Seasonal decline in plant defence is associated with relaxed offensive oviposition behaviour in the viburnum leaf beetle *Pyrrhalta viburni*

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Abstract. 1. Plant defence of *Viburnum* shrubs against oviposition by its specialist herbivore, the viburnum leaf beetle [VLB *Pyrrhalta viburni*(Paykull)], involves an egg-crushing wound response in twigs. Although the response is variable among *Viburnum* species, it can have a strong impact on egg survivorship. Beetles typically aggregate egg masses with conspecifics along infested twigs, forming clusters that can overwhelm the twig response. It was investigated whether twig responses and beetle oviposition behaviour vary seasonally.

2. In a field experiment, twig defences decreased towards the end of the VLB oviposition period: wound response of the North American *Viburnum dentatum* L. and the European *V. opulus* L. was reduced by 100% and 54%, respectively, in September compared with the July to August period.

3. Oviposition trials demonstrated a corresponding behavioural change: VLB females displayed aggregative oviposition in August, but not in September.

4. Further tests revealed that late-season VLB females reverted to aggregative oviposition after being kept on uninfested twigs, whereas females kept on heavily infested twigs did not. This behavioural change suggests that relaxation of aggregative oviposition originates from cues associated with high densities of egg masses.

5. Relaxation of aggregative oviposition may be adaptive (and beneficial for invasion) on shrubs with low levels of defences by reducing intra-specific competition.

Key words. Egg crushing twig defence, herbivore offense, insect–plant interactions, invasion biology, oviposition strategy, wound response.

Introduction

Plant defences are potent agents of natural selection on insect herbivores. As such, very few insects, even among dietary specialists, are completely immune to the defences of their host plants (Ali & Agrawal, 2012). If all other factors are equal (e.g. natural enemies and abiotic conditions), herbivores are expected to preferentially exploit less defended food sources. Examples include insects that prefer plant genotypes or species with low direct defences (Pöykkö *et al.*, 2005; Macel, 2011), exploitation of plant parts with low secondary metabolites (Strauss *et al.*, 2004), and preference for sites whose abiotic conditions do

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not allow plants to express optimal defence (Lavola *et al.*, 1998). While these examples mainly deal with plant variability in constitutive defence, induced responses to herbivory can also affect herbivore preferences (Karban, 2011). Finally, plant defences vary in time, and can be driven by plant ontogeny, owing to seasonal changes, or even diurnal cycles (van Asch & Visser, 2007; Barton & Koricheva, 2010; Goodspeed *et al.*, 2012).

Paul Feeny's classic study (1970) on chemical defences in British oak leaves formulated the hypothesis for how seasonal changes in plant defence can drive herbivore adaptation. He demonstrated that young oak leaves contain less tannins, which are responsible for leaf toughness and reduced digestibility, and hypothesised that lepidopteran herbivores adapted by preferentially exploiting leaves during bud-burst. Additional work

on North American oaks confirmed that specialist lepidopteran communities are negatively impacted by seasonal changes associated with tannins (Forkner *et al.*, 2004). In spite of the many cases of insect phenology being constrained by plant phenology (Kause *et al.*, 2001; van Asch & Visser, 2007), it is essentially unknown to what extent herbivores experiencing temporal changes in the defences of their host plants can adjust their 'offense' tactics accordingly.

We have been studying the Viburnum leaf beetle [VLB, *Pyrrhalta viburni* (Paykull)], a specialist chrysomelid native to Eurasia and invasive in North America where it was first detected in the late 1920s (Majka & Lesage, 2007; Weston *et al.*, 2007). We recently described the oviposition behaviour of VLB and its associations with plant defences. Eggs are laid in round cavities chewed within the pith of young *Viburnum* twigs and covered with a protective 'egg cap' (Fig. 1a,b); in response, twigs can produce undifferentiated wound tissue that physically crushes or expels the eggs during the weeks after oviposition (Desurmont & Weston, 2011) (Fig. 1c). This defence is expressed in a wide range of *Viburnum* species, but is significantly stronger in Eurasian species sharing an evolutionary history with VLB compared with evolutionarily naïve North American species (Desurmont *et al.*, 2011). To overcome the defensive twig response, VLB females aggregate their egg masses with conspecifics, forming clusters that typically kill the twig, preventing production of wound tissue and enhancing egg survivorship in the field (Desurmont & Weston, 2011).

