Secondary predation: quantification of food chain errors in an aphid–spider–carabid system using monoclonal antibodies

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Abstract

‘Secondary predation’ occurs when one predator feeds on a second predator, which has in turn eaten a target prey. Detection of prey remains within predators using monoclonal antibodies cannot distinguish between primary and secondary predation, potentially leading to quantitative and qualitative food chain errors. We report the first fully replicated experiments to measure secondary predation effects, using an aphid–spider–carabid system. Aphids, *Sitobion avenae*, were fed to spiders, *Lepthyphantes tenuis*, which were allowed to digest their prey for a range of time intervals. The spiders were then fed to carabids, *Poecilus (= Pterostichus) cupreus*, which were allowed to digest their prey for set periods. The anti-aphid monoclonal antibody used to identify *S. avenae* remains in *P. cupreus* was one that detected an epitope that increased in availability over the first few hours of digestion, amplifying the signal, extending detection periods and thus increasing the chances of detecting secondary predation. Despite this, and the fact that spiders are known to digest their prey more slowly than many other predators, detection of secondary predation was only possible if the carabids were killed immediately after consuming at least two spiders which were, in turn, eaten immediately after consuming aphids. As this scenario is unlikely to occur frequently in the field it was concluded that secondary predation is unlikely to be a serious source of error during field studies.

Keywords: antigen decay rates, food webs, generalist predators, intraguild predation, *Lepthyphantes tenuis*, *Poecilus cupreus*

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Introduction

Significant advances in our understanding of predator-prey interactions have been made by applying ‘molecular’ techniques to the post mortem analysis of the gut contents of field-caught predators (reviewed in Sunderland 1988; Greenstone 1996; Symondson & Hemingway 1997; Sunderland et al. 2001; Symondson 2001). These techniques have included the detection of prey isoenzymes using electrophoresis (e.g. Murray & Solomon 1978; Giller 1986; Heitmans et al. 1986; Lister et al. 1987; Walrant & Loreau 1995), prey proteins using polyclonal antisera (e.g. Coaker & Williams 1986; Dennison & Hodkinson 1983; Sunderland et al. 1987; Cameron & Reeves 1990; Symondson et al. 1996) and (currently the preferred technique) detection of family, genus, species and even stage-specific protein epitopes using monoclonal antibodies (e.g. Greenstone & Morgan 1989; Hagler et al. 1992; Symondson & Liddell 1996; Symondson et al. 1997, 1999a, 2000; Bacher et al. 1999). Recent experiments have further demonstrated that detection of prey DNA from the guts of such predators is a viable option, using prey-specific primers and the polymerase chain reaction (PCR) (Zaidi et al. 1999; Agustí et al. 1999, 2000; Chen et al. 2000; Hoogendoorn & Heimpel 2001). Data from field experiments analysed by any of these means can be used to model predation rates (Mills 1997), and hence evaluate different species of predator as biocontrol agents, or to address fundamental questions in trophic ecology.

All of these techniques detect the presence of prey remains in the gut of predators but none in fact tells us precisely how the material got there. The assumption is almost
always that direct predation on the prey has taken place. Although in some cases other routes are mentioned, particularly scavenging and secondary predation (food chain errors have been reviewed by Sunderland 1996), serious attempts to quantify these sources of potential error have rarely (if ever) been made. Secondary predation, also termed hyperpredation (Sabelis 1992), is where a (primary) predator, that has consumed the target prey, is consumed by a second predator (or scavenger). The second predator then contains remains of the target prey without directly killing that prey itself. There are many examples in the literature of generalist predators consuming other predators in the field (reviewed in Sunderland 1996). Although the problem of secondary predation is frequently mentioned (Hagler & Naranjo 1996; Sunderland 1996; Symondson & Hemingway 1997; Morris et al. 1999; Sunderland et al. 2001; Symondson 2001), no previous studies have attempted to quantify its significance. Tod (1973) developed a polyclonal antiserum against the slug Dorsena reticulatum (Müller) and speculated that some of the predators she tested may have given a positive reaction due to secondary predation. No experiments to test this hypothesis were performed. In another study, Dennison & Hodkinson (1983) were apparently unable to detect secondary predation using polyclonal antiserum developed against a range of prey using precipitin tests, although no experimental details were provided.

