The effects of invertebrates on the plant communities in upland hay meadows

Sarah Elizabeth Barlow

Doctor of Philosophy

School of Biology

Newcastle University

December 2012

Abstract

Species rich upland hay meadows are of high biodiversity importance and are internationally rare. There is increasing interest in restoring botanically diverse meadows in the uplands but little is known about the role of invertebrates. The purpose of the project was to investigate the role of (i) slugs as seedling herbivores and (ii) pollinators in affecting the plant communities. Research has revealed important interactions between invertebrates and plant life history traits with implications for grassland restoration.

Slugs are known to affect plant communities by the selective seedling removal of more acceptable species, although few studies have considered their impact at restoration sites. A glasshouse feeding trial showed the acceptability of seedlings of different meadow plants to slugs is influenced by plant defensive properties. Field investigations of slug population densities identified a negative correlation with increasing levels of agricultural improvement. This relationship was partly driven by the increase in grass abundance cover in agriculturally improved meadows and may be influenced by the anti-feedant properties of silica-rich grass leaves. A 3-year mesocosm study found evidence that slugs are an important selective force affecting seedling recruitment and community composition. The selective seedling removal of the hemi-parasite and keystone species *Rhinanthus minor* was a key finding of this study. Use of Scanning Electron Microscopy showed the leaf surface of *R. minor* to be characterised by a diverse assemblage of trichomes which may play an important role in anti-herbivore defence.

Parallel declines in pollinator and insect-pollinated plant populations have raised concerns that smaller populations of important pollinator groups such as bumblebees may be reducing seed set by plants. Survey work showed upland hay meadows to be an important forage resource for common and rare/scarce bumblebees. Bumblebees visited a small number of forage plants, most notably *R. minor*, *Trifolium* species and *Geranium sylvaticum*. Pollen supplementation tests did not find significant evidence that reproductive output of *R. minor* and *G. sylvaticum* (a gynodioecious species) was limited by pollination services. Testing of a new system to record pollinator visitors to flowers proved highly effective and

offers the potential to significantly reduce time and labour input into the study of plant-invertebrate interactions.

The project has shown that invertebrates are an important functional group affecting the success of plant species in hay meadows. The findings provide new and important information to the industry in developing management prescriptions for restoring upland hay meadows and other botanically rich plant communities.

Acknowledgements

It is with much appreciation and gratitude that I thank the Perry Foundation for providing funding for this project. I thank and acknowledge Dr. Andrew Close (School of Biology, Newcastle University) for his advice and work in analysing much of the data generated by studies within this thesis, namely: i) in Chapter 2, the analysis of data using CAP, PERMANOVA and PERMDISP performed in PRIMER-E ; ii) in Chapter 3, the analysis of data using GAMM performed in R and writing of the statistical method; iii) in Chapter 4, the analysis of data using db-RDA, db-linear model, PERMANOVA and PERMDISP performed in PRIMER-E and assistance with writing the statistical method; and iv) in Chapter 6, the analysis of data using CAP and PCA performed in PRIMER-E and suggestions for future analyses. I am grateful to the technical staff at Close House Biology Field Station, Alan Craig and Robert Hodgson, for their continued support and advice, and help with setting-up and maintaining experiments. I thank Malcolm Barlow and Alan Craig for assistance in conducting field work in the Yorkshire Dales and landowners for granting access to study sites. For work detailed in Chapter 5, I thank Tracey Davey (Electron Microscopy Research Services, Newcastle University) and Pauline Carrick (Advanced Chemical and Materials Analysis, Newcastle University) for SEM and EDX spectroscopy expertise, respectively. I am grateful to Shaun Hackett (Ranger, Northumberland National Park) for his training in bumblebee identification and assistance with field work conducted at Barrowburn (Chapter 6). For work detailed in Chapter 8, the development of Rana was solely the work of Dr. Mark O'Neill. I also thank Daniel Reed for his assistance with experimental work involved in this Chapter. I thank Dr. Roger Smith (Newcastle University) for first introducing me to upland hay meadows and his help as second supervisor during the early stages of the project. I am also grateful to him for allowing data collected as part of the DIGFOR project (BD1451) to be used within the study detailed in Chapter 2. Finally, I thank my Supervisor Dr. Gordon Port for his much valued support and guidance throughout this project.

Table of Contents

Page

List of Tables

List of Figures

Chapter 1. General Introduction

1.1 General Introduction

In the UK and much of Europe, land use change and increasing agricultural intensification over the past 60 years has caused catastrophic declines in the extent of unimproved semi-natural grassland, such as species-rich hay meadows (van Dijk 1991; Critchley, Burke & Stevens 2004). It is estimated that 97% of unimproved lowland grassland was lost between the 1930s and 1980s in England and Wales leaving an area of 0.2 M ha (Fuller 1987). Later study revealed further declines and indicated that the area of floristically diverse grassland was in the range of 0.05 M ha to 0.1 M ha by the end of the 20th century (Blackstock *et al.* 1999). Currently, only 2% of the UK's grassland area comprises high diversity priority semi-natural grassland (UK: 215, 686 ha; England: 104,525 ha) (Bullock *et al.* 2011). After the Second World War, financial incentives to increase food production saw many sites converted to arable use and a move towards increasingly intensive management practices including increased agrochemical usage, higher stocking densities and, in hay meadows, a switch to silage production involving earlier cutting and reseeding with perennial rye grass (*Lolium perenne* L.). Ultimately these factors have caused a decline in botanical diversity in agriculturally improved sites and threaten the persistence of remaining species-rich sites within a highly fragmented landscape (Jefferson 2005). These habitat changes have caused population declines in other taxonomic groups including farmland birds (Chamberlain *et al.* 2000) and various insect groups such as bumblebees (Goulson *et al.* 2005; Carvell *et al.* 2006; Potts *et al.* 2010). Concerns over the loss of biodiversity in the agricultural landscape have been a major driver of European agri-environment policy development. In response to these concerns, a variety of policies have been implemented in the UK including statutory designation of sites of high conservation value; agri-environment schemes for enhancing biodiversity; and Biodiversity Action Plans (BAPs) for threatened habitats and species of conservation interest. Statutory protection has greatly reduced the importance of agricultural improvement as a driver of loss; for example, in England, 68% (74, 894 ha) of semi-natural grassland is now protected as SSSIs. Primary drivers of loss are now nitrogen deposition, inadequate management and habitat fragmentation (Bullock *et al.* 2011). Agri-environment schemes were first introduced in 1985 and since 2005 have operated within a tiered system under the heading of Environmental Stewardship. In the UK, 441,941 ha of grassland are within agri-environment scheme options. In England, 60,733 ha of grassland are entered into the maintenance and restoration options of either the classic schemes (Countryside Stewardship, Environmentally Sensitive Areas) or Higher Level Stewardship (HLS) and are a key mechanism for achieving targets outlined in BAPs (Critchley, Burke & Stevens 2004; Smith *et al.* 2008). In HLS, 2373 ha have been entered into the creation of species-rich grassland option (Bullock *et al.* 2011; Natural England 2009a). However, despite these conservation efforts, the overall UK trend for semi-natural grassland is still one of (slowing) decline (BARS 2008; Bullock *et al.* 2011).

Species-rich upland hay meadows, conforming to MG3 (*Anthoxanthum odoratum-Geranium sylvaticum*) in the National Vegetation Classification (NVC) (Rodwell 1992), are one of the rarest types of grassland in the UK and are recognised as a UK BAP priority habitat and as an Annex I habitat (H6520 Mountain Hay Meadow) by the EU Habitats Directive (JNCC 2008). Recent estimates indicate that 897 ha remain in the UK, located in upland valleys in northern England (870 ha), with a few outliers in Scotland (27 ha) where traditional hay-making practices still persist (BARS 2008). Meadows are typically small (93% are <5 ha) and are threatened by habitat fragmentation, often persisting as islands in a ‘sea’ of agriculturally improved grassland (Natural England 2009b). The current status is a declining (slowing) trend and unfavourable (bad) but improving condition (BARS 2008; Bullock *et al.* 2011). These meadows differ from their lowland counterparts in including northern-montane species such as *G. sylvaticum* L., *Cirsium heterophyllum* L. and various *Alchemilla* species, as well as woodland species such as *Conopodium majus* (Gouan) Loret. and *Anemone nemorosa* L. reminding us that meadows were once woodland clearings used for livestock grazing during Neolithic times (North Pennines AONB 2009; O'Reilly 2010). In recent decades there has been growing interest in conserving high quality grasslands and restoring botanical diversity of improved sites. Research involving restoration of lowland and upland meadows has identified the optimum management regime (i.e.

no mineral fertilizer, mid-July hay cut, grazing with cattle in autumn and sheep in spring) (Smith *et al.* 1996a; Smith *et al.* 1996b; Smith *et al.* 2000; Smith *et al.* 2003; Smith *et al.* 2008) and constraints to enhancing species richness including high residual soil fertility (phosphate) and low fungal to bacterial ratios (Smith *et al.* 2003; Bardgett *et al.* 2007; Smith *et al.* 2008); propagule limitation (Smith *et al.* 2002); and competition from dominant grasses (Smith *et al.* 2000; Pywell *et al.* 2004). To help combat these constraints, restoration measures must include the addition of seed of species absent from the seed bank of improved sites and which are drivers of above- and below-ground community-level properties, namely the hemi-parasite and keystone species *Rhinanthus minor* L. (Hay rattle) and a variety of early-colonising fungal-facilitating species, including legumes (Bardgett *et al.* 1999; Smith *et al.* 2003; Bardgett *et al.* 2006; Smith *et al.* 2008). *R. minor* selectively parasitizes, thereby weakening, competitively dominant grasses and indirectly enables a wider diversity of forb species to become established (Pywell *et al.* 2004). As such, the use of *R. minor* as a management tool in grassland restoration is well established. The establishment of species, such as *Trifolium pratense* L.*, Anthoxanthum odoratum* L. and *Ranunculus acris* L. is also important during the early stages of restoration through their role in facilitating mycorrhizal populations and associated increases in fungal to bacterial ratios and decreases in residual soil phosphorus (Smith *et al.* 2008). Whilst these constraints and restoration measures have been, and continue to be well studied, the effects of invertebrates on the conservation and restoration of priority grassland have received very little attention.

Invertebrates are integral to the functioning of grassland ecosystems through roles such as herbivory and pollination (Littlewood, Stewart & Woodcock 2012). Invertebrate herbivores and pollinators represent two important functional groups with the potential to influence plant community composition through direct effects on plant function and fitness. These plant-invertebrate interactions are influenced by a multitude of interacting plant traits involved in: i) anti-herbivore defence and tolerance to herbivory such as plant secondary chemicals, silicon-based defences, trichomes and growth rate and form (Levin 1973; Hanley 1998; Carmona, Lajeunesse & Johnson 2011; Johnson 2011); and ii) pollination success, such as the timing of flowering and floral traits which attract pollinators (Goulson 1999; Goulson 2003; Hanley *et al.* 2008), as well as the degree of specialisation of the pollination mechanism (i.e. range of suitable pollen vectors) and pollination system (i.e. self-compatibility and ability to self-fertilise) (Burd 1994; Larson & Barrett 2000; Ashman *et al.* 2004; Knight *et al.* 2005). In the context of grassland restoration and conservation, it is not clear whether the effects of invertebrates on seed supply and the success of germinating seedlings play a role in affecting botanical diversity and community structure. The purpose of this project was therefore to examine the role of: (i) slugs as seedling herbivores, and (ii) pollinators in affecting the plant communities in upland hay meadows.

Previous studies suggest that invertebrate herbivores are likely to have the greatest effect on the restoration of grassland plant communities through effects on seedling recruitment (Brown & Gange 1989; Wardle & Barker 1997; Del-Val & Crawley 2005). This information is particularly important for *R. minor* and fungal-facilitating species as their successful establishment is fundamental to the restoration process (Smith *et al.* 2008). Slugs (Gastropoda: Agriolimacidae, Arionidae and Milacidae) are probably the most important invertebrate herbivores in temperate grassland (Hulme 1994) and have been identified as important regulators of plant community structure during and beyond the establishment phase (Hanley, Fenner & Edwards 1995a; Buckland & Grime 2000; Allan & Crawley 2011). Slugs are abundant in grassland (South 1989b; South 1992) and, as generalist but selective herbivores (Dirzo 1980), may exert a strong selective pressure by affecting seedling recruitment (Hanley, Fenner & Edwards 1995a; Hanley, Fenner & Edwards 1996a) and shifting the competitive balance between coexisting species (Wardle & Barker 1997; Hanley & Sykes 2009). The aim of this part of the project was to determine whether selective grazing by slugs affects plant community development and structure of the plant communities in upland hay meadows. Whether slug grazing affects the success of *R. minor* was of particular interest. To address this research question, studies were conducted concerning: i) the population densities of slugs in hay meadows and their response to increasing management intensity and particular environmental factors (Chapter 2); ii) the relative acceptability of seedlings of different meadow plants to slugs

(Chapter 3); iii) the effects of high and low slug densities on community structure and development as part of a three-year mesocosm study (Chapter 4); and iv) the possible anti-herbivore defensive properties of *R. minor* leaves (Chapter 5).

Parallel declines in pollinator and insect-pollinated plant populations, particularly of bees and bee-pollinated plants, have raised concerns over reductions in pollinator services to crops and wildflowers and subsequent reductions in seed production (Kearns, Inouye & Waser 1998; Biesmeijer *et al.* 2006; Potts *et al.* 2010). Many of the plant species characteristic of species-rich meadows are known to be reliant on pollination by insects, particularly bumblebees. The loss, degradation and fragmentation of flower-rich forage resources, especially unimproved grassland has strongly contributed to declines in bumblebee populations and diversity (Goulson *et al.* 2005), and it is highly likely that MG3 meadows provide a valuable foraging resource to bumblebee populations, including some rare/scarce species, in the uplands of Northern England (Darvill, Mabon & Rolph 2010). The aim of this part of the project was to investigate how the reproductive output of some of the key wildflower species was affected by pollinator services. To address this aim, bumblebee activity and forage use was investigated in a suite of upland hay meadows (Chapter 6); and the reproductive output of *R. minor* and *G. sylvaticum* (a gynodioecious species) was tested for pollen limitation (Chapter 7). In addition, the project includes details of a novel method for recording plant-pollinator interactions using an automated motion detection system (Rana) (Chapter 8). Although in the early stages of development, the system was used to record insect visitors to *R. minor* and *Centaurea nigra* L. and offers great potential for use in future studies of plant-invertebrate interactions.

The results of this project are of importance to policy makers and land managers in developing management prescriptions for improving botanical diversity of upland hay meadows and other grassland communities (e.g. through agri-environment schemes). A fuller understanding of the factors affecting the success of plant species in hay meadows and other botanically rich habitats will pave the way for developing methods to manage the successful restoration or establishment of these plant communities on more sites.

Chapter 2. Effects of agricultural intensification and environmental factors on slug densities in upland hay meadows

2.1 Abstract

Slugs are probably the most important invertebrate herbivores in temperate grassland yet little is known about how agricultural improvement and environmental factors, such as botanical and soil properties influence slug densities. We estimated slug population densities in upland hay meadows of different agricultural improvement types using the quantitative sampling technique of soil flooding. The influence of management intensity (unimproved, semi-improved and improved meadows) and environmental factors (plant species richness, percentage grass cover, herbage N content, dissolved inorganic nitrogen (DIN) and soil bulk density) on slug densities was analysed using a combination of semi-parametric hypothesis testing techniques using permutations (PERMDISP and PERMANOVA) and unconstrained (Principal Coordinates) and constrained (Canonical Analysis of Principal Coordinates) ordination techniques. A significant multivariate effect of management on slug assemblages was identified with results indicating a positive response in slug abundance and species richness to decreasing management intensity. This trend was most apparent at opposite ends of the management gradient. Slug densities in semi-improved meadows were highly variable suggesting that slug densities at restoration sites are likely to be site-specific. Density of all slug species was negatively correlated with percentage grass cover and positively correlated with soil bulk density. The most common and abundant slug species were *Deroceras reticulatum* and *Arion fasciatus* agg.

Key-words: agricultural improvement, *Deroceras reticulatum*, gastropods, grassland restoration, molluscs, soil flooding

2.2 Introduction

Terrestrial molluscs (Gastropoda) are widespread in a variety of ecosystems in all climatic zones (South 1992; Barker 2001; Dallinger *et al.* 2001). Much attention has been paid to slugs (Agriolimacidae, Arionidae and Milacidae) as pests of agricultural and horticultural crops in temperate Europe and North America (Barker 2002). As such, previous studies have attempted to determine slug population densities in cropping systems, including winter wheat (Glen, Wiltshire & Milsom 1984; Glen, Milsom & Wiltshire 1989; Bohan *et al.* 2000b), oilseed rape (Symondson *et al.* 1996; Frank 1998) and potato (South 1973). Mean total slug densities in arable fields have been estimated in excess of 150 m^{-2} depending on crop, cultivation technique and period of sampling (South 1973; Glen, Wiltshire & Milsom 1984; Symondson *et al.* 1996). These studies commonly report *Deroceras reticulatum* (Müller) to be the most abundant species at UK sites, although it may be outnumbered by *Arion lusitanicus* (Mabille) in mainland Europe depending on the availability of cover at field edges (Frank 1998; Honek & Martinkova 2011).

Studies of slug populations in agricultural grassland are predominantly restricted to botanically species-poor sites, particularly ryegrass or grass-legume leys, in which slugs are pests during the establishment phase (Runham & Hunter 1970; Barker 1989; Glen, Cuerden & Butler 1991; Barker & Addison 1992; Cordoba *et al.* 2011). There are few studies of slug populations at more diverse sites and semi-natural communities, although Lutman (1978) reported the abundance of *D. reticulatum* and *Arion intermedius* (Normand) to be associated with species-rich grassland in the UK uplands; and Clements *et al.* (1990) found the application of molluscicide and insecticide to eight permanent grassland sites, consisting of no more than 30% ryegrass (*Lolium perenne* L.), to result in an increased total annual herbage yield of 11% on average over two years. Estimates of annual slug densities in grassland range from 12 to >50 m^{-2} depending on the type of grassland and sampling technique used, amongst other factors (Carrick 1938; Warley 1970; Lutman 1978; South 1989b; South 1992). *D. reticulatum* is also an abundant species in grassland, with several studies reporting it to be the dominant species in grassland in several countries (South 1989b; Barker & Addison 1992; South

1992; Cordoba *et al.* 2011). For example, South (1989b) found the annual mean density of *D. reticulatum* in permanent pasture in Northern England to be 42.4 m^{-2}.

Various sampling techniques are available to investigate slug activity and density; the pros and cons of which have been discussed by South (1992) and compared experimentally by several studies (e.g.Clements & Murray 1991; Archard *et al.* 2004; Cordoba *et al.* 2011). Quantitative techniques include defined area traps (DATs) (Ferguson, Barratt & Jones 1989; Ferguson & Hanks 1990) and hand sorting (Philipson 1983), soil washing and soil flooding of a defined volume of soil (Hunter 1966). Cold-water flooding (South 1964) is an accurate technique to estimate slug population densities and, although it is highly labour intensive, has been used successfully to determine slug densities within arable fields (Glen, Wiltshire & Milsom 1984; Symondson *et al.* 1996; Archard *et al.* 2004), grassland (South 1964; Warley 1970; Lutman 1978; South 1989a; South 1989b; Cordoba *et al.* 2011) and woodland (Jennings & Barkham 1975). The capture-recapture technique can also be used to quantify populations by marking slugs directly (e.g. UV dye, feeze branding) or via contaminated food (e.g. coloured or isotope-labelled food (South 1992; Foltan & Konvicka 2008). McKemey *et al.* (2006) found the use of monoclonal antibodies to be an effective technique to quantify slug densities, generating results equivalent to slug biomass.

Previous studies have investigated the influence of particular biotic and abiotic factors on slug activity and population dynamics such as interspecific competition (Cook & Radford 1988), meteorological conditions (Young & Port 1989; South 1989b; Young *et al.* 1991), different cultivation techniques and predator density (Symondson *et al.* 1996; Bohan *et al.* 2000a; Symondson *et al.* 2002). For example, Symondson *et al.* (1996) showed the density of slugs in oilseed rape fields to be favoured by non-inversion tillage and direct drilling, and established a positive link between increasing slug density and biomass with the activity-density of the predatory ground beetle *Pterostichus melanarius* L. Fountain *et al.* (2009) and Buckland and Grime (2000) suggested a possible negative influence of slug predators on slug populations in grassland plots subject to nutrient enrichment. However, South (1989a) found no evidence that predators, parasites or diseases caused any reduction in the populations of *D. reticulatum* and *A. intermedius* in permanent pasture, although the general negative effect did depend on the timing of attack in the life cycle of slugs. Detailed modelling studies of slug population dynamics have also been conducted (Shirley *et al.* 2001; Choi *et al.* 2006; Willis *et al.* 2006).

There is a paucity of correlative evidence to show the influence of particular vegetation parameters on slug and snail densities in grassland. A notable exception is the study by Dedov *et al.* (2006) which found gastropod assemblages in experimental field plots sown with grass and wildflower seed mixes to be negatively correlated with percentage grass cover, and the abundance of *D. reticulatum* was promoted by plant species richness and moss cover. Boag and Wishart (1982) found that the distribution of gastropods was correlated with vegetation cover of various rangeland habitats in Canada, with maximum abundance found where deciduous trees or shrubs were a major component of the canopy. In addition, Griffiths *et al.* (1998) report the positive effect of tree rows on local slug densities in silvoarable agroforestry landscapes. Other studies have investigated the response of gastropod assemblages to changes in the ground flora and microclimate following felling in conifer plantations (Hawkins *et al.* 1997; Prezio *et al.* 1999; Hylander, Nilsson & Gothner 2004; Strom, Hylander & Dynesius 2009).

Differences in agricultural intensification also affect gastropod assemblages. Neumann (1998) report a negative effect of agricultural improvement on slug densities in wet grassland pastures and meadows in Germany; gastropod species richness and abundance were lower in intensively managed sites characterised by lower plant diversity, soil moisture and soil structure and subject to greater livestock densities. Also, cutting in meadows created a drier microclimate causing greater egg mortality. Numerous studies have addressed the opposite scenario by investigating the influence of slug populations on vegetation dynamics by way of exclusion experiments. Field studies that have manipulated slug populations by use of control measures, such as molluscicides, have found profound effects of slugs on grassland plant community dynamics as a result of the selective grazing of seedlings of preferred forb species (Rees & Brown 1992; Hulme 1994; Hanley, Fenner & Edwards 1995a; Hulme 1996; Hanley, Fenner & Edwards 1996a; Hanley 2004; Pywell *et al.* 2007; Allan & Crawley 2011). Grasses may be more resistant to damage from molluscs due to their relative unacceptability in comparison with forbs (containing low concentrations of secondary metabolites) as a result of silica phytoliths in grass leaves (Speiser 2001). Grasses are also well-adapted to tolerate grazing damage via a basal meristem and fast growth rate (Hanley 1998 and references therein). These studies suggest that slugs are the most important invertebrate herbivore in temperate grassland, an argument that is presumably influenced by slug density; however, there have been few attempts to quantify slug populations in less-intensively managed or more species-rich sites including grassland habitats of high conservation interest. Given the potential adverse effects of selective grazing by slugs on the seedling recruitment of acceptable forb species and the goals of grassland restoration to successfully establish a diversity of wildflower species from seed, it is of interest from both a practical and ecological perspective to determine slug population densities in priority grassland communities as well as their less diverse counterparts, and to identify useful environmental predictor variables of slug density.

The aim of this study was firstly, to quantify slug population densities in upland hay meadows using the soil flooding sampling technique; and secondly, to assess the value of botanical and soil properties as predictors of slug populations. The work was conducted in mesotrophic upland hay meadows in the Yorkshire Dales, UK and incorporated meadows subject to different levels of agricultural improvement at five geographical locations. Species-rich unimproved meadows (i.e. those subject to traditional extensive management practices) had a National Vegetation Classification (NVC) MG3 or MG4 (Rodwell 1992) and most were designated as SSSI (Site of Special Scientific Interest). Semi-improved and improved meadows were MG6 or MG7 (Rodwell 1992). We hypothesised that slug density would be positively correlated with decreasing levels of agricultural improvement because of the greater availability of forbs and reduction in grass cover. It was also predicted that *D. reticulatum* would be the dominant species in all meadows. The findings are discussed in the context of hay meadow restoration and form part of a larger study of the effects of slugs on the plant communities in upland hay meadows.

2.3 Method

Soil samples were collected from fourteen mesotrophic hay meadows located in the Yorkshire Dales National Park, UK (for grid references see Table 2.1) between 27th March and 9th April 2008. Meadows were clustered according to geographical location and agricultural improvement category as previously described by Bardgett *et al.* (2007) (DIGFOR project BD1451). Five locations (Askrigg Bottoms, Thornton Rust, Yockenthwaite, Walden and Muker) were used within a 15 km radius of Hawes, North Yorkshire (SD8789) (approximate area = 308 km^2). At each location three meadows (two at Muker) were present, each representing a different improvement category and a gradient of increasing management intensity (unimproved, semi-improved and improved). Note, there was no semi-improved site at Muker. As far as possible the fields at each location were situated on similar soil types, with similar topography and edaphic characteristics. Permission for work in SSSI meadows was granted by Natural England and all samples were returned to their original location within four weeks of sampling.

Within each meadow a representative 25 x 25 m area was selected and a random number table used to identify five sampling locations. Random numbers causing sampling locations to be within 4 m of each other were ignored. All sampling locations were recorded using a hand-held GPS device (Table 2.1). At each sampling location, a soil sample measuring 0.009 m^3 (30 cm (L) X 30 cm (W) X 10 cm (D)) was removed by pushing a metal frame into the ground, cutting away an outer edge with a spade and lifting the sample with a turf cutter. This size of sample is suitable for agricultural grassland (South 1992) and samples of equal or similar size have been used by previous studies (South 1964; Lutman 1978; Symondson *et al.* 1996; Cordoba *et al.* 2011). Each sample was placed in a plastic box with tight fitting lid and transported to Close House Biology Field Station, Newcastle upon Tyne (NZ 128 659) where they underwent the process of cold water flooding (South 1964). Boxes were gradually flooded with cold water over 7-11 days until water reached the top of the sample and no further slugs appeared. Slugs present on the soil surface were removed daily (for 11 days) and identified to species level where possible using the field key by Cameron, Jackson and Eversham (1983). To aid the recovery of slugs, refuge traps (upturned saucers) were placed on the top of each sample.

The same meadows were used by Bardgett *et al.* (2007) to identify suitable indicators of restorability of plant diversity to grassland ecosystems. Botanical survey work and vegetation and soil sampling was conducted in June-July 2005. Note, meadows at Muker were surveyed in 2005 but are not included in Bardgett *et al.* (2007) because one meadow was absent from the triplet of meadows. For full details of methodology refer to Bardgett *et al.* (2007). Where possible, the area within each meadow used to conduct slug sampling was close to the area surveyed in 2005. The location of the 2005 botanical and soil sampling points and the 2008 slug sampling points are therefore offset spatially and temporally; yet for the analysis they are treated as coincident. Meadows continued to be managed in much the same way during this time period and it is unlikely that significant changes in botanical or soil conditions would have occurred.

Table 2.1. National Grid References of soil samples taken from meadows in the Yorkshire Dales, UK. Meadow classification: UI = unimproved; SI = semi-improved; I = improved. NVC communities of unimproved meadows: MG3 = *Anthoxanthum odoratum-Geranium sylvaticum* grassland; MG4: *Centaurea nigra-Cynosurus cristatus* grassland.

Location	Meadow	GPS co-ordinates of soil samples				
		1	2	3	4	5
Askrigg Bottoms	UI (SSSI) MG3	SD 94669 90817	SD 94669 90809	SD 94655 90809	SD 94661 90812	SD 94663 90815
	SI	SD 94753 90958	SD 94760 90961	SD 94752 90973	SD 94762 90964	SD 94749 90957
	I	SD 95367 90775	SD 95351 90781	SD 95351 90775	SD 95368 90783	SD 95357 90779
Thornton Rust	UI MG3	SD 97161 89632	SD 97032 89589	SD 97039 89589	SD 97036 89591	SD 97039 89586
	SI	SD 97051 89837	SD 97035 89830	SD 97044 89827	SD 97047 89844	SD 97039 89842
	I	SD 97149 89623	SD 97144 89638	SD 97153 89644	SD 97162 89642	SD 97160 89632
Yockenthwaite	UI (SSSI) MG3	SD 91031 79023	SD 91037 79023	SD 91021 79029	SD 91027 79027	SD 91025 79031
	SI	SD 90532 79416	SD 90544 79415	SD 90532 79416	SD 90542 79414	SD 90540 79419
	I	SD 90588 79146	SD 90607 79144	SD 90598 79159	SD 90600 79145	SD 90595 79141
Walden	UI (SSSI) MG4	SE 00506 82863	SE 00511 82882	SE 00518 82880	SE 00523 82880	SE 00512 82872
	SI	SE 00739 83448	SE 00729 83458	SE 00728 83445	SE 00736 83456	SE 00738 83442
	I	SE 00886 83694	SE 00868 83705	SE 00865 83688	SE 00874 83677	SE 00865 83707
Muker	UI MG3	SD 91047 98628	SD 91052 98627	SD 91030 98640	SD 91047 98643	SD 91045 98629
	I	SD 90947 98512	SD 90953 98504	SD 90959 98513	SD 90954 98508	SD 90959 98505

2.3.1 *Statistical analysis*

The abundance of each slug species per soil sample was used as the response variable in the multivariate analysis. There were seven species of slug recorded and 70 soil samples in total (25 unimproved, 20 semi-improved, 25 improved). To explore differences in slug assemblages according to management intensity, we used a combination of hypothesis testing techniques together with unconstrained and constrained ordination as described by Anderson and Willis (2003). The null hypothesis being that there were no differences in slug assemblages between each management group (i.e. unimproved, semi-improved and improved). Hypothesis testing was performed in two ways: Firstly, using semi-parametric permutation tests as described by Anderson (2004a) and Anderson (2005); and secondly, semi-parametric permutation tests incorporated in a constrained multivariate ordination as described by Anderson and Willis (2003). For all analyses, the response data was transformed to $y' = \ln(y + 1)$ to remove large differences in scale among the original variables. Data were then transformed using the Bray-Curtis dissimilarity index.

The underlying effects of management upon the slug assemblages of soil samples were determined using Permutational Multivariate Dispersion (PERMDISP) (Anderson 2004a) and Permutational Multivariate Analysis of Variance (PERMANOVA) (Anderson 2005). PERMDISP was used to determine differences in multivariate dispersion within management groups (meadow improvement categories). PERMANOVA was used to determine differences between management groups due to differences in multivariate location, and is also sensitive to variation in multivariate dispersion. Following the methodology of Anderson (2004a, 2006), pair-wise comparisons between treatment groups were also undertaken in PERMANOVA and PERMDISP. The statistical significance of each test (pseudo *F*-statistic, *P*-value, $\alpha = 0.05$) was determined by means of Monte Carlo permutation (10000 permutations).

Slug assemblages were subjected to an unconstrained ordination using Principal Coordinates (PCO) (i.e. metric MDS). A plot of the first two PCO axes was used to visually interpret patterns in the distribution of samples within multivariate space and to compare visual observations with the results generated by PERMDISP and PERMANOVA.

Two constrained ordinations were performed using Canonical Analysis of Principal Coordinates (CAP) on the same dissimilarity matrix. Unlike traditional canonical methods, CAP can be used with any distance or dissimilarity measure and thus provides a flexible and meaningful constrained ordination of ecological species abundance data (Anderson & Willis 2003). The first CAP analysis included the predictor variable “management” (consisting of the three improvement categories). The number of axes (*m*) used for the canonical analysis was 2. The pattern of samples in multivariate space was displayed graphically using the first two canonical axes. A visual interpretation of sample distribution was made together with tests of hypotheses using permutation procedures on canonical test statistics using 10000 permutations (trace statistic, delta squared statistic). Significant test statistics by both tests indicate that the relationship between canonical axes and explanatory variables is sound. Results of permutation tests can also be compared with those performed in PERMANOVA.

As meadows were placed in three *a priori* groups based on improvement category, the goodness of fit of samples (transformed data) to these groups was investigated to test whether the classification was successful. This was done for every sample in the dataset to calculate the proportion of the samples that were incorrectly classified for each management group and enabled the mis-classification error to be calculated (i.e. one minus the proportion of correct classifications). This approach gives a reasonable measure of how distinct the three management groups are in multivariate space in terms of slug assemblages.

For the second CAP analysis (*m* = 3), five environmental predictor variables were used to constrain sample distribution, namely botanical species richness; grass percentage cover; herbage N content (N mg/kg); soil bulk density (kg/ha (to 7.5 cm)) and dissolved inorganic N (DIN) (µg N g-1Dry soil). We then considered the correlations of each predictor variable with canonical axes 1 and 2 so that those variables that were potentially driving multivariate patterns could be identified. This was an indirect way of identifying which environmental variables were possibly responsible for multivariate patterns as important variables show strong positive or negative correlations with either axis. Correlations of each environmental variable with both CAP axes were superimposed on to a plot of the first two CAP axes to form a biplot. A visual interpretation of sample distribution and variable correlations was made together with tests of hypotheses using permutation procedures on canonical test statistics as described above.

We next considered the partial correlations of each slug species with canonical axes 1 and 2. These relationships were displayed graphically in a second biplot. A major advantage of CAP in comparison with other constrained ordination techniques is that it allows co-linear variables to be examined together. This enabled a direct comparison to be made between the two biplots. As such, it was possible to identify relationships between slug species relative abundances and important environmental variables. All analyses were undertaken using the PRIMER6 and PERMANOVA+ extension (Clarke & Gorley 2006).

2.4 Results

2.4.1 *Slug densities*

A total of 274 slugs were recorded from all soil samples representing 159 (58.0%), 67 (24.5%) and 48 (17.5%) slugs from all unimproved, semi-improved and improved meadows, respectively. Seven species of slug were recorded in total and details of their mean abundance per sample (0.09 m^2) and mean relative abundance (m^{-2}) are provided in Table 2.2. The mean density of slugs in unimproved, semi-improved and improved meadows was 70.7±14.1 m^{-2}, 37.22±8.3 m^{-2} and 21.33±5.4 m^{-2} respectively.

The relative proportions of each species in terms of mean density (mOverall, *D. reticulatum* was the most numerous species accounting for 58.6% of all slugs recorded from soil samples and was the most abundant species within each improvement category of meadow . It was also the most common species being recorded in all meadows except the semi-improved meadow at Askrigg Bottoms and 56% (39/70) of all samples. The mean density of *D. reticulatum* in meadows of each improvement category was 27.6 m^{-2} (unimproved), 32.4 m^{-2} (semi-improved) and 10.2 m^{-2} (improved) (Table 2.2). *Arion fasciatus* agg. was the second most abundant species in each improvement category and accounted for 18.6% of all slugs collected. It was also common, occurring in 34% (24/70) of samples and in all but one improved and two semi-improved meadows . Mean population densities of *A. fasciatus* agg. in each improvement category of meadow (unimproved: 4.9 m^{-2}; semi-improved: 10.2 m^{-2}; improved: 8.4 m^{-2}) were lower in comparison with *D. reticulatum*, but in some meadows it was the more abundant species (Table 2.2). Other slug species were *A. intermedius*, *A. distinctus* (Mabille), *A. subfuscus* (Draparnaud), *A. ater* agg. and *D. caruanae* (Pollonera). The first three species occurred in 17-20% (12-14 out of 70) of samples and were recorded in meadows of each improvement category. *D. caruane* and *A*. *ater* agg. were absent from improved meadows and occurred in <3% (≤2/70) of all samples.

Table 2.2. Mean (±SEM) population densities of slug species extracted from soil samples (0.09 m^2) in meadows of different agricultural improvement categories (unimproved, semi-improved, improved) at five locations in the Yorkshire Dales. Relative abundance (m^{-2}) is shown in parantheses. N = 5 samples per meadow. Location: AB = Askrigg Bottoms; TR = Thornton Rust; Y = Yockenthwaite; W = Walden; M = Muker. Note there was no semi-improved meadow at Muker.

Meadow	Location	*Deroceras reticulatum*	*Arion distinctus*	*Arion fasciatus agg.*	*Arion intermedius*	*Arion subfuscus*	*Deroceras caruanae*	*Arion ater agg.*	Total density
Unimproved	AB	2.4	0	0.2	0.2	0.4	0	0.2	3.4±1.12 (37.77±12.47)
	TR	7.2	0	0.8	0.2	0.2	0	0	1.8±0.20 (93.32±22.66)
	Y	1.6	0.8	0.6	0	1.4	0.2	0	4.6±1.63 (51.12±18.12)
	W	0.6	0	0.2	0.6	0	0	0	1.4±0.87 (15.55±9.69)
	M	6.4	1.6	2.8	2.8	0.4	0	0	14±2.92 (155.54±32.39)
	Combined	2.48 (27.55)	0.16 (1.78)	0.44 (4.89)	0.2 (2.22)	0.4 (4.44)	0.04 (0.44)	0.04 (0.44)	6.36±1.27 (70.66±14.07)
Semi-improved	AB	0	0	1.6	0	0.8	0	0	2.4±0.68 (26.66±7.54)
	TR	5	0.2	0	0.2	0.2	0	0	5.6±3.20 (62.22±35.59)
	Y	1.2	0.6	0.2	0	0.2	0.2	0	2.4±1.25 (26.66±13.88)
	W	2	0	0	1	0	0	0	3±0.89 (33.33±9.94)
	Combined	2.92 (32.44)	0.48(5.33)	0.92 (10.22)	0.8 (8.89)	0.32 (3.56)	0.04 (0.44)	0 (0)	3.35±0.75 (37.22±8.32)
Improved	AB	2.4	0.4	1	0.2	0.4	0	0	4.4±1.6 (48.88±17.78)
	TR	0.2	0	0	0	0	0	0	0.2±0.2 (2.22±2.22)
	Y	1.2	0	2	0	0	0	0	3.2±1.02 (35.55±11.33)
	W	0.4	0	0.8	0.2	0	0	0	1.4±0.40 (15.55±4.44)
	M	0.4	0	0	0	0	0	0	0.4±0.25 (4.44±2.72)
	Combined	0.92 (10.22)	0.08 (0.89)	0.76 (8.44)	0.08 (0.89)	0.08 (0.89)	0 (0)	0 (0)	1.92±0.49 (21.33±5.44)

2.4.2 *Influence of management and environmental factors on slug densities*

Results of the PERMDISP analysis showed a significant effect of management on sample dispersions ($F = 4.8768$, $P < 0.05$). Pair-wise comparison tests of management groups also indicated a significant difference in multivariate dispersions between the improved and semi-improved groups ($t = 2.5177$, $P < 0.05$) and between the improved and unimproved groups ($t = 2.7806$, $P < 0.01$), but not between the semi-improved and unimproved groups ($t = 0.1551$, $P = 0.8969$).

Results of the PERMANOVA analysis showed a significant effect of management on multivariate location of group centroids (Pseudo-$F = 3.2998$, $P < 0.01$) suggesting that slug assemblages differed according to management [Appendix, Table S1.1a], thus rejecting the null hypothesis of no effect of management on slug assemblages. The test result was influenced by differences in multivariate dispersions meaning that the significant effect of management was not solely due to differences in multivariate location. Pair-wise comparison tests indicated a significant difference in slug assemblages of soil samples between the improved and unimproved group ($t = 3.1896$, $P < 0.001$), although the test was influenced by differences in multivariate dispersions between these two groups. Pair-wise tests did not find a significant difference between the improved and semi-improved groups ($t = 0.9835$, $P = 0.4231$) or between the semi-improved and unimproved groups ($t = 0.7726$, $P = 0.6054$) [Appendix, Table S1.1b].

Data were characterised by a large proportion of zero counts with many samples containing few species or no slugs at all (18/70; 25.71%). This finding was not exclusive to a particular management group. Most species occurred infrequently with *D. caruanae* and *A. ater* recorded in ≤2 samples. Samples characterised by very low slug abundance or zero slugs strongly influenced the distribution of samples in multivariate space as depicted by the PCO plot (Figure 2.1). The first two PCO axes explained 80.5% of the variation in the original dissimilarity matrix. Visual interpretation of the plot indicated that samples do not conform to three clear management groups; however, there was some separation in multivariate space on the first PCO axis (57.3%) between samples within the unimproved (increasingly negative axis 1 scores) and improved (increasingly positive axis 1 scores) groups suggesting a possible difference in multivariate location between these groups. This observation is supported by results of the pair-wise comparison tests performed in PERMANOVA. Samples within each management group were scattered throughout the PCO plot demonstrating dispersion within groups. Furthermore, there was some indication of greater heterogeneity between samples present in the improved group in comparison with sample distribution within the semi-improved and unimproved groups. Again, these observations are supported by results of the PERMDISP analysis.

Overlapping samples visible in the PCO plot (having the highest axis 1 scores) are those which contained zero slugs, and samples from each improvement category are represented within this group. Other samples correlated with positive PCO axis 1 scores were also characterised by low slug abundance. In comparison, samples associated with highly negative axis 1 scores were those characterised by high slug abundance and richness, and were predominantly samples classified as unimproved. This pattern indicates a potential separation of samples correlated with PCO axis 1 according to slug abundance and a potential separation of samples within the unimproved and improved groups. There was some correlation of sample distribution with PCO axis 2 (27.2%), with two groups distinguishable at opposite ends of axis 2; however, it was not apparent what factor(s) was (were) responsible for this pattern from visual interpretation of the unconstrained ordination.

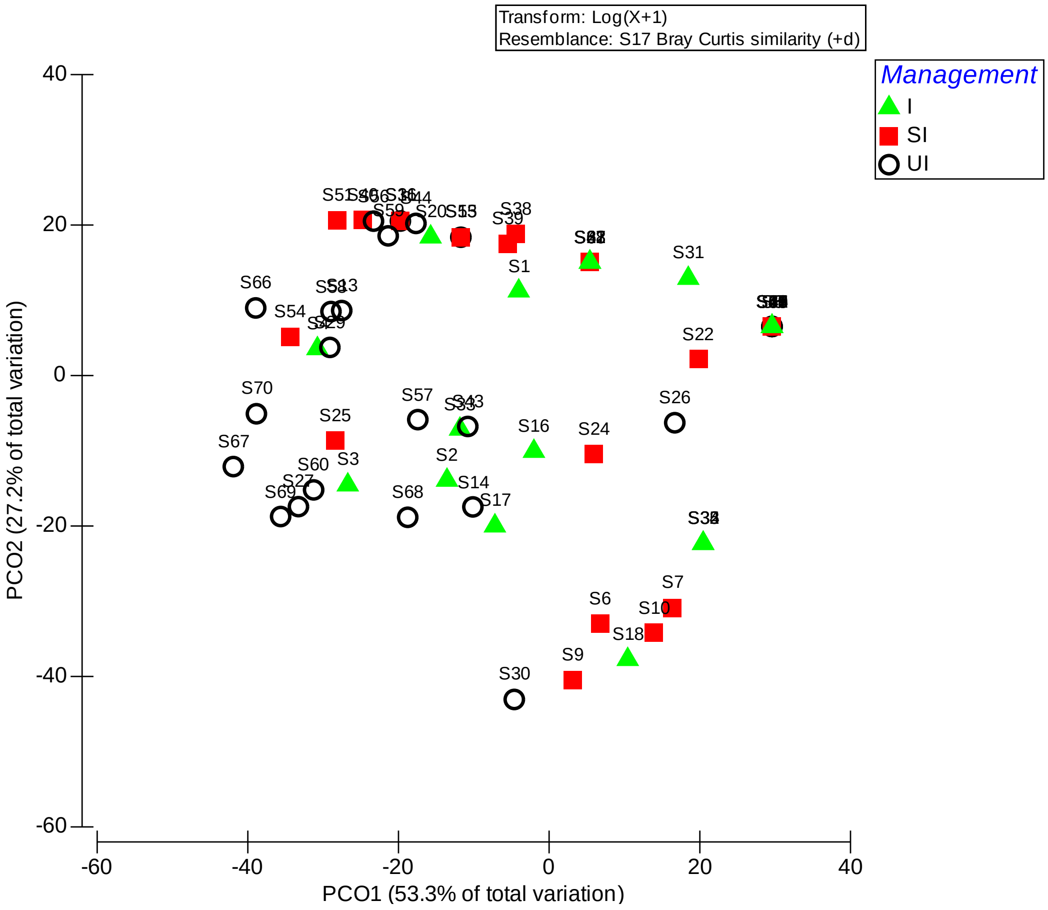


Figure 2.1. Principal Coordinates analysis (unconstrained ordination) of (transformed) slug abundance data classified by meadow management type. I = improved; SI = semi-improved; UI = unimproved.

The multivariate patterns in slug assemblages across sampling sites according to management are shown by a plot of the first two canonical axes of the CAP ordination (Figure 2.2). The canonical analysis (CAP) produced two canonical axes with squared canonical correlations of 0.2013 and 0.0161. Canonical axis 2 therefore explained virtually none of the variation in sample distribution between management groups. Differences in slug assemblages between some management groups were shown by the distribution of samples in multivariate space correlated with canonical axis 1. Samples belonging to the unimproved group were predominantly correlated with positive canonical axis 1 scores, whereas samples belonging to the improved group showed the opposite pattern, being associated predominantly with mid to low canonical axis 1 scores. However, there was clearly some overlap between the two groups and it is important to note that canonical axis 1 explained only 20.13% of variation in the model. Samples belonging to the semi-improved group were scattered over all of canonical axis 1 and were mixed with samples from the other two groups, although there were more SI samples associated with lower than higher canonical axis 1 scores. A general pattern of increasing slug abundance with increasing canonical axis 1 scores is evident in the plot. Samples forming a separate group visible at the extreme negative end of canonical axis 1 (axis 1, -0.0568; axis 2, -0.0119), and represented by each management group, contained zero slugs and clearly influenced the overall pattern of samples in the ordination as demonstrated previously by the unconstrained ordination. In contrast, samples correlated with increasing positive axis 1 scores were characterised by more abundant slug assemblages. The two canonical test statistics were highly significant (trace statistic: *P* = 0.0156; delta squared statistic: *P* = 0.0092, using 10000 permutations) indicating a strong correlation between slug assemblages and management intensity and are consistent with the result obtained by the PERMANOVA global test.

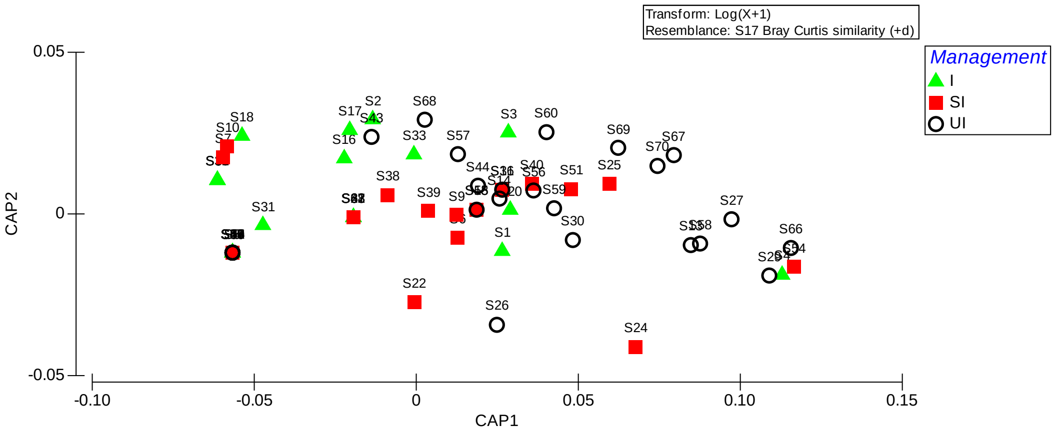


Figure 2.2. Canonical analysis of principal coordinates (CAP) of slug assemblages recorded in soil samples collected from meadows and constrained by meadow management type. The squared canonical correlations of the first two canonical axes are 0.2013 and 0.0161. I= improved; SI = semi-improved; UI = unimproved.

Results of the cross-validation test (Table 2.3) showed that 76% of improved samples and 64% of unimproved samples were classified correctly indicating that they formed more or less distinct groups in multivariate space. In contrast, only 20% of semi-improved samples were classified correctly indicating that samples overlapped with the other groups and slug assemblages were highly variable within this group and did not conform to a distinct group. These findings indicate that slug abundance responds positively to a gradient of decreasing agricultural improvement. The mis-classification error of 44.3% is quite high and was undoubtedly influenced by the high mis-classification of samples within the semi-improved group and variability (i.e. dispersion) between samples within groups as indicated by results of the PERMDISP analyses. This also suggests that management (i.e. different improvement categories) is not, by itself, sufficient to predict slug assemblages within meadows.

Table 2.3. Canonical Analysis of principal coordinates (CAP) for slug densities within upland hay meadows, incorporating a cross validation test using Leave-one-out Allocation of Observations to Groups (for the choice of *m*: 3). Groups are agricultural improvement categories (I= improved; SI = semi-improved; UI = unimproved) representing a gradient of increasing management intensity.

	Classified				
Original group	I	SI	UI	Total	% correct
I	19	2	4	25	76
SI	8	4	8	20	20
UI	6	3	16	25	64
Total correct	39/70 (55.714%)				
Mis-classification error	44.286%				

The influence of environmental variables on the general patterns in slug assemblages, grouped by management class, was investigated by the second CAP analysis. The squared canonical correlations of the first two canonical axes were 0.1984 and 0.0882 – capturing a similar proportion of the variability in the original dissimilarity matrix as when differences in slug assemblages were constrained solely by management, as in Figure 2.2. The trace statistic was significant ($P < 0.05$), although the delta squared statistic was marginally above the significance threshold ($P = 0.0515$) indicating a potentially significant correlation between slug assemblages and environmental variables. The discrepancy between the two canonical test statistics is possibly a product of the variability in abundance between samples, particularly the large proportion of samples which yielded very low slug abundance or no slugs at all, and suggests that a greater sample size may have improved the reliability of the analysis.

The pattern of samples in the second CAP analysis, constrained by environmental factors, was displayed graphically and combined with individual species scores to produce a biplot (Figure 2.3). The first two canonical axes showed that the multivariate pattern in abundance between sampling sites, while still grouped by management type, was determined by the relative abundance of the species themselves. Table 2.4 shows the partial correlations of individual slug species (after transformation) with both CAP axes. Species which characterise the multivariate effect are those with the greatest positive and negative scores.

A pattern in sample distribution was apparent by reference to the strength of correlation between different slug species and canonical axis 1. All slug species were positively correlated with canonical axis 1 indicating that samples associated with negative axis 1 scores were characterised by increasing slug abundances of all species and, consequently, increasing slug species richness. Those samples with increasingly negative axis 1 scores were predominantly classified as unimproved. In contrast, samples associated with increasingly positive axis 1 scores were characterised by decreasing abundance of all species and were predominantly classified as improved or semi-improved. This is consistent with results obtained using PERMANOVA and the previous CAP ordination. There was clear overlap, however, between samples of all groups and this supports the findings of the mis-classification test and again suggests that slug abundance operates on an ecological gradient. Species showing the strongest positive correlations with axis 1 were *D. reticulatum* (-0.62417) and *A. fasciatus* (-0.37715) and were the most abundant species in all samples. However, increases in their relative abundance were associated primarily with unimproved samples. Other species showed weaker correlations with axis 1 and occurred less frequently in the dataset.

Although the distribution of samples in association with canonical axis 2 is random according to management groups, results of the CAP analysis indicate that the variability amongst samples (i.e. variation in slug assemblages) was influenced by differences in the relative abundance of *D. reticulatum* and *A. fasciatus*. These species are diametrically opposed to one another and are the main drivers of canonical axis 2 (*D. reticulatum*: -0.33596; *A. fasciatus*: 0.619391). In other words, samples associated with positive axis 2 scores are characterised by slug assemblages with increasing relative abundance of *A. fasciatus* and decreasing abundance of *D. reticulatum*; and samples associated with negative axis 2 scores are characterised by the opposite trend. This observation may be due to interspecific competition between these species in all improvement classes of meadow, although shifts in relative abundance may be less marked in unimproved meadows as these species do not have diametric correlations with axis 1. However, it is important to remember that axis 2 explains only 8.82% of the variation in sample distribution.

The weak correlations of most slug species with canonical axes may be a product of the infrequent occurrence of some species and the large proportion of samples containing very few slugs. This may be due to issues inherent within the data, namely low sample size, rather than the underlying ecology of the different species. Variability amongst samples is also indicated by results obtained by PERMDISP and warrants an evaluation of the sampling regime.

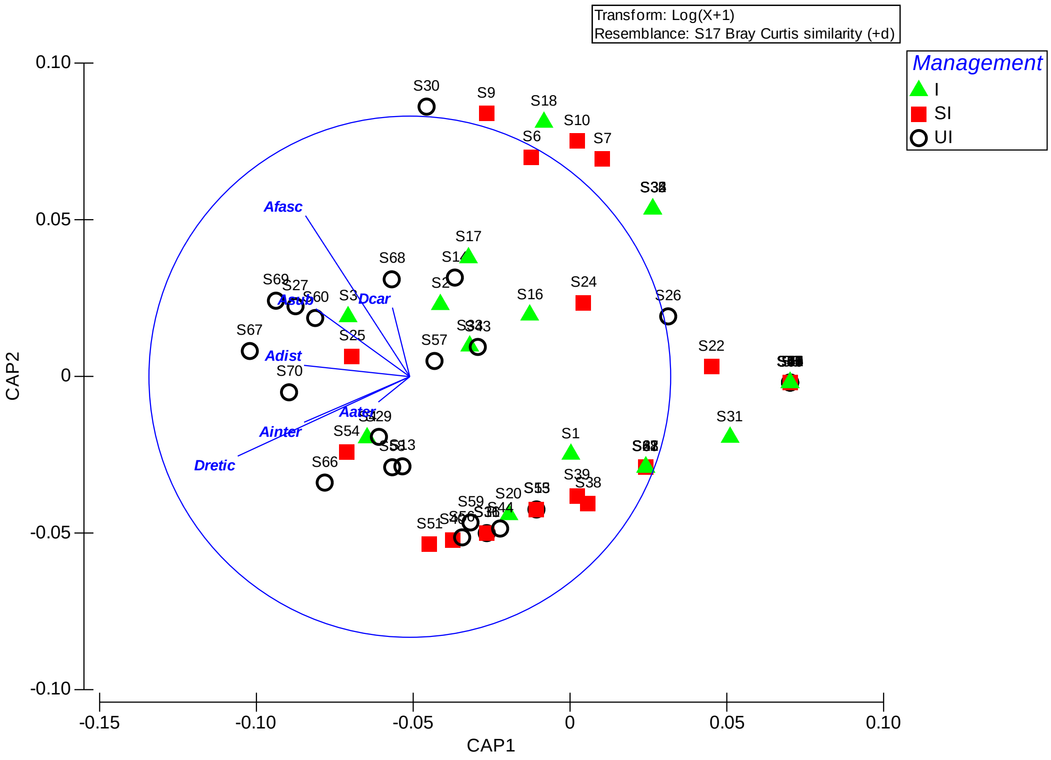


Figure 2.3. Canonical analysis of principal coordinates (CAP) of slug assemblages in meadows classified according to management type and constrained by environmental factors. The squared canonical correlations of the first two canonical axes are 0.1984 and 0.0882. I = improved; SI = semi-improved; UI = unimproved. Partial correlations between slug species and canonical axis are displayed. Slug species: Aater, *Arion ater* agg.; Dretic, *Deroceras reticulatum*; Ainter, *A. intermedius*; Adist, *A. distinctus*; Asub, *A. subfuscus*; Afasc, *A. fasciatus* agg.; Dcar, *D. caruanae*.

Table 2.4. Partial correlations between canonical axes and slug species generated by a Canonical analysis of principal coordinates (CAP) for slug assemblages in meadows classified according to management type and constrained by environmental factors.

	Canonical eigenvectors in the space of x	
Slug species	CAP1	CAP2
Deroceras reticulatum	-0.62417	-0.33596
Arion distinctus	-0.06092	0.196456
Arion fasciatus agg.	-0.37715	0.619391
Arion intermedius	-0.18542	-0.10673
Arion subfuscus	-0.25162	0.274549
Deroceras caruanae	-0.09329	0.117926
Arion ater agg.	-0.0406	-0.02707

Turning now to the biplot displaying the correlations of environmental variables with the first two canonical axes, it is apparent that the pattern of sample distribution, and thereby the variation in slug assemblages between samples, is influenced by particular environmental variables (Figure 2.4). The correlations of each environmental variable with the first two canonical axes are displayed in Table 2.5. As before, environmental variables which characterise the multivariate effect are those with the greatest positive and negative scores.

The most important variables influencing slug assemblages are the main drivers of canonical axis 1, namely percentage grass cover (0.542) and soil bulk density (-0.722) which are diametrically opposed to one another. This observation indicates that slug abundance is strongly negatively correlated with grass abundance cover and strongly positively correlated with bulk density. Slug abundance is also negatively correlated with herbage N content (axis 1 0.316). Comparisons between correlations for slug species and environmental factors with canonical axis 1 show that all slug species are negatively correlated with axis 1, whereas grass cover is positively correlated, indicating that the abundance of all species was lower in meadows with high grass cover. The abundance of *D. reticulatum* (-0.62417) and *A. fasciatus* (-0.37715) was most affected by grass cover, although the scarcity and low abundance of other species hampers the ability of the model to ascertain the true effect of grass cover on all species. Conversely, the relative abundance of all slug species, particularly *D. reticulatum* and *A. fasciatus*, was positively correlated with soil bulk density.

In comparison, the correlations of DIN (-0.037) and plant species richness (0.290) with canonical axis 1 are low and do not influence slug assemblages in different management groups. These variables are shown by the biplot as being directly opposed to grass cover and density. Hence, species richness and DIN explain minimal amounts of variation in slug assemblages between management groups.

The main drivers of canonical axis 2 were percentage grass cover (0.634) and DIN (-0.487). The finding that grass is seen to dominate on both axes suggests that its effect on slug abundance is quite profound. Comparison of biplots also indicates that increasing grass cover (axis 2, 0.634) may be more detrimental to the relative abundance of *D. reticulatum* (axis 2 -0.33596) than *A. fasciatus* (axis 2 0.619391), possibly as an indirect effect on interspecific competition between these species. This trend can also be seen in Table 2.2. In contrast, increasing DIN (-0.487) has the opposite effect on the relative abundance of these species, as a positive correlation with *D. reticulatum* (-0.33596) and a negative correlation with *A. fasciatus* (0.619391) is evident. Although, again, it is important to remember that axis 2 explained only 8.82% of the variation in sample distribution and the large proportion of samples containing very few slugs hinders the ability of the model to ascertain the true variability in slug assemblages due to environmental variables.

Table 2.5. Absolute correlations between canonical axes and environmental variables generated by a Canonical analysis of principal coordinates (CAP) for slug assemblages in meadows classified according to management type and constrained by environmental factors.

	Canonical eigenvectors in the space of x	
Variable	CAP1	CAP2
DIN	-0.037	-0.487
Plant species richness	0.290	-0.322
Percentage grass cover	0.542	0.634
Herbage N content	0.316	0.208
Soil bulk density	-0.722	0.462

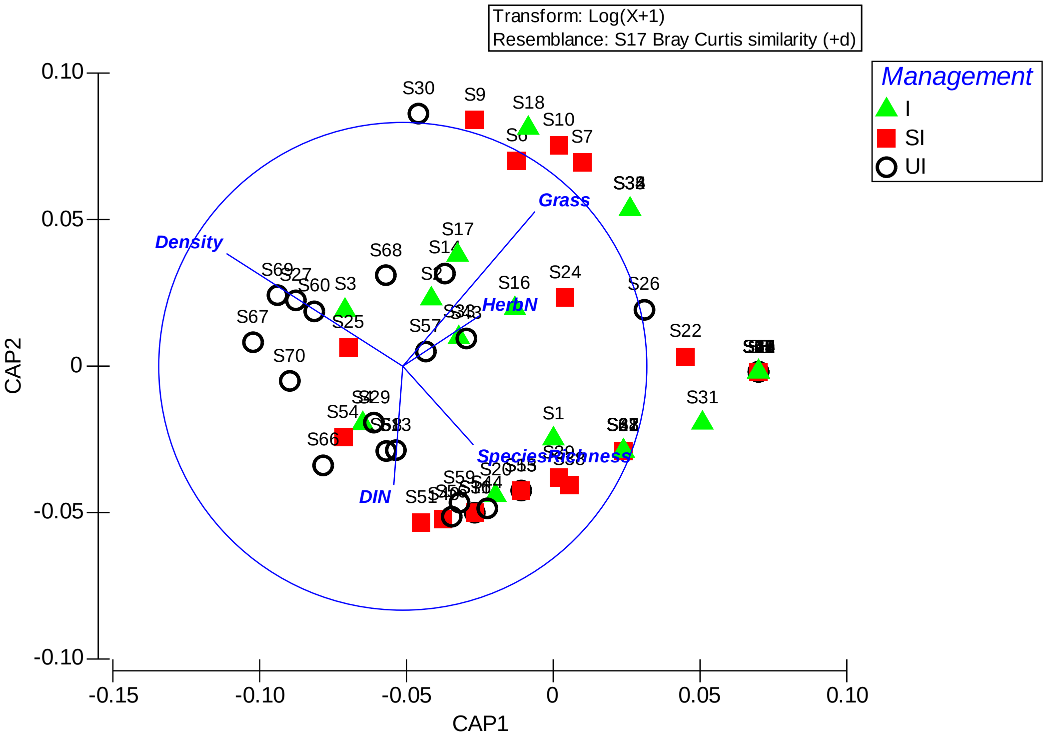


Figure 2.4. Canonical analysis of principal coordinates (CAP) of slug assemblages grouped according to management type and constrained by environmental factors. The squared canonical correlations of the first two canonical axes are 0.1984 and 0.0882. I = improved; SI = semi-improved; UI = unimproved. The pattern of samples is determined by environmental variables: i) percentage grass abundance cover; ii) plant species richness; iii) herbage N content (N mg/kg); iv) dissolved inorganic N soil (DIN) (µg N g-1Dry soil); and v) soil bulk density (kg/ha (to 7.5 cm)). Correlations between environmental variables and canonical axes are displayed.

2.5 Discussion

Mean slug densities in each improvement category of meadow (unimproved: 70.7±14.1 m^{-2}; semi-improved: 37.2±8.3 m^{-2}; improved: 21.3±5.4 m^{-2}) are similar to results obtained by other studies that have used soil flooding to estimate slug densities in grassland. Estimates of annual slug densities in grassland range from >50 m^{-2} in permanent pasture (South 1989b), rough grassland and grass paddock

(Warley 1970) to approximately 30 m^{-2} in upland acid grassland (Lutman 1978). It is reasonable therefore to describe the mean slug densities recorded for improved, semi-improved and unimproved meadows as low, medium and high, respectively. The high frequency and abundance of *D. reticulatum* in this study is also consistent with these studies which report it to be the dominant species in UK grassland. The density of *D. reticulatum* in each meadow improvement category was 27.6 m^{-2} (unimproved), 32.4 m^{-2} (semi-improved) and 10.2 m^{-2} (improved) and can be compared with annual mean densities of 42.4 m^{-2} (South 1989b), 66 m^{-2} (rough grassland) and 23.7 m^{-2} (grass paddock) (Warley 1970), although we recognise the limitations of sampling in just one month of the year. *A. fasciatus* was also recorded by these studies but at lower abundances than in this study. Interestingly, Carrick (1938) reported an annual mean density of 12 m^{-2} for *D. reticulatum* following extraction of slugs using copper sulphate from a rye grass sward. This finding is very similar to results obtained in this study for the density of *D. reticulatum* in improved meadows.

A positive relationship between both total slug density and species richness with decreasing levels of agricultural improvement of hay meadows is evident in this study. This finding supports that of Lutman (1978) who found slugs to be associated with species-rich grassland in Northern England. Opposite ends of the improvement gradient (i.e. improved and unimproved meadows) showed this trend best with semi-improved samples showing much overlap between groups. The pattern of samples in multivariate space shown by both unconstrained and constrained ordinations together with results of the mis-classification test and permutational tests of hypotheses were all consistent with this general trend; however, there was clear variability within each management group, particularly the improved (greater dispersion) and semi-improved (high mis-classification) groups. All management groups contained some samples characterised by very low slug abundance or no slugs at all. This variability was likely influenced by the low sample size (n = 5) for each meadow and we suggest that a greater sample size may have substantially reduced the variability within the dataset. South (1992) suggests that 12-20 samples will give satisfactory results. The decision to use five samples per meadow was based on: i) previous studies that have found five samples to be adequate in estimating slug densities using soil flooding (e.g.

Glen, Milsom & Wiltshire 1989; Kendall *et al.* 1995); and ii) the logistical difficulties of using the soil flooding technique (Symondson *et al.* 1996). Estimating slug populations in this way was a laborious and time-consuming process intensified by the need to transport soil samples from isolated meadows to a vehicle and then to the field station some 80 miles away. The use of other quantitative techniques, such as defined area traps (DAT), were considered but were not practical or feasible alternatives as they would have required daily monitoring over several weeks.

It is also necessary to consider other disadvantages of the sampling technique. Slug species vary in their vertical and horizontal distribution (South 1992). Soil samples captured slugs in the top 10 cm of the soil horizon. The technique is biased towards smaller slugs and species which take refuge in the upper soil horizon. Mature *Arion ater* agg. individuals are relatively large and take refuge within dense vegetation, thus are less likely to be sampled using the technique in this study. This is reflected by the single record for (a juvenile) *A. ater* agg. Most slug species are located in the top 10 cm of the soil horizon, particularly *D. reticulatum* which is predominantly located within 2-3 cm of the soil surface or in the vegetation layer (South 1965; Warley 1970). However, some species are more frequently located at depths exceeding 10 cm (Hunter 1966; Stephenson 1966); for example, *Tandonia budapestensis* (Hazay) is predominantly a root-feeding species and was absent from all samples, but was likely to have been present in meadows at greater depths. Slugs may also be aggregated in their distribution (i.e. show a negative binomial distribution) (South 1992 and references therein) and in this study samples were taken from within a 25 x 25 m square. The location of the square was selected to encompass a representative area of the vegetation but did not control for topographic variables such as slope (and thereby drainage). Another important consideration is that life cycles differ between different slug species (Lutman 1978; South 1989a; South 1992), therefore the relative proportions of each species and the total number of slugs would be expected to fluctuate seasonally or even monthly. The results obtained in this study reflect populations of each species in spring time (March) which was chosen as the sampling time because it did not greatly bias a particular slug species.

Although management was a useful predictor of slug density there was clearly some variability within management groups. CAP analysis revealed percentage grass cover and soil bulk density to be the most important underlying reasons for differences in slug assemblages between samples and were depicted by the biplot as having diametrically opposing correlations with the first canonical axis. A strong negative correlation was evident between percentage grass cover and density of each slug species. Samples characterised by high grass cover and low slug density were mostly classified as improved or semi-improved in accordance with the known positive effects of agricultural intensification on grass cover (e.g. Smith & Rushton 1994; Smith *et al.* 1996a; Smith *et al.* 1996b; Smith *et al.* 2000). Similarly, increasing herbage N content was also associated with decreasing slug density, although the strength of the correlation was not as strong as for grass abundance cover. This was surprising as herbage N content is negatively correlated with increasing grassland management intensity and is associated with forb-rich unimproved meadows (Bardgett *et al.* 2007). It would also be expected that greater herbage N content would benefit slugs as nitrogen is important for slug nutrition (Speiser 2001) and *D. reticulatum* has been shown to select fertilised lettuce leaves with the highest nitrogen contents (Pakarinen, Niemela & Tuomi 1990). Increasing soil bulk density was strongly correlated with increasing total slug density and density of each slug species, particularly *D. reticulatum* and *A. fasciatus* and was associated with samples that were predominantly classified as unimproved. In other words, decreasing grass abundance cover and increasing soil bulk density had a positive effect on slug density and were most associated with environmental conditions in unimproved meadows. However, the variability within the dataset hindered the ability of the model to ascertain the true effect of environmental variables on slug density and the likely influence of low sample size has already been discussed.

The general negative effect of high grass cover on slug densities is consistent with slug diet. Slugs are “acceptability-moderated” generalists (Dirzo 1980), showing distinct food preferences under laboratory (Grime, MacPherson-Stewart & Dearman 1968; Dirzo 1980) and field conditions (Hanley, Fenner & Edwards 1995a; Hanley, Fenner & Edwards 1996a). Studies frequently report that slugsprefer broad-leaved forbs to grasses (Barker & Addison 1992; Hanley 2004;

Pywell *et al.* 2007), although slugs will readily damage grass seedlings (Hulme 1994; Fenner, Hanley & Lawrence 1999) and are a pest of newly sown grass leys (Barker 1990; Glen, Cuerden & Butler 1991). The low acceptability of grass leaves to slugs – at least at the post-seedling stage - is a likely consequence of silica bodies (phytoliths) within grass leaves (Grime, MacPherson-Stewart & Dearman 1968; Dirzo 1980)which which act as an anti-herbivore defence (against insects) by increasing mandible wear and reducing the digestibility of forage carbohydrates and larval growth rates (Massey, Ennos & Hartley 2006; Massey & Hartley 2009). Silica phytoliths in rice (*Oryza sativa* L.) have also been shown to reduce feeding by *D. reticulatum* (Wadham & Wynn-Parry 1981). Although the influence of grass cover explained some of the variation in slug densities, further investigation is required to determine whether slug densities are food limited in meadows dominated by grass (or particular grass species) (Speiser 2001).

A negative correlation between overall gastropod abundance and percentage grass cover was also evident in the study by Dedov *et al.* (2006) which examined the effects of various parameters of the vegetation on gastropod species richness. Gastropod assemblages, in field plots subject to several sowing treatments of grass and wildflower seed mixes, contained seven species dominated by two of them – *D. reticulatum* and a snail, *Cochlicopa lubrica* (Müller)/*lubricella* (Rossmässler) (species complex not distinguished). Dedov *et al.* (2006) found that *D. reticulatum* was more abundant in samples with higher plant diversity and moss cover (associated with several years of natural plant colonisation in experimental plots). *C. lubrica*/*lubricella* responded positively to plant height, more so than diversity, and was also strongly negatively correlated with grass cover, indicating that both species responded negatively to grass cover but showed species-specific preferences for other aspects of the plant community structure. Dedov *et al.* (2006) also observed plant-gastropod species-level associations and it would be of interest to apply this method in future to the results of this study, particularly for abundant grass and slug species. For example, the cover of *L. perenne* may be a better predictor of slug populations than total grass cover.

