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Ecosystem function enhanced by combining four functional types of plant species in intensively managed grassland mixtures: a 3-year continental-scale field experiment

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Summary

1. A coordinated continental-scale field experiment across 31 sites was used to compare the biomass yield of monocultures and four species mixtures associated with intensively managed agricultural grassland systems. To increase complementarity in resource use, each of the four species in the experimental design represented a distinct functional type derived from two levels of each of two functional traits, nitrogen acquisition (N_2-fixing legume or nonfixing grass) crossed with temporal development (fast-establishing or temporally persistent). Relative abundances of the four functional types in mixtures were systematically varied at sowing to vary the evenness of the same four species in mixture communities at each site and sown at two levels of seed density.

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2. Across multiple years, the total yield (including weed biomass) of the mixtures exceeded that of the average monoculture in >97% of comparisons. It also exceeded that of the best monoculture (transgressive overyielding) in about 60% of sites, with a mean yield ratio of mixture to best-performing monoculture of 1·07 across all sites. Analyses based on yield of sown species only (excluding weed biomass) demonstrated considerably greater transgressive overyielding (significant at about 70% of sites, ratio of mixture to best-performing monoculture = 1·18).

3. Mixtures maintained a resistance to weed invasion over at least 3 years. In mixtures, median values indicate <4% of weed biomass in total yield, whereas the median percentage of weeds in monocultures increased from 15% in year 1 to 32% in year 3.

4. Within each year, there was a highly significant relationship ($P < 0{\cdot}0001$) between sward evenness and the diversity effect (excess of mixture performance over that predicted from the monoculture performances of component species). At lower evenness values, increases in community evenness resulted in an increased diversity effect, but the diversity effect was not significantly different from the maximum diversity effect across a wide range of higher evenness values. The latter indicates the robustness of the diversity effect to changes in species' relative abundances.

5. Across sites with three complete years of data (24 of the 31 sites), the effect of interactions between the fast-establishing and temporal persistent trait levels of temporal development was highly significant and comparable in magnitude to effects of interactions between N_2-fixing and nonfixing trait levels of nitrogen acquisition.

6. *Synthesis and applications*. The design of grassland mixtures is relevant to farm-level strategies to achieve sustainable intensification. Experimental evidence indicated significant yield benefits of four species agronomic mixtures which yielded more than the highest-yielding monoculture at most sites. The results are relevant for agricultural practice and show how grassland mixtures can be designed to improve resource complementarity, increase yields and reduce weed invasion. The yield benefits were robust to considerable changes in the relative proportions of the four species, which is extremely useful for practical management of grassland swards.

Key-words: agronomic mixtures, diversity effect, ecosystem function, forage yield, functional groups, monocultures, resource efficiency, sustainable intensification, traits, transgressive overyielding

Introduction

Empirical evidence from semi-natural or extensively managed grasslands generally shows that reductions in plant diversity reduce the yield of above-ground biomass (e.g. Cardinale *et al.* 2007). These benefits have been attributed to improved utilization of resources in total niche space (niche differentiation), positive interspecific interactions and selection effects (Hooper *et al.* 2005). The ability of more diverse plant communities to acquire and convert available resources to above-ground biomass (and provide other ecosystem processes) has considerable relevance to agricultural systems, although the results from extensively managed (low-nutrient) semi-natural grasslands do not necessarily extrapolate to intensively managed grasslands. Thus, we address the question: how can we improve the design of multispecies agricultural mixtures to improve yield efficiency and the sustainability of resource utilization in agriculture?

Intensively managed agricultural grasslands are globally prominent ecosystems in which large amounts of energy and nutrients flow, and associated management options have the potential to supply (or degrade) a range of ecosystem services. They cover large areas and agronomic advice regularly advocates monocultures of high-yielding grass species to maximize yield, especially with the application of high levels of nitrogen fertilizer. One potential strategy to improve agricultural sustainability includes the use of multispecies plant mixtures that can exploit complementarity and interspecific interactions within more intensively managed grassland systems. Although there has been work on agronomic mixtures and intercropping (Trenbath 1974; Vandermeer 1989; Federer 1999; Gibson *et al.* 1999; Connolly, Goma & Rahim 2001; Sanderson 2010), the paucity of multispecies agronomic experiments with >2 species that have been conducted across multiple sites, years and different relative abundances of component species in mixtures means that general predictions about the benefits of multispecies mixtures remain largely untested (Connolly, Goma & Rahim 2001; Sanderson *et al.* 2004; Tracy & Faulkner 2006). Investigations of the role of multispecies mixtures have critical

importance for the sustainability of agricultural systems, which strive to maintain or increase food production from fewer inputs (Tilman *et al.* 2002; Foley *et al.* 2011). To be relevant to agricultural practice as part of sustainable systems, however, the benefits of agronomic multispecies mixtures would need to occur quickly, occur under relatively intensive management conditions, persist across a wide variety of pedo-climatic conditions and outperform (or at least be as good as) the best-performing monoculture (or the prevailing conventional system). In perennial systems, the mixtures would need to demonstrate sufficient temporal stability for the benefits to persist for several years, and it would be a significant practical advantage for the benefits to be robust to changes in relative abundance of the component species (Kirwan *et al.* 2007; Nyfeler *et al.* 2009).

