

Research article

Variation in the response of *Mimulus guttatus* (Scrophulariaceae) to herbivore and virus attack

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Received 26 April 2004; Accepted 30 September 2004

Co-ordinating editor: Steufer

Abstract. Plants are often simultaneously attacked by insect herbivores and plant pathogens, yet relatively few studies have investigated the potential interactive effects of herbivores and pathogens on plant fitness. We studied the effects of simultaneous attack by meadow spittlebugs, *Philaenus spumarius* (Homoptera: Cercopidae), and a plant virus, cucumber mosaic virus (CMV), on the fitness of *Mimulus guttatus* (Scrophulariaceae). We wanted to determine: (1) if trade-offs in defense against meadow spittlebugs and CMV exist, (2) if meadow spittlebugs and CMV interact to affect plant fitness, and (3) if genetic variation is associated with these interactive effects. We found no evidence of trade-offs in defense against meadow spittlebugs and CMV in *M. guttatus* in a greenhouse experiment. Thus, the ability of *M. guttatus* to defend itself against one of these enemies is unlikely to preclude the evolution of adequate defenses against the second enemy. We did, however, find strong evidence that spittlebugs and CMV interacted to affect plant fitness and that genetic variation underlies the degree and direction of this interaction. This suggests that selection can act on the genetic variation underlying the interaction between the two enemies and that strong selection imposed by one will alter the response of *M. guttatus* populations to the second. To our knowledge this is the first study to demonstrate genetic variation associated with the non-additive effect of an herbivore and a pathogen on plant fitness. We suggest that future studies of the mechanisms underlying the defensive properties of plants need to consider variation associated with defense mechanisms and the potential effect of this variation on the response of plant populations to selection by multiple enemies.

Key words: cucumber mosaic virus, genetic variation, herbivory tolerance, *Philaenus spumarius*, virus tolerance

Introduction

An incredibly large number of parasitic organisms attack plants including viruses, bacteria, fungi, other plants, and invertebrate and vertebrate animals (Fritz and Simms, 1992; Karban and Baldwin, 1997; Gilbert, 2002). Many of these organisms simultaneously attack plants and coordinating defenses to counter this onslaught may represent major physiological, ecological, and evolutionary challenges. Although simultaneous attack by parasitic organisms

is likely to be a universal feature of plant ecology, until recently surprisingly few studies have explored the challenges faced by plants when attacked by multiple enemies. Recent work on the potential for ‘cross talk’ or interference among signaling pathways associated with induced resistance (IR) to insects and systemic acquired resistance (SAR) to pathogens has produced exciting results and has generated widespread interest in consequences of simultaneous attack by diverse enemies (e.g., Doherty *et al.*, 1988; Maleck and Dietrich, 1999; Felton and Korth, 2000; Bostock *et al.*, 2001; Dicke and Hilker, 2003; Rostás *et al.*, 2003). In field grown tomato plants, for example, stimulation of SAR interferes with the jasmonate-induced expression of anti-herbivore enzymes and, consequently, host-plant resistance to caterpillars is compromised (Thaler *et al.*, 1999).

Most studies to date, however, have taken a relatively mechanistic approach to this problem (Roda and Baldwin, 2003) and have focused on one variety or genotype of agricultural crops (Dicke and Hilker, 2003). Consequently, we know very little about ecological and genetic variation associated with the response of plants to simultaneous attack by multiple parasites. This is especially true for natural systems, even though such variation is likely to be widespread in nature. In ribwort plantain (*Plantago lanceolata*), for example, fungal pathogens can induce the production of iridoid glycosides that deter attack by generalist insect herbivores. This effect varies, however, among *P. lanceolata* genotypes. Fungal pathogens induced iridoid glycoside production in 67% of genotypes and tissue-specific induction of these anti-herbivore compounds varied among these genotypes (Marak *et al.*, 2002).

