APPLIED ECOLOGY

Multispecies grasslands produce more yield from lower nitrogen inputs across a climatic gradient

James O'Malley[1], John A. Finn[2]*, Carsten S. Malisch[3], Matthias Suter[4], Sebastian T. Meyer[5], Giovanni Peratoner[6], Marie-Noëlle Thivierge[7], Diego Abalos[3], Paul R. Adler[8], T. Martijn Bezemer[9,10], Alistair D. Black[11], Åshild Ergon[12], Barbara Golińska[13], Guylain Grange[1,2], Josef Hakl[14], Nyncke J. Hoekstra[15], Olivier Huguenin-Elie[4], Jingying Jing[16], Jacob M. Jungers[17], Julie Lajeunesse[18], Ralf Loges[19], Gaëtan Louarn[20], Andreas Lüscher[4], Thomas Moloney[21], Christopher K. Reynolds[22], Ievina Sturite[23], Ali Sultan Khan[2], Rishabh Vishwakarma[1], Yingjun Zhang[16], Feng Zhu[24], Caroline Brophy[1]

High-yielding forage grasslands frequently contain low species diversity and receive high inputs of nitrogen fertilizer. To investigate multispecies mixtures as an alternative strategy, the 26-site international LegacyNet experiment systematically varied the diversity of sown grasslands using up to six high-yielding forage species (grasses, legumes, and herbs) managed under moderate nitrogen inputs. Multispecies mixtures outyielded two widely used grassland practices: a grass monoculture with higher nitrogen fertilizer and a two-species grass-legume community. High yields in multispecies mixtures were driven by strong positive grass-legume and legume-herb interactions. In warmer sites, the yield advantage of legume-containing multispecies mixtures over grass monocultures with higher nitrogen fertilizer inputs increased. Improved design of grassland mixtures can inform more environmentally sustainable forage production and may enhance adaptation of productive grasslands to a warming climate.

To feed a growing global population, agricultural production systems have undergone substantial simplification and intensification to increase productivity as well as food and feed quality (*1*). In the context of ongoing resource depletion and the global climate and biodiversity crises, innovative agricultural practices are needed to balance food security with environmental sustainability (*2–5*). Ruminant livestock production is a major contributor to the global agricultural economy, and associated grassland forage production varies from high-input, intensively managed monocultures to more extensive, low-input systems (*6*). Sown grasslands in temperate regions (hereafter "productive grasslands") typically comprise a small number of high-yielding perennial species, often grass monocultures reliant on high nitrogen fertilizer inputs or two species grass-legume communities, typically comprising 70% *Lolium perenne* (perennial ryegrass) and 30% *Trifolium repens* (white clover). Despite promoting yield increases, high-nitrogen inputs contribute to several agri-environmental problems, including greenhouse gas emissions, reduced soil and water quality, and biodiversity loss (*1*). The use of multispecies grasslands has been proposed to achieve high yields and increase sustainability (*7, 8*). However, critical gaps remain in understanding whether higher plant diversity with lower nitrogen inputs can match yields of productive grasslands with higher nitrogen inputs across varying climates and environmental conditions.

Biodiversity and ecosystem function is well studied in seminatural grassland experiments, where higher plant diversity generally increases yield [e.g., (*8–10*)] and enhances resistance and resilience to environmental disturbance (*11*). However, most seminatural grasslands have low yields and/or limited forage quality, and many species mixtures fail to out-yield the most productive monoculture (*12*). Based on ecological principles, combining productive species with complementary traits has the potential to deliver high yields with moderate inputs (*8, 13*). Mixtures of grasses and legumes in productive grasslands at low-to-medium nitrogen fertilizer levels can outyield the component monocultures (at the same level of nitrogen fertilizer) and achieve the same or higher yields as highly fertilized grass monocultures owing to the nitrogen-fixing abilities of legume species (*14–17*). The inclusion of herb species (defined as nonleguminous forbs) in grass-legume mixtures increases functional diversity, which can increase overall resource utilization and ecosystem function (*10, 18*). However, the nitrogen-saving and yield-related benefits in productive grasslands and mixtures with more than two functional groups are largely unexplored.

