

variance imply that most naturally occurring variation in immunocompetence is derived from mutations of minor effect. However, mutations of larger effect (explaining 10 to 15% of the measured phenotypic variance) can be found segregating in intracellular signaling molecules (Fig. 2B). Second, the polymorphic sites most significantly associated with variance in suppression of *S. marcescens* are found in pathogen recognition and intracellular signaling molecules, with antibacterial peptide genes harboring markedly few polymorphisms that produce individually significant effects. The apparent strength of influence of recognition and signaling molecules may result from the regulatory control these proteins exert over a variety of immune-related cellular processes (1, 2, 15). In particular, because activation of *Drosophila* immune signaling pathways induces the expression of components of those pathways (16, 17), small changes in expression or activity of signaling components may show an amplified effect on the phenotype. In contrast, because antimicrobial peptides are the most downstream targets of immune regulation and are partially redundant (18), these genes might be expected to make weaker contributions to phenotypic variability. Third, although polymorphic markers were typed in both transcribed and regulatory regions of candidate genes (fig. S1), many of the significantly associated sites are located upstream of protein-coding sequences. Because the markers may not themselves be the sites causing phenotypic variability, it is possible that upstream markers are in linkage disequilibrium with untyped amino acid variants in the coding region. However, it is also likely that variation in transcriptional regulation of immunity genes may influence a substantial proportion of observed phenotypic variation. This hypothesis is particularly attractive in light of the epistatic interactions observed between intracellular signaling molecules and antimicrobial peptide genes.

D. melanogaster immune competence and the phenotypic effects of genetic polymorphism were found to vary over the circadian day. Notably, a complex of sites upstream of the *imd* gene showed strong associations with bacterial load sustained by AM-infected flies, but not by PM-infected flies, with one site explaining 16.4% of the AM phenotypic variance; *imd* is an important regulator of the response to Gram-negative bacteria. Circadian cycling of the expression of immune-related genes, including *imd*, has been previously documented in the absence of immune challenge (14). The current results suggest that circadian cycling could have an important genetic influence on the immunocompetence of *D. melanogaster* in the wild. Interactions between genotype and sex or environment in *D. melanogaster* quantitative

traits are not unprecedented, as studies of sensory bristle number, wing shape, and longevity have previously identified multiple quantitative trait loci that are either strongly influenced by or completely dependent on sex and/or environment (19–22).

It is not apparent why phenotypically strong mutations in a vital trait such as immune competence are allowed to persist in the population. Potentially, these may exist in mutation-selection-drift balance, as the result of other physiological trade-offs (23, 24) or as polymorphisms balanced against multiple environmental pathogens. Further study of sequence level and phenotypic variability in innate immune systems is warranted to address this question.

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Materials and Methods

Figs. S1 and S2

References

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Impact of Nitrogen Deposition on the Species Richness of Grasslands

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A transect of 68 acid grasslands across Great Britain, covering the lower range of ambient annual nitrogen deposition in the industrialized world (5 to 35 kg N ha⁻¹ year⁻¹), indicates that long-term, chronic nitrogen deposition has significantly reduced plant species richness. Species richness declines as a linear function of the rate of inorganic nitrogen deposition, with a reduction of one species per 4-m² quadrat for every 2.5 kg N ha⁻¹ year⁻¹ of chronic nitrogen deposition. Species adapted to infertile conditions are systematically reduced at high nitrogen deposition. At the mean chronic nitrogen deposition rate of central Europe (17 kg N ha⁻¹ year⁻¹), there is a 23% species reduction compared with grasslands receiving the lowest levels of nitrogen deposition.

Conservation of biodiversity underpins some of the largest and most ambitious environmental legislation in the world (1). Most of the focus of this legislation is on mitigating

damage to ecological communities from direct environmental degradation such as land clearing. Although chronic “low-level” stresses such as air pollution are also considered to damage biodiversity, their actual importance is not well understood.

Because nitrogen (N) is the limiting nutrient for plant growth in many terrestrial ecosystems (2), atmospheric deposition of reactive N has the potential to reduce plant species richness (the number of species in a given area—an important component of biodiversity) through favoring species bet-

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ter adapted to high nutrient levels (3). Through intensive agriculture and fossil fuel combustion, humans have greatly enhanced the global emission and deposition of fixed N over the past 50 years (4). Nitrophilous species have increased, and N-sensitive vegetation has declined in European peatlands, heathlands, grasslands, and forests since the mid-20th century (3, 5, 6). Similar changes can be induced by experimental addition of high levels of N ($>25 \text{ kg N ha}^{-1} \text{ year}^{-1}$) (7–10). Despite these findings, there has as yet been no clear evidence that enhanced deposition of a nonpoint pollutant over a large region has had any impact on terrestrial biodiversity.

