PCR-BASED GUT CONTENT ANALYSIS OF INSECT PREDATORS: A FIELD STUDY

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INTRODUCTION

Identification of the gut contents of predatory insects can provide information on trophic relationships and the dynamics of predator-prey interactions. Several problems may be encountered in determining the diet of predatory insects in the field. Direct observations of predation in the field (Carter and Dixon, 1982; Carter *et al*., 1984; Legaspi, 1996; Heimpel *et al*., 1997; Munyaneza and Obrycki, 1998) can be complicated by the fact that both prey and predator are often small and cryptic. Microscopic analysis of gut contents (Walker *et al*., 1988; Aussell and Linley, 1994; Powell *et al*., 1996; Sleaford *et al*., 1996; Triltsch, 1997) is possible for chewing predators that ingest relatively large prey fragments, but is not suitable for piercing-sucking predators.

Immunological assays using prey-specific protein antibodies (Greenstone and Hunt, 1993; Hagler *et al*., 1995; Powell *et al*., 1996; Hagler and Naranjo, 1997; Hagler, 1998; Symondson *et al*., 1999a, b) are currently widely used to identify predator gut contents. These assays are used to determine absence or presence of prey in the gut. The accuracy of the assay depends on several factors, including temperature, meal size, time since feeding, resistance of the target protein to digestion, and predator species (Hagler *et al*., 1997; Symondson *et al*., 1997, 1999b; Hagler, 1998).

Here, we describe the analysis of gut contents of field-caught coccinellid beetles, using a polymerase chain reaction (PCR). This work builds upon a number of recent studies on the use of PCR in the analysis of predator gut contents (Agustí *et al*., 1999, 2000; Zaidi *et al*., 1999; Chen *et al*., 2000; Hoogendoorn and Heimpel, 2001). We used primer sets that amplify fragments of different lengths that each have a characteristic detection time, which made it possible to estimate the time since feeding from the number of bands that was detectable (Hoogendoorn and Heimpel, 2001). The primers we used are derived from ribosomal RNA genes, which are present in multiple copies in the insect genome (Hoy, 1994).

MATERIALS AND METHODS

The System

The European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae), is an important exotic pest of corn in north central North America (Andow, 1996). It was first detected in North America in 1917 (Baker *et al*., 1949). *Coleomegilla maculata* De Geer (Coleoptera: Coccinellidae) occurs throughout central and eastern North America, where it is a native species. It has a diverse diet, consisting of aphids, insect eggs (including European corn borer eggs), and pollen (Andow, 1990, 1996; Munyaneza and Obrycki, 1998). *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) has been introduced into North America from Asia. It was first detected in 1988 (Chapin and Brou, 1991) and has since spread widely (Colunga-Garcia and Gage, 1998). *Harmonia axyridis*, like *C. maculata*, is a polyphagous predator (Lucas *et al*., 1997; Kalaskar and Evans, 2001).

Molecular Assays

DNA was extracted from whole insects, using a protocol modified from Bender *et al*. (1983), as described in Hoogendoorn and Heimpel (2001). For detection of *O. nubilalis* DNA, a DNA sequence

was used that included the complete sequence of the internal transcribed spacer (ITS-1) and partial sequences of the flanking 5.8S and 18S nuclear ribosomal RNA genes (Marçon *et al*., 1999). We used a set of four primers that were designed to amplify sequences of four different lengths, in addition to a primer that amplified DNA from both predators and prey, which was used to check if DNA extractions were successful. Tests for cross-reactivity were performed with three potential prey species of *C. maculata* and *H. axyridis*: two lepidopteran species, *Diatraea grandiosella* Dyar and *Helicoverpa zea* (Boddie), and an aphid, *Myzus persicae* (Sulzer) (Hoogendoorn and Heimpel 2001).

Feeding Experiments

From a laboratory experiment on feeding by *C. maculata* (Hoogendoorn and Heimpel 2001), we obtained estimations of detection time for each amplified sequence in this species. We repeated this experiment on a smaller scale, using *H. axyridis* to determine if detection times are similar in this species. We allowed *H. axyridis* adults to feed on European corn borer egg masses for 30 minutes, and recorded the number of eggs that were consumed. Four individuals were frozen immediately after feeding. We allowed other individuals to digest the meal at 27 °C for 0, 1, 2, 4, 6, or 8 hours (two individuals for each time period). After these time periods, individuals were frozen at minus 20 °C and their gut contents were assayed as described in Hoogendoorn and Heimpel (2001).

