J Chem Ecol (2016) 42:183–192
DOI 10.1007/s10886-016-0682-2

# The Gastropod Menace: Slugs on Brassica Plants Affect Caterpillar Survival Through Consumption and Interference With Parasitoid Attraction

**Gaylord A. Desurmont**[1] · **Miriam A. Zemanova**[2] · **Ted C. J. Turlings**[1]

Received: 12 October 2015 / Revised: 29 February 2016 / Accepted: 8 March 2016 / Published online: 22 March 2016


**Abstract** Terrestrial molluscs and insect herbivores play a major role as plant consumers in a number of ecosystems, but their direct and indirect interactions have hardly been explored. The omnivorous nature of slugs makes them potential disrupters of predator-prey relationships, as a direct threat to small insects and through indirect, plant-mediated effects. Here, we examined the effects of the presence of two species of slugs, *Arion rufus* (native) and *A. vulgaris* (invasive) on the survivorship of young *Pieris brassicae* caterpillars when feeding on *Brassica rapa* plants, and on plant attractiveness to the main natural enemy of *P. brassicae*, the parasitoid *Cotesia glomerata*. In two separate predation experiments, caterpillar mortality was significantly higher on plants co-infested with *A. rufus* or *A. vulgaris*. Moreover, caterpillar mortality correlated positively with slug mass and leaf consumption by *A. vulgaris*. At the third trophic level, plants infested with slugs and plants co-infested with slugs and caterpillars were far less attractive to parasitoids than plants damaged by caterpillars only, independently of slug species. Chemical analyses confirmed that volatile emissions, which provide foraging cues for parasitoids, were strongly reduced in co-infested plants. Our study shows that the presence of slugs has the potential to affect insect populations, directly via consumptive effects, and indirectly via changes in plant volatiles that result in a reduced attraction of natural enemies. The fitness cost for *P. brassicae* imposed by increased mortality in presence of slugs may be counterbalanced by the benefit of escaping its parasitoids.

**Keywords** Herbivore-induced plant volatiles · Indirect defense · Infochemical networks · Intraguild predation · Molluscan ecology · VOCs · Invasive slug

## Introduction

Along with mammalian herbivores, phytophagous insects and terrestrial molluscs belonging to the class Gastropoda (i.e., snails and slugs) are among the main plant consumers in terrestrial ecosystems (Buschmann et al. 2005; Hulme 1996). Both insect and gastropod herbivores are expected to play an important role in the evolution of plants defenses. Phytophagous insects attack and damage plants in a variety of ways, leading to a broad range of physical and chemical plant defenses (Agrawal 2007). The impact of slugs and snails on plant fitness, on the other hand, appears to be less diverse, but no less dramatic. Indeed, gastropods are major herbivores of seedlings (Barker 2001; Scheidel and Bruelheide 2005) and limit the ability of certain plants to persist in mollusc-rich habitats (Hanley et al. 1995; Hanley and Sykes 2009), and thus are key drivers of the evolution of seedling defenses.

Although they often occupy separate ecological niches, gastropod and insect herbivores may coexist on the same plants. For example, slugs of the genus *Arion* (Gastropoda: Arionidae) and caterpillars of the genus *Pieris* (Lepidoptera: Pieridae) are both considered major leaf-feeding pests of several cultivated crucifer species (Fig. 1; Ahuja et al. 2010;

**Electronic supplementary material** The online version of this article (doi:10.1007/s10886-016-0682-2) contains supplementary material, which is available to authorized users.

✉ Gaylord A. Desurmont
gaylord.desurmont@unine.ch

[1] Institute of Biology, University of Neuchâtel, Rue Emile-Argand 11, CH-2000 Neuchâtel, Switzerland

[2] Institute of Ecology and Evolution, University of Bern, Baltzerstrasse 6, CH-3012 Bern, Switzerland


**Fig. 1** Juvenile *Arion vulgaris* slug foraging on a *Brassica rapa* leaf infested by a group of 1st instar *Pieris brassicae* caterpillars. Photo: Neil Villard

Barker 2002). How these two types of herbivores directly and indirectly interact has been little explored, and most work on insect-mollusc associations focuses on insect predation on molluscs, often in the context of biological control in agroecosystems (Barker 2002; Pianezzola et al. 2013). Because of the inherent feeding characteristics of terrestrial molluscs, the nature and breadth of these herbivore-herbivore interactions may go beyond competition for the same food sources. For example, several species of terrestrial molluscs, including *Arion* spp. slugs, are omnivorous, and direct predation on insect herbivores cannot be excluded (Barker 2001; Furuichi 2014). Moreover, mollusc feeding can affect plant defenses (Falk et al. 2014; Kästner et al. 2014), and the elicitation of specific defensive pathways by slug mucus may have consequences for the performance of insect herbivores, or indirectly affect members of the third trophic level (Desurmont et al. 2014; Gols and Harvey 2009).

To gain more insight into how slugs can directly and indirectly affect insect herbivores, we examined the interactions between caterpillars and slugs when feeding on the same plants, and the consequences of this co-occurrence for natural enemies of caterpillars. The study involved the tri-trophic interactions between the field mustard *Brassica rapa* (Brassicales: Brassicaceae), a native lepidopteran herbivore, *Pieris brassicae*, and its larval parasitoid, the braconid *Cotesia glomerata* (Hymenoptera: Braconidae), and we investigated the effects of herbivory by two slug species, *Arion rufus* and *A. vulgaris* (or *A. lusitanicus* auct. Non-Mabille) (Fig. 1). *Arion rufus* is native to several European countries, including Switzerland where the study was conducted, whereas *A. vulgaris* is considered one of the hundred most invasive species in Europe (DAISIE 2009): its origin is still unclear (Quinteiro et al. 2005), but it has spread and become a serious pest in many European countries since the 1950's (Rabitsch 2006). Because *P. brassicae* has supposedly shared a lomger evolutionary history with *A. rufus* but not with *A. vulgaris*, we hypothesized that the two slug species may have a different impact on caterpillars. Specifically, we measured survivorship of *P. brassicae* caterpillars on control and slug-infested plants, and determined the effects of slug herbivory on the attractiveness of plants to *C. glomerata*. Because we observed a decrease in plant attractiveness to the parasitoid following slug herbivory, we also measured herbivore-induced plant volatiles emitted in response to *P. brassicae* caterpillars with and without co-infestation by slugs. In order to determine if slug traces (i.e., mucus and frass) are involved in the decrease in attractiveness to parasitoids, an additional olfactometer experiment was conducted to specifically test the effects of slug traces on parasitoid attraction.