Variability in slug assemblages within and between management groups was, in part, explained by differences in the relative abundances of the two most common species, *D. reticulatum* and *A. fasciatus*, which were inversely related to one another. Comparisons of CAP biplots suggested that inverse fluctuations in the relative abundance of these species occurred in all improvement categories of meadow, but with *A. fasciatus* potentially gaining a competitive advantage over *D. reticulatum* in sites containing high grass cover. However, this observation was due to correlations with axis 2 which only explained 8% of variability in the CAP analysis. Shifts in the relative abundance of these species may be reduced in unimproved meadows as both species were similarly correlated with unimproved samples containing high slug densities.

D. reticulatum and *A. fasciatus* are likely to experience considerable niche overlap, in terms of diet and temporal and spatial distribution, yet as co-occurring species must possess sufficient niche differentiation in some aspects of their biology. Greater interspecific competition may occur when preferred food plants are less abundant and perhaps less apparent (Cook & Radford 1988; Speiser 2001). Slugs may experience increased search time and reduced foraging efficiency if preferred food plants are less readily available in swards dominated by productive grasses (MacArthur & Pianka 1966). It is possible that the relative acceptability of grasses (or particular grass species) to *D. reticulatum* and *A. fasciatus* may differ, with *A. fasciatus* being potentially better adapted to feed on and/or digest silica-rich leaf tissue. Different responses by sympatric slug species to grass species are apparent in the literature (e.g. Getz 1959; Duval 1971; Duval 1973; Mølgaard 1986; Cook & Radford 1988). The effect of grass cover on these slug species may be more specifically due to particular grass species i.e. those with a tough leaf texture, such as *L. perenne*, *D. glomerata* and *P. trivialis* which are promoted by increasing levels of agricultural improvement and are reported to be largely avoided by slugs (Barker & Addison 1992). Conversely, Boycott (1934) and Runham and Hunter (1970) found no evidence that the quantity or quality of food had any influence on slug populations. Also, *A. fasciatus* is known to prefer wetter soils, whereas *D. reticulatum* is more tolerant of drier conditions (South 1964; South 1992) which may account for some variation in the relative abundance of these species between samples. Unfortunately, soil moisture of samples was not recorded but may be a good predictor of slug populations and may account for some of the variability within management groups.

The positive correlation between soil bulk density and slug density in association with decreasing levels of agricultural improvement is interesting and perhaps somewhat counter-intuitive. Results are in contrast to those of Bardgett *et al.* (2007) who identified a positive correlation between soil bulk density and management intensity of grassland including mesotrophic meadows. Higher soil bulk densities are often the result of compaction by agricultural machinery or livestock poaching/trampling (Greenwood & McKenzie 2001). As such, compaction would be expected to be greater within more intensively managed meadows, but this does not follow in our results. However, not all unimproved samples were equally associated with increasing bulk density. A possible explanation for this variability may lie in differences in soil conditions between unimproved meadow sampling sites (i.e. geographical locations), although inclusion of 'site' as a factor in PERMANOVA and PERMDISP analyses generated non-significant results and was removed from the models presented here.

A possible explanation for CAP results may be the action of plant roots on bulk density (da Silva, Kay & Perfect 1997). The diametric correlation between bulk density and grass cover suggests that soils with high bulk density occurred in meadows with lowest grass cover. High grass cover and dense fibrous grass roots may, over time, have a positive effect on soil structure, lowering bulk density (Imhoff, da Silva & Tormena 2000). Therefore, bulk density, rather than directly influencing slug populations, may be a pseudo-measurement for grass cover. However, direct effects of soil structure on slug populations are also likely. Soils with greater compaction (a positive dependent of bulk density) may retain a well-developed system of cracks which benefit slugs and other soil mesofauna by aiding their ability to move about, forage and take refuge below ground (Stephenson 1966; Duval 1970; Stephenson 1975a; Stephenson 1975b; Glen, Milsom & Wiltshire 1989). Another possible explanation may be that soils with lower porosity (a factor inversely related to bulk density) would be expected to be wetter (less free-draining) (Ball *et al.* 1997) and thus create more favourable abiotic conditions for slugs (Port & Port 1986; Cook 2001). Hypothetically, a greater frequency of fleshy rooted herbs that replace fibrous rooted grasses in less intensively managed meadows may lead to decreased granular structure formation and hence lower soil porosity and higher bulk density. If combined with rather fine loamy or clayey soils or higher groundwater levels, this could favour higher densities of slugs. Overall, slugs may prefer a smooth, denser and wetter soil surface produced by higher soil bulk densities and slightly poorer soil drainage conditions in combination with lower grass cover (R. Payton, personal comment). Further investigation of soil conditions is necessary to confirm or refute the above hypotheses.

There are other factors which may have influenced slug density that were not included in the model but are likely to be co-linear with grass abundance cover. Higher stocking rates of sheep and cattle and possibly longer grazing periods throughout the year are associated with increasing levels of agricultural improvement. Livestock may adversely affect slug densities by greater levels of trampling and poaching, for example Ferguson, Barratt and Jones (1988) describe how so-called mob stocking of sheep drastically reduced *D. reticulatum* populations by 90%. In addition to grazing management, differences in the cutting regime between improvement categories of meadow may influence slug populations. Cutting reduces the available shelter and produces a drier microclimate (South 1992). Agriculturally improved meadows are cut earlier in the year (May-June) and the crop is removed immediately for silage. Improved meadows may also be cut more than once throughout the growing season. This is in contrast with unimproved meadows which are cut later (mid-July-September) and the crop is left *in situ* to make hay. Greater stocking density, earlier cutting and silage production have a negative effect on botanical diversity and a positive effect on grass cover by promoting the abundance of competitive grass species, such as *L. perenne* (Smith *et al.* 1996a; Smith *et al.* 1996b; Smith *et al.* 2000). Our findings support those of Bruijns, Altena and Butot (1959) who found fields cut for hay were able to support a more diverse slug fauna than compared with intensively managed grassland subject to heavy trampling by livestock. Furthermore, South (1989b) found the number of slugs and eggs in permanent grassland to be unaffected by low livestock grazing pressure and a July hay cut. These findings and hypotheses support those of Neumann (1998) who found the species richness and abundance of the gastropod fauna in wet grasslands (pasture and meadow) in Germany to be negatively affected by increasing intensity of land use, and decreasing soil moisture, botanical diversity and soil structure. Neumann (1998) also found evidence that intensive management adversely affected reproduction conditions in two ways: i) trampling by higher stocking densities increased mortality rates, particularly of large species; and ii) earlier cutting caused increased egg desiccation on the open dry ground.

The interacting effects of the numerous factors discussed above on slug densities could be analysed using more advanced statistical techniques. The use of Structural Equation Modelling (SEM) or Bayesian Belief Networks and Decision Graphs (e.g. Arhonditsis *et al.* 2006) would enable the direct and indirect effects of predictor variables on slug densities to be ascertained. These analytical methods would prove fascinating in unravelling the factors influencing slug populations but would require a much larger dataset, and hence sample number, to be worthwhile.

While there is much evidence that slugs exert a strong selective pressure on forb seedling recruitment (Hanley 2004; Buschmann *et al.* 2005), and some studies have shown slugs to negatively affect forb richness up to three years from sowing (Wilby & Brown 2001; Pywell *et al.* 2007) and in the longer term (Allan & Crawley 2011), this study would indicate that high slug densities and slug species richness are associated with established mid-successional meadows of high botanical diversity. This observation supports the prediction made by Tilman (1988) that herbivore numbers will be greatest in mid-successional communities. A direct effect of reduced grass cover (or perhaps particular grass species) in more diverse meadows is linked to a positive response in slug abundance. We do not consider the reverse scenario i.e. the predictive power of slug populations on botanical composition, although it is likely that slugs have little impact on established swards in comparison with factors such as fertiliser addition, cutting date and livestock grazing intensity (Smith *et al.* 1996a; Smith *et al.* 1996b; Smith *et al.* 2003; Smith *et al.* 2008). However, the potential for slug densities to be high or low in semi-improved meadows (as indicated by the variability and high mis-classification within this group) may present a potential limiting factor to the recruitment of desirable forb seedlings in sites targeted for restoration. The successful establishment of a wider diversity of forb species, such as the hemi-parasite *Rhinanthus minor* L., introduced as seed from species-rich donor meadows, may respond to slug densities on a site-specific basis.

Chapter 3. The acceptability of meadow plants to the slug *Deroceras reticulatum* and implications for grassland restoration

3.1 Abstract

Despite the selective pressure slugs may exert on seedling recruitment there is a lack of information in this context within grassland restoration studies. Selective grazing is influenced by interspecific differences in acceptability. Here an assessment of relative acceptability is made for seedlings of meadow plants to the slug, *Deroceras reticulatum* to consider their potential susceptibility to selective grazing at (upland hay meadow) restoration sites.

Slug feeding damage to seedling monocultures of 23 meadow species and *Brassica napus* was assessed under controlled conditions. The severity and rate of damage incurred by each plant species was analysed with a Generalized Additive Mixed Model. Plant species were then ranked for their relative acceptability. The results were considered as a function of selected plant traits collated from the literature and electronic databases.

Species were assigned to one of four *a priori* damage categories based on severity and rate of damage. Interspecific variation in acceptability suggested slugs were influenced by chemical properties and that physical properties were less effective as a deterrent. Most unacceptable species were relatively slow-growing forbs and likely to be chemically well-defended, and are associated with species-rich mid-successional grassland. Highly acceptable species were predominantly fast-growing grasses and were seemingly poorly defended. Seedling growth rate was a good predictor of acceptability and provided some evidence of a growth-defence trade-off.

Highly unacceptable species, or those of low acceptability, were target restoration species (*Geranium sylvaticum*, *Rumex acetosa*, *Leontodon hispidus*, *Anthoxanthum odoratum*, *Sanguisorba officinalis*, *Ranunculus acris, Lotus corniculatus*), indicating that they are unlikely to be selectively grazed by slugs during the seedling recruitment phase. Highly acceptable species may be more vulnerable to selective grazing and the establishment of *Trifolium pratense*, an important fungal-facilitating species, may be negatively affected by slug herbivory. *In situ* investigation is necessary to test the predictive value of the results reported here.

Key-words: acceptability, grassland restoration, growth-defence trade-off, invertebrate herbivory, seedling recruitment, slugs

3.2 Introduction

Herbivory is a fundamental driver of plant community composition (Grime, 2002). Studies of (extensive) vertebrate grazing in temperate grassland have found important beneficial effects on plant diversity as a consequence of animals (livestock, rabbits, horses) consuming competitive grass species (Tansley and Adamson, 1925; Smith and Rushton, 1994); increasing the heterogeneity of sites (Rossignol *et al.*, 2011); preventing succession to woodland (Grubb, 1976); and providing germination niches for seedlings (Smith *et al.*, 2000). Comparatively less is known about the role of invertebrate herbivores; however, some groups or species have the potential to affect plant populations and communities during the establishment phase (Fenner, 1987; Hanley, 1998). Studies involving the addition of insecticides have found mixed effects on plant community composition (Crawley, 1989). For instance, Brown and Gange (1989*a*, *b*; 1992) demonstrated an increase in species richness of early successional vegetation as a likely consequence of a relaxation in herbivory of seeds or seedlings. In contrast, other studies found that arthropods had little effect on seedling recruitment and community development and that the dominant factor regulating recruitment was selective grazing by slugs (Buckland and Grime, 2000; Wilby and Brown, 2001).

Studies reviewed by Hanley (1998) have highlighted slugs as the most important invertebrate herbivore in temperate grassland (Rees and Brown, 1992; Hulme, 1994, 1996; Hanley *et al.*, 1995*a*). Molluscs prefer seedlings to mature plants (Fenner *et al.*, 1999) and selective grazing during the seedling phase is known to exert a strong selective pressure on community assemblages by causing the differential recruitment of species within a community (Hulme, 1994; Hanley *et al.*, 1995a, 1996; Hanley, 2004) and by altering the competitive balance between species (Cottam, 1986; Hanley and Sykes, 2009). More recently, field studies have revealed persistent effects of slugs on community dynamics over the mid to long term as a result of selective grazing during the seedling stage (Wilby and Brown, 2001; Buschmann *et al.*, 2005; Allan and Crawley, 2011).

Slug feeding behaviour is influenced by plant acceptability (Dirzo, 1980). Previous studies have attempted to determine the relative acceptability (or palatability) of a range of plant species to slugs and snails, including *Deroceras reticulatum*, under controlled microcosm conditions (Grime et al., 1968; Duval, 1971, 1973; Dirzo, 1980; Mølgaard, 1986; Cook *et al.*, 1996; Briner and Frank, 1998; Fenner *et al.*, 1999; Keller *et al.*, 1999; Koztowski and Koztowska, 2004). Evidence indicates that the primary factor influencing acceptability is the expression of chemical and physical anti-herbivore defences (Hanley *et al.*, 2007). For instance, molluscs are generally deterred by hard leaf texture (Grime *et al.*, 1968) and by secondary metabolites including tannins and phenolics (Mølgaard, 1986); cyanogenic glycosides (Dirzo and Harper, 1982; Horrill and Richards, 1986) and alkaloids (Speiser and Rowell-Rahier, 1991). Silica bodies (phytoliths) in grass leaves may also function as a defence against molluscs (Grime *et al.*, 1968; Dirzo, 1980; Wadham and Wynn-Parry, 1981; Hanley, 2004) as has been found for insects (Massey *et al.*, 2006; Massey and Hartley 2009) and voles (Massey and Hartley, 2006). However, silica concentrations are known to vary between grass species (Massey *et al.*, 2006) and silica is likely to be a less effective deterrent in seedlings (Hulme, 1996; Fenner *et al.*, 1999). Plant trichomes (glandular and non-glandular) may also serve as a deterrent to molluscs (Stahl, 1888; Westerbergh and Nyberg, 1995).

Defensive properties of seedlings may be subject to ontogenetic effects; whereby the development of an effective defence(s) is delayed following germination (Horrill and Richards, 1986; Elger *et al.*, 2009) or declines with seedling age (Glen *et al.*, 1990; Woodman and Fernandes, 1991). Thus, seedling age can influence acceptability, and in turn, the likelihood of attack and survival (Hanley, 1995*b*).

Experimental approaches to examining the acceptability of plant species may be criticised for a potential lack of applicability at the ecosystem level. However, evidence suggests that plant acceptability may be a useful life history trait to predict plant community development (Burt-Smith *et al.*, 2003; Strauss *et al.*, 2009). According to the Growth-Differentiation-Balance Hypothesis (GDBH), slow-growing species may be better defended against principle herbivores whilst fast-growing species lack defences but tolerate herbivory by investing resources in a fast relative growth rate (Herms and Mattson, 1992). Several studies have examined evidence of a growth-defence trade-off but report conflicting findings (Herms and Mattson, 1992; Hanley, 1998; Kelly and Hanley, 2005). Supporting evidence is often found for plants growing in unproductive habitats or late-successional communities (Grime, 2002). Indeed, there is evidence that molluscs find ruderal species more palatable than stress-tolerating, later successional species (Grime *et al.*, 1968, 1996; Cates and Orians, 1975).

Despite the potential impacts of slug herbivory on seedling recruitment, few studies have considered the effects in the context of achieving grassland restoration targets (but see Keller *et al.*, 1999; Hitchmough, 2003; Pywell *et al.*, 2007; Allan and Crawley, 2011). The seedling recruitment phase is key to initial establishment (Grubb, 1977; Fenner and Thompson, 2005) and represents an important bottle-neck to founding populations introduced as seed from species-rich donor meadows. Studies of meadow restoration have shown that the successful establishment of the hemi-parasite *Rhinanthus minor* and fungal-facilitating species (including legumes, particularly *Trifolium pratense*, and stress-tolerant and stress-tolerant-ruderal species) is important in the early stages of restoration as they act to reduce constraints causing niche limitation to early and later-colonising species (e.g. strong competition from dominant grasses and high residual soil fertility) (Smith *et al.*, 2003, 2008; Pywell *et al.*, 2004; Bardgett *et al.*, 2006, 2007). It is important, therefore, to determine the relative acceptability of species that are likely to be encountered by slugs at restoration sites during the seedling recruitment phase.

This study determines the relative acceptability of seedlings of 23 grassland plant species and a (positive) reference species (*Brassica napus*) to the field slug

Deroceras reticulatum. We hypothesise that slow-growing forbs, typical of the target grassland community will be relatively less acceptable to slugs due to the expression of chemical defences; whereas fast growing species lacking defences at the seedling stage will be more acceptable. To aid explanation of the findings, results were correlated with selected plant traits including seedling growth rate. The implications for grassland restoration are discussed.

3.3 Method

3.3.1 *Deroceras reticulatum*

In temperate climes, *D. reticulatum* is a pest of newly sown arable crops and grass leys (Runham and Hunter, 1970; Barker, 2002). The species is widespread and abundant in temperate grasslands (South, 1992; Hanley *et al.*, 1996) including upland hay meadows (Chapter 2). *D. reticulatum* forages predominantly above ground in the seedling stratum (Cook *et al.*, 1996) feeding on fresh green plant material (Pallant, 1972). Foraging activity is greatest in spring and autumn when weather conditions are cool and wet and coincides with seedling germination.

3.3.2 *Collection and maintenance of slugs*

D. reticulatum individuals were collected from beneath baited hardboard refuge traps from a plot of species-poor grassland at Close House Field Station, Newcastle upon Tyne, UK (NZ 126 661). Slugs were kept for up to 10 days in ventilated plastic boxes lined with damp paper towels and stored in controlled environmental conditions (12^{o}C, high relative humidity, 12 hour dark: 12 hour light). Slugs were fed on a mixed diet of lettuce and wheat grain with fresh material provided and boxes cleaned every two days. Slugs were deprived of food 24 hours prior to being introduced to experimental trays.

3.3.3 *Plant material and relative acceptability*

Plant species were chosen for their occurrence in species-rich upland hay meadows (defined by the National Vegetation Classification as MG3 *Anthoxanthom odoratum*-*Geranium sylvaticum* grassland) and less diverse associates (Rodwell, 1992). Nomenclature follows Stace (2010).

Seedlings of 23 grass and forb species were established from seed (Table 3.2). Seed was obtained from the commercial suppliers Emorsgate Seeds (Norfolk, England) and Herbiseed (Twyford, England). *Brassica napus* 'Lioness' (oilseed rape) was also sown as a positive reference species for comparative purposes and was the only annual species to be tested.

Preliminary tests were undertaken to establish the time to germination and the germination requirements for all species. Species were grouped according to germination times and acceptability tests were performed once for each species between November 2007 and May 2010. The study duration was due to several species failing to germinate in sufficient quantities.

Species monocultures were obtained by sowing seeds in propagator trays (355 mm x 215 mm x 50 mm) filled with John Innes No.2 compost and maintained under glasshouse conditions. Trays of each species were replicated five times. Seedlings were hand-thinned to leave 30 seedlings per tray (arranged in three rows of 10 plants). Five *D. reticulatum* individuals were introduced to each tray once seedlings had reached the two true-leaf stage of development (or equivalent biomass for monocot species). *B. napus* seedlings were tested at the cotyledon stage in order to represent a similar biomass as other test species. Slugs were provided with a refuge shelter (upturned saucer) and retained by the use of clear plastic ventilated propagator lids attached to trays with masking tape. The soil surface was watered every two days using a hand-held pressurised sprayer. Assessments of feeding damage were performed at 1, 2, 4, 7, 10 and 14 days following the introduction of slugs. Damage was described in terms of the proportion of plant biomass consumed by slugs for each seedling and, therefore, did not include biomass removed, but left on the soil surface.

Initial testing was undertaken in a heated glasshouse (min 14.5^{o}C; max 22.7^{o}C). In order to better control environmental variables, remaining testing was undertaken in a controlled temperature room maintained at 15^{0}C and high relative humidity with a time-controlled light-box (12 hour light:12 hour dark). Attempts to germinate sufficient numbers of *Conopodium majus*, *Ranunculus repens* and *R. bulbosus* were unsuccessful. *Rhinanthus minor* seedlings suffered high mortality prior to the introduction of slugs, thus the relative acceptability of *R. minor* could not be assessed. As the Fabaceae species tested are polymorphic for cyanide production, seedlings remaining after the 14 day assessment period were analysed using the sodium picrate method (Corkhill, 1942; Jones, 1966).

3.3.4 *Statistical analysis*

Statistical models were used to determine differences in the acceptability of meadow plant species to grazing damage by *D. reticulatum*. Proportional data describing the degree of damage to each seedling was first arcsine-transformed and then examined visually in order to conceptualise the functional response of each plant species *through time* following exposure to *D. reticulatum* [Appendix, Figure S2.1]. The response of each plant species was found to be non-linear and plant species varied in terms of the magnitude of the response [Appendix, Figure S2.1].

The statistical modelling of non-linear responses may be undertaken using Generalized Additive Models (GAM). GAM's are non-parametric statistical models that are suited to describing the non-linear relationships between a response variable and explanatory variable by means of a smoothing function (Guisan *et al.*, 2002; Zurr *et al.*, 2009). As such, additive models are ideally suited to examining complex non-linear processes that are typically found in biological systems (Torres *et al.*, 2008; Parra *et al.*, 2011). Furthermore, the development of Generalized Additive Mixed Models (GAMM) provides a tool for modelling data generated by hierarchical and longitudinal studies (Faraway, 2006). Such data are characterised by within-subject correlation and the correlation between repeated observations through time. Here, we generated a GAMM to assess the severity of damage incurred by each plant species as a function of time, whilst allowing for differing correlation between observations for each plant species. Models were fit to the data following the methodology described by Zurr *et al.* (2009). The model was used to estimate the susceptibility of each species to damage as a function of time where the optimal amount of smoothing required for each species was calculated by the internal cross-validation procedure (Wood 2006, 2012). Random-effects estimates were then used to rank each plant species according to the severity and rate of slug feeding damage to seedlings and, subsequently, to describe each plant species in terms of relative acceptability. Species were subjectively assigned to one of four *a priori* damage categories according to variation in severity and rate of damage as shown by the GAMM results. Analysis was undertaken via the R package for statistical computing version 2.15.0 (R Development Core Team, 2012) using the package gamm4 (Wood, 2012).

Correlations between plant traits and seedling damage sustained within two and four days exposure to slugs were examined visually. No further analyses were performed for traits other than seedling growth rate. The influence of seedling growth rate on seedling damage incurred after two days exposure to slugs (arcsine transformed) was analysed by a Generalised Linear Model undertaken in the R package for statistical computing version 2.15.0 (R Development Core Team, 2012).

3.4 Results

3.4.1 *Cyanogenesis testing of Fabaceae seedlings*

As seed of each Fabaceae species came from wild populations, the proportion of cyanogenic and acyanogenic seedlings offered to slugs was not known in advance. Following testing by the sodium picrate method, a greater proportion of the *Lotus corniculatus* seedlings remaining after 14 days tested positive for cyanide production suggesting that slugs discriminated against cyanogenic morphs (data not shown). For *Trifolium repens* and *T. pratense*, it was not clear from the remaining seedlings whether slugs were influenced by cyanogenesis.

3.4.2 *Relative acceptability*

The overall model fit was expressed using the adjusted R-squared ($r^2_{adj} = 0.502$; Table 3.1). The estimate for the overall mean proportion of damage (per individual seedling) (Intercept) incurred by all species as a function of time was 0.251 (back-transformed) and was found to be statistically significant ($t = 10.730$, $P < 0.001$; Table 3.1).

Table 3.1. Parametric coefficients of a Generalized Additive Mixed Model for slug feeding damage to seedlings of meadow plant species and *Brassica napus* as a function of time (14 days exposure to slugs) under controlled microcosm conditions. The overall mean (Intercept) level of damage sustained by all species (individual seedlings) as a function of time was 0.525 (back-transformed to 0.251).

Coefficient	Estimate[1]	Standard Error (s.e)	*t*-value	*P*-value	*R*-squared (adjusted)
Intercept	0.251	0.002	10.730	0.000	0.502

Estimate of the overall mean back-transformed to the original units[1].

Plant species clearly differed in terms of the severity and rate of slug feeding damage sustained, indicating that they differed in their acceptability to *D. reticulatum* (Table 3.2; Figure 3.1). The damage incurred by each plant species was assessed as a function of time and the degree of non-parametric smoothing required to describe the underlying relationship is shown in Table 3.2. The relative effect of time on the severity and rate of damage incurred by each plant species is described in Figure 3.1. The non-parametric smoothing functions for each plant species were found to be statistically significant indicating that the damage sustained by each plant species continued to increase non-linearly as a function of time, albeit to a differing degree between species dependent on severity and rate of damage incurred. Species that suffered high levels of damage in the shortest time are shown by a strong non-linear smoothed response curve (Figure 3.1). Increases in damage sustained were notable for *Achillea millefolium* ($F = 137.136$, d.f. = 5.451, $P < 0.001$), *Holcus lanatus* ($F = 127.884$, d.f. = 4.685, $P < 0.001$), *Festuca rubra* ($F = 108.760$, d.f. = 4.760, $P < 0.001$), *Cynosurus cristatus* ($F = 98.568$, d.f. = 4.679, $P < 0.001$), *Poa trivialis* ($F = 146.832$, d.f. = 3.738, $P < 0.001$), *Agrostis capillaris* (F = 131.437, d.f. = 3.695, $P < 0.001$) and *Trifolium pratense* (F = 109.732, d.f. = 3.758, $P < 0.001$). These species suffered high level of damage from early exposure to slugs. By comparison, species that suffered the lowest levels of damage throughout the 14 day exposure period are shown by weak non-linear smoothed response curves (Figure 3.1). As such, while the relative degree of damage sustained by *Geranium sylvaticum* was also found statistically significant, the corresponding F-statistic was comparatively smaller than preceding examples (F = 3.462, d.f. = 1.932, $P < 0.05$) (Table 3.2). In other words, smoothing terms indicate that *G. sylvaticum* and other very low ranking species (*Rumex acetosa*, *Leontodon hispidus*, *Anthoxanthum odoratum*) incurred minimal levels of feeding damage throughout the 14 day exposure period.

Random-effects estimates generated by the model were used to rank each species according to the severity and rate of damage sustained as a function of time (Table 3.3) and to create a caterpillar plot (Figure 3.2). The random-effects estimates reflect the degree to which each species differs in comparison with the overall mean (Intercept) for all species (Gelman and Hill, 2007). The mean level of damage incurred by each species as a function of time was calculated by combining the estimates of the random-effect and the overall mean (Intercept = 0.525, back-transformed estimate = 0.251; Table 3.3). In other words, random-effects estimates account for the *rate* of damage sustained by species for the duration of the study period (i.e. given that the study was constrained by the upper limit of 14 days, the mean rate of damage incurred by each species is calculated for all time points and not discrete time points).

The caterpillar plot showed species to form a hierarchy of acceptability to *D. reticulatum* (Figure 3.2). Back-transformed estimates revealed that *A. odoratum*, *L. hispidus*, *R. acetosa* and *G. sylvaticum* sustained minimal damage (mean damage as a function of time = <6%; Table 3.3) despite slugs having no other fresh plant material available. In contrast, the high estimate for *A. millefolium* (80.7%; Table 3.3) is a consequence of slugs completely consuming the majority of seedlings within a few days of exposure.

Following interpretation of the GAMM results, species were subjectively assigned to one of four damage categories whereby 1 = Fast, severe damage; 2 = Mid-term, severe damage; 3 = Constant, non-severe damage; and 4 = Minimal damage over 14 days. Damage categories are illustrated in the caterpillar plot (Figure 2). Species classified as category 1 (Fast, severe damage) namely *A. millefolium* and the grasses *H. lanatus*, *F. rubra* and *C. cristatus*, sustained severe damage within the first few days of exposure to slugs and generated combined estimates >40% (Table 3). Category 2 (Mid-term, severe damage) contained two further grass species (*P. trivialis* and *A. capillaris*) and five forbs including *T. pratense* and the reference species *B. napus*. These species incurred high levels of damage within seven days exposure to slugs and generated combined estimates of 27>39% (Table 3.3). For species classified as category 3 (Constant, non-severe), such as *Sanguisorba officinalis*, *Ranunculus acris* and *Lotus corniculatus*, slugs continued to consume small amounts of leaf tissue over the 14 day exposure period (combined estimates 10<25%; Table 3.3). Cumulative damage gradually increased, but began to curtail during the latter half of the exposure period. Category 4 species, *A. odoratum*, *L. hispidus*, *R. acetosa* and *G. sylvaticum* sustained minimal levels of feeding damage throughout the 14 day exposure period (combined estimates 2<6%). Alternatively, it is reasonable to describe species within each of the four damage categories as being highly acceptable; acceptable (ranging from moderate to low); and highly unacceptable to *D. reticulatum*.

Table 3.2. Fitted Generalized Additive Mixed Model for slug feeding damage to seedlings of meadow plant species and *Brassica napus* as a function of time (14 days exposure to slugs) using smoothing (cubic regression spline). Smoothing terms (*F*-value, *P*-value, degrees of freedom) indicate significant positive non-linear responses for all species. Species are ranked according to descending severity and rate of damage according to random-effects estimates (Table 3.3).

Coefficient	Smoothing terms		
	DF	*F*-value	*P*-value
Time and *Achillea millefolium*	5.451	137.136	0.000
Time and *Holcus lanatus*	4.685	127.884	0.000
Time and *Festuca rubra*	4.780	108.760	0.000
Time and *Cynosurus cristatus*	4.679	98.568	0.000
Time and *Poa trivialis*	3.738	146.832	0.000
Time and *Agrostis capillaris*	3.695	131.437	0.000
Time and *Trifolium pratense*	3.758	109.732	0.000
Time and *Brassica napus*	4.295	72.200	0.000
Time and *Plantago lanceolata*	4.683	60.194	0.000
Time and *Prunella vulgaris*	4.555	53.562	0.000
Time and *Cerastium fontanum*	4.974	31.598	0.000
Time and *Bellis perennis*	3.548	75.824	0.000
Time and *Trifolium repens*	3.994	40.889	0.000
Time and *Taraxacum officinale*	3.031	82.732	0.000
Time and *Dactylis glomerata*	4.059	28.807	0.000
Time and *Centaurea nigra*	2.989	60.728	0.000
Time and *Lolium perenne*	2.726	75.379	0.000
Time and *Lotus corniculatus*	3.584	33.078	0.000
Time and *Sanguisorba officinalis*	3.398	25.456	0.000
Time and *Ranunculus acris*	2.658	26.908	0.000
Time and *Anthoxanthum odoratum*	2.695	8.745	0.000
Time and *Leontodon hispidus*	1.785	9.828	0.000
Time and *Rumex acetosa*	1.794	9.609	0.000
Time and *Geranium sylvaticum*	1.932	3.462	0.033

Table 3.3. Ranked random-effects estimates generated by a Generalized Additive Mixed Model for the rate and severity of slug feeding damage sustained by seedlings of meadow plant species and *Brassica napus* as a function of time (14 days exposure to slugs). ‘Plant species’ was treated as the random effect and reflects the degree to which each species differs from the Intercept (Gelman and Hill, 2007). The overall mean level of damage (Intercept) sustained by all species as a function of time was 0.525 (back-transformed to 0.251) (Table 3.1). The overall mean level of damage sustained by each individual species as a function of time is calculated as the combined estimate (random-effect estimate + Intercept). Back-transformed estimates are the proportion of overall damage per individual seedling.

Species	Random-effect	Combined Estimate	Combined Estimate (Back-transformed)	
Achillea millefolium	0.591	1.115	0.807	↑ Increasing Acceptability
Holcus lanatus	0.326	0.851	0.566	
Festuca rubra	0.297	0.822	0.537	
Cynosurus cristatus	0.254	0.778	0.494	
Poa trivialis	0.197	0.721	0.437	
Agrostis capillaris	0.140	0.665	0.381	
Trifolium pratense	0.121	0.646	0.362	
Brassica napus	0.108	0.633	0.350	
Plantago lanceolata	0.100	0.625	0.342	
Prunella vulgaris	0.070	0.594	0.314	
Cerastium fontanum	0.024	0.549	0.273	
Bellis perennis	-0.005	0.520	0.247	
Trifolium repens	-0.038	0.486	0.219	
Taraxacum officinale	-0.071	0.454	0.193	
Dactylis glomerata	-0.083	0.442	0.183	
Centaurea nigra	-0.086	0.439	0.181	
Lolium perenne	-0.105	0.419	0.166	
Lotus corniculatus	-0.114	0.411	0.160	
Sanguisorba officinalis	-0.186	0.339	0.111	
Ranunculus acris	-0.190	0.335	0.108	
Anthoxanthum odoratum	-0.289	0.236	0.055	
Leontodon hispidus	-0.344	0.181	0.033	
Rumex acetosa	-0.347	0.178	0.032	
Geranium sylvaticum	-0.373	0.152	0.023	

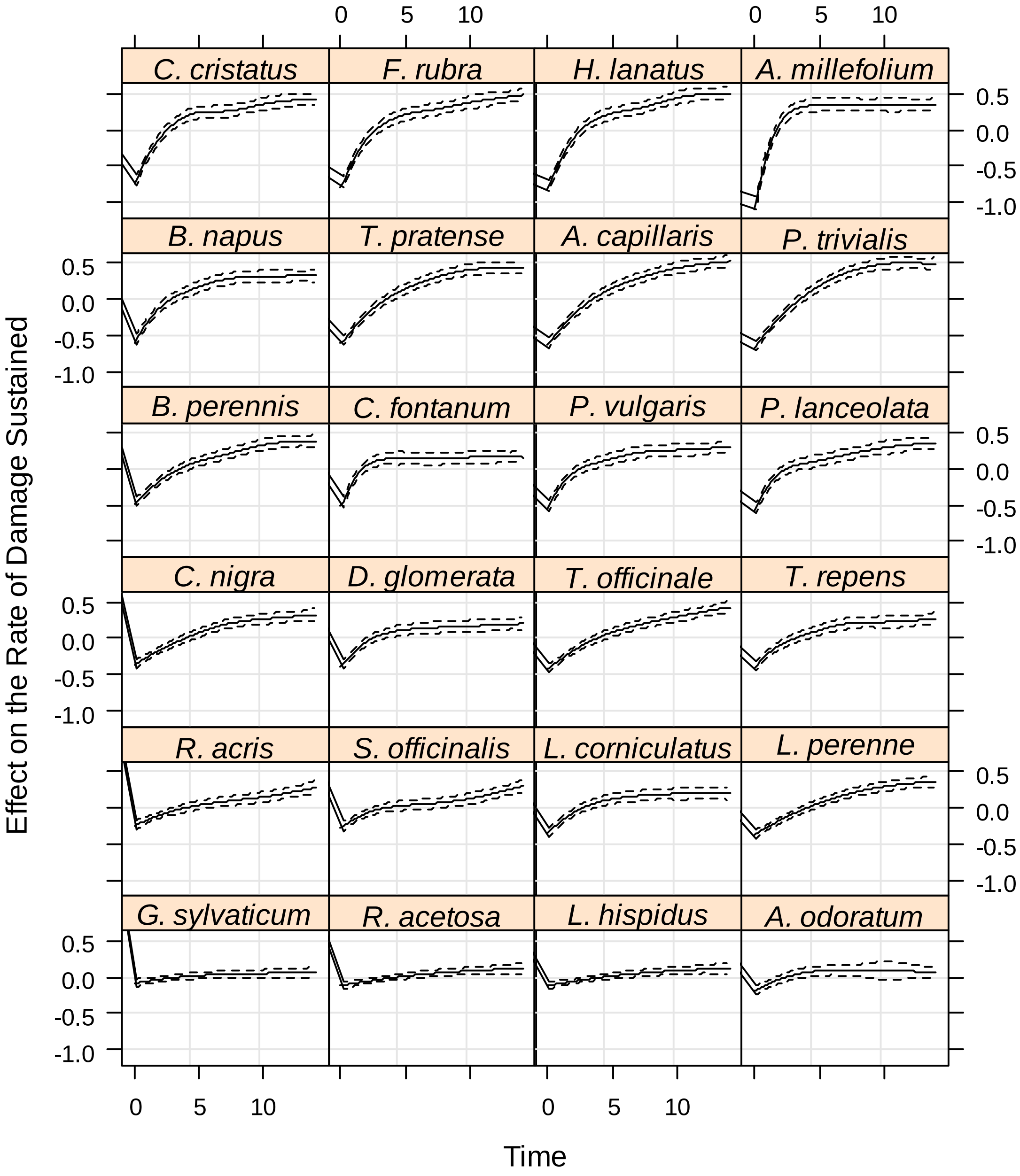


Figure 3.1. Generalized Additive Mixed Model smoothed response curves for slug feeding damage to seedlings of meadow plant species as a function of time. The y-axis describes the positive non-linear trend of damage incurred by each species as a function of time. Response curves that are orientated around zero on the y-axis indicate little or no effect of the predictor variable. The x-axis represents the contribution of time (0-14 days) to the overall trend in damage for each plant species. Solid curves are the cubic regression spline fits. The broken lines correspond to 95% confidence limits for the smoothing. Species are ordered

according to the ranked random-effects (Table 3.3) and, hence, the severity and rate of damage incurred as a function of time. Species that incurred the least mean damage over 14 days to those that incurred the greatest mean damage in the shortest exposure time are arranged from left to right starting at bottom left and show increasingly non-linear responses accordingly. For key to species refer to Table 3.2.

Table 3.4. Generalised Linear Model coefficients for slug feeding damage to seedlings of meadow plant species and *Brassica napus* after two days exposure to slugs (arcsine transformed) as a function of seedling growth rate. The proportion of the deviance explained was 0.41.

Coefficients	Estimate	Standard Error	*t*-value	*P*-value
Intercept	0.002	0.016	0.117	0.908
Seedling Growth Rate	0.225	0.082	2.740	0.015

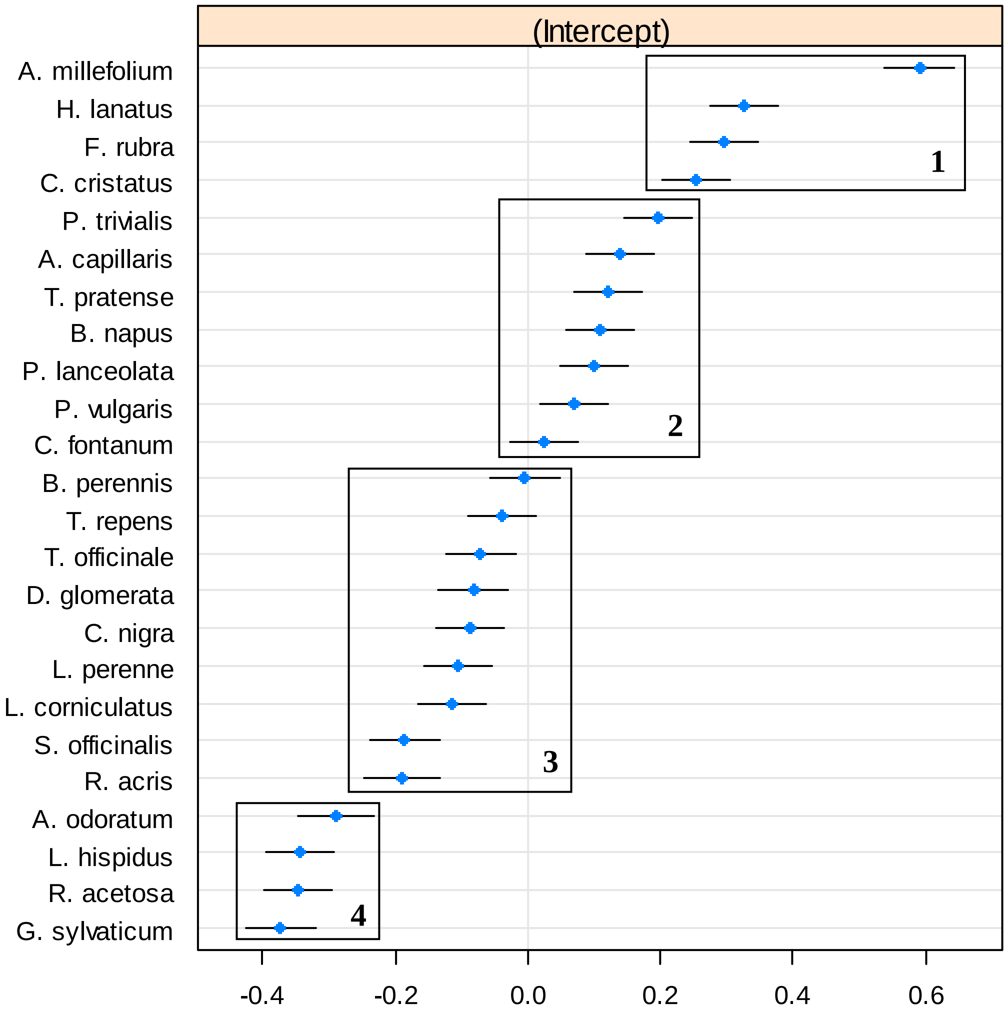


Figure 3.2. A caterpillar plot generated by a Generalized Additive Mixed Model of the ranked random-effects estimates for slug feeding damage to seedlings of meadow plant species and *Brassica napus* as a function of time (14 days exposure to slugs). Plant species were used as the random-effects. The rank-order of random-effects estimates can be interpreted (from top to bottom) as a hierarchy of decreasing acceptability to *Deroceras reticulatum*. Species with a random-effect estimate centred on 0 do not differ from the Intercept (overall mean damage incurred as a function of time). Random-effects estimates are shown in Table 3.3. Damage categories (subjectively assigned) are illustrated whereby: 1 = Fast, severe damage; 2 = Mid-term, severe damage; 3 = Constant, non-severe damage; and 4 = Minimal damage over 14 days. Alternatively, species within these four categories may be described as being highly acceptable; acceptable

(ranging from moderate to low); and highly unacceptable to *D. reticulatum*. For key to species refer to Table 3.2.

3.4.3 *Plant traits*

To aid explanation of the results, plant trait data (relative growth rate, specific leaf area, leaf dry matter content, seed size, seed mass and seedling growth rate) was collated from the literature (Grime *et al.*, 2007) and electronic databases (Fitter *et al.*, 1994; Poschlod *et al.*, 2003; Royal Botanic Gardens Kew, 2008). Data for secondary metabolite concentrations was insufficient for most species. A GLM was used to examine the effect of seedling growth rate on mean damage (arcsine transformed) sustained by seedlings of each plant species within two days exposure to slugs. Seedling damage significantly increased with seedling growth rate ($t = 2.740$, $P < 0.05$; Table 3.4) indicating that slugs found species with fast growing seedlings more acceptable. These results suggest a trade-off between growth and defence whereby slow growing seedlings were less susceptible to severe slug damage. Seedling growth rate information was not available for *G. sylvaticum*, *S. officinalis*, *R. acris* and *Bellis perennis*. Our experience of growing these species from seed, lead us to speculate that the seedling growth rates of these species would likely strengthen the relationship found here.

3.5 Discussion

Given that herbivory is the primary mortality factor for most seedlings (Moles and Westoby, 2004) and that numerous field studies report slugs to exert a selective pressure on seedling recruitment (Hulme, 1994, 1996; Hanley *et al.*, 1995*a*, 1996; Hanley, 2004), it is surprising that the effects of slug herbivory (or invertebrate herbivory in general) have not received more attention by restoration ecologists (but see Hitchmough, 2003; Pywell *et al.*, 2007). Here we have shown how variation in the acceptability of meadow plants to a common temperate grassland slug, *Deroceras reticulatum*, may influence seedling recruitment at restoration sites. Results of a GAMM indicated that 50.2% (adj $R^2 = 0.502$; Table 1) of the variation in slug response (severity and rate of damage) was explained by the species of plant. We found that meadow plant species form a hierarchy of acceptability to *D. reticulatum*. Of the 23 meadow species tested, four were highly acceptable (i.e severe damage incurred in short exposure time) and four were highly unacceptable (i.e. minimal damage over 14 days). Such ‘all or nothing’ feeding responses have been described by previous feeding studies using slugs (Duval, 1971, 1973; Dirzo, 1980) and snails (Grime *et al.*, 1968). The implications of this study for grassland restoration are discussed below, along with the limitations of our approach.

As the purpose of this study was to assess acceptability using seedling monocultures under controlled microcosm conditions, we recognise that extrapolating the findings to field conditions should be done with caution. Certainly a seed tray is not a meadow and, although the acceptability of seedlings influences selection by molluscs (Hanley *et al.*, 1995b), other factors are known to influence selection in field conditions (Fenner, 1987; Hanley 1998) including seedling density and distribution (Root, 1973; Bergelson, 1990; Hanley *et al.*, 1996), frequency (Cottam, 1985) and the identity of nearest neighbours (Hanley *et al.*, 1995*a*, *b*; Hanley, 2004). Furthermore, acceptability may have little implication for seedling survival as the effect of slug feeding may be disproportionate to the amount of biomass removed (Dirzo and Harper, 1980; Fenner *et al.*, 1999). Even low levels of slug damage places a seedling at a competitive disadvantage towards its neighbours due to increased allocation of resources to re-growth. The ability of seedlings to tolerate (i.e. recover from) sub-lethal grazing damage also varies between species (Hulme, 1994) and is influenced by seedling morphology (Hanley *et al.*, 1995*a*, *b*), relative growth rate (Kelly and Hanley, 2005) and meristem location (Glen *et al.*, 1991) amongst other factors. Nevertheless, acceptability has been shown to be an accurate predictor of plant community development (Burt-Smith *et al.*, 2003; Strauss *et al.*, 2009). Our results provide new and useful information as to which species may be more or less susceptible to selective grazing by slugs during the seedling recruitment phase at restoration sites and which inherent properties of seedlings may be driving this pattern of variation in acceptability. Critically, the successful (or otherwise) recruitment and establishment of key species has wider implications for grassland restoration at the ecosystem level as a result of facilitation and competition affects.

The variation in acceptability of the meadow species strongly suggests that slug feeding responses were influenced by the chemical and structural properties of each species in seedling form [Appendix, Table S2.1]. Furthermore, slug responses suggest a potential role of attractive and repellent olfactory cues that is worthy of further investigation. Slugs fed intensively on *A. millefolium* seedlings from initial exposure and caused minimal damage to *G. sylvaticum* seedlings for 14 days despite no other fresh plant material being available. Similarly, Hanley *et al*. (2011) found the snail *Helix aspersa* to make olfactory selections (macerated seedlings) of preferred (highly acceptable) species more rapidly than for less preferred species; however, it was not known whether positive or negative olfactory cues were ultimately responsible.

As we hypothesised, several of the desirable species targeted in the restoration of upland hay meadows (*G. sylvaticum*, *L. hispidus*, *S. officinalis*, *A. odoratum*, *R. acris*, *L. corniculatus*) were highly unacceptable or of low acceptability to *D. reticulatum* at the seedling stage, most likely as a consequence of anti-feedant secondary metabolites (including tannins) [Appendix, Table S3.1]. For *L. corniculatus*, slugs seemingly discriminated against cyanogenic morphs, as shown by (Keymer and Ellis, 1978). It is likely that *D. reticululatum* rejected *G. sylvaticum* seedlings because of “digestibility-reducing” tannins which deter feeding by rodents (Moen *et al.*, 1996) and sheep (Evju *et al.*, 2011). Scheidel and Bruelheide (1999) also found that slugs reject leaves of *G. sylvaticum*. The response of slugs to these species suggests that slugs may have regulated the consumption of potentially toxic concentrations of secondary metabolites by decreasing meal size and the interval between meals, as shown in the study by Torregrossa *et al.* (2011) for the generalist rodent, *Neotoma albigula*. This bodes well for their chances of avoiding severe slug herbivory in the field, although younger seedlings may be at greater risk if the expression of chemical defences is affected by seedling ontogeny.

General comparisons between previous ‘acceptability-testing’ feeding trials reveal slugs and snails are generally deterred by secondary metabolites and hard-leaf texture [Appendix, Table S3.1]. For example, Dirzo (1980) found *D. caruanae* to generally reject leaves of species known to contain glucosides and alkaloids; and Mølgaard (1986) suggests that plants generally avoided by molluscs are those containing tannins and phenolics. The anti-feedant properties of secondary metabolites against molluscs are also suggested by the results of field studies (Hulme, 1994; Hanley *et al.*, 1995*a*; Hanley, 2004).

In contrast, the moderate acceptability of *T. pratense* (the second highest ranking forb species) to *D. reticulatum* presents a potential limiting factor to the recruitment of seedlings at restoration field sites, although it was not clear to what extent slugs were influenced by cyanogenesis. *T. pratense* seedlings are reported by other studies to be acceptable to slugs under laboratory conditions (Briner and Frank, 1998, Fenner *et al.*, 1999; Hanley and Sykes, 2009). However, Hulme (1996) found molluscs did not reduce the survival of *T. pratense* seedlings within mollusc exclusion field plots, although survival was reduced by rodents. The successful recruitment of *T. pratense* seedlings is particularly important during the early stages of upland hay meadow restoration as established plants are associated with facilitating mycorrhizal populations in the soil, promoting increases in fungal to bacterial ratios and consequent reductions in high residual soil (phosphorus) fertility caused by inorganic fertiliser additions (Smith *et al.*, 2003, 2008). *T. pratense* is also an important forage plant for long-tongued bumblebees (Hanley *et al.*, 2008; Goulson *et al.*, 2008). *A. odoratum* and *R. acris* are also beneficial species associated with increasing soil fungal to bacterial ratios (Smith *et al.*, 2008; De Deyn *et al.*, 2012) and were shown here to be of very low acceptability to *D. reticulatum*. The presence of volatiles in *A. odoratum* (Grime *et al.*, 1968), and ranunculin in *R. acris* (Briner and Frank, 1998) may lower seedling acceptability to slugs and reduce their susceptibility to severe grazing damage. The findings of Hanley *et al.* (1995*a*) suggest *R. acris* seedlings are also unacceptable to molluscs under field conditions, with the cover of *R. acris* found to be significantly higher in grazed field plots in comparison with plots subject to molluscicide applications. In contrast, Hanley *et al.* (1996) found molluscicide applications to cause an increase in the abundance of *R. acris* seedlings in experimental field plots. The findings of the latter study may have been an apostatic response by molluscs to aggregated *R. acris* seedlings in experimental gaps. It was disappointing that the relative acceptability of *Rhinanthus minor* was not determined here as results of the three year mesocosm detailed in Chapter 4 suggest that seedlings were selectively grazed by slugs within mixed swards. The successful recruitment and establishment of *R. minor* is critical to the restoration process as plants selectively parasitize competitive grass species and facilitate an increase in forb diversity (Pywell *et al.*, 2004; Smith *et al.*, 2008).

The presence of foliar trichomes may have influenced the acceptability of some species. The markedly hairy species, *H. lanatus*, *T. pratense*, *Cerastium fontanum*, *Centaurea nigra*, *R. acris*, *L. hispidus* and *G. sylvaticum*, were present in each acceptability class with the latter three suffering very low levels of feeding damage. Undoubtedly the type (glandular or non-glandular), morphology, size and density of trichomes, as well as ontogenetic effects, may influence mollusc feeding behaviour. *G. sylvaticum*, for example, has glandular hairs which likely contain volatile oils (geraniol, linalool) (Pedro *et al.*, 1991) and may have a repellent function when damaged. However, despite Grime *et al.*, (1968) recognising the need for further work, we are still no further forward in determining whether particular trichomes function as a defence against molluscs.

High grass species richness is a defining feature of species-rich meadows (Rodwell, 1992). Grasses are reported to deter invertebrate and vertebrate herbivory via silica phytoliths in leaves (Wadham and Wynn-Parry, 1991; Massey and Hartley, 2006; Massey *et al.*, 2006; Massey and Hartley, 2009). Furthermore, silica is known to be an inducible defence against herbivory (Massey *et al.*, 2007; Reynolds *et al.*, 2012). Here, the presence of most grass species in the higher acceptability rankings points to a diminished defensive role of silica in grass seedlings. Accordingly, Fenner *et al.* (1999) report grass seedlings to be more palatable than adult conspecifics when offered as agar discs to *D. reticulatum*. Likewise, Hulme (1996) found grass seedlings to suffer more severe damage than forb seedlings under field conditions, although most grass species demonstrated higher survival rates. Several studies, including this one, report *H. lanatus* to be readily eaten by molluscs, a finding that is likely due to its soft leaf texture (Grime et al., 1968; Pallant, 1972). In contrast, Hanley (2004) found seedlings of *A. capillaris*, *F, rubra*, *H. lanatus* and *P. trivialis* to suffer considerably less slug feeding damage than neighbouring forbs.

Here, slugs did not inflict high levels of damage on the seedlings of *A. odoratum, Dactylis glomerata* or *Lolium perenne* suggesting that slugs are unlikely to dramatically reduce the seedling recruitment of these species. A high abundance of the latter two species is characteristic of agriculturally improved swards and their competitive strategy is a constraint to diversity. It is likely that seedlings of *D. glomerata* and *L. perenne* were of low acceptability to slugs because of their hard leaf texture (Grime *et al.*, 1968; Dirzo, 1980; Scheidel and Bruelheide, 1999; Hanley *et al.*, 2007) or, perhaps, as a consequence of relatively higher silica concentrations (Massey *et al.*, 2006). Hard-leaved (scleromorphic) plants, such as many grass species, contain high levels of digestibility-reducing cellulose, although it is often difficult to separate the effects of cellulose from those of silica (Hanley *et al.*, 2007).

A drawback of this study was that, as seedlings were offered to slugs at the two-leaf stage or, for grasses an equivalent biomass, it did not control for interspecific variation in seedling age or size; factors which are known to influence the expression of chemical and physical properties (Hanley *et al.*, 2007; Elger *et al.*, 2009) and the feeding behaviour of molluscs (Hulme, 1994; Hanley *et al.*, 1995*b*). Newly emerging seedlings may lack the chemical or physical defences of older seedlings and be at greater risk of attack than the results of this study would suggest.

Finally, it was not the intention of this study to test the Growth-Differentiation-Balance Hypothesis (Herms and Mattson, 1992); however, the findings offer some evidence of a growth-defence trade-off in that species which suffered minimal damage were likely to have been well defended, particularly by chemical defences and are relatively slow-growing, perennial stress-tolerating species typical of mid-late-successional grassland (Grime, 2002). Likewise, those species that were readily attacked by slugs were predominantly fast-growing grasses which seemingly lacked physical or chemical defensive properties during early development. This observation was supported by the significant positive effect of seedling growth rate on seedling damage found here.

In conclusion, the study shows how interspecific variation in acceptability may influence slug herbivory of seedlings at grassland restoration sites. Very often grassland restoration sites are seed limited requiring seed of target species to be introduced from species-rich donor sites (Smith *et al.*, 2002). Successful recruitment and establishment is of ecosystem-level importance for species which provide a facilitating function, in particular the facilitation of mycorrhizal populations by *T. pratense* and other stress-tolerating species, and the suppression of grass competition by *R. minor* (Pywell *et al.*, 2004; Smith *et al.*, 2008). As we have already stated, a seed tray is not a meadow. The value of this study (in applied terms) lies in the ability of lab-based acceptability tests to predict herbivory damage under field conditions. The use of specific plant traits as correlates for acceptability is worthy of further investigation. Our initial attempts indicate that seedling growth rate may be a useful predictor and suggest that growth-defence theory may explain plant interspecific variation in seedling herbivory by slugs. It is likely that the best predictors of acceptability will be secondary metabolite concentrations, although this would require a considerable research effort to establish the chemicals and concentrations in seedlings of different species, as well as the ontogenetic effects.

Seedling acceptability is an important trait influencing selection by molluscs, although future work which investigates the effects of slug herbivory on seedling survival and establishment at restoration sites is necessary to establish the predictive value of the results reported here.

Chapter 4. Effects of slug herbivory on meadow plant communities and implications for grassland restoration

4.1 Abstract

The effects of high and low slug densities on plant community structure and development was investigated in a three-year mesocosm experiment. Mesocosms were sown with seed mixes typical of community composition in semi-improved (MG6b) and unimproved (MG3) upland hay meadows.
Results of multivariate analyses confirmed slug herbivory was a significant biotic force regulating experimental plant communities during the three years from establishment. Both communities were affected by variation in slug densities during the establishment phase, primarily as a consequence of slugs altering the relative abundance of common grass species. For the semi-improved community, the importance of slug herbivory on community assemblages declined over time. Results suggested that competition was of overriding importance with increasing sward age and caused community assemblages to become more similar due to the dominance of competitive grasses. The unimproved community assemblages were significantly affected by slug population densities over three years. This study is the first to report a tri-trophic interaction involving the selective grazing by slugs of the keystone species *Rhinanthus minor* and the likely consequences on community composition and development. Herb richness increased over time in association with low slug densities and the presence of *R. minor*, while grass cover declined. The findings are therefore directly relevant to the restoration management of species-rich grassland, particularly those plant communities which contain *R. minor*.

Key-words: agricultural improvement, community composition, *Deroceras reticulatum*, Distance based redundancy analysis (db-RDA), invertebrate herbivory, *Rhinanthus minor*, seedling recruitment

4.2 Introduction

Species-rich upland hay meadows are of high biodiversity importance and are internationally rare. Meadows conforming to MG3 (*Anthoxanthum odoratum-Geranium sylvaticum*) in the National Vegetation Classification (NVC) (Rodwell 1992) are a priority grassland in the UK and are protected as an Annex I habitat by the EU Habitats Directive (JNCC 2008). Agricultural intensification since the 1940s has greatly reduced the area of MG3 meadows resulting in vast areas of species-poor improved and semi-improved meadows (Jefferson 2005). Recent estimates indicate that 897 ha remain in the UK, found in upland valleys in Northern England, with a few outliers in Scotland, where traditional hay-making practices still persist (BARS 2008).

Restoration and re-creation of MG3 meadows, and other species-rich grassland communities, is a target of Habitat Action Plans and a focus of research. A growing body of valuable research has established important requirements for successful meadow restoration including: the optimum management regime (Smith & Jones 1991; Smith & Rushton 1994); the need to introduce seed of missing target species (Smith *et al.* 1996a; Smith *et al.* 1996b; Smith *et al.* 2000; Smith *et al.* 2002) including the hemi-parasitic annual, *Rhinanthus minor* L. (Pywell *et al.* 2004); and the interactions between fertility, the soil microbial community and above-ground vegetation (Smith *et al.* 2003; Bardgett *et al.* 2006; Bardgett *et al.* 2007; Smith *et al.* 2008). However little is known about the effects of invertebrate herbivory on community composition (Jefferson 2005). Of particular interest are the effects of herbivory on keystone species such as *R. minor*. The successful recruitment and establishment of the *R. minor* is fundamental to the early phase of meadow restoration due to plants suppressing the vigour of competitive grasses and enabling a wider diversity of forbs to establish. As such, *R. minor* is used as a management tool in the restoration of species-rich grasslands. The effect of invertebrate herbivores on *R. minor* seedling recruitment is unknown and poses an interesting tri-trophic system for study (i.e. Herbivores-Hemi-parasitic plant-Plant community composition).

Slugs (Gastropoda: Agriolimacidae, Arionidae and Milacidae) are probably the most important invertebrate herbivore in temperate grassland (Hulme 1994) and have been identified as important regulators of plant community structure during and beyond the establishment phase (for review see Hanley 1998). Slugs are abundant in grassland (South 1989b; South 1992) and, as generalist but selective herbivores (Dirzo 1980), may exert a strong selective pressure by affecting seedling recruitment (Hanley, Fenner & Edwards 1995a; Hanley, Fenner & Edwards 1996a) and shifting the competitive balance between coexisting species (Wardle & Barker 1997; Hanley & Sykes 2009). The selection of one species over another is influenced by acceptability (chemical and physical defences, seedling size and age) as well as community/spatial factors including seedling density and distribution and identity of nearest neighbours (Hanley 1998). The likelihood of seedling post-grazing survival also varies between species and functional groups, for example Hulme (1996) found grass seedlings to suffer high levels of mollusc grazing damage, but grasses demonstrated higher survival rates than forb seedlings due to their ability to quickly re-grow from a basal meristem.

Persistent direct and indirect effects of slug herbivory on the abundance and composition of species have been documented by mid-term field and mesocosm studies involving the manipulation of slug numbers through molluscicidal pellet applications (Wardle & Barker 1997; Buckland & Grime 2000; Buschmann *et al.* 2005; Pywell *et al.* 2007). Manifestations of effects differ between studies and are no doubt largely dependent on compositional differences between study plant communities. The effect of slug herbivory on plant diversity may be positive if slugs reduce the contribution of abundant and competitive species (e.g. Buschmann *et al.* 2005). Conversely, the effects may be negative if slugs feed preferentially on scarce, less vigorous species (e.g. Wilby & Brown 2001) or promote the cover of a highly unacceptable, but otherwise sub-dominant species (Hanley, Fenner & Edwards 1995b). Field studies commonly report slugs to preferentially select seedlings of forb species over grasses (Wilby & Brown 2001; Hanley 2004; Buschmann *et al.* 2005) and negative effects of mollusc herbivory on forbs have been reported to persist 3-4 years after sowing (Wilby & Brown 2001; Pywell *et al.* 2007). The manipulation of insect herbivores using insecticides has been shown to produce similar findings (Brown & Gange 1989; Wardle & Barker 1997), although the net effects of herbivory are clearly interactions between different herbivores (Del-Val & Crawley 2005; Allan & Crawley 2011). The effect of slug herbivory on long term community development has received less attention. A recent field study by Allan and Crawley (2011) reported the findings of a 14 year field study investigating the interactive effects of mollusc and insect herbivory and nutrient addition on an MG5 (*Cynosurus cristatus*-*Centaurea nigra*) meadow community. Mollusc exclusion, in the presence of insects, led to increased forb abundance, although this did not become apparent until after 8 years and the effects of mollusc exclusion were much smaller in productive plots where forb abundance was low and grass dominated the sward.

The effects of herbivory cannot be studied without considering plant competition. Herbivore-induced shifts in the competitive balance and relative abundance of coexisting species were apparent in the studies of Wardle and Barker (1997) and Buckland and Grime (2000). Experimental manipulation of competition and herbivory has shown that while both are important interacting factors regulating grassland community structure, the effects of competition are of overriding importance in swards dominated by competitive grasses (Del-Val & Crawley 2004; Del-Val & Crawley 2005).

Few studies have investigated the effects of slug herbivory on the restoration of species-rich grassland (Hitchmough 2003; Pywell *et al.* 2007) and no studies have considered the effect of slugs on upland hay meadow plant communities. In view of this research requirement a mesocosm study was designed to investigate the effects of high and low slug densities on the establishment and development of two upland hay meadow communities sown from seed. Community seed mixes represented typical unimproved and semi-improved upland hay meadows, conforming to the NVC communities MG3 and MG6b (*Lolium perenne*-*Cynosurus cristatus* sub-community *Anthoxanthum odoratum*), respectively. As the MG3 seed mix contained *R. minor*, the potential tri-trophic interaction between slugs, *Rhinanthus* and plant community composition was of particular interest and has not been investigated before. In this study we address three specific questions:

(i) How do slug densities affect plant species diversity, functional groups and total biomass? (ii) How do slug densities affect plant species abundance and composition? (iii) Do the effects of slug herbivory change through time? In answering these questions, the study aims to address the unknown effects of slug herbivory on upland hay meadow plant communities.

4.3 Method

4.3.1 *Experimental design*

In March 2008, a mesocosm experiment was set-up at Close House Biology Field Station, Newcastle upon Tyne, UK (NZ128659), to investigate the effects of slug herbivory on community development of two hay meadow communities established from seed (Figure 4.1). Twenty plastic mesocosms (1 m x 1 m x 0.39 m) were filled with a base layer of hardcore (depth 10 cm) and a top layer of soil (depth 15 cm) and allowed to adjust for four weeks prior to sowing. Soil was sourced from a local mid-secondary successional grassland in order to replicate low-medium fertility field conditions. Hardboard baited refuge traps were used to remove some of the residual slug populations present in the soil. To control slug movement, copper-impregnated matting (Spinout) was used as ground cover between plots and attached to the perimeter of all mesocosms.

Mesocosms were sown with a seed mix typical of either an unimproved or semi-improved upland hay meadow plant community (i.e. 10 mesocosms for each seed mix). Seed mixes for the respective communities were designed to replicate species composition of the NVC grassland communities MG3 and MG6b (Rodwell 1992). Each seed mix was applied at approximately 4 g m^{-2}, with species composition and seed quantity proportions based on quadrat surveys of unimproved and semi-improved hay meadows within the Yorkshire Dales (S.E. Barlow, unpublished data) and NVC floristic tables for MG3 and MG6b grassland communities (Rodwell, 1992) (Table 4.1). Most seed was obtained from Emorsgate Seeds, with the exception of *Dactylis glomerata*, *Ranunculus repens* and *Cerastium fontanum* which were sourced from Herbiseed, and *Rhinanthus minor* which was hand collected from upland hay meadow field sites. All species were sown (with the exception of *G. sylvaticum* which was transplanted as seedlings (10 m^{-2})) on 21st April 2008, apart from *R. minor* which was not sown until 27th August 2008 (30 seeds m^{-2} within the unimproved community only to represent a medium density under natural field conditions (Bardgett *et al.* 2006)). *R. minor* seed requires an obligate vernalisation period over winter and thus was sown later in the year to enable germination the following spring. *G. sylvaticum* was transplanted as seedlings because seed had begun to germinate in the glasshouse following scarification and an 8-week chilling period.

Each plant community was subjected to a 'slug' treatment at two levels (slugs added, no slugs added) with five replicates. Seed mixes and slug treatments were applied in a randomised block design. Mesocosms to which slugs were added received 30 slugs m^{-2} (of the field slug, *Deroceras reticulatum* Müller) on 10th June 2008 and 20 slugs m^{-2} on 2nd July 2008. This density was chosen to represent a medium-high population density as found in field investigations (Chapter 2). *D. reticulatum* was chosen because it is common and highly abundant in temperate grassland (South 1989b; South 1992), including upland hay meadows (Chapter 2). Adult slugs (300-550 mg) were collected from a plot of mixed herbage on-site and kept under controlled laboratory conditions before being introduced to mesocosms once a sufficient amount of green biomass (20% cover) was present in all mesocosms (10th June, 49 days after sowing). All mesocosms contained two upturned plant pot saucers (10 cm diameter) to act as refuge shelters for slugs and to aid monitoring of slug activity levels. Mesocosms in which no slugs were added contained small residual slug populations; regular monitoring and removal maintained these low background populations. A second introduction of slugs (50 m^{-2}) was made on 27th October 2009 as regular monitoring indicated a decline in the populations. Slug treatment groups are henceforth referred to as SL (slugs added to mesocosms to represent high population densities) and NSL (no slugs added to mesocosms, but a residual population maintained a low population density).

An appropriate cutting regime was used to simulate vertebrate grazing in autumn and spring and the annual hay-cut (circa late-July). Unlike some other studies

(Bardgett *et al.* 2006), farmyard manure (FYM) applications and the formation of artificial livestock hoof prints were not included as part of mesocosm management as they are known to have potential negative effects on slug populations (Barker 1991; G.R. Port, personal comment).

Figure 4.1. Mesocosms in June 2009; one year after sowing with seed mixes typical of either a semi-improved or unimproved upland hay meadow and subject to a slug grazing treatment.

Table 4.1. Species composition and seed quantity of seed mixes sown into mesocosms 21st April 2008. Ten *Geranium sylvaticum* seedlings (m^{-2}) (2-true leaf stage) were hand-transplanted at the time of sowing into mesocosms containing the unimproved seed mix only. *Rhinanthus minor* was sown 27th August 2008 (30 seeds m^{-2}) in mesocosms previously sown with the unimproved seed mix.

Species	**Semi-improved seed mix**	**Unimproved seed mix**
	Seed quantity (g m^{-2})	**Seed quantity (g m^{-2})**
Agrostis capillaris L.	0.4	0.4
Anthoxanthum odoratum L.	0.6	0.6
Bellis perennis L.	N/A	0.04
Centaurea nigra L.	N/A	0.2
Cerastium fontanum Baumg.	0.04	0.04
Conopodium majus (Gouan) Loret.	N/A	0.04
Cynosurus cristatus L.	0.12	0.4
Dactylis glomerata L.	0.04	0.04
Festuca rubra L.	0.2	0.2
Geranium sylvaticum L.	N/A	10 transplanted seedlings m^{-2}
Holcus lanatus L.	0.4	0.2
Leontodon hispidus L.	N/A	0.08
Lolium perenne L.	0.8	N/A
Plantago lanceolata L.	0.2	0.32
Poa trivialis L.	0.2	0.2
Prunella vulgaris L.	N/A	0.04
Ranunculus acris L.	0.24	0.24
Ranunculus bulbosus L.	N/A	0.12
Ranunculus repens L.	0.2	N/A
Rhinanthus minor L.	N/A	30 seeds m^{-2} in August 2008
Rumex acetosa L.	0.4	0.12
Sanguisorba officinalis L.	N/A	0.2
Taraxacum officinale F.H.Wigg	0.04	0.04
Trifolium pratense L.	N/A	0.12
Trifolium repens L.	0.08	0.12
Species richness	**15**	**22**

4.3.2 *Data collection*

Vegetation recording

All vascular plant species were identified and their percentage abundance cover visually estimated in mid-summer 2008, 2009 and 2010. Nomenclature follows Stace (2010). An *a posteriori* assessment of *R. minor* seedling abundance per mesocosm (m^{-2}) was performed (unimproved community only) on 6th April 2009 as germination levels were observed to be higher within NSL plots.

Hay biomass

All mesocosms received an annual hay cut (20th August 2008, 27th July 2009, 3rd August 2010), with the crop left to dry *in situ* before removal. Total dry biomass (g m^{-2}) was then recorded.

Slug activity

Slug activity was recorded on an approximately fortnightly basis from study commencement. The number of individuals beneath refuge traps was counted and any slugs found within the NSL treatment group were removed to prevent the build-up of populations.

4.3.3 *Statistical analysis*

All analyses were undertaken separately for each plant community type (semi-improved, unimproved).

Species richness and abundance cover of plant functional groups

The abundance cover and species richness of entire community assemblages and functional groups (grass, forb, sedge and rush, and annual) in individual years were calculated for each slug treatment group and analysed with non-parametric permutational ANOVA using distance matrices. Data (incorporating all years) was transformed prior to analysis (log(x+1), log(x+10)) and the Bray-Curtis distance measure used to calculate the underlying degree of association between samples.

