

# Synergistic Effects of Amides from Two *Piper* Species on Generalist and Specialist Herbivores

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**Abstract** Plants use a diverse mix of defenses against herbivores, including multiple secondary metabolites, which often affect herbivores synergistically. Chemical defenses also can affect natural enemies of herbivores via limiting herbivore populations or by affecting herbivore resistance to parasitoids. In this study, we performed feeding experiments to examine the synergistic effects of imides and amides (hereafter “amides”) from *Piper cenocladum* and *P. imperiale* on specialist (*Eois nympha*, Geometridae) and generalist (*Spodoptera frugiperda*, Noctuidae) lepidopteran larvae. Each *Piper* species has three unique amides, and in each experiment, larvae were fed diets containing different concentrations of single amides or combinations of the three. The amides from *P. imperiale* had negative synergistic effects on generalist survival and specialist pupal mass, but had no effect on specialist survival. *Piper cenocladum* amides also acted synergistically to increase mortality caused by parasitoids, and the direct negative effects of mixtures on parasitoid resistance and pupal mass were stronger than indirect effects via changes in growth rate and approximate digestibility. Our results are consistent with plant defense theory that predicts different effects of plant chemistry on generalist versus adapted specialist herbivores. The toxicity of *Piper* amide mixtures to generalist herbivores are standard bottom-up

effects, while specialists experienced the top-down mediated effect of mixtures causing reduced parasitoid resistance and associated decreases in pupal mass.

**Key Words** Synergy · Imides · *Piper* amides · Lepidoptera · Chemical defense · Generalists · Specialists · Parasitoid resistance

## Introduction

Experiments that examine the effects of plant chemical defenses on herbivores often examine individual compounds, despite evidence that many defenses can be additive or synergistic (Hay et al., 1994; Nelson and Kursar, 1999; Steppuhn and Baldwin, 2007). Synergistic plant defenses are defined as multiple compounds that have effects greater than expected based on projected additive effects of each individual compound (Berenbaum et al., 1991; Jones, 1998; Nelson and Kursar, 1999). Synergy can occur among different classes of compounds or structurally similar compounds, and effects can range from deterrence to toxicity (Jones, 1998; Hagele and Rowell-Rahier, 2000; Scott et al., 2002; Dyer et al., 2003). Since secondary metabolites are present in plants as simple or complex mixtures, widespread synergy is partly responsible for what some have termed “redundancy” in chemical defenses (Jones and Firm, 1991; Romeo et al., 1996). One documented mechanism of synergy is that inactive metabolites synergize with toxins via impeding an herbivore’s or pathogen’s ability to metabolize or eliminate the toxins. Thus, it is possible that many compounds that appear to have no defensive value are actually important for effective defense (Berenbaum and Neal, 1985; Kubo and Muroi, 1993; Stermitz et al., 2000). Synergy may partly explain the

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apparent lack of defensive properties for a variety of plant secondary compounds that have no antiherbivore function when tested separately from complex natural mixtures (Harborne, 1988; Ayres et al., 1997).

Quantification of the tritrophic effects of plant defenses has been the focus of many studies in chemical ecology. The bottom-up or “green desert” model suggests that plant defenses work directly to limit herbivore populations, which in turn limits natural enemy populations (White, 1978; Abe and Higashi, 1991). Plant defenses also have been found to mediate top-down forces by attracting natural enemies to herbivores (Vet and Dicke, 1992; De Moraes et al., 1998; Turlings and Wackers, 2004) or by decreasing herbivore resistance to parasitoids (Zvereva and Rank, 2003; Bukovinszky et al., 2009; Lampert and Bowers, 2010). In the latter example, sequestered toxins negatively affect a caterpillar’s immune response by reducing its ability to encapsulate and kill parasitoid eggs, thus rendering them more vulnerable to successful attacks (Haviola et al., 2007; Smilanich et al., 2009). On the other hand, plant defenses can have detrimental effects on natural enemies. Herbivore sequestered plant defenses can provide resistance by being toxic or deterrent to natural enemies (Campbell and Duffey, 1981; Barbosa et al., 1986; Dyer, 1995; Ode, 2006; Singer et al., 2009). Parasitoid performance often is linked to the host herbivore performance, such that negative effects of plant chemistry on the herbivore are associated with negative effects on the parasitoid (Harvey et al., 2005; Soler et al., 2005; Gols et al., 2008). These deleterious effects of plant defenses on parasitoids, combined with higher toxicity and diversity of plant defenses in the tropics, are suggested to explain why there is no latitudinal diversity gradient for parasitic hymenoptera (“nasty” host hypothesis, Gauld et al., 1992).

While the effects of plant chemistry on the third trophic level vary among study systems, one strong bitrophic pattern is that there are marked differences in the response of specialist herbivores, which are adapted to specific host plant chemistry, and generalist herbivores, which are adapted to compounds from many different host plants but have a comparatively weaker response to specific defenses. Most studies that compare the effects of chemistry on specialists versus generalists have focused on single compounds (Bowers and Puttick, 1989; Van Dam et al., 1995; Wasano et al., 2009) or a broadly measured mixture of compounds (e.g., total phenolics, Bi et al., 1997, also see Coley et al., 2005) as opposed to examining synergy. However, there is growing evidence that synergistic defenses also affect specialist and generalist differently (Hagele and Rowell-Rahier, 2000; Dyer et al., 2003). Whether or not the defensive compounds are acting synergistically, specialists tend to be better adapted to the variety of secondary metabolites within a single host plant

genus or species as measured by feeding efficiency, fecundity, and mortality, while generalists are more likely to be negatively affected when feeding on the same host plant. Moreover, these differences in herbivore performance potentially can cascade up to the third trophic level (Dyer et al., 2004b; Harvey et al., 2005; Utsumi and Ohgushi, 2009).

In this paper, we examine how synergistic imide and amide chemical defenses found in *Piper* spp. (Piperaceae), known collectively as *Piper* amides, affect specialist and generalist herbivores. We specifically test the hypotheses that: (1) *Piper* amides act synergistically to negatively affect herbivore growth and survivorship; (2) synergistic effects vary between specialist and generalist herbivores; and (3) there are negative synergistic effects on a specialist herbivore’s resistance to parasitoids. To test these hypotheses we performed a series of experiments that examined the performance of specialist and generalist lepidopteran larvae on *Piper* amides found in two species of *Piper*—*P. cenocladum* and *P. imperiale*.

