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Olfactory cues mediating prey-searching behaviour in interacting aphidophagous predators: are semiochemicals key factors in predator-facilitation?

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Abstract

Based on their effect on prey populations, predators can interact synergistically, additively, or antagonistically. Predator attraction by semiochemicals in response to herbivory is well documented; however, the possibility of semiochemicals mediating synergistic interactions has not been explored. *Eriopis connexa* (Germar) and *Hippodamia variegata* (Goeze) (both Coleoptera: Coccinellidae) interact synergistically with carabid species in Central Chile, a phenomenon in which semiochemicals may be involved. Moreover, olfactory behaviour in these coccinellids is unknown. Olfactometries contrasting non-infested vs. infested plants with *Acyrthosiphon pisum* Harris (Hemiptera: Aphididae) were performed to study olfactory prey-searching in *E. connexa*, *H. variegata*, and *Trirammatus striatula* (Fabricius) (Coleoptera: Carabidae). To evaluate whether semiochemicals can mediate synergistic predatory interactions, four experiments were established: olfactometries contrasting (1) infested plants with and without a predator, (2) uninfested plants with and without a predator; (3) predator vs. air, and (4) plants with previous physical activity of a predator vs. clean plants (nine combinations of predator species, according to whether they corresponded to the stimulus or focal individual). *Hippodamia variegata* and *T. striatula* were attracted to infested plants when contrasted with non-infested plants. Infested plants with a conspecific and *H. variegata* elicited attraction in *E. connexa*, whereas *T. striatula* preferred infested plants with *E. connexa* or *H. variegata*. Treatments with only predators (with or without the plant) did not elicit responses, except in *E. connexa* which was repelled by conspecifics and *H. variegata*, perhaps indicating an antagonistic interaction between them; plants with previous physical activity of predators did not elicit responses. These results corroborated the importance of semiochemicals produced by herbivory in the prey-searching behaviour of aphidophagous predators. In addition, presence of predators on the foliage may favour emission of aphid alarm pheromones, which could attract *E. connexa* and *T. striatula*. Volatiles can intervene in synergistic interactions between carabids and coccinellids; this should be replicable in other systems where predator-facilitation between aphidophagous predators is observed.

Introduction

Based on the impact of the combined effect of multiple natural enemies, predatory intraguild interactions may be categorized as: (1) additive, if natural enemies of the prey do not interact and the effect on the prey is merely the sum of each specific predator effect; (2) antagonistic, if natural enemies compete for the same prey or display intraguild predation, resulting in a lower rate of prey consumption; or (3) synergistic, if the interaction between natural enemies modifies normal prey behaviour and/or prey niche, making it more susceptible to predator attack.

Interactions between phytophagous insects, their host plants, and their natural enemies are increasingly under-

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stood in a chemical tri-trophic context. Plant volatiles represent cues for phytophagous insects and also for carnivores, mediating the relationship between predators and prey (Steidle & van Loon, 2003; Hatano et al., 2008). In contrast to these tri-trophic interactions, the mediating role of semiochemicals in intraguild interactions between predators has received comparatively little attention. Moreover, chemical-ecology research in this field is mostly focused on antagonist intraguild behaviour, exploring aspects of deterrence and avoidance responses to semiochemicals (e.g., Růžička, 2001; Agarwala et al., 2003; Sarmento et al., 2007; Oliver et al., 2008). Conversely, research evaluating the role of semiochemicals in interactions involving synergistic effects of predation or predator-facilitation (Charnov et al., 1976) has not been reported.

