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The effects of temperature on detection of prey DNA in two species of carabid beetle

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Abstract

PCR-based techniques to investigate predator-prey trophic interactions are starting to be used more widely, but factors affecting DNA decay in predator guts are still poorly understood. Here, we investigated the effects of time since feeding, temperature and amplicon size on the detectability of prey DNA in the gut content of two closely related predator species. Cereal aphids, *Sitobion avenae*, were fed to the carabid beetles *Pterostichus melanarius* and *Nebria brevicollis*. Beetles were allowed to digest their meal at 12°C, 16°C and 20°C, and batches of beetles were subsequently frozen at time periods from 0–72 h after feeding. Aphid DNA was detected within beetles' gut contents using primers amplifying fragments of 85, 231, 317 and 383 bp. Prey DNA detection rates were significantly higher in *N. brevicollis* than in *P. melanarius*, indicating fundamental dissimilarities in prey digestion capacities. High temperatures (20°C) and large amplicons (383 bp) significantly decreased detection rates. The shortest amplicon gave the highest prey DNA detection success, whereas no differences were observed between the 231 bp and the 317 bp fragment. Our results indicate that factors such as ambient temperature, predator taxon and amplicon size should all be considered when interpreting data derived from PCR-based prey detection. Correction for such factors should make calculation of predation rates in the field more accurate and could help us to estimate when predation events occur in the field.

Keywords: Carabidae, DNA decay, gut content analysis, Homoptera, PCR

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Introduction

Predator-prey interactions are important processes driving animal population dynamics and are central to many ecological studies. Identification of trophic links can be difficult without disturbing the system under study, especially in predators which are small, active at night or living in the soil. *Post-mortem* determination of predator diets, using gut content analysis, is an accurate method, as the predators can be assumed to have been behaving naturally prior to their capture. Visual examination of predator gut contents is possible if recognisable prey fragments are ingested by the predator (Ingerson-Mahar, 2002). As most invertebrate predators are at least partly fluid feeders, many trophic links are inevitably missed using this approach.

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Biochemical and molecular techniques overcome these problems and have been rapidly developed over the last two decades (reviewed in Symondson, 2002; Sheppard & Harwood, 2005; Sunderland *et al*., 2005; King *et al*., 2008). PCR-based techniques of *post-mortem* gut content analysis have been widely used and applied to insect predator-prey systems, including Coleoptera, Diptera, Heteroptera, Homoptera, Lepidoptera, Neuroptera and Collembola but also Annelida, Crustacea, Arachnida and Mollusca (Harper *et al*., 2005; Read *et al*., 2006; Gariepy *et al*., 2007; Juen & Traugott, 2007). Although several studies investigate parameters that might affect detection periods and amplification success (Agustí *et al*., 1999; Zaidi *et al*., 1999; Chen *et al*., 2000; Hoogendoorn & Heimpel, 2001; Juen & Traugott, 2005), several factors remain to be explored, such as the effect of ambient temperature and predator taxon. The more we know about which factors affect prey DNA detection success, the better we will be able to interpret field-derived data and assess trophic links and their strength in natural systems.

One of the fundamental parameters affecting prey DNA detectability is the time elapsed since feeding (Symondson, 2002). In general, DNA detectability decreases with increasing digestion time, but considerable differences between predator species have been reported (Chen *et al*., 2000; Hoogendoorn & Heimpel, 2001; Ma *et al*., 2005; Read *et al*., 2006; Greenstone *et al*., 2007; Harwood *et al*., 2007; Traugott & Symondson, 2008). Furthermore, Hoogendorn & Heimpel (2001) showed that an increase of ambient temperature significantly decreases prey DNA detectability within a coccinellid predator, indicating that temperature influences the rate of DNA digestion. Likewise, the size of the target DNA molecule influences the amplification success of prey DNA. This was first recognised by Zaidi *et al*. (1999), who showed longer post-feeding detection of a smaller amplicon (up to 28 h), compared with larger amplicons that were rapidly degraded.

