

# Mites associated with egg masses of the viburnum leaf beetle *Pyrrhalta viburni* (Paykull) on *Viburnum tinus* L.

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## Original article

### ABSTRACT

The viburnum leaf beetle *Pyrrhalta viburni* (Coleoptera: Chrysomelidae) is a specialist *Viburnum* leaf-feeder that is native to Eurasia and invasive in North America. Eggs of *P. viburni* are laid in round cavities excavated by the ovipositing female beetle and covered with a protective secretion. We document in this paper the mite fauna associated with *P. viburni* egg masses on *Viburnum tinus* in southern France. We then report the results of experiments investigating the seasonal patterns of mite infestation and the effects of the most common mite found within egg masses, *Trichoribates trimaculatus* (Oribatida: Ceratozetidae), on *P. viburni* egg survivorship. A diverse mite fauna of 18 species was found on *V. tinus* twigs, often living within *P. viburni* egg masses, including predaceous, phytophagous, and detritivorous species. Mite abundance and diversity were higher on *Viburnum* twigs containing at least one intact egg mass and were positively correlated with the number of intact egg masses per twig. Detritivorous mites were more abundant on twigs nine months after oviposition than one and four months after oviposition. Finally, we found no evidence that *T. trimaculatus* impacts *P. viburni* egg survivorship and overwintering success. These findings show that *P. viburni* egg masses and their associated cavities form a microhabitat favorable for the establishment of several mite species. It seems likely that these associations are cases of commensalism where mites benefit from the presence of food and shelter in these protected cavities, with no direct negative impact on *P. viburni* eggs.

**Keywords** insect-mite interactions; oviposition behavior; Oribatida; Phytoseiidae; community ecology

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## Introduction

Insects and mites have successfully colonized nearly every terrestrial and aquatic habitat on Earth, and their highly diverse feeding habits allow them to occupy a wide range of ecological niches. Interactions between insects and mites are extremely common in nature, and can take a variety of forms (Krantz and Walter 2009). Antagonistic interactions are very common, as many insects feed on mites (Helle and Sabelis 1985) and, conversely, mites of several families prey on small insects or insect eggs, or are insect parasites (Gerson *et al.* 2008). Commensalism (interaction where one partner benefits from the interaction and the other remains unharmed) is also very common. Phoretic mites that use insects for transportation without apparent fitness cost for the insect are a good example of such interactions (Norton 1980; Wilson and Knollenberg 1987). Lastly, mutualisms between mites and insects are less common but have

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been documented. Some insects have evolved morphological structures to host mites on their cuticle (i.e. acarinarium), suggesting an evolutionary benefit to hosting mites. For instance, Okabe and Makino (2008) clearly showed that the mite *Ensliniella parasitica* Vitzthum benefits its host, the wasp *Allopynerus delphinalis* (Giraud), by providing protection against parasitic wasps (Okabe and Makino 2008). However, in many other instances of specialized mite-insect associations, the way in which the presence of mites may benefit the insect has not been fully clarified (Lindquist 1975; Okabe 2013; Hofstetter and Moser 2014).

Mites are often found in structures built by insects, for example those built by social insects, and these associations can be obligate or simply opportunistic (Eickworth 1990; Okabe 2013). Here we investigated the mite fauna associated with the egg masses of the viburnum leaf beetle *Pyrrhalta viburni* (Paykull) (Coleoptera: Chrysomelidae). This insect is a specialist of *Viburnum* (Caprifoliaceae) species, a genus of shrubs and small trees containing approximately 180 species worldwide (Weston and Desurmont 2002; Weston *et al.* 2007). Native to Eurasia, this insect has been accidentally introduced to North America, first in Canada then in the U.S.A., where it causes extensive damage to native and exotic *Viburnum* species in managed landscapes and natural areas (Desurmont and Agrawal 2014). Both larvae and adults are leaf chewers that can extensively defoliate shrubs, often killing them after 2 to 4 years of repeated complete defoliations. In its invaded range, *P. viburni* has an economic impact for nurseries as well as an ecological impact on wild *Viburnum* populations and their associated fauna. For instance, *Viburnum* fruits can be an essential food source for resident and migratory birds in Northeast U.S. and Canada (Sargent 1990; Witmer 2001) and their populations are impacted by *P. viburni* invasion. A six-year study in Pennsylvania recently showed that the avian community declined and that mass gain by birds was negatively affected by the invasion of the viburnum leaf beetle and the disappearance of local *Viburnum* shrubs (Smith and Hatch 2017).

*Pyrrhalta viburni* is a univoltine species overwintering as eggs. The larvae hatch in the early spring and take three to four weeks to develop and reach pupation, which occurs in the soil. Adults typically emerge in May-June and are present in the field through the summer. Females lay eggs in round cavities they excavate in the young twigs of their host plants. These cavities are filled with an average of 8 eggs and covered with a frass-like secretion (i.e. “egg cap”) that protects eggs from desiccation during winter months (Weston *et al.* 2008). Females prefer to lay their eggs adjacent to existing egg masses, a cooperative behavior described as aggregative oviposition (Desurmont and Weston 2011). As a result of aggregative oviposition, egg masses are typically found clustered on the underside of twigs. The main benefit of this behavior is to overcome a plant defensive response. Indeed, infested *Viburnum* twigs often produce wounding tissue in response to viburnum leaf beetle oviposition (wound response), which can crush or expel eggs out of their cavity. High densities of egg masses often kill infested twigs, preventing further wound tissue production and thus enhancing egg survivorship (Desurmont *et al.* 2009; Desurmont and Weston 2011). These two factors, aggregative oviposition and plant wound response, are assumed to play a key role in *P. viburni* invasion success in North America (Desurmont *et al.* 2011; Desurmont *et al.* 2012). The role of enemy release on invasion success remains to be elucidated, and the impact of natural enemies of *P. viburni* in its native range is still poorly known.

In the South of France, *P. viburni* feeds on *Viburnum tinus* L., an evergreen shrub often found in Mediterranean forest understories and managed landscapes. In this area, high levels of egg mortality during winter months as well as the presence of mites in egg masses of *P. viburni* have been observed in recent years (Gaylord A. Desurmont, personal observation), prompting the present study. This study aims at: (i) documenting the mite fauna associated with *P. viburni* egg masses, (ii) understanding the modalities of mite infestation, and (iii) determining whether mites pose a threat to egg survivorship and overwintering success. We first conducted an observational study during spring 2016 to document the abundance and diversity of mites present on naturally infested *V. tinus* twigs, and examined the associations between mite abundance and diversity and egg mass density. We then conducted a field experiment to understand the seasonal patterns of mite infestation by collecting mites on

artificially infested *V. tinus* twigs in summer and fall 2016, and in spring 2017. Finally, we conducted a field experiment between fall 2017 and spring 2018 to measure the impact of one mite species, *Trichoribates trimaculatus* (Koch) (Acari, Oribatida, Ceratozetidae), on the overwintering success of egg masses. The ultimate goal of this research, in addition to documenting interesting new insect-mite interactions, is to determine whether mites can pose a threat to *P. viburni* fitness and to identify potential biological control agents of this invasive pest.

