Biological characterization of Euscelidius variegatus iflavirus 1

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Abstract

Virus-based biocontrol technologies are gaining attention as sustainable alternatives to pesticides and insecticides. Phytoplasmas are prokaryotic plant pathogens causing severe losses to crops worldwide. Novel approaches are needed since insecticide treatments 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*, rising the hypothesis of virus-based application against phytoplasma disease. Here EVV-1 is characterized in its transmission routes and localization within the host to unfold the mechanism of insect tolerance to virus infection. Both vertical and horizontal transmissions occur, and the former resulted more efficient. The virus is ubiquitously distributed in different organs and life-stages, with the highest loads measured in ovaries and first to third instar nymphs. The basic knowledge gained here on biological viral properties is crucial for future application of iflaviruses as biocontrol agents.

Keywords

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

1. Introduction

The onset of next generation sequencing gave a strong impulse in the last few years to the discovery of many novel viruses in arthropods (Dong et al., 2018; dos Santos et al., 2019; O'Brien et al., 2018; Yang et al., 2019). In particular, this technology resulted crucial for the identification of covert viruses, which display no overtly pathological effects on their hosts (Nouri et al., 2018). Within the newly discovered viral entities, positive-sense RNA viruses are preponderant (Bonning, 2020). Among them, iflaviruses form a distinct group in the order *Picornavirales*, justifying their classification in the virus family *Iflaviridae* (Van Oers, 2010). Interestingly, some of these viruses may cause developmental anomalies (Li et al., 2019) behavioural alterations (Wells et al., 2016), histopathological effects (Brettell et al., 2017), and premature death (Geng et al., 2017) of the insect host, while others do not cause any evident symptoms (dos Santos et al., 2019; Murakami et al., 2013; Yang et al., 2019). Transmission modalities of these viruses have been explored, leading however to a multifaceted, not univocal infection model. In some cases, horizontal transmission (oral route) is the most efficient spreading way, such as for Nilaparvata lugens honeydew virus-1 of the brown leafhopper (Murakami et al., 2013) and for Helicoverpa armigera iflavirus (Yuan et al., 2017) and Nora Virus (Yang et al., 2019) of the cotton bollworm. Similarly, the horizontal route is the main transmission modality for Pyrrhocoris apterus virus 1 in red firebugs (Vinokurov and Koloniuk, 2019). On the contrary, vertical transmission (from infected parental specimens to progeny) better explains viral spreading in the cases of the causal agent of Antheraea pernyi Vomit Disease of Chinese oak silkmoth (Geng et al., 2017) and for the Spodoptera exigua iflaviruses-1 and 2 of the beet armyworm (Virto et al., 2014). In the case of Deformed Wing Virus (DWV) of the honeybee both the horizontal route, trough *Varroa destructor* mite as vector, and the vertical viral transmission occur (Chen et al., 2006; Martin and Brettell, 2019; Yue, 2005).

The genomic features, phylogenetic analysis, and prevalence of a new member of the family *Iflaviridae*, named Euscelidius variegatus virus 1 (EVV-1), have been reported (Abbà et al., 2017) as additional result obtained during the *de novo* assembly of trascriptome from the hemipteran leafhopper *Euscelidius variegatus* Kirschbaum (Galetto et al., 2018). EVV-1 does not induce any evident symptom in the *E. variegatus* surveyed population, which is laboratory reared, and shows a viral endemic infection (100% prevalence) (Abbà et al., 2017). EVV-1 forms non-enveloped, icosahedral particles, and the ssRNA(+) genome encodes a single polyprotein of 3132 amino acids, which is post-translationally processed. *Euscelidius variegatus* (Cicadellidae: Deltocephalinae) is a palearctic, multivoltine and polyphagous species, widespread in Europe and North America. High relevance is given to this species since it is a natural vector of the '*Candidatus* Phytoplasma asteris' (chrysanthemum yellows strain) and a laboratory vector of the Flavescence dorée phytoplasma (Caudwell et al., 1972; Rashidi et al., 2014). Phytoplasmas are plant-pathogenic bacteria that cause severe symptoms to affected plants, leading to heavy economic losses to many crops worldwide (Tomkins et al., 2018). In particular, Flavescence dorée phytoplasma of grapevine is a quarantine pest, which strongly limits European viticulture (EFSA Panel on Plant Health (PLH), 2014; EFSA Panel on Plant Health (PLH) et al., 2016). Phytoplasmas are obligate parasites and display a dual life cycle, infecting phloem of host plant as well as insect vector body. Several lines of evidence indicate that interactions between phytoplasmas and vector host cells are very strict and may regulate transmission ability (Arricau-Bouvery et al., 2018; Galetto et al., 2011; Rashidi et al., 2015; Suzuki et al., 2006; Trivellone et al., 2019). Indeed, molecular relationships between pathogens and insect hosts contribute to determining phytoplasma transmission specificity, together with plant susceptibility, as well as feeding behaviour, ecological dispersal and plant host range preference of vectors (Bosco and D'Amelio, 2010; Sugio et al., 2011). Insects play a

key role in phytoplasma epidemiology and therefore the main control strategies to limit these pathogens are the insecticide treatments against vector species (Marcone, 2014).

