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Ph.D. Thesis

Facultative scavenging on invertebrate cadavers

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Annotation:

The Ph.D. thesis enclosed focuses on various implications of scavenging by generalist predators on invertebrate cadavers. Comparison of the retention time for invertebrate cadavers in the field, with the detection period for decaying slug material in the guts of the predators is presented and indicates that PCR-based techniques are not able to distinguish between predated and scavenged food items. Disappearance rates for invertebrate cadavers in the field, together with generealist predator preference for dead prey were estimated and their implication on survival strategies of entomopathogenic and molluscicidal nematodes in the cadavers is discussed. Two different strategies were investigated and are presentd in the thesis.

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Declaration of author:

I hereby declare that I worked out this Ph.D. thesis on my own, or in collaboration with the co-authors of the presented papers and manuscripts, and only using the literature cited.

I further declare that in accordance with § 47b of Act No. 111/1998 Coll. in its valid version, I agrese with the publication of my Ph.D. thesis (in an edition made by removing marked parts archived by the Faculty of Science) in an electronic way in a publicly accessible part of the STAG database, operated by the University of South Bohemia in České Budějovice on its web pages.

In České Budějovice, December 7th 2009 ....

Pavel Foltan

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Content

Introduction……………………………………………………………………………...1

References……………………………………………………………………………..5

Paper 1…………………………………………………………………………………...8

Foltan P, Sheppard S, Konvicka M, Symondson WOC (2005) The significance of facultative scavenging in generalist predator nutrition: detecting decayed prey in the guts of predators using PCR. *Molecular Ecology*, **14**: 4147–4158.

Paper 2…………………………………………………………………………………20

Pechova H, **Foltan P** (2008) The parasitic nematode *Phasmarhabditis hermaphrodita* defends its slug host from being predated or scavenged by manipulating host spatial behaviour. *Behavioural Processes*, **78**: 416-420.

Paper 3…………………………………………………………………………………25

Foltan P, Puza V (2008) To complete their life cycle, pathogenic nematode–bacteria complexes deter scavengers from feeding on their host cadaver**.** *Behavioural Processes*, **80**: 76-79.

Paper 4…………………………………………………………………………………29

Foltan P, Konvicka M (2008) A new method for marking slugs by **UV**-fluorescent dye. *Journal of Molluscan Studies*, **74**: 293-297.

Paper 5…………………………………………………………………………………34

Foltan P (2004) Influence of slug defence mechanisms on the prey preferences of the carabid predator *Pterostichus melanarius* (Coleoptera : Carabidae). *European Journal of Entomology*, **101**: 359-364.

Introduction

Facultative scavenging is one of the most common foraging strategies of both vertebrates (Putman 1983, DeVault et al. 2003; Hunter et al. 2007) and invertebrates (Sunderland and Sutton 1980, Fellers & Fellers 1982, Calder *et al 2005*). Many animals die from causes other than predation including parasitism, disease, dehydration, starvation, environmental conditions or partial consumption by a predator. These processes provide a substantial amount of dead animal biomass for potential scavengers (Putman 1983; Winder et al . 1994; Sunderland 1996; De Vault et al . 2003). Considering that invertebrates comprise much of the global terrestrial animal biomass (Williams 1960, Cornaby 1973; Seastedt et al. 1981; Fellers & Fellers 1982), the importance of invertebrate carrion as a resource for scavenging predators has received surprisingly little attention.

The knowledge of insect scavengers is limited almost entirely to studies carried in ant-dominated communities as a by- product of ant foraging strategy research (Seastedt et al. 1981; Fellers & Fellers 1982; Retana et al. 1991; Bestelmeyer & Wiens 2003), unsurprisingly identyfying ants as the dominant scavengers.

As far as I know, there are no estimates of available dead insect bimass, although its production is huge and thus carrion scavenging can play a key role in nutrition flow and predator-prey interactions in many of the terrestrial communities.

For example, average perennial colony of a honey bee *Apis mellifera* consists of about 14,000 individuals (Free and Racey 1968). Given a mean of 116 mg/worker (Schmolz et al. 2005), a single colony produces more than 1.6 kg of dead insect biomass p.a. (the bees are rarely predated). Considering over 2.3 million managed colonies in the United States alone (USDA 2009), dead individuals of this and other insect species may represent an important and underappreciated food source for scavenging predators. As dead prey does not posses any active defence mechanisms, low risk and null energy expenditure is required in prey capture, similar aggregation and constant production of dead insect individuals may magnify top-down effects of predation as scavenging predators subsidize their diets with carrion (Polis and Strong 1996; Rand et al. 2006).

The importance of defence mechanisms for prey selection is presented in the enclosed Foltan, (2004, paper 5). In a series of two-choice experiments on prey preferences of a generalist carabid predator *Pterostichus melanarius*, and five species of slug prey, conducted under controlled laboratory conditions, significantly different preferences for individual slug species were found. The influence of hunger level, temperature, day/light period, condition of slugs

and beetles, weight of slugs and beetles, and the sex of beetles were controlled. The above parameters had no effect on the prey selection, when the slug weight had an effect only within the species. The significant prey preferences were interpreted as the outcome of differing slug species-specific defence mechanisms. In a latter experiment (unpublished data), the predatory beetles easily fed on all slug species when offered dead and there was no preference for the different cadavers.

Direct observation of predatory events poses severe logistical challenges (Miller et al. 2006; Retana et al. 1991). When the study involves small or highly mobile species, *in situ* predation assessment becomes nearly impossible (Hagler et al. 2004). The time required to capture even a few direct observations of predation, which typically involve multiple species of predators (Heimpel et al. 1997; Rosenheim et al. 1999), makes obtaining sufficient sample sizes infeasible. For these reasons above, different post-mortem gut-contents analyses, involving a range of biochemical and molecular techniques (reviewed in Symondson 2002a,b and King et al. 2009), are used as valuable tools for determining the frequencies or strengths of trophic interactions, or for establishing new trophic links. However, predation is not the only route by which animal DNA enters the gut of a predator. If the other options occur (scavenging, secondary predation) , assuming predation can lead to overestimation of the strength of trophic interactions, identification of false trophic links, and consequently an underestimation of scavenging/secondary predation as a significant ecological process. The effect of secondary predation (a consequence of a predator consuming a second predator, shortly after the latter has consumed the target prey) was found to be negligible in an aphid–spider–carabid system using a highly sensitive monoclonal antibody-based system (Harwood *et al*. 2001) and was suggested to be ignored. However, in a PCR-based study, secondary predation was found to be a significant potential source of error (Sheppard *et al*. 2005). Secondary carabid predator (*Pterostichus melanarius*) was found positive for aphid DNA 4 h after eating the primary spider predator (*Tenuiphantes tenuis*), that itself had been digesting its aphid prey (*S. avenae*) for 4 h.

In the enclosed Foltan et al (2005, paper 1), we have examined scavenging as a potential source of error in the currently dominating PCR-based diagnostic systems. We used PCR to amplify DNA of the cadavers from scavenged material in predator gut homogenates. The rates at which DNA in decaying slugs and aphids became undetectable were estimated. The detectability of DNA from both carrion types in the guts of the generalist carabid predator *P. melanarius* was then determined. The effects of carrion age and weight, as well as beetle sex, on detection periods, were quantified. The period of carrion decomposition on soil and

digestion time within *P. melanarius* significantly decreased amplification success for both slug and aphid DNA. Both variables had independent additive effects on decreasing the detectability, when digestion times exhibited much steeper effect than the time of decay. In the laboratory trials we measured prey preference of beetles between live and decaying prey. Further experiments measured feeding by *P. melanarius* on dead slugs and aphids directly in the field at a farm near Cardiff, UK. In both field and laboratory trials, *P. melanarius* preferentially fed on dead prey if available, but preference changed if the prey became increasingly decayed (for more than 150h). Surprisingly fast disappearance rates (mean and median time periods that the dead slugs remained in place were 11.1 and 6 h) for slug carrion in wheat fields and grasslands were estimated and *P. melanarius* was identified as dominating scavenger (representing > 80 % of predators trapped). Comparison of the retention time for dead slugs in the field, with the detection period for decaying slug material in the guts of the predators, showed that PCR-based techniques are not able to distinguish between predated and scavenged food items.

The extremely fast removal rates of invertebrate cadavers by scavengers may represent a severe threat to commercialy important pathogenic parasites which have to complete their life cycle inside the pest-host-cadaver. In the enclosed studies Pechova & Foltan (2008, paper 2) and Foltan & Puza (2008, paper 3), we deal with different survival strategies of of molluscicidal rhabditid (*Phasmarhabditis Hermaphrodita)* and entomopathogenic steinernematid (*Steinernema affine)* nematodes. Both nematodes adopted a similar life cycle: infective stages carry symbiotic bacteria, which kill their host. Nematodes subsequently finish their life cycle feeding on the proliferating symbiont and the host tissue, which takes typically 7–15 days for *S. affine* (Poinar, 1979; Adams and Nguyen, 2002) and between 7 and 21 days for *P. hermaphrodita*, under ideal environmental conditions. As is evident from the above, the critical factor for successful reproduction of both pesticidal nematodes is that the infected host and the subsequent cadaver remains intact while nematodes complete their life cycle.

In the enclosed Pechova and Foltan (2008) we suggest that *P. hermaphrodita* manipulates the spatial behaviour of its slug host, *Deroceras reticulatum*, preventing the host from being predated or scavenged.We exposed the slugs to the nematodes and four other pathogenic agents. Slugs infected by the nematodes were transported into nematode-free containers, where they died from the infection. The infected slugs would be expected to die on the surface as they were not habituated in the containers and had less time, compared to other treatments, to create shelters in the soil (Hommay et al., 1998; Grewal et al., 2001). However,

the slugs exhibited significant tendency to die in the soil, out of the reach of surface dwelling scavengers, compared to slugs killed by other pathogenic agents.

In the enclosed Foltan and Puza (2008) two-choice trials were used to assess prey choice of *P. melanarius* between *D. reticulatum* slugs or wax moth *Galleria mellonella* larvae killed by infection of *P. hermaphrodita*/*S. affine* and control slugs/larvae killed by freezing. We demonstrate that the presence of either of the two nematodes tested deters the beetles from consuming infected cadavers. As *P. hermaprodita* cannot infect an insect host, we assume the deterrent effect being an evolutionary adaptation of the nematode/bacteria complex rather than the ability of the beetles to avoid potentially infective cadavers.

To enable identification decayed slug cadavers in the soil in Pechova and Foltan (2008) a new method of UV-fluorescent slug marking was used. The method is presented in the enclosed Foltan and Konvicka (2008, paper 4). In this study we demonstrated that the new fast, cheap, harmless and stabile marking by biologically inert UV-fluorescent dye is suitable for mark-release-recapture studies of slug population and spatial dynamics.

General human aversion to decaying substances and difficulties in identification of scavenged material in the guts of predators have resulted into general underestimation of scavenging either as an important ecological process or more specifically as a potentials source of error in the gut content analysis methods.

I suggest that that this understudied topic warrants increased experimental analysis and attention. More empirical work examining availability and exploitation of dead prey by scavenging predators, together with further investigation of prevalence and significance of scavenging in individual species diets, will permit better understanding of food links and nutrition flow in terrestrial habitats under study.

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The significance of facultative scavenging in generalist predator nutrition: detecting decayed prey in the guts of predators using PCR

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Abstract

Gut-content analyses using molecular techniques are an effective approach to quantifying predator–prey interactions. Predation is often assumed but scavenging is an equally likely route by which animal DNA enters the gut of a predator/scavenger. We used PCR (polymerase chain reaction) to detect scavenged material in predator gut homogenates. The rates at which DNA in decaying slugs (Mollusca: Pulmonata) and aphids (Homoptera: Aphididae) became undetectable were estimated. The detectability of DNA from both carrion types in the guts of the generalist predator *Pterostichus melanarius* (Coleoptera: Carabidae) was then determined. The effects of carrion age and weight, as well as beetle sex, on detection periods, were quantified. Laboratory trials measured prey preference of beetles between live and decaying prey. Further experiments measured, for the first time, feeding by *P. melanarius* on dead slugs and aphids directly in the field. In both field and laboratory, *P. melanarius* preferentially fed on dead prey if available, but preference changed as the prey became increasingly decayed. Disappearance rates for slug carrion in wheat fields and grasslands were estimated and *P. melanarius* was identified as the main scavenger. Comparison of the retention time for dead slugs in the field, with the detection period for decaying slug material in the guts of the predators, showed that PCR-based techniques are not able to distinguish between predated and scavenged food items. This could potentially lead to overestimation of the impact of predation on slugs (and other prey) by carabids. Possible implications of facultative scavenging by invertebrate predators for biocontrol and food-web research are discussed.

Keywords: aphid, DNA degradation, gut content, *Pterostichus melanarius*, scavenging, slug

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Introduction

Decomposition of dead organic material is a key process in any ecological system. The decomposers are responsible for more than 95% of the total community metabolism in terms of the overall contribution to the total energetic operation of a terrestrial community (Putman 1983). In the majority of studies that deal with this topic, decomposition of plant material receives considerably more attention than that of animal products. Exceptions include the effects of salmon spawning on nutrient intake by coastal ecosystems (Schindler *et al*. 2003) and fluxes of nutrients associated with hatching of periodical cicadas (Whiles *et al*. 2001). In leaf litter systems true decomposers (Putman 1983) process more than 95% of available material, and only a minute amount is used by scavengers (Putman 1983; Gartner & Cardon 2004). In the degradation of animal material, these proportions are completely different. Scavengers remove between 50% and 100% of animal carcases in the majority of terrestrial ecosystems (Akopyan 1953; Mullen & Pitelka 1972; Seastedt *et al*. 1981; Fellers & Fellers 1982; Putman 1983; Young 1984a; Retana *et al*. 1991; De Vault & Rhodes 2002; Bestelmeyer & Wiens 2003; De Vault *et al*. 2003). Many

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animals die from causes other than predation including parasitism, disease, dehydration, starvation, or partial consumption by a predator. These processes provide a substantial amount of dead animal biomass for potential scavengers (Putman 1983; Winder *et al*. 1994; Sunderland 1996; De Vault *et al*. 2003). Carrion represents an easily available (Lang & Gsödl 2001) and valuable source of energy and nutrition, and carrion scavenging is an important component of energy flow in terrestrial biotopes. As dead prey do not posses any active defence mechanisms, low risk and null energy expenditure in prey capture means that many invertebrate and vertebrate predators regularly scavenge. Difficulties in identification of scavenged material have resulted in a lack of information concerning scavenging behaviour. Consequently, the phenomenon of scavenging is often viewed as a behavioural curiosity rather than an important ecological process (De Vault *et al*. 2003).

Scavenging on vertebrate carcases has been studied more than that on invertebrate corpses, despite the greater amount of dead biomass produced by invertebrates (Cornaby 1973; Seastedt *et al*. 1981; Fellers & Fellers 1982). Carrion decomposition rates, and species composition of vertebrate carrion-feeding scavengers, are well known (Payne 1965; Payne & King 1968; Putman 1983; Kostecke *et al*. 2001). Studies in which scavenging on invertebrate carcases is considered deal mainly with the ability of various predators to feed on dead prey, or with laboratory-based choice (live or dead) experiments (Wheeler 1971, 1974, 1977; Tod 1973; Wolf 1986; Sunderland 1987; Lang & Gsödl 2001). Very few studies consider dead prey as a resource or examine the exploitation of invertebrate carrion in the field (Seastedt *et al*. 1981; Fellers & Fellers 1982; Young 1984a; Retana *et al*. 1991; Obrycki 1992; Bestelmeyer & Wiens 2003). Studies of disappearance rates of invertebrate carrion and identification of scavengers in field are limited almost entirely to ant-dominated biotopes using arthropod carcases as bait (Seastedt *et al*. 1981; Fellers & Fellers 1982; Young 1984a; Retana *et al*. 1991; Bestelmeyer & Wiens 2003). As a consequence (unsurprisingly) ants have been identified as dominant scavengers. Detailed studies of disappearance rates of arthropod carcasses and carrion use by other invertebrates in the field are necessary for a better understanding of trophic interactions in invertebrate ecosystems. Various invertebrate predators (Diptera, Heteroptera, Dermaptera, Formicidae, Carabidae, Coccinellidae, Miridae, Chrysopidae, Anthocoridae) have been observed feeding on dead prey including slugs, aphids, flies, weevils, caterpillars and wide a range of other invertebrates (Smith 1966; Wheeler 1971, 1974, 1977; Young 1984a, b; Sunderland *et al*. 1987; Powell *et al*. 1987; Greenstone & Morgan 1989; Barnes 1990; Symondson & Liddell 1993; Sunderland 1996). Some predators have been shown to prefer dead to live prey (Sunderland & Sutton 1980; Langan *et al*. 2001; Mair & Port 2001).

