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

Running title: zoophytophagous phytoseiid mites can trigger plant defensive 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.

1 INTRODUCTION

Omnivores are consumers that feed on resources at more than one trophic level.[1] In the case of arthropods, Coll and Guershon[2] called true omnivores those species that feed on both plants and prey in nature. This category contains many terrestrial arthropods including plant feeding predators, which are also known as zoophytophagous predators.[3] Among these species, predatory bugs (Hemiptera: Heteroptera), especially Miridae, have recently received attention because of their increasing interest as biological control agents in augmentative releases against important agricultural pests.[4-12] These omnivores have been proven to affect the performance of herbivores not only directly by predation but also through induced plant defense. Zoophytophagy, though, is not restricted to Heteroptera. Phytoseiidae mites (Acari: Mesostigmata) constitute another important group of omnivorous biological control agents.[13-14] Several studies have shown that some phytoseiid species can feed directly on the plant.[15-17] Cheliceral traits typical of phytoseiid plant feeders have been observed in five genera including the genus *Euseius* De Leon, where this feeding habit could be widespread.[17,18] Leaf-feeding, though, may be plant specific. In a study where leaf feeding on plants labeled with radioactive phosphoric acid by the omnivorous predators *Euseius hibisci* (Chant), *E. fructicolus* (Gonzales and Schuster), and *E. stipulatus* (Athias-Henriot) was evaluated, only *E. hibisci* proved to feed from avocado leaves, its natural host, whereas none of them showed evidence of feeding from lemon foliage.[19] The genus *Euseius* is one of the most common genera in citrus worldwide.[20-21] Indeed, *E. stipulatus* is the most abundant phytoseiid species in citrus orchards in the Mediterranean basin.[22] In Spain, this prevalence occurs both in the canopy and in the cover crops associated with citrus, irrespective of the species/cultivar and management practices used in the orchard.[23-25] 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.[26-27] According to Adar et al.[17] phytoseiid direct leaf feeding could be cultivar
74 specific, and this could explain the results of Porres et al.[19] 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.[28-31] 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[30-31]. 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.[29] 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,[28] the effect of induction on mite choice was further studied. *T.*
95 *urticae* still preferred less defended uninfested Cleopatra plants, whereas *E. stipulatus*

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

2 MATERIALS AND METHODS

2.1 Plant material

Sour orange (*C. aurantii*) and Cleopatra mandarin (*C. reshni*), the two citrus rootstocks exhibiting extreme responses to *T. urticae*[30, 32] were used. Three-month-old plants of both species (with about 10 fully developed leaves) were maintained in a climatic chamber at 60 ± 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 ± 2° and 20 ± 2°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[33] (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 ± 2.5°C and 75 ± 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 × 20 × 7 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 [28].

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. [34] 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 ± 2°C and 60 ± 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

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

2.5 Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis

Three assays including 3 plants per treatment each were carried out. For each assay, six sour orange and six Cleopatra mandarin plants were used. For each genotype three plants were infested with *E. stipulatus* as previously explained, whereas the other three were remained uninfested and were used as controls. 48 hours after infestation at the same temperature and RH conditions as before, leaves were cut and immediately introduced into 50 ml Falcon vials, which were immersed in liquid nitrogen and stored at -80° C until extraction. Leaves from the same treatment were pulled together in the same vial. RNA was extracted using a Plant RNA protocol with trizol. For qRT-PCR experiments, 1 µg of total RNA was digested with 0.7 µg of DNase (RNase-free DNase 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' GAACCATATTGCCACTTTCG; 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.[32] 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***

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

2.7 Gas chromatography (GC) instrumentation

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

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

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

2.8 Statistical analyses

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

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

3 RESULTS

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

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

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

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

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

GLM results showed differences in the volatile metabolome of infested relative to uninfested plants, which also suggest that *E. stipulatus* can feed directly on citrus plants. The factor 'replicate' and all the 2- and 3-factor interactions where it was included in the GLM used were significant. The reason is that for each HIPV identified, the TOF-MS-derived peak areas obtained for each replicate could be up to two orders of magnitude apart. However, as the relative differences observed for the other two factors considered (plant genotype and infestation) for each volatile were consistent (Figure 4), results were interpreted in a qualitative manner and according to these two factors only. From the 11 compounds identified in these blends, four of them did not change with infestation and plant genotype. These were 1,4-diethyl-Benzene, 1-(4-ethylphenyl)-Ethanone, 4-Butoxybutanoic acid, and 3,5-di-tert-Butyl-4-hydroxybenzaldehyde. The remaining 7 compounds showed different trends (Table 1, Figure 4). The terpenoid Pinene decreased with infestation irrespective of the genotype (Figure 4A). The production of another terpenoid, Cineole (Figure 4B), and that of the aromatic compound 1-(2,5-dimethylphenyl)-Ethanone (Figure 4C) showed a common trend: they 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.[37-39] 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.[17] 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.[40] 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.[41] 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,[42-44] 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.[32] 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,[29] where some well-known negative cross-talk mechanisms between signaling
349 pathways in plant defense (i.e., JA-SA) do not occur.[29, 32, 45-46] 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.[29, 32] Direct mechanisms include high levels of
354 flavonoids and a fast and effective activation of the JA signaling pathway.[32] 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,[47-48] 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*.[41]

360 Although direct plant feeding by *T. urticae*[32] 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[28] (Table 1). While Pinene was
363 indicative of *E. stipulatus* infestation in both genotypes, for *T. urticae* this volatile was

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

With the exception of Pinene, which was equally induced in *E. stipulatus*-infested plants, the remaining HIPVs could be split in two groups: those which were higher in sour orange and decreased with infestation (Cineole and 1-(2,5-dimethylphenyl) Ethanone), and those which were higher in Cleopatra mandarin and increased with infestation (Table 1). These two groups most probably play an important role in the plant choices observed for this phytoseiid mite. Interestingly, most of the volatiles in the second group are aromatic compounds, which are related to the flavonoids pathway since both originate from the same precursors, including phenylalanine and its derivatives.[50] However, the levels of most of these aromatic volatiles did not change in sour orange [1-methyl-4-(2-propenyl) Benzene escaped to this trend and slightly increased, Figure 4E] while they increased in Cleopatra mandarin, just the opposite of 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.[32] 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.[51-52] 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.[27] 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

428 **ACKNOWLEDGMENTS**

429 The research leading to these results was partially funded by the Spanish Ministry of
430 Economy and Competitiveness (AGL2014-55616-C3; AGL2015-64990-2R). The
431 authors thank M. Piquer (UJI) for technical assistance and Victoria Ibáñez-Gual (UJI)
432 for statistical advice. MC received a pre-doctoral fellowship from the Spanish Ministry
433 of Economy and Competitiveness (BES-2015-074570) and MP was the recipient of a
434 research fellowship from INIA, Spain (subprogram DOC INIA-CCAA).

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592

593

TABLES

Table 1. Volatile profiling in the headspace of sour orange (SO) and Cleopatra mandarin (Cleo) plants either uninfested (clean) or infested (inf). For each volatile, TOF-MS-derived peak areas were compared using a Generalized Linear Model. Plant genotype, infestation status, and replicate 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 the table. As the relative differences observed for the other two factors considered were consistent for each volatile, results were interpreted in a qualitative manner and according to these two factors only. Volatiles were tentatively identified by comparing to the National Institute of Standards and Technology (NIST) Library as described by Wallis et al.[36]

Volatile compounds	GLM results (Wald-χ^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

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**. Lypoxigenase 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 ± 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