 change between 24 and 48 hours post infestation.<sup>32</sup> Three plants per genotype and  
235 infestation status were considered in each of the three replicates of this assay.

## 236 **2.7 Gas chromatography (GC) instrumentation**

237 An Agilent 6890N GC system (Palo-Alto, CA, USA), equipped with an Agilent 7683  
238 autosampler, coupled to a time-of-flight mass spectrometer (TOF-MS), GCT (Waters  
239 Corp., Manchester, UK), operating in electron ionization (EI) mode were used in our  
240 assays. A fused silica DB-5MS capillary column of 30 m length, 0.25 mm internal  
241 diameter and a film thickness of 0.25 µm (J&W Scientific, Folsom, CA, USA) were

242 used for GC separation. The temperature program for this process was the following;  
243 50°C (1min); 5°C min<sup>-1</sup> to 210°C (1 min); 20°C min<sup>-1</sup> to 300°C (2 min); this resulted in  
244 a total analysis run of 40.50 min. Splitless injections were carried out. Helium was used  
245 as carrier gas at 1ml min<sup>-1</sup>. The interface and source temperatures were both set to  
246 250°C and a solvent delay of 3 min was selected. The TOF-MS was operated at 1  
247 spectrum s<sup>-1</sup> acquiring the mass range m/z 50–650 and using a multi-channel plate  
248 voltage of 2800 V. The TOF-MS resolution was c. 8500 (full width at half-maximum,  
249 FWHM) at m/z 614. Heptacose, used for the daily mass calibration as well as lock  
250 mass, was injected via syringe into the reference reservoir at 30°C. The m/z ion  
251 monitored was 218.9856. The application manager ChromaLynx, a module of  
252 MassLynx software, was used to investigate the presence of non-target compounds in  
253 the samples.

254 The retention time and fragmentation spectrum of the following commercial standards  
255 were used to identify volatile compounds: methyl salicylate (MeSA) and methyl  
256 jasmonate (MeJA) (Sigma-Aldrich). Other volatile compounds were tentatively  
257 identified using GC–MS and matching to the National Institute of Standards and  
258 Technology (NIST) Library, using Match values of at least 850 as a threshold for  
259 identification, as described by Wallis et al.<sup>36</sup> Furthermore, for each HIPV identified the  
260 TOF-MS-derived peak areas were calculated and used to estimate their relative  
261 concentration.

## 262 **2.8 Statistical analyses**

263 Statistical analyses were conducted using IBM SPSS Statistics 23. Chi-square and  
264 Student *t*-tests were used to compare the results of the two-choice assays and genetic  
265 expression results, respectively. For each volatile identified in the blends produced by

266 plants, TOF-MS-derived peak areas were compared using a Generalized Linear Model  
267 (GLM) with a normal distribution of the error and identity link function (i.e, linear  
268 regression). Plant genotype, infestation status, and replicate were used as fixed effects.  
269 When necessary, we used Bonferroni post-hoc test ( $P < 0.05$ ) for mean separation.

270

## 271 **3 RESULTS**

### 272 **3.1 *E. stipulatus*-infested citrus plants modify the behavior of conspecifics and also** 273 **of the potential prey *T. urticae*.**

274 In agreement with our initial hypothesis that *E. stipulatus* odors would result repellent  
275 for *T. urticae*, two-spotted spider mite adult females avoided *E. stipulatus* when  
276 exposed to the predator odors alone (Figure 1). However, contrary to our expectations,  
277 when *E. stipulatus* was infesting the plants, these resulted attractive for *T. urticae*  
278 irrespective of the genotype. Indeed, when *T. urticae* was simultaneously exposed to the  
279 two infested citrus genotypes, no preference for any of them was observed whereas a  
280 preference for Clementine mandarin was observed when the same genotypes were  
281 uninfested. Likewise, contrary to our expectations, *E. stipulatus* females did not avoid  
282 conspecifics when exposed to their own odors alone (Figure 2). However, when HIPVs  
283 were at play, their response was genotype dependent. As expected, *E. stipulatus*-  
284 infested Cleopatra mandarin plants resulted attractive, whereas infested sour orange  
285 became repellent. Moreover, when the two genotypes were simultaneously offered, a  
286 preference for Cleopatra mandarin was observed when plants were infested, whereas  
287 sour orange was preferred when plants were uninfested.