Given the aim of agricultural grassland systems to produce forage biomass as efficiently as possible, the greater spatial and temporal complementarity offered by more species-rich communities may provide valuable tools to increase resource use efficiency. Informed by such principles, the choice of species for use in multispecies mixtures can be strategically designed to include traits that maximize complementarity and interspecific interactions to improve resource utilization and yield of above-ground biomass. In contrast to semi-natural grasslands of high biodiversity value, this is particularly relevant for intensively managed agricultural systems that are characterized by their use of highly modified and synthesized plant communities, and in which biomass production of high forage quality is often the single most important ecosystem function used to measure their value.

This experimental investigation of mixtures for intensively managed grasslands focused on two agronomically important functional traits, nitrogen acquisition and temporal development, to inform the design of productive grassland mixtures that would increase effects due to complementarity. In agronomic grasslands, grasses have an excellent ability to acquire and convert available resources (including nitrogen transferred from legumes) into high-quality forage (Sanderson *et al.* 2004; Nyfeler *et al.* 2011). Legumes also provide high-quality forage and, due to their ability to fix atmospheric nitrogen, can contribute significantly to synergistic interactions through interspecific transfer of fixed nitrogen (Boller & Nösberger 1987; Ledgard & Steele 1992; Zanetti *et al.* 1997; Carlsson & Huss-Danell 2003; Nyfeler *et al.* 2011). Patterns of temporal establishment and growth over years and/or within a growing season are another important agronomic trait that would be expected to improve the establishment of mixtures (Teasdale 1998; Tracy & Sanderson 2004) and increase complementarity (Daepp, Nosberger & Lüscher 2001; Lüscher, Fuhrer & Newton 2005).

Here, we build on a previous analysis of the first year of data from this experiment (Kirwan *et al.* 2007) and report the results from 3 years of harvesting of a field experiment across multiple geographical sites. This 3-year experiment allowed us to assess the following: the spatial and temporal persistence of any mixture benefits; whether yield benefits were produced by the combination of species with different traits for temporal development and nitrogen acquisition, and; whether the mixture benefits are robust to changes in the relative abundance of species at sowing. We address the following main objectives:

1. Were there yield benefits (overyielding) from mixtures and, if so, did the benefits persist over 3 years and across sites?

2. Were the yield benefits sufficiently large for transgressive overyielding to occur?

3. Did the benefits of diversity occur and persist across the range of mixture communities used in this experiment?

4. Did both the functional traits of nitrogen acquisition and temporal development contribute to the diversity effect (the excess of mixture performance over that of the monoculture performances of component species)?

Averaged across years, we show that diversity effects persistently occurred across different communities, sites and multiple years. The magnitude of the diversity effects were such that overyielding almost always occurred and significant transgressive overyielding occurred in about 60% of sites for total yield (and 70% of sites for yield of sown species). Mixtures were much more resistant to weed invasion than monocultures. Yield benefits were attributed to strong complementarity, due to the *a priori* design of the mixtures to include a combination of traits related both to nitrogen acquisition and to temporal development.

Materials and methods

EXPERIMENTAL DESIGN

At each of 31 sites (30 European and one Canadian), fifteen grassland communities comprised of four monocultures and 11 mixtures of four functional types of species (each represented by one species) were sown at two seed density levels (see Table S1, Supporting Information for details of each site). To investigate the sensitivity of yield responses to different relative proportions of the functional types, the sowing rate of the same four species was systematically varied according to a simplex design to produce the 11 mixture communities (see Kirwan *et al.* 2007; and Table S2, Supporting Information): four mixtures dominated in turn by each species (sown proportions of 70% of one species and 10% of each of the other three species), six mixtures dominated in turn by pairs of species (40% of each of two species and 10% of each of the other two) and the community with equal proportions (25% of each species). Relative proportions of species in a mixture were manipulated by varying seeding rates of the four species at sowing and resulted in four planned levels of evenness in the design (Table S2, Supporting Information). This design resulted in 30 experimental plots per site (arranged in a completely randomized design) and a total of 930 plots across sites. Special emphasis was given to: first, selection from a small pool of species that were known to produce high-quality forage

in intensive agricultural systems that were typical of the local site, and; second, a planned selection of species to represent four distinct functional types to maximize complementarity in resource use. The four functional types were defined as the factorial combination of the following two levels of each of two functional traits (i) nitrogen acquisition [N_2-fixing (N) associated with legumes, zero (Z) level of N_2-fixing associated with nonfixing grasses] and (ii) pattern of temporal development [fast-establishing (F) and temporally persistent (P)]. Thus, the four functional types of species at each site consisted of a fast-establishing nonfixing grass (ZF), a fast-establishing N_2-fixing legume (NF), a temporally persistent nonfixing grass (ZP) and a temporally persistent N_2-fixing legume (NP) (see Table S1, Supporting Information for details). Although these functional types were consistent across all sites, the chosen species varied across sites, and a total of 11 species were used across all 31 sites (Table S1, Supporting Information). Plots were harvested and subsamples of the harvested vegetation sorted into above-ground biomass of the different sown species and weeds. The results are reported for durations of 3 years for 24 sites, 2 years for six sites and 1 year only for one site.

Here, we report total yield (includes weed biomass), which is a measure of the total forage that is available for feed or ensiling. We summarize weed invasion as the proportion of total yield composed of weed biomass. We also report the yield of sown species, which reflects the relative ability of the monocultures and mixtures to convert available resources into biomass of the sown species only.