The goal of this study was to explore the evolutionary ecology of plant defense of the yellow monkeyflower, *Mimulus guttatus* (Scrophulariaceae), against two diverse types of enemies: an insect herbivore and a plant virus. Our previous work with this system showed that spittlebugs, *Philaenus spumarius* (Homoptera: Cercopidae) (Carr and Eubanks, 2002) and cucumber mosaic virus (CMV) (Carr *et al.*, 2003) independently reduce biomass and flower production of *M. guttatus* plants. There were, however, differences among *M. guttatus* families in defense against spittlebugs and virus. In some families herbivory and infection strongly reduced plant fitness, but in other families herbivory and infection only weakly influenced fitness. Furthermore, the pattern of variation within *M. guttatus* populations suggested there might be trade-offs in defense such that some genotypes are able to resist or tolerate spittlebug herbivory, but not CMV infection and vice-versa. Our specific objectives were to: (1) determine if spittlebugs and CMV interact to affect plant fitness, (2) determine if genetic variation is associated with these interactive effects, and (3) determine if trade-offs in spittlebug and CMV defense exist.

Materials and methods

Study species

Mimulus guttatus ranges throughout western North America from Mexico to Alaska, occupying a variety of moist, open habitats. *M. guttatus* plants can be either perennial or annual depending upon soil moisture. Field-collected *M. guttatus* seed for this experiment came from two annual populations, one (M5) in Santa Clara County, CA (37°17'N, 122°09'W) and the other (M13) in Napa County, CA (38°33'N, 122°22'W) (Carr and Eubanks, 2002).

Meadow spittlebugs are perhaps the most polyphagous of all insect herbivores with over 500 host plant species recorded (Weaver and King, 1954; Hamilton, 1982; Owen, 1988). Meadow spittlebugs are univoltine. Females lay their eggs on plant tissue or in the soil in the fall, eggs overwinter, and first instar nymphs hatch in the spring. In coastal California, meadow spittlebugs are the most abundant herbivore on *M. guttatus*. *M. guttatus* has been used as a model system for studying the evolution of plant mating systems for over a century (e.g., Darwin, 1876), yet very little is known about its field ecology and interactions with other species and that was our motivation for studying this species. Meadow spittlebugs initially attack during the rosette stage in early spring, nymphs develop on the plants throughout spring and into early summer, and infested plants typically host 1–2 spittlebugs (Carr and Eubanks, unpublished data). Once a feeding site is established, nymphs feed on xylem with their straw-like mouthparts and generate the spittle for which they are named (Severin, 1950; Wiegert, 1964; Horsfield, 1978). The spittle mass covers their bodies and primarily protects nymphs from desiccation (Weaver and King, 1954; McEvoy, 1986). Nymphs rarely move to a new plant once they settle and usually complete development (five nymphal instars) on a single plant. In a previous experiment we found that herbivory by a single *P. spumarius* nymph can reduce the biomass and flower production of *M. guttatus* plants by 10–30% (Carr and Eubanks, 2002). There is little information on naturally occurring viruses in *M. guttatus*. CMV was chosen for this study because it is ubiquitous in nature, with one of the broadest host ranges among viruses that infect plants (over 1000 plant species) (Tomlinson *et al.*, 1970; Palukaitis *et al.*, 1992). We have shown in previous experiments that *M. guttatus* is fully susceptible to CMV infection (Carr *et al.*, 2003), that CMV infection reduces biomass and flower production by as much as 15% (Carr *et al.*, 2003), and that there is genetic variation associated with resistance and tolerance to infection (Carr *et al.*, unpublished data). The widespread occurrence of CMV in nature is due, in part, to its nonpersistent mode of aphid transmission, meaning that an aphid can acquire CMV from an infected epidermal cell during a brief probe of the

plant and with no latent period transmit the virus to another plant (Kennedy *et al.*, 1962; Tomlinson, 1987).

Definitions

In this study, we measured defense by comparing plants exposed to spittlebugs, CMV, or both to spittlebug- or virus-free controls. We refer to defense in its broadest sense to mean qualities possessed by some individuals or genotypes that result in the individual or genotype suffering a smaller loss of fitness when attacked by an enemy than a susceptible individual or genotype (*sensu* Kennedy and Barbour, 1992; Karban and Baldwin, 1997). This definition includes components of resistance (plant traits that reduce the survival, reproductive output, or preferences of a natural enemy) and tolerance (plant traits that decrease the negative fitness consequences of a given level of attack) (Karbon and Baldwin, 1997; Strauss and Agrawal, 1999). Because resistance (as measured by body size and development time of spittlebugs or CMV accumulation) is not correlated with tolerance (effect of spittlebugs or CMV on plant fitness) in this system (Carr and Eubanks, 2002; Carr *et al.*, 2003; Eubanks and Carr, unpublished data), it is likely that ‘defense’ against spittlebugs and CMV primarily comprises tolerance traits.