The urgent need for more targeted and sustainable pest management approaches in fighting against phytoplasma diseases encourages the exploration of new research frontiers to envisage potential innovative use of biocontrol agents, such as insect viruses. Nevertheless, many fundamental biological features of EVV-1 were unknown, impairing any potential applications of this agent in biocontrol strategy or as VIGS vector. To fill the gap of knowledge of EVV-1 biology, we investigated here the presence of EVV-1 virus in different developmental stages and organs of *E. variegatus*, as well as both horizontal and vertical transmission of this iflavirus among insect populations.

2. Materials and methods

2.1 Insects

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

2.2 Collection of different insect life stages and organ dissection

To determine the viral abundance in different insect life stages, total RNAs were extracted from laid eggs, I-V instar nymphs and adults of virus infected EvaTo population. Preliminary experiments showed that egg collection was easier when eggs were laid on *Arabidopsis thaliana* plants than on *A. sativa* or *Vicia faba* plants. Therefore, about 100 EvaTo female adults were caged on 15 *A. thaliana* plants for four days and then eggs were collected with sterile forceps under a stereomicroscope. Seven pooled samples, each made of about 30 laid eggs, were obtained. To exclude surface viral contamination, egg samples were sterilized according to Prado et al. (2006) and then stored at -80°C prior to RNA extraction. For every nymphal stage, 10 pooled samples (each with three EvaTo specimens) were collected and stored at -80°C until RNA extraction. Newly emerged EvaTo adults (five males and five females) were also collected and stored.

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

2.3 Virus vertical transmission route test

To determine whether EVV-1 is vertically transmitted, virus free EvaBx adults were injected with a fresh extract of EvaTo individuals. Microinjection was chosen as infection modality to maximize the probability to obtain a high number of infected EvaBx adults. These were then used as F0 parents to test vertical transmission to the offspring (F1 generation). The fresh extract containing virus particles was prepared by crushing 30 EvaTo adults in 900 µL of cold filter-sterilized injection buffer (300 mM glycine, 30 mM MgCl2, pH 8.0) as detailed by Bressan et al. (2006). The extract was clarified by slow centrifugation (10 min, 800 g), and passed through a 0.22 µm pore-size filter. All extraction steps were done at 4°C. Newly emerged EVV-1 free EvaBx adults were CO_2 anaesthetized and injected with 0.5 µL of virus containing solution between two abdominal segments under a stereomicroscope with a Cell Tram Oil microinjector (Eppendorf). As negative control, a group of EvaBx insects was injected with injection buffer only. Injected insects were isolated on fresh oat plants and sampled at 4 and 10 days post injection (dpi). The F1 adults were collected, at 60 dpi. All collected insects (F0 and F1) were stored at -80°C until RNA extraction to check the presence of EVV-1. The experiment was repeated twice.

2.4 Virus horizontal transmission route test

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

After the positive results of co-feeding, three possible horizontal transmission modalities were specifically tested i) fecal-oral route, ii) virus infection via plant, and iii) cuticle penetration. Fecal-oral transmission modality was assessed according to Murakami et al. (2013). About 60 EvaTo adults were confined within a 50-mL conical tube for 1 h. The insects were then removed from the tube, and the excreted honeydews were collected by rinsing the wall of the plastic tube with 4 mL of artificial feeding medium (5% sucrose, 10 mM Tris/Cl, 1 mM EDTA, pH 8.0 (Rashidi et al., 2015)). An aliquot (400 µL) of this solution was stored at -80°C for RNA extraction to confirm the presence of the virus, while the remaining solution was used to artificially feed EvaBx adults. To this purpose, non-viruliferous insects were confined within small chambers according to Rashidi et al. (2015). Six small cages were set up, each with five EvaBx insects allowed to feed for 48h on 600 µL of honeydew solution laid between two layers of stretched Parafilm. As negative controls, three small cages were set up with artificial medium only, devoid of honeydew. Following artificial feeding, alive insects were caged on oat plants for ten days (Murakami et al., 2013), then collected and stored at -80°C, until RNA extraction for virus detection. To assess the plant-mediated horizontal transmission of EVV-1, virus presence was initially tested in *A. sativa* plants exposed to either EvaTo or EvaBx insects. To determine whether EVV-1 virus is able to invade the plant tissue or only remains on the plant surface, samples from plants exposed to EvaTo insects were surface sterilized (Prado et al., 2006) before RNA extraction and EVV-1 diagnosis. Later on, virus transmission through the host plant was tested by isolating about 50 EvaTo adults for