Investigations of the addition of herbs to grass or grass-legume communities in productive grasslands are difficult to synthesize because they vary considerably in their experimental design (*19–26*). Systematic investigation across varying conditions is required for the development of robust agricultural practices intended for wide implementation. We established LegacyNet, a voluntary, coordinated 26-site network of grassland experiments spanning a broad climatic gradient within temperate regions across North America, Europe, Asia, and New Zealand (Fig. 1A and data S1). LegacyNet sites implemented a common field experiment (*27*), where the composition and sown proportions of six high-yielding forage species (two grasses, two legumes, and two herbs, denoted G1, G2, L1, L2, H1, and H2) were manipulated at plot scale (table S1) to assess the suitability of the wide-scale adoption of multispecies mixtures in productive grasslands. Given the range of environmental conditions across sites, the species selected for each site were suited to the locality (*27*). Nevertheless, there was high overlap in species across sites (data S1); the species most commonly used across sites were G1 *L. perenne*, G2 *Phleum pratense* (timothy), L1 *Trifolium pratense* (red clover), L2 *T. repens*, H1 *Cichorium intybus* (chicory), and H2 *Plantago lanceolata* (plantain). Either no (two sites) or moderate levels of nitrogen fertilizer were applied at the sites, over an average duration of 24 months (data S1). Mixture and monoculture performances were compared to a grass monoculture (*L. perenne* at 21 of 26 sites) with a higher level of nitrogen fertilizer (at least twice the moderate rate, hereafter "high N grass") and a two-species community of 70% *L. perenne* and 30% *T. repens*. Thus, LegacyNet investigated (i) whether diversity of plant species and/or functional groups affects the yields of productive grasslands, (ii) whether higher-diversity mixtures can equal or exceed the yields of conventional management practices, and (iii) the extent to which climate alters potential yield benefits of multispecies mixtures.

[1]School of Computer Science and Statistics, Trinity College Dublin, Dublin 2, Ireland. [2]Environment, Soils and Land Use Department, Teagasc, Johnstown Castle, County Wexford, Ireland. [3]Department of Agroecology, Aarhus University, Tjele, Denmark. [4]Agroscope, Reckenholzstrasse, Zurich, Switzerland. [5]School of Life Sciences, Technical University of Munich, Munich, Germany. [6]Research Area Mountain Agriculture, Laimburg Research Centre, Auer-Ora, Italy. [7]Agriculture and Agri-Food Canada, Quebec Research and Development Centre, Quebec City, QC, Canada. [8]US Department of Agriculture, Agricultural Research Service, University Park, PA, USA. [9]Institute of Biology, Leiden University, Leiden, Netherlands. [10]Netherlands Institute of Ecology, NIOO-KNAW, Wageningen, Netherlands. [11]Department of Agricultural Sciences, Lincoln University, Christchurch, New Zealand. [12]Department of Plant Sciences, Norwegian University of Life Sciences, Ås, Norway. [13]Department of Grassland and Natural Landscape Sciences, Poznań University of Life Sciences, Poznań, Poland. [14]Department of Agroecology and Crop Production, Czech University of Life Sciences, Prague, Czech Republic. [15]Louis Bolk Institute, Bunnik, Netherlands. [16]College of Grassland Science and Technology, China Agricultural University, Beijing, China. [17]Department of Agronomy and Plant Genetics, University of Minnesota, Saint Paul, MN, USA. [18]Agriculture and Agri-Food Canada, Research Farm, Normandin, QC, Canada. [19]Institute of Animal Nutrition and Physiology, Group of Organic Farming, Kiel University, Kiel, Germany. [20]INRAE UR4 URP3F, Lusignan, France. [21]DLF, Ballymountain, Ferrybank, County Waterford, Ireland. [22]School of Agriculture, Policy and Development, University of Reading, Earley Gate, UK. [23]Department of Grassland and Livestock, Norwegian Institute of Bioeconomy Research (NIBIO), Ås, station Steinkjer, Norway. [24]Hebei Key Laboratory of Soil Ecology, Key Laboratory of Agricultural Water Resources, Center for Agricultural Resources Research, Institute of Genetic and Developmental Biology, Chinese Academy of Sciences, Shijiazhuang, China. *Corresponding author. Email: john.finn@teagasc.ie
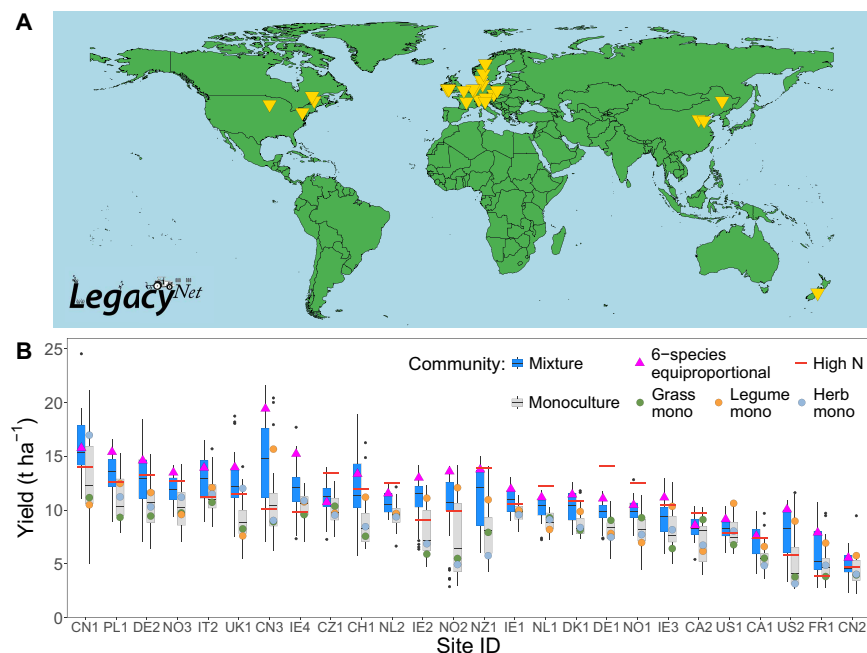
A