Coccinellids and carabids are recognized as important beneficial insects in agricultural systems (Lövei & Sunderland, 1996; Obrycki & Kring, 1998; Kromp, 1999; Symondson et al., 2002). Coccinellids and carabids regularly coexist in crop fields, often establishing synergistic interactions (Losey & Denno, 1998a, 1999; Grez et al., 2007). In this context, the dropping behaviour of aphids in response to attack by foliar predators facilitates predation by opportunistic ground foragers; in addition, given their different foraging strategies on the plant, no interference between these predators is established. In particular, synergistic predatory interactions between the coccinellid species *Eriopis connexa* (Germar) and *Hippodamia variegata* (Goeze) (Coleoptera: Coccinellidae) and ground beetles coexisting in alfalfa crops in Central Chile have been described (Grez et al., 2007), and constitute ideal systems to determine whether semiochemicals are involved in these kinds of interactions, modifying prey-searching behaviour of the aphidophagous beetles involved. Additionally, this system represents an optimal opportunity to obtain first evidence about the potential influence of semiochemicals on the behaviour of *E. connexa* and *H. variegata* (and their biological interactions), species that have not been properly studied under a chemical-ecology framework. To achieve these objectives, we designed olfactometric bioassays in order to (1) evaluate predator olfactory attraction to prey, and (2) to evaluate the effect of prey–predator complexes on the olfactory prey-searching behaviour of a second predator.

Materials and methods

Insects and plants

Adult and immature stages of *H. variegata*, *E. connexa*, and *Acyrthosiphon pisum* Harris (Hemiptera: Aphididae) and adult individuals of *Trirammatus striatula* (Fabricius) (Coleoptera: Carabidae), were collected from alfalfa fields near Pirque (33º40′S, 70º35′W), Central Chile, and reared in the laboratory at 20 °C, 47–50% r.h., and L16:D8 photoperiod. Aphids were reared on broad bean plants [*Vicia faba* L., cv. Sevillana (Fabaceae)] and this same aphid–host plant system served as the rearing substrate for both coccinellid species. *Vicia faba* was chosen as the experimental plant due to its simple cultivation in the laboratory and experimental success acknowledged in similar studies (e.g., Raymond et al., 2000). Bioassays were carried out using pots containing three plants of 14 ± 2 days (height about 10 cm), and experimental plants were infested 24 h prior to the olfactometry with 25 individuals of fourth instars and adults of *A. pisum* (Ojeda-Camacho et al., 2001). After each replicate, focal individuals were sexed to ensure absence of sex bias (no bias was detected).

Olfactometer

A Y-tube olfactometer was employed for all experiments. Each arm of the olfactometer was connected by Teflon pipes to glass bell-jars where stimulus sources were enclosed. For dispersion of volatiles, an air flow of 250 ml per min previously purified by charcoal filters, was injected passing through bell jars and then to the Y-tube, permeating each arm of the olfactometer. To concentrate volatiles, stimulus sources were always enclosed inside bell-jars for 5 min before starting the experiments, and to ensure similar volatile concentrations, plants used as stimuli were weighted considering substrate and canopy for each replicate. The focal individual was gently placed in the central tube of the olfactometer and the time it spent in each arm of the olfactometer was registered using the JWATCHER software version 0.9 (Blumstein et al., 2000). Insects with no reaction longer than 2.5 min were considered as 'unresponsive' and the replicate discarded. Insects used as focal individuals were starved for 24 h before the experiments to maximize predatory behaviour (Mundy et al., 2000), and changed at each replicate to avoid pseudoreplication (Ramírez et al., 2000). After each experiment pipes, bell-jars, and Y-tubes were washed with distillated water and ethanol, and dried at 100 °C. Finally, to avoid bias, connections between the arms of the olfactometer and stimulus sources were periodically alternated, lighting was provided from above, and all experiments took place in a room with a controlled temperature of 20 °C.

Behavioural bioassays and statistical analysis

To explore the olfactory prey-searching behaviour of each of the aphidophagous beetles, *H. variegata*, *E. connexa*, and *T. striatula*, olfactometric bioassays contrasting infested broad bean plants vs. non-infested plants were performed. After 2 min of acclimatization, the time spent in each area of the olfactometer by the focal individual was recorded. Continuous observation lasted 5 min, a sufficient amount of time to evaluate olfactory searching behaviour in coccinellids as suggested by Raymond et al. (2000); in the case of the carabids, an observation time of 5 min was also established after preliminary bioassays.

