

Detection of shield beetle remains in predators using a monoclonal antibody

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Abstract: *Cassida rubiginosa* Muell. (Col., Chrysomelidae) is considered to be a native biocontrol agent of the creeping thistle, *Cirsium arvense* (L.) Scop, one of the world's worst weeds. However, predation is a major mortality factor in *C. rubiginosa* populations and prevents high beetle densities in nature. We determined temperature-dependent detection periods of shield beetle remains within predators by immunological gut analysis, in order to define the time period within which predation must have occurred. We conducted feeding trials with the model predators *Nabis mirmicoides* Costa and *Chrysopa carnea* Stephen at two and three different temperatures, respectively, using *C. rubiginosa* larvae as prey. Indirect enzyme-linked immunosorbent assay (ELISA) was used to examine the predators for the presence of prey antigen. The monoclonal antibody (MAb) CRL5-1 clearly identified predation by *N. mirmicoides* for up to 47 h and by *C. carnea* up to 96 h post-feeding at 15°C. An exponential decay gave a good description of the antigen time course within predators. Calculated detection periods for the antigen were 234.7 h at 15°C and 85.0 h at 20°C in *N. mirmicoides* and 215.3 h (15°C), 91.9 h (20°C) and 79.1 h (25°C) in *C. carnea*. The reported detection periods of prey remains in predators are the longest known in immunology, and are much higher than the recently published detection periods of prey DNA in predators. Therefore MAb CRL5-1 is a highly valuable tool for quantifying predation in the field.

Key words: *Cassida rubiginosa*, detection period, ELISA, feeding experiments, gut content analysis, serology

1 Introduction

Predation is not only one of the major factors affecting population dynamics of insect herbivores, but also one of the most difficult to determine and quantify. Laboratory experiments can be used to evaluate the acceptability of a particular prey and rates of predation. However, these type of studies seldom translate to actual field situations where requirements of predator search are more demanding, a variety of potential prey species in changing abundances are present, and both predator and prey are subject to changing environmental conditions. Immunoassays using monoclonal antibodies (MAb) are an elegant way to evaluate predators in their natural environment (KIDD and JERVIS, 1996).

Serological studies can provide accurate data on field predation rates and the importance of a particular prey for the predator, if the detection times of prey antigen in the gut of predators and accurate estimates of predator and prey densities are obtained (SOPP and SUNDERLAND, 1989; KIDD and JERVIS, 1996). As detection periods provide a measure of the interval in which predation must have occurred, they are important for the construction of models to estimate predation rates in the field. The detection interval is a key parameter in most indices that have been developed to assess predation using immunoassays (reviewed by SOPP et al., 1992).

Cassida rubiginosa Muell. (Col., Chrysomelidae) is considered to be a native biocontrol agent against creeping thistle, *Cirsium arvense* (L.) Scop (BACHER and SCHWAB, 2000), one of the world's worst weeds (HOLM et al., 1977). To augment and sustain the densities of natural populations of *C. rubiginosa* it is essential to investigate the factors responsible for the low population densities of the agents (FRIEDLI and BACHER, 2001). Predation is a major mortality factor in *C. rubiginosa* populations (SCHENK and BACHER, 2002), especially in late larval instars (S. BACHER and E. KAUFMANN unpublished data). The actual predator species and their predation rates are known only from two study sites with the aid of video surveillance (SCHENK and BACHER, 2002). However, while video surveillance gives the most accurate data on the predator complex, it is less suitable for simultaneously screening a large number of field sites. Serology is the method of choice for obtaining a more complete picture of the predator complex of *C. rubiginosa* at a number of different field sites (SYMONDSON, 2002).

We recently developed an MAb to the shield beetle *C. rubiginosa* (BACHER et al., 1999). With this antibody we are able to examine the gut contents of field-collected predators for the presence of prey antigen by enzyme-linked immunosorbent assay (ELISA). This study reports the half-life and detection period of *C. rubiginosa* antigen at different temperatures in two model

predators, *Nabis mirmicoides* Costa (Hem., Nabidae) and *Chrysopa carnea* Stephen (Neurop., Chrysopidae) using the MAb CRL5-1 in indirect ELISA. Nabid bugs, namely *Nabis apterus* (F.) and *Nabis limbatus* (Dahlb-ohm) were also reported to be the predators of *C. rubiginosa* (listed in OLMSTEAD, 1996). We found nabid bugs to be common on creeping thistle plants at field sites of *C. rubiginosa* (D. SCHENK and S. BACHER personal observation). *C. carnea* larvae are generalist predators occurring in the same habitats as *C. rubiginosa* and were occasionally observed preying on *C. rubiginosa* larvae in the field (SCHENK and BACHER, 2002; D. SCHENK and S. BACHER personal observation).