one week on an oat plant. The virus-positive insects were then removed, while the oat plant was used to feed about 50 EvaBx adults for the following week. EvaBx adults were then transferred onto a fresh oat plant and five of them were tested for the presence of EVV-1 every seven days for one month. To assess EVV-1 ability to penetrate through the insect cuticle, EvaBx nymphs were submerged into fresh virus extract. For this experiment, second instar nymphs were used, as the highest virus level was detected at this life stage in EvaTo and the insect dimensions allowed an easier manipulation compared to the smaller and more delicate first instar nymphs. Briefly, EvaBx nymphs were CO_2 anaesthetized and submerged for five minutes in 300 µL of fresh virus solution prepared by crushing ten EvaTo adults in 400 µL of PBS added with a cocktail of protease inhibitors (Pierce Protease Inhibitor Mini Tablets, EDTA-Free, Thermo Scientific). The extract was clarified by slow centrifugation (5 min, 4500 g). Submerged nymphs were then maintained on an oat plant and collected as adults for RNA extraction and virus detection.

2.5 RNA extraction and cDNA synthesis

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

2.6 EVV-1 qPCR assays and statistical analyses

A multiplex qPCR assay was developed to detect and quantify virus presence in *E. variegatus* adults. To detect EVV-1, primers and FAM-labelled TaqMan probe were designed on the virus capsid 1 coding sequence (Evv1Cap1Fw 5'-GACCATTATCGCGCTAATG-3', Evv1Cap1Rv 5'-AGTGCTCATCATAGGACA-3', Evv1Cap1Probe 5'-FAM-ATTCTCGTAGCCAACTGCCAAAC-3'). To check that all the cDNA samples were properly amplifiable, the *E. variegatus* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript, was chosen as endogenous control. To amplify the insect GAPDH, the primer GapFw632 (5'-ATCCGTCGTCGACCTTACTG-3' (Galetto et al., 2013)), was used with the newly designed primer GapRv819 (5'-GTAGCCCAGGATGCCCTTC-3') and the internal HEX-labelled TaqMan probe GapEvProbe (5'-HEX-ATATCAAGGCCAAGGTCAAGGAGGC-3'). One µL of cDNA was used as template in a reaction mix of 10 µL total volume, containing 1X iTaq Universal Probes Supermix (Bio-Rad), 200 nM of each of the four primers and 300 nM of each of the two TaqMan probes. Each sample was run in triplicate in a CFX Connect Real-Time PCR Detection System (Bio-Rad). Cycling conditions were 95°C for 3 min and 40 consecutive cycles at 95°C for 10 s as denaturing step followed by 30 s at 60°C as annealing/extension step. In each qPCR plate, four serial 100-fold dilutions of pGem-T Easy (Promega) plasmids, harbouring the target virus and insect genes, were included to calculate the viral load. For both plasmid standard curves, dilutions included in plates ranged from 10^8 to 10^2 target copy numbers per µL and were prepared taking into account that 1 fg of plasmids harbouring EVV-1 and GAPDH

gene portions contains 138 and 244 number of molecules, respectively. Dilution series of both plasmids were used to calculate qPCR parameters (reaction efficiency and R^2). Mean virus copy numbers in amplified samples were automatically calculated by CFX MaestroTM Software (Bio-Rad) and used to express virus amount as EVV-1 copy numbers/insect GAPDH transcript.

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

SigmaPlot version 13 (Systat Software, Inc., www.systatsoftware.com.) was used for statistical analyses. To compare viral load among different categories (life stages, organs and vertical transmission experiments) ANOVA, followed by the proper post hoc test, or Kruskal Wallis test, when the parametric analysis assumptions were not met, were used. To compare viral load between two groups of samples (male vs. female EvaTo adults, virus exposed EvaBx vs. EvaTo samples in horizontal transmission experiments) t-test was used.

