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Direct and indirect effects of nitrogen deposition on species composition change in calcareous grasslands

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Keywords:	Nitrogen deposition, species diversity, soil acidity, Festuco Brometalia grasslands, management
Abstract:	<p>Atmospheric nitrogen (N) deposition has been identified as a major threat to biodiversity, but field surveys of its effects have rarely focussed on sites which are actively managed to maintain characteristic species. We analysed permanent quadrat data from 106 plots in nature reserves on calcareous grassland sites in the UK collected during a survey between 1990 and 1993 and compared the data with the results from re-survey of 48 of these plots between 2006 and 2009.</p> <p>N deposition showed no significant spatial association with species richness, species diversity, or the frequency of species adapted to low nutrient conditions in the 1990-1993 dataset. However, temporal analysis showed that N deposition was significantly associated with changes in Shannon diversity and evenness. In plots with high rates of N deposition, there was a decrease in species diversity and evenness, a decline in the frequency of characteristic calcareous grassland species, and a lower number of rare and scarce species. Since all sites had active management to maintain a high diversity and characteristic species, our results imply that even focussed management on nature conservation objectives cannot prevent adverse effects of high rates of N deposition.</p> <p>Structural equation modelling was used to compare different causal</p>

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	mechanisms to explain the observed effects. For change in Shannon diversity, direct effects of N deposition were the dominant mechanism and there was an independent effect of change in grazing intensity. In contrast, for change in herb species number, indirect effects on soil acidity, linked to both N and S deposition, were more important than direct effects of N deposition.

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1 **Keywords**

2 Nitrogen deposition, species diversity, soil acidity, *FestucoBrometalia* grasslands,

3 management

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Abstract

Atmospheric nitrogen (N) deposition has been identified as a major threat to biodiversity, but field surveys of its effects have rarely focussed on sites which are actively managed to maintain characteristic species. We analysed permanent quadrat data from 106 plots in nature reserves on calcareous grassland sites in the UK collected during a survey between 1990 and 1993 and compared the data with the results from re-survey of 48 of these plots between 2006 and 2009.

N deposition showed no significant spatial association with species richness, species diversity, or the frequency of species adapted to low nutrient conditions in the 1990-1993 dataset.

However, temporal analysis showed that N deposition was significantly associated with changes in Shannon diversity and evenness. In plots with high rates of N deposition, there was a decrease in species diversity and evenness, a decline in the frequency of characteristic calcareous grassland species, and a lower number of rare and scarce species. Since all sites had active management to maintain a high diversity and characteristic species, our results imply that even focussed management on nature conservation objectives cannot prevent adverse effects of high rates of N deposition.

Structural equation modelling was used to compare different causal mechanisms to explain the observed effects. For change in Shannon diversity, direct effects of N deposition were the dominant mechanism and there was an independent effect of change in grazing intensity. In contrast, for change in herb species number, indirect effects on soil acidity, linked to both N and S deposition, were more important than direct effects of N deposition.

1 Introduction

2

3 Atmospheric deposition of nitrogen (N) and sulphur (S) compounds increased dramatically in
4 Europe in the second half of the 20th century, mainly as a result of increased fossil fuel
5 combustion and intensive agriculture (Galloway, 1995). Despite a strong decline in S
6 emissions and deposition in most parts of Europe since the 1990's (Vestrenget *et al.* 2007), N
7 deposition has not declined to the same extent. Both N and S deposition has continued to
8 increase in other parts of the world, especially in parts of India and China (Galloway &
9 Cowling, 2002; Dentener *et al.* 2006).

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11 N deposition can result in severe eutrophication via the direct input of nutrients but also
12 acidification via indirect processes such as nitrification (Galloway, 1995; Bobbink *et al.*
13 1998). N deposition has been identified as one of the most important global drivers of
14 biodiversity change in terrestrial ecosystems (Vitousek *et al.* 1997; Sala *et al.* 2000, Phoenix *et*
15 *al.* 2006), and has been linked to changes in plant species composition in a wide range of
16 terrestrial biomes from the Arctic to the tropics (e.g. Bobbink *et al.* 2010). Some of the most
17 affected terrestrial habitats are nutrient-poor semi-natural habitats such as heathlands and
18 grasslands. Calcareous grasslands are floristically amongst the most species rich communities
19 of North-Western Europe, containing many rare and endangered plant species (e.g. Willems,
20 1990). Their biodiversity is threatened by changes in management (e.g. Tansley, 1922;
21 Willems, 1978), habitat fragmentation (e.g. Fisher & Stöcklin, 1997) and disturbance (e.g.
22 Smart *et al.* 2003a), among other factors.

23

24 Experimental studies in Dutch calcareous grasslands have shown that increased N deposition
25 results in the loss of characteristic plant species and an increase of highly competitive grass

1 species such as *Brachypodium pinnatum* (e.g. Willems 1990; Bobbink *et al.* 1998). In the UK,
2 changes in species composition, a reduction in flowering, and a reduction in the forb/grass
3 ratio have been shown in a long-term N-addition experiment (Carroll *et al.* 2003), but
4 encroachment by *B. pinnatum* or other highly competitive grasses such as *Bromus erectus*
5 was not observed, possibly due to co-limitation by phosphorus (Morecroft *et al.* 1994; Wilson
6 *et al.* 1995; Carroll *et al.* 2003; Phoenix *et al.* 2003).

7
8 Stevens *et al.* (2004) found a significant negative spatial relationship between N deposition
9 and species richness in a national survey of one acidic grassland community in the UK. More
10 recently, analysis of data from a national survey covering all plant communities in the UK
11 corroborated these findings for both heathlands and acid grasslands, but failed to find a
12 significant relationship between N deposition and species richness in calcareous grasslands,
13 even when interacting environmental factors such as rainfall and altitude were included in the
14 models (Maskell *et al.* 2010). However, significantly more species characteristic of fertile
15 soil conditions, and a higher grass/forb ratio, was found in calcareous grassland plots with
16 higher N deposition. These findings suggest that the responses of calcareous grasslands to
17 increased N deposition differ from those of other semi-natural ecosystems, such as acid
18 grasslands and heathlands. This may be due to several factors, including (a) the importance of
19 co-limitation by phosphorus, (b) active management and conservation to protect
20 characteristic plant species and (c) the occurrence on shallow well buffered soils that are less
21 sensitive to acidification caused by N and S deposition.

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23 In addition, the limitations of the studies undertaken to date need to be considered.

24 Experimental studies measure change over a relatively short period of time with N deposition
25 as the only variable factor. Field surveys which explore the spatial associations between N

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3 1 deposition and species richness, or other response variables, do not allow an assessment of
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5 2 whether these are due to long-term historical deposition or recent changes in deposition, such
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7 3 as those simulated in experimental studies. Although long-term vegetation change in
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9 4 calcareous grasslands in the UK since 1950 has been described by Bennie *et al.* (2006), their
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11 5 analysis was not designed to assess the effect of N deposition, and there are no field surveys
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13 6 relating recent changes in species composition or species numbers to atmospheric N
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15 7 deposition, using plots that can be relocated with confidence. A further limitation to the
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17 8 interpretation of field survey data is the multi-collinearity between predictor variables such as
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19 9 N deposition and climate variables, and earlier studies have highlighted the limitations that
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21 10 multiple regression or covariance analysis have in predicting the relative effects of the
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23 11 explanatory variables on dependent variables such as species richness (e.g. Stevens *et al.*
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25 12 2004, Maskell *et al.* 2010).

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34 14 We investigated the direct, indirect and interacting effects of atmospheric N deposition, other
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36 15 confounding environmental variables, and site management on species richness and species
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38 16 diversity in calcareous grasslands in the UK. We based our study on a network of 128
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40 17 permanently marked plots in areas of calcareous grassland spread across the UK that were
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42 18 specifically established in the early 1990s, with the aim of monitoring long-term impacts of
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44 19 climate change and air pollution, by John Rodwell and colleagues (Rich *et al.* 1993). Between
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46 20 2006 and 2009, 48 plots were revisited and additional soil measurements were made. This
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48 21 allowed us to assess the effect of N deposition and other factors on both spatial variation and
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50 22 recent temporal changes in species richness and other relevant variables.