Crossing design

In the fall of 1998, field-collected seed from *M. guttatus* populations M5 and M13 were sown in a pollinator-free greenhouse at the Blandy Experimental Farm (BEF) in Boyce, VA, USA. Population M5 was represented by 13 full-sib families and population M13 was represented by 12 full-sib families. One individual grown from each of these families served as the maternal plant for the production of seed. All flowers were emasculated in bud prior to hand-pollination. To produce seed, individuals from the same population were randomly paired and pollen was applied directly from the anthers of the pollen donor to the receptive stigma of the recipient. Seeds were harvested when fruits were mature and stored at room temperature.

Experimental design

Forty seeds per full-sib family were sown on 13 March, 2001 into 72 mm² plastic pots filled with Pro Mix growing medium in a greenhouse at Auburn University, Auburn, AL, USA. On 27 March, 20 randomly selected seedlings per treatment per full-sib family were transplanted into individual 72 mm pots. The experiment was a randomized complete block design with population (M5 and M13), full-sib family (13 families from M5 and 12 families from M13),

herbivore (present or absent), and virus (inoculated or not inoculated) replicated twice within each of five blocks (1000 total plants). Plants were not fertilized during the experiment.

CMV strain Fny was used throughout this project (kindly provided by Peter Palukautis, Scottish Crop Research Institute, Invergowrie, Scotland). On 9 April, 2001, *M. guttatus* plants were inoculated with CMV by rub-inoculation onto the oldest two to three leaves. Inoculum consisted of systemically infected *Nicotiana tabacum* cultivar Kentucky 14 tissue ground in 50 mM potassium phosphate buffer, pH 7.0, 10 mM sodium sulfite (1 g tissue: 10 ml buffer). Control plants were mock inoculated with healthy Kentucky 14 tissue ground in buffer. *M. guttatus* plants were inoculated in blocks with fresh inoculum used for each block. A young Kentucky 14 plant was inoculated upon completion of inoculation of each block to confirm inoculum infectivity. In each case the plant developed typical systemic infection symptoms, confirming infectivity of the inoculum.

On 12–13 April, 2001, second and third instar *P. spumarius* nymphs were collected from Point Reyes National Seashore, CA, where it is a common herbivore on *M. guttatus* (Carr and Eubanks, 2002). Spittlebugs were packed in plastic bags and provided fresh plant material for transport to Auburn University. On 15 April, one randomly selected spittlebug (a typical number for infested plants) was applied to each of the herbivore-designated plants. All plants were in a large rosette stage. Most nymphs immediately established feeding sites on the plants. On 16–18 April, nymphs that failed to establish a feeding site were replaced. We chose to infest plants with spittlebug nymphs 6 days after CMV inoculation because that is when *M. guttatus* plants begin to develop the first symptoms of CMV infection (Carr *et al.*, 2003).

The experiment was monitored daily for adult spittlebug emergence. The total number of flowers produced by each plant was counted on 6 May, immediately after the emergence of the last spittlebug. Each plant was harvested at ground level, wrapped in aluminum foil, dried to a constant weight at 50 °C, and weighed to the nearest 0.01 g. In *M. guttatus*, biomass and flower production are highly correlated ($r = 0.89$; Galloway, 1995), and biomass is highly correlated with total seed production (r ranging from 0.68 to 0.80; Fenster and Ritland, 1994).

Statistical analysis

The effect of herbivore and virus attack on flower production and above-ground biomass were analyzed by mixed-model ANOVA using SAS Proc Mixed (version 8.2). The significance of the random effects and interactions with random effects was tested by running models with and without the effect and calculating likelihood ratio statistics (Littell *et al.*, 1996). Flower number

was square root-transformed and biomass was log-transformed prior to analyses.

To test for a trade-off in defense, we correlated the reduction in flower and biomass production due to spittlebug infestation with the reduction due to CMV infection (fitness of controls minus fitness of attacked plants). We used full-sib family means for the correlations; these are commonly used as estimates of genetic correlations and, because these traits were measured on different individuals, they are unbiased (Lynch and Walsh, 1998). Caution must be taken in interpretation of these data, however, because they include maternal effects.