B

Fig. 1. Yields varied substantially across the LegacyNet sites. (A) The locations of the 26 LegacyNet sites, indicated by inverted yellow triangles (see data S1 for the site location details and ID codes). **(B)** The yield per growing season (t ha^{-1}) based on plot-level measurements for each site are shown in boxplots that are grouped by mixtures (blue; 29 plots per site) and monocultures (gray; 18 plots per site), at the moderate nitrogen fertilizer level. The average yield of the six-species equiproportional mixture plots (three plots per site) is shown as a magenta triangle. The average yield of the monoculture plots from each functional group is highlighted with a circle (green for grass, orange for legume, and light blue for herb; six monoculture plots per functional group per site). The average yield of the high N grass monoculture (five plots per site) is shown by a red horizontal line. Sites are arranged on the *x* axis in order of decreasing median yield. The center line represents the median, box limits are upper and lower quartiles, and whiskers extend to the minimum and maximum of nonoutlier values.

Mixing of functional groups underpinned a high degree of overyielding

Yield per growing season (*27*) varied substantially across sites, with median values for plots at each site ranging from 4.65 to 14.89 tonnes per hectare (t ha^{-1}), reflecting the range of climates, soil conditions, and management practices. Many mixture plots yielded higher than monoculture plots at individual sites (Fig. 1B).

Across sites, yields of the six forage species in monoculture were highest for L1, and all other species were similarly high to each other [Fig. 2; predicted from a Diversity-Interactions model (*28–30*); tables S2 and S3]. Overyielding occurs when a mixture outyields the weighted average of the component monocultures (hereafter "weighted average monoculture") (*31*). The six-species grass-legume-herb equiproportional mixture and four-species equiproportional mixtures of grass-legume, legume-herb, and grass-herb all displayed overyielding by outperforming their weighted average monoculture by 3.13 t ha^{-1} (grass-legume-herb, +34%), 2.90 t ha^{-1} (grass-legume, +31%), 2.46 t ha^{-1} (legume-herb, +26%), and 0.78 t ha^{-1} (grass-herb, +9%), respectively [*P* < 0.0001 for each mixture, testing whether the net interactions (Fig. 2) differed from zero]. Strong overyielding occurred across a wide range of sown proportions of grasses, legumes, and herbs (Fig. 3, A and B). We identified other six-species mixtures that outperformed the equiproportional six-species mixture [e.g., see Fig. 3A; the optimal mixture, grass:legume:herb = 0.24:0.59:0.17, yielded 12.83 t ha^{-1} (+4%) compared with 12.31 t ha^{-1} for the equiproportional mixture, cyan versus magenta circle; *P* < 0.0001]. Thus, we found overwhelming evidence of overyielding in mixtures (Fig. 3B) that was robust across a wide range of sown functional group proportions; this was due to strong interaction effects

(Fig. 3A and table S2), with interactions being important even when some functional groups had low sown proportions [partially due to the non-linear term theta (*29*, *32*) in eq. S3 (*27*) and table S2; see also fig. S1].