Because the twig response takes several weeks and may be associated with sap flow dynamics within twigs (Desurmont, 2009), it is possible that the potency of twig responses as a defence mechanism decreases towards the end of the growing season. In this study, we examined the twig defence expressed by two susceptible *Viburnum* species, North American *Viburnum dentatum* L. and European *V. opulus* L., during the entire oviposition period of VLB (July–September). In parallel, oviposition choice tests were conducted to determine whether VLB oviposition behaviour varied (aggregative or not aggregative) across the growing season. Because we found a relaxation of VLB aggregative oviposition associated with declining twig defence, and because late-season females originated from shrubs with high densities of egg masses built up over the oviposition period, we additionally investigated the importance of cues associated with high levels of infestation for oviposition.

Material and methods

Plant and insect material

The viburnum leaf beetle is a univoltine insect that overwinters as eggs. Larvae develop in the spring, skeletonising young expanding leaves of *Viburnum shrubs*. They can develop on a wide range of *Viburnum* species, although several species are suboptimal or unsuitable for their development (Desurmont *et al.*, 2011). Adults, which are also folivores, typically emerge in June (from pupae in the soil) and are typically present in the field during the summer months. Females lay eggs during their entire lifetime (Weston *et al.*, 2008). The two *Viburnum* species included in this study, *V. dentatum* and *V. opulus*, are deciduous and leaf senescence typically occurs in November for these two species.

All *Viburnum* shrubs and VLB adults used for the field experiment came from an unmanaged common garden in Ithaca, New York (U.S.A.). Shrubs were 8–10 years old and breeding populations of VLB had been present at the site since 2001. Insects and plant material used for the oviposition choice tests came from a second site located in Ithaca containing only umanaged *V. dentatum* shrubs.

Seasonal variation in twig response to VLB oviposition

In order to document the production of defensive wound tissue over the period of VLB oviposition, randomly selected non-infested terminal twigs of *V. dentatum* and *V. opulus* shrubs were bagged with 20 VLB females and five males in fine-mesh bags for a period of 2 weeks. The number of egg masses laid on the twig was then recorded and the beetles removed from the bags. The bags were sealed again to prevent further infestation by naturally occurring VLB females, and the wound response of each twig was calculated at the end of the growing season (November) as the number of egg masses encased in wound tissue divided by the total number of egg masses laid on the twig. Hereafter we use this number $\times\,100$ as per cent wound response. We previously showed that encased or expelled egg masses almost never survive (Desurmont & Weston, 2011). This experiment was repeated three times over the VLB ovipostion period: in July, August, and September (7–13 twigs used per species and per month). We assessed the effects of infestation period (i.e. July, August, and September) and egg masses per twig on the per cent wound response using ANCOVA (JMP v9). Interactions between these two predictors were tested and removed from the final model if not significant. Per cent wound response data were arcsine transformed, which improved the normality of the residuals.