2 Materials and Methods

2.1 Feeding trials

Cassida rubiginosa and *C. carnea* larvae were reared in the laboratory and *N. mirmicoides* nymphs and adults were collected in August 1998 at an ecological compensation area near Bern, Switzerland. Both predator species were maintained at a photoperiod of 16.00 : 8.00 hours (L : D) and temperature of 20°C. *C. carnea* and *N. mirmicoides* were not starved before the tests, but kept on alternative, non-target food: aphids (*Acyrtosiphon pisum* Harris) and Mediterranean flour moth (*Ephestia kuehniella* Zell.) eggs. Fresh water was provided every day. *N. mirmicoides* were maintained at least 1 week prior to testing on non-target food in order to ensure that no *C. rubiginosa* antigens remained in their guts.

Feeding trials were conducted in plastic Petri dishes of 90 mm diameter with a filter paper on the bottom. One individual of *N. mirmicoides* or *C. carnea* was placed together with one fifth instar *C. rubiginosa* larva as prey in the Petri dish. The predators were removed after finishing their meal. Feeding trials took place in a controlled temperature room at 15 or 20°C (both predators) and additionally at 25°C with *C. carnea* larvae. Both *C. rubiginosa* larvae and their predators were weighed before and after feeding (± 0.1 mg, Mettler Toledo, Greifensee, CH) and the feeding time was registered. After feeding, the predators were placed individually in plastic containers (10 × 5 × 5 cm) with water and aphids or *E. kuehniella* eggs *ad libitum* in incubators at constant temperatures until killing (*N. mirmicoides*: 15 and 20°C; *C. carnea*: 15, 20 and 25°C). SYMONDSON and LIDDELL (1995) found that unrestricted feeding on alternative prey, following consumption of the target prey, significantly increased the detection period of their antigen in the gut of the predators. We chose a set up in which predators had access to alternative prey after feeding on the target prey because it reflects the field situation more realistically (GREENSTONE, 1996). Both predators are known to be generalist predators, and it is quite likely that they encounter and feed on alternative prey in a natural situation.

Predators were killed by deep freezing at different times post-feeding (up to 96 h; figs 1 and 2) and kept at -30°C until ELISA testing (see below).

Because of the limited number of *N. mirmicoides*, feeding trials with this predator species were performed with one individual at different times post-feeding (15°C: $n = 19$; 20°C: $n = 17$). On the contrary, the larger number of *C. carnea* larvae available allowed us to test up to 18 individuals at each trial (15°C: $n = 87$; 20°C: $n = 55$; 25°C: $n = 77$). Twenty-six individuals of *C. carnea* and five individuals of *N. mirmicoides* were fed only with non-target food and deep-frozen immediately after feeding (controls).