3. Results

3.1 EVV-1 qPCR diagnostic and quantitative assay

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

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

EVV-1 was detected in the eggs and all the insect developmental stages (I-V instar nymphs and adults) of EvaTo population. Male and female EvaTo individuals were considered together for the analyses, as no significant differences were found between their viral loads (t-test t=1.448, P=0.186) (Supplementary Table S1). Significant differences were found among developmental stages (ANOVA F=10.367, P<0.001) and I, II, III instar nymphs showed the highest viral loads, as indicated by pairwise multiple comparison according to Duncan's multiple range test (Fig. 1 and Supplementary Table S1).

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

3.3 Vertical transmission

Virus injection was selected as the most effective infection modality to obtain a high number of newly infected EvaBx individuals (F0) and test their ability to transmit the virus to progeny (F1). Virus presence was detected in 95 and 100 % injected insects sampled at 4 and 10 dpi, respectively, as well as in all the F1 tested insects (Supplementary Table S3), demonstrating that vertical viral transmission occurs with very high frequency. Interestingly, mean EVV-1 loads measured in F1 samples were significantly higher than those detected in injected F0 insects sampled at 4 dpi and in EvaTo adults (ANOVA on Ranks, Kruskal-Wallis test H=15.999, P=0.001) (Fig. 3). The virus was detected in EvaTo fresh extract injected in EvaBx specimens, while no amplification was obtained from EvaBx adults injected with buffer only (negative control).

3.4 Horizontal transmission

The co-feeding experiment, in which EvaTo insects were isolated together with EvaBx specimens on the same plant, indicated that horizontal viral transmission may occur. The overall mean rate of virus transmission was 8.0% (6/75), comprehensively considering the three different co-feeding experimental approaches (indicated in Table 1). The mean viral load, measured in the infected EvaBx insects three weeks after co-feeding with EvaTo, was 0.23 EVV-1 copy numbers/insect GAPDH transcript ± 0.10 (SEM, N=6), with values ranging from 6.27E-04 to 5.94E-01. The mean viral load measured in these virus-exposed EvaBx samples did not significantly differ from that measured in EvaTo adults, which was 0.44 EVV-1 copy numbers/insect GAPDH transcript ± 0.14 (SEM, N=10) (t-test t=1.815, P=0.091).

When exploring the possible routes of EVV-1 horizontal transmission, none of the EvaBx insects artificially fed on virus-infected honeydew solution resulted positive for EVV-1 presence (Table 1). Therefore, despite the presence of EVV-1 in EvaTo honeydew was confirmed by qPCR, the possibility of virus transmission via fecal-oral route could not be confirmed under our experimental conditions.

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

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

4. Discussion

In this work, distribution and transmission routes of the Euscelidius variegatus iflavirus 1 were explored to show that the virus is ubiquitous in the insect body and life stages, and it is vertically transmitted to offspring with high efficiency. Unveiling these biological parameters is a prerequisite to explore the potential application of iflaviruses as biocontrol agents of phytoplasma vectors. This work also paves the road to exploit iflaviruses as

molecular tools to study insect genes involved in pathogen transmission through the construction of infectious viral clones.

Iflaviruses have different ways of colonizing their arthropod hosts. In some cases, viruses do not show tropism for specific organs or tissues, such as the Deformed Wing Virus (DWV) and Antheraea pernyi Iflavirus (ApIV). These can be detected in all parts of the bee body, DWV (Martin and Brettell, 2019), or in the head, epidermis, hemocytes, gut, fat body, ovary and testis of the Chinese oak silkmoth, ApIV (Geng et al., 2017) . On the contrary, other iflaviruses show some organ tropism, such as the Nora Virus of the cotton bollworm (Yang et al., 2019) or the Helicoverpa armigera iflavirus (Yuan et al., 2017), which show higher abundance in midgut tissues or higher prevalence in fat bodies compared to other insect body districts, respectively. Molecular analysis showed that EVV-1 is present in different *E. variegatus* tissues and organs as well as in different life stages (from egg to adult). The significantly highest viral load detected in I-III instar nymphs may indicate that these stages are probably supporting the peak of viral replication. Indeed, over-time increase of viral load has been observed in pupae of the Chinese oak silkmoth following the injection of ApIV purified particles (Geng et al., 2017). Analogously, the high viral load measured in ovaries suggests a key role of this organ in the viral replication cycle. Indeed, this feature may be common to iflaviruses infecting Hemiptera, as the Laodelphax striatellus Iflavirus 1 (LsIV1) is present in eggs and ovaries of the small brown planthopper (Wu et al., 2019). Evidence from different iflaviruses seems therefore to delineate an assorted model of insect body colonization, that requires case-by-case characterization.