Results

Symptoms of CMV infection were conspicuous in all inoculated plants. Chlorosis and leaf curling were observed in most plants and more than half of the inoculated plants had severe flower deformation, including reduced size, a pale corolla, and petals that failed to fuse into the characteristic zygomorphic corolla. These symptoms were consistent with our previous study (Carr *et al.*, 2003).

Interactive effects of herbivore and virus

We found strong evidence that spittlebugs altered the effect of CMV infection on the fitness of *M. guttatus* plants and vice-versa (three-way interactions between family, spittlebug, and CMV Tables 1 and 2). In some cases, attack by one enemy appeared to ameliorate the negative effects of the other enemy. In family 21 of population M5, for example, it appeared that CMV infection ameliorated the negative effects of spittlebugs (Fig. 1). Spittlebugs alone dramatically reduced flower production, but CMV infection alone did not. Likewise, flower production of plants in this family attacked by both spittlebugs and CMV was similar to control plants (Fig. 1). This pattern was also evident in flower production of family 2 in population M13 (Fig. 2) and biomass production of family 18 in population M13 (Fig. 3). The opposite pattern, where spittlebugs ameliorated the negative effects of CMV, was less prevalent, but this appeared to be the case for flower production in family 19 of population M5 (Fig. 1).

In several cases, the combined negative effect of spittlebugs and CMV on flower or biomass production was greater than either spittlebugs or CMV alone. This happened for biomass production of all families in population M5 (spittlebug \times virus interaction, Table 1; Fig. 4) and in population M5 for flower production in families 22, 33, and especially 42 (Fig. 1). This pattern

Table 1. Treatment effects and variance components (for random effects) for flower production, and above-ground biomass in Population M5

Source	Flower production		Above-ground biomass	
	VarComp	χ^2 or F	VarComp	χ^2 or F
Block	0.187	54.50***	0.022	48.1***
Family	0.671	13.70***	0.032	6.70*
Spittlebug	–	12.68**	–	12.36**
CMV	–	0.86	–	5.57*
Family \times Spittlebug	0.00	0.00	0.007	1.20
Family \times CMV	0.026	0.20	0.000	0.00
Spittlebug \times CMV	–	1.69	–	7.07*
Fam \times Spit \times CMV	0.121	4.00*	0.006	1.00
Error	1.063	–	0.142	–

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The χ^2 tests associated with family and interactions involving family have one degree of freedom. All F-tests have degrees of freedom of one and 12 (numerator and denominator, respectively).

was also evident in flower production for family 20 in population M13 (Fig. 2) and for biomass production in families 7, 8, 16, and 20 (Fig. 3).

Trade-offs

We found no evidence of trade-offs in defense against spittlebugs and CMV. In population M5, there was a statistically significant, positive relationship between the effect of spittlebugs and CMV on flower production ($r = 0.83$, $p = 0.007$) and a statistically non-significant, positive relationship between

Table 2. Treatment effects and variance components (for random effects) for flower production, and above-ground biomass in Population M13

Source	Flower production		Above-ground biomass	
	VarComp	χ^2 or F	VarComp	χ^2 or F
Block	0.000	0.00	0.003	1.40
Family	0.367	6.70*	0.083	6.6*
Spittlebug	–	11.49*	–	16.33**
CMV	–	0.61	–	2.97
Family \times Spittlebug	0.059	0.60	0.007	0.20
Family \times CMV	0.011	0.10	0.000	0.00
Spittlebug \times CMV	–	0.17	–	0.16
Fam \times Spit \times CMV	0.127	4.80*	0.043	10.6*
Error	0.993	–	0.230	–

* $p < 0.05$, ** $p < 0.01$, $p < 0.001$. The χ^2 tests associated with family and interactions involving family have one degree of freedom. All F-tests have degrees of freedom of one and 11 (numerator and denominator, respectively).

