Eur. J. Entomol. **110**(4): 567–576, 2013
http://www.eje.cz/pdfs/110/4/567
ISSN 1210-5759 (print), 1802-8829 (online)

Molecular analysis of the gut contents of *Harmonia axyridis* (Coleoptera: Coccinellidae) as a method for detecting intra-guild predation by this species on aphidophagous predators other than coccinellids

BRECHT INGELS[1], ALEXANDRE AEBI[2, 3], LOUIS HAUTIER[4], THOMAS VAN LEEUWEN[1] and PATRICK DE CLERCQ[1]

[1]Laboratory of Agrozoology, Department of Crop Protection, Faculty of Bioscience Engineering, Ghent University, Coupure links 653, 9000 Ghent, Belgium; e-mails: brecht.ingels@ugent.be; thomas.vanleeuwen@ugent.be; patrick.declercq@ugent.be

[2]Agroscope Reckenholz-Tänikon, Research Station ART, Reckenholzstrasse 191, 8046 Zürich, Switzerland; e-mail: alexandre.aebi@unine.ch

[3]Laboratory of Soil Biology, University of Neuchâtel, Rue Emile-Argand 11, 2000 Neuchâtel, Switzerland

[4]Biological Control and Plant Genetic Resources Department, Walloon Agricultural Research Centre, Rue de Liroux 2, 5030 Gembloux, Belgium; e-mail: hautier@cra.wallonie.be

Key words. Coleoptera, Coccinellidae, *Harmonia axyridis*, Neuroptera, Chrysopidae, *Chrysoperla carnea*, Diptera, Syrphidae, *Episyrphus balteatus*, intraguild predation, gut-content analysis

Abstract. Several studies have demonstrated that the invasive ladybird *Harmonia axyridis* is a strong intra-guild predator of native species of ladybird. Laboratory studies have shown that *H. axyridis* can be an intra-guild predator of aphid predators other than coccinellids, including the hoverfly *Episyrphus balteatus* and lacewing *Chrysoperla carnea*. However, little is known about the effect of intra-guild predation (IGP) by *H. axyridis* on hoverfly and lacewing populations in the field. In the present study molecular analyses were used to detect the DNA of *E. balteatus* and *C. carnea* in the gut contents of *H. axyridis*. Primers for the syrphid and chrysopid prey were designed and feeding experiments performed to determine how long prey DNA remains detectable in the guts of this ladybird. DNA detection was influenced by the life stage of the predator and species of prey. Meal size did not affect detection time, except when fourth instar individuals of *H. axyridis* were fed 10 eggs or one second instar of *C. carnea*. Predator weight, sex and morpho-type (melanic/non-melanic) did not influence DNA detection. The half-life of the time for which the DNA of the prey remained detectable was calculated for each predator-prey combination, and ranged from 8.9 to 52.4 h. This method can be used to study the ecological importance of IGP by *H. axyridis* on aphidophagous predators other than coccinellids in the field.

INTRODUCTION

Intra-guild predation (IGP) is a widespread phenomenon, which occurs when one predator consumes another that is competing for the same prey. It differs from classical predation because it reduces the incidence of scramble competition (Polis et al., 1989). IGP occurs in a variety of ecosystems at different trophic levels. It affects the distribution, abundance and evolution of the intra-guild predator, intra-guild prey and their common prey (Polis et al., 1989; Arim & Marquet, 2004). Because of its importance in regulating predator communities, IGP has been investigated extensively in a variety of ecosystems, including systems of aphidophagous predators (reviewed by Lucas, 2005; Hemptinne et al., 2012).

A large number of IGP studies have focused on the harlequin ladybird, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae). It is native to large parts of Eastern Asia, but has spread at a very fast rate through North America, Europe, and parts of Africa and South America over the past 25 years (Brown et al., 2011b). Its establishment in North America and Europe is believed to have had an adverse effect on certain native species (Convention of Biological Diversity, http://www.cbd.int; Roy et al., 2011). In the UK, Belgium and Switzerland, invasion by *H. axyridis* is associated with the decline in the abundance of several native species of ladybird (Brown et al., 2011a; Roy et al., 2012), ostensibly due to direct competition and IGP.

Consequently, IGP involving *H. axyridis* has been widely studied under laboratory conditions (reviewed by Pell et al., 2008). The majority of these studies investigated interactions with other ladybird species. Generally, *H. axyridis* is a strong intra-guild predator, successfully consuming other coccinellid species with which it is paired (e.g. Ware & Majerus, 2008). There are also studies on intra-guild interactions with predators other than coccinellids. In interactions with the hoverfly *Episyrphus balteatus* DeGeer (Diptera: Syrphidae) *H. axyridis* was the intra-guild predator in almost all combinations of life stages. Young larval instars of this hoverfly were especially vulnerable to predation (Putra et al., 2009; Alhmedi et al., 2010; Ingels & De Clercq, 2011). When paired with larvae of *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae), fourth instar and adult *H. axyridis* are the intra-guild predator, although third instar lacewing larvae are able to feed on small larvae of this ladybird (Gardiner & Landis, 2007; Wells, 2011).

Most studies on IGP, including those discussed above, were performed under controlled laboratory conditions, often in Petri dishes or on small potted plants. Such

experiments are useful for determining the potential outcome of predatory interactions, but the results may not be relevant to field situations (Harwood & Obrycki, 2005). Therefore, in order to obtain an insight into the importance of IGP for insect communities field studies are crucial.

Predation is one of the most difficult inter-specific interactions to study in the field (Sunderland, 1988; Symondson, 2002). Direct visual observations have been used in some studies (e.g. Rosenheim et al., 1999), but are very time-consuming and likely to disturb the system (Symondson, 2002). To overcome these difficulties, several techniques for post-mortem gut-content analysis of field collected predators have been developed, which have the advantage of revealing past predation events without any form of experimental interference (Sunderland, 1988; Harwood & Obrycki, 2005). Early application of gut-content analysis consisted of dissection and visual identification of prey remains (Triltsch, 1999; Ricci & Ponti, 2005). In recent years, predator-prey studies have focused on identifying prey remains in the gut of a predator by using monoclonal antibodies, gas chromatography mass spectrometry (GC-MS) or DNA-based technology (Michaud & Harwood, 2012). Each technique has its strengths and weaknesses, which have been reviewed by Symondson (2002), Sheppard & Harwood (2005) and Aebi et al. (2011).

