The influence of time and temperature on molecular gut content analysis: *Adalia bipunctata* fed with *Rhopalosiphum padi*

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Abstract Gut content analysis is a useful tool when studying arthropod predator-prey interactions. We used polymerase chain reaction (PCR) technique to examine how detection of prey DNA in the gut content of predators was influenced by digestion time and temperature. Such knowledge is critical before applying PCR-based gut content analysis to field collected predators. Larvae of the two-spotted ladybeetle (*Adalia bipunctata* L.) were fed with the bird cherry-oat aphid (*Rhopalosiphum padi* L.) at either 21°C or 14°C. After consuming one aphid, the predators were allowed to digest the prey for a range of time periods up to 24 hours. The influence of temperature on *A. bipunctata* feeding behavior was also recorded. From the fed larvae, total DNA was extracted and PCR reactions with *R. padi* specific primers were run. The number of *A. bipunctata* that tested positive for *R. padi* DNA was negatively related to the length of digestion time. Temperature influenced larval feeding behavior but did not have a significant effect on *R. padi* DNA detection. After pooling the data from both temperature treatments we estimated the time point when *R. padi* DNA could be amplified from 50% of the fed *A. bipunctata* by PCR to be 4.87 hours. With such a rapid decrease in prey DNA detection success, positive PCR reactions will most likely be the result of predation events occurring shortly before capture. If a defined digestion temperature range has proven not to influence prey detection, PCR data obtained from predators collected within that particular range can be interpreted in the same way.

Key words *Adalia bipunctata*, aphidophagous predator, prey DNA detection success, gut content analysis, PCR, *Rhopalosiphum padi*
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Introduction

Predaceous ladybeetles (Coleoptera: Coccinellidae) can cause significant mortality of insect pests in various agro-ecosystems. A well-known example of classic biological control is the successful introduction of the Vedalia beetle (*Rodolia cardinalis* Mulsant) for suppression of the cottony cushion scale (*Icerya purchasi* Maskell) in Californian citrus groves at the end of the nineteenth century (Caltagirone & Doutt, 1989). Today, several coccinellid species, including the two-spotted ladybeetle (*Adalia bipunctata* L.) used in this study, are commercially available and have, for example, been successfully used against aphids in apple orchards (Wyss, 1999). Predaceous coccinellids are also frequently suggested to play an important role in conservation biological control of aphids (reviewed by Obrycki & Kring, 1998). However, the true pest-suppressing efficacy of coccinellids in natural systems is difficult to determine given their high mobility and polyphagous nature (Hodek, 1973).

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One approach for studying arthropod predator-prey interactions is predator gut content analysis using polymerase chain reaction (PCR)-based techniques (e.g., Zaidi *et al*., 1999; Chen *et al*., 2000; Agustí *et al*., 2003a, b; Ma *et al*., 2005; De León *et al*., 2006). In the PCR reaction, prey-specific DNA fragments are detected and amplified from the predator's gut content. To ensure successful detection of partly digested prey DNA most PCR-based studies have focused on the amplification of relatively short prey fragments, approximately 100−500 base pairs (bp), from genes being present in multiple copies in each cell such as the mitochondrial cytochrome oxidase I (COI) and II (COII) genes (Agustí *et al*., 2003a; Chen *et al*., 2000) and nuclear ribosomal RNA genes (Hoogendoorn & Heimpel, 2001; Ma *et al*., 2005). PCR has turned out to be an advantageous method for arthropod gut content analysis as it is highly sensitive and because application requires skills and equipment that are common in many labs (Greenstone & Shufran, 2003; Sheppard & Harwood, 2005). Another positive feature is that post-mortem gut content analysis reveals predation events that occurred without any experimental interference (Sunderland, 1988).