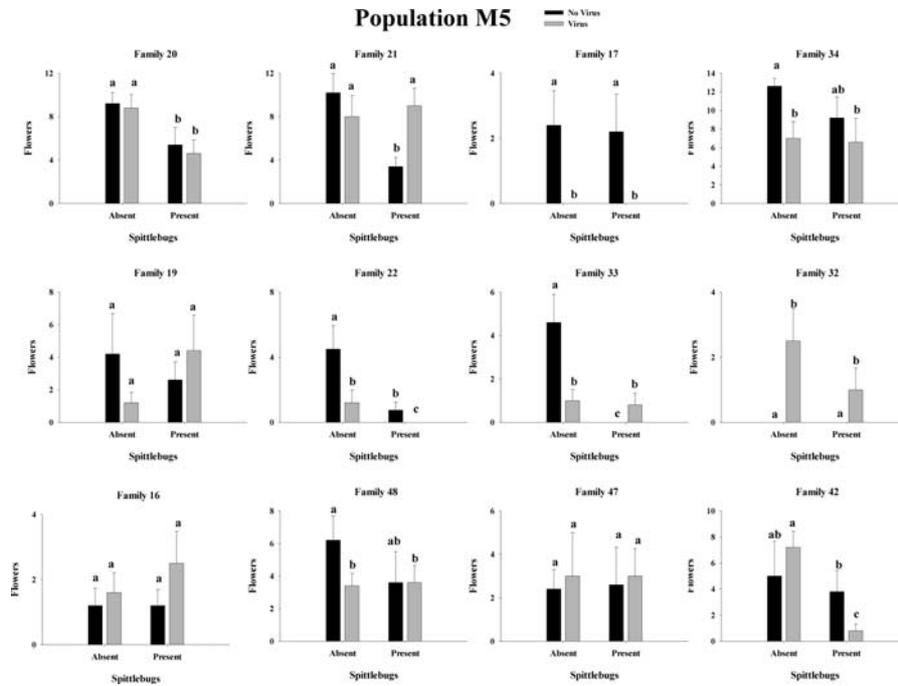


Figure 1. Effect of spittlebugs, CMV, and their interaction on flower production in 12 full-sib families of *M. guttatus* from population M5. Bars represent one standard error. Letters indicate differences among treatment means within families ($p < 0.05$, Bonferroni-adjusted means separation test).

their effects on biomass production ($r = 0.35$, $p = 0.24$). In population M13, there were statistically non-significant, positive relationships between the effect of spittlebugs and CMV on flower production ($r = 0.39$, $p = 0.21$) and on biomass production ($r = 0.487$, $p = 0.11$).

Discussion

We found evidence of complicated, non-additive effects of spittlebugs and CMV on *M. guttatus* fitness. In several cases, simultaneous attack by spittlebugs and CMV dramatically reduced plant performance, even when neither alone had an effect. In a few cases, attack by both spittlebugs and CMV appeared to ameliorate the negative effects of one of them on plant fitness, suggesting that the effects of one enemy somehow ‘cancelled out’ or masked the negative effects of the second enemy.

The interactive effect of spittlebugs and CMV could be the result of several mechanisms. First, spittlebug feeding induces changes in plant morphology

