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Intraguild predation by *Harmonia axyridis* on coccinellids revealed by exogenous alkaloid sequestration

Louis Hautier[1], Jean-Claude Grégoire[2], Jérôme de Schauwers[3], Gilles San Martin[3,4], Pierre Callier[3], Jean-Pierre Jansen[1], Jean-Christophe de Biseau[3]

[1] Biological Control and Plant Genetic Resources Department, Walloon Agricultural Research Centre, Rue de Liroux, 2, 5030 Gembloux, Belgium
[2] Biological Control and Spatial Ecology, Université Libre de Bruxelles, CP 160/12, Av. F.D. Roosevelt 50, 1050 Bruxelles, Belgium
[3] Behavioural and Evolutionary Ecology, Université Libre de Bruxelles, CP 160/12, Av. F.D. Roosevelt 50, 1050 Bruxelles, Belgium
[4] Present address: Behavioural Ecology and Conservation Group, Biodiversity Research Centre, Université catholique de Louvain, Croix du Sud 4, 1348 Louvain-la-Neuve, Belgium

Summary. Under laboratory conditions, the multicolored Asian lady beetle, *Harmonia axyridis* is well known as an intraguild predator of other ladybirds. However the real impact of this exotic species on native species was poorly investigated in the field. Because many ladybird species produce alkaloids as defensive compounds, we propose here a new method of intraguild predation monitoring in coccinellids based on alkaloid quantification by GC-MS. In laboratory experiments, adaline was unambiguously detected in fourth instar larvae of *H. axyridis* having ingested one egg or one first instar larva of *Adalia bipunctata.* Although prey alkaloids in the predator decreased with time, traces were still detected in pupae, exuviae and imagines of *H. axyridis* having ingested one prey when they were fourth instar larvae. Analysis of *H. axyridis* larvae collected in two potato fields shows for the first time in Europe the presence of exogenous alkaloids in 9 out of 28 individuals tested. This new method of intraguild predation detection could be used more widely to follow the interactions between predators and potential chemically defended insect preys.

Key words. Adalia bipunctata – alkaloids – coccinellids – Coccinella septempunctata – detection – Harmonia axyridis – intraguild predation – Propylea quatuordecimpunctata

Correspondence to: Louis Hautier, e-mail hautier@cra.wallonie.be

Introduction

After its purposeful introduction for biological control, the multicolored Asian lady beetle, *Harmonia axyridis* (Pallas), has rapidly invaded large parts of Europe and America (Koch *et al.*, 2006; Brown *et al.*, 2008). Its success in newly colonised areas is partly attributed to its high capacities as an interspecific competitor, particularly as an intraguild predator (Cottrell and Yeargan 1998; Yasuda and Ohnuma 1999; Dixon 2000; Snyder *et al.,* 2004). However, its impact in this respect, especially on coccinellid species, is still poorly known. In the laboratory, several studies showed that *H. axyridis* predates numerous ladybirds, among which *Coccinella septempunctata* (L.) (Yasuda and Ohnuma 1999; Yasuda *et al.,* 2001; Snyder *et al.,* 2004) and *Adalia bipunctata* (L.) (Kajita *et al.,* 2000; Santi *et al.,* 2003; Burgio *et al.,* 2005). Field studies also suggest that native ladybirds were declining or displaced (Colunga-Garcia and Cage, 1998; Dixon 2000, Snyder *et al.*, 2004) but, although these authors generally mentioned intraguild predation (IGP) by *H. axyridis*, this was never really quantified except by visual observations in Japan (Hironori and Katsuhiro, 1997).

To follow the spatial and/or temporal dynamics of interactions between a predator and a prey, many techniques have been developed (Harwood and Obrycki, 2005) : analysis of food remains by gut dissection (Triltsch, 1999), use of monoclonal antibodies (Hagler, 2006) and molecular analysis with detection of species-specific DNA sequences (Hoogendoorn and Heimpel, 2001). This latter technique has been used to detect IGP in coccinellids but with some limitations in detection time because of the rapid digestion of the DNA of the prey (Gagnon *et al.*, 2005).

A promising method to study IGP in coccinellids could be the use of prey-produced alkaloids for tracing

prey consumption. All instars (from egg to imago) of many species in Coccinellinae, Scymninae, Chilocorinae and Epilachninae produce distinctive alkaloids (Daloze *et al.*, 1995; Glisan King and Meinwald, 1996), as a protection against predation by other coccinellids (Agarwala and Dixon, 1992; Hemptinne *et al.*, 2000) but also by ants (Pasteels *et al.*, 1973; Ayer and Browne, 1977; Marples, 1993) and by birds (Marples *et al.*, 1989).