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58 24 In order to test if changes in species richness and composition are significantly affected by
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60 25 atmospheric N deposition in a matrix of effects of factors such as management, climate, S

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3 1 deposition and soil conditions, we analysed the data using both mixed effect models and
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6 2 structural equation modelling (SEM). The SEM models allow explanatory variables to be
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8 3 placed in 'series' rather than 'parallel', using sequential arrangements that reflect
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10 4 hypothetical causal chains linking the effects. Such models provide insights that are not
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12 5 possible with standard covariance analyses into the relative importance of different direct and
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14 6 indirect effects of N deposition.
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20 8 We tested three major hypotheses:
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24 10 1) Ndeposition is a significant explanatory driver of spatial variation in species richness,
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26 11 species diversity and the frequency of individual species in calcareous grasslands.
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31 13 2) Ndeposition is a significant explanatory driver of change over the last 15 years in species
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33 14 richness, species diversity and the frequency of individual species in calcareous grasslands.
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38 16 3) Recent changes in species richness, species diversity and the frequency of individual
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40 17 species in calcareous grasslands can be explained by a combination of the effects of
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42 18 management, direct effects of N deposition, and indirect effects of S and N deposition via soil
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44 19 acidification.
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1 **Materials and Methods**

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3 *Calcareous grassland survey: 1990-1993 survey*

4 A large national survey of calcareous grasslands was carried out between 1990 and 1993
5 (Rich *et al.* 1993). The survey method was specifically designed to be repeatable with a
6 minimum of observer bias. All the selected sites were nature reserves with active management
7 to maintain species diversity and characteristic species; 56 sites were selected, with 1-6 plots
8 on each site, resulting in a total of 128 plots. Sites were chosen to cover a wide geographical
9 range (Fig. 1), a large climatic gradient, a wide range in deposition of air pollutants and all
10 types of calcareous grasslands known in the UK, using the National Vegetation Classification
11 (NVC) (Rodwell, 1998). On most sites, plots were chosen on both south and north-facing
12 slopes to account for effects of aspect. Each plot covered an area of 144 m² (typically
13 measuring 12×12 m or more rarely 6×24 m) and was established in a floristically
14 homogeneous stand of calcareous grassland. Individual plots were permanently marked at
15 unique places with buried copper coils. Detailed field notes, describing the exact location of
16 the plots, photographs, sketch maps and measurements from fixed features were also
17 recorded, to ensure successful relocation of each plot in future surveys. The presence/absence
18 of all vascular plants was recorded in 36 50×50 cm quadrats positioned within each permanent
19 plot. Soil pH of the top 10 cm was determined in the field by mixing soil into a slurry with
20 distilled water (1:1 vol:vol), equilibrating for 5 minutes, and measuring using a calibrated pH
21 meter. Soil type, soil depth, slope, aspect and sward height were recorded for each plot. For
22 each site, the size, its protection status and detailed information on local management
23 (grazing intensity) were noted. Data from the original reports could not be recovered for 7
24 plots (4 sites), resulting in available data for 121 plots.

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7 2 *2006-2009 survey*
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10 3 In 2006, 35 out of the 56 sites were selected for re-survey (48 plots), covering a wide range of
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12 4 Ndeposition. Sites were selected to reduce the effects of spatial autocorrelation in atmospheric
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14 5 N deposition (e.g. by selecting high and low Ndeposition sites at similar latitudes). Sites and
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16 6 plots with NVC communities CG1, CG2, CG10, CG11 and CG12 were targeted where
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18 7 possible to reduce variation due to initial vegetation composition. Only six of the plots selected
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20 8 for re-survey were identified as supporting different NVC communities (CG3, CG8, CG9),
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22 9 mainly due to the presence of grasses such as *Bromus erectus* (CG3) or *Sesleria albicans*
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24 10 (CG8&9). In broad terms, plots in the lowlands of Wales and England focussed on stands of
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26 11 CG1 and CG2, while those in the uplands of Wales and Scotland focussed on stands of
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28 12 CG10, CG11 or CG12.
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34 13 Metal detectors were used for relocation of the plots. In each plot, 36 quadrats were
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36 14 positioned in exactly the same pattern as in 1990-1993 and in each quadrat the
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38 15 presence/absence of all vascular plants was recorded. One soil sample, composed of 5 sub-
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40 16 samples, was taken from the top 10 cm of each plot using an auger (3 cm diameter). Soil
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42 17 samples were stored at 4 °C until further analysis. Sward height and soil depth were recorded
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44 18 at each plot and detailed information on the local management (grazing intensity) for the past
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46 19 15 years was obtained from local managers at the plot level.
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55 21 *Soil analysis*
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58 22 Soil samples from the 2006-2009 survey were thoroughly mixed prior to extraction.
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60 23 Extractions were performed on fresh soil and corrected for moisture content after drying for

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3 1 24h at 105°C. A portion of 35g of fresh soil was mixed with 100ml bi-distilled water for
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5 2 water soluble extraction or 100ml 0.2M NaCl for the exchangeable fraction. Extracts were
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7 3 shaken for 1h at 100rpm after which the pH was measured in the water fraction with a
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9 4 Radiometer type PHM 82 pH meter. Extracts were filtered (0.2µm) and stored at 4°C until
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11 5 analysis. Plant available P was measured after Olsen-P extraction (Olsen *et al.* 1954); 5 g of
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13 6 fresh soil was mixed with 100 ml NaHCO₃ and shaken for 30 minutes at 100 rpm. Extracts
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15 7 were filtered (0.2µm) and stored at 4°C until analysis. NO₃⁻ and NH₄⁺ were analysed using a
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17 8 Bran and Luebbe Autoanalyser 3 (Bran and Luebbe, Norderstedt, Germany). Concentrations
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19 9 of base cations (Ca²⁺, Na⁺, Mg²⁺ and K⁺) and Al³⁺ and plant available P were determined in
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21 10 the NaCl and Olsen extracts respectively using an ICP Spectrometer (IRIS Intrepid II,
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23 11 Thermo Electron Corporation, Franklin, MA). Organic matter contents of the soils were
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25 12 determined by loss on ignition (LOI, 4h, 550°C).
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34 *Plant attributes*

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36 15 The names of all plant species in the 1990-1993 survey were checked and if necessary
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38 16 updated to enable a comparison with the recent 2006-2009 survey. All data were then linked
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40 17 to the attribute list PLANTATT (Hill *et al.* 2004). Stace (1997) was followed for
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42 18 nomenclature. We used Ellenberg indicator values modified for the UK (Hill *et al.* 2004) to
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44 19 characterise plant species. For each plot the average, frequency weighted, Ellenberg value
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46 20 was calculated in order to characterise plots according to the species they contained. Species
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48 21 were grouped into eutrophic (Ellenberg N>5), oligotrophic (Ellenberg N<3), acidophobic
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50 22 (Ellenberg R>5), and acidophilic (Ellenberg R<3) species. The total number of scarce and
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52 23 rare species, as denoted in the PLANTATT database, was recorded for each plot.
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54 24 Species richness (S) was recorded as the total number of vascular plant species in each plot.
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56 25 For each plot the species diversity was calculated as the Shannon index (H'): $H' = -\sum(p_i \ln p_i)$,
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1 where p_i is the number of occurrences of the i^{th} species in the 36 quadrats expressed as a
2 proportion of the total number of occurrences of all species in those quadrats (Magurran
3 2004). In addition, Shannon's equitability or the Evenness (E_H) was calculated as $E_H = H'/\ln$
4 (S). For each species the frequency of occurrence was calculated as $n_i/36$, where n_i is the
5 number of quadrats in which the species occurred in plot i .

