# Biological characterization of Euscelidius variegatus iflavirus 1

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## Abstract

Virus-based biocontrol technologies represent sustainable alternatives to pesticides and insecticides. Phytoplasmas are prokaryotic plant pathogens causing severe losses to crops worldwide. Novel approaches are needed since insecticides against their insect vectors and rogueing of infected plants are the only available strategies to counteract phytoplasma diseases. A new iflavirus, named EVV-1, has been described in the leafhopper phytoplasma vector *Euscelidius variegatus*, raising the potential to use virus-based application strategies against phytoplasma disease. Here transmission routes of EVV-1 are characterized, and localization within the host reveals the mechanism of insect tolerance to virus infection. Both vertical and horizontal transmission of EVV-1 occur and vertical transmission was more efficient. The virus is systemic and occurs in all life-stages, with the highest loads measured in ovaries and first to third instar nymphs. The basic knowledge gained here on the biology of the virus is crucial for possible future application of iflaviruses as biocontrol agents.

***Keywords***

Leafhopper, phytoplasma vector, insect virus, vertical transmission, horizontal transmission

45 **1. Introduction**

46 The use of next generation sequencing in the last few years allowed the discovery of
47 many novel viruses in arthropods (Dong et al., 2018; dos Santos et al., 2019; O'Brien et al.,
48 2018). In particular, this technology proved crucial for the identification of covert viruses,
49 those that do not cause overtly pathological effects on their hosts (Nouri et al., 2018). Of the
50 newly discovered virus types, positive-sense RNA viruses are dominant (Bonning, 2020).
51 Among them, iflaviruses (Iflaviridae) form a distinct group in the order *Picornavirales*, (Valles
52 et al., 2017). Effects of some of these viruses include developmental anomalies (Li et al., 2019)
53 behavioural alterations (Wells et al., 2016), tissue pathologies (Brettell et al., 2017), and
54 premature death (Geng et al., 2017) of the insect host, while others do not cause any evident
55 symptoms (dos Santos et al., 2019; Murakami et al., 2013). Transmission modalities of these
56 viruses have been explored, leading to a multifaceted infection model. Horizontal
57 transmission (oral route) is the most efficient means of spread of some species, for example
58 Nilaparvata lugens honeydew virus-1 of the brown leafhopper (Murakami et al., 2013),
59 Helicoverpa armigera iflavirus (Yuan et al., 2017), and Nora Virus of the cotton bollworm
60 (Yang et al., 2019), and Pyrrhocoris apterus virus 1 in red firebugs (Vinokurov and Koloniuk,
61 2019). In contrast, Antheraea pernyi Vomit Disease of Chinese oak silkmoth (Geng et al.,
62 2017) and Spodoptera exigua iflaviruses-1 and 2 of the beet armyworm (Virto et al., 2014)
63 appear to be primarily spread by vertical transmission. The Deformed Wing Virus (DWV) of
64 the honeybee is transmitted both horizontally and vertically, and by *Varroa destructor* mite as
65 vector (Chen et al., 2006; Yue and Genersch, 2005).

66 The genomic features, phylogenetic analysis, and prevalence of a new member of the
67 family *Iflaviridae*, Euscelidius variegatus virus 1 (EVV-1), were previously reported (Abbà et
68 al., 2017) as an additional result obtained during the *de novo* assembly of transcriptome from
69 the hemipteran leafhopper *Euscelidius variegatus* Kirschbaum (Galetto et al., 2018). EVV-1 did

70 not induce any evident symptoms in the *E. variegatus* population surveyed but, when
71 laboratory-reared, produced infections of 100% prevalence (Abbà et al., 2017). EVV-1 forms
72 non-enveloped, icosahedral particles, and the ssRNA(+) genome encodes a single polyprotein
73 of 3132 amino acids, which is post-translationally processed.

74 *Euscelidius variegatus* (Cicadellidae: Deltocephalinae) is a palearctic, multivoltine and
75 polyphagous species, widespread in Europe and North America. This species is economically
76 important because it is a natural vector of '*Candidatus* Phytoplasma asteris' (chrysanthemum
77 yellows strain) and a laboratory vector of the Flavescence dorée phytoplasma (reviewed in
78 EFSA Panel on Plant Health (PLH), 2014. Phytoplasmas are plant-pathogenic bacteria that
79 cause severe symptoms in affected plants, leading to heavy economic losses of many crops
80 worldwide (Tomkins et al., 2018). In particular, Flavescence dorée phytoplasma of grapevine
81 is a quarantine pathogen, which strongly limits European viticulture (EFSA Panel on Plant
82 Health (PLH), 2014). Phytoplasmas are obligate parasites with a dual life cycle, infecting
83 phloem of host plant as well as the body of the insect vector. Several lines of evidence indicate
84 that interactions between phytoplasmas and vector host cells are very strict and may regulate
85 transmission ability (reviewed in Rossi et al., 2019). Molecular relationships between
86 pathogens and insect hosts contribute to determining phytoplasma transmission specificity
87 and plant susceptibility, as well as feeding behaviour, ecological dispersal and plant host
88 range vectors (Bosco and D'Amelio, 2010). Insects play a key role in phytoplasma
89 epidemiology and, therefore, the main control strategies to limit these pathogens are
90 insecticide treatments against the vector species (Tomkins et al., 2018).

91 The urgent need for more targeted and sustainable pest management approaches in
92 fighting phytoplasma diseases includes the exploration for alternative biocontrol agents such
93 as insect viruses. Many fundamental biological features of EVV-1 were unknown, impairing
94 potential applications of this agent in biocontrol strategies or as a virus-induced gene

95 silencing (VIGS) vector. To fill the gap of knowledge about EVV-1 biology, we investigated the
96 presence of EVV-1 virus in different developmental stages and organs of *E. variegatus*, as well
97 as both horizontal and vertical transmission of this iflavirus among insect populations.

