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Detection of scavenged material in the guts of predators using monoclonal antibodies: a significant source of error in measurement of predation?

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Abstract

Molecular detection systems used to analyse the gut contents of invertebrate predators have enhanced our understanding of trophic interactions, but do not distinguish between the methods of consumption. Many predators regularly scavenge, which could have profound implications for quantitative analyses of the dynamics of predation. We report the first quantified assessment of the potential error caused by scavenging in post-mortem measurements of predation in a slug–carabid system. An anti-slug monoclonal antibody was able to detect antigens from decayed slugs after surprisingly long periods, significantly longer on relatively sterile peat than on natural soil. On soil the half-life of antibody-detectable slug proteins was 8.2 days while on peat it was 11.5 days. When slugs that had decayed on soil for 100 h were fed to the carabid predator *Pterostichus melanarius*, slug proteins could still be identified after 6 h (but not 12 h) digestion. Fresh and decayed slug was eaten in equal quantities by the beetles suggesting no aversion to the latter. The results suggest that significant errors may be caused by scavenging leading to inaccurate interpretation of predation rates in the field.

Introduction

The detection of prey remains within the guts of invertebrate predators, using antibodies and prey-specific molecular markers, has been invaluable for obtaining a greater understanding of trophic interactions between predators and prey in the field (reviewed by Symondson, 2002a). Recent work using monoclonal antibodies has, for example, examined the spatial and/or temporal dynamics of interactions between predators (carabid beetles and spiders) and prey such as earthworms (Symondson *et al*., 2000), slugs (Bohan *et al*., 2000) and aphids (Harwood *et al*., 2004, 2005; Winder *et al*., in press). Increasingly now, having been tried and tested in the laboratory, PCR-based approaches are also being used to study predation by invertebrates in the field (Agustí *et al*., 2003; Dodd *et al*., 2003; Kaspar *et al*., 2004) but their detection periods (Zaidi *et al*., 1999; Chen *et al*., 2000; Hoogendoorn & Heimpel, 2001; Greenstone & Shufran, 2003; Agustí *et al*., 2003; Cuthbertson *et al*., 2003; Sheppard *et al*., 2004) tend to be shorter than antibody-based techniques (Symondson *et al*., 1999, 2000; Harwood *et al*., 2001a, 2004, 2005; Schenk & Bacher, 2004). Although indicating the presence of prey proteins or DNA in predators, these techniques fail to account for the state of the prey when it was consumed. If the prey was dead then the predator will have had no direct effect on the prey population and no predation link will have been made (indirect effects are still possible if scavenged prey help to sustain or retain a higher predator population). Such sources of error have been recognized for some time but, to date, nobody has been able to design a molecular detection system that can distinguish between scavenged remains within predators and those that got there as a result of predation.

Sunderland (1996) reviewed the potential significance of secondary predation and scavenging. Secondary predation is where a predator eats a second predator after the latter has eaten the target prey species. Molecular analysis would

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suggest, falsely, that the first predator had consumed the target prey directly. Secondary predation has been assessed experimentally only once (Harwood *et al.*, 2001a) and revealed that it was a negligible source of error in an aphid–spider–carabid system using a monoclonal antibody-based approach. However, while secondary predation might reveal false trophic links, scavenging on dead prey could either lead to the false conclusion that predation is taking place or, more likely, to an overestimation of the importance of predation on live prey. For some predator groups, such as many spiders, scavenging may not be significant since they usually rely on movement of the prey to trigger an attack (Persons & Uetz, 1997), either through visual observation or vibratory detection, and locate to areas where active prey species are particularly abundant (Harwood *et al.*, 2001b, 2003). However, for most other predators, scavenging is common for simple reasons of energetics; a lion would always prefer to eat a dead zebra, when available, rather than go to the trouble of, and potential dangers associated with, capturing a live one. Scavenging is common among carabid beetles (Lövei & Sunderland, 1996; Lang & Gsödl, 2001) which have been shown to choose freshly killed over live slug prey (Langan *et al.*, 2001; Mair & Port, 2001). Despite this potentially significant source of error, no previous immunological or DNA-based molecular studies have attempted to assess its significance and Sunderland (1996) reported that of 72 relevant publications between 1956 and 1994 only 15% mentioned scavenging and none attempted to measure it.

