



TITLE: Nitrogen yield advantage from grass-legume mixtures is robust over a wide range of legume proportions and environmental conditions

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1 **Nitrogen yield advantage from grass-legume mixtures is robust over a wide range of**  
2 **legume proportions and environmental conditions**

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4 Running head: Nitrogen yield from grass-legume mixtures

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19

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21 replacement, climate change mitigation, climatic gradient, N uptake, protein, symbiotic N<sub>2</sub>

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23

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## 25 Abstract

26 Current challenges to global food security require sustainable intensification of agriculture  
27 through initiatives that include more efficient use of nitrogen (N), increased protein self-  
28 sufficiency through home-grown crops, and reduced N losses to the environment. Such  
29 challenges were addressed in a continental-scale field experiment conducted over three years,  
30 in which the amount of total nitrogen yield ( $N_{\text{tot}}$ ) and the gain of N yield in mixtures as  
31 compared to grass monocultures ( $N_{\text{gainmix}}$ ) was quantified from four-species grass-legume  
32 stands with greatly varying legume proportions. Stands consisted of monocultures and  
33 mixtures of two  $N_2$  fixing legumes and two non-fixing grasses.

34 The amount of  $N_{\text{tot}}$  of mixtures was significantly greater ( $P \leq 0.05$ ) than that of grass  
35 monocultures at the majority of evaluated sites in all three years.  $N_{\text{tot}}$  and thus  $N_{\text{gainmix}}$   
36 increased with increasing legume proportion up to one third of legumes. With higher legume  
37 percentages,  $N_{\text{tot}}$  and  $N_{\text{gainmix}}$  did not continue to increase. Thus, across sites and years,  
38 mixtures with one third proportion of legumes attained ~95% of the maximum  $N_{\text{tot}}$  acquired  
39 by any stand and had 57% higher  $N_{\text{tot}}$  than grass monocultures.

40 Realized legume proportion in stands and the relative N gain in mixture ( $N_{\text{gainmix}}/N_{\text{tot}}$  in  
41 mixture) were most severely impaired by minimum site temperature ( $R = 0.70$ ,  $P = 0.003$  for  
42 legume proportion;  $R = 0.64$ ,  $P = 0.010$  for  $N_{\text{gainmix}}/N_{\text{tot}}$  in mixture). Nevertheless, the relative  
43 N gain in mixture was not correlated to site productivity ( $P = 0.500$ ), suggesting that, within  
44 climatic restrictions, balanced grass-legume mixtures can benefit from comparable relative  
45 gains in N yield across largely differing productivity levels.

46 We conclude that the use of grass-legume mixtures can substantially contribute to resource-  
47 efficient agricultural grassland systems over a wide range of productivity levels, implying  
48 important savings in N fertilizers and thus greenhouse gas emissions and a considerable  
49 potential for climate change mitigation.

## 50 Introduction

51 Global food security is currently challenged by the increasing demands for food, including  
52 meat and milk, which arise through the continuing growth of the world's population and  
53 consumption (Godfray *et al.*, 2010; Smith & Gregory, 2013). At the same time, food  
54 production is significantly affected by competition between food, feed and bioenergy,  
55 demands from other economic sectors for land and water, and the need to maintain and  
56 preserve ecosystem services and biodiversity (Thornton, 2010). Moreover, current food  
57 production is highly nitrogen (N) limited (Cassman *et al.*, 2002), while the provision of  
58 industrial N is largely based on fossil energy and its multiple impacts on the environment  
59 (Galloway *et al.*, 2008; Davidson, 2009; Canfield *et al.*, 2010). Under a business-as-usual  
60 scenario, it must be assumed that any increase in food provision will further aggravate the  
61 pressure regarding the divergent demands for food security and environmental integrity  
62 (Tilman *et al.*, 2002; Foley *et al.*, 2005). This challenge has recently reinforced the need for  
63 more sustainable agriculture and sustainable intensification (Godfray *et al.*, 2010; Foley *et al.*,  
64 2011).

65

66 Sustainable intensification of agriculture aims to raise productivity while at the same time  
67 reduce its environmental impacts (Godfray *et al.*, 2010; Foley *et al.*, 2011; Lüscher *et al.*,  
68 2014; Taube *et al.*, 2014). In the face of the prevailing N limitation, rising costs of inorganic  
69 N fertilizers, and deleterious side-effects of excessive N application (Galloway *et al.*, 2008;  
70 Canfield *et al.*, 2010), increased sustainability and improved N self-sufficiency can be gained  
71 through home-grown N<sub>2</sub> fixing crops. Currently, grassland-based livestock production of  
72 medium to high management intensity depends largely on high-yielding pure grass stands  
73 requiring large inputs of mineral N fertilizers. Production and distribution of mineral N  
74 fertilizers need large amounts of energy (Kitani *et al.*, 1999) and their application can result in

76 environment (Schils *et al.*, 2013; Schmeer *et al.*, 2014). For example, each kg of N produced  
77 as ammonium nitrate in the industrial Haber-Bosch process consumes 58 MJ of energy and  
78 emits 8.6 kg CO<sub>2</sub> equivalents (Kitani *et al.*, 1999; Ecoinvent Centre, 2010). Moreover,  
79 according to the guidelines of the IPCC (2006), for every 100 kg of N fertilizer added to the  
80 soil, on average 1.0 kg of N is emitted as N<sub>2</sub>O, a GHG that is approximately 300 times more  
81 potent than CO<sub>2</sub>. At the same time, European livestock systems depend strongly on protein  
82 imported from overseas. A major challenge, therefore, is to increase home-grown forage  
83 protein with reduced input of mineral N fertilizers and at the same time reduce N losses to the  
84 environment (Peyraud *et al.*, 2009; Lüscher *et al.*, 2014; Taube *et al.*, 2014).