In the present study, we investigated, in a full-factorial experimental design, whether ambient temperature and fragment length affect the post-feeding detectability of prey DNA in two different but closely related predator species. We hypothesised that: (i) the detectability of prey DNA differs even between closely related predator species (within one family), and (ii) the post-feeding prey detection period is affected by ambient temperature and target fragment size. Based on these results, we discuss whether PCR-based prey detection might allow us to calculate the time at which predation occurred in the field.

Materials and methods

Insects

During October 2005, adult carabid beetles, *Nebria brevicollis* (F.) and *Pterostichus melanarius* (Ill.), were collected from two fields at Burdens farm, Wenvoe, near Cardiff, UK by pitfall trapping and hand searching. Beetles were transferred individually into plastic containers (8.5 cm diameter, 4.5 cm height) filled with 80 g moist sphagnum peat and maintained in a controlled environment (L : D 16 : 8; 16°C). They were fed with one *Calliphora vomitoria* (L.) larva twice a week. Prior to the feeding experiments, beetles were starved for five days to ensure the same nutritional status in all individuals. Grain aphids, *Sitobion avenae* (F.), were reared on wheat plants in fine mesh cages. From these cultures, adult aphids were removed and frozen as prey for the subsequent feeding experiments at −80°C. Both carabid species are known to feed on cereal aphids in the field (Sunderland, 2002).

Feeding experiments

Feeding experiments were carried out in controlled climate chambers at 12°C, 16°C and 20°C (L : D 16 : 8). Experiments were conducted separately for the two carabid species but simultaneously at all three temperature levels for each species. Petri dishes, lined with filter paper, were used as feeding arenas. Within each arena, one carabid beetle was allowed to feed for 1 h on five freeze-killed adult aphids. Beetles consuming fewer than three aphids were discarded from the experiment. After the one-hour feeding period, aphid remains were removed and arenas provided with fresh filter paper. Additionally, a piece of damp filter paper was added, serving as a shelter for the beetles. Beetles were frozen at −80°C after digesting their meal for 0, 3, 6, 12, 24, 36, 48 and 72 h from the end of the feeding period. At each time point post-feeding, seven individuals were frozen, except for *P. melanarius* at 12°C/0 h ($n = 6$) and *N. brevicollis* at 12°C/72 h ($n = 6$), at 16°C/72 h ($n = 6$) and at 20°C/72 h ($n = 5$). Due to problems with maintaining *N. brevicollis* at 20°C for extended time periods post feeding (36 h, 48 h and 72 h), an additional set of beetles was used for these three time points where carabids were kept in Petri dishes filled with damp soil.

Sequencing and primer design

For sequencing, DNA of aphids was extracted using a Chelex protocol. Whole aphids where homogenised separately in 20 µl PBS, mixed with 5 µl Proteinase K and 200 µl 10% Chelex solution and incubated at 56°C for 4 h on a rocking platform. After a final incubation at 94°C for 15 min, samples were stored at −24°C. Universal invertebrate primers LCO-1490 and HCO-2198 (Folmer *et al*., 1994) were used to amplify part of the mitochondrial cytochrome oxidase subunit I gene. PCR was carried out in a GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, CA, USA) in 20 µl reaction volumes containing 3 µl of extracted DNA, 0.25 mM dNTPs (Invitrogen GmbH, Karlsruhe, Germany), 1 µM of each primer, 2 µl 10× buffer (Invitrogen), 3 mM $MgCl_2$, and 1.5 U *Taq* DNA polymerase (Invitrogen). Initial denaturation was done at 94°C for 2 min, followed by 35 cycles at 94°C for 15 s, 50°C for 30 s, 72°C for 45 s, and final elongation at 72°C for 2 min. PCR products were purified using ExoSAP-IT (USB, Staufen, Germany), subjected to sequencing PCR using Big-Dye Terminator mix (version 1.3, Applied Biosystems) and sequenced in both forward and reverse directions. Sequences were aligned using BioEdit (Hall, 1999) and corrected manually.