Statistical significance of slug treatment, year and the interaction of treatment and time was determined by means of Monte-Carlo permutation test (10000 simulations) (pseudo F-statistic, P-value) with a significance level of 0.05. The best fitting models (i.e with or without the interaction term included) are presented in the results. Analyses were undertaken within the R environment for statistical computing (v. 2.9.2) (R Development Core Team 2011) utilising the community ecology statistical library vegan.

Species diversity

Species diversity and evenness was calculated using the Shannon-Weiner index and Shannon evenness based on abundance cover data within each year. The following equations were used:

Shannon-Wiener ind $$H' = -\sum_{i=1}^{S} p_i \ln p_i$$

Shannon evenness: $E' = H/\ln S$

Where, S is the total number of species, P_{i} is the proportional contribution of the i-th species.

A General Linear Model was used to analyse the effect of year and slug treatment on species diversity and evenness, with year included as a covariate (*F*-value, *P*-value). All data was tested for normality using the Anderson-Darling test. The analysis was undertaken in MINITAB 16 (Minitab 16 Statistical Software 2010).

Treatment effects on community assemblages

Unconstrained and constrained ordination techniques were used in conjunction with semi-parametric tests of hypotheses. Non-metric Multi-dimensional Scaling (nMDS) and Distance-based Redundancy Analysis (Legendre & Anderson 1999) were used to partition sources of variation and explore structural patterns within data.

Species abundance data was first standardised to z-scores (normalised), before being transformed using the Hellinger distance (dissimilarity) measure (Legendre & Gallagher 2001). Stress tests were used to assess the variation between sample distances on the nMDS plot and those distances in the original distance matrix. Stress values were less than 0.2 indicating that the method of transformation gave a reasonable representation of the original data matrix (Anderson & Underwood 1997 and authors therein) and all subsequent multivariate analyses were undertaken using the Hellinger distance measure.

Non-metric multidimensional scaling (nMDS) provided a visual means of identifying patterns of variation among samples (mesocosm plots). A plot of the first two nMDS axes was used to visually interpret patterns in the distribution of samples in multivariate space and was useful in identifying potential differences in multivariate location (i.e. group centroids) and multivariate dispersion between slug treatment groups. Variation between years could also be identified if replicates within individual years formed distinct gregarious distributions.

Constrained ordination, namely distance-based redundancy analysis (db-RDA) was undertaken using the PRIMER6 and PERMANOVA+ extension (Clarke & Gorley 2006). Distance based redundancy analysis, incorporating slug treatment and time as experimental factors, was used to further develop hypotheses and for identifying important plant species in relation to experimental factors. Partial correlations between db-RDA axes with plant species were superimposed on to a plot of the first two db-RDA axes to form a biplot. The partial correlations were used to identify those species which were driving multivariate patterns, as important species show strong positive or negative correlations with either axis. Due to *a posteriori* findings generated by PERMANOVA results (see below) a db-RDA analysis was repeated using samples from 2008 only. Integral to the flexibility of db-RDA is the ability to incorporate distance measures that are non-Euclidean in their mathematical properties. Here, the Hellinger distance measure was used to determine the degree of association between community samples. Community ecology datasets, and in particular species abundance data, rarely adhere to the statistical assumptions of multivariate normality. As such, semi-parametric permutation tests of hypothesis were used to determine the statistical significance of the underlying multivariate patterns within the study system. Furthermore, the above approach also allows interaction terms to be tested within semi-parametric permutation tests (Legendre & Anderson 1999; Anderson 2004a; Anderson 2005).

The underlying effects of treatment and time upon the composition of plant community assemblages were determined using Permutational Multivariate Analysis of Variance (PERMANOVA) (Anderson 2005) and Permutational Multivariate Dispersion (PERMDISP) (Anderson 2004a). The latter is considered analogous to Levene's test of homogeneity of variances. The statistical significance of each test is determined by means of Monte Carlo permutation (pseudo *F*-statistic, *P*-value). Here, PERMANOVA was used to determine the differences in multivariate locations between treatment groups. However, differences in multivariate locations may be sensitive to variation in multivariate dispersion. As such, PERMDISP seeks to determine differences in multivariate dispersion and may be used in conjunction with the assessment of differences between multivariate locations.

Following the methodology of Anderson (Anderson 2004a; Anderson 2006), pair-wise comparisons between treatment groups in individual years was undertaken. A Monte Carlo permutation test (10000 permutations) was used to determine statistical significance at 0.05.

A further multivariate analysis was performed using a distance-based linear model (db-linear model) and incorporated permutation tests (pseudo *F*-statistic, *P*-value), using the computer program DISTLM (Anderson 2004b). The significance of treatment, year and block were tested by permutation tests, however it was not possible to test the interaction between treatment and time using this technique. Visual interpretation of the db-linear model findings was also used as a means of interpretation.

Rhinanthus minor seedling recruitment

The effect of slug treatment on *R. minor* seedling recruitment (log(y+1) transformed) was analysed using One-Way ANOVA performed in MINITAB 16 (Minitab 16 Statistical Software 2010).

Hay biomass

The effect of slug treatment and year on hay biomass was analysed using a General Linear Model with year as a covariate (*F*-value, *P*-value). Prior to analysis, data was tested for normality using the Anderson-Darling test and subsequently transformed as appropriate (semi-improved, square root transformed; unimproved, log transformed). The analysis was undertaken using MINITAB 16 (Minitab 16 Statistical Software 2010).

4.4 Results

The majority of species germinated within six weeks of sowing. When slugs were first introduced, all mesocosms contained approximately 20% vegetation cover at mostly the seedling (or early post-seedling) stage (Hanley *et al.* 2004). Germination failed (*Festuca rubra*, *Conopodium majus, Ranunculus bulbosus* and *Sanguisorba officinalis*) or was very low (*Centaurea nigra, Ranunculus acris, Rumex acetosa, Dactylis glomerata* and *Poa trivialis*) for several species . These species were not present as planned, as all were intended for the unimproved community and five in the semi-improved community. It is likely that, despite being newly purchased, the seed had lost its viability prior to sowing as subsequent germination tests with new seed from a different supplier in an unheated glasshouse proved successful for all but *R. bulbosus* and *C. majus*. These species were not re-sown, as different sowing and germination times would have introduced unknown variation into the experimental analysis. Numerous species emerged from the existent seed/propagule bank (9 annuals and 12 perennials in total) although most were infrequent, transient and/or at very low abundance (<2%) and were not weeded out. A few species were more frequent and abundant in particular years including *Stellaria media* in 2008 and *Luzula campestris* in 2009 and 2010.

4.4.1 *Species richness and abundance cover of plant functional groups*

Species richness and percentage cover, in each plant community, are summarised in terms of total, grass, forb, sedges and rushes, and annuals (Figure 4.2a, b; Figure 4.3a, b). In both plant communities there were major changes in total vegetation cover between the first and second growing season as swards developed from being low and relatively open with some small gaps, to tall,dense communities.

For the semi-improved community the mean total vegetation cover approximately doubled between 2008 and 2009 with some decline evident in 2010. Grass and forb cover followed a similar pattern (Figure 4.2a). Total species richness declined between 2008 (SL: 17.8±0.86, NSL: 19±0.71) and 2009 (SL: 14.4±0.51, NSL: 14±0.55), but showed little change in 2010 (SL: 14.2±0.73, NSL: 14.8±1.11). A similar pattern was observed for grass and forb species richness. Although within year statistical comparisons were not made, it is noteworthy that in the first year low slug densities may have benefitted forb richness (SL: 9±0.55; NSL: 11.2±0.49) (Figure 4.2b). Permutation tests (Table 4.2a) confirmed a significant positive effect of time on the abundance cover of grasses ($P < 0.001$), sedges and rushes ($P < 0.01$) and total vegetation cover ($P < 0.001$), but not forbs ($P = 0.1018$); and a significant negative effect of time on the species richness of all groups (total: $P < 0.001$; grasses: $P < 0.01$; forbs: $P < 0.05$), except sedges and rushes ($P = 0.1294$); however, slugs did *not* have a significant effect on any functional group (including total) in terms of abundance cover or species richness over the experimental period. The abundance cover ($P < 0.001$) and species richness ($P < 0.001$) of annuals also significantly declined post-2008 in both slug treatment groups. Annual species that did not persist to 2009 included *Stellaria media*, *Juncus bufonius*, *Persicaria maculatum* and *Myosotis discolour* and contributed to the overall decline in total species richness and forb richness.

The development of the vegetation in the unimproved community demonstrated similar directional change. Total vegetation cover increased substantially between the first and second year but with little further change in the third year (Figure

4.3a). Permutation tests (Table 4.2b) showed time had a significant positive effect on the abundance cover of all functional groups (total: $P < 0.001$; grasses: $P < 0.001$; forbs: $P < 0.001$; sedges and rushes: $P < 0.001$), except annuals ($P = 0.0563$). The species richness of grasses ($P < 0.01$) and annuals ($P < 0.001$) significantly declined over time, but total ($P = 0.1410$) and forb ($P = 0.9778$) richness were unaffected by time. The abundance cover of annuals showed divergent trends over time dependent on slug treatment group; cover increased in the NSL treatment but declined in the SL treatment. In contrast to the semi-improved community, slugs *were* found to significantly reduce the abundance cover ($P < 0.001$) and species richness ($P < 0.001$) of annuals, with no annual species recorded in SL plots in 2010. The abundance cover of annuals within the unimproved community was also significantly affected by the interaction of slugs and year ($P < 0.01$), suggesting that the effect of slugs was inconsistent in each year. These findings are due to the response of the annual species, *Rhinanthus minor* to slug treatment: *R. minor* was absent from all plots in 2008 (as it was not sown until August and germinated in spring 2009), but increased in abundance in 2009 and 2010 in NSL plots only. Furthermore, forb species richness was significantly higher within low slug density conditions over the three-year experimental period ($P < 0.01$). In contrast to the semi-improved community, results do not indicate a possible positive effect of low slug densities on forb richness in the first year (Figure 4.3b). The statistical significance for the effect of slugs on the abundance cover of forbs was $P = 0.060$, indicating an emergent trend in the increase of forb abundance cover over time within low density slug environments in comparison with those with high slug densities. The effect of slug treatment on grass cover over the three years was not significant ($P = 0.5357$), although grass cover declined in NSL plots between 2009 and 2010. By the third year, forb cover was on average 23% higher (and grass cover 14% lower) within the low slug density treatment group (Fig. 4.3a). The effect of slugs on grass species richness was close to the significance threshold of 0.05 with a P value of 0.0577 suggesting that high slug densities also promoted a greater richness of grass species. The increase in forb cover was predominantly due to the increasing contribution of *R. minor* between 2009 (7%±3.45) and 2010 (16%±6.16) in some NSL plots, although other species, such as *T. pratense*, also increased in cover in all NSL plots. Results therefore show that the observed increases in forb species richness and abundance cover are associated not only with low slug density environments, but also with swards that contain *R. minor*.

Table 4.2. Permutational univariate analysis of variance for transformed (log+1 or log+10 transformed followed by transformation using bray-curtis distance matrix) abundance cover and species richness data of key functional groups within two experimental plant communities sown with a seed mix typical of: a) a semi-improved; and b) an unimproved upland hay meadow. Statistical significance of factors was determined by means of Monte-Carlo permutation test (10000 simulations) (pseudo *F*-statistic, *P*-value) with a significance level of 0.05. Results of the best fitting model (with or without the interaction) are presented for each response variable.

Group	**Transformation**	**Factors**	**d.f**	**SS**	**F.Model**	**R2**	***P***
a) semi-improved							
i) Abundance cover (%)							
Total	Log(x+1)	Slugs	1	4.7797e-04	0.75865	0.0156	0.3864
		Year	1	1.3113e-02	20.814	0.4285	0.0002
		Residuals	27	1.7011e-02		0.5559	
		Total	29	3.0602e-02		1.0000	
Grass	Log(x+1)	Slugs	1	1.2159e-03	1.4236	0.0249	0.2384
		Year	1	2.4633e-02	28.840	0.5036	0.0001
		Residuals	27	2.3062e-02		0.4715	
		Total	29	4.8911e-02		1.0000	
Forb	Log(x+1)	Slugs	1	6.6885e-04	0.45825	0.0151	0.5079
		Year	1	4.2328e-03	2.9000	0.0955	0.1018
		Residuals	27	3.9409e-02		0.8894	
		Total	29	4.4310e-02		1.0000	
Sedge+Rush	Log(x+10)	Slugs	1	5.1301e-03	2.0037	0.0472	0.1687
		Year	1	3.4525e-02	13.4847	0.3174	0.0016
		Residuals	27	6.9127e-02		0.6355	
		Total	29	1.0878e-01		1.0000	
Annual	Log(x+10)	Slugs	1	2.8883e-04	1.1907	0.0142	0.2813
		Year	1	1.3569e-02	55.937	0.6649	0.0001
		Residuals	27	6.5495e-03		0.3209	
		Total	29	2.0407e-02		1.0000	

Table 4.2. *continued*.

Group	**Transformation**	**Factors**	**d.f**	**SS**	**F.Model**	**R2**	***P***
a) semi-improved							
ii) Species richness							
Total	Log(x+1)	Slugs	1	1.1599e-04	0.27923	0.0060	0.5883
		Year	1	8.0611e-03	19.406	0.4157	0.0003
		Residuals	27	1.1216e-02		0.5783	
		Total	29	1.9393e-02		1.0000	
Grass	Log(x+1)	Slugs	1	9.1314e-06	7.2046e-03	0.0002	0.9404
		Year	1	1.4236e-02	11.232	0.2937	0.0029
		Residuals	27	3.4221e-02		0.7061	
		Total	29	4.8466e-02		1.0000	
Forb	Log(x+1)	Slugs	1	5.5993e-04	0.36534	0.0108	0.5582
		Year	1	9.9806e-03	6.5121	0.1922	0.0153
		Residuals	27	4.1381e-02		0.7970	
		Total	29	5.1921e-02		1.0000	
Sedge+Rush	Log(x+10)	Slugs	1	2.3612e-07	2.5102e-03	0.0001	0.7806
		Year	1	2.5404e-04	2.7007	0.0909	0.1294
		Residuals	27	2.5397e-03		0.9090	
		Total	29	2.7940e-03		1.0000	
Annual	Log(x+10)	Slugs	1	3.2107e-04	2.0099	0.0175	0.1673
		Year	1	1.3707e-02	85.805	0.7473	0.0001
		Residuals	27	4.3130e-03		0.2352	
		Total	29	1.8341e-02		1.0000	

Table 4.2. *continued*.

Group	**Transformation**	**Factors**	**d.f**	**SS**	**F.Model**	**R2**	***P***
b) unimproved							
i) Abundance cover (%)							
Total	Log(x+1)	Slugs Year Residuals Total	1 1 27 29	1.8562e-04 1.5597e-02 8.1770e-03 2.3960e-02	0.61292 51.502	0.0077 0.6510 0.3413 1.0000	0.4392 0.0001
Grass	Log(x+1)	Slugs Year Residuals Total	1 1 27 29	3.5064e-04 1.4375e-02 2.5086e-02 3.9812e-02	0.37740 15.472	0.0088 0.3611 0.6301 1.0000	0.5357 0.0005
Forb	Log(x+1)	Slugs Year Residuals Total	1 1 27 29	2.3605e-03 2.1286e-02 1.6672e-02 4.0318e-02	3.8229 34.473	0.0585 0.5279 0.4135 1.0000	0.060 0.0001
Sedge+Rush	Log(x+10)	Slugs Year Residuals Total	1 1 27 29	8.3176e-05 2.5694e-02 3.6612e-02 6.2389e-02	6.1340e-02 18.948	0.0013 0.4118 0.5868 1.0000	0.8048 0.0004
Annual	Log(x+10)	Slugs Year Slugs x Year Residuals Total	1 1 1 26 29	0.0433339 0.0083789 0.0270657 0.0551986 0.1339770	20.4114023 3.9466654 12.7486776	0.3234 0.0625 0.2020 0.4120 1.0000	0.0001 0.0563 0.0011

Table 4.2. *continued*.

Group	**Transformation**	**Factors**	**d.f**	**SS**	**F.Model**	**R2**	***P***
b) unimproved							
ii) Species richness							
Total	Log(x+1)	Slugs	1	8.4062e-04	2.0223	0.0647	0.1659
		Year	1	9.2578e-04	2.2272	0.0713	0.1410
		Residuals	27	1.1223e-02		0.8640	
		Total	29	1.2989e-02		1.0000	
Grass	Log(x+1)	Slugs	1	0.0059773	3.8801	0.0944	0.0577
		Year	1	0.0157323	10.2126	0.2485	0.0051
		Residuals	27	0.0415930		0.6571	
		Total	29	0.0633026		1.0000	
Forb	Log(x+1)	Slugs	1	4.7970e-03	8.2755	0.2346	0.0082
		Year	1	1.2448e-06	2.1475e-03	0.0001	0.9778
		Residuals	27	1.5651e-02		0.7654	
		Total	29	2.0449e-02		1.0000	
Sedge+Rush	Log(x+10)	Slugs	1	6.0445e-05	0.47964	0.0171	0.4139
		Year	1	6.3510e-05	0.50397	0.0180	0.4887
		Residuals	27	3.4026e-03		0.9649	
		Total	29	3.5265e-03		1.0000	
Annual	Log(x+10)	Slugs	1	1.8548e-03	17.425	0.1809	0.0005
		Year	1	5.2798e-03	49.600	0.5150	0.0001
		Slugs x Year	1	3.4940e-04	3.2824	0.0341	0.0797
		Residuals	26	2.7676e-03		0.2700	
		Total	29	1.0252e-02		1.0000	

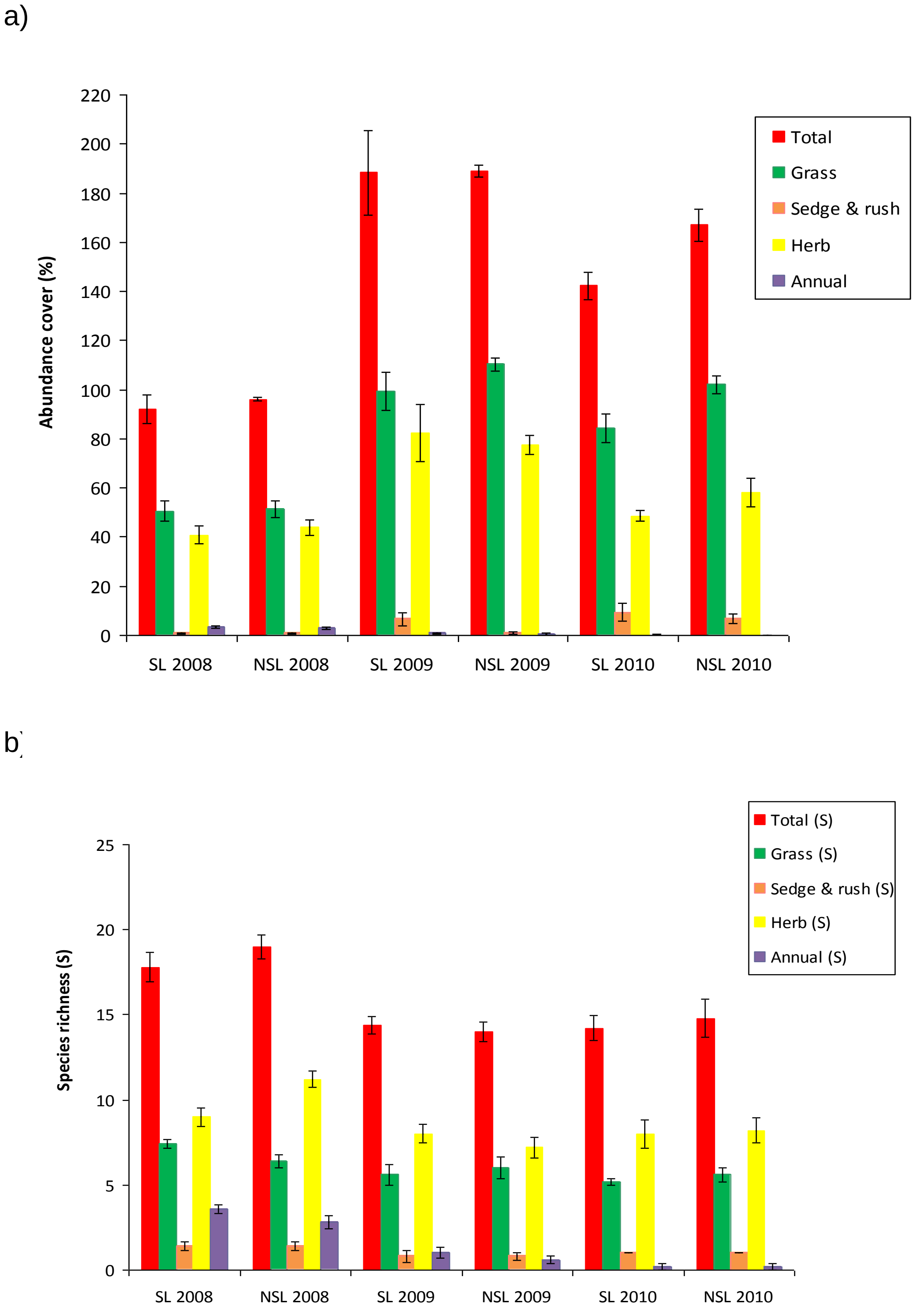
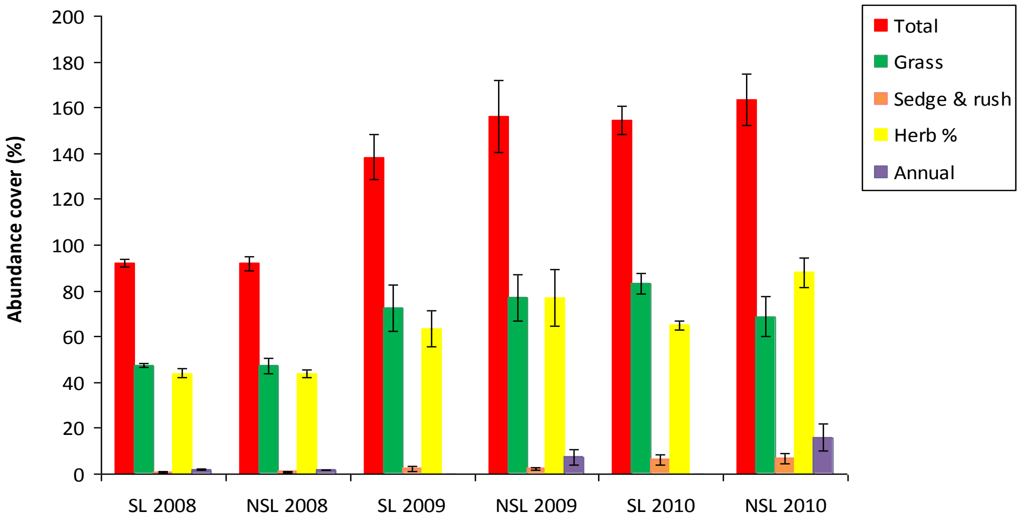


Figure 4.2. Summary of community composition in the semi-improved community, in terms of a) mean (±SEM) abundance cover (%), and b) mean (±SEM) species richness of whole assemblages and functional groups (n = 5 mesocosms per treatment group). Legend: SL, slugs added; NSL, no slugs added.

a)



b)

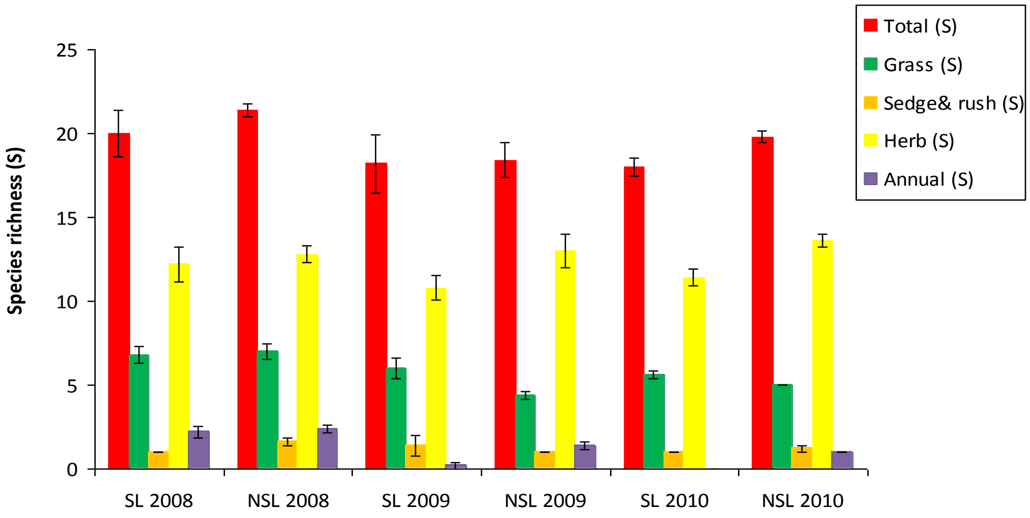


Figure 4.3. Summary of community composition in the unimproved community, in terms of a) mean (±SEM) abundance cover (%), and b) mean (±SEM) species richness of whole assemblages and functional groups (n = 5 mesocosms per treatment group). Legend: SL, slugs added; NSL, no slugs added.

4.4.2 *Species diversity*

Shannon-Weiner diversity indices (H) and evenness (E) for the semi-improved and unimproved communities are summarised in Table 4.3. For the semi-improved community, species diversity and evenness were affected by time (H: $F = 4.25$, $P < 0.05$; E: $F = 12.01$, $P < 0.001$), but not slug treatment (H: $F = 0.50$, $P = 0.450$; E: $F = 1.71$, $P = 0.202$). However, the changes in diversity and evenness over time were very small in ecological terms. In contrast, species diversity in the unimproved community was reduced at high slug density ($F = 4.38$, $P < 0.05$), but unaffected by time (F=0.82, $P = 0.372$). This may indicate a potential increase in diversity in association with low slug density conditions during the latter stages of the study, although the difference between slug groups was minimal in terms of ecological significance. Evenness was not significantly affected by either slugs ($F = 1.13$, $P = 0.297$) or time ($F = 0.60$, P= 0.446).

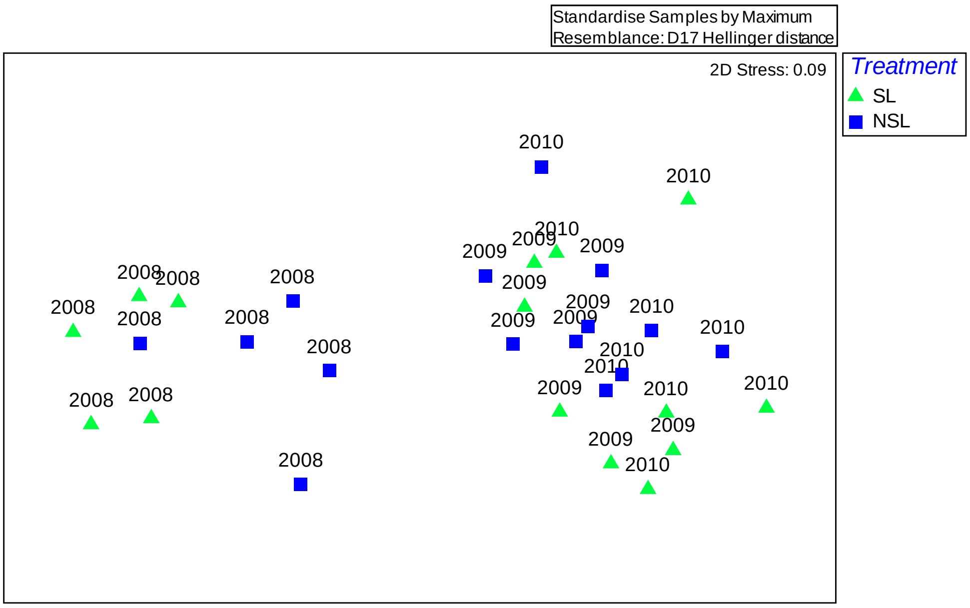
Table 4.3. Mean (+SEM) Shannon-Wiener index (H) and mean (+SEM) Shannon evenness (E) calculated using abundance cover data for species recorded in the semi-improved and unimproved community from 2008 to 2010 (n = 5 mesocosms per treatment group). SL, slugs added; NSL, no slugs added.

	Semi-improved				**Unimproved**			
	Shannon-Weiner Index		Shannon Evenness		Shannon-Weiner Index		Shannon Evenness	
	SL	NSL	SL	NSL	SL	NSL	SL	NSL
2008	2.3±0.02	2.31±0.05	0.80±0.01	0.78±0.01	2.44±0.05	2.57±0.02	0.82±0.01	0.84±0.01
2009	2.22±0.03	2.18±0.02	0.83±0.00	0.83±0.01	2.29±0.06	2.39±0.02	0.80±0.02	0.82±0.01
2010	2.25±0.04	2.22±0.03	0.85±0.01	0.83±0.03	2.44±0.03	2.47±0.05	0.84±0.00	0.83±0.01

4.4.3 *Treatment effects on community assemblages*

Non-metric multidimensional scaling (nMDS) plots and 2D stress tests (SI: stress = 0.09; UI: stress = 0.11) indicated that the method of transforming species abundance data was sound and provided a reasonable representation of the original distance matrices (Figure 4.4a, b).

a) semi-improved community



b) unimproved community

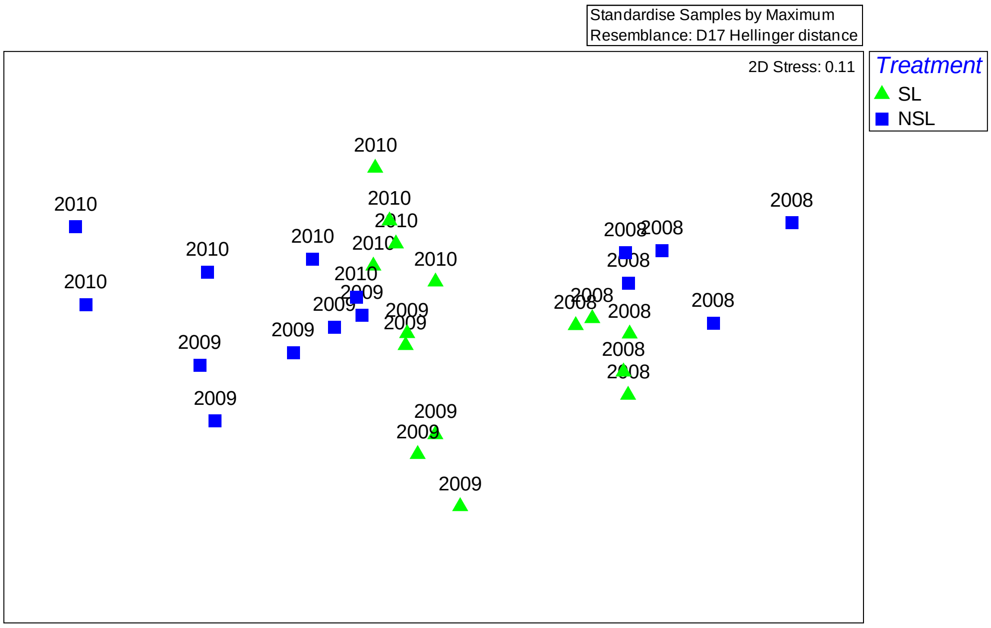


Figure 4.4. Non-metric multidimensional scaling analysis based on a Hellinger distance matrix of standardized plant community assemblages subject to high or low slug densities over a three year period. 2D stress tests of model fit indicate interpretable plots for the: a) semi-improved community (2D stress = 0.09); and b)

unimproved community (2D stress = 0.11). Legend: triangle SL = slugs added; square NSL = no slugs added.

SEMI-IMPROVED COMMUNITY

Visual interpretation of the nMDS plot (Figure 4.4a) shows that 2008 samples form a distinct cluster, whilst 2009 and 2010 samples are not separated according to year, but form one large mixed group. Within the 2008 cluster there appears to be a difference in multivariate location between treatment groups as SL and NSL samples form two groups, albeit with some overlap. In contrast, samples from 2009 and 2010 show no distinctions in terms of slug treatment group location; however, there is some indication that SL samples are more dispersed than NSL samples in 2009.

The db-RDA biplot (incorporating all years) (Figure 4.5) shows the pattern of samples and species observations within the ordination and reveals that the first two db-RDA axes explain 62.9% of the variation in the model. The pattern of samples largely supports the visual assessments made from the nMDS plot. 2008 samples form a distinct cluster and are correlated with positive axis 1 scores (axis 1 explains 49.8% of variation), indicating that community assemblages in 2008 were considerably different to those in 2009 and 2010. Despite some overlap, 2008 samples are separated according to slug treatment as SL samples are correlated with highest axis 1 scores. 2009 and 2010 samples are correlated with negative axis 1 scores and show overlapping distributions according to year and slug treatment group. However, there is some evidence of a separation of samples according to year as 2009 samples are predominantly correlated with negative axis 2 scores and 2010 samples are predominantly correlated with positive axis 2 scores (axis 2 explains 13.1% of variation). There is also some evidence that samples are separated according to slug treatment as samples correlated with increasingly positive axis 2 scores belong to the SL treatment group, particularly in 2010. Potential differences in heterogeneity between slug groups are also discernible in 2009 and 2010, with greater dispersion between SL replicates implied by sample distribution.

The db-RDA biplot shows that plant species varied in their response to slug treatment and time. Plant species which characterised the multivariate effect (i.e. those predominantly responsible for variation in community assemblages between samples) are those showing the strongest correlations with the first two db-RDA axes (Table 4.4). Species showing the strongest positive correlations with axis 1 were *L. perenne* (0.381) and *P. trivialis* (0.189), indicating that they were positively associated with all 2008 samples and their abundance declined over time. In contrast, species showing the strongest negative correlations with axis 1 were the grasses *H. lanatus* (-0.467), *C. cristatus* (-0.399), *A. capillaris* (-0.379) and *A. odoratum* (-0.304), and the sedge *L. campestris* (-0.380). 2009 and 2010 samples were also negatively correlated with db-RDA axis 1 meaning that the abundance cover of these species was positively correlated with time and was diametrically opposed to *L. perenne* and *P. trivialis*. This inverse relationship indicates that a decline in abundance of *L. perenne* and *P. trivialis* over time was associated with a convergent increase in the abundance of these competitor species. In addition, strong positive species correlations with axis 1 also indicate a bias towards the SL treatment group, suggesting that the abundance of *L. perenne* was promoted by high slug densities in the first year.

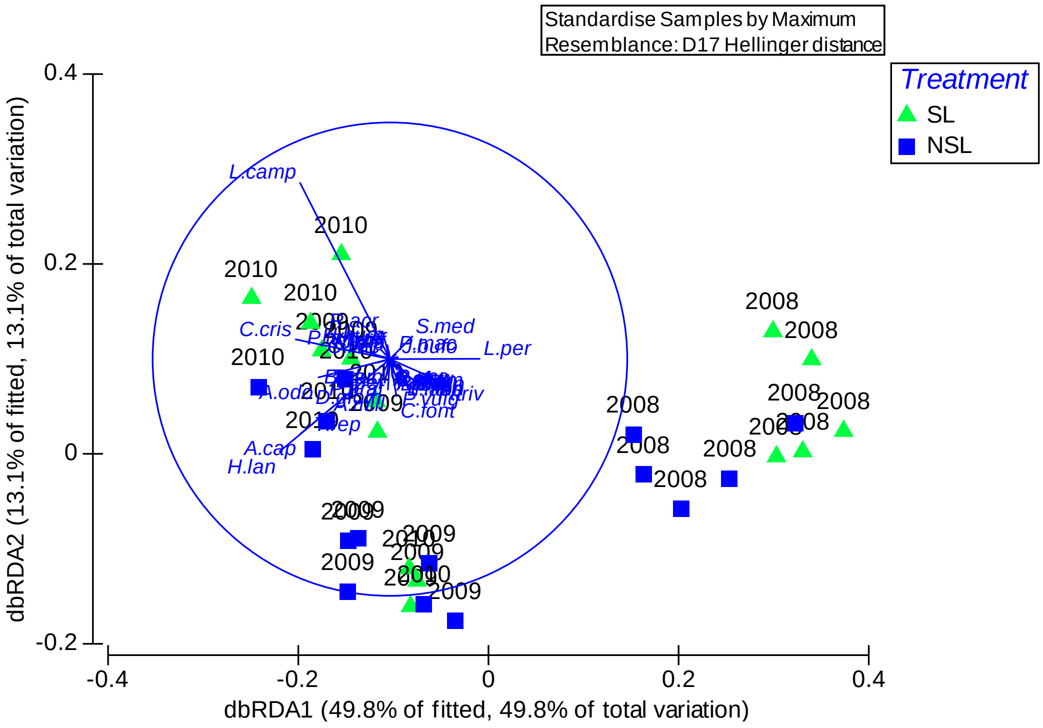


Figure 4.5. Distance based redundancy analysis biplot of (transformed) species abundance cover (2008-2010) for the semi-improved community conditioned by treatment. Abundance cover values were first standardized to Z scores before being transformed by the Hellinger distance (dissimilarity) measure. 62.9% of variation is explained by the first two ordination axes. Legend: triangles, slugs added; squares, no slugs added; L. camp, *Luzula campestris*; S. med, *Stellaria media*; J. bufo, *Juncus bufonius*; L. per, *Lolium perenne*; P. triv, *Poa trivialis*; P. vulg, *Prunella vulgaris*; C. font, *Cerastium fontanum*; T. rep; *Trifolium repens*; A. cap, *Agrostis capillaris*; H. lan, *Holcus lanatus*; A. odo, *Anthoxanthum odoratum*; C. cris, *Cynosurus cristatus*; all remaining species cannot be deciphered but include *Poa annua*, *Deschampsia cespitosa*, *Alopercurus pratensis*, *Dactylis glomerata*, *Phleum pratense*, *Carex flacca*, *Plantago lanceolata*, *Ranunculus acris*, *Rumex acetosa*, *Taraxacum officinale*, *Achillea millefolium*, *Leontodon hispidus*, *Hypericum humifusum*, *Plantago major*, *Ranunculus repens*, *Rumex obtusifolius*, *Alchemilla glabra*, *Capsella bursa-pastoris*, *Myosotis discolor*, *Persicaria maculosa*, *Veronica chamaedrys*, *Bellis perennis*, *Trifolium dubium* and *Veronica serpyllifolia*.

Species with the strongest negative correlations with axis 2 were *H. lanatus* (-0.392), *A. capillaris* (-0.318) and *T. repens* (-0.215). Samples that were also highly negatively correlated with axis 2 were predominantly classified as NSL 2010, indicating that the abundance of these species in some samples was promoted by low slug density conditions by the third year. In comparison, the only species with a strong positive correlation with axis 2 was *L. campestris* (0.747). Samples showing the greatest positive correlations with axis 2 were classified as SL 2010, indicating that *L. campestris* abundance benefited from the presence of a consistently large slug population. Furthermore, the diametric correlation evident between *L. campestris* with *H. lanatus*, *A. capillaris* and *T. repens* suggests that slug treatments influenced the relative abundance of these species three years after sowing. Although *L. campestris* was not sown, investigation of the raw data revealed it to have been present in similar frequency and abundance in each slug treatment group in 2008, yet was considerably more abundant in some SL plots in later years. In 2010, cover of *L. campestris* had also increased in some NSL plots to similar levels as in SL plots (data not shown) and likely reduced the degree of variation in overall assemblages between treatment groups. In addition, *Cyanosurus cristatus* shows a weak correlation with db-RDA axis 2 (0.084) indicating that its abundance cover was largely unaffected by slug densities or the relative abundance of other species.

Partial correlations between remaining species and db-RDA axes 1 and 2 were weak, indicating that the majority of species in the community assemblages were largely unaffected by slug treatment and time. However, care should be taken in interpreting the response of these species as most occurred at low abundance cover levels (<2%) typical of field conditions, and some species occurred infrequently and/or transiently within community assemblages. Species showing positive but weak correlations with axis 1 were present in the first year but declined in abundance or became extinct over time and included four annual species (*Stellaria media*, *Phleum maculatum*, *Poa annua* and *Juncus bufonius*). Some herb species were relatively abundant in all mesocosms, namely *Plantago lanceolata* and *Cerastium fontanum*, but were weakly correlated with experimental factors.

Table 4.4. Partial correlations between db-RDA coordinate axes and plant species for the semi-improved community. Important species are emboldened.

Variable (plant species)	Semi-improved community	
	dbRDA1	dbRDA2
Achillea millefolium L.	-0.009	-0.131
Agrostis capillaris	**-0.379**	**-0.318**
Alchemilla glabra Neygenf	0	-0.02
Alopercurus pratensis L.	0.013	-0.042
Anthoxanthum odoratum	**-0.304**	-0.077
Bellis perennis	-0.054	-0.015
Capsella bursa-pastoris (L.) Medik.	-0.006	0.024
Carex flacca Schreb.	-0.029	0.012
Cerastium fontanum	0.028	-0.159
Cynosurus cristatus	**-0.399**	0.084
Dactylis glomerata	-0.023	-0.102
Deschampsia cespitosa (L.) P.Beauv.	0.032	-0.047
Holcus lanatus	**-0.467**	**-0.392**
Hypericum humifusum L.	-0.011	0.062
Juncus bufonius L.	0.037	0.009
Leontodon hispidus	0.058	-0.062
Lolium perenne	**0.381**	0.002
Luzula campestris L.	**-0.38**	**0.747**
Myosotis discolour Pers.	-0.015	0.027
Persicaria maculosa L.	0.027	0.021
Plantago lanceolata	-0.133	0.042
Plantago major L.	0.013	-0.033
Poa annua L.	0.077	-0.065
Poa pratensis L.	-0.013	-0.068
Poa trivialis	**0.189**	-0.084
Prunella vulgaris	0.036	-0.108
Ranunculus acris	-0.032	0.12
Ranunculus repens	0.014	-0.035
Rumex acetosa	0.01	-0.018
Rumex obtusifolius L.	0.006	-0.019
Stellaria media (L.) Vill.	0.097	0.098
Taraxacum officinale	-0.005	0.036
Trifolium dubium Sibth.	-0.015	-0.016
Trifolium repens	-0.104	**-0.215**
Veronica chamaedrys L.	0.006	-0.029
Veronica persica Poiret.	-0.008	-0.035
Veronica serpyllifolia L.	-0.039	0.041

Permutation tests performed in PERMANOVA [Appendix, Table S3.1a] confirmed the size of slug populations had a highly significant effect on community assemblages during the period 2008-2010 (Pseudo-$F = 3.1933$, $P \leq 0.001$). Time also had a highly significant effect (Pseudo-$F = 19.869$, $P < 0.001$), accounting for the greatest variation in the model. There was also a significant multivariate interaction between the factors slugs and year (Pseudo-$F = 1.6829$, $P < 0.05$), indicating an inconsistent effect of slugs in each year. Block also accounted for a significant proportion of the variation within the model (Pseudo-$F = 2.4503$, $P < 0.01$).

Pair-wise PERMANOVA tests [Appendix, Table S3.1b] showed that community assemblages significantly differed according to slug treatment over the experimental period (2008-2010) ($t = 1.787$, $P < 0.01$). However, the pair-wise PERMANOVA tests within years were not significant (2008: $t = 1.8086$, $P = 0.0552$; 2009: $t = 1.3401$, $P = 0.1922$; 2010: $t = 0.99497$, $P = 0.4377$). Note, however, that the statistical significance of the test in 2008 was $P = 0.0552$, suggesting that slug treatment had the greatest impact on community assemblages during the early establishment phase and accounts for the significant interaction effect of treatment and time. This finding also indicates that community assemblages in each slug treatment group became more similar over time. The results therefore support the visual interpretation of the nMDS plot and db-RDA biplot that community assemblages present in the first year were distinct from those in later years and that slug treatment affected early community assemblages, predominantly due to affecting the abundance of *L. perenne*. Furthermore, PERMANOVA results confirmed that variability in community assemblages in later years, driven by the response of key species evident in the db-RDA analysis, were not sufficient to cause significant differences in multivariate location in individual years but contributed to the differences between treatment groups when viewed over all years.

Pair-wise comparison tests undertaken in PERMDISP [Appendix, Table S3.2] confirmed a significant difference in multivariate dispersions between slug treatments for the period 2008-2010 ($t = 2.4296$, $P < 0.05$), and in 2009 ($t = 3.9526$, $P < 0.01$), but not in 2008 ($t = 0.93378$, $P = 0.4206$) or 2010 ($t = 1.0855$, P

= 0.4312). Results indicate that the pair-wise difference between slug treatment groups over all years as shown by PERMANOVA results is influenced by the greater dispersion between replicates within the SL treatment group in comparison with the NSL treatment group. Likewise, as pair-wise PERMDISP results did not find a significant difference in 2008, this implies that the difference detected by the pair-wise PERMANOVA test in 2008 was due to a difference in multivariate location rather than dispersion. The PERMDISP analysis verifies the visual assessment of the nMDS plot, confirming that replicates experiencing high slug grazing pressure were more variable in terms of the abundance and composition of species over the experimental period as a whole (2008-2010) and in 2009. This finding is likely to have been influenced by the strong positive response by *L. campestris* to high slug densities in 2009 in some, but not all replicates and the associated changes in relative abundance of those species diametrically opposed to *L. campestris* in the biplot. The increase in the abundance of *L. campestris* in some NSL plots in 2010 seemingly reduced the variation in whole assemblages between treatment groups in the third year (i.e. dispersion increased in NSL treatment group to similar levels as in SL group, coupled with a further reduction in differences in multivariate location between groups).

As the results of permutation tests performed in PERMANOVA suggested an effect of slug treatment on community assemblages in 2008, which could not be satisfactorily explained by differences between functional groups or by use of the db-RDA biplot incorporating all years, a second db-RDA biplot was produced incorporating samples from 2008 only (Figure 4.6). The db-RDA biplot incorporating all years provides information on the response of individual species to slug treatment over the whole study period but does not indicate how the majority of species responded in the first growing season. Note the 2008 db-RDA biplot can not be directly compared with the biplot of all years because some species were not present in all years. Results show that the first two axes explained 52.9% of total variation in the model. Samples are distributed according to slug treatment group in relation to axis 1 (35% of total variation) as all SL samples are negatively correlated with axis 1 and the majority of NSL samples are positively correlated with axis 1. The distribution of samples according to treatment group in correlation with axis 2 is random. Of the 29 species recorded, 16 species were not sown and included 7 annual species. Some of the constant and more abundant species responded to slug treatment. Partial correlations between species and axis 1 suggests that variation in community assemblages was primarily driven by opposing responses of the most abundant grass species (*L. perenne* and *A. capillaris*) and, to a lesser extent, *T. repens* to slug treatment. *L. perenne* was strongly negatively correlated with axis 1 (-0.712) indicating that high slug densities promoted the cover of *L. perenne*. Conversely, *A. capillaris* (0.567) and *T. repens* (0.208) were positively correlated with axis 1 indicating that high slug densities reduced the cover of these species. The diametric correlation between *L. perenne* and both *A. capillaris* and *T. repens* suggests that slug herbivory caused shifts in their relative abundance depending on slug grazing pressure. In plots containing low slug densities, the rank abundance of *L. perenne* and *A. capillaris* switched as *A. capillaris* abundance was greater and *L. perenne* abundance lower than under conditions of intensive herbivory. *T. repens* was the most abundant forb species in both treatment groups and was promoted by low slug densities, but remained competitively subordinate to the two dominant grass species. In addition, the relative abundance of *A. capillaris* (0.395) and *L. perenne* (0.368) to community assemblages was influenced by the cover of *P. lanceolata* (-0.460) and *C. fontanum* (-0.437) which are shown by the biplot as being directly opposed in correlation with axis 2.

C. cristatus (0.185), *H. lanatus* (0.128) and *C. fontanum* (0.135) were relatively abundant in all plots in 2008 but showed weak positive correlations with axis 1 suggesting some bias towards low slug density conditions. *P. lanceolata* was also relatively abundant in all plots and showed the opposite pattern (-0.107). The remaining species were sparse (<2% cover) and/or infrequent as some sown species failed to germinate as expected and generated weak partial correlations with axis 1.

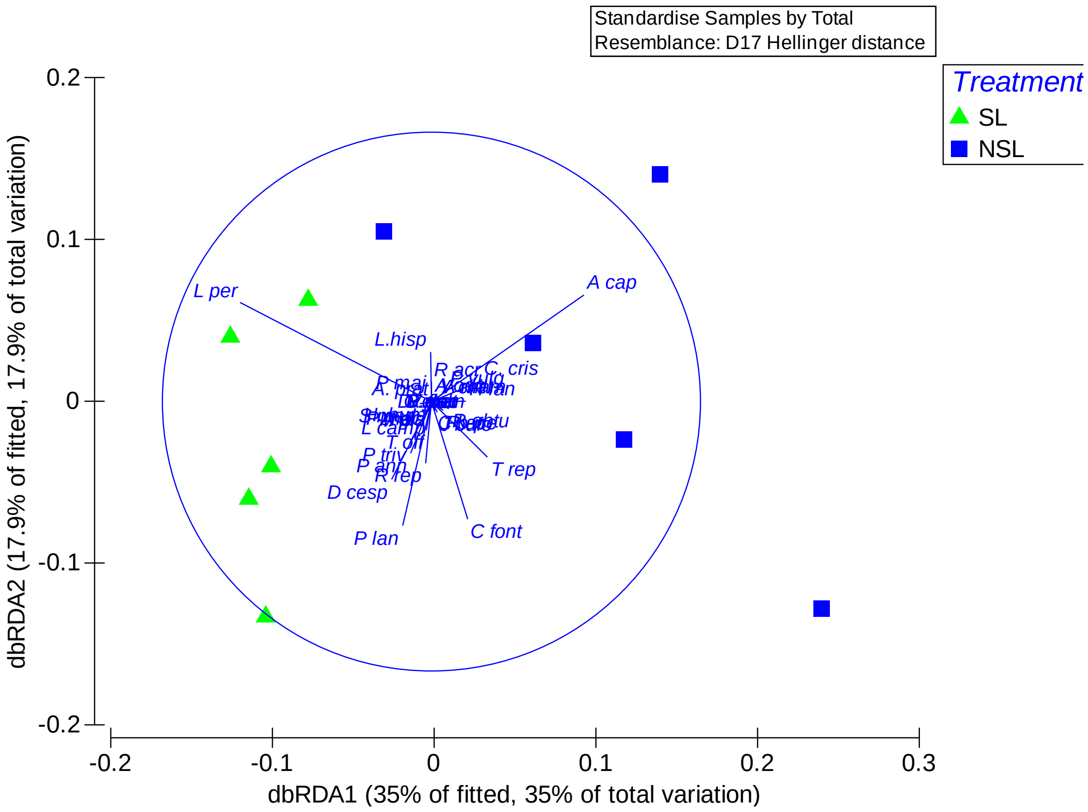


Figure 4.6. Distance based redundancy analysis biplot of species abundance cover in 2008 for the semi-improved community conditioned by treatment. Abundance cover values were first standardized to Z scores before being transformed by the Hellinger distance (dissimilarity) measure. 52.9% of variation is explained by the first two ordination axes. Legend: triangles, slugs added; squares, no slugs added; L. hisp, *Leontodon hispidus*; R. acr, *Ranunculus acris*; A, cap, *Agrostis capillaris*; C. cris, *Cynosurus cristatus*; H. lan, *Holcus lanatus*; T. rep, *Trifolium repens*; C. font, *Cerastium fontanum*; P. lan, *Plantago lanceolata*; R. rep, *R. repens*; D, cesp, *Deschampsia cespitosa*; P. ann, *Poa annua*; P. triv, *P. trivialis*; T. off, *Taraxacum officinale*; L. cam, *Luzula campestris*; P. maj, *Plantago major*; L. per, *Lolium perenne*; all remaining species cannot be deciphered but include *Anthoxanthum odoratum*, *Juncus bufonius*, *Prunella vulgaris*, *Rumex ace*tosa, *Achillea millefolium*, *Hypericum humifusum*, *Rumex obtusifolius*, *Stellaria media*, *Alchemilla glabra*, *Capsella bursa-pastoris*, *Myosotis discolour*, *Persicaria maculosa* and *Veronica chamaedrys*.

The first two axes of the db-linear model (Figure 4.7) explained 43.3% of the total variation in the model (compared with 62.9% for the db-RDA model). The plot of the first two axes shows samples are distributed according to slug treatment groups in association with axis 2; however, axis 2 only accounts for 4.1% of total variation. Samples are also distributed according to year (time) in association with axis 1 (axis 1 explained 39.2% of total variation). Permutation tests [Appendix, Table S3.3] confirmed the highly significant effect of time on community assemblages (Pseudo-*F* = 17.932, *P* < 0.001), but in contrast to the PERMANOVA tests, slug treatment had no significant effect on community assemblages over the experimental period (Pseudo-*F* = 1.2264, *P* = 0.255). The effect of block was again found to be a significant influencing factor on community assemblages (Pseudo-*F* = 0.70753, *P* < 0.01).

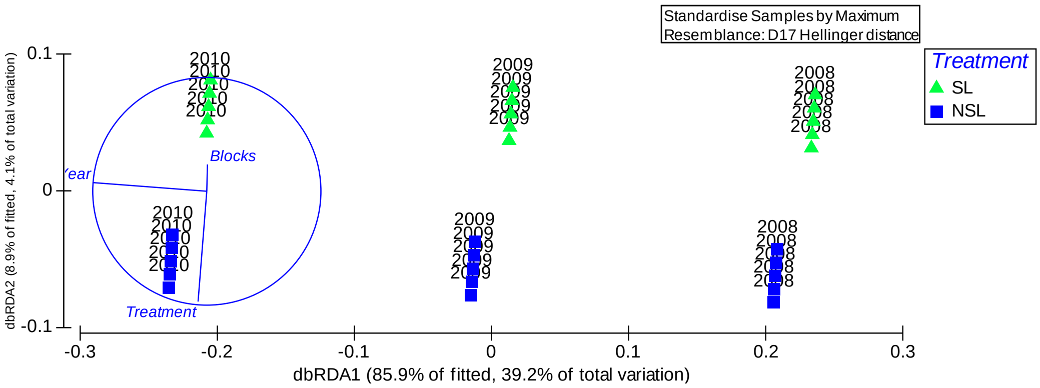


Figure 4.7. Distance based linear model of (transformed) species abundance cover within the semi-improved community mesocosms subject to high and low slug population densities over three years. 43.3% of variation is explained by the first two ordination axes. Legend: triangles, slugs added; squares, no slugs added.

In summary, permutations tests performed in PERMANOVA confirmed a significant effect of treatment, time and the interaction of treatment and time on community assemblages over the study period. Permutation tests incorporated in a db-linear model also found time to be a significant factor, but did not confirm a significant effect of treatment on community assemblages. Pair-wise permutation tests performed in PERMANOVA and PERMDISP showed that variation between slug treatment groups over the experimental period was due to both differences in multivariate location and multivariate dispersion. However, the underlying effect of slug treatment on community assemblages in individual years was dependent on the age of the community. Variation in community assemblages over time and between slug treatment groups was primarily driven by species-level responses, namely: i) the positive response by *L. campestris* to high slug densities; and ii) the negative response by *H. lanatus*, *A. capillaris* and *T. repens* to high slug densities and effects on the relative abundance of these species

UNIMPROVED COMMUNITY

The nMDS plot for the unimproved community (Figure 4.4b) shows similar patterns to those of the semi-improved community: Between year variation is evident with all 2008 samples forming a separate cluster to all 2009 and 2010 samples. Likewise, 2008 slug treatment groups are visually distinct from one another, suggesting a potential difference in multivariate location between treatment groups. The 2009 and 2010 samples overlap somewhat; however, samples from each year are scattered in vertically opposing directions (2009 samples are distributed from the middle to the bottom of the plot and 2010 samples are distributed from the middle towards the top of the plot). Contrary to the semi-improved community, a potential difference in multivariate location between slug treatments is discernible in both 2009 and 2010. In further contrast there appears to be greater dispersion between NSL samples, particularly in 2010, than between SL samples.

These visual observations are supported by the patterns discernible in the db-RDA biplot (Figure 4.8). The biplot shows that 55.11% of variation is explained by the first two ordination axes. Sample distribution is associated with both time and slug treatment in relation to axis 1 (41.8% of total variation), and with only slug treatment in relation to axis 2 (13.3% of total variation). All 2008 samples form a distinct group (high positive axis 1 scores) from all 2009 and 2010 samples (negative axis 1 scores). Distribution of 2008 samples differ according to slug treatment group in association with axis 2: 2008 NSL samples are associated with negative axis 2 scores; whereas, 2008 SL samples are predominantly associated with positive axis 2 scores. The distribution of 2009 and 2010 samples is random according to year, indicating little variation in community assemblages between these years. However, these samples do show some separation according to slug treatment; all SL samples are located in a group at the top-middle of the biplot (mid axis 1, high/positive axis 2) and NSL samples are scattered along axis 2 but are always associated with lower (i.e. negative) axis 1 scores. This indicates that community assemblages differed between slug treatment groups but that assemblages within each treatment group experienced little change between 2009 and 2010. It is also evident that the difference in community assemblages between treatment groups was greater in some NSL samples than others (associated with increasingly negative axis 1 and axis 2 scores). NSL samples showing the greatest separation in multivariate space from SL samples were recorded in 2010 (and had the lowest axis 1 and 2 scores). Accordingly, dispersion between replicates appears to be greatest within the NSL treatment group over the experimental period and particularly in 2010.

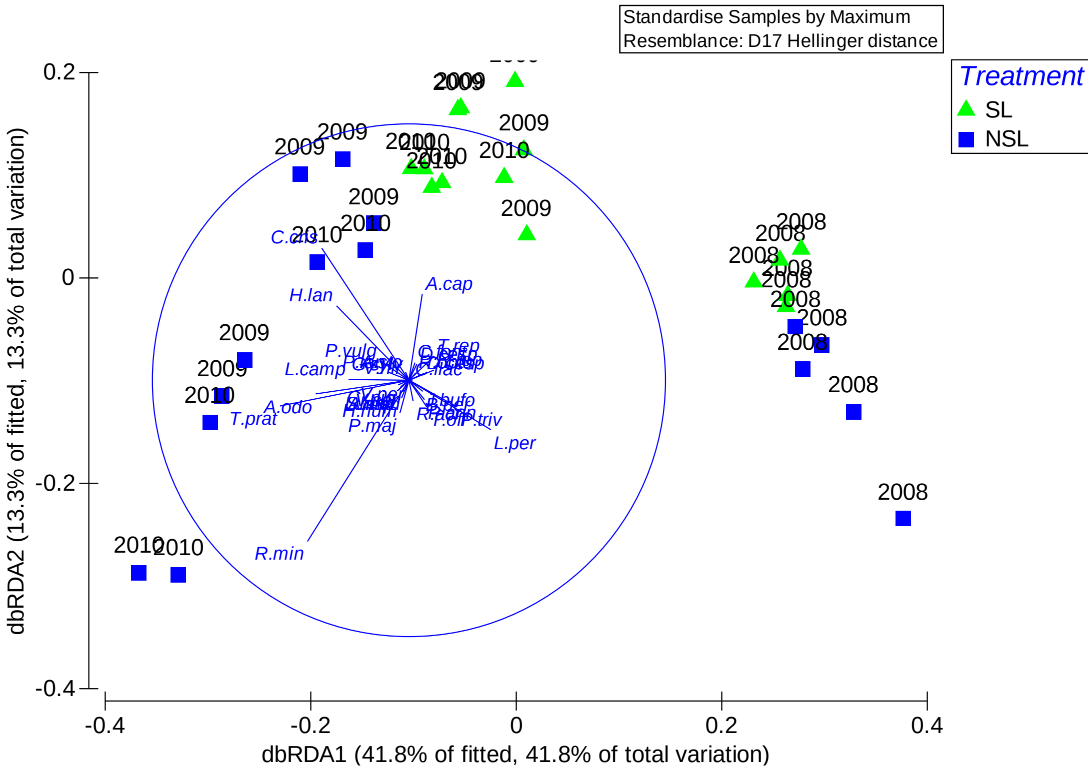


Figure 4.8. Distance based redundancy analysis biplot of species abundance cover within the unimproved community subject to different slug population densities over three years. 55.11% of variation is explained by the first two ordination axes. Legend: triangles, slugs added; squares, no slugs added. A. cap, *Agrostis capillaris*; T. rep, *Trifolium repens*; P. triv, *Poa trivialis*; L. per, *Lolium perenne*, R. min, *Rhinanthus minor*; P, maj, *Plantago major*; T. prat, *Trifolium pratense*; A. odo, *Anthoxanthum odoratum*; L. camp, *Luzula campestris*; G. sylv, *Geranium sylvaticum*; P. vulg, *Prunella vulgaris*; H. lan, *Holcus lanatus*; C. cris; *Cynosurus cristatus*; the remaining species can not be deciphered but include *Poa annua*, *Deschampsia cespitosa*, *Bellis perennis*, *Cerastium fontanum*, *Plantago lanceolata*, *Ranunculus acris*, *Taraxacum officinale*, *Achillea millefolium*, *Leontodon hispidus*, *Hypericum humifusum*, *Ranunculus repens*, *Rumex obtusifolius*, *Stellaria media*, *Vicia hirsuta*, *Centaurea nigra*, *Juncus bufonius*, *Carex flacca*, *Veronica persica*, *V. serpyllifolia* and *Agrostis stolonifera*.

The db-RDA biplot and species partial correlations with ordination axes (Table 4.5) showed the abundance of several species to be notably affected by experimental factors. Species driving axis 1 responded strongly to time and results were highly similar to those of the semi-improved community. Species with the strongest positive correlations with axis 1 were *L. perenne* (0.319) and *P. trivialis* (0.188) indicating that they declined in abundance over time. Conversely, species with strong negative correlations with axis 1 were *T. pratense* (-0.503), *R. minor* (-0.396), *A. odoratum* (-0.363), *C. cristatus* (-0.340), *H. lanatus* (-0.282) and *L. campestris* (-0.236) indicating that they responded positively to time. As found for the semi-improved community, the decline in abundance of *L. perenne* and *P. trivialis* is associated with a convergent increase in abundance of competitor grass and legume species. Although in contrast to the semi-improved community, *L. perenne* is also negatively correlated with axis 2 (-0.193) as are NSL plots in 2008, suggesting that its abundance may have been positively affected by low slug densities in the first year. However, *L. perenne* was not sown within the unimproved community and was also comparatively less abundant in the unimproved community compared with the semi-improved community in 2008.

The main drivers of axis 2 were *R. minor* (-0.628), *C. cristatus* (0.515), *A. capillaris* (0.336) and *H. lanatus* (0.290) and were the key species responsible for variation in community assemblages between treatment groups and within the NSL treatment group. It is clear that slug treatment had a profound effect on the abundance of *R. minor* and the response of *R. minor* to experimental factors is fundamental to deciphering the pattern of samples in multivariate space. *R. minor* shows a strong negative correlation with both axis 1 and axis 2, as do several NSL samples, particularly in 2010, indicating that *R. minor* abundance was markedly greater when slug grazing pressure was low and, in these conditions, its abundance increased in its second year within the sward (*N.B:- R. minor* first appeared in the sward in 2009). *T. pratense* (axis 1, -0.503; axis 2, -0.100) and *A. odoratum* (axis 1, -0.363; axis 2, -0.053) also show negative correlations with both axis 1 and 2 indicating that they too may have benefitted from low slug densities *and* in swards which contained a greater abundance of *R. minor*. *R. minor* was present within all NSL plots in 2009 and 2010 and showed some variation in cover between NSL samples (2009: 7% ±3.45 SEM; 2010: 16% ±6.16 SEM). This is apparent in the variation in distribution of NSL samples in association with axis 2. NSL samples characterised by a lesser abundance cover of *R. minor* are associated with negative axis 1 scores and positive axis 2 scores. *C. cristatus* (axis 1, -3.40; axis 2, 0.515) and *H. lanatus* (axis 1, -0.282; axis 2, 0.290) show the same correlation with each axes indicating that the abundance of these species was greater in low slug density conditions in comparison with high slug densities, but that their abundance was suppressed by the cover of *R. minor* either by competition or parasitism. The inverse relationship between *R. minor* and these grass species is apparent by their diametric correlations with axis 2.

R. minor is also diametrically opposed to *A. capillaris* indicating that the abundance of *A. capillaris* was suppressed according to the abundance of *R. minor* in NSL plots. Furthermore, *A. capillaris* (axis 1, 0.052; axis 2, 0.336) shows the same correlation with each axes as SL samples (mid axis 1 and positive axis 2 scores) indicating that the abundance of *A. capillaris* was greater within high slug density conditions *and* in the absence of *R. minor*.

Abundance of remaining species was weakly correlated with experimental variables and they occurred infrequently, transiently and/or at low abundance cover scores (<2%). Eight species showed weak negative correlations with axis 1 and axis 2, all of which were forbs, suggesting that they may have benefitted from low slug density conditions and the presence of *R. minor* throughout the study period. In comparison, nine species showed weak positive or negative correlations with axis 1 and weak positive correlations with axis 2, suggesting that they may have benefitted from high slug density conditions and the absence of *R. minor*, and included two grasses, one sedge, two legumes and four non-leguminous forbs.

Table 4.5. Partial correlations between db-RDA coordinate axes and plant species for the unimproved community. Important species are emboldened.

Variable (plant species)	dbRDA1	dbRDA2
Achillea millefolium	-0.042	-0.039
Agrostis capillaris	**0.052**	**0.336**
Agrostis stolonifera	-0.013	0.032
Anthoxanthum odoratum	**-0.363**	-0.053
Bellis perennis	0.054	-0.043
Carex flacca	0.014	0.001
Centaurea nigra	-0.042	-0.018
Cerastium fontanum	0.024	0.069
Cynosurus cristatus	**-0.34**	**0.515**
Deschampsia cespitosa	0.058	0.027
Geranium sylvaticum	-0.014	0.019
Holcus lanatus	**-0.282**	**0.29**
Hypericum humifusum	-0.029	-0.07
Juncus bufonius	0.052	-0.029
Leontodon hispidus	-0.046	-0.041
Lolium perenne	**0.319**	**-0.193**
Luzula campestris	**-0.236**	0.003
Plantago lanceolata	-0.081	0.032
Plantago major	-0.035	-0.127
Poa annua	0.06	-0.076
Poa trivialis	**0.188**	-0.104
Prunella vulgaris	-0.11	0.074
Ranunculus acris	0.016	-0.08
Ranunculus repens	0.097	0.039
Rhinanthus minor	**-0.396**	**-0.628**
Rumex obtusifolius	0.027	0.029
Stellaria media	-0.024	-0.041
Taraxacum officinale	0.069	-0.104
Trifolium pratense	**-0.503**	-0.1
Trifolium repens	0.099	0.094
Veronica persica	-0.009	-0.005
Veronica serpyllifolia	-0.032	-0.026
Vicia hirsuta (L.) Gray	-0.017	0.006

PERMANOVA results [Appendix, Table S3.4a] confirmed a highly significant effect of slugs (pseudo-*F* = 5.5399, *P* < 0.001) and time (pseudo-*F* = 0.0001, *P* < 0.001) on community assemblages during the period 2008-2010. Results also indicated a significant interaction effect of slugs and year (pseudo-*F* = 2.6681, *P* < 0.01), suggesting that the effect of slugs was inconsistent in each year. Block also caused a significant proportion of the variation within the model (pseudo-*F* = 1.4953, *P* < 0.05).

Pair-wise tests performed in PERMANOVA [Appendix, Table S3.4b] confirmed a significant difference in multivariate location between slug treatment groups over the three year experimental period (*t* = 2.3537, *P* < 0.001), and in 2008 (*t* = 1.8635, *P* < 0.05) and 2010 (*t* = 2.1036, *P* < 0.05) but not in 2009 (*t* = 1.5336, *P* = 0.1118). Evaluation of these results and the db-RDA biplot indicate that differences in community assemblages in 2010 were predominantly driven by the responses of the species mentioned above, particularly *R. minor*.

Pair-wise tests performed in PERMDISP [Appendix, Table S3.5] found no evidence of differences in multivariate dispersion within slug treatment groups in individual years (2008: *t* = 1.9197, *P* = 0.1016; 2009: *t* = 1.4483, *P* = 0.2854; 2010: *t* = 1.0675, *P* = 0.3771). PERMDISP results also indicate that significant differences between slug treatment groups suggested by PERMANOVA results for 2008 and 2010 were due to variation in multivariate location and not multivariate dispersion. The significance of the test incorporating all years was *P* = 0.0594 (*t* = 2.1684), suggesting that heterogeneity within the NSL treatment was greater than within the SL treatment as predicted by visual interpretation of the nMDS plot and db-RDA biplot.

As PERMANOVA results confirmed a significant difference between community assemblages in 2008, a db-RDA biplot was once more produced for this year (Figure 4.9). The first two axes of the 2008 db-RDA biplot explained 51.8% of the total variation in the model and shows replicates to be distributed according to treatment group in association with axis 1 (31.4% of total variation). SL samples are negatively correlated with axis 1 and NSL samples are positively correlated with axis 1. The same caveats for interpretation apply as described for the semi-improved community. Of the 29 species recorded, 13 were not sown including 5 annual species and *L. perenne*. The separation of samples according to treatment group in relation to axis 1 was predominantly driven by the responses of the abundant grass species *L. perenne* (0.726), *A. capillaris* (-0.474) and *C. cristatus* (-0.430). Despite not being sown, *L. perenne* was present in all plots but was positively correlated with axis 1 indicating that it was more abundant in NSL plots. *A. capillaris* and *C. crisitatus* were also abundant in NSL plots, but were negatively correlated with axis 1 indicating that they were more abundant within SL plots and were associated with a reduction of *L. perenne* in this treatment group. This does not necessarily imply that large slug densities directly reduced the cover of *L. perenne*, but is more likely to suggest that the relatively low abundance of *L. perenne* was unable to compete with *A. capillaris* and *C. cristatus* in both treatment groups and the abundance of *A. capillaris* and *C. cristatus* were further promoted by their ability to avoid or tolerate slug grazing.