85

86 Grass-legume mixtures offer the benefit of symbiotic N<sub>2</sub> fixation by legumes, which are able  
87 to utilize atmospheric N<sub>2</sub> for their requirements and thereby produce more protein with less N  
88 input. The amount of symbiotic N<sub>2</sub> fixation by legumes can be substantial and ranges from  
89 100 to 380 kg ha<sup>-1</sup> year<sup>-1</sup> in northern temperate/boreal regions (Ledgard & Steele, 1992;  
90 Carlsson & Huss-Danell, 2003). Grown in mixtures with grasses, legumes meet their own N  
91 demand by deriving more than 80% from symbiosis (Boller & Nösberger, 1987; Zanetti *et al.*,  
92 1997; Nyfeler *et al.*, 2011) and consequently, the relative availability of soil N increases for  
93 grasses (“N sparing”, Temperton *et al.*, 2007). Besides symbiotic N<sub>2</sub> fixation, other processes  
94 have been found to increase yield and efficiency in resource uptake by grass-legume mixtures.  
95 These include facilitation, that is, N transfer from legumes to grasses (Høgh-Jensen &  
96 Schjoerring, 1997; Pirhofer-Walzl *et al.*, 2012; Rasmussen *et al.*, 2013) and increased  
97 exploitation of soil resources through spatial (deep- and shallow-rooting) or temporal niche  
98 complementarity in resource uptake (van Ruijven & Berendse, 2005; Mueller *et al.*, 2013).  
99 All of these processes can lead to considerable gains in N yield of mixtures compared to grass

100 monocultures; consequently, the use of such mixtures in agricultural grassland systems could  
101 allow substantial reductions in the application of industrial N fertilizers.

102

103 Inclusion of legumes in grassland management has been proposed as an important strategy for  
104 climate change mitigation in the agricultural sector (Smith *et al.*, 2008; Smith & Gregory,  
105 2013; Bustamante *et al.*, 2014). The use of grass-legume mixtures in temporary grassland is  
106 widely applicable and does not necessarily compromise harvest yield, thereby improving  
107 agricultural efficiency (Kirwan *et al.*, 2007; Finn *et al.*, 2013). Moreover, the application is  
108 practical and robust, as demonstrated over many years by the “Swiss Standard Mixtures” that  
109 use up to eight species of grasses and legumes in temporary grassland to improve legume  
110 persistence under various pedo-climatic conditions (Frey, 1955; Suter *et al.*, 2012). Grass-  
111 legume mixtures offer also a sustainable farm management practice: there is no evidence of  
112 significant emissions of N<sub>2</sub>O arising from the process of symbiotic N<sub>2</sub> fixation (Rochette &  
113 Janzen, 2005; Barton *et al.*, 2011) and thus, N<sub>2</sub> fixation has been removed as a direct source  
114 of N<sub>2</sub>O in the revised GHG guidelines of the IPCC (2006). Finally, mixing grasses with  
115 legumes is a mitigation measure that can be implemented in the near future, which is an  
116 important feature for meeting emission reduction targets (Smith *et al.*, 2013; Bustamante *et*  
117 *al.*, 2014).

118

119 Although amounts of total N yield and N from symbiotic sources have been quantified in  
120 temperate grassland (Ledgard & Steele, 1992; Carlsson & Huss-Danell, 2003), data from  
121 arctic or continental ecosystems are rare. In such environments, low winter temperatures  
122 and/or precipitation may hamper the legumes' growth, and accordingly, the benefit of  
123 including legumes in mixtures to achieve high gains in N yield may be limited. Absolute  
124 amounts of N from symbiosis seem to decrease at higher latitudes of Europe (Nesheim &

125 Oyen, 1994); however, there is no simple correlation between latitude and the N performance  
126 of legumes (Carlsson & Huss-Danell, 2003). Any large-scale geographic influence acts in  
127 concert with local pedo-climatic conditions that also interact with the type and intensity of  
128 management, all of which may strongly affect the legumes' growth.