## Methods and Materials

**Piper** The genus *Piper* is diverse with high intraspecific diversity of secondary metabolites, including the *Piper* amides (reviewed by Parmar et al., 1997; Kato and Furlan, 2007). These compounds contain a phenyl moiety with a variable length carbon side chain (typically with at least one unsaturation) ending in a carbonyl carbon. The nitrogen containing portion of the amide is derived from piperidine, pyrrole, or an isobutyl group, and may contain an unsaturation, an epoxide, or a carbonyl group. *Piper cenocladum* and *P. imperiale* are two species of particular interest due to their high densities in forest understories, high diversity of consumers, and previously recorded synergy among some of the *Piper* amides present in the leaves (Dyer et al., 2004b; Fincher et al., 2008).

*Piper imperiale* is a large shrub or small tree found in the lowland tropical wet forest of Costa Rica and is morphologically and ecologically similar to *P. cenocladum* (Burger, 1971). It contains three imides: 5'-desmethoxydihydropiartine (an analog of piplartine found in *P. cenocladum*), pipiaroxide (an epoxide derivative of 5'-desmethoxydihydropiartine), and an alkene (Fincher et al., 2008); these compounds deter arthropods (Fincher et al., 2008). *Piper imperiale* also contains at least five different sesquiterpenes, and their role as a plant defense has not yet been determined (Fincher et al., 2008). Several facultative ant species have been found inhabiting *P. imperiale*. However, these plants do not produce food rewards for the ants, and it is unknown if the plants benefit from the presence of ants.

*Piper cenocladum* is a tall understory plant with large, long-lived leaves and is common in the lowland wet forests of Costa Rica (Burger, 1971). This shrub is defended by a combination of *Piper* amides and a mutualism with *Pheidole bicornis* Forel ants. Plants uninhabited by ants increase amide production by at least a factor of three (Dodson et al., 2000; Dyer et al., 2001). *Piper cenocladum* contains two imides and an amide at high concentrations (total amide content can be as high as 3.8% dry weight): 4'-desmethylpiplartine (0.18% dry weight with an ant colony and 0.45% without ants), piplartine (0.14% with ants and 0.58% without ants), and cenocladamide (0.09% with ants and 0.33% without ants, Dodson et al., 2000). Other experiments have demonstrated that these *Piper* amides deter arthropods and act synergistically against some herbivores (Dyer et al., 2003, 2004b).

**Herbivores** The species-rich genus, *Eois* (Geometridae), is specialized on the genus *Piper*, with *Eois* species specializing on an average of two *Piper* species (Connahs et al., 2009). *Eois nympha* feeds on both *P. cenocladum* and *P. imperiale* (Dyer and Gentry, 2002). In Costa Rica, *Eois* are parasitized at a rate of 23.4% by a combination of wasps (Braconidae and Ichneumonidae) and flies (Tachinidae) (Connahs et al., 2009). *Spodoptera frugiperda* (Noctuidae) is a *Piper* naïve generalist herbivore native to tropical regions of the western hemisphere and is a major crop pest in several regions of North America. Eggs were purchased from Agripest, North Carolina, USA and a lab colony was maintained at Tulane University.

**Experiments** The synergistic effects of *Piper* amides from *P. cenocladum* and *P. imperiale* were examined on specialist and generalist caterpillars. For experiments with *P. imperiale* imides, we used both a specialist and a generalist caterpillar, and for experiments with *P. cenocladum* amides, we used only the specialist. In each experiment, larvae were fed diets containing different concentrations of single amides or a combination of amides. The amides were synthesized in the laboratory at Mesa State College (Richards et al., 2001). Amide concentrations for experimental diets were chosen based on concentrations found in *Piper* leaves, and were calculated as the % dry weight of the leaf disk or artificial diet. These experiments were labor and material (field-collected larvae and synthetic amides) intensive. Thus, it was difficult to conduct them at the same time and with identical methods.

***Piper imperiale* Imides** Before initiating experiments, we quantified the imide concentrations in fully expanded new leaves of 39 individual *P. imperiale* shrubs, using methods previously described by Dodson et al. (2000). Leaves were collected at 39 different sites, spread evenly across the

forest at La Selva Biological Station, Heredia province, Costa Rica. The *P. imperiale* leaves contained 0.13% ( $\pm 0.021$  SE) dry weight total imides. Dry weight of individual imides was distributed as follows: 0.006% ( $\pm 0.001$  SE) alkene, 0.12% ( $\pm 0.021$  SE) piplaroxide, and 0.003% ( $\pm 0.001$  SE) 5'-desmethoxydihydropiplartine. We performed leaf disk bioassays on the specialist, *E. nympha*, at La Selva (Dec 2007–Feb 2008), using concentrations similar to these natural concentrations (but slightly lower). We collected early instar (1–2) larvae of *E. nympha* from the field, and randomly assigned them to petri dishes with 9 cm diam disks of fresh *P. imperiale* leaf cuttings. Before adding the caterpillars, we pipetted 1 ml of methanol solution containing amides onto the underside of each leaf disk, covering the entire surface, and allowed the methanol to evaporate. Control diets included methanol only. Imide concentrations added to the leaf disks were alkene (0.001, 0.003, 0.01, 0.05% dry weight), 5'-desmethoxydihydropiplartine (0.001, 0.003, 0.01, 0.05% dry weight), piplaroxide (0.001, 0.01, 0.1, 0.2%), and mixtures which were equal parts of each compound with total imide dry weights of 0.003, 0.06, 0.12, and 0.3% (0.001, 0.02, 0.04, 0.1% of each compound). These added imides supplemented existing leaf defensive chemistry. We placed six individuals on each diet concentration and recorded the survival; for caterpillars that reached pupation, we recorded pupal mass, which is a good measure of potential fecundity (Tamaru et al., 1996; Thiery and Moreau, 2005; Hazarika et al., 2007).

In a second synergy experiment with *P. imperiale* imides, we used newly hatched caterpillars from a *S. frugiperda* lab colony. The control diet consisted of 35 g powdered Fall Armyworm Diet (Southland Products, Inc. Lake Village, AR, USA) mixed with 201 ml distilled water and 1.5 ml linseed oil. For experimental diets, a single compound or a combination of compounds was added to the powdered control diet as follows, alkene: 0.001, 0.006, 0.01, 0.1%, 5'-desmethoxydihydropiplartine: 0.001, 0.003, 0.01, 0.1%, piplaroxide: 0.001, 0.12, 0.25, 0.5%, and mixtures that were equal parts of each compound with total imide dry weights of 0.003, 0.15, 0.3, and 0.9% (0.001, 0.05, 0.1, 0.3% of each compound). Each treatment started with 310 larvae and culled to 300 at Day 3 to have the same starting number across treatments. Survival was recorded daily from days 9–40 and again on days 43, 45 and 53. We used Day 53 as a cutoff since over 95% of the larvae had pupated by then (those that were still alive were assumed to make it to pupation). Due to high mortality on the mixed diets, we were unable to compare pupal mass for this treatment.