Insect Sampling

Fresh egg masses (1 day old) of European corn borer on wax paper circles (purchased from French Agricultural Research, Lamberton, Minnesota) were pinned on corn plants that were planted in plots of 3,600 m^2 (1999) or 400 m^2 (2000). Egg mass density in 1999 was 1 per 45 m^2 and was 1 per 4 m^2 in 2000. In both years, plots were located in a field that was planted with soybeans. Plots were approximately 60 m apart.

In 1999, a total of 80 egg masses were present in each plot, and in 2000 there were 100 egg masses per plot, spaced evenly. The egg masses were left in the plots for 3 days, during which *C. maculata* and *H. axyridis* adults and larvae were collected from each plot. In 1999, one outplant of egg masses was carried out, on July 27. Beetles were collected on July 28 and 29. In 2000, two outplants were carried out: the first on July 12 (beetles were collected on July 12, 13, and 14) and the second on August 23 (beetles were collected on August 23 and 25). Individuals were collected in vials of 70% ethanol, and they were stored on ice. Upon return to the laboratory, beetles were frozen at minus 20 °C. Their gut contents were assayed as described in Hoogendoorn and Heimpel (2001). For the initial assays only the primer set that amplifies the shortest sequence (of 150 bp) was used. This fragment remained detectable for up to 12 hours *in C. maculata* (Hoogendoorn and Heimpel 2001). If prey DNA was detected using this set of primers, the PCR was repeated using the primer sets that amplify longer sequences to obtain an estimate of the minimum and the maximum time since feeding.

RESULTS

Detection Time for Amplified Sequences

In a laboratory experiment, the number of bands detectable in *C. maculata* gut contents decreased with time, and this relationship could be described by a quadratic function for the range of detectability from 0 to 12 hours (Hoogendoorn and Heimpel, 2001). The shortest sequence was always detectable for the longest period of time. Sex, weight, and meal size did not affect detectability.

The average detection time for each band in *C. maculata* and the 95% confidence intervals based on 1,000 bootstrap iterations are listed in Table 1 (from Hoogendoorn and Heimpel, 2001). Beetle weight, sex, and meal size had no effect on detection time.

Table 1. Average detection time and confidence interval (CI) for each number of bands in *Coleomegilla maculata.*

	4 bands	3 bands	2 bands	1 band
Average time span (h)	1.83	4.43	4.0	6.41
95% CI	1.36-2.29	3.00-5.78	3.08-4.89	5.46-7.35

In *H. axyridis*, detection times are similar, with the shortest sequence always detectable for the longest time period. Weight and meal size had no influence on detectability, and time had a significant influence on the number of bands that was detectable (R^2 = 13.27, p = 0.0071). In Table 2 a comparison is shown of detection times at 27° C in *C. maculata* and *H. axyridis*.

Table 2. Detection times of sequences of four different lengths at 27°C for *Coleomegilla maculata* and *Harmonia axyridis.*

Time Since Feeding (h)	Average No. of Bands Visible in H. axyridis (two observations per time)	Average No. of Bands Visible in C. maculata (four observations per time)
0	4	4
1	4	3
2	2.5	2
4	1.5	2.5
6	1	0.75
8	1.5	0.5

In Table 3, the results are shown of the multiple regression on the data from Hoogendoorn and Heimpel (2001), with the addition of the data from the feeding experiment on *H. axyridis*. There is no effect of species on the number of bands that is detectable, and as in Hoogendoorn and Heimpel (2001) the only significant effects on the number of bands detected are time since feeding and temperature (note that *H. axyridis* digestion rate was tested only at 27°C).