129

130 The COST Action 852 entitled "Quality legume-based forage systems for contrasting  
131 environments" ([www.cost.eu/domains\\_actions/fa/Actions/852](http://www.cost.eu/domains_actions/fa/Actions/852)) aimed at increasing the  
132 quantity and quality of home-grown protein from regionally adapted legume-based forage  
133 systems. To this aim, a coordinated continental-scale field experiment was established, the  
134 "Agrodiversity Experiment", focusing on intensively managed, mown temporary grassland as  
135 a model system. Monocultures and mixtures comprised of two grasses (non N<sub>2</sub> fixing) and  
136 two legumes (N<sub>2</sub> fixing), and were set up to result in a broad range of legume proportion in  
137 stands (Kirwan *et al.*, 2007). Here, we report on the benefits of legumes to total N production  
138 in these grass, legume, and mixed swards by analyzing the total N yield ( $N_{\text{tot}}$ ) and realized  
139 legume proportion of swards, and the potential N yield gain in mixtures as compared to grass  
140 monocultures ( $N_{\text{gainmix}}$ ). To our knowledge, this is the first study to directly relate N yields in  
141 mixtures and monocultures to greatly differing stand legume proportions across largely  
142 contrasting pedo-climatic conditions, which will reveal the potential of these grassland  
143 systems for resource-efficient, sustainable agriculture through savings in N fertilizers and thus  
144 GHG emissions. The following specific questions were addressed:

- 145 1. Is  $N_{\text{tot}}$  consistently higher in grass-legume mixtures as compared to grass monocultures?
- 146 2. Is  $N_{\text{tot}}$  and  $N_{\text{gainmix}}$  affected by legume proportion in the sward? If yes, how much does the  
147 effect of legume proportion on  $N_{\text{tot}}$  and  $N_{\text{gainmix}}$  vary across sites?
- 148 3. Is the effect of legume proportion on  $N_{\text{tot}}$  and  $N_{\text{gainmix}}$  persistent over three years? Does  
149 legume proportion itself persist over time?

- 150 4. Are realized legume proportion,  $N_{tot}$  in mixture, and the relative N gain in mixture  
151 ( $N_{gainmix}/N_{tot}$  in mixture) related to climatic variables and to productivity levels across  
152 sites?



## 153 **Material and Methods**

### 154 *Experimental design*

155 At each of sixteen sites and following a common protocol, four monocultures and eleven  
156 mixtures were established at two levels of seed density for a total of 30 stands per site. The  
157 sites spanned a gradient of climate from Atlantic to continental and from temperate to arctic  
158 (Fig. 1, Table S1, Supporting Information). Regarding climate and productivity, mean annual  
159 temperature of the sixteen sites ranged between 4.2 and 10.9 °C, precipitation between 492  
160 and 1556 mm year<sup>-1</sup>, and productivity of aboveground biomass between 2 and 14 t DM ha<sup>-1</sup>  
161 year<sup>-1</sup> (see below). Full information to all sites is given in Kirwan *et al.* (2014), where  
162 geographic coordinates and further details on climate and soils are provided. The selection of  
163 species used for experimentation focused on i) species known to achieve high forage quantity  
164 and quality in systems of intensive grassland management, and ii) functional types of species  
165 that were expected to maximise complementarity in resource use. Four functional types of  
166 species were initially defined as the factorial combination of traits being associated with the  
167 manner of N acquisition (non N<sub>2</sub> fixing grasses *vs.* N<sub>2</sub> fixing legumes), and temporal pattern  
168 of species' growth (fast establishment *vs.* temporally persistent), resulting in the following set:  
169 fast establishing grass (G1), temporally persistent grass (G2), fast establishing legume (L1),  
170 temporally persistent legume (L2). These four functional types were consistent across all  
171 sixteen sites although the species selected at sites varied depending on geographical region  
172 (Table 1).

173

174 The four monocultures consisted of one of each of G1, G2, L1, or L2, and mixtures contained  
175 all four functional types of species in varying relative abundances following a simplex design  
176 (Cornell, 2002). This was achieved by systematically varying the sown species proportions in  
177 mixtures (Table S2, Supporting Information) to result in four mixtures dominated in turn by

178 one species (70% of one species, 10% of the three others), six mixtures co-dominated in turn  
179 by pairs of species (40% of each of two species, 10% of the two others), and one mixture with  
180 equal species proportions (25% of each species). Doing so, sown legume proportions across  
181 different communities were 0, 20, 50, 80, and 100% (see also Kirwan *et al.*, 2007 for further  
182 details on the design).

183

#### 184 *Maintenance and measurements*

185 Plots were fertilized with commercial N fertilizer. The amount was constant for all plots and  
186 for all years of experimental duration within individual sites, but varied among sites to range  
187 from 0 to 150 kg N ha<sup>-1</sup> year<sup>-1</sup> (Table S1, Supporting Information). This range of N  
188 application reflected background productivity levels across the large climatic gradient as well  
189 as variations in types and fertility of soils. Note that the maximum level of 150 kg N ha<sup>-1</sup> year<sup>-1</sup>  
190 is far below highest levels of N application to grass monocultures in intensive grassland  
191 management and that N application of up to 150 kg ha<sup>-1</sup> year<sup>-1</sup> did not impair positive grass-  
192 legume interactions at one of the study sites (Nyfeler *et al.*, 2009). Background levels of  
193 phosphorus and potassium in experimental plots were adjusted to non-limiting amounts  
194 (Kirwan *et al.*, 2014). Aboveground biomass of plots was harvested several times per year  
195 following the agronomic practice at each site (Table S1, Supporting Information), and  
196 representative subsamples of harvested yield were sorted into the four sown and pooled  
197 unsown species. Drying to constant weight and summing over harvests allowed computing of  
198 the total harvested dry mass per year and species' fractional contributions. Importantly,  
199 measurements were only recorded in the first three full years of production in this temporary  
200 grassland; thus, the sowing year was not considered. This restriction was imposed to evaluate  
201 the fully established system.