Five forward (S101–S105) and three reverse primers (A101–A103) were designed using PrimerPremier (PREMIERE Biosoft) following the guidelines for primer design given by Hawkins (1997). The resulting 15 primer pair combinations were tested for their sensitivity using DNA from *S. avenae* and for their specificity using DNA from the two carabid species. Optimisation of the PCR protocol included determination of optimum annealing temperatures by temperature gradient PCR, testing different concentrations of primers and adjusting cycling conditions.

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Table 1. Primers (5′–3′) designed from the cytochrome oxidase subunit I mtDNA of *Sitobion avenae* and expected product sizes of each forward primer combined with the reverse primer Sit-ave-A103.

Primer name	Primer sequence	Product size (bp)
forward		
Sit-ave-S 101	att aga ttt tga yta cta cca cca	383
Sit-ave-S 102	aca ggt aca gga tga act att tac	317
Sit-ave-S 103	aca ttt agc agg aat ctc atc a	231
Sit-ave-S 105	tac cag ttt tag ctg gtg ct	85
reverse		
Sit-ave-A103	tct cct cct cct gct gga	

Table 2. Results of cross-tabulation tables testing for differences in DNA detectability of aphid prey between *Pterostichus melanarius* and *Nebria brevicollis* with respect to fragment length and temperature.

	χ^2 df = 1	*P*
383 bp	12.89	< 0.001
317 bp	13.64	< 0.001
231 bp	10.39	0.001
85 bp	9.46	< 0.01
12°C	8.18	< 0.01
16°C	7.06	< 0.01
20°C	38.83	< 0.001

Screening predators for prey DNA

The beetles from the feeding trials were thawed, their complete digestive tract removed and homogenised in 50 µl of PCR water (distilled and autoclaved water) using a plastic pistil. For each beetle, separate gloves were used to avoid sample-to-sample contamination. As beetles often regurgitated as a defence reaction when they were transferred from the feeding arenas into the Eppendorf tubes, their gut was homogenized in the same tube the beetle was stored in, avoiding loss of any regurgitated material. Twenty-five microlitres of the homogenate were used for DNA extraction with DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions and 200 µl of the DNA extracts were stored at −24°C.

The extracts were analysed for the presence of aphid DNA using four primer pairs (table 1), amplifying fragments from 85 to 383 bp. PCRs were performed in 10 µl reactions containing 3 µl of extracted DNA, 0.25 mM dNTPs (Fermentas GmbH, St. Leon-Rot, Germany), 1 µM of each primer, 1 µl 10 × buffer (Invitrogen), 3 mM $MgCl_2$, 0.12 µg bovine serum albumin (BSA) and 1.5 U *Taq* DNA polymerase (Invitrogen). Amplifications were carried out in an Eppendorf Mastercycler Gradient PCR machine (Eppendorf AG, Hamburg, Germany); cycling conditions were 2 min at 94°C, 40 cycles of 15 s at 94°C, 30 s at 61°C, 45 s at 72°C, and final elongation of 2 min at 72°C. PCR water, as well as aphid and carabid DNA, were included within each PCR to test for DNA carry-over contamination, false-negative and false-positive amplifications. PCR products were visualised on a multi-channel capillary gel electrophoresis system HDA-GT12 (eGene, Inc., Irvine, CA, USA).