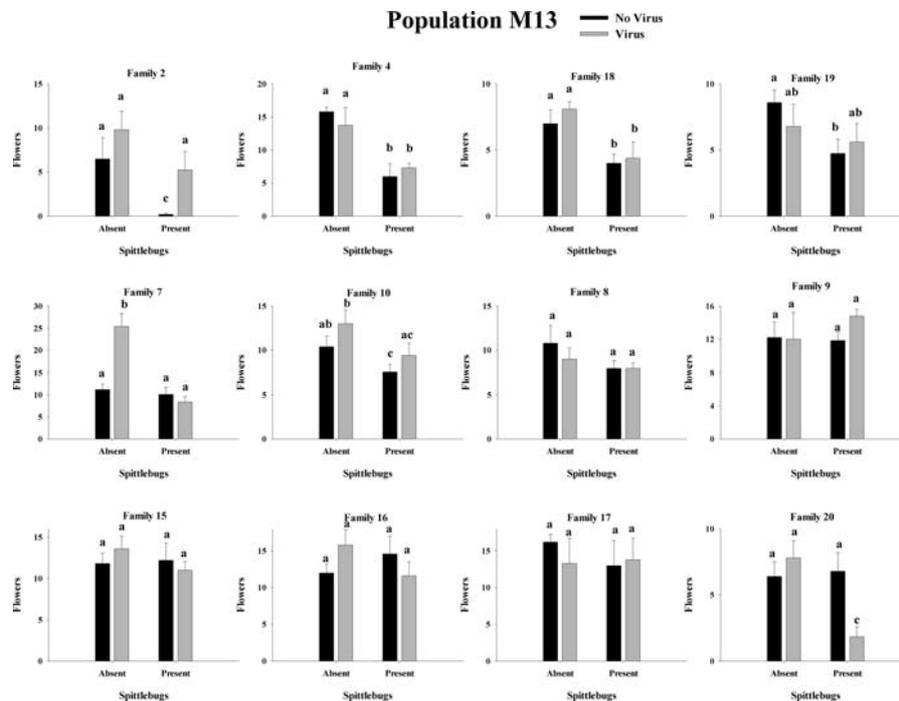


Figure 2. Effect of spittlebugs, CMV, and their interaction on flower production in 12 full-sib families of *M. guttatus* from population M13. Bars represent one standard error. Letters indicate differences among treatment means within families ($p < 0.05$, Bonferonni-adjusted means separation test).

and physiology that almost certainly alters the resistance and tolerance of *M. guttatus* to CMV. For example, spittlebug feeding reduces photosynthesis and photoassimilate movement, both of which are positively correlated with the CMV infection process (Bawden and Kassanis, 1950; Cheo *et al.*, 1952; Navas *et al.*, 1998). Thus, spittlebug feeding may reduce the quality of *M. guttatus* plants as hosts for CMV, thereby reducing the negative effect of CMV on plant performance. Conversely, CMV alters the morphology and physiology of plants in ways that almost certainly affect spittlebugs. CMV, for example, reduces stem elongation and turgor pressure of plants, both of which are associated with spittlebug performance and their consequent impact on host plants (Horsfield, 1977; McEvoy, 1986). Second, it is possible that defensive pathways associated with spittlebugs and CMV in *M. guttatus* interfere with each other (e.g., Thaler *et al.*, 1999, 2002). CMV stimulates the salicylic acid pathway that results in systemic acquired resistance (SAR) in some plants (Murphy *et al.*, 2001), but we do not know if this happens in *M. guttatus*. It is also not known if spittlebugs activate the jasmonic acid

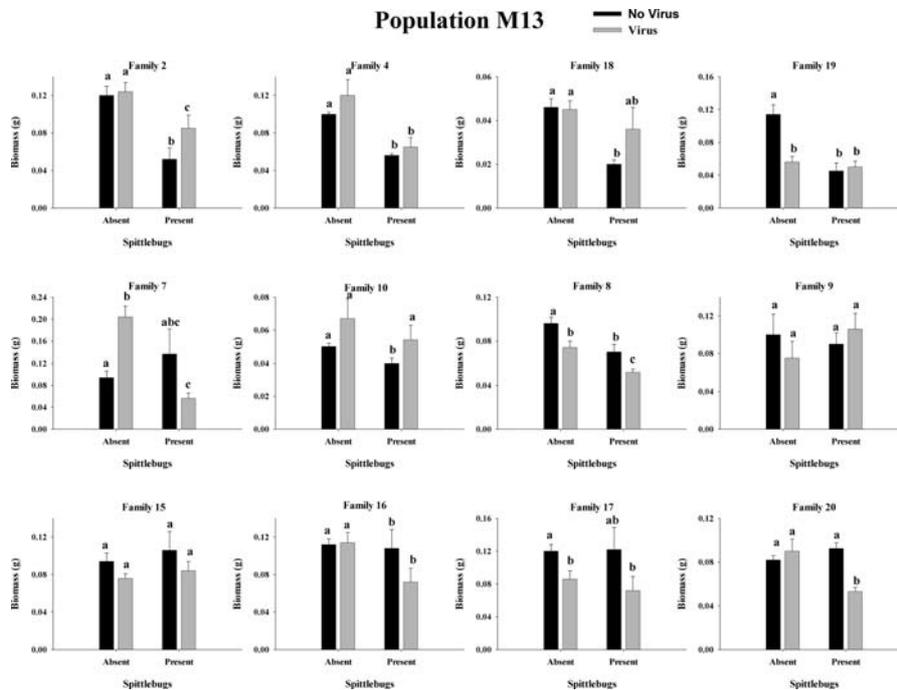


Figure 3. Effect of spittlebugs, CMV, and their interaction on biomass production in 12 full-sib families of *M. guttatus* from population M13. Bars represent one standard error. Letters indicate differences among treatment means within families ($p < 0.05$, Bonferroni-adjusted means separation test).

pathway that leads to induced resistance against insect herbivores. If both pathways are triggered, then physiological interference among the pathways may explain the response of some *M. guttatus* families. Clearly, we need to investigate in detail the defensive responses of *M. guttatus* plants to both of these enemies to better understand the complex interactive effects that they have on plant fitness.