7 *Survey data selection*

8 In the soils of the plots revisited in 2006-9, pH varied widely, ranging from shallow rendzinas
9 of pH 8.5 to peaty soils overlaying calcareous rock of pH 4.2. The six plots with the lowest
10 pH had in general very different vegetation (e.g. more acidophilic species such as *Calluna*
11 *vulgaris* or *Juncuseffusus*), a very high soil organic matter and low base cation content. In
12 order to relate atmospheric N deposition and other environmental parameters to species
13 composition and richness in calcareous grasslands which share similar characteristics and to
14 reduce confounding effects, only those plots with a soil $\text{pH}_{(1990-1993)} > 6.0$ were included in the
15 analysis. This resulted in 106 plots from the 1990-1993 data and 42 plots which could be used
16 for the comparison between 1990-1993 and 2006-2009. For analysis at the individual species
17 level, changes in species frequency were analysed on a subset of the data; only those species
18 which occurred in at least 11 (25%) or more of the 42 plots in both surveys were included.
19 This resulted in a dataset for only 52 species. Grass to herb ratio was calculated as the ratio
20 between the numbers of grass and herb species at the plot level.

22 *Plot and site specific parameters*

23 Modelled N and S deposition data for each site were obtained from the Centre of Ecology and
24 Hydrology (CEH) for two periods: 1993-1995 and 2006-2008 for the surveys of 1990-1993

1 and 2006-2009 respectively. The Ndeposition ranged from 7.3 to 40.7 kg N ha⁻¹ y⁻¹ while
2 Sdeposition ranged from 8.0 to 27.6 kg S ha⁻¹ y⁻¹. Climate data for each site were obtained
3 from the 5km gridded climate database held by the UK Meteorological Office
4 (www.metoffice.co.uk). 10 year averaged data for the periods 1981-1991 and 1997-2006
5 respectively were used for the 1990-1993 and 2006-2009 surveys, including mean minimum
6 winter temperature, mean maximum summer temperature, mean annual temperature and
7 mean annual rainfall. The aspect of each plot was converted to a 0-1 scale using
8 $\sin(a/(360/\pi))$, where a = the aspect in degrees. This resulted in a gradient from North =0 to
9 South =1, with East and West both at 0.5. Where information on stocking was available from
10 local site managers, grazing intensity for each plot was calculated in terms of livestock units
11 (LU) per hectare per year using the conversion factors provided in Attwood & Heavey (1964).
12 For the 9 plots where detailed stocking was not provided we estimated the grazing intensity
13 from the sward height, based on the highly significant correlation ($r^2=0.58$) between sward
14 height and livestock units obtained from the other plots.

16 *Statistical analysis*

17 Principal component analysis (PCA) in CANOCO (TerBraak, 1988) was used to determine
18 the variables explaining most of the variation between the plots. For each survey, we selected
19 the strongest, non-correlated, variables for further analysis.

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21 Possible combinations of predictor variables were explored to assess the effects of
22 Ndeposition, soil conditions, climate data and grazing pressure on species richness and
23 species composition. Data from the 1990-1993 survey were used in the spatial analysis, while
24 data from both surveys were used in the temporal analysis. All models were explicitly tested

1 for spatial autocorrelation in the response variable and residuals by inspection of semi-
 2 variograms. In the presence of spatial autocorrelation, generalized linear mixed-effect models
 3 (GLMM) were used to control for this spatial autocorrelation. In these models, a correlation
 4 structure was added to correct for spatial autocorrelation. Correlation structures such as
 5 corExp, and corSpher were used with the “form=~Easting+Northing” argument in the
 6 correlation option to calculate the Euclidean distances (using Pythagoras theorem) between
 7 sites with coordinates given by Easting and Northing. The models were ranked according to
 8 the Akaike Information Criterion (AIC) and only the models and corresponding correlation
 9 structure with the lowest AIC, representing the ‘best fitting models’, were selected. When no
 10 spatial autocorrelation was observed, multiple linear regression models were used. Again,
 11 models were ranked according to AIC and only the models with the lowest AIC were
 12 selected. To test for multicollinearity, variance inflation factors (VIF) were computed for
 13 each predictor variable in the model. Predictor variables that were highly correlated (VIF>10:
 14 Gujarati, 1995) were not analysed in the same model; in those situations only the predictor
 15 variable that explained most of the variance was selected.

16
 17 For the change in Shannon diversity H', the ecological significance of the effect was
 18 quantified by dividing the data into two groups, one with N deposition below the lowest, and
 19 one with N deposition above the highest, critical load of N deposition for this vegetation type
 20 (<15 and >25 kg N ha⁻¹y⁻¹ respectively, Bobbink *et al.*, 2003). The Cohen d-value was then

$$d = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{(s_1^2) + s_2^2}{2}}}$$

21 calculated according to: , where \bar{x}_1 and \bar{x}_2 are the means of the data for sites
 22 with high and low N deposition respectively and s_1 and s_2 the standard deviations in these
 23 groups. The calculated value of d was checked against Cohen's classification (Cohen,
 24 1988). We also compared the predicted change in H' from our models at sites with N

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3 1 deposition above $25 \text{ kg ha}^{-1} \text{ y}^{-1}$ with the value of H' in a reference dataset representing an
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5 2 highly undesirable endpoint that could be realised given continued high N deposition when
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7 3 grazing and/or cutting is maintained. We selected botanical data ($n=327$) from within the
8
9 4 randomly located plots of the Countryside Survey (CS) of Great Britain which independent
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11 5 land cover mapping showed could be referenced to the Neutral Grassland Broad Habitat
12
13 6 (Jackson 2000). The change in H' from 1990-93 to 2006-09 in the high N deposition plots
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15 7 was then expressed as a percentage of the difference between the 1990-93 value and that of
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17 8 the reference dataset, an approach similar to that used by Smart *et al.* (2003b). The analyses
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19 9 were done in the 'R' (version 2.9.0) statistical and programming environment (R
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21 10 Development Core Team, 2008).
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30 12 Structural equation models (SEM) were used to further examine the relationships between N
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32 13 deposition, soil conditions, climate data and grazing pressure on species richness and species
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34 14 composition. The software package AMOS 17 was used to design the SEM models and
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36 15 calculate path coefficients, squared multiple correlations, direct and indirect effects and
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38 16 model fit. A significant χ^2 -statistic indicates that the SEM model provides a poor fit. χ^2 -
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40 17 statistics and four non- χ^2 -distributed fitting functions (Comparative Fit Index, CFI; Normed
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42 18 Fit Index, NFI; Tucker-Lewis Index, TLI; Incremental Fit Index, IFI) were compared in order
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44 19 to test the goodness-of-fit of the models. In the SEM models, soil nutrient concentrations
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46 20 were represented by "mineral N" ($\text{NH}_4^+ + \text{NO}_3^-$).
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1 Results

2 3 *Analysis of 1990-1993 survey*

4 Linear regression between the total number of vascular plant species and Ndeposition did not
5 reveal a significant relationship in the 1990-1993 dataset (Fig. 2a), but Ndeposition did show
6 a slight positive relationship with the grass to herb ratio (Fig. 2b). Almost no scarce and rare
7 species were observed at plots with Ndeposition above $25 \text{ kg N ha}^{-1}\text{y}^{-1}$ (Fig. 2c); the average
8 number of scarce and rare species was 1.6 in the 77 plots with deposition below $25 \text{ kg N ha}^{-1}\text{y}^{-1}$
9 compared to 0.3 in the 29 plots with deposition above $25 \text{ kg N ha}^{-1}\text{y}^{-1}$ (ANOVA, $df = 1$,
10 $F=13.62$, $p<0.001$).

11
12 Minimum winter temperature and maximum summer temperature were highly
13 correlated; only maximum summer temperature was included in the model, as this was the
14 stronger predictor according to the PCA. For similar reasons, organic content of the soil, Olsen
15 P and slope (which were all strongly correlated with pH) and mean annual rainfall (which
16 was strongly correlated with maximum summer temperature) were excluded from the
17 analysis. Grazing pressure entered the analysis, but was not a significant explanatory variable
18 for any of the dependant parameters (Table 1a).