202

203 *Record of climatic data*

204 Data on climatic conditions were monitored by weather stations at each of the sixteen sites.  
205 Daily precipitation was summed and mean daily temperature was averaged across all days of  
206 the year to give annual precipitation and mean annual temperature per site. Moreover, daily  
207 minimum and maximum temperatures were used to compute the annual minima and maxima  
208 per site as the average of the ten days with most extreme values. This was of specific interest  
209 because extreme temperatures are assumed to especially impair legumes' growth and N<sub>2</sub>  
210 fixation (Lynch & Smith, 1993; Zhang *et al.*, 1995).

211

212 *Analysis of N concentration in plant material*

213 Nitrogen concentration ( $N_{\text{conc}}$ ) was measured at each harvest from a representative subsample  
214 of total harvested biomass from each plot, with biomass being dried to constant weight at 65  
215 °C and ground to pass through a 1 mm sieve. The value of  $N_{\text{conc}}$  of all samples was  
216 determined by near-infrared reflectance spectroscopy (NIRS) at one lab (Christian-Albrechts  
217 University, Kiel, Germany) using a NIRSystems 5000 monochromator (FOSS, Silver Spring,  
218 USA). See Appendix A, Supporting Information, for validation of the NIRS method. For a  
219 subset of sites,  $N_{\text{conc}}$  was not measured at all three experimental years and from all plots of the  
220 design (see Table S1, Supporting Information). Note that temporary grassland in crop rotation  
221 systems is generally maintained for a sowing year and one or two subsequent production  
222 years. Here, we measured  $N_{\text{conc}}$  for two production years for most sites (year 1 and 2), while  
223 half the sites also had data for a third year (year 3), resulting in a total of 350, 304, and 167  
224 analyzed plots in years 1, 2, and 3, respectively. Importantly, included sites covered the full  
225 pedo-climatic range in all years. There were a total of 36 site-years of data.

226

227 *Data analyses*

228 We aimed to analyze the total annual N yield ( $N_{tot}$ ) per plot. To achieve this, values of  $N_{conc}$  of  
 229 each harvest were first multiplied by total dry matter per harvest to receive the N yield per  
 230 harvest and plot. Annual  $N_{tot}$  per plot was then computed as the sum over all harvests and  
 231 reflects therefore an N output of the system that is seasonally weighted for variability in  $N_{conc}$   
 232 and biomass yield.

233

234 *i) Comparisons of mixtures against grass monocultures*

235 Annual  $N_{tot}$  of mixtures was initially compared against  $N_{tot}$  of the average *grass* monoculture,  
 236 as this comparison reflected a test of mixed stands (all of which included legumes) against  
 237 stands sown only with grass species. Wilcoxon rank sum tests were applied for inference.  
 238

239 *ii) Testing the effect of legume proportion on total nitrogen yield*

240 The effect of legume proportion ( $PL_{eg}$ ) the on annual  $N_{tot}$  was analyzed applying a regression  
 241 approach following Nyfeler *et al.* (2011), which used the following basic notation:

$$242 N_{tot} = f_{l_0} + p_1 P_{,eg} + p_2 PL_{eg}^2 + p_3 PL_{eg}^3 + aD + y\Delta Biomass + E \quad \text{eqn. 1}$$

243 In this formulation,  $PL_{eg}$  denotes the summed proportions of the two legumes L1 and L2,  
 244 using the harvested biomass proportions of the previous year (sown proportions were used for  
 245 year 1) as predictor variables to overcome confounding effects of year-to-year changes in  
 246 community composition on  $N_{tot}$ . Thus,  $N_i$  to )63 estimate the linear and non-linear effects of  
 247 stand legume proportion on  $N_{tot}$ . The intercept,  $P_0$ , gives the estimate of  $N_{tot}$  if  $PL_{eg} = 0$ , i.e.  
 248 for grass monocultures including potential weeds. The effect of seed density is estimated by  
 249  $a$ , with  $D$  denoting the level of seed density coded as -1 and +1 for low and high density,  
 250 respectively, so all other terms are estimated at average density. The effect of fluctuations in  
 251 stand biomass (*DeltaBiomass*) on the response variable is estimated by the  $y$  coefficient (for