We report the first fully replicated and quantitative study of secondary predation using any of the ‘molecular’ approaches. We used an aphid-specific monoclonal antibody (Symondson et al. 1999b) within a three species system (aphid–spider–beetle) to investigate detection limits following secondary predation. Our hypothesis was that if beetles ate spiders that had fed recently on aphids, aphid proteins would be detectable in the guts of the beetles. Our aim was to quantify the limits of detectability within this system and thus determine whether secondary predation is likely to be a significant source of error in subsequent field experiments.

Materials and methods

Predator preparation and antigen decay rates

Spiders, Lepthyphantes tenuis (Blackwall) (Araneae: Linyphiidae), were collected from winter wheat fields at Horticulture Research International, Wellesbourne, UK. They were maintained in a controlled environment at 16 °C on a 16:8 light/dark cycle, a temperature comparable with average summer temperatures in UK cereal crops (Harwood et al. 2001). Following collection, spiders were fed for ~1 month on a diet of live Drosophila melanogaster Meigen. Each spider was maintained separately in a 50 × 15 mm triple-vented Petri dish containing a damp Plaster of Paris and charcoal base to ensure high humidity. Following starvation for 2 weeks, 16 spiders (eight male and eight female) were frozen as starved controls. All remaining spiders were fed ad libitum on live aphids, Sitobion avenae (F.) (Hemiptera: Aphididae), for 2 h. Any spiders that failed to eat aphids were excluded from the experiment. In total, 16 spiders (eight male and eight female) were frozen immediately after the feeding period. The remaining spiders were transferred to clean Petri dishes (prepared as above). Further batches of spiders (eight male and eight female) were killed by freezing at 2, 4, 8, 16, 24, 36, 48, 72, 96, 120, 144, 168, 192, 216, 240 and 288 h after the feeding period and stored at ~20 °C. All samples, including starved controls, were tested by enzyme-linked immunosorbent assays (ELISA) (see below).

Poccilus (= Pnuenicola) cupreus (L.) (Coleoptera: Carabidae) were collected from winter wheat fields at Long Ashton Research Station, Bristol, UK, and maintained under the same environmental conditions as the spiders. Following collection, P. cupreus were fed for ~1 month on a diet of Calliphora vomitoria (L.) larvae that had been killed by freezing. All predators were maintained in separate 90 × 15 mm triple-vented Petri dishes on damp filter paper. Following starvation for 2 weeks, 16 beetles (eight male and eight female) were frozen as starved controls. The remaining beetles were observed to feed ad libitum on freshly killed (by freezing) S. avenae for 2 h and any non-feeding individuals were excluded from the experiment. We were not interested in predatory behaviour in this experiment and killing the aphids in this way ensured (and co-ordinated) rapid consumption by the beetles. Sixteen beetles (eight male and eight female) were frozen as starved controls. The remaining beetles were transferred into clean Petri dishes and a further eight males and eight females were killed by freezing at 2, 4, 8, 16, 24, 36, 48, 72, 96, 120, 144, 168 and 192 h after the feeding period and stored at ~20 °C. All samples, including starved controls, were tested by ELISA (see below).