Additionally, to evaluate the possible mediating role of semiochemicals in the intraguild interaction between these aphidophagous beetles, four olfactometric bioassays were established: (1) one adult individual of an aphidophagous species on an infested plant as the first stimulus source vs. an infested plant only (no predator) as the second stimulus source; (2) one adult individual of an aphidophagous species on a non-infested plant vs. a non-infested plant only; (3) one adult individual of an aphidophagous species only vs. air (empty bell-jar); and (4) a non-infested plant vs. a non-infested plant with previous physical activity of a predator. Whereas bioassay 1, explores potential olfactory behavioural responses of each aphidophagous species to the various tri-trophic interactions, bioassays 2 and 3, dissect the various trophic levels involved in these interactions to explore their particular olfactory effects and which ones trigger responses. Finally, bioassay 4, allowed us to explore possible repellent olfactory semiochemicals left by predators on the plant that could interfere with predatory synergistic cues.

For each of these experiments, nine treatments were set up with the various combinations of natural enemy species, whether they corresponded to the focal individual or the stimulus source. Predator species corresponding to the stimulus source were enclosed in bell jars 10 min before the experiment took place, to ensure the occurrence of interactions between prey and the predator, and to concentrate volatiles. Similarly, for experiments assaying predator tracks, one individual was allowed to walk on the plant for 10 min and then removed from the plant prior to the bioassay, consistent with the previous time set for all preceding experiments. As for experiments of olfactory prey-searching behaviour continuous observation of the focal individual lasted 5 min and the time it spent in each area of the olfactometer was recorded, after 2 min of acclimatization. Total times spent in the areas corresponding to stimulus 1 and stimulus 2 of the olfactometer were compared by a Wilcoxon matched pairs test using the software SigmaPlot version 11 (Systat Software, 2008). Each treatment was replicated 10 times.

Results

When confronted with infested vs. non-infested plants, both *H. variegata* and *T. striatula* were significantly attracted to volatiles emitted by infested plants (Z = 2.293, P = 0.022, and Z = 2.803, P = 0.005, respectively), whereas *E. connexa* did not show any preference (Figure 1). On the other hand, in olfactometries with natural enemies on one stimulus source, *E. connexa* showed a significant olfactory preference for infested plants with a conspecific (Z = 2.395, P = 0.017) and to infested plants with *H. variegata* (Z = 2.293, P = 0.022); no attraction was found for infested plants with the presence of *T. striatula* (Figure 2A). When the focal species was *H. variegata*, no significant olfactory attraction was observed to infested plants with a conspecific or to infested plants with heterospecific aphidophagous beetles (Figure 2B). Finally, for *T. striatula* as focal species, significant olfactory attraction to infested plants with either *E. connexa* (Z = 2.803, P = 0.005) or *H. variegata* (Z = 2.803, P = 0.005) was observed, whereas no attraction to infested plants with presence of a conspecific was observed (Figure 2C).

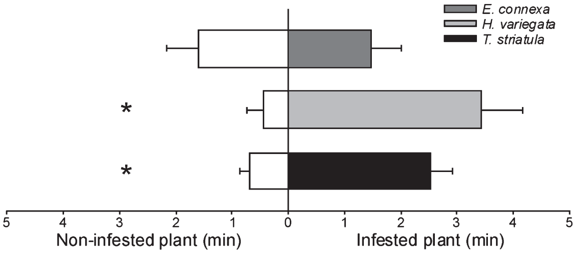

Figure 1 Mean (+ SE) time spent by adult individuals of *Eriopis connexa*, *Hippodamia variegata*, and *Trirammatus striatula* in zones of the olfactometer permeated by stimulus 1 and 2 (Wilcoxon matched pairs test: *P<0.05; n = 10). Stimuli used were a *Vicia faba* plant infested with 25 individuals of *Acyrthosiphon pisum* (filled bars) and non-infested plants of *V. faba* (white bars).

In experiments with uninfested plants with the presence of a predator species, *E. connexa* was significantly deterred by plants with the predator when the latter was a conspecific (Z = 2.803, P = 0.005) or *H. variegata* (Z = 2.089, P = 0.037), whereas no deterrence was elicited by uninfested plants with *T. striatula* (Figure 3A). On the contrary, no significant attraction was observed in the *H. variegata* (Figure 3B) and *T. striatula* olfactometric bioassays (Figure 3C). Likewise, for all olfactometric bioassays with the occurrence of only a predator in one stimulus source contrasted with air and for olfactometries contrasting clean plants vs. plants with previous physical activity of a predator, no significant attraction was elicited in any of the predator species (all Z<1.890 and P>0.05; Figures 4 and 5).