Post-mortem gut-contents analyses, using a range of biochemical and molecular techniques (reviewed in Symondson 2002a), are valuable tools for determining the frequencies or strengths of trophic interactions, or for establishing new trophic links (Sunderland 1987, 1996; Symondson & Liddell 1996b; Symondson 2002a, b). However, such analyses do not discriminate between prey that was predated or scavenged (Sunderland 1996; Symondson 2002a; De Vault *et al*. 2003). The effect of secondary predation was found to be negligible in an aphid–spider–carabid system using a highly sensitive monoclonal antibody-based system (Harwood *et al*. 2001) and can probably be ignored. Prey is usually assumed to be scavenged only in cases when it is too big to be subdued by the predator or when it is found in a herbivore. The inability to distinguish between predation and scavenging could lead to overestimation of the strength of trophic interactions, identification of false trophic links, and consequently an underestimation of scavenging as a significant ecological process.

Two recent studies have examined scavenging in the laboratory as a potential source of error in molecular diagnostic systems. Calder *et al*. (2005) used a slug species-specific monoclonal antibody, developed by Symondson & Liddell (1996a), to measure the decay of dead slugs on soil and peat. On soil it took 8.2 days of decay for the concentration of the slug protein detected by this antibody (using enzyme-linked immunosorbent assays: ELISA) to be reduced by 50%. On relatively sterile peat this point was reached after 11.5 days. Carabid predators, *Pterostichus melanarius* Illiger, were then fed on slug that had decayed on soil for 100 h. After 6 h of digestion the slug proteins were clearly detectable using ELISA. These laboratory results suggest that if scavenging were occurring in the field, many 'positives' recorded using this antibody detection system could be caused by consumption of dead prey and not predation. A second laboratory study, by Juen & Traugott (2005), showed that PCR-based (polymerase chain reaction-based) detection systems are potentially equally vulnerable to this source of error. The 'prey' were white grubs (Coleoptera: Scarabaeidae), which were allowed to decay for 9 days. DNA was amplified from decaying grubs and also from the guts of beetles fed on decayed grubs. Perhaps because of the extreme sensitivity of PCR, and the fact that, unlike ELISA, only positive/negative results were obtained over this time period, there was no significant difference in detectability between fresh and decayed grubs, irrespective of age, whether in the guts of the carabid predators or on the soil.

A critical factor, which has not been addressed to date, is how readily available dead invertebrates may be in the field and, more specifically, how rapidly they are removed after death and by which predators. We quantified the attractivity of dead slugs and aphids for a carabid predator. In a separate experiment, we assessed removal rates of

dead slugs in the field. Carabids such as *P. melanarius* are known to feed on aphids and may be capable of affecting their spatial and temporal dynamics (Winder *et al*. 2001, 2005). In the UK, and probably elsewhere in Europe and North America, *P. melanarius* are the main invertebrate predators of slugs in arable crops, capable of affecting their temporal and spatial dynamics (Symondson *et al*. 1996; Bohan *et al*. 2000; Buckland & Grime 2000; Symondson 2002b; Thomas 2002; McKemey *et al*. 2003; Paill 2004). We also tested, for the first time, the hypothesis that decay rates for invertebrate carrion on soil are prey specific. This was carried out by comparing the loss of our ability to detect slug vs. aphid DNA over time. As in the study by Juen & Traugott (2005), the aim was to calibrate the ability to detect semidecayed slug DNA in the guts of the beetles using PCR. Uniquely, however, we wished to obtain a more meaningful and accurate measure of the potential significance of scavenging by relating PCR data to both prey choices by the beetles (between fresh and decayed slug remains) and the mean residence time of decaying slugs in the field.

Materials and methods

Deroceras reticulatum slugs and *Pterostichus melanarius* beetles were collected from arable fields around Cardiff, UK. The weight of each slug was recorded immediately before killing by freezing at –80 °C. The beetles were housed individually in plastic containers (8.5 cm diameter, 4.5 cm deep) with 80 g moist sphagnum peat and maintained in a controlled environment (L:D 16:8; 16 ± 2 °C). The beetles were fed on a diet of *Calliphora vomitoria* (L.) larvae. Prior to the experiment, the beetles were fed and then starved for 7 days, to ensure the same nutritional state in all individuals. *Sitobion avenae* (F.) aphids were reared in multiclonal cultures on wheat plants and killed by freezing at –80 °C. Only adult aphids were used. Characterized well-drained sandy loam topsoil for laboratory experiments was obtained from Long Ashton Research Station, Long Ashton, Somerset, UK. Field experiments were carried out in two wheat fields (Wheat1, 2.7 ha and Wheat2, 3.5 ha) and two pastures (Pasture1, 5.4 ha and Pasture2, 2.2 ha) at Burdens farm, Wenvoe, near Cardiff.

Decay rate of DNA in slug and aphid carrion

Eighty grams of topsoil was added to 260 clear, uncovered plastic containers (as described above). Freshly killed slugs or aphids were placed on the substrate (one per container) and decayed for 0, 1, 3, 6, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240 and 264 h in a controlled environment room (L:D 16:8; 16 ± 2 °C). The samples were then frozen in batches of eight slugs and aphids for each time period. Eight control containers were kept in the same conditions as above. The weight of the control containers was recorded every 24 h during the experiment. Mean weight reduction in the control containers was replaced by water addition both in experimental and control containers. This ensured preservation of the same moisture level (~30%) in each experimental container during the experiment.

Slugs from this experiment were homogenized prior to DNA extraction in 350 µL of distilled H_2O and centrifuged for 10 min at 16 000 *g*. Twenty-five microlitres of the supernatant was used for the DNA extraction and further PCR amplification. This both enabled better separation of the DNA from the soil and slime and, by reducing the quantity of DNA, improved amplifications. Whole aphids were used for the DNA extraction.

Detectability of slug and aphid remains in the guts of carabids

Batches of 30 slugs and aphids were frozen after 0, 3, 6, 12, 24, 48, 72 and 120 h of decomposition on the soil under the same conditions as described above. Decayed material was offered to the starved *P. melanarius* beetles, which were observed to feed over a 20-min period. Any nonfeeding beetles were discarded. Five beetles were killed by freezing at –80 °C for each decomposition period for slugs and aphids at 0, 1, 3, 6, 12, 24 and 48 h after the feeding period. Beetle guts were dissected, their gut contents removed and DNA was extracted and used for PCR.

DNA extraction and PCR

QIAamp® DNA Stool Mini Kits (QIAGEN GMBH) were used to extract slug or aphid DNA from the decaying invertebrates and from beetle gut samples. The DNA was resuspended in 200 µL 1 mM TE buffer and stored at –20 °C.

General aphid primers were used to amplify aphid DNA from the cytochrome oxidase I (*COI*) mitochondrial gene. Primers Aph14F (5′-AATCAAAATAAATGTTGATA-3′) and Aph236R (5′-TCAATTTTAGGRGCAATAA-3′) amplified a 245-bp fragment (Harper *et al*. 2005). Amplification was performed in 25-µL reaction volumes containing 4 µL of the sample, 0.625 unit of *Taq* polymerase (Promega) and final concentrations of 0.5 µM dNTPs (Boehringer Manheim), 3 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl (pH 8.0) and 0.15 µM of each of the primers. PCR was carried out in a Hybaid thermal cycler (Hybaid). Cycling parameters were as follows: 95 °C for 4 min, then 35 cycles of 94 °C for 45 s, 50 °C for 45 s, and 72 for 1min and 15 s, with a final extension step at 72 °C for 10 min, after which the machine cooled to 4 °C. PCR products were visualized by ultraviolet transillumination following electrophoresis at 110 V (for 40 min) on an ethidium bromide-stained 1.5% agarose gel.

Deroceras reticulatum species-specific primers were used to amplify slug DNA targeting a 109-bp fragment of the

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12S rDNA gene (Harper *et al*. 2005). Primer sequences were DR11F (5′-CTATACACAATTTTTAAATAAGC-3′) and DRF29RC (5′-GTCTCTGGTTTATCTATTATTGGT-3′). Amplification was performed in 25-μL reaction volume containing 4 μL of the sample DNA, 0.625 unit of *Taq* polymerase (Promega) and final concentrations of 0.5 μM dNTPs (Boehringer Manheim), 3 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl (pH 8.0) and 0.25 μM of each of the primers. PCR was carried out in a Hybaid thermal cycler (Hybaid). Cycling parameters were as follows: 95 °C for 3 min, then 35 cycles of 94 °C for 45 s, 46 °C for 1 min, and 72 for 1 min and 15 s, with a final extension step at 72 °C for 10 min, after which the machine cooled to 4 °C. The products were electrophoresed at 100 V in a 1.5% agarose gel for approximately 50 min.

Appropriate precautions were taken against contamination and negative and positive controls were included in all PCRs.

Disappearance rate of slug carcasses in the field

Fifteen *D. reticulatum* carrion were placed directly on the ground in the middle of each of the four experimental fields. Within each field each of the 15 slugs was placed 10 m apart, in a grid. Presence, absence, or partial consumption of the slugs was checked every 3 h over a 24-h period. The experiment was repeated after 12 h. Scavengers attracted by the slugs were identified *in situ*.

Pterostichus melanarius *response to slug and aphid carrion in the field*

In parallel with the previous experiment, 10 slug-baited, 10 aphid-baited and 10 unbaited control pitfall traps, 6.5 cm diameter, were inserted, 10 m apart, in each of the four above-mentioned fields (120 traps in total). The order of trap types was randomized in rows (each row of three traps contained one slug-baited, one aphid-baited and one unbaited trap). A plastic tube (1.5 cm in diameter and of the same height as the trap) was filled with soil from each of the experimental sites and placed in the centre of each of the traps. Bait of one slug or 2–3 aphids was placed on the top of the soil in the tubes. This ensured natural decay of the carrion at ground level on soil but protected the carrion from consumption by ground predators (Fig. 1). The number of *P. melanarius* beetles (by far the dominant species in all of the experimental sites) in the traps was recorded and traps were emptied every 3 h for 36 h. Frequent emptying minimized the possibility that the beetles would be attracted to other (live) invertebrates that had fallen into the traps.

Choice experiments with decayed and live prey

Batches of eight freshly killed slugs and aphids were decayed for 0, 1, 3, 6, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240 and 264 h under same conditions as in previous experiments, and frozen. Two hundred and fifty-six Petri dishes (9 cm diameter) were lined with moist filter paper. A single dead slug (< 80 mg) or aphid was placed in each Petri dish, together with a live slug or aphid. Single *P. melanarius* beetles (equal sex proportions) were placed in each of the dishes and observed continuously for 1.5 h. First attack by the beetle on the live prey or the carrion and feeding on the live prey or the carrion were recorded as separate parameters. An attack was recorded when the beetle bit the prey. Feeding was recorded when it occurred continuously for more than 2 min on one prey or when the whole prey item was consumed. Any nonfeeding beetles were discarded and the experiment was repeated with new beetles, until the final number of eight trials was reached. All attacked aphids were consumed, thus only consumption was measured for aphids.

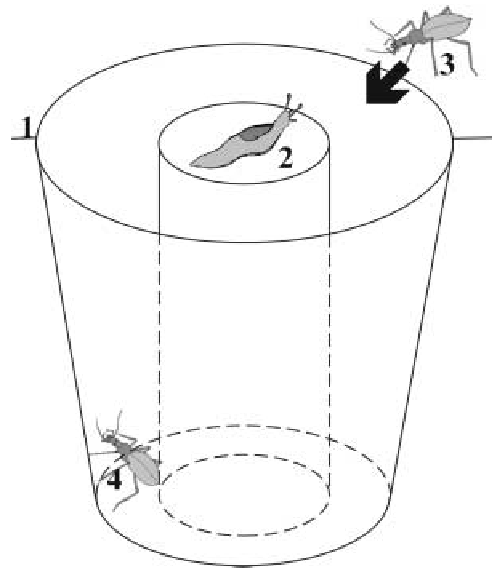

Fig. 1 Pitfall trap (6.5 cm diameter, 10 cm depth) used for assessing *Pterostichus melanarius* response to slug and aphid carrion. (1) Rim of the trap at soil level; (2) dead slug, aphids (n = 2–3) or nothing (in case of control traps) on top of a glass tube (1.5 cm diameter) filled with soil; (3) carabid approaching trap at the same level as prey; (4) captured carabid in the trap.

Analysis

Decay rate of DNA in slug and aphid carrion. To assess the effect of slug/aphid weight, and period of carrion decomposition, on the probability of detection by PCR, we used multiple regressions with detection (+/−) as the binary response variable (logit link), and time of exposure and slug weight as numeric independent variables. We fitted the regressions using generalized linear modelling (GLM) procedures in S-PLUS 2000 (1999), using the quasi-Akaike information criterion (qAIC) for selecting the most parsimonious models, and F values for comparing fitted models with null model.

Detectability of slug and aphid carrion in the guts of predators. As above, multiple GLM regressions with binary response variable (detected vs. undetected) were used to analyse the data. Independent variables were period of carrion decomposition, time until the beetle was killed (digestion time), sex of the beetle, weight (for slugs only). After fitting single-term regressions, we used both forward term addition and backward term elimination to construct multiple-regression models. Single terms, as well as their interactions, were considered while building the models. Model parsimony was assessed using qAIC.

Disappearance rate of slug carrion in the field. The appropriate approach to analyse the results of this experiment were those used for survival analysis, because the data were censored, i.e. some slugs did not disappear after the entire duration of the observation. To estimate the probability of retention of the slugs over time, we used the Kaplan–Meier estimator, which redefines the estimates beyond the last observations to be zero (S-PLUS 2000, 1999). Since there were two repetitions of the experiment, we computed tree estimates, one for each repetition and one for pooled data. Subsequently, we compared slopes of the survival curves for individual fields, and the two separate experiments, using the chi-squared based method of Fleming & Harrington (1981).

To further explore the factors affecting the disappearance rate of the carrion, we used Cox's proportional hazard regressions, which test the relative contribution of factors to the slope of survival curves. Following single-term regressions, we computed multiple regressions for each repetition of the experiment and for the pooled data, based on selection from the factors carrion weight, crop type (wheat or pasture), experiment repetition (1 or 2), and field (the four individual fields). Since the individual fields were significant predictors in all multiple regressions, we finally defined a stratified model that estimated mean and median survival for each field separately. All analyses were computed using S-PLUS 2000.

Pterostichus melanarius *response to slug and aphid carrion in the field.* To analyse the experiment, we used analysis of covariance with independent variables Crop (wheat or pasture) and Bait (slug, aphid, control), and covariable time (linear term). The effect of time was assessed using within-cell regression. Post hoc comparison, via least significant difference (LSD) test, was used to compare the three types of Bait.

Choices between decayed and live prey by Pterostichus melanarius. The experimental design had two factors: sex of the beetle and dead or living prey, and the additional linear component of time. We again used GLM regressions, where the dependent variables were counts of attacks or observed feeding on prey items in these categories. As response variables were normally distributed (Kolmogorov–Smirnov tests, aphids $d = 0.04$, slugs attacked $d = 0.05$, slugs consumed $d = 0.06$, all $P > 0.1$), we used identity link. Because there was no reason to test for differences in beetle sex and time (equal counts of prey items were attacked and consumed each time and by both beetle sexes), we only searched for differences in preferences between dead and living prey, and for interactions with the other two variables. Again, best model was selected on the basis of qAIC.