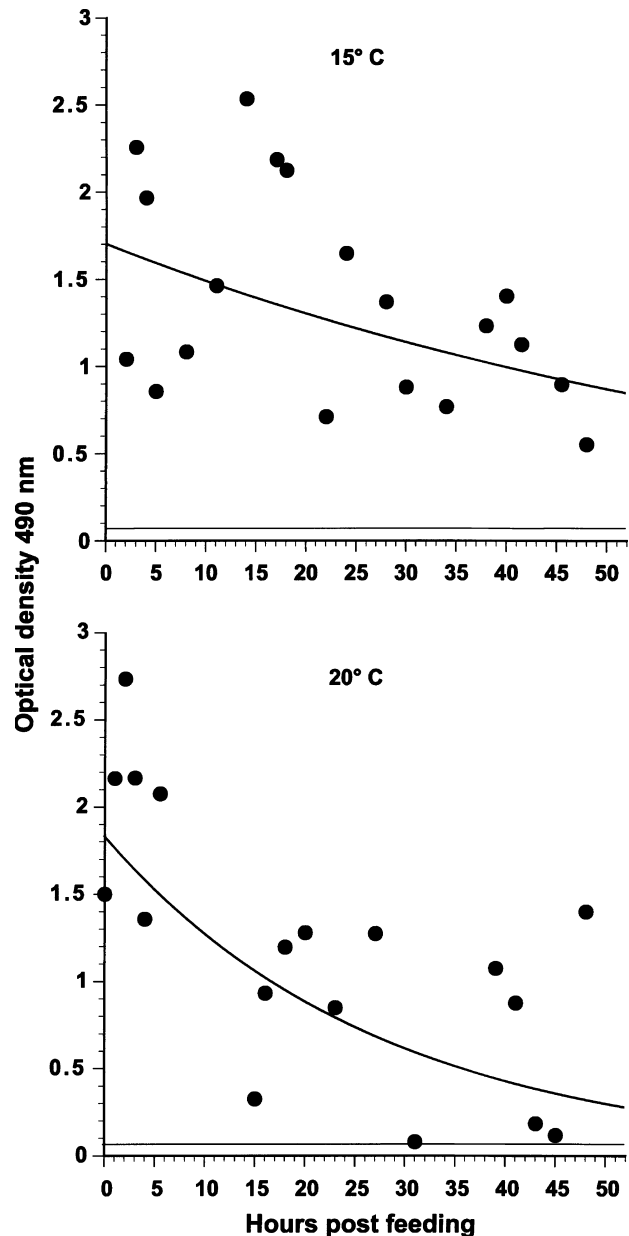


Fig. 1. ELISA results (optical density) for 19 *Nabis mirmicoides* feeding on *Cassida rubiginosa* larvae and killed at different hours (up to 48 h) post-feeding at 15°C. $OD = 1.69 * \exp(-0.013 * t)$; $r^2 = 0.221$; $P = 0.042$. Antibody response (OD) for 17 *N. mirmicoides* feeding on *C. rubiginosa* larvae and killed at different hours (up to 48 h) post-feeding at 20°C. Regression: $OD = 1.83 * \exp(-0.037 * t)$; $r^2 = 0.341$; $P = 0.01$

2.2 Sample preparation and ELISA tests

Individual predators were homogenized with a tissue grinder in 1 ml of a 0.2-M carbonate buffer pH 9.4. Supernatants were collected by centrifugation at 10 000 g for 2 min. Tests were performed using the monoclonal antibody CRL5-1 in an indirect ELISA as described in detail by BACHER et al. (1999). In brief, each well of the 96-well assay plates (Immulon 4 flat bottom plate; Dynatech Laboratories Inc., Chantilly, VA) was incubated with 100 μ l of the predator homogenate supernatant overnight at 4°C with agitation. After incubation, the plates were emptied and washed five times with a