## Methods and Materials

**Animal and Plant Material** *Arion rufus* and *A. vulgaris* are among the most common slug species found in the canton of Bern, Switzerland. Individuals used in the study were four-month-old juveniles reared at 18 °C, 16/8 h L/D and fed with lettuce, carrots, and mushrooms. The parental slugs had been collected during summer months of 2013 in the Bern area, Switzerland. As closely related *Arion* species might be difficult to distinguish morphologically, the identity of the parental slugs had been determined with a molecular marker. DNA was extracted using a proteinase K and a phenol-chloroform (Sambrook et al. 1989) or a high-salt extraction method (Aljanabi and Martinez 1997). After dilution in double-distilled water, the DNA was stored at −20 °C. Locus-specific primers developed by Quinteiro et al. (2005) were used to amplify a 400 bp long fragment: MOL-NAD1F (5′-CGRAARGGMCCTAACAARGTTGG-3′) and MOL-NAD1R (5′-GGRGCACGATTWGTCTCNGCTA-3′). PCRs contained ca. 100 ng of DNA, 1 U of GoTaq polymerase (Promega), 1 μM of each primer, 10X Buffer, 200 μM of dNTPs, and 3 mM $MgCl_2$ in total volume of 25 μl. The PCR thermal profile consisted of an initial denaturation step at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min. A last extension step at 72 °C for 10 min was added. The amplified fragments were cleaned with a Wizard SV Gel and PCR Clean-Up system (Promega). The purified products were placed in 40 μl of sterile water. Sequencing was performed in both directions. A 10-μl reaction volume, which contained 2.5 μl of Big Dye Terminator Kit® version 3.1 (Applied Biosystems), 1 μM primer, and approximately 100 ng of purified DNA, was submitted to the following conditions: denaturation at 96 °C for 1 min, 35 cycles of denaturation at 96 °C for 15 s, annealing at 50 °C for 15 s, and extension at 60 °C for 3 min. The sequencing reactions were visualized on ABI Prism 3100 (Applied Biosystem). Sequences were aligned manually in BioEdit 7.1.3.0 (Hall 1999) and compared with published reference sequences available in GenBank (http://www.ncbi.nlm.nih.gov/genbank/): *A. vulgaris* – accession numbers AY316239,

KJ843125, and KJ843127; *A. rufus* – accession numbers KJ843138, KJ843139, and KJ843140.

*Pieris brassicae* caterpillars originated from a laboratory rearing that was started with field individuals collected in the Zürich area, Switzerland. Insects were kept at ambient temperature under a standardized photoperiod (16 h L/8 h D, 240–260 μmol light intensity) with *B. rapa* var. *pekiniensis* as oviposition and food material, and young caterpillars were used for experimental purposes within 48 h after hatching (1st larval instar). *Cotesia glomerata* parasitoids came from a laboratory rearing started with field-collected individuals from the Netherlands and from the Neuchâtel area, Switzerland, and were reared on *P. brassicae* caterpillars. Newly-emerged adult parasitoids were left at ambient temperature for 48 h to feed and mate, and were provided water and honey ad libitum. Then, they were placed in an incubator at 13 °C until needed for experimental purposes. Parasitoids were 2 to 4-wk.-old at the time of the experiments, and parasitoid age was not randomized among experiments; a similar parasitoid age was used in previous studies that focused on *C. glomerata* in our laboratory, and parasitoids were found to show consistent olfactory preferences in behavioral bioassays (Chabaane et al. 2015; Desurmont et al. 2015). Only naïve females were used for experimental purposes. Plants used for the olfactometer tests and chemical analyses came from a wild accession of *Brassica rapa* highly attractive to *C. glomerata* (Chabaane et al. 2015), for which seeds were collected in 2009 and 2012 near Maarsen, the Netherlands. Plants used for the predation experiments came from commercially purchased *B. rapa* var. *cima de rapa* seeds for the first experiment and *B. rapa* var. *pekiniensis* for the second experiment. All plants were kept in growth chambers (25 °C, 16/8 h L/D, 240–260 μmol light intensity), and were grown in cylindrical plastic pots (4*10 cm), with fertilized commercial soil (Ricoter Aussaaterde, Aarberg, Switzerland). Plants were watered every other day without supplemental nutrients and had 4–6 fully developed leaves at the time of the experiments (3–4-wk.-old).

**Predation Experiments** Two separate predation experiments were conducted. In the first, six *B. rapa* plants were placed in glass aquaria (60 × 30 × 30 cm) covered with a thin removable Plexiglas sheet, and two of these plants were randomly selected and infested with 15 1st instar *P. brassicae* caterpillars. Eight juvenile slugs of either species (*A. rufus* or *A. vulgaris*) were then placed in a petri dish in the center of the aquaria, except in control aquaria in which no slugs were placed. Slugs were free to move to all plants. We only infested two out of six *B. rapa* plants with caterpillars in order to add to the realism of the experiment: in nature, it is unlikely that slugs would encounter *Pieris* caterpillars on every plant they forage on. In this setting, both caterpillars and slugs had an abundance of leaf material on which to feed and were not artificially forced to interact. To account for the possibility that larger slugs may predate more on young caterpillars, the weight of each slug was measured prior to the experiment (but not afterwards). After one, three, and five days, the number of living caterpillars remaining on the *B. rapa* plants was counted. This experiment was repeated twice, with four replicates (1 replicate =1 aquarium) of each treatment (*A. rufus*, *A. vulgaris*, control; $N = 8$). The second experiment was designed to measure the predation risk associated with each slug species, while also measuring plant damage, and was conducted under more artificial conditions. In this second setting, three slugs of either *A. rufus* or *A. vulgaris* and 10 1st instar *P. brassicae* caterpillars were randomly deposited on single 3-wk.-old *B. rapa* var. *pekiniensis* plants in closed glass containers (28 × 9 cm). The weight of each slug was measured prior to the experiment (but not afterwards). Fifteen replicates for each slug species and five controls without slugs were used. After 24 h, the number of living and dead caterpillars remaining on the plants was counted. Damaged leaf area was quantified after the experiment using Abode PhotoShop CC (Adobe Systems, Inc., San Jose, CA, USA). Because this second experiment was conducted in smaller containers, it allowed us to carefully determine the presence or absence of caterpillar cadavers at the end of the experiment.