Results

Decay rate of DNA in dead slugs and aphids

In both slugs and aphids, the probability of detection via PCR significantly decreased with time of carrion decomposition (null vs. fitted model, slugs: $F_{1,126} = 70.86$, $P < 0.0001$; aphids, $F_{1,126} = 184.40$, $P < 0.0001$). In slugs, for which the data were available, the probability did not depend on weight ($F_{1,126} = 0.38$, $P = 0.54$), and neither adding slug weight to the time model nor fitting interaction among the terms improved the deviance explained by the time model. Therefore, time of decay alone suffices to account for probability of detection.

The fitted regressions (Fig. 2) suggest that the decrease of detectability was steeper in aphids. To test whether they indeed differ, we pooled data from both organisms and constructed common regressions containing the additive terms time and organism (it was highly significant when compared with null model: $F_{3,252} = 114.6$, $P < 0.0001$). We then compared it with a model containing the interaction term 'time*organism'. The latter model achieved a lower qAIC value (167.5 vs. 169.2) and differed significantly from the regression without interaction ($F_{1,252} = 4.49$, $P < 0.05$). Therefore, the decline of detectability was indeed steeper in aphids.

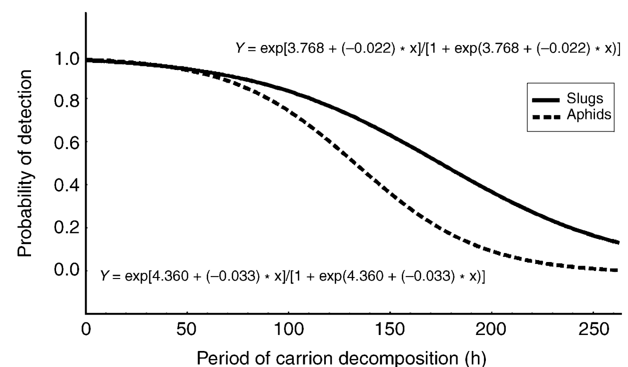

Fig. 2 Fitted relationship between detectability of the DNA and period of decomposition for dead slugs ($\chi^2 = 60.03$, d.f. = 1, $P < 0.001$) and aphids ($\chi^2 = 99.6$, d.f. = 1, $P < 0.0001$) on soil.

Term	Dir.	d.f.	Deviance*	qAIC	F	P
Aphid						
Null		269		293.0		
Beetle sex		1, 268	< 0.01	294.9	0.1	0.77
Decay period	–	1, 268	13.0	257.6	34.9	< 0.0001
Digestion time	–	1, 268	15.4	202.7	137.3	< 0.0001
Slug						
Null		269		376.3		
Beetle sex		1, 268	< 0.01	377.5	0.7	0.40
Decay period	–	1, 268	8.4	346.7	31.2	< 0.0001
Digestion time	–	1, 268	36.9	239.6	162.0	< 0.0001
Slug weight	+	1, 268	1.7	371.9	6.4	< 0.05

Dir., direction of the relationship.
*Percentage of deviance explained by fitted model.

Table 1 Single-term regressions of factors affecting PCR detectability of slug and aphid DNA in the gut of the predatory beetle *Pterostichus melanarius*. Fitted as generalized linear models with assumed binary distribution of response variable and logit link

Model terms (coefficients ± SD)	d.f.	Deviance*	F	P
Aphids				
−0.03 (± 0.007) period of decay				
−0.31 (± 0.056) digestion time	2, 267	49.1	113.2	< 0.0001
Slugs				
−0.02 (± 0.004) period of decay				
−0.20 (± 0.026) digestion time	2, 267	51.6	133.1	< 0.0001

*Percentage of deviance explained by fitted model.

Table 2 Multiple regression models (GLM with assumed binary response variable and logit link) for PCR detectability of slug and aphid DNA in the gut of the predatory beetle *Pterostichus melanarius*

Detection of slug and aphid remains in predator guts

The period of carrion decomposition on soil and digestion time within *Pterostichus melanarius* significantly decreased amplification success for both slug and aphid DNA. Slug weight also had a positive significant effect but beetle sex did not (Table 1).

The multiple regressions showed that, in both slugs and aphids, digestion time and the period of carrion decomposition had independent additive effects on decreasing the detectability. They were positively correlated (r = 0.29 and 0.46 in aphids and slugs, respectively), and digestion times exhibited steeper slopes than the effects of time of decay (Table 2 and Fig. 3a, b). Slug weight did not enter into the final models, perhaps because of autocorrelation with time of decay (Table 2).

Disappearance rate for dead slugs in the field

The estimates of mean and median time periods that the dead slugs remained in place were 10.5 and 6 h for first run of the experiment, 11.7 and 6 h for the second run, and 11.1 and 6 h for the pooled data. However, the slopes of the curves for individual fields differed in both experimental runs (first run χ^2 = 18.1, 3 d.f., P < 0.001; second run χ^2 = 20.9, 3 d.f., P < 0.001), and in the pooled data (χ^2 = 33.8, 3 d.f., P < 0.0001). In all three cases, the steepest slope was in Wheat1, followed by Wheat2, Pasture1 and Pasture2. However, if the data from both experiment runs were pooled together, the two runs did not differ (χ^2 = 1, 1 d.f., P = 0.32).

The Cox's proportional hazard regressions for pooled data showed that the time that a dead slug remained in place depended on slug weight [heavier slugs disappeared later: Wald statistic (W) = 21.3, 1 d.f., P < 0.001], crop type (faster disappearance in wheat: W = 25.7, 1 d.f., P < 0.001), as well as on individual sites (W = 54.7, 3 d.f., P < 0.001). The best-fitting stratified model contained the explanatory variable weight, as well as the four fields as strata (R^2 = 0.24, W = 29, 1 d.f., P < 0.0001). It pointed to large differences in disappearance rate between the fields (Fig. 4).

Response of Pterostichus melanarius *to dead slugs and aphids in the field*

Overall, *P. melanarius* represented more than 80% of identified scavengers recorded *in situ* in the field and was the only predator found in significant numbers (more than 90% of trapped insect individuals) in the pitfall traps. The four fields differed in total numbers of carabids captured

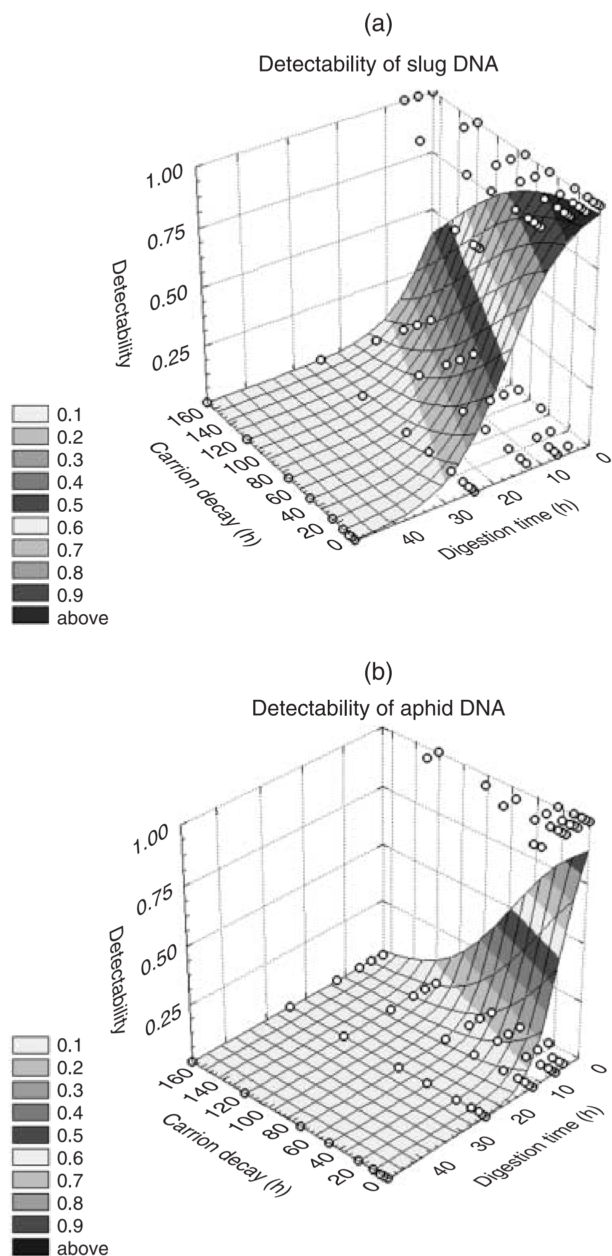

Fig. 3 Effects of period of carrion decay, and period of carrion digestion in the gut of the beetle *Pterostichus melanarius*, upon the detectability of carrion DNA. (a) Slug carrion decay and digestion; (b) aphid carrion decay and digestion.

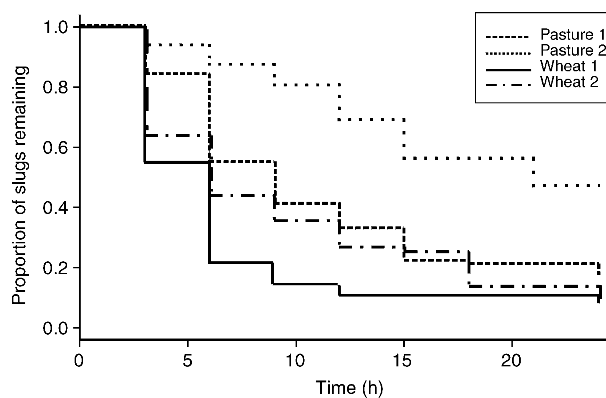

Fig. 4 Retention rates (proportions present for a given time period) of slug carrion in the four experimental fields.

Fig. 5 Total numbers of *Pterostichus melanarius* trapped in the slug-baited, aphid-baited and control traps in the four experimental fields. Duration of the trapping session was 36 h.

during the experiment (comparison with equal catches, $\chi^2 = 26.13$, 3 d.f., $P < 0.001$; Fig. 5).

In the ANCOVA, both crop type ($F_{1,137} = 39.13$, $P < 0.0001$) and bait (slugs or aphids) ($F_{2,137} = 6.95$, $P < 0.01$) significantly affected numbers of captured carabids, whereas neither their interaction nor the covariable time had significant effects. Traps with dead slug bait captured more carabids than either traps with aphids (LSD: $P < 0.01$) or empty controls ($P < 0.001$), whereas traps baited with aphids did not differ from controls ($P = 0.44$). More carabids were captured in wheat than in the pasture. It follows that (i) the carabids were attracted to dead slugs, (ii) the pattern of the attraction did not differ between crops and (iii) the attraction neither declined nor increased, during the time that the traps were exposed.

Choice experiments

No significant differences in preference for dead or living prey were found in carabids feeding on aphids. In slugs, no significant difference applied to first attacks but dead slugs were consumed significantly more often than living ones (Table 3). Furthermore, the interaction status (dead/live)*time was highly significant and performed as the best-fitting combination of explanatory variables for consuming aphids as well as for attacking and consuming slugs (Table 3). Time therefore systematically and nonlinearly influenced the preference. Dead prey was preferentially attacked/consumed in the early stages of decay (up to 96 h in aphids and 168 h in slugs). Later on the predators changed their preference to living prey (Fig. 6).

Table 3 Regression analysis of the two-choice experiment assessing selection of living versus dead slugs and aphids by the predatory beetle *Pterostichus melanarius*. The beetles were simultaneously offered living prey and prey decayed for a period of time. The best fitting multiple-regression models are in bold

		Attack				Consumption			
Term*	d.f.	Deviance†	qAIC	*F*	*P*	Deviance	qAIC	*F*	*P*
Aphid									
Null	63						71.2		
Status	1, 62					0.14	73.3	0.12	0.72
Status*sex	3, 60					0.18	78.0	0.05	0.99
Status*time	3, 60					31.6	42.4	16.87	< 0.0001
Status*sex*time	7, 64					32.3	47.2	7.04	< 0.0001
Slug									
Null	63		82.1				116.9		
Status	1, 62	1.66	83.1	1.24	0.27	30.8	87.8	30.82	< 0.0001
Status*sex	1, 62	1.8	88.7	0.43	0.73	31.3	93.4	7.14	< 0.001
Status*time	3, 60	49.2	56.2	11.48	< 0.0001	22.0	25.1	152.78	< 0.0001
Status*sex*time	7, 64	32.2	61.8	5.06	< 0.001	91.5	28.0	31.73	< 0.0001

*Status, status of prey, i.e. dead or living; sex, sex of the beetle; time, decay time of the prey (i.e. decayed for 0, 1, 3, 6, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240 and 264 h).
†Deviance fitted by individual models.

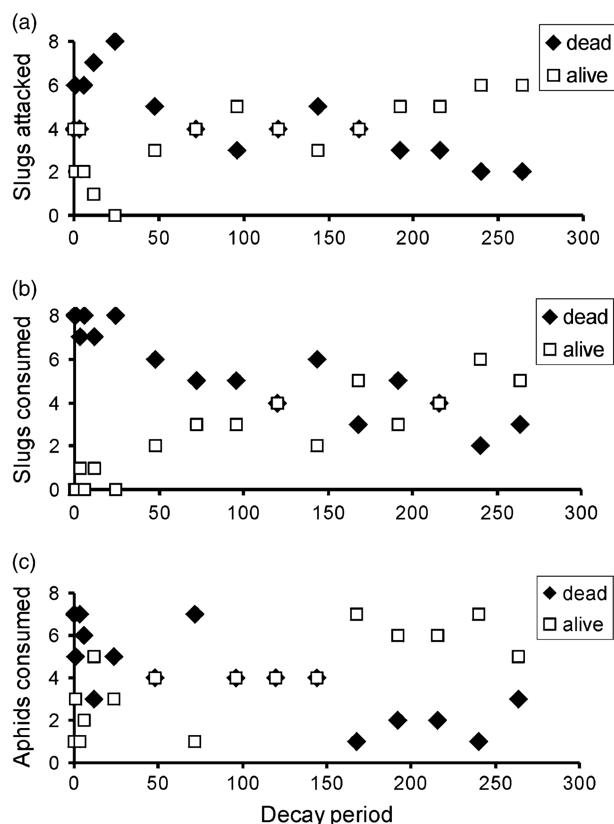

Fig. 6 Preference of the predatory beetle *Pterostichus melanarius* for dead vs. living prey in two-choice experiments. (a) First attacks on dead vs. living slugs; (b) consumption of dead vs. living slugs; and (c) consumption of dead vs. living aphids. Note the steeply increasing length of decay periods on *x*-axis.

Discussion

Analysis of the rates at which DNA in dead slugs and aphids (on soil) became incapable of amplification by PCR enabled us to characterize DNA degradation in decaying invertebrate corpses. These rates were then used to establish parameters for the design of the later experiments to detect both types of carrion in the guts of predators. In both slugs and aphids, the probability of detection via PCR significantly decreased with the duration of carrion decomposition. The decay rate of the DNA was slow in slugs but significantly faster in decaying aphids. Similar surprisingly slow decay rates in slug corpses were found by Calder *et al*. (2005), using monoclonal antibodies to detect slug antigens, and by Juen & Traugott (2005) who used PCR to measure the decay of DNA in *Melolontha melolontha* larvae (Coleoptera: Scarabaeidae). DNA from more than 50% of the dead invertebrates was amplifiable after decomposition for 174.5 h in slugs and for 133.5 h in aphids, respectively. Faster degradation in aphid corpses was possibly related to their smaller size. However, since in slugs the probability of detection did not depend on the animal weight, it is more likely that longer detection periods for slug DNA resulted from the use of primers that amplified a smaller DNA fragment (109 bp, compared with primers that amplified a 236-bp fragment from aphids). Other studies have shown that, where DNA has become degraded, for example in faecal analyses (Kohn *et al*. 1995; Farrell *et al*. 2000), gut-content analyses (Zaidi *et al*. 1999; Hoogendoorn & Heimpel 2001; Sheppard *et al*. 2004) or the decay of invertebrates on soil (Juen & Traugott 2005),

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shorter amplicons are more robust, and can be detected for a longer period, than longer amplicons.