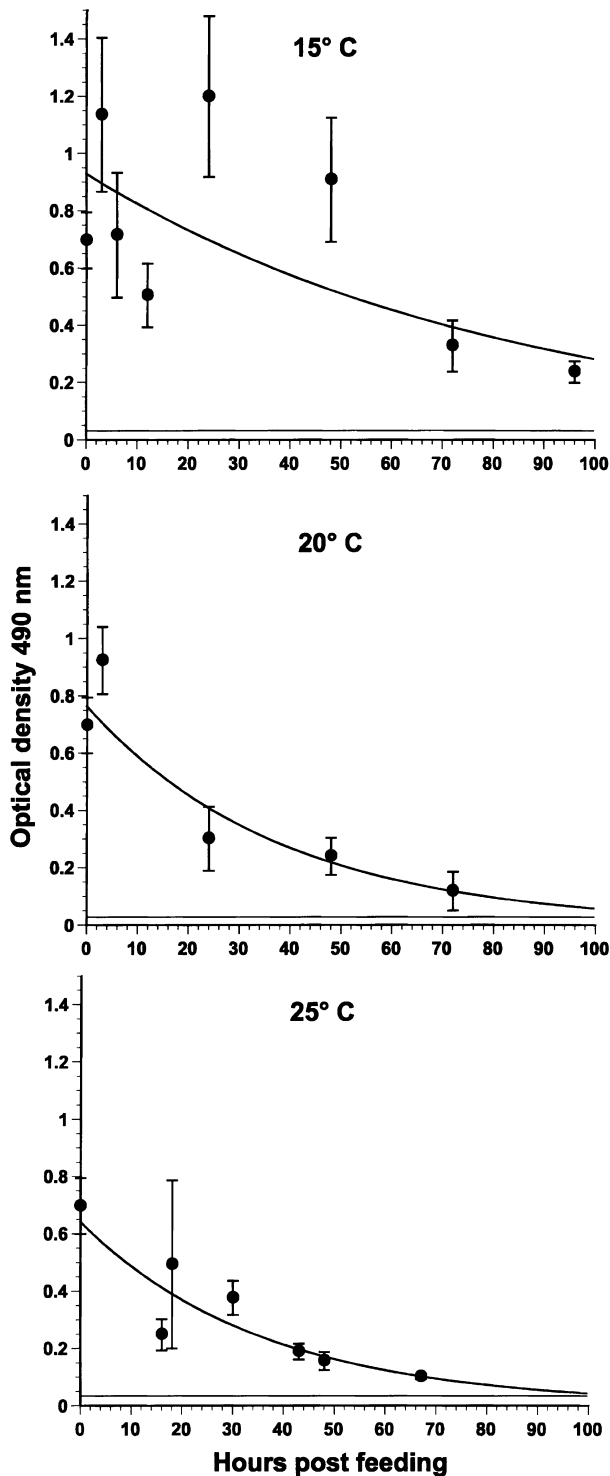


Fig. 2. ELISA results (optical density) for *Chrysopa carnea* feeding on *Cassida rubiginosa* larvae and killed at different hours (up to 96 h) post-feeding at three different temperatures (mean \pm SE): 15°C: $OD = 0.927 * \exp(-0.012 * t)$, $r^2 = 0.54$, $P < 0.036$; 20°C: $OD = 0.763 * \exp(-0.026 * t)$, $r^2 = 0.93$, $P = 0.007$; 25°C: $OD = 0.641 * \exp(-0.028 * t)$, $r^2 = 0.85$, $P = 0.003$. Sample sizes were: 15°C: 0 h $n = 18$; 3 h $n = 10$; 6 h $n = 8$; 12 h $n = 5$; 24 h $n = 9$; 48 h $n = 10$; 72 h $n = 7$; 96 h $n = 10$; 20°C: 0 h $n = 18$; 3 h $n = 10$; 24 h $n = 8$; 48 h $n = 12$; 72 h $n = 7$; 25°C: 0 h $n = 18$; 16 h $n = 7$; 18 h $n = 5$; 30 h $n = 15$; 43 h $n = 9$; 48 h $n = 13$; 67 h $n = 10$

Dynex AM60 multi-reagent washer (Dynex Technologies Inc., Chantilly, VA), three times with 0.9% NaCl, 0.1% Tween 20, and two times with 0.9% NaCl. A volume of 200 μ l of a blocking solution (50 mM Tris-HCl; 150 mM NaCl; 1% bovine serum albumin; pH 7.5) was added to each well for at least 1 h at 25°C and then washed as above. Hybridoma supernatants containing the antibody CRL5-1 were diluted 1 : 10 with 50 mM Tris-HCl; 150 mM NaCl; 0.5% bovine serum albumin (BSA); 0.05% Tween 20; pH 7.5, filled (100 μ l per well) in the prepared plates and incubated for 1 h. After washing, 100 μ l of horseradish peroxidase-conjugated goat anti-mouse IgG (GAM^P; Dako A/S, Zug, CH, Denmark), diluted 1 : 1000 in the same buffer as the supernatant, was added to each well and incubated for 1 h at 25°C. After washing, the wells were filled each with 100 μ l of 0.17 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as substrate in 3.25 mM sodium perborate; 39.8 mM citric acid; 60 mM sodium hydrogencarbonate; pH 4.5 solution. After 60 min of incubation at 25°C in the dark, the absorbance [optical density (OD)] of each well was measured using a Dynex MRX microplate reader set at 405 nm with a reference reading at 490 nm.

In order to test for the accuracy of antigen concentrations in our assay and to ensure comparability between plates, we included a dilution series of pure haemolymph (1 : 500 000, 1 : 625 000, 1 : 833 333, 1 : 1 250 000, 1 : 2 500 000, 1 : 5 000 000) on a series of ELISA plates. With the dilution series we also tested that the colour reaction in the range of interest in ELISA is linearly related to the amount of antigen in the wells. The highest dilution (1 : 5 000 000) was set as standard (1 unit haemolymph) and the other dilutions were re-calculated on units haemolymph. We standardized the OD values by first subtracting the mean values for the negative controls and then dividing by the number of units haemolymph. Differences in the accuracy of the detection at different dilutions and between different plates were tested by a two-way analysis of variance (ANOVA; SPSS 11) with the factors dilution and plate.