Recently, DNA-based field studies of IGP by *H. axyridis* on native coccinellids have been carried out in soybean fields in Canada (Gagnon et al., 2011b) and on linden trees in eastern England (Thomas et al., 2012). Hautier et al. (2008, 2011) used GC-MS to provide evidence of IGP by *H. axyridis* on other ladybirds in potato fields and on linden trees in Belgium. However, there are no field studies on IGP by *H. axyridis* on aphid predators other than coccinellids.

The hoverfly *E. balteatus* and lacewing *C. carnea* are both the most widespread species of their family in Europe (McEwen et al., 2001; Stubbs & Falk, 2002). Furthermore, their larvae are important aphidophagous predators and are used as biological control agents in various agro-ecosystems (Sadeghi & Gilbert, 2000; McEwen et al., 2001). Because of their wide distribution, both species co-occur with *H. axyridis* in different crops and semi-natural habitats (Alhmedi et al., 2009; Wells, 2011). Thus, *H. axyridis* is likely to interact with both *E. balteatus* and *C. carnea*.

The aim of the present study was to develop a DNA-based gut-content analysis to detect the DNA of *E. balteatus* and *C. carnea* in the gut of *H. axyridis*, which can be used to determine the incidence of IGP among these species in the field. Factors known to influence the level at which the DNA of prey can be detected (Hosseini et al., 2008) were investigated, including digestion time, meal size and *H. axyridis* developmental stage, weight, sex and morpho-type.

MATERIAL AND METHODS

Insects

Adults and larvae of *H. axyridis* were collected in "De Groene Vallei", a public park in which the vegetation consists mainly of maple trees (*Acer* sp.), in Ghent, Belgium. The individuals collected were used to initiate two laboratory populations. The first consisted of non-melanic *succinea* individuals (approximately 100), and will hence forth be referred to as the "non-melanic population". Melanic *spectabilis* and *conspicua* individuals (approximately 100) were used to start a second population, called the "melanic population" (Osawa & Nishida, 1992). Non-melanic individuals appearing in the first few laboratory generations of the melanic population were removed. Both populations were reared on frozen *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs, as described by De Clercq et al. (2003). Eggs of *E. kuehniella* were obtained from Koppert BV (Berkel en Rodenrijs, The Netherlands).

A culture of *E. balteatus* was established with individuals (approximately 50) collected in July 2009 in cabbage fields in Sint-Katelijne-Waver, Beitem and Kruishoutem, Belgium. New field collected individuals were added to the laboratory population in 2010 and 2011 (approximately 30 each year). Adults were kept in Plexiglas cages (60 × 60 × 60 cm) and provided with pollen and honey water. Ground dry honey bee pollen was presented on a tray (5 cm in diameter). Honey water was provided by placing a piece of cotton wool previously soaked in water in a dish (5 cm in diameter). Broad bean plants (*Vicia faba* L.) infested with pea aphid *Acyrthosiphon pisum* (Harris) (Hemiptera: Aphididae) were placed in the cages to stimulate the syrphids to oviposit. On hatching larvae were individually transferred to small Petri dishes (5 cm in diameter, 1.5 cm high) and fed ad libitum with pea aphids.

A culture of *C. carnea* was initiated with individuals obtained from Koppert BV. Adults were placed in Petri dishes (15 cm in diameter, 2 cm high) and provided with pollen and honey water (in the same way as the population of *E. balteatus*). Eggs laid in the Petri dishes were removed every two days. When the eggs hatched the larvae were placed individually in small Petri dishes (5 cm in diameter, 1.5 cm high) and fed frozen eggs of *E. kuehniella*. All laboratory colonies were maintained at 23 ± 1°C, 65 ± 5% RH and a 16L : 8D photoperiod.

DNA extraction

DNA in the *H. axyridis* used in the feeding experiments (see below) was extracted using whole insects in the case of larvae. For adult specimens from these feeding experiments, the elytra, wings and legs were removed prior to extraction. All the other individuals from which DNA was extracted were either starved for 72 h or had their abdomen removed prior to extraction, to avoid contamination from the gut contents.

DNA was extracted using an EZNA® Insect DNA Isolation Kit (Omega Bio-tek), following the protocol described by the manufacturer, with some minor changes as described below. 350 µl of CTL buffer and 25 µl of Proteinase K (20 g/l) were added to a 1.5 ml micro-centrifuge tube in which the insect was placed. Then, the insects were ground with a pestle and incubated at 60°C for 2 to 3 h. Pestles were rinsed with acetone, washed with soap, rinsed with distilled water and autoclaved before use. To remove RNA from the samples, they were incubated at 70°C for 10 min after adding 2 µl RNase A. DNA was eluted from the HiBind® DNA column using 100 µl elution buffer. The micro-centrifuge tubes containing the extracted DNA were stored at –20°C until required.

TABLE 1. Species (one individual per species) used to test the specificity of the primers selected.