We surveyed a grassland community in Great Britain to determine whether any significant variability in plant species richness could be detected and, if so, whether it was related to regional-scale levels of atmospheric pollution (inorganic N, inorganic S) deposition. The location allowed us to consider as large an area as possible while remaining within one climatic region with well-described vegetation communities. We chose an *Agrostis-Festuca* grassland (11), because this vegetation type is common throughout Europe, Australia, and North America and makes up economically valuable pastureland. Sixty-eight sites were sampled in 2 m by 2 m quadrats (5 replicate quadrats per site) along the gradient of atmospheric N deposition (5 to 35 $\text{kg N ha}^{-1} \text{ year}^{-1}$ wetfall and dryfall) during the summers of 2002 and 2003 (Fig. 1A) (12, 13). Most (a mean of 70%) of the inorganic N deposited is as reduced N (NH_3 , NH_4^+) (Fig. 1B). Plant species presence and abundance were measured in each quadrat, and from these data we calculated site species richness. We also considered the Shannon diversity index (H), which takes into account both species number and relative abundance (13, 14).

For each site, we compiled a data set of the potential drivers on plant species richness, including all of those described as globally important (15): nine chemical environmental factors [deposition of reduced inorganic N (NH_3 , NH_4^+), oxidized inorganic N (NO , NO_2 , NO_3^-), and total inorganic N; deposition of SO_4^{2-} ; acid deposition (total inorganic N + SO_4^{2-}); topsoil (A or O horizon) pH and subsoil (30 to 40 cm) pH; topsoil percentage of N; and topsoil C:N ratio]; nine physical environmental factors (mean annual temperature and precipitation, actual and potential evapotranspiration, soil moisture deficit, litter cover, altitude, slope, and aspect); and two human modifications (grazing intensity and enclosures) (table S1). These variables were entered into a stepwise multiple regression with site species richness as the dependent variable.

Species richness showed large variability, ranging from a mean of 7.2 to 27.6 species per quadrat. Species richness was highest in four sites in western Scotland, then showed high variability from west to east along an overall negative trend (fig. S1A). There was a clearer latitudinal gradient, with some sites of high species richness in the extreme south, and a gradual increase in species richness from south to north (fig. S1B). N deposition showed the opposite trends (fig. S1, C and D).

Of 20 variables measured to account for the variability in species richness, total deposition of inorganic N (N_{dep} , $\text{kg N ha}^{-1} \text{ yr}^{-1}$) was the most important predictor, explaining more than half of the variation in the number of species per quadrat (Fig. 2A and Eq. 1). The trend was linear and negative, indicating that for every 2.5 $\text{kg N ha}^{-1} \text{ year}^{-1}$ of inorganic N deposited on an acid grassland, a mean of one additional species is excluded from a randomly placed 4-m² quadrat:

$$\text{Plant Species Richness} =$$

$$23.3 - 0.408(N_{dep})$$

$$(r^2 = 0.55, P < 0.0001)$$

(1)

After accounting for N deposition, mean annual precipitation (MAP, mm) explained an additional 8% of variability in species richness. A further 5% was explained by the A horizon soil pH ($ToppH$, Fig. 2B) and 3% by altitude (Alt , m). In total, 70% of the variability in species richness could be explained by these four variables:

$$\text{Plant Species Richness} = 2.25 -$$

$$0.165(N_{dep}) + 0.00286(MAP) +$$

$$3.23(ToppH) - 0.00703(Alt)$$

$$(r^2 = 0.70, P < 0.02)$$

(2)

The secondary variables—precipitation (+), pH (+), and altitude (—)—have all been identified in previous studies as potential influences on vegetation biodiversity (15, 16). A closer examination of the effect of these variables revealed that the addition of mean annual precipitation and altitude is due to a group of seven grasslands in western and central Scotland, with a mean annual precipitation exceeding 2700 mm. Removing these sites from the database and re-running the stepwise regression with the 20 predictor variables eliminated both MAP and altitude as significant, leaving N deposition ($r^2 = 0.44$, $P < 0.0001$) together with pH as the most significant model ($r^2 = 0.50$, $N = 61$, $P < 0.02$). No other variable was significant in this regression.