### 288 **3.2 The generalist predator *E. stipulatus* triggers defensive responses in sour** 289 **orange and Cleopatra mandarin plants.**

290 The JA, SA, and flavonoid signaling pathways homologous marker genes *LOX2*, *PR5*,  
291 and *CHS*, respectively, were analyzed in uninfested and *E. stipulatus*-infested plants.  
292 *LOX2* relative expression was 2.5 times higher in infested than in uninfested plants  
293 irrespective of the plant genotype (Figure 3A). However, for the other two marker  
294 genes, plant-genotype differences were observed. *PR5* and *CHS* expressions were  
295 enhanced in sour orange ( $\times 2.2$  and  $\times 1.2$ , respectively), whereas *PR5* did not change  
296 and *CHS* was downregulated ( $\times 0.7$ ) in Cleopatra mandarin (Figures 3B and 3C).

### 297 **3.3 The generalist predator *E. stipulatus* triggers the production of volatiles** 298 **(HIPVs) in sour orange and Cleopatra mandarin plants.**

299 GLM results showed differences in the volatile metabolome of infested relative to  
300 uninfested plants, which also suggest that *E. stipulatus* can feed directly on citrus plants.  
301 The factor ‘replicate’ and all the 2- and 3-factor interactions where it was included in  
302 the GLM used were significant. The reason is that for each HIPV identified, the TOF-  
303 MS-derived peak areas obtained for each replicate could be up to two orders of  
304 magnitude apart. However, as the relative differences observed for the other two factors  
305 considered (plant genotype and infestation) for each volatile were consistent (Figure 4),  
306 results were interpreted in a qualitative manner and according to these two factors only.  
307 From the 11 compounds identified in these blends, four of them did not change with  
308 infestation and plant genotype. These were 1,4-diethyl-Benzene, 1-(4-ethylphenyl)-  
309 Ethanone, 4-Butoxybutanoic acid, and 3,5-di-tert-Butyl-4-hydroxybenzaldehyde. The  
310 remaining 7 compounds showed different trends (Table 1, Figure 4). The terpenoid  
311 Pinene decreased with infestation irrespective of the genotype (Figure 4A). The  
312 production of another terpenoid, Cineole (Figure 4B), and that of the aromatic  
313 compound 1-(2,5-dimethylphenyl)-Ethanone (Figure 4C) showed a common trend: they  
314 were higher in sour orange than in Cleopatra mandarin and decreased with infestation.

315 The other 4 HIPVs, the Green Leaf Volatile 2,6,10-Dodecatrienoic acid, and the  
316 aromatic compounds 1-methyl-4-(2-propenyl)-Benzene, 1-ethyl-4-(1-methylethyl)-  
317 Benzene, and 4-(1-methylethyl)-Benzaldehyde (Figures 4D, 4E, 4F and 4G,  
318 respectively), also showed another common trend. In this case, they increased with  
319 infestation and were higher in Cleopatra mandarin.

320

#### 321 **4 DISCUSSION**

322 To our knowledge, this is the first study to demonstrate that zoophytophagous  
323 phytoseiid mites can trigger defensive responses in plants. The presence of *E. stipulatus*  
324 was perceived by the plant, which reacted to it in a genotype-dependent way, with sour  
325 orange exhibiting a stronger and more diversified response than Cleopatra mandarin.  
326 Although phytophagy remains the most likely trigger for these responses, other causes,  
327 including the physical presence of the predatory mite on the plant, its footsteps and its  
328 deposition of feces or eggs, cannot be discarded.<sup>37-39</sup> Direct plant feeding by the closely  
329 related phytoseiids *Euseius scutalis* (Athias-Henriot) and *Iphiseius degenerans*  
330 (Berlese) entails crimping and piercing the feeding surface.<sup>17</sup> In the case of *E. scutalis*,  
331 plant cell-sap uptake in pepper plants is performed by penetrating the leaf epidermis,  
332 leaving discrete holes in its surface surrounded by intact cells.<sup>40</sup> This type of wounding  
333 is completely different from the injury produced by *T. urticae*. This herbivore uses its  
334 stylets to penetrate leaves, either in between epidermis pavement cells or through a  
335 stomatal opening, to feed from individual mesophyll cells without damaging the  
336 epidermal cell layer.<sup>41</sup> Assuming that *E. stipulatus* most likely produces a wounding  
337 similar to that described for *E. scutalis*, which also occurs in citrus in the  
338 Mediterranean,<sup>42-44</sup> the plant responses expected after feeding would be different from