Statistical analysis

Chi-square tests were performed to test for differences in detection rates for aphid DNA within each of the three temperature levels and each of the four fragment lengths between the two carabid species. To investigate the effects of temperature, fragment length and digestion time on detectability of aphid DNA within both predator species, a three-variable logistic regression was performed including prey DNA detectability (yes/no) as the dependent variable and temperature (12, 16 and 20°C), fragment length (85, 231, 317 and 383 bp) and time since feeding (0, 3, 6, 12, 24, 36, 48 and 72 h) as the independent variables. A logistic regression model was chosen because of the dichotomous and nominal character of the response variable. As the design is experimental and the independent variables are not correlated, we were testing all variables simultaneously instead of using variable selection methods (Cody & Smith, 2006). In case of significant effects of independent variables, single logistic regressions were calculated comparing two factor levels at a time in all possible combinations within the variable, equivalent to performing protected ANOVAs following a significant MANOVA (Scheiner & Gurevitch, 2001). Logistic regressions and Chi-square tests were calculated using SAS 9.1 (SAS Institute Inc., Cary, NC, USA) and Statistica 7.1 (StatSoft, Tulsa, OK, USA), respectively. For the *N. brevicollis* feeding experiment at 20°C, all statistics were calculated without data from digestion times of 36, 48 and 72 h because too few beetles survived under these conditions. Instead of calculating the detectability half-life as described by Greenstone & Hunt (1993), using an exponential model, the real time points, as defined by the experimental design, were determined where more than 50% of the beetles tested positive for aphid DNA.

Results

The four primer pairs S101/A103, S102/A103, S103/A103 and S105/A103 successfully amplified DNA fragments of *S. avenae* of 85, 231, 317 and 383 bp, respectively. The optimal annealing temperature identified by temperature gradient PCR was 61°C for all four primer pairs. All amplifications were optimised to run at the same cycle conditions and PCR reagent concentrations. The PCR assay proved to be (for our purposes) specific for *S. avenae* DNA, as no amplicons were obtained with DNA of the two carabid species.

Detectability rates of aphid DNA in the predators' gut contents differed significantly between *P. melanarius* and *N. brevicollis*, with overall detection rates being significantly higher ($\chi^2 = 41.63$; $P < 0.001$) in *N. brevicollis* (61%; $n = 145$) than in *P. melanarius* (42%; $n = 146$). These higher detection rates in *N. brevicollis* were significant within each of the three temperature levels and each of the four fragment lengths (table 2).

Detectability of aphid DNA in *P. melanarius* significantly decreased with increasing digestion time, temperature and fragment length (table 3). Mean detection rates were similar at 12°C (44%) and at 16°C (43%) but were significantly lower at 20°C (29%) (table 4). These lower detection rates were due to a rapid decline in detectability of aphid DNA within digestion times 0–12 h at 20°C compared to a moderate decline in detectability in beetles maintained at 12°C and 16°C (fig. 1a).

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Table 3. Summary of logistic regression analysis for the effect of temperature, fragment length and time on aphid DNA detectability in the guts of *Pterostichus melanarius* ($n = 668$) and *Nebria brevicollis* ($n = 420$).

	Pterostichus melanarius			*Nebria brevicollis*		
factor	Wald χ^2	df	*P*	Wald χ^2	df	*P*
time	81.02	7	< 0.001	19.04	7	< 0.001
temperature	16.05	2	< 0.001	0.005	2	*n.s.*
fragment length	37.58	3	< 0.001	13.52	3	< 0.01
temperature* fragment length	1.78	6	*n.s.*	7.62	6	*n.s.*
temperature* time	25.81	14	0.027	18.08	14	0.021
fragment length* time	15.68	21	*n.s.*	8.51	21	*n.s.*

n.s., not significant.

Table 4. Results of single logistic regressions for the effect of each factor level combination of the factors temperature and fragment length on aphid DNA detectability in the guts of *Pterostichus melanarius* and *Nebria brevicollis*.

	Pterostichus melanarius		*Nebria brevicollis*	
	Wald $\chi^2(1)$	*P*	Wald $\chi^2(1)$	*P*
12°C vs. 16°C	0.03	*n.s.*	–	–
12°C vs. 20°C	11.43	< 0.001	–	–
16°C vs. 20°C	10.44	0.001	–	–
383 bp vs. 317 bp	9.92	0.0016	8.10	< 0.001
383 bp vs. 231 bp	10.62	0.001	5.80	0.016
383 bp vs. 85 bp	60.03	< 0.001	25.25	< 0.001
317 bp vs. 231 bp	0.01	*n.s.*	0.21	*n.s.*
317 bp vs. 85 bp	25.73	< 0.001	6.55	0.01
231 bp vs. 85 bp	24.68	< 0.001	8.84	0.003

n.s., not significant.