252 details on computation of  
*DeltaBiomass* see Appendix A, Supporting Information). The error  
253  $E$  is assumed normally distributed with zero mean and variance  $a^2$ .  
254  
255 Equation 1 was extended to a linear mixed model where all coefficients were estimated for  
256 each of three years, and where random coefficients were added to estimate the general stand  
257 performance while allowing for variation across sites for each of the three years (Pineiro &  
258 Bates, 2009) (see eqn. S1, Appendix A, Supporting Information, for detailed notation).  
259 Inference on fixed main effects of the linear mixed model was based on single term deletion  
260 from a main effects model as specified in eqn. 1 (each effect in turn, including  $\sigma^2$  and  
261  $\sigma_{eg}^3$ ) and subsequent  $F$ -tests for comparison of models while applying the Kenward-Roger  
262 method to determine the approximate denominator degrees of freedom (Kenward & Roger,  
263 1997); interactions were similarly tested but from a model that included all effects. The range  
264 of legume proportion for which  $N_{tot}$  was significantly different from its maximum was  
265 computed using the Johnson Neyman technique (Johnson & Neyman, 1936) as applied in  
266 Suter *et al.* (2007).  
267  
268 In regressions of eqs. 1 and S1 (Appendix A, Supporting Information), species G1, G2, L1,  
269 and L2 are grouped into grasses and legumes according to their functional ability to fix  $N_2$  (or  
270 not), and such analysis does not include interactions between species regarding temporal  
271 pattern of species' growth. This grouping was justifiable because preliminary analyses  
272 revealed that interactions affecting  $N_{tot}$  were mainly between non-fixing grasses and  $N_2$  fixing  
273 legumes, but to a far lesser extent between fast establishing and temporally persistent species  
274 within grasses and legumes (see Appendix A, Supporting Information, for details and  
275 inference on pooling individual species performances).  
276

277     iii) *Computing gain of N yield in mixture*

278 Total N yield in mixed grass-legume stands and the respective monocultures can be  
279 subdivided into different parts to illustrate the role of legumes and their interactions with  
280 grasses. In grass monocultures (legume proportion = 0),  $N_{\text{tot}}$  accumulated by the sward  
281 derives solely from the soil and from fertilizer N (quantity A, dashed white line for reference,  
282 Fig. 2). In legume monocultures (legume proportion = 1),  $N_{\text{tot}}$  is often greater (A + B), due  
283 mainly to symbiotic  $N_2$  fixation of legumes. Mixing grasses with legumes should lead  
284 therefore at first to a linear increase of accumulated N by the stand with increasing mixture  
285 legume proportion through the contribution of the legume component, i.e. through a constant  
286 amount of N added per unit of legumes (continuous white line). In addition, positive grass-  
287 legume interactions such as stimulation of the rate of symbiotic  $N_2$  fixation (% N derived  
288 from symbiosis in the legume) when grass is present (Nyfeler *et al.*, 2011), N transfer (Høgh-  
289 Jensen & Schjoerring, 1997; Zanetti *et al.*, 1997) or increased utilization of fertilizer and soil  
290 N resources through temporal and/or spatial niche complementarity (Mueller *et al.*, 2013) can  
291 result in a nonlinear surplus of N yield (C, Fig. 2), resulting in a total N yield of A+B+C in  
292 mixed swards.

293

294 This study aimed to quantify the amount of N yield gain in mixtures as compared to grass  
295 monocultures. This was achieved by computing the difference between  $N_{\text{tot}}$  in mixture and  
296  $N_{\text{tot}}$  in grass monoculture using the estimates of the regression model (based on eqn. S1,  
297 Appendix A, Supporting Information). This quantity of N yield gain in mixture is hereafter  
298 termed  $N_{\text{gainmix}}$  (=  $N_{\text{tot}}$  in mixture minus  $N_{\text{tot}}$  in grass monoculture, quantity B + C in Fig. 2),  
299 and was calculated both for the mean across all sites (using the fixed parameter estimates of  
300 the regression) and for each of the sixteen individual sites (using the variation around the  
301 fixed mean). Note that the quantity of  $N_{\text{gainmix}}$  reflects the total of N gain in mixture compared

302 to grass monoculture, but it does not allow quantification of the individual contributions from  
303 symbiotic N<sub>2</sub> fixation, N transfer, more efficient exploitation of soil and fertilizer N, or N  
304 from any other source (e.g. decaying roots of legumes).

305

306 *iv) Relating N data to productivity and climate*

307 To evaluate patterns in N dynamics over the environmental gradient, realized legume

308 proportion,  $N_{\text{tot}}$  in mixture, and the relative N yield gain in mixture ( $N_{\text{gainmix}}/N_{\text{tot}}$  in mixture)

309 were related to site productivity, annual precipitation, mean annual temperature, and

310 minimum and maximum temperature. To increase the robustness of results, these calculations

311 were based on site means across the first two experimental years. For consistent comparison,

312 both  $N_{\text{tot}}$  in mixture and the relative N gain were calculated for a mixture with one third

313 proportion of legumes and two thirds of grasses using a simplified linear mixed model (eqn.

314 S2, Appendix A, Supporting Information). The ratio of proportions (1/3:2/3 legumes:grasses)

315 could be justified from analyses that regressed  $N_{\text{tot}}$  on legume proportion (see results), and

316 amounts of total N yield for this representative mixture are hereafter termed  $N_{\text{totmix}}$ . Site

317 productivity was estimated by averaging across the biomass yields of all grass monocultures.