DATA ANALYSIS

Objectives 1 and 2: persistence of yield benefits across years and sites

We compared the yields of the 11 mixture communities against the average monoculture yield (to test for overyielding, objective 1) and against the monoculture that performed best across all years (to test for transgressive overyielding, objective 2) using a permutation test (Kirwan *et al.* 2007). For each site, these comparisons were conducted for each separate year as well as for the average yields over the duration of the experiment. Statistical testing of transgressive overyielding is complicated by a number of biases (Kirwan *et al.* 2007; Schmid *et al.* 2008) that result in our test method being conservative. The size of these biases partly relates to the variation among monocultures, the bias from this source being smaller for a smaller range of this variation (see Appendix S1, Supporting Information). For the above analyses, data for annual yield at each site were averaged across the two levels of seed density for each community and (for relevant analyses) also averaged across years.

Objective 3: persistence of yield benefits across mixture communities

For both objectives 3 and 4, the contributions of the different functional types to the diversity effect were quantified using a regression-based approach that relates ecosystem function to species identity and interaction effects (diversity-interaction modelling, Kirwan *et al.* 2009). The identity effect represents the ecosystem function of the monoculture of a species. The interaction effect for a pair of species represents the net effects of their interspecific interactions on the ecosystem function of interest, which may be positive, negative or neutral for a specific pair of species (O'Hea, Kirwan & Finn 2010). The net effect of all of the separate interaction effects is the diversity effect (the difference between the actual performance of a mixture and its expected performance from proportional contribution of the constituent monocultures). Thus, the performance of a mixture with differing proportions of species can be estimated from the appropriate proportional combination (using species' relative abundances) of the identity effects and interaction effects. Inspection of individual pairwise interactions allows one to assess the contribution to the diversity effect of interactions among functional trait levels (Kirwan *et al.* 2009; Nyfeler *et al.* 2009). This flexible modelling system facilitates identification of patterns in species interaction effects related to properties such as community evenness and species' functional traits (see Appendix S2, Supporting Information for details).

In the analysis of year 1 data in Kirwan *et al.* (2007), a quadratic form of sward evenness (which assumes that each of the separate pairwise interaction terms have equal values) best represented the diversity–function relationship. This model has the practical community-level property of directly relating sward evenness to the diversity effect and can reflect the robustness of the relationship (Kirwan *et al.* 2007, 2009). Here, we investigated whether this relationship persisted over time as a test of the robustness of the diversity effect to changes in the relative abundance of the component species (objectives 4 and 1 above). Because the evenness model is applied to data from multiple sites, it also tests objective 2. In this model, the diversity effect = $\delta E + \delta_1 E^2$, where E = evenness of the mixture. We define the evenness (E) of the community as

$$E = (2s/(s-1))\sum_{i<j}^{s} P_i P_j,$$

where there are up to s species in a community and P_i is the relative abundance of the ith species (Kirwan *et al.* 2007). Our design resulted in four levels of evenness (E) at sowing, with $E = 0$ for monocultures and $E = 0{\cdot}64$, $0{\cdot}88$ and 1 for different mixtures (Table S2, Supporting Information). The full model also included species identity effects, seed density and year as a factor and was specified as a random coefficients model (see Appendix S2, Supporting Information for details). As an estimate of realized proportions, yield data in year 1 were regressed on sown proportions, whereas yield data in years 2 and 3 were regressed on proportions of species in annual biomass in years 1 and 2, respectively (further details in Appendix S2, Supporting Information). An implication of this model is that all pairwise interactions among the functional types are estimated as equal. For completeness, a series of models were fitted to test whether biologically meaningful patterns were evident in the interaction effects. The models included linear evenness, quadratic evenness, separate pairwise interactions and three-species interactions. The goodness-of-fit of the models was calculated, and the model based on quadratic evenness was the best fitting model (Appendix S2, Tables S3 and S4, Supporting Information).

Objective 4: contribution of the functional trait levels to the diversity effect

The contributions of the different functional types to the diversity effect were modelled using the initial sown proportions as

regression predictors for the analysis, with the mean yield of a plot over 3 years being the response. These data are most appropriate for this analysis, as we expected the interactions among slow-establishing and temporally persistent trait levels to operate over a multiyear time-scale. There were 720 yield values in the analysis (30 plots at each of 24 sites). Plot yields were not averaged over seed density in these analyses. The basic model of yield (y) was:

$$
\begin{aligned}
y = & \sum_{i=1}^{9} \beta_i P_i && \text{identity effects} \\
& + \delta_{\mathrm{ZF.ZP}} P_{\mathrm{ZF}}.P_{\mathrm{ZP}} + \delta_{\mathrm{NF.NP}} P_{\mathrm{NF}}.P_{\mathrm{NP}} && \text{fast} * \text{persistent effects (FP)} \\
& + \delta_{\mathrm{NF.ZF}} P_{\mathrm{NF}}.P_{\mathrm{ZF}} + \delta_{\mathrm{NP.ZP}} P_{\mathrm{NP}}.P_{\mathrm{ZP}} && \mathrm{N_2}\text{-fixing} * \text{nonfixing effects (NZ)} \\
& + \delta_{\mathrm{ZF.NP}} P_{\mathrm{ZF}}.P_{\mathrm{NP}} + \delta_{\mathrm{ZP.NF}} P_{\mathrm{ZP}}.P_{\mathrm{NF}} && \text{FP} * \text{NZ effects} \\
& + \alpha M + \varepsilon && \text{seed density}
\end{aligned}
$$