We report the first quantitative study to assess the potential effects of scavenging on measurement of predation within a carabid–slug system. We hypothesized that scavenging on partially decayed slug was likely to be a significant source of error and that such material would be detectable within the guts of *Pterostichus melanarius* (Illiger) (Coleoptera: Carabidae) for significant periods post-feeding. This species of carabid, though a generalist predator known to feed on a wide variety of prey (reviewed in Sunderland, 2002; Symondson, 2002b), has been shown in a long-term study over five years to have a loosely-coupled relationship with slugs in arable fields (Symondson *et al.*, 1996) and to be capable of affecting their spatial dynamics (Bohan *et al.*, 2000). We also tested the hypothesis that slug proteins (and hence antibody-detectable antigens) would break down more rapidly on soil from the field, with all its associated saprophytic bacteria and fungi, than slugs left to decay on relatively inert peat.

Materials and methods

Slug decay on soil and peat

Slugs, *Deroceras reticulatum* (Müller) (Mollusca: Pulmonata), were collected from arable fields at Long Ashton Research Station, Bristol, UK, and killed by freezing at −20°C. Prior to the experiment, 150 clear plastic containers (9.5 cm diameter, 5 cm depth) were filled with 80 g topsoil from Long Ashton, and a further 150 with 80 g Scotts® Sphagnum Peat Moss (Scotts Company (UK) Ltd, Godalming, Surrey, UK). Freshly killed slugs were placed on the substrate (one per container) and the containers sealed with a loose-fitting lid. Samples were maintained in a controlled environment of 16°C on a 16:8 h light:dark cycle, a temperature comparable to UK field temperatures (Harwood *et al.*, 2001b). In addition to ten control slugs being frozen at −20°C prior to the experiment and therefore not subjected to decay (t = 0), ten dead slugs from each substrate were frozen after 2 h (t = 2). Further batches of ten dead slugs from both topsoil and peat were frozen at 4, 8, 16, 24, 36, 48, 72, 96, 120, 144, 168, 192 and 216 h after the start of the experiment.

Consumption of decayed slugs by carabid beetles

Female *P. melanarius* were collected from fields at Long Ashton by pitfall trapping. The beetles were housed individually in plastic containers (9.5 cm diameter, 5 cm depth) with 80 g moist peat and maintained in a controlled environment as above. The beetles were fed on a diet of freshly killed fly larvae, *Calliphora vomitoria* (L.) (Diptera: Calliphoridae).

Deroceras reticulatum were prepared on Long Ashton topsoil (as above) and left to decay for 100 h at 16°C on a 16:8 h light:dark cycle. Following a two-week starvation period, the beetles were allowed to feed during a 3 h period on decayed slug, whilst being observed. Any non-feeding individuals were discarded. After feeding, five beetles were killed by freezing. All other beetles were transferred into clean containers and maintained under controlled conditions as described above for slugs. Further batches of five beetles were killed by freezing at 6, 12, 24, 48 and 96 h after feeding. In addition, to compare the consumption of freshly killed versus decayed slug, beetles were fed on freshly killed slug for 3 h and, as above, beetles were killed by freezing at −20°C at both 24 h and 96 h after feeding.

Sample processing and screening

All fresh and decayed slugs were thawed, diluted ×10 (w/v) in phosphate-buffered saline, pH 7.4 (PBS), and homogenized. The homogenate was dispersed for 1 min on a vortex mixer and then centrifuged at 8000 *g* for 15 min at room temperature. The supernatants were transferred into clean Eppendorf tubes and stored at −20°C. All particulate remains were discarded.

The foregut of each beetle was removed, diluted ×20 in PBS, homogenized, dispersed and centrifuged as above. Particulate matter was discarded, the supernatant transferred to clean Eppendorf tubes and stored at −20°C.

Samples were subsequently screened by indirect ELISA at room temperature after being diluted in PBS to a final concentration of ×20000 (w/v). Samples were added in duplicate to two ELISA plate wells, at 200 µl per well, and left to incubate overnight. Each plate also included a duplicate 1.5× dilution series of *D. reticulatum* standards that provided absorbance readings for protein concentrations between 985.75 and 25.64 ng 200 μl^{-1}. Protein concentrations were calculated using the Bio-Rad Protein Assay System (Bio-Rad Laboratories, Germany). The slug standards were diluted with heterologous protein (starved carabid beetle guts) to stabilize protein concentrations and regression of absorbance readings against concentration enabled the calculation of slug protein equivalents for each decayed slug or beetle gut sample.

