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Molecular analysis of predation by carabid beetles (Carabidae) on the invasive Iberian slug *Arion lusitanicus*

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Abstract

The invasive Iberian slug, *Arion lusitanicus*, is spreading through Europe and poses a major threat to horticulture and agriculture. Natural enemies, capable of killing *A. lusitanicus*, may be important to our understanding of its population dynamics in recently invaded regions. We used polymerase chain reaction (PCR) to study predation on *A. lusitanicus* by carabid beetles in the field. A first multiplex PCR was developed, incorporating species-specific primers, and optimised in order to amplify parts of the mitochondrial cytochrome c oxidase subunit 1 (*cox1*) gene of large *Arion* slugs, including *A. lusitanicus* from the gut contents of the predators. A second multiplex PCR, targeting *12S rRNA* mtDNA, detected predation on smaller *Arion* species and the field slug *Deroceras reticulatum*. Feeding trials were conducted to measure the effects of digestion time on amplicon detectability. The median detection times (the time at which 50% of samples tested positive) for *A. lusitanicus* and *D. reticulatum* DNA in the foreguts of *Carabus nemoralis* were 22 h and 20 h, respectively. Beetle activity-densities were monitored using pitfall traps, and slug densities were estimated using quadrats. Predation rates on slugs in the field by *C. nemoralis* in spring ranged from 16–39% (beetles positive for slug DNA) and were density dependent, with numbers of beetles testing positive being positively correlated with densities of the respective slug species. *Carabus nemoralis* was shown to be a potentially important predator of the alien *A. lusitanicus* in spring and may contribute to conservation biological control.

Keywords: *Carabus nemoralis*, *Deroceras reticulatum*, gut content analysis, multiplex PCR, predator-prey interactions

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Introduction

Predator-prey interactions are an essential part of any understanding of the population dynamics of the species involved. The prey preferences of non-specialist generalist predators are determined by complex interactions between factors such as encounter rates, switching behaviour, relative densities of potential prey, functional responses, availability of refugia and the defensive capabilities of the prey (Begon *et al.*, 2000; Symondson *et al.*, 2002a). Analysis of predation in the field, where multiple prey species are available, can provide

essential background information for evaluating potential biological control agents for pest species. Such analyses are particularly useful for identifying natural enemies that are capable of attacking alien species, such as the Iberian slug *Arion lusitanicus* Mabille (*sensu* Altena 1955) in Norway.

Arion lusitanicus (also recognised as *A. vulgaris* Moquin-Tandon 1855 (Anderson, 2005)) is spreading through large parts of northern Europe (von Proschwitz, 1992, 1994; Dolmen & Winge, 1997; Grimm *et al*., 2000) and was first recorded in Norway in 1988 (von Proschwitz & Winge, 1994). The species is causing major concerns for gardeners, horticulturalists and farmers (Hofsvang, 2003), causing damage to crops such as strawberries and devaluing grass fodder for cows. There is also a concern that these slugs may have negative effects on native slugs such as the closely related *A. ater*, by invading their habitats (von Proschwitz, 1997). The success of alien pests is sometimes explained by the enemy release hypothesis (Keane & Crawley, 2002; Mitchell & Power, 2003; Torchin *et al*., 2003), in which the pests thrive when released from native natural enemies that have evolved to feed on them. Populations of *A. lusitanicus* are reaching plague proportions in some areas (von Proschwitz, 1992), thus development of new pest management is necessary. Molluscicidal baits are the most commonly used control against gastropods, although formulations have sometimes been either too weak, so that gastropods do not ingest a lethal dose before they are sated, or too strong, which means they reject the baits immediately (Barker, 2002). Furthermore, molluscicides cause collateral damage to other organisms, especially methiocarb, which is poisonous for mammals such as dogs and hedgehogs (Bailey, 2002). Thus, there is a need to identify native predators that are capable of killing and consuming these alien slugs. Only then can pest management strategies be devised that foster these potentially useful natural enemies.

Many carabid beetles are opportunistic generalist predators and have been shown to be beneficial to agriculture as predators of pest species (Thiele, 1977; Luff, 1987; Lövei & Sunderland, 1996; Kromp, 1999; Holland, 2002). Symondson (2004) reviewed carabids and other beetle groups as natural enemies of molluscs, concluding that many polyphagous predators in these groups are important gastropod predators. Many studies have focused on the generalist *Pterostichus melanarius* (Ayre, 2001; McKemey *et al*., 2001, 2003, 2004; Oberholzer & Frank, 2003; Langan *et al*., 2004), which is a major predator of slugs in arable fields (Symondson *et al*., 1996, 2002a; Bohan *et al*., 2000). *Pterostichus melanarius* has been shown to aggregate to areas of high slug densities (Bohan *et al*., 2000) and to be capable of developing a semi-coupled relationship with slugs between years (Symondson *et al*., 2002a). Most work to date has been on the field slug *Deroceras reticulatum*, while only a few studies have dealt with *A. lusitanicus* (Paill, 2000, 2004; Paill *et al*., 2002; Oberholzer & Frank, 2003).

The versatility of PCR-based methods for detecting predator-prey interactions is now well recognised and is the most practical approach where prey are soft bodied, leaving no recognisable hard parts in the guts or faeces of predators (Symondson, 2002). These techniques are highly sensitive and have been used to detect predation on a wide range of species (Zaidi *et al*., 1999; Agusti *et al*., 2003; Harper *et al*., 2005; Traugott & Symondson, 2008). Slugs such as *D. reticulatum* and *A. hortensis* have been found to be detectable after approximately 30 h in the gut of the carabid beetle *P. melanarius* (Harper *et al*., 2005). Multiplex PCRs, in particular, provide an efficient means of disentangle trophic interactions by detecting many species at the same time in each predator gut sample (Harper *et al*., 2005; King *et al*. 2010a,b).