**Olfactometer Tests** The preferences of *C. glomerata* females toward certain odor blends were investigated using a 4-arm olfactometer setting (D'Alessandro and Turlings 2005). In this setting, wasps were given the choice between 4 odor sources (= treatments) contained in separated glass bottles, each connected to an individual air flow, and all converging to a central glass piece where the wasps were released. After 30 min spent in the olfactometer, wasps were recollected and the treatment they chose was recorded. Wasps that did not make a choice were recorded as "no choice" in the analysis of the results. One olfactometer test (= 1 replicate) consisted in 5 consecutive releases of 5 wasps (25 wasps tested in total). Plants were changed, and glassware was cleaned between replicates, and a minimum of 5 replicates was conducted for each of the experiments. To investigate the effects of separate slug herbivory (i.e., caterpillars and slugs feeding on separate plants) on parasitoid attraction, wasps were given the choice between: empty odor source (blank), undamaged plant (control), plant damaged by *P. brassicae*, and plant damaged by slugs either belonging to *A. rufus* or *A. vulgaris*. To investigate effects of simultaneous herbivory (i.e., caterpillars and slugs feeding on the same plant), wasps were given the choice between: empty odor source (blank), undamaged plant (control), plant damaged by *P. brassicae*, and plant damaged simultaneously by *P. brassicae* and slugs. To prepare the herbivory treatments, 15 1st instar *P. brassicae* or three juvenile slugs were placed on the plant 12–18 h before the test. To prevent slug-induced caterpillar mortality on co-infested plants, caterpillars were enclosed in a clip cage on the plants whereas slugs were left free to forage on the entire plant. Caterpillars also were

enclosed in a clip cage (21 mm inner diam) for the plants infested solely by *P. brassicae* caterpillars, and a clip cage was added to the control plant. Clip cages were removed from the plant just before the olfactometer tests, but not the caterpillars nor the slugs. Leaf area damaged at the end of the experiments was readily quantified using Abode PhotoShop CC (Adobe Systems, Inc., San Jose, CA, USA). Finally, to test for the effects of slug traces (i.e., mucus and frass) on parasitoid preferences, an additional olfactometer experiment was designed. For this experiment, parasitoids were given the choice between: a plant damaged by *P. brassicae* caterpillars, a plant damaged by *P. brassicae* with the addition of slug traces, an undamaged plant, and an empty arm. To prepare the slug traces treatment, 3 juvenile slugs were left with no food in a closed plastic container with three standard microscope slides (75 × 25 mm) for 12 h prior to the olfactometer test. Water was sprayed in the container before closing it to insure high humidity inside the container. Slides were removed from the containers immediately before the tests. The presence of mucus and frass was checked visually on the slides, and the slides were added to a bottle containing a plant infested by *P. brassicae*. For this experiment, *P. brassicae* were left free to forage on the plant (no clip cages).

**Chemical Analyses** To identify and quantify the blends of volatile organic compounds (VOCs) emitted by undamaged *B. rapa* plants ($N = 7$) or plants under different herbivory treatments, potted plants were placed in a VOC collection setup (Ton et al. 2007) for 5 h. VOCs were collected using a trapping filter containing 25 mg of 80–100 mesh SuperQ absorbent. Before use, trapping filters were cleaned with 300 µl of methylene chloride (HPLC grade). After each collection, VOCs were extracted from the filters with 150 µl of methylene chloride. Two internal standards (200 ng of n-octane and nonyl acetate in 10 µl methylene chloride) were added to each sample. VOCs were analyzed with an Agilent 6890 gas chromatograph series GC system G1530A coupled to a mass spectrometer (GC-MS, Agilent 5973 Network Mass Selective Detector; transfer line 230 °C, source 230 °C, ionization potential 70 eV). A 2-µl aliquot of each sample was injected in the pulsed splitless mode onto a non-polar column (HP-1 ms, 30 m, 0.25 mm ID, 0.25 µm film thickness, Agilent J&W Scientific, USA). Helium was used as carrier gas at constant pressure (15 psi). After injection, temperature was maintained at 40 °C for 3 min, then increased to 100 °C at 8 °C/min, and then to 220 °C at 5 °C/min. The quantities of the major components of the blends were estimated based on the peak areas of the compounds compared to the peak areas of the internal standards. Compounds were identified tentatively by comparing the spectra obtained from the samples with those from a reference database (NIST mass spectral library), and classified in different categories according to their structure. Five categories of compounds were found: alkanes, aromatics, glucosinolates breakdown products (isothiocyanates and nitriles), green leaf volatiles (GLVs), and terpenoids. Herbivory treatments were prepared by infesting the plants with 15 first instar *P. brassicae* ($N = 8$) or with 15 first instar *P. brassicae* and three juvenile *A. vulgaris* slugs ($N = 9$), following the infestation procedure of the olfactometer experiments. In addition, VOCs from three *A. vulgaris* slugs in a glass bottle with no plant material ($N = 6$) and from empty glass bottles ($N = 6$) were collected using the same setup. Compounds that were only present in a few samples (<50 % of the samples of a single treatment) or were associated with the presence of a clip cage on the plant were discarded from the analysis.

**Statistical Analyses** Effects of presence of slugs on caterpillar survivorship were analyzed using a *parametric survival analysis* with a Weibull distribution (JMP9). In this model, the response variable was the number of days that individual caterpillars survived, and this variable was censored if caterpillars were still alive at the end of the experiment. The fixed effect in this model was the treatment (*A. rufus*, *A. vulgaris*, or control). In the second experiment, effects of slugs on caterpillar survivorship were analyzed using a *One-way ANOVA* with caterpillar survivorship as the dependant variable, and means were compared with the *post hoc Tukey-Kramer HSD all-pairwise comparison* procedure ($\alpha = 0.05$, JMP9). Associations between slug weight, leaf damage, and caterpillar survivorship were then investigated for each slug species by conducting *linear regression analyses*. Differences in foliar damage at the end of the experiment were analyzed using a *one-way ANOVA* ($\alpha = 0.05$, JMP9). Results of the olfactometer tests were analyzed by conducting a *One-way ANOVA* with number of wasps (mean per replicate) as the dependent variable, and treatment as the independent variable, and means were compared with *the post hoc Tukey-Kramer HSD all-pairwise comparison* procedure ($\alpha = 0.05$, JMP9). Regarding the results of the chemical analyses, the total amounts of plant volatiles emitted and the amounts of volatiles belonging to each category identified (green leaf volatiles, terpenoids, isothiocyanates, aromatics, and alkanes) also were compared among treatments using *One-way ANOVA* and *the post hoc Tukey-Kramer HSD all-pairwise comparison* procedure ($\alpha = 0.05$, JMP9). Differences in amounts of individual compounds were analyzed using non-parametric *Wilcoxon each pair* tests. Results of the second predation experiment were arcsin transformed, and data of the olfactometer tests and of the chemistry analysis with groups of compounds were square root transformed to meet the assumptions of the models.