Following overnight incubation, all wells were washed three times with PBS-Tween (0.05% Tween® 20, Sigma-Aldrich, Poole, UK). The anti-mollusc monoclonal antibody 1C9 (Dodd, 2004) was diluted 1:250 in PBS-Tween and 200 µl added to one of the duplicate wells for each sample to allow

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binding between antibodies and antigen. Alternate wells were simultaneously incubated for 2 h with PBS-Tween (containing no antibody) to quantify any non-specific binding by the enzyme conjugate. Following incubation, all wells were washed three times with PBS-Tween and goat anti-mouse IgM (μ-chain specific) peroxidase conjugate (Sigma-Aldrich, Poole, UK), diluted 1:2000 in PBS-Tween, was added to all wells. Following 1 h incubation, all wells were washed three times in PBS-Tween, the enzyme substrate *o*-phenylenediamine in a citrate-phosphate buffer was added at 200 μl per well, and the ELISA plates allowed to incubate in the dark. After 30 min, 50 μl of 2.5 M H_2SO_4 was added to stop the reaction. Absorbance readings were taken at 492 nm using an ELISA plate spectrophotometer (Thermomax Plate Reader, Molecular Devices, California, USA). Readings from duplicate wells, to which no antibody was added, were subtracted from the wells to which antibody was added to eliminate effects of non-specific binding.

Results

Rate of slug protein decay

The rates of decay of antibody-recognizable *D. reticulatum* proteins are shown in fig. 1 where, for clarity, untransformed data are presented. The data suggest little sign of decay until the last two samples at 192 and 216 h. To compare the decay rates on soil and peat the data were transformed, and the best fitting model proved to be $\log_e$ slug protein concentration against time squared (on soil, $\log_e$ concentration = 6.39–0.000018 h^2, R^2 0.75, $P < 0.001$; on peat, $\log_e$ concentration = 6.314–0.000009 h^2, R^2 0.79, $P < 0.001$). Analysis of covariance was performed on the means for each time period and treatment and showed (as expected) no significant difference between the y-axis intercepts ($P > 0.05$), but the slopes were significantly different from each other ($P = 0.008$) and from zero ($P < 0.001$). The slugs decayed on the soil, therefore, significantly more rapidly than on the peat. The regression equations were then used to calculate how long it would take for the concentration of antibody-recognizable slug protein to decline to half the value at the start of the experiment (the half life), with the start value calculated from the regression line. On soil the half-life was 196 h while on peat it was 278 h.

Detection of scavenging by Pterostichus melanarius

The decline in detectability of decayed slug in the guts of *P. melanarius* is presented in fig. 2. The results show that although decayed slug was detectable immediately after and 6 h post-feeding, after 12 h it was not possible to detect the presence of antibody-recognizable antigens. By comparison, 24 h after the consumption of freshly-killed *D. reticulatum*, this anti-slug monoclonal was still able to detect significant levels in the guts of *P. melanarius*, but not after 96 h (fig. 2). Comparison between uneaten slug that had decayed for 100 h versus recently consumed 100 h decayed slug from within the gut of *P. melanarius* (immediately after the 3 h feeding period) indicated 86% lower concentrations of detectable slug protein following scavenging ($F_{1,13} = 24.34$, $P < 0.001$).

Beetle gut weight was also recorded to ensure that *P. melanarius* was not rejecting or preferentially feeding on fresh/decayed slug. A comparison of gut weight at 24 h and 96 h after feeding indicated no significant difference between those beetles fed freshly killed slug and those that consumed decayed material (24 h, $F_{1,9} = 0.82$, $P = 0.386$; 96 h, $F_{1,9} = 1.93$, $P = 0.198$).