Before investigating any particular predator-prey system it is essential to determine for how long after a feeding event it is possible to detect the target prey in the predator of interest (Greenstone & Hunt, 1993; Greenstone & Shufran, 2003). This can be achieved by determining the time period after feeding when the probability of detecting DNA from the ingested prey in the predator equals 50% (e.g., Chen *et al*., 2000; Agustí *et al*., 2003b; Ma *et al*., 2005). In addition to determining this time period it is essential to investigate if it is influenced by temperature, which is a critical abiotic factor affecting many aspects of insect biology, physiology and ecology. Because insects are poikilotherms, their body temperature and basic metabolism vary with the temperature of their surrounding environment (Speight *et al*., 1999). It is therefore reasonable to assume that digestion rate, as driven by enzymatic reactions, may be temperature-dependent and that temperature could affect any method of insect gut content analysis and consequently the interpretation of data obtained from natural systems. Previous studies have shown that serological detection of prey proteins in predator guts can be significantly affected by the surrounding environment (Hagler & Naranjo, 1997; Hagler & Cohen, 1990). In one study it was also demonstrated that PCR-based detection of lepidopteran DNA in adult coccinellids was significantly affected by temperature (Hoogendoorn & Heimpel, 2001). We consider that this topic needs more attention and here present the first study in which the influence of temperature on PCR-based gut content analysis of insect predators is specifically addressed. The aim of our study was to investigate the influence of temperature on feeding behavior and the detection success of aphid DNA ingested by coccinellid larvae. As model predator-prey organisms we used the bird cherry-oat aphid (*Rhopalosiphum padi* L.) and *A. bipunctata* larvae. The temperatures chosen, 14 and 21°C, are considered to be relevant in field situations in Sweden. Our hypothesis was that detection time and the time period when *R. padi* DNA could be amplified from 50% of the fed *A. bipunctata* by PCR would be longer when the predators were kept in a cooler environment.

Materials and methods

Insects

The bird cherry-oat aphids used in these experiments came from colonies maintained at the Department of Entomology, Swedish University of Agricultural Sciences. They were reared on oats (*Avena sativa* L.) at 18°C, and a photoperiod of 16 : 8 L : D. The aphids were removed from the colony immediately prior to their use in the feeding trials.

The study predators, *A. bipunctata*, were purchased as batches containing 100 mixed second to fourth instar larvae from Biobest (Westerlo, Belgium). Compared to other ladybeetles, *A. bipunctata* consumes a relatively wide range of aphid prey, including *R. padi* (Hodek, 1973; Rana *et al*., 2002). The travel time and quarantine restrictions meant that the larvae were starved for a minimum of 48 hours prior to arrival, so no further starvation was necessary to eliminate gut contents. The larvae were always kept under the same conditions and used in the same manner within 3 days after arrival; they were not given any supplemental foods but were provided moistened filter paper daily.

Feeding trials

The feeding trials were conducted either in a laboratory at room temperature (≈ 21°C) or in a climate-controlled room at 14°C (± 2°C). All larvae were maintained at a photoperiod of 16 : 8 L : D. About 30 coccinellids were randomly selected from one batch of larvae and carefully transferred to individual 47-mm Petri dishes containing a moistened filter paper. The larvae were allowed to acclimatize to the environment for approximately 5 minutes prior to the addition of one live apterous *R. padi* (either 4th instar or adult) into the Petri dish. The behavior of the predators; the time until attack and the feeding time, was then observed and recorded to within 1 minute. Time until attack was the amount of time that the aphid and larva were in the Petri dish until feeding was initiated. Feeding time

was the length of time between the initiation and completion of aphid consumption. The coccinellids that did not attack the aphid within 120 minutes were excluded from all further trials. Immediately after consumption of the aphid, the larvae were transferred to a new sterile Petri dish to avoid external contamination by any aphid remains left after the meal. These fed larvae were then allowed to digest the aphid for various time periods ranging from 0, 1, 2, 3, 4, 6, 8, 10 and 12 up to 24 hours. Following the designated digestion periods the larvae were frozen and stored at −70°C until DNA extraction. As a check against false positives, unfed *A. bipunctata* larvae were maintained in identical conditions to those in the feeding trial and were frozen after 120 minutes.

To obtain 10 fed larvae for each digestion period and temperature treatment, the above described procedure had to be repeated on seven different occasions at 14°C and 21°C respectively. We used two batches of larvae for each temperature treatment and the coccinellids from each batch were always used within a period of 3 days after arrival to the laboratory. By assigning the fed larvae from each replicate to various digestion periods, we compensated for any variation in larval physiological state between replicates. In summary, for each digestion period (0−24 h) at both temperatures, 10 *A. bipunctata* larvae that had consumed one *R. padi* were frozen and later analyzed by PCR resulting in a total of 200 tested larvae (exclusive of unfed controls).