Topsoil pH, which ranges from 3.7 to 5.5, shows a fairly uniform distribution across both longitude and latitude and across the gradient of N deposition (Fig.

2B). Soil pH can be considered to modify the effect of N deposition: At any level of N deposition, sites with a higher soil pH are predicted (based on this analysis) to show higher species richness. Soil pH is in part due to the local site characteristics (parent material, organic matter content, management history) and in part to the long-term rate of acid deposition (16). If soil pH is "forced" as the first variable in the regression, N deposition is still highly significant ($P < 0.007$).

All of the other independent variables in our study were either not significantly correlated to species richness or were not significant after accounting for the effect of N deposition (table S1). Temperature and evapotranspiration have been shown in other studies to positively correlate with global or regional species richness (15). Our study found the opposite effect, with mid- and lower-latitude sites generally showing lower species richness than higher latitude sites (fig. S1B). This indicates that temperature is not responsible for the observed variability in species richness.

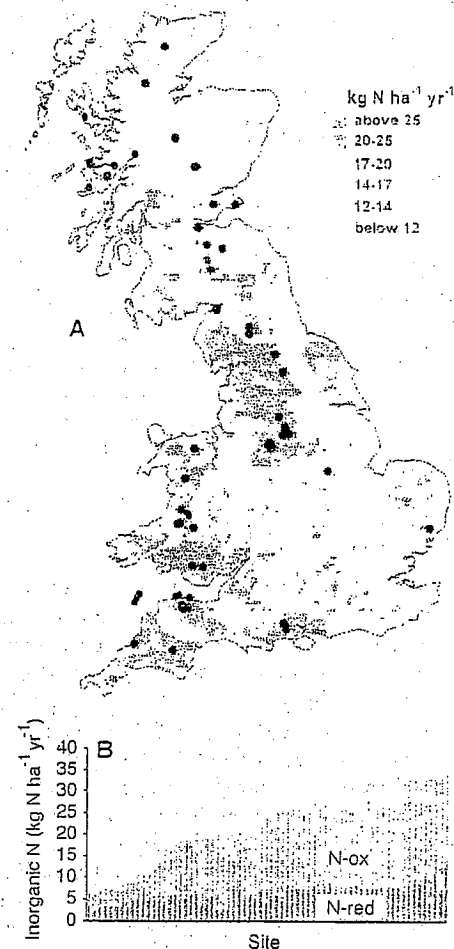


Fig. 1. (A) Total inorganic N deposition ($\text{kg N ha}^{-1} \text{ year}^{-1}$) to the United Kingdom (72), with field site locations shown. (B) The deposition of reduced (NH_3 , NH_4^+) and oxidized (NO , NO_2 , NO_3^-) N across the sites.