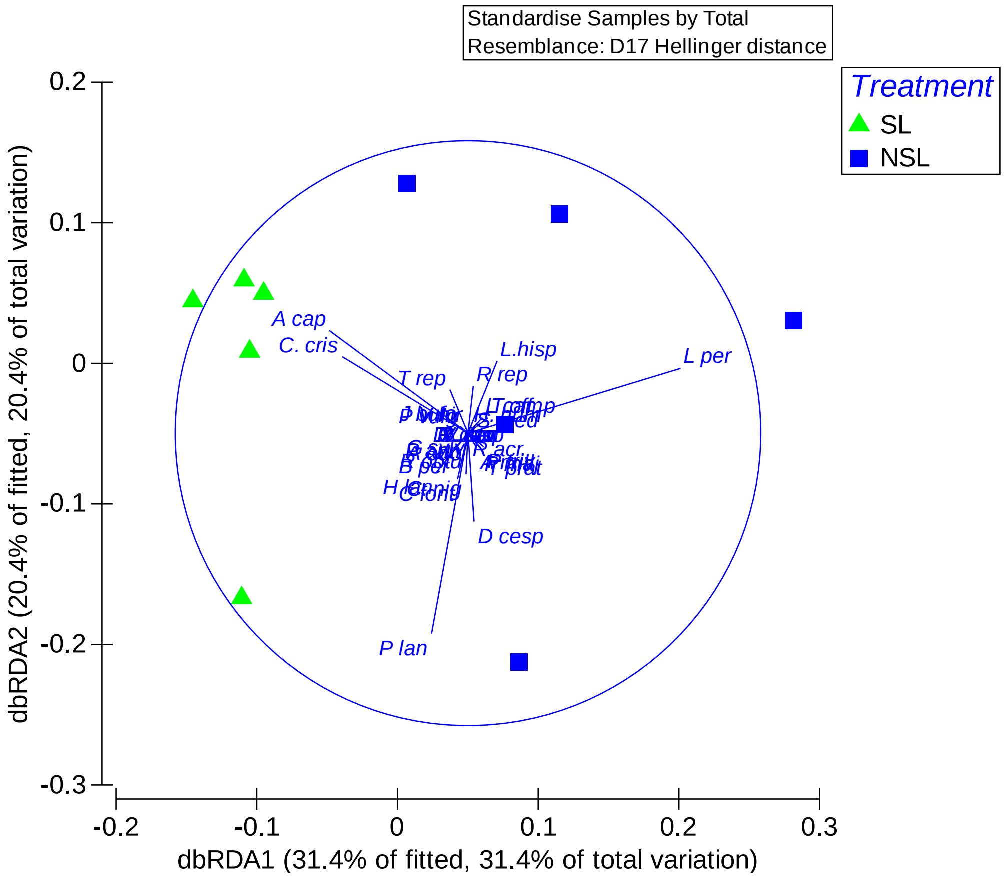


Figure 4.9. Distance based redundancy analysis biplot of (transformed) species abundance cover in 2008 for the unimproved community conditioned by treatment. Abundance cover values were first standardized to Z scores before being transformed by the Hellinger distance (dissimilarity) measure. 51.8% of variation is explained by the first two ordination axes. Legend: triangles SL, slugs added; squares NSL, no slugs added; R. rep, *Ranunculus repens*; L. hisp, *Leontodon hispidus*; L. per, *Lolium perenne*; R. acr, *Ranunculus acris*; T. prat, *Trifolium pratense*; D. cesp, *Deschampsia cespitosa*; P. lan, *Plantago lanceolata*; C. fon, *Cerastium fontanum*; H. lan, *Holcus lanatus*; B. per, *Bellis perennis*; C. cris, *Cynosurus crist*atus; A. cap, *Agrostis capillaris*; T. rep, *Trifolium repens*; all remaining species cannot be deciphered but include *Anthoxanthum odoratum*, *Poa trivialis*, *Poa annua*, *Geranium sylvaticum*, *Prunella vulgaris*, *Achillea millefolium*, *Hypericum humifusum*, *Plantago major*, *Rumex obtusifolius*, *Stellaria media*, *Veronica hirsuta*, *Centaurea nigra*, *Juncus bufonius* and *Luzula campestris.*

As in the semi-improved community, *P. lanceolata* (axis 1 -0.125) was largely unaffected by slug treatment, but showed some bias towards high slug densities, and was influenced by the cover of the three common grasses. All of the remaining common and relatively abundant species (*H. lanatus*, *B. perennis*, *C. fontanum*, *T. pratense*, *T. repens*) were unaffected by slug populations during the early establishment phase (evidenced by weak correlation with db-RDA axis 1).

The first two axes of the db-linear model explained 40% of the total variation in the model. The plot of the first two axes shows that replicates are distributed according to slug treatment in association with axis 2 (6.8% of total variation) and by year in association with axis 1 (33.2% of total variation) (Figure 4.10). Permutation tests [Appendix, Table S3.6] confirm a significant effect of slug treatment (pseudo-*F* = 2.6479, *P* < 0.05) and year (pseudo-*F* = 12.428, *P* ≤ 0.001) on community assemblages, and support the findings of the permutation tests performed in PERMANOVA.

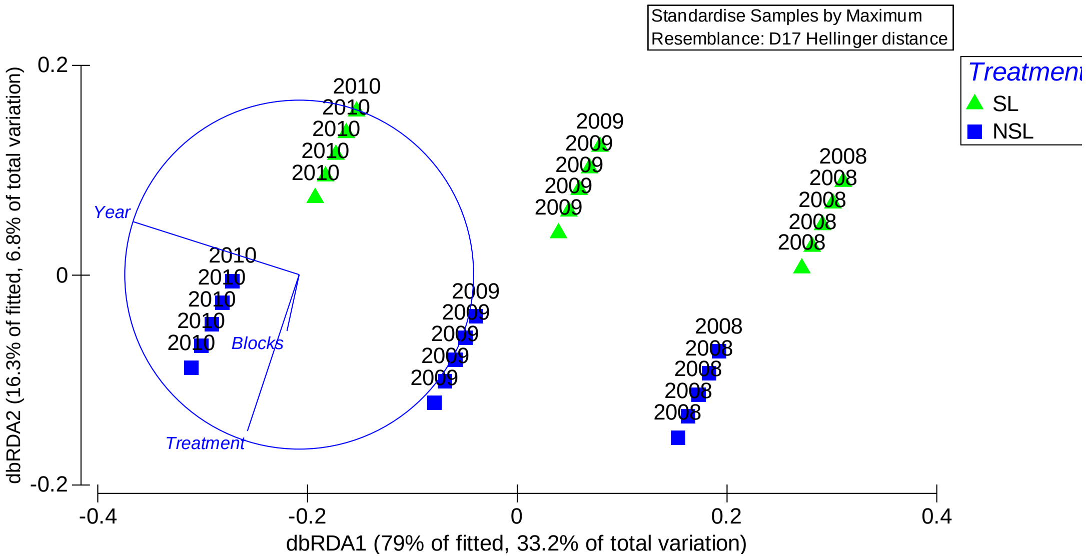


Figure 4.10. Distance based linear model of (transformed) species abundance cover within the unimproved community mesocosms subjected to high and low slug population densities over three years. Db-RDA axis 1 and db-RDA axis 2 cumulatively explain 40% of the total variation in the model. Legend: triangles SL, slugs added; squares NSL, no slugs added.

In summary, multivariate permutation tests performed in PERMANOVA confirmed a significant effect of treatment, time and the interaction of treatment and time on community assemblages during the period of study (2008-2010). These findings were also supported by permutation tests incorporated in a db-linear model (N.B:- an interaction effect could not be tested within the db-linear model). Significant pair-wise differences between slug treatment groups within years were due to differences in multivariate location and not multivariate dispersions. The significant (PERMANOVA) pair-wise difference between slug treatment groups over all years was predominantly due to variation in multivariate location but was influenced by the greater dispersion between replicates within the NSL treatment group. Variation in community assemblages between slug treatment groups was predominantly driven by the negative response of *R. minor* to large slug populations.

4.4.4 Rhinanthus minor *seedling recruitment in the unimproved community*

A statistical comparison of *R. minor* seedling recruitment was significantly lower (in some replicates all seedlings were absent) in mesocosms containing large slug populations (ANOVA, F = 53.17; P < 0.001) (Figure 4.11). Mean seedling recruitment in the NSL and SL treatment groups represents a seed to seedling conversion rate of 51.33% and 1.33%, respectively. Only two seedlings were found within two SL replicates, and both exhibited signs of slug damage (S.E. Barlow, personal observation) and did not survive to contribute to the mid-summer assessment of abundance cover. All *R. minor* seedlings lacked the visual symptoms of host attachment and the majority of seedlings were at the approximately 4-6 true leaf stage indicating that mortality was not caused by slug herbivory to host plants.

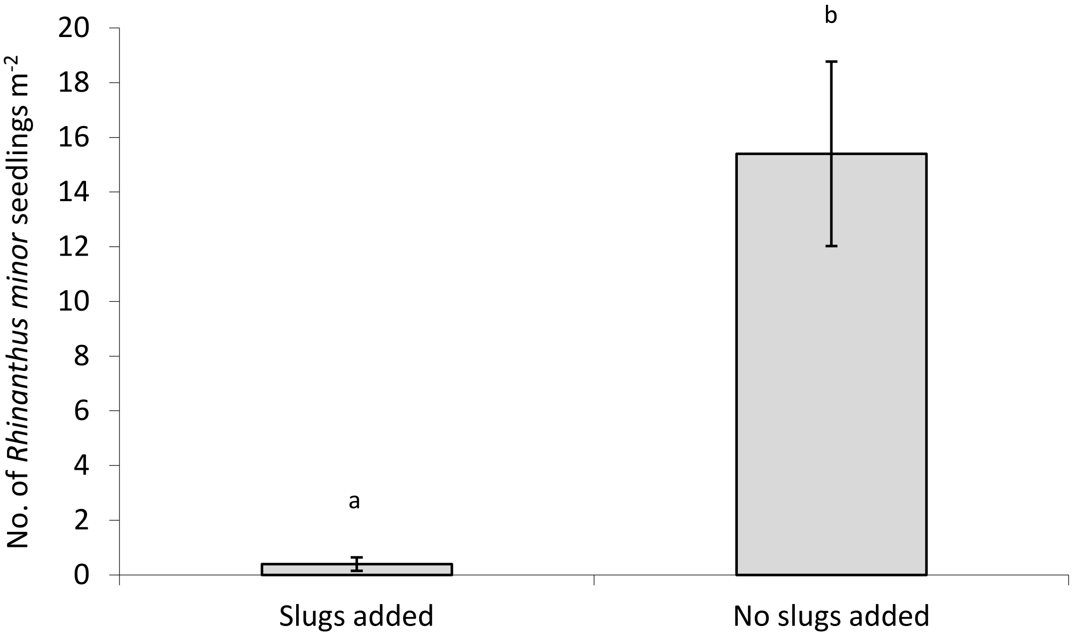


Figure 4.11. Mean(±SEM) number of *Rhinanthus minor* seedlings recorded in April 2009 (253 days after sowiug) within mesocosms (1 m^2) containing mixed swards subject to high or low slug population densities (n = 5 mesocosms per treatment). Means that do not share a letter are significantly different.

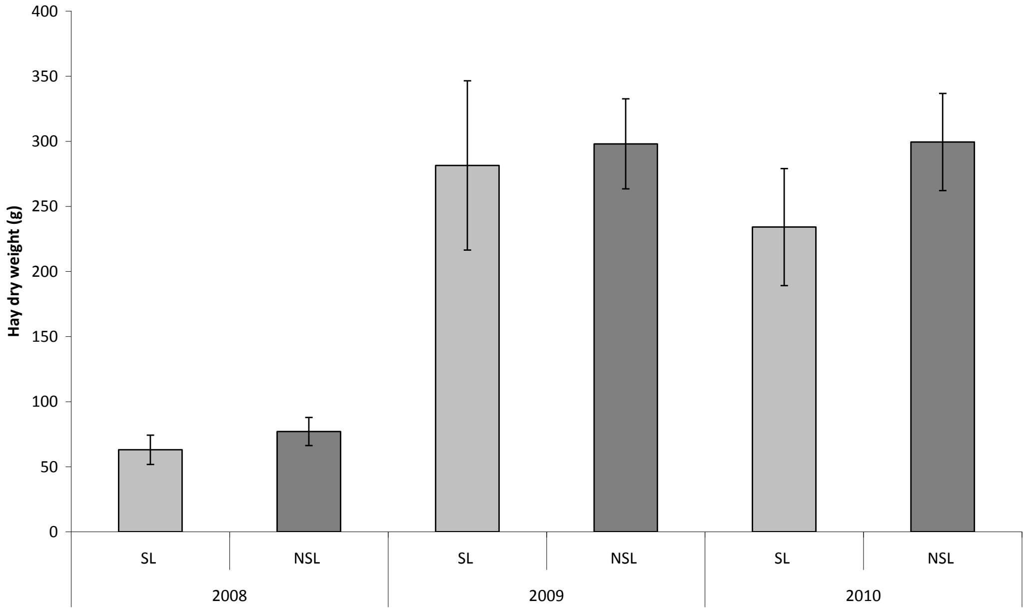
4.4.5 *Hay biomass*

Results of the GLM showed that hay biomass was not affected by slug treatment in either plant community (semi-improved: $F = 1.07$, $P = 0.311$; unimproved: $F = 1.10$, $P = 0.304$); however, time had a highly significant effect on biomass in both communities (semi-improved: $F = 24.99$, $P < 0.001$; unimproved: $F = 17.64$, $P < 0.001$). Hay biomass greatly increased between 2008 and 2009 in both communities, with little change in 2010 (Figure 4.12a, b). Visual interpretation of the data suggests that by the third year, hay biomass in the unimproved community was lower under low slug density conditions, and this reflected the increase in forb cover (predominantly *R. minor*) and parallel reduction in grass cover in comparison with SL plots. In terms of the semi-improved community, there was no indication that hay biomass was affected by slug treatment within years.

4.4.6 *Slug activity*

Slug activity is summarised as the mean number of slugs recorded beneath refuge traps throughout the experimental period. All slugs recorded within SL plots were removed at the time of recording. It should be born in mind that refuge traps are a relative method for assessing slug activity and are heavily dependent on weather conditions (seasonal and daily) and time of day, and are biased towards larger slugs and more surface-active species. This method therefore does not quantify slug density. Figure 4.13 a, b shows slug activity within SL plots (semi-improved and unimproved communities) to constantly track higher than activity levels within NSL plots. Peaks in activity represent introductions described in the method. Several slug species were present in the residual slug community. In addition to the introduced *D. reticulatum* individuals, *D. reticulatum*, *Arion hortensis*, *A. fasciatus* and *D. caruanae* were also recorded in SL and NSL plots.

a) semi-improved



b) unimproved

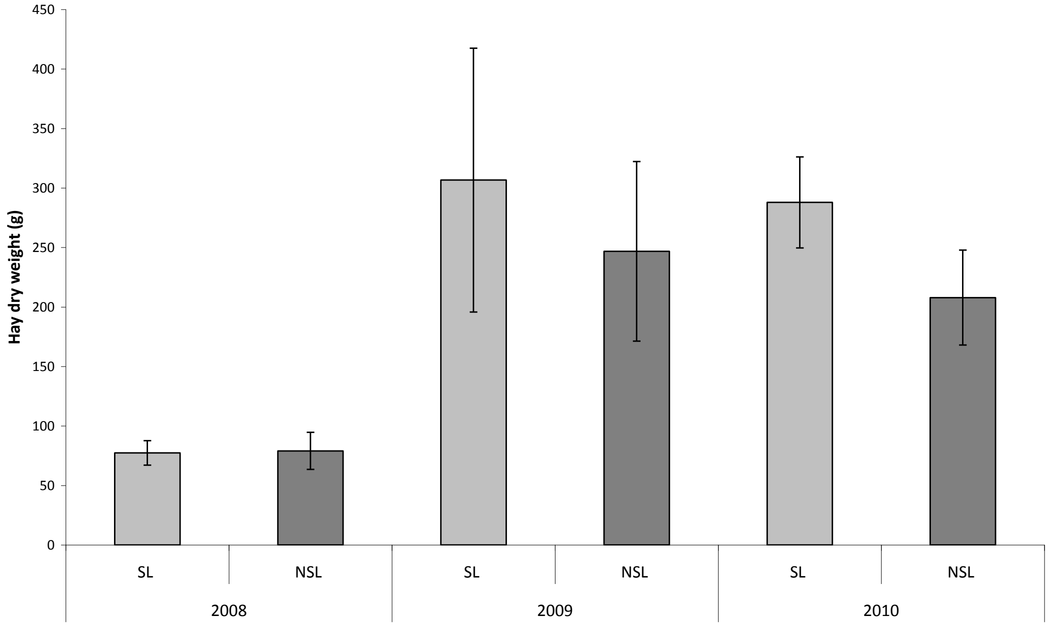
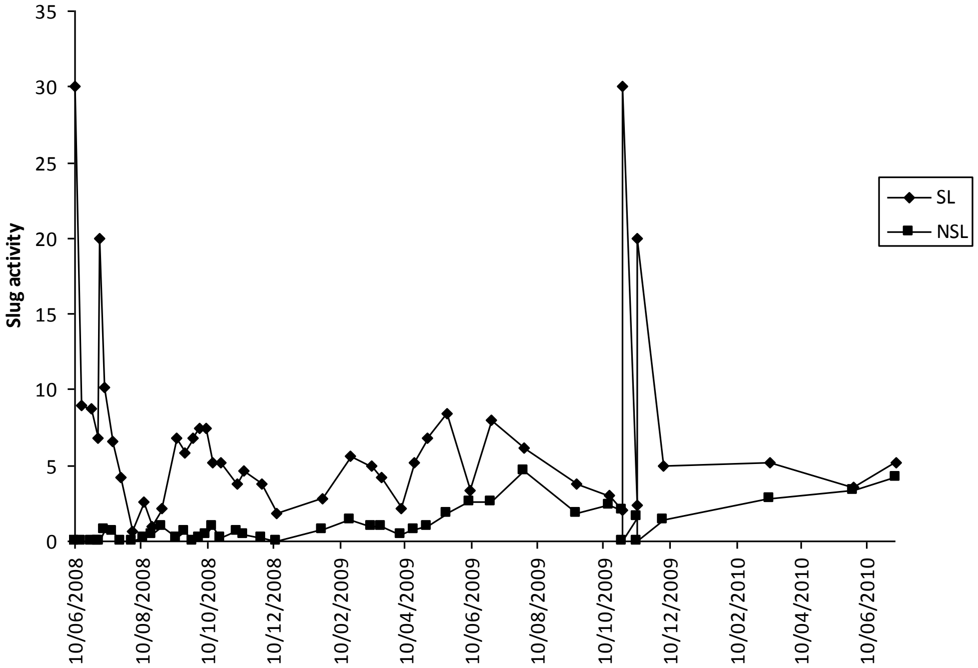


Figure 4.12. Mean (±SEM) hay biomass recorded within the a) semi-improved and b) unimproved plant communities subject to high or low slug densities over three years (n = 5 mesocosms per treatment group). Legend: SL, slugs added; NSL, no slugs added.

a) semi-improved community



b) unimproved community

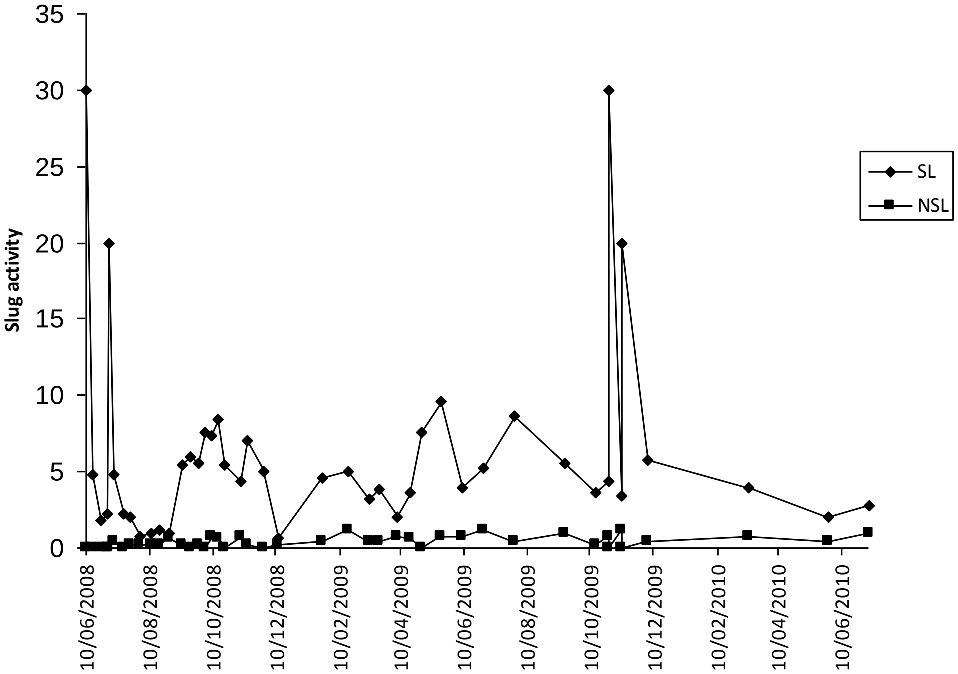


Figure 4.13. Mean slug activity within the a) semi-improved and b) unimproved plant communities containing high and low slug densities from June 2008 to July 2010. Slug activity is the number of slugs present beneath two upturned small plant pot saucers within experimental mesocosms (1 m^2) (n = 5 mesocosms per

treatment group). Slugs were introduced to SL plots on 10th June 2008 (30 m^{-2}), 2nd July 2008 (20 m^{-2}) and 27th October 2009 (50 m^{-2}). Legend: SL, slugs added; NSL, no slugs added.

4.5 Discussion

This study has identified the significant influence of slug herbivory on population and community dynamics in upland hay meadow plant communities. Multivariate results demonstrated several synonymous findings in each experimental plant community (semi-improved and unimproved). The development of community assemblages showed directional change with increasing age, developing from relatively low and open swards with some small gaps remaining in the first year, to tall and dense closed swards in the summer of the second year. Swards were also characterised by the decline in the number of pioneering annual species typical of early successional seres (Brown & Gange 1989; Wilby & Brown 2001). The size of slug population was confirmed as a highly significant factor in shaping community assemblages throughout the three year period of study for both the semi-improved and unimproved communities. Importantly, the interaction of slugs and year was also highly significant, signifying that the effect of slugs was inconsistent in each year (Anderson 1999) and was dependent community age.

For both community types, pair-wise PERMANOVA tests confirmed that slug treatment had an important impact on whole community assemblages in the first year, during the establishment phase when plants were present as seedlings or juveniles and the sward was relatively low and open. These findings are consistent with the general principle that slugs have the potential to cause the greatest ecological impact at this early stage of development (Fenner 1987; Hulme 1994; Hanley, Fenner & Edwards 1995a; Hanley, Fenner & Edwards 1996a; Hanley 1998; Strauss *et al.* 2009).

Despite these findings, there was little evidence to suggest that slugs fed preferentially on any functional group in the first year. Some studies have found slugs to show clear preferences for seedlings of acceptable forb species over grasses (Hanley, Fenner & Edwards 1996a; Wilby & Brown 2001; Hanley 2004;

Pywell *et al.* 2007; Strauss *et al.* 2009) and to reduce total plant cover in the first year (Buschmann *et al.* 2005). A possible explanation for the discordance between this study and others is the growth stage of plants when first subjected to slug treatment. In the studies cited above, slugs were present from seedling emergence; however, in this study slugs were first introduced seven weeks after sowing when many plants had already developed beyond the (early) seedling stage (Hanley *et al.* 2004) before slugs were introduced. Forb seedlings may be highly acceptable to molluscs if they lack the chemical or physical defences of their older conspecifics but often such defences are subject to positive ontogenetic effects (Fenner, Hanley & Lawrence 1999; Elger *et al.* 2009). For example, Horrill and Richards (1986) found that the slug *Arion hortensis* (Férrusac) did not discriminate between cyanogenic and acyanogenic phenotypes of *Trifolium repens* seedlings that were less than 5 days old, but did discriminate towards acyanogenic seedlings more than 15 days old. Furthermore, the likelihood of attack and survival is influenced by seedling age, size, growth rate and morphology and the identity of nearest neighbours (see Hanley 1998 for review). Hulme (1994) found that seedling exploitation by molluscs decreased with increasing seedling age. Hanley, Fenner and Edwards (1995b) demonstrated that the timing of slug grazing can determine the survivorship of a cohort of seedlings: Slugs attacked seedling monocultures of *T. officinale* less frequently with increasing seedling age, and younger seedlings of all species tested were more likely to suffer lethal damage than older conspecifics. However, slugs selectively grazed *T. officinale* seedlings regardless of seedling age when grown in mixed assemblages with a less acceptable species (*Senecio vulgaris*). Hence, there may be a short window of opportunity for invertebrate herbivores to affect forb seedling recruitment and functional groups. In addition, the assessment of abundance cover was performed approximately 3 months after sowing (8 weeks after the introduction of slugs) and it is possible that effects of treatment on functional groups or species may have diminished by this stage.

Given the lack of evidence that slug treatment affected functional groups in the first year, multivariate differences in location between treatment groups must have been driven by changes in the relative abundance of individual species but without affecting the cover of functional groups. Multivariate differences between community assemblages in 2008 became clearer with the aid of within-year biplots which enabled the response of individual species to slug treatment in the first year to be investigated. For each community, the main effect of slug treatment on community assemblages in 2008 was seemingly to alter the relative abundance of the most common grass species. Individual grass species could respond either positively or negatively to intensive herbivory (depending on the plant community) suggesting that the species which best avoided or tolerated grazing damage gained a selective competitive advantage and suppressed the cover of the main competitors. Results therefore indicate that the competitive relationship between common grass species is dynamic and susceptible to variation in slug grazing intensity during the establishment phase. Buckland and Grime (2000) also found slug (and aphid) herbivory to alter the relative abundance of some grass species, particularly at low soil fertility, when slugs (*D. reticulatum*) were introduced into mesocosm plant communities some time after establishment (April-June). In general, grasses are less acceptable than broad-leaved forbs due to the presence of silica phytoliths within their leaves (Grime, MacPherson-Stewart & Dearman 1968; Hanley 2004); but slugs can negatively influence grass abundance at the species-level. For example, Hanley, Fenner and Edwards (1995a) found populations of *A. capillaris* to be significantly higher in plots treated with molluscicidal pellets for several months following sowing in artificially created gaps in a grassland sward; yet in a similar experiment Hanley, Fenner and Edwards (1996a) found seedling recruitment of *A. capillaris* and *H. lanatus* to be unaffected by mollusc herbivory. Hulme (1996) found grass seedlings to suffer more severe slug feeding damage than forb seedlings but seedlings of most grass species demonstrated higher survival rates than forbs, particularly legumes, even when grazed down to ground-level, doubtless by virtue of their basal meristem (e.g. Dirzo & Harper 1980).

In both communities, *P. lanceolata* (a moderately abundant species) was largely unaffected by slug densities in 2008. This may have been due to the presence of iridoid glycosides in leaves reducing its acceptability to slugs (Bowers & Stamp 1993) and its rosette morphology conferring some protection against severe grazing damage (Hanley, Fenner & Edwards 1995b). However, the abundance of *P. lanceolata* was not substantially greater in high slug density conditions and was ultimately controlled by competition with more abundant grass species in both treatment groups.

In the semi-improved community, results indicated that high slug densities promoted the cover of *L. perenne* and reduced the cover of *T. repens* and the ability of *T. repens* to compete with *L. perenne*. Glen, Cuerden and Butler (1991) also found grazing by *D. reticulatum* to reduce the establishment of *T. repens* (of three cultivars) relative to *L. perenne*, and noted that *L. perenne* seedlings were able to recover from attack even when grazed to ground level whilst *T. repens* seedlings were often killed.

Slug foraging behaviour may have been influenced by the density (Root 1973; Cottam 1985) and distribution (Bergelson 1990) of the common grasses. During the early establishment phase, the fast-growing grass and legume species would have offered concentrated areas for feeding and also provided cover for slugs foraging on the predominantly bare soil surface. Shelter, provided by vegetation, is critical for slugs to reduce water loss and risk of predation (Port & Port 1986).

The 2008 biplots also showed that the majority of species were weakly correlated with slug treatment groups. This can be explained by the fact that most species, particularly forbs, were present at low abundance cover values and some were infrequent (i.e. did not occur in all replicates). Using abundance cover to assess differences between treatment groups may have disguised any effects of treatment on the scarce forb species and we recognise that recording the number of individual seedlings from emergence (in subsample plots) would have provided a more accurate indication of the effects of treatment on seedling recruitment.

In addition to the multivariate pair-wise differences evident in the first year for both communities, pair-wise PERMANOVA and PERMDISP results confirmed that the density of slugs caused significant variation in community assemblages throughout the study period, indicating that the effects of slugs extended beyond the first growing season. Multivariate results showed that the underlying effect of slug treatment on community assemblages in subsequent years differed according to the type of plant community and was dependent on the age of the sward (i.e.

year). Species-specific responses were also often unique to the type of plant community. Therefore, in order to effectively discuss the findings, each community is discussed separately.

SEMI-IMPROVED

Although PERMANOVA global and pair-wise test results confirmed that slug treatment significantly affected community assemblages throughout the 3-yr period of study, the difference between slug treatment groups was influenced by the greater dispersion between SL samples over the whole study period and in 2009. Dispersion within the SL group indicated that the effect of high slug densities on community assemblages was variable and not consistent across all samples. Despite PERMANOVA (within year) pair-wise results finding a significant difference between slug treatment groups in 2008, results did not show a significant difference in the second or third year indicating that treatment differences in multivariate location declined over time. The declining effect of slug treatment at the community level was supported by the absence of any treatment effects on the abundance cover or species richness of functional groups, species diversity, or hay biomass throughout the study.

Results for SI communities are similar to those of Hulme (1996) who found that although molluscs had a general negative effect on plant survival and above-ground biomass (10 months after sowing), the difference was not significant. Buschmann *et al.* (2005) also found total plant cover to be unaffected by the manipulation of slug grazing intensity after three years, despite a negative effect operating in the first year during the seedling establishment phase. However, Buschmann *et al.* (2005) did find slugs to increase diversity by the third year because slugs reduced the biomass of the two most abundant species (*L. perenne* and *T. repens*), allowing less competitive species to establish in grazed plots. Other studies have found slugs to cause persistent effects beyond the early establishment phase. Hanley, Fenner and Edwards (1995a) found that slugs had a negative effect on total cover over 8 months from germination and Wilby and Brown (2001) found molluscs to reduce seedling recruitment of forbs and promote the transition to a grass-dominated community within the first three years of secondary succession. Allan and Crawley (2011) found molluscs to have a negative effect on forb abundance up to 16 years after the start of the experiment, although an effect was not apparent until after 8 years. As Allan and Crawley (2011) rightly point out, differences between study findings are clearly in part attributable to compositional differences between study plant communities, and theory predicts that herbivores will have more effect where preferred food plants are more abundant (Root 1973). The effects of herbivory may also depend on the abundance of species adapted to tolerate grazing damage, and also the productivity of the plant community and importance of competitive interactions. Both Allen and Crawley (2011) and del-Val and Crawley (2005) demonstrate that the effects of mollusc herbivory are diminished in productive swards where forb abundance is constrained by competitive grass dominants.

Multivariate differences between slug treatment groups over all years were explained by the general positive response of *L. campestris*, and the negative response of *H. lanatus*, *A. capillaris* and *T. repens*, to high slug densities and indirect effects of herbivory on interspecific competition, although the effect of slug treatment on *L. campestris* was less marked in 2010 due to an increase in NSL plots. Slug treatment altered the relative abundance of species and inverse relationships between species associated with different treatment groups could be clearly identified; however, slug treatment had a more pronounced effect in some samples than others. This is consistent with numerous studies that have manipulated mollusc or insect grazing pressure (Wardle & Barker 1997; Buckland & Grime 2000; Hanley & Sykes 2009) and found herbivory to modify plant community structure by influencing the competitive balance between coexisting species.

The association of *L. campestris* with high slug density conditions during the latter stages of the study suggests that it is highly unacceptable to slugs and benefited from persistently large slug populations. It is likely that *L. campestris* was not only able to deter grazing by slugs but was also able to better capture and retain resources when competition with grasses was reduced by intensive slug grazing (Buckland & Grime 2000). There are no published data regarding its acceptability to molluscs, although *L. campestris* is most likely protected by its long leaf hairs and hard, toughened leaves and stem (i.e. sclerophylly). Leaves may also contain silica and chemical defences, as found in other members of the Juncaceae (Guang *et al.* 2007; Borrelli *et al.* 2011). By avoiding grazing damage *L. campestris* gained a selective advantage which was associated with some decline in cover of *H. lanatus*, *A. capillaris* and *T. repens*, within high slug density conditions (although *L. campestris* remained less abundant than these species throughout the study). Del-Val and Crawley (2005) report *L. campestris* biomass to be greater in grassland plots grazed by rabbits, although mollusc grazing had no effect; rather molluscs promoted the biomass of *A. odoratum* and suppressed *H. lanatus*.

Although *L. campestris* was a key driver of variation between treatment group community assemblages, the cover of *L. campestris* was <15% in all plots throughout the study and swards of both slug treatment groups were characterised by a few fast-growing perennial grass species and *T. repens*. The increasing similarity of community assemblages subject to large and small slug suggests that competition, rather than herbivory, was of overriding importance in determining community composition with increasing sward age and density (Wardle & Barker 1997; Schädler *et al.* 2003; Buschmann *et al.* 2005; Del-Val & Crawley 2005).

It is interesting that the grass species which came to dominate swards after three years occur in communities varying in soil fertility. In comparison, *L. perenne*, a species typical of high fertility swards, declined in cover over time. Despite being more abundant within SL plots in 2008, *L. perenne* failed to capitalise on its avoidance or tolerance of herbivory in the first year and declined over time to similar levels in both slug treatment groups (data not shown). Its decline in cover was therefore not influenced by slugs but was seemingly suppressed by the convergent increase in cover of competitor species (*H. lanatus*, *A. capillaris*, *T. repens*, *A. odoratum, C. cristatus* and *L. campestris*) and likely suffered reduced vigour under nutrient stress. This is similar to the findings of Buckland and Grime (2000) who reported that the contribution of *L. perenne* to mesocosm plots was reduced at low soil fertility (in the absence of herbivores), and slug (and aphids) herbivory to reduce the contribution of *L. perenne* (in all fertility treatments) and promote slower-growing grasses and relatively fast-growing, but unpalatable forbs.

The db-RDA analysis indicated that the majority of species were unaffected by slug treatment throughout the 3-year period. For most species this was due to their being either sparse (<3%), infrequent or transient within community assemblages. Although only weak correlations were evident it is noteworthy that species which may have benefited from high slug density conditions were those likely to be unacceptable to slugs as a consequence of physical or chemical defences (Duval 1971; Hanley *et al.* 2007).

In conclusion, the study shows that slug herbivory (under natural population densities) was an important biotic factor influencing plant community structure during the establishment phase but as plant cover increased, swards became dominated by competitive perennial species and competition was the main driver of community composition. This indicates that the effects of slug herbivory are reduced in communities dominated by competitive fast-growing grass species and competition is the overriding factor influencing the persistence of most forb species in semi-improved meadows. Effects of slug herbivory on plant community structure and development operated at the individual species level by causing changes in relative abundance of the common species through direct interactions and shifts in the competitive balance with other species, but did not affect functional groups. A key driver of changes in species composition was the promotion of a highly unacceptable species under high slug density conditions.

UNIMPROVED

Multivariate results showed that slug treatment had no effect on community assemblages in 2009, but in 2010 a significant difference in multivariate location was evident. This finding indicates that the significant effect of slug treatment on community assemblages in the first year (2008) did not cause a strong divergent pattern of community development by 2009. We hypothesise that the effects of slugs on community assemblages in the third year were primarily indirect, through the selective seedling removal of the keystone hemi-parasitic species, *Rhinanthus minor* by large slug populations during the previous year, and subsequent shifts in the relative abundance of functional groups and individual species. However, the outcome of the experiment renders it impossible to separate the effects of slugs from those of *R. minor*. A similar scenario was faced by Anderson and Underwood (1997) who found a significant effect of gastropod grazers on estuarine community assemblages and on individual species, but hypothesised that the effect of grazers was primarily indirect due to their removal of algal species. Anderson (1999) expanded the experiment to include an additional binary algal treatment, whereby algae were either left untreated or removed using a selective herbicide. Distance-based RDA was used to test for an interaction effect of grazers and algae and confirmed that the effect of grazers was primarily indirect via their removal of algal species and the subsequent effects on the recruitment and succession of other organisms. Anderson's (1999) experimental design could be extrapolated to this study in the future by adding a binary treatment involving the addition of *R. minor* seed and testing for a significant interaction between slugs and *Rhinanthus*.

The direct negative effect of large slug populations on R. minor seedling recruitment is an important finding and provides strong evidence that slugs are a major factor affecting *R. minor* seedling survival – a finding that has rarely been reported before (Westbury 2004). Indeed, van Hulst, Shipley and Theriault (1987) is the only study we are aware of that attempts to quantify *R. minor* seedling losses to slugs, reporting 47% of juvenile mortality to be attributable to herbivores, mainly slugs.

Slug herbivory occurred before the majority of *R. minor* seedlings had reached the 6-true leaf stage and prior to attachment with host plants (seedlings lacked visual symptoms of parasitism (S.E. Barlow, personal observation), indicating that *R. minor* seedlings are highly acceptable to slugs and their acceptability is not a product of parasitic attachment with host plants. The ability of seedlings to survive grazing damage pre-attachment is likely to be poor given their limited ability to acquire and assimilate inorganic compounds from the soil (Seel, Parsons & Press 1993). Slugs selectively grazed seedlings even when 'hidden' within a relatively species-rich (19 species m^{-2}) and dense sward. However, *R. minor* is likely to have been the most abundant forb species present in seedling form, as the majority of forbs did not flower in the previous year, suggesting that slug grazing behaviour may have been apostatic (Hanley, Fenner & Edwards 1996a).

The conversion rate for *R. minor* seed to seedling survival within the low slug density treatment was 51.3%, compared with just 1.3% within the SL treatment. Those seedlings that were recorded within SL plots showed visual signs of invertebrate grazing damage (most likely, slugs) and did not survive past mid-summer. The successful establishment of *R. minor* is known to be highly variable (Gibson 2000; Westbury 2004) and most likely site-specific (Westbury *et al.* 2006). Establishment is affected by vegetation density (Westbury *et al.* 2006) and accordingly, competition for light (Westbury 2004) and moisture (van Hulst, Shipley & Theriault 1987). Westbury *et al.* (2006) showed that scarification and gap creation increased survival rates of *R. minor* seedlings, although smaller gaps containing relatively greater seedling densities (1000 seeds m^{-2}) suffered greater mortality. Westbury *et al.* (2006) hypothesised that this may have been due to greater seed predation or fungal pathogens, or closer proximity of the surrounding vegetation, but seedling predation by slugs was not considered. This is a valid explanation as slugs aggregate feeding in small pockets of high seedling density (Bergelson 1990; Hanley, Fenner & Edwards 1996a). Westbury *et al.* (2006) suggested that sowing seed over a large area (rather than in small inoculation gaps) may have improved establishment rates. This strategy proved successful in the study by Pywell *et al.* (2004) which found persistent populations of *R. minor* to establish following sowing over areas of 100 m^{2} at significantly lower sowing densities (75 seeds m^{-2}). Parallels can also be drawn with the study by Hitchmough (2003) who found slug herbivory to be the critical factor determining the survival of *Trollius europaeus* L. seedlings (a species targeted in restoration) in gaps within wet meadow grassland.

The use of *R. minor* as a management tool in the restoration of species-rich grassland is well established (Smith *et al.* 1996b; Davies *et al.* 1997; Pywell *et al.* 2004; Westbury 2004; Westbury *et al.* 2006; Smith *et al.* 2008). *R. minor* operates as a keystone species in many grassland communities as populations may suppress the abundance of competitive grass species, and enable a wider diversity of less competitive forb species to develop. As such, the presence of *R.*

minor would be predicted to have a positive influence on forb richness and cover, and a negative influence on grass cover and total hay yield. These trends are visible in the results: The cover and richness of forbs was significantly positively affected by low slug densities throughout the 3-year period and by the third year forb cover was on average 23% greater in plots subject to small slug populations. The positive effect of low slug densities on forb cover was primarily driven by the increasing contribution of *R. minor*, although *T. pratense* also increased under these conditions. The finding that forb richness was significantly greater over the course of the study in association with *R. minor* (and low slug densities) supports those of Westbury *et al.* (2006) who found that forb species richness was higher in plots sown with *R. minor* in the second year following sowing. Pywell *et al.* (2004) also found the frequency of *R. minor* to be a strong determinant of forb number in the subsequent year.

Field studies investigating mollusc herbivory in grasslands, without the confounding influence of *R. minor*, have also found positive effects of mollusc control on forbs over the mid- (Wilby & Brown 2001) to long-term (Allen & Crawley 2011) and support those of del-Val and Crawley (2005) that the effects of mollusc exclusion are greater in swards that are not dominated by competitive grasses. Allan and Crawley (2011) found molluscs to feed preferentially on forb species and to have a compensatory effect with insects which reduced the abundance of the dominant grass species (*Holcus mollis*) in unfertilised MG5 grassland plots, highlighting the importance of interaction effects between herbivores. The same study corroborated our findings that molluscs (in the presence of insects) cause an increase in total biomass in a relatively species-rich sward by promoting the biomass of particular grass species; however, the effect of slugs on forbs and biomass were evident in our study in much shorter timescales. In contrast, Del-Val and Crawley (2005) found molluscs in combination with rabbits to reduce grass biomass over two years although individual grass species showed species-specific responses and could be promoted by herbivory. Results can also be compared with studies involving only insect herbivores, for example Brown and Gange (1989) found the addition of insecticide to result in an increase in species richness and forb richness, and the difference increased with successional age (3yrs). Wardle and Barker (1997) also report that while total biomass was unaffected by insecticide application, selective herbivory caused important shifts in community structure including a reduction in forb richness.

The study by Pywell *et al.* (2007) is particularly interesting and relevant as it investigates the effects of multiple treatments, including *Rhinanthus minor* and mollusc control, on the establishment of sown (and unsown) species over four years. High *Rhinanthus* frequency was associated with higher numbers and frequency of sown forbs and grasses and total species richness in the following year; whilst mollusc control significantly increased the number and abundance of sown forbs in either the first or second year depending on the field site. Furthermore, Pywell *et al.* (2007) detected significant negative effects of slug herbivory on forbs up to four years after sowing and found molluscs to have a greater impact on sward restoration than sheep grazing during this time.

The greater abundance of *A. capillaris* in high slug density plots throughout the study may have been a consequence of seedlings (or juveniles) benefiting from high slug density conditions in the first year, and gaining an early competitive advantage over other grass species. However, it is also likely that *A. capillaris* benefitted from the absence of *R. minor* within SL plots. The opposing distributions of *A. capillaris* and *R. minor* visible in the biplot indicated that *Agrostis* was suppressed by the presence of *R. minor* within low slug density plots, either as a result of parasitism or competition. In contrast, *C. cristatus* was apparently unable to capitalise on the positive effect of large slug populations in the first year. The abundance cover of *C. cristatus* and *H. lanatus* was greater in association with consistently low slug densities but was also inversely related to *R. minor*, indicating that *R. minor* had an important negative effect (via parasitism or competition) on these species within the NSL treatment group. *R. minor* is known to selectively parasitize grass and legume species over non-leguminous forbs due to their poor ability to resist attack (Cameron & Seel 2007; Rumer *et al.* 2007; Jiang *et al.* 2008). In this study there was no evidence of an inverse relationship with *T. pratense* and results support those of Davies *et al.* (1997) who found the cover of legumes to increase in association with *Rhinanthus* spp. This may be due to the ability of legumes to compensate for parasitism due to their symbiotic relationship with nitrogen-fixing bacteria and the advantage gained through reduced competition with grasses. Davies *et al.* (1997) also report an increase in the proportion of dicotyledons coupled with a decrease in the proportion of grasses in the presence of *Rhinanthus spp*. The decline in grass cover within low slug density plots in the third year indicates an emergent trend in the suppression of grass cover by *R. minor* as an indirect consequence of low slug densities. It is also hypothesised that a concomitant increase in forb cover (of species other than *R. minor*) and richness will occur in subsequent years in association with *R. minor* and low slug densities, in accordance with the findings of Smith *et al.* (2003). These predicted changes are also likely to result in higher species diversity relative to plots with high slug densities and without *R. minor*. Some studies report a negative effect of *R. minor* on species diversity when competitively subordinate species are the main hosts in a community (Gibson & Watkinson 1992) or by promoting non-host species such as *P. lanceolata* (Cameron & Seel 2003), but we found no evidence of this.

The response of *R. minor* to slug treatment also influenced the finding that slugs and the interaction of slugs and time had a significant effect on annuals. The declining contribution of annual species was evident over the three year period in both treatment groups, with only *R. minor* remaining in the third year in NSL plots. Buschmann *et al.* (2005) also found slugs to reduce the cover of annuals (but not the number of species). The persistence of *R. minor* at this age of the experimental plant community would be expected as it is reported to be a good invader in unproductive swards where other annuals are rare or absent (van Hulst, Shipley & Theriault 1987); however, results showed that selective grazing by large slug densities prevented populations from establishing in experimental mesocosms.

The selective grazing of *R. minor* by slugs and the potential indirect effects on community composition has not been reported before and is of direct interest to grassland restoration management. The successful early establishment of *R. minor* and species with high mycorrhizal associations, such as *T. pratense*, is a critical early milestone in the long-term (>20 years) restoration process (Smith *et al.* 2003; Bardgett *et al.* 2006; Smith *et al.* 2008). Clearly, selective grazing by slugs (particularly in sites with large slug densities) may significantly limit *R. minor* seedling recruitment and slow down the rate of succession in grassland restoration communities.

In conclusion, the results have highlighted the importance of slug grazing on the recruitment and establishment of R*. minor* populations and the likely indirect negative consequences for forb species composition and species diversity within swards where the aim is to increase conservation value. The direct and indirect effects of slug herbivory and *R. minor* on whole community assemblages cannot be distinguished in this study but it is likely that they are interactive. The study also supports the hypothesis that the effects of slug herbivory are more pronounced in swards with greater forb cover and where the importance of competition from grass species is reduced.

Chapter 5. Foliar trichomes of *Rhinanthus minor*: an adaptation for herbivore defence?

5.1 Abstract

Plant trichomes are either glandular or non-glandular and occur in a wide array of morphological types. For many species, trichomes are known to function as physical or chemical defences against herbivores; however, trichomes have been shown to provide other functions including reducing leaf temperature and water loss, climbing and fruit dispersal. Some plant families, such as Solanaceae and Lamiaceae, have received more attention than others and there remains a multitude of species yet to be described. Hemi-parasites present an interesting group from a plant-herbivore co-evolutionary perspective as they are poorly adapted to survive the loss of leaf tissue during the pre-attachment phase of growth and may invest heavily in trichome defences. The leaf surface morphology of *Rhinanthus minor* (Orobanchaceae), a hemi-parasitic herb, was investigated using scanning electron microscopy and energy-dispersive X-ray spectroscopy. Glandular and nonglandular trichomes were identified on the adaxial (upper) surface of mature and seedling leaves and the abaxial (lower) surface of seedling leaves. Silicon was the predominant element within nonglandular trichomes. Our results provide the most detailed imagery and descriptions to date of the leaf trichomes of *R. minor*. The function of each type of trichome identified is not clear, although the abundance and variety of trichomes present on the leaf surface suggest that they may function in physical and chemical defence against herbivores. It is also possible that some glandular trichomes may function in the effective supply of nutrients from the host to the parasite – a phenomenon termed active guttation. Further investigation of trichome function, particularly the potential anti-herbivore defensive role, is an intriguing direction for further study.

Key-words: active guttation, energy-dispersive X-ray (EDX) spectroscopy, hemi-parasite, herbivory, plant defences, plant-herbivore interactions, scanning electron microscopy, silicon

5.2 Introduction

Plant trichomes are hair-like appendages extending from specialised epidermal cells and may be located on the aerial surface of vegetative or reproductive organs (Levin 1973). They occur in almost all plant groups and are present in most angiosperms (Jeffree 1986). Trichomes may be either glandular or nonglandular and occur in a diverse array of morphological forms. They may vary in density and distribution on different plant parts and are useful identification features for taxonomic classification (Edmonds 1982). The genus *Lycopersicon* (tomatoes) has been particularly well studied in this regard with trichome type and distribution used in the classification of wild and cultivated hybrid species (Luckwill 1943). Nonglandular trichomes vary in shape (simple, branched, stellate, dendritic, hooked, spiral, etc), texture (smooth, verrucose), alignment (erect, procumbent, etc), number of cells and size. Glandular trichomes also show considerable morphological variation, but can be broadly classified into two types: peltate trichomes which have a short stalk that is sunk into the epidermis and a large globular head; and capitate trichomes which have a stalk of variable length and a small globular head. Glandular trichomes contain mainly secondary metabolites or essential oils and have received much attention for their wide-ranging commercial uses in the pesticide, pharmaceutical, cosmetic and fragrance and flavour industries. For example, the antimalarial drug artemisinin, a sesqueterpernoid lactone, is produced by the glandular trichomes of *Artemisia annua* L. (Klayman 1985). Various widely used pesticides are the result of glandular trichome exudates, such as the pyrethroids of *Chrysanthemum* species (Casida 1980) and the neo-nicotinoids derived from *Nicotiana* species (tobacco) (Thurston, Smith & Cooper 1966; Tomizawa & Casida 2009). The Lamiaceae are particularly rich in essential oils of commercial value (Werker 1993; Ascensão, Marques & Pais 1995; Ascensão & Pais 1998) such as those produced by various *Mentha* and *Salvia* species (Baran, Ozdemir & Aktas 2010). For some species, different types of glandular trichome on individual plants are known to secrete different products (Ascensao, Marques & Pais 1997; Ascensão & Pais 1998) and may also differ in terms of the mode and timing of secretion (Werker 1993). A number of studies have included histochemical testing of trichome secretory products (e.g. Ascensao *et al.* 1998; Ascensão, Mota & Castro 1999; Sacchetti *et al.* 1999; Grassi *et al.*

2004; Marin *et al.* 2010). Various compounds have been identified including terpenes (Wagner 1991; Ascensao, Marques & Pais 1997; Sacchetti *et al.* 1999), phenols and, to a lesser extent, alkaloids as well as other substances such as polysaccharides and proteins. Some studies have attempted to determine the biosynthetic and secretory pathways (Gershenzon, Maffei & Croteau 1989; Gershenzon *et al.* 1992; Duke 1994; Ascensao, Marques & Pais 1997; Ascensão & Pais 1998; Gang *et al.* 2001; Schilmiller, Last & Pichersky 2008).

Trichomes are known to have several functions. Some studies have reported the physiological significance of trichomes in acting as an evapotranspirative barrier against water loss, reducing leaf temperature and increasing light reflectance (Woodman & Fernandes 1991; Fahn & Shimony 1996). Trichomes may also reduce the absorption of harmful UV radiation and also conserve heat during the night (Jeffree 1986). However, there are examples of species from xerophytic climes that are densely hairy only on the abaxial (lower) side. Hooked or recurved trichomes are also important in climbing and anchoring (e.g. *Phaseolus* species), or may facilitate the dispersal of fruit and seeds by animals, such as those possessed by *Galium aparine* L..

Despite the multifunctional role of trichomes, their primary function is likely to be as defence mechanisms that have evolved out of the complex relationship between plants and animals. Duke (1994) considers the concomitant evolution of chemical defences and their sequestration and secretion by glandular trichomes to be the result of interacting selection pressures, most likely insects and other herbivores or pathogens and competitor plant species (i.e. allelopathy) and the need to avoid autotoxicity. Although the literature concerning the anti-pathogen role of trichomes is less extensive than that concerning an anti-herbivore role, some studies provide experimental evidence of an antibacterial (e.g. Ascensao *et al.* 1998; Williams *et al.* 2003) and antifungal effect (e.g. Valkama *et al.* 2005). However there are also examples of a pathogen facilitating effect (Calo *et al.* 2006).

Many studies have considered the physical and chemical defensive properties of trichomes for use against herbivores, particularly phytophagous invertebrates (Levin 1973; Simmons & Gurr 2005). The majority of previous studies have focused on pest resistance in agronomically important crop plants. Consequently, the defensive role of trichomes of members of the Solanaceae, in particular, species of *Lycopersicon* and *Solanum* (potato), against a wide variety of pest species, have been intensely studied since the 1970^{s} (Duffey, Juniper & Southwood 1986; Gregory *et al.* 1986; Wagner 1991; Simmons & Gurr 2005; Kang *et al.* 2010). The aim of this research was (and is) to develop highly resistant varieties via selective breeding and genetic modification. Current research operates at the genome-scale and is led by the Solanum Trichome Project (Michigan State University 2012), which focuses on the identification of genes that control the development and biochemical function of glandular trichomes (McDowell *et al.* 2011). This research has also produced the “omics” database TrichOME, to facilitate the study of plant trichomes (Dai *et al.* 2010).

Experimental and correlative evidence have linked increasing pubescence with pest resistance, in terms of reduced infestation (Brown & Simmonds 2006), oviposition (Handley, Ekbom & Ågren 2005) and feeding damage, increased larval and adult mortality, and with limiting larval development (Agrawal 1999; Pelletier, Grondin & Maltais 1999; Malakar & Tingey 2000) (for examples of trichome-based resistance of *Lycopersicon* species to arthropods see the review by Simmons and Gurr (2005)). Most of these studies have concentrated on insects and there is a paucity of information in regard to molluscs (Westerbergh & Nyberg 1995; Tuberville, Dudley & Pollard 1996; Puustinen, Koskela & Mutikainen 2004).

Nonglandular trichomes may provide a physical barrier or deterrent against herbivores. They may hook or impale insects, particularly young larvae, leading to death via exhaustion, starvation or loss of haemolymph, as in the case of Heliconiine butterfly larvae on the leaves of *Passiflora adenopoda* DC. (Gilbert 1971) and aphids on *Phaseolus vulgaris* L. (Johnson 1953). Furthermore, they may reduce suitable oviposition sites, increase the susceptibility of eggs to desiccation, provide obstructions to feeding (Wilkens *et al.* 1996) and if consumed offer little nutrititive value. However, there are numerous studies providing contrasting examples of insects that are well adapted to life on hirsute or pubescent plants (Southwood 1986; Simmons & Gurr 2005 and references therein).

The physical or mechanical defensive properties of trichomes may also be influenced by the accumulation of silicon. Some studies report the accumulation of silicon in and around trichomes on leaves (Lanning & Eleuterius 1985; Lanning & Eleuterius 1987; Lanning & Eleuterius 1989; Cherif *et al.* 1992; Frantz *et al.* 2008) and reproductive organs (Hayward & Parry 1973; Sangster *et al.* 1983). These studies have mainly focused on grass species, although Frantz *et al.* (2008) report the accumulation of silicon in leaf trichomes for several non-graminaeae species. Silicon has multiple biochemical and physical functions including the capacity to reduce many abiotic pressures, such as water stress and heavy metal toxicity, and is also an effective constitutive and inducible herbivore defence (Epstein 1999; Ma 2003; Epstein 2009; Cooke & Leishman 2011). Silicon in grass leaves has been shown to act as an inducible defence against some vertebrate herbivores, such as voles (Massey & Hartley 2006; Massey, Ennos & Hartley 2007) but is more widely effective against invertebrate herbivores causing reduced leaf consumption and digestion (nitrogen absorption) and negative effects on herbivore growth and development (Massey, Ennos & Hartley 2006; Massey & Hartley 2009). Furthermore, Cooke and Leishman (2011) provide evidence of a negative correlation between silicon concentration and leaf longevity measured as a binary trait (annual vs perennial) for 672 species within a wide range of plant groups and orders. This relationship was particularly strong for leaves of deciduous dicotyledons. The authors suggest that silicon may contribute to a more favourable carbon strategy in leaves with shorter life spans in several ways including providing protection from herbivores in place of costly phenolic and other carbon-based anti-herbivore compounds.

The biological activity of glandular trichome secretory products may have numerous effects. They may be toxic to herbivores, such as the alkaloids produced by *Nicotiana* trichomes that are toxic to aphids (Thurston, Smith & Cooper 1966); or repellent such as volatiles released by some glandular trichomes of Lycopersicon that repel whitefly (Muigai *et al.* 2002) and spidermites (Zhang, Thacker & Snyder 2008), and aphid repellent sesquiterpenes released by glandular trichomes of *Solanum* species (Ave, Gregory & Tingey 1987). Glandular secretions may also cause behaviour-modifying effects or serve as feeding deterrents to invertebrate herbivores. In addition to their biological effects, the secretions may have mechanical effects. Some trichomes operate to trap pests in sticky secretions rendering them immobile and may also block insect spiracles and “clog-up” mouthparts (Southwood 1986). Some *Solanum* species produce (following oxidative polymerization) phenolic quinones that stick insects to the leaf surface or render their mouthparts unusable (Kowalski, Tingey & Steffens 1990). Some carnivorous plants, such as *Drosera* and *Pinguicula* species, have also evolved to trap pests in sticky secretions, although these plants have gone a step further by using the trapped insect as a nutrient source (Juniper 1986; Alcalá *et al.* 2010). It is noteworthy that other carnivorous species that employ alternative methods of entrapment (i.e. trigger, pitcher, bladder or lobster-pot) also rely, to some extent, on the adaptive nature of trichomes.

Trichome development on very young leaves has been reported by several authors (Seithe 1979; Werker & Fahn 1981; Duke & Paul 1993; Bourett *et al.* 1994; Kobayashi *et al.* 2008) and may support the optimal defence theory (Feeny 1976) if younger, more valuable leaves are both more defended and less frequently attacked than older leaves. Franceschi and Giaquinta (1983) noted the decline in density of glandular trichomes on mature leaves of *Glycine max* L. (Merr.) (soybean) in comparison with very young leaves and suggested this to be a consequence of the greater susceptibility of young developing leaves (or whole plants) to foraging insects. Evidence of age-specific defence was reported by Woodman and Fernandes (1991) who found young, more pubescent leaves of *Verbascum thapsus* L. (non-parasitic Scrophulariaceae) to be attacked less frequently by insect herbivores than older, less pubescent leaves. In addition, some studies have shown an increase in leaf trichome density to be an inducible response to herbivore damage (Dahlin, Brick & Ogg 1992; Agrawal 1999; Bjorkman, Dalin & Ahrne 2008).

The species of interest in this study is the facultative root-hemiparasite *Rhinanthus minor* L. (Yellow rattle), belonging to the Orobanchaceae family of parasitic plants and formerly included in the Scrophulariaceae (Figure 5.1). *R. minor* parasitizes a wide range of hosts, particularly grasses and legumes, by forming haustorial connections via the root system that enable the transfer of water and solutes from the host (Cameron & Seel 2007). It is associated with low to medium fertility grasslands and is distributed throughout Britain, North America, Asia and most of

Europe (Westbury 2004). *R. minor* is used as a management tool in the restoration of species-rich grasslands (Pywell *et al.* 2004) where it is recognised as a keystone species for its role as a driver of community-level competitive interactions (Bardgett *et al.* 2006; Smith *et al.* 2008).

Figure 5.1. *Rhinanthus minor*

The trichomes of members of the Orobanchaceae and Scrophulariaceae have received some attention for their use in taxonomy (Barthlott 1980; Raman 1986; Kaplan & Inceoglu 2003). Neumann *et al.* (1997) described the trichomes and their distribution for several genera of parasitic Orobanchaceae (formerly Scrophulariaceae), including *Rhinanthus*, for primarily taxonomic purposes; all species possessed glandular and nonglandular trichomes of varying morphological types, some of which were unique to particular plant organs and specific genera or species. Neumann *et al.* (1997) reported a scutiform (shield-shaped) glandular trichome with a four-celled head on the abaxial surface of leaves of *R. minor* but do not provide images. The only study to examine and provide images of *R. minor* trichomes is that of Ponzi and Pizzolongo (1992), but depicts only the abaxial leaf surface. Glandular trichomes with a four-celled head were found to occur in association with the minor veins on the abaxial leaf surface and were interpreted by the authors as hydathodes involved in active guttation (i.e excreting water and solutes from the xylem and acting as a pump to draw up solutes from host xylem), although the excretory products were not identified. A previous study by Govier, Brown and Pate (1968) used radioisotopic labelling to demonstrate this phenomenon for the root-hemiparasitic herb, *Odontites verna* Dumortier.

No studies have conducted a histochemical analysis on the trichome secretions of *Rhinanthus* species, although tests on other parasitic members of the Orobanchaceae have proved positive for terpenes and flavonoids (Sacchetti *et al.* 2003). There are no studies that we are aware of that have experimentally tested the resistance of parasitic plants within the Orobanchaceae to invertebrate herbivores, although Kobayashi *et al.* (2008) describe the trichomes of *Paulownia tomentosa* (Thunb.) Steud. (non-parasitic Scrophulariaceae) in relation to their role in herbivore defence; and Woodman and Fernandes (1991) provide evidence of the dual function of leaf hairs in herbivore (insect) defence and reducing water loss for *V. thapsus*.

The work reported here is part of a larger study of the role of invertebrate herbivores in structuring upland hay meadow communities in which *R. minor* is a keystone species. We wished to investigate the plant-herbivore interactions of *R. minor* by incorporating research on the physical and chemical properties of the leaf epidermis. The foliar morphology of *R. minor* was of interest because the study presented in Chapter 4 showed seedlings to be selectively grazed by slugs. Furthermore, an additional mesocosm study intended to investigate grazing of *R. minor* in more depth was abandoned because slugs did not feed on young plants that had already formed parasitic attachments. This, in itself, was highly interesting and led to the hypothesis that *R. minor* may undergo ontogenetic changes from the early seedling stage which cause it to be well defended (i.e. unacceptable) against slugs beyond the early seedling stage. The aim of this study was to investigate the epidermis of *R. minor* seedling and mature leaves using Scanning Electron Microscopy (SEM). In doing so, we provide morphological descriptions of the foliar trichomes of seedling and mature *R. minor* plants, using scanning electron micrographs, and relate the findings to their possible functional roles. A further aim was, for the first time, to analyse the elemental composition of the leaf surface of *R. minor* using energy-dispersive X-ray (EDX) spectroscopy. Within this aim, it was predicted that silicon would be a predominant element.

5.3 Method

5.3.1 *Plant material*

The leaves of mature (flowering) *Rhinanthus minor* plants were collected in July 2010. Plants had been grown at Close House Biology Field Station, Newcastle upon Tyne (NZ128659) from seed collected from a species-rich upland hay meadow (Askrigg Bottoms SSSI) in the Yorkshire Dales, UK, during the previous summer. A single leaf from six plants was taken and a section cut from the centre of each leaf (approximately 1 cm^2) including the leaf edges.

To establish whether foliar morphology differed between mature and immature plants the leaves of seedlings at various developmental stages were collected in January and February 2011, following germination in December 2010. Seed had been collected from a species-rich upland hay meadow (Barrowburn SSSI) in Northumberland, UK, in July 2010, sown in seed trays and stored in a chilling cabinet at 4^{o}C. Upon germination trays were removed to a heated glasshouse with artificial light. Whole leaves were taken from seedlings possessing their first, second and third pair of true leaves at approximately weekly intervals between 14th January and 6th February (Table 5.1).

A single leaf from each pair of leaves was taken at each developmental stage and three replicate seedlings were used on each sampling occasion to give a total of 27 leaf samples.

Table 5.1. Leaf sampling methodology for *Rhinanthus minor* seedlings.

Sampling date	Seedling developmental stage	Leaf samples (n=3)
14/01/11	First pair of true-leaves developed	One leaf from first true pair of leaves
20/01/11	Second pair of true leaves beginning to develop	One leaf taken from the first and second pair of true leaves
31/01/11	Third pair of true leaves beginning to develop	One leaf taken from the first, second and third pair of true leaves
06/02/11	Third pair of true leaves fully developed	One leaf taken from the first, second and third pair of true leaves

5.3.2 *Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray (EDX) Spectroscopy*

Three mature leaf samples and all seedling leaves were fixed in 2% Gluteraldehyde in Sorensons Phosphate buffer for a minimum of 24 hours. Samples were rinsed twice in Sorensons Phosphate Buffer for 15 minutes before being dehydrated in a graded series of ethanol (25% for 30 min, 50% for 30 min, 75% for 30 min, 2 X 100% for 1 hr). This was followed by critical point drying using a Baltec Critical Point Dryer. All mature and seedling leaf samples were mounted on aluminium stubs with carbon discs and coated with 15 nm of gold using a Polaron SEM Coating Unit. The adaxial (upper) surface of mature and seedling leaves and the abaxial (lower) surface of some seedling leaves were observed using a Cambridge Stereoscan 240 scanning electron microscope with digital image collection. Mature leaves were viewed in July 2010 and seedling leaves in March 2011.

For analysis with SEM-EDX, three mature leaf samples were used that had been stored in 100% ethanol for two months. A Hitachi S2400 scanning electron microscope fitted with EDX light element analyser was used to detect and quantify elements. Only the adaxial surface of mature leaf samples was analysed. Seedling leaves were not analysed by SEM-EDX.

5.4 Results

Scanning electron micrographs showed the surface of mature and seedling leaves to be characterised by several morphological types of glandular and nonglandular trichomes.

5.4.1 *Mature leaves*

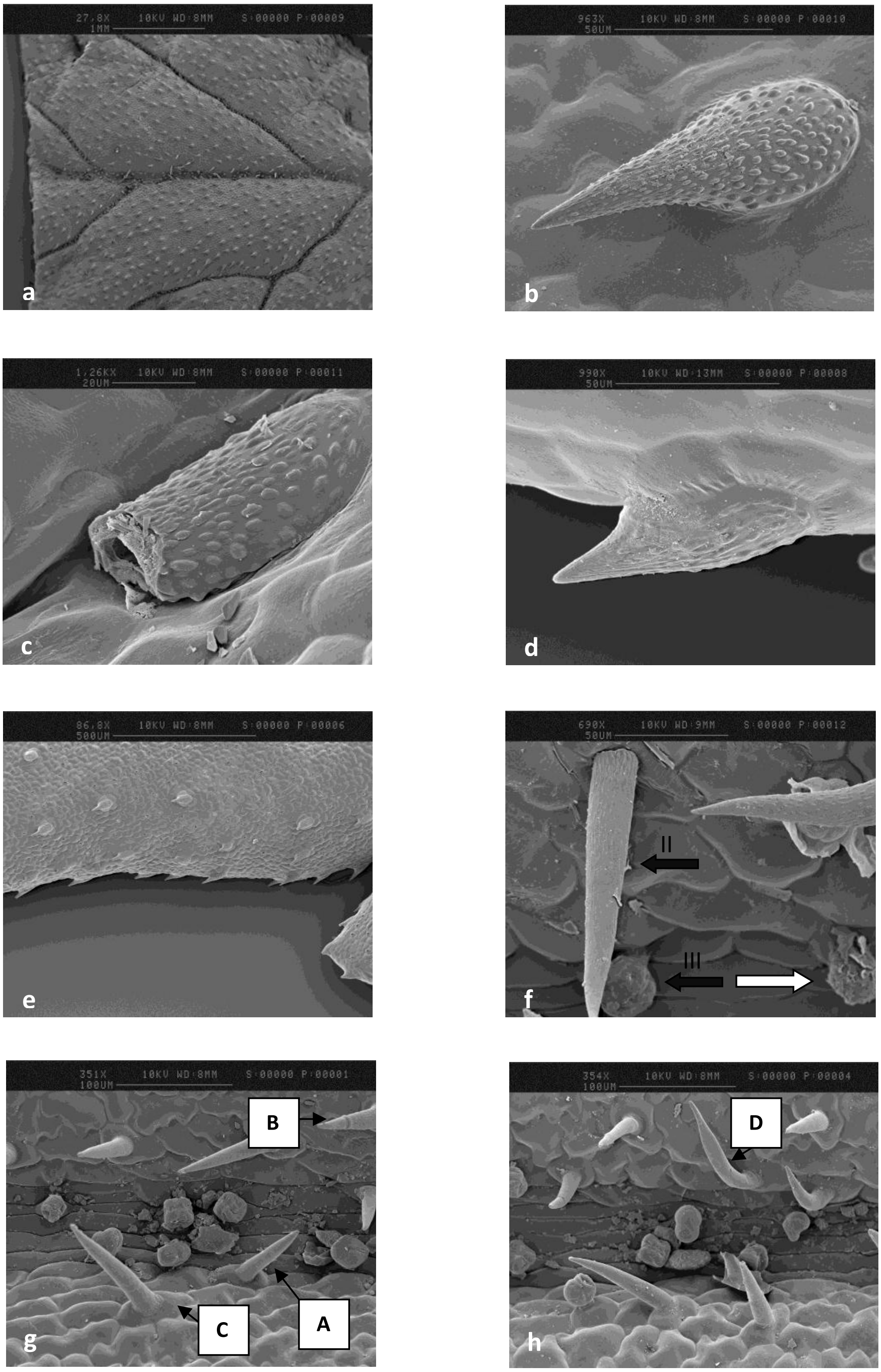
Three different morphological types of trichome were identified on the adaxial surface of mature leaves (Figure 5.2a-n). Type I are nonglandular with a multicellular foot and a single body cell consisting of a bulbous base and short hooked point (approximately 100 µm from base to tip) and are covered in cuticular warts (i.e. verrucose surface) (Figure 5.2b). Figure 5.1c shows the hollow structure of a broken Type I trichome. These trichomes are abundant on the epidermal surface, interspersed between stomata, but are absent from the midrib and pinnate network of lateral major veins (Figure 5.2a). All Type I trichomes are orientated in parallel with the leaf surface and point towards the leaf tip (apex) or edge (i.e away from the midvein). Type I trichomes are also present at the leaf edge but have a shorter point and are less verrucose than those on the main epidermal surface (Figure 5.2d, e). A count of Type I trichomes in a similar area of the epidermis of three leaves found there to be 33.3±17.5 (SD) mm^{-2}. Type II are also nonglandular but have a single foot cell and a predominantly unicellular body that is thin, straight, smooth and pointed (approximately ≤100 µm in length) (Figure 5.2f). However, body cells were variable and could be hooked (Figure 5.2h, j), bicellular (Figure 5.2g, k), or verrucose (to a greater or lesser extent) (Figure 5.2f-l). These trichomes are concentrated at the perimeter of the midrib and major veins (Figure 5.2a, g, h). Type III are capitate glandular trichomes with a large globular secretory head and short unicellular stalk and a single foot cell (Figure 5.2m, n). The glandular heads appeared to be predominantly bi-cellular although some were unicellular. The arrow depicted in Figure 5.2f points to a ruptured Type III trichome. These trichomes are abundant in the sunken midrib and major veins and are also present at the perimeter of these veins (Figure 5.2a, g, h). A count of Type II and III trichomes within a 1 mm section of the midvein of three mature leaves showed there to be approximately 13.7±5.9 (SD) mm^{-1} and 23.3±2.9 (SD) mm^{-1}, respectively.