Discussion

Prey-searching behaviour in aphidophagous beetles

Adult ladybird beetles usually orient their movement towards prey using olfactory cues (Seagraves, 2009). Our

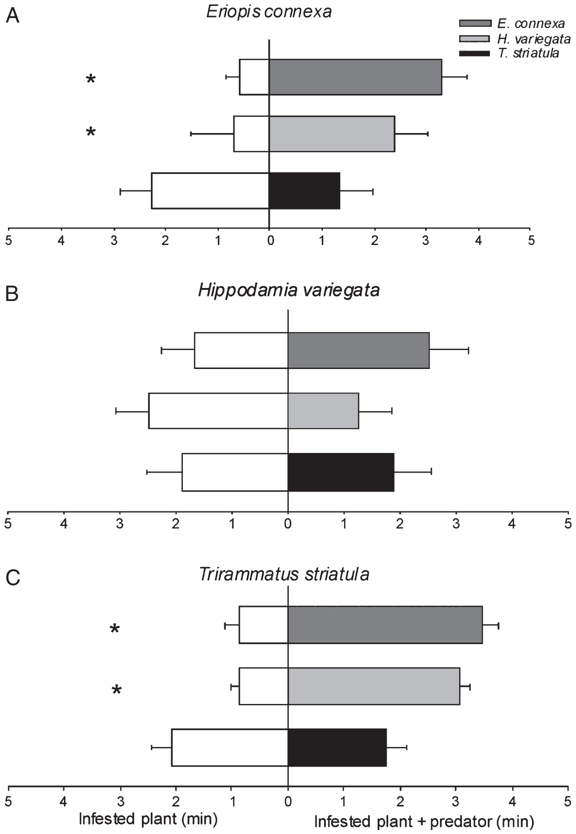

Figure 2 Mean (+ SE) time spent by adult individuals of (A) *Eriopis connexa*, (B) *Hippodamia variegata*, and (C) *Trirammatus striatula* in zones of the olfactometer permeated by stimulus 1 and 2 (Wilcoxon matched pairs test: *$P<0.05$; $n = 10$). Stimuli used were a *Vicia faba* infested plant (white bars) and a *V. faba* infested plant + one individual of either of the three predator species studied (filled bars).

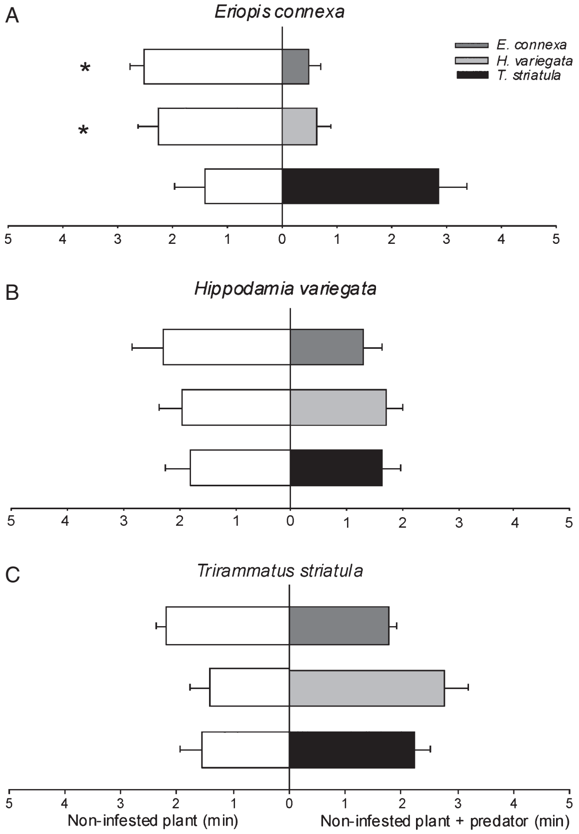

Figure 3 Mean (+ SE) time spent by adult individuals of (A) *Eriopis connexa*, (B) *Hippodamia variegata*, and (C) *Trirammatus striatula* in zones of the olfactometer permeated by stimulus 1 and 2 (Wilcoxon matched pairs test: *$P<0.05$; $n = 10$). Stimuli used were a non-infested plant of *Vicia faba* (white bars) and a non-infested plant of *V. faba* + one individual of either of the three predator species studied (filled bars).