318 Pearson correlation and ordinary least squares regression were used to quantify relationships

319 between legume proportion,  $N_{\text{totmix}}$ , the relative N yield gain in mixture ( $N_{\text{gainmix}}/N_{\text{totmix}}$ ), and

320 environmental variables (predictors) (see Table S4, Supporting Information, for site values of

321 the five environmental variables, and Table S5 for their correlation matrix). All analyses were

322 performed with the statistics software R (R Development Core Team, 2014).

## 323 Results

### 324 *Positive effect of grass-legume mixtures on total N yield*

325 Annual  $N_{\text{tot}}$  of mixtures was significantly greater than that of grass monocultures at the  
326 majority of evaluated sites in all years (Fig. 3), and approximated the values of  $N_{\text{tot}}$  in legume  
327 monocultures. In general, the positive mixture effect on  $N_{\text{tot}}$  was already evident in the first  
328 year and persisted over years (Fig. 3); however, no mixture effect on  $N_{\text{tot}}$  was apparent at the  
329 continental sites Lithuania\_a (site 18; years 1 and 2), and Poland\_a and Poland\_b (sites 26  
330 and 27, all years).

331

### 332 *Positive effect of legume proportion on total N yield and N yield gain in mixture*

333  $N_{\text{tot}}$  was significantly affected by the proportion of legumes in mixtures. The effects of  
334 legume proportion on  $N_{\text{tot}}$  were similar across years and were maximal when legume  
335 proportions in mixtures were 60, 42, and 40% in years 1, 2, and 3, respectively (Fig. 4). More  
336 importantly, because  $N_{\text{tot}}$  was non-linearly affected by legume proportion (Table 2, Table S6,  
337 Supporting Information),  $N_{\text{tot}}$  was not significantly different from the maximum amount in  
338 mixture over a wide range of legume proportion (Fig. 4), and mixtures with approximately  
339 one third proportion of legumes attained 95% of maximum  $N_{\text{tot}}$  (Table 3).  $N_{\text{tot}}$  varied  
340 substantially among sites, with greatest (predicted) values in mixtures being as high as 480 kg  
341  $\text{N ha}^{-1} \text{ year}^{-1}$  at Ireland (site 15, year 1) and Switzerland (site 34, year 2), and smallest values  
342 in Iceland (sites 13 and 14: around 50  $\text{kg N ha}^{-1} \text{ year}^{-1}$ ; Fig. 4).

343

344 Because mixtures with approximately one third proportion of legumes attained 95% of the  
345 maximum  $N_{\text{tot}}$  (Table 3), further values for  $N_{\text{tot}}$  <sup>(and  $N_{\text{gainmix}}$ , below)</sup> were estimated for this  
346 representative mixture (1/3:2/3 legumes:grasses). Doing so,  $N_{\text{totmix}}$  decreased by 12% between



347 years 1 and 2 ( $P = 0.09$  for the difference in  $N_{\text{totmix}}$ ), and by a further 23% between years 2  
348 and 3 ( $P = 0.002$ ).

349

350 Average annual values of  $N_{\text{gainmix}}$  across sites ranged between 108 and 76  $\text{kg ha}^{-1} \text{ year}^{-1}$  (years  
351 1 and 3, respectively), resulting in a relative N gain in mixture ( $N_{\text{gainmix}}/N_{\text{totmix}}$ ) that exceeded  
352 0.3 in all years (Table 3). Again, there was a large variation of  $N_{\text{gainmix}}$  among sites with  
353 amounts being as high as 280  $\text{kg ha}^{-1} \text{ year}^{-1}$  at Norway\_a (site 22, year 1) but being close to  
354 zero at the continental sites Lithuania\_a (site 18, year 1), Poland\_a, and Poland\_b (sites 26  
355 and 27, all years), suggesting that at these sites legumes in mixtures induced no gain in total N  
356 yield (Fig. 4). Compared to grass pure stands and averaged across sites,  $N_{\text{gainmix}}$  was 61, 46,  
357 and 64% in years 1, 2, and 3, respectively (calculated from Table 3), meaning that mixtures  
358 with only one third proportion of legumes had, on average across all years, 57% higher total  
359 N yield than pure grass stands.

360

### 361 *Declining stand legume proportion over time*

362 Realized legume proportion in stands decreased generally over time: across sites, realized  
363 legume proportion was 32, 21, and 9% in years 1, 2, and 3, respectively. Regarding site  
364 variation, almost the full possible range was covered: over all swards with highly varying  
365 legume proportion in the seed mixture, Norway\_a (site 22), Wales\_a (35), and Switzerland  
366 (34) still had average legume proportions around 50% and maximal values exceeding 80% by  
367 year 2; in contrast, legumes were absent from the second year onward in Ireland (15) and had  
368 disappeared by the third year in Lithuania\_c (20) (Fig. 4).