98

99 **2. Materials and methods**

100 *2.1 Insects*

101 *Euscelidius variegatus* in the Turin laboratory colonies were originally collected in
102 Piedmont Italy and reared on oats, *Avena sativa* (L.), inside plastic and nylon cages in growth
103 chambers at 20–25 °C, L16:D8 photoperiod. A French population of *E. variegatus* was kindly
104 provided by Dr. Xavier Foissac (INRA, UMR1332 Biologie du Fruit et Pathologie, Bordeaux,
105 France) and reared in Turin laboratory under the above described conditions. The original
106 Turin colony (EvaTo), was naturally infected with EVV-1 virus with a 100% prevalence,
107 whereas the French colony (EvaBx) appeared to be EVV-1 free, according to molecular
108 detection and electron microscopy observation (Abbà et al., 2017). The two colonies were
109 routinely confirmed to be EVV-1 infected (EvaTo) and free (EvaBx) by RT-qPCR, as described
110 below.

111 *2.2 Collection of different insect life stages and organ dissection*

112 To determine the viral loads in different insect life stages, total RNAs were extracted
113 from laid eggs, I-V instar nymphs and adults of virus infected EvaTo population. Preliminary
114 experiments showed that egg collection was easier when eggs were laid on *Arabidopsis*
115 *thaliana* plants than on *A. sativa* or *Vicia faba*. Therefore, approximately 100 EvaTo female
116 adults were caged on 15 *A. thaliana* plants for 4 d and then eggs were collected with sterile
117 forceps using a stereomicroscope. Seven pooled samples, each containing approximately 30

118 eggs, were obtained. To exclude surface viral contamination, egg samples were sterilized
119 according to Prado et al., (2006) and then stored at -80°C prior to RNA extraction. For each
120 nymphal stage, 10 pooled samples (each with three EvaTo specimens) were collected and
121 stored at -80°C until RNA extraction. Newly emerged EvaTo adults (five males and five
122 females) were also collected and stored.

123 To estimate the viral presence in different insect organs, total RNAs were extracted
124 from Malpighian tubules, salivary glands, guts, ovaries, testes and hemolymph of newly
125 emerged EvaTo adults. Three hemolymph samples (each with hemolymph collected from 10
126 specimens) were obtained by removing the head of a $CO_2$-anaesthetized adult and drawing
127 0.5 μL hemolymph from the inner thorax cavity with a Cell Tram Oil microinjector
128 (Eppendorf), using a stereomicroscope. For the remaining organs, three pooled samples (each
129 made of five dissected organs) were obtained. Organs were carefully separated with forceps
130 and needles using a stereomicroscope, rinsed with phosphate-buffered saline (PBS) solution
131 and stored at -80°C until RNA extraction.

132

133 *2.3 Virus vertical transmission route test*

134 To determine whether EVV-1 is vertically transmitted, virus free EvaBx adults were
135 injected with a fresh extract of EvaTo individuals. Microinjection was chosen as infection
136 modality to maximize the probability of obtaining a high number of infected EvaBx adults.
137 These were then used as F0 parents to test vertical transmission to the offspring (F1 and F2
138 generations). The fresh extract containing virus particles was prepared by crushing 30 EvaTo
139 adults in 900 μL of cold filter-sterilized injection buffer (300 mM glycine, 30 mM MgCl2, pH
140 8.0). The extract was clarified by slow centrifugation (10 min, 800 g), and passed through a
141 0.22 μm pore-size filter. All extraction steps were done at 4°C. Newly emerged EVV-1 free

142 EvaBx adults were $CO_2$ anaesthetized and injected with 0.5 μL of the virus-containing extract
143 between two abdominal segments using a stereomicroscope with a Cell Tram Oil
144 microinjector (Eppendorf). As negative control in order to exclude any procedural
145 contamination, a group of EvaBx insects was injected with injection buffer only. Injected
146 insects were isolated on fresh oat plants and sampled at 4 and 10 days post injection (dpi).
147 The F1 and F2 adults were collected at 60 and 120 dpi, respectively. All collected insects (F0,
148 F1 and F2) were stored at -80°C until RNA extraction to check the presence of EVV-1. The
149 experiment was repeated twice.

150 *2.4 Virus horizontal transmission route test*

151 To determine whether EVV-1 is horizontally transmitted, EVV-1 free EvaBx adults
152 were caged together with infected EvaTo. In order to distinguish individuals belonging to the
153 two original populations, three strategies were carried out: EvaBx third instar nymphs caged
154 with EvaTo adults (Experiment 1); EvaBx female adults caged with EvaTo male adults
155 (Experiment 2); and EvaBx male adults caged with EvaTo female adults (Experiment 3). For
156 each experiment, 50 EvaBx insects were co-fed on the same oat plant together with 50 EvaTo
157 individuals for two weeks, then transferred to a new plant in a new clean and sampled three
158 weeks later for virus detection. Each experiment was repeated twice.

159 After the positive results of co-feeding, three possible horizontal transmission
160 modalities were specifically tested i) fecal-oral route, ii) virus infection via plant, and iii)
161 cuticle penetration. Fecal-oral transmission modality was assessed according to Murakami et
162 al. (2013). About 60 EvaTo adults were confined in a 50-mL conical tube for 1 h. The insects
163 were then removed from the tube, and the excreted honeydew was collected by rinsing the
164 wall of the plastic tube with 4 mL of artificial feeding medium (5% sucrose, 10 mM Tris/Cl, 1
165 mM EDTA, pH 8.0. An aliquot (400 μL) of this solution was stored at -80°C for RNA extraction

166 to confirm the presence of the virus, while the remaining solution was used to artificially feed
167 EvaBx adults. To this purpose, non-viruliferous insects were confined within small chambers.
168 Six small cages were set up, each with five EvaBx insects allowed to feed for 48 h on 600 μL of
169 honeydew solution laid between two layers of stretched Parafilm. As negative controls, three
170 small cages were set up with artificial medium only, devoid of honeydew. Following artificial
171 feeding, live insects were caged on oat plants for 10 d (Murakami et al., 2013), then collected
172 and stored at -80°C, until RNA extraction for virus detection.