Oviposition choice-tests

To test whether females actively aggregate their egg masses, a choice test was designed as follows: two mated *P. viburni* females were placed in a plastic cylindrical container $(21 \times 9.5\,cm^2)$ with a screen lid containing two *V. dentatum* twigs uniform in length (12 cm) and diameter (*c*. 2.5 mm), and bearing a pair of leaves as a food source. One twig was uninfested, and the other was infested with 1–4 egg masses laid by conspecifics in the laboratory 24 h preceding the choice trial. The number of egg masses on both twigs was recorded after 48 h. This experiment was repeated in August and September ($n = 10$ paired replicates at each date). Twigs were collected in the field a few days before the trials and kept at 17 °C in floral water picks until used. Differences in numbers of egg masses laid on uninfested and infested twigs after 48 h were analysed with a two-tailed paired *t*-test (JMP v9). Additionally, we performed effect size calculation for the results of the choice-tests: *d* statistics were calculated using equations for dependent groups (Dunlap *et al.*, 1996). Values > 0.2 indicate a
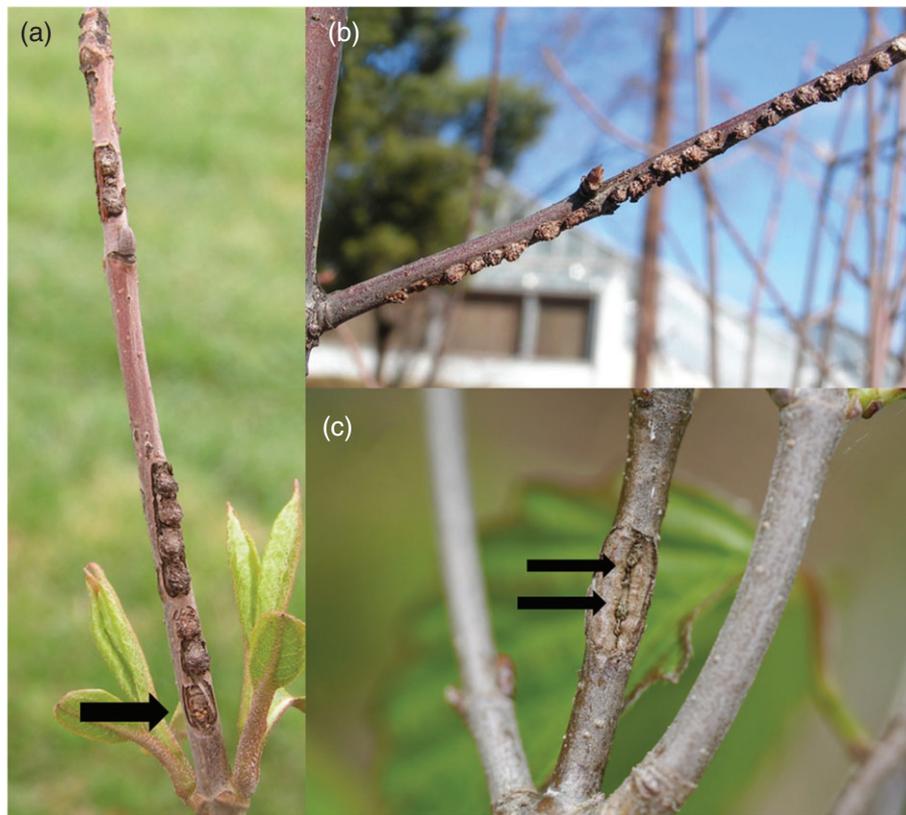
Fig. 1. (a) Cluster of viburnum leaf beetle egg masses along a *Viburnum lentago* twig. The distal portion (i.e. above the egg masses) of the twig is dead. The protective egg cap of the lowest egg mass was manually removed, revealing the eggs (indicated by the black arrow). (b) High density of egg masses reached in September on a *V. dentatum* twig. (c) *Viburnum dentatum* showing a typical defensive twig response several weeks after oviposition. Undifferentiated tissue grows and crushes or expels the eggs, especially when egg mass density is low. Fibrous remnants of the protective cap over the egg masses can be seen in the centre of the slit (indicated by the black arrows).

small effect, values > 0.5 indicate a medium effect, and values > 0.8 indicate a large effect (Nakagawa & Cuthill, 2007).

To test whether aggregative oviposition was dependent on exposure to high densities of conspecific egg masses and could be reverted in their absence, 30 females were collected in the field in September and kept separated in individual plastic cylindrical containers. Half of the females were provided a twig already infested with >20 egg masses ('saturation' treatment), whereas the other half received a twig free of egg masses ('isolation' treatment). Twigs were changed in each container every 2 days for a period of 10 days. Twigs used for the two treatments originated from the same site, but not necessarily from the same shrubs: shrubs with highly infested twigs rarely held twigs free of egg masses, and vice versa. After this period, females were individually tested in the choice assay described above ($n = 15$ for each treatment).