## Material and methods

### Observational study of the mite fauna associated with *P. viburni* egg masses on *V. tinus*

In April 2016, *V. tinus* twigs were collected from five field sites located in Southern France (Montpellier area) (Table 1). Five *V. tinus* shrubs were selected for each site. The level of damage caused by *P. viburni* was assessed by visually estimating the overall defoliation of each shrub and was classified in three categories: shrubs with defoliation levels higher than 15 % were considered heavily infested, shrubs with 5-15 % defoliation were considered moderately infested, and shrubs with defoliation levels lower than 5 % were considered lightly infested. For each shrub, five young twigs from the current year's growth were collected. The sampling of twigs on each shrub was conducted as follows: one twig that contained *P. viburni* egg masses (intact or damaged) and one twig that did not contain any *P. viburni* egg masses were selected. The other three twigs were chosen randomly. In total, 125 twigs were collected in the field and brought to the laboratory in individual Ziploc™ bags placed in a plastic cooler used for transportation. Bags with twigs were left at ambient temperature in the laboratory and the twigs were carefully inspected for presence of mites within the next 24 h. For each twig, leaves were removed before inspection and the following parameters were recorded: length of the twig, number of intact and damaged *P. viburni* egg masses present on the twig, total number of mites present on the twig, total number of mite morphotypes (i.e. mites showing morphological differences and a priori belonging to different species) present on the twig. We considered that an egg mass was intact if the egg cap was still present and if the egg mass cavity was not covered by wounding tissue. We considered that an egg mass was damaged if

**Table 1** Coordinates of the study sites used for the observational study (2016) and the timing of mite infestation experiment (2017).

| Observational study 2016     | Coordinates (Latitude, Longitude) |
|------------------------------|-----------------------------------|
| Site 1                       | 43.683325, 3.874779               |
| Site 2                       | 43.667094, 3.851299               |
| Site 3                       | 43.769778, 3.787764               |
| Site 4                       | 43.716070, 3.848204               |
| Site 5                       | 43.681688, 3.878901               |
| Timing mite infestation 2017 |                                   |
| Site 1                       | 43.682444, 3.880156               |
| Site 2                       | 43.676900, 3.874368               |
| Site 3                       | 43.714209, 3.861153               |
| Site 4                       | 43.708495, 3.837012               |

the egg cap was absent and if the egg mass cavity was at least partially covered by wounding tissue. Intact egg masses present on the twigs were opened using a scalpel or forceps and carefully inspected using a dissection microscope for presence of mites ( $\times 40$ ). Mites from the different morphotypes observed were then placed in 70 % ethanol for subsequent identification. The taxonomic identification of the mites was based on several published identification keys (Bolland *et al.* 1998; Weigmann and Miko 2006; Chant and McMurtry 2007; Krantz and Walter 2009; Mesa *et al.* 2009). If for any reason twigs could not be examined within 24 h after collection, they were discarded from the analysis.

## Timing of mite infestation on *V. tinus* twigs infested with *P. viburni* egg masses

The aim of this experiment was to understand how quickly mites colonize *V. tinus* twigs with newly-laid *P. viburni* egg masses and the patterns of seasonal abundance of mites on those twigs. In order to control for the timing of *P. viburni* oviposition, we selected and artificially infested *V. tinus* twigs with *P. viburni* egg masses in July 2016 in four study sites in the Montpellier area (Table 1). The process for artificial infestation went as follows: twigs from the year's growth that did not contain any *P. viburni* egg mass were selected and bagged with a fine-mesh sleeve net (200  $\mu\text{m}$  mesh diameter), and five females and two males of *P. viburni* were released inside the bags for 72 h. After 72 h, the bags and insects were removed and the number of egg masses laid on each twig was counted. In total three *V. tinus* shrubs were selected for each of the four study sites, and nine twigs were selected per shrub, for a grand total of 108 twigs artificially infested. We then collected one third of the twigs artificially infested on each shrub (i.e. three twigs) one month after infestation (August 2016), a second third four months after infestation (November 2016), and the last third nine months after infestation (April 2017). The last collection time (April 2017) corresponds to the period of egg hatch for *P. viburni* in the Montpellier area. Collected twigs were brought to the laboratory and examined for presence of mites in the conditions described in the previous paragraph. The number of intact and damaged egg masses and the number of mites belonging to each of the morphotypes observed were recorded for each twig.

## Impact of *T. trimaculatus* on overwintering success of *P. viburni* eggs

*Trichoribates trimaculatus* was the most common mite observed within *P. viburni* egg masses (see results section). Although *T. trimaculatus* is a detritivorous species unlikely to predate directly on *P. viburni* eggs, its presence might deteriorate the secretion protecting the eggs, reduce the quality of the cavity as a protection against desiccation and natural enemies, and ultimately decrease egg overwintering success. To test this hypothesis, we enclosed *V. tinus* twigs with newly-laid egg masses in nylon sleeve nets with and without an addition of *T. trimaculatus* adults in September 2017. We then left the twigs in the sleeve nets under field conditions during the overwintering period and then measured *P. viburni* egg survivorship after the overwintering period in March-April 2018. The process for twig infestation with *P. viburni* egg masses went as follows: in July 2017, 10 young *V. tinus* shrubs (25–50 cm tall) with no sign of *P. viburni* infestation were transplanted from a field in the Montpellier area and potted in plastic pots (22.5 cm diameter  $\times$  18 cm height) with potting soil kept in a growth chamber with a 22 °C constant temperature and a 12/12 (l:d) photoperiod. In August 2017, these shrubs were placed in large mesh cages (90  $\times$  60  $\times$  60 cm, 500  $\mu\text{m}$  mesh diameter) with 25 *P. viburni* individuals (20 females and 5 males) per cage for a period of one week. After a week, *P. viburni* individuals were removed from the cages and the number of twigs infested and the number of egg masses per twig were counted on each shrub (without damaging/removing the twigs from the shrubs). A total of 54 twigs containing between 1 and 20 egg masses were selected for the experiment and each twig was enclosed in a sleeve net (50 cm long, 200  $\mu\text{m}$  mesh diameter, Diatex Co): 26 twigs each received an addition of 30 *T. trimaculatus* adults (mite