173 To assess the plant-mediated horizontal transmission of EVV-1, virus presence was
174 tested in *A. sativa* plants exposed to either EvaTo or EvaBx insects, following leaf surface
175 sterilization or no sterilization (Prado et al., 2006). Virus transmission through the host plant
176 was tested by isolating about 50 EvaTo adults for 1 week on an oat plant. The virus-positive
177 insects were then removed, while the oat plant was used to feed about 50 EvaBx adults for the
178 following week. EvaBx adults were then transferred onto a fresh oat plant and five were
179 tested for the presence of EVV-1 every 7 d for 1 mo.

180 To assess EVV-1 ability to penetrate through the insect cuticle, EvaBx nymphs were
181 submerged into fresh virus extract. For this experiment, second instar nymphs were used, as
182 the highest virus level was detected at this life stage in EvaTo and the insect dimensions
183 allowed an easier manipulation compared to the smaller and more delicate first instar
184 nymphs. Briefly, EvaBx nymphs were $CO_2$ anaesthetized and submerged for 5 min in 300 μL
185 fresh virus solution prepared by crushing ten EvaTo adults in 400 μL of PBS with a cocktail of
186 protease inhibitors added (Pierce Protease Inhibitor Mini Tablets, EDTA-Free, Thermo
187 Scientific). The extract was clarified by slow centrifugation (5 min, 4500 g). Submerged
188 nymphs were then maintained on an oat plant and collected as adults for RNA extraction and
189 virus detection.

190 *2.5 RNA extraction, cDNA synthesis, EVV-1 qPCR assays and statistical analyses*

191 Total RNAs were extracted from samples of single *E. variegatus* adults, and pooled
192 samples of i) 30 eggs, ii) three nymphs, iii) three dissected organs, iv) hemolymph collected
193 from 10 insects, v) honeydew collected from 60 insects and vi) 500 mg of oat leaves, with
194 Direct-zol RNA Mini Prep kit (Zymo Research), following manufacturer's protocol. For each
195 sample, cDNA was synthesized from total RNA (1 μg) with random hexamers using a High
196 Capacity cDNA reverse transcription kit (Applied Biosystems).

197 A multiplex qPCR assay was developed to detect and quantify virus presence in *E.*
198 *variegatus* adults. To detect EVV-1, primers and FAM-labelled TaqMan probe were designed
199 on the virus capsid 1 coding sequence (Evv1Cap1Fw 5'-GACCATTATCGCGCTAATG-3',
200 Evv1Cap1Rv 5'-AGTGCTCATCATAGGACA-3', Evv1Cap1Probe 5'-FAM-
201 ATTCTCGTAGCCAACTGCCAAAC-3'). To check that all the cDNA samples were properly
202 amplifiable, the *E. variegatus* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript,
203 was chosen as endogenous control (GapFw632 5'-ATCCGTCGTCGACCTTACTG-3', GapRv819
204 5'-GTAGCCCAGGATGCCCTTC-3', GapEvProbe 5'-HEX-ATATCAAGGCCAAGGTCAAGGAGGC-3').
205 Standard conditions were used for qPCR assay and detailed in Supplementary Method File.
206 Mean virus copy numbers were used to express virus amount as EVV-1 copy numbers/insect
207 GAPDH transcript.

208 For comparison of viral load in different insect life stages and organs, virus load was
209 expressed as EVV-1 copy numbers/ng of cDNA, as GAPDH transcript levels varied among the
210 different organs and life stages (Supplementary Tables S1 and S2).

211 SigmaPlot version 13 (Systat Software, Inc., www.systatsoftware.com.) was used for
212 statistical analyses. To compare viral load among different categories (life stages, organs and
213 vertical transmission experiments) ANOVA, followed by the proper post hoc test, or Kruskal

214 Wallis test, when the parametric analysis assumptions were not met, were used. To compare
215 viral load between two groups of samples (male vs. female EvaTo adults, virus exposed EvaBx
216 vs. EvaTo samples in horizontal transmission experiments) t-test was used.

217

218 **3. Results**

219 *3.1 EVV-1 qPCR diagnostic and quantitative assay*

220 A multiplex qPCR assay was optimized to detect and quantify the EVV-1 virus in *E.*
221 *variegatus* adults, using primers and TaqMan probes targeting viral EVV-1 capsid 1 and insect
222 GAPDH coding sequences. The viral genomic portion coding the virus capsid 1 protein was
223 selected to maximize the specificity of EVV-1 detection, as this genomic portion was poorly
224 conserved among other iflaviruses according to BLAST analysis (not shown). Standard curves
225 of plasmids harbouring viral and insect target amplicons showed 96.5 and 103.1% qPCR
226 efficiencies, respectively, with a 0.998 $R^2$ for both curves. The most diluted standard point
227 ($10^2$ copy numbers/μL) was detected before the $35^{th}$ amplification cycle by both viral and
228 insect primer sets. The test successfully detected EVV-1 in single EvaTo individuals (n=10),
229 whereas no amplification was obtained from any of the 10 tested EvaBx. Quantification of
230 EVV-1 in the EvaTo adults ranged from 0.21 to 0.84 EVV-1 copy numbers/insect GAPDH
231 transcript.

232 *3.2. EVV-1 distribution in different insect life stages and organs*

233 EVV-1 was detected in the eggs and all insect developmental stages (I-V instar nymphs
234 and adults) of the naturally infected EvaTo population. Male and female EvaTo individuals
235 were considered together for the analyses, as no significant differences in viral loads were
236 found (t-test t=1.448, P=0.186) (Supplementary Table S1). Significant differences were found

237 among developmental stages (ANOVA F=10.367, P<0.001) and I, II, III instar nymphs showed
238 the highest viral loads, as indicated by pairwise multiple comparison according to Duncan's
239 multiple range test (Fig. 1 and Supplementary Table S1).