2.3 Half-life and detection period

We assumed the amount of antigen y in the predator's gut to decline exponentially with time t ($y = a * \exp^{-b*t}$). Regressions were calculated for the mean OD at each census time. The half-life of the antigen, defined as the time period in which the concentration in the gut had declined to half its initial value, was determined by regression analysis. In order to calculate a detection period – the time from ingestion to non-detectability – threshold values for positive scoring samples were chosen. We defined threshold values as the mean of the negative controls ($n = 23$ for *C. carnea* and $n = 5$ for *N. mirmicoides* fed with non-target food) adding three times the corresponding standard deviation. This is a procedure commonly used for this purpose (e.g. SUTULA et al., 1986; HAGLER et al., 1992, 1994; HAGLER and NARANJO, 1994). Detection periods were calculated only for comparative purposes; they have no biological meaning (SYMONDSON, 2002).

3 Results

3.1 Feeding trials

The feeding time of *N. mirmicoides* was relatively long, with a mean (\pm SD) of 241.6 \pm 64.6 min at 15°C and 233.1 \pm 65.9 min at 20°C (range: 120.0–399.6 min). The mean weight gain during feeding at 15°C was 3.7 \pm 1.2 mg and at 20°C 4.7 \pm 1.9 mg.

Table 1. ANOVA table of weight gain (mg) and feeding time (min)

	Weight gain			Feeding time		
	d.f.	F-value	P-value	d.f.	F-value	P-value
Species	1	136.8	<0.001	1	15.067	<0.001
Temperature	1	2.79	0.097	1	1.24	0.267
Species * temperature	1	3.81	0.053	1	1.87	0.173

Chrysopa carnea larvae gained on average 1.3 ± 1.07 mg (15°C), 1.2 ± 1.1 mg (20°C) and 2.7 ± 2.3 mg (25°C). The feeding time of *C. carnea* larvae was 100.0 ± 63.3 min at 15°C, 99.8 ± 54.9 min at 20°C and 132.4 ± 69.7 min at 25°C.

Two-way ANOVA of weight gain and feeding time revealed significant differences between species but not between temperatures or interactions (table 1). Because there were no differences in the weight gain, i.e. the amount of antigen ingested, at different temperatures, ELISA results for $t = 0$, i.e. directly after feeding, were pooled across temperatures for regression.

3.2 Immunological results

No significant difference in the OD values between different plates (d.f. = 6; $F = 0.256$; $P = 0.953$) and between dilutions 1 : 500 000 and 1 : 2 500 000 ($P = 0.539$, Tukey's HSD) was found. This and the fact that all procedures were conducted at well-defined temperatures for well-defined time periods ensure that the OD values of different plates are directly comparable and that the colour reaction in the range of interest in ELISA is linearly related to the amount of antigen in the wells.

CRL5-1 clearly detected *C. rubiginosa* antigen within the predators *C. carnea* and *N. mirimicoides*. ELISA results for 19 (at 15°C) and 18 (at 20°C) *N. mirimicoides* up to 48 h post-feeding are presented in fig. 1. We found an exponential decay of the antigen at 15°C ($OD = 1.69 * \exp(-0.013 * t)$; $r^2 = 0.22$; $P = 0.042$) and at 20°C (regression: $OD = 1.83 * \exp(-0.037 * t)$; $r^2 = 0.34$; $P = 0.01$; fig. 1).

The feeding experiments with *C. carnea* also showed significant exponential decay at the temperatures tested (fig. 2): 15°C: $OD = 0.927 * \exp(-0.012 * t)$, $r^2 = 0.54$, $P < 0.036$; 20°C: $OD = 0.763 * \exp(-0.026 * t)$, $r^2 = 0.93$, $P = 0.007$; 25°C: $OD = 0.641 * \exp(-0.028 * t)$, $r^2 = 0.85$, $P = 0.003$.

We calculated a half-life for the antigen in *N. mirimicoides* of 51.4 h (26.7–69.3 h) at 15°C and 18.7 h (11–69.3 h) at 20°C, and in *C. carnea* of 57.8 h (30.1–69.3 h) at 15°C, 26.7 h (17.8–53.3 h) at 20°C and 24.8 h (16.9–49.5 h) at 25°C. A detection threshold for positive records in ELISA for *N. mirimicoides* at an absorbance of 0.08 and for *C. carnea* of 0.07 was applied when calculating the detection periods. The calculated detection periods were 234.7 h (117.7–3060 h) at 15°C and 85.0 h (49.5 h–312 h) at 20°C for *N. mirimicoides* and 215.3 h (112.3–2584 h; 15°C), 91.9 h (61.3–183.8 h; 20°C) and 79.1 h (54–158.2 h; 25°C) for *C. carnea*.