DNA extraction and PCR conditions

Total DNA was extracted from whole larvae using Qiagen's DNeasy Tissue Kit (Qiagen, Germantown, MD, US) following the manufacturer's instructions. All samples were stored at −20°C prior to PCR amplification. PCR reactions, using *R. padi* primers BcoaCOIIF1 and BcoaCOIIR1, were performed according to Chen *et al*. (2000) with minor modifications. These primers amplify a 331 bp long fragment of the mitochondrial COII gene. All reactions (25 μL) were run with a Mastercycler® gradient (Eppendorf AG, Hamburg, Germany) in a mix containing a PCR Buffer (10 mmol/L Tris- HCl pH 8.3, 50 mmol/L KCl), 1 mmol/L $MgCl_2$, 0.1 mmol/L of each dNTP, 1 μmol/L of each primer and 1.25 U of TaKaRa Taq™ polymerase (Takara Bio Inc, Shiga, Japan). Each reaction contained 1 μL of template with an average DNA concentration of 91 ng/μL. After 3 minutes at 94°C, 35 amplification cycles were run including denaturing at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 1 minute. The final extension was carried out at 72°C for 2 minutes. Samples always included pure *R. padi* DNA and autoclaved distilled water as positive and negative controls respectively. PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, and then visualized and photographed under UV light. Each PCR setup was always repeated and a particular individual showing a PCR product of expected size (331 bp) at least once was scored as positive. Individuals showing no PCR product in the two attempts were scored as negative.

Statistical analyses

For each feeding trial replicate, a mean was calculated for the two behavior variables: time until attack and feeding time. These means were used in a *t*-test to see if there was a difference in larval behavior between the two temperature treatments.

In order to test for a difference in data between the two temperatures we used proc GENMOD in SAS version 9.0. As the independent variable we used digestion time transformed by ln (digestion time +1) and as the dependent variable we used a logit transformation of the proportion of coccinellid larvae that tested positive for the target aphid DNA fragment. To see if a single line could be used to describe the data we first tested if the two slopes were parallel and then tested to see if the same model could describe data from both temperatures.

To obtain the time period when *R. padi* DNA could be amplified from 50% of the fed *A. bipunctata* by PCR we subjected the data to analysis by proc PROBIT in SAS version 9.0. We then solved the obtained equation for the digestion time that corresponded to 50% detection success. This value was then back-transformed into hours.

Results

Behavior

Temperature (14°C *vs*. 21°C) clearly influenced larval feeding behavior. At the lower temperature the larvae were significantly slower to attack the aphids, and once they did, they took a significantly longer time to complete feeding. Mean time until attack and standard error in minutes were 36.3 ± 3.9 at 21°C, and 47.9 ± 2.6 at 14°C (*t*-test; $t = 2.44$, df = 12, $P = 0.03$). Mean feeding time and standard error in minutes were 28.3 ± 1.7 at 21°C, and 46.6 ± 2.4 at 14°C (*t*-test; $t = 6.25$, df = 12, $P < 0.000\ 1$).

Detection success of prey DNA

We could amplify the target aphid DNA fragment from all larvae that were killed immediately (0 hours) after having consumed one *R. padi* individual at both 14°C and 21°C. It was possible to detect the aphid DNA for up to 24