Here, our aim was to design and optimize multiplex PCRs to detect predation on arionid slugs, including the invasive Iberian slug *A. lusitanicus*, and to measuring rates of predation in the field. Symondson (2004) emphasized the need to identify key predator species that are numerous enough to affect prey populations. *Carabus* species are generally regarded as oligophagous specialists on earthworms and gastropods, with mouthparts adapted for killing and feeding on such prey (Hengeveld, 1980a,b; Evans & Forsythe, 1985). In many parts of Europe, including Scandinavia, *Carabus nemoralis* Müller is widespread and common in agricultural landscapes (Lindroth, 1985; Turin *et al*., 2003). It is also active during the spring (Lindroth, 1985) when individuals of *A. lusitanicus* are small enough for the beetles to be able to prey upon them (Hatteland *et al*., 2010). Laboratory experiments have found that *C. nemoralis* is capable of killing and consuming *A. lusitanicus* up to 1.3 g, with a preference for slugs <1 g (Hatteland *et al*., 2010). These experiments found no preference for *D. reticulatum* over the more recently introduced *A. lusitanicus*. We, therefore, hypothesised that *C. nemoralis* has no specific preferences for any slug species within the *Arion* and *Deroceras* genera and that rates of predation, detected using PCR, would simply reflect relative population densities of different slug prey.

Materials and methods

Field site

The carabid beetles *C. nemoralis*, *P. melanarius* and *P. niger* were sampled at two sites, a meadow in Bergen (60°38′N, 5°34′E) and a site with strawberry patches surrounded by set-aside land in Askøy (60°28′N, 5°12′E), western Norway. The molluscicide Ferramol® and the herbicide Roundup® were used in the latter field by the farmer. Temperature was measured hourly in the field by data logger (Dickson HT100) during the sampling periods.

Beetles collected for gut content analysis

Sampling was by dry pitfall traps arranged in arrays of eight traps at each sample point. Beetles trapped in any of the eight traps were pooled as one sample. In the meadow, the traps were arranged in a total of 21 circles randomly spread out across the field, where each circle of traps represented one sample with minimum distance of three meters between circles. However, at the site with strawberry patches, we used nine transects from surrounding set-aside land into the strawberry patches. Each transect consisted of 4–8 sampling points, depending on the size of the respective strawberry patch, with four meters between each sampling point (fig. 1). Half of the sample points were set up in the set-aside (meadow or grassland) and half were set up within a strawberry patch. A total of 57 sample points were used at this site, each comprised of eight traps in double lines (456 pitfall traps in total).

Traps were plastic cups, 7-cm wide × 9.5-cm deep, buried to the rim and covered by a metal roof *ca*. 2 cm above the rim. Each trap had a 2.4 mm mesh insert through which smaller prey could fall, reducing potential predation by the beetles after being caught. The traps were emptied twice per day, in

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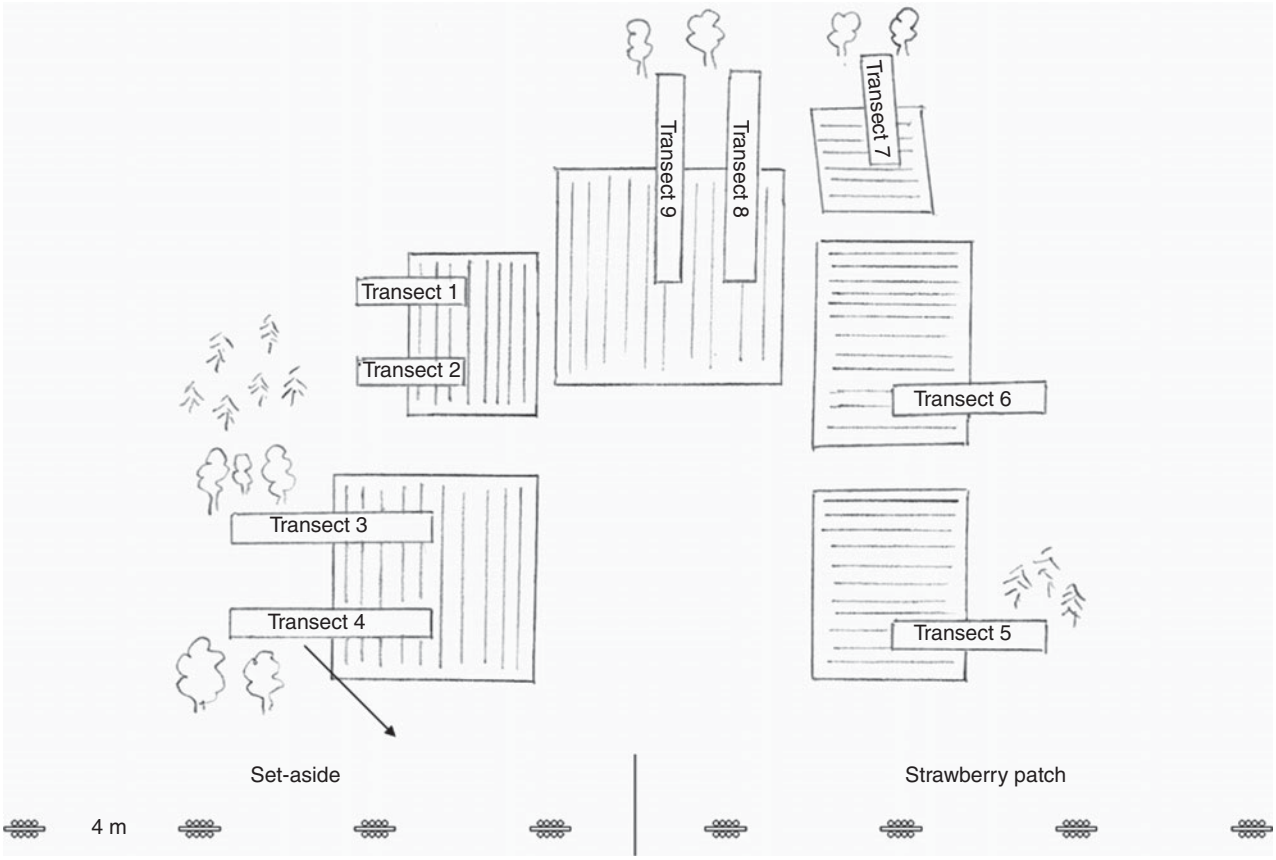