Here, using *A. bipunctata* as a prey for *H. axyridis*, we show that prey alkaloids can be identified in the predator by GC-MS, even several days after the predation event. We also demonstrate that this technique can be used to detect other coccinellid preys such as *C. septempunctata.* Finally, we present a first assessment of IGP between *H. axyridis* and native coccinellids in Belgium, using this IGP detection technique with coccinellid larvae sampled from potato fields.

Material and methods

Insect cultures

Ladybirds and aphids were reared in air-conditioned rooms at 20±1°C, 16L:8D photoperiod and 60–90% relative humidity. The ladybirds were fed with pea aphids, *Acyrthosiphon pisum* (Harris) reared on horse bean plants, *Vicia faba minor* L., and with honeybee pollen ("Gabriel Perronneay", APIDIS sa, Dijon, France). Adult *H. axyridis, A. bipunctata* and *C. septempunctata* were kept separately in clear polystyrene boxes (40x30x20 cm). Aphids and pollen were renewed in each box three times a week and, at the same time, egg batches laid on absorbent paper were removed and placed separately in 90 mm Petri dishes. After egg hatching, the first instar larvae were isolated in 55 mm Petri dishes and fed *ad libitum* with aphids. The larvae were closely monitored so that their exact developmental stage was known at any moment.

Alkaloid extraction from predator larvae

Each larva was put in a 1.5 ml Eppendorf microtube filled with a 200 µl solution of bidistilled methanol and an internal standard (see below), and was then crushed with a pestle. After 10 minutes of extraction by soaking, the mixture was filtrated in a Pasteur pipette containing a 1 cm thick layer of compressed cotton wool. To maximize alkaloid recovery, the pipette was rinsed several times with additional methanol. The filtrate was then evaporated under nitrogen. The residue was dissolved in 300 µl of bidistilled hexane and vortexed until complete homogenization.

GC/MS analyses

Aliquots of 1 µl of the hexane extracts were analysed by GC/MS using a Finnigan Polaris Q ion trap mass spectrometer linked to a Finnigan Trace GC equipped with a split/splitless injector and a DB-5MS column (30 m X 0.25 mm diam X 0.25 µm film thickness) from J&W Scientific. The injection port and transfer line were set at 240°C and 310°C respectively. Splitless mode was used. Oven temperature was programmed from 50°C (isothermal for 1 min) to 310°C at 20°C/min, then isothermal for 1 minute, using helium as carriers gas (1.2 ml/min). Alkaloids were identified by analysis of their mass spectra produced by electron impact (ion source operating at 250°C with an ionization energy of 70eV, scan range m/z 20–400) and by comparison of their GC retention times with those of reference compounds. Alkaloid peak areas were calculated with the help of Xcalibur software 1.4 SR1 (Thermo Electron Corporation).

Alkaloid quantification

Euphococcinine was selected as internal standard because its structure is very similar to that of adaline. Euphococcinine was extracted from 14 imagines of *Cryptolaemus montrouzieri* Mulsant (BioBest, Westerloo, Belgium) either by collecting hemolymph obtained by reflex-bleeding on a filter paper placed subsequently in methanol (ISTD 1) or by soaking specimens directly in bidistilled methanol (ISTD 2). At the time of extraction, 5 µl of ISTD 1 or 20 µl of ISTD2 were added to the samples.

Euphococcinine was not purified but a constant quantity of the internal standard solution was added to each sample at the time of extraction. A reference solution was prepared in which the same quantity of internal standard was added to a known quantity of synthetic adaline provided by the Laboratory of Organic Chemistry (ULB).

From areas of euphoccocine and adaline in the GC spectra of the reference solution, it was possible to estimate adaline concentration in each extract analysed by GC/MS on the basis of the ratio between peak areas of adaline and euphoccocine. Since adaline undergoes a thermal degradation in the injection port of the GC (Chan, 1995), adaline concentration was estimated by pooling the peak areas of both adaline and degraded adaline (see Fig. 1). NMR analysis did not allow an accurate identification of the chemical structure of degraded adaline (Chan, 1995).

Assessment of IGP by alkaloid detection in the laboratory

Different IGP experiments were performed with *H. axyridis* fourth instar larvae as predators and *A. bipunctata* as preys. Firstly, adaline was quantified in *H. axyridis* that had consumed one egg or one first instar prey larva. These stages contain the smallest alkaloid quantities that could be consumed by a predator in our experimental system.