Digestion rates of slug and aphid DNA in predator guts were again significantly faster in aphids than in slugs and were the strongest factor influencing detectability. The two factors (digestion time and the period of carrion decomposition) had independent additive effects, whereas their interaction was not significant. Surprisingly, in contrast to the preliminary experiment, slug weight also had a significant effect on detectability, with heavier slugs detectable longer. The mass of consumed slug tissue could possibly differ among different weight categories of slug carrion. The slug carrion used, however, were big enough so that the whole slug was not in any instance consumed and the slug weight did not enter into the final regression model.

In experiments to measure *Pterostichus melanarius* responses to dead slugs and aphids in the field it was clear that beetles were significantly more attracted to slug-baited traps than to control or aphid-baited traps in all fields. McKemey *et al*. (2004), using electroantennograms, found that antennal receptors of *P. melanarius* responded to volatiles produced by slugs that had decayed for 48 h, but not to fresh slugs. The beetles also responded to mucus from fresh slugs in behavioural assays, changing their rates of turning and moving greater distances within such patches. The baited-trap experiment appears to confirm that direct contact and mucus trail following are not necessary and that beetles can find dead slugs simply by olfaction. *P. melanarius* has been shown in other studies to orientate towards other prey species by olfaction (Wheater 1989; Kielty *et al*. 1996). However, beetle numbers in aphid-baited traps did not significantly differ from those in control traps. It is likely that finding aphid carrion by carabids is a random process or that the beetles are attracted from shorter distances than the radius of traps.

The disappearance rate for dead slugs in the field revealed that they are rapidly removed by scavengers. The chance of a dead slug being consumed significantly decreased with slug weight. This response is to be expected since a smaller number of scavengers are capable of eating or moving the whole prey the larger it is. Both crop type and field also had highly significant effects and explained more variation in the model than the carrion weight. On sites where there was higher activity/density of *P. melanarius*, there were higher disappearance rates for dead slugs (Figs 4 and 5). Carrion disappearance was significantly faster in wheat fields than in pastures, possibly because there were more beetles present in wheat, but also in part because activity may have been impeded in pasture by the thicker vegetation. *P. melanarius* dominated trap catches, suggesting that it acted as the major invertebrate scavenger of dead slugs at our experimental sites.

For both slugs and aphids, dead prey was preferentially attacked/consumed in the early stages of decay. Later on, the preference shifted towards live prey as the dead prey on offer became increasingly putrid. Carrion was ultimately refused when completely enveloped by fungi. Under natural conditions, the difference in preference between dead and live prey would have been even greater since such live prey have effective escape and defence mechanisms, including climbing up the plants, prey toxins and the production of mucus (slugs) (Pakarinen 1993; Symondson 1993, 1997; Winder *et al*. 1994; Losey & Denno 1998; Foltan 2004). The limited height of the Petri dishes precluded escape by climbing.

In both field and laboratory experiments, *P. melanarius* readily and preferentially fed on dead prey if available, such preferences only changing as the prey decayed. The field assessment of availability of slug carrion showed that 50% of carrion was present after 6 h of exposure. Fifty per cent of the DNA from carrion that decayed for this time was detectable in beetle gut after 17.5 h (slugs) and 5.7 h (aphids) of digestion. In the case of freshly killed prey (as in predation), the values were 183 h and 63 h, respectively. Detection of food remains obtained by scavenging and predation were evidently very similar and this clearly demonstrates that PCR-based gut-content analyses are not able to distinguish between predated and scavenged food items. Assuming that material in the gut of a predator was predated could therefore lead to overestimation of the impact of predation. The significance of this potential error can only be quantified if carrion availability is estimated in the field, as demonstrated here. The availability of live and dead aphids to ground predators in cereal crops has been measured and more live aphids fall to the ground than dead ones (Winder *et al*. 1994). The availability of live aphids, however, was low since aphids quickly returned to the canopy, increasing the relative availability of dead aphids. The availability of live aphids on the ground increases in the presence of foliar foraging parasitoids and/or predators (Ruth *et al*. 1975). Aphids release alarm pheromones when in contact with predators/parasitoids (Nault *et al*. 1973) which lead to synchronized defence behaviour. Dropping, leaping or falling from the host plant is the most common defence response among aphids (Ruth *et al*. 1975; Clegg & Barlow 1982; Dixon 1985; Losey *et al*. 1998). However, during hot weather live aphids falling from the host plant onto the soil die within seconds (Ruth *et al*. 1975). Many predators feed facultatively upon dead aphids (Smith 1966; Wheeler 1977; Powell 1987) and gut-contents analyses are useful for estimating the contribution of aphids to predator nutrition. Assessment of the impact of generalist predators upon aphid population dynamics should only be attempted if density and removal rates of dead aphids can be measured. Estimation of death rates, and availability of dead slugs, has only been carried out for those treated with molluscicides or parasitic nematodes (i.e. Langan *et al*. 2001, 2004; Iglesias *et al*. 2003) and further work is

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needed to find methods for measuring slug mortality (other than through predation) in natural systems.

The consumption of carrion by invertebrate predators and scavengers has important implications for food-web research. Among invertebrate scavengers, many arthropod species are specialized for feeding upon large mammal carcasses (Akopyan 1953; Putman 1978, 1983). Some invertebrates specialized to feed on small vertebrates carcases, such as rodents, have evolved mechanisms that enable them to monopolize their prey, for example by burying it (e.g. sexton beetles, Coleoptera: Silphidae). Small carcasses disappear much quicker than the large ones because large items of carrion cannot be consumed by a small scavenger. There is no evidence in terrestrial systems of the existence of obligate scavengers of invertebrate carcasses. Distribution of animal carcases is unpredictable in time and space; thus the energy cost of discovering a prey item is the main energetic constraint for the scavengers (Ruxton & Houston 2004). It is likely that a scarcity of invertebrate carrion biomass as a food resource has limited the evolution of obligate scavenging behaviour and thus invertebrate corpses are removed entirely by facultative scavengers, principally generalist predators, in a short period of time. This hypothesis is supported by observations of scavenging of invertebrate cadavers in ant-dominated ecosystems. Arthropod corpses (size 2–8 mm) placed in a temperate Mediterranean grassland remained in the field for less than 5 min on average (Retana *et al*. 1991). Disappearance was most rapid during the middle hours of the day, precisely during the peak time of ant activity. Dead crickets, Diptera, Lepidoptera and termites in pine and oak woodlands and semiarid grasslands in the USA disappeared within a few minutes (Seastedt *et al*. 1981; Fellers & Fellers 1982; Bestelmeyer & Wiens 2003). All scavengers that took the cadavers were ants. Other studies have developed this work by placing five different types of insect carrion onto the soil surface in corn and sphagnum fields in Georgia, USA, together with control and baited pitfall traps (Young 1984a). Eighty percent of insect cadavers disappeared within 24 h. Small grasshopper carcasses were removed more rapidly than the other four larger insect types. Comparison of data from baited and control pitfall traps demonstrated that ants, earwigs and scarabs were the principal carcass-consuming groups.

So, what is the future of gut-content analysis in ecological research, given our inability to distinguish between predation and scavenging? It very much depends upon the questions asked. If taxonomic identity of the food resources sustaining a predator is the primary aim, then scavenging does not necessarily constitute a problem. Small predators cannot usually kill large prey items, yet such prey may constitute a valuable food resource in the form of carrion and be an important component of predator fitness. The distribution of dead invertebrates is likely to be spatially correlated with the distribution of live prey and may well provide cues whereby predators find such live prey. If the primary focus is identifying and/or quantifying predation per se, gut-content analysis should be accompanied by as detailed as possible an understanding of the systems under study. Some predators, such as spiders, rarely scavenge, whereas, as we have shown, the carabid beetle *P. melanarius* has a preference for dead prey (when fresh). Most carabids probably behave in a similar manner but this should be confirmed through laboratory and (ideally) field experiments. Although we can measure predator numbers and carcass removal rates in the field, the one factor that is hard to determine is availability of dead prey. Slugs are rarely found dead on the soil surface, unless killed by pesticides, but this may simply be because they are removed rapidly by facultative scavengers. It is also probable that moribund invertebrates are killed and eaten in preference to healthy prey, so the distinction between predation and scavenging is itself not always clear (Sunderland 1996). Where possible availability and exploitation of dead prey should be quantified which, when combined with gut-content analyses, should permit a better understanding of food links and energy flow in terrestrial ecosystems.

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The parasitic nematode *Phasmarhabditis hermaphrodita* defends its slug host from being predated or scavenged by manipulating host spatial behaviour

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Abstract

Infective stages of commercially used molluscicidal rhabditide nematodes *Phasmarhabditis hermaphrodita* contain bacterial symbionts which kill their host by septicaemia. The nematodes feed on the multiplying bacteria and entire host tissue, develop and repeatedly reproduce. Invertebrate cadavers are rapidly (from minutes to hours) removed by scavengers. However nematodes need days to complete their life cycle inside the host.

The post mortem locations of slugs killed by six different treatments (three types of molluscicides, a simulation of unsuccessful predation and two *P. hermaphrodita* nematode treatments) were compared.

In comparison to other pathogenic states, significantly more slugs killed by the nematodes died within the soil, where the scavenging pressure is weaker than on the soil surface (where most of the slugs died regardless treatment). We suggest that this is an outcome of behavioural manipulation, which prevent the parasites from being predated or scavenged together with their host until the nematodes complete development inside the host cadaver.
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Keywords: Manipulation; Slug; Nematode; *Phasmarhabditis*; Deroceras; UV-fluorescent

1. Introduction

The rhabditid nematode *Phasmarhabditis hermaphrodita* (Schneider, 1859) has been shown to be a successful biocontrol agent of different pest slugs (i.e. Wilson et al., 1993; Wilson et al., 1995; Glen and Wilson, 1997; Glen et al., 2000; Iglesias et al., 2003). The infective third-stage juveniles of *P. hermaphrodita* invade the slug host (Tan and Grewal, 2001) and release the associated bacterial symbiont, which then multiplies in the slug body and kills the host by septicaemia (Tan and Grewal, 2002). After host death, typically within a week from infection (Grewal et al., 2001), the nematodes colonize the entire cadaver and feed on the bacterial symbiont and the host tissue, developing into adult self-fertilizing hermaphrodites and repeatedly reproducing. As food resources in the cadaver are depleted, new infective juveniles carrying symbiotic bacteria leave the cadaver in search for a new host (Wilson et al., 1993).

While inhabiting the cadaver, the nematodes are under serious threat from predatory and scavenging invertebrates, which can destroy them while feeding on their host. This threat is particularly high because the susceptibility of dying hosts to predation increases as their defence efficiency declines (Pakarinen, 1993; Winder et al., 1994; Symondson, 1997; Foltan, 2004). Invertebrate cadavers are rapidly, usually in minutes or hours (Seastedt et al., 1981; Fellers and Fellers, 1982; Young, 1984; Retana et al., 1991; Bestelmeyer and Wiens, 2003; Foltan et al., 2005) and preferentially (Sunderland and Sutton, 1980; Mair and Port, 2001; Langan et al., 2001; Foltan et al., 2005) removed by predators and scavengers, while nematodes require several days to complete their reproduction in the cadaver.

Different parasitic groups have been shown to manipulate feeding, social, spatial or dispersal behaviour or physiological functions of their vectors, which results in an increased probability of their transmission to other hosts (Barnard and Behnke, 1990; Combes, 1991, 1998; Poulin, 1995, 1998; Lafferty et

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al., 2000; Moore, 2002; Lion et al., 2006). Such manipulations expose the hosts to increased predation risks, facilitating transmission of the parasites across the food chains. Several studies have shown that manipulation can sometimes evolve as a strategy to protect the infected host from environmental dangers (Brodeur and McNeil, 1989; Eberhard, 2000; Médoc et al., 2006) However, not all cases of reported host behavioural change are necessarily a result of the parasite actively manipulating host behaviour; they may rather be the 'by-products' of pathology caused by the parasites (Thomas et al., 2005; Leung and Poulin, 2006).

In this study, we report that *P. hermaphrodita* manipulates the spatial behaviour of its slug host, *Deroceras reticulatum* (Müller, 1774), preventing the host from being predated or scavenged until the nematodes complete their development. We exposed the slugs to the nematodes and four other pathogenic agents. Although all treatments ultimately killed the slugs, nematode treatment resulted into spatial distribution of the cadavers differing from the spatial distribution of slugs that have died due to action of molluscicides or mechanical damage. As far as we are aware, this is a rare case where host manipulation protects the infected host (and parasite) from predation and scavenging, rather than manipulating the host to increase the probability of consumption by a consequent host species.

2. Methods

2.1. Slugs

D. reticulatum slugs (100–250 mg/individual) were collected from arable fields around Ceske Budejovice, Czech Republic (49°0′N, 14°30′, alt. 400 m). The slugs were housed individually in plastic containers containing moist soil substrate (AROS Ltd.) and fresh lettuce leaves, and maintained in a controlled environment (L:D 16:8; 20 ± 1 °C) for at least 7 days prior to the experiment to become established and equally hydrated. Ninety slugs were labelled by a UV-fluorescent dye (10 μl/individual) 2 days before the experiment (Foltan and Konvicka, in press). The labelling enabled later identification of slug cadavers on the soil surface or in the soil, which otherwise would not be possible.

2.2. UV-fluorescent dye

10% liquid solution of biologically inert powder Radglo JST UV-fluorescent pigment (Radiant colour N.V.) was made up by dilution in Polyethylene glycol, Av. mol. wt.: 400 (Sigma–Aldrich) The solution was subsequently diluted in distilled water 1:1 (Foltan and Konvicka, in press).

2.3. Mini-plots

The experiment was conducted under controlled conditions in 90 mini-plots consisting of translucent plastic cylinders (7.5 cm dia. and 27 cm deep) half filled with 15 cm of moist soil substrate and covered with mosquito-curtain to prevent slugs from escaping. Each container was coated in black paper tape from outside, up to the soil surface, to prevent side illumination through the container wall. To simulate field conditions, barley seeds were sown at a depth of 1 cm (approx. 25 seeds per container) in the soil and allowed to grow up to 10 cm before the experiment. Equal soil moisture of approx. 30% was maintained in all the containers used.

2.4. Experimental design

The 90 slugs were divided into six groups, each consisting of 15 individuals (kept individually in 15 containers) and subjected to six different treatments. Three treatments were different types of molluscicides, Ferramol (iron phosphate-based, W. Neuedorff GmbH KG), Mesurol (methiocarb-based, Bayer AG) and Vanish slug pellets (metaldehyde-based, TransChem Professional BV). The slugs to be treated with molluscicides were introduced into the containers 5 days before treatment to become established and find natural refugia. Two treatments were by the nematodes (commercially available *P. hermaphrodita* nematodes (Nemaslug®): one treatment simulated exposure to infected soil, consisted of treating the 5-day established slugs with commercially recommended dose of the parasite. In the second treatment, the slugs were infested by placing them into water containing a high concentration of the nematode for 25 min, washed and placed individually into the containers. Finally, one treatment simulated deaths from unsuccessful predation: the slugs were cut by scissors into the hepatopancreas region (slugs were able to move for several hours before the death) and placed individually into the experimental containers.

After the slugs had died, the post mortem position (vegetation/walls, surface, and soil) of each individual was identified, using a portable UV-lamp (according to Foltan and Konvicka, in press). All slugs that died under the two nematode treatments were checked for the presence of nematodes using a stereomicroscope.

Contingency table (with six treatments and three response levels) was used to compare distribution of post mortem locations.

3. Results

Slugs exposed to all six treatments were able to move for some period of time before they all died regardless of treatment. There were significant differences between the distributions of the slug post mortem locations between all treatments (M–L $\chi^2 f = 24.31$, df = 10, $p < 0.01$) Treatments without nematodes (simulation of predation and the three types of molluscicides) did not differ in their distribution of the slug post mortem positions (M–L $\chi^2 f = 6.30$, df = 6.0, $p = 0.39$). Thus, for further analyses, this group of treatments was chosen as the control group.