Under our experimental conditions, EVV-1 was efficiently transmitted to the offspring, and complete colonization of the virus-free population occurred in one generation. Vertical transmission is a second common feature between EVV-1 and LsIV1 (Wu et al., 2019). Indeed, following injection of virus-free parents, the newly infected F1 progeny always showed 100% EVV-1 incidence. The development of an efficient multiplex fluorescent qPCR assay, based on glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous gene for normalization of quantitative data and as internal control of cDNA quality, allowed the quantitative detection of EVV-1, a prerequisite to characterize its distribution and transmission modalities. Interestingly, the mean viral load of the F1 individuals was significantly higher than that of naturally infected EvaTo adults. This result supports the hypothesis of a higher viral replication rate in the experimentally infected than in the naturally infected insects, in the absence of evident fitness costs. Indeed, in several mosquito species, a state of tolerance response against covert arboviral infections has been described leading to persistent infection in the host with few fitness costs (Goic et al., 2016).

Horizontal transmission has also been reported for some iflaviruses (Murakami et al., 2013; Yuan et al., 2017), and indeed, it occasionally occurred for EVV-1 under our experimental conditions, although less efficiently than vertical route. In particular, the Nilaparvata lugens iflavirus 1 (NHLV-1) is efficiently transmitted to virus-free brown leafhoppers through feeding on infected honeydews (Murakami et al., 2013). On the other hand, despite consistent EVV-1 presence in EvaTo honeydew added to the artificial diet, the virus was not transmitted to EvaBx individuals through feeding, suggesting this virus is not able to cross the gut barrier. EVV-1 could be sporadically detected in not-sterilized leaves from plants exposed to virus-infected insects, probably due to honeydew contamination, but it was never detected after sterilization of leaf surface. Consistently, the phloem feeding habit of *E. variegatus* may explain its inability to acquire EVV-1 through the host plant.

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

Although the idea behind the application of entomopathogens as biocontrol agent is more than 60 years old (Steinhaus, 1956), the use of insect-specific viruses in plant protection is lately gaining attention. As an example, nowadays Baculoviruses applications range from fitness decreasing (Gramkow et al., 2010), to synergic approach (Haase et al., 2015) to compete with pathogen acquisition. Iflaviruses may often infect their hosts and transcribe high amounts of their RNAs without inducing visible symptom in the infected population (dos Santos et al., 2019), but in the case of *Spodoptera exigua* larvae, infection with an iflavirus increases the susceptibility of the larvae to a baculovirus occlusion body-based insecticide (Carballo et al., 2017). This intriguing cross-talk among different partners of a complex microbiome has been evoked to justify the lower load of EVV-1 in *E. variegatus* individuals infected with Flavescence dorée phytoplasma (Abbà et al., 2017). The urgent need for more precise and sustainable pest management approaches to fight against phytoplasma diseases encourages the exploration of new research frontiers to envisage viruses as biocontrol agents able to compete with phytoplasmas transmission and/or acquisition. This pioneering hypothesis is well supported by the cases of West Nile virus vectored by mosquitoes co-infected with Nhumirim virus (Nouri et al., 2018) or with Wolbachia (Glaser and Meola, 2010), and by the cases of Dengue, Chikungunya, and Plasmodium transmitted by *Aedes* co-infected with Wolbachia (Moreira et al., 2009). Several fundamental biological features of EVV-1 were addressed in this work, in view of an ongoing exploration of this virus to express heterologous genes in *E. variegatus* and regulate the expression of insect genes by virus-induced gene silencing (VIGS). To reach this goal, preliminary results in the synthesis of an EVV-1 infectious clone have been gathered (Marzachì et al., 2019) and RNAi mediated by injection of dsRNA molecules has been proven to work efficiently in *E. variegatus* with a long-lasting effect (Abbà et al., 2019). Elucidating the mechanisms of insect tolerance to iflavirus infection paves the way to conceptualize new anti-vectorial strategies to selectively control plant pathogen-transmitter Hemipteran insects.

Author contributions

Concept & financing (LG, SA, CM, DB); Experimental design (LG, MR, CM); Execution (SO, AP, MR, MV, GM); Analysis (SO, MR, LG, CM); Publication (LG, MR, SA, MV, SP, DB, RB, CM).

All authors read and approved the final manuscript. The authors declare no competing financial interests.