Results

For both *Viburnum* species tested, infestation period had a significant effect on twig defensive wound response. For *V. opulus*, the twig response was highest in July, intermediate in August, and lowest in September ($F_{2,26} = 11.1$, $P < 0.001$, Fig. 2). For

V. dentatum, the twig response was substantially lower than in *V. opulus*, and a similar pattern of seasonal decrease in responsiveness was observed: it was high in July and August, but was entirely absent in September ($F_{2,21} = 7.50$, $P < 0.005$, Fig. 2). The number of egg masses per twig did not correlate with the extent of twig response for either species ($F_{1,26} = 1.17$, $P = 0.2$ and $F_{1,21} = 3.44$, $P = 0.08$ for *V. opulus* and *V. dentatum*, respectively). There was no interaction between infestation period and egg masses ($F_{2,26} = 1.95$, $P = 0.16$ and $F_{2,21} = 1.14$, $P = 0.34$ for *V. opulus* and *V. dentatum*, respectively).

In August, VLB females showed an aggregative oviposition behaviour and laid three-fold more eggs on twigs previously infested by conspecifics than on non-infested twigs (paired $t = 3.7$, $P = 0.005$, $d = 1.95$), but this preference was not found in September (paired $t = 1.47$, $P = 0.17$, $d = -0.85$) (Fig. 3a,b). Females collected in September and subjected to the 'saturation' treatment again showed a lack of preference (paired $t = 0.14$, $P = 0.88$, $d = -0.05$), but females subjected to the 'isolation' treatment, and thus only provided with non-infested twigs for 10 days, tended to revert to aggregative oviposition and laid nearly three-fold more eggs on twigs infested by conspecifics than on non-infested twigs (paired $t = 1.92$, $P = 0.07$, $d = 0.77$) (Fig. 3c,d).
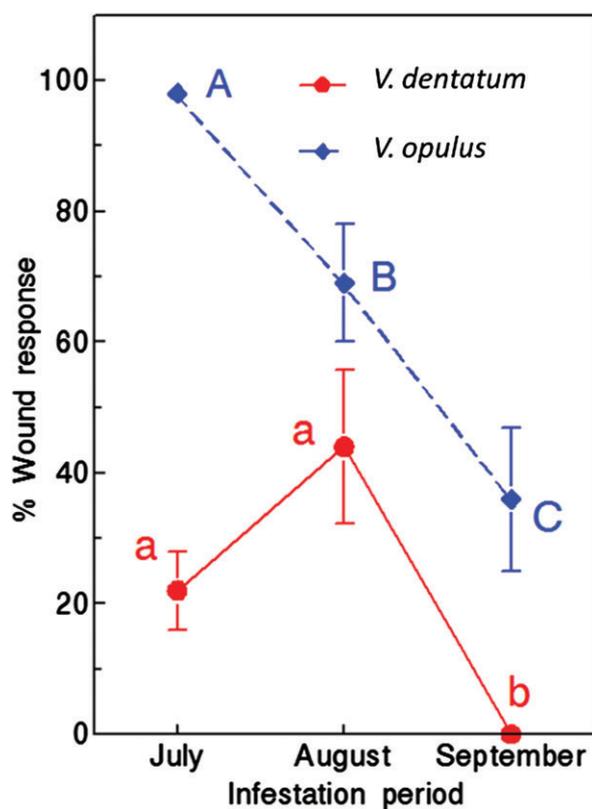
Fig. 2. Per cent wound response of *Viburnum dentatum* (North American species) and *V. opulus* (European species) twigs in response to viburnum leaf beetle oviposition, during the July–September period. For each species, means followed by a different letter are statistically different ($P < 0.05$, ANCOVA, Tukey's all-pairwise comparisons procedure, JMP9). Each data point represents the mean ($\pm$SE) for a sample of 7–13 twigs.

Fig. 3. Number of egg masses laid by viburnum leaf beetle (VLB) females on *Viburnum dentatum* twigs infested by conspecifics and non-infested twigs in laboratory choice tests in (a) August, (b) September, and after (c) isolation (i.e. exposure to non-infested twigs) or (d) saturation (i.e. exposure to heavily infested twigs) treatments for 10 days. *P*-values from the paired *t*-test ($\alpha = 0.05$) and *d* statistics are shown.