results demonstrated that broad bean plants after being infested with aphids elicited attraction in *H. variegata* and also in the carabid *T. striatula* (Figure 1), as generally recognized for several other species of aphidophagous beetles (Raymond et al., 2000; Ninkovic et al., 2001; James, 2005; Zhu & Park, 2005; Verheggen et al., 2007; Bahlai et al., 2008). As a result of oviposition or damage by herbivory, plants change their volatile profiles, releasing compounds synthesized de novo (Paré & Tumlinson, 1999) which behave as specific cues that guide predators and parasitoids towards their prey. Cornicle secretions and cuticular chemicals are also attracting cues for coccinellids (Seagraves, 2009) and may be responsible for these responses.

By contrast, the lack of attraction to infested plants in *E. connexa* represents a contra intuitive outcome, which may indicate that integration with other stimuli (i.e., visual stimuli; Harmon et al., 1998; Mondor & Warren, 2000; Bahlai et al., 2008) and/or orientation to volatiles emanating from other stimulus sources (Schaller & Nentwig, 2000) are needed when foraging. Also, attraction to herbivory infochemicals might occur only at certain densities of aphids feeding on the plant, given that responsiveness in searching and oviposition behaviour in coccinellids are related to prey densities (Seagraves, 2009). This work represents the first report of olfactory searching behaviour in these coccinellids species, and with no previous information about *E. connexa*, more accurate conclusions and explanations are constrained. Nevertheless, we can conclude that *H. variegata* should be more efficient than *E. connexa* in prey-searching if no other stimulus is present, which under the perspective of biological control may represent a relevant outcome. Interestingly, Mancilla (2010) indicated that *H. variegata* showed a greater consumption rate of *A. pisum* than *E. connexa*.

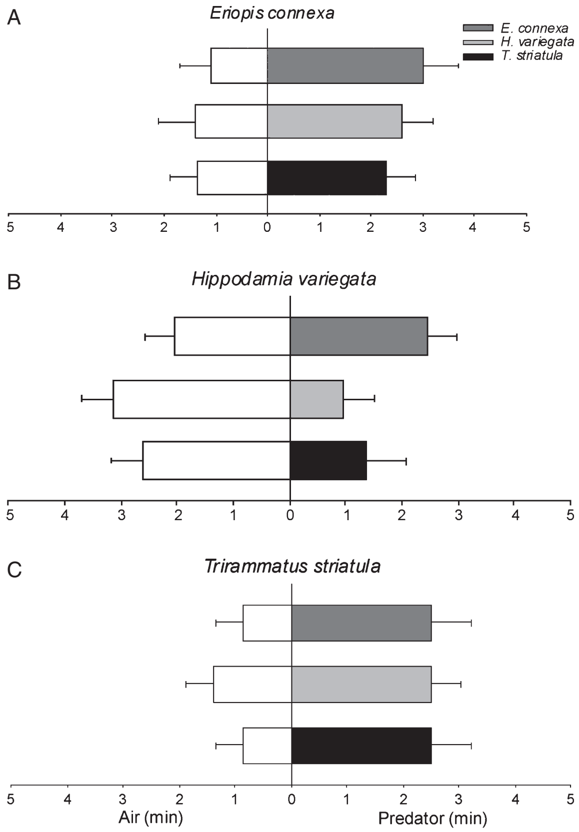

Figure 4 Mean (+ SE) time spent by adult individuals of (A) *Eriopis connexa*, (B) *Hippodamia variegata*, and (C) *Trirammatus striatula*, in zones of the olfactometer permeated by stimulus 1 and 2 (Wilcoxon matched pairs test; n = 10). Stimuli used were air (empty bell-jar; white bars) and one individual of either of the three predator species studied (filled bars).