SEM-EDX analysis revealed the elemental composition (measured in counts per second) of Type I, II and III trichomes and the leaf epidermis (non-vein and vein area) (Figure 5.3a-f). Silicon was the predominant element for all point samples, except for Type III trichomes, although silicon content varied depending on point location and was approximately twice as abundant in Type I trichomes as in Type II (Figure 5.4); O and C were also prominent and various other elements were detected in lesser amounts. A similar elemental composition was evident for Type I trichomes on the main epidermal surface (Figure 5.3a) and on the leaf edge (Figure 5.3b), and the epidermal surface between these trichomes (Figure 5.3e), with peaks for Si at 45 cps, 37 cps and 47 cps, respectively. The second most important element was O (20 cps, 26 cps, 17 cps) followed by C (6 cps, 11 cps, 4 cps) and Ca, Cl and K occurred in minor amounts. Mg was also detectable, although was not detected in the Type I trichome at the leaf edge. The Type II trichome was predominantly composed of Si and O, with peaks at 20 cps and 14 cps, respectively (Figure 5.3c). Other detectable elements were C, Ca, Mg, Mn, K, Cl, S and P, all with peaks ≤5 cps. The Type III trichome was predominantly composed of C (10.5 cps) and O (7 cps), where as Si was much less prominent in comparison with Type I and II trichomes, with a peak at 4.5 cps (Figure 5.3d). Other less important elements were K, Al, P, Ca, S and Fe, all with peaks ≤3 cps. The epidermis covering the midrib (between Type II and III trichomes) also generated low counts for all elements (Figure 5.3f). The main elements were Si (6.75 cps), O (4.75 cps) and Al (4.75 cps), with C, K, P, S, Ca and Fe also detectable (≤2 cps). The concentration of Si for all point samples is summarised in Figure 5.4.

5.4.2 *Seedling leaves*

Figure 5.5a-g shows the adaxial leaf surface of seedlings possessing their first pair of true leaves. Type I, II and III trichomes were identified on the adaxial leaf surface of seedlings (Figure 5.5a-d) as described for mature leaves. In comparison with mature leaves, Type I trichomes exhibited more pronounced multiple foot cells forming a raised pedestal for the body cell (Figure 5.5f). Furthermore, Type I and Type II trichomes were typically longer than found on mature leaves (Type I: mature, approximately 100 µm; seedling, approximately 135 µm; Type II: mature, approximately ≤100 µm; seedling, approximately ≥120 µm). Type I trichomes were similarly abundant on the epidermal surface (approximately 26 mm^{-2}) as for mature leaves, but Type II trichomes appeared to be less abundant (approximately 9 mm^{-1} section of midrib) in association with the midrib and major veins. Type III trichomes were also less abundant in the midrib (approximately 15 mm^{-1}) but not in the major veins (approximately 23 mm^{-1}) in comparison with mature leaves. The glandular head was visible in single (Figure 5.5d) and bicellular (Figure 5.5e) forms and many were noticeably ruptured (Figure 5.5e). Type II and III trichomes most likely increase in abundance as the young leaves continue to expand. In contrast to mature leaves a second type of glandular trichome was also identified: Type IV are capitate and uniseriate with a single foot cell and smooth multicellular stalk consisting of a long conical basal cell, 1-2 intermediary cells, a short subapical (neck) cell and a small unicellular glandular head at the apex (tip) (approximately 75-125µm in length) (Figure 5.5g). Their distribution pattern is similar to Type I but they are sparsely distributed on the epidermal surface (Figure 5.5a) (approximately 8 mm^{-2}) and more concentrated at the leaf edge (Figure 5.5f) and close to the midrib (Figure 5.5g). Leaf samples from seedlings possessing their second and third pair of true leaves showed similar findings (images not shown except for oldest leaf of a seedling possessing three pairs of true leaves) (Figure 5.6a).

The oldest leaf of seedlings possessing their third pair of true leaves showed very different patterns of trichome distribution and density on the adaxial leaf surface (Figure 5.6a) compared with the abaxial leaf surface (Figure 5.6b-g). The abaxial surface was characterised by a dense covering of Type III glandular trichomes on the epidermal surface closely associated with the network of minor veins but, in contrast to the adaxial surface, were absent from the midrib and major veins (Figure 5.6b-e). Figure 5.6f, g shows the majority of Type III trichomes on the abaxial surface to have a 4-celled glandular head, although some were uni- or bi-cellular. An additional type of trichome was also observed in lesser abundance interspersed between Type III trichomes in association with the minor veins: Type V trichomes are peltate and have a very short stalk that is sunk into the epidermis. The unicellular globular head is dome-shaped and notably larger than that of Type III (Figure 5.6f). Figure 5.5h shows a leaf cross-section with Type III and V trichomes on the abaxial surface. Type I trichomes were also present on the main epidermal surface, but were less abundant than on the adaxial surface (Figure 5.6b). Type II and IV trichomes were abundant on the midrib and major leaf veins, but appeared to be absent form the main epidermal surface (Figure 5.6b-e). In addition, leaves could be seen to be amphistomatal (stomata present on both sides of the leaf), although the density of stomata was presumably lower on the abaxial surface due to the high density of Type III trichomes.


27.8X 10KV WD:8MM S:00000 P:00009
1MM
a
963X 10KV WD:8MM S:00000 P:00010
50UM
b
1.26KX 10KV WD:8MM S:00000 P:00011
20UM
c
990X 10KV WD:13MM S:00000 P:00008
50UM
d
86.8X 10KV WD:8MM S:00000 P:00006
500UM
e
690X 10KV WD:9MM S:00000 P:00012
50UM
II
III
f
351X 10KV WD:8MM S:00000 P:00001
100UM
B
C
A
g
354X 10KV WD:8MM S:00000 P:00004
100UM
D
h

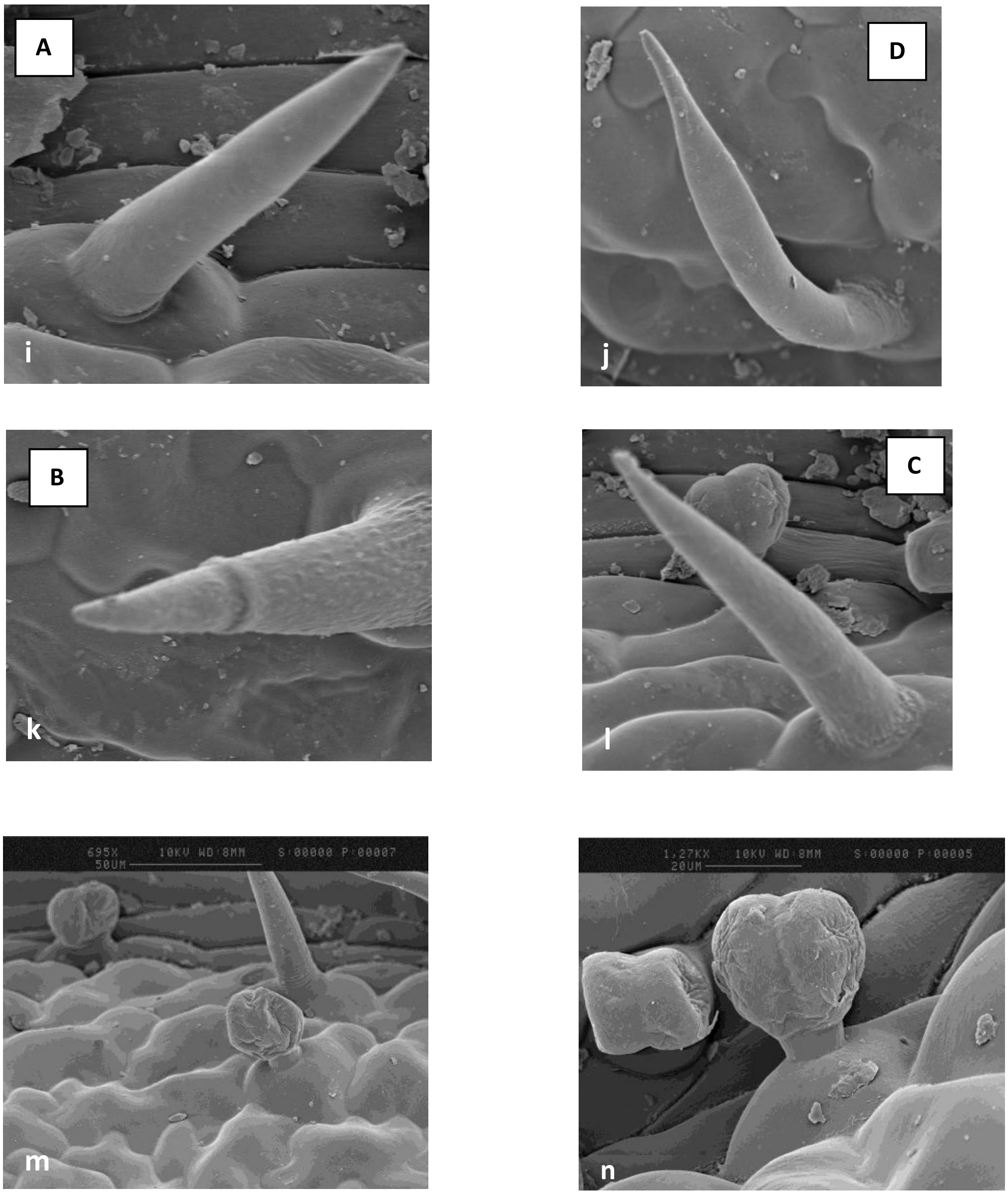
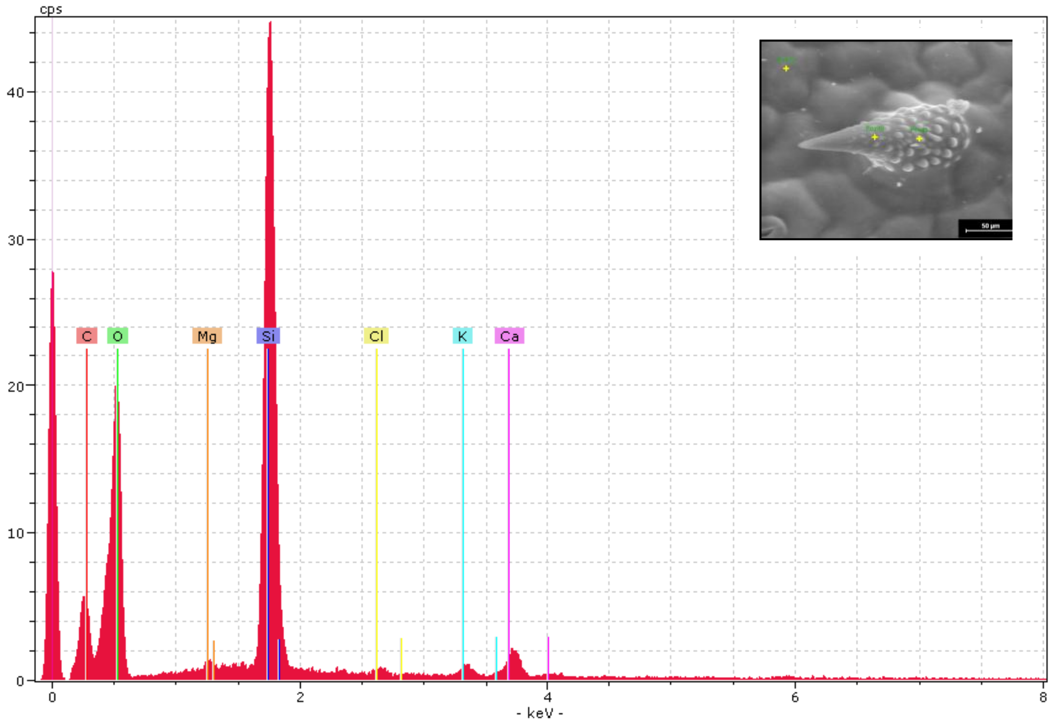
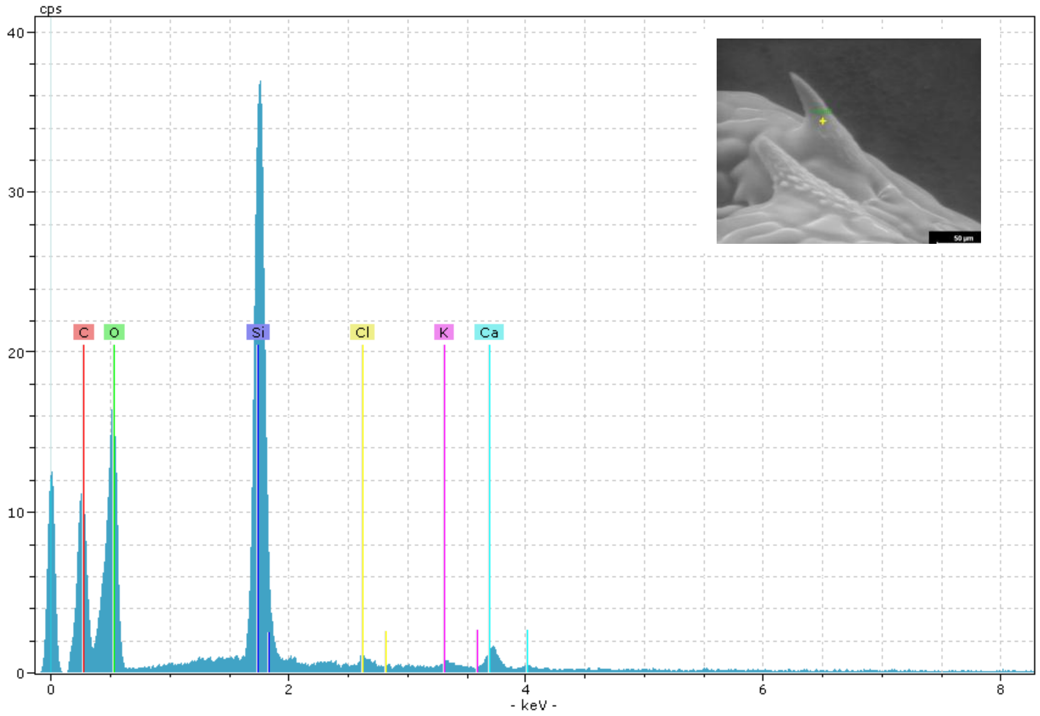


Figure 5.2. SEM micrographs of trichomes on the adaxial (upper) leaf surface of mature (in flower) *Rhinanthus minor* plants. a) Adaxial surface showing Type I trichomes located on main epidermal surface (i.e. inter-vein area) and leaf edge, and Type II and III trichomes associated with midrib and major veins. b) Type I nonglandular trichome located on main epidermal surface, exhibiting multiple foot cells and a single body cell with bulbous base and hooked point and covered in cuticular warts (verrucose). c) A broken Type I trichome revealing a hollow centre. d) Type I trichome located at leaf edge showing a typically less verrucose body cell and shorter hooked point. e) Type I trichomes at leaf edge and main epidermal surface. f) Type II trichome located at perimeter of midrib, exhibiting a single foot cell and long, thin, smooth body cell (black arrow). Type II trichome in right of image shows a verrucose body cell. Type III glandular capitate trichomes are also present (black arrow); white arrow points to a ruptured head. g) and h) Type II and III trichomes located in or near to midrib. Morphological variation of Type II trichomes are highlighted by labels A, B, C and D and cropped images shown in Fig. 1i-l. i) A - Type II trichome with a smooth, straight unicellular body. j) D – Type II trichome with a smooth, hooked unicellular body. k) B – Type II trichome with a straight, verrucose, bicellular body. l) C – Type II trichome with a straight, partly verrucose, unicellular body. m) Type III glandular capitate trichomes in midrib and at perimeter, exhibiting a single foot cell, short stalk cell and large bicellular globular head. n) Type III trichomes with bicellular globular head.

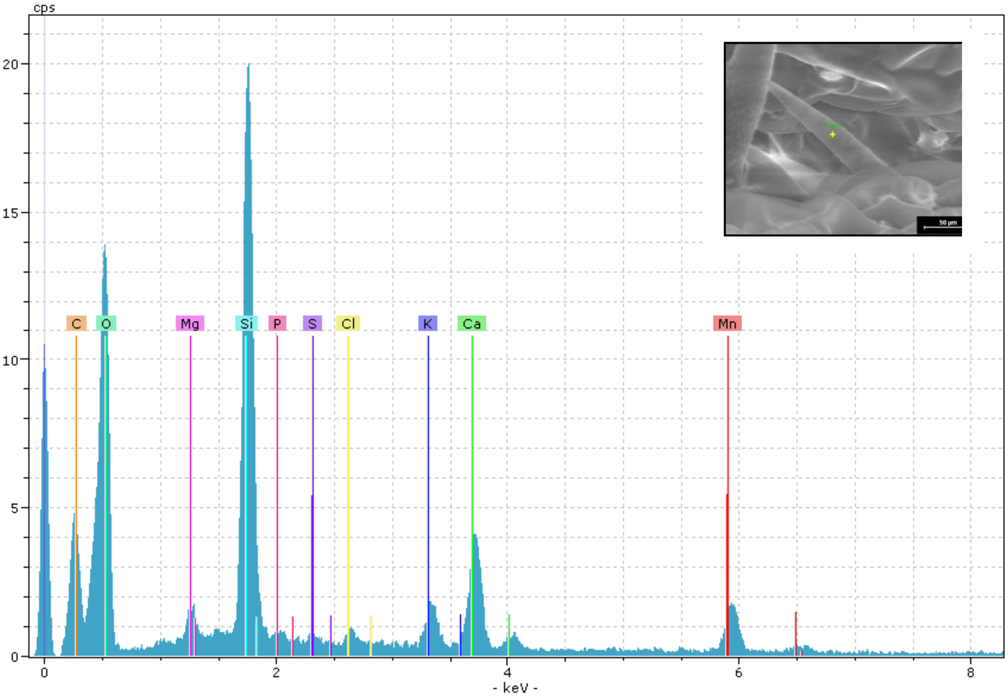
a)


cps
40
30
20
10
0
C
O
Mg
Si
Cl
K
Ca
0
2
4
6
8
- keV -
50 µm

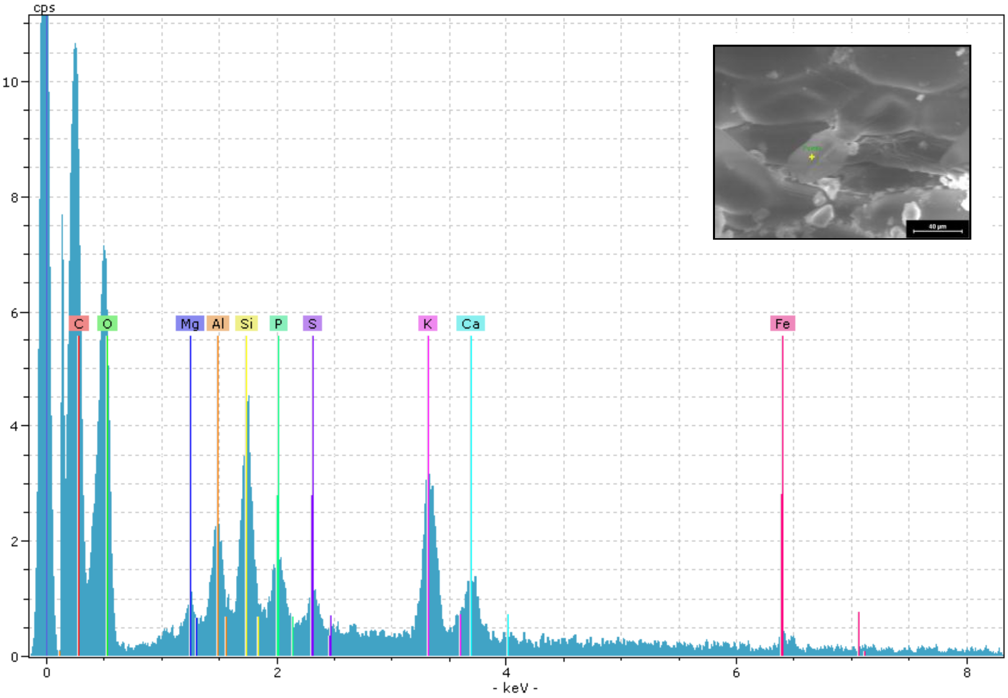
b)


cps
40
30
20
10
0
C
O
Si
Cl
K
Ca
0
2
4
6
8
- keV -
50 µm

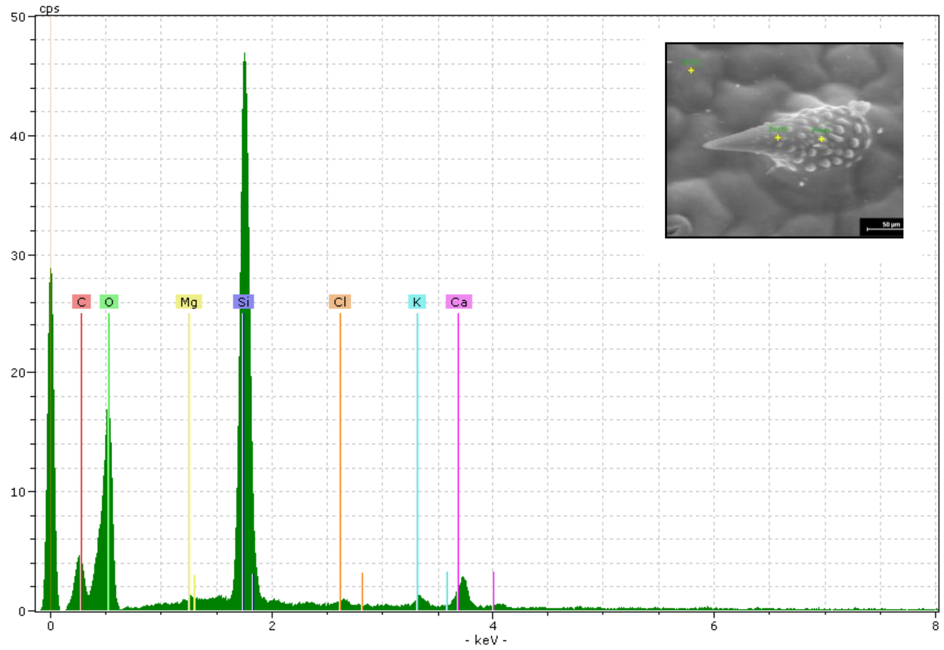
c)


cps
20
15
10
5
0
C
O
Mg
Si
P
S
Cl
K
Ca
Mn
50 µm
0
2
4
6
8
- keV -

d)


cps
10
8
6
4
2
0
C
O
Mg
Al
Si
P
S
K
Ca
Fe
40 µm
0
2
4
6
8
- keV -

e)



f)

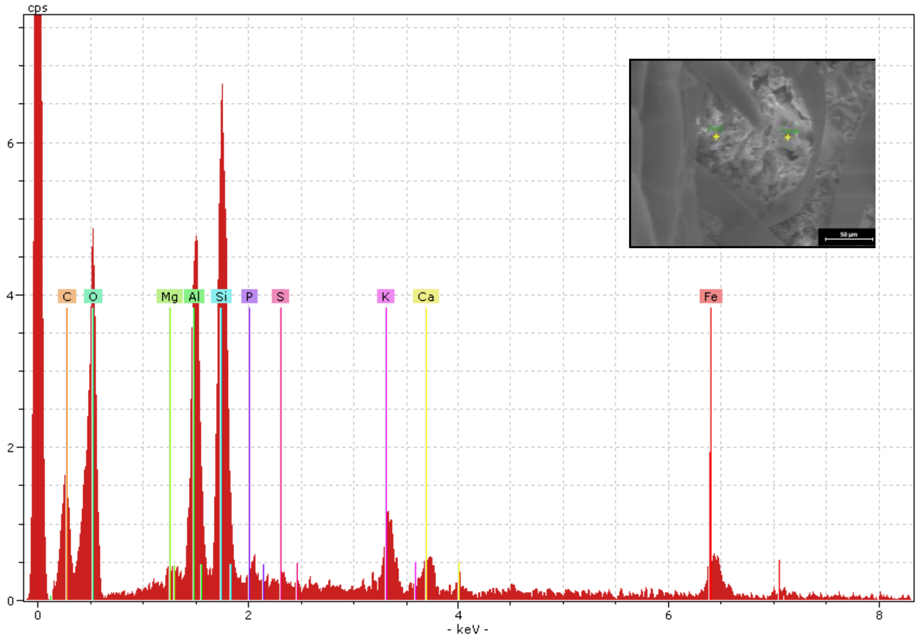


Figure 5.3. Energy dispersive X-ray spectroscopy (EDX) analyses and scanning electron micrographs of the adaxial leaf surface of mature *Rhinanthus minor* plants. EDX analyses show elemental composition (peak x-ray counts per second) of: a) Type I trichome on epidermal surface (point 1); b) Type I trichome at

leaf edge (point 0); c) Type II trichome near leaf vein (point 0); d) Type III trichome in leaf vein (point 0); e) Epidermal surface between Type I trichomes (point 2); f) Leaf vein between Type III trichomes (point 1). Y axis = counts per second (cps).

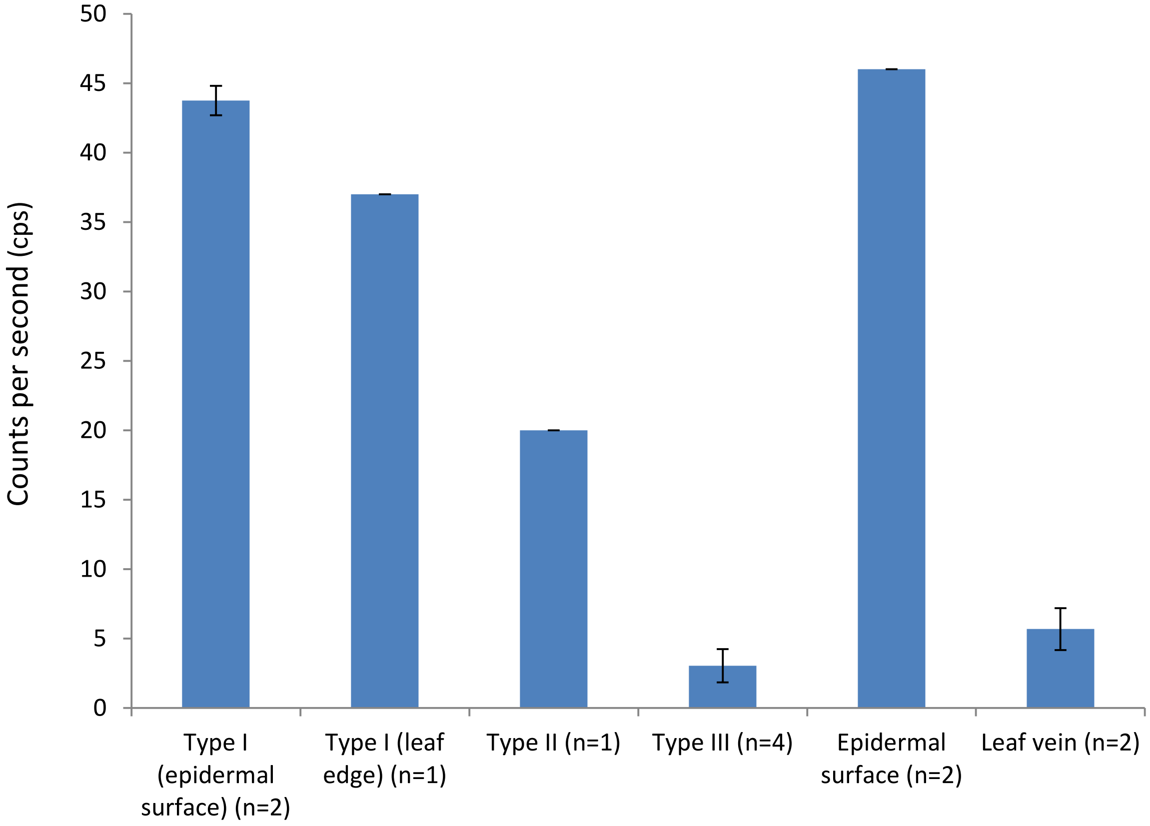
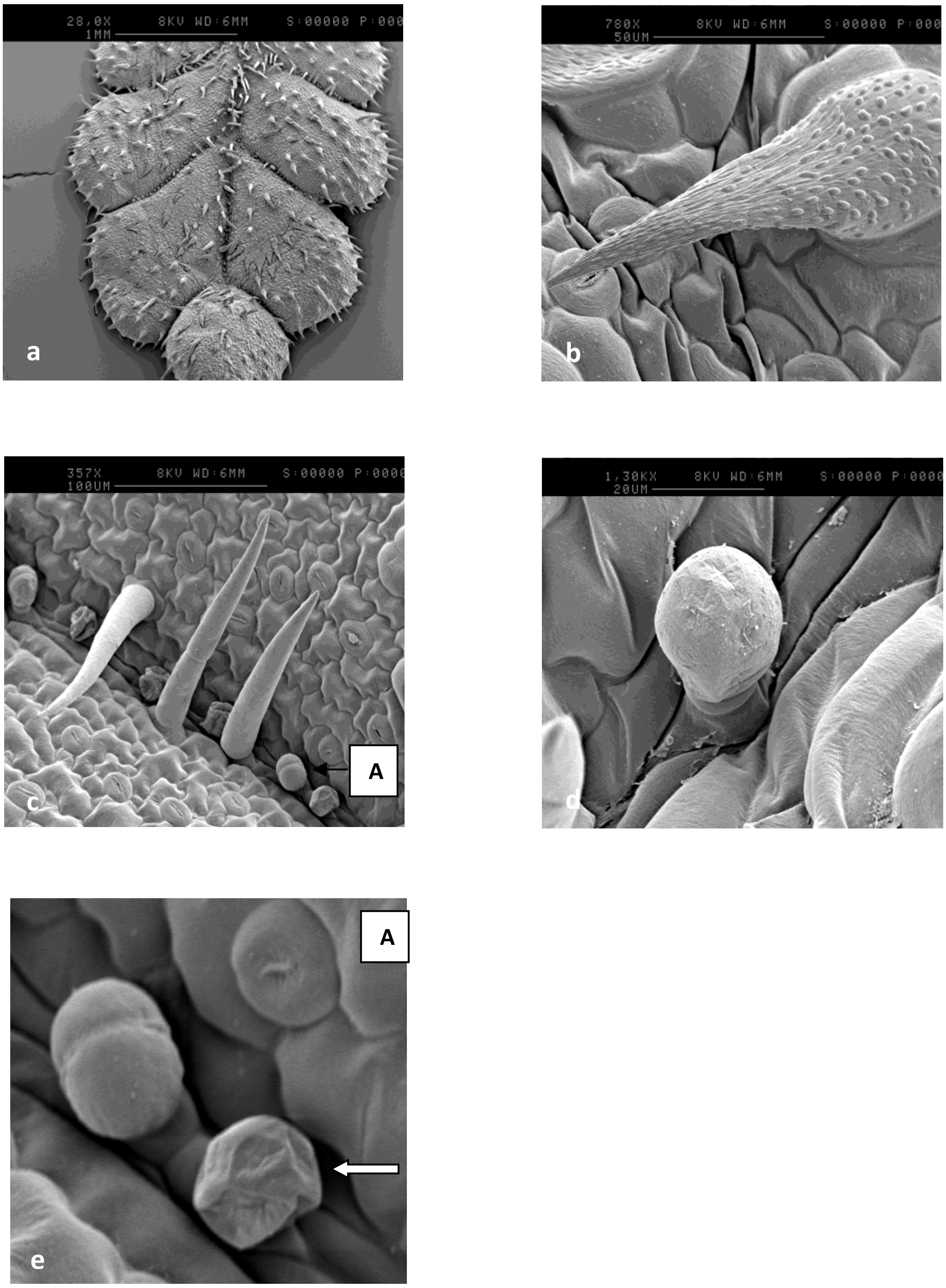


Figure 5.4. Mean (±SD) silicon content (X-ray cps) of different types of trichome and the epidermal surface of the adaxial leaf surface of mature (in flower) *Rhinanthus minor* plants; n represents the number of point samples analysed for each leaf structure.


28.0X 8KV WD:6MM S:00000 P:000
1MM
a
780X 8KV WD:6MM S:00000 P:000
50UM
b
357X 8KV WD:6MM S:00000 P:0000
100UM
A
c
1.30KX 8KV WD:6MM S:00000 P:0000
20UM
d
A
e

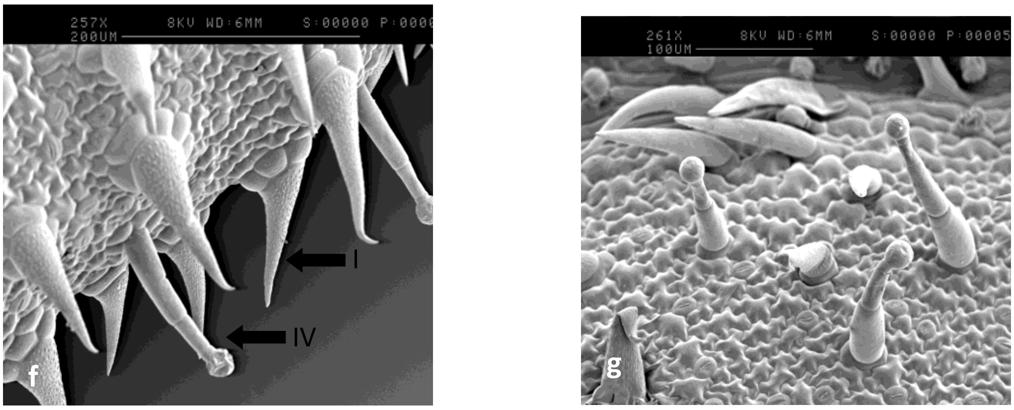
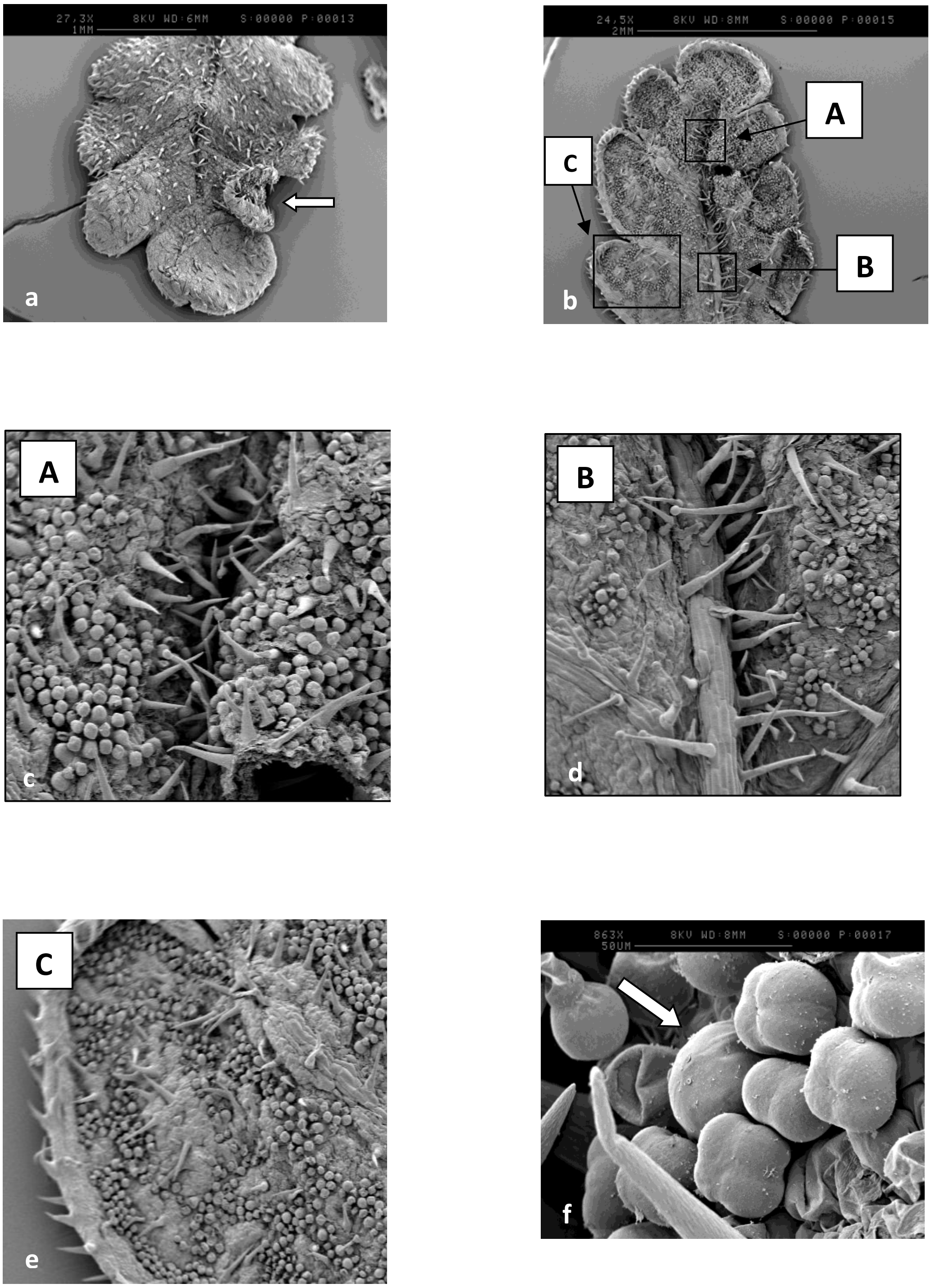


Figure 5.5. SEM micrographs of trichomes on the adaxial leaf surface of *Rhinanthus minor* seedlings possessing the first pair of true leaves. a) Adaxial surface of seedling leaf. b) Type I nonglandular trichome located on main epidermal surface exhibiting multiple foot cells and a single verrucose body cell with bulbous base and long hooked point. c) Type II nonglandular and Type III glandular trichomes located in and near to a major vein. Type II trichomes exhibit a single foot cell and either a uni- or bi-celluar body that is smooth, straight, long and pointed. Type III capitate trichomes are clearly visible in the sunken vein and have a bicellular globular head, though many are ruptured. Type I trichomes are visible in the right of the image showing the multicellular foot forming a raised pedestal for the body cell. d) A Type III capitate trichome with unicellular globular head. e) A – cropped image taken from Fig. 4c showing Type III capitate trichomes with a bicellular or (white arrow) ruptured head. f) Type I nonglandular and Type IV capitate glandular trichomes at leaf edge. The raised multicellular foot of Type I trichomes is clearly visible. Type IV capitate trichomes exhibit a single foot cell, a smooth multicellular stalk consisting of a long conical basal cell, 1-2 intermediary cells, a short subapical (neck) cell and a small unicellular glandular head at the apex. g) Type IV trichomes on main epidermal surface.


27.3X 8KV WD:6MM S:00000 P:00013
1MM
a
24.5X 8KV WD:8MM S:00000 P:00015
2MM
A
C
B
b
A
c
B
d
C
e
863X 8KV WD:8MM S:00000 P:00017
50UM
f

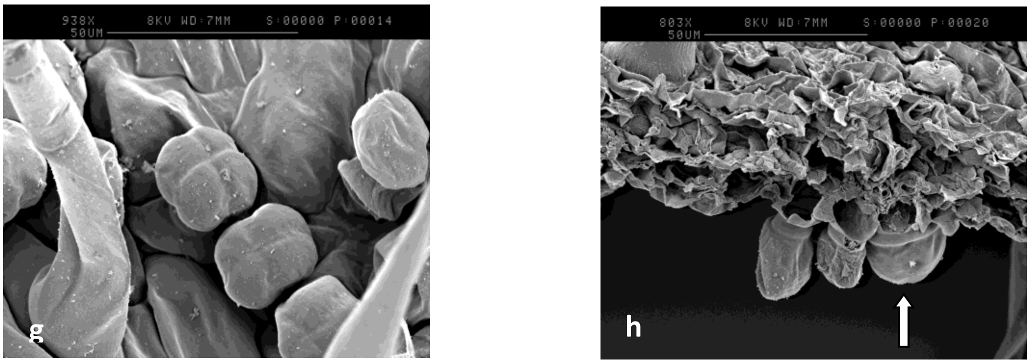


Figure 5.6. SEM micrographs of trichomes on the adaxial (upper) and abaxial (lower) leaf surface of *Rhinanthus minor* seedlings. Leaves shown are the oldest leaf of a seedling possessing three pairs of true leaves. a) Adaxial surface of whole leaf; white arrow pointing to folded section revealing abaxial surface. b) Abaxial surface of whole leaf showing a dense covering of Type III and V glandular trichomes closely associated with the network of minor veins. c)-e) A, B and C – cropped images taken from Fig. 5b showing Type III and V glandular trichomes associated with minor veins. Type II nonglandular and Type IV capitate glandular trichomes are visible on the midrib and major veins. Type I nonglandular trichomes are sparsely distributed on the main epidermal surface between minor veins. f) Type III and Type V (white arrow) glandular trichomes on abaxial surface. Type III capitate trichomes exhibit a single foot cell and short stalk with a tetra-cellular or bicellular globular head. Note the predominance of the 4-celled head type. A Type V peltate trichome is visible with a stalk cell sunk into the epidermis and a large, dome-shaped unicellular globular head. g) Type III glandular trichomes with tetracellular globular head. h) Cross section showing two Type III trichomes and a Type V trichome (white arrow) on the abaxial surface.

5.5 Discussion

This study is the first to provide scanning electron micrographs and full morphological descriptions of *Rhinanthus minor* leaf trichomes for mature and seedling plants, and to detail their elemental composition using EDX analysis. Various types of glandular and nonglandular trichomes were abundant on the epidermal surface of mature and seedling leaves, with variation evident between the adaxial and abaxial surface of seedling leaves.

The adaxial surface of mature leaves was characterised by two different types of nonglandular trichome (Types I and II) and a glandular short-stalked capitate trichome (Type III). Type I trichomes exhibited a hooked morphology and were abundant on the main epidermal surface and leaf edge, but were absent from veins. Type II trichomes were also simple and unbranched and could be straight or hooked and verrucose or smooth, although the predominant morphological type appeared to be straight and smooth. These trichomes were abundant in, and at the perimeter of, the midrib and major veins. Type III glandular trichomes consisted of a short stalk and 1-2 celled head and were abundant within the furrows of the midrib and major veins. Our observations correlate well with those of Neumann *et al.* (1997) who showed similar trichome types to occur on some other parasitic Orobanchaceae (formerly Scrophulariaceae) species. The Type I trichomes observed on the main epidermal surface of *R. minor* leaves are similar to those found on the stem of *Striga hermonthica* (Del.), although in the latter species the body cell is erect rather than hooked; the Type I trichomes located at the leaf edge are similar to those found at the leaf margin of *Rhamphicarpa fistulosa* (Hochst.). *R. fistulosa* is also characterised by short glandular trichomes located in the furrows of the major veins on the adaxial surface of leaves, similar to Type III trichomes found on *R. minor*, but exhibiting an 8-10 celled globular head. Type II trichomes are similar to those present on some floral parts and organs of various *Striga* species. Unfortunately, Neumann *et al.* (1997) do not describe all trichome types on the leaves of the investigated species, but concentrate their study on the trichomes of taxonomic importance. The nonglandular trichomes observed on *R. minor* leaves are probably ubiquitous amongst other plant families and have been found to occur, for example, on leaves of *Solanum* species (Edmonds 1982). An additional issue raised by this study is that further investigation of *R. minor* trichomes, may provide useful means of identifying the numerous subspecies.

The morphology, abundance and distribution pattern of trichome Types I and II suggests that they function as physical deterrents against invertebrate herbivores. The defensive role of trichomes has been demonstrated by previous studies and numerous ways have been identified by which nonglandular trichomes may affect invertebrate herbivores, namely by inhibiting feeding and causing deleterious effects on growth and development and fecundity (Levin 1973; Juniper & Southwood 1986; Simmons & Gurr 2005; Hanley *et al.* 2007). The high density of Type I trichomes on the main epidermal surface and at the leaf margin may provide a physical obstruction to feeding by chewing/rasping invertebrate herbivores, whereas Type II trichomes, located in and around the major veins, may be more effective against phloem feeders, such as aphids. Type I trichomes may also reduce suitable colonisation and oviposition sites on the adaxial surface (Baur, Binder & Benz 1991) and increase the susceptibility of eggs to desiccation, although some studies report trichome density to positively affect oviposition rates (Heinz & Zalom 1995). Some insects may be susceptible to becoming trapped or impaled on Type I and II hooked trichomes leading to death by exhaustion or loss of haemolymph as has been shown with aphids, bedbugs and leafhoppers on *Phaseolus* leaves (Johnson 1953; Jeffree 1986). Furthermore, both Type I and II trichomes may increase the duration of feeding and exposure to predators, and if trichomes are consumed offer little nutritive benefit, which in turn may limit herbivore growth and development, particularly of larvae. This hypothesis is supported by the findings of the EDX analysis which confirmed that trichome Types I and II on mature leaves are predominantly composed of silicon, as was the main epidermal surface. This finding is similar to previous studies which report the accumulation of silicon in or at the base of nonglandular trichomes and at the leaf edge (Cherif *et al.* 1992; Frantz *et al.* 2008; Epstein 2009). Previous studies have shown silica phytoliths in grass leaves to act as an anti-herbivore defence against lepidopteran and orthopteran folivores by reducing the digestibility of leaves and increasing mandible wear leading to reduced insect growth rates (Massey, Ennos & Hartley 2006; Massey & Hartley 2009). Furthermore, silicon is thought to be a factor in reducing the acceptability of grass species compared with broad-leaved forbs to molluscs (Grime, MacPherson-Stewart & Dearman 1968; Dirzo 1980; Wadham & Wynn-Parry 1981; Hanley, Fenner & Edwards 1996a). The very low concentration of silicon within Type III glandular trichomes was expected as the secretory globular heads presumably require a less rigid structure than the nonglandular trichomes. Furthermore, low silicon content is likely to increase herbivore exposure to chemical defences (if present) via rupture of the secretory head. Unfortunately, as seedling leaves were not analysed with EDX, a comparison cannot be made with Type IV trichomes.

Seedlings with only their first pair of true leaves were found to possess fully developed indumenta, characterised by trichome Types I-III and an additional Type IV long-stalked capitate glandular trichome. A similar ontogenetic effect has been described for *Solanum* species: Edmonds (1982) writes that Seithe (1979) described the presence of “gland-tipped finger hairs” for numerous *Solanum* species in the seedling stage, but on successive leaves these gland-tipped trichomes were replaced by simpler non-glandular forms, and adds that for *S. nigrum* this transition was complete within a few leaf stages. From a taxonomic point of view it is therefore essential to examine mature leaves, but presents a more interesting observation from the point of view of the evolution of plant defence against herbivores. The absence of Type IV trichomes on mature leaves and the findings of previous studies would suggest that secretory products may function in the chemical defence of young leaves (Levin 1973). The capitate trichome found in some members of the Solanaceae, including *Solanum fragile* Wedd. (Edmonds 1982, Fig.8); *Nicotiana tabacum* L. (Akers, Weybrew & Long 1978) and some *Lycopersicon* (tomato) and *Solanum* (potato) species (Juniper & Southwood 1986; Simmons & Gurr 2005) are comparable to the Type IV trichome in this study, albeit with some morphological variation. In tomato and potato species, this type of trichome is known to contribute to resistance against a variety of insect pests (Duffey, Juniper & Southwood 1986; Gregory *et al.* 1986). A similar capitate trichome with a multicellular head is present on the flowers of the hemiparasitic species, *Orobanche ramosa* subsp. *nana* Reut. (Cout.), and is known to secrete terpenes and flavonoids (Sacchetti *et al.* 2003). Kaplan and Inceoglu (2003) report an equivalent trichome to occur on the mature leaves of some members of the Rhinantheae tribe.

The presence of trichomes at this early developmental stage further suggests that they function to protect young, vulnerable leaves from invertebrate herbivore attack. This hypothesis is supported not only by the presence of Type IV trichomes on seedling leaves only, but also the apparent greater length of Type I and II trichomes on seedling leaves in comparison with mature leaves. Investment in defence by young plants may be particularly important for hemi-parasitic species, as prior to attachment they are less able to compensate for lost leaf tissue than non-parasitic species (Seel, Parsons & Press 1993). Some previous studies have demonstrated the early development of trichomes on leaves (Werker & Fahn 1981; Franceschi & Giaquinta 1983; Duke & Paul 1993; Bourett *et al.* 1994; Kobayashi *et al.* 2008) but few have demonstrated their efficacy in reducing herbivore damage in comparison with older leaves. Woodman and Fernandes (1991) showed that the young leaves of *Verbascum thapsus*, characterised by abundant nonglandular stellate trichomes, suffered less frequent damage by chewing insects than older, less pubescent leaves and offer their findings as support for the optimal defence theory (Feeny 1976).

Histochemical tests were not performed in this study, therefore It is not known what chemicals are present within glandular trichome secretions or if they confer any biological activity against herbivores (i.e. toxic, repellent, immobilising). In his review of *R. minor*, Westbury (2004) summarises the biochemistry and known herbivores and diseases. The leaves of *R. minor* contain the iridoid glycoside rhinanthin, also known as aucubin, which may be harmful to livestock if sufficient quantities are consumed but it is not known whether rhinanthin is present in glandular trichome secretory products. There are no feeding assay studies involving *R. minor* and invertebrates, although aucubin within *Plantago* leaves is known to have toxic, deterrent and growth limiting effects on insect herbivores (Bowers & Stamp 1993; Pankoke, Bowers & Dobler 2010) and may also serve as oviposition stimuli (Nieminen *et al.* 2003). According to Westbury’s (2004) review, leaf herbivores are few, although mature plants are attacked by aphids and seedlings by molluscs. Various fungal pathogens are also reported including mildews, British rust fungi, British stem and leaf fungi and other European species. The presence of foliar trichomes may influence the susceptibility of *R. minor* to fungal attack. Previous studies report mixed effects of trichomes on fungal pathogens, for example Calo *et al.* (2006) report the nonglandular trichomes of *Arabidopsis* facilitate the colonisation of fungi by acting as physical adhesion points for hyphae; whereas, Valkama *et al.* (2005) showed the leaf glandular trichomes of *Betula* species to reduce fungal infection. It is possible that, for *R. minor*, the likely role of nonglandular trichomes in herbivore defence may backfire in increasing the plants susceptibility to fungal attack.

The adaxial and abaxial surfaces of seedling leaves revealed marked variation in trichome distribution, abundance and morphology. Type III trichomes with uni- or bi-cellular heads were present in the furrows of the major veins on the adaxial surface whereas trichomes with a tetracellular head were highly abundant on the abaxial surface in association with the minor veins. The latter type have previously been described as scutiform or ‘shield-shaped’ and are known to occur on the abaxial surface of leaves, bracts and sepals of *Euphrasia rostkoviana* Hayne and *Melampyrum arvense* L. as well as *R. minor* (Neumann *et al.* 1997 and references therein). The Type V peltate trichome, observed on seedling leaves, was also associated with the minor veins on the abaxial surface only. This description corresponds well to the studies by Ponzi and Pizzolongo (1992) on *R. minor* and Govier, Brown and Pate (1968) on the root-hemiparasitic herb, *Odontites verna* (Orobanchaceae), which describe glandular trichomes to be abundant on the abaxial leaf surface in association with the minor veins. Both of these studies suggest that trichomes function as hydathodes involved in active guttation. Chemin (1920) also reported glandular hairs in leaf cavities of the holo-parasite, *Lathraea squamaria* L. to function as hydathodes. Ponzi and Pizzolongo (1992) described numerous rows of glandular trichomes exhibiting a 4-celled head, but the authors did not identify the secretory products nor did they identify the peltate (Type V) trichome evident in this study: It is possible that they failed to observe it or it may be absent from mature leaves. Govier, Brown and Pate (1968) used radioisotopic labelling to demonstrate the involvement of short-stalked capitate glandular trichomes in guttation for *O .verna* attached to hosts, and suggested that in hemi-parasitic species glandular trichomes may function to: i) allow the withdrawal of solutes from host xylem when the latter have closed stomata; ii) provide a mechanism whereby metabolites are diverted to young shoots; and/or iii) act as excretory organs to rid the hemi-parasite of excess solutes arriving in the transpiration stream. Govier, Brown and Pate (1968) also describe a peltate glandular trichome associated with the minor leaf veins indicating that the abaxial surface of *O. verna* is highly similar in terms of trichome morphology and distribution to that of *R. minor*. Although Govier, Brown and Pate (1968) analysed the amino compounds in the guttation fluid of *O. verna*, they did not perform a full histochemical analysis of all glandular trichome secretory products. In contrast to *R. minor* and *O. verna*, the glandular trichomes of various other confamilial parasitic species are separated from the vascular bundles by more than one layer of parenchymatous cells, making their role in active guttation unclear (Neumann *et al.* 1997). It is possible, therefore, that Type III and V trichomes serve multiple functional roles in active guttation and chemical defence against herbivores. It also remains to be seen whether the Type III trichomes located on the adaxial and abaxial surface differ in terms of their secretory products and function. Comparisons can be made with similar short-stalked capitate trichomes on leaves of tomatoes and potatoes (Juniper & Southwood 1986; Simmons & Gurr 2005) that are known to secrete bioactive exudates that function in anti-herbivore defence.

In conclusion, the indumenta of *R. minor* leaves are characterised by a dense covering of glandular and nonglandular trichomes from very early stages of plant development. The morphology, abundance and distribution of trichome Types I, II and IV strongly indicates a role in defence against invertebrate herbivores, and testing is required to establish the effects of each type of trichome on a variety of invertebrate herbivores. Furthermore, the absence of Type IV trichomes on mature leaves suggests that young plants are better defended against herbivore attack in comparison with mature plants; an attribute that is perhaps particularly beneficial to *R. minor* during the pre-attachment phase. Histochemical tests are necessary to identify the chemical composition of glandular trichome exudates in combination with tests to discover the biological activity, if any, towards herbivores. The description of glandular trichome Types III and V correlates well with other studies and indicates that these trichomes may function in active guttation, although this phenomenon requires experimental confirmation. To gain a clear understanding of trichome function future research will require an interdisciplinary approach incorporating research on ecology, chemistry and genetics.

Chapter 6. Bumblebee activity and forage use in upland hay meadows

6.1 Abstract

Recent studies of British and European bumblebees have shown that the decline of many species is linked to loss and degradation of suitable foraging habitat particularly unimproved species-rich grassland. The availability of Fabaceae forage plants possessing protein-rich pollen has also been identified as a critical factor and may affect rare, long-tongued species with a short colony life cycle in particular. Species-rich upland hay meadows are likely to support populations of common and rare bumblebees which in turn provide essential pollination services to many wildflowers. Bumblebee activity and forage use was surveyed in five connected upland hay meadows in Northumberland, UK in June and July using a standard transect walk methodology. Seven bumblebee species were active in the meadows, including the rare/scarce species *Bombus monticola*, *B. jonellus* and *B. muscorum* (the latter a UKBAP species). Despite the floristic diversity in the meadows, forage use was directed predominantly towards four plant species: *Rhinanthus minor*, *Trifolium repens*, *T. pratense* and *Geranium sylvaticum*. Of the 15 species visited by foraging bumblebees, seven were Fabaceae. Although many forage plant species were visited by most bee species, visitation frequency was differentiated between short- and long-tongued species. *R. minor* was visited frequently by most species, but visits to *T. pratense* and *T. repens* were markedly greater by long- and short-tongued species, respectively. The study provides supporting evidence that protection and restoration of upland hay meadows will benefit common and rare bumblebee populations and help to maintain pollination services to fragmented populations of native wildflowers.

Key-words: agricultural improvement, *Bombus*, Fabaceae, floral reward, *Geranium sylvaticum*, grassland restoration, pollination services, *Rhinanthus minor*, tongue-length, *Trifolium*

6.2 Introduction

Populations of most social bumblebees (*Bombus* species) have undergone major declines in recent decades across Europe and North America (Williams 1982; Goulson 2003; Colla & Packer 2008; Grixti *et al.* 2009; Williams & Osborne 2009). Using results of the Bumblebee Distribution Maps Scheme (1970-1976), Williams (1982) reported the serious range contraction of all but six species of the UK (true/social) bumblebee fauna since pre-1960. Species distributions were defined in terms of three biogeographic elements and four biogeographic regions. The six 'Mainland Ubiquitous Species' (*Bombus terrestris* L., *B. lucorum* L., *B. hortorum* L., *B. pratorum* L., *B. lapidarius* L. and *B. pascuorum* Scopoli) have suffered small declines in distribution and remain well represented throughout mainland Britain. The 'Widespread Local Species' (*B. muscorum* L., *B. jonellus* Kirby, *B. soroeensis* Fabricius and *B. monticola* Smith) have become more localised in their distribution and are found on outer Scottish Islands and in mainland Britain outside of central and eastern England (the so-called central impoverished region). The north of Britain is particularly characterised by these, and the ubiquitous, species. *B. monticola* has a unique upland distribution. The third group are the 'Southern Local Species' which have suffered severe range contractions post-1960 and are associated with Southern England and Wales. One species, *B. distinguendus* (Morawitz) does not conform to these three groups having dramatically declined throughout northern Britain and is now largely confined to Hebridean Islands. Extinctions and one new arrival (*B. hypnorum* L.) place the current UK fauna at 24 bumblebee species including six cuckoo species. Seven species are designated UKBAP priority species including five of the Southern Local Species, *B. distinguendus* and *B. muscorum* (JNCC 2011). A BAP exists for *B. subterraneus* (L.) although it is believed to be extinct in the UK and reintroductions from Sweden are planned for 2012 (BBCT 2011).

The principal cause of these declines is likely to have been the vast reduction, degradation and fragmentation of suitable foraging habitat and nesting and hibernation sites due to changes in land-use and agricultural practices (Kells & Goulson 2003; Goulson *et al.* 2005; Williams 2005) such as drainage, greater fertiliser and herbicide usage, silage production and field consolidation. Pyrethroid-based insecticides may also have contributed to declines although effects of pesticides on bumblebee biology remain largely unknown (Goulson 2003). Analyses of long-term datasets have identified dramatic declines in flower-rich habitats, particularly unimproved grassland (Fuller 1987; Blackstock *et al.* 1999; Haines-Young *et al.* 2003; Howard *et al.* 2003), and forage plants for bumblebees (Carvell *et al.* 2006) at a national scale. For example, over 90% of unimproved grassland has been lost in Britain since the 1940s (Blackstock *et al.* 1999). While the causes of rarity still require further investigation, a combination of factors including the degree of forage, habitat and climatic specialisation are likely to influence bumblebee abundance and diversity (Williams 1988; Goulson *et al.* 2005; Williams 2005). More specifically the abundance of the most rewarding forage species may be more important than forage diversity to the rare bumblebee species and overall bumblebee diversity. Several studies have identified the importance of Fabaceae and Fabaceae-rich habitat to bumblebees, particularly to most long-tongued and rare species (Goulson & Darvill 2004; Goulson & Hanley 2004; Goulson *et al.* 2005; Carvell *et al.* 2006; Goulson, Lye & Darvill 2008). Members of the Fabaceae, such as *Trifolium pratense* (L.), are highly rewarding to bumblebees as they provide a good source of nectar and have highly protein-rich pollen (Hanley *et al.* 2008). High quality pollen may be of particular benefit to those species with late emerging queens (which tend to be long-tongued and rare species) as the colony has less time to rear larvae and produce sexual reproductives (Goulson & Darvill 2004). Late emerging species are also associated with unimproved grassland (a Fabaceae-rich habitat), as opposed to woodland or woodland edge like some early-emerging species, and are likely to have been more severely affected by agricultural improvement of grassland (Edwards & Williams 2004; Goulson *et al.* 2005). In most of Europe, changing agricultural practices have seen a dramatic decline in clover-leys, sown for horse fodder, which is thought to have had a serious negative impact on many bumblebee species (Rasmont 1988; Goulson 2006).

Declines in bumblebee and wildflower populations have raised valid concerns over potential reductions in pollination services and pollen limitation of plant female fitness (fruit and seed set) (Ghazoul 2005; Biesmeijer *et al.* 2006; Potts *et al.* 2010; Potts *et al.* 2011). Disruptions to plant-pollinator mutualisms may also lead to genetic drift and inbreeding depression of both parties, particularly in small isolated populations (Kearns, Inouye & Waser 1998). The ecological consequences of declining seed quantity and quality will depend on the role of seeds in population dynamics and whether populations are limited by seedling recruitment. Several meta-analyses reveal pollen limitation of reproductive output to be a common phenomenon, particularly for self-incompatible plant species (Burd 1994; Larson & Barrett 2000; Knight *et al.* 2005), although for most species we do not know if their reproductive output is pollen limited as nobody is studying them, and long-term studies would be needed to determine whether reduced seed quantity or quality is causing effects at the population and community level.

Species-rich upland hay meadows are one of the rarest types of grassland in the UK and Northern Europe (Jefferson 2005). In the UK, 897 ha remain and are found predominantly in upland valleys in northern England (27 ha in Scotland) where traditional hay-making practices still persist (BARS 2008). Upland hay meadows, conforming to MG3 (*Geranium sylvaticum-Anthoxanthum odoratum* grassland) in the National Vegetation Classification (Rodwell 1992), are an Annex I habitat under the EU Habitats Directive and a UKBAP priority habitat (JNCC 2008). Protection and restoration of botanical diversity has many advantages for wildlife and it is likely that upland hay meadows are an important forage resource and nesting habitat for bumblebees including several rare/scarce species, namely *B. monticola*, *B. jonellus*, *B. soroeensis* and *B. muscorum* (the latter a BAP priority species). Equally, healthy bumblebee populations provide important pollination services for many wildflowers in the meadows.

This study involved a collaborative survey effort with the Northumberland National Park Authority to investigate bumblebee activity and forage use in five connected upland hay meadows, two of which are designated as SSSI (Site of Special Scientific Interest). This information was previously lacking and sought to narrow the gap between conservation research and practical on-the-ground conservation management, as highlighted recently by Goulson *et al.* (2011). Information can be transferred into practical conservation by targeting relevant agri-environment scheme options, such as grassland restoration and recreation, uncut margins, and pollen and nectar wildflower strips in areas likely to be of most benefit to bumblebees and biodiversity in general.

6.3 Method

Transect walks were used to record bumblebee activity and forage use in five connected mesotrophic upland hay meadows at Barrowburn, Northumberland, UK (NT 868 107) (Figure 6.1). Barrowburn meadows are situated in a valley in upper Coquetdale at approximately 250 m elevation. Descriptions of meadow vegetation and management are provided in Table 6.1 and briefly comprised two meadows of intermediate species richness (meadows 1 and 2), two highly species-rich (SSSI, MG3) meadows (meadows 3 and 4) and a species-rich uncut stream bank within a semi-improved meadow (meadow 5).

Table 6.1. Meadows used to survey bumblebee activity and forage use.

Meadow	Vegetation	Management
1	Small (approx. 1 ha) flat meadow, intermediate species-richness, characteristic forbs *Rhinanthus minor*, *Trifolium pratense*, *T. repens*, occasional *Geranium sylvaticum*	No spring grazing, no FYM, hay-cut early August
2	Large (approx. 4 ha) +/- flat meadow, intermediate species-richness, characteristic forbs *T. repens* (highly abundant), *T. pratense* and *R. minor*	Spring grazing (sheep), shut-up mid-May, no FYM, hay-cut early August
3	Two adjacent meadows situated on a west-facing valley side, total area 5.4 ha, designated as SSSI in 1988, MG3, characterised by *G. sylvaticum*, *T. pratense*, *R. minor* and rich diversity of other forbs	Spring grazing (sheep), shut-up early May, no FYM, hay-cut early August
4		Spring grazing (sheep), shut-up mid-May, no FYM, hay cut early August
5	SE facing streamside bank within rank grassy meadow (approx. 0.5 ha); bank vegetation tall and characterised by *G. sylvaticum*, *Geum urbanum*, *Filipendula ulmaria* and *Cirsium heterophyllum*	No grazing, no FYM, no cut

For meadows 1-4, a linear 100 m transect was positioned in a representative area of the main vegetation. For meadow 5, a 100 m transect followed the contour of the uncut streamside bank. Meadow and transect locations are marked in Figure 6.1. Transects were walked in one direction recording all bumblebees and forage use in the area 2 m to the left before repeating the survey in the opposite direction. Transects were walked at a slow and steady pace (20 minutes per 200 m) during suitable weather conditions (sunny, warm and dry) between 0900 and 1800. The abundance of all *Bombus* species was recorded, as well as the behaviour of the bee specified as in flight, at rest or foraging. Foraging bees were observed until they left a transect or for a maximum of one minute.

The plant species used as forage and the number of plants visited were recorded. No distinctions were made between pollen and nectar collection. Observations of nectar robbing were also noted.

Air temperature (^{0}C) was recorded at the start of each transect walk. All meadows were surveyed on six assessment days between 15.06.09 and 23.07.09, with the exception of meadow 5 which could not be surveyed on the final assessment day due to flood damage. Meadows were surveyed in a randomised order in both the morning and afternoon, except on 15.06.09 when only morning surveys were possible due to adverse weather conditions. On 01.07.09 all meadows were surveyed three times (once in the morning and twice in the afternoon).

Figure 6.1. Ordnance survey map of Barrowburn, upper Coquetdale, Northumberland, UK (NT 868 107). Scale 1:1500. Meadows and transect locations used for bumblebee surveys are marked. For further details of meadows refer to Table 6.1.

6.3.1 *Statistical analysis*

BUMBLEBEE ACTIVITY

As the number of transects per assessment day varied, activity data were summarised to give mean values for each meadow on individual assessment days. Bumblebee activity data included species richness and abundance of all bees observed in transects i.e. bees in flight, resting and actively foraging.

Bumblebee activity was analysed using unconstrained and constrained ordination techniques. Prior to analysis data were subject to a fourth root transformation to reduce the impact of kurtosis arising from the zero-inflated data. Data were then transformed using the Bray-Curtis dissimilarity index. An unconstrained ordination (Principal Coordinates (PCO)) was applied to the transformed dataset to evaluate the suitability of the transformation and to visually interpret patterns in sample distribution in multivariate space.

The constrained ordination technique, Canonical Analysis of Principal Coordinates (CAP) as described by Anderson and Willis (2003) was used to investigate how overall bumblebee activity in each meadow was influenced by temperature and time (i.e. sampling date). The number of axes (*m*) used for the canonical analysis was 3. Correlations of environmental predictor variables (temperature and time) with canonical axes were superimposed on to a plot of the first two CAP axes to form a biplot. In order to investigate how different bumblebee species influenced overall activity levels, a second biplot was produced by superimposing partial correlations for each bumblebee species on to a plot of the first two canonical axes. The magnitudes of partial correlations were used to ascertain which bumblebee species characterised the multivariate effect. A visual interpretation of sample distribution was made together with tests of hypotheses using permutation procedures on canonical test statistics using 10000 permutations (trace statistic, delta squared statistic). Permutation tests explored the multivariate relationship between environmental variables and patterns in bumblebee activity. All analyses were performed using PRIMER-E (Clarke & Gorley 2006).

FORAGE USE

Data concerning bumblebee foraging behaviour was restricted to bees that were observed to be actively foraging within transects. The number of visits by workers of each bee species to each plant species was examined using Principal Components Analysis (PCA) performed within PRIMER-E (Clarke & Gorley 2006). Mean tongue-length of each bee species was taken from Goulson *et al.* (2005) and compared with the first principal component using a regression analysis performed in Minitab 16 (Minitab 16 Statistical Software 2010). As the subspecies of *B. muscorum* was not known, the shorter tongue length cited (that of *B. muscorum sladenii* (a southern race)) was used so as not to overestimate the influence of tongue-length on forage use. Analyses were performed with and without the inclusion of *B. jonellus* as only two individuals were recorded throughout the sampling period and both bees visited only *G. sylvaticum*. As the purpose of the analysis was to assess forage use by each bee species it was not helpful to include *B. jonellus* based on the behaviour of only two bees.

6.4 Results

Seven species of bumblebee (235 individuals) were recorded (all transects combined) including four ‘Mainland Ubiquitous’ species (*B. lucorum*, *B. pascuorum*, *B. hortorum* and *B. pratorum*) and three ‘Widespread Local’ species (*B. monticola*, *B. muscorum* and *B. jonellus*) (Williams 1982) (Figure 6.2). Other ubiquitous species, namely *B. terrestris* and *B. lapidarius* were not recorded, nor were any species of cuckoo bumblebee. We did not distinguish the species complex of *B. lucorum* as not all individuals could be caught; however, some individuals were caught for which the thoracic collar extended below the wing base indicating that they were *B. magnus* (Northern white-tail). Abundance varied markedly between species with *B. lucorum* (43.4%) and *B. pascuorum* (20.9%) individuals being recorded the most frequently; *B. hortorum* (11.1%), *B. monticola* (11.1%), *B. muscorum* (7.7%) and *B. pratorum* (5.1%) were recorded less frequently; and *B. jonellus* (0.9%) accounted for just two observations.