In *N. brevicollis* prey DNA, detection rates significantly decreased with increasing fragment length and digestion time. Detection rates differed significantly between the three temperature levels depending on digestion time. Detectability of aphid DNA decreased markedly between 6 h and 12 h and between 12 h and 24 h at 20°C and 16°C, respectively, compared to a more constant detectability of aphid DNA up to 24 h in beetles maintained at 12°C (fig. 1b).

A clear effect of fragment length on prey detection in *P. melanarius* was observed: for the three larger fragments (231, 317, 383 bp) amplification success decreased below 50% between 0 h and 24 h post feeding at all temperature levels (fig. 2a). In contrast, the shortest fragment (85 bp) was detectable in over 50% of the beetles up to 24, 36 and 72 h at 20, 16 and 12°C, respectively. Prey DNA detection rates differed significantly among the different-sized amplicons except for the 231 bp and 317 bp fragments (table 4).

In *N. brevicollis*, the effect of fragment length on prey detection was more distinctive between temperatures than in *P. melanarius*. The two larger fragments (317 and 383 bp) were successfully detectable in more than 50% of the beetles up to between 6 h and 24 h digestion time at all temperatures (fig. 2b). For the second shortest fragment (231 bp), 50% amplification success was similar at 16°C and 20°C (12 h), whereas even at 48 h post feeding more than 50% of the beetles tested positive at 12°C. For the shortest fragment (85 bp), more than 50% of the beetles still tested positive after 72 h at 12°C and 16°C and up to 24 h at 20°C. As in *P. melanarius*, detection rates among the different-sized amplicons in *N. brevicollis* differed significantly, except for the 231 bp and 317 bp fragments (table 4).

Discussion

Within the present study, we found that aphid prey DNA detection rates differed significantly in two carabid species, with a higher detectability in *N. brevicollis* compared to *P. melanarius*. It has been shown in previous studies that detection rates of prey protein (Sunderland *et al.*, 1987; Symondson & Liddell, 1993; Hagler & Naranjo, 1997) and prey DNA (Chen *et al.*, 2000; Hoogendoorn & Heimpel, 2001; Ma *et al.*, 2005; Read *et al.*, 2006; Greenstone *et al.*, 2007; Traugott & Symondson, 2008) can differ considerably in different predator species. Results from enzyme-linked immunosorbent assay (ELISA) suggest that staphylinids digest prey proteins faster than carabids and spiders (Sunderland *et al.*, 1987). The spiders' prolonged detection times for prey protein and prey DNA (Harwood *et al.*, 2004; Sheppard *et al.*, 2005; Traugott & Symondson, 2008) are possibly due to their ability to vary their metabolic rates in response to starvation (Anderson, 1970) and/or to their use of gut diverticula to store partially digested food (Nakamura & Nakamura, 1977). The longer detection times in carabids, compared with staphylinids, may possibly result from the intake of solid prey remains together with fluids, whereas rove beetles are mainly fluid feeders (Sunderland & Vickerman, 1980; Lovei & Sunderland, 1996). Note that fluid-feeding carabid larvae were found to have extended prey DNA detection times as well, which, however, were significantly influenced by prey species (Juen & Traugott, 2005, 2006, 2007). Comparing detectability of potato beetle DNA in pentatomid nymphs and ladybird larvae, Greenstone *et al.* (2007) found significantly higher mean prey DNA detection times in the former, which has been ascribed to the bug's spider-like hunting style and feeding mode. In contrast to these studies, we, here, compared detection rates of the same prey species in taxonomically-related predators. Hoogendorn & Heimpel (2002) compared, in a similar experiment, prey DNA detection rates between two coccinellid species, *Coleomegilla maculata* De Geer and *Harmonia axyridis* (Pallas), both of which are within the Cocciellini. Having fed both predators on eggs of *Ostrinia nubilalis*