Significantly more slugs (M–L $\chi^2 f = 9.0$, df = 2, $p < 0.05$) died on the vegetation or container walls above ground level in the treatment, where slugs were infected inside the experimental containers than in the control group. In the treatment where slugs were infected outside the experimental container, significantly more cadavers were found within the soil compared to the control (M–L $\chi^2 f = 6.39$, df = 2, $p < 0.05$). Slug cadavers from the two "nematode treatments" differed in distribution from each other

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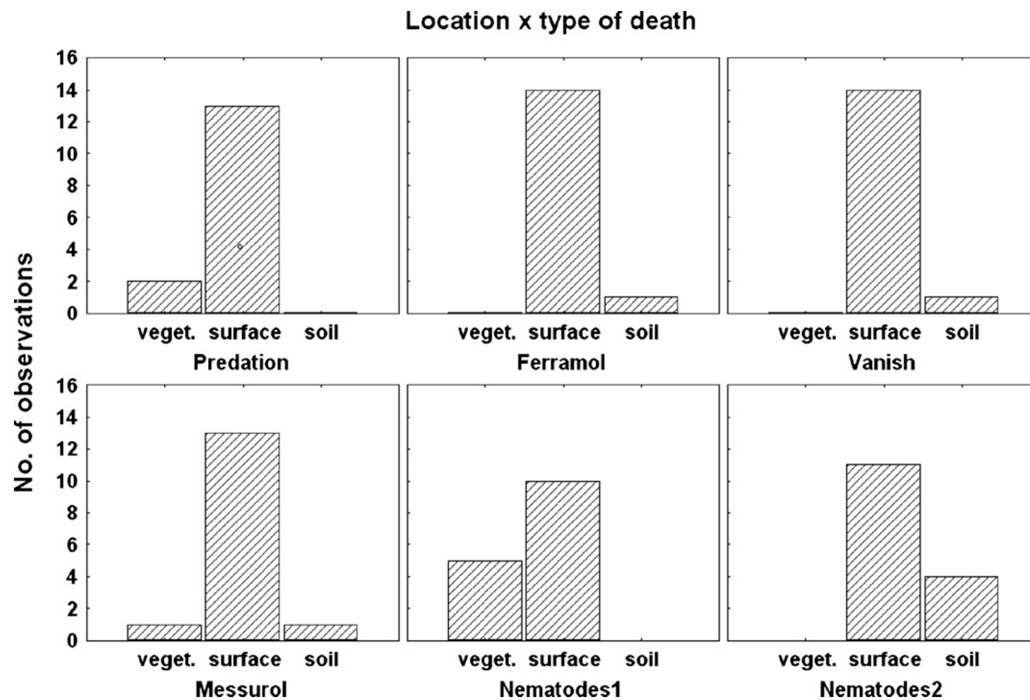

Fig. 1. Counts and position (vegetation/walls, soil surface, soil) of slug cadavers found in the experimental containers ($N = 90$) where six different treatments were applied to slugs placed individually into the containers: three different molluscicides, simulation of predation and two nematode treatments were used. Nematodes I: slugs were infected by nematodes inside the experimental containers. Nematodes II: slugs were infected outside the containers and introduced into nematode-free containers.

(M–L $\chi^2 f = 12.52$, df = 2, $p < 0.05$), see Fig. 1. All slugs that died under the two nematode treatments contained high numbers of reproducing nematodes.

4. Discussion

In the first nematode treatment, a considerable proportion of slugs added to containers containing infective nematodes died on plants or walls of experimental containers, probably trying to escape from the reach of the nematodes present in the soil. Under natural conditions, the nematode spatial distribution varies (Stuart and Gaugler, 1994). The escaping infected slugs thus can reach some nematode-free areas before their death. Slugs have been shown to survive infection to some level (Wilson et al., 2000; Wilson and Gaugler, 2000), thus escaping from the soil inhabited by infective nematodes remains meaningful even for already infected hosts. This observation is supported by findings of Wilson et al. (1999), who placed *D. reticulatum* and *Arion ater* slugs into boxes, where half the soil surface area was treated by *P. hermaphrodita* and the second half remained untreated. Both slug species were more likely to rest and feed on the nematode untreated than nematode treated soil, but showed no preference between the treatments if low (<38/cm^2) nematode densities where used. However in a mini-plot experiment using *P. hermaphrodita* to control slugs in Chinese cabbage, Hass et al. (1999) found no evidence of slugs being repelled. In the second nematode treatment, slugs infected by the nematodes were transported into nematode-free containers, where they died from the infection. The slugs would be expected to die on the surface as they were not habituated in the containers and had less time, compared to other treatments excluding the simulation of predation, to create shelters in the soil (Hommay et al., 1998; Grewal et al., 2001). However, the slugs exhibited significant tendency to die in the soil compared to other pathogenic states.

The prevalent post mortem location on the soil surface in molluscicide treatments and consequent significant difference from the two nematode treatments can be potentially caused by extremely rapid death of slugs after direct contact with the molluscicide. However, in all such treatments, at least some slugs were found elsewhere than on the soil surface, which indicates that they moved before they died. Furthermore, in the “predation” treatment, where injured slugs were able to move for a sufficient period of time before their death, the distribution of their post mortem positions did not differ from the molluscicide treatments.

Invertebrate cadavers present on the soil surface are open to extreme scavenging pressure of generalist predators/scavengers (Seastedt et al., 1981; Fellers and Fellers, 1982; Young, 1984; Retana et al., 1991; Bestelmeyer and Wiens, 2003; Foltan et al., 2005), whereas the removal of invertebrate cadavers is much lower under the soil surface (Seastedt et al., 1981). Apart from scavenging, the cadavers present on the soil surface are exposed to high temperatures (Ruth et al., 1975) and quickly desiccate.

It would be beneficial, to the nematode/bacteria complex manipulating slugs, for the slugs to die in soil. The manipulation enables the nematodes to resist scavenging pressure (at least at a certain level) and also to avoid desiccation on the soil surface. Foltan and Puza, submitted for publication revealed significant deterrent effect of slugs infected by *P. hermafrodita* to a

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carabid predator *Pterostichus melanarius*, a prevalent predator of slugsin the majority of cropscapable of affecting their temporal and spatial dynamics (Symondson et al., 1996; Bohan et al., 2000; McKemey et al., 2003; Paill, 2004). Combination of the two effects potentially facilitates successful development of the nematodes inside their host cadavers. However, it would be essential to experimentally compare the success of nematode development both on and under the soil surface with and without predator/scavenger presence in order to be able to claim that the manipulation has evolved as a strategy against predators and scavengers. Our experimental design unfortunately excludes to decide whether it is the nematodes, the associated bacterial symbionts or the interaction of both who is responsible for the suggested behavioural manipulation. Further experiments with bacterial and nematode isolates are necessary to test this.

Extrapolation of laboratory mini-plot data to the field situation is also problematic and only further field experiments can reveal whether the manipulation hypothesis can be adopted in the context presented above.

Previously, many different gut content analysis techniques have been developed as a valuable tool for the study of predation on pest slug species (Sunderland et al., 1987; Sunderland, 1996; Symondson and Liddell, 1996a,b; Symondson, 2002a,b).

Foltan et al. (2005) and Calder et al. (2005) used a slug-carabid model to show that scavenging represents a significant biasing factor when molecular (PCR-based) or biochemical (monoclonal antibody-based) gut content analysis is used to assess impact of predation on prey populations. Neither analytical approach was able to distinguish between material that was predated or scavenged. To assess the size of error coming from scavenging, estimation of carrion availability for scavengers is necessary. In our experiment, regardless of the treatment, a majority of the slug cadavers remained on the surface, easily accessible to scavengers. As many predators regularly scavenge (Sunderland, 1996) or prefer dead to live prey (Sunderland and Sutton, 1980; Mair and Port, 2001; Langan et al., 2001), we assume that many false positive results of predation can be obtained when gut content analyses are used for its assessment.

In terms of biological control, the effects of parasites and predators are often negatively correlated. The predators are likely to destroy parasites within their prey. On the other hand, a parasitic infection can quickly reduce pest abundance, which can be critical for maintaining predators in the field. Success of entomopathogenic and molluscicidal nematodes in reducing pest populations is significantly influenced by persistence of infective juveniles in the soil and the ability of a parasitic stage to develop within the cadaver (Baur et al., 1998). In the case of *P. hermafrodita*, the suggested manipulation of slug behaviour so that they die in the soil, together with the repellent effect of infected carrion (Foltan and Puza, submitted for publication), reduces the vulnerability of the parasites to predation however potentially beneficial predators are restricted in their access to a valuable source of energy which originates from scavenging. Further research on predator–parasite–host and scavenger–parasite–cadaver interactions is needed to improve application success and commercial use of pesticidal nematodes as well as to maintain pest predators in the field as an essential part of the integrated pest management.

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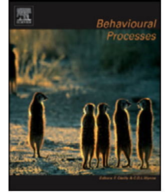

To complete their life cycle, pathogenic nematode–bacteria complexes deter scavengers from feeding on their host cadaver

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ABSTRACT

The life cycle of commercially used molluscicidal rhabditid nematodes *Phasmarhabditis hermaphrodita* and entomopathogenic steinernematid nematodes is similar: infective stages carry symbiotic bacteria, which kill their host. Nematodes complete their life cycle feeding on the proliferating symbiont and the host tissue. After 1–2 weeks, new infective stages carrying the bacteria leave the host cadaver in search of new hosts. The removal of invertebrate cadavers by scavengers is extremely fast and represents a severe threat to the developing nematodes.Two-choice trials were used to assess prey choice of the generalist predator/scavenger *Pterostichus melanarius* (Coleoptera: Carabidae) between *Deroceras reticulatum* (Mollusca: Agriolimacidae) slugs or wax moth *Galleria mellonella* (Lepidoptera: Pyralidae) larvae killed by infection of *P. hermaphrodita*/*Steinernema affine* and control killed by freezing. We demonstrate that the presence of either of the two nematodes tested deters the beetles from consuming infected cadavers. As *P. hermaprodita* cannot infect an insect host, we hypothesise the deterrent effect being an evolutionary adaptation of the nematode/bacteria complex rather than the ability of the beetles to avoid potentially infective cadavers.

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1. Introduction

Entomopathogenic nematodes from the Steinernematidae and Heterorhabditidae families are lethal parasites of various soil-dwelling insects and are used commercially in biological control (Mracek, 2002; Shapiro-Ilan et al., 2002; Arthurs et al., 2004; Georgis et al., 2006). Similarly the rhabditid nematode *Phasmarhabditis hermaphrodita*, is a lethal parasite of terrestrial molluscs (predominantly slugs) and has recently been commercialised in the EU as a biological molluscicide against slug pest.

Both nematodes appear to adopt a similar life cycle including development and reproduction inside a host and a free-living infective stage (IJ) carrying symbiotic bacteria, which kills their host. Nematodes subsequently finish their life cycle feeding on the proliferating symbiont and the host tissue, which takes typically 7–15 days for entomopathogenic nematodes (Poinar, 1979; Adams and Nguyen, 2002) and between 7 and 21 days for *P. hermaphrodita*, respectively. As food resources in the cadaver are depleted, new IJs, carrying symbiotic bacteria, are formed that leave the cadaver in search of new hosts (Poinar, 1979; Wilson et al., 1993; Tan and Grewal, 2001, 2002).

As is evident from the above, the critical factor for successful reproduction of both pesticidal nematodes is that the infected host and the subsequent cadaver remains intact while nematodes complete their life cycle. Dying hosts are vulnerable to predation as their defence efficiency declines (Pakarinen, 1993; Winder et al., 1994; Symondson, 1997; Foltan, 2004). The removal of invertebrate cadavers by scavenging invertebrates is typically rapid, taking only minutes or hours (Seastedt et al., 1981; Fellers and Fellers, 1982; Young, 1984; Retana et al., 1991; Bestelmeyer and Wiens, 2003; Foltan et al., 2005). However, nematodes need days to complete their life cycle.

There is evidence to suggest that nematode/bacteria infestation can reduce cadaver attractiveness to ant scavengers. Baur et al. (1998) placed 2-days and 8-days old cadavers (4 and 10 days after infection) of *Galleria mellonella* (L.)(Lepidoptera: Pyralidae) larvae infected by different heterorhabditid and steinernematid nematodes on the ground and 2 cm under the ground in a horticultultural habitat in California. Different ant species removed more steinernematid-killed (60–80%) than heterorhabditid-killed (10–20%) insects within 24 hours.

In another study, Zhou et al. (2002) placed 4-days old *G. mellonella* larvae killed by different strains of *Xenorhabdus*

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nematophilus and *Photorhabdus luminescens* or by freezing, into ant colonies or near to ant foraging trails. Significantly more non-infected cadavers were removed by the ants.

The critical period for "survival" of invertebrate cadavers in the field is the first 24 h after death as cadavers are typically scavenged during this period (Seastedt et al., 1981; Fellers and Fellers, 1982; Young, 1984; Retana et al., 1991; Bestelmeyer and Wiens, 2003; Foltan et al., 2005). Thus, estimating removal rates of several-days old cadavers infected by pathogenic nematodes, even when their repellent effect is evident (Baur et al., 1998; Zhou et al., 2002), does not fully answer the question as to how nematodes survive the extreme scavenging pressure on inhabited cadavers. Moreover, as the attractiveness of invertebrate cadavers declines with time (Foltan et al., 2005), comparing removal rates of rapidly decaying nematode infected cadavers with freshly killed controls (Baur et al., 1998; Zhou et al., 2002) does not demonstrate the deterrent effect of the infection.

This study is the first to address the significant difference between the fast removal rates of invertebrate cadavers from the field and the relatively long period which is necessary for successful reproduction of molluscicidal and entomopathogenic nematodes within the cadavers. We demonstrate for the first time the strong antifeedant effect of rhabditid nematode infected slug cadavers as well as the significant anti-feeding effect of steinernematid infected insect cadavers on invertebrate non-ant scavengers.

We discuss whether such an effect enables the nematodes to survive in cadavers which would otherwise be removed through the extreme pressure from scavenging.

2. Materials and methods

Two-choice laboratory experiments were used to assess the prey choice of the common generalist predator/scavenger *Pterostichus melanarius* (Coleoptera: Carabidae) between slugs *Deroceras reticulatum* killed by infection with *P. hermaphrodit* or wax moth *G. mellonella* larvae killed by infection with *Steinernema affine* and non-infested controls.

D. reticulatum slugs and *P. melanarius* beetles were collected from arable fields around Ceske Budejovice, Czech Republic. The beetles were kept individually in plastic containers (9.5 cm × 7 cm × 5 cm) with 80 g moist sphagnum peat (Agro CS a.s.) and maintained in a controlled environment (16 h light:8 h dark photoperiod; 20 ± 1 °C). They were maintained on a diet of mixed pork and beef. Prior to the experiment, the beetles were fed *ad libitum* and then starved for 7 days, to ensure the same nutritional state in all individuals.

G. mellonella larvae were reared in multiclonal cultures according to Haydak (1936). Forty slugs and forty moth larvae were killed by freezing at −80 °C and weighed. Another forty slugs/larvae were killed by the infection with *P. hermaphrodita*/*S. affine* nematodes. The slugs were infected by exposure to a superabundance of *P. hermaphrodita* in a plastic container (approximately 10 million IJ's per 30 cm × 20 cm × 20 cm container) filled with moist filter paper. Moth larvae were infected in Petri dishes (7 cm diameter) with moist filter paper on the bottom by adding 2000 IJ's of *S. affine* in 1 ml of tap water per 10 moth larvae. The weight of freshly killed nematode infected slugs/larvae and the weight of the control slugs/larvae was recorded before storage at −80 °C (avg. w. slugs: 167.8 mg, SD 39.8; avg. w. larvae: 163.1 mg, SD 39.4).