***Piper cenocladum* Amides** We performed similar leaf disk bioassays of *E. nympha* with amides from *P. cenocladum*.

Diet treatments consisted of cenocladamide, piplartine, 4'-desmethylpiplartine and equal part mixtures. Added concentrations varied from 0.01 to 0.8% dry weight, with 7–9 different concentrations per diet. Fresh leaves were collected from plants with ants in the field that had a mean total amide dry weight of 0.41% ( $\pm 0.02$  SE), while concentrations without ants was 1.36% ( $\pm 0.02$  SE) (Dodson et al., 2000). Thus, the amide concentrations added were likely within the natural range of the plant. Caterpillars had been naturally exposed to parasitoids in the field prior to collection, and since they were randomly assigned treatments in the lab, we assumed equal parasitism frequency before placement on diets. Therefore, differences in mortality caused by the emergence of a parasitoid are due to post-treatment differences in the development of the immature parasitoid, which may be correlated with the immune response as well as nutritional indices (Smilanich et al., 2009). During the fourth instar, we measured weight gain, average weight, food consumed, and frass produced. Using dry weight conversions, we calculated relative growth rate (GR = larval dry weight gain / average larval dry weight during interval) and approximate digestibility (AD = (dry weight of food consumed – dry weight of frass) / dry weight of food consumed) (Waldbauer, 1968). In summary, the response variables for this experiment were: survival, pupal mass, parasitoid related mortality, GR, and AD.

**Analysis** For both experiments, we used linear and logistic regressions to estimate the effects of diet concentration on pupal weights, survivorship, and parasitism rates. We further examined the relationships between *P. cenocladum* amide concentrations and *E. nympha* pupal mass and parasitism frequency with path analysis (Proc CALIS, SAS Institute Inc., NC, USA). Based on previous path models (Smilanich et al., 2009), we chose simple models *a priori* in which amide concentration had direct and indirect (via GR and AD) effects on pupal mass and parasitism frequency. Path analyses were conducted for all diets (three single amide diets and one mixed diet) and models yielding goodness of fit *chi-squares* with *P*-values greater than 0.05 were considered a good fit to the data.

An interaction index ( $\alpha$ ) is usually calculated in standard synergy studies (Tallarida, 2000). It is calculated as,  $\alpha = Z / \sum (f_i A_i)$ . Where  $f_i$  represents the fraction of a compound in the mixed diet and  $A_i$  is the concentration of that compound necessary to achieve a certain level of response when tested individually;  $Z$  is the concentration of the mixture of compounds necessary to achieve a certain level of response. Values of  $\alpha$  significantly less than one demonstrate a synergy. However, our data were inappropriate for these calculations because in all experiments there were single amides that did not significantly affect the

response variables, making it impossible to determine  $A_i$ . Thus, any increase in effect resulting from including these ineffective compounds was considered evidence of synergy.

## Results

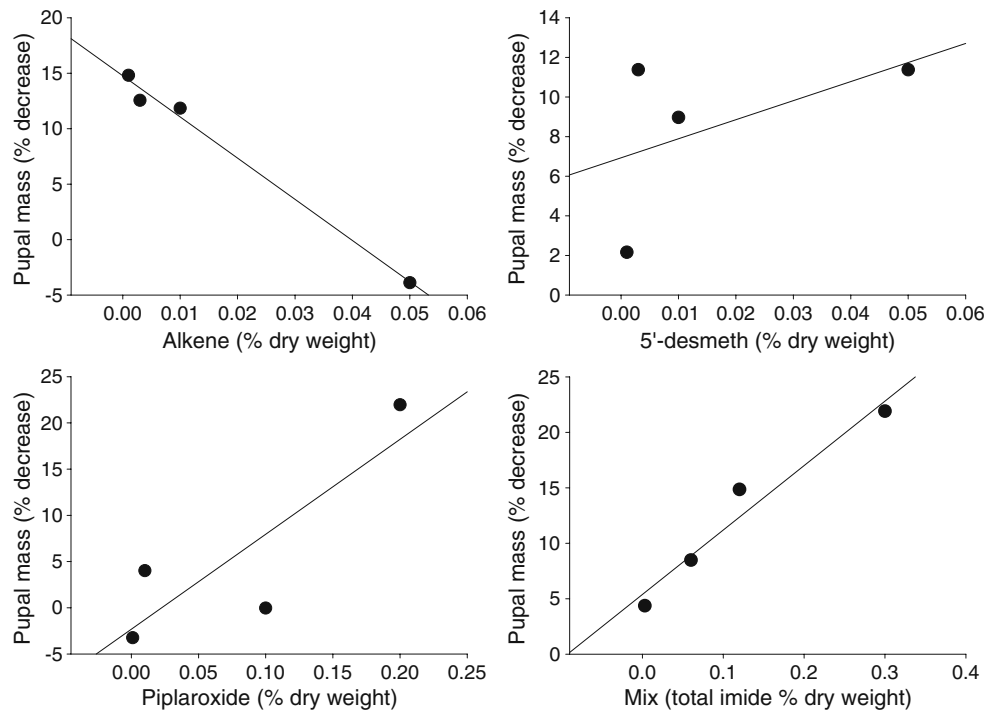
***Piper imperiale* Imides** *Piper* imides had a synergistic effect on *E. nympha* pupal mass, but not on survival. The mixed imide diets reduced pupal mass ( $r^2=0.95$ ,  $F_{1,3}=38.77$ ,  $P<0.05$ , Fig. 1). However, there was no significant relationship between change in pupal mass and piplartine ( $r^2=0.72$ ,  $F_{1,3}=5.11$ ,  $P=0.15$ ) or 5'-desmethoxydihydropiplartine ( $r^2=0.70$ ,  $F_{1,3}=4.66$ ,  $P=0.16$ ) concentrations. The alkene appeared to have a positive effect on *E. nympha*, since increasing the concentrations of alkene caused increases in pupal mass ( $r^2=0.99$ ,  $P<0.01$ ,  $F_{1,3}=219.93$ ). There were no significant associations between *Piper* amide concentration and survival for any single or mixed diets (logistic regression,  $P>0.05$ ). Mean survivorships were 100% for the control, 96% for the alkene diet, 92% for the 5'-desmethoxydihydropiplartine diet, 75% for the piplartine diet, and 75% for the mixture diet.

*Piper* imides had a synergistic effect on *S. frugiperda* survival. All concentrations of 5'-desmethoxydihydropiplartine produced results similar to the control (Fig. 2). The highest concentration piplartine diet (0.5% dry wt) and all concentrations of alkene caused reduced larvae survival. In contrast, larvae experienced 100% mortality on all mixed diets.