19
20 Maximum summer temperature and soil depth were the most important variables explaining
21 variation in species composition and species richness in the 1990-1993 dataset (Table 1a).
22 Spatial variation in species richness, the number of eutrophic species, the number of herb
23 species, and H' could be explained solely by soil depth and maximum summer temperature,
24 with positive relationships with both variables in all cases.

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3 1 Ndeposition did not have a significant effect on speciesrichness or on the number of
4
5 2 eutrophic or oligotrophic species. The frequency weighted average Ellenberg N value was
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7 3 also not significantly related toN deposition, but was best explained by the maximum summer
8
9 4 temperature.The only variable for which Ndeposition entered the final model was the grass to
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11 5 herb ratio, for which a positive effect, albeit at the $p < 0.1$ level,was recorded.A significant
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13 6 negative effect of soil pH was found on the number of acidophilic species, in a model that
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15 7 also included a negative effect of maximum summer temperature. The frequency weighted
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17 8 Ellenberg R values increased with soil pH, in a model that also included a positive effect of
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19 9 maximum summer temperature.
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26 27 11 *Analysis of change between surveys*

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29 12 The average soil pH in the 1990-1993 dataset was 7.41. The average soil pH in the more
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31 13 recent survey wasmeasured more accurately and was 7.14. The significant difference (paired
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33 14 t-test: $t=2.52$, $df=35$, $p=0.016$)was most likely due to different measurement methods and
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35 15 cannot be interpreted in terms of environmental change.
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41 17 A significant, but slight, decrease in speciesrichness, independent of Ndeposition, was
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43 18 observed between 1990-1993 (average = 41) and 2006-2009 (average=39) (paired t-test $t=-$
44
45 19 2.274 , $p=0.028$, $df=41$).The change in both H' and E_H over this period was significantly
46
47 20 negatively related to Ndeposition,with a general increase in H' and E_H at low Ndeposition and
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49 21 a decrease at high Ndeposition (Fig. 3).Ndeposition was not significantly related to the
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51 22 change in any of the other dependent variables.
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58 24 Instrong contrast to the spatial analysis for the 1990-93 data, maximum summer temperature
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60 25 and aspect did not enter the best fit model for the change in any of the dependent variables

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3 1 (Table 1b). In contrast to the spatial models, soil depth entered the models for change in
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5 2 number of oligotrophic, acidophilic and acidophobic species, and was never significant.
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10 4 Soil pH showed a significant positive relationship with the change in the species richness, i.e.
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12 5 there tended to be an increase in plots with a higher soil pH. In addition, soil pH was
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14 6 associated positively with change in the number of herb species and negatively with change in
15
16 7 grass to herb ratios. The mineral N content was also weakly positively associated with change
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18 8 in the number of herb species. However, the changes in the number of acidophilic species, the
19
20 9 number of acidophobic species, and the cover weighted Ellenberg R index were not related
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22 10 significantly to soil pH or any other variable.
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29 12 The models showed a significant negative effect of N deposition on changes in H' and
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31 13 evenness (Table 1b). However, N deposition was not significantly related to change in the
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33 14 number of eutrophic or oligotrophic species, or change in Ellenberg N index. The change in
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35 15 grazing was significantly negatively related to the change in frequency weighted Ellenberg N
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37 16 index, while the change in the number of oligotrophic species showed a positive relationship
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39 17 to the mineral N content of the soil.
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44 45 46 19 *Structural equation modelling*

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48 20 Structural equation models were used to further investigate the direct and indirect effects of
49
50 21 N deposition for two variables for which change between 1990-1993 and 2006-2009 was not
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52 22 spatially auto-correlated: H' and number of herb species. Using maximum likelihood
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54 23 estimation, we observed a χ^2 of 4.488 for the model for H' ($df=7$, $p=0.722$) for a goodness-of-
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56 24 fit test of the null hypothesis that the covariance matrix implied by the (expected) model
57
58 25 reproduces the observed covariance matrix. Failure to reject the null hypothesis indicates that
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3 1 the model was a good fit. In addition, the CFI of 1.00, the NFI of 0.94, the TLI of 1.14 and the
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5
6 2 IFI of 1.04 that we calculated all indicated a good model fit.
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10 4 As expected from the linear models (Table 1b), the SEM model showed that N deposition is a
11
12 5 significant explanatory parameter for the changes in H' of the plots (Fig. 4a, Table 2a). A
13
14 6 direct negative standardised path coefficient of -0.302 (Fig. 4a) on the change in H' was
15
16 7 observed. Standardised path coefficients represent the standardised change in a response
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18 8 variable per standardised unit change in a predictor variable, i.e. when N deposition
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20 9 increased by one standard deviation, change in H' decreased by 0.302 standard deviation. In
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22 10 terms of ecological significance, this can be regarded as a moderate effect (Chin 1998). In
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24 11 addition, there was a positive relationship between soil pH and increase in H'. Mineral N
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26 12 content exerted an indirect effect on the changes via soil pH, as well as a direct effect. N
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28 13 deposition was positively related to mineral N content. S deposition had no direct effect but a
29
30 14 negative indirect effect via soil pH (Fig. 4a, Table 2a). Grazing pressure had an independent
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32 15 direct negative effect on change in H'.
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41 17 The SEM model for herb species showed that N deposition only weakly explains the
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43 18 change between 1990-1993 and 2006-2009 (Fig. 4b, Table 2b) and that this was an indirect
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45 19 effect via soil pH. A much stronger negative effect of S deposition on the change in herb
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47 20 species was observed (Table 2b), also indirectly via soil pH. Grazing pressure did not have an
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49 21 effect and was eliminated from this model. Mineral N content exerted an indirect effect via
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51 22 soil pH (Fig. 4b, Table 2b).
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24 *Change in frequency of individual species*

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3 1 Someof the 52 individual species that met our selection criteria showed very distinct changes
4
5 2 in frequency between the two surveys. We divided the plots into three groups based on the
6
7 3 2006-8 Ndeposition data,and calculated the mean change in frequency of individual species
8
9 4 for plots in each deposition class.Species which declined or increased in frequency over all
10
11 5 plots by more than 15% are listed in Table 3, with the change in frequency split between the
12
13 6 three deposition categories.For the three species with an increase in frequency at the highest
14
15 7 Ndeposition, the overall relationship with Ndeposition was not significant. However, the five
16
17 8 species which declined in frequency in the highest class of N deposition (*Galiumsternerii*,
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19 9 *Linum catharticum*, *Briza media*, *Gentianella amarella* and *Campanula rotundifolia*) all
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24 10 showed a significant correlation between the decline in frequency and Ndeposition.
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1 Discussion

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8 A key finding from our study is that the effects of N deposition, and of other driving
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10 variables, are quite different when considering the spatial variation between sites in 1990-93,
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12 and when considering the temporal change between 1990-93 and 2006-9. Here we consider
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14 the results from the spatial analysis and the analysis of temporal change in turn, placing our
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16 findings into the wider context of the role of Ndeposition and other factors in influencing the
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18 species richness, composition and diversity of calcareous grasslands.
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24 *Spatial variation*

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27 We found that N deposition did not explain spatial variation in species richness, diversity, or
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29 the frequency of eutrophic species. This is consistent with a study in 1998 of 94 calcareous
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31 grassland plots in the UK, in which there was no significantrelationship between N
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33 deposition, or climatic variables, and species richness (Maskell *et al.* 2010), even though
34
35 significant relationships between N deposition and species richness were found in the same
36
37 survey in acid grasslands and heathlands. These findings contrast with results from
38
39 experimental studies in which clear N fertilization effects have been shown in calcicolous
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41 habitats, with fast-growing eutrophic species outcompeting slow growing sensitive species
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43 (e.g. Bobbink, 1991; Carroll *et al.* 2003; van den Berg *et al.* 2005). Maskell *et al.* (2010)
44
45 argued that their relatively small sample size (94 plots),and the correlation between N
46
47 deposition and climatic parameters, may have masked the significance of Ndeposition as an
48
49 explanatory variable. Our sample size is similar (106 plots) to that of Maskell *et al.* (2010),
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51 but a more likely explanation for the lack of significant relationships with Ndeposition is that
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53 all of our sites are nature reserves, at which the management is aimed specifically at the
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55 conservation of high species diversity and in which grazing regimes are implemented to
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1 prevent the dominance of eutrophic species. Grazing at moderate stocking rates maintains
2 species diversity by reducing the number of competitive species (Collins *et al.* 1998; Ollf &
3 Ritchie, 1998; Klimek *et al.* 2007). The absence of a management effect may result from the
4 management regimes at all the sites being successfully focussed on preserving species
5 richness.