Order	Family	Species tested	Origin of sample
Diptera	Syrphidae	*Sphaerophoria scripta* (Linnaeus)	Belgium
		Eupeodes corollae (Fabricius)	Belgium
		Platycheirus peltatus Meigen	Belgium
		Syrphus ribesii Linnaeus	Belgium
		Syrphus vitripennis Meigen	Belgium
Neuroptera	Chrysopidae	*Chrysopa perla* Linnaeus	Netherlands
	Hemerobiidae	*Hemerobius* sp.	Belgium
Coleoptera	Coccinellidae	*Harmonia quadripunctata* (Pontoppidian)	Belgium
		Propylea quatuordecimpunctata (Linnaeus)	Belgium
		Calvia quatuordecimguttata (Linnaeus)	Denmark
		Exochomus quadripustulatus Linnaeus	Belgium
		Adalia bipunctata (Linnaeus)	Denmark
		Adalia decempunctata (Linnaeus)	Denmark
		Coccinella septempunctata Linnaeus	Belgium
		Coccinella undecimpunctata Linnaeus	Belgium
		Hippodamia variegata (Goeze)	Belgium
		Halyzia sedecimguttata (Linnaeus)	Belgium
		Oenopia conglobata (Linnaeus)	Belgium
Hemiptera	Anthocoridae	*Anthocoris nemoralis* (Fabricius)	United Kingdom
		Anthocoris nemorum (Linnaeus)	United Kingdom
		Orius minutus (Linnaeus)	Belgium
	Aphididae	*Acyrthosiphon pisum* (Harris)	Belgium
		Sitobion avenae (Fabricius)	Belgium
		Periphyllus sp.	Belgium

Sequencing and primer design

Part of the mitochondrial cytochrome oxidase subunit I (COI) gene was targeted for the design of primers for *E. balteatus* and *C. Carnea*, because this gene is present in thousands of copies in arthropod cells (Hoy, 1994). The primers also amplified sequences shorter than 250 bp. Amplifying a short DNA sequence of a target gene present in multiple copies in the cell has resulted in an increase in the likelihood of detecting the DNA of prey in the gut of a predator (Zaidi et al., 1999; Agusti et al., 2003).

The COI-region of three individuals from different populations of *E. balteatus*, *C. carnea* and *H. axyridis* was amplified using the universal COI-primers UEA5 and UEA10 in the case of *E. balteatus* and UEA3 and UEA10 for the other species (Zhang & Hewitt, 1996), using the PCR conditions described by the authors. The double stranded PCR products were purified using an EZNA® Cycle Pure Kit (Omega Bio-tek) and sequenced by LGC Genomics (Germany). The newly obtained sequences were submitted to GenBank (GenBank ID: JQ715423 to JQ715425 for *E. balteatus*; JQ715426 to JQ715428 for *C. carnea*; JQ715429, JQ715430 and JQ740172 for *H. axyridis*). Sequences available in GenBank for *E. balteatus* (EU241740), *C. carnea* (AY743794) and *H. axyridis* (GU073896 and GU073932) were also used.

The obtained COI-sequences were aligned using the BioEdit sequence alignment editor 7.0.5 (Hall, 1999). After identifying areas with species-specific signatures, several primer pairs for both *E. balteatus* and *C. carnea* were designed using the software Primer3 (Rozen & Skaletsky, 2000). All the primers we designed were then tested for specificity (as described in King et al., 2008) and performance in PCR.

PCR conditions

All reactions (50 µl) were run on a TProfessional standard gradient thermo-cycler (Biometra). The PCR-mix contained a PCR-buffer (20 mmol/l Tris-HCl pH 8.4, 50 mmol/l KCl), 2 mmol/l $MgCl_2$, 0.2 mmol/l of each dNTP, 0.14 mmol/l of each primer and 2.5 U of Invitrogen™ Taq DNA polymerase (Life Technologies, California, USA). Each reaction solution contained 5 µl of template DNA, extracted as described above. The PCR-cycle was the same for the primers for *E. balteatus* and *C. carnea*. It started with 1 min and 30 s at 95°C, followed by 35 amplification cycles including denaturation at 95°C for 30 s, annealing at 55°C for 1 min and elongation at 72°C for 30 s. The final elongation was carried out at 72°C for 10 min. Two positive (dilutions of 1 part prey DNA in 1 or 100 parts *H. axyridis* DNA) and two negative controls (pure *H. axyridis* DNA and autoclaved distilled water) were always included. 15 µl of the PCR-product was loaded and separated on a 2% agarose gel stained with ethidium bromide. The PCR-products on the gel were visualized and photographed under UV light.

The universal invertebrate primers BD1 and 4S, which amplify the internal transcribed spacer (ITS) region (Bowles & Mcmanus, 1993) were used to ascertain the presence of DNA of sufficient quality in the samples that did not yield a PCR-product in the first reaction, in order to avoid false negative results. The PCR-mix contained the same compounds as described above. The PCR-cycle consisted of 2 min at 95°C, followed by 30 cycles including denaturation at 95°C for 20 s, annealing at 55°C for 30 s and elongation at 72°C for 1 min. The final elongation was carried out at 72°C for 10 min. Samples that did not yield a PCR-product in this second reaction were discarded.

Specificity tests

To ensure specificity for the target species, the primers that we designed were tested in two PCR experiments. In the first, six individuals of *E. balteatus*, *C. carnea* and *H. axyridis* that originated from at least three different populations collected in different countries (Belgium, UK, Switzerland and Denmark) were used. In the second, the PCR assays of both *E. balteatus* and *C. carnea* were tested for cross-reactivity against a number of non-target species (listed in Table 1). We focused on species

TABLE 2. Name, sequence, annealing temperature (Ta) and amplicon size of the primer pairs (targeting the COI-gene) selected for *E. balteatus* and *C. carnea*.

Species	Primers		T_a	Amplicon size
	Name	Sequence (5' to 3')		
Episyrphus balteatus	EB.F6	CTTTCTGCTGGTATTGCTCATGG	55°C	160 bp
	EB.R5	CAAATAAAGGTATTCGATCATAAG		
Chrysoperla carnea	CC.F4	CGAGCTGAATTAGGTCAACCAG	55°C	244 bp
	CC.R4	CTACTATAGAAGAAGCAAGTAATAAAG		

that are expected to co-occur with *E. balteatus*, *C. carnea* and *H. axyridis* in the field (Alhmedi et al., 2009).

Sensitivity test

To assess the ability of the primers we designed to detect small amounts of DNA of the prey within a large quantity of predator DNA, a sensitivity test was performed. For this test, a series of tenfold dilutions of prey to *H. axyridis* DNA (i.e. 1 part prey to 1 part *H. axyridis* DNA, diluted to 1 part prey to 106 parts *H. axyridis* DNA) was used as a template in PCR-reactions. The diluted samples always contained a total concentration of 200 ng/µl DNA, and only 1 µl of this DNA-solution was used per PCR reaction. This kind of test is only a simulation of the natural situation, but provides useful information on the sensitivity of the primers that can be used for determining which primer set to use (Chen et al., 2000; Traugott et al., 2006; Thomas et al., 2012).