Detection of secondary predation

The structure of the experiment is described below and summarized in Fig. 1. L. tenuis and P. cupreus were collected and maintained under laboratory conditions for ~1 month (as described above). Approximately 1000 female L. tenuis were fed ad libitum on live S. avenae for 2 h following a 2-week starvation period. Any spiders that failed to feed were excluded, leaving a total of 808 female L. tenuis that were used for the quantification of secondary predation. Two hundred and eight spiders were frozen before feeding on S. avenae, eight of which were reserved as starved controls. A further 200 were frozen immediately after feeding. All remaining spiders were transferred to clean Petri dishes, prepared as
described above, and batches of 200 spiders frozen 2 and 4 h after end of the feeding period. The 200 spiders, which were frozen immediately after feeding on *S. avenae*, were fed *ad libitum* to 24 male and 24 female *P. cupreus* (which had been starved for 2 weeks prior to the experiment). All beetles were observed to feed on the spiders and consumed either two or three adult *L. tenius* (any that ate fewer than two spiders were rejected and none ate more than three). Following a 2-h feeding period, 16 beetles (eight male and eight female) were killed by freezing. All remaining beetles were transferred to clean 90 × 15 mm triple-vented Petri dishes on damp filter paper. Further batches of eight male and eight female beetles were frozen 2 and 4 h after the feeding period. Three further groups of 48 *P. cupreus* were fed on either two or three *L. tenius*, which had been killed either 2 or 4 h after feeding on *S. avenae*, or which had been starved. Batches of 16 beetles from each of the groups were then frozen 0, 2 and 4 h after feeding on the spiders.

**Sample processing and ELISA**

All spiders were weighed, homogenized and diluted ×40 (w/v) in phosphate-buffered saline, pH 7.4 (PBS). The homogenate was dispersed for 1 min on a vortex mixer and centrifuged at 8000 × g for 15 min at room temperature. The particulate remains were discarded and the supernatants transferred into clean 0.5 mL Eppendorf tubes and stored at −20 °C.

Each *P. cupreus* was allowed to thaw at room temperature and the foregut, or crop, removed as described in detail in Symondson et al. (2000). The beetle was carefully teased apart between the thorax and abdomen, allowing the foregut to be pulled away and removed from the body. The foregut was homogenized in PBS to a ×20 dilution (w/v) then dispersed for 1 min on a vortex mixer and centrifuged at 8000 × g for 15 min at room temperature. The supernatant was transferred to a clean 1.5 mL Eppendorf tube and stored at −20 °C.

All invertebrate samples were screened by indirect ELISA at room temperature (Symondson & Liddell 1995). The homogenized samples were diluted to a final concentration of ×20 000 (w/v) in PBS. Each sample was added to two ELISA plate wells, at 200 μL per well, and left to incubate overnight. All wells of the ELISA plate were washed three times with PBS–TWEEN (Sigma-Aldrich, Poole, UK). The anti-aphid ascites, MdW-7C1G1 (Symondson et al. 1999b), was diluted 1:4000 in PBS–TWEEN and 200 μL added to one only of the duplicate wells for each test sample, whereas 200 μL of PBS–TWEEN was added to the second well. The plates were allowed to incubate for 2 h to enable binding between the antigen and antibody. Plates were washed three further times with PBS–TWEEN. ImmunoPure® goat antimouse IgG horseradish peroxidase enzyme conjugate (Pierce, Rockford, IL, USA) was diluted 1:4000 in PBS–TWEEN and 200 μL added to all ELISA plate wells and allowed to incubate for 1 h so that the conjugate could bind to the mouse antibodies. Plates were washed a further three times with PBS–TWEEN, and 200 μL of the enzyme substrate, o-phenylenediamine in a citrate–phosphate buffer, added to wells and placed in the dark for 30 min to allow colour development. The reaction was stopped by adding 50 μL per well of 2.5 M H₂SO₄. Absorbance readings were recorded at 492 nm using an ELISA plate spectrophotometer (Thermomax Plate Reader, Molecular Devices, CA, USA). Absorbance readings for duplicate wells, to which no ascites were added, were subtracted from readings for wells to which antibody was added, to eliminate the effects of non-specific binding (Symondson et al. 2000).

Each ELISA plate also included a dilution series (×1.5) of *S. avenae* standards that provided absorbance readings for aphid protein concentrations between 265.5 and 4.6 ng per 200 μL. Aphid protein concentrations were calculated following a protein assay using the Bio-Rad Protein Assay System (Bio-Rad Laboratories Ltd, GmbH, Munich, Germany). In order to stabilize protein concentrations throughout the dilution series, the aphid standards were diluted with heterologous protein, also diluted 1:20 000 (w/v) in PBS (Symondson & Liddell 1995). In these samples, the heterologous proteins were either starved *L. tenius* (for the *L. tenius* antigen decay experiment) or...
Results