240 EVV-1 was detected in all collected insect organs (Malpighian tubules, salivary glands,
241 guts, ovaries, testes, and hemolymph) of the EvaTo population. Viral loads ranged from 8606
242 in the ovaries to 1 EVV-1 copy numbers/ng of cDNA in the testes. The high viral load found in
243 ovaries significantly differed from that found in the testes (ANOVA on Ranks, Kruskal-Wallis
244 test H=15.415, P=0.009), (Fig. 2 and Supplementary Table S2).

245 *3.3 Vertical transmission*

246 Virus injection was selected as the most effective infection modality to obtain a high
247 number of newly infected EvaBx individuals (F0) and test their ability to transmit the virus to
248 progeny (F1 and F2). Virus presence was detected in 95 and 100 % injected insects sampled
249 at 4 and 10 dpi, respectively, as well as in all the F1 and F2 tested insects (Supplementary
250 Table S3), demonstrating that vertical viral transmission occurs with very high frequency.
251 Interestingly, mean EVV-1 loads measured in F1 and F2 samples were significantly higher
252 than those detected in injected F0 insects sampled at 4 dpi and in EvaTo adults (ANOVA on
253 Ranks, Kruskal-Wallis test H=22.769, P<0.001) (Fig. 3). The virus was detected in EvaTo fresh
254 extract injected in EvaBx specimens, while no amplification was obtained from EvaBx adults
255 injected with buffer only (negative control).

256 *3.4 Horizontal transmission*

257 The co-feeding experiment, in which EvaTo insects were isolated together with EvaBx
258 specimens on the same plant, indicated that horizontal viral transmission may occur. The
259 overall mean rate of virus transmission was 8.0% (6/75), comprehensively considering the
260 three different co-feeding experimental approaches (indicated in Table 1). The mean viral

261 load, measured in the infected EvaBx insects three weeks after co-feeding with EvaTo, was
262 0.23 EVV-1 copy numbers/insect GAPDH transcript ± 0.10 (SEM, N=6), with values ranging
263 from 6.27E-04 to 5.94E-01. The mean viral load measured in these virus-exposed EvaBx
264 samples did not significantly differ from that measured in EvaTo adults, which was 0.44 EVV-
265 1 copy numbers/insect GAPDH transcript ± 0.14 (SEM, N=10) (t-test t=1.815, P=0.091).

266 When exploring the possible routes of EVV-1 horizontal transmission, none of the
267 EvaBx insects artificially fed on virus-infected honeydew solution were positive for EVV-1
268 presence (Table 1). Although the presence of EVV-1 in EvaTo honeydew was confirmed by
269 qPCR, the possibility of virus transmission via fecal-oral route was not confirmed under our
270 experimental conditions.

271 None of the 20 analysed EvaBx adults tested positive for EVV-1 presence (Table 1)
272 following isolation on *A. sativa* plants that were exposed to EvaTo insects, thus excluding the
273 plant as efficient transmission route. Indeed, while EVV-1 was detected only in one of six
274 samples of *A. sativa* exposed to EvaTo insects, the virus was absent from all the six analysed
275 plants following surface sterilization of the leaf before RNA extraction, indicating that viral
276 contamination of the leaves may occur. The absence of EVV-1 in plants exposed to EvaBx
277 insects was also confirmed, as expected (0 positive samples out of six total analysed samples).

278 Viral transmission through insect cuticle of second instar EvaBx nymphs occurred in
279 9.5% of the treated insects (Table 1). The viral loads measured in these infected EvaBx insects
280 ranged from 0.09 to 1.79 EVV-1 copy numbers/insect GAPDH transcript. No significant
281 differences were found between the mean viral loads measured in these virus-exposed EvaBx
282 samples and those measured in EvaTo adults (t-test t=-1.414, P=0.174).

283

284 **4. Discussion**

285 We studied the distribution and transmission routes of the Euscelidius variegatus
286 iflavirus 1 and showed that infection with the virus is systemic and occurs in all life stages. As
287 well, it is vertically transmitted to offspring with high efficiency. Elucidating these biological
288 parameters is a prerequisite to exploring the potential application of iflaviruses as biocontrol
289 agents of phytoplasma vectors. Our work also provides information needed to exploit
290 iflaviruses as molecular tools to study insect genes involved in pathogen transmission through
291 the construction of infectious viral clones.

292 Iflaviruses colonize their arthropod hosts in different ways. In some cases, viruses do
293 not exhibit tropism (restriction to specific organs or tissues). Deformed Wing Virus (DWV)
294 can be detected in all parts of the bee body (Martin and Brettell, 2019), and Antheraea pernyi
295 Iflavirus (ApIV) in the head, epidermis, hemocytes, gut, fat body, ovary and testis of the
296 Chinese oak silkmoth, ApIV (Geng et al., 2017). On the contrary, other iflaviruses show some
297 organ tropism, such as the Nora Virus of the cotton bollworm which show higher loads in
298 midgut tissues (Yang et al., 2019) or the Helicoverpa armigera iflavirus, which shows higher
299 levels in fat bodies compared to other insect body tissues. Molecular analysis showed that
300 EVV-1 is present in different *E. variegatus* tissues and organs as well as in different life stages
301 (from egg to adult). The highest viral load was detected in I-III instar nymphs and may
302 indicate that these stages support the peak of viral replication, even though northern blot
303 analysis did not detect an accumulation of negative strands in nymphal stages nor in adults
304 (Abbà et al., 2017). The presence of detectable replicative intermediates may be limited in
305 time and difficult to detect. In line with our results, increase of viral load over time was
306 observed in pupae of the Chinese oak silkmoth following the injection of ApIV purified
307 particles (Geng et al., 2017). The high EVV-1 viral load measured in the ovaries suggests a key
308 role of this organ in the viral replication cycle. Indeed, this feature may be common to
309 iflaviruses infecting Hemiptera. For example, like EVV-1, the Laodelphax striatellus Iflavirus 1

310 (LsIV1) is present in eggs and ovaries of the small brown planthopper (Wu et al., 2019).
311 Infection data from different iflaviruses seems, therefore, to delineate an assorted model of
312 insect body colonization, that requires case-by-case characterization.