## Results

**Predation Experiment** In the first experiment, survivorship of young *P. brassicae* caterpillars was significantly impacted by the presence of slugs (*d.f.* = 2, $\chi^2$ = 107.4, $P < .001$). In presence of *A. rufus*, caterpillar survivorship decreased by 27.0 % after one day, 39.7 % after three days, and 53.4 % after five days compared to control. In presence of *A. vulgaris*, caterpillar survivorship decreased by 22.5 % after one day, 54.3 % after three days, and 62.6 % after five days compared to control (Fig. 2a). Slugs of both species were very active, and we observed regular movement from plant to plant within each aquarium. In the second experiment, survivorship of *P. brassicae* significantly declined after 24 h in presence of slugs on *B. rapa* leaves ($F_{2,32} = 9.6$, $P < .001$), and *A. vulgaris* had a stronger effect on caterpillar survivorship than *A. rufus* (Fig. 2b). Overall caterpillar survivorship was 23.9 % and 42.1 % lower in presence of *A. rufus* and *A. vulgaris* compared to control plants, respectively. That represents a total of 103 caterpillars missing (out of 300 initially deposited) on slug-infested plants after 24 h. Only four dead caterpillars were found on slug-infested plants: these four appeared to be caught in slug mucus. Thus, we conclude that the vast majority of missing caterpillars was consumed by slugs. Leaf damage (%) at the end of the experiment was significantly lower on plants infested by *P. brassicae* (3.0 ± 0.7, mean ± SE) than on plants infested by *P. brassicae* and *A. rufus* (11.7 ± 1.3) or *P. brassicae* and *A. vulgaris* (14.6 ± 1.3; $F_{2,31} = 6.5$, $P < 0.01$). There was no significant difference in leaf damage between plants infested by *A. rufus* and *A. vulgaris* ($F_{1,25} = 2.3$, $P = 0.14$). Initial weight was different for the two slug species: *A. rufus* slugs were significantly heavier than *A. vulgaris* at the beginning of the experiment (2.2 ± 0.1 and 1.7 ± 0.2 mg, respectively; $F_{1,28} = 4.3$, $P = 0.048$). For plants infested by *A. vulgaris*, we found negative associations between caterpillar survivorship and initial slug weight ($R^2 = 0.23$, $F_{1,13} = 5.2$, $P = 0.03$), and between caterpillar survivorship and leaf damage ($R^2 = 0.19$, $F_{1,13} = 4.3$, $P = 0.05$; Fig. 3a, b), and we found a positive association between initial slug weight and leaf damage ($R^2 = 0.53$, $F_{1,13} = 16.7$, $P < 0.001$). These associations were absent in plants infested by *A. rufus* (*P*s > 0.05; Fig. 3c, d).

**Olfactometer Tests** When testing the effects of separate slug herbivory, parasitoids showed a similar pattern of preferences with both slug species: with *A. rufus*, *P. brassicae*-infested plants were by far the most attractive treatment (81.4 ± 8.7 % attractiveness) followed by slug-damaged plant (17.4 ± 4.9), undamaged plant (1.2 ± 1.2), and empty arm ($F_{3,16} = 46.7$, $P < 0.001$); with *A. vulgaris*, *P. brassicae*-infested plants were again the most attractive treatment (71.0 ± 6.6), whereas slug-infested plant (16.7 ± 4.9), undamaged plants (9.6 ± 3.6), and empty arm (2.6 ± 1.9) did not significantly differ ($F_{3,16} = 27.9$, $P < 0.001$; Fig. 4 a, b). When testing the effects of simultaneous slug herbivory, parasitoids showed the following patterns of preferences: with *A. rufus*, *P. brassicae*-infested plants were the most attractive treatment (53.3 ± 6.8), and plants damaged by slugs and caterpillars (14.3 ± 4.1), undamaged plants (18 ± 4.6), and empty arm (14.3 ± 4.9) did not differ in attractiveness ($F_{3,16} = 5.4$, $P < .01$). With *A. vulgaris*, *P. brassicae*-infested plants were again the most attractive treatment (44.4 ± 5.1), and plants damaged by slugs and caterpillars (21.7 ± 3.7), undamaged plant (16.2 ± 3.2), and empty arm (17.6 ± 3.2) did not differ in attractiveness ($F_{3,36} = 3.8$, $P = 0.02$; Fig. 4 c, d). In other words, the presence of slugs on the same plant as *P. brassicae* reduced its attractiveness to parasitoids by 73.1 % in the case of *A. rufus*, and by 51.1 % in the case of *A. vulgaris*. Finally, olfactometer tests with slug traces gave the following results: *C. glomerata* wasps equally preferred plants infested by *P. brassicae* (42.1 ± 7.6) and plants infested by *P. brassicae* with the addition of *A. vulgaris* traces (38.2 ± 9.6), whereas undamaged plants (11.8 ± 4.9) and empty arm (7.8 ± 3.4) were the least attractive treatments ($F_{3,16} = 4.1$, $P = 0.02$).

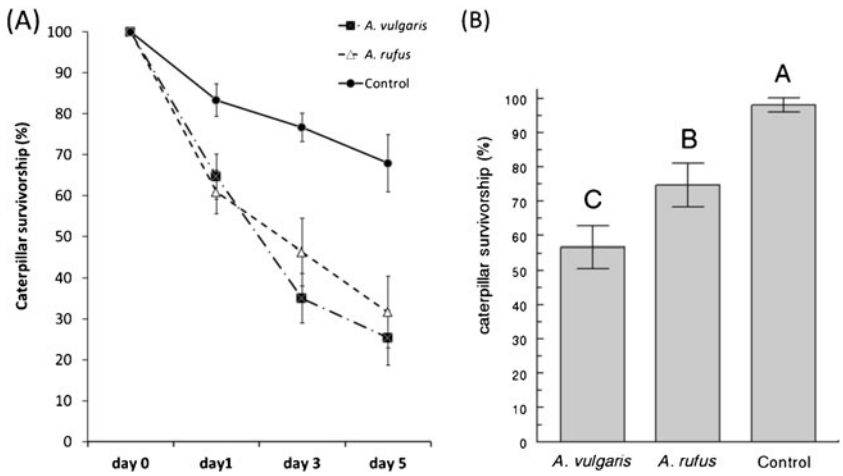


**Fig. 2** Survivorship (%, mean ± SE) of 1st instar *Pieris brassicae* caterpillars on *Brassica rapa* plants in the presence of *Arion rufus*, *A. vulgaris*, or in the absence of slugs (*Control*) in two separate experiments: **a** after 1, 3, and 5 d in an aquarium setting with six plants, and **b** in glass bottles after 24 h on individual plants. Treatments followed by a different letter are significantly different (One-way ANOVA, $\alpha = 0.05$, JMP 9)