339 those triggered by the tetranychid. These differences would be related to the targeted  
340 plant cell/tissue type. This was the case for Cleopatra mandarin but not for sour orange,  
341 where the defense pathways triggered by these two mite species were quite similar. On  
342 the one hand, the oxylipin pathway was upregulated in both citrus genotypes in a similar  
343 manner (Figure 3A), whereas for *T. urticae* infestation, this upregulation was observed  
344 in sour orange only.<sup>32</sup> On the other hand, the SA (Figure 3B) and flavonoids (Figure 3C)  
345 pathways presented the same trends as observed for *T. urticae* infestation. As the  
346 response of sour orange is based on the simultaneous activation of different types of  
347 defense (JA, SA, flavonoids), our results confirm that this genotype may be a jack-of-  
348 all-trades,<sup>29</sup> where some well-known negative cross-talk mechanisms between signaling  
349 pathways in plant defense (i.e., JA-SA) do not occur.<sup>29, 32, 45-46</sup> However, the solely  
350 upregulation of the JA pathway in Cleopatra mandarin may indicate that some of these  
351 negative cross-talks are functional in this genotype. The ability of sour orange to resist  
352 *T. urticae* was attributed in former studies to a combination of basal and inducible direct  
353 and indirect defense mechanisms.<sup>29, 32</sup> Direct mechanisms include high levels of  
354 flavonoids and a fast and effective activation of the JA signaling pathway.<sup>32</sup> Because  
355 LOX proteins are a family of enzymes involved in the synthesis of JA that play  
356 important roles in the metabolic responses to wounding,<sup>47-48</sup> we hypothesize that the  
357 activation of this pathway in both genotypes (Figure 3) may be a response to the  
358 wounding produced to epidermal cells by *E. stipulatus*. Such damage, as explained  
359 above, does not occur for *T. urticae*.<sup>41</sup>

360 Although direct plant feeding by *T. urticae*<sup>32</sup> and presumably by *E. stipulatus* activated  
361 the same defensive pathways in sour orange, Pinene was the only common compound  
362 found in the HIPV blends elicited by these two mites<sup>28</sup> (Table 1). While Pinene was  
363 indicative of *E. stipulatus* infestation in both genotypes, for *T. urticae* this volatile was

364 differentially produced upon infestation in sour orange, only.<sup>28</sup> As a consequence,  
365 Pinene, together with 2,6,10-Dodecatrienoic acid, 1-methyl-1-4-(2-propenyl)-Benzene,  
366 1-ethyl-4-(1-methylethyl)-Benzene, and 4-(1-methylethyl)-Benzaldehyde, which  
367 followed similar increasing trends upon *E. stipulatus* infestation in Cleopatra mandarin  
368 (Figure 4), could be the key volatiles for the observed attraction of *T. urticae* for *E.*  
369 *stipulatus*-infested plants (Figure 1). The result that Pinene and 1-methyl-1-4-(2-  
370 propenyl)-Benzene were the only volatiles which increased in sour orange upon  
371 infestation (Table 1; Figures 4A and 4E) could be taken as indicative of the crucial role  
372 of these two HIPVs. Whether *T. urticae* attraction could be the result of these volatiles  
373 masking *E. stipulatus* own odors deserves further research. Remarkably, the fact that  
374 upon *T. urticae* feeding, sour orange became repellent for conspecific mites,<sup>28</sup>  
375 highlights the importance of considering the whole blend of volatiles and no single  
376 specific compounds when assessing this type of behavioral responses.<sup>49</sup>

377 With the exception of Pinene, which was equally induced in *E. stipulatus*-infested  
378 plants, the remaining HIPVs could be split in two groups: those which were higher in  
379 sour orange and decreased with infestation (Cineole and 1-(2,5-dimethylphenyl)  
380 Ethanone), and those which were higher in Cleopatra mandarin and increased with  
381 infestation (Table 1). These two groups most probably play an important role in the  
382 plant choices observed for this phytoseiid mite. Interestingly, most of the volatiles in the  
383 second group are aromatic compounds, which are related to the flavonoids pathway  
384 since both originate from the same precursors, including phenylalanine and its  
385 derivatives.<sup>50</sup> However, the levels of most of these aromatic volatiles did not change in  
386 sour orange [1-methyl-4-(2-propenyl) Benzene escaped to this trend and slightly  
387 increased, Figure 4E] while they increased in Cleopatra mandarin, just the opposite of  
388 what we observed for *CHS* gene expression (Figure 3C). This observation may be



389 explained by the enhanced levels of flavonoids observed in sour orange following  
390 infestation by *T. urticae*, since this genotype seems more efficient in the biosynthesis of  
391 these flavonoid derivatives, such as naringenine, than Cleopatra mandarin.<sup>32</sup> As the  
392 biosynthesis of the aromatic volatiles and flavonoids, which are directly related to direct  
393 defense, share a common origin, *E. stipulatus* could exploit these aromatic volatiles to  
394 select plants with relatively lower levels of direct defense (Figure 2).