Fig. 1. Map of the strawberry patches surrounded by set-aside land at the Askøy site outside Bergen. The strawberry patches are represented as squares while the sampled transects are represented as rectangles. A schematic view of a transect is given below the map with pitfall traps represented as circles and searching plots for gastropods represented as rectangles. The line in the middle of the transect represents the edge of the strawberry patch.

the morning and in the evening, to minimise digestion times and possible predation within the traps. In the meadow, pitfall trapping was performed from 13 May to 30 June and 4 September to 7 October 2006, plus a short supplementary period from 21 June to 28 June in 2007. The strawberry site in Askøy was sampled from 24 April to 1 May and from 18 September to 27 September 2007. Beetles were transported to the laboratory and killed at −20°C in spacious plastic boxes with *ca*. 2 cm of sphagnum moss peat to reduce regurgitation due to stress. The beetles were then removed from the boxes into individual plastic tubes and stored at −80°C. Since pitfall trapping is not an absolute measure of density (Thiele, 1977; Luff, 1982, 2002), we use the term 'activity-density' thorough this paper when referring to the abundance of beetles.

Molluscs sampled for field density analyses

The field density of slugs and snails were determined in 109 quadrats associated with each cluster of pitfall traps. In addition, eggs of *A. lusitanicus* were counted in the field. Eleven, ten, ten and 21 quadrats were sampled in the meadow site at 11 May 2006, 8 June 2006, 12 October 2006 and 1 July 2007, respectively. All the quadrats used in the meadow site were 50 × 50 cm. Furthermore, 57 quadrats, each 20 × 125 cm, were sampled in the strawberry site, including set-aside land. Each quadrat was situated 50 cm from each set of eight pitfall traps representing one sample point. The vegetation within the quadrat was thoroughly searched for molluscs and was then cut *ca*. 2 cm above ground level and the ground searched. Finally, the remaining moss was stripped away, and we searched carefully around any plant bases remaining. We made an attempt at the strawberry site to locate molluscs lying below the soil surface by taking one circular soil sample of 10 cm depth and 10 cm in diameter from each quadrat, which was then hand sorted. All molluscs were counted and weighed to the nearest 0.1 g except slugs <0.1 g, which were denoted as 0.05 g. The slugs were released after weighing at the points where they were collected to avoid locally reducing populations.

Feeding experiments

Deroceras reticulatum slugs and juvenile slugs of *A. lusitanicus*, as well as *C. nemoralis* beetles, were sampled along the perimeter of the meadow field. Slugs were maintained in plastic bags with *ca*. 2 cm of sphagnum moss peat at 4 – 6°C prior to the feeding experiments. The beetles were kept at 14°C in a climate chamber (RUMED® Rubarth Apparate GmbH), simulating the light conditions from the field following a light intensity regime of 50% from 6:00 to

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Table 1. Primers (5′–3′) for detection of slugs in carabid beetles foreguts.

Multiplex	Species	Primer	Primer sequence	Fragment size (bp)	Annealing temperature (°C)	Reference
Cox1	*Arion lusitanicus*	A.l.-Co1-F1	GCCCCCATCTTTACTTTTACTTATTTGCTCC	310	51	New
		A.l.-Co1-R2	GTATGGTAATAGCCCCCGCCAATACG - FAM			
	A. ater	A.a.-Co1-F-new	CACCACTGAGAGGAGCC	225	51	New
		A.a.-Co1-R1	GCTCCAGCCAATACAGGTAAAG			
	A. rufus	A.r.-Co1-F1	MTTACTTATCGGTGCGC	362	51	New
		A.a.-Co1-R1	GCTCCAGCCAATACAGGTAAAG - FAM			
12S rRNA	*Deroceras reticulatum*	DR11F	CTATACACAATTTTTAAATAAGC	109	53	Dodd, 2004
		DRF29RC	GTCTCTGGTTTATCTATTATTGGT			
	Arion spp.	Ai1F	CACATAAATGATAGTCACC	221	53	Dodd, 2004
		AR2R	ATACTTACAAGTCCATCTTT			

FAM, fluorescent label.

8:00, 100% from 8:00 to 20:00 and 50% from 20:00 to 22:00 followed by darkness from 22:00 to 6:00. The beetles were maintained on earthworms (*Lumbricus rubellus*) then starved for ten days prior to the feeding experiments. Feeding experiments were performed in Petri dishes containing moistened filter paper with two live slugs per dish under the same climate conditions as the starvation period. Two slugs were chosen to increase the probability of predation within a 2-h feeding period. Beetles were killed in batches of eight beetles (five males and three females) at 10 h, 20 h, 40 h and 60 h post feeding, and stored at −80°C. Unfed beetles were kept as negative controls and non-feeding beetles were discarded from the experiment.

Beetle dissections

The beetles were defrosted then dissected. The foreguts were removed, weighed and stored in microfuge tubes at −80°C prior to DNA extraction. Forceps and scalpel was sterilized between beetles by cleaning in 96% ethanol and open flame.

DNA extractions

DNA was extracted from beetle foreguts and non-target organisms for the cross-amplification test using the DNeasy Blood & Tissue Kit (Qiagen), following the manufacturer's instructions. Extraction negatives (no tissue) were included for all sets of extractions to test for possible contamination during the extraction process. Extractions were stored in elution buffer at −80°C. DNA was extracted from mollusc foot fringes using the E.Z.N.A. tissue kit (Omega Bio-Tek) following manufacturer's instructions and stored in elution buffer at 4°C.