Secondly, adaline subsistence in the predator was quantified 1 h, 24 h, 48 h, 72 h and 96 h after the consumption of one *A. bipunctata* first instar larva. Pupae, imagines and exuviae of *H. axyridis* having ingested one prey during their fourth larval stage were also analysed.

The predator was starved at least 24 h before the start of the experiments. The predator and the prey were put together in 55 mm Petri dishes. After complete consumption of the prey (or after a given time of digestion), the predators were killed at -20°C in a freezer and kept at this temperature until alkaloid extraction. In each experiment, some predator larvae were not fed and were used as controls. In addition, to assess the risk of false positives following a contact with reflex-bleeding but without bite, ten *H. axyridis* larvae each kept in contact with a first instar *A. bipunctata* larva during 2 minutes without bite were analysed.

In order to determine whether the detection of IGP by alkaloid analysis can be generalized to other coccinellid species, 10 fourth instar larvae of *H. axyridis* having ingested one first instar of *C. septempunctata* were submitted to GC/MS analysis. Only precoccinelline was quantified because coccinelline was not detected in our GC conditions.

IGP assessment on native coccinellids by H. axyridis in potato field

Coccinellid larvae were collected in two potato fields in Belgium (Nivelles and Gembloux) between 27 July and 4 August 2005. In each field, four samples were taken by shaking 10 potato plants above a 40x50x18 cm plastic tray during 30 sec. Insects collected were kept in a cold box to avoid cannibalism or intraguild predation. In the laboratory, larvae of all species were individually separated and identified. *H. axyridis* larvae were put at –20°C in a freezer until alkaloid extraction in 2007.

In addition to adaline and precoccinelline, propyleine was detected in some *H. axyridis* larvae (see results). Propyleine identification in field samples was performed by comparison (mass spectra and reten-