Feeding experiments

In order to determine for how long the DNA of prey species can be detected in the gut of a predator, feeding experiments were conducted with *H. axyridis* as the predator and either *E. balteatus* or *C. carnea* as prey. For each species of prey there were two experiments. In the first, fourth instar individuals of *H. axyridis* were used within 24 h of moulting and collected at random from the melanic and non-melanic populations. In the second experiment both melanic and non-melanic adults of *H. axyridis* of both sexes were used in similar proportions. Sex was determined using the method described by McCornack et al. (2007). The adults were approximately 1 week old and had mated.

Prior to the start of each feeding test, the predators were starved for 24 h in a Petri dish (9 cm in diameter, 1.5 cm high) containing only a piece of moistened paper. Just before the start of the experiment, larvae and adults of *H. axyridis* were weighed on a balance (Sartorius Genius ME 215 P, ± 0.01 mg) and then placed in a ventilated plastic Petri dish (5 cm in diameter, 1.5 cm high). To investigate the effects of meal size and prey developmental stage on the detection of the DNA of the prey, either 5 eggs, 10 eggs or 1 second instar individual of each prey species (*E. balteatus* or *C. carnea)* were provided as food in each dish. Eggs and larvae of both prey species were weighed (N = 20) to determine the actual mass of each meal. One egg of *E. balteatus* weighed on average 0.10 ± 0.01 mg, while a second instar of *E. balteatus* weighed 1.50 ± 0.12 mg. On average the weight of one egg of *C. carnea* was 0.09 ± 0.002 mg, and of a second instar larva 1.21 ± 0.09 mg. Thus, compared to the smallest meal of 5 eggs, meal size doubled in the case of 10 eggs and tripled in the case of a second instar larva for both species of prey.

The eggs of both species of prey used in the experiments were stored at 4°C for a maximum 3 days before testing and were less than 24 h old when transferred to cold storage. Eggs of *E. balteatus* were presented on a piece of a leaf of broad bean. Eggs of *C. carnea* were cut from their stalk and placed on the bottoms of the Petri dishes. Second instar individuals of both prey species were used within 24 h of moulting and starved for 16 h prior to the test.

The predators were allowed to feed for 30 min, after which the number of eggs consumed was recorded. Predators that ate less than 80% of the eggs or did not feed on the second instar larvae of either prey were not included in the analysis.

After feeding, individuals were transferred to a new Petri dish (9 cm in diameter, 1.5 cm high) containing only a moistened piece of paper. The Petri dishes were placed in a growth chamber at 23 ± 1°C, 60 ± 5% RH and a 16L : 8D photoperiod. The predators were allowed to digest their meals for either: 0, 2, 4, 6, 8, 12, 18, 24 or 36 h after which they were killed by immersion and then stored in 70% ethanol (pre-chilled at –20°C) at –20°C until the DNA was extracted. For each food type and digestion period, 5 individuals were frozen. Since there was a high probability of detecting the DNA of prey after 36 h when fourth instar *H. axyridis* fed on *C. carnea*, a 48 h digestion period was also investigated for this combination. The samples of adults of *H. axyridis* that had fed on *C. carnea* and then left to digest the meal for 36 h were not analyzed because the probability of detecting prey DNA was already very low after 24 h.

For each predator-prey combination, the half-life of the period for which the DNA of the prey could be detected was calculated. This value is defined as the time after which the DNA of the prey they have consumed can be detected in only 50% of cases, and is considered an appropriate standardized measure of the interval for which the DNA remains detectable (Greenstone et al., 2007).

Statistical analysis

Data was analyzed using SPSS 19.0 (SPSS, 2010). To investigate the influence of different factors on the detection of *E. balteatus* or *C. carnea* DNA, a generalized linear model was used with a Binomial error distribution and a logit link function (McCullagh & Nelder, 1989). Analysis always started with a saturated model. Interactions and non-significant main factors were deleted if their significance level was 0.05 or less. For the initial analysis of the whole dataset, the saturated model contained two factors: developmental stage of *H. axyridis* (fourth instar or adult) and species of prey (*E. balteatus* or *C. carnea*). When the data was analyzed for each predator-prey combination separately, the saturated model contained three factors in the case of combinations with fourth instar *H. axyridis*: meal size (5 eggs, 10 eggs or 1 second instar), digestion time (0, 2, 4, 6, 8, 12, 24, 36 or 48 h) and predator weight. For the experiments with adult *H. axyridis*, the saturated model contained five factors: meal size (5 eggs, 10 eggs or 1 second instar), digestion time (0, 2, 4, 6, 8, 12, 24, 36 or 48 h), sex (male or female), morpho-type (melanic or non-melanic) and predator weight. The most parsimonious model is reported, using likelihood ratios to ensure model fit. To calculate the detectability half-life of the DNA of the prey, the Probit Analysis procedure in SPSS 19.0 was used.

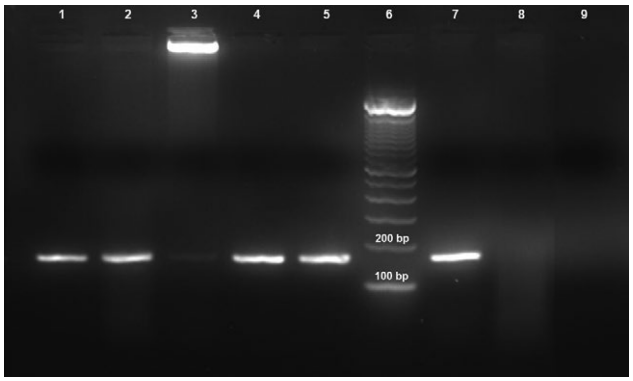

Fig. 1. Agarose gel showing PCR-amplified DNA using primers EB.F6 and EB.R5 (160 bp). lane 1: *Sphaerophoria scripta*, lane 2: *Eupeodes corolla*, lane 3: *Platycheirus peltatus*, lane 4: *Syrphus ribesii*, lane 5: *Syrphus vitripennis*, lane 6: molecular size marker (Invitrogen 100 bp ladder), lane 7: positive control (*Episyrphus balteatus*), lane 8: negative control (*Harmonia axyridis*), lane 9: negative control (H_2O).