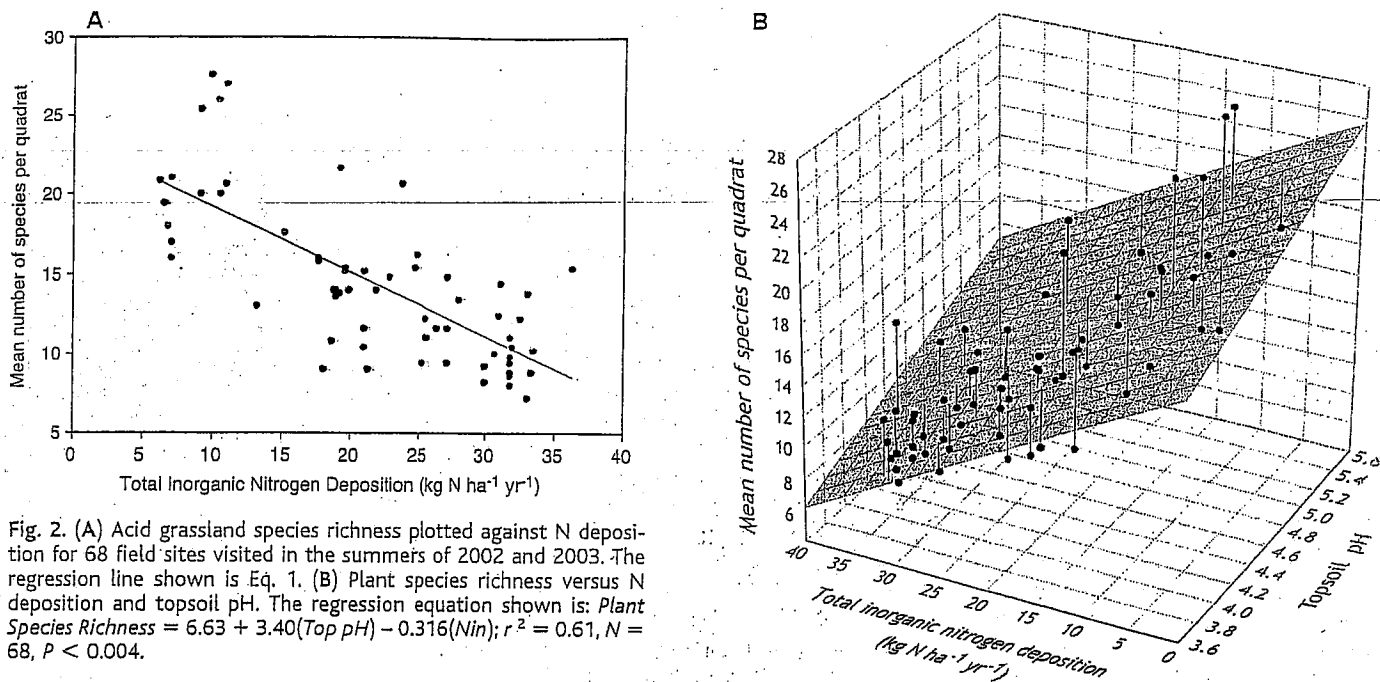


Fig. 2. (A) Acid grassland species richness plotted against N deposition for 68 field sites visited in the summers of 2002 and 2003. The regression line shown is Eq. 1. (B) Plant species richness versus N deposition and topsoil pH. The regression equation shown is: $Plant\ Species\ Richness = 6.63 + 3.40(Top\ pH) - 0.316(Nin)$; $r^2 = 0.61$, $N = 68$, $P < 0.004$.

Somewhat surprisingly, there was no relationship between species richness and topsoil percentage of N or C:N, and there was no relationship between these variables and the rate of N deposition. This may be due to the wide variability in organic matter content of these soils, which ranged from organic-poor mineral soils to peat (%C = 2.3 to 40%). There was no relationship between species richness and percentage of litter cover, aspect, slope, presence/absence of enclosures, or level of grazing. SO_4^{2-} deposition was weakly correlated to species richness, but again was not significant if N deposition was accounted for. Following this, acid deposition ($SO_4^{2-} + N$) was a poorer predictor of species richness than N deposition alone.

The statistical analyses together with examination of individual trends show that, of the variables considered, the observed variability in species richness is best explained by N deposition. We next determined whether certain species are systematically missing from the low species-richness plots, and if so, if these species share any common characteristics. We determined, using canonical correspondence analysis (13), and confirmed by reviewing the relevant data, that there is in fact a consistent loss of certain species: the forbs *Plantago lanceolata* (ribwort plantain), *Campanula rotundifolia* (harebell), and *Euphrasia officinalis* (eye-bright); the grass *Molinia caerulea* (purple moor grass); the shrub *Calluna vulgaris* (heather); and the moss *Hylocomium splendens* (mountain fern moss). These six species constitute 21% of the maximum species number we observed. The only plant found to substantially increase in occurrence and cover at higher levels of N is *Hypnum*

cupressiforme, a moss that can survive relatively high levels of air pollution (17).

Campanula, *Euphrasia*, and *Calluna* are indicators of infertile sites (18), and *Campanula* is recognized as intolerant of competition with vigorous grasses (19). *Plantago* (7), *Calluna* (20), *Molinia* (20, 21) and *Hylocomium* (22) have all been shown to decline under experimental N applications. The identity of these species suggests that the decline in species richness is probably due to N-demanding species growing at the expense of less competitive species (23).

Equation 1 gives the mean species richness of an acid grassland, affected by the lowest levels of N we measured ($5\text{ kg N ha}^{-1}\text{ year}^{-1}$) to be 21.3 species per quadrat. Using this as a conservative estimate of the species richness of a "pristine" grassland, and substituting into Eq. 1 the approximate mean N deposition in the eastern United States [$7.7\text{ kg N ha}^{-1}\text{ year}^{-1}$ (24)] and the United Kingdom or central Europe [$17\text{ kg N ha}^{-1}\text{ year}^{-1}$ (25)], gives a current average reduction of 5.2% and 23% of acid grassland species richness, respectively. The currently accepted critical load for N deposition to grasslands in Europe [$25\text{ kg N ha}^{-1}\text{ year}^{-1}$ (26)] gives a reduction of 38.4%.