treatment), and 28 did not receive any mites (control treatment). A thin metal wire was used to close the extremities of each net. The number of twigs selected for the experiment varied between 2 and 10 per shrub and the number of twigs allocated to each treatment was equally distributed within each shrub (i.e. each shrub had an equal number of twigs with mites and twigs without mites). All *T. trimaculatus* adults used for infesting sleeve nets were collected locally during the days preceding the infestations. They were kept on *V. tinus* twigs with a moistened piece of cotton at 17 °C and a 12:12 (l:d) photoperiod until needed for the experiment. Mite addition in the sleeve nets was done as a two-step process. Step 1, on September 1 2017, 20 *T. trimaculatus* adults were added to the sleeve nets enclosing the twigs selected for the mite treatment. All shrubs were kept in a growth chamber with a 22 °C constant temperature and a 12/12 (l:d) photoperiod until the second mite addition. Step 2, on October 10 2017, 10 more *T. trimaculatus* adults were added to the same sleeve nets. At the time of the second mite addition, 5 twigs from each treatment were cut and inspected using a dissection microscope to check for mite presence and survival: these twigs were discarded from the rest of the analyses. After the second mite addition, shrubs were moved outside in a shaded location near naturally-growing *V. tinus* shrubs, and were left undisturbed until February 2018. The number of *T. trimaculatus* individuals added to twigs of the mite treatment (30) was purposely higher than the natural densities of mites we observed in egg masses during the observational study, in order to account for potential mite mortality during the transfer and early establishment of the mites on the twigs. On February 5 2018, all twigs with sleeve nets were cut and brought back to the laboratory. Portions of each twig containing egg masses were transferred to petri dishes (9 cm diameter) with a thin layer of agar (0.5 cm) covered with a filter paper. Portions of each twig containing egg masses were placed on top of the filter paper. Each petri dish was then sealed with a strip of Parafilm® (American National Can Co), and all petri dishes were monitored until late April 2018 for *P. viburni* larval emergence. The number of larvae emerging from each twig was recorded. The sleeve nets that enclosed the twigs and the twigs themselves were carefully inspected for mite presence when twigs were transferred to petri dishes, and all twigs and egg masses were examined again for mite presence at the end of the experiment after *P. viburni* larval emergence. The total numbers of dead and live *T. trimaculatus* adults and nymphs that were recovered from the sleeve nets and from the examination of egg masses were recorded.

## Statistical analysis

Results from the observational study were analyzed using a generalized linear model (GLM) with a poisson distribution with abundance of mites (i.e. total number of mites observed) per twig and diversity of mites (i.e. total number of mite morphotypes observed) per twig used as dependent variables, and site, level of shrub infestation (light, moderate, high), twig length (cm), number of intact egg masses and number of damaged egg masses used independent variables ( $\alpha = 0.05$ ). The second degree interactions between the terms were initially included in the model, then removed if not significant. We further explored the link between intact egg masses and mite diversity and abundance by running two additional analyses. For the first analysis, we divided the twigs collected in three categories: twigs with no egg masses present, twigs containing only damaged egg masses, and twigs containing at least one intact egg mass. We then ran a GLM test ( $\alpha = 0.05$ ), testing the hypothesis that the abundance and diversity of mites (dependent variables) depended on the category of the twig. Differences between the means were then analyzed by running a post-hoc test. For the second analysis, we directly tested the association between the number of intact egg masses and the abundance and diversity of mites present on the twigs collected by running linear regression models ( $\alpha = 0.05$ ).

Results of the timing of mite infestation experiment were analyzed using general ANOVAs. For these models, we tested the effects of study site, time of collection (August, November, and April), twig length and the interactions between these terms on the following dependent variables: total mite abundance (i.e. total number of mites observed), phytophagous/detritivorous mite abundance, predaceous mite abundance, diversity of mites (i.e. total number of mite

morphotypes observed). The second degree interactions between the terms were initially included in the model then removed if not significant. If a categorical factor was found to have a significant effect on one of the dependent variables, a Tukey post-hoc test was performed to compare the means. If a continuous factor was found to have an effect on one of the dependent variables, a complementary linear regression analysis was performed to investigate the direction and strength of the correlation.

Results from the experiment on the impact of *T. trimaculatus* on *P. viburni* overwintering success were analyzed using a general ANOVA, testing the effects of treatment and total number of egg masses (independent variables) on the number of larvae emerging per egg mass (dependent variable). To account for possible plant-related effects on egg survivorship, a factor “shrub”, representing the shrub each twig used in the study originated from, was added to the model as an independent categorical variable. This analysis was performed with the full dataset of twigs first, then was repeated after excluding twigs from the mite treatment that did not contain any live mites at the end of the experiment (3 twigs out of 21) and twigs from the control treatment that did contain live mites at the end of the experiment (7 twigs out of 23). The mean number of live mites found on twigs at the end of the experiment was compared between the mite treatment and the control treatment using a non-parametric Wilcoxon test on sum of ranks ( $\alpha = 0.05$ ).

For all ANOVA analyses, a Shapiro-Wilk goodness of fit test ( $\alpha = 0.05$ ) was performed to insure that the data were normally distributed, and non-normal data were square-root or log-transformed in order to meet the assumption of normality. The only data that still did not meet the assumption of normality after transformation were the data on total number of live mites inside the sleeve nets at the end of the experiment on the impact of *T. trimaculatus* on *P. viburni* overwintering success: these data were analyzed using a non-parametric Wilcoxon test on sum of ranks.