Our results provide strong evidence of genetic variation among full-sib families in the response of *M. guttatus* to simultaneous attack by spittlebugs and CMV. These results are consistent with our earlier work with this system (Carr and Eubanks, 2002; Carr *et al.*, 2003) and with many other studies that have demonstrated genetic variation within and among plant populations for defense against insect herbivores and pathogens (Fritz and Simms, 1992). Moreover, our study suggests that genetic variation underlies the interactive effect of spittlebugs and CMV on the performance of *M. guttatus* plants. Thus, the mechanism or mechanisms responsible for these interactions vary among *M. guttatus* genotypes. The existence of genetic variation suggests that selection can act on the interaction between the two enemies and that strong

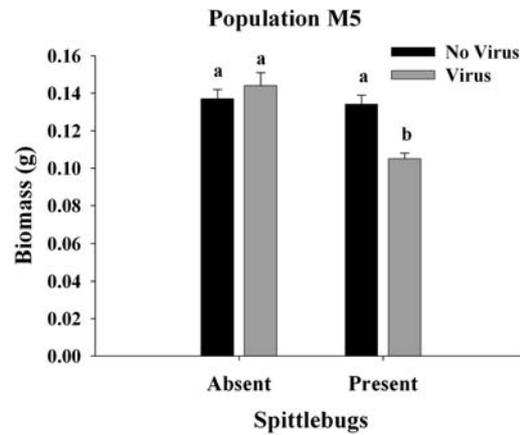


Figure 4. Effects of spittlebugs, CMV, and their interaction on biomass production in population M5. Bars represent one standard error. Letters indicate differences among treatment means within families ($p < 0.05$, Bonferonni-adjusted means separation test).

selection imposed by one of these enemies may alter a population's response to the second enemy.

Despite our previous work that suggested that trade-offs between plant defense against spittlebugs and CMV might exist in *M. guttatus* plants (Carr and Eubanks, 2002; Carr *et al.*, 2003), we found no evidence of trade-offs in this study. This result was somewhat surprising because trade-offs between herbivore and pathogen defense are thought to be widespread (e.g., Felton and Korth, 2000). Trade-offs in defense against multiple enemies are predicted to occur when defense is costly. Several studies, however, suggest that tolerance of herbivory is not costly (e.g., Agrawal *et al.*, 1999) and costs associated with defense are notoriously difficult to experimentally detect (Stamp, 2003). The results of our study suggest that trade-offs in defense against herbivores and pathogens may not be widespread, but rather that simultaneous attack by both of these enemies may result in a wide variety of plant responses.

In conclusion, this is one of the first studies to demonstrate genetic variation associated with the non-additive effect of an herbivore and a pathogen on plant fitness. Given the widespread occurrence of genetic variation underlying defense against herbivores and pathogens in plants and the ubiquity of both types of enemies in nature, it seems likely that genetic variation for their interactive effects on plant fitness will be widespread. We suggest that future studies of the mechanisms underlying the defensive properties of plants consider variation associated with defense mechanisms and the potential effect of this variation on the response of plant populations to selection by multiple enemies.

Acknowledgements

We thank S. Blackwell, B. Clark, Z. DeLamar, J. Eubanks, C. Ivey, C. Harvey, H. Hull-Sanders, I. Kaplan, and M. Patrick for assistance in the field, greenhouse, and/or laboratory. Point Reyes National Seashore, the Wantrup Wildlife Sanctuary, and the Midpeninsula Open Space Preserve kindly provided field sites and accommodations for collecting in California. We thank H. Hull-Sanders, C. Ivey, I. Kaplan, M. Wise, J. Styrsky, C. Fenster, and J. Thaler for thoughtful comments on earlier versions of the manuscript. This work was supported by National Science Foundation Grants DEB-0075225 to MDE, DEB-0074556 to DEC, REU supplements to both grants, the Department of Entomology and Plant Pathology at Auburn University, and the University of Virginia's Blandy Experimental Farm.

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