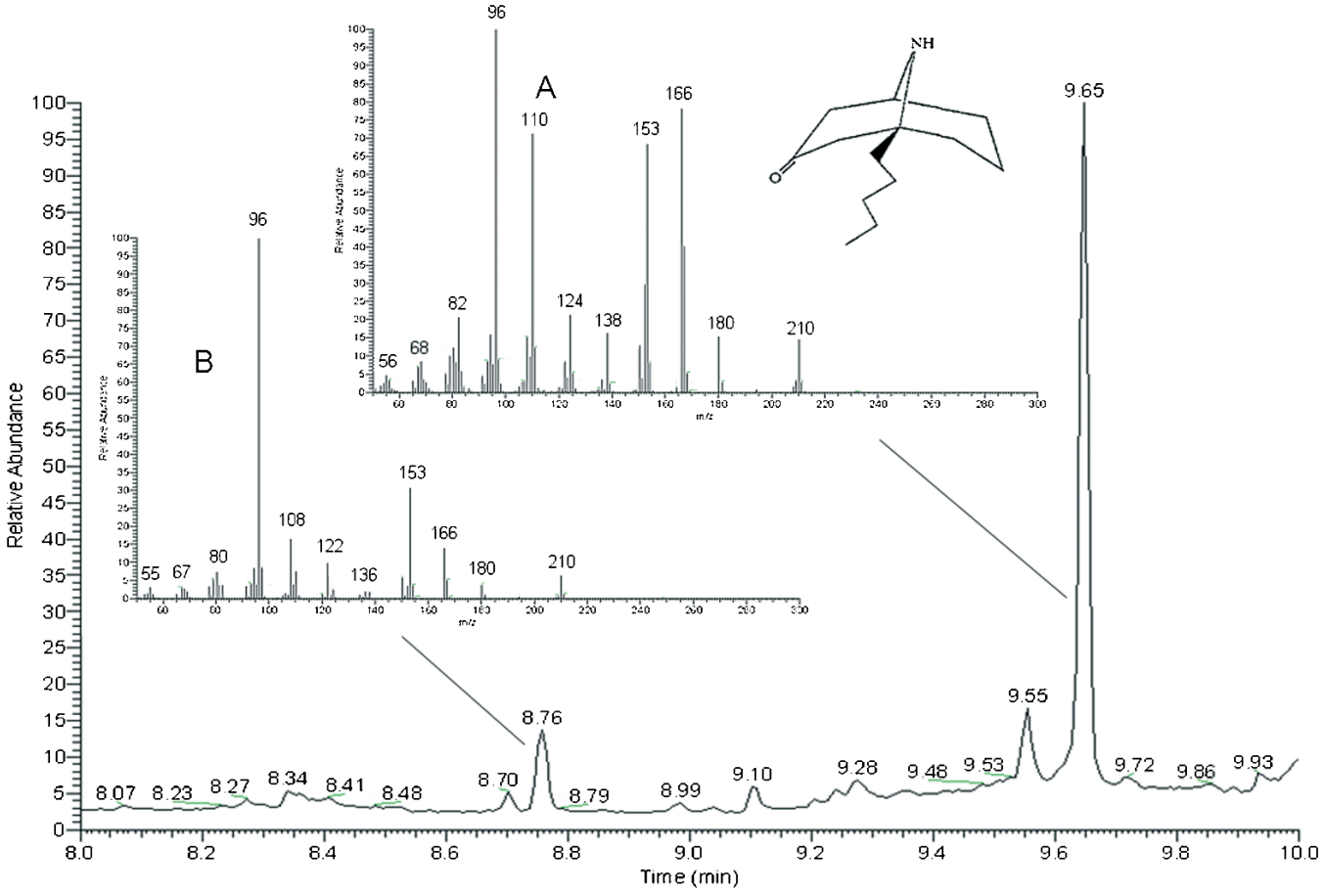

Fig. 1. GC-MS analysis of an extract prepared from one *Harmonia axyridis* fourth instar larva having ingested one *Adalia bipunctata* first instar larva. Mass spectra of adaline (A) and degraded adaline (B). Harmonine is not detected by GC because of the two very polar primary amines of the molecule.

tion time) with reference extracts of *Propylea quatuordecimpunctata* (L.) imagines.

Results

Influence of prey instar : egg vs. first instar larva

Adaline was unambiguously detected in *H. axyridis* larvae after the ingestion of one egg or of one first instar larva of *A. bipunctata* (Fig. 1). The estimated quantities were significantly different : 1.147 µg ± 0.260 (n = 5) and 1.555 µg ± 0.186 (n = 10), respectively (Student's t test, $t_6 = -3.13$; $p = 0.020$).

Evolution of prey alkaloid content in the predator with time

Adaline quantity in *H. axyridis* larvae decreased exponentially with time after ingestion (adaline quantity = $1.767\ e^{-0.0181*\text{time after ingestion}}$, $R^2 = 0.615$; $F_{1,63} = 100.65$, $p < 0.0001$). After 24 h, the quantity of adaline in the predator decreased by half but was still detected in pupae (144 h after ingestion) and also in imagines and exuviae of *H. axyridis* (216 h after ingestion) (Fig. 2).

False positives

After contacts without bite between *H. axyridis* and *A. bipunctata* larvae, the mean amount (±SD) of adaline measured in the predator extracts was 0.000406 µg ± 0.000682 (n=10) and was significantly lower (about 4000x) than after the consumption of one *A. bipunctata* larva : 1.67 µg ± 1.14 (n = 10) ($t_9 = -4.62$; $p = 0.001$).

IGP detection of another coccinellid prey (C. septempunctata)

Precoccinelline was clearly detected in each fourth instar larva of *H. axyridis* having ingested one first instar larva of *C. septempunctata.*

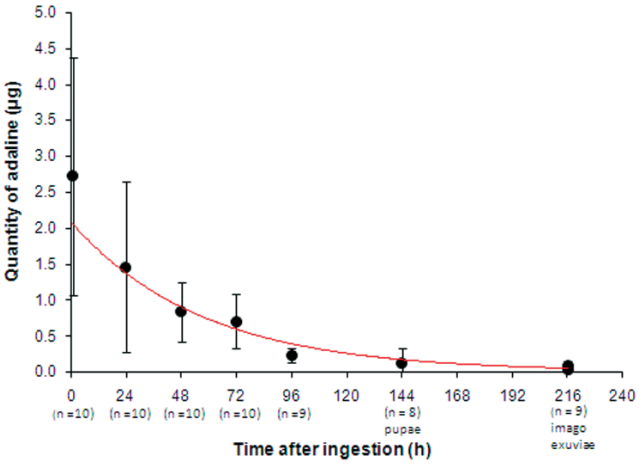

Fig. 2. Quantity of adaline (µg) detected in larvae, in pupae and in imagines of *H. axyridis* (mean ± SD), according to the time after ingestion of one *A. bipunctata* fisrt instar larva by the fourth instar larvae of *H. axyridis* (n = number of samples analysed)

one first instar of *A. bipunctata* when they were fourth instar larvae. Alkaloid detection is therefore a sensitive method for intraguild predation monitoring, allowing IGP detection both when the prey is very young (first instar larva or even egg) and several days after the predation event. In addition, the risk of false positive detections is limited because the quantity of alkaloids in predator extractions is about 4000 times higher after actual predation than after a simple contact with the prey. This risk could also be reduced by washing the larvae of the predator in a methanol bath before the extraction process.