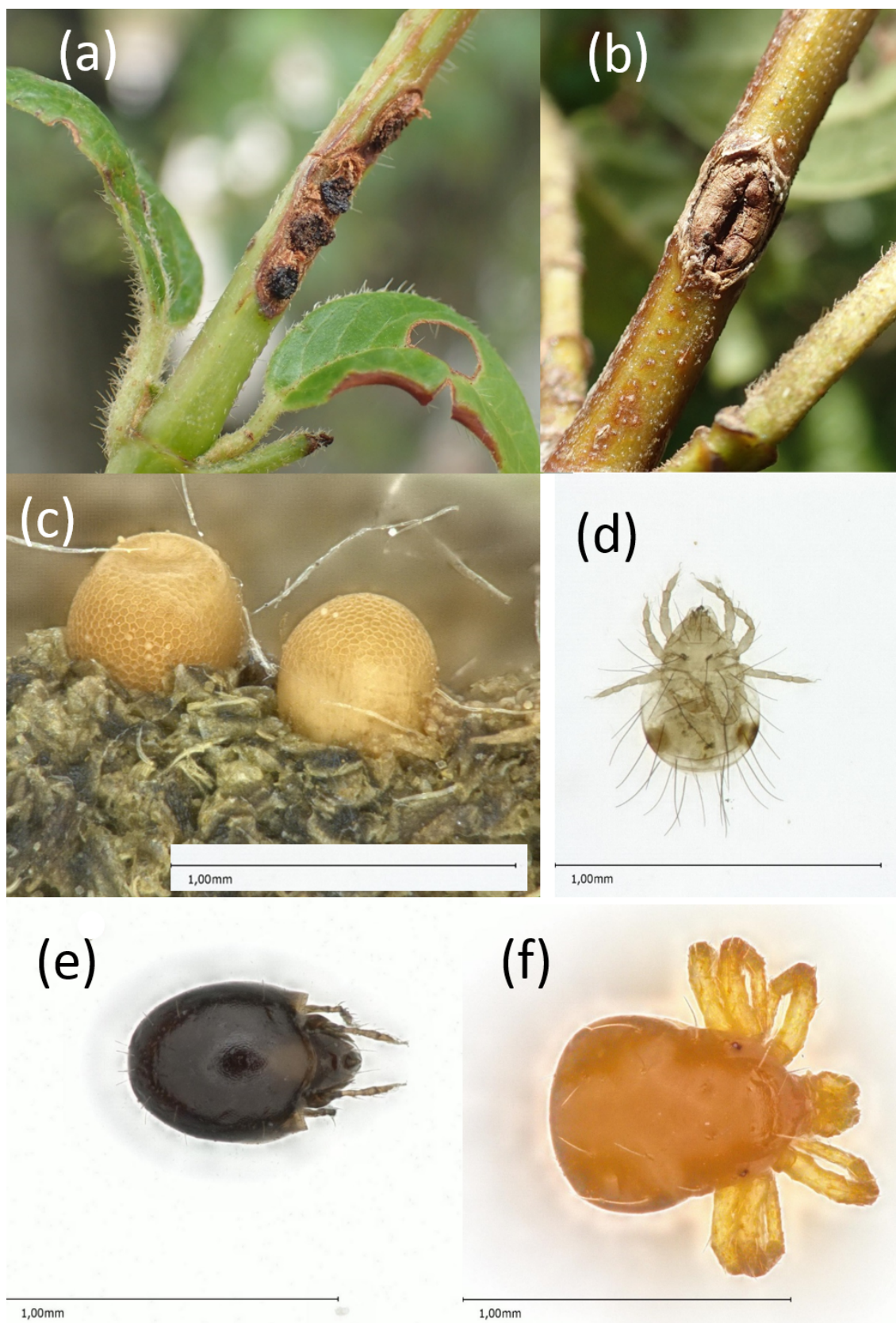
The statistical software used to perform all the analyses was JMP® version 12 (SAS Institute 2015).

## Results

### Observational study of the mite fauna associated with *P. viburni* egg masses on *V. tinus*

A total of 100 twigs were examined in April 2016 (25 twigs could not be examined within 24 h after collection and were discarded). The mean number of *P. viburni* egg masses (damaged or intact) per twig collected was  $6.8 \pm 7.8$  (mean  $\pm$  SE), ranging from 0 to 35 egg masses. Twenty of the twigs collected did not contain any egg mass, 22 contained only damaged egg masses, and 48 contained at least one intact egg mass. The overall percentage of twig wounding response (i.e. number of egg masses damaged  $\times$  100 / total number of egg masses observed) was 43.6 % ( $300 \times 100 / 688$ ). A total of 18 mite species were found (Table 2, Fig. 1): nine were predaceous species, two were phytophagous species, and seven were detritivorous species (Table 2, Fig. 1).

Predaceous and phytophagous mites, on the one hand, were encountered both on the twig itself and on or inside *P. viburni* egg masses. Detritivorous mites, on the other hand, were more often found inside the egg masses, deeply encased within the egg mass cavity, foraging among the wood debris surrounding the eggs. The most common detritivorous mite observed in our studies was by far the oribatid *Trichoribates trimaculatus* (family Ceratozetidae), which often occurred in groups of nymphs that could gather up to 20 individuals within the egg masses. This species is often found on trees, especially on oaks, and was also reported from mosses and meadows (Weigmann and Miko 2006). The other three detected detritivorous oribatid mite species, *Micreremus brevipes* (Michael), *Camisia segnis* (Hermann) and *Scapheremaeus patella* (Berlese), are also predominantly arboricolous taxa that are known to feed on fungi and lichen growing on the bark (Weigmann and Miko 2006). We found a strong significant effect of



**Figure 1** Pictures of (a) four intact *Pyrrhalta viburni* egg masses along a *Viburnum tinus* twig, with the protective “egg cap” visible; (b) one damaged *P. viburni* egg mass following plant wounding response, with the egg cap removed and the egg mass cavity partially covered by wounding tissue; (c) two *P. viburni* eggs; (d) Detritivorous mite *Trichoribates trimaculatus* nymph; (e) *T. trimaculatus* adult; (f) predatory mite *Anystis baccarum*. Photo credit: (a)(b) Gaylord Desurmont; (c)(d)(e)(f) Elven Kerdellant.

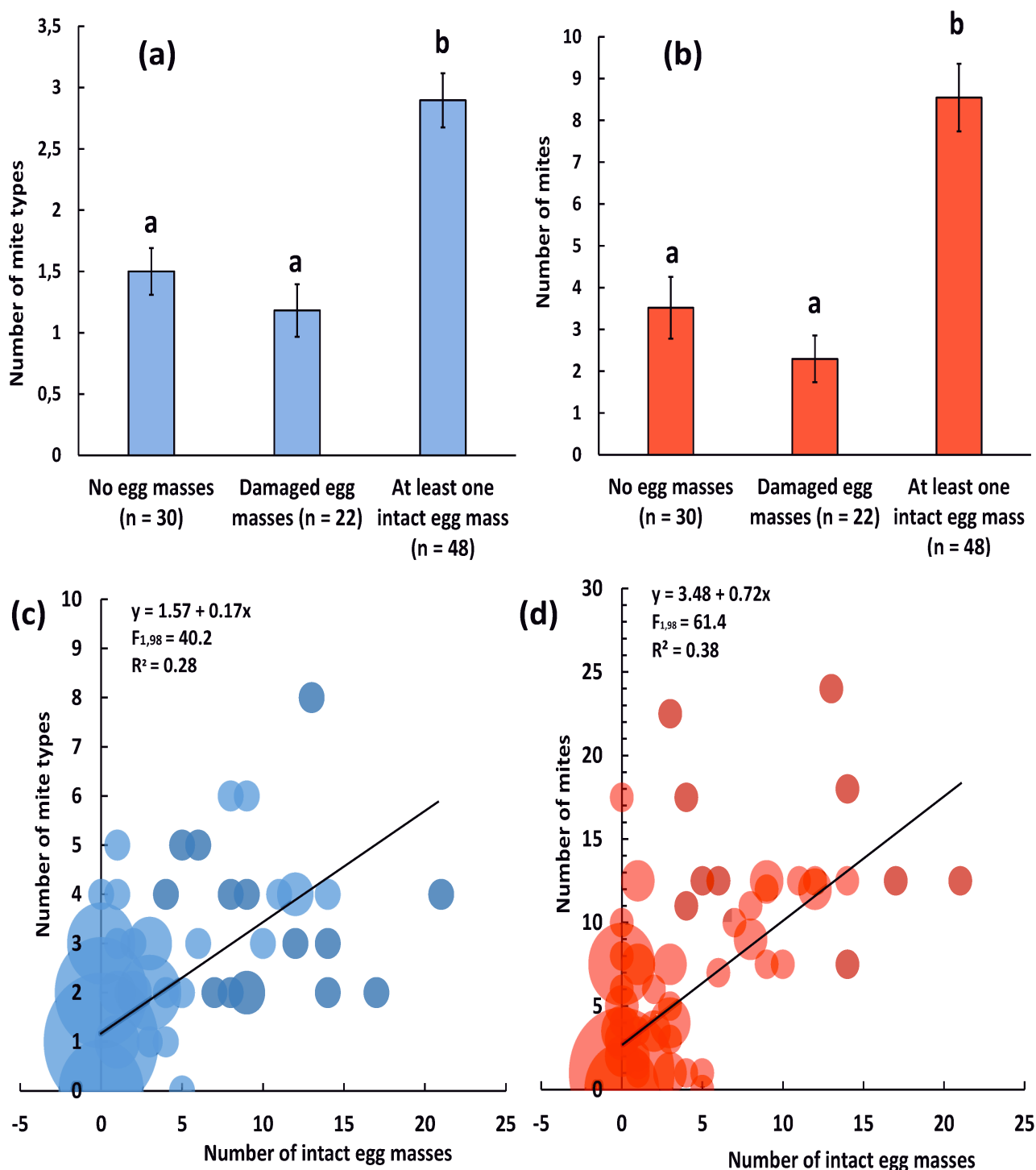
**Table 2** List of the mite species found on *Viburnum tinus* twigs during the study (sp. = species).