The identity effect (β_i) of each species is the model estimate of the yield of the species in monoculture. Here, identity effects are added to the model through the inclusion of the species' proportions in a community, P_i for the nine species. Two of the 11 species used across all sites did not occur in sites with 3 years of harvest data. The interaction between two species (i and j) enters the model by including the products of their proportions (P_iP_j). At any particular site, the value P_i represents the proportions of the functional types (NF, NP, ZF and ZP). To test the general effect of the functional traits on yield, we include pairwise interactions among the four functional types in the model rather than species' pairwise interactions. For example, $\delta_{\mathrm{ZF.ZP}}$, the coefficient of the product of the proportions of ZF and ZP ($P_{\mathrm{ZF}}.P_{\mathrm{ZP}}$), estimates the interaction effect of a fast-establishing nonfixing grass with a slow-establishing nonfixing grass, even though these functional types could be represented by different species across different sites. The diversity effect of a mixture community is the sum of the six interaction terms. M is the level of seed density and is coded -1 for low density and +1 for high density. The model was fitted to the data using a random coefficients approach (Kirwan *et al.* 2007), in which we estimate the general or population relationship, while allowing for variation about this relationship across sites (details in Appendix S2, Supporting Information). All analyses were conducted with SAS (9.1; SAS Institute Inc., Cary, NC, USA).

Results

Sites differed considerably in their productivity, and annual averages of total yield (dry matter) per site ranged from about 18 t ha^{-1} year^{-1} to about 3 t ha^{-1} year^{-1} (Fig. 1a). Across all sites, total yield (averaged across years and seed density) of mixtures exceeded that of the mean monoculture in 98% of mixture communities with an average (across all mixtures and sites) ratio of mixture/monoculture yield of 1·32 (Fig. 1a, Table 1, see also Fig. S1, Supporting Information). Transgressive overyielding occurred in 65% of mixture communities and was significant at 58% of sites, with an average yield ratio of mixture/monoculture of 1·07 (Fig. 1, Table 1a). At nine sites, all of the mixture communities yielded more than the best monoculture community, although all mixture communities yielded less than the best monoculture at four sites. At the 24 sites with 3 years of data, transgressive overyielding occurred in 65% of mixtures and was significant at 15 (63%) of the sites, with a mean ratio of mixture/monoculture of 1·07 (Table 1). The yield benefit of mixtures was already evident in year 1 and persisted for the 3 years of the experiment (Table 1a).

In general, there was an initially high occurrence of fast-establishing species that changed to dominance by the temporally persistent species by year 3 (Fig. 2a). There was successful establishment of the initial sown proportions of legume species (Fig. 2b) that then decreased over time. Mixtures with sown evenness values of 0·64, 0·88 and 1·0 had mean evenness values of the sown species in year 3 of 0·62 (±0·190), 0·72 (±0·218) and 0·79 (±0·245), respectively.

Across all sites, monocultures displayed much higher levels (and variability) of weed invasion than mixtures (Fig. 3). The median percentage of weed biomass in the total yield of monocultures increased over time (15% in year 1, 20% in year 2 and 32% in year 3); in contrast, the median percentage of weed biomass in the mixtures remained consistently low (4% in year 1, 3% in year 2 and 3% in year 3).

Looking at comparisons based on yield of the sown species (Table 1b), there was a more pronounced incidence of overyielding and transgressive overyielding than found for total yield. Across years, the values for the ratio of yield benefit were considerably higher than those based on total yield (on average *c.* 1·18 across all sites). All mixture communities yielded more than the best monoculture community at 16 sites. Because of the higher biomass of weeds in the monoculture plots (Fig. 3), there is a larger reduction in monoculture yields than in mixture yields when weed biomass is removed in the analyses of yield of sown species (cf. Fig. 1a,b).

The relationship between evenness and the diversity effect that was evident in year 1 persisted in years 2 and 3 (Fig. 4, Table S4, Supporting Information). The diversity effect declined in year 3 (at least partially reflecting a general decline in yield across sites over time (e.g. Table S4, Supporting Information) but was highly significantly related to evenness within each year. Within each year, increased evenness resulted in a greater diversity effect at lower evenness values, but the diversity effect did not differ significantly across a wide range of higher evenness values (Fig. 4). The range of evenness across which the diversity effect was not significantly different from the maximum diversity effect was as follows: year 1, E = 0·67 –1; year 2, E = 0·48–0·94; year 3, E = 0·32–1. Overall, these results indicate the persistence of the diversity effect across communities with different relative abundances within a year, across years, across species and across sites. In addition, the results were qualitatively similar for an analysis based on yield of sown species (see Tables S5 and S6 and Fig. S2, Supporting Information).

For the 24 sites with 3 years of data, there were significant effects of species' identity and pairwise interspecific interactions on total yield averaged across years (Table 2). The combinations of the fast-establishing and temporally persistent levels of the functional trait for temporal development were significant regardless of whether they were

among N_2-fixing legumes (NF*NP), nonfixing grasses (ZF*ZP) or among legumes and grasses (NP*ZF, NF*ZP). The interaction effects were all positive and did not differ in magnitude ($P > 0{\cdot}1$). The estimated diversity effect for the mixture community with equal proportions was 2·77 t ha^{-1} year^{-1}. It is no surprise that the N_2-fixing*nonfixing interactions were significant, but these data on the fast-establishing*temporally persistent interactions support the original hypothesis that both types of functional traits would generate positive interspecific interactions that contribute to yield benefits. Although more pronounced in magnitude, qualitatively similar results were found for the analysis of yield of sown species (Table S7, Supporting Information).