***Piper cenocladum* Amides** *Piper* amides of *P. cenocladum* had synergistic effects on the survival of *E. nympha*. While there was no relationship between individual *Piper* amide concentrations and *E. nympha* survival (logistic regression, piplartine failed goodness-of-fit test, 4'-desmethylpiplartine  $\chi^2=4.44$ ,  $df=1$ ,  $P<0.05$ ,  $\beta=-15.31\pm 8.25$ ,  $Wald=3.44$ ,  $df=1$ ,  $P>0.05$ , cenocladamide  $\chi^2=0.59$ ,  $df=1$ ,  $P>0.05$ ), a mixture of all three amides significantly affected survivorship ( $\chi^2=25.50$ ,  $df=1$ ,  $P<0.001$ ,  $\beta=-4.62\pm 1.07$ ,  $Wald=18.691$ ,  $df=1$ ,  $P<0.001$ ). Mean survivorship for control, piplartine, cenocladamide, 4'-desmethylpiplartine, and mix diets were 83, 85, 89, 81 and 33%, respectively.

There was a significant treatment effect on parasitoid success (ANOVA,  $F_{4, 138}=6.78$ ,  $P<0.01$ , Fig. 3). The mixture was the only treatment associated with an increased parasitism frequency (Logistic regression,  $\chi^2=12.64$ ,  $df=1$ ,  $P<0.001$ ,  $\beta=3.47\pm 1.10$ ,  $Wald=9.91$ ,  $df=1$ ,  $P<0.01$ ). No larvae died of parasitism on piplartine or control diets, and there was no relationship between cenocladamide and parasitism, and 4'-desmethylpiplartine and parasitism (cenocladamide  $\chi^2=0.01$ ,  $df=1$ ,  $P>0.05$ , 4'-desmethylpiplartine

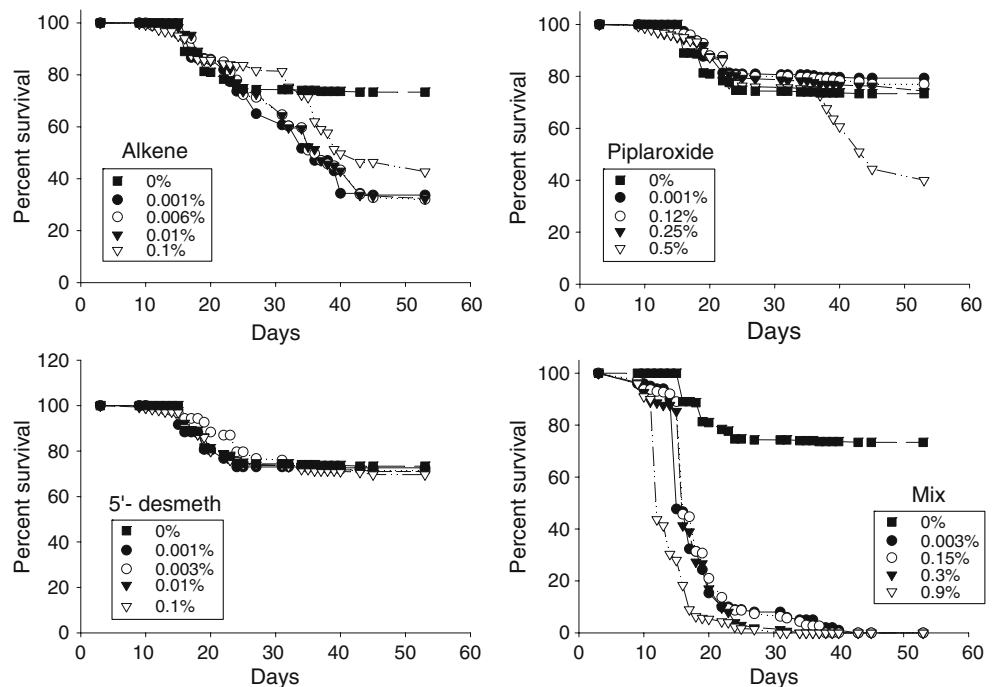
**Fig. 1** Percent decrease in pupal mass of *Eois nympha* from control when reared on *Piper imperiale* leaf disks with alkene, 5'-desmethoxydihydropiartine (5'-desmeth), piplaroxide and a mixture of all three imides added

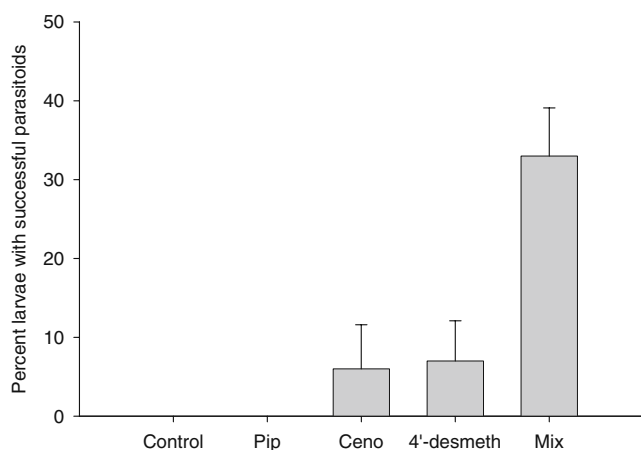


$\chi^2=5.43$ ,  $df=1$ ,  $P<0.05$ ,  $\beta=21.87\pm 11.32$ ,  $Wald=3.73$ ,  $df=1$ ,  $P>0.05$ ). The parasitoid frequency path model demonstrated that mixed *Piper* amide concentration had a greater direct positive effect on parasitoid success than any single amide treatment (Fig. 4). Parasitoid success also was negatively affected via GR and AD, but there were no significant effect of amide treatments on GR and AD (Fig. 4).

There were negative effects of 4'-desmethylpiplartine and mixed amide concentrations on pupal mass of *E. nympha* (4'-desmethylpiplartine diet,  $r^2=0.36$ ,  $P<0.05$ ,  $F_{1,11}=6.18$ , mixed diet  $r^2=0.76$ ,  $P<0.001$ ,  $F_{1,10}=31.64$ ), but pupal mass did not change with piplartine and cenocladamide concentrations ( $r^2=0.001$ ,  $r^2=0.09$ , respectively,  $P>0.05$ ). The pupal mass path model showed that

**Fig. 2** Percent survival of *Spo-doptera frugiperda* feeding on artificial diets with alkene, 5'-desmethoxydihydropiartine (5'-desmeth), piplaroxide and a mixture of all three imides. Mix concentrations indicate the total imide concentrations