6
7 Despite this, the frequency of rare and scarce species was significantly reduced, by a factor of
8 5, at sites with N deposition above 25 kg ha⁻¹ y⁻¹. These relationships are difficult to test in a
9 rigorous way in multivariate models, as rare species, such as *Epipactis atrorubens*,
10 *Triniaglauca* and *Pulsatilla vulgaris*, not only have a highly restricted geographic distribution
11 but are also typically highly localised on the sites where they do occur. Fisher and Stöcklin
12 (1997) and Bennie *et al.* (2006) both found a negative correlation between the initial
13 frequency of a plant species and the extinction rate in calcareous grasslands over a period of
14 several decades, over which Bennie *et al.* (2006) also found an increase in Ellenberg fertility
15 index, although there was not a significant relationship between extinction rates and the
16 Ellenberg fertility index. Our results suggest a link between N deposition and the occurrence
17 of scarce and rare species, which may be due to local extinctions over a period of decades.

18
19 Maximum summer temperature, along with soil depth, explained most of the variation in
20 species richness and the frequency of different types of species; species richness was higher
21 in plots with a higher maximum temperature. Plots with deeper soils tended to occupy
22 moderate to low slopes and had a higher organic content ($r^2 = -0.14$, $p = 0.022$) and a higher
23 moisture content ($r^2 = 0.35$, $p < 0.001$). The higher organic content and the fact that deeper
24 soils are more resistant to drought stress may be important factors in explaining the higher
25 species richness. Duckworth *et al.* (2000) found that temperature and soil organic matter are

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3 1 amongst the strongest parameters explaining spatial variation in vegetation composition of
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6 2 Atlantic European calcareous grasslands. In addition, Bennie *et al.* (2006) found that
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8 3 calcareous grassland plots on deeper soils were more likely to be invaded by (often
9
10 4 mesotrophic) plant species, which may explain the higher species richness.
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13 5 14 15 6 *Comparison between surveys*

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18 7 The species richness was lower in 2006-2009 than in 1990-1993 with an average loss of 2
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20 8 species over 15 years; this small overall change may represent differences between surveyors
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22 9 in the two periods. Change in species richness was not associated with N deposition,
23
24
25 10 but changes in H' and evenness were significantly negatively related to N deposition. The
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27 11 Cohen's d value for the change in H' was 0.99, representing a medium to strong ecological
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29 12 effect (Cohen 1988). Progression towards an undesirable reference dataset in high N
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31 13 deposition plots showed the reduction in H' between 1990-1993 and 2006-2009 represented a
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33 14 6.4% movement towards the H' value typical of British Neutral Grasslands. This implies that
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35 15 a much longer period would be needed to reach the low values of H' typical of neutral
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37 16 grasslands. However, many typical calcareous grassland species may be lost well before this
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39 17 value of H' is reached, and we conclude that the change in H' at high N deposition sites does
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41 18 represent a significant ecological change.
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49 20 H' is a measure of diversity that accounts for both species richness and evenness of the
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51 21 species present in the sample (Magurran, 2004). N deposition did not significantly affect
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53 22 changes in species richness but did significantly affect changes in evenness, indicating that
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55 23 changes in H' were driven primarily by changes in the evenness of the plots.
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3 1 In addition, there was a significant decline in the frequency of five individual species when N
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5 2 deposition exceeded $25 \text{ kg ha}^{-1} \text{ y}^{-1}$. All five individual species (*Linum catharticum*, *Briza*
6
7 3 *media*, *Gentianella amarella*, *Campanula rotundifolia* and *Galium sternerii*) have a low
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9 4 Ellenberg nutrient score. The decline in frequency in these high N deposition plots was
10
11 5 substantial (on average 22%) over a relatively short period of 15 years despite the focussed
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13 6 management on these nature reserves, and indicates that local extinctions of these species
14
15 7 could occur in the future unless N deposition declines. Both *C. rotundifolia* and *B. media* are
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17 8 regarded as poor competitors (Leishman, 1999; Dixon, 2002; Hutchings *et al.* 2003) and for
18
19 9 *C. rotundifolia*, lower frequencies have been found at sites with high N deposition (Stevens *et*
20
21 10 *al.* 2006; Maskell *et al.* 2010). *Linum catharticum* and *G. amarella* are respectively annual
22
23 11 and bi-annual species which may strongly depend on gap formation in the swards for
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25 12 germination (e.g. Turnbull *et al.* 2005). These results are corroborated by an earlier study on
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27 13 calcareous grasslands by Bennie *et al.* (2006) who found strong decreases in frequency over
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29 14 the period 1950-2000 of *C. rotundifolia*, *L. catharticum* and *B. media*.

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39 16 The change in species richness was positively related to soil pH, as was herb species number,
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41 17 indicating that plots of higher buffering capacity tended to gain species and plots with slightly
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43 18 lower pH tended to lose species. Despite the fact that calcareous soils are highly buffered,
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45 19 experimental studies have shown that calcareous grasslands also leach base cations when N
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47 20 deposition is increased, as a result of soil exchange processes and plant uptake of nitrogen
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49 21 (Horswillet *et al.* 2008). An increase of soil pH due to the reduction in acid deposition over the
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51 22 last 15 years (Emmett *et al.* 2010) may have contributed to increased species richness,
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53 23 especially in those plots which were least affected by base cation depletion.
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25 *Importance of direct and indirect effects of N deposition*

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3 1 The use of structural equation models allowed us to identify and compare the comparative
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5 2 strengths of direct effects of N deposition and indirect effects of both N and S deposition,
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7 3 through soil pH, using hypothesised causal relationships that are consistent with experimental
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9 4 studies. The responses of vegetation to changing N deposition are complex and involve
10
11 5 several mechanisms (Bobbink *et al.* 2010), and therefore statistical methods, such as
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13 6 structural equation modelling, that incorporate this complexity and aim to disentangle
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15 7 different direct and indirect effects through potential causal structures provide powerful new
16
17 8 tools to complement more conventional regression analysis.
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24 10 The results show very different models for change in H' and for change in herb species
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26 11 numbers. Changes in H' were dominated by a direct negative effect of N deposition; although
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28 12 indirect negative effects of S and N deposition via soil pH were also significant, they had a
29
30 13 much smaller contribution. The negative direct effect of N deposition on change in H' was
31
32 14 also found for changes in evenness in the mixed effect models, suggesting that eutrophication
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34 15 rather than acidification is the dominant mechanism. In contrast, the direct effect of N
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36 16 deposition on change in herb species numbers was not significant, and was small compared to
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38 17 indirect effects via soil pH, indicating that acidification was the dominant mechanism.
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45 19 Soil pH, which had a significant positive relationship with both changes in H' and changes in
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47 20 herb species numbers, was itself negatively influenced by S deposition and mineral N
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49 21 content. The strong acidifying nature of S in deposition compared to N was revealed by the
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51 22 stronger negative effects that were exerted by S deposition on soil pH. Calcareous grasslands
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53 23 have been shown to retain most of the deposited N in the system with little leaching to deeper
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55 24 soil layers or groundwater, thereby increasing mineral N content of the top soils (Phoenix *et*
56
57 25 *al.* 2008). The increased mineral N in the soils in turn can have a significant negative effect

1 on soil pH (e.g. Horswillet *et al.* 2008). This is consistent with the positive relationships
2 between N deposition and mineral N content and the significant negative relationships
3 between the mineral N content and soil pH in our study. These findings, using a different
4 method of analysis, are consistent with earlier studies of acid grasslands in which negative
5 effects of soil acidity on herb species numbers were recorded (e.g. Stevens *et al.* 2006; Duprè
6 *et al.* 2010, Stevens *et al.* 2010).