RESULTS

Primer specificity and sensitivity

The primer pair that gave the best results in terms of both specificity and sensitivity was selected for each species of prey (see Table 2). The pair of primers EB.F6 and EB.R5 was not entirely species-specific, as they also amplified DNA of other species of hoverfly. Except for *Platycheirus peltatus* Meigen, the bands for the other hoverflies were of similar size and intensity as that for *E. balteatus* (Fig. 1). For primer pair CC.F4 and CC.R4, there was no amplification for any of the species listed in Table 1, indicating it is species specific for *C. carnea*. Both the *E. balteatus* and *C. carnea* primer pairs detected their target DNA in a mix of 1 part prey DNA to 104 parts of *H. axyridis* DNA.

Feeding experiments

In total, 540 samples of *H. axyridis* were analyzed in the PCR assays. All DNA extractions led to high quality DNA as for every sample tested a PCR product was obtained in a reaction with general ITS primers. The number of samples in which prey DNA was detected in each predator-prey combination is listed in Table 3.

Whether the DNA of both of the species of prey was detected depended on the developmental stage of *H. axyridis* ($\chi^2 = 14.72$; df = 1; $P < 0.001$ and $\chi^2 = 68.03$; df = 1; $P < 0.001$ for *E. balteatus* and *C. carnea*, respectively). The DNA of the prey was detected for longer in fourth instar than adult *H. axyridis*. Furthermore, the species of prey had a significant effect on the detection of the DNA of the prey in fourth instar and adult *H. axyridis* ($\chi^2 = 9.89$; df = 14; $P = 0.002$ and $\chi^2 = 10.41$; df = 1; $P = 0.001$, respectively). The level of detection was lower for *E. balteatus* than *C. carnea* in experiments with fourth instar *H. axyridis* and the reverse in experiments with adults. Because of the differences in detection success in the different predator-prey combinations, the data for each combination were analyzed separately.

Feeding experiments with fourth instar *H. axyridis*

Mean predator weight (± SE) before feeding was 18.99 ± 0.30 mg and 19.40 ± 0.27 mg when *E. balteatus* and *C. carnea* were the prey, respectively. When *E. balteatus* was the prey, no interactions were found between factors ($\chi^2 = 0.99$; df = 1 or 2; $P = 0.610$ for all contrasts). Neither meal size nor predator weight affected the detection of syrphid DNA ($\chi^2 = 3.81$; df = 2; $P = 0.149$ and $\chi^2 = 0.003$; df = 1; $P = 0.956$, respectively). In contrast, the detection of syrphid DNA was significantly dependent on how long ago it was ingested ($\chi^2 = 41.67$; df = 1; $P < 0.001$). DNA of *E. balteatus* could be detected in 80 to

TABLE 3. Number of positive samples (out of 5) in which the DNA of prey was detected in each predator-prey combination and different digestion periods. L2 = second instar.

H. axyridis	Digestion time (h)	*E. balteatus*			*C. carnea*		
		5 eggs	10 eggs	1 L2	5 eggs	10 eggs	1 L2
Fourth instar	0	5	5	5	5	5	5
	2	5	5	5	5	5	5
	4	5	5	4	5	5	5
	6	4	4	4	5	5	5
	8	5	5	5	5	5	3
	12	5	5	3	5	5	4
	18	4	4	2	2	4	4
	24	2	2	1	5	3	3
	36	2	1	2	3	5	2
	48	–	–	–	2	4	1
Adult	0	5	5	5	5	5	5
	2	5	5	5	5	5	5
	4	4	5	3	4	5	5
	6	4	5	5	3	2	2
	8	3	4	2	1	2	3
	12	3	0	4	1	2	0
	18	2	0	3	0	1	0
	24	1	2	0	0	0	1
	36	0	0	1	–	–	–

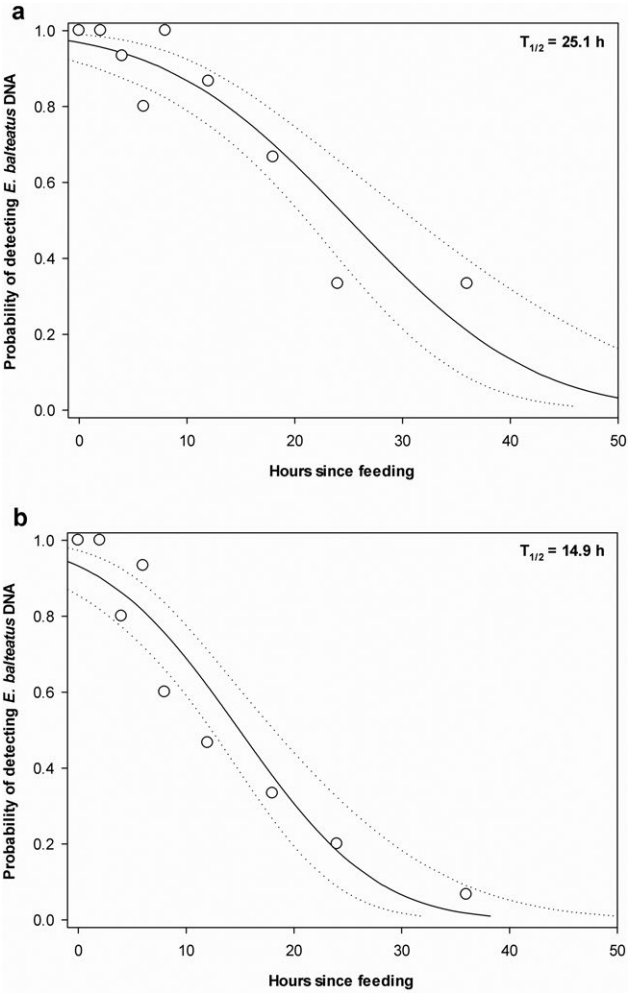

Fig. 2. Results of the feeding experiments in which *E. balteatus* was the prey and a: fourth instar *H. axyridis* and b: adult *H. axyridis* the predator. Circles are PCR data; n = 15 individual predators used for each data point. The solid lines represent the fitted probit model, the dotted lines the 95% confidence limits. T1/2: half-life of the period for which the DNA of the prey could be detected.