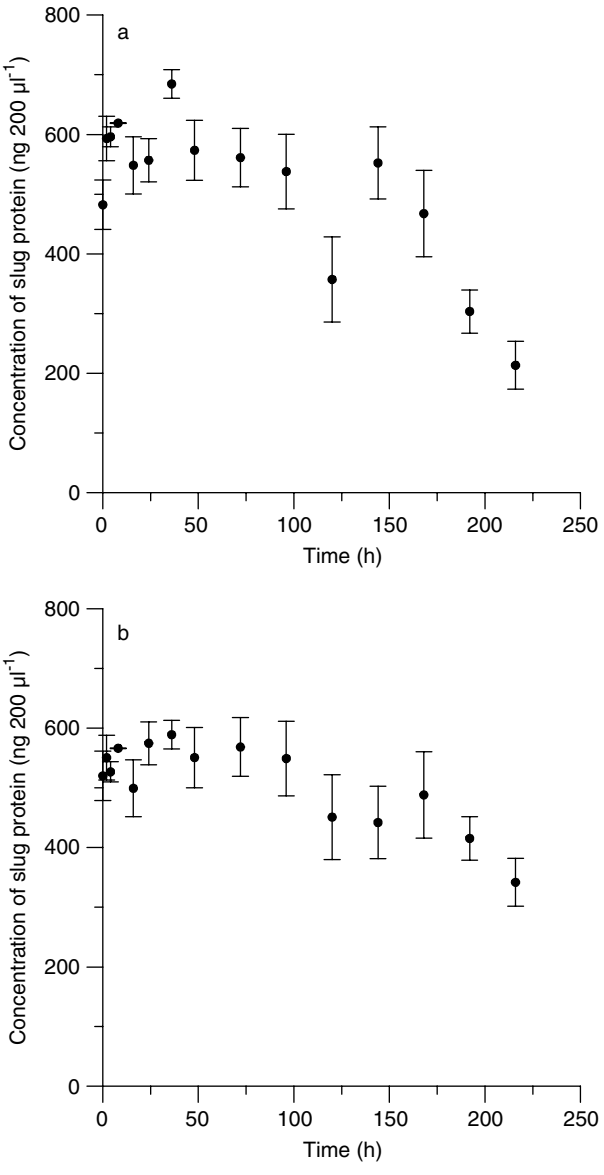

Fig. 1. The response to slug antibody of samples of dead *Deroceras reticulatum* taken at different times after exposure on (a) topsoil and (b) sterilized peat.

Discussion

Slug mortality through disease, physical damage during cultivation, and the application of control measures (nematode biopesticides or synthetic molluscicides) can leave a large number of dead slugs on the ground surface and available for scavenging. However, stochastic events aside, it is rare to see dead slugs on the soil surface and no data are available on this (Langan *et al*., 2001). Interestingly, Symondson *et al*. (1996), using an anti-slug polyclonal

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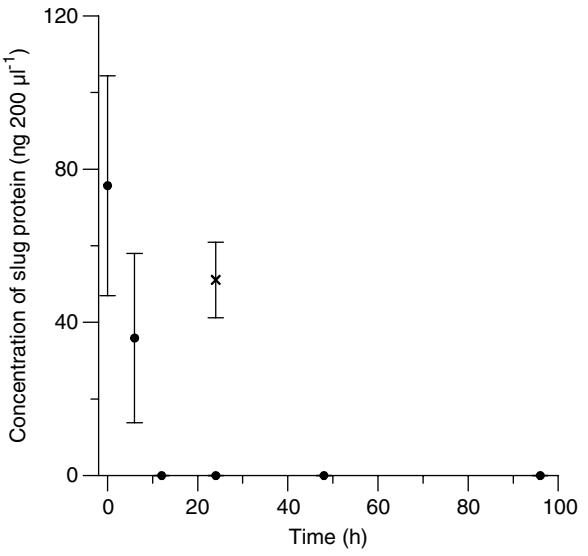

Fig. 2. Detection of decayed remains of *Deroceras reticulatum* in the guts of the carabid beetle *Pterostichus melanarius* following scavenging (●). For comparison, antibody-recognizable slug protein concentration of beetle guts after 24 h from individuals fed freshly killed slug are included (×).

antiserum, found no evidence of an increase in consumption of slugs by *P. melanarius* immediately following cultivation. This does not necessarily mean that the carabids were uninterested in the dead slugs that must have been present, rather they may have been preferentially feeding on other casualties of the cultivation process such as earthworms, on which they are also known to feed in the field (Symondson *et al*., 2000). Thus, although these experiments have shown that scavenging could potentially confound attempts to measure predation, many other dynamic factors must operate to influence scavenging rates in the field. It should also be remembered that in many studies the relevant factor is the value of a particular prey species as food for a predator, in which case it is irrelevant whether that prey species is alive or dead when consumed (Farrell *et al*., 2000; Symondson *et al*., 2000; Sutherland, 2000). However, in many (perhaps most) instances we are interested in the effects of predation on prey populations, and not being able to distinguish predation from scavenging is potentially a significant problem. It is also relevant to question whether scavenging or predation are the only relevant categories, given that predation on moribund and diseased prey also either have no significant effect on prey numbers or could, by breaking disease transmissions cycles, even lead to an increase in prey numbers.