7
8 A recent survey of changes in a local UK flora over the last 50 years showed the positive
9 effects of the designation of conservation sites on maintaining biodiversity, especially in
10 calcareous grasslands (Walker *et al.* 2009). In our structural equation model, change in
11 grazing also significantly improved the model fit, with a decrease in grazing pressure being
12 directly associated with a decrease in H'. A decrease in grazing pressure was also associated
13 with an increase in Ellenberg N score. These results may reflect an effect of poor
14 management on some sites over recent years, since the spatial analysis suggested that grazing
15 management in 1990-93 was optimal overall and hence did not affect spatial variation in
16 species richness or diversity.

17 18 *Conclusions*

19 Critical loads for adverse effects of N deposition for calcareous grasslands, based on field
20 experiments, have been set as a range of 15-25 kg N ha⁻¹y⁻¹ (Bobbink *et al.* 2003; Bobbink *et*
21 *al.* 2010), with the range indicating the variation in sensitivity. Our results provide the first
22 support for this critical load range from field data, as, in the plots where the critical load
23 range is exceeded, there is evidence of a decrease in species diversity and evenness, a decline
24 in the frequency of characteristic species, and a lower number of rare and scarce species.
25 These effects are likely to be due to both direct effects of N deposition and indirect effects

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3 1 through changes in soil pH. Since all sites had active management to maintain a high diversity
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6 2 and characteristic species, our results imply that even focussed management on nature
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8 3 conservation objectives cannot prevent adverse effects of high rates of N deposition. Given
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10 4 the high and increasing rates of N deposition in many parts of the world, N deposition is a
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13 5 wide-scale threat to the conservation of species-rich grassland communities, and their
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15 6 characteristic species.
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For Review Only

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2
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5
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9
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11
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13
14 6 granting permission to access and sample the sites.
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3 **1 Tables**
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5 **2 Table 1:**
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7
8 Effect sizes of the predictor variables (expressed as the coefficient \pm s.d.), for the subset of predictors that were included in the model with the
9
10 lowest AIC for each response variable, for (a) the survey in 1990-93 and (b) the change between the surveys in 2006-9 and 1990-93. Predictors
11
12 significant at $p < 0.1$, $p < 0.05$, $p < 0.01$ and $p < 0.001$ are indicated by +, *, ** and ***, respectively. Analyses were based on a maximum of 106
13
14 calcareous grassland surveys in 1993 and a maximum of 42 comparisons between 2009 and 1993. All analyses were based on vascular plants
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17 only. The number of acidophilic species was $\log(x+1)$ transformed.
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a) 1990-1993		Predictor variable					df ^{1,2}	AIC
	pH	Soil Depth	Aspect	Ndeposition	Max Temp summer	Grazing		
Species richness ¹		0.338*			1.442*		1, 103	770
		±0.121			±0.616			
Eutrophic species ¹		0.274***			1.530		1, 103	715
		±0.093			±0.467***			
Oligotrophic species ¹			1.715				1, 104	706
			±1.218					
Acidophylic species	-0.507***				-0.166***		1, 103	176
	±0.123				±0.025			
Acidophobic species ¹			2.815		2.296***		1, 103	714
			±1.095		±0.513			
Herb species ¹		0.266**			1.501**		1, 103	726
		±0.097			±0.503			
Grass:herb ratio ¹				0.004+	-0.028***		1, 103	-119
				±0.002	±0.007			
WA Ellenberg N ¹					0.028**		1, 104	-79
					±0.009			
WA Ellenberg R	0.395***				0.115***		1, 103	109
	±0.090				±0.018			
Shannon index		0.0133***			0.049***		1, 103	32
		±0.004			±0.013			
Evenness ¹		0.001					1, 104	-460
		±3.390E-4						

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b) Change between 2006-2009-and 1990-1993	Predictor variable							df _{1,2}	AIC
	pH	Soil Depth	Mineral N soil	Aspect	Ndepositio n	Max Temp summer	Grazing		
Species richness	2.634*							1,35	227
	±1.242								
Eutrophic species							-1.434 ± 1.326	1,35	170
Oligotrophic species		-0.106 ±0.056	3.773E-4+ ± 2.003E-4					1,34	149
Acidophylic species		-0.032 ±0.021						1,35	77
Acidophobic species		0.089 ± 0.077						1,35	174
Herb species	2.427*		8.085E-04+ ±4.253E-04					1,34	203
	±0.9348								
Grass:herb ratio ¹	-0.056+ ±0.021							1,35	-61
WA Ellenberg N ¹							-0.213** ±0.073	1,35	-32
WA Ellenberg R ¹			-2.039E-5 ±1.505E-5					1,35	-38
Shannon index					-0.006* ±0.003			1,35	-48
Evenness ¹			-3.110E-6 ± 1.939E-6		-0.001* ±0.0004			1,34	-141

df_{1,2} is degrees of freedom of the tested effect term and the error term, respectively

¹ models tested with lme rather than lm function

Table 2:

Summary of results from structural equation modelling, showing the decomposition of correlations into standardised direct, indirect and total effects of seven explanatory variables on (a) the difference in Shannon diversity index and (b) the difference in the number of herb species between 2006-2009 and 1990-1993. Total effects are the sum of direct and indirect effects. Direct effect coefficients are equal to the path coefficients shown in Fig. 4a and b.

a)	Difference in Shannon diversity between 2009 and 1993 (H2009-H1993)		
	Direct	Indirect	Total
Ndeposition	-0.302	-0.008	-0.310
SOx deposition	-	-0.037	-0.037
Mineral N soil	-0.022	-0.018	-0.040
Soil pH	0.075	-	0.075
Grazing pressure	0.063	-	0.063
b)	Difference in herb species between 2009 and 1993 (n2009-n1993)		
	Direct	Indirect	Total
Ndeposition	-	-0.014	-0.014
SOx deposition	-	-0.147	-0.147
Mineral N soil	-	-0.072	-0.072
Soil pH	0.298	-	0.298

Table 3:

Effect of N deposition class on the mean change in frequency of individual species (expressed as the difference of frequency between 2006-2009 and 1990-1993), for those species with a change in frequency of more or less that 0.15 (15%) in the highest Ndeposition class. Shown are the correlation coefficients (Cor.) between the change in relative frequency of the species and modelled Ndeposition based on 2006-8 data and the level of significance of the correlation.

Species	N deposition class (kgN ha ⁻¹ y ⁻¹)			Cor.	p
	0-15	15-25	25-35		
<i>Cynosurus cristatus</i>	0.08	0.12	0.53	0.04	0.809
<i>Holcus lanatus</i>	0.14	0.20	0.25	-0.08	0.611
<i>Polygala vulgaris</i>	0.23	0.06	0.17	-0.03	0.812
<i>Linum catharticum</i>	0.27	-0.02	-0.18	-0.34	0.026
<i>Briza media</i>	0.17	0.05	-0.21	-0.37	0.015
<i>Gentianella amarella</i>	-0.03	0.12	-0.24	-0.30	0.050
<i>Campanula rotundifolia</i>	0.05	-0.15	-0.25	-0.61	<0.001
<i>Galium sternerii</i>	0.11	0.04	-0.26	-0.53	<0.001

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3 1 **Figure legends**
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5 2 **Fig. 1**
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8 3 Map of the UK showing the location of the 56 calcareous grassland sites that were surveyed.
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10 4 Closed circles are sites that were surveyed between 1990 and 1993 only, open circles are sites
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12 5 which were re-surveyed between 2006 and 2009. Outline map from Ordnance Survey UK.
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2 **Fig. 2**

3 The relationship between total Ndeposition ($\text{kg ha}^{-1}\text{y}^{-1}$) in 1993-1995 and (a) the total number
4 of vascular plant species in 1990-1993; (b) grass to herb ratio in 1990-1993; and (c) the total
5 number of scarce and rare vascular plant species in 1990-1993.). The equation of the fitted
6 line in Figure 2b is $y = 0.004x + 0.40$ ($r^2=0.03$; $p=0.053$).