395 The results of the olfactometer assays only partially match our initial hypotheses. In the  
396 case of *T. urticae* and in agreement with them, it was repelled by the odor of its  
397 potential predator and it chose less defended uninfested Cleopatra mandarin rather than  
398 uninfested sour orange (Figure 1). However, the forecasted over-ruling of its predator  
399 associated odors (including *E. stipulatus*-triggered HIPVs) leading to repellence proved  
400 wrong. In the case of *E. stipulatus*, in agreement with our hypotheses, the phytoseiid  
401 always chose the plants producing higher levels of aromatic volatiles (Figures 2 and 4),  
402 which according to what we exposed in the previous paragraph, could be perceived as  
403 those containing less flavonoids. However, this mite was attracted by conspecifics and  
404 the over-ruling of the odors associated with its presence on the plant proved wrong as  
405 well. On the one hand, these failures may be the result of these two mites making  
406 decisions based not only on volatiles but refined later on with tactile stimuli, both  
407 chemical and physical, on the surface of the host plant, which could change the sign of  
408 the attraction.<sup>51-52</sup> On the other hand, they may be a consequence of *E. stipulatus* posing  
409 a relatively low predation/cannibalism risk to *T. urticae* and conspecific hungry adult  
410 females, respectively. In a field study where *E. stipulatus* was subjected to gut-content  
411 analysis, Pérez-Sayas et al.<sup>27</sup> demonstrated that this phytoseiid significantly preferred  
412 non-tetranychid food sources over both *T. urticae* and *P. citri*, independently of the  
413 densities of these two potential tetranychid preys. Indeed, only 28.4 % of the individuals

414 analyzed proved positive for *T. urticae* feeding, whereas this figure increased to 75.7 %  
415 for the co-occurring *Tetranychus* spp. specialist predator *Phytoseiulus persimilis*  
416 (Athias-Henriot).

417

## 418 **5 CONCLUSION**

419 Although the net effects of the interactions described herein for herbivore pest  
420 populations should be assessed in the field under more realistic conditions, our results  
421 prove that zoophytophagous phytoseiid mites may affect their prey beyond predation  
422 through plant-mediated effects. The characterization of such effects may help refining  
423 current biological control practices. Because the HIPV blends identified in this study  
424 proved to effectively attract *T. urticae* and *E. stipulatus*, opportunities for the  
425 exploitation of these semiochemicals to increase the efficacy of biological control exist  
426 and should be explored.

427

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592

593

594 **TABLES**

595 **Table 1.** Volatile profiling in the headspace of sour orange (SO) and Cleopatra mandarin (Cleo) plants either uninfested (clean) or infested (inf).  
 596 For each volatile, TOF-MS-derived peak areas were compared using a Generalized Linear Model. Plant genotype, infestation status, and replicate  
 597 were used as fixed effects. As replicate and all the interactions including this factor were significant ( $P < 0.05$ ), these results are not presented in  
 598 the table. As the relative differences observed for the other two factors considered were consistent for each volatile, results were interpreted in a  
 599 qualitative manner and according to these two factors only. Volatiles were tentatively identified by comparing to the National Institute of  
 600 Standards and Technology (NIST) Library as described by Wallis et al.<sup>36</sup>

Volatile compounds	GLM results (Wald- $\chi^2$ ; $P$ )		
	Plant genotype (A)	Infestation status (B)	A*B
Pinene	0.004; 1; 0.951 SO = Cleo	153.60; 1; <0.001 clean < inf	0.174; 1; 0.677
Cineole	3.82; 1; 0.051 SO > Cleo	19.17; 1; <0.001 clean > inf	5.29; 1; 0.021 SO clean > SO inf = Cleo clean = Cleo inf
Ethanone, 1-(2,5-dimethylphenyl)	52.92; 1; <0.001 SO > Cleo	12.00; 1; 0.001 clean > inf	12.00; 1; 0.001 SO clean > SO inf > Cleo clean = Cleo inf
2,6,10-Dodecatrienoic acid	35.28; 1; <0.001 SO < Cleo	6.92; 1; 0.009 clean < inf	26.91; 1; <0.001 SO clean = SO inf = Cleo clean < Cleo inf
Benzene, 1-methyl-4-(2-propenyl)-	37.94; 1; <0.001 SO < Cleo	61.04; 1; <0.001 clean < inf	27.30; 1; <0.001 SO clean < SO inf = Cleo clean < Cleo inf
Benzene, 1-ethyl-4-(1-methylethyl)-	65.34; 1; <0.001 SO < Cleo	57.82; 1; <0.001 clean < inf	49.11; 1; <0.001 SO clean = SO inf = Cleo clean < Cleo inf
Benzaldehyde, 4-(1-methylethyl)-	131.05; 1; <0.001 SO < Cleo	123.62; 1; <0.001 clean < inf	124.51; 1; <0.001 SO clean = SO inf = Cleo clean < Cleo inf