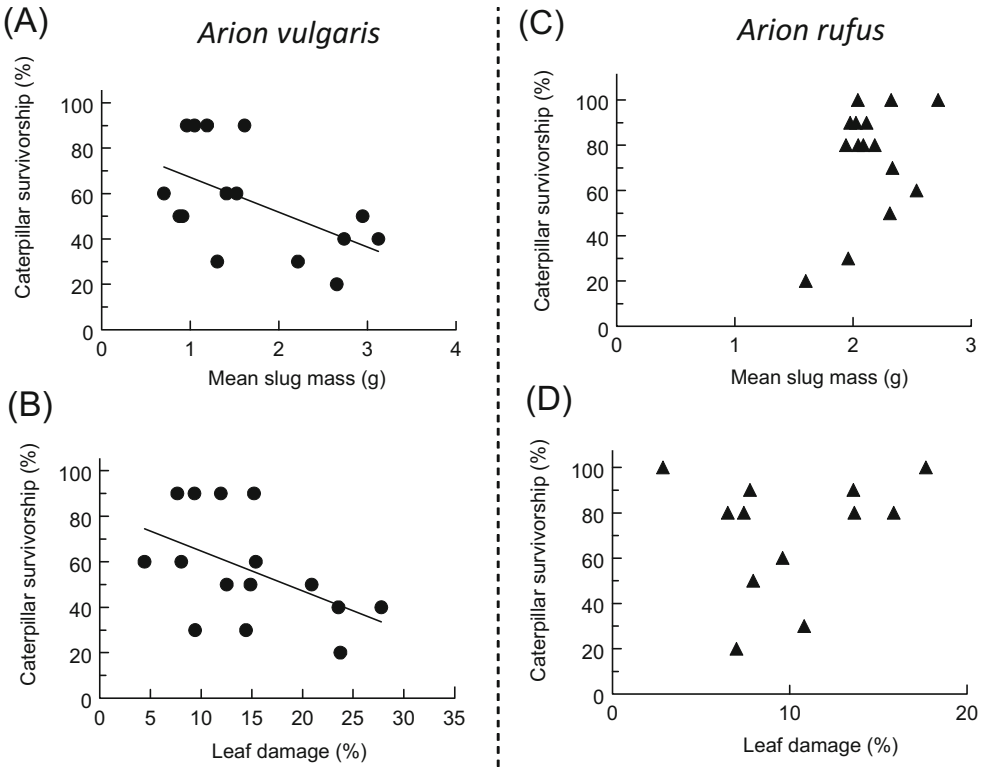


**Fig. 3** Associations between slug mass (g) and caterpillar survivorship (%), and between leaf damage (%) and caterpillar survivorship for **a**,**b** *Arion vulgaris* ($N = 15$), and **c**,**d** *A. rufus* ($N = 15$). Solid lines indicate significant correlations ($\alpha = 0.05$, JMP 9)

**Chemical Analyses** Five categories of compounds were consistently found and identified in the samples: alkanes (5 compounds), aromatics (1), glucosinolate breakdown products (isothiocyanates and nitriles, 3), green leaf volatiles (GLVs, 4), and terpenoids (1; Table 1). Total volatile emission was the highest for plants infested by *P. brassicae* 1st instars (19.3 ± 5.9 μg internal standard equivalents, mean ± SE), was lower for plants infested by caterpillars and slugs simultaneously (9.9 ± 2.6), and was the lowest for undamaged plants (1.9 ± 1.0), bottles containing slugs alone (0.1 ± 0.1), and empty bottles (0.4 ± 0.4; Table 1, Fig. S1). In other words, volatile emission was reduced by 49.0 % in plants infested by both slugs and caterpillars compared to plants infested only by caterpillars. This pattern was consistent for green leaf volatiles (64.7 % reduction) and glucosinolate breakdown products (69.1 % reduction), but less obvious for the other three categories of compounds measured (Table 1). Differences in individual compounds revealed the same trends, with the majority of individual glucosinolate breakdown products and green leaf volatiles compounds being produced in substantially larger amounts by *Pieris* infested plants than by dually infested plants (Table S1). In particular, the nitrile methallyl cyanide was found to be produced over 6 times more in *Pieris*-infested plants than in dually infested plants (1.3 ± 0.6 vs 0.2 ± 0.1, Table S1). The only volatiles that were found in bottles containing slugs only were traces of green leaf volatiles that also were present in empty bottles, and therefore are considered to be contaminants.

## Discussion

It is well accepted that terrestrial molluscs play a fundamental role in various aspects of soil and vegetation ecology, including but not limited to: direct effects on plant fitness (Buschmann et al. 2005; Fritz et al. 2001; Moshgani et al. 2014), seedling herbivory (Hanley and Sykes 2009; Hulme 1996), seed dispersal (Türke et al. 2010), litter decomposition and nutrient cycles (Theenhaus and Scheu 1996). However, their effects on insect-plant interactions have rarely been examined. Here, we discovered not only that the presence of two species of slugs on *B. rapa* directly affects the survivorship of *P. brassicae* caterpillars, but also that slugs indirectly affect the caterpillars by interfering with the attraction of their main natural enemy, the parasitoid *C. glomerata*, through changes in herbivore-induced plant volatiles.

There was a strong negative effect of slug presence on caterpillar survivorship in both predation experiments after one or a few days of coexistence within the same arena (Fig. 2). Caterpillar cadavers rarely were found on the plants, suggesting that the missing caterpillars were consumed, but predation was not directly observed. Both *Arion* species used in the study as well as other species of terrestrial molluscs are known to be omnivorous, and the presence of insect material in excrements commonly occurs in nature (Barker 2001). Direct predation of molluscs on live insect material has been observed for a range of insects, including: egg masses of the lepidopteran *Homona magnanima* (Kosugi 2011), aphids