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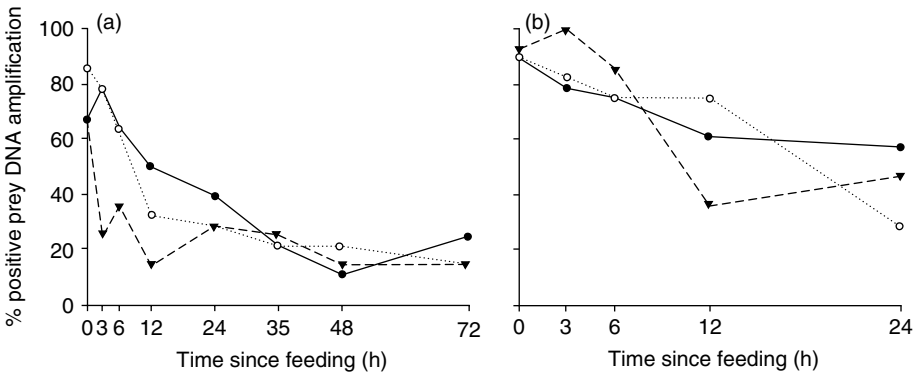

Fig. 1. Mean aphid prey DNA detection rates (%) in the gut of (a) *Pterostichus melanarius* and (b) *Nebria brevicollis* up to 72 h post-feeding of beetles kept at 12°C (–●–), 16°C (···○···) and 20°C (--▼--).

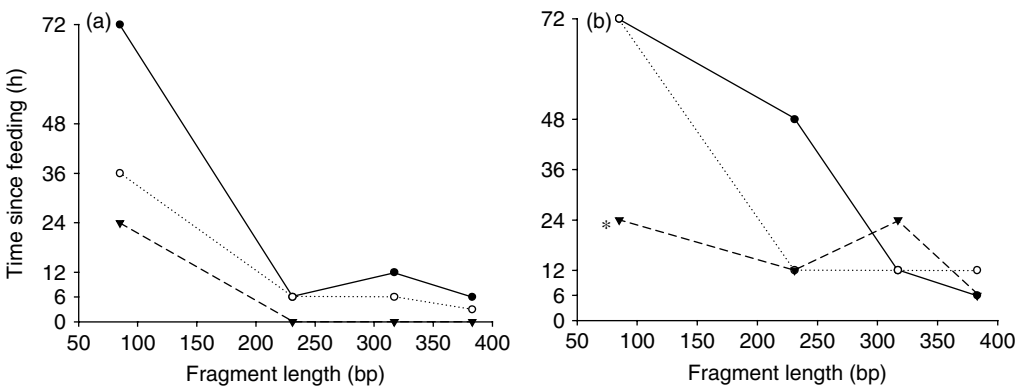

Fig. 2. Maximum time since feeding with more than 50% positive tested beetles for the four different sized amplicons (85, 231, 317 and 383 bp) of aphid DNA at 12°C (–●–), 16°C (···○···) and 20°C (--▼--) in (a) *Pterostichus melanarius* and (b) *Nebria brevicollis*. *Data for the detection times of aphid DNA in *Nebria brevicollis* at 20°C is only available up to 24 h.

(Hübner) (Lepidoptera: Crambidae) and allowed them to digest their prey from between 0 and 8 h, no significant differences in prey DNA detection rates were found. On the other hand, in a similar study using antibodies to measure digestion of prey proteins in two related species of carabid, large differences were found in digestion rates between the smaller *Pterostichus madidus* and the larger *Abax parallelepipedus* (Symondson & Liddell, 1993). *Pterostichus melanarius* and *N. brevicollis*, belonging to the carabid tribes Pterostichini and Nebriini, respectively, are both night-active autumn breeders, which hunt on the soil surface and have a similar feeding mode (Williams, 1959; Greenslade, 1963; Chapman *et al*., 1999). Despite these similarities, considerable differences in prey DNA detection rates were found between these two species. Interestingly, these differences were not altered by ambient temperature or prey amplicon length, indicating a fundamental dissimilarity in prey digestion capacities. These findings underscore the need to assess prey DNA detectability not only for predator taxa showing different feeding modes (Chen *et al*., 2000; Greenstone *et al*., 2007; Traugott & Symondson, 2008) but also for closely related species sharing the same feeding mode to allow correct interpretation of field-derived data. Perhaps, closely-related species within the same taxonomical tribe (Hoogendorn & Heimpel, 2002) are more similar in their prey DNA digestion than species belonging to different tribes, such as the carabids investigated here, a hypothesis which needs to be tested in future studies.