Primer design

The universal invertebrate primers LCO1490 and HCO2198 designed by Folmer *et al.* (1994) were used to amplify the cytochrome c oxidase subunit 1 (*cox1*) gene of *A. lusitanicus*, *A. ater* and *A. rufus*. Each PCR was conducted in 25 µl, containing 12.5 µl Promega PCR Mastermix (Promega), 1.0 µl of each primer (10 µM), 2.0 µl DNA (10:1 diluted) and 5.5 µl distilled H_2O. PCRs were carried out in a MJ Research PTC220 Peltier thermal cycler, with cycling conditions of 94°C for 2 min, followed by five cycles of 92°C for 60 s, 45°C for 1.5 min, 72°C for 1.5 min, then 30 cycles of 92°C for 30 s, 50°C for 60 s and 72°C for 50 s and a final cycle of 72°C for 7 min. PCR products were checked and visualized on 1% agarose gels following standard procedures, before purification with ExoSap-IT (GE Healthcare) and sequenced with version 3.1 Big Dye terminator chemistry on an ABI3700 capillary sequencer (Applied Biosystems). The sequences were aligned using the software BioEdit, including the multiple alignment function of CLUSTAL W (Hall, 1999). Species-specific primers were designed for *A. lusitanicus*, *A. ater* and *A. rufus* (table 1) by using the software PRIMER 3 (Rozen & Saletsky, 1996–1998).

PCR amplification and optimization

To avoid false negatives, extractions were tested by PCR for the presence of amplifiable DNA using general invertebrate primers for a 710 bp fragment of the mitochondrial *cox1* gene (Folmer *et al.*, 1994). PCRs were performed in 12.5 µl, containing 6.25 µl GoTaq (Promega) Mastermix, 0.5 µl of each primer (10 µM), 0.125 µl BSA, 1.0 µl DNA and 4.125 µl dH_2O. PCRs were carried out in a MJ Research PTC220 Peltier thermal cycler, with cycling conditions of 94°C for 1.5 min, followed by five cycles of 94°C for 30 s, 45°C for 1.5 min, 72°C for 1 min, then 35 cycles of 94°C for 30 s, 51°C for 1.5 min and 72°C for 1 min and a final cycle of 72°C for 5 min. Any samples that failed to amplify were excluded from further analyses.

Two diagnostic multiplex PCRs were used to simultaneously screen each predator gut sample for the presence of multiple slug species. The first multiplex PCR (*cox1* multiplex PCR) amplified short fragments of the *cox1* gene from *A. lusitanicus*, *A. ater* and *A. rufus*. The assay was optimized for gut content analysis by adding the PCR facilitator bovine serum albumin (BSA), which has been found to overcome PCR-inhibition in gut-content samples (Juen & Traugott, 2006). The second multiplex PCR targeting the small mitochondrial ribosomal gene (*12S rRNA* multiplex PCR) and contained primers for *D. reticulatum* and an *Arion* genus-specific primer pair (Dodd, 2004: table 1). The latter primer pair amplifies different sized fragments for each *Arion*

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Table 2. Cross-amplification tests on non-target prey, target prey and predators using each of the two multiplex PCRs (*cox1* and *12S rRNA*) separately.

Order	Family	Species	*Cox1*	*12S rRNA*
OLIGOCHAETA	Enchytraeidae		N	N
	Lumbricidae	*Lumbricus rubellus*	N	N
		Aporrectodea caliginosa	N	N
PULMONATA	Helicidae	*Cepaea hortensis*	N	N
		Arianta arbustorum	N	N
	Cochliocopidae	*Cochlicopa lubrica*	N	N
	Endodontidae	*Discus rotundatus*	N	N
	Arionidae	*Arion distinctus*	N	221 bp
		A. fasciatus	N	N
		A. silvaticus	N	N
		A. circumscriptus	N	N
		A. fuscus	N	N
		A. lusitanicus	310 bp	230–231 bp
		A. ater	225 bp	230 bp
		A. rufus	362 bp	250, 231 bp
	Agriolimacidae	*Deroceras reticulatum*	N	109 bp
		D. laeve	N	N
	Limacidae	*Limax maximus*	N	N
	Boettgerillidae	*Boettgerilla pallens*	N	N
MESOSTIGMATA			N	–
ORIBATIDA	Euzetidae	*Euzetes globulus*	N	N
	Damaeidae		N	N
ARANEAE	Lycosidae	*Pardosa* sp.	N	N
		Trochosa sp.	N	N
	Linyphiidae		N	N
OPILIONES	Nemastomatidae	*Nemastoma lugubre*	N	N
		Mitostoma chrysomelas	N	N
PSEUDOSCORPIONES	Neobisiidae	*Neobisium carcinoides*	N	N
CRUSTACEA	Oniscidae	*Oniscus asellus*	N	N
DIPLOPODA	Julidae		N	–
	Polydesmidae	*Polydesmus* sp.	N	–
CHILOPODA	Lithobiidae	*Lithobius* sp.	N	N
COLLEMBOLA	Isotomidae		N	N
	Entomobryidae		N	N
	Tomoceridae	*Tomocerus* sp.	N	N
	Sminthuridae		N	N
HETEROPTERA	Petatomidae		N	N
	Reduviidae		N	N
HOMOPTERA			N	N
COLEOPTERA	Carabidae	*Pterostichus melanarius*	N	N
		P. niger	N	N
		P. diligens	N	N
		Nebria brevicollis	N	N
		Carabus nemoralis	N	N
		Trichocellus placidus	N	N
		Harpalus latus	N	N
	Staphylinidae	*Staphylinus erythropterus*	N	N
		Hygronoma sp.	N	N
		Stenus sp.	N	N
		Atheta sp.	N	N
		Quedius sp.	N	N
	Silphidae	*Phosphuga atrata*	N	N
	Byrrhidae		N	N
	Chrysomelidae		N	N
	Geotrupidae	*Geotrupes stercorarius*	N	N
	Ptilidae		N	N
	Curculionidae		N	N
DIPTERA	Tabanidae	*Haematopota* sp.	N	N
	Tipulidae		N	N
HYMENOPTERA	Formicidae	*Formica* sp.	N	N