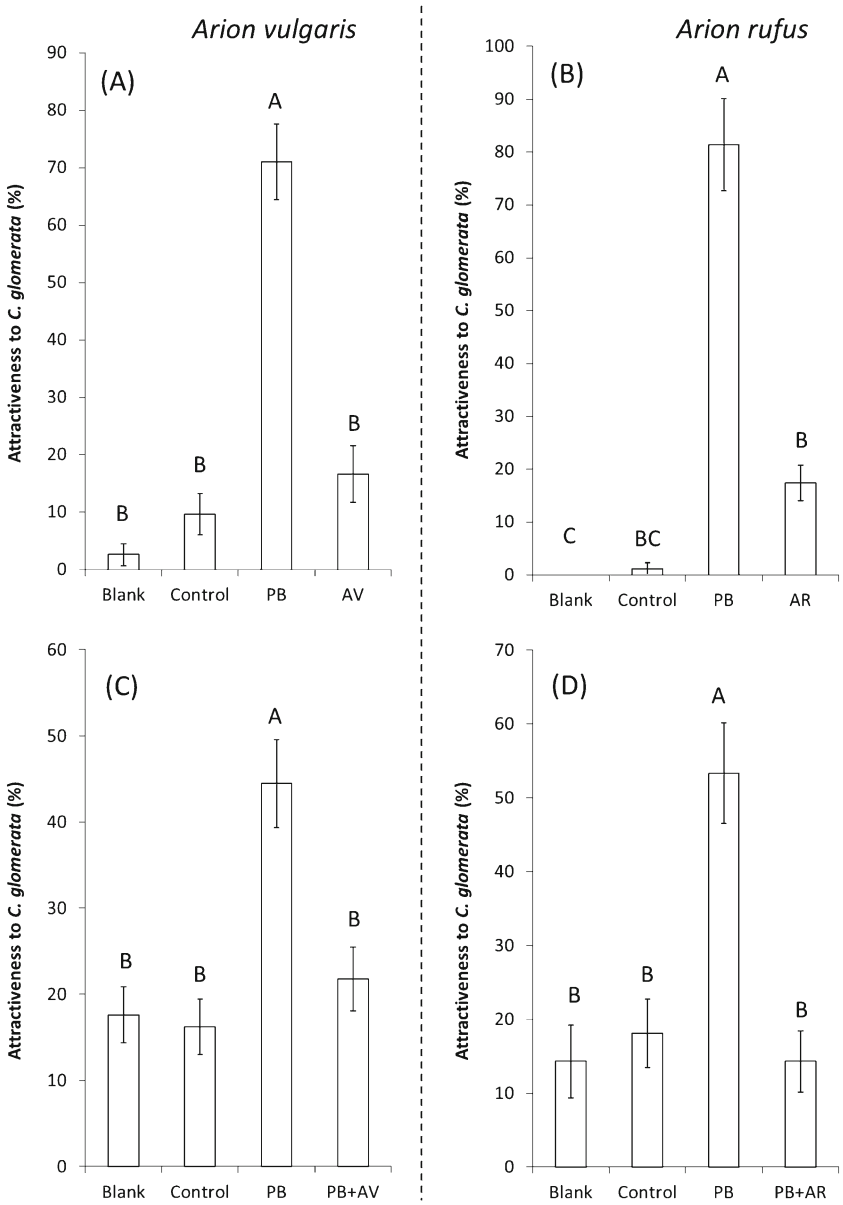


**Fig. 4** Percentage of *Cotesia glomerata* females attracted towards different treatments in a 4-arm olfactometer (Mean ± SE). Treatments represent: Blank = empty odor source, Control = non-infested plant, PB = *Pieris brassicae*-infested plant, AV = *Arion vulgaris*-infested plant, AR = *Arion rufus*-infested plant, PB + AV = plant infested by *P. brassicae* ± *A. vulgaris*, PB + AR = plant infested by *P. brassicae* ± *A. rufus*. Treatments followed by a different letter are significantly different (One-way ANOVA, $\alpha = 0.05$, JMP 9). **a** Effects of separate herbivory by *A. vulgaris* on *C. glomerata* preferences ($N = 5$), **b** Effects of separate herbivory by *A. rufus* ($N = 5$), **c** Effects of simultaneous herbivory with *A. vulgaris* ($n = 10$), and **d** Effects of simultaneous herbivory with *A. rufus* ($N = 5$)

(Fox and Landis 1973), grubs of the turnip gall weevil (Fox and Landis 1973), and paper wasp larvae (Furuichi 2014). Some of these observations may be simple cases of "accidental" predation, but some cases seem indicative of active predation targeted at live insects (e.g., predation on paper wasp larvae within a nest). In our system, both accidental and active predation may have occurred. A third possibility is that caterpillars died in slug mucus and that their corpses were later consumed by slugs, but video recorded observations revealed that *P. brassicae* 1st instars can survive the passing of a slug without getting caught in mucus (Fig. S3). In the case of *A. vulgaris*, larger slugs fed more on *B. rapa* leaves, and also caused higher caterpillar mortality (Fig. 3): a possible interpretation of this result is that young caterpillars were ingested while the slug was feeding on plant material, hence supporting the idea of accidental predation. An alternative explanation could be that slugs that fed more were simply more active and also more prone to prey upon live insects to supplement their diet. However, these correlations were absent for *A. rufus*, and more precise observations are required to determine the exact circumstances of slug-induced mortality of *P. brassicae* caterpillars. Overall, the invasive *A. vulgaris* had a greater effect on caterpillar survivorship than the native *A. rufus*, although this effect was less obvious in the first predation experiment. *Arion vulgaris* is considered an invasive species in several European countries, displacing native slug species such as *A. rufus* and affecting plant communities in a variety of environments (Kozlowski 2007, 2012). The

**Table 1** Volatile organic compounds (mean ± se, μg internal standard equivalents/5 hour collection) emitted by undamaged *Brassica rapa* plants, plants infested by *Pieris brassicae*, plants infested by *P. brassicae* and *Arion vulgaris* simultaneously, *A. vulgaris* alone (without a plant), and empty odor sources (blank)

| Compound class | *F*-value $F_{4,31}$ | *P*-value | *B. rapa* | | | *A. vulgaris* | Blank |
|---|---|---|---|---|---|---|---|
| | | | Undamaged | *P. brassicae* | *P. brassicae* ± *A. vulgaris* | | |
| Aromatics | 3.4 | **0.01** | 0.0 ± 0.0 b | 0.3 ± 0.3 a | 0.2 ± 0.2 ab | 0.0 ± 0.0 b | 0.0 ± 0.0 b |
| Green leaf volatiles | 7.1 | **<.001** | 0.1 ± 0.1 b | 6.5 ± 2.0 a | 2.3 ± 0.5 b | 0.1 ± 0.1 b | 0.4 ± 0.4 b |
| Glucosinolate breakdown products | 3.6 | **0.01** | 0.3 ± 0.3 b | 5.5 ± 2.4 a | 1.7 ± 0.7 ab | 0.0 ± 0.0 b | 0.0 ± 0.0 b |
| Terpenoids | 2.4 | 0.06 | 0.0 ± 0.0 | 0.8 ± 0.4 | 1.7 ± 0.5 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Alkanes | 16.5 | **<.001** | 1.6 ± 0.7 b | 5.9 ± 0.8 a | 4.3 ± 0.7 a | 0.0 ± 0.0 b | 0.0 ± 0.0 b |
| Total compounds | 14.1 | **<.001** | 1.9 ± 1.0 bc | 19.3 ± 5.9 a | 9.9 ± 2.6 b | 0.1 ± 0.1 c | 0.4 ± 0.4 c |

Compounds with significant differences among treatments are indicated in bold. Means followed by a different letter within a same line are statistically different (One-Way ANOVA, Tukey-Kramer HSD all-pairwise comparison, $\alpha = 0.05$, JMP9)

observed greater impact of *A. vulgaris* on native insect populations may add to the harmfulness of this invader under natural conditions.

First instar *P. brassicae* caterpillars are very small and their mobility is limited (Fig. 1): vulnerability to slugs certainly decreases as caterpillars develop and get larger and more mobile. Results of our first predation experiment seem to support this hypothesis, as most of slug-induced caterpillar mortality occurred within the first three days of the experiment. However, the short temporal window corresponding to the 1st larval instar, which typically lasts no more than 48 h, is critical because it corresponds to the period of peak susceptibility to parasitism by *C. glomerata* (Benrey and Denno 1997).