Transgressive overyielding occurs when a mixture outperforms the best-performing monoculture (*31*). Across sites, a wide range of sown proportions of grasses, legumes, and herbs performed better than (the blue region in Fig. 3C) or comparably to (the three gray regions in Fig. 3C) the best-performing monoculture. This high degree of transgressive overyielding is only possible because of positive net effects of interspecific interactions (illustrated in gray in Fig. 2 and quantified in table S2). There were strong positive interactions between grasses and legumes, between herbs and legumes, and, to a lesser degree, between grasses and herbs (table S2). The interaction between the two legume species was moderate, but interactions between the two grasses and between the two herbs were nonsignificant. Although we cannot discern among the various mechanisms that underpin these interactions, they likely include the ability of mixtures to use a wider range of soil, nutrient, and light resources, especially nitrogen (*33*), as well as a reduced susceptibility to pests and diseases. Strong grass-legume interactions are consistent with previous research at single sites (*16*, *33*), and across environmental conditions (*14*, *34*), and reflect the importance of nitrogen fixation by legumes and the ability of other functional groups to transform the additional available nitrogen into biomass. Some evidence for interactions of herbs with grasses or legumes has been shown in single-site studies (*22*, *23*), but our results provide large-scale evidence that multispecies mixtures that include herb species can also deliver high yields.

Increasing species diversity within functional groups can benefit yield

When two species are functionally redundant (*28*), they are interchangeable at sowing without any impact on yield. At the 26 individual sites, the two species within each functional group were only functionally redundant at seven sites for grasses, four sites for legumes, and seven sites for herbs (fig. S2). Functional redundancy, however, may have even lower incidence if multiple responses are considered, and/or over different timescales (*35*).

We further investigated whether yields could be optimized by sowing one rather than two species per functional group. Across sites, equiproportional mixtures with three species spanning three functional groups yielded, on average, 0.91 t ha^{-1} less (−7%; fig. S3) than the equiproportional six-species mixture, clearly indicating a yield advantage of the latter. As richness increased from three to six species, any increase in yield strongly depended on species composition (fig. S4). At individual sites, most of the equiproportional three-species combinations yielded lower than (125 of 208 comparisons) or comparably to (81 of 208 comparisons) the equiproportional six-species mixture and yielded higher in only two comparisons (fig. S3). In the absence of prior knowledge on local species' performances, sowing two species within each functional group, rather than one, is a more reliable strategy to optimize yield and reduce sensitivity to changes in sown functional group proportions (fig. S5). Thus, even with a targeted choice of high-performing forage species, six-species grass-legume-herb mixtures are better than three-species grass-legume-herb mixtures.

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Fig. 2. Yields of monocultures and multispecies mixtures with varying functional group richness. Predicted yield per growing season (t ha^{-1}) for selected communities across sites (predicted from the model estimates in table S2, $n = 1382$). For each bar, yield predictions are broken down by the components of the model prediction (model species' identity effect estimates, each scaled by the respective sown proportion, plus scaled net interactions for mixtures). The error bars show 95% confidence intervals for each prediction. The monoculture bars show predicted yield for the six monoculture communities. The "Mixtures (1 functional group)" bars show predictions for the two-species equiproportional mixtures where the two species are from the same functional group. For the G1:G2 equiproportional mixture, the interaction term is negative and therefore not shown (effect = −0.09t ha^{-1}, SE = 0.17); the height of the bar represents the contribution of G1 and G2 to the yield minus the