Table 3. Results of multiple regression on the number of bands detectable in *Coleomegilla maculata* and *Harmonia axyridis.*

Factor	d.f.	Sum of squares	F ratio	Prob. > F
Time since feeding	1	75.933	75.687	< 0.0001
Temperature	1	6.2172	6.1971	0.0169
Beetle weight (mg)	1	0.4489	0.4475	0.5055
Eggs eaten	1	2.3973	2.3896	0.1262
Species	1	0.0002	0.0002	0.9889

Cross reactivity tests showed that no DNA of *M. persicae* or *H. zea* was amplified. In *D. grandiosella*, sequences were amplified that differed substantially in size from those amplified in *O. nubilalis* (Hoogendoorn and Heimpel, 2001).

Field Samples

None of the individuals collected from the field in 1999 tested positive for European corn borer DNA (the total number of individuals collected was 86 for *C. maculata* and 21 for *H. axyridis*). In 2000, 117 *C. maculata* individuals and 338 *H. axyridis* individuals were analyzed. Of these individuals, two (one larva and one adult) *C. maculata* (1.7%), and four (adult) *H. axyridis* (1.2%) tested positive for European corn borer DNA. Both *C. maculata* individuals that tested positive showed four bands, and this suggests that they had fed less then 2 hours before collection. Of the *H. axyridis* individuals that tested positive, two showed three bands and two showed one band.

DISCUSSION

Laboratory Studies

We showed that detection of prey DNA is dependent on fragment size, and that shorter fragments can be detected for a longer time span after ingestion of the prey than longer fragments. The rate of digestion increased with temperature, which has also been shown in studies by Hagler and Cohen (1990) and Loder *et al*. (1998). Detection time does not depend on meal size, sex, or predator weight, which indicates that this method may be suitable for use in the analysis of field-caught predators, for which these factors cannot be controlled. However, the detection times we estimated in the laboratory may not necessarily reflect detection times in the field. Temperature fluctuations, age of egg masses, and satiation of predators are factors that may influence detection time of prey DNA in field-caught predators.

The relationship between time since feeding and number of bands detected is similar in adult *C. maculata* and *H. axyridis*, which suggests that digestion rate of prey DNA does not differ between adults of these species. However, *H. axyridis* larvae seem to feed more, and suffer more from low prey densities than *C. maculata*, and growth rate of *H. axyridis* larvae also appears to be higher in the field. Only adult *H. axyridis* were included in this laboratory study. However, larvae and adults were collected from the field. It is possible that digestion rate in *H. axyridis* larvae is higher than in *C. maculata* larvae.

Detection of DNA in Field-Collected Individuals

Of the large number of individuals that was analyzed, only six individuals tested positive for DNA of European corn borer. Although it is possible that the maximum detection time of DNA is shorter in the field than in the laboratory, our data suggest a low encounter rate of predators with European corn borer egg masses in the field. In 1999 and 2000 there was a very low natural infestation rate of European corn borer in our research plots. The densities of European corn borer egg masses we achieved during outplants (one egg mass per 45 m^2 in 1999, one egg mass per 4 m^2 in 2000) were low compared with the number of beetles present, which from our samples we estimated to be 8 to 20 beetles (*C. maculata* and *H. axyridis*) per m^2. The chance of sampling an individual that had been feeding on European corn borer thus seemed very small, especially in 1999. However, our results show that it is possible to detect European corn borer DNA in the guts of predators that are collected from the field.

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REFERENCES

Agustí, N., M. C. de Vicente, and R. Gabarra. 1999a. Development of sequence amplified characterized region (SCAR) markers of *Helicoverpa armigera*: a new polymerase chain reaction-based technique for predator gut analysis. *Molecular Ecology* 8: 1467-1474.

Agustí, N., M. C. de Vicente, and R. Gabarra. 2000. Developing SCAR markers to study predation on *Trialeurodes vaporariorum*. *Insect Molecular Biology* 9: 263-268.

Andow, D. A. 1990. Characterization of predation on egg masses of *Ostrinia nubilalis* (Lepidoptera: Pyralidae). *Annals of the Entomological Society of America* 83: 482-486.

Andow, D. A. 1996. Augmenting natural enemies in maize using vegetational diversity, pp. 137-153. *In* Grey, G. (ed.). *FFTC Book Series 47: Biological Pest Control in Systems of Integrated Pest Management*. FFTC, Taipei, Taiwan.

Aussel, J.-P. and J. R. Linley. 1994. Natural food and feeding behavior of *Culicoides furens* larvae (Diptera: Ceratopogonidae). *Journal of Medical Entomology* 31: 99-104.