Results of our olfactometer tests show not only that plants infested by slugs are not attractive to parasitoids but also that plants co-infested by caterpillars and slugs are remarkably less attractive than plants infested only by caterpillars (Fig. 4). This result cannot be explained by the presence of odors associated with slug traces (mucus + excrements), which, when added to *Pieris*-infested plants, did not affect parasitoid attraction. Odors originating from the slugs themselves could potentially have played a role (Schroeder et al. 1999), although none were consistently detected in our volatile collection bioassays. Remarkably, the presence of slugs had a drastic effect on the production of plant volatiles: co-infested plants suffered on average four times more damage but released only half the amount of volatile compounds of plants only infested by *Pieris* caterpillars (Table 1, Fig. S1, Table S2). This decrease was particularly significant for two classes of compounds, green leaf volatiles and glucosinolate breakdown products, which are known to play a role in the attraction of natural enemies (Allmann and Baldwin 2010; Desurmont et al. 2014; Pichersky and Gershenzon 2002), including parasitoids from the *Cotesia* genus (Mumm et al. 2008). Green leaf volatiles are compounds known to be released by a wide range of plants upon cellular damage (Matsui 2006). The fact that these compounds were released in lesser amounts in co-infested plants is surprising considering that co-infested plants had much higher amounts of leaf damage than *Pieris*-infested plants. Furthermore, the suppression of glucosinolates breakdown products, including the nitrile methallyl cyanide (Table S2) is of particular importance for the attraction of *Cotesia* parasitoids. *Pieris* caterpillars are able to form nitriles instead of toxic isothiocyanates upon the ingestion and breakdown of glucosinolates due to a larval gut protein, designated nitrile-specifier protein, which appears to be a specific key biochemical counteradaptation to plant defenses (Wittstock et al. 2004). Nitriles are highly volatile and have been hypothesized to be a reliable indicator of the presence of *Pieris* caterpillars for *Cotesia* parasitoids (Mumm et al. 2008). The strong reduction in emission of both green leaf volatiles and glucosinolates breakdown products following slug herbivory may thus have caused decreased attraction of *C. glomerata* parasitoids to co-infested plants.

How does slug herbivory affect volatile emission? It is possible that the mucus deposited by slugs at the sites of feeding has proprieties that impact the emission and dispersion of plant volatiles. Alternatively, it may be that slugs affect plant responses to herbivory in ways that alter the production of plant volatiles. The effects of slug herbivory on direct defenses have received increasing attention (Falk et al. 2014; Fritz et al. 2001; Kästner et al. 2014). Falk et al. (2014) showed that application of *A. vulgaris* mucus on *Arabidopsis thaliana* leaves elicits the induction of the jasmonic acid (JA) defense pathway, which is a typical plant response to leaf-chewing herbivores such as *Pieris* caterpillars. Glucosinolates, which are JA-mediated defensive compounds, were shown to affect herbivory from gastropods in the field. The association between glucosinolate levels and resistance to slug herbivory was also demonstrated by Moshgani et al. (2014). These findings suggest that gastropod mucus should induce the JA pathway in *B. rapa* and, consequently, that the emission of volatiles associated with the JA pathway should increase in co-

infested plants. However, results of our volatile collection bioassays seem to indicate the opposite trend. Direct measurements of JA levels and JA-associated metabolites in *B. rapa* leaves before and after slug herbivory would help clarify the defensive pathways induced by slugs in *B. rapa*. Interestingly, a comparative study of the mucus composition of several snail and slug species revealed that the mucus of the slug *Deroceras reticulatum* contains salicylic acid (SA), an elicitor of the SA defensive pathway in plants, which may interfere with the JA pathway through cross-talk (Kästner et al. 2014). This finding shows that terrestrial mollusks may have complex ways of manipulating plant induced defenses.

Our results imply that caterpillars on slug-infested plants are less detectable to parasitoids, and will suffer reduced parasitism rates compared to caterpillars present on slug-free plants under natural conditions, although reduction in plant attractiveness does not necessarily mean complete disruption of parasitoid attraction (Fatouros et al. 2012; Pangesti et al. 2015). This adds to the body of studies documenting parasitoid preferences in presence of plants infested by multiple herbivores, which present contrasting effects of non-hosts herbivores ranging from attraction to non-host infested plants to deterrence (de Rijk et al. 2013). Avoidance of slug-infested plants may have an adaptive value for *C. glomerata*: by preferentially choosing plants where slugs are absent, parasitoids may reduce slug-induced mortality for parasitized caterpillars, and therefore maximize their own fitness. From the insect herbivore's perspective, the fitness cost imposed by increased mortality in presence of slugs may be counterbalanced by the benefit of escaping parasitic wasps. Additional studies in more realistic settings, including field experiments in slug-rich and slug-poor environments, are needed to further explore the ecological and evolutionary significance of these interactions. In addition to predation risk on young caterpillars, predation risks on *P. brassicae* eggs also should be examined, as cases of terrestrial mollusks predating on insect eggs have been documented (Kosugi 2011).

In summary, the study constitutes a first step to integrate terrestrial molluscs in the general picture of insect-plant interactions. Results show for the first time that the presence of slugs poses a direct threat to the survivorship of young insect caterpillars and that slug presence also affects the third trophic level and interferes with the foraging behavior of parasitic wasps. Studies of plant-insect interactions and plant-molluscs interactions have historically often been treated as separate entities, but the interactions between these two types of herbivores do commonly occur in nature, and their importance should not be underestimated. The effects of herbivores on plant defenses and their indirect consequences at the third trophic level have mostly been investigated with insects, but the prevalence of terrestrial molluscs and the multiple ways they can affect communities in terrestrial habitats make them a possible key player in multitrophic interactions in native foodwebs.

**Acknowledgments** The authors thank Diane Laplanche, Antoine Guiguet, Leslie Mann, and Gabriel Marcacci for their help with rearing the animals used in the study and performing the predation experiments. Xu Hao and Marie-Jeanne Tschudi helped with growing the plants used, and Neil Villard helped with taking pictures and videos of the bioassays. M. A. Z. is grateful to Gerald Heckel for encouraging her to explore different aspects of the *A. vulgaris* invasion within a PhD project.

**Compliance with Ethical Standards**

**Conflict of Interest** The authors declare that they have no conflict of interest.

## References

Agrawal AA (2007) Macroevolution of plant defense strategies. Trends Ecol Evol 22:103–109

Ahuja I, Rohloff J, Bones AM (2010) Defence mechanisms of *Brassicaceae*: implications for plant-insect interactions and potential for integrated pest management. A review. Agron Sustain Dev 30: 311–348

Aljanabi SM, Martinez I (1997) Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. Nucl Acids Res 25:4692–4693

Allmann S, Baldwin IT (2010) Insects betray themselves in nature to predators by rapid isomerization of green leaf volatiles. Science 329:1075–1078

Barker GM (2001) The biology of terrestrial molluscs. CABI publishing, Wallingford UK

Barker GM (2002) Molluscs as crop pests. CABI publishing, Wallingford UK

Benrey B, Denno RF (1997) The slow-growth-high-mortality hypothesis: A test using the cabbage butterfly. Ecology 78:987–999

Buschmann H, Keller M, Porret N, Dietz H, Edwards PJ (2005) The effect of slug grazing on vegetation development and plant species diversity in an experimental grassland. Funct Ecol 19:291–298

Chabaane Y, Laplanche D, Turlings TCJ, Desurmont GA (2015) Impact of exotic insect herbivores on native tritrophic interactions: A case study of the African cotton leafworm, *Spodoptera littoralis* and insects associated with the field mustard *Brassica rapa*. J Ecol 103:109–117