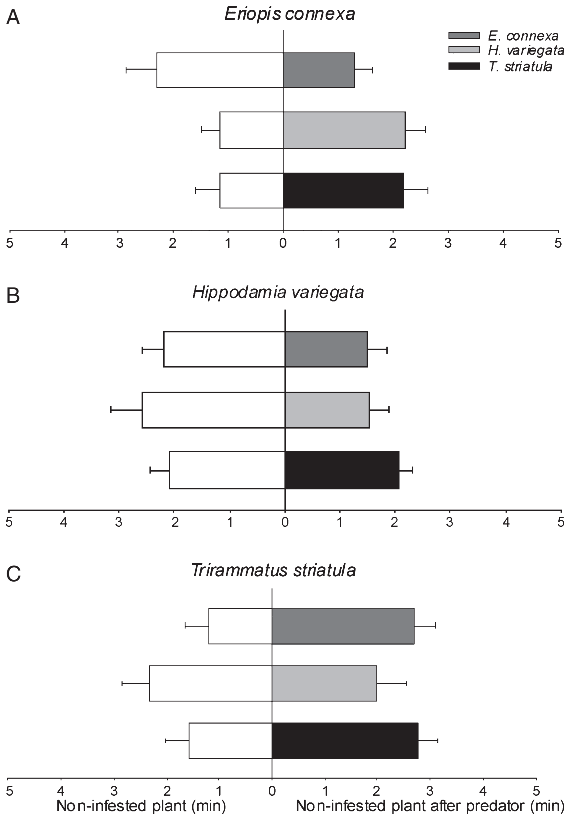

Figure 5 Mean (+ SE) time spent by adult individuals of (A) *Eriopis connexa*, (B) *Hippodamia variegata*, and (C) *Trirammatus striatula* in zones of the olfactometer permeated by stimulus 1 and 2 (Wilcoxon matched pairs test; n = 10). Stimuli used were a non-infested plant of *Vicia faba* (white bars) and a non-infested plant of *V. faba* after 10 min of previous physical activity of one individual of either of the three predator species studied (filled bars).

Olfactory intraguild interactions among aphidophagous beetles

In response to attack by natural enemies, aphids abandon the plant essentially by the dropping behaviour, thus facilitating predation by ground foragers, a phenomenon associated with predator-facilitation (Losey & Denno, 1998b). In concordance, this study represents the first chemical approach to non-antagonistic intraguild predatory interactions and suggests that synergistic predatory interactions can be mediated by semiochemicals. This situation may be replicable in other systems, especially where *A. pisum* is involved, given that its dropping behaviour is more frequent than in other aphids (Losey & Denno, 1998b; Mancilla, 2010).

In our olfactometric bioassays, both *E. connexa* and *T. striatula* were attracted to infested plants of *V. faba* when a foliar aphidophagous beetle (i.e., *H. variegata* or *E. connexa*) was present (Figure 2A and C). When disturbed by natural enemies, aphids release an alarm pheromone through their cornicles, which is perceived by their conspecifics nearby and as a result, they cease feeding and disperse by dropping to other plants or to the ground (Verheggen et al., 2008). As a consequence, and taking into account that all treatments with carabids as stimulus sources (which did not physically disturb the aphids) and those with only predators (with or without the plant) did not elicit attraction, we suggest that chemical attraction in *E. connexa* and *T. striatula* (and therefore, intraguild interactions between them) could be explained by an attraction to volatiles released in response to disturbing and predation on aphids, and particularly to the aphid alarm pheromone emitted in presence of a natural enemy on the plant. In concordance with this, olfactory attraction and electrophysiological responses to aphid alarm pheromone have been previously reported in other coccinellids, such as *Harmonia axyridis* Pallas (Verheggen et al., 2007), *Coccinella septempunctata* L. (Pettersson et al., 2008), *Hippodamia convergens* Guérin-Méneville (Acar et al., 2001), and *Adalia bipunctata* (L.) (Francis et al., 2004), among other species (see also Hatano et al., 2008), and in the carabids *Pterostichus melanarius* Illiger and *Harpalus rufipes* DeGeer (Kielty et al., 1996). Particularly its main component, (*E*)-β-farnesene, has been considered to be responsible for eliciting such behavioural and electrophysiological responses. Furthermore, Mancilla (2010) experimentally demonstrated that dropping behaviour in *A. pisum* increased in response to the occurrence of *E. connexa* and especially *H. variegata*, given its greater mobility. Even though *E. connexa* did not respond to volatiles released by herbivory, attraction to the tri-trophic complex is expected as perturbing aphids or preying upon them can improve prey-detection and intensify searching in predators (Hatano et al., 2008). In this context, lack of attraction in *H. variegata* may reflect that only the emission of volatiles produced by herbivory is relevant. However, we cannot rule out the possibility that this coccinellid may be indeed unresponsive to aphid alarm pheromone or that more time of infestation could be necessary for concentrating volatiles to observe significant responses.