The present experiment showed that DNA detection success in *N. brevicollis* prey was negatively correlated with increasing ambient temperature, whereas in *P. melanarius* prey detection rates were significantly reduced only at 20°C, compared to rates at 12° and 16°C. This suggests a non-linear relationship between DNA digestion rates and ambient temperature for this predator-prey system. Similar results were found by Read (2007), who found that only at a temperature of 24°C were detection rates of nematode prey DNA in the guts of the collembolan *Folsomia candida* affected, whereas no significant differences in mean detection times occurred between ambient temperatures ranging from 4°C to 20°C. Significantly higher detectability rates of DNA from lepidopteran eggs in the ladybird *Coleomegilla maculata* (DeGeer) were observed at 20°C compared to 27°C (Hoogendoorn & Heimpel, 2001). These results indicate that the effects of temperature on prey digestion depend on the specific predator-prey system investigated and the environmental conditions within which these trophic interactions happen. Perhaps temperature effects can be neglected in systems where temperatures fluctuate only within a small range, e.g. in soil-dwelling predators (Juen & Traugott, 2007)

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or epigeic predators hunting under a dense plant canopy. In contrast, effects of ambient temperature on prey DNA digestion rates need to be considered in predators which are exposed to considerable temperature fluctuations. Clearly, further experimental work is needed on this topic to allow better interpretation of field-derived data on prey DNA detection rates.

Within the present study, we found that, in most cases, prey detection rates were positively correlated with decreasing prey DNA fragment length. Several studies have shown that this relationship holds true also in other predator-prey systems, with short amplicons allowing detection of prey DNA for longer periods post feeding (Agustí *et al*., 1999; Zaidi *et al*., 1999; Hoogendoorn & Heimpel, 2001). However, we also found, within the present experiments, that prey DNA detection rates were not significantly different between the 231 bp and the 317 bp fragment. Similarly, Chen *et al*. (2000) found no differences in detectability half lives for fragments of 246 bp and shorter. No differences in detection rates of DNA fragments between 127 and 585 bp were found within 24 h post-feeding intervals, when scarabaeid larvae were fed to carabid larvae (Juen & Traugott, 2005, 2006, 2007). These results indicate that the efficiency of PCR to amplify semi-digested prey DNA fragments is, besides amplicon length, also determined by factors such as the quality of the template DNA extract, PCR reagents, cycle conditions and the efficiency of the primers (King *et al*., 2008). In our study, the impact of those effects was diminished by optimising all primers to amplify at the same PCR cycle conditions, using the same PCR reagent concentrations and by combining all forward primers with the same reverse primer. Therefore, differences in detection rates can be mainly ascribed to the factors investigated, strengthening the significance of our results.

By using a set of fragments that can be detected for different lengths of time, Hoogendoorn & Heimpel (2001) aimed to estimate the time since feeding in a ladybird beetle-lepidopteran egg predator-prey system. The authors suggested that sequences increasing in length can be used to estimate a minimum and a maximum time since the predators consumed prey. This approach, however, demands a clear relationship between fragment length and amplification success at post-feeding intervals. Considering the results of the present study and of those discussed above, a set of primers amplifying fragments of considerably different amplicon size should be used to estimate the time point since feeding. Given all the confounding factors, the precision of such a system, based principally upon multiple amplicons of different sizes, is likely to be somewhat restricted. Nevertheless, ecologically relevant questions could be answered, for example differentiation between night and diurnal predator activity or temporal niche differentiation of predators.

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