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610 ## Table

611 **Table 1.** Detection and quantification results of horizontal transmission of Euscelidius
612 variegatus virus 1 (EVV-1) to EVV-1 free EvaBx population. Mean quantification cycles (Cq)
613 obtained from insect glyceraldehyde-3-phosphate dehydrogenase (GAPDH) qPCR assay
614 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	2639 ± 385 (10)	17.85 ± 0.16 (105)

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

616

617

618 ## Figure captions

619

620 **Figure 1. Distribution of Euscelidius variegatus virus 1 (EVV-1) in different insect life stages.**
621 Different letters indicate significant differences in mean viral loads measured in the sample
622 groups. First to fifth instar nymphs are indicated by Roman numerals and different life stages
623 are depicted beneath the graph.

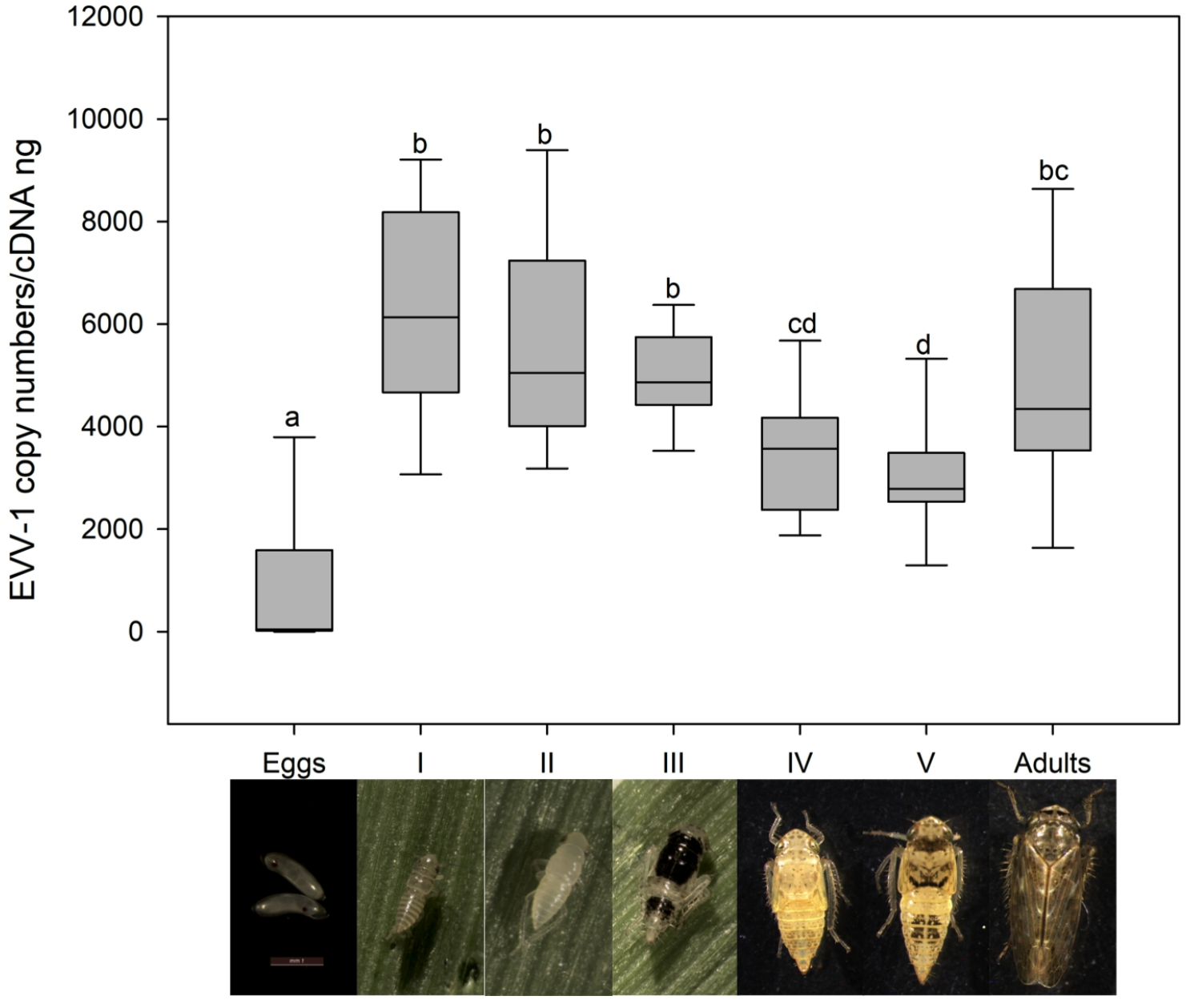
624

625 **Figure 2. Distribution of Euscelidius variegatus virus 1 (EVV-1) in different insect organs.**
626 Different letters indicate significant differences in mean viral load measured in the sample
627 groups. The different insect organs are depicted beneath the graph.