| Feeding guild | Family            | Genus                                       | Species                        |
|---------------|-------------------|---------------------------------------------|--------------------------------|
| Predaceous    | Phytoseiidae      | <i>Typhlodromus</i> ( <i>Typhlodromus</i> ) | <i>T. phialatus</i>            |
|               |                   | <i>Typhlodromus</i> ( <i>Anthoseius</i> )   | <i>T. recki</i>                |
|               |                   |                                             | <i>T. rhenanoides</i>          |
|               |                   | <i>Euseius</i>                              | <i>E. gallicus</i>             |
|               |                   | <i>Kampimodromus</i>                        | <i>K. aberrans</i>             |
|               |                   | <i>Amblyseius</i>                           | sp.                            |
|               | Cunaxidae         | <i>Neocunaxoides</i>                        | sp.                            |
|               | Cheyletidae       | <i>Cheletogenes</i>                         | <i>C. ornatus</i>              |
|               | Anystidae         | <i>Anystis</i>                              | <i>A. baccharum</i>            |
|               |                   |                                             |                                |
| Phytophagous  | Tetranychidae     | <i>Tetranychus</i>                          | sp. ( <i>T. urticae</i> group) |
|               | Tenuipalpidae     | <i>Brevipalpus</i>                          | sp.                            |
| Detritivorous | Ceratozetidae     | <i>Trichoribates</i>                        | <i>T. trimaculatus</i>         |
|               | Micreremidae      | <i>Micreremus</i>                           | <i>M. brevipes</i>             |
|               | Camisiidae        | <i>Camisia</i>                              | <i>C. segnis</i>               |
|               | Cymbaeremaeidae   | <i>Scapheremaeus</i>                        | <i>S. patella</i>              |
|               | Acaridae          | <i>Tyrophagus</i>                           | <i>T. putrescentiae</i>        |
|               | Winterschmidtidae | <i>Calvolia</i>                             | sp.                            |
|               | Tydeidae          | unknown                                     | sp.                            |

the number of intact egg masses on both mite abundance and mite diversity, as well as a small but significant positive effect of twig length on mite abundance (total number of mites =  $0.06 \times \text{twig length} + 3.95$ ,  $R^2 = 0.01$ ) and an effect of shrub level of infestation on mite diversity: mite diversity was higher on twigs coming from shrubs with high levels of infestation by *P. viburni* ( $2.58 \pm 0.24$  mite types) and moderate levels of infestation by *P. viburni* ( $2.13 \pm 0.21$ ) than on shrubs with light infestation by *P. viburni* ( $1.20 \pm 0.32$ ) (Table 3). There were no significant effects of the other variables included in the models (study site and number of damaged egg masses), and there were no significant second degree interactions between the terms of the models (Table 3). The abundance and diversity of mites were dependent on whether *V. tinus* twigs contained no egg mass, damaged egg masses only, or at least one intact egg mass (mite abundance:  $\chi^2 = 31.38$ ,  $P < 0.0001$ , d.f. = 2; mite diversity:  $\chi^2 = 27.01$ ,  $P < 0.0001$ , d.f. = 2): more mites and more mite morphotypes were observed on twigs that contained at least one intact egg mass than on twigs that did not contain egg masses or contained only damaged egg masses (Fig. 2a,b). There was a direct positive correlation between the number of intact egg masses present on a twig and both mite abundance ( $F_{1,98} = 61.4$ ,  $P < 0.0001$ ,  $R^2 = 0.38$ ) and mite diversity ( $F_{1,98} = 40.2$ ,  $P < 0.0001$ ,  $R^2 = 0.28$ ) (Fig. 2c,d).

### Timing of mite infestation on *V. tinus* twigs infested with *P. viburni* egg masses

The artificial infestation of *V. tinus* twigs by *P. viburni* adults in the field was highly successful. The 103 twigs collected for this experiment (five twigs went missing or were damaged in the field and were discarded from the analysis) contained a total of 741 egg masses ( $7.3 \pm 0.6$  egg masses per twig), ranging from 0 to 32 egg masses per twig. The percentage wounding response was noticeably higher than for the observational study:  $488 \times 100 / 741 = 65.9\%$ .



**Figure 2** Observational study 2016. (a) abundance and (b) diversity of mites found on *V. tinus* twigs depending on *P. viburni* infestation: twigs with no *P. viburni* egg masses, twigs with only damaged egg masses, and twigs with at least one intact egg mass (mean  $\pm$  SE). For each graph, means followed by a different letter are statistically different ( $\alpha = 0.05$ ); (c) correlation between mite abundance and number of intact egg masses on *V. tinus* twigs; (d) correlation between mite diversity (i.e. number of mite types observed) and number of intact egg masses on *V. tinus* twigs. Bubble size corresponds to the frequency of occurrences (i.e. multiple data points with the same coordinates appear larger).

**Table 3** Factors affecting the (a) abundance of mites and (b) diversity of mite morphotypes present on *Viburnum tinus* twigs infested with *Pyrrhalta viburni* egg masses (Generalized Linear Model, poisson distribution,  $\alpha = 0.05$ ) during an observational study (2016). Bold values indicate significant effects.