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5 2 **Fig. 3**

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7 3 The relationship between the total Ndeposition ($\text{kg ha}^{-1}\text{y}^{-1}$) in 2006-2008 and the decrease
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9 4 between 1990-1993 and 2006-2009 of (a) the plot species diversity (H'); and (b) the plot
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11 5 species evenness. The equation of the fitted line in Figure 3a is $y = -0.008x + 0.19$ ($r^2=0.15$;
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13 6 $p=0.011$). The equation of the fitted line in Figure 3b is $y = -0.002x + 0.03$ ($r^2=0.21$;
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15 7 $p=0.002$).
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5 2 **Fig. 4a**
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7 3 Structural equation model depicting direct and indirect effects of N and S deposition, soil pH,
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9 4 mineral N soil and grazing on the change in Shannon diversity between 2006-2009 and 1990-
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11 5 1993. The width of each arrow is proportional to the standardised path coefficient
12
13 6 (coefficients shown in *italic*). Positive pathcoefficients are indicated by solid lines and
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15 7 negative pathcoefficients are represented by dashed lines. Asterisks indicate effects that are
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17 8 significant at $p < 0.1$. Estimates of the proportion of total variance explained (squared
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19 9 multiple correlations) are shown in **bold** for each dependent variable. Variables and paths
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21 10 representing unmeasured residual variation are not indicated for simplicity.
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5 2 **Fig. 4b**
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7 3 Structural equation model depicting direct and indirect effects of N and S deposition, soil pH,
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9 4 and soil mineral N soil on the change in herb species numbers between 2006-2009 and 1990-
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11 5 1993. The width of each arrow is proportional to the standardised path coefficient
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13 6 (coefficients shown in *italic*). Positive pathcoefficients are indicated by solid lines and
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15 7 negative pathcoefficients are represented by dashed lines. Asterisks indicate effects that are
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17 8 significant at $p < 0.1$. Estimates of the proportion of total variance explained (squared
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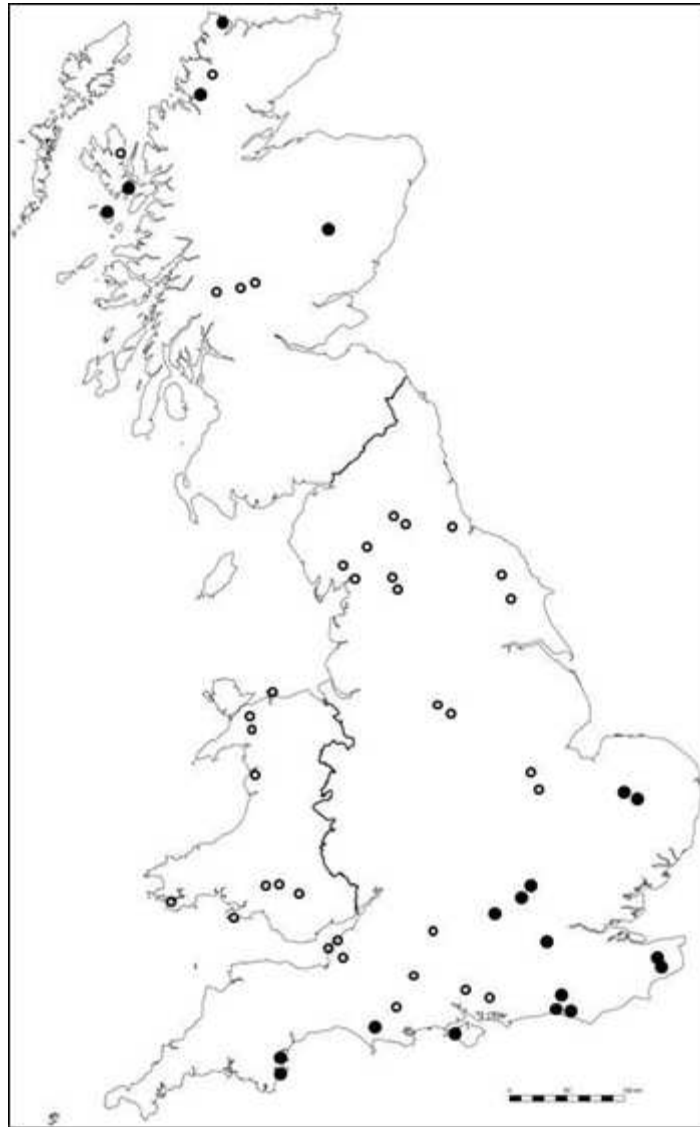
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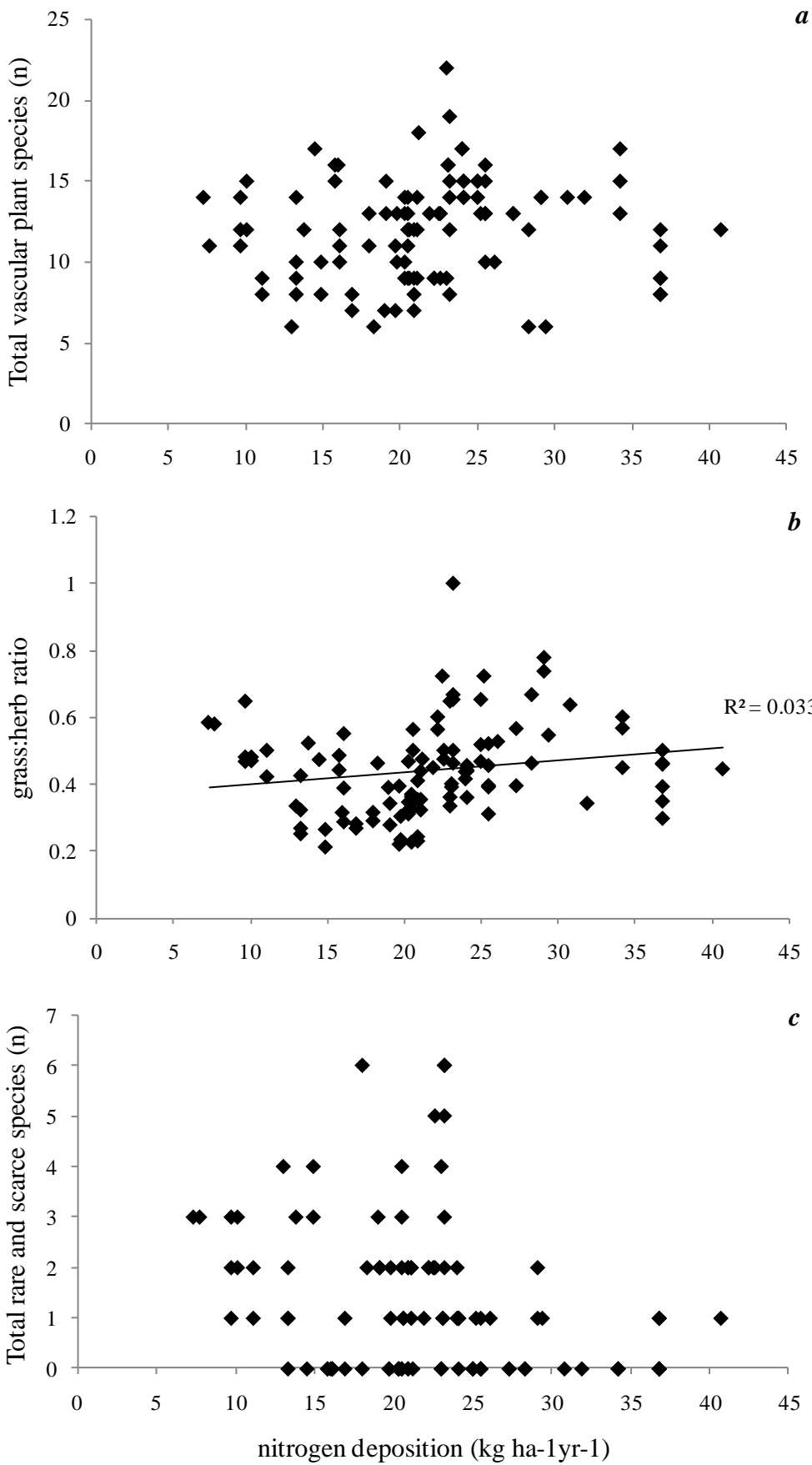
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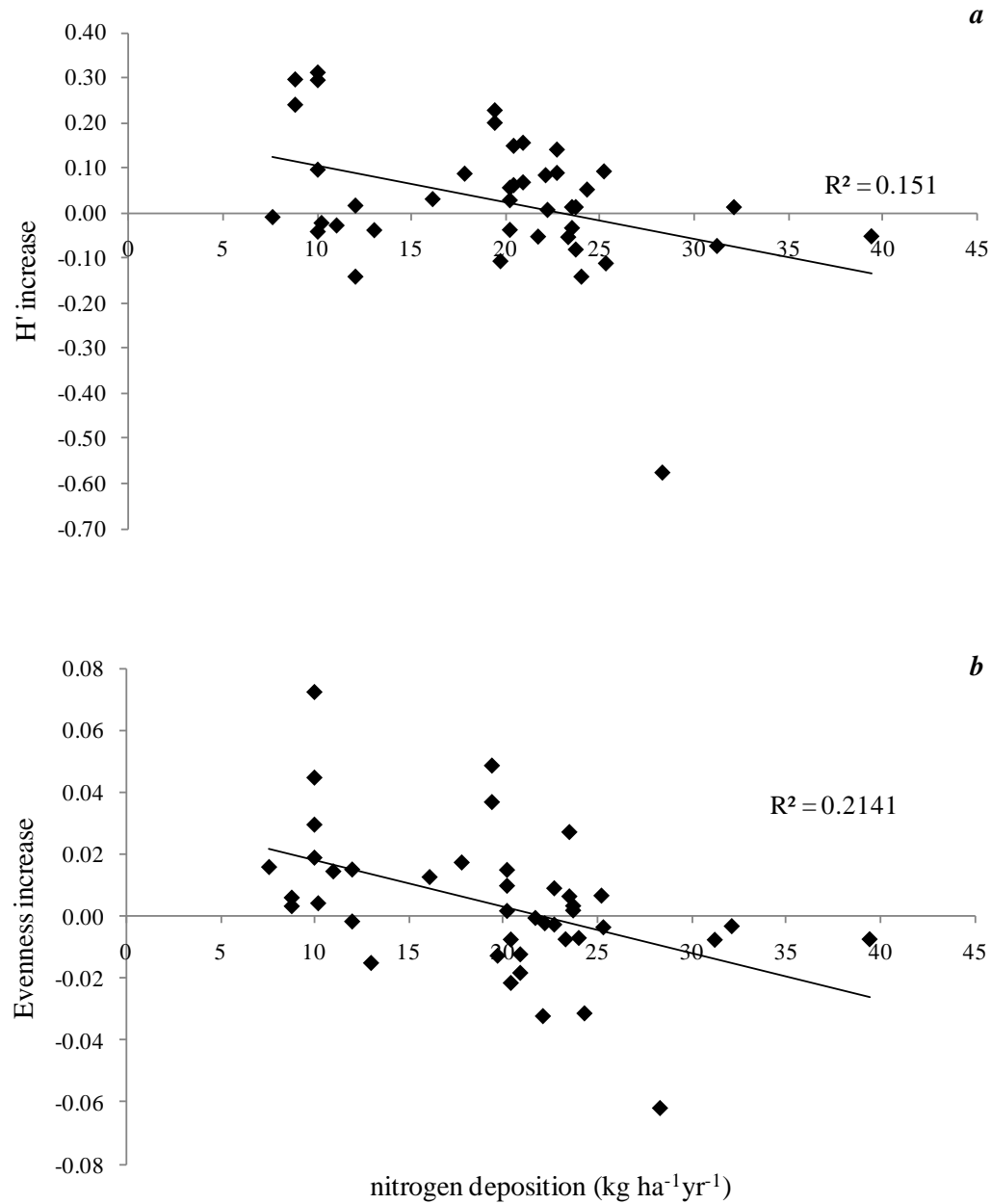
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Figure 2