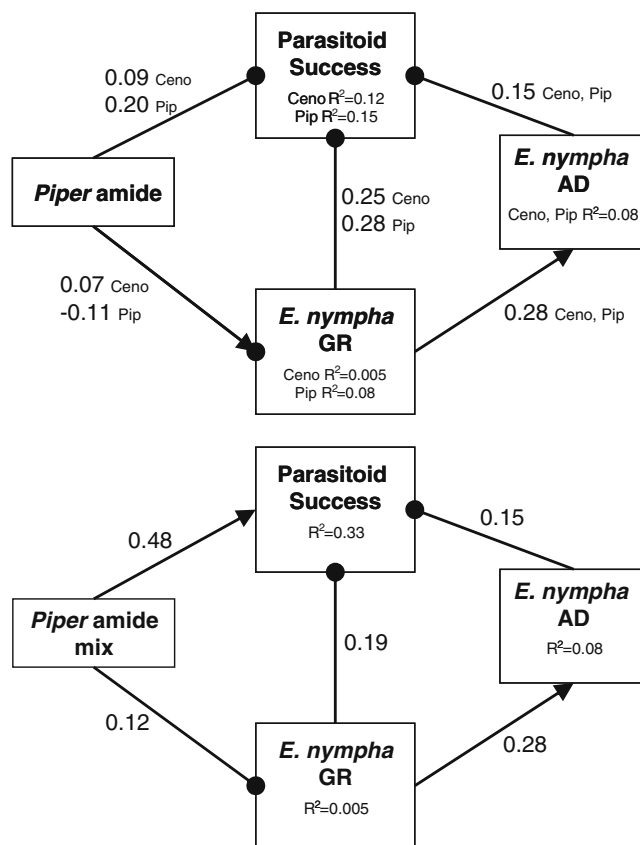
**Fig. 3** Percentage of *Eois nympha* with successful parasitoids when reared on *Piper cenocladum* leaf disks containing methanol without amides (control,  $N=12$ ) or with piplartine (Pip,  $N=26$ ), cenocladamide (Ceno,  $N=18$ ), 4'-desmethylpiplartine (4'-desmeth,  $N=27$ ) and a mixture of the three amides ( $N=60$ ) added. The mixture had significantly higher parasitoid success ( $LSD, P<0.01$ )

the mixed *Piper* amide treatment had the greatest direct negative effect on pupal mass compared to any of the single amide treatments (Fig. 5), and there was an indirect effect of amides on pupal mass via GR and AD. Again this relationship was strongest for the mixed treatment (Fig. 5).

## Discussion

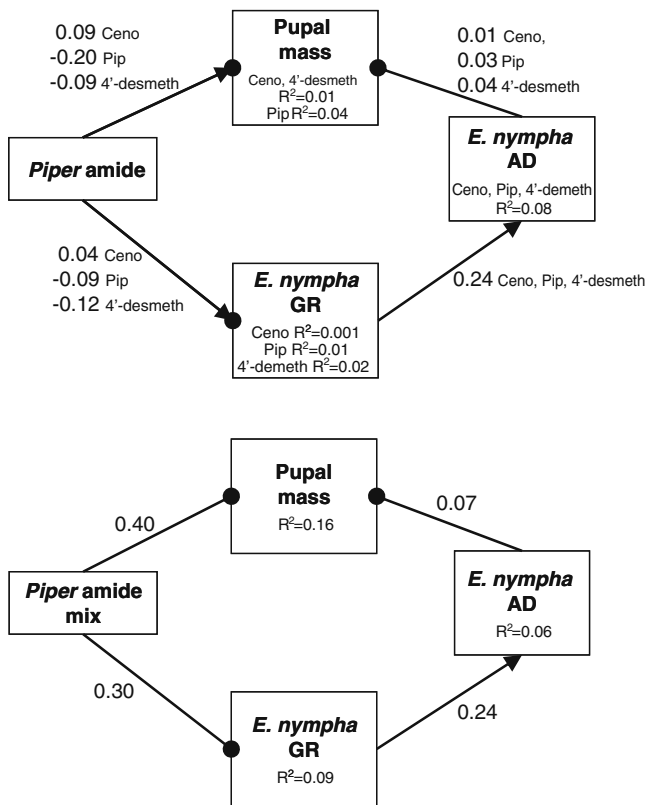
*Piper* amides synergistically affected both generalist and specialist herbivores via different mechanisms. Similar to a previous study with *P. cenocladum* amides (Dyer et al., 2003) and studies with other compounds (e.g., Roslin and Salminen, 2008), the mixture of amides in *P. imperiale* had dramatic toxic effects on survivorship of the naïve generalist *Spodoptera* caterpillars, while the negative effects on the specialist *Eois* caterpillars were more subtle. For *Eois*, increasing *Piper* amides caused increased parasitoid success and lower pupal mass, and this effect was more than doubled in response to increasing mixtures of amides as opposed to individual amides.

The synergistic effects of *Piper* amides on parasitism have interesting tritrophic implications. The traditional view of plant chemical defenses usually focuses on sequestration of toxins that confer a defensive benefit to the herbivore (Brower, 1984; Singer et al., 2009). However, our results are consistent with other recent studies that have shown an indirect positive effect of plant chemical defenses on parasitoids via disrupting the caterpillar immune response (Haviola et al., 2007; Smilanich et al., 2009). Since *Eois* species sequester *Piper* amides (Dyer et al., 2004a), the mechanisms for affecting parasitism include a



**Fig. 4** Effects of *Piper* amides on parasitoid success in *Eois nympha* larvae reared on leaf disks with *Piper cenocladum* amides added (Parasitoid Frequency Model). Positive effects between variables are indicated by an arrow and negative effects are indicated by a bullet. Relationship strengths are indicated by the standardized path coefficient.  $R^2$  values indicate the total variance explained in the model. The top diagrams the relationships for larvae on single amide treatments. 4'-desmethylpiplartine is not represented due to the lack of fit of the model ( $\chi^2=41.84, df=1, P<0.001$ ). The model was a good fit for cenocladamide (Ceno,  $\chi^2=0.22, df=1, P=0.64$ ) and piplartine (Pip,  $\chi^2=0.17, df=1, P=0.81$ ). The bottom diagrams the relationships for larvae on the mixed amide treatment ( $\chi^2=0.04, df=1, P=0.84$ )

direct negative effect on hemocytes responsible for the immune response or an indirect effect via decreasing overall herbivore vigor. Since the feeding efficiency measurements were not affected by ingestion of *Piper* amides, it is more likely that the increases in parasitism were due to direct toxicity of amides to immune cells. We did not directly measure the immune response, thus, there may be alternative explanations for these results, and further investigation is warranted. Interestingly, this *Piper-Eois*-parasitoid system is not consistent with the assumption of the “nasty” host hypothesis that herbivore sequestered plant defenses reduce host suitability for parasitoids (Gauld et al., 1992). We found the opposite; parasitoids were more successful in herbivores on more toxic mixed diets, which is consistent with previous studies that demonstrate that specialist parasitoids are less susceptible to changes in host



**Fig. 5** Effects of *Piper* amides on pupal mass in *Eois nympha* larvae reared on leaf disks with *Piper cenocladum* amides added (Pupal Mass Model). For diagram description refer to Fig. 4. Model fit: Cenocladamide (Ceno)  $\chi^2=2.23, df=2, P=0.33$ , Piplartine (Pip)  $\chi^2=0.73, df=2, P=0.69$ , 4'-desmethylpiplartine (4'-desmeth)  $\chi^2=3.52, df=2, P=0.17$ , Mix  $\chi^2=0.15, df=2, P=0.93$

chemistry than generalists (Gunasena et al., 1990; Barbosa et al., 1991; Harvey et al., 2003, 2005).