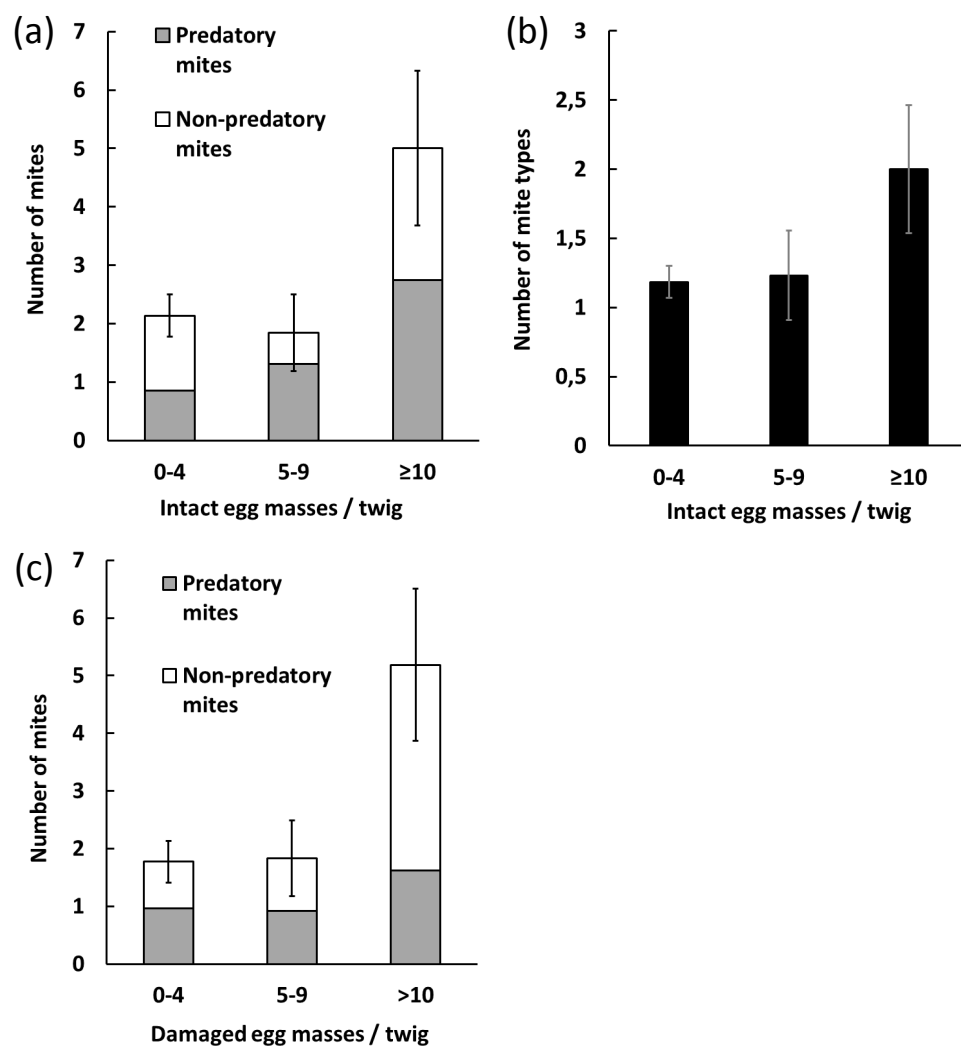
| (a) Mite abundance |                              | df    | $\chi^2$ -value | P-value            |
|--------------------|------------------------------|-------|-----------------|--------------------|
| Full model         |                              | 9, 90 | 49.77           | <b>&lt; 0.0001</b> |
| Effects tested     | Site                         | 4     | 6.03            | 0.2                |
|                    | Twig length (cm)             | 1     | 4.09            | <b>0.04</b>        |
|                    | Shrub infestation            | 2     | 0.91            | 0.63               |
|                    | Number of intact egg masses  | 1     | 16.22           | <b>&lt; 0.0001</b> |
|                    | Number of damaged egg masses | 1     | 0.37            | 0.54               |
| (b) Mite diversity |                              | df    | $\chi^2$ -value | P-value            |
| Full model         |                              | 9, 90 | 53.06           | <b>&lt; 0.0001</b> |
| Effects tested     | Site                         | 5     | 8.5             | 0.07               |
|                    | Twig length (cm)             | 1     | 0.77            | 0.38               |
|                    | Shrub infestation            | 2     | 8.6             | <b>0.01</b>        |
|                    | Number of intact egg masses  | 1     | 28.88           | <b>&lt; 0.0001</b> |
|                    | Number of damaged egg masses | 1     | 1.59            | 0.2                |

Several factors had a significant effect on the abundance and diversity of mites found on the collected twigs. First of all, the number of intact egg masses was found to affect the total abundance and diversity of mites, as well as the abundance of predaceous mites on the twigs (Table 4). These three variables were positively correlated with the number of intact egg masses, supporting the results of the observational study (Fig. 3a,b).

Time of collection (summer 2016, fall 2016, and spring 2017) was found to have an effect on total mite abundance: mites were more numerous on twigs collected in spring 2017 ( $3.3 \pm 0.7$ ) than in summer 2016 ( $1.8 \pm 2.9$ ) and in fall 2016 ( $1.5 \pm 0.3$ ) (Table 4). This effect was mostly driven by the abundance of phytophagous/detritivorous mites, which followed the same pattern and were more abundant in spring 2017 ( $2.2 \pm 0.4$ ) than in summer 2016 ( $0.6 \pm 0.5$ ) and in fall 2016 ( $0.6 \pm 0.5$ ) (Table 4). In addition, the number of damaged egg masses was found to affect total mite abundance and predaceous mite abundance (Table 4): these variables were positively correlated with the number of damaged egg masses (Fig. 3d). Finally, study site was found to have an effect on mite diversity (Table 4), indicating that richness of the mite fauna varied locally during the study.