A single frozen dead slug or larva, killed by freezing (=non-infected), was placed in a Petri dish (7 cm diameter) lined with moist filter paper, together with a frozen slug/larva killed by the nematodes (=infected). The prey items were placed opposite to each other approximately 1.5 cm from the edge of the Petri dish. Every following Petri dish was turned through 90° to prevent any effect of prey preference due to spatial orientation of the prey items. The slugs/larvae were let to thaw for several minutes. Single *P. melanarius* beetles (equal sex proportions) were placed in the middle of each dish and observed continuously for 30 min. The first attack by the beetle on the infected or non-infected cadaver, and first feeding on the infected or non-infected cadaver, were recorded as separate parameters. An attack was recorded when the beetle bit the prey. Feeding was recorded when biting occurred continuously for more than 30 s on one prey. After 30 min, any non-feeding beetles were discarded and the experiment was repeated with a fresh beetle, until the final number of 40 replicate trials for both the slugs and larvae was reached.

To assess the effect of beetle weight, beetle sex, weight of infected and non-infected cadaver and difference in weight between infected/non-infected cadaver on beetle choice, binomial regressions with preference as the dependent variable (and remaining variables as potential predictors) were used, separately for response variables (attack and consumption) and for different types of prey (slugs, larvae); using GLM (link binomial) in S-plus 2000 (1999).

3. Results

Separate effects of all potential predictors were compared with null models (that containing only the response variable) for potential decrease of deviance, measured by Cp criterion (Table 1). The only predictor that exhibited a decrease of deviance was beetle

Table 1
Effect of beetle sex, cadaver weight and difference in weight between infected/non-infected cadavers on the choice of the predatory beetle *P. melanarius* between slugs killed by *P. hermaphrodita*, *G. mellonella* larvae killed by *S. affine* and control slugs/larvae killed by freezing. Binomial regressions with preference as dependent variable (and remaining variables as potential predictors) were used, separately for attack and consumption and for different types of prey; using GLM (link binomial).

Term	Df	Sum of Sq	RSS	Cp	Df	Sum of Sq	RSS	Cp
	Attack on moth larva cadaver				Consumption of moth larva cadaver			
Null			40.00	42.05			40.00	42.05
Beetle sex	1	0.10	39.80	44.00	1	0.17	39.83	43.93
Beetle weight	1	0.25	39.75	43.85	1	0.57	39.43	43.53
Infected larvae weight	1	0.07	39.93	44.03	1	0.00	39.99	44.10
Non-infected larvae weight	1	0.11	39.89	43.99	1	0.16	39.84	43.94
Difference in larvae weights		0.68	39.32	43.42	1	0.00	39.99	44.10
	Attack on slug cadaver				Consumption of slug cadaver			
Null			40.00	42.05			40.00	42.05
Beetle sex	1	0.48	39.52	43.63	1	0.00	40.00	44.10
Beetle weight	1	0.00	39.99	44.10	1	2.32	37.68	41.79
Infected slug weight	1	0.62	39.38	43.48	1	0.01	39.99	44.09
Non-infected slug weight	1	0.68	39.32	43.42	1	1.07	38.93	43.04
Difference in slug weights	1	0.27	39.73	43.83	1	1.01	39.99	44.09

weight on non-infected slugs. However, the model containing this predictor did not differ significantly from the null model $F(1, 38) = 2.59$, $p = 0.12$; Cp_null 0: 46.99, Cp_fitted: 46.40). As none of the potential predictors had any significant effect on beetle choice, they were not used in the following analyses.

There were four possibilities for the beetles attacking and consuming the cadavers tested: (i) to attack infected, to consume infected, (ii) to attack infected, to consume non-infected, (iii) to attack non-infected, to consume non-infected, (iv) to attack non-infected and to consume infected cadaver (Fig. 1). Using the χ^2 test, we could reject the null hypothesis that the four options occurred with the same frequency (slugs: χ^2 (1) = 4.52, $p = 0.03$; larvae: χ^2 (1) = 18.28, $p < 0.001$). There was only a slight tendency for beetles to attack non-infected rather then infected slugs (χ^2 (1) = 3.33, $p = 0.067$), while the moth larvae were attacked at random (χ^2 (1) = 0.45, $p = 0.50$). However, both cadaver types were consumed significantly more often if non-infected (slugs: χ^2 (1) = 5.33, $p = 0.02$; larvae χ^2 (1) = 9.45, $p = 0.002$). Furthermore, the beetles switched significantly more often from having tasted/attacked an infected larvae to consuming a non-infected larvae than vice versa; 17 vs. 1 ($N = 40$), $p < 0.0001$ (exact binomial test). This difference was not significant for beetles attacking slug cadavers; 4 vs. 2 ($N = 40$), $p = 0.13$.

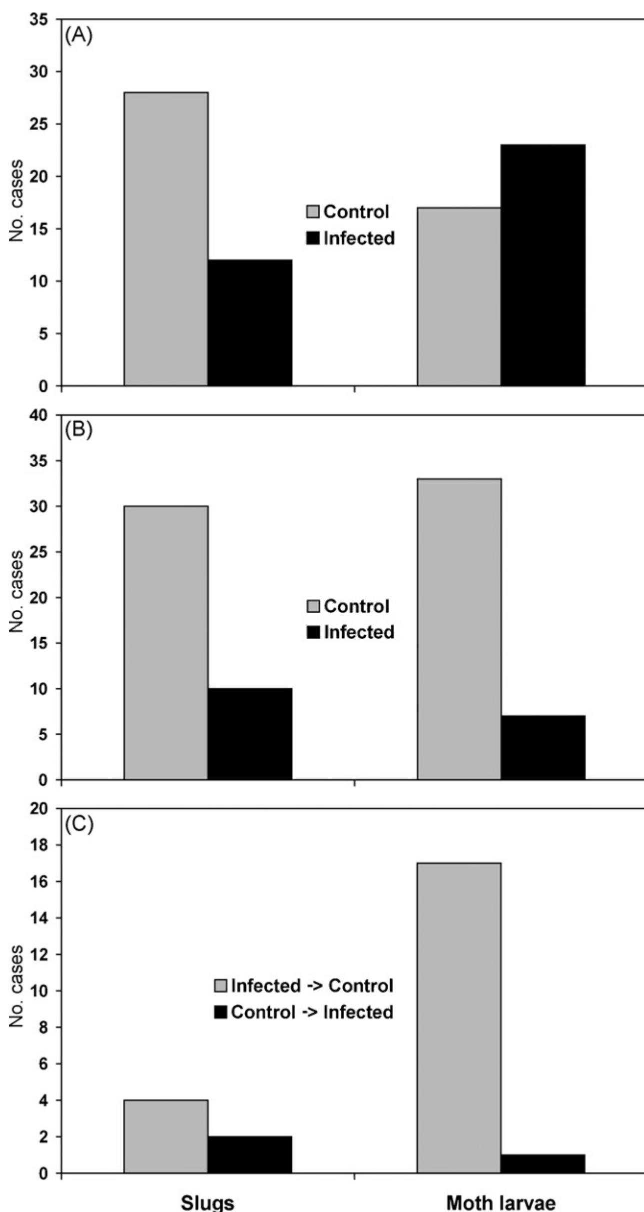

Fig. 1. Number of nematode infected and control cadavers attacked (A) and consumed (B) by *P. melanarius* and a number of switches between infected and non-infected cadavers (The beetles firstly attacked/tasted one type, but later consumed different one) (C) in a two-choice laboratory experiment.

4. Discussion

In our study, we demonstrate a significant antifeedant effect of both types of nematode infected cadavers, even though the effects differed in their mechanism. Whereas attacks on moth larvae were not affected by infection, the beetles were more likely to attack non-infected slugs. The beetles switched significantly more often from infected to non-infected larvae, which suggests that they avoid infected larvae after tasting. Such switching was not significant for beetles attacking slug cadavers because the beetles were deterred from attacking infected cadaver, thus causing the total number of switches to be very low ($N = 6$). Both situations had the same outcome: the beetles consumed significantly fewer cadavers infested by nematodes, which may enable the nematodes to resist scavenging pressure and to successfully complete their life cycle inside cadavers.

Insect scavengers consuming insect cadavers (Seastedt et al., 1981; Fellers and Fellers, 1982; Young, 1984; Retana et al., 1991; Bestelmeyer and Wiens, 2003; Foltan et al., 2005) infected by entomopathogenic nematodes are theoretically under the threat of infection. However, there is no evidence of any important insect-scavenging guild feeding obligately on invertebrate cadavers. The chance of a scavenger coming across an infective cadaver is further minimalised by freshly killed cadavers containing no IJs and by the insect cadavers being a scarce resource in general.

Thus, we would suggest that the evolutionary pressure on facultative scavengers to evolve mechanisms enabling successful recognition of infective cadavers to be negligible. On the other hand, cadaver-inhabiting nematodes are under serious threat of predators and scavenging invertebrates, which can easily destroy the nematodes together with consumption of their host There is a strong selection pressure to evolve a way to deter predators and scavengers from the infected hosts. Our results support such a hypothesis as carabid beetles were deterred by *P. hermaphrodita*, which are able to successfully parasitize only in mollusc hosts (Wilson et al., 1994; Rae et al., 2005; Iglesias et al., 2003), and do not therefore represent any threat to insect scavengers.

The significant deterrent effect of freshly nematode-killed cadavers suggests that the decrease in the defensive capabilities of the host from infection to death may be compensated for by the increasing feeding deterrent effect of the nematode/bacteria complex.

Pechova and Foltan (2008) compared post-mortem locations of slugs killed by six different pathogens. The slugs were manipulated by *P. hermaphrodita* to die under the soil surface and out of the reach of surface dwelling scavengers. A combination of the two effects may further increase the chances of the nematodes avoiding scavenging.

Under natural conditions, scavengers are usually more food deprived than under laboratory conditions as in our study. However, in our experiment, a small proportion of infected cadavers were consumed when offered over a relatively short period of time even in the presence of an uninfected control. High quantities of nematodes used for host infection may have amplified the deterrent effect of the nematode/bacteria complex. This effect is likely to be weaker in the field, where we assume the nematodes may often be destroyed by scavengers. A further study using naturally

occurring levels of infestation would reveal the true extent of the deterrence of scavengers in the field.

The efficacy of pathogenic nematodes in reducing pest populations is dependent on the persistence of IJs in the soil and on their ability to develop within the cadaver (Baur et al., 1998). The demonstration of a deterrent effect of the nematode/bacteria complex supports the application of EPNs in infected insect cadaver, which has been shown to be more effective than conventional application methods (Jansson et al., 1993; Perez et al., 2003; Shapiro-Ilan et al., 2003).

The methods we have used during the present study do not allow us to discriminate which of the two symbiotic partners is responsible for the deterrent effect. However, ant-deterrent factors from two different bacterial isolates from heterorhabditid and steinernematid nematodes were isolated by Zhou et al. (2002), suggesting that the deterrent effect is caused by bacterial metabolites.

Further studies using diverse nemato–bacterial associations in different host species exposed to different scavengers would improve our understanding of the mechanisms involved.

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A NEW METHOD FOR MARKING SLUGS BY ULTRAVIOLET-FLUORESCENT DYE

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ABSTRACT

We introduce a new, cheap, fast and reliable method for marking slugs, using biologically inert UV-fluorescent dye. The dye remained visible for more than 3 months in all individuals marked. Use of the dye did not affect survival, spatial behaviour or daily activity of the slugs, nor the prey choice of the predatory beetle *Pterostichus melanarius*, under controlled semi-natural conditions. The new method will enable further investigations of absolute slug densities, diurnal activity, mobility and dispersal, using capture-recapture techniques. The resulting data would allow the construction of more exact forecasting models for the population dynamics of pest slugs, potentially leading to a reduction in the use of control agents, particularly chemical molluscicides.

INTRODUCTION

Slugs are important pests that attack a wide range of agricultural and horticultural crops (Godan, 1983; Port & Port, 1986; Glen, 1989; Willis *et al.*, 2006). Slug control is typically achieved by treating crops with biologically effective quantities of molluscicides (containing either metaldehyde, methiocarb or iron phosphate) or more recently by pathogenic nematodes (Ester & Wilson, 2005). Only the construction of reliable forecasting models for slug population dynamics can facilitate successful biological and integrated control of the slug pests and reduce common mass usage of molluscicides as a precautionary measure, rather than their prophylactic release (Bohan *et al.*, 1997). In the studies of slug spatial and population dynamics (Barker, 1991; Symondson *et al.*, 1996, 2002; Bohan *et al.*, 1997, 2000; Shirley *et al.*, 2001; Schley & Bees, 2002, 2003; Choi *et al.*, 2004, 2006; Willis *et al.*, 2006) the basic data originate mainly from either laboratory experiments, including gut-content analyses of slug predators, or from different sampling and trapping surveys, of which soil sampling and flooding techniques (Glen, Wiltshire & Milsom, 1992) have recently become the most commonly used. Slugs are usually strongly spatially aggregated (Bohan *et al.*, 2000), and thus estimating slug density, dispersal or other population characteristics from randomized sampling in the field introduces important bias into the data.

Until now, studies of slug population dynamics, as well as studies of treatment effects on pest populations, have suffered a serious limitation resulting from the lack of reliable methods of individual marking for mark-recapture studies. Mark-recapture methods have undergone remarkable progress during the last 15 years, owing to the introduction of regression-based analytical techniques (Lebreton *et al.*, 1992; Schwarz & Seber, 1999; Brooks, Catchpole & Morgan, 2000) and the development of appropriate software (e.g. White & Burnham, 1999). These developments now allow more flexible and reliable model selection and hence more detailed analyses of survival rate, capture probability, recruitment and movement probabilities, including testing the influences of external effects on animal demography parameters.

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Crucial prerequisites of reliable marking are equal survival of marked and unmarked individuals, easy readability and no loss of marks, as well as quick and easy execution of marking and low cost (Petersen, 1896; Krebs, 1989; Schwarz & Seber, 1999; Williams, Nichols & Conroy, 2001). None of the methods used for slugs have so far met all these criteria. Richter (1976) marked slugs using liquid nitrogen. The mortality of treated animals was about 30% (Hogan & Steele, 1986) and the method was recommended for marking adult individuals only, as juveniles had to be remarked due to their rapid growth. Müller & Ohensorge (1985) applied methylene blue, but found this to be ineffective as the duration of the dye was only 6 weeks. Hogan & Steele (1986) used a Panjet dental inoculator for dye-marking of *Deroceras reticulatum* in the laboratory, but the marking of dark individuals proved very problematic. Moreover, in the field experiment, the slugs had to be intensively examined to reveal whether they were marked or not. Finally, Grimm (1996) proposed the use of magnetic transponders for individual marking of large arionid slugs. Advantages of her method included high number of different codes, long durability and reliable readability. However, the method is extremely expensive for mass usage and cannot be used for smaller species such as *D. reticulatum* (O. F. Müller, 1774), one of the most economically important slug pests in temperate climates (South, 1992).

In our study, we introduce a new, cheap, fast and reliable method for marking slugs, using biologically inert UV-fluorescent dye. We report on three experiments demonstrating that the marking technique does not influence survival, prey choice between marked slugs and unmarked controls by a generalized slug predator, the beetle *Pterostichus melanarius* (Illiger, 1798), or spatial behaviour or daily activity of marked slugs, under controlled semi-natural conditions.

METHODS

Sample localities, collection and experimental preparations

Adult slugs *Deroceras reticulatum*, *Arion vulgaris* Moquin-Tandon, 1855 (= *lusitanicus* auct. non Mabille, 1868) and *Limax cinereoniger* (Wolf, 1803), as well as *Pterostichus melanarius* beetles, were collected from arable fields around Ceske Budejovice, Czech Republic (49°02′N, 14°30′E). Both slugs and beetles

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were housed individually in clear plastic containers ($8 \times 10 \times 5$ cm deep) half filled with moist soil substrate (Aros Ltd) and maintained in a controlled environment (light:dark 12:12; 20 ± 1°C) prior to the experiment. The beetles were fed *ad libitum* on a diet of mixed pork and beef and then starved for 7 days, to ensure the same nutritional state in all individuals. The slugs were fed on fresh vegetables, also *ad libitum*.