Monoclonal antibodies can be highly sensitive and the target epitopes are often resistant to digestion (Symondson & Liddell, 1996; Symondson *et al*., 1999, 2000; Harwood *et al*., 2001a). However, the rate of decay without exposure to digestive enzymes, but under the influence of soil microorganisms, could not be predicted. Slug antigens were detectable in decaying slugs on both the soil and peat for remarkably long periods, with half-lives of 8.2 and 11.5 days respectively. If the regression lines are projected to the point where slug antigens disappeared entirely (for convenience calculated at 1 ng of antibody-recognizable slug protein), it would take 24.8 days on soil and 34.9 days on peat to reach that point. These figures are almost certainly unrealistic. At some point, saprophytic bacteria would invade and then increase at an exponential rate, leading to an abrupt breakdown of remaining tissues. It is also highly likely that other predators, such as birds, remove moribund or dead slugs rapidly. Sphagnum peat is irradiated prior to packaging, hence many biological agents would be rendered sterile.

The detection period for partially decayed slug tissues (100 h), further broken down by digestion within carabids, was between 6 and 12 h. By 12 h no antigens could be detected, a time period considerably shorter than found for prey remains in beetles that had consumed freshly killed slug. In the latter, the slug was still reacting strongly with the antibody after 24 h of digestion. Despite this, scavenging may still be a significant source of error. The fact that slug proteins were detectable after 6 h in part reflected the high sensitivity of this assay system. We have maximized the sensitivity of our ELISA tests and used an antibody that targets an epitope that is relatively resistant to digestion. Had our antibody been less sensitive, such as many reported in the literature that have detection period of less than 24 h (reviewed in Symondson, 2002a,b), it would have been impossible to detect the decayed slug after 6 h digestion. In field studies carabids which are subsequently assayed by gut-content analysis are usually dry-pitfall trapped overnight (Symondson *et al*., 1996, 2000; Bohan *et al*., 2000; Winder *et al*., in press), resulting in a delays of 0 h to around 16 h between the times at which they fall into the trap and are transferred into a freezer, halting all further digestion. As the beetles are nocturnal, few would have eaten slug during the day before the overnight trapping. Thus, the mean point at which they are frozen would be 8 h after they had eaten a dead slug, and few if any would test positive after this length of time (fig. 2). There are not enough data points to model this precisely, but it could be done, and a threshold established to exclude most of the beetles that had eaten decayed material overnight. However, this would inevitably reduce the sensitivity of the overall detection system and some false negatives (beetles that had eaten live slugs, but had done so a long time ago, or had consumed very small amounts), would occur. Further work is therefore needed, to look at how these and other factors affect detectability, including temperature and humidity. Many dead slugs available in the field may have decayed for less than 100 h when found and consumed by beetles, potentially increasing the number of positive reactions.

Carabids appeared to be well-adapted scavengers as well as predators (Lövei & Sunderland, 1996; Lang & Gsödl, 2001) and will readily consume dead slugs. The comparison between gut weights of freshly killed versus 100 h decayed slug (that was clearly beginning to putrify) confirmed that *P. melanarius* had no compunction about eating such highly decayed material. There must be a limit to how putrid a slug must be before it is rejected by a beetle, and that point is probably affected by initial beetle hunger and the availability of alternative prey (including live slugs), but this was beyond the scope of our study. An earlier study, using the carabid *Abax parallelopipedus* (Piller & Mitterpacher), looked at the rates at which slugs were killed when: (i) dead slugs were removed every third day, leaving the beetles with access to a live slug only, and (ii) the remains of dead slugs were not removed, but a live slug introduced every time a dead slug was found (Symondson, 1989). The rate at which

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slugs were killed was, against expectation, higher where the remains of dead slugs were not removed, suggesting some aversion by this carabid to decaying prey.

Apart from weather and soil conditions, the method of death (e.g. pesticide, age-related, partial consumption by other predators, disease, nematode infection, cultivation) could also alter the rates at which the remains would decay. Whether the detection of prey DNA would be subject to similar errors has yet to be established, but it is probable. These results highlight the value of determining, as far as possible, the potential significance of scavenging when using molecular approaches to dietary analyses, ideally through a combination of laboratory choice experiments, field monitoring of both dead and living food resources, and calibratory experiments such as those described here. Ultimately, scavenging will only be a significant source of error if there are significant numbers of dead slugs in the field. There are no data on this, and no effective methodology has been proposed for gathering such information.

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