313 Under our experimental conditions, EVV-1 was efficiently transmitted to the offspring.
314 Indeed, following injection of virus-free parents, 100% of the F1 and F2 offspring were
315 infected. The development of an efficient multiplex fluorescent qPCR assay, based on
316 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous gene for
317 normalization of quantitative data and as internal control of cDNA quality, allowed the
318 quantitative detection of EVV-1, a prerequisite to characterize its distribution and
319 transmission modalities. Interestingly, the mean viral load of the F1 and F2 individuals was
320 significantly higher than that of naturally infected EvaTo adults. This result supports the
321 hypothesis of a higher viral replication rate in the experimentally infected insects than in the
322 naturally infected insects. The absence of evident fitness costs was in line with preliminary
323 results showing that longevity and fecundity were not negatively affected by EVV-1 infection.
324 Similarly, in several mosquito species, a state of tolerance response against covert arboviral
325 infections has been described leading to persistent infection in the host with little fitness cost
326 (Goic et al., 2016).

327 Horizontal transmission also has been reported for some iflaviruses (Murakami et al.,
328 2013; Yuan et al., 2017), and occasionally occurred for EVV-1 under our experimental
329 conditions, although less efficiently than vertical route. While Nilaparvata lugens iflavirus 1
330 (NHLV-1) is efficiently transmitted to virus-free brown leafhoppers by feeding on infected
331 honeydew (Murakami et al., 2013), the EVV-1 virus was not transmitted to EvaBx individuals
332 by feeding, despite EVV-1 presence in EvaTo honeydew added to the artificial diet. This
333 suggests that EVV-1 is not able to cross the gut barrier. EVV-1 could be sporadically detected
334 in unsterilized leaves from plants exposed to virus-infected insects, probably due to

335 honeydew contamination, but it was never detected after sterilization of leaf surface. The
336 phloem feeding habit of *E. variegatus* may explain its inability to acquire EVV-1 through the
337 host plant.

338 Silencing of insect genes by RNA interference is a promising approach to control insect
339 pests, and it has been applied to several Hemipteran species including aphids (Yu et al., 2016),
340 psyllids (Lu et al., 2019), and whiteflies (Shi et al., 2019). Efficient delivery of RNA interfering
341 molecules is difficult to achieve for phloem feeders, and microinjection (Mutti et al., 2006) or
342 plant-mediated silencing strategies (Jaubert-Possamai et al., 2007; Pitino et al., 2011) have
343 been explored. In particular, soaking of nymphs of the psyllid *Diaphorina citri*, vector of
344 '*Candidatus* Liberibacter solanacearum', in a solution containing dsRNA silencing molecules
345 provides efficient silencing of different target genes (Killiny et al., 2014; Yu et al., 2017). EVV-1
346 was transmitted to nymphs following their immersion in a solution containing the virus,
347 suggesting that the viral particles are able to penetrate the nymphal cuticle or to pass through
348 the tracheae and then replicate in the insect body. This soaking-based strategy of viral
349 delivery may be further explored as a high-throughput technique to increase the horizontal
350 transmission efficiency of phloem feeder viruses. Also, the ability of EVV-1 to penetrate the
351 nymphal cuticle or the tracheae may also explain the occasional transmission of the virus to
352 some EvaBx individuals, following co-feeding together with virus infected EvaTo individuals.
353 In conclusion, both vertical and cuticle/tracheal horizontal transmission routes may explain
354 the 100% EVV-1 prevalence observed in the mass reared EvaTo lab colony.

355 Although the idea behind the application of entomopathogens as biocontrol agent
356 dates back to the nineteenth century (Metchnikoff, 1880), the use of insect-specific viruses in
357 plant protection is lately gaining attention. Iflaviruses may infect their hosts and transcribe
358 high amounts of RNA without inducing visible symptoms in the infected population (dos
359 Santos et al., 2019) but, for example, infection with an iflavirus increases the susceptibility of

360 *Spodoptera exigua* larvae to a baculovirus insecticide (Carballo et al., 2017). Interactions such
361 as this among different species in a complex microbiome may explain the lower load of EVV-1
362 in *E. variegatus* individuals infected with Flavescence dorée phytoplasma (Abbà et al., 2017).
363 The urgent need for more precise and sustainable pest management approaches to control
364 phytoplasma diseases encourages the exploration of viruses as biocontrol agents able to
365 compete with phytoplasma transmission and/or acquisition. The hypothesis that pathogen
366 transmission may be suppressed or reduced by the concurrent vector infection with other
367 microorganisms is supported by cases of West Nile virus vectored by mosquitoes co-infected
368 with Nhumirim virus (Nouri et al., 2018) or with Wolbachia (Glaser and Meola, 2010), and by
369 the cases of Dengue, Chikungunya, and Plasmodium transmitted by *Aedes* co-infected with
370 Wolbachia (Moreira et al., 2009).

371 The biological information we provided here will be useful in the future to exploit EVV-
372 1 for VIGS to regulate the expression of insect metabolic genes and, possibly, of genes involved
373 in phytoplasma transmission. Also, an iflavirus-based vector will be a valuable tool to explore
374 the role of potential phytoplasma effectors in the insect host. To reach this goal, preliminary
375 results in the synthesis of an EVV-1 infectious clone have been obtained (Simona Abbà,
376 personal communication) and RNAi mediated by injection of dsRNA molecules has been
377 proven to work efficiently in *E. variegatus* with a long-lasting effect (Abbà et al., 2019).
378 Elucidating the mechanisms of insect tolerance to iflavirus infection paves the way to
379 conceptualize new anti-vectorial strategies to selectively control plant pathogen-transmitter
380 Hemipteran insects.