Discussion

Because constitutive and induced plant defences are variable in time through ontogenetic, seasonal, and even diurnal changes (van Asch & Visser, 2007; Barton & Koricheva, 2010; Goodspeed *et al.*, 2012), insect herbivores may benefit from adjusting their strategies to avoid or aggressively overcome these defences. Our results show that there is a decrease in the defensive ability of *Viburnum* twigs towards the end of growing season, which also corresponds to the end of the VLB oviposition period: in both *V. dentatum* and *V. opulus*, the twig wound response was drastically lower in September compared with July to August. These results are consistent with the body of literature showing ontogenetic variation in the defences of woody plants (Boege, 2005; Barton & Koricheva, 2010). Interestingly, twig defence was consistently higher, independently of the seasonal period, in *V. opulus* (a species that shares an evolutionary history with VLB) versus *V. dentatum* (a North American species naïve to VLB), confirming our previous results (Desurmont *et al.*, 2011). Because the wound response takes several weeks to be complete, it is possible that *Viburnum* shrubs lack the time necessary to produce wound tissue in response to oviposition by
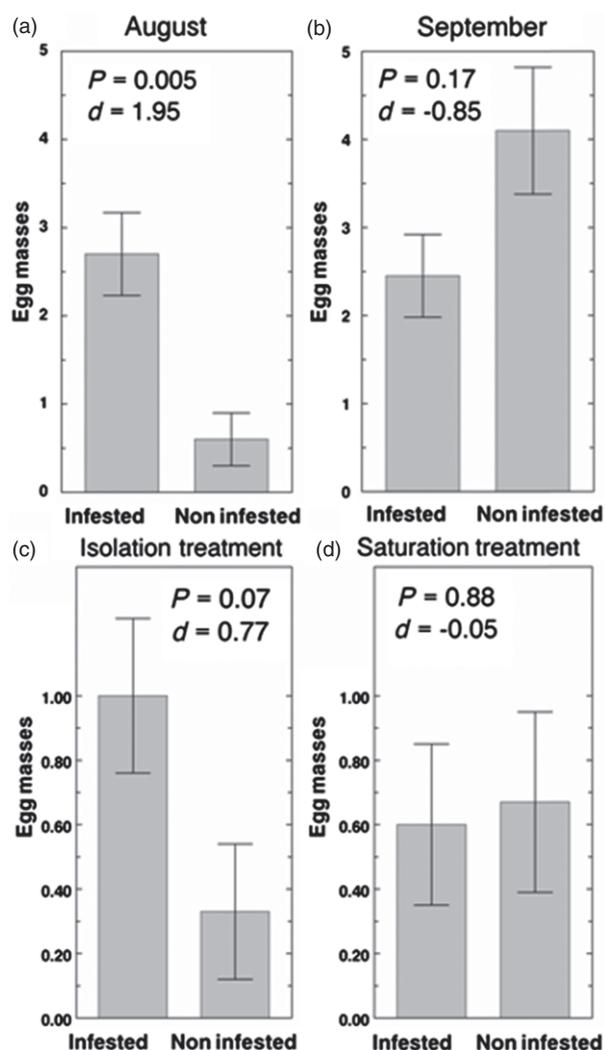
late season females before leaf senescence. Further research is needed to investigate the consistency of wound response dynamics over the growing season, and to examine the possible effect of different environmental factors (e.g. shrub age and growing conditions) on this seasonal variation.

The relaxation of aggregative oviposition at the end of the season that we observed (Fig. 3a,b) may have an adaptive value if this behaviour is costly in the absence of plant defence. It has been suggested that a potential cost of aggregative oviposition is increased intra-specific competition among larvae (Desurmont & Weston, 2011). By not aggregating at a time when plant defences are low, VLB females may minimise competition. Our choice tests with late-season females kept in the presence or absence of heavily infested twigs ('saturation' vs. 'isolation' treatment) suggest that the seasonal change in VLB oviposition