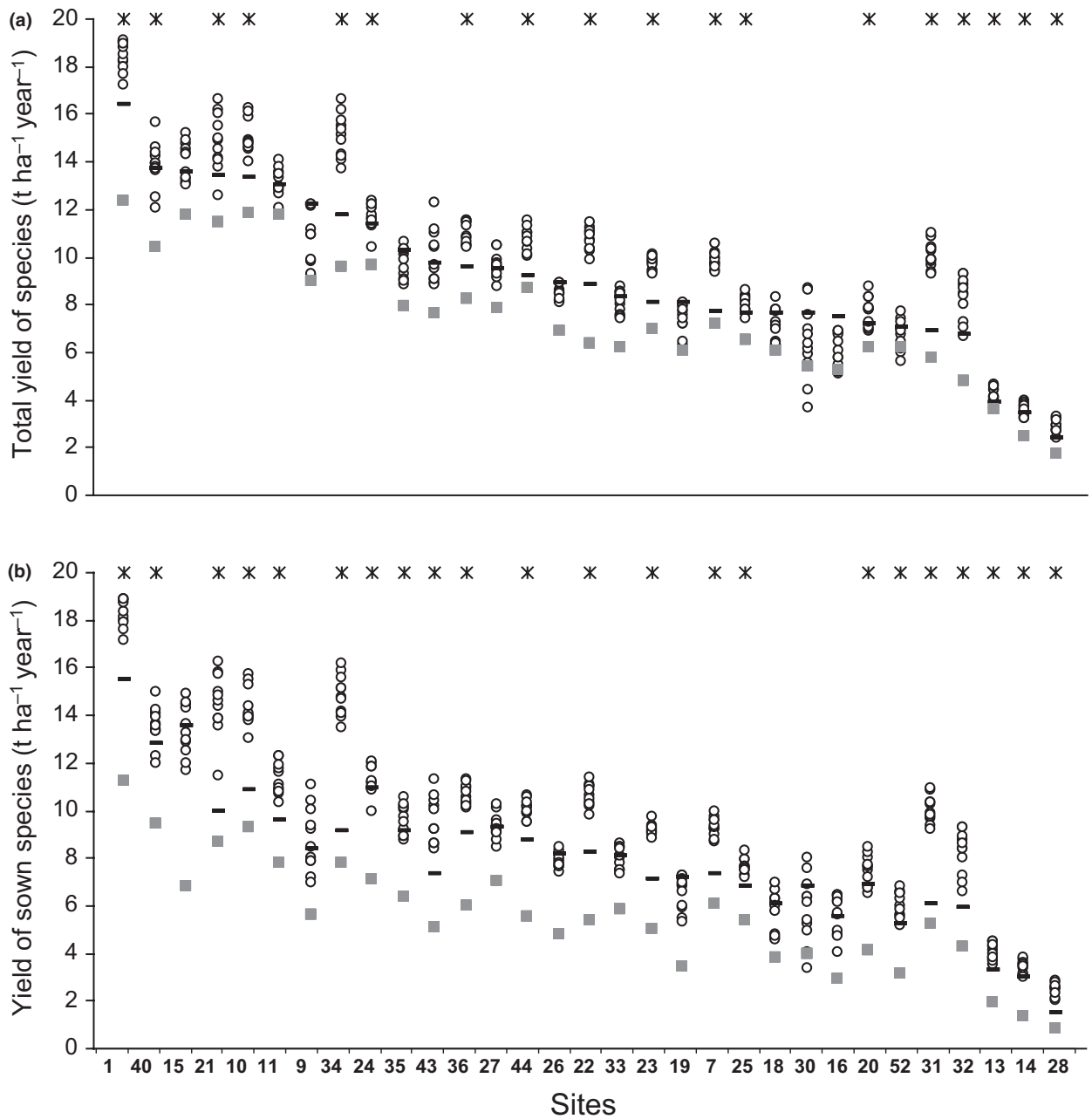

Fig. 1. Average annual yield (dry matter) over the whole experimental duration of (a) total yield and (b) yield of sown agronomic species only (excludes weeds) at each of 31 sites. For all communities, these data are averaged across seed density and across years per site. Sites are arranged in order of decreasing total yield of the best-performing monoculture. Open circles represent each of the 11 mixture communities that differed in their relative abundance at sowing; horizontal bars represent the yield of the best-performing monoculture; squares represent the mean monoculture performance. Significant transgressive overyielding is indicated by the symbol over a site at the top of each panel. Note that not all of these sites had 3 years of yield data (see Table S1, Supporting information; sites are presented in same order as in Table S1, Supporting information).