4 Discussion

Our results clearly show that the MAb CRL5-1 is able to detect prey antigen in the guts of arthropod predators for ecologically relevant time intervals following ingestion. The reported detection periods of prey remains in predators are the longest known for MAbs used in serological predator gut analysis (SYMONDSON and LIDDELL, 1993, 1995, 1996; SYMONDSON et al., 1999, 2000), and are higher than the recently published detection periods of prey DNA in predators (AGUSTÍ et al., 1999; ZAIDI et al., 1999; CHEN et al., 2000; HOOGENDOORN and HEIMPEL, 2001). The total nymphal development time of two species closely related to *N. mirimicoides*, namely *Nabis americanus* Carayon and *Nabis roseipennis* Reuter, is 80 and 71 days at 15°C; and 25.2 and 29.2 days at 21°C, respectively (BRAMAN et al., 1984). BAY et al. (1993) found a total development time of 29.2 days for *C. carnea* at 15°C and CANARD and PRINCIPI (1984) a total time of 14.8 days at 20°C and 10.7 days at 25°C. Assuming that these development times are representative for our predators, prey remains can be detected for 12–14% and 25–30% of the total development time of *N. mirimicoides* and *C. carnea*, respectively. Thus, the detection period of prey remains after consumption of a single prey individual covers a relatively large part of the life span of these predators. A long detection time increases the likelihood of detecting predation in field-collected predators and decreases the number of individuals necessary to be tested to accurately quantify predation rates.

HAGLER and NARANJO (1997) found a large discrepancy in the sensitivity of a gut content immunoassay developed to detect pink bollworm egg remains in whole-body homogenized predators. The predator species examined, a predator's exposure temperature, the quantity of prey consumed, and post-meal time all affected the qualitative and quantitative outcome of the indirect ELISA. We also found no obvious predator species independent rules of antigen decay rates. With the data available we conclude that at present it is impossible to infer generalizations in antigen decay rates from immunological analysis of predator gut contents. Therefore all potential predator species have to be considered when calibrating immunoassays. However, it may not be feasible to include all predator species present at a site in calibration studies. In most cases, this will probably not be necessary, because not all may feed on the prey. However, the real predator-prey relationships are, in general, not known. In order to keep the amount of labour, time and money spent at a reasonable level, it may be advisable to limit the number of predator species to be tested when calibrating serological and other gut analysis assays as far as possible to the real predators of the prey species considered. Recent advances in direct observation techniques of individual prey with the aid of remote video surveillance (MEYHÖFER, 2001; SCHENK and BACHER, 2002) may substantially help in achieving this goal. Direct observation of predation events is a technique complementary to predator gut analysis. The former is focused on the prey and is appropriate for identifying the predators and assessing the impact of different

predators on the prey, while the latter is focussed on the predators and also suited to assess the importance of a single prey species for different predators.

We found a large variability in ELISA between individual predators. Standardizing the data with the amount of food ingested and with the weight of the individual predators yielded no better fit of regressions (results not shown). It may be that individual differences of predators in digestion rates related to e.g. sex, age, developmental or physiological state or feeding habit are responsible for the observed large variability. Other authors also found similar large unexplained variation between individuals throughout their studies, regardless of the detection method used [serology, polymerase chain reaction (PCR) of prey DNA; e.g. FICHTER and STEPHEN, 1981; SOPP and SUNDERLAND, 1989; HOOGENDOORN and HEIMPEL, 2001]. It appears that there is an inherent high variability of antigen titres in predators, which cannot be reduced further even under controlled conditions. We expect that predators show at least the same degree of variability in the field. In order to obtain estimates of predation with a reasonable degree of precision, it will be necessary to collect large numbers of predators in the field for gut content testing. However, it may be difficult to obtain sufficiently high numbers of field-collected predators in predator-prey systems with large inherent variability of individual predators in prey digestion and short detection periods of the associated assay, which also increases the number of predators to be collected. Development of assays with a long detectability of prey remains, like the assay presented in this study, may therefore be a vital prerequisite for studying predator-prey relationships in the field by predator gut analysis.

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