–, not tested; N, no amplification.

species (Dodd, 2004; Harper *et al.*, 2005). PCRs were conducted in 12.5 µl volumes, containing 6.25 µl GoTaq (Promega) or Qiagen Multiplex PCR Mastermix, 0.25 µl of each primer (10 µM), 0.125 µl BSA, 1.0 µl DNA and 3.875 µl dH_2O. Cycling conditions for the *cox1* multiplex PCR (table 1) were 94°C for 2 min, followed by 30 cycles of 92°C for 30 s, 51°C for 1 min

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and 72°C for 50 s. A final cycle of 68°C for 5 min was used when applying Qiagen Multiplex PCR Mastermix, while denaturing for 2 min, annealing for 30 s, and extension at 70°C was carried out when using the GoTaq PCR Mastermix. Cycling conditions for the *12S rRNA* multiplex PCR were 94°C for 15 min, followed by 35 cycles of 94°C for 30 s, 53°C for 30 s and 72°C for 1 min, and a final cycle of 72°C for 10 min. All PCRs included positive (target prey) and negative controls consisting of PCR reagents with distilled water as substitute for DNA. PCR products were visualized on 1–2% agarose gels following standard procedures. In some cases, we checked the absolute amplicon size by using Hi-Di formamide and 0.25 µl GESESCAN 350 (ROX) size standard (both Applied Biosystems) and then separated using a 3130xl Genetic Analyzer (Applied Biosystems). The software Genemapper 4.0 (Applied Biosystems) was used to score electropherograms.

Cross-amplification tests on non-target organisms

All primers were tested for cross-amplification using DNA extracted from 54 potential prey taxa plus the three carabid species analysed in this study (table 2). Most of the taxa were common in the field where the beetles were sampled. Others were selected in order to increase the diversity and breadth of taxa. The non-target organisms were tested individually with one multiplex PCR at a time. At least one specimen was tested for each taxon.

Statistical analysis

All statistical analyses were performed using R version 2.8.0 (R Development Core Team, 2008). Generalized linear models (GLMs) were used to analyze the data from the controlled feeding experiments. As the data consisted of PCR-negatives and PCR-positives, a binomial distribution was used in the models. Median detection times (the time at which 50% of beetles tested positive, equivalent to the detectability half-life of Chen *et al.*, 2000) were calculated from the binomial regression equations. GLMs were also applied to test if the detection of slug DNA in beetle foreguts were significantly different between species of slugs and between sexes of beetles. The latter analyses were carried out using presence and absence of DNA as the response variables and slug species and beetle sex as explanatory variables.

GLM was also used to analyse predation in the field by using a binomial distribution of the presence-absence data of slug DNA in field-caught beetles. The proportions of slug-positive beetles were used as the response variable, while the abundance of *A. lusitanicus* (defined as the abundance of *A. lusitanicus* as a proportion of the total abundance of all target slug species: *A. lusitanicus*, *A. distinctus*, *A. ater* × *A. rufus* and *D. reticulatum*) was used as the explanatory variable.

Finally, generalised linear mixed-effect models (GLMMs) were used to test the differences in density of slugs and beetles in strawberry patches versus set-aside land. This was done by using the function 'glmmPQL' available in the package 'MASS' in R. The different transects were used as a random factor. GLMM approximation for analysing non-normal data such as counts has recently been reviewed by Bolker *et al.* (2008). The quasipoisson distribution was used due to over-dispersion in the count data, as suggested by diagnostic plots (leverage, normal Q-Q, fitted and scale location), and by comparing the residual deviance with degrees of freedom.

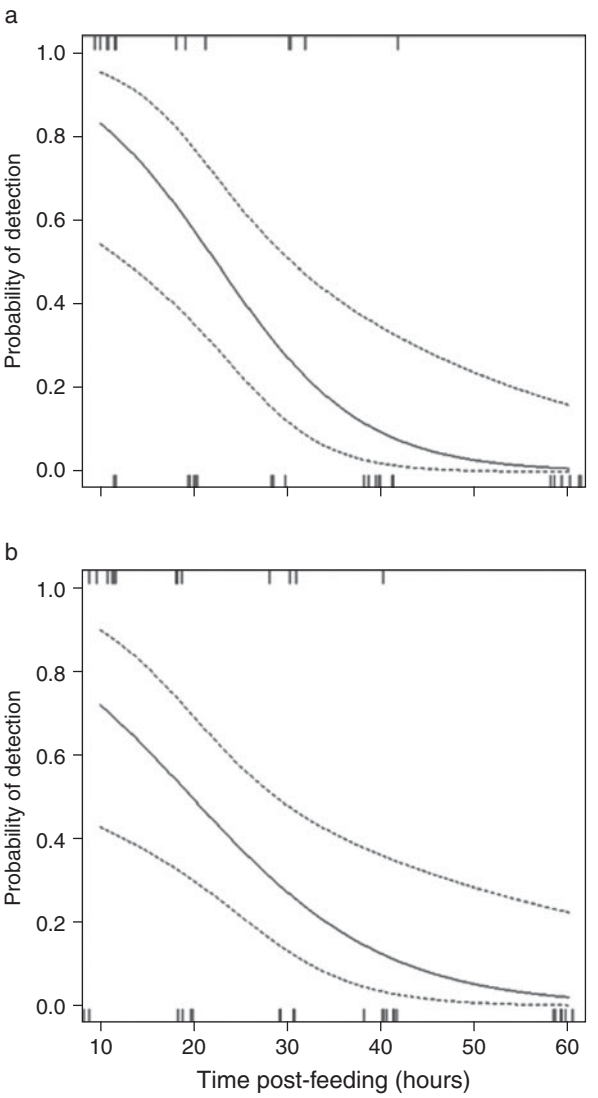

Fig. 2. Detection period of prey DNA in the foreguts of *Carabus nemoralis* fed with *Arion lusitanicus* and *Deroceras reticulatum* using the *cox1* and *12S rRNA* multiplex PCR, respectively. The solid line represents the binomial model, while the dotted lines represent the upper and lower 95% confidence limits. The vertical lines represent the replicates and one line may in some cases consist of more than one replicate. (a) The detection of *A. lusitanicus* (median detection period = 22.4 h). (b) The detection of *D. reticulatum* (median detection period = 19.7 h).