100% of cases within up to 12 h of digestion decreasing to between 20 to 40% after 36 h.

When *C. carnea* was the prey, there were no significant interactions between factors (χ^2 = 2.96; df = 1 or 2; P = 0.228 for all contrasts). The detection of the DNA of *C. carnea* was not affected by the weight of the predator (χ^2 = 1.26; df = 1; P = 0.261), but there was a significant effect of meal size. When one second instar of the chrysopid was eaten, the level of detection was lower than when 10 eggs were eaten (χ^2 = 7.34; df = 1; P = 0.007). There was no difference between one second instar and 5 eggs (χ^2 = 2.40; df = 1; P = 0.121) or between the two egg treatments (χ^2 = 1.89; df = 1; P = 0.169). Again, the period for which the prey was digested significantly affected the level of detection of the DNA of the prey (χ^2 = 31.07; df = 1; P < 0.001). In this case, DNA of *C. carnea* could be detected in 80 to 100% of cases up to 12 h after ingestion and there was still a 40% chance of detection after 48 h.

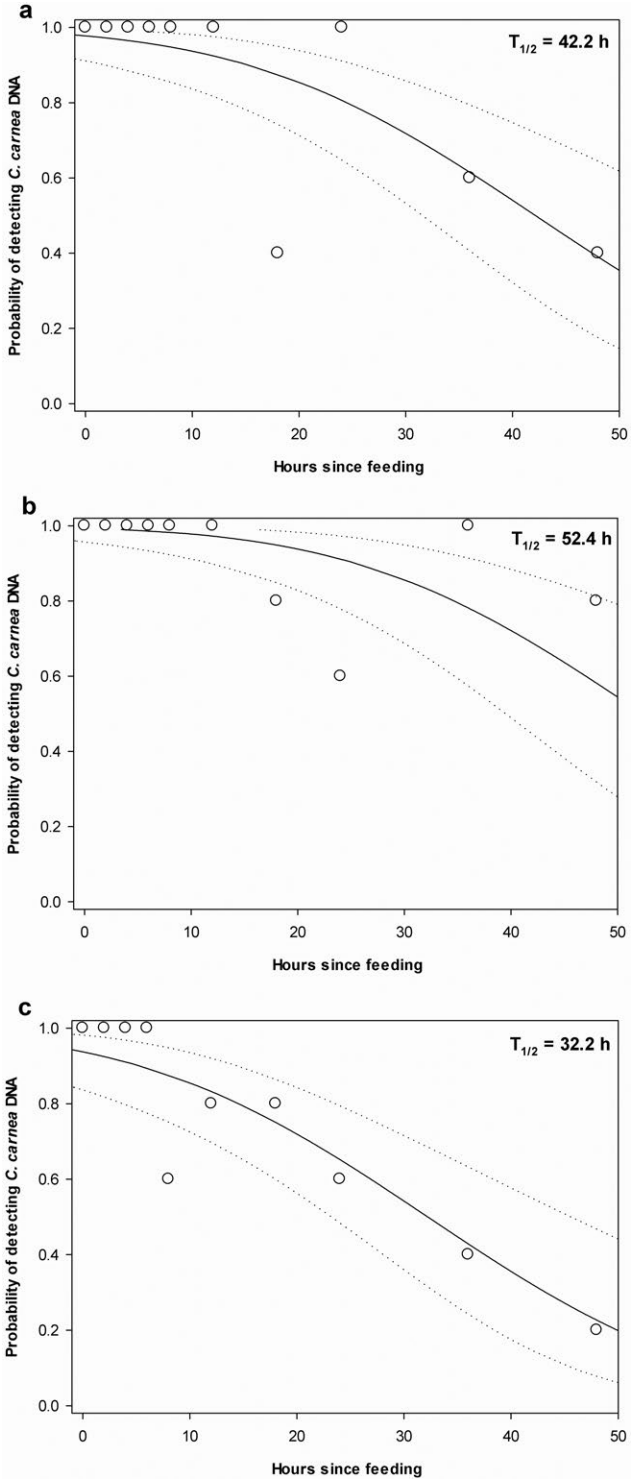

Fig. 3. Results of the feeding experiments in which a fourth instar *H. axyridis* was the predator and a: 5 eggs; b: 10 eggs; c: one second instar of *C. carnea* the prey. Circles are PCR data; n = 5 individual predators used for each data point. The solid lines represent the fitted probit model, the dotted lines the 95% confidence limits. T1/2: half-life of the period for which the DNA of the prey could be detected.

Feeding experiments with adult *H. axyridis*

Mean predator weight (± SE) before feeding was 37.11 ± 0.57 mg and 35.39 ± 0.67 mg for adult *H. axyridis* fed *E. balteatus* and *C. carnea*, respectively. Results for

approximately equal numbers of males and females, and melanic and non-melanic adults were analyzed (121 males and 134 females, and 137 non-melanic and 118 melanic individuals).

When *E. balteatus* was the prey, there were no interactions between factors ($\chi^2 = 3.13$; df = 1 or 2; $P = 0.077$ for all contrasts) and no effect of predator weight ($\chi^2 = 0.04$; df = 1; $P = 0.840$), sex ($\chi^2 = 1.76$; df = 1; $P = 0.184$) or morpho-type ($\chi^2 = 0.66$; df = 1; $P = 0.417$). Furthermore, meal size did not affect the level of detection of the DNA of the prey ($\chi^2 = 0.48$; df = 2; $P = 0.787$), although there was again a strong effect of the length of the period since the prey was ingested ($\chi^2 = 65.50$; df = 1; $P < 0.001$). Detection in 80 to 100% of the cases was obtained within up to 6 h of digestion, whereas after 24 h, it was only 20% of cases.