Results from the *P. cenocladum* experiments demonstrate that the effectiveness of different natural enemies is context dependent (Bronstein, 1994; Heil et al., 2002; McKey et al., 2005). The control leaves, which were collected from plants containing ants, had low amide concentration (Dodson et al., 2000; Dyer et al., 2001), high survival, and no parasitoid related mortality. Thus, when ants are present, they should be the main source of mortality and primary mechanism of *Eois* control. In comparison, the mixed amide leaves, which had concentrations similar to those found in plants without ants present (Dodson et al., 2000; Dyer et al., 2001), had high mortality from parasitoids. These two strategies of biotic and chemical plant defense may differ in their overall effectiveness and costs to the plants. For instance, producing high concentrations of amides may be costly to *P. cenocladum*, but the ecological cost of herbivory is lessened due to lower generalist herbivory and high mortality from parasitism on specialist herbivores. The same trade-off between physiological and ecological costs applies to *P. cenocladum*

individuals that house ants, which produce low concentrations of amides while maintaining high levels of costly lipid and protein rich food bodies for the ants (Dyer et al., 2004b); in this case, it is the ants that maintain low levels of generalist and specialist herbivory (Letourneau et al., 2004). In contrast, *P. imperiale* does not have ant mutualists and suffers higher levels of herbivory by generalists and specialists (Fincher et al., 2008), even though relatively low levels of amides are necessary for synergistic defense against generalist caterpillars (a 0.9% mixture caused 100% mortality in 31 days) compared to *P. cenocladum* (a 1.34% mixture of *P. cenocladum* amides caused 100% mortality in 54 days, Dyer et al. 2003).

In summary, we found that *Piper* amides in two species of *Piper* have negative synergistic effects on specialist and generalist herbivores. *Piper* amides were highly toxic and acted as a strong bottom-up force on generalist herbivores, while specialists were indirectly affected by positive synergistic effects of amides on parasitoids, which can be an important top-down force in herbivore regulation. When considering these responses of generalist and specialist herbivores to synergistic plant defenses, some interesting questions arise: Did synergistic compounds evolve in response to specialist herbivores that circumvented the toxicity of individual defenses? What are the physiological mechanisms that result in effective defensive synergy of secondary metabolites? In our experiments, specialists were able to circumvent the toxicity and developmental effects of diets spiked with single amides via unknown physiological mechanisms, but were strongly affected by synergistic mixes via effects on parasitoid development. Because the fresh leaf disks already contained *Piper* amides, all treatments in the specialist experiments were to some degree mixed diets. This means that in response to selective pressures from herbivores, an increase in the concentration of a single amide would have little adaptive value. In contrast, a small increase in several *Piper* amides would result in significantly reduced pressure from *Eois* populations, through decreased pupal mass, fecundity, parasitoid resistance, and survival. Similar results in other plant-insect systems would have interesting implications for tritrophic theories of coevolution and specialization (e.g., Singer and Stireman, 2005).