Rate of decay of aphid remains within the gut of Lepthyphantes tenuis

The rates of aphid antigen decay in male and female *Lepthyphantes tenuis* are presented in Fig. 2. The anti-aphid monoclonal antibody MdW-71G1 was shown in an earlier experiment, using the predator *Pterostichus melanarius* (Illiger), to detect an initial increase in binding to antigen, probably as digestion revealed internal binding sites within target molecules (Symondson et al. 2000). This pattern was repeated here during digestion by *L. tenuis*. As prey consumption by the predators (both spiders and carabids) could have taken place at any time during the 2 h feeding period, consumption was deemed to have taken place at the mid-point (after 1 h) and thus 1 h has been added to each time interval in Figs 2 and 3 and during regression analyses (Symondson & Liddell 1993).

The rate at which aphid protein within the gut of these spiders decayed showed a linear relationship between 17 and 169 h after feeding, for both female and male spiders. Using the regression equations, the antigenic half-life (the time taken for antigen concentration to decrease to half its original level, which was recorded immediately after feeding) was 101.1 h in females and 105.4 h in males. Analysis of covariance indicated that there was no significant difference between the rates at which the two slopes declined ($F_{1,14} = 0.13, \ P > 0.05$) or the $y$-axis intercepts ($F_{1,14} = 0.14, \ P > 0.05$). As the weights of spiders fed *ad libitum* on aphids differed significantly between the sexes [mean (± SE) males, 1.55 ± 0.086 mg; females 3.17 ± 0.363 mg; $F_{1,14} = 18.96, \ P < 0.001$], but $y$-axis intercepts did not, the ratios between the mean size of each sex, and the quantity of prey each sex consumed, must have been similar. Mann–Whitney U-tests were used to compare aphid protein equivalents for starved spiders with those that had digested their prey for 169 h or more. Aphid was shown to be detectable in female *L. tenuis* for up to 193 h ($U = 3.0, \ n = 16, \ P < 0.001$) and in males for up to 169 h ($U = 7.0, \ n = 16, \ P < 0.01$).

Rate of decay of aphid remains within the gut of *Poecilus cupreus*

The rates of aphid antigen decay in male and female *P. cupreus* are shown in Fig. 3. As with *P. melanarius* and *L. tenuis*, detection of antigen increased initially. There was a linear rate of antigen decay between 17 and 97 h after feeding, for both male and female beetles. The half-lives of the antigen in female (70.8 h) and male (70.8 h) beetles were considerably less than those calculated for the spiders. Analysis of covariance indicated that there was no significant difference between the rates at which the antigen decayed
in male and female beetles ($F_{1,8} = 4.10$, $P > 0.05$) or between the y-axis intercepts ($F_{1,8} = 2.35$, $P > 0.05$). The foregut weights of the two sexes, fed ad libitum on aphids, were not significantly different either (mean ± SE males 9.06 ± 2.28 mg; females 9.55 ± 1.03 mg; $F_{1,14} = 0.04$, $P > 0.05$) indicating that males and females ate the same quantity of aphids. Mann–Whitney U-tests were used to compare aphid protein equivalents for starved $P$. cupreus with those that had digested their prey for 145 h or more. Aphid was shown to be detectable in female beetles for up to 169 h ($U = 9.0$, $n = 16$, $P < 0.05$) and in males for up to 145 h ($U = 6.0$, $n = 16$, $P < 0.01$).

As there was no significant difference between aphid protein decay within the two sexes of $L$. tenuis or $P$. cupreus, data were pooled before between-species comparisons were made. Analysis of covariance indicated that there were highly significant differences between the rates of antigen decay in the two species ($F_{1,11} = 78.66$, $P < 0.001$) and the y-axis intercepts ($F_{1,11} = 17.73$, $P < 0.001$). Higher concentrations of aphid protein in the dissected guts of the beetles would be expected in comparison with whole-body extracts from the spiders.