### Impact of *T. trimaculatus* on overwintering success of *P. viburni* eggs

The infestation of *V. tinus* twigs by *P. viburni* inside cages in August 2017 was highly successful. A total number of 463 egg masses were laid on the 54 twigs selected for the study: the 26 twigs selected for the mite treatment contained in average  $9.4 \pm 1.0$  egg masses / twig, and the 28 twigs selected for the control treatment contained in average  $7.8 \pm 0.9$  egg masses / twig. On October 10 2017, five weeks after the addition of 20 *T. trimaculatus* inside the sleeve nets, the five twigs from the mite treatment that were cut and inspected for mite presence contained an average of  $7.6 \pm 3.4$  *T. trimaculatus* individuals, showing that some mites had successfully established inside the nets. At the end of the experiment in April 2018, both adult and immature nymphs of *T. trimaculatus* were found inside the sleeve nets, indicating that the mites had produced a new generation. Some of the mites were found on the netting material (anecdotal observation), but most of them were found on the twigs, inside the egg mass cavities. An average of  $5.3 \pm 1.2$  live *T. trimaculatus* individuals (adults + nymphs) per twig was found in twigs from the mite treatment, and an average of  $1.3 \pm 0.6$  individuals was found in twigs from



**Figure 3** Timing of mite infestation experiment 2017. (a) abundance and (b) diversity of mites found on *V. tinus* twigs depending on the number of intact *P. viburni* egg masses present on the twigs (mean  $\pm$  SE). Data are pooled in three categories: twigs with 0 to 4 intact egg masses ( $n = 81$ ), twigs with 5 to 9 intact egg masses ( $n = 13$ ), and twigs with  $\geq 10$  intact egg masses ( $n = 8$ ); (c) abundance of mites found on *V. tinus* twigs depending on the number of damaged *P. viburni* egg masses present on the twigs. Data are pooled in three categories: twigs with 0 to 4 damaged egg masses ( $n = 62$ ), twigs with 5 to 9 damaged egg masses ( $n = 24$ ), and twigs with  $\geq 10$  damaged egg masses ( $n = 16$ ).

the control treatment. This difference was highly significant ( $\chi^2 = 13.9$ ,  $P < 0.001$ ). Egg hatch of *P. viburni* occurred in the petri dishes between mid-March and early April 2018. The mean number of larvae that emerged per egg mass was  $2.8 \pm 0.3$  for twigs from the mite treatment ( $N = 21$ ) and  $2.9 \pm 0.5$  for twigs from the control treatment ( $N = 23$ ). Considering that there is an average of 8 eggs per egg mass (Weston *et al.* 2008), these numbers represent 35% and 36% of egg survivorship, respectively. The variables included in the model explained a significant amount of variation in larval emergence (full model:  $F_{10,31} = 3.1$ ,  $P < 0.01$ ,  $R^2 = 0.34$ ), but neither the presence of mites inside sleeve nets ( $F_{1,31} = 0.6$ ,  $P = 0.5$ ), the number of egg masses on twigs ( $F_{1,31} = 0.1$ ,  $P = 0.7$ ), nor the interaction between these two terms ( $F_{1,31} = 0.4$ ,  $P = 0.5$ ) had an effect on larval emergence per egg mass. In other words, the presence of mites did not impact *P. viburni* egg overwintering success. Shrub was the only factor that had a significant effect on larval emergence ( $F_{7,31} = 4.3$ ,  $P < 0.01$ ), indicating a plant-related effect on egg survivorship. This effect could have been twig wounding response, which was not measured

**Table 4** Factors affecting (a) total mite abundance, (b) phytophagous/detritivorous mite abundance, (c) predaceous mite abundance, and (d) diversity of mite morphotypes on *Viburnum tinus* twigs artificially infested with *Pyrrhalta viburni* egg masses (ANOVA,  $\alpha = 0.05$ ) during the timing of mite infestation experiment (2016-2017). Bold values indicate significant effects.

| (a) Total mite abundance                      |                              | df     | F-value | P-value            |
|-----------------------------------------------|------------------------------|--------|---------|--------------------|
|                                               |                              | 8, 93  | 5.84    | <b>&lt; 0.0001</b> |
| Effects tested                                | Time of collection           | 2      | 6.04    | <b>&lt; 0.01</b>   |
|                                               | Site                         | 3      | 1.73    | 0.16               |
|                                               | Twig length (cm)             | 1      | 0.62    | 0.43               |
|                                               | Number of intact egg masses  | 1      | 13.85   | <b>&lt; 0.001</b>  |
|                                               | Number of damaged egg masses | 1      | 6.73    | <b>0.01</b>        |
| (b) Phytophagous/Detritivorous mite abundance |                              | df     | F-value | P-value            |
|                                               |                              | 8, 93  | 3.52    | <b>&lt; 0.01</b>   |
| Effects tested                                | Time of collection           | 2      | 5.53    | <b>&lt; 0.01</b>   |
|                                               | Site                         | 3      | 1.88    | 0.14               |
|                                               | Twig length (cm)             | 1      | 0.2     | 0.65               |
|                                               | Number of intact egg masses  | 1      | 3.39    | 0.07               |
|                                               | Number of damaged egg masses | 1      | 1.19    | 0.27               |
| (c) Predaceous mite abundance                 |                              | df     | F-value | P-value            |
|                                               |                              | 10, 89 | 3.38    | <b>&lt; 0.01</b>   |
| Effects tested                                | Time of collection           | 2      | 0.61    | 0.54               |
|                                               | Site                         | 3      | 1.74    | 0.16               |
|                                               | Twig length (cm)             | 1      | 0.58    | 0.44               |
|                                               | Number of intact egg masses  | 1      | 15.54   | <b>&lt; 0.001</b>  |
|                                               | Number of damaged egg masses | 1      | 8.52    | <b>&lt; 0.01</b>   |
| (d) Total mite diversity                      |                              | df     | F-value | P-value            |
|                                               |                              | 10, 89 | 3.93    | <b>&lt; 0.001</b>  |
| Effects tested                                | Time of collection           | 2      | 1.04    | 0.36               |
|                                               | Site                         | 3      | 3.27    | <b>0.02</b>        |
|                                               | Twig length (cm)             | 1      | 0.52    | 0.47               |
|                                               | Number of intact egg masses  | 1      | 13.07   | <b>&lt; 0.001</b>  |
|                                               | Number of damaged egg masses | 1      | 2.98    | 0.09               |

during this experiment. These results remained consistent after excluding the twigs from the control treatment that contained live mites at the end of the experiment (7 twigs out of 23) and twigs from the mite treatment that did not contain any live mites at the end of the experiment (3 twigs out of 21).

## Discussion

Our results show that a diverse fauna of detritivorous, phytophagous, and predaceous mites is associated with *P. viburni* egg masses on *V. tinus* in southern France. Predatory mites were observed running both onto the stems and within *P. viburni* egg masses, but detritivorous mites were more commonly observed within *P. viburni* egg masses, sometimes deeply encased within the egg mass cavity. Results of the observational study showed that both mite abundance and mite diversity were positively correlated with the number of intact egg masses present per twig, but were not affected by the damaged egg masses present per twig (Table 3, Fig. 2). These results probably illustrate that mites find shelter within the cavity of intact *P. viburni* egg masses, where detritivorous species probably sustain on wood debris. *Micreremus brevipes*

Michael, one of the oribatid mites found in this study, is known to seek shelter in old coccid scales, possibly as a protection against desiccation and predators while they are molting and ovipositing (Wunderle 1992). Similarly, intact egg masses may also provide these mites protection from desiccation and their natural enemies. Egg masses damaged by the wounding response of the plant, on the other hand, seem less valuable for mites. However, we found a positive correlation between the number of damaged egg masses and the abundance of predaceous mites during the timing of mite infestation study (Table 4), suggesting that damaged egg masses may still be valuable for predaceous mites. Results of the timing of infestation experiment did not reveal a regular increase in mite numbers and diversity as more time has passed since *P. viburni* oviposition, but detritivorous mites were more abundant nine months after oviposition than one or four months after oviposition, suggesting that populations of these mites may build up within *P. viburni* egg masses. This idea found further support in the results of the experiment on the impact of *T. trimaculatus* on egg survivorship, which showed that a new generation of *T. trimaculatus* developed in the sleeve nets over the duration of the experiment (August 2017 – April 2018). Overall, results of our study show that *P. viburni* egg masses form a microhabitat favorable to the establishment of several mite species and document a previously unreported occurrence of insect-mite interactions.