UV Fluorescent dye

A 10% liquid solution of biologically inert powder Radglo® JST UV-fluorescent pigment (Radiant colour N.V.) was created by dilution in polyethylene glycol (av. mol. wt.: 400; Sigma–Aldrich). The solution was subsequently diluted 1:1 in distilled water.

Labelling

The dye was injected directly under the mantle using an insulin syringe at a very low angle to prevent injury to internal organs. To simplify manipulation, the slugs were cooled at 4°C for 20 min before dye application and held in a dry paper napkin during the application procedure. Injection into non-cooled individuals was also tested, but the manipulation was much complicated due to slugs rapidly escaping from the touch of the needle. When illuminated, using a mobile UV-lamp in the dark, the dye shone prominently through the mantle in the all marked individuals.

Deroceras reticulatum slugs were marked by an injection of 10 μl of the dye into the tail region. The larger species, *A. vulgaris* and *L. cinereoniger*, were injected twice into the mantle shield, twice into the tail region and twice into the middle of the foot (20 μl per single injection, six injections per individual). When illuminated by UV, all marking points were clearly visible.

Experiment 1: Does UV marking affect slug survival?

Twenty individuals of each slug species were marked. A further 20 individuals of each species were used as an untreated control. The slugs were continually housed individually in plastic containers half-filled with moist soil and fed on fresh vegetables, as described above. Numbers of surviving *L. cinereoniger* and *A. vulgaris* slugs were counted daily for 30 days, *D. reticulatum* for 3 weeks. Ten labelled *A. vulgaris* (having the lowest mortality in both the labelled and control groups) were then observed weekly for another 9 weeks to provide information on the stability of the dye in the slug body.

Experiment 2: Does UV marking influence defence abilities of the slug, or affect prey choice by a slug predator?

Experiments 2 and 3 were conducted under controlled conditions in mini-plots consisting of a glass container ($40 \times 60 \times 40$ cm deep) filled with a 10 cm layer of moist soil substrate and covered with mosquito netting on the top to prevent slugs from escaping. Barley seeds (approx. 500 per mini-plot) were inserted 1 cm deep into the soil and the barley was grown to a height of 10 cm before the experiment started.

Twenty *D. reticulatum* slugs were introduced into each of two containers for a 2-day period and allowed to become established and to locate possible refuges. Ten slugs were then removed from each container, labelled by the UV-dye and replaced 1 h before six *P. melanarius* beetles (3 males and 3 females) were introduced into each of the experimental containers. The experiment was then run for 7 days. At its termination, the beetles were removed and the surviving slugs collected from the surface and extracted from the soil by sieving.

Experiment 3: does UV marking influence daily activity or spatial behaviour of the slugs?

The experimental set-up is described under Experiment 2. Ten dye-labelled and ten control *D. reticulatum* were introduced into each of the two experimental containers. The number of labelled and control slugs apparent on the surface and vegetation or walls was counted after 1, 3, 6, 12, 24, 48, 72, 96, 120, 144 and 168 h using a mobile UV-lamp. The experiment was initiated 3 h after the start of the dark period (L:D 12:12; 20 ± 1°C). Thus, all observations, except the one after 12 h, were made during the dark period, when the maximum number of slugs can be expected to be active and feeding above the ground (Hommay, Lorvelec & Jacky, 1998; Grimm & Schaumberger, 2002).

RESULTS

Experiment 1

Numbers of surviving slugs were compared using Cox proportional hazard regressions for two groups (labelled *vs* not-labelled); the Cox–Mantel test was used to assess statistical significance.

Survival did not differ between the labelled and non-labelled individuals of all three slug species tested: *Deroceras reticulatum* ($P = 0.48$), *Limax cinereoniger* ($P = 0.69$) (Fig. 1). As 100% of *Arion vulgaris* survived in the both the labelled and control groups for the whole experimental period of 3 months, no statistical evaluation of their data was undertaken. The dye remained stable in 100% of surviving labelled individuals, with no signs of having been metabolized.

Experiment 2

Numbers of slugs surviving at the end of experiment, i.e. those that did not fall prey to the beetles, were compared using log-linear analysis. There was no difference in numbers of surviving/predated slugs between the experimental containers (partial/marginal $\chi^2 = 0.82/0.84$; partial/marginal $P = 0.36/0.36$) and between the UV-labelled/non-labelled individuals (partial/marginal $\chi^2 = 0.1/0.1$; partial/marginal $P = 0.76/0.76$).

Experiment 3

Repeated-measure ANOVA with repeated observations as 11 levels of dependent variable was used to compare the numbers of observed slugs with respect to slug position surface/vegetation and labelling.

As all slugs were placed on the surface at the beginning of the experiment and the slugs gradually became established and found refugia, numbers of observed slugs predictably declined in time (Fig. 2). Moreover, there was no difference in numbers of labelled and non-labelled individuals observed either on the surface or on the vegetation during the experimental period (Table 1, Fig. 2).

DISCUSSION

Previously developed marking techniques for slugs have several major disadvantages, such as low durability (Müller & Ohnesorge, 1985), poor readability of markings (Richter, 1976; Hogan & Steele, 1986), altered animal survival (Richter, 1976) or high cost (Grimm, 1996).

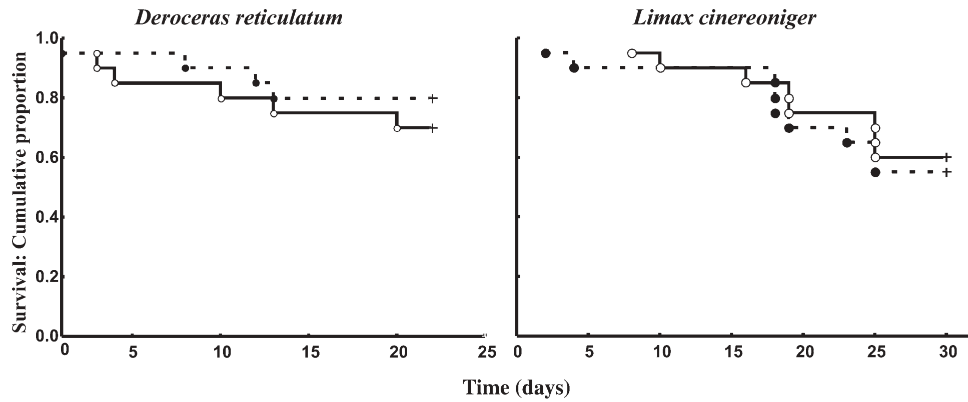

Figure 1. Survival of UV-fluorescent dye labelled (——) and non-labelled (------) *Deroceras reticulatum* and *Limax cinereoniger* slugs over a period of 25 or 30 days, respectively. The data for *Arion vulgaris* are not shown in the figure as 100% of the slugs survived for the whole experimental period.

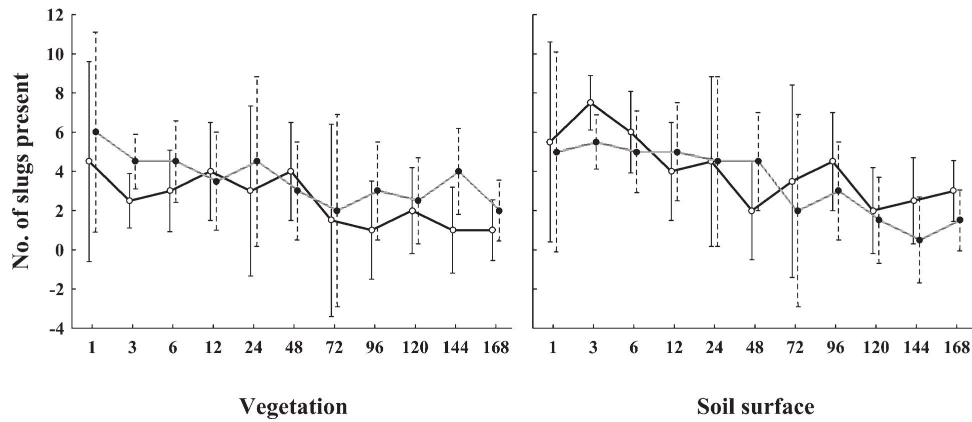

Figure 2. Number of UV-fluorescent-dye-labelled (——) and control slugs (------) apparent on the surface and vegetation/walls of two experimental containers, from the total number of 40 slugs added. The slugs were counted after 1, 3, 6, 12, 24, 48, 72, 96, 120, 144 and168 h by using mobile UV-lamp. Vertical bars denote 0.95 confidence intervals.

We have demonstrated that our cheap and fast marking technique using UV-fluorescent dye does not affect the survival of the three different slug species tested, including *Deroceras reticulatum* which cannot be marked using transponders because of its small body size. The pigment is non-toxic, safe and stable and has previously been used for tracking mammals (e.g. Lemen & Freeman, 1985) and frogs (Eggert, 2002) without causing any apparent harm. Nor does the minor injury caused by insertion of the needle seem to pose any threat to the slug's long-term survival, and the dye remained stable in all surviving individuals. Moreover, our marking technique did not increase susceptibility to predation by *Pterostichus melanarius*, which is probably because the UV-marking does not appear to affect the slugs' spatial behaviour or daily rhythms. Moreover, as there were six marking points present on larger *Limax cinereoniger* and *Arion vulgaris* slugs and there are eight recognizably different colours of dye produced, combining colour positions on the marking points would allow the individual marking of large numbers of individuals, at least in the larger species. However, it is important that new data on absolute densities obtained using our technique should be compared with the results of sampling using other techniques such as baiting (Duthoit, 1961; Webley, 1962; Bailey & Wedgewood, 1991) or trapping (Ökland, 1929; Purkhauser, 1991), in order to test its reliability.

As has previously been demonstrated by Grimm, Paill & Kaiser (2000), Grimm & Paill (2001) and Grimm (2001) for *A. vulgaris*, data on spatial position over time, and other aspects of behaviour of mark-recaptured slugs, can provide new insights into diurnal activity and resource use, hence opening a wide array of slug research possibilities so far largely

Table 1. Comparison of numbers of slugs observed with respect to slug position (surface/vegetation) and labelling by UV-fluorescent dye (repeated-measure ANOVA).

	SS	DF	MS	*F*	*P*
	1,022.73		1,022.73		<0.001
Intercept		1		79.65	
Marking: UV-labelled/not	1.14	1	1.137	0.09	0.78
Position: surface/ vegetation	11.64	1	11.64	0.91	0.39
Marking*Position	16.41	1	16.41	1.28	0.32
Error	51.36	4	12.84		
Time	131.02	10	13.10	8.94	<0.001
Time*Marking	3.114	10	0.31	0.21	0.99
Time*Position	26.61	10	2.66	1.82	0.09
Time*Position*Marking	29.34	10	2.93	2.0	0.06
Error	58.63	40	1.47		

Deroceras reticulatum slugs were introduced in two experimental containers. Number of UV-labelled and control slugs apparent on the surface and vegetation/walls was counted after 11 different time periods, from 1 to 168 h, using mobile UV-lamp. SS, sum of squares; MS, model sum of squares.

available only to those studying vertebrates (e.g. Ramsey, 2005) or insects (e.g. Nowicki *et al*., 2005).

The combination and comparison of existing and new methodologies could in turn permit the construction of more reliable forecasting models for slug populations, thus potentially leading to a reduction in the use any control agents, particularly chemical pesticides.

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Influence of slug defence mechanisms on the prey preferences of the carabid predator *Pterostichus melanarius* (Coleoptera: Carabidae)

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Abstract. Two-choice experiments on prey preferences of a generalist predator *Pterostichus melanarius*, and five species of slug prey, were conducted in the laboratory. Different preferences of *P. melanarius* for each of the slug species are described. They are interpreted as the outcome of differing slug species-specific defence mechanisms. The influence of hunger level, temperature, day/light period, condition of slugs and beetles, weight of slugs and beetles, and the sex of beetles were controlled experimentally or statistically. The order of slug species preference for predation by *P. melanarius* was: *Deroceras reticulatum* (Agriolimacidae), *Malacolimax tenellus*, *Lehmania marginata* (Limacidae), *Arion distinctus* and *A. subfuscus* (Arionidae). Efficiency of slugs' species-specific defence mechanisms reflected their phylogeny. Defence mechanisms of slugs from the superfamily Arionoidea were significantly more effective at deterring an attack of non-specialised ground beetles than the defence mechanisms of slugs from Limacoidea superfamily. *P. melanarius* significantly preferred Agriolimacidae to Limacidae, and Limacidae to Arionidae. Slug species was the strongest factor influencing prey preferences of *P. melanarius* amongst slug prey. Surprisingly, this preference was much more significant than the slug weight. Weight and sex of *P. melanarius* had no impact on its prey preference.

INTRODUCTION

Slugs are important pests of many agricultural crops which are killed and consumed by a range of invertebrate and vertebrate predators (Stephenson, 1965; Tod, 1973; South, 1980; Symondson et al., 1997; Nyffeler & Symondson, 2001). Ground beetles (Coleoptera: Carabidae) are the major natural enemies of slugs that are being considered as biological control agents, an alternative to molluscocides (Altieri et al., 1982; Asteraki 1993; Symondson & Lidell, 1993a, b; Symondson, 1994; Kromp, 1999; Cross et al., 2001). Since slugs posses defence mechanisms which may deter some predator attacks, slug defences are studied intensively as the role of potential predators in pest control can only be fully evaluated with a detailed understanding of slug behaviour (Mair & Port, 2001). The crucial assumption of this study was that slug species vary in behavioural defences (Deyrup-Olsen et al., 1986; Pakarinen, 1993a, b). Hence, I expected that they might vary in efficiency of their defence mechanisms as well. This should influence prey preference of the ground beetles. Former studies have investigated single defence mechanisms (Martin et al., 1983; Deyrup-Olsen et al., 1986; Pakarinen, 1992, 1993a, b; Symondson, 1997; Mair & Port, 2002) or dealt with separate factors influencing prey preferences of ground beetles (Pollet & Desender, 1985; Digweed, 1993; Ayre, 2001; McKemey et al., 2001; Langan et al., 2001). This study compares the preference of *Pterostichus melanarius* Illiger, 1798 among five slug species while controlling for the hunger level of the predator, temperature, day/light period, condition of slugs and beetles, weights of slugs and beetles, sex of the beetles and predator-prey encounter rates (Wareing & Bailey, 1985; Ernstig & Vanderwerf, 1988; Wheater, 1988a, 1991; Wallin & Ekbom, 1994; Ayre, 2001; McKemey et al., 2001, McKemey et al., 2003). Different prey preferences by *P. melanarius* are interpreted as outcomes of differing species-specific defence mechanisms of slugs.

MATERIAL AND METHODS

Choice of a suitable model species of ground beetle

P. melanarius is a readily available, non-specialised and well known predator of slugs and therefore was chosen as a suitable model for testing efficiency of slug defence mechanisms through its prey preferences amongst slug-prey. *P. melanarius* has proved to be a potentially suitable control agent for controlling slugs (Symondson & Liddell, 1993a; Symondson et al., 1996) and has been chosen as a model species for investigating the relationship between ground beetles and their slug-prey in several former studies (Wheater, 1988a; Symondson, 1997; Symondson et al., 1996, 1997, 2000; Bohan et al., 2000; McKemey et al., 2001, 2003). Specialised predators like *Carabus violaceus* or *Cychrus caraboides* are unsuitable for testing the efficiency of slug defence mechanisms because they paralyze/kill slugs in the majority of their first attacks and so prevent slugs from exuding mucus and the use of other active defence mechanisms (Pakarinen, 1993b). Other species of non-specialised ground beetles have been examined in former studies as control agents or used as model predators of slugs – i.e. *Abax parallelepipedus* (Symondson, 1989, 1994; Asteraki, 1993; Symondson & Lidell, 1993a), *Scaphinotus striatopunctatus* (Altieri, 1982; Digweed, 1994), *S. marginatus* (Digweed, 1993), *Pterostichus madidus* (Asteraki, 1993; Ayre, 2001; Mair & Port, 2001, 2002), *P. niger* (Pakarinen, 1993b), *P. adstrictus*, *P. pensylvanicus*, *P. protractus*, *P. surgens* (Digweed, 1993), *Nebria brevicollis* (Ayre, 2001; Mair & Port, 2001, 2002), *Harpalus rufipes*, *H. aeneus* (Ayre, 2001). However, these species

either do not occur in the Czech Republic or were not available in as high numbers as *P. melanarius*.