Field sampling showed the presence of exogenous alkaloid in 32 % of *H. axyridis* larvae. These results demonstrate for the first time in Europe that in the field *H. axyridis* acts as an intraguild predator of native coccinellid species like *C. septempunctata, P. quatuordecimpunctata* or *A. bipunctata*, the mainly collected species in potato fields in Belgium (Jansen and Hautier, 2008).

Table 1. Analysis of IGP by *H. axyridis* larvae from potato fields.

Location	Sampling date	Number of *H. axyridis* analysed	Number of *H. axyridis* with exogenous alkaloids	Exogenous alkaloids detected		
				Propyleine	Precoccinelline	Adaline + Precoccinelline
Nivelles	27 July 2005	10	3	1	2	0
Nivelles	2 Aug 2005	10	3	0	2	1
Gembloux	4 Aug 2005	8	3	3	0	0

IGP assessment on native coccinellids by H. axyridis in potato field

Alkaloids from native ladybird species were detected in 9 out of 28 *H. axyridis* larvae coming from potato fields (Table 1). In Nivelles, adaline, propyleine and precoccinelline were found in *H. axyridis* larvae while only propyleine was found in larvae coming from Gembloux. One *H. axyridis* larva from Nivelles contained at the same time adaline and precoccinelline. Species identified in these fields during sampling were, in order of abundance, in Nivelles : *H. axyridis*, *C. septempunctata*, *A. bipunctata* and *P. quatuordecimpunctata* and in Gembloux : *H. axyridis*, *C. septempunctata*, *P. quatuordecimpunctata* and *A. bipunctata.*

Discussion

Our study shows that, under both laboratory and field conditions, detection of IGP between coccinellids is possible using alkaloids as tracers. Alkaloids of *A. bipunctata* and *C. septempunctata* are unambiguously detected in the predator larvae just after the predation event. Adaline is still detectable 96 h after ingestion and even in pupae and imagines of *H. axyridis* having ingested

In addition, detection of adaline and precoccinelline in the same *H. axyridis* larva shows that the multicoloured asian ladybird can eat several coccinellid species during its larval development, confirming that *H. axyridis* could be a top predator (Dixon 2000). However, field studies of IGP by alkaloid detection need to be performed at large scale in order to assess the frequency of predation by *H. axyridis* on other coccinellid species.

The detection of exogenous alkaloids in the pupae and the imagines of *H. axyridis* raises the question of alkaloid sequestration from the coccinellid prey. It is known that carnivorous ladybirds can sequester plant derived defense compounds such as cardenolides or pyrrolizidine alkaloids (Rothschild *et al.*, 1973; Witte *et al.*, 1990) but it is the first time that sequestration of alkaloids from another coccinellid species is detected several days after an IGP event. The acquisition of exogenous alkaloids, if they are not toxic for the coccinellid predator, could be an additional line of defence. Therefore, IGP could be favoured by natural selection not only in terms of energy gain but also in terms of increase in chemical protection. To test this hypothesis, the effects of exogenous alkaloids on the efficiency of defence in *H. axyridis* should be assessed.

This new method of detection of IGP between coccinellids shows some useful advantages. Alkaloids of dif-

ferent coccinellid preys can be detected in one GC/MS analysis. Alkaloids are more stable compounds than food remains (Hagler, 2006) or DNA (Hoogendoorn and Heimpel, 2001). In addition, the alkaloid analysis method is easier and faster because it does not require gut dissection, and is also inexpensive compared with molecular techniques. The main limitation of this technique for coccinellids is that prey identification is possible only at the genus level, and only when the sympatric species studied produce different alkaloids. A correct estimation of predation frequency by *H. axyridis* is also limited by a possible previous IGP between the preys (for example, it is not possible to differentiate one *H. axyridis* having eaten one *C. septempunctata* and one *A. bipunctata* from one *H. axyridis* having ingested one *C. septempunctata* which previously ingested one *A. bipunctata*).

Despite these limitations, this method could be used more widely to follow the interactions between predators and potential insect prey with chemical defences such as chrysomelids, ants, aposematic lepidopterans, pentatomids, forficulids, … (Laurent *et al.*, 2005).

This novel method should allow a better understanding of the interactions in coccinellid communities in the field or in semi-controlled conditions through the quantification of intraguild predation by *H. axyridis* on native ladybirds and the assessment of its real impact on other coccinellid species. It could also provide an approach to pre-release risk estimation of exotic aphidophagous coccinellids use for biological control.

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