negative interaction effect. The "Mixtures (2–3 functional groups)" bars show predicted yields for the four-species equiproportional grass-legume (GL), legume-herb (LH), and grass-herb (GH) mixtures and the predicted yield of the six-species equiproportional grass-legume-herb (GLH) mixture. The boxplots show the estimated distributions of predicted responses for all possible GL mixtures, LH mixtures, GH mixtures, and GLH mixtures. The dashed red line is the predicted yield of the high N grass monoculture. The dashed black line is the predicted yield of a 70:30 G1:L2 two-species community, where G1 was *L. perenne* and L2 was *T. repens* at most of the sites. In the boxplots, the center line represents the median, box limits are upper and lower quartiles, and whiskers extend to the minimum and maximum of nonoutlier values.
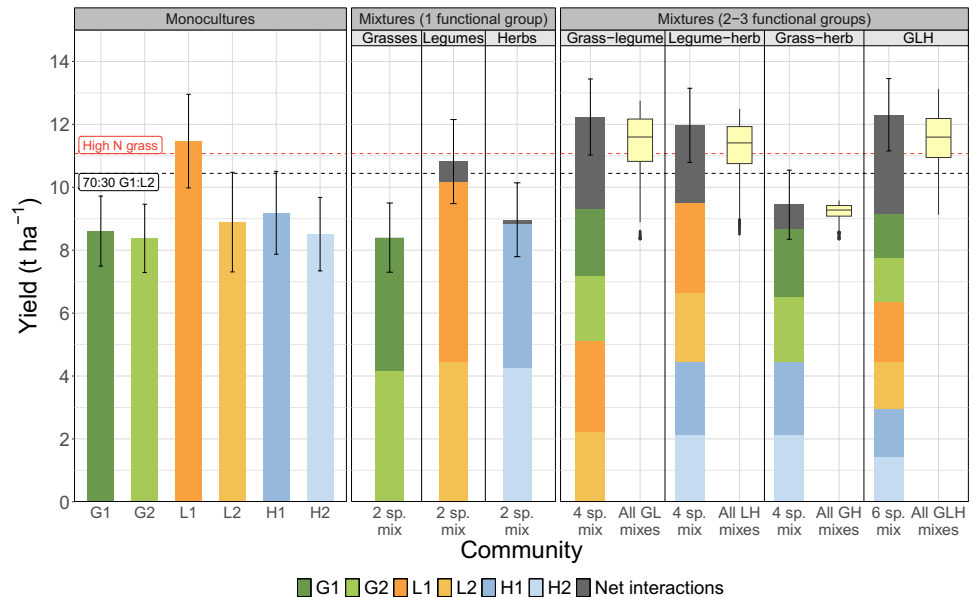
Fig. 3. Higher yields from increased plant diversity. (**A**) Ternary diagram illustrating predicted yield (t ha^{-1}) across all sites as the sown proportions of the three functional groups vary and with the two species within each functional group sown in equal proportions to each other. Note that the vertices of the ternary are two-species communities of grasses (G), legumes (L), or herbs (H), not monocultures of individual species. The highest predicted yield is marked by a cyan circle (with sown functional group proportions: G:L:H = 24:59:17 and corresponding sown species proportions equal to 0.12:0.12:0.295:0.295:0.085:0.085). In the yield legend, values are marked for the predicted responses of the 70:30 G1:L2 community (black line), the high N grass monoculture (red line), and the highest yielding monoculture (blue line). The predicted yield for the six-species equiproportional mixture is shown by a

magenta circle. The remaining panels show the difference (measured in standard errors) of the predicted yield in (A) from (**B**) the weighted average monoculture (for the component species at each point in the ternary; overyielding), (**C**) the highest yielding monoculture (i.e., L1, which was *T. pratense* at most sites; transgressive overyielding), (**D**) the high N grass monoculture, and (**E**) the 70:30 G1:L2 two-species community, where G1 was *L. perenne* and L2 was *T. repens* at most sites. For example, a point on (D) within the highest contour level (blue region) represents a mixture whose predicted yield is more than two standard errors greater than the predicted yield of the high N grass monoculture. The six-species equiproportional mixture and the optimal mixture are marked in (B) to (E) for comparison to (A).
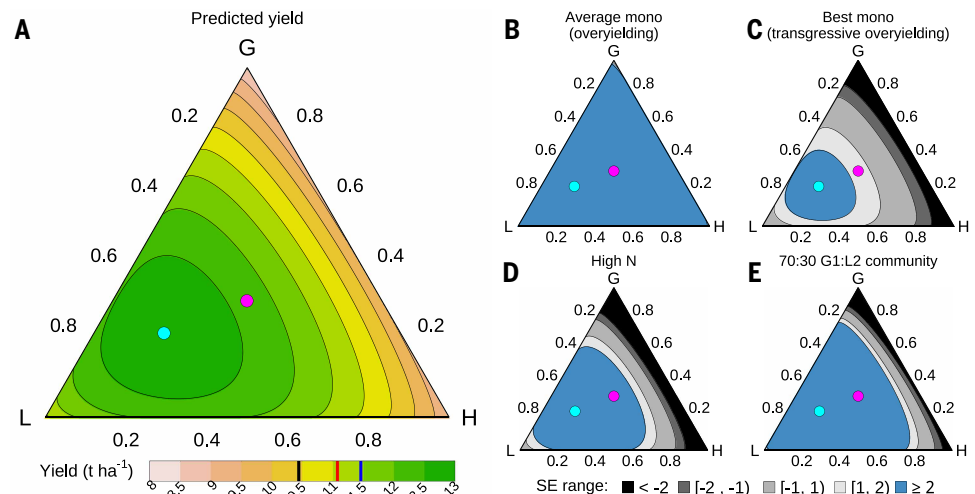
Multispecies mixtures with moderate nitrogen inputs outperformed a grass monoculture with high nitrogen fertilizer inputs