Table 1. Comparisons of (a) total yield (weed biomass is included) and (b) yield of sown species (weed biomass is excluded) in mixtures and monocultures. The tables present the total number of plots in the analysis, the percentage of all mixture communities that demonstrated overyielding or transgressive overyielding within each year, the mean magnitude (ratio) of yield benefit and the number of sites where significant effects occurred. Data for each community were averaged across the two plots with different sowing density (as in Fig. 1). For both (a) and (b), the first two rows present results on both overyielding and transgressive overyielding based on comparisons against the best-performing monoculture within a site both for sites with 3 years of data and for all available years (some sites had <3 years of data)

		Overyielding vs. average of monocultures			Transgressive overyielding vs. best monoculture on average across years		
Year of harvest	No. of mixture plots	% Mixtures	Ratio of yield benefit	Sites	% Mixtures	Ratio of yield benefit	Sites
(a) Total yield							
Sites with 3 years	528	98·1	1·34	24/24	65·5	1·07	15/24
All available years	682	97·9	1·32	31/31	65·1	1·07	18/31
Year 1	682	97·4	1·35	30/31	62·5	1·07	16/31
Year 2	660	94·8	1·33	27/30	69·4	1·12	16/30
Year 3	528	90·9	1·32	21/24	57·6	1·11	12/24
(b) Yield of sown species							
Sites with 3 years	528	100	1·83	24/24	80	1·19	17/24
All available years	682	99·7	1·77	31/31	79	1·18	22/31
Year 1	682	98·5	1·62	31/31	73	1·20	21/31
Year 2	660	99·7	1·82	30/30	76	1·20	21/30
Year 3	528	98·9	2·16	24/24	69	1·35	15/24

Discussion

A number of key findings result from this multisite experiment. Virtually all mixture communities outyielded the average monoculture within each site. The yield benefit of mixtures was established almost immediately and was maintained for at least 3 years, and mixtures were far more resistant to weed invasion than monocultures. Diversity effects were sufficiently strong such that mixtures at the majority (*c.* 60%) of sites were better than even the best monocultures to convert available resources into above-ground biomass, regardless of the site differences in species identities, soil type, soil fertility and climate. The diversity effects within each of 3 years were significantly related to sward evenness and indicated the robustness of the diversity effect to changes in the relative abundance of the four functional types of species. Diversity effects were attributed to the combinations of functional traits and their levels, and the functional trait for temporal development provided a significant diversity effect that was comparable in magnitude to that of the functional trait for nitrogen acquisition. The pattern of results for yield of sown species was similar to that presented for total yield, although the yield benefits were of greater magnitude.

TRANSGRESSIVE OVERYIELDING AND CHOICE OF REFERENCE LEVEL AGAINST WHICH MIXTURES ARE COMPARED

In perennial systems, species that maximize performance over several years are preferred. Although comparison against the maximum-performing monoculture within each year is the most stringent test of transgressive

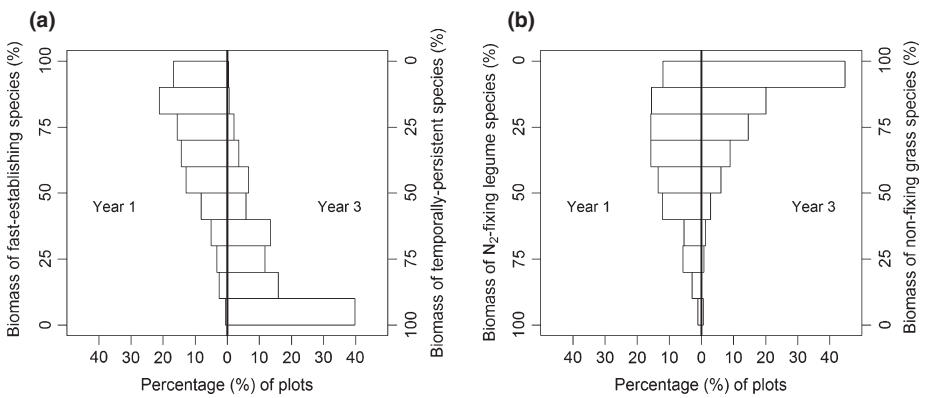

Fig. 2. Interannual changes in composition of the functional trait levels in mixture communities. We calculated the percentage of yield of sown species in a community that was comprised of (a) biomass from each of fast-establishing species and temporally persistent species and (b) biomass from each of N_2-fixing legumes and nonfixing grasses. These percentage data are presented as two separate percentage frequency distributions (panels a and b). Data from all mixture plots are compared between year 1 (left of each panel) and year 3 (right of each panel).

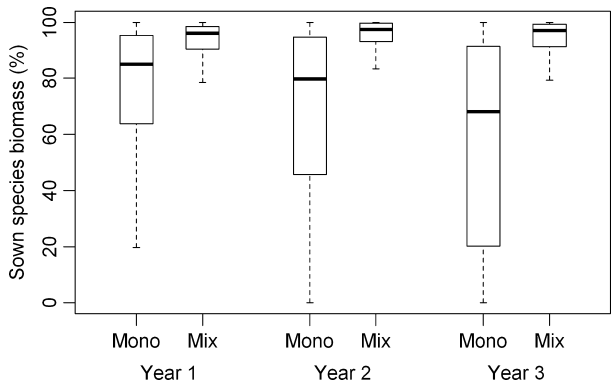

Fig. 3. Box plots of the percentage of total yield (yield of sown species + weed yield) that was composed of the sown species, presented for monocultures (mono) and mixtures (mix) in each year. Data are averaged across all 31 sites. At each site, there were four monoculture communities (n = 8 plots in total) and 11 mixture communities (n = 22 plots in total).