Results

Primer specificity and sensitivity

None of the 57 non-target prey and predator species were amplified using either the *cox1* or *12S rRNA* multiplex PCRs (table 2). The *12S rRNA* multiplex PCR yielded 221 bp and 109 bp amplicons for the target species *A. distinctus* and *D. reticulatum*, respectively, when using fluorescent primers, which are the same as for British specimens (Harper *et al.*, 2005). However, no amplification of other small arions like *A. silvaticus* was achieved. Furthermore, the general *Arion* primers in the *12S rRNA* multiplex PCR could not accurately

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Table 3. Predation in both fields by *Carabus nemoralis* (percentages of PCR-positives) compared with densities and mass of *Arion lusitanicus*, *A. ater* introgressed with *A. rufus*, *A. distinctus* and *Deroceras reticulatum*.

Locality	Season	Slug species	No. of slug-positive *C. nemoralis* (%)	No. of slugs per m^2	Mean weight of slugs (g)
	Spring 2006	*A. lusitanicus*	2 (13)	18 ± 4.4	0.61 ± 0.06
		D. reticulatum	5 (31)	50 ± 8.8	0.31 ± 0.01
		A. distinctus	0	1 ± 0.9	0.30 ± 0.07
		A. silvaticus	–	9 ± 2.6	0.20 ± 0.11
		A. ater x A. rufus	1 (5)	3 ± 0.9	1.40 ± 0.16
Meadow	Autumn 2006	*A. lusitanicus*	1 (3)	45 ± 9.2	0.12 ± 0.06
		D. reticulatum	1 (3)	46 ± 14.2	0.08 ± 0.07
		A. distinctus	0	5 ± 2.0	0.29 ± 0.06
		A. silvaticus	–	15 ± 3.0	0.13 ± 0.03
	Summer 2007	*A. lusitanicus*	1 (11)	17 ± 2.9	1.67 ± 0.19
		D. reticulatum	0	3 ± 0.9	0.30 ± 0.01
		A. distinctus	0	1 ± 0.4	0.18 ± 0.02
		A. silvaticus	–	4 ± 0.1	0.16 ± 0.01
		A. ater x A. rufus	0	1 ± 0.3	0.05 ± 0.01
	Spring 2007	*A. lusitanicus*	4 (16)	39 ± 6.4	0.32 ± 0.02
		D. reticulatum	1 (4)	3 ± 0.8	0.62 ± 0.03
Strawberry		*A. distinctus*	0	2 ± 0.8	0.18 ± 0.08
field and		*A. silvaticus*	–	6 ± 1.3	0.16 ± 0.01
set-asides	Autumn 2007	*A. lusitanicus*	0	6 ± 1.1	3.41 ± 0.18
		D. reticulatum	0	2 ± 0.5	0.35 ± 0.06
		A. distinctus	0	2 ± 0.6	0.10 ± 0.02
		A. silvaticus	–	6 ± 0.7	0.09 ± 0.02

–, no data available; ±, standard errors.

separate the large *Arion* species; hence, the design and inclusion of the *cox1* multiplex PCR. The latter provided clear separation of the three closely related, large arionids, *A. ater*, *A. rufus* and *A. lusitanicus*.

Feeding trials with Arion lusitanicus *and* Deroceras reticulatum

The maximum detection period of *A. lusitanicus* and *D. reticulatum* DNA in the foreguts of *C. nemoralis* was up to 40 h post-feeding with median detection periods of 22.4 and 19.7 h, respectively (fig. 2). Detectability of the two slug species over time was not significantly different ($P = 0.995$, binomial GLM); neither was there a difference between male and female beetles ($P = 0.455$, binomial GLM).

Slug densities

The most common slug species in the present study were *A. silvaticus*, *A. lusitanicus*, *A. distinctus* and *D. reticulatum* (table 3). Other molluscs found in the study sites were *Arion ater* introgressed with *A. rufus* (based on a combination of morphology and the *cox1* multiplex PCR Hatteland *et al.*, in preparation), *A. circumscriptus*, *A. fasciatus*, *A. intermedius*, *Deroceras panormitanum*, *D. laeve*, *Boettgerilla pallens*, *Arianta arbustorum*, *Cepaea hortensis*, *Cochlicopa lubrica*, *Nesovitrea hammonis*, *Trochulus hispidus* and *Discus rotundatus*. In the meadow, *D. reticulatum* was the most numerous species, but *A. silvaticus* and *A. lusitanicus* were also common. On the other hand, *A. lusitanicus* was more numerous compared with other species at the strawberry site, co-occurring with *C. nemoralis* in higher numbers within the surrounding set-aside land (fig. 3). However, only the density of *A. lusitanicus* was significantly higher in the set-asides compared with the strawberry patches ($P < 0.001$, GLMM). These findings are mainly based on sampling the vegetation down to the soil surface. We only found six slugs in the subsurface soil samples at the strawberry site; three specimens of *B. pallens* plus single specimens of *A. lusitanicus*, *A. silvaticus* and *D. reticulatum*.

Predation in the field

In total, we collected 113 adult carabids, of which *C. nemoralis* was the most numerous (table 4). The mean foregut weights of 40 field-collected *C. nemoralis* were 12 ± 2 mg and 19 ± 4 mg for males and females, respectively.

Predation rates by *C. nemoralis* in all the fields together ranged from 16% to 39% slug-positive beetles in spring, of which 7% contained DNA from two slug species. In addition, predation by *P. niger* ranged from 10% to 20%. Furthermore, predation by *C. nemoralis* was density related with beetles feeding on the most abundant slug species (GLM, $P = 0.0340$; fig. 4), which was often *A. lusitanicus* (table 3). In autumn 2006, only 3% of *C. nemoralis* were positive for *A. lusitanicus* and *D. reticulatum*, even though both species were present in high numbers as eggs (82 ± 40 per m^2 s.e.) and newly hatched juveniles.