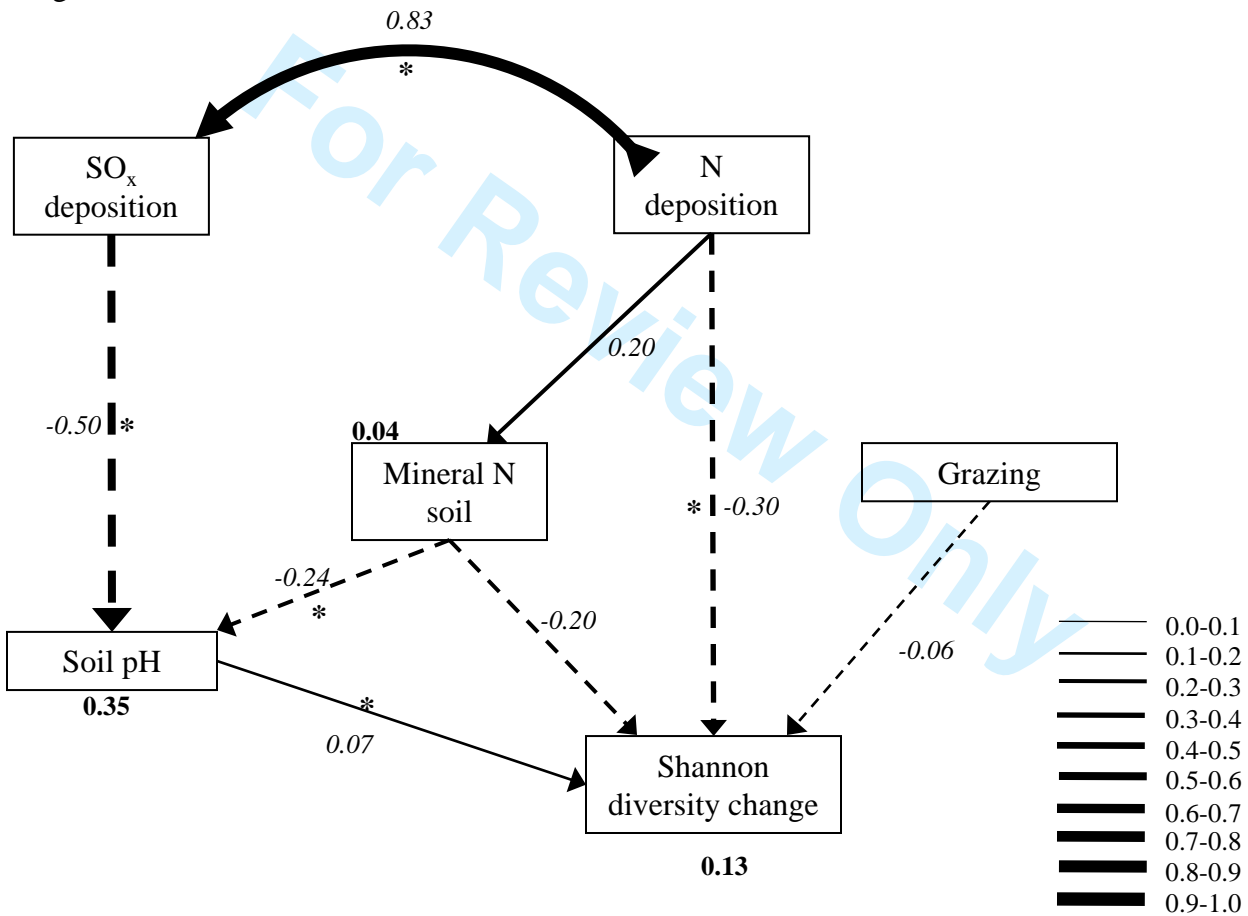
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Figure 3



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Figure 4a



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Figure 4b