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## References

- ABE, T., and HIGASHI, M. 1991. Cellulose centered perspective on terrestrial community structure. *Oikos* 60:127–133.
- AYRES, M. P., CLAUSEN, T. P., MACLEAN, S. F., REDMAN, A. M., and REICHARDT, P. B. 1997. Diversity of structure and antiherbivore activity in condensed tannins. *Ecology* 78:1696–1712.
- BARBOSA, P., SAUNDERS, J. A., KEMPER, J., TRUMBULE, R., OLECHNO, J., and MARTINAT, P. 1986. Plant Allelochemicals and insect parasitoids effects of nicotine on *Cotesia congregata* (Say) (Hymenoptera, Braconidae) and *Hyposoter annulipes* (Cresson) (Hymenoptera, Ichneumonidae). *J. Chem. Ecol.* 12:1319–1328.
- BARBOSA, P., GROSS, P., and KEMPER, J. 1991. Influence of plant allelochemicals on the tobacco hornworm and its parasitoid, *Cotesia congregata*. *Ecology* 72:1567–1575.
- BERENBAUM, M., and NEAL, J. J. 1985. Synergism between myristicin and xanthotoxin, a naturally cooccurring plant toxicant. *J. Chem. Ecol.* 11:1349–1358.
- BERENBAUM, M., NITAO, J. K., and ZANGERL, A. R. 1991. Adaptive significance of furanocoumarin diversity in *Pastinaca sativa* (Apicaceae). *J. Chem. Ecol.* 17:207–215.
- BI, J. L., FELTON, G. W., MURPHY, J. B., HOWLES, R. A., DIXON, R. A., and LAMB, C. J. 1997. Do plant phenolics confer resistance to specialist and generalist insect herbivores? *J. Agric. Food Chem.* 45:4500–4504.
- BOWERS, M. D., and PUTTICK, G. M. 1989. Iridoid glycosides and insect feeding preferences: gypsy moths (*Lymantria dispar*, Lamiinae) and buckeyes (*Junonia coenia*, Nymphalidae). *Ecol. Entomol.* 14:247–256.
- BRONSTEIN, J. L. 1994. Conditional outcomes in mutualistic interactions. *Trends Ecol. Evol.* 9: 214–217.
- BROWER, L. P. 1984. Chemical defense in butterflies, pp. 109–134, in R. I. Vane Wright and P. R. Ackery (eds.). *The Biology of Butterflies*. Academic Press, London.
- BUKOVINSZKY, T., POELMAN, E. H., GOLS, R., PREKATSAKIS, G., VET, L. E. M., HARVEY, J. A., and DICKE, M. 2009. Consequences of constitutive and induced variation in plant nutritional quality for immune defence of a herbivore against parasitism. *Oecologia* 160:299–308.
- BURGER, W. 1971. Flora costaricensis family 40 Casuarinaceae-D family 41 Piperaceae-D. *Fieldiana Bot.* 35:1–227.
- CAMPBELL, B. C., and DUFFEY, S. S. 1981. Alleviation of alpha-tomatine-included toxicity to the parasitoid, *Hyposoter exiguae*, by phytoosterols in the diet of the host, *Heliothis zea*. *J. Chem. Ecol.* 7:927–946.
- COLEY, P. D., LOKVAM, J., RUDOLPH, K., BROMBERG, K., SACKETT, T. E., WRIGHT, L., BRENES-ARGUEDAS, T., DVORETT, D., RING, S., CLARK, A., BAPTISTE, C., PENNINGTON, R. T., and KURSAR, T. A. 2005. Divergent defensive strategies of young leaves in two species of *inga*. *Ecology* 86:2633–2643.
- CONNAHS, H., RODRÍGUEZ-CASTAÑEDA, G., WALTERS, T., WALLA, T., and DYER, L. A. 2009. Geographic variation in host-specificity and parasitoid pressure of an herbivore (Geometridae) associated with the tropical genus *Piper* (Piperaceae). *J. Insect Sci.* 9:1–11.
- DE MORAES, C. M., LEWIS, W. J., PARE P. W., ALBORN, H. T., and TURLINSON, J. H. 1998. Herbivore-infested plants selectively attract parasitoids. *Nature* 393:570–573.
- DODSON, C. D., DYER, L. A., SEARCY, J., WRIGHT, Z., and LETOURNEAU, D. K. 2000. Cenoelamide, a dihydropyridone alkaloid from *Piper cenocladum*. *Phytochemistry* 53:51–54.
- DYER, L. A. 1995. Tasty generalists and nasty specialists—Antipredator mechanisms in tropical lepidopteran larvae. *Ecology* 76:1483–1496.
- DYER, L. A., and GENTRY, G. L. 2002. Caterpillars and parasitoids of a tropical lowland wet forest. <http://www.caterpillars.org>.
- DYER, L. A., DODSON, C. D., BEIHOFFER, J., and LETOURNEAU, D. K. 2001. Trade-offs in antiherbivore defenses in *Piper cenocladum*: Ant mutualists versus plant secondary metabolites. *J. Chem. Ecol.* 27:581–592.
- DYER, L. A., DODSON, C. D., STIREMAN, J. O. III, TOBLER, M. A., SMILANICH, A. M., FINCHER, R. M., and LETOURNEAU, D. K. 2003. Synergistic effects of three *Piper* amides on generalist and specialist herbivores. *J. Chem. Ecol.* 29:2499–2514.
- DYER, L. A., DODSON, C. D., and RICHARDS, J. 2004a. Isolation, synthesis, and evolutionary ecology of *Piper* amides, pp 117–139, in L. A. Dyer and A. N. Palmer (eds.). *Piper. A Model Genus for Studies of Evolution, Chemical Ecology, and Trophic Interactions*. Kluwer Academic, Boston.
- DYER, L. A., LETOURNEAU, D. K., DODSON, C. D., TOBLER, M. A., STIREMAN, J. O., and HSU, A. 2004b. Ecological causes and consequences of variation in defensive chemistry of a Neotropical shrub. *Ecology* 85:2795–2803.
- FINCHER, R. M., DYER, L. A., DODSON, C. D., RICHARDS, J. L., TOBLER, M. A., SEARCY, J., MATHER, J. E., REID, A. J., ROLIG, J. S., and PIDCOCK, W. 2008. Inter- and Intraspecific comparisons of antiherbivore defenses in three species of rainforest understory shrubs. *J. Chem. Ecol.* 34:558–574.
- GAULD, I. D., GASTON, K. J., and JANZEN, D. H. 1992. Plant allelochemicals, tritrophic interactions and the anomalous diversity of tropical parasitoids: the “nasty” host hypothesis. *Oikos* 65:353–357.
- GOLS, R., BUKOVINSZKY, T., VAN DAM, N. M., DICKE, M., BULLOCK, J. M., and HARVEY, J. A. 2008. Performance of generalist and specialist herbivores and their endoparasitoids differs on cultivated and wild *Brassica* populations. *J. Chem. Ecol.* 34:132–143.
- GUNASENA, G. H., VINSON, S. B., and WILLIAMS, H. J. 1990. Effects of nicotine on growth, development, and survival of the tobacco budworm (Lepidoptera, Noctuidae) and the parasitoid *Campoplex sonorensis* (Hymenoptera, Ichneumonidae). *J. Econ. Entomol.* 83:1777–1782.
- HAGELE, B. F., and ROWELL-RAHIER, M. 2000. Choice, performance and heritability of performance of specialist and generalist insect herbivores towards cacalol and seneciphylline, two allelochemicals of *Adenostyles alpina* (Asteraceae). *J. Evol. Biol.* 13:131–142.
- HARBORNE, J. B. 1988. *Introduction to Ecological Biochemistry*. Academic, San Diego, p. 356.
- HARVEY, J. A., VAN DAM, N. M., and GOLS, R. 2003. Interactions over four trophic levels: foodplant quality affects development of a hyperparasitoid as mediated through a herbivore and its primary parasitoid. *J. Anim. Ecol.* 72:520–531.
- HARVEY, J. A., VAN NOUHUYS, S., and BIERE, A. 2005. Effects of quantitative variation in allelochemicals in *Plantago lanceolata* on development of a generalist and a specialist herbivore and their endoparasitoids. *J. Chem. Ecol.* 31:287–302.
- HAVIOLA, S., KAPARI, L., OSSIPOV, V., RANTALA, M. J., RUUHOLA, T., and HAUKIOJA, E. 2007. Foliar phenolics are differently associated with *Epirrita autumnata* growth and immunocompetence. *J. Chem. Ecol.* 33:1013–1023.
- HAY, M. E., KAPPEL, Q. E., and FENICAL, W. 1994. Synergisms in plant defenses against herbivores—interactions of chemistry, calcification, and platy-quality. *Ecology* 75:1714–1726.
- HAZARIKA, U., SARKAR, B. N., BARAH, A., and CHAKRAVORTY, R. 2007. Association of fecundity with larval and pupal weight in different eco-races of eri silkworm *Samia ricini* Donovan. *J. Adv. Zool.* 28:67–70.
- HEIL, M., DELSINNE, T., HILPERT, A., SCHURKENS, S., ANDARY, C., LINSINMAIR, K. E., SOUSA, M. S., and MCKEY, D. 2002. Reduced chemical defence in ant-plants? A critical re-evaluation of a widely accepted hypothesis. *Oikos* 99:457–468.
- JONES, D. G. (ed.). 1998. *Piperonyl Butoxide: The Insect Synergist*. Academic, London, p. 323.