381

382 **Author contributions**

383 Concept & financing (LG, SA, CM, DB); Experimental design (LG, MR, CM); Execution
384 (SO, AP, MR, MV, GM); Analysis (SO, MR, LG, CM); Publication (LG, MR, SA, MV, SP, DB, RB, CM).
385 All authors read and approved the final manuscript. The authors declare no competing
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387

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396

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526

527

528 **Table**

529 **Table 1.** Detection and quantification results of horizontal transmission of Euscelidius
530 variegatus virus 1 (EVV-1) to uninfected colony EvaBx. Mean quantification cycles (Cq)
531 obtained from insect glyceraldehyde-3-phosphate dehydrogenase (GAPDH) qPCR assay
532 confirmed that all samples were amplifiable.

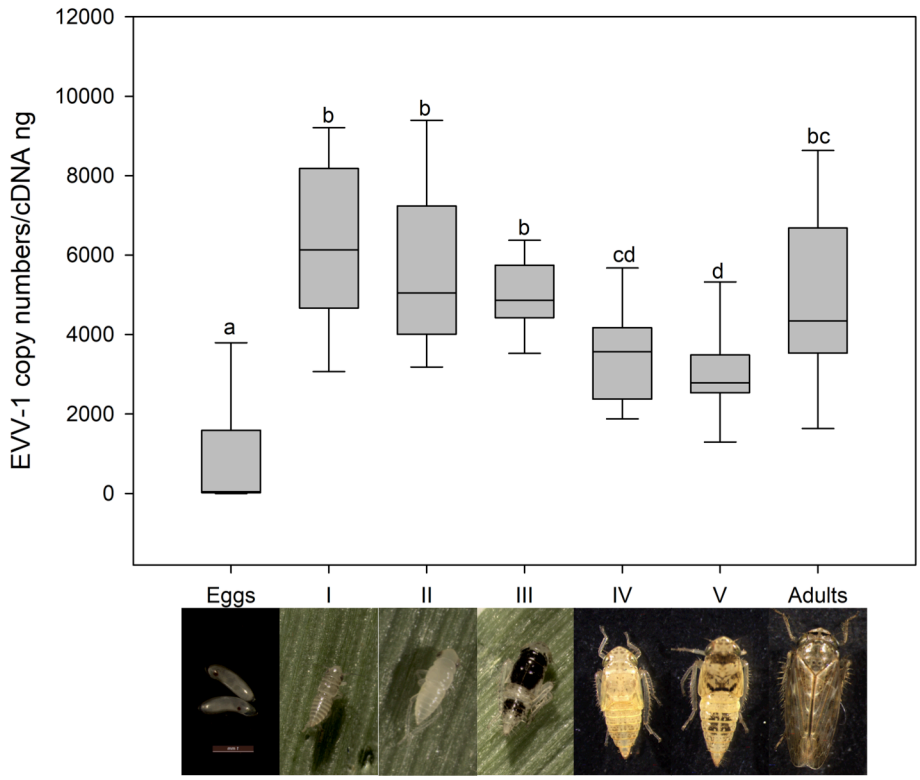
| Virus infection modality | Sample description | N° EVV-1 positive/analysed samples | Mean EVV-1 viral load* ± SEM (N) | Mean Cq ± SEM (N) of GAPDH assay |
|---|---|---|---|---|
| Co-feeding | EvaBx nymphs + EvaTo adults | 3/47 | 0.27 ± 0.11 (3) | 18.93 ± 0.36 (47) |
| | EvaBx females + EvaTo males | 2/17 | 2.56E−03 ± 1.94E−03 (2) | 23.45 ± 0.36 (17) |
| | EvaBx males + EvaTo females | 1/11 | 0.59 (1) | 19.44 ± 0.84 (11) |
| Fecal-oral route | EvaBx adults fed on EvaTo honeydews | 0/12 | / | 20.30 ± 0.28 (12) |
| Plant-mediated route | EvaBx adults fed on plant exposed to EvaTo adults | 0/20 | / | 20.79 ± 0.44 (20) |
| Cuticle penetration | EvaBx nymphs submerged in EvaTo extract | 10/105 | 0.68 ± 0.35 (10) | 17.85 ± 0.16 (105) |

533 *Viral load expressed as EVV-1 copy numbers/insect GAPDH transcript.

534

535

536




537

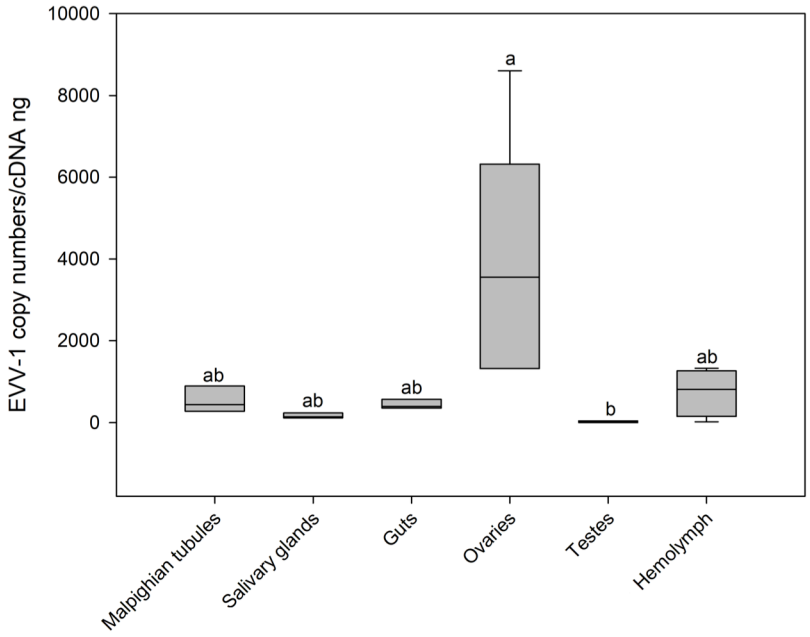
538 **Figure 1. Euscelidius variegatus virus 1 (EVV-1) load in different host life stages.**

539 Different letters indicate significant differences in mean viral load measured in the sample

540 groups. First to fifth instar nymphs are indicated by Roman numerals and different life stages

541 are depicted beneath the graph.