369

370 *Strong effect of minimum site temperature on legume proportion and N yield gain in mixture*

371 Out of the environmental variables tested, minimum site temperature most strongly impaired  
372 both stand legume proportion and the relative N yield gain in mixture (Fig. 5a & c), indicating  
373 that harsh environmental conditions hampered legumes' growth (see Table S4, Supporting  
374 Information, for site values of  $N_{\text{totmix}}$  and  $N_{\text{gainmix}}$ , and Table S7 for a summary of all  
375 regressions). Although to a weaker degree, low annual precipitation also impacted on both  
376 stand legume proportion and  $N_{\text{gainmix}/N_{\text{totmix}}}$  (Fig. 5b & d). The parallel responses of legumes'  
377 growth and relative N yield gain in mixture to these environmental parameters suggested that  
378 legume proportion and  $N_{\text{gainmix}/N_{\text{totmix}}}$  were correlated to each other, which indeed was the  
379 case (Fig. 5e). Furthermore,  $N_{\text{totmix}}$  was positively correlated to site productivity ( $R = 0.703$ ,  $P$   
380  $= 0.003$ , Table S7, Supporting Information); however, the relative N yield gain in mixture was  
381 not (Fig. 5f). Taken together, this suggested that the relative N gain of a balanced grass-  
382 legume mixture was affected more by climatic conditions than by the largely differing  
383 productivity levels across sites.

## 384 Discussion

385 Mixing grasses and legumes in agricultural grassland systems yielded considerable benefits to  
386 total N yield ( $N_{\text{tot}}$ ) over a wide range of European environmental conditions. Averaged across  
387 sites and years, mixtures with only one third proportion of legumes had as much as 57%  
388 higher  $N_{\text{tot}}$  than pure grass stands, indicating a substantial N yield gain in mixtures ( $N_{\text{gainmix}}$ ),  
389 and such mixtures attained amounts of  $N_{\text{tot}}$  close to the maximum  $N_{\text{tot}}$  acquired by any stand.

390 The relative N yield gain in mixture ( $N_{\text{gainmix}}/N_{\text{totmix}}$ ) was not affected by the great differences  
391 in productivity levels across sites. This clearly highlights the potential of grass-legume  
392 mixtures as a practical management option for sustainable agriculture, and we elaborate on a  
393 number of relevant points below.

394

### 395 *Substantial N yield advantage in mixtures as compared to grass monocultures*

396 Amounts of  $N_{\text{tot}}$  from the temperate sites of this study corresponded well to reports from  
397 comparable grass-legume systems (Boller & Nösberger, 1987; Høgh-Jensen & Schjøerring,  
398 1997; Carlsson & Huss-Danell, 2003 for review); however, this experiment also covered  
399 pedo-climatic regions that have been poorly investigated so far. At the majority of sites, the  
400 positive mixture effect on  $N_{\text{tot}}$  appeared in the first year, was maintained for the second year  
401 and was still apparent in five out of eight evaluated sites in the third year. In particular, the  
402 positive mixture effect on  $N_{\text{tot}}$  was apparent also at the Nordic sites in Iceland (sites 13 and  
403 14) and Tromsø-Norway (site 23), where absolute amounts of  $N_{\text{tot}}$  (Fig. 3) and harvested  
404 biomass yield (Finn *et al.*, 2013) were comparably small. Highest N advantages of mixtures  
405 *versus* grass monocultures were more than 250 kg N ha<sup>-1</sup> year<sup>-1</sup> (Belgium (site 1), Switzerland  
406 (site 34), Fig. 3a) and resulted in a more than two-fold higher N output in mixed swards than  
407 in stands with grass only. Considering the substantial differences in site productivity, ranging  
408 between 2 and 14 t DM ha<sup>-1</sup> year<sup>-1</sup>, and the substantial gain of  $N_{\text{tot}}$  in mixtures across sites and

409 years, the data clearly demonstrate the great potential of mixed grass-legume swards for  
410 resource-efficient production in varying pedo-climatic conditions. Using mixed swards  
411 instead of pure grass stands, more N yield can be expected for a given amount of N fertilizer  
412 applied. Alternatively, if the aim is to reduce fertilizer N application for financial, regulatory  
413 and/or environmental reasons (Godfray *et al.*, 2010), our data show the potential to do so  
414 without necessarily compromising N yield and total harvested biomass (Nyfeler *et al.*, 2009;  
415 Finn *et al.*, 2013).

416

417 *N yield gain in mixtures is evident over a wide range of legume proportion*

418 With few exceptions,  $N_{tot}$  demonstrated a considerable increase with increasing legume  
419 proportion up to about 30%. With higher percentages of legumes,  $N_{tot}$  did not continue to  
420 increase (Fig. 4). This is a highly valuable result. It indicates that almost all (~95%) of the  
421 maximum benefit to  $N_{tot}$  from mixing grasses and legumes can be achieved with a modest  
422 (~30%) legume proportion in the mixture. Moreover and equally relevant, the wide range for  
423 which  $N_{tot}$  was not different from maximum values indicates that the benefits of legumes  
424 regarding N gain in mixtures can prevail despite considerable fluctuations of grass-legume  
425 proportions in swards. This is an important feature for practical grassland management.