- JONES, C. G., and FIRN, R. D. 1991. On the evolution of plant secondary chemical diversity. *Philos. Trans. R. Soc. B.* 333:273–280.
- KATO, M. J., and FURLAN, M. 2007. Chemistry and evolution of the Piperaceae. *Pure and Appl. Chem.* 79:529–538.
- KUBO, I., and MUROI, H. 1993. Combination effects of antibacterial compounds in green tea flavor against *Streptococcus mutans*. *J. Agric. Food Chem.* 41:1102–1105.
- LAMPERT, E. C., and BOWERS, M. D. 2010. Host plant species affects the quality of the generalist *Trichoplusia ni* as a host for the polyembryonic parasitoid *Copidosoma floridanum*. *Entomol. Exp. Appl.* 134:287–295.
- LETOURNEAU, D. K., DYER, L. A., and VEGA, G. 2004. Indirect effects of top predator on rain forest undersory plant community. *Ecology* 85:2144–2152.
- MCKEY, D., GAUME, L., BROUAT, C., DI GIUSTO, B., PACAL, L., DEBOUT, G., DALECKY, A., and HEIL, M. 2005. The trophic structure of tropical ant–plant–herbivore interactions: community consequences and coevolutionary dynamics, pp. 386–413, in D. F. R. P. Burslem, M. A. Pinard and S. E. Hartley (eds.). *Biotic Interactions in the Tropics. Their Role in the Maintenance of Species Diversity*. Cambridge University Press, Cambridge.
- NELSON, A. C., and KURSAR, T. A. 1999. Interactions among plant defense compounds: a method for analysis. *Chemoecology* 9:81–92.
- ODE, P. J. 2006. Plant chemistry and natural enemy fitness: Effects on herbivore and natural enemy interactions. *Annu. Rev. Entomol.* 51:163–185.
- PARMAR, V. S., JAIN, S. C., BISHT, K. S., JAIN, R., TANEJA, P., JHA, A., TYAGI, O. D., PRASAD, A. K., WENGEL, J., OLSEN, C. E., and BOLL, P. M. 1997. Phytochemistry of the genus *Piper*. *Phytochemistry* 46:597–673.
- RICHARDS, J. L., MYHRE, S. M., and JAY, J. I. 2001. Total synthesis of piplartinelartine, 13-desmethylpiplartinelartine, and cenocladamide: Three compounds isolated from *Piper cenocladum*. *Abstr. Pap. Am. Chem. S.* 221:522.
- ROMEO, J. T., SAUNDERS, J. A., and BARBOSA, P. 1996. *Phytochemical Diversity and Redundancy in Ecological Interactions*. Plenum, New York.
- ROSLIN, T., and SALMINEN, J.-P. 2008. Specialization pays off: contrasting effects of two types of tannins on oak specialist and generalist moth species. *Oikos* 117:1560–1568.
- SCOTT, I. M., PUNIANI, E., DURST, T., PHELPS, D., MERALI, S., ASSABGUI, R. A., SÁNCHEZ-VINDAS, P., POVEDA, L., PHILOGÈNE, B. J. R., and ARNASON, J. T. 2002. Insecticidal activity of *Piper tuberculatum* Jacq. extracts: synergistic interaction of Piperamides. *Agric. For. Entomol.* 4:137–144.
- SINGER, M. S., and STIREMAN, J. O. III. 2005. The tri-trophic niche concept and adaptive radiation of phytophagous insects. *Ecol. Lett.* 8:1247–1255.
- SINGER, M. S., MACE, K. C., and BERNAYS, E. A. 2009. Self-medication as adaptive plasticity: increased ingestion of plant toxins by parasitized caterpillars. *PLoS ONE* 4.
- SMILANICH, A. M., DYER, L. A., CHAMBERS, J. Q., and BOWERS, M. D. 2009. Immunological cost of chemical defence and the evolution of herbivore diet breadth. *Ecol. Lett.* 12:612–621.
- SOLER, R., BEZEMER, T. M., VAN DER PUTTEN, W. H., VET, L. E. M., and HARVEY, J. A. 2005. Root herbivore effects on above-ground herbivore, parasitoid and hyperparasitoid performance via changes in plant quality. *J. Anim. Ecol.* 74:1121–1130.
- STEPPUHN, A., and BALDWIN, I. T. 2007. Resistance management in a native plant: nicotine prevents herbivores from compensating for plant protease inhibitors. *Ecol. Lett.* 10:499–511.
- STERMITZ, F. P., LORENZ, P., TAWARA, J. N., ZENEWICZ, L. A., and LEWIS, K. 2000. Synergy in a medicinal plant: Antimicrobial action of berberine potentiated by 5'-methoxyhydnocarpin a multidrug pump inhibitor. *Proc. Nat. Acad. Sci. USA* 97:1433–1437.
- TALLARIDA, R. J. 2000. *Drug Synergism and Dose-effect Data Analysis*. Chapman & Hall/CRC, Boca Raton.
- TAMMARU, T., KAITANIEMI, P., and RUOHOMAKI, K. 1996. Realized fecundity in *Epirrita autumnata* (Lepidoptera: Geometridae): Relation to body size and consequences to population dynamics. *Oikos* 77:407–416.
- THIERY, D., and MOREAU, J. 2005. Relative performance of European grapevine moth (*Lobesia botrana*) on grapes and other hosts. *Oecologia* 143:548–557.
- TURLINGS, T. C. J., and WACKERS, F. 2004. Recruitment of predators and parasitoids by herbivore-injured plants, pp. 21–75, in R. T. Cardé and J. G. Millar (eds.). *Advances in insect chemical ecology*. Cambridge University Press, Cambridge.
- UTSUMI, S., and OHGUSHI, T. 2009. Community-wide impacts of herbivore-induced plant regrowth on arthropods in a multi-willow species system. *Oikos* 118:1805–1815.
- VAN DAM, N. M., VUISTER, L. W. M., BERGSHOEFF, C., DE VOS, H., and VAN DER MEIJDEN, E. 1995. The “Raison D’Etre” of Pyrrolizidine Alkaloids in *Cynoglossum officinale*: Deterrent effects against generalist herbivores. *J. Chem. Ecol.* 21:507–523.
- VET, L. E. M., and DICKE, M. 1992. Ecology of infochemical use by natural enemies in a tritrophic context. *Annu. Rev. Entomol.* 37:141–172.
- Waldbauer, G. P. 1968. The consumption and utilization of food by insects. *Adv. Insect Physiol* 5:229–288.
- WASANO, N., KONNO, K., NAKAMURA, M., HIRAYAMA, C., HATTORI, M., and TATEISHI, K. 2009. A unique latex protein, MLX56, defends mulberry trees from insects. *Phytochemistry* 70:880–888.
- WHITE, T. C. R. 1978. Importance of a relative shortage of food in animal ecology. *Oecologia* 33:71–86.
- ZVEREVA, E. L., and RANK, N. E. 2003. Host plant effects on parasitoid attack on the leaf beetle *Chrysomela lapponica*. *Oecologia* 135: 258–267.