To compare mixture performance with a grass monoculture with high nitrogen, the LegacyNet design included a high-nitrogen application (on average across sites, 260.5 and 108.7 kg ha^{-1} year^{-1} for high and moderate nitrogen respectively, i.e., 2.4 times higher; data S1 and table S1) on additional monocultures of G1 ("high N grass" hereafter). Across sites, yields of multispecies mixtures across a wide range of sown functional group proportions and managed with moderate nitrogen fertilizer either outyielded or were comparable to the

high N grass yield (blue region and the three gray regions, respectively, in Fig. 3D). The high N grass yielded 11.07 t ha^{-1}, and the equiproportional six-species mixture yielded 1.24 t ha^{-1} more ($P = 0.0002$, +11%; in Fig. 2, the six-species equiproportional mixture versus the dashed red line), despite the average nitrogen rate being 152 kg ha^{-1} lower for the multispecies mixture. Therefore, functionally diverse mixtures with much lower nitrogen application can outyield a high N grass monoculture. Mixtures that contained little to no legumes had lower yields than the high N grass (grass-grass, herb-herb, and grass-herb predictions in Fig. 2 and the black region in Fig. 3D). At 21 of 26 sites, the yield of

the six-species equiproportional mixture was higher (12 sites) or comparable (nine sites) to the high N grass and significantly lower only at five sites (fig. S6A and table S4). The extent of these effects provides evidence that farmers can use multispecies mixtures in lieu of highly fertilized grass monocultures to reduce their financial costs and environmental impacts while simultaneously maintaining or improving their yields.

Multispecies mixtures outperformed a two-species grass-legume community

Twenty-one of 26 LegacyNet sites sowed *L. perenne* as their G1 species, and 20 of 26 sowed *T. repens* as their L2 species (data S1). Therefore, we used our model to estimate the performance of a two-species community of 70% *L. perenne* and 30% *T. repens* (hereafter "70:30 G1:L2 community"). Across sites, the equiproportional six-species mixture and the equiproportional four-species grass-legume and legume-herb mixtures outyielded the 70:30 G1:L2 community (10.44 t ha^{-1}; dashed black line in Fig. 2) by 1.86 t ha^{-1} (+18%), 1.79 t ha^{-1} (+17%), and 1.53 t ha^{-1} (+15%), respectively ($P < 0.0001$ each) (Fig. 2). Similar results could also be seen at the individual-site level (fig. S6B and table S4). A wide range of four- and six-species mixtures of grasses, legumes, and herbs (where species within each functional group were sown in equal proportion) significantly outyielded the two-species 70:30 G1:L2 community (Fig. 3E). These results underline that increases in species richness (from two to four to six) and manipulation of relative abundances of four-species grass-legume and six-species grass-legume-herb mixtures can result in higher yields than those from the two-species 70:30 G1:L2 community. Although legumes play a key role (Fig. 3A), six-species mixtures of grasses, legumes, and herbs always out-performed two- and four-species grass-legume communities when comparing across communities with the same sown legume percentage, and for values less than 70% (fig. S7).