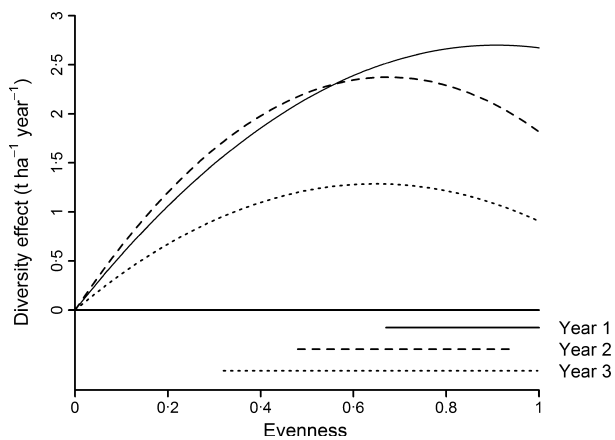

Fig. 4. Predicted diversity effect (for annual total yield) from quadratic evenness model over 3 years and across all sites. Evenness values in year 1 are based on sown proportions, whereas evenness values in years 2 and 3 are based on proportions of annual biomass in years 1 and 2, respectively. Horizontal lines below the regression curves indicate the range of evenness over which the diversity effect is not significantly ($P > 0{\cdot}05$) smaller than the maximum diversity effect.

overyielding, its agronomic relevance is considerably diminished when the identity of the best-performing crop changes from year to year. In this study, there was a 0·52 probability of a switch in the identity of the best-performing species in monoculture in successive years (see Table S8, Supporting Information). The widespread occurrence of switching in the interannual rank order of species' performance in monoculture is highly relevant to the stability of agronomic yield and to risk aversion strategies associated with the choice of monoculture species. Indeed, if such rates of switching continue after >3 years, then the relative benefit of mixtures would increase when compared against the monoculture with the best mean yield over the time period (see also Cardinale *et al.* 2007; Reich *et al.* 2012). We retrospectively selected the best-performing monoculture across years at each site for testing against mixture yield. Our test of transgressive overyielding is a conservative one, which is only made more conservative by the best-performing monoculture not necessarily being the monoculture that would have been chosen by farmers at the time of sowing. This is because farmers may not be able to correctly predict in advance of sowing which of the available species will perform best over several years, but also because they may use selection criteria other than highest yield (such as economic return, forage quality and/or digestibility).

INTERSPECIFIC INTERACTIONS UNDERPIN THE DIVERSITY EFFECT

Only synergistic interactions can produce transgressive overyielding, and transgressive overyielding is only achieved when the net effects of interspecific interactions are sufficiently strong (Trenbath 1974; Cardinale *et al.* 2007; Kirwan *et al.* 2007, 2009). In a meta-analysis of diversity–function experiments with plants, Cardinale *et al.* (2007) found that significant transgressive overyielding occurred in only 10 of 83 comparisons (12%) and, on average, took about 5 years to become evident. Even though overyielding occurred in 79% of comparisons, transgressive overyielding was a relatively rare event in that meta-analysis. Thus, it is remarkable that we find such a very high incidence of transgressive overyielding occurring in a relatively short period of time. This incidence and magnitude of transgressive overyielding in mixtures is even more impressive given its occurrence with agronomic species that already provide high yields in monoculture, and the comparison against the best of these monocultures represents a high benchmark for mixture performance to exceed.

The occurrence of transgressive overyielding is incontrovertible evidence of strong resource complementarity and is also a very important practical criterion for the recommendation of mixtures as one strategy for sustainable agriculture. We consider the most important explanation of the incidence and magnitude of transgressive overyielding to be the *a priori* design of the species composition of the mixtures. The design involved selection of species with differences in agronomically important functional traits, which was intended to maximize the positive effects of interspecific interactions on yield (see Introduction, and below). Grass–legume interactions (the synergy between the two levels of nitrogen acquisition) are well known in agricultural systems and can produce strong effects even at low to moderate levels of nitrogen application. For example, Nyfeler *et al.* (2009) showed that four species mixtures of grasses and legumes (with legume proportions from 50% to 70%) with 50 kg ha^{-1} year^{-1} of nitrogen fertilizer produced yields that were comparable with yields from grass monocultures with 450 kg ha^{-1} year^{-1} of nitrogen fertilizer. This is consistent with symbiotic N_2 fixation of up to 300 kg ha^{-1} year^{-1} of nitrogen in comparable systems

Table 2. Contributions of the separate pairwise interactions among the four functional types to the diversity effect. These results are from an analysis of annual average of total yield (including weed biomass) regressed on the initial design proportions of species. The analysis was based on 24 sites with 3 years of data. The identity effects are presented for each species, and the number of sites at which a species occurred is in brackets. The functional type of each species is also indicated. The pairwise interaction effects for the four functional types are indicated by the relevant combination of trait levels (N, Z, F and P) for N_2-fixing, nonfixing, fast-establishment and temporal persistence, respectively, and the text in brackets indicates the trait levels being tested (see Appendix S2, Supporting information). In the column of model estimates (±SE), the pairwise interaction effects are represented by the values of the δ coefficients. The pairwise interaction effects are also scaled for the mixture with equal proportions of all four species ($\delta_{ij}p_i.p_j$, where p_i = 0·25), for which the diversity effect is the sum of the pairwise interaction effects = 2·77 t ha^{-1} year^{-1}