behaviour is not genetically fixed but dependent on environmental cues associated with highly-infested twigs: late-season females reverted to aggregative oviposition after 10 days in the absence of infested twigs (Fig. 3c,d). The environmental cue could simply be the egg masses themselves. Previous research showed that early-season VLB females prefer heavily infested twigs to lightly infested twigs (Desurmont & Weston, 2010), but it is conceivable that prolonged exposure to heavily-infested twigs may attenuate the value of conspecifics' egg masses as an oviposition stimulus. Learning through habituation to certain stimuli is common in phytophagous insects and is in some cases reversible (Papaj & Prokopy, 1989). In particular, it has been documented for a number of lepidopteran larvae exposed to feeding deterrents (Huang & Renwick, 1995). Alternatively, cues related to twig response to egg masses may play a role in the VLB behavioural change. For example, VLB females prefer to lay eggs on twigs that previously produced wound tissue, as such twigs are less defended against subsequent oviposition (Desurmont & Weston, 2010). Because the twigs provided to the females during the 'isolation' and 'saturation' treatments came from shrubs with different levels of infestation (i.e. uninfested twigs came mainly from shrubs lightly infested and heavily infested twigs came from heavily infested shrubs), it is possible that these shrubs varied in their overall expression of defence. Thus, females could have sensed cues from the twigs indicative of their defensive capacity (or overall plant quality), and adjusted their oviposition strategy accordingly. However, this speculation about twig-related cues remains to be explored.

For VLB, prolonged exposure to high densities of conspecifics' egg masses may occur towards the end of the oviposition period (as illustrated by our study), but also during population outbreaks, which are common in its invasive range (Weston *et al.*, 2007): egg masses can accumulate quickly on *Viburnum* twigs when adult densities are high early summer (G. A. Desurmont, pers. obs.). It is possible that high levels of damage or high numbers of egg masses may decrease the overall defensive capacity of infested shrubs (Desurmont & Agrawal, 2014). Relaxation of aggregative oviposition on weakened shrubs during outbreaks may thus potentially have the same adaptive value (reduced competition) as on late season shrubs. Finally, it is worth mentioning that late-season VLB populations are relatively rare in northeastern United States, where adult densities usually drop significantly in August for reasons not well elucidated (G. A. Desurmont, pers. obs.), but commonly occur in western Europe (Balachowsky, 1962), within the native range of VLB.

The interactions between *Viburnum* plant defences and VLB behaviour have often been compared with bark beetles (Coleoptera: Curculionidae: Scolytinae) targeting live trees, which actively aggregate to overcome the defences of their host trees and successfully colonise them (Raffa *et al.*, 1993). Interestingly, behavioural changes for response to high densities of conspecifics have also been documented for those systems. It is known that adult bark beetles can switch from the production of aggregative pheromones to the production of anti-aggregation pheromones once a certain density of adults has been reached on the colonised host. Indeed, the production of anti-aggregation pheromones is thought to have evolved in bark beetles to limit

intra-specific competition (Blomquist *et al.*, 2010). Similarities in herbivore offense between bark beetles and VLB may thus go beyond comparable modalities of attack and colonisation of hosts, and also encompass behavioural adaptations in response to changes in environmental conditions that alter plant defence.

In summary, our study shows that a specialised insect herbivore can modulate its offense strategy to follow the seasonal changes in the defences of its host plant. These results complement previous research showing that VLB oviposition is a highly plastic behaviour, depending on several factors and environmental conditions including: twig and leaf traits (Weston *et al.*, 2008; Desurmont & Weston, 2011; Desurmont *et al.*, 2011), presence and quality of conspecifics (Desurmont & Weston, 2010), and presence of dead twigs on the infested shrub (Desurmont *et al.*, 2009; Desurmont & Agrawal, 2014). Adaptive plasticity certainly played a role in shaping the interactions between VLB and its host plants in its native range: previous research showed that VLB populations coexisting with different Eurasian *Viburnum* species have adapted their oviposition strategy (aggregative vs. non-aggregative) in accordance with the defensive capacity of their host plant, and are able to adjust their strategy when transferred on a different host plant (Desurmont *et al.*, 2012). In its invasive range, owing to the generally lower defences of evolutionarily naive North American *Viburnum* species (Desurmont *et al.*, 2011), VLB's offensive arsenal is even more efficient than in its native range, which probably contributed to the success of its invasion. Phenotypic plasticity and behavioural flexibility are thought to have played a role for other invasive species (Engel & Tollrian, 2009; Wilson *et al.*, 2009), and should not be underestimated in dynamic offense–defence interactions between plants and herbivores.

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