When adult *H. axyridis* were fed *C. carnea* there were no interactions between factors ($\chi^2 = 4.89$; df = 1 or 2; $P = 0.087$ for all contrasts). Again, there was no effect of predator weight ($\chi^2 = 0.83$; df = 1; $P = 0.361$), sex ($\chi^2 = 0.50$; df = 1; $P = 0.481$) morpho-type ($\chi^2 = 0.13$; df = 1; $P = 0.716$) or meal size ($\chi^2 = 1.06$; df = 2; $P = 0.590$). Period of time for which the prey had been digested strongly affected the level of detection of the DNA of the prey ($\chi^2 = 61.08$; df = 1; $P < 0.001$); it could only be detected in 80 to 100% of *H. axyridis* adults within 4 h of digestion and only in about 20% after 12 h.

The half-life of the period for which the DNA of prey can be detected

The half-life of the period for which the DNA of the prey can be detected for each predator-prey combination, was calculated using probit regression. Because there was no influence of meal size for both treatments with *E. balteatus* and for the treatment with adult *H. axyridis* and *C. carnea* as prey, the data for different meals (5 eggs, 10 eggs or one second instar) were pooled and only one probit model was calculated. Since meal size affected the level of detection of the DNA of the prey in the treatment in which fourth instar *H. axyridis* were fed *C. carnea*, the half-life was calculated for each meal size.

The probit models for *E. balteatus* represent the data well ($\chi^2 = 9.96$; df = 7; p = 0.191 for fourth instar and $\chi^2 = 10.26$; df = 7; p = 0.175 for adult *H. axyridis*) (Fig. 2). From these models, the half-life (T1/2) of the period for the DNA of the prey can be detected was 25.1 h for fourth instar and 14.9 h for adult *H. axyridis*. For the treatments in which *C. carnea* was the prey the estimated models represent the data well ($\chi^2 = 29.43$; df = 26; p = 0.292 for fourth instar and $\chi^2 = 7.93$; df = 6; p = 0.244 for adult *H. axyridis*) (Figs 3 and 4). The half-life for fourth instar *H. axyridis* was 42.2, 52.4 and 32.2 h for 5 eggs, 10 eggs or one second instar larva of *C. carnea*, respectively. For adult *H. axyridis*, the half-life was estimated to be 8.9 h regardless of meal size or life stage of the prey.

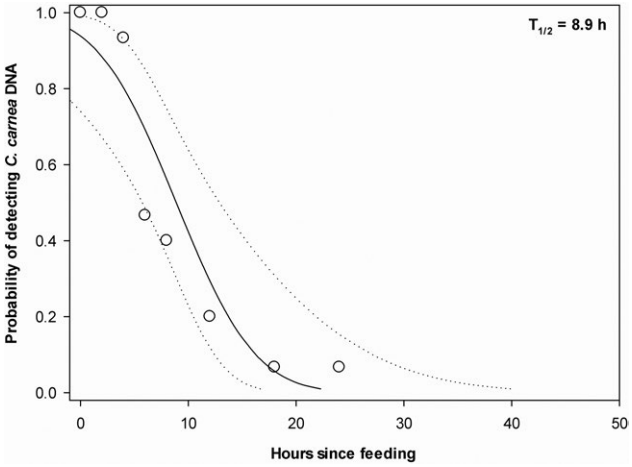

Fig. 4. Results of the feeding experiments in which an adult *H. axyridis* was the predator and *C. carnea* the prey. Circles are PCR data; n = 15 individual predators used for each data point. The solid line represents the fitted probit model, the dotted lines the 95% confidence limits. T1/2: half-life of the period for which the DNA of the prey could be detected.

DISCUSSION

If DNA-based gut-content analysis is to be successfully used in the field the primers need to be both specific and sensitive (King et al., 2008). Although the primers for *C. carnea* fulfilled these requirements, those for *E. balteatus* were not entirely species-specific, as they also amplified DNA of a few other species of hoverfly. Nonetheless, these primers may still be useful for assessing the incidence of IGP in the field under certain circumstances, for example, if *E. balteatus* outnumbers other hoverfly species at the investigated site. This is the case in several crops in Belgium where *E. balteatus* often represents 68 to 82% of all Syrphidae (Alhmedi et al., 2009). Furthermore, species identity could be confirmed by sequencing the PCR-product of a positive sample and comparing this sequence with an alignment of COI sequences obtained from all the species of hoverfly present. This approach, which has been used in studies on marine invertebrates (Blankenship & Yayanos, 2005), would allow an individual of *H. axyridis* to be screened for the presence of different species of intra-guild prey in its gut using a single PCR reaction, a single sequencing reaction and comparing the sequence using a reference barcode system. Using species-specific primers, several subsequent PCR-reactions or a multiplex PCR-assay (e.g. Harper et al., 2005) are needed to obtain the same result.

Both the *E. balteatus* and *C. carnea* primer pairs were able to detect their target DNA in a mixture of 1 part prey DNA to 10^4 parts *H. axyridis* DNA, which corresponds to a sensitivity of approximately 20 pg of prey DNA per PCR reaction. This represents an intermediate sensitivity level, as other studies report primer pairs with higher sensitivity by a factor 2 to 10^3 (Chen et al., 2000; Traugott et al., 2006; Thomas et al., 2012). However, when considering the weight of both the predator and prey, there was a maximum difference of a factor 80 (when an adult of *H. axyridis*, approximately 36 mg, consumed 5 eggs of *C.*

carnea, approximately 0.45 mg). Taking into account that fresh weight is not a perfect predictor of DNA content and the amount of prey DNA decreases over time, this primer sensitivity of $1 : 10^4$ is still believed to be sufficient to reliably detect prey DNA in the gut of *H. axyridis* within the time frames identified.