542

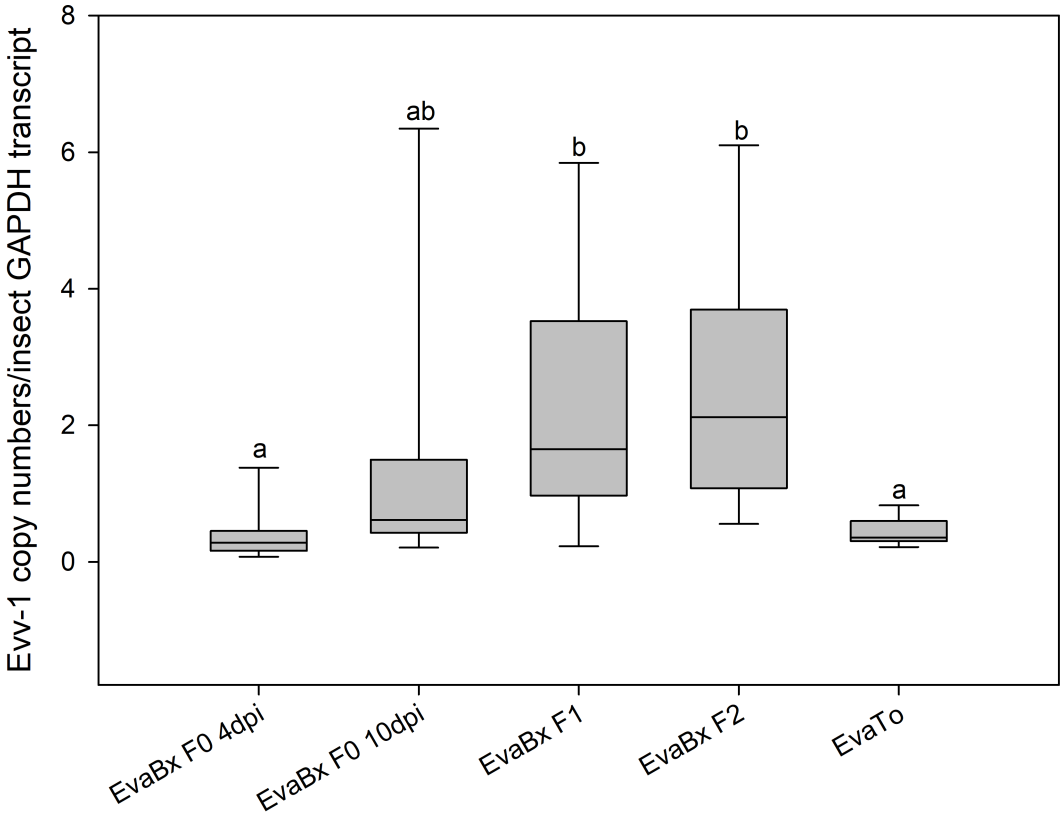



543

544 **Figure 2. Euscelidius variegatus virus 1 (EVV-1) load in different insect organs.** Different

545 letters indicate significant differences in mean viral load measured in the sample groups.

546




547

548 **Figure 3. Vertical transmission of Euscelidius variegatus virus 1 (EVV-1) injected into**

549 **uninfected *Euscelidius variegatus* (EvaBx).** Different letters indicate significant differences

550 in mean viral load measured in the sample groups. Days post injection (dpi) indicate the

551 sampling date after viral injection. Viral load measured in infected colony *Euscelidius*

552 *variegatus* (EvaTo) adults was used for comparison.

553

554 **Supplementary methods**

555 *2.5 RNA extraction, cDNA synthesis and EVV-1 qPCR assays*

556 Total RNAs were extracted from samples of single *E. variegatus* adults, and pooled
557 samples of i) 30 laid eggs, ii) three nymphs, iii) three dissected organs, iv) hemolymph
558 collected from 10 insects, v) honeydew collected from 60 insects and vi) 500 mg of oat leaves.
559 The samples were frozen with liquid nitrogen, crushed with a micropestle in sterile
560 Eppendorf tubes, and homogenized in Tri-Reagent (1 mL for plant samples and 0.5 mL for all
561 other samples). Samples were centrifuged 1 min at 12.000 g at 4°C and RNAs were extracted
562 from supernatants with Direct-zol RNA Mini Prep kit (Zymo Research), following
563 manufacturer's protocol and including the optional DNAse treatment step. Concentration,
564 purity, and quality of extracted RNA samples were analysed through a Nanodrop
565 spectrophotometer (Thermo Scientific). For each sample, cDNA was synthesized from total
566 RNA (1 μg) with random hexamers using a High Capacity cDNA reverse transcription kit
567 (Applied Biosystems). Yields of reverse transcription reactions were estimated by reading a
568 1:10 cDNA dilution in a Nanodrop spectrophotometer (Thermo Scientific).