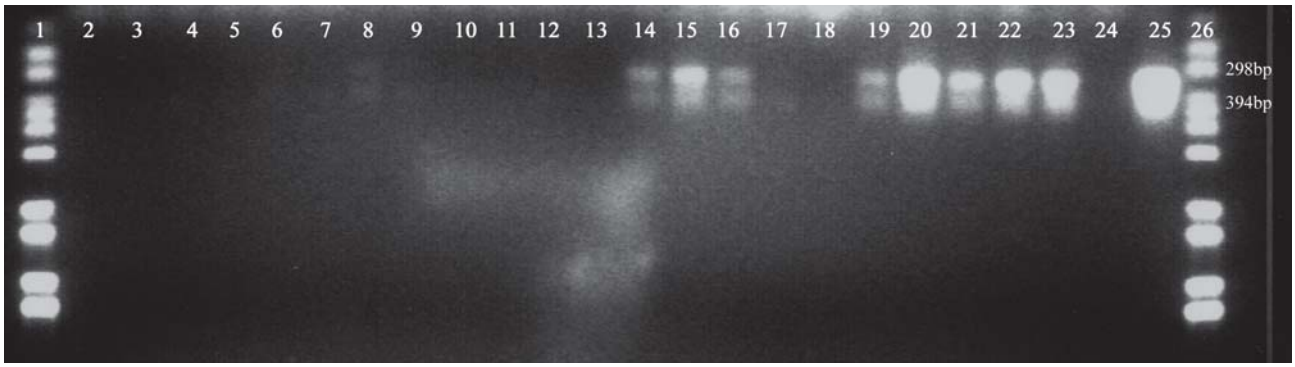

Fig. 1 Agarose gel showing polymerase chain reaction-amplified DNA using *Rhopalosiphum padi* primers BcoaCOIIFI and BcoaCOIIR1 (331 bp). Lanes 1 and 26: DNA size marker; lane 2: starved unfed *Adalia bipunctata* larvae; lanes 3−19: *A. bipunctata* larvae fed one *R. padi* and frozen 1−12 hours after prey ingestion; lanes 20−23: *A. bipunctata* larvae fed one *R. padi* and frozen immediately after prey ingestion; lane 24: water; lane 25: *R. padi*.

hours post-feeding but the number of positive larvae decreased with the length of the digestion time. There were no positive reactions with the unfed control larvae, demonstrating that there was no amplification of coccinellid DNA, no aphid contamination of the larvae during shipping or while conducting the experiment, and that the starvation periods were adequate. Figure 1 shows an agarose-gel with PCR products from one particular run.

The statistical analysis revealed no influence of digestion temperature on PCR-based detection of *R. padi* DNA in *A. bipunctata* gut contents. There was no significant difference between the slopes of the two lines calculated for each temperature treatment ($\chi^2 = 1.88$, df = 1, $P = 0.17$) nor was there a significant difference between the two models ($\chi^2 = 0.95$, df = 1, $P = 0.33$). We therefore pooled the data from both temperature treatments, which revealed a significant negative relation ($\chi^2 = 35.6$, df = 1, $P < 0.000\ 1$) between proportion-positive larvae and time. The estimated intercept was 2.26 and estimated slope was 1.27. The untransformed data are shown in Figure 2.

The time period when *R. padi* DNA could be amplified from 50% of the fed *A. bipunctata* larvae by PCR was equal to 4.87 hours with a 95% confidence interval of 3.52 to 6.62 hours. In practical terms, 50% detection time of 4.87 hours means that a field-collected, immediately frozen predator has a 50% probability of showing a positive reaction if it consumed at least one aphid 4.87 hours prior to capture.

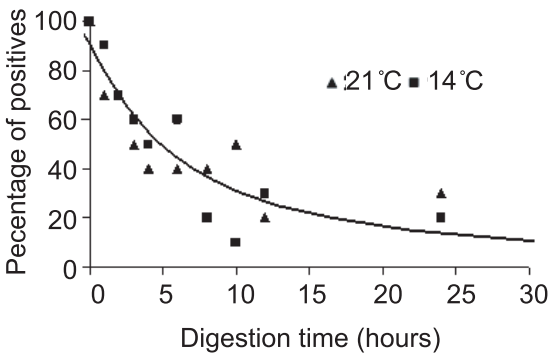

Fig. 2 Relationship between the percentage of laboratory-fed *Adalia bipunctata* larvae that tested positive for the presence of *Rhopalosiphum padi* DNA remains, using polymerase chain reaction-based gut analysis, in relation to digestion time of the aphid meal. All larvae consumed a single *R. padi* individual and the number of analyzed predators for each digestion time (0−24 hours) was 10. The solid line is the back-transformed equation for the pooled data from experiments conducted at two different temperatures (14°C and 21°C). Data were combined because there was no significant difference between the two temperatures.

Discussion

Our results show that it is possible to detect aphid mitochondrial DNA in *A. bipunctata* larvae using the PCR-technique and that detection success of prey DNA was negatively related to the length of digestion time. No significant difference in prey DNA detection success between temperatures was found. This was despite the fact that larvae took a significantly longer time to attack and eat the aphid at the lower temperature, which demonstrated that the temperature treatments were effective. Pooling the data from both temperatures resulted in a time period of 4.87 hours when *R. padi* DNA could be amplified from 50% of the fed *A. bipunctata* by PCR.