Our results indicate that the relationship between the detection of prey DNA and digestion time is negatively exponential. This is in agreement with similar studies on different predator groups (e.g. Agusti et al., 2003; Hosseini et al., 2008). The half-life of the period for which prey DNA could be detected for each predator-prey combination ranged from 8.9 to 52.4 h; the long half-life for fourth instar *H. axyridis* feeding on *C. carnea* indicates it is possible to detect relatively rare predation events (Gagnon et al., 2011a). However, long detection periods could obscure the frequency of predatory events in the field, since recent feeding events are not distinguishable from older ones, and could lead to overestimation of predation rates. Relatively short detection periods, like the ones found for the other predator-prey combinations in this study, facilitate a clearer interpretation of field data (Hagler & Naranjo, 1997; Sheppard & Harwood, 2005).

When comparing detection times of prey DNA in the gut of different Coccinellidae, McMillan et al. (2007) argued that the half-life of the period for which prey DNA can be detected is specific for each predator-prey combination, rather than specific for a particular predator or prey. This was confirmed by Gagnon et al. (2011a), who used this method to investigate IGP interactions between different ladybirds, including *H. axyridis*. Despite close taxonomic relations between the different predators and prey, the half-life of the period for which the DNA of the prey could be detected recorded in the latter study ranged from 5.2 to 19.3 h. In our study, the level of detection of the DNA of prey also differed for the different species of prey, supporting the conclusion of McMillan et al. (2007) and Gagnon et al. (2011a) that detection times cannot be generalized even if predators and prey are closely related and DNA fragments from the same region and of similar length are targeted.

One interesting finding of this study was that the level of detection of the DNA of prey over time declined faster for adult than fourth instar *H. axyridis*. In contrast to our findings, Hoogendoorn & Heimpel (2001) found no effect of predator stage on the detection of prey DNA for the ladybird *Coleomegilla maculata* De Geer (Coleoptera: Coccinellidae) fed eggs of *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae). Greenstone et al. (2010) report a difference in half-life of the period for which the DNA of prey can be detected in adults and immature stages of several predators, including *C. maculata*, fed one egg of *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae), however coccinellid larvae digested the egg DNA more rapidly than adults, which is the opposite of what we found. This strengthens our assumption that the level of detection of prey DNA is predator-, prey- and life stage-specific. This also supports the contention that coccinellid adults and larvae have different digestive capabilities and nutritional requirements (Michaud, 2005).

The results from our study and that of Greenstone et al. (2010) indicate that, at least in some cases, the rate of digestion of larval and adult ladybirds can differ. This may be explained by the different feeding mode of ladybird larvae and adults. In contrast to adults, digestion by the larvae of most predatory coccinellids is extra-oral and characterized by periodic regurgitation of fluid into the chewed up prey and the sucking back of the pre-digested prey (Hodek & Honek, 1996). Further Lundgren & Weber (2010) show that the rate of digestion of certain foods increases during larval development in *C. maculata*, which indicates a change in the quantity or quality of the digestive enzymes. Differences in the composition of the digestive enzymes of fourth instar and adult individuals may thus also account for the different rates of digestion rates recorded in this study.

In general, there was no influence of meal size on the detection of *E. balteatus* or *C. carnea* DNA in the gut of *H. axyridis*. Only when fourth instar larvae were fed *C. carnea* did meal size affect the level of detection. In most studies that use PCR to detect prey DNA in the guts of predators, the amount of prey eaten did not affect the detection of prey DNA (Zaidi et al., 1999; Hoogendoorn & Heimpel, 2001; Juen & Traugott, 2005). In contrast, Weber & Lundgren (2009) found a significant relation between the quantity of target DNA detected and the number of *L. decemlineata* eggs consumed by *C. maculata* when using quantitative PCR. It thus appears that, due to its high sensitivity, conventional PCR cannot be used to measure meal-size-related differences in prey DNA, at least not for the small range of meal sizes used in the present study. The single predator-prey combination in which we found an influence of meal size was also the combination for which the DNA of the prey was detectable for the longest and probably reflects the increased statistical power to separate digestion curves under these circumstances. The fact that the level of detection of the DNA of the prey was lower after the consumption of a single second instar of *C. carnea* than 10 eggs, despite the former being 30% heavier, may be because the larvae were not completely consumed. That is, the amount of DNA ingested was lower and therefore the detection time would have been shorter. Furthermore, the relatively low number of replicates could also have contributed to the observed effect of meal size.

Despite the many advantages of using molecular gut-content analysis to investigate predator-prey relationships and IGP in the field (Aebi et al., 2011), this method has limitations. In addition to the factors investigated in this study, temperature, time for which the predators are starved and what they eat subsequently are reported to affect the level at which prey can be detected (Hosseini et al., 2008; Weber & Lundgren, 2009). Furthermore, this technique cannot discriminate primary and secondary predation (Sheppard et al., 2005), scavenging behaviour (Juen & Traugott, 2005) or prey DNA obtained via cannibalism (Sheppard & Harwood, 2005).

The method of DNA-based gut-content analysis developed in this study may improve our understanding of the ecological importance of IGP by this invasive ladybird on populations of both *E. balteatus* and *C. carnea* in the field. As a first in-field validation of this method, larvae of *H. axyridis*, mainly collected from lime trees (*Tilia* x *europaea* L.) in different European countries, were analyzed using the molecular markers developed in this study (Brown et al., in prep.). Approximately 180 individuals of *H. axyridis* were tested, with detection rates varying between 1.1 and 2.8%. The knowledge obtained from this and future field studies may also assist in unraveling the mechanisms underlying the invasive success of *H. axyridis*.

ACKNOWLEDGEMENTS. The funding for this project came from a grant from the Research Foundation – Flanders (FWO Vlaanderen) awarded to B. Ingels. T. Van Leeuwen is a postdoctoral fellow of the Research Foundation – Flanders. The authors would like to thank P. Brown and A. Howe for supplying insect samples, F. Bigler and F. Widmer for providing access to research infrastructures at ART, and A. Thomas and R. Zindel for helpful suggestions.

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Received November 5, 2012; revised and accepted April 8, 2013