**Rate of decay of aphid remains following secondary predation**

The rates of aphid antigen decay following secondary predation are shown in Fig. 4. Statistical comparisons using the Mann–Whitney U-test were made with starved $L$. tenuis controls (Fig. 4). The results show clear detection of aphid protein equivalents only in beetles that were frozen immediately after feeding on spiders that were in turn frozen immediately after feeding on Sitobion avenae ($P < 0.001$).

However, significant detection was also found for female (but not male) beetles killed 2 h after feeding on spiders that were themselves killed immediately after feeding on aphids ($P < 0.05$) (Fig. 4a). However, when thresholds for significance in relation to other non-aphid prey, that might be consumed by the beetles in the field, are applied (Symondson et al. 1999b), none of this latter group of beetles would be deemed to contain aphid (see Discussion). If the beetles have 2 h or more to digest the spiders, aphid within those spiders could not be detected, even if the spiders were killed immediately after feeding on aphids (Fig. 4b,c). Beetle foreguts containing starved spiders did not react with the monoclonal antibody (Fig. 4d).

The mean foregut weight of $P$. cupreus fed aphids (mean weight ± SE = 9.31 ± 1.20 mg) was significantly heavier than that of beetles that were fed spiders (mean weight ± SE = 5.87 ± 0.41 mg; $t_{18} = 2.68$, $P < 0.05$). Both groups of beetles were fed ad libitum with prey (rejecting beetles that ate fewer than two prey items), and therefore over the 2-h feeding period a greater quantity of aphid material was consumed.

**Discussion**

Secondary predation has been largely ignored in studies using antibodies to assess predation, despite the obvious implications in terms of food chain errors (Sunderland 1996). That food chain errors could lead to misinterpretation of results was acknowledged in only 7% of 72 studies reporting
Fig. 4 Detection of *Sitobion avenae* proteins following secondary predation. Information is presented for *Poecilus cupreus* fed *Lepthyphantes tenuis* which were killed (a) 0 h, (b) 2 h, (c) 4 h after feeding on *S. avenae*, and (d) *P. cupreus* which were fed starved *L. tenuis* and frozen at set time intervals following feeding. Mean (±SE) are presented for starved *P. cupreus* (Pc) and starved *L. tenuis* (Lt) (and therefore do not relate to time periods on the x-axis). Significance compared against aphid protein equivalent for starved *L. tenuis* and presented as ***P < 0.001, *P < 0.05*. 1Mann–Whitney U = 0.0, n = 16, P < 0.001; 2U = 11.0, n = 16, P < 0.05; 3U = 0.0, n = 16, P < 0.001.
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the use of antibody techniques published between 1956 and 1994 (Sunderland 1996). Should secondary predation be a significant factor, generalist predators collected from the field would yield unknown numbers of false positives, resulting in both quantitative and qualitative misinterpretation of trophic links. Our experiments were designed to test rigorously the hypothesis that secondary predation was detectable, using a system that would maximize the possibility of such detection. This was achieved by, first, using a monoclonal antibody that bound with an epitope that had been shown, in a previous study, to increase in availability and hence antibody–antigen binding, over the first few hours of digestion (Symondson et al. 1999b; Figs 2 and 3). By contrast, most other monoclonal antibodies and polyclonal antisera have demonstrated exponential rates of antigen decay that are most rapid immediately after prey consumption (e.g. Sopp & Sunderland 1989; Symondson & Liddell 1995; Hagler & Naranjo 1997; Symondson et al. 1997, 1999a). Second, we chose web-building spiders as the first predator. It has been shown in previous work that antigen decay rates in such spiders can be particularly long, possibly because they use the branching of the midgut to store, for extended periods, excess food following feeding on a particularly large meal (Nakamura & Nakamura 1977) and/or can vary their metabolic rates in response to starvation (Anderson 1970). Indeed, the rate of antigenic decay within the carabid Poecilus cupreus was found to be ~60% faster than within the spider Lepthyphantes tenuis. Thus, the properties of both the monoclonal antibody and the first predator should have maximized overall detection periods, and hence enhanced our ability to detect aphid remains within the spiders after the latter had been consumed by carabids.