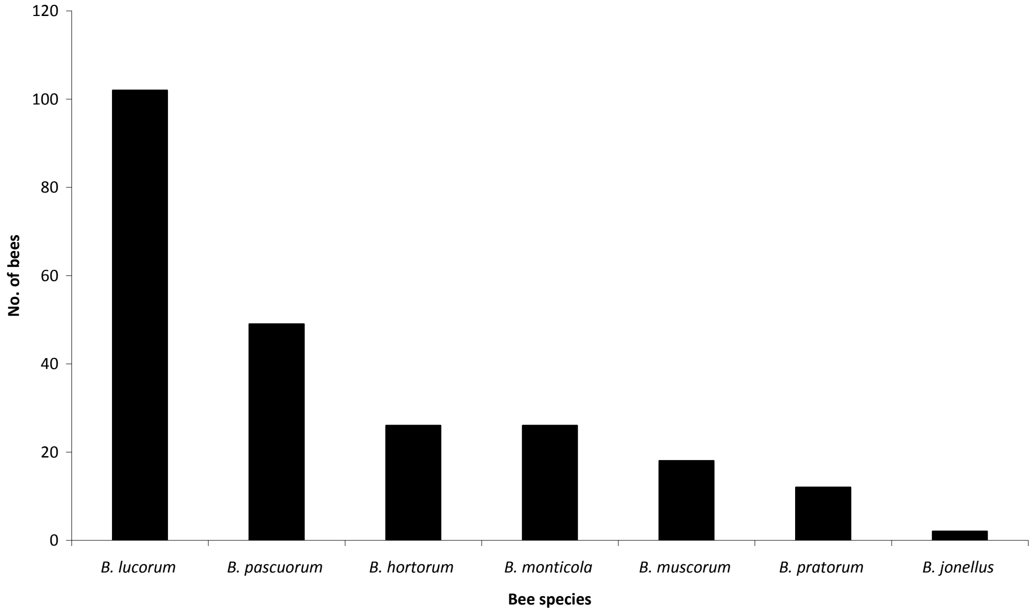


Figure 6.2. Total number of each bumblebee (*Bombus*) species recorded at five upland hay meadow sites in Northumberland between mid-June and late July 2009.

6.4.1 *Bumblebee activity*

Summarised activity data used in the PCO and CAP analyses consisted of 128 bees (*B. lucorum*, 40.3%; *B. pascuorum*, 24.8%; *B. hortorum*, 9.5%; *B. monticola*, 11.6%; *B. muscorum*, 8.1%; *B. pratorum*, 5.1%; *B. jonellus*, 0.5%). The dataset suffered from zero inflation due to frequently low species richness per sample (i.e. transect). A characteristic of the data was that rarer species (i.e. those recorded less frequently) are recorded in fewer meadows and at fewer time points. The small dataset prevented this issue from being corrected using the technique of rarefaction as described by Williams (2005).

The first two axes of the PCO ordination explained 78% of the variation in the original data matrix. The plot of the first two PCO axes (Figure 6.3) shows that the majority of samples are associated with positive axis 1 scores and are not separated according to meadow, suggesting that bumblebee activity did not vary between meadows throughout the assessment period. Some samples are outliers from the main group and are associated with negative axis 1 scores. These samples belong to meadows 1, 4 and 5. The multivariate distribution appears to be random according to meadow type in association with axis 2. This suggests that dispersion between samples within each meadow group is smallest for meadow 2, indicating that bumblebee activity remained relatively constant between sampling times. Dispersion within other meadow groups indicates that bumblebee activity was more variable between sampling times.

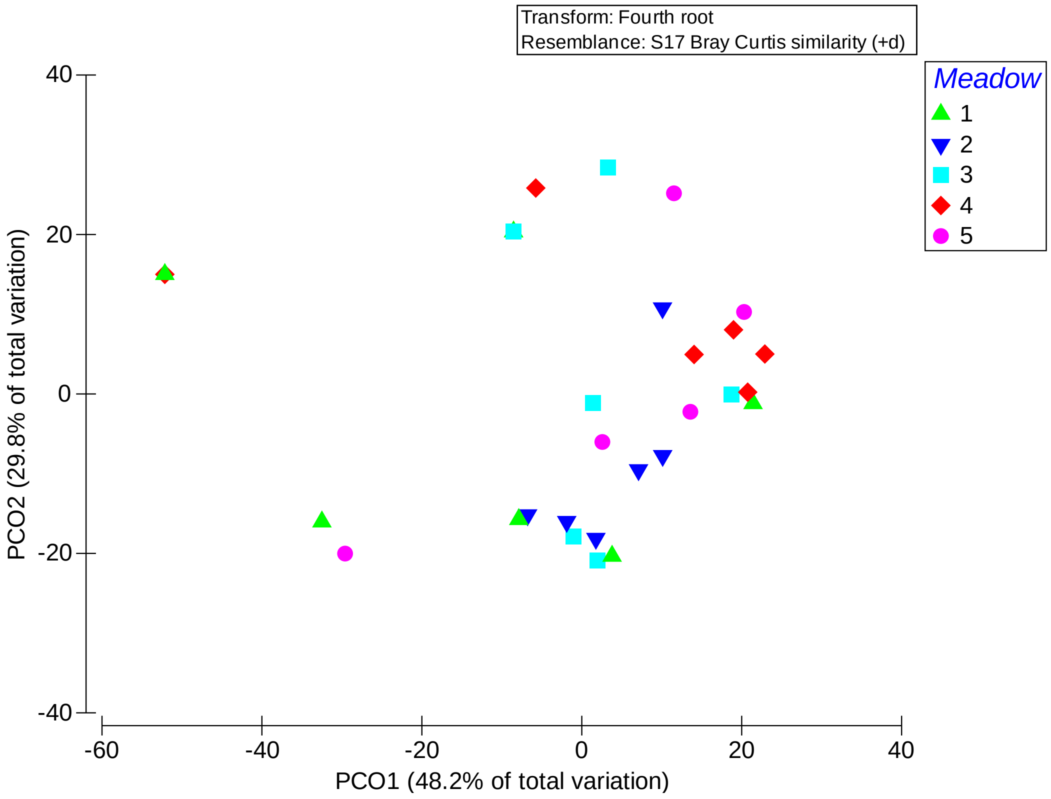


Figure 6.3. Principal Coordinates (PCO) analysis of bumblebee activity within five upland hay meadows in Northumberland between mid-June and late July 2009.

Bumblebee activity recorded in each meadow on individual assessment days is represented as samples in the CAP biplot (Figure 6.4). Correlations between temperature and time with the first two canonical axes are displayed graphically in the biplot. The squared canonical correlations of the first two canonical axes were

0.1022 and 0.0674 indicating a weak relationship between environmental variables and activity. The relationship between the environmental variables and bumblebee activity was tested using canonical test statistics. These were not statistically significant (trace statistic: *P* = 0.3287; delta squared statistic: *P* = 0.5544, using 1000 permutations).

Despite the findings of the permutation tests, correlations between environmental factors and canonical axes suggested that temperature and time did affect bee activity. Temperature ranged from 14.7^{0}C (07.07.09) to 27.7^{0}C (01.07.09). Time (0.947) and temperature (0.322) are positively correlated with axis 1. Temperature (0.947) is positively correlated with axis 2, while time (-0.322) is negatively correlated with axis 2. The majority of samples from all meadows are associated with negative axis 1 scores and positive axis 2 scores indicating a general decline in bumblebee activity over time (15th June-26th July) and with declining temperature. More samples were associated with positive axis 2 scores indicating that temperature had a stronger influence on bee activity than time, with increasing temperature having a strong positive effect on bumblebee activity in all meadows. Samples associated with positive axis 2 scores and negative axis 1 scores were characterised by greater bumblebee activity later in the assessment period, but activity levels were still positively affected by temperature. Samples with negative axis 2 scores were all negatively correlated with temperature and could be distinguished by time according to their axis 1 scores. Overall, bees continued to be active in all meadows throughout the sampling period although time had a general negative effect on bumblebee activity and temperature was the more important factor influencing activity levels on individual assessment days.

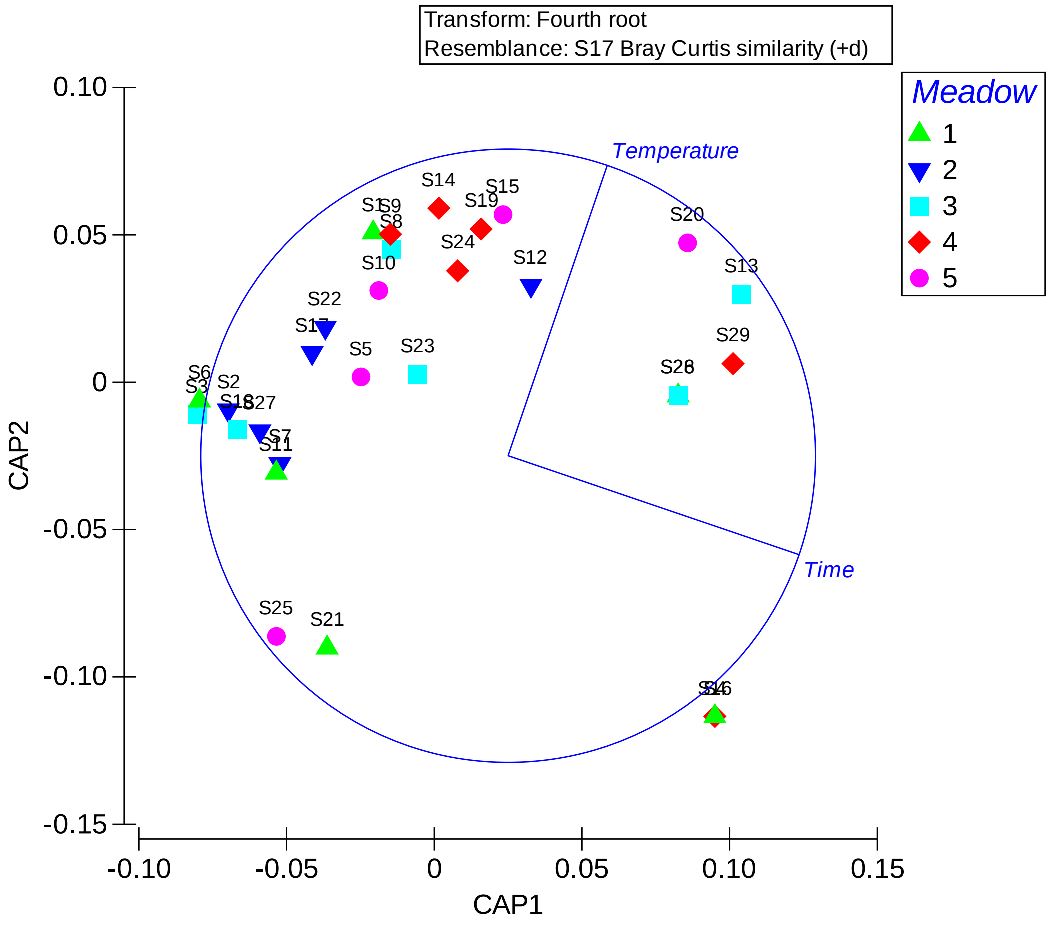


Figure 6.4. Canonical analysis of principal coordinates (CAP) of (transformed) bumblebee abundance in five upland hay meadows between June and July 2009 and constrained by temperature and time (date). Correlations for environmental predictor variables with canonical axis are superimposed on to sample distribution.

The effect of environmental factors on bumblebee activity in different meadows is discernible in the biplot. Meadow 1 samples are associated with both positive and negative scores of both canonical axes indicating that: i) bumblebee activity in meadow 1 declined steeply over time and ii) activity was little affected by temperature. This was likely to have been a consequence of an earlier reduction in the number of inflorescences in this meadow (S.E. Barlow, personal observation) and inclusion of this factor would likely have increased the predictive value of the model. The spread of samples belonging to meadows 3 and 4 in association with axis 1 also suggests a more marked decline in activity levels between early (June) and later (July) sampling dates. In contrast, meadow 2 samples are all associated with mid to low (negative) axis 1 scores indicating that activity levels remained relatively constant over time. As the majority of samples formed a large group and were all, to a greater or lesser extent, negatively correlated with time, this suggests that bumblebee activity was similar on later assessment days (July).

The contribution of individual bumblebee species to activity levels is visible in the second biplot (Figure 6.5). Partial correlations of bumblebee species with canonical axes are displayed graphically in Figure 6.5 and are provided in Table 6.2. The magnitude of the correlations indicates which bumblebee species are the drivers of the variability in activity levels between samples and can be compared with correlations of environmental factors with canonical axes. However, some species are recorded less frequently and this is reflected by fewer observations in meadows through time. For instance, *B. pratorum* and *B. jonellus* were recorded in too few transects to draw conclusions as to the effect of environmental factors on their activity levels (Table 6.2). As the frequency of observations varied between species this will have affected the strength of the correlations with canonical axes and hindered the ability of the analysis to ascertain the true effect of environmental factors on the activity of the rarer species.

With this in mind, comparisons of the two biplots indicate that the activity of all bumblebee species responded positively to temperature but differed in response to time. All bumblebee species are positively correlated with axis 2 indicating a positive correlation with temperature; however, the strength of correlation varies between species. Species showing the strongest positive correlations with axis 2 were *B. lucorum* (0.5702), *B. hortorum* (0.4594) and *B. monticola* (0.3334). Other species showed weak positive correlations with axis 2 (*B. muscorum*, 0.1644; *B. jonellus*, 0.1547; *B. pratorum*, 0.1013; *B. pascuorum*, 0.0778) indicating that they were relatively less affected by variation in temperature. Bumblebee species were either positively or negatively correlated with axis 1 and hence, time. Strong negative correlations with axis 1 were evident for *B. pascuorum* (-0.5484) and *B. muscorum* (-0.2592) indicating that they were negatively correlated with time and their activity levels declined throughout the assessment period. *B. lucorum* (-

0.1190) was weakly correlated with axis 1 suggesting that its activity levels remained relatively constant throughout the assessment period and demonstrated only a marginal decline over time. Conversely, *B. hortorum* (0.3253) was positively correlated with axis 1 indicating that it tended to be recorded more frequently during the early part of the assessment period. Remaining species (*B. monticola*, *B. pratorum* and *B. jonellus*) showed no correlation with time (axis 1 <+/-0.07).

The activity levels of *B. lucorum* and *B. monticola* may have been influenced by sampling site (i.e. meadow). The correlation of *B. lucorum* and *B. monticola* with canonical axes is associated with the distribution of meadow 4 samples, indicating that activity levels of these species were consistently higher in this SSSI meadow. The activity of other species, including *B. muscorum*, is not associated with particular meadows.

Table 6.2. Partial correlations of bumblebee species with canonical axis 1 and 2.

Bumblebee species	Correlation with CAP 1	Correlation with CAP 2	Frequency of observations (%) (of 128 bees)
Bombus lucorum	-0.1190	0.5702	40.3
Bombus pascuorum	-0.5484	0.0778	24.8
Bombus monticola	-0.0145	0.3334	11.6
Bombus hortorum	0.3253	0.4594	9.5
Bombus muscorum	-0.2592	0.1644	8.1
Bombus pratorum	0.0644	0.1013	5.1
Bombus jonellus	-0.0145	0.1547	0.5

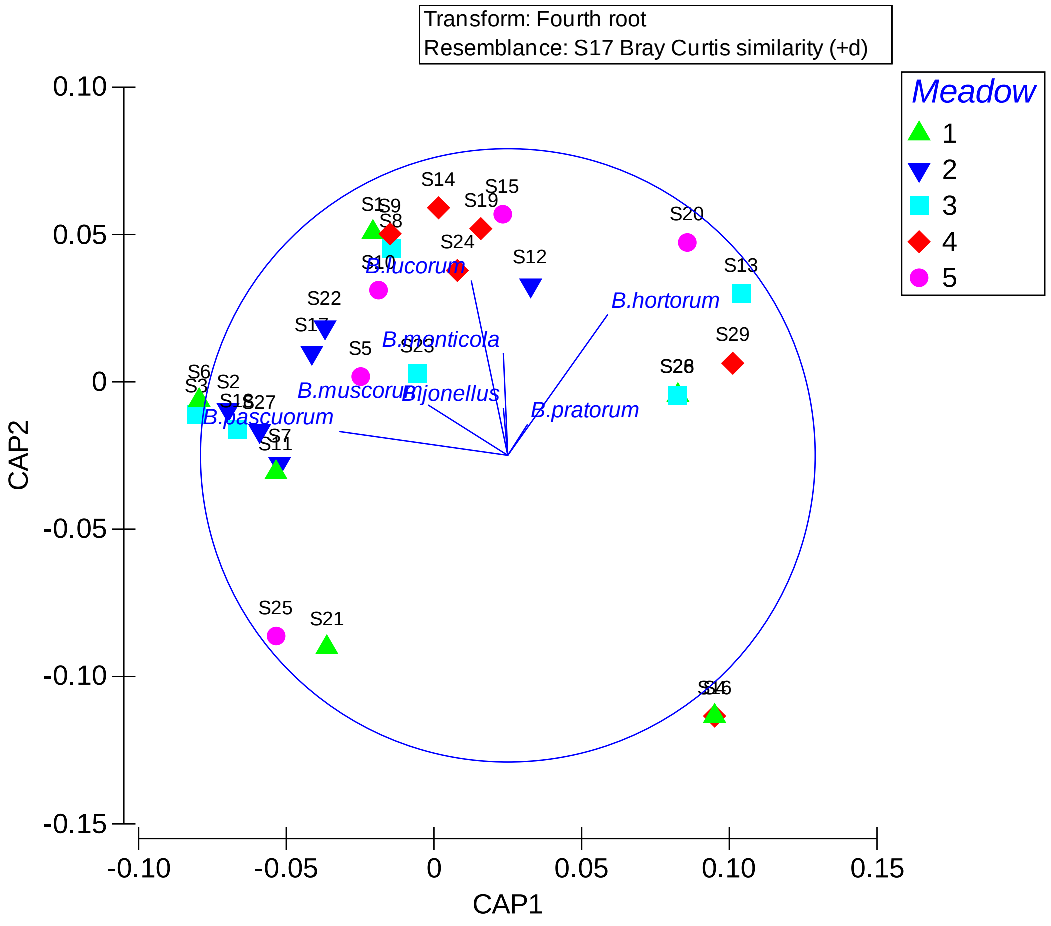


Figure 6.5. Canonical analysis of principal coordinates (CAP) of (transformed) bumblebee activity (species richness and abundance) in five upland hay meadows between June and July 2009 and constrained by temperature and time (date). Partial correlations for bumblebee species with canonical axis are superimposed on to sample distribution.

6.4.2 *Forage use*

In total 201 bees were recorded foraging from one or more of 15 plant species. Due to the small geographical scale of the study, all bees were subject to the same array of options. The frequency of visits to forage plant species by each bumblebee species (meadows and sampling times combined) is summarised in Table 6.3. Seven bumblebee species were recorded, although frequency of observations varied between species (Table 6.3). Samples were characterised by a large proportion of zero counts as most plant species received very few foraging visits by bees. Again, a statistical artefact of the data is that rarer species (i.e. those recorded less frequently) were recorded visiting fewer plants and plant species. This does not imply that rarer species are more specialised than common species because they have a narrower forage niche (i.e. diet breadth) and vice versa. Mean tongue length of each bumblebee species according to Goulson *et al.* (2005) is also included in Table 6.3.

When combining data for all bee species, sampling sites and times 87.6% of all foraging visits were to four plant species: *Rhinanthus minor* (26.5%), *Trifolium repens* (26.1%), *T. pratense* (18.4%) and *Geranium sylvaticum* (16.5%) (Table 6.3). The short-tongued species, *B. lucorum* and *B. monticola* showed similar forage use patterns. The majority of foraging visits by *B. lucorum* were to *T. repens* (40.4%) followed by *R. minor* (21.5%), *Filipendula ulmaria* (14.0%), *T. pratense* (13.1%) and *G. sylvaticum* (8.4%). *B. monticola* visited *T. repens* (47.5%) most often, followed by *R. minor* (30.4%), *G. sylvaticum* (14.3%) and, less frequently, *T. pratense* (5.4%). In contrast, the long-tongued species *B. pascuorum*, *B. hortorum* and *B. muscorum* frequently visited *T. pratense* (*B. pascuorum*, 29.7%; *B. hortorum*, 48.1%; *B. muscorum*, 60.0%) but rarely utilised *T. repens* or not at all (*B. pascuorum*, 0.5%, *B. hortorum*, 5.9%; *B. muscorum*, 0%) as a forage resource. All of these short- and long-tongued species frequently (20%>45%) visited *R. minor* and was the primary forage species used by *B. pascuorum* (43.2%). *B. pratorum* visited *G. sylvaticum* (83.3%) most often followed by *R. minor* (8.3%) and *T. repens* (5%). Only two *B. jonellus* individuals were observed foraging and both bees visited only *G. sylvaticum*. Note also the low number of observations for *B. pratorum* and *B. muscorum* and the variation in number of observations between all species. The number of plant species correlates with the number of observations thus, findings are not evidence of differences in dietary breadth between species. Seven members of the Fabaceae are represented in the list of forage species and account for 47.1% of all bee foraging visits. Other plant families were Rosaceae, Asteraceae, Orobanchaceae (formerly Scrophulariaceae), Plantaginaceae (formerly Scrophulariaceae), Geraniaceae and Campanulaceae.

Table 6.3. Forage use by bumblebees within five upland hay meadows between 15th June and 23rd July 2009. Mean tongue length of each bumblebee species is according to Goulson *et al.* (2005).

		Bombus lucorum	*Bombus pascuorum*	*Bombus hortorum*	*Bombus monticola*	*Bombus muscorum*	*Bombus pratorum*	*Bombus jonellus*	All bees
	No. of observations	92	36	24	23	13	11	2	201
	No. of plant species	8	11	7	5	3	3	1	15
	No. of plants visited	344	118	77	112	30	60	24	765
	Mean tongue length (mm)	7.5	8.5	12.5	7.5	8.7	7.3	6.4	
Frequency (%) of foraging visits to each plant species	*Rhinanthus minor* L.	21.5	43.2	37.7	30.4	33.3	8.3	0	26.5
	Trifolium repens L.	40.41	0.9	5.2	47.3	0	5.0	0	26.1
	Trifolium pratense L.	13.1	29.7	48.1	5.7	60.0	0	0	18.4
	Geranium sylvaticum L.	8.4	4.2	0	14.3	6.7	83.3	100.0	16.5
	Filipendula ulmaria L. (Maxim)	14.0	0	1.3	0	0	3.3	0	6.7
	Cirsium heterophyllum L.	0	1.7	5.2	2.7	0	0	0	1.2
	Geum riviale L.	1.2	5.1	0	0	0	0	0	1.3
	Lathyrus pratensis L.	0	6.8	0	0	0	0	0	1.1
	Vicia sepium L.	0	5.9	0	0	0	0	0	0.9
	Campanula rotundifolia L.	1.2	0	0	0	0	0	0	0.5
	Lotus corniculatus L.	0.3	0	1.3	0	0	0	0	0.3
	Leontodon autumnalis L.	0	0.9	0	0	0	0	0	0.1
	Digitalis purpurea L.	0	0	1.3	0	0	0	0	0.1
	Vicia cracca L.	0	0.9	0	0	0	0	0	0.1
	Lathyrus linifolius L.	0	0.9	0	0	0	0	0	0.1

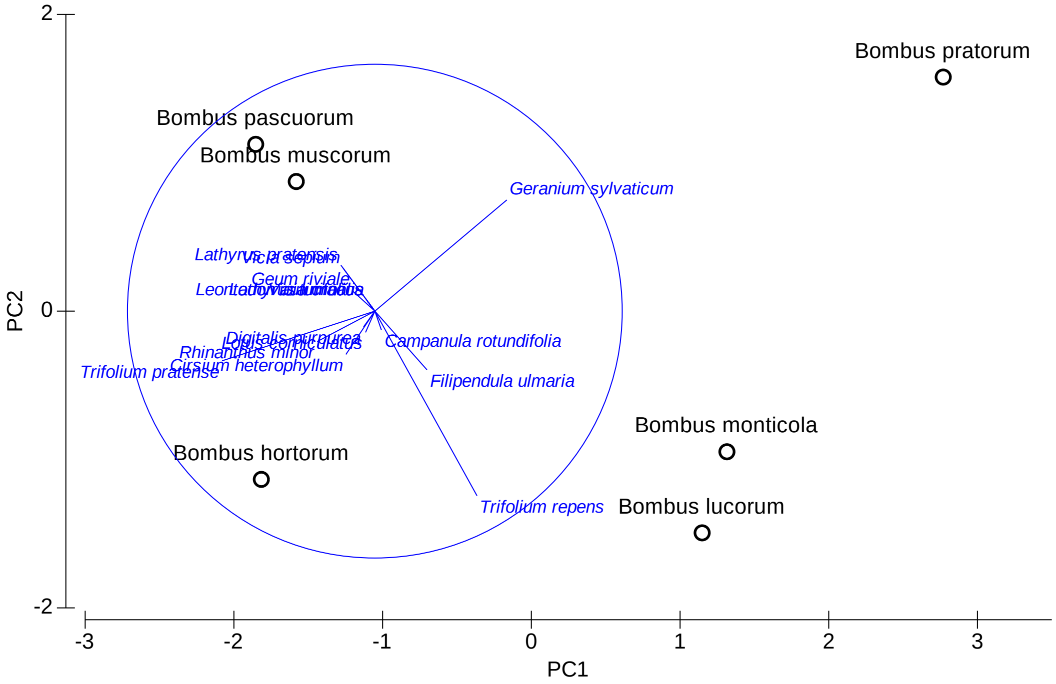


Figure 6.6. Principal Components Analysis (PCA) of the proportion of visits to different plant species by each bumblebee species. PCA axis 1 and axis 2 explain 53.8% and 24.1% of variation, respectively. *Bombus jonellus* is excluded from the analysis as only two observations were recorded.

The first two axes of the PCA analysis explained 77.9% of the variation in visitation patterns to different plant species (Figure 6.6). The first principal component (53.8% of variation) separated species on their usage of *T. pratense*, *T. repens* and *G. sylvaticum* and by tongue-length. *T. pratense* (-0.618) has a strong negative correlation with axis 1 and is associated with long-tongued species (*B. hortorum*, *B. muscorum* and *B. pascuorum*). Other plant species negatively correlated with axis 1 are typically those with deep corollae and include *R. minor* and five Fabaceae species. The weak negative correlation between *R. minor* (-0.233) and axis 1 indicates that overall it was more regularly visited by long-tongued species. In contrast, *T. repens* (0.413) and *G. sylvaticum* (0.534) are positively correlated with axis 1 and are associated with short-tongued species (*B. lucorum* and *B. pratorum*). The main drivers of axis 2 are *T. repens* (-0.748) and

G. sylvaticum (0.451) indicating that *B. lucorum* and *B. monticola* visited *T. repens* more often than *G. sylvaticum* and *B. pratorum* showed the opposite visitation pattern. In addition, *B. hortorum* also occasionally visited *T. repens* causing it to be separated from *B. pascuorum* and *B. muscorum* on axis 2. Tongue-length and PCA axis 1 were negatively correlated although the relationship was not significant (r^2 = 0.46; Pearson's correlation coefficient -0.680, $P = 0.137$). This finding was likely influenced by *B. hortorum* which has the longest tongue but visited *T. repens* unlike *B. muscorum* or *B. pascurorum*.

Note that the inclusion of *B. jonellus* in the analysis (PCA plot not shown) increased the strength of the positive correlation between *G. sylvaticum* and axis 1 as expected because 100% of all foraging visits were to *G. sylvaticum*. While this was true, data was based on observations of just two bees and does not constitute a valid assessment of forage use. *B. jonellus* also has the shortest tongue-length and increased the negative correlation between tongue length and axis 1 accordingly (r^2 = 0.58; Pearson's correlation coefficient -0.759, $P < 0.05$).

6.5 Discussion

Barrowburn SSSI meadows and other meadows connected in the landscape supported an important array of bumblebee species. Of the seven species (235 individuals) recorded, four of the six common species in mainland Britain were present: *B. lucorum*, *B. pascuorum*, *B. hortorum* and *B. pratorum*. The other common species, *B. terrestris* and *B. lapidarius* were conspicuous by their absence. Three scarce/rare species characteristic of Northern Britain were also recorded namely, *B. monticola*, *B. jonellus* and, the UKBAP priority species, *B. muscorum* (Williams 1982). The most frequently recorded species throughout the sampling period were *B. lucorum* and *B. pascuorum* accounting for 43.40% and 20.85% of all bees, respectively. These findings add support to numerous studies that have identified the importance of species-rich grassland to bumblebees (Carvell 2002; Goulson 2003; Kells & Goulson 2003; Goulson, Lye & Darvill 2008), and their particular importance in upland areas in Northern England to the long-term conservation of *B. muscorum* (Darvill, Mabon & Rolph 2010).

B. jonellus was rarely seen in the meadows and is known to be strongly associated with moorland and heathland and Ericaceae forage plants such as heather (*Calluna vulgaris* L. (Hull)) and bilberry (*Vaccinium myrtillus* L.) (Goulson *et al.* 2005; Darvill, Mabon & Rolph 2010); however, at the time of the survey most Ericaceae plants were not in flower. The rarity of *B. jonellus* in the meadows may have been influenced by foraging range as nests are likely to have been in nearby heathland.

Permutation tests performed within the CAP analysis did not find a significant relationship between temperature and time with bumblebee activity. These findings were expected as the sampling period (June-July) was within the main period of activity for workers of all species and did not span the entire period of activity (i.e. spring to late-summer). However, correlations with canonical axes indicated that activity levels of all species were positively affected by temperature (within the range 14.7^{0}C to 27.65^{0}C). Furthermore, a general decline in activity levels was evident between mid-June and late-July, although variability in the number of observations of each species hindered the ability of the model to ascertain the true effect of environmental factors on each species. The inclusion of botanical variables in the CAP analysis would have been beneficial as it is likely that changes in the abundance of inflorescences throughout the summer would have explained a greater amount of variation in bee activity than time. Results also suggested that activity levels of *B. lucorum* and *B. monticola* were consistently higher in one of the SSSI meadows throughout the sampling period. There was no other evidence to suggest that the activity of bee species differed between meadows, indicating that all meadows were important for bees during June and July; however, any habitat preference by rarer species would not have been apparent. Similar findings were reported by Darvill, Mabon and Rolph (2010) who found the absolute number of bumblebees was not correlated with plant species richness, and abundance was similar in species-rich and species-intermediate meadows in Upper Teesdale. The design of this study did not enable us to assess whether the connectivity of species-rich and species-intermediate meadows influenced the activity of bees, although it is likely that bumblebee activity in species-intermediate sites is promoted by the increasing proximity of forage-rich sites (Carvell *et al.* 2011).

Despite the floristic diversity in Barrowburn meadows (especially in the SSSI meadows) all bumblebees predominantly visited a small number of plant species. The plant species visited most frequently by all bumblebee species were *R. minor*, *T. repens*, *T. pratense* and *G. sylvaticum*, accounting for 87.58% of all foraging visits. Furthermore, 7 of the 15 plant species used as forage were members of the Fabaceae and accounted for 47.06% of all foraging visits. Results of the PCA ordination also identified a distinction in forage use according to tongue-length with *T. pratense* and other leguminous species visited predominantly by long-tongued species, particularly *B. pascuorum* and *B. muscorum*. The exception being *T. repens* which was visited more frequently by short-tongued species, in particular *B. lucorum* and *B. monticola*. Results are in accord with previous studies which have shown that although bumblebees will visit a wide range of plant species, they have clear preferences for a relatively short list of key forage plants, most notably members of the Fabaceae, Lamiaceae, (former) Scrophulariaceae, Boraginaceae and Ericaceae (Goulson *et al.* 2005; Darvill, Mabon & Rolph 2010). It is well known that members of the Fabaceae particularly *T. pratense* and *T. repens* are important forage plants for bumblebees (Goulson 2003; Goulson & Darvill 2004; Goulson & Hanley 2004; Goulson *et al.* 2005; Carvell *et al.* 2006); for example, Goulson *et al.* (2005) found the Fabaceae to be the most highly visited family by bumblebees collecting pollen and nectar; and *T. pratense* and *T. repens* to be the most frequently visited plants for pollen and were ranked in the top three species for nectar collection by all bee species studied. Members of the Fabaceae, especially *T. pratense*, have highly protein-rich pollen (Hanley *et al.* 2008) which is preferentially collected by bumblebees, particularly long-tongued and rare species including *B. muscorum* (Carvell 2002; Goulson & Darvill 2004; Goulson & Hanley 2004; Goulson *et al.* 2005; Carvell *et al.* 2006; Goulson, Lye & Darvill 2008). A possible reason why some bumblebee species may forage predominantly from plants with high quality pollen is their short colony cycle. Species with short colony cycles, such as *B. muscorum*, have less time in which to develop and rear reproductives (males and new queens) and may require high quality pollen to do this successfully (Goulson & Darvill 2004). Most late emerging species are medium or long tongued and have declined in association with the loss of Fabaceae-rich unimproved grassland and clover leys in the UK and much of Europe (Rasmont 1988; Goulson *et al.* 2005; Carvell *et al.* 2006; Goulson 2006;

Goulson, Lye & Darvill 2008). Areas in Europe where Fabaceae specialists remain abundant are those where traditional farming and clover leys still persist (Goulson, Lye & Darvill 2008).

Previous studies also report that bumblebees frequently visit *R. minor* for nectar and pollen indicating that it is a relatively highly rewarding forage species (Goulson & Darvill 2004; Goulson *et al.* 2005; Hanley *et al.* 2008). Carvell *et al.* (2006) also found *R. minor* to rank highly as a forage plant and notes that visitation was somewhat greater by long-tongued species. Kwak (1979) described how bumblebees would visit flowers of *R. minor* in different orientations depending on tongue-length, whereby long-tongued bees visited flowers in an upright position with pollen transferred to the bee's dorsal surface (nototribic position), and short-tongued bees visited flowers in a downwards facing position with pollen transferred to the bee's ventral surface (sternotribic position). We did not observe any bees visiting flowers in a sternotribic position, although *B. pascuorum* tended to visit flowers at a 90^0 angle with pollen likely to be transferred to the dorsal side of the bee. As very few bumblebee studies have been conducted in upland hay meadows little is known about the frequency of use of *G. sylvaticum* by different bumblebee species or its floral reward in comparison with other forage plants. In addition to *G. sylvaticum*, bumblebees visited *Cirsium heterophyllum* (Melancholy thistle) and *Lathyrus linifolius* (Bitter-vetch), all northern and upland plant species characteristic of species-rich meadows and reliant on traditional hay-making practices.

The findings of this study would suggest that floristic diversity *per se* was not of overriding importance with bumblebee activity and fidelity of forage use probably due to the presence and relatively high abundance of highly rewarding forage plants in all meadows which has been reported by a number of previous studies (Williams 1989; Carvell 2002; Carvell *et al.* 2007). Bumblebees are able to select rewarding flowers (in terms of nectar sugar content) through learned associations between the nectar reward and flower scent, colour or shape (Goulson 1999; Stout & Goulson 2002; Goulson *et al.* 2007). As a result bees tend to exhibit flower constancy, making repeated visits to the more rewarding plant species. Whether bees can make learned associations between pollen reward and floral characteristics is as yet unclear, though recent studies indicate that pollen quality (protein content) may be the more critical determinant of floral reward to many species (Hanley *et al.* 2008 and references therein).

A diverse assemblage of bumblebee species in upland hay meadows will provide important pollinator services to a high diversity of plant species. Many wildflower species occurring in traditionally managed meadows rely predominantly or exclusively on pollination by bumblebees and there is strong evidence that plants with an obligate breeding system also have higher quality pollen (Hanley *et al.* 2008). Declines in pollination services to these species in particular, may reinforce further declines in bumblebee populations in a positive feedback cycle if seed production is pollen-limited and plant populations are limited by seedling recruitment. Future research is necessary to establish whether reproductive output of insect-pollinated plants in upland hay meadows is suffering from pollen limitation as a result of declining pollinator populations.

In conclusion, continuing efforts to restore and protect botanically diverse upland hay meadows will be important in providing rewarding forage resources to populations of common and rare/scarce bumblebee species in the uplands, and in supporting adequate pollination services to native wildflowers within a scarce plant community. As *T. pratense* and *R. minor* are amongst the first species to be introduced to species-poor meadows targeted for restoration, the benefits to bumblebees are likely to be fast-acting. Furthermore, targeting relevant agri-environment scheme options, such as uncut margins and pollen and nectar wildflower strips within or near to meadows will benefit bumblebees and biodiversity in general.

6.5.1 Study limitations and future recommendations

The main problem with analysis of the data was uneven sample size of different bumblebee species and therefore comparisons between species were problematic. In order to make comparisons between species in terms of habitat and forage preference, future work should include a replicated design of species-rich and species-intermediate meadows (and other habitats) and data analyses should account for uneven sample size and forage availability. As foraging is resource-limited and foraging behaviour is adaptive, it would be necessary to collect data concerning the presence/absence and abundance of (flowering) forage species and bee species. A CAP analysis could be used to analyse this type of data. Alternatively matrix comparison methods could be used such as a Mantel Matrix Randomization Test and Procrustean Randomizations Test (Legendre & Legendre 1998). The techniques are sophisticated and determine the relationships between two or more data matrices. A matrix quantifying the foraging activity of bumblebees within each meadow and at each sampling point is generated. This may then be related to a matrix of forage plant species which describes plant phenology; for example 0 or 1 to indicate the presence of a flowering forage plant species in that meadow at a given point in time. This would provide the means to determine the relationships between the presence of flowering forage plants and the frequency of bumblebee visits. Without this information we cannot accurately determine variability in the frequency of visits by bumblebees to forage plants and forage preferences by different bumblebee species.

Chapter 7. Is plant reproductive output limited by pollinator services in a species-rich upland hay meadow? A case study of *Rhinanthus minor* and *Geranium sylvaticum*

7.1 Abstract

World-wide declines in pollinators and insect-pollinated plants warn of likely reductions in pollination services and increased pollen limitation for many wildflower and crop species. Pollen limitation of plant reproductive output may have demographic consequences for plant populations depending on plant life history traits and should be considered in the conservation of diverse and fragmented grassland such as unimproved upland hay meadows, which contain many wildflowers reliant on insect pollination services for their reproduction. In this study, the effects of supplemental hand-pollination on the reproductive output of the hemi-parasitic annual, *Rhinanthus minor* and the gynodioecious perennial, *Geranium sylvaticum* were tested for populations present in a species-rich upland hay meadow in Northern England. Pollen availability did not limit fruit set, seed production, seed mass or seedling emergence of *R. minor* or either sex morph of *G. sylvaticum*. Greater reproductive output of female *G. sylvaticum* plants was evident, regardless of pollination treatment in terms of seed quantity and quality, as is common in gynodioecious species and acts to maintain gynodioecious populations. Results suggest that neither species is currently pollen limited at the study site and may reflect their relatively general breeding system and attractive traits, such as floral phenotype, although alternative possibilities are discussed including resource-limitation and problems associated with hand-pollination.

Key-words: female fitness, fruit set, gynodioecy, hand-pollination, pollen limitation, pollinator declines, seed set

7.2 Introduction

Land use change and agricultural intensification have caused parallel declines in pollinator and plant abundance and diversity (Potts *et al.* 2010). After the Second

World War, increasing emphasis on enhancing agricultural productivity saw a decline in traditional farming methods and a rapid increase in intensive management including the greater usage of fertilisers and pesticides, a switch to silage production and increasingly consolidated fields. This caused the dramatic loss, degradation and fragmentation of semi-natural habitat such as species-rich hay meadows and consequently a reduction in forage diversity and connectivity (Pywell *et al.* 2005). For example, in Britain, over 90% of unimproved species-rich grassland has been lost since the mid-20th Century (Blackstock *et al.* 1999). Social (non-parasitic) bumblebees are particularly vulnerable to reduced forage availability, as workers must forage for themselves and developing larvae in order for sexual reproductives to be produced (Goulson 2006). Analyses of long-term datasets have shown declines in many British bumblebee populations to be associated with a 76% decline in frequency of bumblebee forage plants between 1978 and 1998 (Carvell *et al.* 2006). For bumblebees, there is some evidence to suggest that dietary and habitat specialism may also contribute to declines (Goulson *et al.* 2005; Williams 2005) with many rare bumblebee species showing strong associations with members of the Fabaceae and unimproved grassland (a Fabaceae-rich habitat), probably due to a dependency on protein-rich pollen (Goulson, Lye & Darvill 2008; Hanley *et al.* 2008).

Parallel declines in pollinator and insect-pollinated plant populations, particularly of bees and bee-pollinated plants, have raised concerns over reductions in pollinator services to crops and wildflowers (Kearns, Inouye & Waser 1998; Biesmeijer *et al.* 2006; Potts *et al.* 2010). The concern being that a decline in pollinator services may cause a reduction in plant reproductive output (i.e female fitness) and a subsequent reduction in the size of plant populations, thus reinforcing further pollinator declines in a positive feedback loop. For wildflower populations, the demographic consequences (and magnitude of positive feedback to pollinator populations) of lower female fitness will depend on the role of seeds in population dynamics and this role will depend on life history traits of the species in question; for example, annual species that do not form a persistent seed bank will be affected sooner than long-lived iteroparous species capable of vegetative reproduction. However, few studies have investigated whether pollen limitation of seed set has effects at the population level (but see Ashman *et al.* 2004; Hegland & Totland 2007). A reduction in reproductive output would also be of ecological importance to populations of seed-eating animals (Young 1982), while plant population declines would have wider reaching consequences for biodiversity at large. It is therefore essential to conserve and restore botanically diverse habitat to provide forage resources for pollinators and to maintain adequate pollination services to wildflowers; thereby supporting a positive feedback mechanism that benefits, rather than diminishes, both parties. This is particularly important for the conservation of small and fragmented populations of native plants. Declining pollinator services are a global concern and have led to special initiatives by the Convention on Biological Diversity (CBD 2011). Currently, there are several national and international programmes aimed at researching the complex causes and consequences of pollinator declines and appropriate mitigation strategies, such as the STEP Project (Status and Trends of European Pollinators) (STEP Project 2012) and, in the UK, the Insect Pollinators Initiative (LWEC 2012).

For pollination services to be described as limiting plant reproductive output requires evidence of pollen limitation i.e. female fitness is limited by the quantity (or quality) of pollen received (Knight *et al.* 2005). Pollen limitation may occur through decreased visitation or altered pollinator efficiency (Hegland & Totland 2007) and is most commonly tested by way of pollen-supplementation experiments whereby the reproductive output of naturally-pollinated (control) plants is compared with plants given supplemental pollen (e.g. Burd 1994; Agren 1996; Zimmerman & Pyke 1998; Ashman *et al.* 2004; Knight *et al.* 2005; Aguilar *et al.* 2006). Several meta-analyses (Burd 1994; Larson & Barrett 2000; Knight *et al.* 2005; Aguilar *et al.* 2006) suggest that pollen limitation is a common phenomenon, for example Burd's (1994) review of 258 wildflower species in 77 families found that 62% of species were significantly pollen limited with significant results being found more frequently for fruit set rather than seed set, and for self-incompatible compared with self-compatible species. Burd (1995) proposes that plants may be adapted to a stochastic pollination environment by producing an excess of flowers or over-packing of ovules in flowers in order to take advantage of unpredictably high pollinator visitation and pollen receipt. It is no surprise therefore that some flowers remain unpollinated or under fertilized and such a strategy would come hand in hand with common pollen limitation results.

A criticism of pollen limitation studies is that several factors may contribute to ambiguous results and the possibility of Type I or Type II error, including: i) resource allocation to hand-pollinated flowers if not all flowers receive supplemental pollen (Knight, Steets & Ashman 2006; Wesselingh 2006); ii) hand-pollinated flowers may receive an excess of out-cross pollen above naturally obtainable levels (Young & Young 1992); iii) resource limitation may be operating in conjunction with pollen limitation (Asikainen & Mutikainen 2005a); iv) hand-pollination may have negative effects such as damage to reproductive structures, pollen-tube overcrowding due to excessive pollen loads, or attraction of pollen thieves (Young & Young 1992); v) the measure of female fitness used as the response variable may influence results; and, vi) the experimental design may suffer from low statistical power as a consequence of inadequate sample size (Young & Young 1992; Thomson 2001). Furthermore, meta-analyses may be misleading as reviews by Thomson (2001) and Knight, Steets and Ashman (2006) suggest an existing publication bias towards significant findings (i.e. hand>natural). Results are also complicated by the ability of some plants to control the quantity and quality of their seeds in response to available resources by aborting some flowers fertilised by self-pollen, or fruits with under-fertilised ovules (Stephenson 1981).

Obligate out-crossing insect-pollinated plants, particularly those which rely on specialist pollinators are most vulnerable to declines in pollination services (Burd 1994; Waser *et al.* 1996; Larson & Barrett 2000; Aguilar *et al.* 2006; Biesmeijer *et al.* 2006). Furthermore, pollinator visitation is known to be influenced by plant attractiveness, and previous studies have found evidence of positive correlations between pollinator visitation and the following factors: floral phenotype and floral reward (nectar and pollen quality and quantity) (Goulson 1999; Stout & Goulson 2002; Hanley *et al.* 2008); plant population size (Agren 1996) and density (Kunin 1993; Kunin 1997) and presence of co-flowering species (Knight *et al.* 2005).

In this study, a pollen supplementation experiment was performed on the hemi-parasitic annual *Rhinanthus minor* L. and the gynodioecious (hermaphrodite and female flowers on separate plants) perennial *Geranium sylvaticum* L. to investigate whether reproductive output (quantity and quality) was pollen limited. These species were frequently visited by foraging bumblebees in upland hay meadows in Northumberland (Chapter 6). We also conducted a germination experiment using seed from hand-pollinated and control plants to test whether supplemental pollen had subsequent effects on seed quality. The gynodioecious breeding system of *G. sylvaticum* presented an opportunity to compare reproductive output between genders. As hermaphrodite flowers are reported to be larger and attract a greater proportion of pollinator visits than females (Asikainen & Mutikainen 2005b and see below for further details), flower size was also compared between sex morphs. Plants were growing within a species-rich upland hay meadow conforming to the National Vegetation Classification (NVC) grassland community MG3 (*Geranium sylvaticum-Anthoxanthum odoratum* grassland) (Rodwell 1992) – an internationally rare grassland community of high conservation interest threatened by agricultural intensification and habitat fragmentation (Jefferson 2005).

7.2.1 *Study species*

Rhinanthus minor

Figure 7.1. *Rhinanthus minor* L. (floralimages.co.uk 1997)

Rhinanthus minor (Yellow rattle) (Onobranchaceae) is an erect (to 50 cm) annual, facultative root hemi-parasite (Westbury 2004) (Figure 7.1). It has been recorded in 22 NVC plant communities but is most abundant in MG3 meadows (Rodwell 1992; Westbury 2004) where it functions as a keystone species by suppressing the abundance of competitive grass species and enabling a wider diversity of wildflower species to develop and persist in the sward (Smith *et al.*

2003; Pywell *et al.* 2004; Bardgett *et al.* 2006). *R. minor* responds negatively to agricultural improvement and has declined nationally in recent decades as a result (Westbury 2004; Carvell *et al.* 2006). Flowers are in short leafy spikes and are solitary, zygomorphic[1], hermaphrodite, didynamous[1] and self-compatible. Stamens are located in the upper lip of the yellow to yellow-brown corolla (12-15(-17) mm). Fruit is a many seeded capsule containing large seeds which 'rattle' inside the calyx when ripe. Seed dispersal is aided by hay-cutting machinery. *R. minor* has a transient seed bank with the majority of seedlings emerging the following spring, although Thompson, Bakker and Bekker (1996) report seed to persist for over three years. Flowers are insect-pollinated by bees, particularly bumblebees (*Bombus*) (Kwak 1979; Gibson 1986; Proctor, Yeo & Lack 1996), or selfed. Long-tongued bumblebees are reported to visit flowers nototribically, landing on the lower lip of the corolla facing upwards and are likely to affect cross-pollination. In contrast, short-tongued bumblebees may visit flowers sternotribically, landing on the upper lip facing downwards and may promote self-pollination owing to poor pollen transfer to the inaccessible stigmas (Kwak 1979). Short-tongued nectar thieves may also promote self-pollination by dislodging pollen (Gibson 1986).

The resources available for reproduction are largely dependent on the quality of the parasitized host species. For the purposes of the study, *R. minor* plants were randomly selected but were not used in areas atypical of the meadow vegetation at large or if individuals were exhibiting morphological symptoms such as stunting or lack of vigour. Those plants used in the study were therefore presumed to be parasitizing hosts of similar quality.

Geranium sylvaticum

Geranium sylvaticum (Wood Crane's-bill) (Geraniaceae) is a perennial herb, characteristic of MG3 meadows (Rodwell 1992) and has declined in recent decades. Pacha and Petit (2008) found that *G. sylvaticum* populations had disappeared from 40% of sites previously occupied in 1980 due to agricultural

---

[1] See glossary

improvement. Plants are hemicryptophytes[1] and effective clonal dispersers, capable of rhizomatous growth, with one individual consisting of several ramets[1] belonging to a single rhizome (Asikainen & Mutikainen 2003). *G. sylvaticum* has a gynodioecious breeding system whereby female flowers and hermaphroditic flowers exist on separate individuals (Darwin 1877) (Figure 7.2a,b). The solitary actinomorphic[1] flowers are typically light purple (although range from dark purple to white) with an ultraviolet light-absorbing centre and visible-light nectar guiding marks. Females are usually pistillate[1] but some plants may have female flowers with vestigial[1] anthers. Intermediate plants also exist with flowers consisting of 1-9 functional anthers and are functionally hermaphrodite. Typical hermaphrodite flowers contain five pistils and ten anthers, arranged in two whorls of five (Asikainen & Mutikainen 2003). Hermaphrodite flowers are self-compatible but protandrous, meaning anthers produce pollen before stigmas are receptive. This dichogamy (i.e. temporal separation) is a strategy to prevent self-fertilization via autogamous[1] pollination, but does not stop geitonogamous[1] pollination which is likely to be common. Pollination is predominantly by bees, particularly bumblebees, syrphid flies and other dipterans (Asikainen & Mutikainen 2003; Asikainen & Mutikainen 2005a). Fruit is a schizocarp, containing five locules with each locule bearing two ovules. The maximum number of seeds per fruit is therefore ten. Studies of *G. sylvaticum* populations in Finland report that fruit usually contain less than five seeds, but as many as seven seeds have been found within a fruit (Asikainen & Mutikainen 2003; Asikainen & Mutikainen 2005a).

Gynodioecious species often display morphological differences between sex morphs with hermaphrodite plants commonly having larger, and more, flowers than females. Hermaphrodites may also offer a greater level of nectar reward, as well as pollen reward, to pollinators; for example Delph and Lively (1992) found hermaphrodite flowers of *Hebe stricta* (Benth.) to produce over four times as much nectar as females. For gynodioecy to persist, female plants must compensate for the lack of male fitness (i.e. pollen production) and lower pollinator attractiveness by allocating resources (that may otherwise be used in pollen production and pollinator attraction) to increasing female fitness i.e. by producing more seeds or

---

[1] See glossary

better quality seeds relative to hermaphrodites (Lloyd 1975; Lloyd 1976; Charlesworth & Charlesworth 1978). Females may also benefit in terms of seed quality, as a result of cross-fertilization and reduced inbreeding depression (Charlesworth & Charlesworth 1978; Mutikainen & Delph 1996). Female frequency is also affected by sex-determining genes which affect the fecundity advantage needed by females for sufficient compensation (Lewis 1941; Charlesworth & Charlesworth 1978; Gouyon & Couvet 1988). A number of studies of gynodioecious species have found females to produce more seeds than hermaphrodites (e.g. Shykoff 1988; Agren & Willson 1991; Lopez-Villavicencio *et al.* 2005), but some studies have found no differences (e.g. Delph & Lively 1992), or the opposite trend (e.g. Alonso, Mutikainen & Herrera 2007) including an early study of *G. sylvaticum* (Vaarama & Jaaskelainen 1967). Previous studies of *G. sylvaticum* populations in Finland have found females within most study populations to display higher seed quantity but not quality (Asikainen & Mutikainen 2003; Ramula & Mutikainen 2003) despite females having fewer and smaller flowers than hermaphrodites and attracting fewer pollinator visitors (Asikainen & Mutikainen 2005b). Tests of pollen limitation have shown female plants to be no more pollen limited than hermaphrodite plants, rather hermaphrodites were more pollen limited than females in terms of fruit set, suggesting that hermaphrodites are equally dependent on pollinator services as females. However, whereas hermaphrodites may invest more resources on extra and larger flowers and thus attract more pollinators, females may benefit by lower expenditure of resources on pollinator attraction (fewer and smaller flowers) and allocation of resources towards seed production (Asikainen & Mutikainen 2005a). In the same study, Asikainen and Mutikainen (2005a) found that reproductive output was simultaneously pollen-limited and resource-limited for both hermaphrodite and female plants, although seed production was more limited by resources than pollen availability. The potential female advantage arising from more abundant resources is therefore more likely to be maintained when resources, rather than pollen, limit reproductive output.

a) **b)**

Figure 7.2. *Geranium sylvaticum* L. a) A hermaphrodite flower showing the outer whorl of stamens (male) and the stigmas (female) at the centre. The protandrous flower is in its male, pollen producing phase prior to the opening-up of the stigmas. b) A female flower showing the open receptive stigmas. (floralimages.co.uk 1997)

7.3 Method

7.3.1 *Study site*

In Britain, MG3 meadows are found only in the uplands of Northern England with a few outliers in Scotland and are reliant on traditional hay-making practices. The meadow used in the study (Askrigg Bottoms SSSI) is located in the Yorkshire Dales National Park (SD 948903) on the banks of the River Ure and was designated as a SSSI in 1984. The surrounding landscape is predominantly semi-improved and improved grassland managed for livestock grazing and silage production.

7.3.2 *Experimental design*

Within the meadow, 60 *R. minor* plants and 40 hermaphrodite and 40 female *G. sylvaticum* plants were randomly selected at the onset of flowering in May 2009. Plants were randomly assigned to a pollination treatment group (natural-pollination, hand-pollination) in equal proportions. As *G. sylvaticum* plants consisted of several ramets, one flowering stem of similar size was selected for each plant and used for further study. For plants within the hand-pollination treatment group, an attempt was made to provide supplemental pollen to all flowers throughout the flowering period. Plants were visited weekly and all receptive flowers were provided with out-cross pollen from nearby donor plants by brushing newly dehisced (pollen-loaded) anthers across the receptive stigmas (pistillate phase of protandry). Sepals of all hand-pollinated flowers were marked with acrylic paint so that flowers did not receive supplemental pollen on more than one occasion and the proportion of flowers receiving supplemental pollen could be calculated. As *G. sylvaticum* flowers open synchronously and hermaphrodite flowers are protandrous, it was inevitable that some receptive flowers escaped hand-pollination between weekly visits. At the end of flowering and prior to dehiscence, all *R. minor* plants and all *G. sylvaticum* marked stems were bagged using perforated bread bags and left *in situ* until all fruit had reached maturity.

DATA COLLECTION

Plants (or stems) were removed to the laboratory for assessment of the following parameters of female fitness:

1. Fruit set (%) = (Total number of fruits/Total number of flowers)*100
2. Number of seeds per fruit = Total number of seeds/Total number of fruits
3. Number of seeds per flower = Total number of seeds/Total number of flowers
4. Seed mass = Total seed mass/Total number of seeds

In addition to seed mass, seed quality was assessed in terms of the number of emerged seedlings. Sixty seeds were randomly selected from each pollination treatment group for each plant species (and for *G. sylvaticum*, each gender). Prior to sowing, *G. sylvaticum* seeds were scarified with sandpaper. All seed was sown in seed trays in John Innes no. 2 compost and stratified in a chilling cabinet at 4^0C for approximately 2-3 months. At the first signs of emergence for each species, trays were removed to a glasshouse and the subsequent number of emerged seedlings recorded weekly for two (*R. minor*) or three (*G. sylvaticum*) weeks. Note slug grazing of *R. minor* seedlings prevented accurate assessments of emergence after two weeks.

As hermaphrodite flowers of *G. sylvaticum* are reported to be larger and attract more pollinators than females (Asikainen & Mutikainen 2003; Asikainen & Mutikainen 2005b; Asikainen & Mutikainen 2005a), we also measured petal length of hermaphrodite and female flowers. Samples of 15 hermaphrodite and 15 female plants, from which 5 flowers per plant and 2 petals per flower were randomly selected and petal length measured using digital callipers.

7.3.3 *Statistical analysis*

For *R. minor*, each measure of female fitness, except seedling emergence, was compared between pollination treatment groups using two-sample t-tests. For *G. sylvaticum*, the effect of gender and pollination treatment on female fitness was determined by nested ANOVA. Petal length was also analysed with nested ANOVA using gender, flower and petal as factors. All data were tested for the assumptions of ANOVA prior to analysis and transformed where appropriate. Analyses were performed in Minitab 16 (Minitab 16 Statistical Software 2010).

7.4 Results

7.4.1 Rhinanthus minor

Results of two-sample t-tests showed there were no significant differences between hand-pollinated and naturally-pollinated plants in terms of percentage fruit set, seeds per fruit, seeds per flower or seed mass indicating that reproductive output was not pollen limited (Table 7.1). *R. minor* plants demonstrated high percentage fruit set (Hand: 99.1%±0.7; Natural: 96.0%±2.2) indicating that nearly all flowers developed into mature fruit. On average, fruit contained approximately nine seeds (Hand: 9.1±0.4; Natural: 9.7±0.5) and average seed mass was approximately 3 mg (Hand: 3.0±0.1; Natural: 3.1±0.1). The similar number of seeds per flower as seeds per fruit also indicates that the majority of flowers produced mature fruit. In addition, there was no evidence to suggest that seedling emergence rate was affected by the pollination treatment (Figure 7.3). After 14 days in a glasshouse, 30% (18 of 60) of seedlings had emerged in each pollination treatment group.

Table 7.1. Mean (±SEM) reproductive output of naturally-pollinated and hand-pollinated *Rhinanthus minor* plants and results of two-sample t-tests.

Pollination treatment	Fruit set (%)	Seeds per fruit	Seeds per flower	Seed mass (mg)
Natural-pollination (n = 30)	96.0±2.2	9.7±0.5	8.8±0.5	3.1±0.1
Hand-pollination (n = 28)	99.1±0.7	9.1±0.4	8.9±0.4	3.0±0.1
95% CI	(-1.48, 7.66)	(-1.865, 0.635)	(-1.170, 1.398)	(-0.271, 0.224)
t-value	1.37	-0.99	0.18	-0.19
P-value	0.178	0.329	0.859	0.852
DF	56	56	56	56

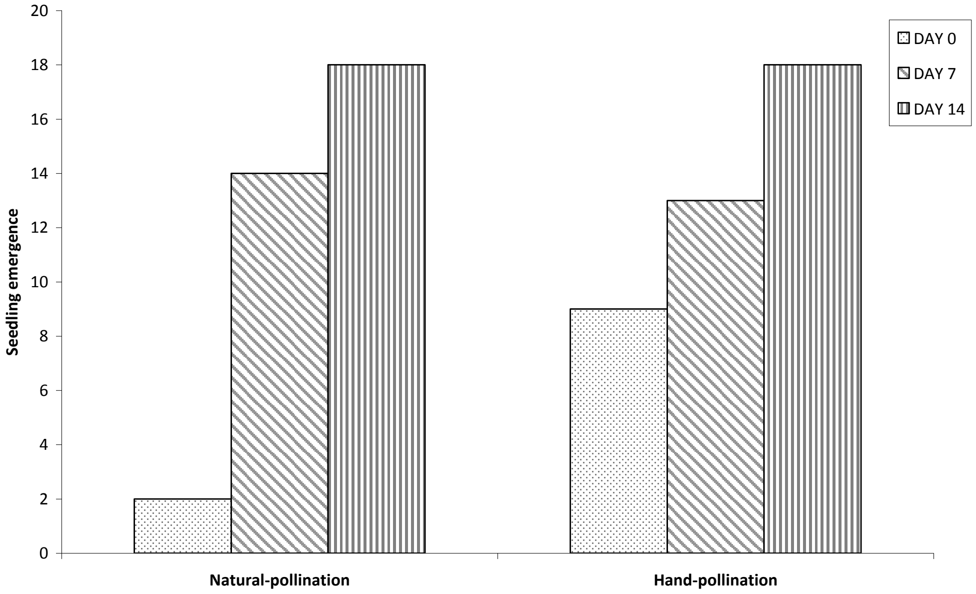


Figure 7.3. Total number of emerged seedlings from seed collected from naturally-pollinated and hand-pollinated plants of *Rhinanthus minor* (n = 60 seeds per treatment group).

7.4.2 Geranium sylvaticum

Results of nested ANOVAs did not show a significant effect of pollination treatment on any measure of reproductive fitness indicating that neither gender was pollen limited; however, reproductive output was found to differ according to gender (Table 7.2). Female plants produced a significantly greater number of seeds per fruit than hermaphrodite plants ($F = 36.52$, $P < 0.05$); and the P values for percentage fruit set ($P = 0.064$) and number of seeds per flower ($P = 0.055$) were marginally above the significance threshold of 0.05 indicating a trend towards greater fruit and seed production by female plants, and a greater flower and fruit abortion rate by hermaphrodites. Although fruits contain 10 ovules, they typically contained 2-3 seeds indicating that hermaphrodite and female flowers regularly produced fruit containing many unfertilised ovules (i.e. ≤30% of ovules fertilised). Gender had no effect on seed quality in terms of seed mass ($F = 0.020$, $P = 0.901$); however, there was some evidence to suggest seed quality may have been greater for females in terms of seedling emergence (Figure 7.4). After 21 days in a glasshouse, seedling emergence rate of hermaphrodite progeny was

27% (natural-pollination) and 30% (hand-pollination), in comparison with 55% (natural-pollination) and 42% (hand-pollination) for female progeny.

Petal length was found to differ between genders (*F* = 3158.11, *P* < 0.001) and between flowers (*F* = 5.156, *P* < 0.01) confirming that hermaphrodite flowers (15.3±0.1 mm) were significantly larger than female flowers (9.7±0.1 mm).

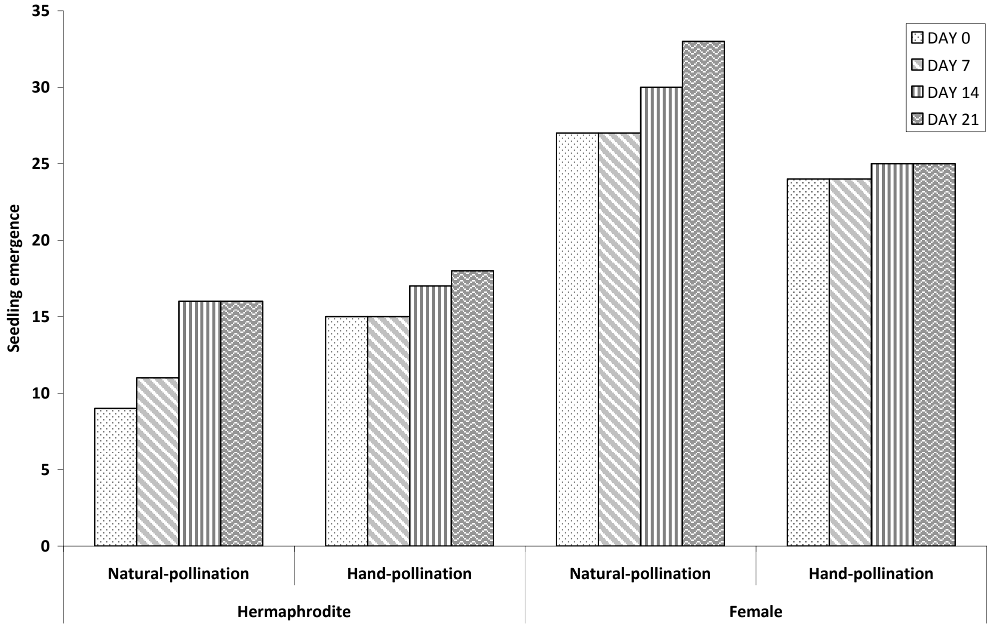


Figure 7.4. Total number of emerged seedlings from seed collected from naturally-pollinated and hand-pollinated hermaphrodite and female plants of *Geranium sylvaticum* (n = 60 seeds per treatment group.

Table 7.2. Mean (±SEM) reproductive output of naturally-pollinated and hand-pollinated hermaphrodite and female plants of *Geranium sylvaticum*, and results of nested ANOVAs.

Reproductive output	Gender and Pollination treatment		Mean±SEM	Source	df	*F*	*P*
Fruit set (%)	Hermaphrodite	Natural	74.4±4.8	Gender	1	14.123	0.064
		Hand	71.3±6.8	Treatment	2	0.511	0.602
	Female	Natural	90.7±2.3	Error	72		
		Hand	83.7±6.4	Total	75		
Seeds per fruit	Hermaphrodite	Natural	2.1±0.1	Gender	1	36.520	**0.026**
		Hand	1.7±0.2	Treatment	2	0.167	0.847
	Female	Natural	2.7±0.2	Error	72		
		Hand	2.3±0.3	Total (SQ transformed)	75		
Seeds per flower	Hermaphrodite	Natural	1.6±0.2	Gender	1	16.533	0.055
		Hand	1.3±0.2	Treatment	2	0.665	0.517
	Female	Natural	2.3±0.2	Error	72		
		Hand	2.0±0.3	Total	75		
Seed mass (mg)	Hermaphrodite	Natural	5.2±0.1	Gender	1	0.020	0.901
		Hand	4.8±0.4	Treatment	2	1.575	0.214
	Female	Natural	5.5±0.1	Error	72		
				Total (SQ transformed)	75		

7.5 Discussion

This study has found no evidence that reproductive output (i.e. female fitness) of *R. minor* and *G. sylvaticum* was pollen limited in an isolated species-rich upland hay meadow. Percentage fruit set, number of seeds per fruit and per flower and seed mass were similar for plants pollinated naturally and those which received supplemental pollen. There are several possible reasons why supplemental pollen may have failed to increase reproductive output. Firstly, natural pollination services may have been sufficient; however, this does not necessarily imply that pollinator populations are healthy as vulnerability to pollen limitation is known to reflect plant breeding systems and plant traits which attract, and compete for, pollinator services. Several meta-analyses have established a link between pollen limitation and breeding system, revealing that while significant pollen limitation results are common (hand>natural), so too are non-significant findings (hand=natural) with self-incompatible species more likely to benefit from supplemental pollen than self-compatible species (Burd 1994; Larson & Barrett 2000; Knight *et al.* 2005). For example, Burd (1994) reports 62% of 258 species to be significantly pollen limited and 35% to generate non-significant findings. It is therefore not wholly surprising that as self-compatible species, *R. minor* and *G. sylvaticum* (hermaphrodite morph only) were not found to be pollen limited. However, we found no evidence that the reproductive output of female flowers of *G. sylvaticum* were pollen limited either. Both species are visited by generalist pollinators; more specifically, *G. sylvaticum* is visited by bees (particularly bumblebees), syrphid flies and other dipterans (Asikainen & Mutikainen 2003; Asikainen & Mutikainen 2005b), and *R. minor* is visited predominantly by bumblebees (Kwak 1979; Gibson 1986). A generalised pollination system is likely to enable *R. minor* and *G. sylvaticum* to buffer declines in pollination services by some species if services continue to be provided by other pollinators with equal effectiveness (Waser *et al.* 1996). The review by Knight *et al.* (2005) found pollen limitation to decrease with an increase in the number of pollinating taxa, and in addition, found no differences in pollen limitation between perfect-flowered species and species with unisexual flowers. Furthermore, pollination services may be adequate because *R. minor* and *G. sylvaticum* represent an attractive forage resource to pollinators. Flowers of both species display certain traits typical of a 'pollination syndrome', notably the yellow and zygomorphic flowers of *R. minor* flowers and the UV nectar guide marks present on flowers of *G. sylvaticum* (Corbet, Williams & Osborne 1991; Proctor, Yeo & Lack 1996; Goulson 2003). Flowers of each species are also likely to be attractive in terms of floral reward (nectar and pollen quality and quantity). In particular, *R. minor* is reported to be a preferred forage plant by bumblebees (Goulson & Darvill 2004; Goulson *et al.* 2005; Carvell *et al.* 2006). *R. minor* was also an abundant species in the meadow (S.E. Barlow, personal observation) and *G. sylvaticum* formed dense patches dues to its clonal growth behaviour; and both large population size (Agren 1996) and density (Kunin 1993; Kunin 1997) have been shown to increase pollinator visitation. Furthermore, plants may have benefited from the presence of co-flowering species within the meadow (Moeller 2004; Knight *et al.* 2005). However, clearly not all of these factors apply equally to hermaphrodite and female *G. sylvaticum* plants as female plants were less abundant than hermaphrodites in the meadow (S.E. Barlow, personal observation), as is typical of sex ratios in gynodioecious populations (Asikainen & Mutikainen 2003), and flowers were smaller than hermaphrodites and lacked a pollen reward. Indeed, smaller female flowers of *G. sylvaticum* have been shown to attract 25% fewer pollinator visits than hermaphrodites (Asikainen & Mutikainen 2005b). Given a reduced ability to attract pollinators and a reliance on insect pollinators for receipt of pollen, reproductive output of female plants would be predicted to be more vulnerable to pollen limitation than hermaphrodites; however we found no evidence of this. In a study of several *G. sylvaticum* populations in Finland, Asikainen and Mutikainen (2005a) reported pollen limitation in hermaphrodite and female plants in most populations; however contrary to expectations, female plants were no more pollen-limited than hermaphrodites, rather hermaphrodites were more pollen-limited than females in terms of fruit set. The authors suggest that females may benefit by lower expenditure of resources on pollinator attraction (fewer and smaller flowers) and greater allocation of resources towards seed production. Results also corroborate findings of the meta-analysis by Shykoff *et al.* (2003), which found no difference in pollen limitation between hermaphrodite and female morphs of gynodioecious species.