Discussion

The main finding was that *C. nemoralis* was a consumer of the invasive *A. lusitanicus*, the 'plague' slug that is causing severe damage to crops through much of northern Europe. Importantly, the data showed that alien species were not avoided and that the different slug species were eaten seemingly in proportion to their density, with little discrimination between species.

Previous studies have shown that many factors affect detection times of prey DNA in the guts of predators. The

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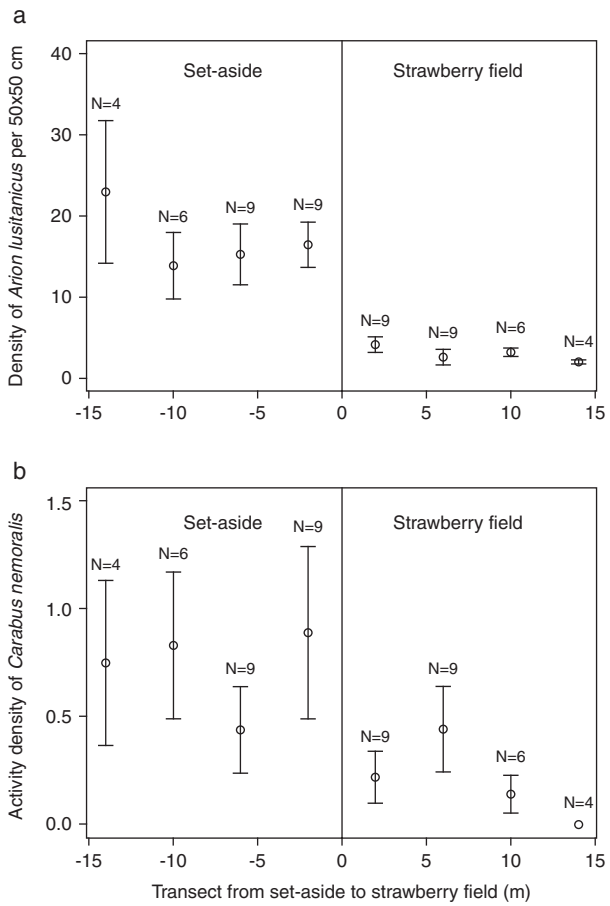

Fig. 3. Densities of slugs and beetles in nine transects from surrounding set-asides to the strawberry patches in spring 2007. The plots along transects were 4 m apart and were sampled once in late April. (a) Density of *Arion lusitanicus* per 0.25 m^2. (b) Activity-density of *Carabus nemoralis* per sample point (eight traps for one week). The vertical line represents the edge of the strawberry patch. Error bars are given as standard errors.

Table 4. The total number of adult carabid beetles used for screening of slug DNA.

	Pterostichus melanarius	*Pterostichus niger*	*Carabus nemoralis*
Spring 2006	2	0	16
Autumn 2006	0	1	31
Spring 2007	1	5	25
Summer 2007	3	10	9
Autumn 2007	0	7	3
Total	6	23	84

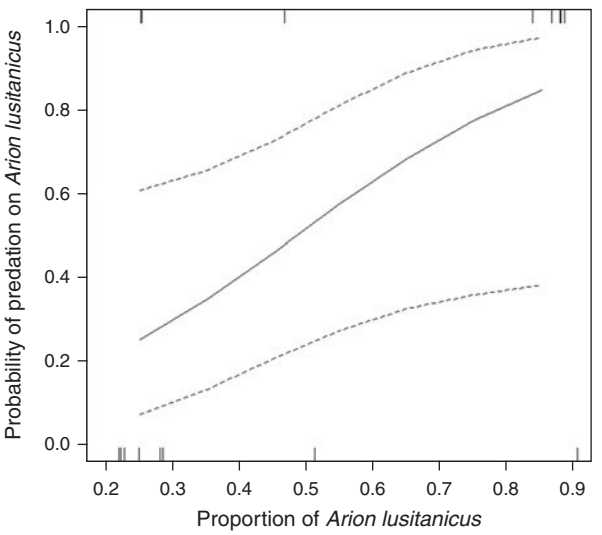

Fig. 4. Predation by *Carabus nemoralis* on *Arion lusitanicus* in response to the proportion of *A. lusitanicus* compared with the total abundance of all target slug species (*Arion distinctus, A. lusitanicus, A. ater, A. rufus* and *Deroceras reticulatum*) across all dates and both field sites. The solid line represents the binomial model, while the dotted lines represent the 95% confidence limit. The vertical ticks represent the replicates (number of positives and negatives for each dataset), and one line may in some cases consist of more than one replicate.

fragments amplified by primers used in the present study were about 300 bp in length, which is set as a preferred upper limit, since larger fragments degrades faster than shorter fragments (Symondson, 2002; King *et al.*, 2008). The choice of predator taxa has also been found to significantly influence the detectability (Chen *et al.*, 2000; Ma *et al.*, 2005; Read *et al.*, 2006; Greenstone *et al.*, 2007; Harwood *et al.*, 2007; Traugott & Symondson, 2008), even in related species within the Carabidae family (von Berg *et al.*, 2008a). However, the detection period of *D. reticulatum* in *C. nemoralis* in the present study was similar to the results obtained in other studies (Dodd, 2004; Harper *et al.*, 2005) when using *P. melanarius*. Many other factors also influence the detection of prey DNA, such as temperature (Hoogendoorn & Heimpel, 2001; von Berg *et al.*, 2008a), weather (von Berg *et al.*, 2008b), combination of prey DNA (Harper *et al.*, 2005) and the amount of prey DNA ingested (de Leon *et al.*, 2006; King *et al.*, 2010b). Our calibrating feeding experiment with *A. lusitanicus* and *D. reticulatum* makes it possible to compare predation on these species in the field directly without adjustment since detection times were not significantly different. Furthermore, the detection time in male and female *C. nemoralis* was not significantly different; thus, predation by field-caught beetles may be interpreted without considering sex. This is in accordance with Dodd (2004), who did not find any significant differences in detection of *D. reticulatum* in male and female *P. melanarius*.