Field site and sample collection

The study was carried out in South Bohemia, Czech Republic, from July to September 2002. The beetles were collected by pitfall trapping from a cornfield near České Budějovice, South Bohemia (49°0´N, 14°30´E, alt. 400 m). Males and females were kept apart in glass containers (20 × 20 × 30 cm) filled with 5 cm of plant litter in a controlled environment (16L : 8D; 20 ± 2°C). They were fed with darkling beetle maggots (*Tenebrio molitor*) for several days. Prior to the experiment, to ensure that the beetles were of a similar nutritional state, they were put into individual container and fed ad libitum. They were than starved for 7 days (Symondson, 1997). Slow moving or injured beetles were not used for the experiment.

The following slugs were tested: *Deroceras reticulatum* Müller, 1774, *Lehmania marginata* Müller, 1774, *Malacolimax tenellus* Müller, 1774, *Arion distinctus* Mabille, 1868, *A. subfuscus* Draparnaud, 1805. They were collected in the vicinity of České Budějovice from an oilseed-rape field (*D. reticulatum*), coniferous or mixed forest (*L. marginata*, *M. tenellus*) or suburban gardens (*A. distinctus*, *A. subfuscus*). The species were kept separately in round glass containerss (8 cm in diameter, 25 cm deep), because they (especially *M. tenellus*) showed a surprisingly large mortality when kept in mixed-species groups. The containers were filled with plant litter and stored in a controlled environment (16L : 8D; 20 ± 2°C). *A. distinctus*, *A. subfuscus* and *Deroceras reticulatum* were fed on cabbage leaves, *Lehmania marginata* and *M. tenellus* on mushrooms of *Russula* spp. Food supply was replaced every 3rd day. Placing the slugs on saturated filter paper (>1h) prior to weighing ensured that all slugs were uniformly and fully hydrated. Usage of fully hydrated slugs maximised their mucus production and hence their defensive ability. Injured slugs were not used for the experiment.

Two choice experiment

At the start of the experiment a single beetle was placed in a 9-cm diameter Petri dish with two slugs. The dish was lined with filter paper and moistened daily. The sex and weight of the beetle and weight of the experimental slugs were recorded before the experiment. The experiment was carried out in a controlled environment (16L : 8D; 20 ± 2°C) and the status of the slugs, eaten or not eaten, was checked every 7 h for 3 days. Each trial was considered completed when the first slug was eaten. The slug was also considered eaten, if it was dead and heavily damaged by the beetle. The trials in which both slugs were eaten or one of them was dead but undamaged were disregarded, as were trials where the beetle died. In total, there were 10 pairwise combinations of slug species, corresponding to 400 slug and 200 beetle individuals in 200 successfully completed trials. The sex ratio of ground beetles was equal for each combination of slugs (10 males and 10 females). The relatively small Petri dishes did not enable the slugs to fully employ their species-specific antipredator strategies. For instance, the tail-wagging and fast escaping species such as *D. reticulatum* were disadvantaged compared to *Arion* spp., that rely on their skin and mucus when being attacked by *P. melanarius* (Pakarinen, 1993a, b). However, the Petri dishes highlighted species-specific differences in defence mechanisms and so ensured that the results were not disturbed by other external factors.

Statistical processing

The numbers of eaten slug individuals for all pairs of slug species were compared (against equal proportions) by Chi-square tests. 2 × 5 Contingency table was used to test total numbers of eaten slug individuals of each species against the null hypothesis that all slug species were eaten in equal proportions.

To assess if slug species, weight, or sex and weight of the beetle affected whether the slug was eaten, generalised linear modelling (GLM) multiple-regressions were used. GLMs allow for both continuous and categorical predictors, and for non-linear responses of the dependent variables, which are transformed using specific link-functions (Venables & Ripley, 2002). The regressions were computed in the program S-plus (S-Plus, 1999, 2000), assuming a binary distribution of the dependent variable (eaten vs. not-eaten) and using the link function "logit". The model construction was as follows: (1) defining a null model, which did not contain any explanatory predictors; (2) constructing single-term regressions for all potential explanatory variables; (3) constructing multiple regressions, using stepwise addition of individual terms, and comparing them with the null model using both F-test and the Akaike information criterion (AIC, weights increasingly better fit of higher-order models by their increasing complexity). Models for effects of slug family and superfamily were constructed by the same routine.

RESULTS

Total numbers of eaten/uneaten slugs

Numbers of slugs eaten in each pairwise experiment are shown in Table 1. *D. reticulatum* was eaten in significantly greater numbers than all other slugs tested. *M. tenellus* was eaten in significantly greater numbers than other slug species except *D. reticulatum*. There were no significant differences in the number of slugs eaten amongst *L. marginata*, *A. distinctus* and *A. subfuscus*.

Significant differences in the total numbers of slugs of each species eaten were found for *A. subfuscus*, *D. reticulatum* and *M. tenellus*. Differences in total numbers of eaten individuals of *L. marginata* and *A. distinctus* eaten were only marginally significant ($p = 0.060$ and $p = 0.075$; see Table 1).

TABLE 1. Numbers of slug individuals eaten for each combination of slug species in the pairwise experiments (compared by Chi-square tests against equal proportions). TN – total numbers of slugs of each species eaten in the whole experiment (2 × 5 Contingency was used to test against the null hypothesis that all slug species were eaten in equal proportions). Significant differences are indicated by an asterix.

	A. subfuscus	*L. marginata*	*D. reticulatum*	*M. tenellus*	TN	p
A. subfuscus					20*	< 0.01
L. marginata	13/7				34	0.06
D. reticulatum	18/2*	16/4*			65*	< 0.01
M. tenellus	17/3*	15/5*	5/15*		52*	< 0.01
A. distinctus	12/8	8/12	4/16*	5/15*	29	0.08

TABLE 2. Regression models showing the influences of slug species and weight, and sex and weight of the predatory beetle *Pterostichus melanarious*, on prey preferences of the beetle. A generalised linear modelling procedure is used with assumed binary distribution of the dependent variable (a slug either eaten or not eaten). The three multiple regression models (constructed via stepwise addition of significant predictors) consider slug species, slug family and slug superfamily as possible explanatory variables.

model	df	residual[1] deviance	fitted[2] deviance	AIC[3]	F[4]	P[4]
null model	399	554.5	–	556.5	–	–
single-term regressions						
slug species	4, 395	484.6	69.9	494.8	17.3	****
slug weight	1, 398	554.5	0.03	558.5	0.03	0.87
beetle sex	1, 398	554.5	< 0.01	558.5	< 0.01	0.99
beetle weight	1, 398	554.5	< 0.01	558.5	< 0.01	0.99
multiple regression models						
slug species + slug weight	5, 394	475.4	79.1	487.4	15.8	****
slug family + slug weight	3, 396	484.9	69.6	492.9	23.2	****
slug superfamily	1, 398	513.7	40.9	517.7	40.7	****

[1]Deviance in the data not accounted for by the model; [2]Deviance in the data accounted for by the model; [3]Akaike information criterion [4]F-test of null vs. fitted model. ****p < 0.0001

Multiple regressions

The single-term regressions of slug being/not-being eaten against all possible predictors revealed that slug species influenced the prey preferences of *P. melanarius* very significantly (Table 2). The slugs were again preferred in the following order: *D. reticulatum*, *M. tenellus*, *L. marginata*, *A. distinctus*, *A. subfuscus* (Fig. 1A). On the other hand, there was no separate effect of slug or beetle weight, or sex of the beetle. However, the best model constructed by the multiple regression contained slug weight in addition to slug species. This, together with the fact that slug weight was not significant in single-term regression, suggests that *within* species the beetle preferred lighter slug individuals (Fig. 1B). Inclusion of further predictors (beetle sex and weight) increased the AIC values, indicating that the predictors were redundant (see full model in Table 2). Moreover, a comparison of the fits of the final and full models did not reveal any significant differences (F = 0.53, d.f. = 2, 392, p = 0.59, delta AIC = 2.97). This indicated that the species of slug had a strong separate effect on prey preferences of the beetle even when the variability due to weight of the slug (and sex and weight of the beetle) were included in the model, acting in effect as co-variables. This indicates the importance of species-specific defence mechanisms of the slugs in carabid beetle prey selection.

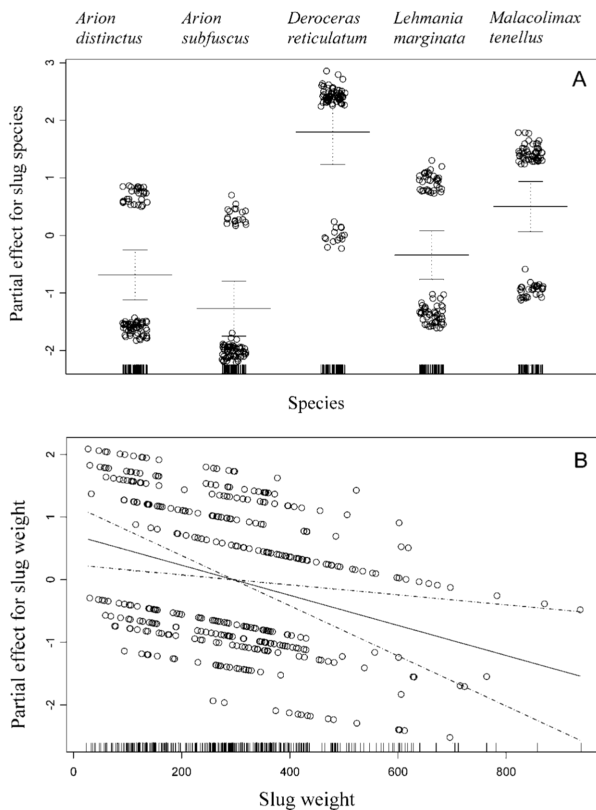

Fig. 1. Multiple GLM regression of the effects of (A) slug species (categorical predictor) and (B) slug weight (continuous predictor) on the slug being either eaten or not eaten (with assumed binary distribution). Model with slug species as one of the independent variables, see Table 2 for model statistics. The plots show partial effects of individual predictors (full lines) with respective standard errors (dotted lines). The empty circles are individual cases, the internal tickmarks on x-axis show frequencies of respective values of independent variables. Note that the values on y-axis are fitted using the logit-link function.

The models in which family/superfamily were considered instead of slug species (Table 2) showed significant effects of these taxonomic rankings. Slugs from the superfamily Limacoidea (Limacidae + Agriolimacidae) were significantly preferred to slugs from the superfamily Arionoidea (Arionidae). Slugs from the Agriolimacidae family (*D. reticulatum*) were significantly preferred to slugs from the Limacidae family (*L. marginata* and *M. tenellus*). Slugs from the Limacidae family were significantly preferred to the slugs from the Arionidae family (*A. distinctus* and *A. subfuscus*). Interestingly, slug weight was a variable in the model in which slug family was considered, but was redundant in the model in which superfamily level was the focus. This suggested that the difference in readiness of the beetle to prey on Limacids vs. Arionids outweighed the preferences due to prey weights.

DISCUSSION

The present study documents how a non-specialised ground beetle preyed upon different slug species selectively as a consequence of species-specific defence mechanisms of the slugs. Results of this study seem to be consistent with the hypothesis that the efficiency of slug defence mechanisms varies amongst species. Importantly, all other known (Wareing & Bailey, 1985; Ernsting & Vanderwerf, 1988; Wheater, 1988a, b, 1991; Wallin & Ekbom, 1994; Honěk, 1997; Ayre, 2001; McKemey et al., 2001) or possible confounding influences (i.e., hunger level, temperature, day/light period, condition of slug or beetle, weights of slugs and beetles, sex of the beetles) were controlled for experimentally or statistically. This further supports the notion that the different preferences of *P. melanarius* were due to differences in efficiency of species-specific defence mechanisms of slugs. In several earlier studies (Thiele, 1977; Ernsting & Vanderwerf, 1988; Wheater, 1988a; Ayre, 2001; McKemey et al., 2001), the carabids were considered to be generalist predators, which feed on almost any suitable prey they meet and are able to subdue. The authors mentioned above considered weight of the slug to be the strongest factor influencing prey preferences of the ground beetles eating slug-prey.

P. melanarius preferred slugs from superfamily Limacoidea (Limacidae, Agriolimacidae) to slugs from superfamily Arionoidea (Arionidae); Agriolimacidae (*D. reticulatum*) to Limacidae (*M. tenellus*, *L. marginata*); and Limacidae to Arionidae (*A. distinctus*, *A. subfuscus*). It follows that efficiency of slug defence mechanisms reflects phylogenetic positions of the slugs as postulated by Wiktor (1984) and Reischütz (1999). This should be taken into account, especially in studies examining the potential of ground beetles as biocontrol agents against slug pests. In particular, *D. reticulatum*, as the most preferred slug prey in this study, might not be a suitable model for other slug pests.

Most limacids are fast-moving slugs with soft skin and a slender body (Godan, 1983; Pakarinen, 1993b) that react to an attack by the production of alarm mucus and tail-wagging, accompanied by a burst of speed. The tail-wagging engages attackers attention giving the victim time to escape or hide. Limacids lift up the anterior part of their body and distend their tentacles to orientate towards shelters. On the other hand, slow moving Arionids respond by stopping and withdrawing the head under the mantle (Rollo & Wellington, 1979), relying on their tough skin for protection (Godan, 1983, Pakarinen, 1992). Heparan sulphate was identified in mucus of *Arion lusitanicus*, *A. ater* and *A. rufus* (Wright et al., 1997; Ditrich, 2001). In the Arionidae, autotomy has been demonstrated only in the Anadeninae subfamily (Pakarinen, 1993a).

In this study, the slugs had no chance to hide effectively and their escape strategies were constrained by the 9cm diameter of the Petri dish. The most preferred species (*D. reticulatum* and *M. tenellus*) are able to autotomize their tail when attacked (Pakarinen, 1993a). Wagging of body parts can decrease their defence ability if their escape fails and the predator attacks them again in the dish. However, the significant differences in preference of *P. melanarius* between *D. reticulatum* and *M. tenellus* can not be explained in this way, because these species are similar in their autotomy/escape strategies (Pakarinen, 1993a). Moreover, attempted escapes of a slug from the place of attack in Petri dish increased the probability of *P. melanarius* encountering another slug species, which could be regarded as a successful use of escape strategy by the attacked slug. Despite this bias, usage of relatively small Petri dishes reduced the efficiency of hide/escape strategies of the slugs. This emphasized species-specific differences in the efficiency of slug defences, and highlighted their importance for Carabid-beetle prey selection.

The species-specific defence of slugs had a stronger effect on prey preference of *P. melanarius* than the prey weight, which had a significant effect only within slug species. The probability that the predator will eat a slug decreased log-linearly with slug weight. This corresponds with findings in other studies (Ernsting & Vanderwerf, 1988; Wheater, 1991; Ayre, 2001; McKemey et al., 2001). However, all these studies dealt in fact only with a partial prey-size preference. The rates of feeding on slugs of different sizes differ between the field and laboratory conditions as a consequence of slug-size related behavioural differences and other factors, which can only be simulated in the laboratory (Mckemey et al., 2003).

Sex of *P. melanarius* had no effect on its prey-preference. This corroborates the findings of Kielty et al. (1999) and Digweed (1993), who studied feeding preferences of carabid beetles on aphids and collembolans, and terrestrial snails. The missing effect of sex might liberate further researchers from considering it in experimental design, and thus considerably reduce the requirements for material.

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