Secondly, reproductive output may have been limited by resources, thus hand-pollinated plants were unable to capitalise on supplemental pollen receipt and evidence of pollen limitation could not be seen. If this were the case, results would constitute a Type II error (false negative). Percentage fruit set was high for both *R. minor* (mean>95%) and *G. sylvaticum* particularly for female plants (mean hermaphrodite >75%; mean female >90%), suggesting that percentage fruit set was not resource limited. Mean *R. minor* seed mass was approximately 3 mg for both natural and hand pollinated plants, similar to the 2.84 mg mean seed mass reported by Grime, Hodgson and Hunt (1988) . The parasitic behaviour of *R. minor* may buffer its sensitivity to resource limitation as plants typically parasitize several hosts at one time (Gibson & Watkinson 1989). In the absence of a host and on low nutrient substrates plants produce only 1-2 seed capsules (Westbury 2004). In this study, both naturally-pollinated and hand-pollinated *R. minor* plants produced 6 capsules per plant (data not shown) and approximately nine seeds per capsule for both treatments , results similar previous studies in Canada (van Hulst, Shipley and Theriault (1987))and the UK (Westbury (2004)). However, Kelly (1989) reported plants growing on chalk grassland in southern England to have 9.63±1.71 seeds per capsule but only 3.14 capsules per plant. Fruit set in subsequent years was similarly low and almost absent during a very dry year indicating that fruit production, but not the number of seed per capsule, may be limited by resources (nutrients, water). Kwak and Jennersten (1991) reported the seed set of the confamilial species *Melampyrum pratense* L. to be pollen limited in a meadow in Sweden in one of two years but plants may also have been limited by resources as seed set decreased throughout the season; however, only plants hand-pollinated on several occasions demonstrated higher seed set whereas plants hand-pollinated only once had a seed set similar to caged flowers, thus demonstrating that sequential pollinations were necessary to increase seed set. A similar finding was observed for *Rhinanthus angustifolius* (C.C. Gmel.) and *Melampyrum arvense* (L.) and may be an adaptation for promoting out-crossing in self-compatible homoganous plants (Kwak & Jennersten 1986; Kwak 1988). *R. minor* has a high germination rate, depending on stratification conditions, with a number of studies claiming germination rates of >80% (Westbury 2004 and references therein) suggesting that the germination rate of seedlings in this study was atypically low (30% after 14 days in glasshouse for both natural-pollinated and hand-pollinated parent plants); however a longer assessment time would have benefited the test. Low emergence rate may be a consequence of low pollen quality due to high selfing rates and may also be affected by resources available to parent plants as well as germination conditions. For *G. sylvaticum*, the average seed set of natural and hand-pollinated (hermaphrodite and female) plants was approximately 2-3 seeds per fruit (i.e. <30% of ovules fertilised). Asikainen and Mutikainen (2005a) investigated the occurrence of pollen and resource limitation in several populations of *G. sylvaticum* and also report mean seed set of both sex morphs was less than 3 seeds per fruit; results of their study showed both hand-pollination and resource addition increased fruit set, but only resource addition caused an increase in seed production (and a slight increase in seed mass) suggesting that *G. sylvaticum* is generally more resource- than pollen-limited. Results are in accord with Burd's (1995) 'oversupply hypothesis' which suggests that plants may possess flowers with more ovules than would usually be fertilised in a given environment; however, flowers would be predicted to respond to the addition of supplemental pollen accordingly and may suggest that seed production was subject to simultaneous resource and pollen limitation.

Thirdly, hand-pollination may have decreased reproductive output thus disguising evidence of pollen limitation. Again, this would constitute a Type II error. Young and Young (1992) suggest a number of reasons why hand-pollination may reduce reproductive output including: stigma damage; pollen tube overcrowding at high pollen densities; and a failure to coincide pollen donation with peak stigma receptivity. Burd (1994) suggests that problems associated with hand-pollination are relatively uncommon because only 4% of 258 species studied showed a significant reduction in reproductive output following hand-pollination (i.e. hand<natural) but it is logical that species showing no effect of hand-pollination (34% of species in Burd (1994)) could also be influenced in this way.

Finally, lack of significant results may be subject to Type II error caused by insufficient sample size. In this study sample sizes were similar or greater than that used by previous studies that identified significant pollen limitation in populations of wildflower species (of some aspects of female fitness) (e.g. Zimmerman & Pyke 1998; Asikainen & Mutikainen 2005a; Hegland & Totland 2007) and was a reflection of the logistical difficulties involved in hand-pollinating high numbers of flowers throughout the flowering period. However, the review by Thomson (2001) suggested that the likelihood of a non significant result is high for studies with this degree of statistical power.

The gynodioecious breeding system of *G. sylvaticum* presented an opportunity to compare reproductive output between genders. In general, female reproductive output was greater in comparison with hermaphrodite plants as predicted, particularly for number of seeds per fruit and marginally so for percentage fruit set and number of seeds per flower. Results of seedling emergence tests also indicated that females may produce greater quality seed in terms of germination viability. These findings are in accord with the findings of Asikainen and Mutikainen (2003) in their study of *G. sylvaticum* and with studies of other gynodioecious species (e.g. Shykoff 1988; Agren & Willson 1991; Williams, Kuchenreuther & Drew 2000; Lopez-Villavicencio *et al.* 2005), and support the theory that female plants may compensate for lack of male fitness, and thus persist in a co-sexual population by: i) investing resources from lack of pollen production into greater fruit and seed production (Lloyd 1975; Lloyd 1976; Charlesworth & Charlesworth 1978); and ii) producing higher quality offspring either by greater provisioning of resources to individual seeds (Koelewijn 1996), or by lower level of inbreeding depression due to out-crossing (Mutikainen & Delph 1996). Results also indicate that hermaphrodite plants more frequently aborted flowers or fruits, possibly due to under-fertilised ovules or because ovules were fertilised with self-pollen, as suggested by Stephenson (1981). In conjunction with findings of pollen limitation tests, results suggest that environmental factors were acting in favour of the maintenance of gynodioecy within the study population.

7.5.1 *Conclusions and suggestions for future research*

Reproductive allocation of *R. minor* and *G. sylvaticum* is not currently pollen limited at this study site, most likely due to there being sufficient pollinator populations within the study area. If reproductive output were pollen limited, the demographic consequences would be expected to vary between the two species given their different life history traits. *R. minor* is an annual and does not form a persistent seed bank. Yearly populations are therefore reliant on the seed output of the population from the previous year. As a keystone species, smaller populations of *R. minor* would likely have negative consequences for botanical diversity. In contrast, *G. sylvaticum* is a long-lived perennial and a highly effective clonal disperser. As such, plants may reallocate resources from sexual reproduction to vegetative growth (Thomson 2001) and effects of declining seed set on population dynamics would not be apparent in the short-term. Further investigation of pollen limitation in other meadow wildflower species, in particular those with a self-incompatible breeding system would be worthwhile and should include investigation of multiple populations over more than one year, in combination with tests of resource limitation. Comparisons between species-rich meadows and restoration sites, and investigation of the influence of habitat fragmentation on pollen limitation could also form part of future work.

7.6 Glossary

ACTINOMORPHIC = flowers which are radially symmetrical. An insect can take up numerous positions in relation to the axis of the flower.
AUTOGAMY = self-pollination involving transfer of pollen to stigma of same flower
DICHOGAMY = a separation in time between pollen release and stigma receptivity. However, this does not necessarily prevent geitonogamous self-fertilisation. How likely this is depends on how many flowers are open on a plant at the same time, pollinator behaviour and the level of pollen carryover.
DIDYNAMOUS = a flower with four stamens in two pairs of unequal length
GEITONOGAMY = self-pollination involving transfer of pollen to stigma of another flower on the same plant
GYNODIOECIOUS = A plant breeding system where hermaphrodite and female flowers exist on separate individuals.

HEMICRYPTOPHYTE = A plant with perennating (overwintering) buds situated at or just below the soil surface. Hemicryptophytes are usually herbaceous perennials and are commonly found in cold moist climates.

PISTILLATE = Flower has pistil(s) but no stamen(s) i.e. female

PROTANDROUS = flowers which shed pollen before the stigmas are ready for pollination i.e. male before female.

RAMET = a physiologically independent individual i.e. plants that repeat themselves in a modular fashion.

VESTIGIAL = rudimentary or degenerate structure e.g. vestigial stamens in female flowers of gynodioecious species that do no produce pollen.

ZYGOMORPHIC = flowers which are bilaterally symmetrical and are often pollinated by bumblebees. An insect tends always to take up a single position. Such flowers are usually placed horizontally.

Chapter 8. Recording pollinator visitation to flowers using an automated motion sensitive detection system (Rana)

8.1 Abstract

Investigation of plant-pollinator relationships is often a difficult and time-consuming process. As such, a more efficient methodology would be desirable and valuable to researchers. To meet this demand we have developed an automated motion sensitive detection system (Rana) to record pollinator visitors to flowers, enabling pollinators to be observed with significant reductions in time and labour. The usability and efficacy of Rana was tested under field conditions using the wildflower species, *Rhinanthus minor* and *Centaurea nigra* and proved highly effective in recording pollinator visitors including Hymenoptera, Lepidoptera, Diptera and Coleoptera whilst suppressing extraneous events, such as the effects of breeze and shadow. High resolution images enabled manual identification of most insect visitors to species level. All pollinator visits during a period of several hours were typically condensed into a few minutes of recorded material. Rana offers great potential to significantly reduce time and labour input into the study of plant-invertebrate interactions, and biological interactions in a wider context, although the current set-up requires highly technical know-how and needs to be more user-friendly before it can be made available to researchers.

Key-words: *Bombus*, bumblebees, *Centaurea nigra*, plant-invertebrate interactions, *Rhinanthus minor*

8.2 Introduction

A detailed understanding of plant-pollinator interactions has become pressing given increasing concerns over pollinator declines and the pollination services they provide to wildflower and crop plants (Kearns, Inouye & Waser 1998; Biesmeijer *et al.* 2006; Carvell *et al.* 2006; Potts *et al.* 2010). The standard methodologies for observing pollinator visitors to flowers (e.g. transect walks and timed observations of a plant or group of plants) is a time consuming process and is often combined with working in remote locations (Delph & Lively 1992; Steffan-Dewenter & Tscharntke 1999; Dupont & Olesen 2009; Hegland *et al.* 2010). Other problems associated with recording pollinator visits to plants are: being present to record visits when pollinators are active (i.e. during suitable weather conditions); being there all day (i.e. from dawn to dusk); and, observer effects on pollinator behaviour. To address these issues we have developed an automated motion sensitive detection system (Rana) to record insect visitors to flowers, enabling pollinators to be observed with significant reductions in time and labour. Rana was developed through extensive modification of the open source package “Motion” (Lavrsen 2011) with the aim of detecting a target object (e.g. a pollinating insect) using the features of motion, contrast, colour and size, whilst suppressing extraneous environmental events such as the effects of breeze and shadow. As such, the system is loosely modelled on frog (*Rana* sp.) motion detection of prey (Maturana *et al.* 1960; Lettvin *et al.* 1968).

The aim of this study was to test Rana and develop a fully operational system in the field. Several plant species with different floral morphologies and flowering phenologies were used as test plants and the pollinator visitors to two species are presented here. Firstly, the hemi-parasite *Rhinanthus minor* L. (Hay rattle) (Orobanchaceae) was of interest for several reasons; *R. minor* flowers exhibit certain ‘pollination syndrome’ morphological characteristics (Proctor, Yeo & Lack 1996) and are visited predominantly by Hymenoptera, particularly bumblebees (*Bombus* spp.) (Kwak 1979; Gibson 1986; Goulson *et al.* 2005). *R. minor* is also a keystone species in several grassland communities, due to its behaviour of suppressing competitive grass species, and is used as a management tool in the restoration of species-rich grassland (Pywell *et al.* 2004). Secondly, *Centaurea nigra* L. (Knapweed) (Asteraceae) was used as it flowers later in the summer, has a contrasting floral morphology to *R. minor*, and is highly attractive to a range of pollinators includingLepidoptera, Diptera and Hymenoptera (Lack 1982; Pywell *et al.* 2011), particularly bumblebees (Goulson, Lye & Darvill 2008; Darvill, Mabon & Rolph 2010).

8.3 Materials and Method

8.3.1 *Monitoring set-up*

Trials were conducted at Newcastle University's Close House Field Station (NZ128659) during summer 2010. The basic experimental set-up consisted of a data logger equipped with Rana and linked to a webcam (Phillips SPC1330) positioned above or adjacent to an inflorescence with the aid of a clamp stand (Figure 8.1). The data logger was stored in a weather proof box out of direct sunlight and the web camera was fitted with a rain guard. An Ethernet cable was run from the data logger to the field station laboratory to enable live recording to be viewed on a desktop PC and the camera settings to be altered remotely. Details of all logged visits could also be viewed remotely in real-time.

The open source package "Motion" (Lavrsen 2011) was modified by adding tuneable blob detection and automated tracking. The addition of these features turned motion into a powerful tool for detecting flower pollination visits while suppressing extraneous events, such as the effects of shadow and flower motion due to breeze. Recordings of insect visitors to flowers were stored as an avi (audio video interleave) and converted to mpeg using the open source conversion package avitompeg (avitompeg 2010). Insect visits were viewed as a movie (mpeg) in the vitualdub movie editing suite and identified manually. Individual mpeg files consisted of all insect visits in a single day condensed into one continuous movie with time and date visible on screen. It is therefore possible to gather data concerning the number of insect visits, the length of each visit and the behaviour of insects. Mpeg recordings can also be slowed down and edited to aid visual inspection, and still images can be converted to jpeg files. Rana functioning was part-validated by moving needle-mounted bumblebees and other similar sized (non-insect) objects under the web camera. Only movement of bees triggered the system to record movie footage.

Figure 8.1. Experimental set-up for recording pollinator visits to flowers using a web camera and data-logger equipped with the automated motion detection system, Rana.

8.3.2 *Recording pollinator visitors to* Rhinanthus minor *and* Centaurea nigra

R. minor has an erect, simple or branched stem up to 500 mm. Flowers are solitary in terminal spike-like racemes, zygomorphic, hermaphrodite, didynamous (4 stamens in 2 pairs of different length) and self-compatible. The corolla (12-15(-17) mm) is yellow to yellow-brown with a three-lobed down-turned lower lip and a compressed upper lip with two violet teeth below the apex. Stamens are located in the upper lip. The stigma is included or slightly exserted. Flowers are either insect-pollinated or selfed (Westbury 2004).

Pollinator visitors to *R. minor* were recorded daily between 7th and 22nd June 2010. Plants were present in experimental mesocosms (1 m^2) sown with a species-rich meadow mix two years previously. The webcam was positioned to the side of an individual plant to capture visits to all flowers in the raceme, and moved to newly flowering plants every few days. To reduce excessive movement, plants were tied to a short cane. As technical issues inevitably arose during the recording period, the duration of recording varied on different days, although attempts were made to cover an uninterrupted 24 hour period with a new avi file created at 23:01 each day. Using the mpeg files, all insects were identified to species level where possible and the number of visits by individuals of different species was counted on a daily basis. The behaviour of insect visitors was also noted, such as nectar robbing and whether *Bombus* species visited flowers nototribically (i.e. landing on the lower lip of the corolla and facing upwards with pollen transferred to the dorsal surface of the insect) or sternotribically (i.e. landing on the upper lip and facing downwards with pollen transferred to the ventral surface of the insect).

C. nigra has an erect branched stem 15-60 cm tall. Flower heads are a composite inflorescence, 2-4 cm across, terminal and solitary; involucre globose, florets red-purple (Rose & O'Reilly 2006). Flowers are rich in pollen and nectar (Goulson, Lye & Darvill 2008; Pywell *et al.* 2011) and protein content of pollen is relatively high (Hanley *et al.* 2008). Pollinator visitors were recorded over 8 days between 24th July and 19th August as previously described, except two webcams were employed at one time. *C. nigra* plants were present in a small plot of mixed herbage and were the most abundant species in flower at the time. The webcams were positioned above flowers looking down onto the composite head of 1-2 flowers. Flowering stalks were again tied to canes to reduce excessive movement and flowers were replaced every two days. All insect visitors were counted and identified to species where possible. Most hoverflies were identified to family (although this was more due to identifier inexperience rather than image quality).

8.4 Results & Discussion

8.4.1 Rhinanthus minor

In total, 104 separate visits to *R. minor* were recorded between 7th and 22nd June 2010 consisting of four species: *Bombus pascuorum* Scopoli (49%), *B. hortorum* L. (48%), *B. terrestris* L. (2%) and a solitary leaf-cutter bee, *Megachile willughbiella* Kirby (1%). The latter species (identified by C. O'Toole) is a new record for

Northumberland (BWARS n.d) (Figure 8.2d). The important pollinators were the long-tongued bumblebees, *B. pascuorum* (Figure 8.2a) and *B. hortorum* (Figure 8.2b) which regularly visited *R. minor* flowers for nectar in a nototribic position (bee facing upwards); a behaviour which may promote cross-pollination (Kwak 1979). Nectar is secreted at the base of the ovary and short-tongued *B. terrestris* visitors were observed nectar-robbing (Figure 8.2c), a strategy which may promote self-pollination (Kwak 1979). Contrary to the findings of this study, Kwak (1979) and Gibson (1986) did not record *B. terrestris*, but reported the common short-tongued bumblebees, *B. lucorum* L., *B. lapidarius* L. and *B. pratorum* L. to be frequent sternotribic visitors (bee facing downwards) or nectar thieves. These species were not recorded despite being present in the study area (S.E. Barlow, personal observation). Access to pollen is not restricted by bee tongue-length and *R. minor* is often cited as a frequently used forage plant by bumblebee studies (e.g. Goulson *et al.* 2005; Carvell *et al.* 2006). Gibson (1986) also reported *Megachile* sp. to be occasional visitors.

Figure 8.2. Bee visitors to *Rhinanthus minor* flowers recorded using a motion sensitive detection package (Rana): a) *Bombus pascuorum* (worker) and b) *B. hortorum* (worker) visiting flowers in a nototribic position; c) *B. terrestris* (worker) nectar robbing at the base of a flower; and d) *Megachile willughbiella* (female) visiting a flower nototrically.

8.4.2 Centaurea nigra

A total of 2635 separate insect visits to *C. nigra* flowers were recorded over 8 days between 24th July and 19th August 2010, consisting predominantly of *Bombus* species (41.6%) and Syrphids (57.7%) and occasional other Hymenoptera, Diptera and Lepidoptera (<2%) (Table 8.1; Figure 8.3). It was possible to record motion by insects ranging in size from pollen beetles to bumblebees and butterflies. Pollen beetles (*Pollenia* sp.) were abundant in *C. nigra* inflorescences and were prevented from triggering the recording process by altering the settings of the package to record larger insects only. Bumblebee visitors included a mixture of short- and long-tongued species (*B. lapidarius*, *B. pascuorum*, *B. lucorum*, *B. terrestris* and cuckoo species *B. vestalis* Geoffroy/*bohemicus* Seidl), although the most frequent visitor was the short-tongued species *B. lapidarius*. In comparison with *R. minor*, *C. nigra* attracted a greater diversity of pollinating insects due to differences in floral morphology and accessibility of pollen and nectar. Differences in the quality of forage reward may also be important to some insect groups, for example *C. nigra* pollen has a greater protein content than *R. minor* and pollen protein content is positively correlated with frequency of bumblebee visitation (Hanley et al. 2008). Several studies have shown *C. nigra* to be an important forage plant for short- and long-tongued bumblebees (Pywell *et al.* 2005; Carvell *et al.* 2006; Goulson, Lye & Darvill 2008; Darvill, Mabon & Rolph 2010) and other pollinator groups in late summer, and is an important component of seed mixtures used within relevant agri-environment scheme options (Carvell *et al.* 2007; Pywell *et al.* 2011).

It was also possible to pass an image of an insect visitor to *C. nigra* (extracted from an mpeg file) through DAISY (Digital Automated Identification SYstem) (O'Neill 2007) thus completely automating the detection and subsequent identification processes**.** Note, however, that we used images that clearly displayed the key identification features of an insect and DAISY is not suitable for all images or all species.

Figure 8.3. Insects visiting *Centaurea nigra*: a) *Bombus lapidarius*, pollen beetles (*Pollenia* sp.) and a Syrphid; and b) *Syrphus ribesii* (female).

Table 8.1. Insect visitors to *Centaurea nigra*. Other Hymenoptera are a solitary bee species and *Vespula vulgaris* L.; Diptera (non-Syrphidae) include *Sarcophaga carnaria* L. and other unidentified species; Syrphid species include *Episyrphus balteatus* De Geer (35.9%), *Epistrophe* sp. (6.3%), *Eupeodes corollae* Fabricus, *Volucella bombylans* L., *Syrphus ribesii* L. and other unidentified species. M = male; F = female

Order	Insect	24.07.10	25.07.10	03.08.10	04.08.10	06.08.10	07.08.10	10.08.10	19.08.10
Hymenoptera	*Bombus lapidarius*								
	M	194	165	12	10	100	70	48	116
	F	0	1	0	0	0	4	0	0
	Bombus pascuorm								
	M	18	11	32	5	18	7	11	19
	F	10	6	1	0	2	2	0	7
	Bombus lucorum								
	M	0	1	0	0	0	0	0	0
	F	1	0	0	0	0	0	0	0
	Bombus terrestris								
	M	0	0	0	0	0	0	0	0
	F	2	1	0	0	0	0	0	0
	Bombus vestalis/ bohemicus	67	24	21	8	15	17	4	30
	Other	1	0	1	1	6	2	0	0
Diptera	Syrphidae	72	84	176	250	273	200	110	344
	Non-Syrphidae	0	0	0	1	1	1	1	1
Lepidoptera	*Pieris brassicae* L.	0	0	3	0	1	0	0	0
	Lasiommata megera L.	0	0	0	0	2	5	0	4
	Pyronia tithonus L.	2	1	0	0	0	0	0	0
	Total	365	293	247	305	416	303	174	518

8.4.3 *Conclusions*

Testing Rana in the field has shown it to be highly effective in recording pollinator visitors, whilst suppressing the effects of extraneous events such as flower movement and shadow. However, further testing should include comparisons with manual observations to verify whether Rana records all pollinator visits. Multiple pollinator visits to flowers over the course of a day were condensed into approximately 20 minutes of recorded material, thus Rana offers a time- and labour-saving alternative to manual field observations. The system is also likely to be cost-effective as we estimate that a unit (data logger, webcam and Rana software) would cost between £600 and £800. Furthermore, Rana produces high resolution images and is compatible with DAISY to completely automate the identification process. It was, however, necessary to regularly monitor the video stream by remote access to ensure that the flower had not been blown out of shot or out of focus. The data logger occasionally required rebooting and, to re-establish functioning of Rana, required the skills of the system developer. Therefore, operation of Rana needs to be more user-friendly before it can be made available to research biologists. The potential uses of Rana are wide-ranging and are far from limited to plant-invertebrate interactions, but extend to studies of biological interactions in general.

Since completion of the initial trials described in this study, it is now possible to operate the system using a wireless connection, thus removing the need for an Ethernet cable connection. The system has also been tested with off mains operation using 80AH car batteries and can be run for three weeks or more. Furthermore, attempts to get tracking and ad-hoc radio communication to data-loggers proved successful and enabled the data to be transferred to a laptop within 25-50 m of the logger (M.A. O’Neill, personal comment).

Chapter 9. General Discussion

9.1 General Discussion

This thesis increases our understanding of how plant-invertebrate interactions may influence the restoration of MG3 upland hay meadows and other semi-natural grasslands. Studies have revealed several mechanisms by which invertebrate herbivores (slugs) and pollinators (focusing mainly on bumblebees) may affect the conservation, restoration and enhancement of grassland botanical diversity. The implications of these studies for grassland restoration are discussed, along with management recommendations and directions for future research.

Results of this thesis support those of previous studies highlighting slugs as the most important invertebrate herbivore in temperate grassland (Hulme 1994) through exerting an important selective pressure on seedling recruitment (e.g. Hanley, Fenner & Edwards 1995a; Hulme 1996; Hanley, Fenner & Edwards 1996a) and shifting the balance of competition between coexisting plant species (Wardle & Barker 1997; Buckland & Grime 2000).

The study described in Chapter 2 is the first to quantify slug population densities in species-rich upland hay meadows and their less diverse counterparts. Slug densities in meadows classified as semi-improved (species-intermediate) were highly variable highlighting that slug densities at restoration sites are likely to be site-specific (Chapter 2). Restoration of semi-improved grasslands towards the target MG3 community may be more difficult at sites with high slug densities due to slugs reducing the seedling recruitment of desirable forb species, specifically *Rhinanthus minor* and *Trifolium pratense* (Chapters 2, 3 and 4). The successful recruitment and establishment of these species is fundamental to the early stages of restoration because they positively affect wider ecosystem-level functioning. Namely, *R. minor* selectively parasitizes grasses, thus reducing their vigour and enabling less competitive desirable forb species to establish within the sward (Pywell *et al.*, 2004). *T. pratense* facilitates the fungal component of the soil microbial community; increases fungal to bacterial ratios as found in species-rich grasslands (Bardgett *et al.*, 2007); and is associated with reductions in residual soil fertility, particularly phosphorus (Smith *et al.*, 2008). These species, therefore, pave the way for the establishment of later colonising plants which further increase the similarity of the restored sward with the target community.

Selective grazing of seedlings was a key interest of this project. Chapter 3 revealed meadow plants to form a hierarchy of acceptability to slugs and suggested seedlings of several stress-tolerant species typical of secondary successional grassland were highly unacceptable, most likely due to a defensive function of plant secondary chemicals (including tannins). Some species, identified by Smith *et al.* (2003, 2008) as promoting fungal populations, were found to be highly unacceptable to slugs (*Ranunculus acris*, *Anthoxanthum odoratum*), but *Trifolium pratense* was highly acceptable. This study therefore indicated that the recruitment and establishment of *T. pratense* seedlings may be negatively affected by slug herbivory in the field with potential wider implications for the restoration process. Most grass species were highly acceptable to slugs suggesting that the efficacy of silica-based defences may be subject to positive ontogenetic effects. It was disappointing that *R. minor* was not included in the final analysis of this study as results detailed in Chapters 4 and 5 suggested that information regarding its relative acceptability in seedling form would have been highly interesting and valuable. Further work is necessary to assess the value of the acceptability results as a predictor of slug damage to seedlings under field conditions.

A key finding of this thesis is the selective grazing of *R. minor* seedlings by slugs within mixed swards under mesocosm conditions (Chapter 4). High slug densities and the absence of *R. minor* had important community-level effects including greater grass cover and lower forb species richness. This tri-trophic interaction has not been reported before and is one of the main advances that the thesis makes to understanding the importance of invertebrate herbivory to the restoration of upland hay meadows (and species-rich grasslands containing *R. minor* or other parasitic plants). Likewise, *T. pratense* seedling recruitment and establishment may be negatively affected by slug herbivory (Chapter 3 and 4) with wider ecosystem-level implications. Chapter 4 also showed that the effects of slugs on community development and structure were reduced in swards dominated by competitive grasses. These findings were in accord with similar studies involving molluscs that have found competition from dominant grasses to be of overriding importance with increasing age of sown swards (Wardle & Barker 1997; Buschmann *et al.* 2005; Del-Val & Crawley 2005). Further study is recommended of how the establishment of *R. minor* and *T. pratense* populations (and thus interactions with other plant species) are affected by slug grazing at restoration field sites.

In addition to observations made within the mesocosm study (Chapter 4), it was noted during the course of the project that slugs rarely damaged older *R. minor* plants (S.E. Barlow, personal observation) (data not included in this thesis). This led to the hypothesis that *R. minor* may possess effective defences against slug herbivory that are subject to positive ontogenetic effects.   Chapter 5 sought to investigate this further by examining the leaf surface of seedlings and older leaves using scanning electron microscopy and EDX analysis. Results revealed a diversity of trichome types and confirmed that the elemental composition of nonglandular trichomes and the epidermal surface was predominantly composed of silicon. Furthermore, SEM images identified ontogenetic variation between seedling and older leaves but, contrary to expectations, revealed that seedlings also possessed trichomes. The foliar trichomes of older *R. minor* plants may function in defence against invertebrate herbivores, but be ineffective against slugs during the early seedling stage. A possible explanation may be that chemicals present in glandular trichomes are subject to positive ontogenetic effects. The potential function of trichomes and silicon in herbivore defence is an intriguing direction for further study. Further work would be useful in understanding the interactions between slugs and *R. minor* at restoration field sites.

Overall, results have highlighted the likely role of plant traits involved in direct anti-herbivore defence including plant secondary chemicals, silica-based defences and trichomes in influencing interactions between plants and slugs (Chapters 2, 3, 4, 5). The outcome of these interactions has been shown to have implications at the species- and community-level in upland hay meadow plant communities (Chapter

4). In light of the findings reported here, it is recommend that slug herbivory is considered within management recommendations for grassland restoration, with the specific agenda of improving the establishment of key forb species at the seedling stage, especially *R. minor* and *T. pratense*. The 'best' way forward is debatable and there are several possible options. Firstly, is control necessary? Chapter 4 suggests that targeted control of high slug densities during seedling emergence would assist the establishment of *R. minor* populations and thus influence the associated community-level interactions. Also, Chapter 3 suggests that slug control may be beneficial for *T. pratense* establishment if the relatively high acceptability of seedlings to slugs under laboratory conditions translates into high damage under field conditions. However, slug densities at restoration sites are likely to be site-specific (Chapter 2) and should be assessed prior to any direct control action. Two types of control are currently available: i) chemical control using molluscicidal pellets; and ii) biological control using nematode worms. Molluscicidal pellets can effectively reduce seedling losses to slugs (Hanley, Fenner & Edwards 1995a; Frank & Barone 1999; Wilby & Brown 2001; Pywell *et al.* 2007) and Natural England recommend the use of pellets where necessary in their Technical Information Notes for sward enhancement (Natural England 2010a; Natural England 2010b; Natural England 2010c), yet, to the best of our knowledge pellets have not been used in upland hay meadow restoration. Targeting pellet application during seedling emergence may be complicated by the presence of livestock which firstly, may consume pellets and secondly, trample pellets into the soil rendering them less effective against slugs. Furthermore, the use of heavy machinery to apply pellets is likely to cause unacceptable damage to the sward in springtime. Molluscicidal pellets have other disadvantages, namely: financial cost; potential phytotoxic effects on seed germination and seedling performance (Hanley & Fenner 1997); potential adverse environmental and non-target effects (Getpelletwise 2010); and social criticism. The latter three points are more so associated with the active ingredient metaldehyde and may be reduced by using pellets containing ferric phosphate and registered for organic use. Biological control of slugs using the nematode worm, *Phasmarhabditis hermaphrodita* (A. Scheider), can be an effective control method (Morand, Wilson & Glen 2004) and is likely to be more socially acceptable than pellets; however worms must be mixed in water and sprayed over the target area, requiring specialist equipment.

Water cannons are available to distribute nematodes over large areas but are expensive and the alternative approach of using tractors fitted with spraying equipment would damage plants in early spring.

An alternative approach is to avoid exposing seedlings to slugs altogether through *ex situ* propagation using locally sourced seed and transplanting young plants into restoration field sites. This method is a good one (e.g. Hitchmough 2003) but requires time and labour and is not feasible for *R. minor* as transplants suffer very high mortality levels, most likely due to root disturbance during the transplanting process (S.E. Barlow, personal comment). Another option is to increase the quantity of seeds for species of particular ecological significance and which are likely to be selectively grazed by molluscs such as *R. minor* and *T. pratense* (as indicated in Chapters 3 and 4). This strategy was suggested by Frank (2003) who found slugs to selectively graze seedlings of *Centaurea cyanus*, a species important to flower visiting insects within wildflower strips. Finally, it is recommended that seed be distributed randomly over large areas to avoid dense seedling pockets and the likelihood of apostatic grazing.

Chapter 6 corroborated previous studies that showed bumblebees to more frequently visit highly rewarding forage plants (Williams 1989; Goulson 1999; Hanley *et al.* 2008) and the strong feature of the Fabaceae in the diet of long-tongued and rare species associated with unimproved grassland (Goulson *et al.* 2005). Despite the diversity of plant species available in Barrowburn meadows, bumblebees foraged predominantly from just four species: *Trifolium repens*, *T. pratense*, *R. minor* and *G. sylvaticum*. High slug densities may reduce the availability of these preferred forage plants to bumblebees via (direct or indirect) negative effects on *R. minor* and *T. pratense* seedling recruitment and abundance cover (Chapters 3 & 4).   The pollinator food plant preferences reported in Chapter 6 also tells us that the abundance of these species within the restoration plant community are particularly important for the conservation of local bumblebee populations, including *Bombus monticola*, *B. jonellus* and the UK BAP listed *B. muscorum*. The establishment of *T. pratense* and *R. minor* within the first few years of meadow restoration is likely to be important, not just for botanical diversity, but also in providing a new, high quality forage resource for bumblebees.

The provision of highly rewarding forage plants for pollinators is important not simply to benefit pollinator populations and diversity, but also to support sustainable pollination services to insect-pollinated plants. The importance of pollination services in affecting the reproductive output (i.e. female fitness) of *R. minor* and *G. sylvaticum* was investigated by way of a pollen supplementation experiment (Chapter 7). These species were selected because they were amongst those most frequently visited by foraging bumblebees (evidenced in Chapter 6) and, unlike *T. pratense* and *T. repens*, their floral morphology was suitable for pollen to be applied by hand. Furthermore, *R. minor* was selected for its keystone function and because it is bee-pollinated; and *G. sylvaticum* was chosen because it is insect-pollinated, is characteristic of MG3 upland meadows, and its gynodioecious breeding system presented an opportunity to compare obligate-outcrossing female plants with self-compatible hermaphrodites. The female fitness of both species was not pollen limited at the single study site. *R. minor* and hermaphrodite plants of *G. sylvaticum* may be less vulnerable to pollen limitation because they each have a self-compatible breeding system, although frequent self-pollination will increase the risk of inbreeding depression (Kearns, Inouye & Waser 1998). In terms of female *G. sylvaticum* plants, results corroborated previous studies that suggest females to maintain their reproductive advantage over hermaphrodites when resources are limited, as females may reallocate resources away from pollen production and pollinator attraction (by producing smaller flowers) and towards seed production (Asikainen & Mutikainen 2003; Asikainen & Mutikainen 2005a; Asikainen & Mutikainen 2005b). In concluding this study, it was recommended that future work should involve an improved experimental design by incorporating replicate study sites across a gradient of increasing management intensity and a treatment to test for resource-limitation and interactive effects. Furthermore, future work involving the study of insect visitation to flowers could be greatly assisted by the use of Rana, although further verification of the efficacy of Rana is required (Chapter 8).

Studies highlighted that concerns over pollen limitation in wild plant populations should consider plant traits involved in pollinator attraction; and the degree of specialisation of the pollination mechanism (i.e. range of suitable pollen vectors) and pollination system (i.e. self-compatibility and ability to self-fertilise). Isolated plant populations of species with self-incompatible breeding systems and flowers which offer lower rewards to pollinators compared with conspecifics are likely to be most at risk of pollen limitation (Burd 1994; Knight *et al.* 2005). Studies must also consider the role of seeds in plant population dynamics (Ashman *et al.* 2004). Furthermore, the likely implications of pollinator limitation and inbreeding depression are not solely reduced reproductive output (i.e. lower seed set) but extend to offspring fitness. A component of fitness may be the ability of seedlings to resist or tolerate herbivore attack and thus govern their ability to establish and persist in the face of intense slug herbivory within meadows undergoing restoration.

Agri-environment scheme options aimed at enhancing or prolonging botanical diversity and pollinator forage availability, such as wildflower strips and uncut margins, should be targeted within or near to species-rich and restoration sites with the aim of increasing the connectivity of forage habitat and being of greatest benefit to pollinators and pollination services to wildflowers (Carvell 2002; Pywell *et al.* 2005). At upland hay meadow sites and the surrounding landscape, it is recommended that wildflower seed mixes contain Fabaceae species (particularly *T. pratense* and *T. repens*) and *R. minor* (Chapter 6) and that the establishment of these species is assisted through management options aimed at reducing the impact of slug herbivory as discussed above.

9.1.1 *Conclusions*

The successful establishment of desirable meadow plants and their reproduction is fundamental to the restoration/creation of diverse and sustainable semi-natural grasslands such as MG3 upland hay meadows.

This thesis has identified functional and applied aspects of plant-invertebrate interactions that have implications for grassland restoration. The findings provide a better understanding of how invertebrates may affect the success of plant species in hay meadows and have highlighted important interactions between invertebrates and keystone plants. The impact of this research lies in applying the findings to developing better methods and management prescriptions for restoring botanical diversity of upland hay meadows and other grassland communities (e.g. through agri-environment schemes). This will require publication of the research and dissemination of the results beyond direct academic circles in order to reach policy makers and conservation practitioners who deliver land management. The project has highlighted several areas for future research, particularly the further study of herbivore-parasitic plant interactions.

APPENDIX - Supplemental Information

1. Supplemental information to Chapter 2: Effects of agricultural intensification and environmental factors on slug densities in upland hay meadows

Table S1.1. Semi-parametric permutational MANOVA for slug assemblages of soil samples including: a) a global test of management as group factor (sum of squares [SS], pseudo-F statistic, *P* value) and b) pair-wise comparisons of management groups (meadow improvement categories) (t statistic, *P* value). Statistical significance is determined by Monte Carlo permutation tests (10000 permutations) at 0.05.
**P* < 0.05, ***P* < 0.01, ****P* < 0.001

a) Global test of factors

Factor	d.f	SS	MS	*Pseudo-F*	*P*	Unique perms
Management	2	6462.6	3231.3	3.2998	0.0077**	9952
Residual	67	65609	979.24			
Total	69	72072				

b) Pair-wise comparison of management groups. I = Improved; SI = Semi-improved; UI = Unimproved.

Groups	*t*	P(perm)	Unique perms
I, SI	0.9835	0.4231	9967
I, UI	3.1896	0.0002***	9961
SI, UI	0.7726	0.6054	9962

2. Supplemental information to Chapter 3: The acceptability of meadow plants to the slug *Deroceras reticulatum* and implications for meadow restoration

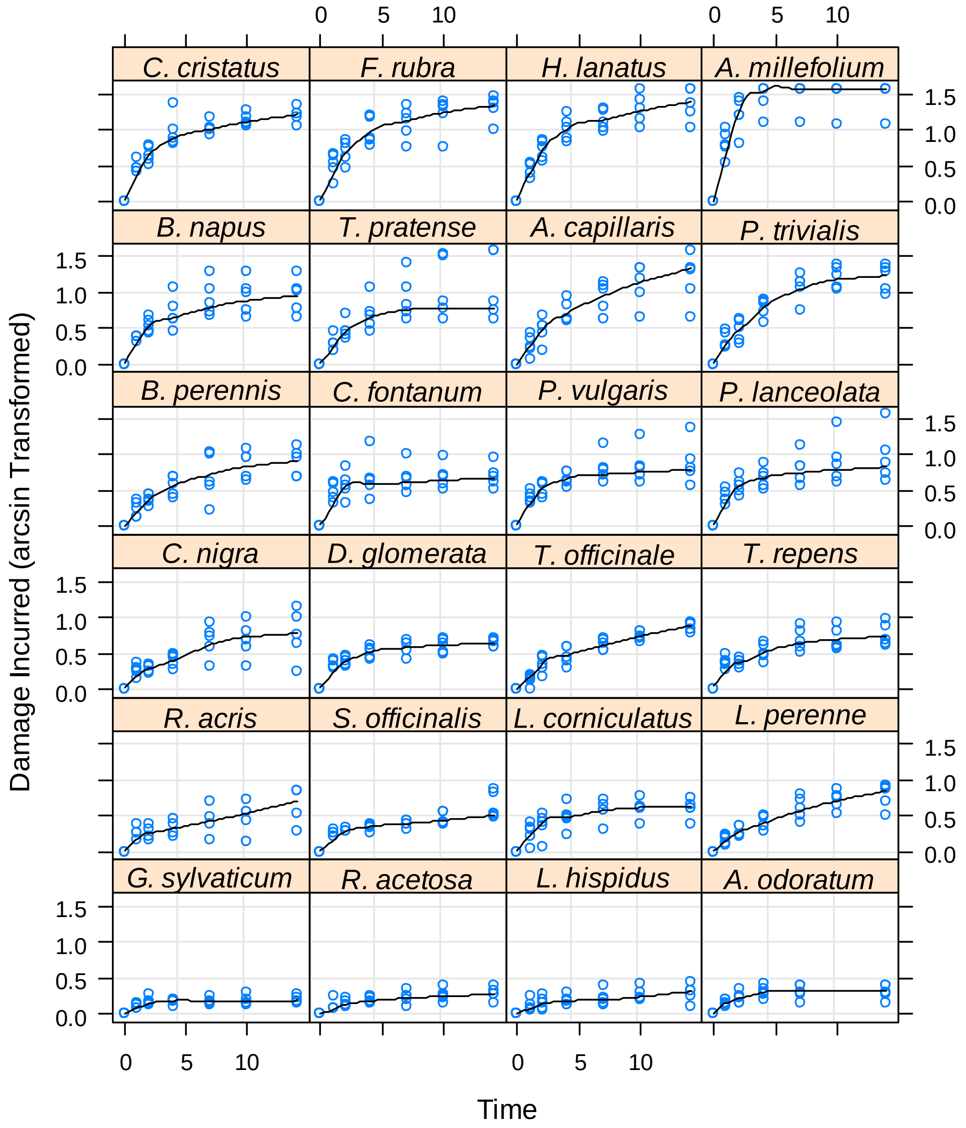


Figure S2.1. Response curves for slug feeding damage incurred by seedlings of meadow plant species and *Brassica napus* as a function of time. Proportional data were arcsine transformed to facilitate interpretation. The y-axis describes the positive non-linear trend of damage incurred by each species. The x-axis represents the contribution of time (0-14 days) to the overall trend in damage for each plant species. Response curves are non-linear for each species and show species to vary in terms of the degree of damage sustained through time. Increasing non-linearity of response is ordered from bottom left to top right. For key to species refer to Table 3.2 in main text.

Table S2.1. The chemical and physical properties of plant species offered as seedling monocultures to *Deroceras reticulatum*. The findings of previous laboratory-based mollusc feeding studies are summarised for each plant species. Mollusc species: *Arion lusitanicus*, *A. hortensis*, *A. subfuscus*, *A. ater*, *Cepea nemoralis*, *Deroceras reticulatum*, *D. caruanae*, *D. agreste*. Acceptability class: H = highly acceptable (highly palatable); A = acceptable (palatable); U = highly unacceptable (highly unpalatable).

Species	**Family**	**Physical properties**	**Chemical properties**	**Literature review**			
				Study	Mollusc species	Plant material	Acceptability class
Achillea millefolium	Asteraceae	Downy	Terpenes, phenols	Duval (1971)	*D. reticulatum*	Adult leaves	H
					A. hortensis	Adult leaves	U
				Briner and Frank (1998)	*A. lusitanicus*	Adult leaves	A
Agrostis capillaris	Poaceae	Silica phytoliths		Fenner *et al*. (1999)	*D. reticulatum*	Agar discs of seedlings and adult leaves	A
Anthoxanthum odoratum	Poaceae	Silica phytoliths	Aromatics	Grime *et al*. (1968)	*C. nemoralis*	Adult leaves	U
Bellis perennis	Asteraceae			Duval (1971)	*D. reticulatum*	Adult leaves	A (low)
					A. hortensis	Adult leaves	A (low)
				Dirzo (1980)	*D. caruanae*	Adult leaves	U
				Briner and Frank (1998)	*A. lusitanicus*	Adult leaves	A (low)
Brassica napus	Brassicaceae		Glucosinolates, sinapic acid	Dirzo (1980)	*D. caruanae*	Adult leaves	H
				Mølgaard (1986)	*A. ater*	Adult leaves	H
				Briner and Frank (1998)	*A. lusitanicus*	Adult leaves	H

Table S2.1. *Continued*.

Species	Family	Physical properties	Chemical properties	Literature review			
				Study	Mollusc species	Plant material	Acceptability class
Centaurea nigra	Asteraceae	Hairy, rough					
Cerastium fontanum	Caryophyllaceae	Densely hairy	Alkaloids	Fenner *et al*. (1999)	*D. reticulatum*	Agar discs of seedlings and adult leaves	A
Cynosurus cristatus	Poaceae	Silica phytoliths					
Dactylis glomerata	Poaceae	Silica phytoliths		Grime *et al*. (1968)	*C. nemoralis*	Adult leaves	U
				Duval (1971)	*D. reticulatum*	Adult leaves	H
					A. hortensis	Adult leaves	U
				Briner and Frank (1998)	*A. lusitanicus*	Adult leaves	U
				Fenner *et al*. (1999)	*D. reticulatum*	Agar discs of:	
						Seedlings	A
						Adult leaves	U
Festuca rubra	Poaceae	Silica phytoliths		Briner and Frank (1998)	*A. lusitanicus*	Adult leaves	A
Geranium sylvaticum	Geraniaceae	Hairy	Tannins	Scheidel and Bruelheide (1999)	*A. lusitanicus*	Adult leaves	U
					A. subfuscus	Adult leaves	U
					D. agreste	Adult leaves and seedlings	U
Holcus lanatus	Poaceae	Silica bodies, hairy		Grime *et al*. (1968)	*C. nemoralis*	Adult leaves	A
Leontodon hispidus	Asteraceae	Hispid (rough hairs)		Grime *et al*. (1968)	*C. nemoralis*	Adult leaves	H

Table S2.1. *Continued*.

Species	**Family**	**Physical properties**	**Chemical properties**	**Literature review**			
				Study	Mollusc species	Plant material	Acceptability class
Lolium perenne	Poaceae	Silica phytoliths					
Lotus corniculatus	Fabaceae		Cyanogenic glycosides (polymorphic)	Grime *et al*. (1968)	*C. nemoralis*	Adult leaves	U
				Dirzo (1980)	*D. caruanae*	Adult leaves	U
				Briner and Frank (1998)	A. lusitanicus	Adult leaves	A
				Fenner *et al*. (1999)	*D. reticulatum*	Agar discs of seedlings and adult leaves	A
Plantago lanceolata	Plantaginaceae		Iridoid glycosides (catapol and aucubin), phenolics	Grime *et al*. (1968)	*C. nemoralis*	Adult leaves	U
				Duval (1973)	*A. hortensis*	Adult leaves	U
				Dirzo (1980)	*D. caruanae*	Adult leaves	U
				Briner and Frank (1998)	*A. lusitanicus*	Adult leaves	A
				Fenner *et al*. (1999)	*D. reticulatum*	Agar discs of seedlings and adult leaves	A
Poa trivilais	Poaceae	Silica phytoliths		Briner and Frank (1999)	*A. lusitanicus*	Adult leaves	A
Prunella vulgaris	Lamiaceae						

Table S2.1. *Continued*.

Species	Family	Physical properties	Chemical properties	Literature review			
				Study	Mollusc species	Plant material	Acceptability class
Ranunculus acris	Ranunculaceae	Hairy	Ranunculin (protoanemonin)	Grime *et al*. (1968)	*C. nemoralis*	Adult leaves	U
				Dirzo (1980)	*D. caruanae*	Adult leaves	H
				Briner and Frank (1998)	*A. lusitanicus*	Adult leaves	A (low)
Rumex acetosa	Polygonaceae		Quinones, oxalic acid	Grime *et al*. (1968)	*C. nemoralis*	Adult leaves	A
Sanguisorba officinalis	Roseaceae						
Taraxacum officinale	Asteraceae		Triterpene, bitter principles, alkaloids	Duval (1971)	*D. reticulatum*	Adult leaves	H
					A. hortensis	Adult leaves	H
				Dirzo (1980)	*D. caruanae*	Adult leaves	H
				Cook *et al*. (1996)	*D. reticulatum*	seedlings	H
				Fenner *et al*. (1999)	*D. reticulatum*	Agar discs of:	
						Seedlings	A
						Adult leaves	A (low)
Trifolium pratense	Fabaceae	Hairy	Cyanogenic glycosides (polymorphic)	Dirzo (1980)	*D. caruanae*	Adult leaves	U
				Briner & Frank (1998)	*A. lusitanicus*	Adult leaves	A
				Fenner *et al*. (1999)	*D. reticulatum*	Agar discs of:	
						Seedlings	H
						Adult leaves	A
				Hanley & Sykes (2009)		Seedlings	H

Table S2.1. *Continued*.

Species	**Family**	**Physical properties**	**Chemical properties**	**Literature review**			
				Study	Mollusc species	Plant material	Acceptability class
Trifolium repens	Fabaceae		Cyanogenic glycosides (polymorphic)	Dirzo (1980)	*D. caruanae*	Adult leaves	U
				Cook *et al*. (1996)	*D. reticulatum*	Seedlings	H
				Briner and Frank (1998)	*A. lusitanicus*	Adult leaves	A
				Fenner *et al*. (1999)	*D. reticulatum*	Agar discs of: Seedlings	
						cyanogenic	H
							A (low)
						acyanogenic	
						Adult leaves	A
						cyanogenic	H
						acyanogenic	
				Hanley and Sykes (2009)		Seedlings	A (low)

3. Supplemental information to Chapter 4: Effects of slug herbivory on meadow plant communities and implications for grassland restoration

Table S3.1. Semi-parametric permutational MANOVA for community assemblages within the semi-improved community including: a) a global test of factors (sum of squares [SS], pseudo-F statistic, *P* value), and b) pair-wise comparisons of slug treatments incorporating all years and within years (t statistic, *P* value). Statistical significance is determined by Monte Carlo permutation tests (10000 permutations) at 0.05. **P* < 0.05, ***P* < 0.01, ****P* < 0.001

a) global test of factors

Factor	d.f	SS	*Pseudo-F*	*P*
Slugs	1	0.1046	3.1933	0.0039 **
Year	2	1.3017	19.869	0.0001 ***
Block	4	0.32106	2.4503	0.0009 ***
SlugsxYear	2	0.11025	1.6829	0.0462 *
Residual	20	0.65514		
Total	29	2.4928		

b) pair-wise comparison of slug treatments incorporating all years and within years

Groups	*t*	*P*
SL, NSL (all years)	1.7870	0.0035 **
SL, NSL (2008)	1.8086	0.0552
SL, NSL (2009)	1.3401	0.1922
SL, NSL (2010)	0.9950	0.4377

Table S3.2. Semi-parametric permutational analysis of multivariate dispersions for community assemblages within the semi-improved community, including pair-wise comparisons of slug treatments incorporating all years and within years (*t* statistic, *P* value). Statistical significance is determined by Monte Carlo permutation tests (10000 permutations) at 0.05. **P* < 0.05, ***P* < 0.01, ****P* < 0.001

Groups	*t*	*P*
SL, NSL (all years)	2.4296	0.0408 *
SL, NSL (2008)	0.9338	0.4206
SL, NSL (2009)	3.9526	0.0075 **
SL, NSL (2010)	1.0855	0.4311

Table S3.3. Permutation tests incorporated in a distance-based linear model of (transformed) species abundance cover within the semi-improved community. Statistical significance is determined by Monte Carlo permutation tests (10000 permutations) at 0.05. **P* < 0.05, ***P* < 0.01, ****P* < 0.001

Variable	Pseudo-F	*P*
Slugs	1.2264	0.255
Year	17.932	0.0001***
Block	0.70753	0.0025**

Table S3.4. Semi-parametric permutational MANOVA for community assemblages within the unimproved community including: a) a global test of factors (sum of squares [SS], pseudo-F statistic, *P* value); and b) pair-wise comparisons of slug treatments incorporating all years and within years (t statistic, *P* value). Statistical significance is determined by Monte Carlo permutation tests (10000 permutations) at 0.05. **P* < 0.05, ***P* < 0.01, ****P* < 0.001

a) global test of factors

Factor	d.f	SS	Pseudo-F	*P*
Slugs	1	0.3093	5.5399	0.0005 ***
Year	2	1.522	13.632	0.0001 ***
Block	4	0.3339	1.4953	0.0427 *
SlugsxYear	2	0.2979	2.6681	0.0020 **
Residal	20	1.1165		
Total	29	3.5797		

b) pair-wise test between slug treatments

Year	Groups	*t*	*P*
All years	slugs, no slugs	2.3537	0.0003 ***
2008	slugs, no slugs	1.8635	0.0499 *
2009	slugs, no slugs	1.5336	0.1118
2010	slugs, no slugs	2.1036	0.0413 *

Table S3.5. Semi-parametric permutational analysis of multivariate dispersions for community assemblages within the unimproved community including pair-wise comparisons of slug treatments incorporating all years and within years (*t* statistic, *P* value). Statistical significance is determined by Monte Carlo permutation tests (10000 permutations) at 0.05. **P* < 0.05, ***P* < 0.01, ****P* < 0.001

Groups within years	*t*	*P*
SL, NSL (all years)	2.1684	0.0594
SL, NSL (2008)	1.9197	0.1016
SL, NSL (2009)	1.4483	0.2854
SL, NSL (2010)	1.0675	0.3771

Table S3.6. Permutation tests incorporated in a distance-based linear model of (transformed) species abundance cover within the unimproved community. Statistical significance is determined by Monte Carlo permutation tests (10000 permutations) at 0.05. **P* < 0.05, ***P* < 0.01, ****P* < 0.001

Variable	Pseudo-F	*P*
Slugs	2.6479	0.035 *
Year	12.428	0.001 ***

References

Agrawal, A.A. (1999) Induced responses to herbivory in wild radish: Effects on several herbivores and plant fitness. *Ecology,* **80,** 1713-1723.

Agren, J. & Willson, M.F. (1991) Gender variation and sexual differences in reproductive characters and seed production in gynodioecious *Geranium maculatum*. *American Journal of Botany,* **78,** 470-480.

Agren, J. (1996) Population Size, Pollinator Limitation, and Seed Set in the Self-Incompatible Herb *Lythrum salicaria*. *Ecology,* **77,** 1779-1790.

Aguilar, R., Ashworth, L., Galetto, L. & Aizen, M.A. (2006) Plant reproductive susceptibility to habitat fragmentation: review and synthesis through a meta-analysis. *Ecology Letters,* **9,** 968-980.

Akers, C.P., Weybrew, J.A. & Long, R.C. (1978) Ultrastructure of glandular trichomes of leaves of *Nicotiana tabacum* L, CV XANTHI. *American Journal of Botany,* **65,** 282-292.

Alcalá, R.E., Mariano, N.A., Osuna, F. & Abarca, C.A. (2010) An experimental test of the defensive role of sticky traps in the carnivorous plant *Pinguicula moranensis* (Lentibulariaceae). *Oikos,* **119,** 891-895.

Allan, E. & Crawley, M.J. (2011) Contrasting effects of insect and molluscan herbivores on plant diversity in a long-term field experiment. *Ecology Letters,* **14,** 1246-1253.

Alonso, C., Mutikainen, P. & Herrera, C.M. (2007) Ecological context of breeding system variation: Sex, size and pollination in a (predominantly) Gynodioecious shrub. *Annals of Botany,* **100,** 1547-1556.

Anderson, M.J. & Underwood, A.J. (1997) Effects of gastropod grazers on recruitment and succession of an estuarine assemblage: A multivariate and univariate approach. *Oecologia,* **109,** 442-453.

Anderson, M.J. & Willis, T.J. (2003) Canonical analysis of principal coordinates: A useful method of constrained ordination for ecology. *Ecology,* **84,** 511-525.

Anderson, M.J. (1999) Distinguishing direct from indirect effects of grazers in intertidal estuarine assemblages. *Journal of Experimental Marine Biology and Ecology,* **234,** 199-218.

Anderson, M.J. (2004a) PERMDISP: a FORTRAN computer program for permutational analysis of multivariate dispersions (for any two-factor ANOVA design) using permutation tests. Department of Statistics, University of Aukland, New Zealand.

Anderson, M.J. (2004b) DISTLM v.5: a FORTRAN computer program to calculate a distance-based multivariate analysis for a linear model. Department of Statistics, University of Aukland, New Zealand.

Anderson, M.J. (2005) PERMANOVA: a FORTRAN computer program for permutational multivariate analysis of variance. Department of Statistics, University of Aukland, New Zealand.

Anderson, M.J. (2006) Distance-based tests for homogeneity of multivariate dispersions. *Biometrics,* **62,** 245-253.

Anderson, M.J. (2006) Distance-based tests for homogeneity of multivariate dispersions. *Biometrics,* **62,** 245-253.

Angseesing, J.P.A. (1974) Selective eating of acyanogenic form of *Trifolium repens*. *Heredity,* **32,** 73-83.

Archard, G.A., Bohan, D.A., Hughes, L. & Wiltshire, C.W. (2004) Spatial sampling to detect slug abundance in an arable field. *Annals of applied Biology,* **145,** 165-173.

Arhonditsis, G.B., Stow, C.A., Steinberg, L.J., Kenney, M.A., Lathrop, R.C., McBride, S.J. & Reckhow, K.H. (2006) Exploring ecological patterns with structural equation modeling and Bayesian analysis. *Ecological Modelling,* **192,** 385-409.

Ascensão, L. & Pais, M.S. (1998) The leaf capitate trichomes of *Leonotis leonurus*: Histochemistry, ultrastructure and secretion. *Annals of Botany,* **81,** 263-271.

Ascensao, L., Figueiredo, A.C., Barroso, J.G., Pedro, L.G., Schripsema, J., Deans, S.G. & Scheffer, J.J.C. (1998) *Plectranthus madagascariensis*: Morphology of the glandular trichomes, essential oil composition, and its biological activity. *International Journal of Plant Sciences,* **159,** 31-38.

Ascensão, L., Marques, N. & Pais, M.S. (1995) Glandular Trichomes on Vegetative and Reproductive Organs of *Leonotis leonurus* (Lamiaceæ). *Annals of Botany,* **75,** 619-626.

Ascensao, L., Marques, N. & Pais, M.S. (1997) Peltate glandular trichomes of *Leonotis leonurus* leaves: Ultrastructure and histochemical characterization of secretions. *International Journal of Plant Sciences,* **158,** 249-258.

Ascensão, L., Mota, L. & Castro, M.D.M. (1999) Glandular trichomes on the leaves and flowers of *Plectranthus ornatus*: Morphology, distribution and histochemistry. *Annals of Botany,* **84,** 437-447.

Ashman, T., Knight, T.M., Steets, J.A., Amarasekare, P., Burd, M., Campbell, D.R., Dudash, M.R., Johnston, M.O., Mazer, S.J., Mitchell, R.J., Morgan, M.T. & Wilson, W.G. (2004) Pollen limitation of plant reproduction: Ecological and evolutionary causes and consequences. *Ecology,* **85,** 2408-2421.

Asikainen, E. & Mutikainen, P. (2003) Female frequency and relative fitness of females and hermaphrodites in gynodioecious *Geranium sylvaticum* (Geraniaceae). *American Journal of Botany,* **90,** 226-234.

Asikainen, E. & Mutikainen, P. (2005a) Pollen and resource limitation in a gynodioecious species. *American Journal of Botany,* **92,** 487-494.

Asikainen, E. & Mutikainen, P. (2005b) Preferences of Pollinators and Herbivores in Gynodioecious *Geranium sylvaticum*. *Annals of Botany,* **95,** 879-886.

Ave, D.A., Gregory, P. & Tingey, W.M. (1987) Aphid repellent sesquiterpenes in glandular trichomes of *Solanum berthaultii* and *Solanum tuberosum*. *Entomologia Experimentalis Et Applicata,* **44,** 131-138.

avitompeg (2010) Avi to Mpeg v. 3.2 [online] Available at: http://www.avitompeg.net [Accessed 12 Feb 2012].

Ayoub, N.A. (2003) Unique phenolic carboxylic acids from *Sanguisorba minor*. *Phytochemistry,* **63,** 433-436.

Ball, B.C., Campbell, D.J., Douglas, J.T., Henshall, J.K. & O'Sullivan, M.F. (1997) Soil structural quality, compaction and land management. *European Journal of Soil Science,* **48,** 593-601.

Baran, P., Ozdemir, C. & Aktas, K. (2010) Structural investigation of the glandular trichomes of *Salvia argentea*. *Biologia,* **65,** 33-38.

Bardgett, R.D., Mawdsley, J.L., Edwards, S., Hobbs, P.J., Rodwell, J.S. & Davies, W.J. (1999) Plant species and nitrogen effects on soil biological properties of temperate upland grasslands. *Functional Ecology,* **13,** 650-660.

Bardgett, R.D., Smith, R.S., Shiel, R.S., Peacock, S., Simkin, J.M., Quirk, H. & Hobbs, P.J. (2006) Parasitic plants indirectly regulate below-ground properties in grassland ecosystems. *Nature,* **439,** 969-972.

Bardgett, R.D., Smith, R.S., Shiel, R.S., Tallowin, J.R.B., Mortimer, S.R., Brown, V.K., Pilgrim, E.S., Millward, D., Lawson, C., Harrison, K.A., Edwards, A.E., Hobbs, P.J., Sanderson, R., Harris, S.J., Beaumont, D.A., Murray, A.M., Wright, D.G. & Quirk, H. (2007) Confirming the reported association of high fungal to bacterial ratios with species-rich grasslands relative to improved and semi-improved grasslands. Diversification of grassland through the manipulation of plant-soil interactions (BD1451): report to Defra on objective 1.

Barker, G.M. & Addison, P.J. (1992) Pest status of slugs (Stylommatophora: Mollusca) in two New Zealand pastures. *Crop Protection,* **11,** 439-442.

Barker, G.M. (1989) Slug problems in New Zealand pastoral agriculture. *Slugs and Snails in World Agriculture* (ed. I.F. Henderson), pp. 59-68. British Crop Protection Council Monograph No. 41.

Barker, G.M. (1990) Pasture renovation: interactions of vegetation control with slug and insect infestations. *Journal of Agricultural Science,* **115,** 195-202.

Barker, G.M. (1991) Biology of slugs (Agriolimacidae and Arionidae: Mollusca) in New Zealand hill country pastures. *Oecologia,* **85,** 581-595.

Barker, G.M. (2001) *The Biology of Terrestrial Molluscs.* CABI Publishing, Wallingford.

Barker, G.M. (2002) *Molluscs as Crop Pests.* CABI Publishing, Wallingford.

Barker, G.M. (2002) *Molluscs as Crop Pests.* CABI Publishing, Wallingford.

BARS (2008) National action plan - Biodiversity Action Reporting System. [online] Available at: http://ukbars.defra.gov.uk/plans/national_plan.a...HAP=%7B03279B7B-2C03-443E-A6DB-54726F7BBDC8%7D [Accessed: 11 Feb 2012].

Barthlott, W. (1980) Development and micro-morphology of complex cuticular fold patterns on floral trichomes of *Antirrhinum* L (Scropulariaceae). *Berichte Der Deutschen Botanischen Gesellschaft,* **93,** 379-390.

Baur, R., Binder, S. & Benz, G. (1991) Nonglandular leaf trichomes as short-term inducible defense of the gray alder, *Alnus incana* (L.), against the chrysomelid beetle, *Agelastica alni* L. *Oecologia,* **87,** 219-226.

BBCT (2011) The Short-haired bumblebee reintroduction project. [online] Available at: http://www.bumblebeeconservation.org/subt_project.html [Accessed: 5 Feb 2012].

Bergelson, J. (1990) Spatial patterning in plants: opposing effects of herbivory and competition. *Journal of Ecology,* **78,** 937-948.

Biesmeijer, J.C., Roberts, S.P.M., Reemer, M., Ohlemueller, R., Edwards, M., Peeters, T., Schaffers, A.P., Potts, S.G., Kleukers, R., Thomas, C.D., Settele, J. & Kunin, W.E. (2006) Parallel declines in pollinators and insect- pollinated plants in Britain and the Netherlands. *Science,* **313,** 351-354.

Bjorkman, C., Dalin, P. & Ahrne, K. (2008) Leaf trichome responses to herbivory in willows: induction, relaxation and costs. *New Phytologist,* **179,** 176-184.

Blackstock, T.H., Rimes, C.A., Stevens, D.P., Jefferson, R.G., Robertson, H.J., Mackintosh, J. & Hopkins, J.J. (1999) The extent of semi-natural grassland communities in lowland England and Wales: a review of conservation surveys 1978-96. *Grass and Forage Science,* **54,** 1-18.

Boag, D.A. & Wishart, W.D. (1982) Distribution and abundance of terrestrial gastropods on a winter range of bighorn sheep in southwestern Alberta. *Canadian Journal of Zoology-Revue Canadienne De Zoologie,* **60,** 2633-2640.

Bohan, D.A., Bohan, A.C., Glen, D.M., Symondson, W.O.C., Wiltshire, C.W. & Hughes, L. (2000a) Spatial dynamics of predation by carabid beetles on slugs. *Journal of Animal Ecology,* **69,** 367-379.

Bohan, D.A., Glen, D.M., Wiltshire, C.W. & Hughes, L. (2000b) Parametric intensity and the spatial arrangement of the terrestrial mollusc herbivores *Deroceras reticulatum* and *Arion intermedius*. *Journal of Animal Ecology,* **69,** 1031-1046.

Borrelli, N., Fernández Honaine, M., Altamirano, S.M. & Osterrieth, M. (2011) Calcium and silica biomineralizations in leaves of eleven aquatic species of the Pampean Plain, Argentina. *Aquatic Botany,* **94,** 29-36.

Bourett, T.M., Howard, R.J., Okeefe, D.P. & Hallahan, D.L. (1994) Gland development on leaf surfaces of *Neptea racemosa*. *International Journal of Plant Sciences,* **155,** 623-632.

Bowers, M.D. & Stamp, N.E. (1993) Effects of plant age, genotype, and herbivory on *Plantago* performance and chemistry. *Ecology,* **74,** 1778-1791.

Boycott, A.E. (1934) The habitats of land mollusca in Britain. *Journal of Ecology,* **22,** 1-38.

Briner, T. & Frank, T. (1998) The palatability of 78 wildflower strip plants to the slug *Arion lusitanicus*. *Annals of applied Biology,* **133,** 123-133.

Brown, A.S.S. & Simmonds, M.S.J. (2006) Leaf morphology of hosts and nonhosts of the thrips *Heliothrips haemorrhoidalis* (Bouche). *Botanical Journal of the Linnean Society,* **152,** 109-130.

Brown, V.K. & Gange, A.C. (1989a) Differential effect of above- and below-ground insect herbivory during early plant succession. *Oikos,* **54,** 67-76.

Brown, V.K. & Gange, A.C. (1989b) Herbivory by Soil-Dwelling Insects Depresses Plant Species Richness. *Functional Ecology,* **3,** 667-671.

Brown, V.K. & Gange, A.C. (1992) Secondary plant succession: how is it modified by insect herbivory? *Vegetatio,* **101,** 3-13.

Bruijns, M.F.M., Altena, C.O.v.K. & Butot, L.J.M. (1959) The Netherlands as an environment for land Mollusca. *Basteria Suppl,* **23,** 132-162.

Buckland, S.M. & Grime, J.P. (2000) The effects of trophic structure and soil fertility on the assembly of plant communities: A microcosm experiment. *Oikos,* **91,** 336-352.

Bullock, J.M et al. (2011) Semi-natural Grasslands. In: *The UK National Ecosystem Assessment Technical Report*. UK National Ecosystem Assessment, UNEP-WCMC, Cambridge.

Burd, M. (1994) Bateman's Principle and Plant Reproduction: The role of Pollen Limitation in Fruit and Seed Set. *The Botanical Review,* **60,** 83-139.

Burd, M. (1995) Ovule packaging in stochastic pollination and fertilization environments. *Evolution,* **49,** 100-109.

Burt-Smith, G.S., Grime, J.P. & Tilman, D. (2003) Seedling resistance to herbivory as a predictor of relative abundance in a synthesised prairie community. *Oikos,* **101,** 345-353.

Buschmann, H., Keller, M., Porret, N., Dietz, H. & Edwards, P.J. (2005) The effect of slug grazing on vegetation development and plant species diversity in an experimental grassland. *Functional Ecology,* **19,** 291-298.

BWARS (n.d) Distibution Maps. [online] Available at: http://www.bwars.com/maps_bees.htm [Accessed 13 Feb 2012].

Calo, L., Garcia, I., Gotor, C. & Romero, L.C. (2006) Leaf hairs influence phytopathogenic fungus infection and confer an increased resistance when expressing a Trichoderma alpha-1,3-glucanase. *Journal of Experimental Botany,* **57,** 3911-3920.

Cameron, D.D. & Seel, W.E. (2003) Variable resistance to the grassland hemi-parasite *Rhinanthus minor*, and consequences for community composition. *Comparative Biochemistry and Physiology,* **134,** 158-159.

Cameron, D.D. & Seel, W.E. (2007) Functional anatomy of haustoria formed by *Rhinanthus minor*: linking evidence from histology and isotope tracing. *New Phytologist,* **174,** 412-419.

Cameron, R.A.D., Jackson, N. & Eversham, B. (1983) A field key to the slugs of the British Isles. *Field Studies,* **5,** 807-824.

Candan, F., Unlu, M., Tepe, B., Daferera, D., Polissiou, M., Sokmen, A. & Akpulat, H.A. (2003) Antioxidant and antimicrobial activity of the essential oil and methanol extracts of *Achillea millefolium* subsp *millefolium* Afan. (Asteraceae). *Journal of Ethnopharmacology,* **87,** 215-220.

Carmona, D., Lajeunesse, M.J. & Johnson, M.T.J. (2011) Plant traits that predict resistance to herbivores. *Functional Ecology,* **25**.

Carrick, R. (1938) The life history and development of *Agriolimax agrestis* L. the grey field slug. *Transactions of the Royal Society of Edinburgh,* **59,** 563-597.

Carvell, C. (2002) Habitat use and conservation of bumblebees (*Bombus* spp.) under different grassland management regimes. *Biological Conservation,* **103,** 33-49.

Carvell, C., Meek, W.R., Pywell, R.F., Goulson, D. & Nowakowski, M. (2007) Comparing the efficacy of agri-environment schemes to enhance bumble bee abundance and diversity on arable field margins. *Journal of Applied Ecology,* **44,** 29-40.

Carvell, C., Osborne, J.L., Bourke, A.F.G., Freeman, S.N., Pywell, R.F. & Heard, M.S. (2011) Bumble bee species' responses to a targeted conservation measure depend on landscape context and habitat quality. *Ecological Applications,* **21,** 1760-1771.

Carvell, C., Roy, D.B., Smart, S.M., Pywell, R.F., Preston, C.D. & Goulson, D. (2006) Declines in forage availability for bumblebees at a national scale. *Biological Conservation,* **132,** 481-489.

Casida, J.E. (1980) Pyrethrum flowers and pyrethroid insecticides. *Environmental Health Perspectives,* **34,** 189-202.

Cates, R.G. & Orians, G.H. (1975) Successional status and palatability of plants to generalized herbivores. *Ecology,* **56,** 410-418.

CBD (2011) Partners & Initiatives. [online] Available at: http://www.cbd.int/agro/pollinatorspartners.shtml [Accessed: 24 Feb 2012].

Chamberlain, D.E., Fuller, R.J., Bunce, R.G.H., Duckworth, J.C. & Shrubb, M. (2000) Changes in the abundance of farmland birds in relation to the timing of agricultural intensification in England and Wales. *Journal of Applied Ecology,* **37,** 771-788.

Charlesworth, B. & Charlesworth, D. (1978) A model for the evolution of dioecy and gynodioecy. *American Naturalist,* **112,** 975-997.

Chemin, E. (1920) Observations anatomiques et biologiques sur le genre *Lathraea*. *An. Sci. Nat. B.,* **10,** 125-272.

Cherif, M., Menzies, J.G., Benhamou, N. & Belanger, R.R. (1992) Studies of silicon distribution in wounded and *Pythium ultimum* infected cucumber plants. P*hysiological and Molecular Plant Pathology,* **41,** 371-385.