Experimental addition of nitrogen to grasslands in Great Britain (7) and North America (10) has resulted in a loss of 3 to 4% of species richness for each 100 kg N ha^{-1} added over the course of the experiments (27). If we make the conservative assumption that N deposition has a fully cumulative effect on vegetation [i.e., no N is lost because of seepage, denitrification, etc. (28)], and taking a value of 3.5% species richness loss for

each cumulative 100 kg N ha^{-1} deposited, the total amount of N required to decrease species richness by 25% is 714 kg N ha^{-1} . At the mean nitrogen deposition of central Europe, the time to reach 714 kg N ha^{-1} is 42 years at a constant level of deposition, and longer if N deposition has increased from lower levels in the past or if some N is not taken up by vegetation. This time frame is consistent with the enhanced emission of reactive N that began around the start of the 20th century and accelerated in its latter half (29). Although N deposition is beginning to decline in many areas of Europe (29, 30), the potential importance of cumulative N deposition and the alteration of soil pH (which may take many decades to recover) may mean that a return to an "unpolluted" condition of high species richness may not be achieved in the foreseeable future.

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Supporting Online Material

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Materials and Methods

Fig. S1

Table S1

References

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Comparative Losses of British Butterflies, Birds, and Plants and the Global Extinction Crisis

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There is growing concern about increased population, regional, and global extinctions of species. A key question is whether extinction rates for one group of organisms are representative of other taxa. We present a comparison at the national scale of population and regional extinctions of birds, butterflies, and vascular plants from Britain in recent decades. Butterflies experienced the greatest net losses, disappearing on average from 13% of their previously occupied 10-kilometer squares. If insects elsewhere in the world are similarly sensitive, the known global extinction rates of vertebrate and plant species have an unrecorded parallel among the invertebrates, strengthening the hypothesis that the natural world is experiencing the sixth major extinction event in its history.

Large-scale attempts to quantify recent losses of biodiversity are hindered by inconsistencies in the quality of data available for different taxa (1–3). For example, reported rates of global and national extinction in insect species are typically two orders of magnitude lower than those

recorded for birds, large mammals, and certain fish, plant, and snail groups (1, 2). This difference might be merely an artifact of undersampling of the known insect species, exacerbated by the probability that a disproportionate number of the most acutely threatened insects belong to the majority (estimated at 90% globally) of species that have yet to be described (2, 4–6). Models that account for sampling effort do indeed generate more even extinction rates across taxa (6), although others conclude that the available data are inadequate for any comparisons to be made (7). Whatever the validity of these predictions, the assumption that mammals and birds serve as indicator groups for wider species losses remains untested (1, 2, 6, 8, 9). Furthermore, the problem of underrecording of invertebrates is amplified by recent recommendations that biologists focus on population extinctions, albeit at less-than-global scales, as more sensitive measures of decline than species extinctions (8–10).

The repetition of comprehensive surveys of plants, birds, and butterflies over the past 20 to 40 years in Britain generated six very large data sets that allow accurate comparisons (11) of the fate of these three groups at a large (228,073 km²) spatial scale. With colleagues, we organized two surveys of the distributions in Britain of all 1254 native species of vascular plant in 1954 to 1960 (12) and 1987 to 1999 (13); of all 201 native breeding bird species in 1968 to 1972 (14) and 1988 to 1991 (15); and of all 58 native breeding butterfly species in 1970 to 1982 (16) and 1995 to 1999 (17). Each survey achieved 98 to 100% cover of the 2861 10-km (10 km by 10 km) grid squares of England, Wales, and Scotland; in total, >20,000 volunteer recorders submitted >15 million records of species during the six surveys (11), providing the most comprehensive data sets in the world of changing status for each taxon (2, 18).

For every species, change in status was measured as the difference in the total number of 10-km grid squares occupied in each census period [the second butterfly data set being subsampled to equalize recorder effort (11)]. We then ranked species by the percentage change (from the first survey) in their occupancy of squares (Fig. 1). Although this coarse-grained sampling may underemphasize more local changes in status (19), we have previously shown that range changes expressed at this scale are closely correlated with trends in the mean size of individual populations of butterfly (20) and bird (21) species across Britain. Range changes (Fig. 1) are thus a surrogate for abundance, making each survey effectively a population census.

We found (Fig. 1A) that 28% of native plant species have decreased in Britain over the past 40 years, that 54% of native bird species have decreased over 20 years, and

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