628

629 **Figure 3. Vertical transmission of Euscelidius variegatus virus 1 (EVV-1) injected into EVV-1**
630 **free EvaBx rearing.** Different letters indicate significant differences in mean viral load
631 measured in the sample groups. Days post injection (dpi) indicate the sampling date after
632 viral injection. Viral load measured in infected EvaTo adults (EvaTo) was used for comparison.

633


EVV-1 copy numbers/cDNA ng
12000
10000
8000
6000
4000
2000
0
a
b
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b
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d
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Eggs
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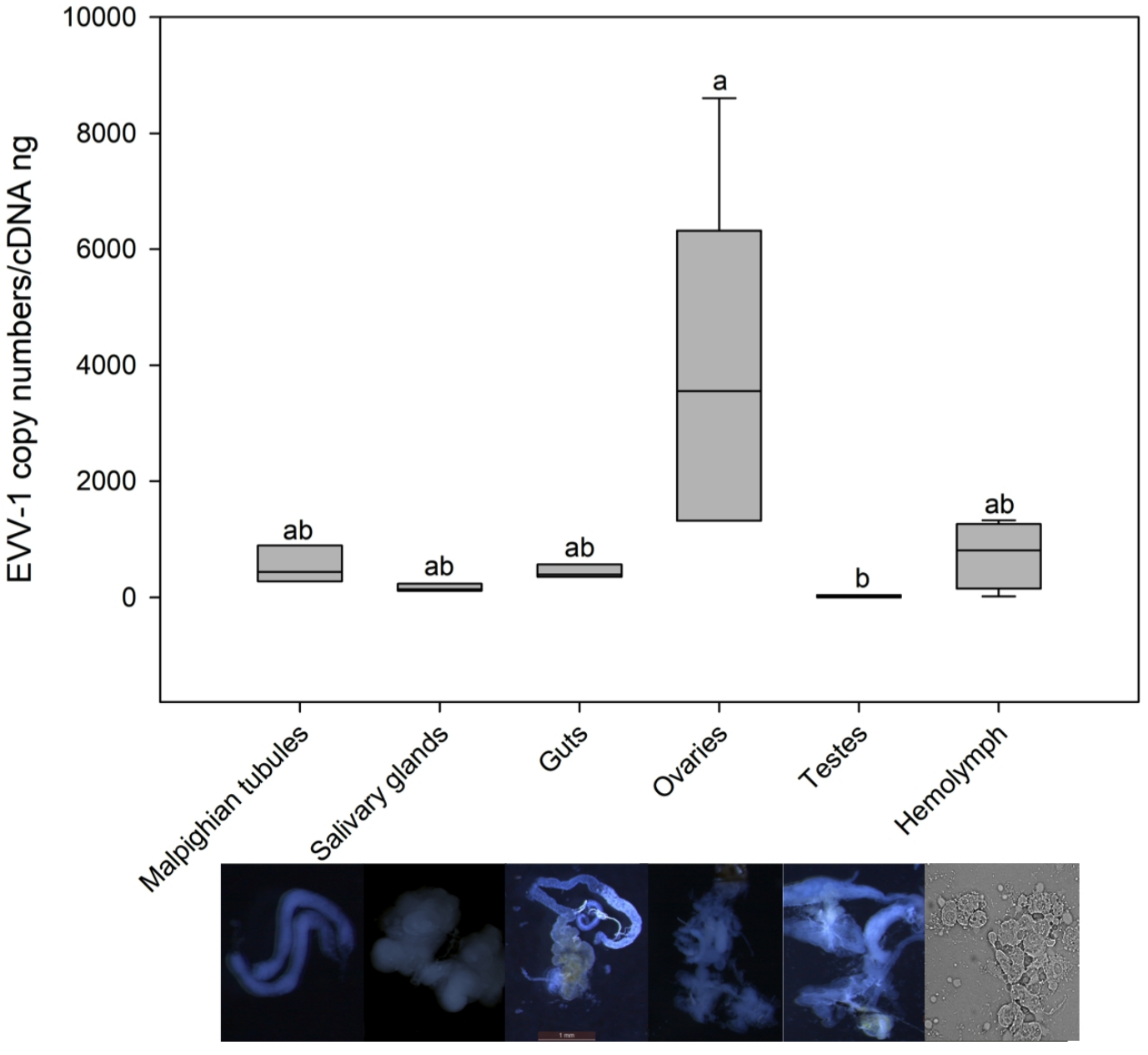
Adults