Baker, W. A., W. G. Bradley, and C. A. Clark. 1949. Biological control of the European corn borer in the United States. *Technical Bulletin 983,* U.S. Department of Agriculture, Washington, D.C.

Bender, W., P. Spierer, and D. S. Hogness. 1983. Chromosomal walking and jumping to isolate DNA from the *Ace* and *Rosy* loci and the bithorax complex in *Drosophila melanogaster*. *Journal of Molecular Biology* 168: 17-33.

Carter, M. C. and A. F. G. Dixon. 1982. Habitat quality and the foraging behaviour of coccinellid larvae. *Journal of Animal Ecology* 51: 865-878.

Carter, M. C., D. Sutherland, and A. F. G. Dixon. 1984. Plant structure and the searching efficiency of coccinellid larvae. *Oecologia* 63: 394-397.

Chapin , J. B. and V. A. Brou. 1991. *Harmonia axyridis* (Pallas), the third species of the genus to be found in the U.S. (Coleoptera: Coccinellidae). *Proceedings of the Entomological Society of Washington* 93: 630-635.

Chen, Y., K. L. Giles, M. E. Payton, and M. H. Greenstone. 2000. Identifying key cereal aphid predators by molecular gut analysis. *Molecular Ecology* 9: 1887-1898.

Colunga-Garcia, G. M. and S. H. Gage. 1998. Arrival, establishment, and habitat use of he multicolored Asian lady beetle (Coleoptera: Coccinellidae) in a Michigan landscape. *Environmental Entomology* 27: 1574-1580.

Greenstone, M. H. and J. H. Hunt. 1993. Determination of prey antigen half-life in *Polistes metricus* using a monoclonal antibody-based immunodot assay. *Entomologia Experimentalis et Applicata* 68: 1-7.

Hagler, J. R. 1998. Variation in the efficacy of several predator gut content immunoassays. *Biological Control* 12: 25-32.

Hagler, J. R. and A. C. Cohen.1990. Effects of time and temperature on digestion of purified antigen by *Geocoris punctipes* (Hemiptera: Lygaeidae) reared on artificial diet. *Annals of the Entomological Society of America* 83: 1177-1180.

Hagler, J. R. and S. E. Naranjo. 1997. Measuring the sensitivity of an indirect predator gut content ELISA: detectability of prey remains in relation to predator species, temperature, time, and meal size. *Biological Control* 9: 112-119.

Hagler, J. R., S. L. Buchmann, and D. A. Hagler. 1995. A simple method to quantify dot blots for predator gut analyses. *Journal of Entomological Science* 30: 95-98.

Hagler, J. R., S. E. Naranjo, M. L. Erickson, S. A. Machtley, and S. F. Wright. 1997. Immunological examinations of species variability in predator gut content assays: effects of predator:prey protein ratio on immunoassay sensitivity. *Biological Control* 9: 120-128.

Heimpel, G. E., J. A. Rosenheim, and M. Mangel. 1997. Predation on adult *Aphytis* parasitoids in the field. *Oecologia* 110: 346-352.

Hoogendoorn, M. and G. E. Heimpel. 2001. PCR-based gut content analysis of insect predators: using ribosomal ITS-1 fragments from prey to estimate predation frequency. *Molecular Ecology* 10: 2059-2067.

Hoy, M. A. 1994. *Insect Molecular Genetics: An Introduction to Principles and Applications.* Academic Press, San Diego, California, U.S.A.

Kalaskar, A. and E. W. Evans. 2001. Larval responses of aphidophagous lady beetles (Coleoptera: Coccinellidae) to weevil larvae versus aphids as prey. *Annals of the Entomological Society of America* 94: 76-81.

Legaspi, J. C., B. C. Legaspi, R. L. Meaghher, and M. A. Ciomperlik. 1996. Evaluation of *Serangium parcesetosum* (Coleoptera: Coccinellidae) as a biological control agent of the silverleaf whitefly (Homoptera: Aleyrodidae). *Environmental Entomology* 25: 1421-1427.