Model term	Functional type	Model estimates (t ha^{-1} year^{-1})	Interaction effects on yield for equi-proportional mixture (t ha^{-1} year^{-1})	*P*
Lolium perenne (*n* = 16)	ZF	7·92 ± 0·75	–	<0·0001
Phleum pratense (*n* = 7)	ZF*	6·82 ± 1·13	–	0·0009
Lolium rigidum (*n* = 2)	ZF	3·36 ± 2·11	–	0·3572
Dactylis glomerata (*n* = 17)	ZP	7·83 ± 0·73	–	<0·0001
Poa pratensis (*n* = 6)	ZP	4·65 ± 1·22	–	0·0125
Trifolium pratense (*n* = 22)	NF	8·49 ± 0·67	–	<0·0001
Medicago polymorpha (*n* = 2)	NF	3·32 ± 2·21	–	0·3738
Trifolium repens (*n* = 21)	NP	6·84 ± 0·68	–	<0·0001
Medicago sativa (*n* = 3)	NP	5·67 ± 1·80	–	0·0878
Seed density	–	0·04 ± 0·07	–	0·5297
ZF*ZP (fast, persistent)	–	4·72 ± 1·56	0·295	0·0060
NF*NP (fast, persistent)	–	8·01 ± 1·56	0·501	<0·0001
NF*ZF (N2-fixing, nonfixing)	–	6·78 ± 1·65	0·424	0·0004
NP*ZP (N2-fixing, nonfixing)	–	8·99 ± 1·65	0·562	<0·0001
NP*ZF (all four trait levels)	–	8·11 ± 1·87	0·507	0·0002
NF*ZP (all four trait levels)	–	7·70 ± 1·87	0·481	0·0004

*In this analysis, an exception occurred at site 1, where *P. pratense* was used as a nonfixing, temporally persistent species (ZP).

(Boller & Nösberger 1987; Zanetti *et al.* 1997; Nyfeler *et al.* 2011) and with transfer of up to 60 kg ha^{-1} year^{-1} of fixed nitrogen from legumes to grasses (Boller & Nösberger 1987; Zanetti *et al.* 1997). Thus, grass–legume mixtures at lower levels of nitrogen application could exceed (or be comparable to) the yield of grass monocultures with high (nonlimiting) levels of nitrogen (Nyfeler *et al.* 2009; Weigelt *et al.* 2009). We also exploited known patterns of temporal dynamics of the species and, as expected, there was a clear overall trend for the replacement of fast-establishing species by temporally persistent ones (Fig. 2b). For example, this helped ensure greater temporal persistence of N_2-fixing legumes, despite some anticipated turnover of individual legume species over time (Black *et al.* 2009). In addition, the early establishment of ground cover is important so that the sown species can establish well and inhibit the growth of weed species (Teasdale 1998; Tracy & Sanderson 2004). The functional traits we used to characterize functional types do not exclude other trait differences among plant species from also contributing to synergistic effects (van Ruijven & Berendse 2005). Such other synergistic effects may include the ability of species with different root systems to access different niche spaces for soil moisture and nutrients (Skinner, Gustine & Sanderson 2004; Mommer *et al.* 2010). Similarly, combinations of species with different shoot characteristics can result in a greater overall use of incident light (Spehn *et al.* 2000), and mixtures can result in reduced incidence of pests and/or diseases (Zhu *et al.* 2000).

ROBUSTNESS OF YIELD BENEFITS

Comparisons of the ratio values in Tables 1 and 2 are based on comparisons of the *average* diversity effect across a range of mixture communities that differed in relative abundance. Irrespective of this, we found widespread evidence of overyielding and transgressive overyielding, which emphasizes the robustness of yield benefits across the different mixtures. This is reflected in the persistence of the evenness effect over time and the maintenance of the diversity effect across a considerable range of the higher values of evenness (Fig. 4; Connolly *et al.* in review). This highlights both the temporal stability of the diversity effect and its robustness over species proportions. This stability of ecosystem function in the face of compositional change is an important feature for land managers and farmers, because the benefit of the diversity effect can be achieved despite a considerable range of fluctuation in the relative proportions of the four species, for example, the diversity effect in communities dominated (70%) by one species is comparable to that of the most even community. This robustness of the yield benefits to considerable changes in the relative proportions of the four species is an extremely useful property for practical management of grassland swards.

The above issues are highly relevant from a farmer's perspective, and the robust response to evenness also affords more flexibility for the implementation of adaptive management techniques to maintain the community composition at higher levels of evenness. As the global human population approaches 10 billion, the high environmental and economic costs of nitrogen fertilizer (Gruber & Galloway 2008; Peyraud, Le Gall & Lüscher 2009) and other inputs will require future agricultural systems to not just deliver more food, but to do so more sustainably. The design of grassland mixtures provides opportunities to increase resource efficiency (e.g. improving yield for a given amount of fertilizer and other inputs) and is relevant to farm-level strategies to achieve sustainable intensification.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Appendix S1. Supporting text and information on monoculture variation.

Appendix S2. Supporting text and information on the methods and analyses.

Fig. S1. Ratio values of mixture/monoculture for total yield and yield of sown species.

Fig. S2. Relationship between the diversity effect and evenness for annual yield of sown species.

Table S1. Detailed information on sites and species.

Table S2. The relative abundances of communities used in the simplex design.

Table S3. Analysis of total annual yield: model selection.

Table S4. Analysis of total annual yield: parameter estimates.

Table S5. Analysis of annual yield of sown species: model selection.

Table S6. Analysis of annual yield of sown species: parameter estimates.

Table S7. Analysis of mean yield (averaged across 3 years) of sown species.

Table S8. Further information on switching of the identity of the best-performing monoculture.