The lack of significant impact of temperature could be due to the relatively narrow temperature interval studied (14−21°C). Hagler and Naranjo (1997) similarly found that digestion at 15, 20 or 25°C had little or no effect on prey

protein detectability in *Orius insidiosus* Say when analysing gut content using serological techniques. At post-meal temperatures $\geqslant 30^{o}$C there was, on the other hand, a clear decline in the proportion of individuals scoring positive. Comparable results were also found by Hagler and Cohen (1990). It is possible that high temperatures might influence detection success of aphid DNA in *A. bipunctata*. However, we have investigated a temperature range that is reasonable for field conditions in Sweden and we conclude that PCR data obtained from predators collected at temperatures within the investigated range can be interpreted in the same way. In agreement with our results, Hoogendoorn and Heimpel (2001) found no effect of temperature on PCR-based detection of lepidopteran eggs in larvae of the coccinellid *Coleomegilla maculata* De Geer. However, they did observe an effect of temperature in the adults; digestion at 27^{o}C significantly decreased the number of detectable prey fragments compared to digestion at 20^{o}C. Therefore, the influence of temperature on prey DNA detection success needs to be investigated for all coccinellid life stages before comparing predation rates between larvae and adults.

To date, four studies have analyzed the gut content of coccinellids by PCR-technique (Chen *et al*., 2000; Hoogendoorn & Heimpel, 2001, 2002; Sheppard *et al*., 2004). Each of these has utilized different species and developmental stages of coccinellids feeding on different prey. In addition, they have targeted prey DNA fragments of different length and been conducted at different temperatures. Given these differences, direct comparisons between the studies are not possible, but they can offer insight and information. For example, DNA remains of the corn leaf aphid (*Rhopalosiphum maidis* Fitch) in third instar convergent ladybeetle (*Hippodamia convergens* Guerin) larvae could be detected in 50% of the predators 7.72 hours after ingestion when Chen *et al*. (2000) amplified a 339 bp COII fragment, which is almost 3 hours longer than the result found in our experiment. This suggests that generalization of detection times cannot be made even if predators from the same family have consumed prey from the same genus and a DNA fragment of almost identical length from the same region has been targeted.

In another coccinellid study, Sheppard *et al*. (2004) investigated detection of lepidopteran larvae in *Curinus coeruleus* Mulsant. Detection times were long, with 67%−100% of *C. coeruleus* testing positive for prey DNA 24 hours after ingestion. As the coccinellids in Sheppard *et al*. (2004) consumed a large lepidopteran larva (compared to one aphid in the present work) there would be a greater amount of target DNA relative to the predator DNA, possibly allowing a greater chance of successful detection for longer time periods.

In previous work using PCR-based gut content analysis, primer pairs amplifying shorter fragments have resulted in longer prey detection times (Zaidi *et al*., 1999; Agustí *et al*., 1999). This was true for the coccinellid study of Hoogendoorn and Heimpel (2001) where four fragments of different lengths (150 bp, 256 bp, 369 bp, 492 bp) were compared. The shortest fragment could be detected for the longest time. Consequently another possible explanation for the longer detection times in Sheppard *et al*. (2004) is that prey fragments of 140 and 170 bp were examined whereas the pair of primers used in the present work amplifies a sequence of 331 bp.

Hagler and Naranjo (1997) pointed out that relatively short detection intervals of prey proteins may be favorable compared to longer ones ($\geqslant$24 h) when analyzing predators feeding under natural conditions, and we believe that a short prey DNA detection interval shares the same advantages. At present, PCR-based estimation of gut contents is not a quantitative measure of how much prey an individual predator has consumed. It can only indicate that a predation event has occurred within a specific time frame. Keeping this in mind, a rapid decrease in prey DNA detection success allows a much more specific indication of the frequency of predation compared to prey DNA that can be detected over very long time intervals. Consequently, if prey DNA can be detected for several days after ingestion (e.g. Ma *et al*., 2005) predation rates on specific prey items might be overestimated. On the other hand, when prey DNA detection success is low after only a few hours one might underestimate predation rates or have problems when sampling only every 24 hours. But by using tailored sampling programs where collected predators are frozen directly in the field, this shortcoming can be overcome (Hagler & Naranjo, 1997). Shorter prey detection intervals may also allow a better understanding of diel feeding cycles (Chen *et al*., 2000). For example, predators may be collected at different times and frozen immediately upon capture to determine whether a particular predator consumes prey items more frequently during a specific part of the day.

The use of molecular methods for determining arthropod gut content is increasing rapidly and PCR-based techniques will undoubtedly play a critical role in gaining new insight into trophic interactions. A better understanding of the frequency and diversity of prey consumed by predators will improve our ability to identify key predators of pests. However, to use these molecular methods effectively the influence of environmental variables such as temperature on the outcome of detection tests needs to be considered.

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