601 For volatiles for which the Plant\*Infestation interaction is significant, means were separated according to Bonferroni ( $P < 0.05$ ).

602 **FIGURE LEGENDS**

603

604 **Figure 1.** Olfactory responses of *T. urticae* adult females to *E. stipulatus*. Five different  
605 combinations, in which *T. urticae* had to choose between two odor sources, were tested.  
606 A minimum of 40 adult females per choice combination was tested. From top to bottom  
607 these combinations were: empty glass versus *E. stipulatus*, Cleopatra mandarin  
608 uninfested plants (Cleo) vs sour orange uninfested plants (SO), SO vs SO-infested  
609 plants (SO inf), Cleo vs Cleo-infested plants (Cleo inf), and Cleo inf vs SO inf. Infested  
610 plants had been exposed to 25 *E. stipulatus* adult females for 48 h before the onset of  
611 the assay. Asterisks indicate significant differences from a 1:1 distribution (chi-square  
612 test;  $P < 0.05$ ).

613

614 **Figure 2.** Olfactory responses of *E. stipulatus* adult females to conspecific mites. Five  
615 different combinations, in which *E. stipulatus* had to choose between two odor sources  
616 were tested. A minimum of 40 adult females per choice combination was tested. From  
617 top to bottom these combinations were: empty glass versus *E. stipulatus*, Cleopatra  
618 mandarin uninfested plants (Cleo) vs sour orange uninfested plants (SO), SO vs SO-  
619 infested plants (SO inf), Cleo vs Cleo-infested plants (Cleo inf), and Cleo inf vs SO inf.  
620 Infested plants had been exposed to 25 *E. stipulatus* adult females for 48 h before the  
621 onset of the assay. Asterisks indicate significant differences from a 1:1 distribution (chi-  
622 square test;  $P < 0.05$ ).

623

624 **Figure 3.** Relevance of: **A.** Lipoxygenase 2, *LOX2* (cit16759.1S1), **B.** Pathogenesis-  
625 related protein 5, *PR5* (BAI63287.1), and **C.** Chalcone synthase, *CHS* (CF417078), in

626 citrus defense triggered by *E. stipulatus*. Total RNA was extracted from the leaves of  
627 three plants per genotype (sour orange, SO, and Cleopatra mandarin, Cleo) and  
628 infestation status (uninfested and infested with 25 mites, inf) 48 hours after infestation,  
629 converted to cDNA and subjected to quantitative RT-PCR analysis. Transcript levels  
630 were normalized to the expression of the housekeeping gene glyceraldehyde 3-  
631 phosphate dehydrogenase (*GAPDH*) measured in the same sample. For each genotype,  
632 data are presented as a mean of transcript expression relative to uninfested plants  $\pm$  SE  
633 ( $n = 3$ ). Significant differences between uninfested and infested plants were estimated  
634 performing a *t*-test for each genotype. Asterisks indicate statistically significant  
635 differences ( $P < 0.05$ ).

636

637 **Figure 4.** Relative signal (TOF-MS-derived peak areas) of the volatiles differentially  
638 produced in infested (inf) and uninfested (clean) sour orange (SO) and Cleopatra  
639 mandarin (Cleo) plants during the first 24 hours of infestation with 25 *E. stipulatus*  
640 adult females. **A.** Pinene; **B.** Cineole; **C.** 1-(2,5-dimethylphenyl)-Ethanone; **D.** 2,6,10-  
641 Dodecatrienoic acid; **E.** 1-methyl-4-(2-propenyl)-Benzene; **F.** 1-ethyl-4-(1-  
642 methylethyl)-Benzene; **G.** 4-(1-methylethyl)-Benzaldehyde. For each figure, bars with  
643 the same letter are not significantly different ( $P < 0.05$ ).

644