Mixtures with legumes increased yield advantages across a temperature gradient

Yield generally showed a quadratic relationship with average daily temperature across sites (Fig. 4A and table S2). Owing to the correlation between temperature and growing-season length, the temperature effects in our model also reflect some of the site-to-site variation in growing-season duration. Yields were reduced considerably at lower temperatures (Fig. 4A); for example, the equiproportional six-species mixture yielded 10.47 t ha^{-1} at 5°C compared with 12.50 t ha^{-1} at 11°C. Although the effect of temperature was similar on all monocultures and grass-herb mixtures (Fig. 4A), any mixture containing legumes yielded proportionately more as temperature increased, because the grass-legume and legume-herb interactions were stronger at higher temperatures (see climate terms in table S2). Thus, yield patterns across the equiproportional grass-legume, legume-herb, and grass-legume-herb communities were quite consistent from 9° to 13°C (Fig. 4A and fig. S8A); for example, at 9°, 11°, and 13°C in Fig. 4A, the six-species equiproportional mixture yielded 12.21, 12.50, and 12.39 t ha^{-1}, respectively. Importantly, at higher temperatures, these legume-related interactions caused an increased yield difference between the six-species equiproportional mixture and both the high N grass monoculture (Fig. 4B) and all the other monocultures (Fig. 4A). There were combinations of sown grass-legume-herb proportions with clear evidence
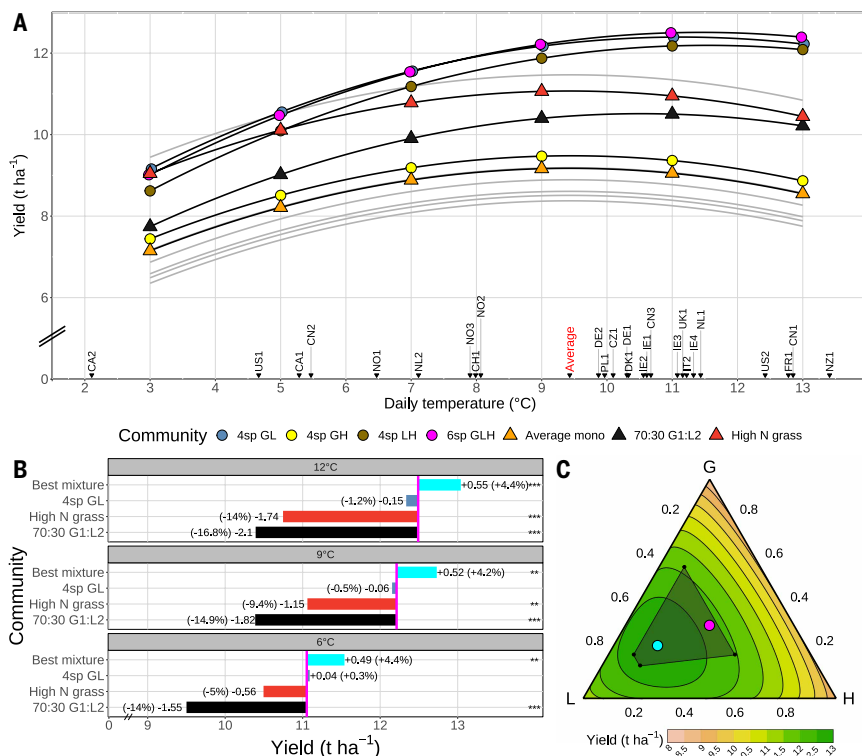

Fig. 4. As mean site temperatures increased, multispecies mixtures best maintained yield. (A) Predicted yields across the gradient of 3° to 13°C average daily temperature for selected communities: the four-species equiproportional mixtures of grass-legume, grass-herb, and legume-herb (blue, yellow, and brown circles, respectively), the six-species equiproportional mixture of grass, legume, and herb (magenta circle), the average of the six monocultures (orange triangle), the two-species 70:30 G1:L2 community (black triangle), and the high N grass monoculture (red triangle). Gray curves show the predicted yields of the six monoculture species (from highest- to lowest-yielding, these are L1, H1, L2, G1, H2, and G2). Circles on the top two curves are jittered. Site ID labels (data S1) are included along the *x* axis to indicate the average daily temperature at each site during the experimental period; the across-site average of daily temperatures is indicated as "Average" (in red). **(B)** The predicted yield (t ha^{-1}) for the six-species equiproportional mixture for three average daily temperatures: 6°, 9°, and 12°C (vertical magenta line in each panel). For each temperature, differences are shown between the six-species equiproportional mixture and each of four selected communities (as labeled on the *y* axis): 70:30 G1:L2 community (black), high N grass (red), four-species equiproportional grass-legume mixture (blue), and the optimal "best" mixture (cyan). Significant differences are highlighted on the right-hand side (***$P < 0.0001$; **$P < 0.001$). **(C)** Ternary diagram showing predicted yield (t ha^{-1}) across all sites. The shaded region identifies a range of sown proportions of grasses, legumes, and herbs for multispecies communities that optimize yield.

of (i) overyielding (fig. S8B) across the temperature gradient, (ii) transgressive overyielding at and above 9°C (fig. S8C), (iii) outperformance of the high N grass at and above 7°C (fig. S8D), and (iv) outperformance of the 70:30 G1:L2 community at all temperatures (fig. S8E).