Despite the diverse mite fauna discovered roaming within *P. viburni* egg masses, none of the species observed seem likely to pose a direct threat to *P. viburni* eggs, due to their small size and/or lack of mouthparts strong enough to pierce through the egg cuticle. The biggest predatory mite observed, *Anystis baccarum* (L.), is known to be able to prey upon small soft bodied insects such as aphids (Cuthbertson *et al.* 2003), but was very uncommon on *V. tinus* twigs compared to the very common and much smaller species from the family Phytoseiidae. These Phytoseiidae species, which are all endemic to Europe and are known to be generalist predators (Demite *et al.* 2014), are unlikely to pose a predation risk on *P. viburni* eggs due to their small size.

The possibility that the presence of mites indirectly impact *P. viburni* egg survivorship through feeding on wood debris and/or the protective “egg cap”, making the eggs more exposed to desiccation risks during the overwintering period, was tested with the mite *T. trimaculatus*, which was the most commonly found mite occurring within *P. viburni* egg masses. Results showed no negative impact of mite presence on *P. viburni* egg overwintering success. A few twigs from the mite treatment were found empty of mites at the end of the experiment, indicating that mites failed to establish on these twigs. In contrast, live mites were found on several twigs from the control treatment, suggesting that mites managed to infest these twigs despite the protection provided by the sleeve nets or that they were already present on the twigs at the moment of installing the sleeve nets but went unnoticed. The main result of the experiment (no impact of mites on egg survivorship) remained robust after excluding these twigs. Therefore, it seems likely that associations between *P. viburni* egg masses and mites on *V. tinus* are cases of commensalism where mites benefit from food and shelter without causing noticeable harm to *P. viburni* egg masses. However, another potential impact of mites on *P. viburni* fitness that was not explored in this study is that mites may attract predators able to feed on *P. viburni* eggs (Wilson 2005; Jaloszynski and Olszanowski 2015), or may make the eggs more exposed to parasitoids (Desurmont 2009).

Associations between mites and *P. viburni* egg masses are highly unlikely to be obligate. The mite species observed within egg masses are not habitat specialists and are commonly found on different kinds of trees as well as in other habitats. It is thus very likely that they exploit *P. viburni* egg masses opportunistically but do not depend on them to survive. However, they may still have been selected to actively search for egg masses on *Viburnum* shrubs if the benefits they derive from inhabiting egg masses have remained significant and stable over evolutionary time. Different parts of the *P. viburni* egg mass (e.g. egg cap, wounding tissue) have been found to be attractive cues to *P. viburni* females and to play a role in the behavior of aggregative oviposition (Desurmont and Weston 2010). Investigating how the mites found in our study respond to these cues may give some insight into the strength of their association

with *P. viburni*. Highly specialized parasitic or predaceous mites have sometimes been found to be attracted to host-specific chemical cues (Jalil and Rodriguez 1970; Sabelis and Van de Baan 1983).

What are the ecological consequences of *P. viburni* infestation for plant-insect-mite interactions on *V. tinus*? Interestingly, the genus *Viburnum* is well known for its rich and complex plant-mite interactions (Parolin *et al.* 2011; Weber *et al.* 2012). The leaves of *V. tinus* possess domatia, which consist of small tufts usually found at vein junctions on the undersides of leaves. The main function of leaf domatia is thought to provide protection for predatory or fungivorous mites and their eggs, which in turn help reduce the damage caused by phytophagous mites or plant pathogens (Romero and Benson 2005; Weber *et al.* 2016). Several of the phytophagous (e.g. *Tetranychus* sp.) and predatory (e.g. *Amblyseius* sp.) mite genera observed in our study have been previously documented in *V. tinus* domatia (Grostal and O'Dowd 1994; Parolin *et al.* 2011). Manipulative experiments have shown that *V. tinus* leaves whose domatia were removed suffered higher infestation and oviposition by phytophagous mites (Grostal and O'Dowd 1994). Thus, *V. tinus* and predaceous mites are often cited as a classic example of indirect defense involving tritrophic interactions between plants, herbivores, and natural enemies (Heil 2008). Infestation by *P. viburni* may add a layer of complexity to these interactions. By providing a shelter to phytophagous mites but also to predaceous mites, *P. viburni* egg masses may directly impact mite-mite interactions on *V. tinus*. In addition, *P. viburni* is known to impact the defenses of its host plants on the long term: extensive defoliations by larvae and adults have been shown to cause partial shrub dieback, which makes infested shrubs more vulnerable to *P. viburni* oviposition the following year, creating a self-sustaining loop of infestation (Desurmont and Agrawal 2014). Shrub dieback can also be expected to favor populations of mites by providing plenty of decaying wood material and potential refuges. In our study, shrubs that were the most heavily infested by *P. viburni* also carried the most diverse fauna of mites, providing support for this hypothesis. Finally, *P. viburni* defoliations often cause infested shrubs to produce a second flush of new leaves. In the case of *V. tinus*, an evergreen shrub, it would be interesting to investigate how leaf damage and the production of young fresh leaves affect leaf domatia, whose structures and abundance can be dependent on leaf age and plant ontogeny (Boege and Marquis 2005), and the mite-mite interactions associated with these domatia.

The viburnum leaf beetle is an invasive pest in North America, and its present and future impact on North American forest ecosystems should not be underestimated (Weston *et al.* 2007; Smith and Hatch 2017). Finding possible biological control agents against this pest is a critical challenge and complementary manipulative experiments should be conducted to determine whether or not mites from other parts of *P. viburni* native range may pose a direct or indirect threat to *P. viburni* egg survivorship. The native range of *P. viburni* is vast, and this insect has been hypothesized to originate from Southeast Asia, an area that is a center of diversity for the genus *Viburnum* and for other *Viburnum*-feeding *Pyrrhalta* species (Desurmont 2009): this area may therefore be home for more specialized mite-*P. viburni* interactions.

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