Despite using a system strongly biased in favour of our hypothesis, the results suggest that secondary predation will rarely be detected in the field using this methodology. To detect clearly such an effect in our aphid/P. cupreus system, a beetle must be caught and killed immediately after it has eaten at least two spiders, which had, in turn, just consumed aphids. This is an unlikely scenario. Additionally positive results were obtained from female beetles killed within 2 h of feeding on at least two spiders that were eaten immediately after feeding on aphids. When the spiders were given 2 h or more to digest their aphid prey before being ingested by the beetles, aphid proteins could not be detected. If the beetles had more than 2 h to digest the spiders, aphid within those spiders could not be detected either. In addition, it must be remembered that, in these experiments, the beetles were fed spiders containing aphids, or nothing. In the field the beetles may have eaten other non-aphid and non-spider prey. To guard against the possibility of false positives, a significance level must be set, defined as the mean (plus 2. 5 SD) aphid protein equivalent (ng/200 mL) generated in an ELISA by the non-aphid prey giving the strongest non-specific cross-reaction. During characterization of the MdW-7(1)G1 antibody this proved to be the whitefly Trialeurodes vaporariorum (Westwood) (Symondson et al. 1999b). Subsequent screening of potential prey from the field has, to date, revealed no other species that gives a stronger reaction (JD Harwood, unpublished data). Based upon this calculation, a beetle gut sample would only be considered significant if it gave an aphid protein equivalent of 16.5 ng/200 mL at the x20 000 ELISA dilution. When this is used as the cross-reaction significance level, only two female and three male P. cupreus gave a positive reaction when killed immediately after feeding on at least two spiders that were killed immediately after feeding on aphids. None of the beetles killed 2 h after feeding on spiders that were killed immediately after feeding on aphids (Fig. 4a) would have been positive using this criterion.

Very few studies have compared the rates at which antigens decay in male and female predators. Symondson et al. (1999a), using a species-specific monoclonal antibody, reported that the detection period for male P. melanarius was ~30% longer than for females feeding on the slug Arion hortensis (Férussac). This contrasts with the results presented here which showed no evidence for differential antigen decay rates between male and female L. tenuis or P. cupreus. Given that male spiders were considerably smaller than the females it appears that both sexes consumed a similar proportion of aphid prey relative to their size, hence y-axis intercepts and antigen decay rates were very similar. In the field, however, males of most species tend to be more active than females (Sunderland 1987; Topping & Sunderland 1992; Harwood et al. 2001), which could affect the rates at which antigens decay. Laboratory experiments do not take such factors into account.

In conclusion, although it is conceivable that secondary predation might be detected within our particular aphid–spider–carabid system in the field, it is highly unlikely to be a significant factor. In most other studies using antibody–antigen systems, antigenic recognition decreases exponentially immediately after the predator has fed on the prey, making detection of prey after it has gone through two predators even less likely. The antigen half-lives and detection periods recorded in these experiments were considerably longer than those reported in any previous study using a monoclonal antibody. Although these results cannot be directly extrapolated to other predator food webs, or the use of alternative molecular detection systems (e.g. PCR), they do provide strong evidence that secondary predation will not be a major source of error in field studies using the MdW-7(1)G1 antibody, despite the extended detection periods that it provides. A situation in which secondary predation might, possibly, cause a measurable food chain error is where a predator species is known to be feeding intensively on another predator species which is, in turn, feeding predominantly on the target prey.
example would be heteropteran predators consuming the lacewing larvae Chrysoperla carnea (Stephens), that were controlling the aphid *Aphis gossypii* Glover on cotton (Rosenheim et al. 1993). Also, in systems operating at temperatures significantly below the 16 °C used here (Lister et al. 1987), rates of antigen inactivation are slower (temperature-dependent in poikilothersms) thus potentially extending detection periods and the opportunity to detect secondary predation. If detection of secondary predation is found within any food chain it could be eliminated from analyses either by using antibodies targeted at more labile epitopes or by statistically raising the threshold for significance following ELISA. The former approach would result in extra costs, the latter in a loss of information. It is encouraging therefore that our results suggest that such measures are unlikely to be necessary.

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**References**


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