The method we applied for sampling of slugs does not give absolute numbers, since only the vegetation down to the soil surface was sampled. Thus, the accuracy of the methods is probably affected by the vegetation and soil structure. There are reasons to assume that slugs will be found deeper down in the soil in arable fields than in meadows (lower vegetation cover and coarser soil particles in arable fields as compared to meadows). However, very few slugs were found in the analysed soil samples in the present study, leading us to conclude that most slugs were located on the soil surface or in the vegetation, at least in the fields included in our study.

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As the soil at the study sites was compacted, we believe that sampling down to the soil surface recovered most slugs in the area, at least during the part of the year when they are active.

The present study is the first to demonstrate that *C. nemoralis* and *P. niger* feed on the invasive Iberian slug, *A. lusitanicus*, as well as *D. reticulatum* in the field. *Carabus nemoralis* fed on slugs with no obvious prey preference, and predation on *A. lusitanicus* occurred mainly in spring when juveniles were present. Hence, *C. nemoralis* has potential in conservation biological control since this carabid is most active in spring (Lindroth, 1985). In addition, *P. niger* was found to be a predator of *A. lusitanicus*, as also suggested by laboratory experiments (Hatteland *et al*., 2010). Paill (2000, 2004) found the same to be true for *C. violaceus* and *P. melanarius*, respectively, feeding on *A. lusitanicus* in June and in early autumn. However, *Pterostichus* species are more restricted in the size of prey taken compared to larger and more specialised predators, such as *Carabus* spp. (Hatteland *et al*., 2010). The threshold of slug size for *P. niger* and *P. melanarius* seems to be approximately 100 mg, based on laboratory experiments (McKemey *et al*., 2001; Hatteland *et al*., 2010) and field studies (Paill, 2004), while *C. nemoralis* consumes slugs of up to 1 g (Hatteland *et al*., 2010). Surprisingly, we did not find many positive beetles in autumn, although slugs were abundant as newly hatched juveniles or eggs. The explanation might be that the beetles find it difficult to find newly hatched slugs, and their eggs (reviewed in Symondson, 2004), and/or alternative prey, may have been abundant at this time.

Temperature can also affect rates of predation on slugs (Ayre, 2001). The activity threshold of *C. nemoralis* has been found to be 4°C, and activity is greater when temperatures rise in spring, while activity is not correlated with temperature later in the season (Weber & Heimbach, 2001). In our study area, the diel mean temperature at ground level was 10.6 ± 0.2°C (s.e.) in the spring and 14.2 ± 0.2°C in the summer of 2007 as measured by a data logger, which suggests sufficient temperatures for beetle activity. The activity-density of beetles was low at the strawberry site compared with the meadow, which might reflect the different densities of these carabids in the two fields surveyed. However, many factors tend to affect pitfall catches which make it hard to compare different results (Adis, 1979; Spence & Niemelä, 1994; Luff, 2002). Activity of beetles may often be related to satiation levels; thus, pitfall catches can be used as a measure of prey availability (Szyszko *et al*., 2004). Satiation, however, is not always linked to predation. *Pterostichus melanarius* has been found to even kill prey when satiated (Hagley *et al*., 1982). In addition, pesticides were used in our strawberry site. This, as well as a much higher degree of disturbance in the field compared to the meadow, may explain the lower numbers of beetles in the strawberry patches and the surrounding set-asides.

Unfortunately, our method of detecting prey remains in the predators does not discriminate between predation and scavenging (Foltan *et al*., 2005). *Pterostichus melanarius* has been found to prefer dead slugs over live slugs, although preference changed to living prey the longer the decaying process progressed (Calder *et al*., 2005; Foltan *et al*., 2005). Another finding indicating carrion preference was that *P. melanarius* seems to orientate towards slugs by responding to volatiles produced by the decay process (McKemey *et al*., 2004), although *P. melanarius* has been found to orientate towards fresh slugs as well as following mucus trails (McKemey *et al*., 2004). *Carabus nemoralis* has also been found to follow mucus of *D. reticulatum* (Ayre, 1995). Furthermore, the detection period for DNA from decaying slug in the gut of a predator was very similar compared to live slugs (Foltan *et al*., 2005). This means that the beetles testing positive for prey consumption in the present study may be from feeding on dead as well as live slugs. However, as Sunderland (1996) and Juen & Traugott (2005) state, carrion may attract predators and may even signal that living prey is also available (Griffith *et al*., 1985; Chiverton, 1988; Winder *et al*., 1994). Furthermore, slug-positive beetles in the present study were clearly linked with the density of juvenile slugs in the spring, which suggests that predation is probably the main explanation for these results.

Future work should focus more on spatial rather than temporal data to further test the hypothesis that carabid beetles like *C. nemoralis* feed on slugs in proportion to numbers and biomass present. It may be possible to enhance the effectiveness of carabids as slug control agents by habitat management (Pickett & Bugg, 1998). Provision of shelters and overwintering sites, lowering the amount of indiscriminate pesticides used as well as manipulating carabid activity by semiochemicals, may all have potential (Altieri *et al*., 1982; Kromp, 1999; Halaj *et al*., 2000; Symondson *et al*., 2002b). However, predator-prey interactions are complex and affected by additional factors, such as microhabitat structure, feeding history, cannibalism, fertilizers, crop, season and alternative prey (Symondson *et al*., 2002b). On the other hand, native predators, including carabids, are clearly able to suppress or reduce indigenous and exotic pests (Sunderland, 2002; Symondson *et al*., 2002b), and future studies should explore the efficiency of biological control of slugs.

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