Loder, P. M. J., J. W. Hargrove, and S. E. Randolph. 1998. A model for blood meal digestion and fat metabolism in male tsetse flies (Glossinidae). *Physiological Entomology* 23: 43-52.

Lucas, E., D. Coderre, and C. Vincent. 1997. Voracity and feeding preferences of two aphidophagous ladybeetles against European red mite (Acarina: Tentranychidae). *Phytoprotection* 78: 117-123.

Marçon, P. C. R. G., D. B. Taylor, C. E. Mason, R. L. Hellmich, and B. D. Siegfried. 1999. Genetic similarity among pheromone and voltinism races of *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae). *Insect Molecular Biology* 8: 213-221.

Munyaneza, J. and J. J. Obrycki. 1998. Searching behavior of *Coleomegilla maculata* larvae feeding on Colorado potato beetle eggs. *Biological Control* 13: 85-90.

Powell, W., M. P. Walton, and M. A. Jervis. 1996. Populations and communities, pp. 223-293. *In* Jervis, M. and N. A. C. Kidd (eds.). *Insect Natural Enemies*. Chapman and Hall, London.

Sleaford, F., D. E. Bignell, and P. Eggleton. 1996. A pilot analysis of gut contents in termites from the Mbalmayo Forest Reserve, Cameroon. *Ecological Entomology* 21: 279-288.

Symondson, W. O. C., M. L. Erickson, and J. E. Liddell. 1997. Species-specific detection of predation by Coleoptera on the Milacid slug *Tandonia budapestensis* (Mollusca: Pulmonata). *Biocontrol Science and Technology* 7: 457-465.

Symondson, W. O. C., M. L. Erickson, and J. E. Liddell. 1999a. Development of a monoclonal antibody for the detection and quantification of predation on slugs within the *Arion hortensis* agg. (Mollusca: Pulmonata). *Biological Control* 16: 274-282.

Symondson, W. O. C., M. L. Erickson, J. E. Liddell, and K. G. I. Jayawardena. 1999b. Amplified detection, using a monoclonal antibody, of an aphid-specific epitope exposed during digestion in the gut of a predator. *Insect Biochemistry and Molecular Biology* 29: 873-882.

Triltsch, H. 1997. Gut contents in field sampled adults of *Coccinella septempunctata* (Col.: Coccinellidae). *Entomophaga* 42: 125-131.

Walker, E. D., E. J. Olds, and R. W. Merritt. 1988. Gut content analysis of mosquito larvae (Diptera: Culicidae) using DAPI stain and epifluorescence microscopy. *Journal of Medical Entomology* 25: 551-554.

Zaidi, R. H., Z. Jaal, N. J. Hawkes, J. Hemingway, and W. O. C. Symondson. 1999. Can multiple-copy sequences of prey DNA be detected amongst the gut contents of invertebrate predators? *Molecular Ecology* 8: 2081-2087.

DNA/PCR based molecular gut analysis of Coccinella septempunctata, Cheilomenes sexmaculata, Hippodamia convergens, Camponotus pennsylvanicus and Solenopsis invicta, for six hemipteran pest species namely Macrosiphum miscanthi, Aphis maidis, Schizaphis graminum, Bemisia tabaci, Empoasca kerri and Drosicha mangiferae was done in the laboratory.Â Gut content analysis allow prey consumption to be assessed after the feeding event (or events) occurred naturally in the field.Â The prey DNA detection of a single species is not affected by the sex of a predator, or the presence of other prey species in the gut contents (Hosseini et al., 2008). Protein-based gut content analysis are well-represented in literature (Pollard 1968, Boreham and Ohiagu 1978, Lister et al. 1987, Sunderland et al.Â 1992), and pathogens in gut contents of insects (Hill and Crampton 1994, Pinto et al. 2000). Wolbachia, a bacterial endosymbiont, was tracked by amplifying prey mite microorganisms in predatory mites (Johanowicz and Hoy 1996). Marine biologists identified stone flounder mitochondrial DNA from sand shrimp (Asahida et al.Â Investigators compared the results of PCR gut content analysis to those obtained by ELISA by running both tests to detect slug predation on each field-collected Pterostichus melanarius individual. Slug remains were detected for 4.3% of beetles using PCR, contrasting with the 8% positive by ELISA (Dodd et al., 2003).