Implications for agronomic practice

To optimize yield and nitrogen-saving benefits, we identified a range of functional group proportions defined by 30 to 70% of legumes, a minimum of 15% of grasses, and a minimum of 10% of herbs (the dark shaded region in Fig. 4C). Many diverse mixtures within this region exhibited transgressive overyielding by outperforming the highest-yielding monoculture (L1, red clover, in 23 of 26 sites). In practice, red clover monocultures would not be sown in productive grasslands, given the risk of disease in fields repeatedly cropped with it, bloat risk for grazing ruminants, and negative environmental effects on nitrous oxide emissions and nitrate leaching (36, 37). Despite being an overly stringent comparison, it is all the more impressive that many mixtures outperformed red clover, the highest-yielding monoculture.

We retrospectively identified the optimal-yielding communities at each site (fig. S9), which were generally within or close to the optimal

region of community proportions across sites (Fig. 4C). However, the site-specific sown proportions of these optimal communities would not be known in advance and, furthermore, would likely vary depending on the weather conditions in the year of establishment and subsequent years. Therefore, aiming for sown proportions of the functional groups within the specified region across sites (Fig. 4C) is a more dependable approach to optimize yield. The wide range of species proportions in this region also allows more targeted design of grassland communities to match different farming systems, without any compromise in yields; for example, lower legume proportions (~30%) would be more appropriate for a grassland grazed by ruminants, whereas higher legume proportions may better suit an ungrazed grassland ley in a rotation.

Productive grasslands are expected to deliver more than just forage yield, and communities in the optimal region (Fig. 4C) can also reduce agri-environmental harms associated with nitrogenous gaseous emissions and leaching (36–39), leading to benefits across multiple ecosystem functions (multifunctionality), including agronomic (40, 41), livestock (21, 23, 42), and other environmental (13, 43) responses.

The duration of the LegacyNet experiments generally matches well with that of grassland leys as implemented within rotational cropping systems, where they can enhance soil carbon, disrupt pest and weed life cycles, and reduce greenhouse gas emissions and nitrate leaching (44). Even within a short multispecies grassland ley, savings from reduced nitrogen fertilizer far exceed additional seed costs. Productive grassland systems can have a 4- to 8-year duration that exceeds our experimental length. Although we show yield benefits of mixtures in the initial years, species' proportions may shift away from the optimal region (Fig. 4C) over several years; however, adaptive farming management [e.g., oversowing or slot-seeding of seeds of declining species, rather than ploughing and reseeding, e.g., (45)] can direct grassland composition back to the optimal region. The insights provided by this study on the value of multispecies mixtures can incentivize longer-term agronomic and grazing studies as well as improved selection of forage-plant species and cultivars that are more persistent, maintain functional diversity over time, and improve synergistic interactions among species in mixtures.

Grassland production contributes greatly to livelihoods in rural regions and is threatened by the climate crisis (46, 47). Our study strengthens the evidence that legume-containing multispecies mixtures are an adaptation option for future climate scenarios (48, 49). Climate change also increases the incidence of extreme weather events, for which our results do not necessarily provide insight. However, multispecies mixtures can enhance yield resilience to weather-related stresses, including drought (11, 24, 50).

More diverse multispecies mixtures can generally deliver higher yields than either a grass monoculture with higher nitrogen input or a two-species grass-legume community. Overall, we highlight the strong potential of combining high-performing plants from different functional groups to enhance forage yields across a wide gradient of temperate climates and soil conditions while achieving reductions in nitrogen fertilizer inputs.

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SUPPLEMENTARY MATERIALS

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Materials and Methods; Figs. S1 to S9; Tables S1 to S4; References (52–61);
MDAR Reproducibility Checklist; Data S1