569 A multiplex qPCR assay was developed to detect and quantify virus presence in *E.*
570 *variegatus* adults. To detect EVV-1, primers and FAM-labelled TaqMan probe were designed
571 on the virus capsid 1 coding sequence (Evv1Cap1Fw 5'-GACCATTATCGCGCTAATG-3',
572 Evv1Cap1Rv 5'-AGTGCTCATCATAGGACA-3', Evv1Cap1Probe 5'-FAM-
573 ATTCTCGTAGCCAACTGCCAAAC-3'). To check that all the cDNA samples were properly
574 amplifiable, the *E. variegatus* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript,
575 was chosen as endogenous control. To amplify the insect GAPDH, the primer GapFw632 (5'-
576 ATCCGTCGTCGACCTTACTG-3' (Galetto et al., 2013)), was used with the newly designed
577 primer GapRv819 (5'-GTAGCCCAGGATGCCCTTC-3') and the internal HEX-labelled TaqMan
578 probe GapEvProbe (5'-HEX-ATATCAAGGCCAAGGTCAAGGAGGC-3'). One μL of cDNA was used
579 as template in a reaction mix of 10 μL total volume, containing 1X iTaq Universal Probes
580 Supermix (Bio-Rad), 200 nM of each of the four primers and 300 nM of each of the two
581 TaqMan probes. Each sample was run in triplicate in a CFX Connect Real-Time PCR Detection
582 System (Bio-Rad). Cycling conditions were 95°C for 3 min and 40 consecutive cycles at 95°C
583 for 10 s as denaturing step followed by 30 s at 60°C as annealing/extension step. In each qPCR
584 plate, four serial 100-fold dilutions of pGem-T Easy (Promega) plasmids, harbouring the
585 target virus and insect genes, were included to calculate the viral load. For both plasmid
586 standard curves, dilutions included in plates ranged from $10^8$ to $10^2$ target copy numbers per
587 μL and were prepared taking into account that 1 fg of plasmids harbouring EVV-1 and GAPDH
588 gene portions contains 138 and 244 number of molecules, respectively. Dilution series of both
589 plasmids were used to calculate qPCR parameters (reaction efficiency and $R^2$). Mean virus
590 copy numbers in amplified samples were automatically calculated by CFX MaestroTM
591 Software (Bio-Rad) and used to express virus amount as EVV-1 copy numbers/insect GAPDH
592 transcript.

593

594 **Reference**

595 Galetto, L., Bosco, D., Marzachì, C., 2013. Selection of reference genes from two leafhopper
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598 **Supplementary Tables**

599 **Supplementary Table S1**. Presence and loads of Euscelidius variegatus virus 1 (EVV-1) in
600 different insect life stages of the virus infected EvaTo rearing and corresponding mean
601 transcript levels of insect glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All samples
602 were amplifiable.

| Life stage | Mean EVV-1 viral load* ± SEM (N) | Mean GAPDH transcript levels** ± SEM (N) |
|---|---|---|
| Egg | 944 ± 534 (7) | 848 ± 295 (7) |
| 1st instar nymph | 6335 ± 654 (10) | 2841 ± 297 (10) |
| 2nd instar nymph | 5656 ± 638 (10) | 3397 ± 365 (10) |
| 3rd instar nymph | 5005 ± 289 (10) | 4516 ± 304 (10) |
| 4th instar nymph | 3441 ± 387 (10) | 6350 ± 541 (10) |
| 5th instar nymph | 3045 ± 364 (9) | 6349 ± 430 (9) |
| Adult Female | 5810 ± 1105 (5) | 10707 ± 254 (5) |
| Adult Male | 3945 ± 662 (5) | 11564 ± 1684 (5) |

603 *Viral load expressed as EVV-1 copy numbers/ng of cDNA.
604 **Insect GAPDH level expressed as GAPDH transcript numbers/ng of cDNA.
605

606 **Supplementary Table S2**. Presence and loads of Euscelidius variegatus virus 1 (EVV-1) in
607 different insect organs of the virus infected EvaTo population and corresponding mean
608 transcript levels of insect glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All samples
609 were amplifiable.

| Organs | Mean EVV-1 viral load* ± SEM (N) | Mean GAPDH transcript levels** ± SEM (N) |
|---|---|---|
| Malpighian tubules | 537 ± 185 (3) | 2665 ± 870 (3) |
| Salivary glands | 164 ± 36 (3) | 234 ± 41 (3) |
| Guts | 437 ± 66 (3) | 5118 ± 249 (3) |
| Ovaries | 3768 ± 1332 (5) | 5491 ± 1617 (5) |
| Testes | 16 ± 13 (3) | 132 ± 87 (3) |
| Hemolymph | 741 ± 290 (4) | 57 ± 21 (4) |

610 *Viral load expressed as EVV-1 copy numbers/ng of cDNA.
611 **Insect GAPDH level expressed as GAPDH transcript numbers/ng of cDNA.
612

613

614 **Supplementary Table S3**. Presence and loads of Euscelidius variegatus virus 1 (EVV-1)
615 following injection into the EVV-1 free EvaBx population. Mean quantification cycles (Cq)
616 obtained from insect glyceraldehyde-3-phosphate dehydrogenase (GAPDH) qPCR assay
617 confirmed that all samples were amplifiable.

| Sample description | Sampling date (dpi, days post injection) | N° EVV-1 positive/ analysed samples | Mean EVV-1 viral load* ± SEM (N) | Mean Cq ± SEM (N) of GAPDH assay |
|---|---|---|---|---|
| F0 (EvaBx adults injected with EVV1 extract) | 4 | 8/9 | 0.41 ± 0.15 (8) | 19.97 ± 0.64 (9) |
| | 10 | 10/10 | 1.37 ± 0.63 (10) | 20.20 ± 1.49 (10) |
| F1 (Progeny of injected EvaBx adults) | 60 | 16/16 | 2.40 ± 0.49 (16) | 17.41 ± 0.21 (16) |
| F2 | 120 | 10/10 | 2.53 ± 0.60 (10) | 16.91 ± 0.17 (10) |

618 *Viral load expressed as EVV-1 copy numbers/insect GAPDH transcript.