Choi, Y.H., Bohan, D.A., Potting, R.P.J., Semenov, M.A. & Glen, D.M. (2006) Individual based model of slug population and spatial dynamics. *Ecological Modelling,* **190,** 336-350.

Clarke, K.R. & Gorley, R.N. (2006) PRIMER V6: User Manual/Tutorial. PRIMER-E, Plymouth.

Clements, R.O. & Murray, P.J. (1991) Comparison between defined-area slug traps and other methods of trapping slugs in cereal fields. *Crop Protection,* **10,** 152-154.

Clements, R.O., Murray, P.J., Bentley, B.R., Lewis, G.C. & French, N. (1990) The impact of pests and diseases on the herbage yield of permanent grassland at eight sites in England and Wales. *Annals of applied Biology,* **117,** 349-357.

Coles, S.M. (1971) The *Ranunculus acris* D complex in Europe. *Watsonia,* **8,** 237-262.

Colla, S.R. & Packer, L. (2008) Evidence for decline in eastern North American bumblebees (Hymenoptera: Apidae), with special focus on *Bombus affinis* Cresson. *Biodiversity and Conservation,* **17,** 1379-1391.

Cook, A. & Radford, D.J. (1988) The comparative ecology of four sympatric limacid slug species in Northern Ireland (UK). *Malacologia 28(1-2): 131-146.*

Cook, A. (2001) Behavioural Ecology: On Doing the Right Thing, in the Right Place at the Right Time. *The Biology of Terrestrial Molluscs* (ed. G.M. Barker), pp. 447-488. CABI Publishing, Wallingford.

Cook, R.T., Bailey, S.E.R. & McCrohan, C.R. (1996) Slug preferences for winter wheat cultivars and common agricultural weeds. *Journal of Applied Ecology,* **33,** 866-872.

Cooke, J. & Leishman, M.R. (2011) Silicon concentration and leaf longevity: is silicon a player in the leaf dry mass spectrum? *Functional Ecology,* **25,** 1181-1188.

Corbet, S.A., Williams, I.H. & Osborne, J.L. (1991) Bees and the pollination of crops and wildflowers in the European community. *Bee World,* **72,** 47-59.

Cordoba, M., Iglesias, J., Ribadulla, P. & Castillejo, J. (2011) Performance of permanent refuge traps for the assessment of slug populations in pastureland. *Annals of applied Biology,* **159,** 130-140.

Corkill, L. (1942) Cyanogenesis in white clover (*Trifolium repens* L.). V. The inheritance of cyanogenesis. *New Zealand Journal of Science and Technology,* **23,** 178B-193B.

Cottam, D.A. (1985) Frequency-dependent grazing by slugs and grasshoppers. *Journal of Ecology,* **73,** 925-933.

Cottam, D.A. (1986) The effects of slug-grazing on *Trifolium repens* and *Dactylis glomerata* in monoculture and mixed sward. *Oikos,* **47,** 275-279.

Crawley, M.J. (1989) Insect herbivores and plant-population dynamics. *Annual Review of Entomology,* **34,** 531-564.

Critchley, C.N.R., Burke, M.J.W. & Stevens, D.P. (2004) Conservation of lowland semi-natural grasslands in the UK: a review of botanical monitoring results from agri-environment schemes. *Biological Conservation,* **115,** 263-278.

da Silva, A.P., Kay, B.D. & Perfect, E. (1997) Management versus inherent soil properties effects on bulk density and relative compaction. *Soil & Tillage Research,* **44,** 81-93.

Dahlin, R.M., Brick, M.A. & Ogg, J.B. (1992) Characterization and Density of Trichomes on Three Common Bean Cultivars. *Economic Botany,* **46,** 299-304.

Dai, X., Wang, G., Yang, D.S., Tang, Y., Broun, P., Marks, M.D., Sumner, L.W., Dixon, R.A. & Zhao, P.X. (2010) TrichOME: A Comparative Omics Database for Plant Trichomes. *Plant Physiology,* **152,** 44-54.

Dallinger, R., Berger, B., Triebskorn-Kohler, R. & Kohler, H. (2001) Soil Biology and Ecotoxicology. *The Biology of Terrestrial Molluscs* (ed. G.M. Barker), pp. 489-525. CABI Publishing, Wallingford.

Darrow, K. & Bowers, M.D. (1999) Effects of herbivore damage and nutrient level on induction of iridoid glycosides in *Plantago lanceolata*. *Journal of Chemical Ecology,* **25,** 1427-1440.

Darvill, B., Mabon, G. & Rolph, N. (2010) Teesdale bumblebee survey: A survey of upland hay meadows and nearby habitats in the North Pennines AONB. Bumblebee Conservation Trust.

Darwin, C.R. (1877) *The different forms of flowers on plants of the same species*. Murray, London.

Davies, D.M., Graves, J.D., Elias, C.O. & Williams, P.J. (1997) The impact of *Rhinanthus* spp. on sward productivity and composition: implications for the restoration of species-rich grasslands. *Biological Conservation,* **82,** 87-93.

De Deyn, G.B., Quirk, H. & Bardgett, R.D. (2012) Plant species richness, identity and productivity differentially influence key groups of microbes in grassland soils of contrasting fertility. *Biology Letters,* **7,** 75-78.

Dedov, I., Stoyanov, I.L., Penev, L., Harvey, J.A., Van der Putten, W.H. & Bezemer, T.M. (2006) Long-term effects of sowing high or low diverse seed mixtures on plant and gastropod diversity. *Acta Oecologica-International Journal of Ecology,* **30,** 173-181.

DEFRA (2012) Environmental Stewardship Defra. [online]
Available at: http://www.defra.gov.uk/food-farm/land-manage/stewardship
[Accessed: 18 Feb 2012].

Delph, L.F. & Lively, C.M. (1992) Pollinator visitation, floral display, and nectar production of the sexual morphs of a gynodioecious shrub. *Oikos,* **63,** 161-170.

Del-Val, E. & Crawley, M.J. (2004) Importance of tolerance to herbivory for plant survival in a British grassland. *Journal of Vegetation Science,* **15,** 357-364.

Del-Val, E. & Crawley, M.J. (2005) What limits herb biomass in grasslands: Competition or herbivory? *Oecologia,* **142,** 202-211.

Dirzo, R. (1980) Experimental studies on slug-plant interactions I. The acceptability of thirty plant species to the slug *Agriolimax Carunae*. *Journal of Ecology,* **68,** 981-998.

Dirzo, R. & Harper, J.L. (1980) Experimental studies on slug-plant interactions. II. The effect of grazing by slugs on high density monocultures of Capsella bursa-pastoris and Poa annua. *Journal of Ecology,* **68,** 999-1011.

Dirzo, R. & Harper, J. (1982a) Experimental studies on slug-plant interactions III. Differences in the acceptability of individual plants of *Trifolium repens* to slugs and snails. *Journal of Ecology,* **70,** 101-117.

Dirzo, R. & Harper, J. (1982b) Experimental studies on slug-plant interactions IV. The performance of cyanogenic and acyanogenic morphs of *Trifolium repens* in the field. *Journal of Ecology,* **70,** 119-138.

Duffey, S.S., Juniper, B. & Southwood, R. (1986) Plant glandular trichomes: their partial role in defence against insects. *Insects and the plant surface.* (eds B. Juniper & R. Southwood), pp. 151-172. Edward Arnold, London.

Duke, S.O. & Paul, R.N. (1993) Development and fine-structure of the glandular trichomes of *Artemisia annua* L. *International Journal of Plant Sciences,* **154,** 107-118.

Duke, S.O. (1994) Glandular Trichomes - A Focal Point of Chemical and Structural Interactions. *International Journal of Plant Sciences,* **155,** 617-620.

Dupont, Y.L. & Olesen, J.M. (2009) Ecological modules and roles of species in heathland plant-insect flower visitor networks. *Journal of Animal Ecology,* **78,** 346-353.

Duval, D.M. (1970) Some aspects of the behaviour of pest species of slugs. *Journal of Conchology,* **27,** 163-170.

Duval, D.M. (1971) A note on the acceptability of various weeds as food for *Agriolimax reticulatus* (Müller). *Journal of Conchology,* **27,** 249-251.

Duval, D.M. (1973) A note on the acceptability of various weeds as food for *Arion hortensis*. *Journal of Conchology,* **28,** 37-39.

Edmonds, J.M. (1982) Epidermal hair morphology in *Solanum* L. section *Solanum*. *Botanical Journal of the Linnean Society,* **85,** 153-167.

Edwards, M. & Williams, P. (2004) Where have all the bumblebees gone, and could they ever return? *British Wildlife,* **15,** 305-312.

Elger, A., Lemoine, D.G., Fenner, M. & Hanley, M.E. (2009) Plant ontogeny and chemical defence: Older seedlings are better defended. *Oikos,* **118,** 767-773.

Epstein, E. (1999) Silicon. *Annual Review of Plant Physiology and Plant Molecular Biology,* **50,** 641-664.

Epstein, E. (2009) Silicon: its manifold roles in plants. *Annals of applied Biology,* **155,** 155-160.

Evju, M., Halvorsen, R., Rydgren, K., Austrheim, G. & Mysterud, A. (2011) Effects of sheep grazing and temporal variability on population dynamics of the clonal herb *Geranium sylvaticum* in an alpine habitat. *Plant Ecology,* **212,** 1299-1312.

Fahn, A. & Shimony, C. (1996) Glandular trichomes of *Fagonia* L (Zygophyllaceae) species: Structure, development and secreted materials. *Annals of Botany,* **77,** 25-34.

Faraway, J.J. (2006) *Extending the Linear Model with R: Generalized Linear, Mixed Effects and Nonparametric Regression Models*. USA: Chapman & Hall/CRC.

Feeny, P.P. (1976) Plant apparency and chemical defense. *Recent Advances in Phytochemistry,* **10,** 1-40.

Fenner, M. (1987) Seedlings. *New Phytologist,* **106,** 35-47.

Fenner, M., Hanley, M.E. & Lawrence, R. (1999) Comparison of seedling and adult palatability in annual and perennial plants. *Functional Ecology,* **13,** 546-551.

Fenner, M., Thompson, K. (2005) *The ecology of seeds*. Cambridge: Cambridge University Press.

Ferguson, C.M. & Hanks, C.B. (1990) Evaluation of defined-area trapping for estimating the density of the field slug *Deroceras reticulatum* (Muller). *Annals of applied Biology,* 117, 451-454.

Ferguson, C.M., Barratt, B.I.P. & Jones, P.A. (1988) Control of the grey field slug (*Deroceras reticulatum* (Müller)) by stock management prior to direct-drilled pasture establishment. *Journal of Agricultural Science,* **111,** 443-449.

Ferguson, C.M., Barratt, B.I.P. & Jones, P.A. (1989) A new technique for estimating density of the field slug (*Deroceras reticulatum* (Müller)). *Slugs and Snails in World Agriculture BCPC Mongraph No. 41* (ed. I.F. Henderson), pp. 331-336.

floralimages.co.uk (1997) Floralimages [online] Available at: http://www.floralimages.co.uk [Accessed: 24 Feb 2012].

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

Fountain, M.T., Thomas, R.S., Brown, V.K., Gange, A.C., Murray, P.J. & Symondson, W.O.C. (2009) Effects of nutrient and insecticide treatments on invertebrate numbers and predation on slugs in an upland grassland: A monoclonal antibody-based approach. *Agriculture Ecosystems & Environment,* **131,** 145-153.

Fitter, A.H & Peat, H.J. (1994) The Ecological Flora Database. *Journal of Ecology* **82**: 415-425. http://www.ecoflora.co.uk [Accessed: 14 Oct. 2012].

Franceschi, V.R. & Giaquinta, R.T. (1983) Glandular trichomes of soybean leaves - cytological differentitation from initiation through senescence. *Botanical Gazette,* **144,** 175-184.

Frank, T. & Barone, M. (1999) Short-term field study on weeds reducing slug feeding on oilseed rape. *Zeitschrift-fuer-Pflanzenkrankheiten-und-Pflanzenschutz.Sept., 1999; 106 (5): 534-538.*

Frank, T. (1998) Slug damage and numbers of the slug pests, *Arion lusitanicus* and *Deroceras reticulatum*, in oilseed rape grown beside sown wildflower strips. *Agriculture, Ecosystems and Environment,* **67,** 67-78.

Frank, T. (2003) Influence of slug herbivory on the vegetation development in an experimental wildflower strip. *Basic and Applied Ecology,* **4,** 139-147.

Frantz, J.M., Locke, J.C., Datnoff, L., Omer, M., Widrig, A., Sturtz, D., Horst, L. & Krause, C.R. (2008) Detection, Distribution, and Quantification of Silicon in Floricultural Crops utilizing Three Distinct Analytical Methods. *Communications in Soil Science and Plant Analysis,* **39,** 2734-2751.

Fuller, R.M. (1987) The changing extent and conservation interest of lowland grasslands in England and Wales – a review of grassland surveys 1930-1984. *Biological Conservation,* **40,** 281-300.

Gang, D.R., Wang, J.H., Dudareva, N., Nam, K.H., Simon, J.E., Lewinsohn, E. & Pichersky, E. (2001) An investigation of the storage and biosynthesis of phenylpropenes in sweet basil. *Plant Physiology,* 125, 539-555.

Gelman, A. & Hill, J. (2007) *Data Analysis Using Regression and Multilevel/Hierarchical Models*. Cambridge: Cambridge University Press.

Gerhold, D.L., Craig, R. & Mumma, R.O. (1984) Analysis of trichome exudate from mite-resistant *Geraniums*. *Journal of Chemical Ecology,* **10,** 713-722.

Gershenzon, J., Maffei, M. & Croteau, R. (1989) Biochemical and histochemical localization of monoterpene biosynthesis in the glandular trichomes of spearmint (*Mentha spicata*). *Plant Physiology,* **89,** 1351-1357.

Gershenzon, J., McCaskill, D., Rajaonarivony, J.I.M., Mihaliak, C., Karp, F. & Croteau, R. (1992) Isolation of secretory cells from plant glandular trichomes and their use in biosynthetic studies of monoterpenes and other gland products. *Analytical Biochemistry,* **200,** 130-138.

Getpelletwise (2010) Get Pelletwise. [online] Available at: http://www.getpelletwise.co.uk [Accessed: 22 Feb2012].

Getz, L.L. (1959) Notes on the ecology of slugs. *American Midland Naturalist,* **61,** 485-498.

Ghazoul, J. (2005) Buzziness as usual? Questioning the global pollination crisis. *Trends in Ecology & Evolution,* **20,** 367-373.

Gibson, C.C. & Watkinson, A.R. (1989) The host range and selectivity of a parasitic plant: *Rhinanthus minor* L. *Oecologica,* **78,** 401-406.

Gibson, C.C. & Watkinson, A.R. (1992) The role of the hemiparasitic annual *Rhianthus minor* in determining grassland community structure. *Oecologia,* **89,** 62-68.

Gibson, C.C. (1986) The population and community biology of *Rhinanthus minor* L. PhD Thesis. The University of East Anglia, Norwich, UK.

Gibson, C.C. (2000) Use of hay rattle. *Enact,* **8,** 14.

Gibson, D.J., Urban, J. & Baer, S.G. (2011) Mowing and fertilizer effects on seedling establishment in a successional old field. *Journal of Plant Ecology-Uk,* **4,** 157-168.

Gilbert, L.E. (1971) Butterfly-plant coevolution: Has *Passiflora adenopoda* won the selectional race with heliconiine butterflies? *Science,* **172,** 585-586.

Glen, D.M., Cuerden, R. & Butler, R.C. (1991) Impact of the field slug *Deroceras reticulatum* on establishment of ryegrass and white clover in mixed swards. *Annals of applied Biology,* **119,** 155-162.

Glen, D.M., Jones, H. & Fieldsend, J.K. (1990) Damage to oilseed rape seedlings by the field slug *Deroceras reticulatum* in relation to glucosinolate concentration. *Annals of applied Biology,* **117,** 197-207.

Glen, D.M., Milsom, N.F. & Wiltshire, C.W. (1989) Effects of seed-bed conditions on slug numbers and damage to winter wheat in a clay soil. *Annals of applied Biology,* **115,** 177-190.

Glen, D.M., Wiltshire, C.W. & Milsom, N.F. (1984) Slugs and straw disposal in winter wheat. *BCPC Conference – Pests and Diseases*, pp. 139-144. British Crop Protection Council.

Goulson, D. & Darvill, B. (2004) Niche overlap and diet breadth in bumblebees; are rare species more specialized in their choice of flowers? *Apidologie,* **35,** 55-63.

Goulson, D. & Hanley, M.E. (2004) Distribution and forage use of exotic bumblebees in South Island, New Zealand. *New Zealand Journal of Ecology,* **28,** 225-232.

Goulson, D. (1999) Foraging strategies of insects for gathering nectar and pollen, and implications for plant ecology and evolution. *Perspectives in Plant Ecology Evolution and Systematics,* **2,** 185-209.

Goulson, D. (2003) *Bumblebees: Their Behaviour and Ecology*. Oxford University Press, New York, USA.

Goulson, D. (2006) The demise of the bumblebee in Britain. *Biologist,* **53,** 294-299.

Goulson, D., Cruise, J.L., Sparrow, K.R., Harris, A.J., Park, K.J., Tinsley, M.C. & Gilburn, A.S. (2007) Choosing rewarding flowers; perceptual limitations and innate preferences influence decision making in bumblebees and honeybees. *Behavioral Ecology and Sociobiology,* **61,** 1523-1529.

Goulson, D., Hanley, M.E., Darvill, B., Ellis, J.S. & Knight, M.E. (2005) Causes of rarity in bumblebees. *Biological Conservation,* **122,** 1-8.

Goulson, D., Lye, G.C. & Darvill, B. (2008) Diet breadth, coexistence and rarity in bumblebees. *Biodiversity and Conservation,* **17,** 3269-3288.

Goulson, D., Rayner, P., Dawson, B. & Darvill, B. (2011) Translating research into action; bumblebee conservation as a case study. *Journal of Applied Ecology,* **48,** 3-8.

Gouyon, P.H. & Couvet, D. (1988) A conflict between two sexes, females and hermaphrodites. *The evolution of sex and its consequences* (ed. S.C. Stearns), pp. 245-261. Birkhauser Verlag, Basel, Switzerland.

Govier, R.N., Brown, J.G.S. & Pate, J.S. (1968) Hemiparasitic nutrition in angiosperms. *New Phytologist,* **67,** 963-972.

Grassi, P., Novak, J., Steinlesberger, H. & Franz, C. (2004) A Direct Liquid, Non-equilibrium Solid-phase Micro-extraction Application for Analysing Chemical Variation of Single Peltate Trichomes on Leaves of *Salvia officinalis*. *Phytochemical Analysis,* **15,** 198-203.

Greenwood, K.L. & McKenzie, B.M. (2001) Grazing effects on soil physical properties and the consequences for pastures: a review. *Australian Journal of Experimental Agriculture,* **41,** 1231-1250.

Gregory, P., Ave, D.A., Bouthyette, P.J. & Tingey, W.M. (1986) Insect-defensive chemistry of potato glandular trichomes. *Insects and the Plant Surface* (eds B. Juniper & R. Southwood), pp. 173-183. Edward Arnold, London.

Griffiths, J., Phillips, D.S., Compton, S.G., Wright, C. & Incoll, L.D. (1998) Responses of slug numbers and slug damage to crops in a silvoarable agroforestry landscape. *Journal of Applied Ecology,* **35,** 252-260.

Grime, J.P. & Blythe, G.M. (1969) An investigation of the relationships between snails and vegetation at the Winnats Pass. *Journal of Ecology,* **57,** 45-66.

Grime, J.P. (2002) *Plant Strategies, Vegetation Processes, and Ecosystem Properties (2nd Ed.)*. John Wiley & Sons, Chichester.

Grime, J.P., Cornelissen, J.H.C., Thompson, K. & Hodgson, J.G. (1996) Evidence of a causal connection between anti-herbivore defence and the decomposition rate of leaves. *Oikos,* **77,** 489-494.

Grime, J.P., Hodgson, J.G. & Hunt, R. (1988) *Comparative Plant Ecology - A Functional Approach to Common British Species*. Unwin-Hyman, London.

Grime, J.P., Hodgson, J.G. & Hunt, R. (2007) *Comparative Plant Ecology: A Functional Approach to Common British Species*, 2nd edn. London: Unwin-Hyman.

Grime, J.P., MacPherson-Stewart, S.F. & Dearman, R.S. (1968) An investigation of leaf palatability using the snail C*epaea nemoralis* L. *Journal of Ecology,* **57,** 45-66.

Grixti, J.C., Wong, L.T., Cameron, S.A. & Favret, C. (2009) Decline of bumble bees (*Bombus*) in the North American Midwest. *Biological Conservation,* **142,** 75-84.

Grubb, P.J. (1976) A theoretical background to the conservation of ecologically distinct groups of annuals and biennials in the chalk grassland ecosystem. *Biological Conservation,* **10,** 53-76.

Grubb, P.J. (1977) Maintenance of species-richness in plant communities - importance of regeneration niche. *Biological Reviews of the Cambridge Philosophical Society,* **52,** 107-145.

Guang, Z.Y., Hong, X.L., Fa, J.S. & Chen, Y. (2007) Diterpenoid and phenolic compounds from *Juncus effusus* L. *Helvetica Chimica Acta,* **90,** 1289-1295.

Guisan, A., Edwards, T.C. & Hastie, T. (2002) Generalized linear and generalized additive models in studies of species distributions: setting the scene. *Ecological Modelling* **157**: 89-100.

Haddad, N.M. & Hicks, W.M. (2000) Host pubescence and the behavior and performance of the butterfly *Papilio troilus* (Lepidoptera : Papilionidae). *Environmental Entomology,* **29,** 299-303.

Haines-Young, R., Barr, C.J., Firbank, L.G., Furse, M., Howard, D.C., McGowan, G., Petit, S., Smart, S.M. & Watkins, J.W. (2003) Changing landscapes, habitats and vegetation diversity across Great Britain. *Journal of Environmental Management,* **67,** 267-281.

Handley, R., Ekbom, B. & Ågren, J. (2005) Variation in trichome density and resistance against a specialist insect herbivore in natural populations of *Arabidopsis thaliana*. *Ecological Entomology,* **30,** 284-292.

Hanley, M.E. & Fenner, M. (1997) Effects of molluscicides on seedlings of four grassland plant species. *Journal of Applied Ecology,* **34,** 1479-1483.

Hanley, M.E. & Lamont, B.B. (2002) Relationships between physical and chemical attributes of congeneric seedlings: how important is seedling defence? *Functional Ecology,* **16,** 216-222.

Hanley, M.E. & Sykes, R.J. (2009) Impacts of seedling herbivory on plant competition and implications for species coexistence. *Annals of Botany,* **103,** 1347-1353.

Hanley, M.E. (1998) Seedling herbivory, community composition and plant life history traits. *Perspectives in Plant Ecology, Evolution and Systematics,* **1,** 191-205.

Hanley, M.E. (2004) Seedling herbivory and the influence of plant species richness in seedling neighbourhoods. *Plant Ecology,* **170,** 35-41.

Hanley, M.E., Collins, S.A. & Swann, C. (2011) Advertising acceptability: is mollusk olfaction important in seedling selection? *Plant Ecology,* **212,** 727-731.

Hanley, M.E., Fenner, M. & Edwards, P.J. (1995a) An Experimental Field-Study of the Effects of Mollusk Grazing On Seedling Recruitment and Survival in Grassland. *Journal of Ecology,* **83,** 621-627.

Hanley, M.E., Fenner, M. & Edwards, P.J. (1995b) The effect of seedling age on the likelihood of herbivory by the slug *Deroceras reticulatum*. *Functional Ecology,* **9,** 754-759.

Hanley, M.E., Fenner, M. & Edwards, P.J. (1996) The effect of mollusc grazing on seedling recruitment in artificially created grassland gaps. *Oecologia,* **106,** 240-246.

Hanley, M.E., Fenner, M., Whibley, H. & Darvill, B. (2004) Early plant growth: Identifying the end point of the seedling phase. *New Phytologist,* **163,** 61-66.

Hanley, M.E., Franco, M., Pichon, S., Darvill, B. & Goulson, D. (2008) Breeding system, pollinator choice and variation in pollen quality in British herbaceous plants. *Functional Ecology,* **22,** 592-598.

Hanley, M.E., Lamont, B.B., Fairbanks, M.M. & Rafferty, C.M. (2007) Plant structural traits and their role in anti-herbivore defence. *Perspectives in Plant Ecology Evolution and Systematics,* **8,** 157-178.

Hastie, T.J. & Tibshirani, R.J. (1990) *Generalized Additive Models: Monographs on statistics and applied probability*. Chapman & Hall, London.

Hawkins, J.W., Lankester, M.W., Lautenschlager, R.A. & Bell, F.W. (1997) Effects of alternative conifer release treatments on terrestrial gastropods in northwestern Ontario. *Forestry Chronicle,* **73,** 91-98.

Hayward, D.M. & Parry, D.W. (1973) Electron-probe Microanalysis Studies of Silica Distribution in Barley (*Hordeum sativum* L.). *Annals of Botany,* **37,** 579-591.

Hegland, S.J. & Totland, O. (2007) Pollen limitation affects progeny vigour and subsequent recruitment in the insect-pollinated herb *Ranunculus acris*. *Oikos,* **116,** 1204-1210.

Hegland, S.J., Dunne, J., Nielsen, A. & Memmott, J. (2010) How to monitor ecological communities cost-efficiently: The example of plant-pollinator networks. *Biological Conservation,* **143,** 2092-2101.

Heinz, K.M. & Zalom, F.G. (1995) Variation in trichome-based resistance to Bemisia argentifolii (Homoptera, Aleyrodidae) oviposition on tomato. *Journal of Economic Entomology,* **88,** 1494-1502.

Herms, D.A. & Mattson, W.J. (1992) The dilemma of plants: To grow or defend. *Quarterly Review of Biology,* **67,** 283-335.

Hitchmough, J.D. (2003) Effects of sward height, gap size, and slug grazing on emergence and establishment of *Trollius europaeus* (Globeflower). *Restoration Ecology,* **11,** 20-28.

Honek, A. & Martinkova, Z. (2011) Body size and the colonisation of cereal crops by the invasive slug *Arion lusitanicus*. *Annals of applied Biology,* **158,** 79-86.

Hopkins, A., Pywell, R.F., Peel, S., Johnson, R.H. & Bowling, P.J. (1999) Enhancement of botanical diversity of permanent grassland and impact on hay production in Environmentally Sensitive Areas in the UK. *Grass and Forage Science,* **54,** 163-173.

Horrill, J.C. & Richards, A.J. (1986) Differential grazing by the mollusc *Arion hortensis* Fer. on cyanogenic and acyanogenic seedlings of the white clover, *Trifolium repens* L. *Heredity,* **56,** 277-281.

Howard, D.C., Watkins, J.W., Clarke, R.T., Barnett, C.L. & Stark, G.J. (2003) Estimating the extent and change in broad habitats in Great Britain. *Journal of Environmental Management,* **67,** 219-227.

Hulme, P.E. (1994) Seedling herbivory in grassland: Relative impact of vertebrate and invertebrate herbivores. *Journal of Ecology,* **82,** 873-880.

Hulme, P.E. (1996) Herbivores and the performance of grassland plants: A comparison of arthropod, mollusc and rodent herbivory. *Journal of Ecology,* **84,** 43-51.

Hunter, P.J. (1966) The distribution and abundance of slugs on an arable plot in Northumberland. *Journal of Animal Ecology,* **35,** 543-557.

Hylander, K., Nilsson, C. & Gothner, T. (2004) Effects of buffer-strip retention and clearcutting on land snails in boreal riparian forests. *Conservation Biology,* **18,** 1052-1062.

Imhoff, S., da Silva, A.P. & Tormena, C.A. (2000) Spatial heterogeneity of soil properties in areas under elephant-grass short-duration grazing system. *Plant and Soil,* **219,** 161-168.

Jefferson, R.G. (2005) The conservation management of upland hay meadows in Britain: a review. *Grass and Forage Science,* **60,** 322-331.

Jeffree, C.E. (1986) The cuticle, epicuticular waxes and trichomes of plants, with reference to their structure, functions and evolution. *Insects and the Plant Surface* (eds B. Juniper & R. Southwood), pp. 23-64. Edward Arnold, London.

Jennings, T.J. & Barkham, J.P. (1975) Food of slugs in mixed deciduous woodland. *Oikos,* **26,** 211-221.

Jiang, F., Jeschke, W.D., Hartung, W. & Cameron, D.D. (2008) Does legume nitrogen fixation underpin host quality for the hemiparasitic plant *Rhinanthus minor*? *Journal of Experimental Botany,* **59,** 917-925.

JNCC (2008) UK Biodiversity Action Plan Priority Habitat Descriptions: Upland Hay Meadows. [Online] Available at: http://jncc.defra.gov.uk/Docs/UKBAP_BAPHabitats-60-UplandHayMeadows.doc [Accessed: 11 Feb 2012]. (ed. A. Maddock).

JNCC (2011) UK BAP Priority Terrestrial Invertebrate Species. [online] Available at: http://jncc.defra.gov.uk/page-5169 [Accessed: 5 Feb 2012].

Johnson, B. (1953) The injurious effects of the hooked epidermal hairs of French beans (*Phaseolus vulgaris* L.) on *Aphis craccivora* Koch. *Bulletin of Entomological Research,* **44,** 779-788.

Johnson, M.T.J. (2011) Evolutionary ecology of plant defences against herbivores. *Functional Ecology,* **25,** 305-311.

Jones, D.A. (1966) On the polymorphism of cyanogenesis in *Lotus corniculatus*. I. Selection by animals. *Canadian Journal of Genetics and Cytology,* **8,** 556-557.

Juniper, B. & Southwood, R. (1986) Insects and the plant surface. Edward Arnold, London.

Juniper, B.E. (1986) The path to plant carnivory. *Insects and the Plant Surface* (eds B. Juniper & R. Southwood), pp. 195-218. Edward Arnold, London.

Kang, J.H., Shi, F., Jones, A.D., Marks, M.D. & Howe, G.A. (2010) Distortion of trichome morphology by the hairless mutation of tomato affects leaf surface chemistry. *Journal of Experimental Botany,* **61,** 1053-1064.

Kaplan, A. & Inceoglu, O. (2003) Leaf anatomy and morphology of 14 species belonging to the Turkish Rhinantheae (Scrophulariaceae) tribe. *Israel Journal of Plant Sciences,* **51,** 297-305.

Kearns, C.A., Inouye, D.W. & Waser, N.M. (1998) Endangered mutualisms: The conservation of plant-pollinator interactions. *Annual Review of Ecology and Systematics,* **29,** 83-112.

Keller, M., Kollman, J. & Edwards, P. (1999) Palatability of weeds from different European origins to the slugs *Deroceras reticulatum* Muller and *Arion lusitanicus* Mabille. *Acia Oecologica,* **20,** 109-118.

Kells, A.R. & Goulson, D. (2003) Preferred nesting sites of bumblebee queens (Hymenoptera: Apidae) in agroecosystems in the UK. *Biological Conservation,* **109,** 165-174.

Kelly, C.K. & Hanley, M.E. (2005) Juvenile growth and palatability in co-occurring, congeneric British herbs. *American Journal of Botany,* **92,** 1586-1589.

Kelly, D. (1989) Demography of short-lived plants in chalk grassland. I. Life cycle variation in annuals and strict biennials. *Journal of Ecology,* **77,** 747-769.

Kendall, D.A., Chinn, N.E., Glen, D.M., Wiltshire, C.W., Winstone, L. & Todbold, C. (1995) Effects of soil management on cereal pests and their natural enemies. *Ecology and Integrated Arable Farming Systems* (eds D.M. Glen, M.P. Greaves & H.M. Anderson), pp. 83-102. Wiley, London.

Keymer, R. & Ellis, W.M. (1978) Experimental studies on plants of *Lotus corniculatus* L. from Anglesey polymorphic for cyanogenesis. *Heredity,* **40,** 189-206.

Klayman, D.L. (1985) Quinghosu (artemisinin): an antimalarial drug from China. *Science,* **228,** 1049-1055.

Knight, T.M., Steets, J.A. & Ashman, T. (2006) A quantitative synthesis of pollen supplementation experiments highlights the contribution of resource reallocation to estimates of pollen limitation. *American Journal of Botany,* **93,** 271-277.

Knight, T.M., Steets, J.A., Vamosi, J.C., Mazer, S.J., Burd, M., Campbell, D.R., Dudash, M.R., Johnston, M.O., Mitchell, R.J. & Ashman, T. (2005) Pollen Limitation of Plant Reproduction: Pattern and Process. *Annual Review of Ecology and Evolutionary Systematics,* **36,** 467-497.

Kobayashi, S., Asai, T., Fujimoto, Y. & Kohshima, S. (2008) Anti-herbivore Structures of *Paulownia tomentosa*: Morphology, Distribution, Chemical Constituents and Changes During Shoot and Leaf Development. *Annals of Botany,* **101,** 1035-1047.

Koelewijn, H.P. (1996) Sexual differences in reproductive characters in gynodioecious *Plantago coronopus*. *Oikos,* **75,** 443-452.

Kowalski, S.P., Tingey, W.M. & Steffens, J.C. (1990) Inheritance of polyphenol oxidase in type A glandular trichomes of *Solanum berthaultii*. *Journal of Heredity,* **81,** 475-478.

Koztowski, J. & Koztowska, M. (2004) Food preferences of *Deroceras reticulatum*, *Arion lusitanicus* and *Arion rufus* for various medicinal herbs and oilseed rape. *Journal of Plant Protection Research,* **44,** 239-250.

Kunin, W.E. (1993) Sex and the Single Mustard: Population Density and Pollinator Behaviour Effects on Seed-Set. *Ecology,* **74,** 2145-2160.

Kunin, W.E. (1997) Population size and density effects in pollination: pollinator foraging and plant reproductive success in experimental arrays of *Brassica kaber*. *Journal of Ecology,* **85,** 225-234.

Kwak, M.M. & Jennersten, O. (1986) The significance of pollination time and frequency and of purity of pollen loads for seed set in *Rhinanthus angustifolius* (Scrophulariaceae) and *Viscaria vulgaris* (Caryophyllaceae). *Oecologia,* **70,** 502-507.

Kwak, M.M. & Jennersten, O. (1991) Bumblebee visitation and seedset in *Melampyrum pratense* and *Viscaria vulgaris* - heterospecific pollen and pollen limitation. *Oecologia,* **86,** 99-104.

Kwak, M.M. (1979) Effects of bumblebee visits on the seed set of *Pedicularis*, *Rhinanthus* and *Melampyrum* (Scrophulariaceae) in the Netherlands. *Acta Botanica Neerlandica,* **28,** 177-195.

Kwak, M.M. (1988) Pollination ecology and seed-set in the rare annual species *Melampyrum arvense* L. (Scrophulariaceae). *Acta Botanica Neerlandica,* **37,** 153-163.

Lack, A.J. (1982) Competition for pollinators in the ecology of *Centaurea scabiosa* L. and *Centaurea nigra* L. 1. Variation in flowering time. *New Phytologist,* **91,** 297-308.

Lanning, F.C. & Eleuterius, L.N. (1985) Silica and Ash in Tissues of some plants growing in the coastal area of Mississippi, USA. *Annals of Botany,* **56,** 157-172.

Lanning, F.C. & Eleuterius, L.N. (1987) Silica and ash in native plants of the Central and Southeastern regions of the United States. *Annals of Botany,* **60,** 361-375.

Lanning, F.C. & Eleuterius, L.N. (1989) Silica deposition in some C3 and C4 species of grasses, sedges and composites in the USA. *Annals of Botany,* **63,** 395-410.

Larson, B.M.H. & Barrett, S.C.H. (2000) A comparative analysis of pollen limitation in flowering plants. *Biological Journal of the Linnaen Society,* **69,** 503-520.

Lavrsen, K. (2011) Webhome < Motion < Foswiki [online] Available at: http://www.lavrsen.dk/foswiki/bin/view/Motion/WebHome [Accessed 12 Feb 2012].

Legendre, P. & Anderson, M.J. (1999) Distance-based redundancy analysis: Testing multispecies responses in multifactorial ecological experiments. *Ecological Monographs,* **69,** 1-24.

Legendre, P. & Gallagher, E.D. (2001) Ecologically meaningful transformations for ordination of species data. *Oecologia,* **129,** 271-280.

Legendre, P. & Legendre, L. (1998) *Numerical Ecology*. Elsevier, Amsterdam.

Lettvin, J.Y., Maturana, H.R., McCulloch, W.S. & Pitts, W.H. (1968) What the frog's eye tells the frog's brain. *The Mind: Biological Approaches to its Functions* (eds W.C. Corning & M. Balaban), pp. 233-258.

Levin, D.A. (1973) Role of trichomes in plant defense. *Quarterly Review of Biology,* **48,** 3-15.

Lewis, D. (1941) Male sterility in natural populations of hermaphroditic plants. *New Phytologist,* **40,** 56-63.

Littlewood, N.A., Stewart, A.J.A. & Woodcock, B.A. (2012) Science into practice - how can fundamental science contribute to better management of grasslands for invertebrates? *Insect Conservation and Diversity,* **5,** 1-8.

Lloyd, D.G. (1975) The maintenance of gynodioecy and androdioecy in angiosperms. *Genetica,* **45,** 325-339.

Lloyd, D.G. (1976) The transmission of genes via pollen and ovules in gynodioecious angiosperms. *Theoretical Population Biology,* **9,** 299-316.

Lopez-Villavicencio, M., Genton, B.J., Porcher, G.E. & Shykoff, J.A. (2005) The role of pollination level on the reproduction of females and hermaphrodites in the gynodioecious plant *Gypsophila repens* (Caryophyllaceae). *American Journal of Botany,* **92,** 1995-2002.

Luckwill, L.C. (1943) *The genus Lycopersicon: An historical biological and taxonomic survey of the wild and cultivated tomatoes.* Aberdeen University Press, U.K.

Lutman, J. (1978) The role of slugs in an Agrostis-Festuca grassland. *Production Ecology of British Moors and Montane Grasslands* (eds O.W. Heal & D.F. Perkins), pp. 332-347. Springer-Verlag, New York and Berlin.

LWEC (2012) Insect Pollinators Initiative **|** LWEC. [online] Available at: http://www.lwec.org.uk/activities/insect-pollinators-initiative [Accessed: 24 Feb 2012].

Ma, J.F. (2003) Functions of silica in higher plants. *Progress in Molecular Subcellular Biology,* **33,** 127-147.

MacArthur, R.H. & Pianka, E.R. (1966) On the optimal use of a patchy environment. *American Naturalist,* **100,** 603-609.

Malakar, R. & Tingey, W.M. (2000) Glandular trichomes of *Solanum berthaultii* and its hybrids with potato deter oviposition and impair growth of potato tuber moth. *Entomologia Experimentalis Et Applicata,* **94,** 249-257.

Marin, M., Jasnic, N., Lakusic, D., Duletic-Lausevic, S. & Ascensao, L. (2010) The micromorphological, histochemical and confocal analysis of Satureja subspicata Bartl. Ex Vis. glandulat trichomes. *Arch. Biol. Sci., Belgrade,* **62,** 1143-1149.

Massey, F.P. & Hartley, S.E. (2006) Experimental demonstration of the anti-herbivore effects of silica in grasses: impacts on foliage digestibility and vole growth rates. *Proceedings of the Royal Society B-Biological Sciences,* **273,** 2299-2304.

Massey, F.P. & Hartley, S.E. (2009) Physical defences wear you down: Progressive and irreversible impacts of silica on insect herbivores. *Journal of Animal Ecology,* **78,** 281-291.

Massey, F.P., Ennos, A.R. & Hartley, S.E. (2006) Silica in grasses as a defence against insect herbivores: Contrasting effects on folivores and a phloem feeder. *Journal of Animal Ecology,* **75,** 595-603.

Massey, F.P., Ennos, A.R. & Hartley, S.E. (2007) Herbivore specific induction of silica-based plant defences. *Journal of Ecology,* **95,** 414-424.

Maturana, H.R., Lettvin, J.Y., McCulloch, W.S. & Pitts, W.H. (1960) Anatomy and Physiology of Vision in the Frog (*Rana pipiens*). *The Journal of General Physiology,* **43,** 129-175.

McDowell, E.T., Kapteyn, J., Schmidt, A., Li, C., Kang, J.H., Descour, A., Shi, F., Larson, M., Schilmiller, A.L., An, L., Jones, D.A., Pichersky, E., Soderlund, C.A. & Gandg, D.R. (2011) Comparative Functional Genomic Analysis of *Solanum* Glandular Trichome Types. *Plant Physiology,* **155,** 524-539.

McKemey, A., Glen, D., Wiltshire, C. & Symondson, W. (2006) Molecular quantification of slug density in the soil using monoclonal antibodies. *Soil Biology & Biochemistry,* **38,** 2903-2909.

Michigan State University (2012) The Solanum Trichome Project | Michigan State University, University of Michigan and University of Arizona. [online] Available at: http://www.trichome.msu.edu [Accessed 13 Feb 2012].

Minitab 16 Statistical Software (2010) [Computer software]. Minitab, Inc. (www.minitab.com), State College, PA.

Moeller, D.A. (2004) Facilitative interactions among plants via shared pollinators. *Ecology,* **85,** 3289-3301.

Moen, J., Gardfjell, H., Ericson, L. & Oksanen, L. (1996) Shoot survival under intense grazing for two broad-leaved herbs with different chemical defense systems. *Oikos,* **75,** 359-364.

Moles, A.T. & Westoby, M. (2004) Seedling survival and seed size: a synthesis of the literature. *Journal of Ecology,* **92**: 372-383.

Mølgaard, P. (1986) Food plant preferences by slugs and snails: A simple method to evaluate the relative palatability of the food plants. *Biochemical Systematics and Ecology,* **14,** 113-121.

Morand, S., Wilson, M.J. & Glen, D.M. (2004) Nematodes (Nematoda) Parasitic in Terrestrial Gastropods. *Natural Enemies of Terrestrial Molluscs* (ed. G.M. Barker), pp. 525-558. CABI Publishing, Wallingford.

Muigai, S.G., Schuster, D.J., Snyder, J.C., Scott, J.W., Bassett, M.J. & McAuslane, H.J. (2002) Mechanisms of resistance in Lycopersicon germplasm to the whitefly Bemisia argentifolii. *Phytoparasitica,* **30,** 347-360.

Mutikainen, P. & Delph, L.F. (1996) Effects of herbivory on male reproductive success in plants. *Oikos,* **75**.

Natural England (2009a) Agri-environment schemes in England 2009. A review of results and effectiveness. Natural England, Sheffield. [online] Available at: <http://www.naturalengland.org.uk/Images/AE-schemes09_tcm6-14969.pdf> [Accessed 09.02.11].

Natural England (2009b) UK BAP priority habitat inventories version 2.01. Natural England.

Natural England (2010a) Natural England Technical Information Note TIN063 Sward enhancement: diversifying grassland by spreading species-rich green hay.

Natural England (2010b) Natural England Technical Information Note TIN064 Sward enhancement: diversifying grassland by oversowing and slot seeding.

Natural England (2010c) Natural England Technical Information Note TIN067 Arable reversion to species-rich grassland: establishing a sown sward.

Neumann, F. (1998) The effects of various land use of wet grasslands on the gastropod fauna. *Faunistisch-Oekologische Mitteilungen,* **0,** 5-43.

Neumann, U., Pare, J., Raynal-Roques, A., Salle, G. & Weber, H.C. (1997) Characteristic Trichomes and Indumentum Specialization in African and European Parasitic Scrophulariaceae. *Botanica Acta,* **110,** 150-158.

Nieminen, M., Suomi, J., van Nouhuys, S., Sauri, P. & Riekkola, M. (2003) Effect of iridoid glycoside content on oviposition host plant choice and parasitism in a specialist herbivore. *Journal of Chemical Ecology,* **29,** 823-844.

North Pennines AONB (2009) Hay Time Walk 2 - Weardale. [online] Available at: http://www.northpennines.org.uk/Pages/PublicationItem.aspx?DocRef=55 [Accessed: 15 Feb 2012].

O'Neill, M.A. (2007) DAISY: A Practical Computer Based Tool for Semi Automated Species Identification. *Automated Taxon Identification Systematics, Theory Approaches and Applications* (ed. M. N), pp. 101-114. CRC Press.

O'Reilly, J. (2010) The state of upland hay meadows in the North Pennines. *British Wildlife,* **21,** 184-192.

Pacha, M.J. & Petit, S. (2008) The effects of landscape structure and habitat quality on the occurence of *Geranium sylvaticum* in fragmented hay meadows. *Agriculture Ecosystems & Environment,* **123,** 81-87.

Pakarinen, E., Niemela, P. & Tuomi, J. (1990) Effect of fertilization, seaweed extracts and leaf-damage on palatability of lettuce to *Deroceras*-slugs. *Acta Oecologica,* **11,** 113-119.

Pallant, D. (1972) The food of the grey field slug, *Agriolimax reticulatus* (Muller), on grassland. *Journal of Animal Ecology,* **41,** 761-769.

Pankoke, H., Bowers, M.D. & Dobler, S. (2010) Influence of iridoid glycoside containing host plants on midgut beta-glucosidase activity in a polyphagous caterpillar, Spilosoma virginica Fabricius (Arctiidae). *Journal of Insect Physiology,* **56,** 1907-1912.

Parra, J.L., Rakbeck, C., McGuire, J.A. & Graham, C.H. (2011) Contrasting patterns of phylogenetic assemblage structure along the elevational gradient for major hummingbird clades. *Journal of biogeography***, 38**, 2358-2361.

Pedro, L., Campos, P. & Pais, M.S.S. (1991) Ultrastructure of the apical cell of procumbent (Type I) trichomes in *Geranium robertianum* L. (Geraniaceae). *Israel Journal of Botany,* **40,** 209-217.

Pelletier, Y., Grondin, G. & Maltais, P. (1999) Mechanism of resistance to the Colorado potato beetle in wild *Solanum* species. *Journal of Economic Entomology,* **92,** 708-713.

Philipson, J. (1983) Slug numbers, biomass and respiration metabolism in a beech woodland. *Oecologia,* **60,** 38-45.

Ponzi, R. & Pizzolongo, P. (1992) Structure and function of *Rhinanthus minor* L. trichome hydathode. *Phytomorphology,* **42,** 1-6.

Port, C.M. & Port, G.R. (1986) The biology and behaviour of slugs in relation to crop damage and control. *Agricultural Zoology Reviews,* **1,** 253-299.

Poschlod, P., Kleyer, M., Jackel, A-K., Dannemann, A., Tackenberg, O. (2003) BIOPOP– a database of plant traits and internet application for nature conservation. *Folia Geobotanica,* **38**: 263-271. http://www.landeco.uni-oldenburg.de/Projects/biopop/biopop_en.htm [Accessed: 4 Oct. 2012].

Potts, S.G., Biesmeijer, J.C., Bommarco, R., Felicioli, A., Fischer, M., Jokinen, P., Kleijn, D., Klein, A.-M., Kunin, W.E., Neumann, P., Penev, L.D., Petanidou, T., Rasmont, P., Roberts, S.P.M., Smith, H.G., Sorensen, P.B., Steffan-Dewenter, I., Vaissiere, B.E., Vila, M., Vujic, A., Woyciechowski, M., Zobel, M., Settele, J. & Schweiger, O. (2011) Developing European conservation and mitigation tools for pollination services: approaches of the STEP (Status and Trends of European Pollinators) project. *Journal of Apicultural Research,* **50,** 152-164.

Potts, S.G., Biesmeijer, J.C., Kremen, C., Neumann, P., Schweiger, O. & Kunin, W.E. (2010) Global pollinator declines: trends, impacts and drivers. *Trends in Ecology & Evolution,* **25,** 345-353.

Prezio, J.R., Lankester, M.W., Lautenschlager, R.A. & Bell, F.W. (1999) Effects of alternative conifer release treatments on terrestrial gastropods in regenerating spruce plantations. *Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere,* **29,** 1141-1148.

Proctor, M., Yeo, P. & Lack, A. (1996) *The Natural History of Pollination,* 1st edn. Collins, London.

Puustinen, S., Koskela, T. & Mutikainen, P. (2004) Direct and ecological costs of resistance and tolerance in the stinging nettle. *Oecologia,* **139,** 76-82.

Pywell, R.F., Bullock, J.M., Tallowin, J.B., Walker, K.J., Warman, E.A. & Masters, G. (2007) Enhancing diversity of species-poor grasslands: an experimental assessment of multiple constraints. *Journal of Applied Ecology,* **44,** 81-94.

Pywell, R.F., Bullock, J.M., Walker, K.J., Coulson, S.J., Gregory, S.J. & Stevenson, M.J. (2004) Facilitating grassland diversification using the hemiparasitic plant *Rhinanthus minor*. *Journal of Applied Ecology,* **41,** 880-887.

Pywell, R.F., Meek, W.R., Hulmes, L., Hulmes, S., James, K.L., Nowakowski, M. & Carvell, C. (2011) Management to enhance pollen and nectar resources for bumblebees and butterflies within intensively farmed landscapes. *Journal of Insect Conservation,* **15,** 853-864.

Pywell, R.F., Warman, E.A., Carvell, C., Sparks, T.H., Dicks, L.V., Bennett, D., Wright, A., Critchley, C.N.R. & Sherwood, A. (2005) Providing foraging resources for bumblebees in intensively farmed landscapes. *Biological Conservation,* **121,** 479-494.

R Development Core Team (2011) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

R Development Core Team. (2012) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/ (12 Oct. 2012).

Raman, S. (1986) A Code Proposed for the Classification of Trichomes as Applied to the Scrophulariaceae. *Beitrage zur Biologie der Pflanzen,* **62,** 349-367.

Ramula, S. & Mutikainen, P. (2003) Sex Allocation of Females and Hermaphrodites in the Gynodioecious *Geranium sylvaticum*. *Annals of Botany,* **92,** 207-213.

Rasmont, P. (1988) Monographie ecologique et zoogepgraphique des bourdons de France et de Belgique (Hymenoptera, Apidae, Bombinae). pp. 309 + Ixi. Faculte des Sciences agronomiques de l'Etat, Gembloux.

Raudenbush, S.W. & Bryk, A.S. (2002) *Hierarchical Linear Models: Applications and Data Analysis Methods. Second Addition. Advanced Quantitative Techniques in the Social Sciences: Series 1.* Sage Publications Limited, London.

Rees, M. & Brown, V.K. (1992) Interactions between invertebrate herbivores and plant competition. *Journal of Ecology,* **80,** 353-360.

Reitalu, T., Johansson, L.J., Sykes, M.T., Hall, K. & Prentice, H.C. (2010) History matters: village distances, grazing and grassland species diversity. *Journal of Applied Ecology,* **47,** 1216-1224.

Reynolds, J.J.H., Lambin, X., Massey, F.P. *et al.* (2012) Delayed induced silica defences in grasses and their potential for destabilising herbivore population dynamics. *Oecologia* 170: 445-456.

Richardson, B. & Whittaker, J.B. (1982) The effect of varying the reference material on ranking of acceptability indices of plant species to a polyphagous herbivore, Agriolimax reticulatus. *Oikos,* **39,** 237-240.

Rodwell, J.S. (1992) *British Plant Communities. Volume 3. Grassland and montane communities*. Cambridge University Press, Cambridge.

Root, R.B. (1973) Organization of a plant-arthropod association in simple and diverse habitats - fauna of collards (*Brassica oleracea*). *Ecological Monographs,* **43,** 95-120.

Rose, F. & O'Reilly, C. (2006) *The Wildflower Key*. Frederick Warne & Co., China.

Rossignol, N., Bonis, A. & Bouzill, J. (2011) Impact of selective grazing on plant production and quality through floristic contrasts and current-year defoliation in a wet grassland. *Plant Ecology,* **212,** 1589-1600.

Royal Botanic Gardens Kew. (2008) Seed Information Database (SID). Version 7.1. http://data.kew.org/sid/ [Accessed: 14 Oct. 2012].

Rumer, S., Cameron, D.D., Wacker, R., Hartung, W. & Jiang, F. (2007) An anatomical study of the haustoria of *Rhinanthus minor* attached to roots of different hosts. *Flora,* **202,** 194-200.

Runham, N.W. & Hunter, P.J. (1970) *Terrestrial Slugs*. Hutchinson University Library, London.

Sacchetti, G., Ballero, M., Serafini, M., Muzzoli, M., Tosi, B. & Poli, F. (2003) Morphological and Histochemical Investigation on Glandular Tichomes of *Orobanche ramosa* subsp. *nana* (Orobanchaceae). *Phyton-Annales Rei Botanicae,* **43,** 207-214.

Sacchetti, G., Romagnoli, C., Nicoletti, M., Di Fabio, A., Bruni, A. & Poli, F. (1999) Glandular Trichomes of Calceolaria adsendens Lidl. (Scrophulariaceae): Histochemistry, Development and Ultrastructure. *Annals of Botany,* **83,** 87-92.

Sangster, A.G. (1970) Intracellular silica deposition in immature leaves in three species of the Gramineae. *Annals of Botany,* **34,** 245-257.

Sangster, A.G., Hodson, M.J., Wynn-Parry, D. & Rees, J.A. (1983) A developmental study of silicification in the trichomes and associated epidermal structures of the inflorescence bracts of the grass, *Phalaris canariensis* L. *Annals of Botany,* **52,** 171-187.

Schädler, M., Jung, G., Auge, H. & Brandl, R. (2003) Palatability, decomposition and insect herbivory: Patterns in a successional old-field plant community. *Oikos,* **103,** 121-132.

Scheidel, U. & Bruelheide, H. (1999) Selective slug grazing on montane meadow plants. *Journal of Ecology,* **87,** 828-838.

Schilmiller, A.L., Last, R.L. & Pichersky, E. (2008) Harnessing plant trichome biochemistry for the production of useful compounds. *Plant Journal,* **54,** 702-711.

Scott, G.C., Pickett, J.A., Smith, M.C., Woodcock, C.M., Harris, P.G.W., Hammon, R.P. & Koetecha, H.D. (1984) Seed treatments for controlling slugs in winter wheat. *British Crop Protection Conference-Pests and Diseases*, pp. 133-138.

Seel, W.E., Parsons, A.N. & Press, M.C. (1993) Do inorganic solutes limit growth of the facultative hemiparasite *Rhinanthus minor* L. in the absence of a host? *New Phytologist,* **124,** 283-289.

Seithe, A. (1979) Hair types as taxonomic charcetrs in Solanum. *The Biology and Taxonomy of the Solanaceae* (ed. J.G. Hawkes), pp. 307-319. Academic Press, London.

Shirley, M.D., Rushton, S.P., Young, A.G. & Port, G.R. (2001) Simulating the long-term dynamics of slug populations: a process-based modelling approach for pest control. *Journal of Applied Ecology,* **38,** 401-411.

Shykoff, J.A. (1988) Maintenance of gynidioecy in *Silene acaulis* (Caryophyllaceae) - stage-specific fecundity and viability selection. *American Journal of Botany,* **75,** 844-850.

Shykoff, J.A., Kolokotronis, S.O., Collin, C.L. & Lopez-Villavicencio, M. (2003) Effects of male sterility on reproductive traits in gynodioecious plants: a meta-analysis. *Oecologia,* **135,** 1-9.

Simmons, A.T. & Gurr, G.M. (2005) Trichomes of *Lycopersicon* species and their hybrids: effects on pests and natural enemies. *Agricultural and Forest Entomology,* **7,** 265-276.

Smith, R.S. & Jones, L. (1991) The phenology of mesotrophic grassland in the Pennine Dales, Northern England – Historic hay cutting dates, vegetation variation and plant-species phenologies. *Journal of Applied Ecology,* **28,** 42-59.

Smith, R.S. & Rushton, S.P. (1994) The effects of grazing management on the vegetation of mesotrophic (meadow) grassland in northern England. *Journal of Applied Ecology,* **31,** 13-24.

Smith, R.S., Buckingham, H., Bullard, M.J., Shiel, R.S. & Younger, A. (1996a) The conservation management of mesotrophic (meadow) grassland in northern England.1. Effects of grazing, cutting date and fertilizer on the vegetation of a traditionally managed sward. *Grass and Forage Science,* **51,** 278-291.

Smith, R.S., Corkhill, P., Shiel, R.S. & Millward, D. (1996b) The conservation management of mesotrophic (meadow) grassland in Northern England .2. Effects of grazing, cutting date, fertilizer and seed application on the vegetation of an agriculturally improved sward. *Grass and Forage Science,* **51,** 292-305.

Smith, R.S., Shiel, R.S., Bardgett, R.D., Millward, D., Corkhill, P., Evans, P., Quirk, H., Hobbs, P.J. & Kometa, S.T. (2008) Long-term change in vegetation and soil microbial communities during the phased restoration of traditional meadow grassland. *Journal of Applied Ecology,* **45,** 670-679.

Smith, R.S., Shiel, R.S., Bardgett, R.D., Millward, D., Corkhill, P., Rolph, G., Hobbs, P.J. & Peacock, S. (2003) Soil microbial community, fertility, vegetation and diversity as targets in the restoration management of a meadow grassland. *Journal of Applied Ecology,* **40,** 51-64.

Smith, R.S., Shiel, R.S., Millward, D. & Corkhill, P. (2000) The interactive effects of management on the productivity and plant community structure of an upland meadow: an 8-year field trial. *Journal of Applied Ecology,* **37,** 1029-1043.

Smith, R.S., Shiel, R.S., Millward, D., Corkhill, P. & Sanderson, R.A. (2002) Soil seed banks and the effects of meadow management on vegetation change in a 10-year meadow field trial. *Journal of Applied Ecology,* **39,** 279-293.

South, A. (1964) Estimation of slug populations. *Annals of applied Biology,* **53,** 251-258.

South, A. (1965) Biology and ecology of *Agriolimax reticulatus* Muller and other slugs: Spatial distribution. *Journal of Animal Ecology,* **34,** 403-417.

South, A. (1973) Degats causes par les limaces en Grande-Bretagne (Slug damage in Great Britain). *Haliotis,* **3,** 19-25.

South, A. (1989a) A comparison of the life cycles of the slugs *Deroceras reticulatum* (Muller) and *Arion intermedius* Normand on permanent pasture. *Journal of Molluscan Studies,* **55,** 9-22.

South, A. (1989b) The effect of weather and other factors on numbers of slugs in permanent pasture. *Slugs and Snails in World Agriculture* (ed. I.F. Henderson), pp. 355-360. British Crop Protection Council Mongraph No. 41.

South, A. (1992) *Terrestrial Slugs: Biology, ecology and control*. Chapman & Hall, London.

Southwood, R. (1986) Plant surfaces and insects - an overview. *Insects and the Plant Surface* (eds B. Juniper & R. Southwood), pp. 1-22. Edward Arnold, London.

Speiser, B. & Rowell-Rahier, M. (1991) Efects of food availability, nutritional value, and alkaloids on food choice in the generalist herbivore *Arianta arbustorum* (Gastropoda, Helicidae). *Oikos,* **62,** 306-318.

Speiser, B. (2001) Food and Feeding Behaviour. *The Biology of Terrestrial Molluscs* (ed. G.M. Barker), pp. 259-288. CABI Publishing, Wallingford.

Stace, C.A. (2010) *New Flora of the Bristish Isles*. Cambridge University Press, Cambridge.

Stahl, E. (1888) Pflanzen und Schnecken: Biologische Studie uber die Schutzmittel die Planzen gegen Schneckenfrass. *Jena. Z. Naturw.,* **22,** 557-684.

Steffan-Dewenter, I. & Tscharntke, T. (1999) Effects of habitat isolation on pollinator communities and seed set. *Oecologia,* **121,** 432-440.

STEP Project (2012) STEP Project. [online] Available at: http://www.step-project.net [Accessed: 24 Feb 2012].

Stephenson, A.G. (1981) Flower and fruit abortion: proximate causes and ultimate functions. *Annual Review of Ecology and Systematics,* **12,** 253-279.

Stephenson, J.W. (1966) Notes on the rearing and behaviour in soil of *Milax budapestensis* (Hazay). *Journal of Conchology,* **26,** 141-145.

Stephenson, J.W. (1975a) Laboratory observations on the distribution of Agrolimax reticulatus (Mull.) in different aggregate fractions of garden loam. *Plant Pathology,* **24,** 12-15.

Stephenson, J.W. (1975b) Laboratory observations on the effect of soil compaction on slug damage to winter wheat. *Plant Pathology,* **24,** 9-11.

Stout, J.C. & Goulson, D. (2002) The influence of nectar secretion rates on the responses of bumblebees (Bombus spp.) to previously visited flowers. *Behavioral Ecology and Sociobiology,* **52,** 239-246.

Strauss, S.Y., Stanton, M.L., Emery, N.C., Bradley, C.A., Carleton, A., Dittrich-Reed, D.R., Ervin, O.A., Gray, L.N., Hamilton, A.M., Rogge, J.H., Harper, S.D., Law, K.C., Pham, V.Q., Putnam, M.E., Roth, T.M., Theil, J.H., Wells, L.M. & Yoshizuka, E.M. (2009) Cryptic seedling herbivory by nocturnal introduced generalists impacts survival, performance of native and exotic plants. *Ecology,* **90,** 419-429.

Strom, L., Hylander, K. & Dynesius, M. (2009) Different long-term and short-term responses of land snails to clear-cutting of boreal stream-side forests. *Biological Conservation,* **142,** 1580-1587.

Symondson, W.O.C., Glen, D.M., Ives, A.R., Langdon, C.J. & Wiltshire, C.W. (2002) Dynamics of the relationship between a generalist predator and slugs over five years. *Ecology,* **83,** 137-147.

Symondson, W.O.C., Glen, D.M., Wiltshire, C.W., Langdon, C.J. & Liddell, J.E. (1996) Effects of cultivation techniques and methods of straw disposal on predation by *Pterostichus melanarius* (Coleoptera: Carabidae) upon slugs (Gastropoda: Pulmonata) in an arable field. *Journal of Applied Ecology,* **33,** 741-753.

Tansley, A. & Adamson, R. (1925) Studies of the vegetation of the English Chalk. III. The chalk grasslands of the Hampshire-Sussex border. *Journal of Ecology,* **13,** 177-223.

Thompson, K., Bakker, J.P. & Bekker, R.M. (1996) *The soil seed banks of north west Europe*. Cambridge University Press, Cambridge.

Thomson, J.D. (2001) Using Pollination Deficits to Infer Pollinator Declines: Can Theory Guide Us? *Conservation Ecology,* **5,** 1-12.

Thurston, R., Smith, W.T. & Cooper, B.P. (1966) Alkaloid secretion by trichomes of *Nicotiana* species and resistance to aphids. *Entomologia Experimentalis Et Applicata,* **9,** 428-432.

Tilman, G.D. (1988) Plant strategies and the dynamics of plant communities. *American Journal of Botany,* **75,** 67.

Tomizawa, M. & Casida, J.E. (2009) Molecular Recognition of Neonicotinoid Insecticides: The Determinants of Life or Death. *Accounts of Chemical Research,* **42,** 260-269.

Torregrossa, A., Azzara, A.V. & Dearing, M.D. (2011) Differential regulation of plant secondary compounds by herbivorous rodents. *Functional Ecology,* **25,** 1232-1240.

Torres, L.G., Read, A.J. & Halpin, P. (2008) Fine-scale habitat modelling of a top marine predator: do prey data improve predictive capacity? *Ecological Applications,* **18,** 1702-1717.

Tuberville, T.D., Dudley, P.G. & Pollard, A.J. (1996) Responses of invertebrate herbivores to stinging trichomes of *Urtica dioica* and *Laportea canadensis*. *Oikos,* **75,** 83-88.

Vaarama, A. & Jaaskelainen, O. (1967) Studies in gynodioecism in the Finnish populations of *Geranium sylvaticum* L. *Annales Acadamiae Scientiarum Fennicae. Series A. IV. Biologica,* **108**.

Valkama, E., Koricheva, J., Salminen, J.P., Helander, M., Saloniemi, I., Saikkonen, K. & Pihlaja, K. (2005) Leaf surface traits: overlooked determinants of birch resistance to herbivores and foliar micro-fungi? *Trees-Structure and Function,* **19,** 191-197.

van Dijk, G. (1991) The status of semi-natural grasslands in Europe *The Conservation of Lowland Dry Grassland Birds in Europe. Proceedings of an International Seminar held at the University of Reading 20-22 March 1991*, pp. 15-36.

van Hulst, R., Shipley, B. & Theriault, A. (1987) Why is *Rhinanthus minor* *(*Scrophulariaceae) such a good invader? *Canadian Journal of Botany,* **65,** 2373-2379.

Wadham, M.D. & Wynn-Parry, D. (1981) The silicon content of *Oryza sativa* L. and its effect on the grazing behaviour of *Agriolimax reticulatus* Müller. *Annals of Botany,* **48,** 399-402.

Wagner, G.J. (1991) Secreting glandular trichomes - more than just hairs. *Plant Physiology,* **96,** 675-679.

Walters, D.S., Grossman, H., Craig, R. & Mumma, R.O. (1989) Geranium defensive agents. 4. Chemical and morphological bases of resistance. *Journal of Chemical Ecology,* **15,** 357-372.

Wardle, D.A. & Barker, G.M. (1997) Competition and herbivory in establishing grassland communities: Implications for plant biomass, species diversity and soil microbial activity. *Oikos,* **80,** 470-480.

Warley, A.P. (1970) Some aspects of the biology, ecology and control of slugs in S.E. Scotland with particular reference to the potato crop. Ph.D. thesis, University of Edinburgh.

Waser, N.M., Chittka, L., Price, M.V., Williams, N.M. & Ollerton, J. (1996) Generalization in pollination systems, and why it matters. *Ecology,* **77,** 1043-1060.

Werker, E. & Fahn, A. (1981) Secretory hairs of *Inula viscosa* (L.) Ait. - development, ultrastructure, and secretion. *Botanical Gazette,* **142,** 461-476.

Werker, E. (1993) Function of essential oil-secreting glandular hairs in aromatic plants of the Lamiaceae. A review. *Flavour and Fragrance Journal,* **8,** 249-255.

Wesselingh, R.A. (2006) Pollen limitation meets resource allocation: towards a comprehensive methodology. *New Phytologist,* **174,** 26-37.

Westbury, D.B. (2004) *Rhinanthus minor* L. *Journal of Ecology,* **92,** 906-927.

Westbury, D.B., Davies, A., Woodcock, B.A. & Dunnett, N.P. (2006) Seeds of change: The value of using *Rhinanthus minor* in grassland restoration. *Journal of Vegetation Science,* **17,** 435-446.

Westerbergh, A. & Nyberg, A.B. (1995) Selective grazing of hairless *Silene dioica* plants by land gastropods. *Oikos-.1995; 73 (3) 289-298..*

Wilby, A. & Brown, V.K. (2001) Herbivory, litter and soil disturbance as determinants of vegetation dynamics during early old-field succession under set-aside. *Oecologia,* **127,** 259-265.

Wilkens, R.T., Shea, G.O., Halbreich, S. & Stamp, N.E. (1996) Resource availability and the trichome defenses of tomato plants. *Oecologia,* **106,** 181-191.

Williams, C.F., Kuchenreuther, M.A. & Drew, A. (2000) Floral dimorphism, pollination, and self-fertilization in gynodioecious *Geranium richardsonii* (Geraniaceae). *American Journal of Botany,* **87,** 661-669.

Williams, L.A.D., Vasques, E., Reid, W., Porter, R. & Kraus, W. (2003) Biological activities of an extract from *Cleome viscosa* L. (Capparaceae). *Naturwissenschaften,* **90,** 468-472.

Williams, P. (2005) Does specialization explain rarity and decline among British bumblebees? A response to Goulson et al. *Biological Conservation,* **122,** 33-43.

Williams, P.H. & Osborne, J.L. (2009) Bumblebee vulnerability and conservation world-wide. *Apidologie,* **40,** 367-387

Williams, P.H. (1982) The distribution and decline of British bumblebees (*Bombus* Latr). *Journal of Apicultural Research,* **21,** 236-245.

Williams, P.H. (1988) Habitat use by bumble bees (*Bombus* spp). *Ecological Entomology,* **13,** 223-237.

Williams, P.H. (1989) Why are there so many species of bumblebees at Dungeness. *Botanical Journal of the Linnean Society,* **101,** 31-44.

Willis, J.C., Bohan, D.A., Choi, Y.H., Conrad, K.F. & Semenov, M.A. (2006) Use of an individual-based model to forecast the effect of climate change on the dynamics, abundance and geographical range of the pest slug *Deroceras reticulatum* in the UK. *Global Change Biology,* **12,** 1643-1657.

Wood, S. (2006) *Generalized Additive Models: An Introduction with R*. Chapman & Hall/CRC Press.

Wood, S. (2012) gamm4: Generalized additive mixed models using mgcv and lme4. R package version 0.1-2 http://CRAN.R-project.org/package=gamm4.

Woodman, R.L. & Fernandes, G.W. (1991) Differential Mechanical Defense - Herbivory, Evapotranspiration, and Leaf-hairs. *Oikos,* **60,** 11-19.

Young, A.G. & Port, G.R. (1989) The effect of microclimate on slug activity in the field. *Slugs and Snails in World Agriculture, BCPC Mongraph No. 41* (ed. I.F. Henderson), pp. 263-269.

Young, A.G., Port, G.R., Emmett, B.J. & Green, D.I. (1991) Development of a forecast of slug activity **-** Models to relate slug activity to meteorological conditions. *Crop Protection,* **10,** 413-415.

Young, H.J. & Young, T.P. (1992) Alternative outcomes of natural and experimental high pollen loads. *Ecology,* **73,** 639-647.

Young, T.P. (1982) Bird visitation, seed-set, and germination rates in 2 species of *Lobelia* on Mount Kenya. *Ecology,* **63,** 1983-1986.

Zhang, X., Thacker, R.R. & Snyder, J.C. (2008) Occurrence of 2,3-dihydrofarnesoic acid, a spidermite repellent, in trichome secretions of *Lycopersicon esculentum* x *L-hirsutum* hybrids. *Euphytica,* **162,** 1-9.

Zimmerman, M. & Pyke, G.H. (1998) Reproduction in *Polemonium*: Assessing the factors limiting seed set. *The American Naturalist,* **131,** 723-738.

Zuur, A.P., Elena, N.I., Walker, N.J., Saveliev, A.A. & Smith, G.M. (2009) *Mixed Effects Models and Extensions in Ecology with R: Statistics for Biology and Health*. Springer, New York.