EVV-1 copy numbers/cDNA ng
10000
8000
6000
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2000
0
a
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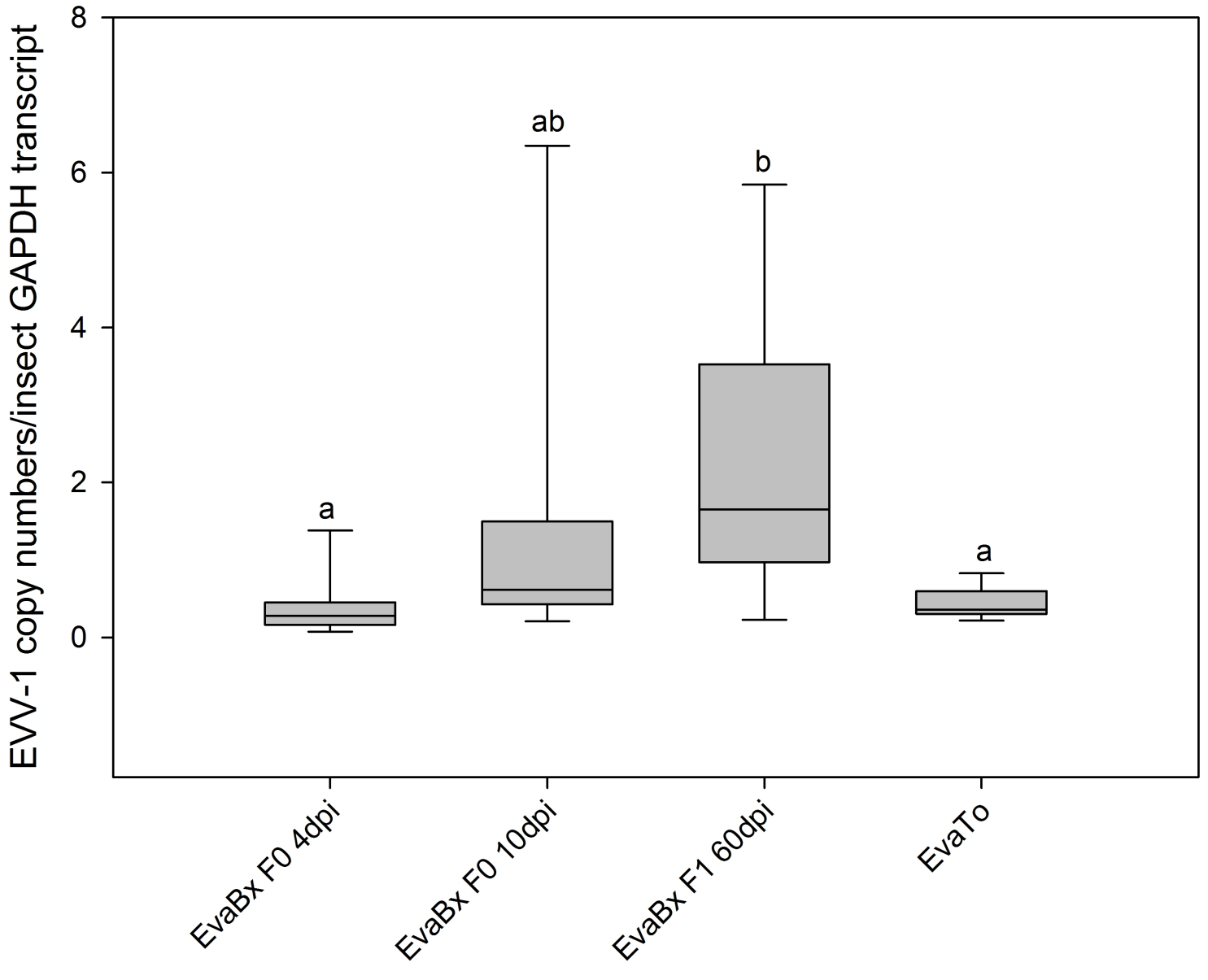
Malpighian tubules
Salivary glands
Guts
Ovaries
Testes
Hemolymph


EVV-1 copy numbers/insect GAPDH transcript
8
6
4
2
0
a
ab
b
a
EvaBx F0 4dpi
EvaBx F0 10dpi
EvaBx F1 60dpi
EvaTo

Supplementary Tables

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

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

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

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

Supplementary Table S3. Presence and loads of Euscelidius variegatus virus 1 (EVV-1) following injection into the EVV-1 free EvaBx population. Mean quantification cycles (Cq) obtained from insect glyceraldehyde-3-phosphate dehydrogenase (GAPDH) qPCR assay 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)

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