By contrast, several studies have demonstrated chemical deterrence among coccinellid beetles (e.g., Hemptinne et al., 2001; Růžička, 2002, 2003; Oliver et al., 2006; Klewer et al., 2007) and have especially focused on oviposition-deterring pheromones (ODP). Our results showed repellent effects by conspecifics and *H. variegata* exclusively on *E. connexa*, and that these effects seemed to be overcome when aphids were present. This trade-off between intraguild repellent and prey-attractive volatiles, where the latter suppressed the former, may represent a novel result in terms of the type of semiochemicals involved and the nature of the outcome, and in the case of *E. connexa*, emphasizes the importance of volatiles emitted by the prey under these conditions for foraging. Conversely, although *E. connexa* exhibited deterrence, these effects were not appreciated in *H. variegata*, and although congeneric species (i.e., *H. convergens*) do exhibit deterrence in presence of con- and heterospecific individuals (Michaud & Jyoti, 2007), it is known that different species from the same genus can exhibit different levels of response to repellent semiochemicals (Magro et al., 2007). No research on pheromones or chemical interactions mediated by volatiles has been published for *E. connexa* or *H. variegata*, this work being the first attempt in this matter; thus, we have limited evidence to explain the behavioural differences between these species. Our results suggest that *H. variegata* responds exclusively to semiochemicals released by herbivory and that both coccinellid species may produce repellent volatiles, whose effects are apparent only in *E. connexa* and which do not interfere with predator-synergistic cues. Additionally, in the light of our results, these coccinellids would require more time of physical activity on the plant, more individuals, larval attachment, or oviposition on the plant, to leave repellent marks (Růžička, 2002, 2003; Klewer et al., 2007). Accordingly, more studies are necessary to achieve conclusive support for these explanations, including experiments with larvae of coccinellids.

On the other hand, the deterrence elicited in *E. connexa* to the uninfested plant–*H. variegata* complex increases our interest in exploring the coexistence between these two coccinellids in Central Chile, where the former species is native and the latter introduced. Introductions of foreign species of coccinellids have led to competitive interactions with native species in other countries (i.e., Kajita et al., 2000; Evans, 2004; Snyder et al., 2004; Yasuda et al., 2004), and also chemical avoidance between competing coccinellids has been reported (Yasuda et al., 2000; Magro et al., 2007). *Eriopis connexa* and *H. variegata* present overlapping habitats in Central and southern Chile (Zaviezo et al., 2004; Rebolledo et al., 2007) and important disparities in their density trends when coexisting, with seasonal divergences at the time when each species reaches maximal densities (Grez, 1997). Thus, studies evaluating behavioural and demographic attributes of these coccinellids in shared habitats can reveal the actual state of their coexistence, plus natural history traits, strategies implicated in coexistence, and how the trade-off between repellent and attractant semiochemicals discussed above influence it. Moreover, possible differences in searching efficiency between *E. connexa* and *H. variegata*, as we proposed above, should be explored more deeply to have a complete view of their competitive abilities as well as their potentials as controllers for pests.

Our results describing olfactory attraction in aphidophagous beetles, which can be elicited by the aphid alarm pheromone, is evidence that semiochemicals cannot be ruled out as moderating factors in intraguild synergistic interactions, as they occur in antagonistic interactions, even in the presence of repellent volatiles. However, it is crucial to assess its validity under natural conditions and in other systems in which predator-facilitation has been described, and to complement this information with the actual identity of the compounds involved in this outcome.

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