D'Alessandro M, Turlings TCJ (2005) *In situ* modification of herbivore-induced plant odors: A novel approach to study the attractiveness of volatile organic compounds to parasitic wasps. Chem Sens 30:739–753

DAISIE (2009) Handbook of alien species in Europe. Springer, Dordrecht

DE Rijk M, Dicke M, Poelman EH (2013) Foraging behaviour by parasitoids in multiherbivore communities. An Behav 85:1517–1528

Desurmont GA, Harvey J, VAN Dam NM, Cristescu SM, Schiestl FP, Cozzolino S, Anderson P, Larsson MC, Kindlmann P, Danner H, Turlings TCJ (2014) Alien interference: disruption of infochemical networks by invasive insect herbivores. Plant Cell Environ 37: 1854–1865

Desurmont GA, Laplanche D, Schiestl FP, Turlings TCJ (2015) Floral volatiles interfere with plant attraction of parasitoids: ontogeny-dependent infochemical dynamics in *Brassica rapa*. BMC Ecol 15:1–11

Falk KL, Kaestner J, Bodenhausen N, Schramm K, Paetz C, Vassao DG, Reichelt M, VON Knorre D, Bergelson J, Erb M, Gershenzon J, Meldau S (2014) The role of glucosinolates and the jasmonic acid

pathway in resistance of *Arabidopsis thaliana* against molluscan herbivores. Mol Ecol 23:1188–1203

Fatouros NE, Lucas-Barbosa D, Weldegergis BT, Pashalidou FG, VAN Loon JJ, Dicke M, Harvey JA, Gols R, Huigens ME (2012) Plant volatiles induced by herbivore egg deposition affect insects of different trophic levels. PLoS one 7:e43607

Fox L, Landis BJ (1973) Notes on the predaceous habits of the gray field slug *Deroceras laeve*. Environ Entomol 2:306–307

Fritz RS, Hochwender CG, Lewkiewicz DA, Bothwell S, Orians CM (2001) Seedling herbivory by slugs in a willow hybrid system: developmental changes in damage chemical defense and plant performance. Oecologia 129:87–97

Furuichi S (2014) Field observation of predation on paper wasp nests by introduced terrestrial slugs. Insect Soc 61:95–96

Gols R, Harvey JA (2009) Plant-mediated effects in the *Brassicaceae* on the performance and behaviour of parasitoids. Phytochem Rev 8: 187–206

Hall TA (1999) BioEdit: A user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucl Acids S 41:95–98

Hanley ME, Sykes RJ (2009) Impacts of seedling herbivory on plant competition and implications for species coexistence. Ann Bot-London 103:1347–1353

Hanley ME, Fenner M, Edwards PJ (1995) An experimental field study of the effects of mollusk grazing on seedling recruitment and survival in grassland. J Ecol 83:621–627

Hulme PE (1996) Herbivores and the performance of grassland plants: a comparison of arthropod mollusc and rodent herbivory. J Ecol 84: 43–51

Kästner J, VON Knorre D, Himanshu H, Erb M, Baldwin IT, Meldau S (2014) Salicylic acid a plant defense hormone is specifically secreted by a molluscan herbivore. PLoS one 9:e86500

Kosugi Y (2011) Predation of egg masses of oriental tea *Tortrix homona magnanima* diakonoff and smaller tea *Tortrix adoxophyes honmai* Yasuda by Valencia slug *Lehmannia valentiana* (Férussac). Tea Research J 2008:27–34

Kozlowski J (2007) The distribution biology population dynamics and harmfulness of *Arion lusitanicus* mabille 1868 (gastropoda: pulmonata: arionidae) in Poland. J Plant Protection Res 47:219–230

Kozlowski J (2012) The significance of alien and invasive slug species for plant communities in agrocenoses. J Plant Protection Res 52:67–76

Matsui K (2006) Green leaf volatiles: hydroperoxide lyase pathway of oxylipin metabolism. Curr Opin Plant Biol 9:274–280

Moshgani M, Kolvoort E, DE Jong TJ (2014) Pronounced effects of slug herbivory on seedling recruitment of *Brassica* cultivars and accessions, especially those with low levels of aliphatic glucosinolates. Basic Appl Ecol 15:607–615

Mumm R, Burow M, Bukovinszkine'Kiss G, Kazantzidou E, Wittstock U, Dicke M, Gershenzon J (2008) Formation of simple nitriles upon glucosinolate hydrolysis affects direct and indirect defense against the specialist herbivore, *Pieris rapae*. J Chem Ecol 34:1311–1321

Pangesti N, Weldegergis BT, Langendorf B, VAN Loon JJ, Dicke M, Pineda A (2015) Rhizobacterial colonization of roots modulates plant volatile emission and enhances the attraction of a parasitoid wasp to host-infested plants. Oecologia 178:1169–1180

Pianezzola E, Roth S, Hatteland BA (2013) Predation by carabid beetles on the invasive slug *Arion vulgaris* in an agricultural semi-field experiment. B Entomol Res 103:225–232

Pichersky E, Gershenzon J (2002) The formation and function of plant volatiles: perfumes for pollinator attraction and defense. Curr Opin Plant Biol 5:237–243

Quinteiro J, Rodriguez-Castro J, Castillejo J, Iglesias-Pineiro J, Rey-Mendez M (2005) Phylogeny of slug species of the genus *Arion*: evidence of of Iberian endemics and of the existence of relict species in Pyrenean refuges. J Zool Syst Evol Res 43:139–148

Rabitsch W (2006) *Arion vulgaris* (moquin-tandon 1855) fact sheet. Online database of delivering alien invasive species inventories for Europe. http://www.europe-aliens.org/pdf/Arion_vulgaris.pdf.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Scheidel U, Bruelheide H (2005) Effects of slug herbivory on the seedling establishment of two montane *Asteraceae* species. Flora 200:309–320

Schroeder FC, Gonzàlez A, Eisner T, Meinwald J (1999) Miriamin, a defensive diterpene from the eggs of a land slug (*Arion* sp.). Proc Natl Acad Sci U S A 96:13620–13625

Theenhaus A, Scheu S (1996) The influence of slug (*Arion rufus*) mucus and cast material addition on microbial biomass respiration and nutrient cycling in beech leaf litter. Biol Fert Soils 23:80–85

Ton J, D'Alessandro M, Jourdie V, Jakab G, Karlen D, Held M, Mauch-Mani B, Turlings TCJ (2007) Priming by airborne signals boosts direct and indirect resistance in maize. Plant J 49:16–26

Türke M, Heinze E, Andreas K, Svendsen SM, Gossner MM, Weisser WW (2010) Seed consumption and dispersal of ant-dispersed plants by slugs. Oecologia 163:681–693

Wittstock U, Agerbirk N, Stauber EJ, Olsen CE, Hippler M, Mitchell-Olds T, Gershenzon J, Vogel H (2004) Successful herbivore attack due to metabolic diversion of a plant chemical defense. Proc Natl Acad Sci U S A 101:4859–4864