426

427 The method to compute  $N_{gainmix}$  has been previously used to calculate the amount of apparent  
428 symbiotic  $N_2$  fixation ( $N_{sym}$ ) in grass-legume mixtures (“N-difference method“, e.g. Ledgard  
429 & Steele, 1992). The N-difference method has been criticized because it assumes that the  
430 cumulative uptake of N from soil and fertilizer of grasses and legumes in mixture would be  
431 the same as for the reference grass monoculture, which may not necessarily be the case  
432 (Boller & Nösberger, 1987; Nyfeler *et al.*, 2011). The N-difference method therefore can lead  
433 to biased estimates of  $N_{sym}$ . At the Swiss site of our study, Nyfeler *et al.* (2011) used the more

434 accurate  $^{15}\text{N}$  dilution procedure to quantify  $N_{\text{tot}}$ ,  $N_{\text{sym}}$  and N uptake from non-symbiotic  
435 sources (N derived from the soil and/or fertilizer). They demonstrated that, for a 50:50  
436 mixture of grasses and legumes and fertilizer N application of  $50 \text{ kg ha}^{-1} \text{ year}^{-1}$ , the N-  
437 difference calculation resulted in  $N_{\text{sym}}$  values that were 26% higher than the  $^{15}\text{N}$  dilution  
438 procedure in the first production year, while overestimation was 24% in the second year. For  
439 N applications of  $150 \text{ kg ha}^{-1} \text{ year}^{-1}$ , overestimation by the N-difference method became  
440 smaller, and was 17% and 7% in the first and second year, respectively. In Northern mixed  
441 grassland (Vågønes, Norway,  $69^\circ\text{N}$ ) of *Trifolium repens* L. (white clover) and *Phleum*  
442 *pratense* L. (timothy) and no fertilizer N application, the overestimation of the N-difference  
443 method compared to  $^{15}\text{N}$  dilution was 7% and 36% in two production years, respectively  
444 (Nesheim & Oyen, 1994). Regarding our continental-scale experiment,  $N_{\text{gainmix}}$  corresponds to  
445 values of the N-difference calculation. It is important to realize that this difference method is  
446 appropriate to the purpose used here, namely to measure the total N gain achieved by the  
447 grass-legume mixture compared to the pure grass stand. However, because it appears that this  
448 difference method does not allow quantification of symbiotic  $\text{N}_2$  fixation and N uptake from  
449 non-symbiotic sources with acceptable accuracy, we do not aim to specify these different  
450 fractions. Nevertheless, the above assessment suggests that the great majority of  $N_{\text{gainmix}}$  is  
451 derived from  $\text{N}_2$  fixation of legumes (see also Boller & Nösberger, 1987; Zanetti *et al.*, 1997).  
452

453 This gain of N yield in mixtures due to the presence of legumes can be explained by various  
454 mechanisms. A linear increase in  $N_{\text{tot}}$  with increasing legume proportion can be expected  
455 solely through a constant additional N input to the system per unit of legumes (see B in Fig.  
456 2). However, because we found a highly nonlinear response of  $N_{\text{tot}}$  (Fig. 4, C in Fig. 2),  
457 positive interactions between grasses and legumes must have played a role, and we suggest  
458 four possible mechanisms. First, positive mixing effects have been attributed to stimulation of

459 the rate of symbiotic N<sub>2</sub> fixation by N-demand from co-occurring grasses (Høgh-Jensen &  
460 Schjoerring, 1997). At the Swiss site, Nyfeler *et al.* (2011) revealed that the rate (%) of N  
461 derived from symbiosis in the clover plants was significantly higher at low legume proportion  
462 than at high legume proportion. This pattern could be explained by strong competition from  
463 grasses for N from non-symbiotic sources: even if the grass component in mixture was  
464 reduced to 50%, grass still took up the same total amount of N from non-symbiotic sources as  
465 a stand with 100% grass (Nyfeler *et al.*, 2011). Second, the presence of both grass and legume  
466 components in mixture allows for transfer of symbiotically fixed N from legumes to grasses.  
467 Quantification of apparent N transfer between legumes and grasses shows that more than 40%  
468 of the N nutrition of the grass component in mixture can derive from N<sub>2</sub> fixation of the  
469 legumes (Boller & Nösberger, 1987; Høgh-Jensen & Schjoerring, 1997; Nyfeler *et al.*, 2011)  
470 with N transfer being greatest in equilibrated mixtures. Third, the nonlinear response of N<sub>tot</sub>  
471 can be attributed to an increased capability of the grass component to acquire N from non-  
472 symbiotic sources in the presence of legumes (Nyfeler *et al.*, 2011). Grasses have a denser  
473 root system (i.e. comparably higher root length density per unit soil volume and high root  
474 surface area of active absorption, Craine *et al.*, 2002; Hill *et al.*, 2006), which they use to  
475 outcompete the legumes in accessing the available mineral and fertilizer N. Fourth, increased  
476 utilization of fertilizer and soil N resources through temporal and/or spatial niche  
477 complementarity between grasses and legumes can also explain the positive mixing effects  
478 (van Ruijven & Berendse, 2005; Mueller *et al.*, 2013). To conclude, the benefit of mixed  
479 swards to total N output of the system comes through significant grass-legume interactions.  
480  
481 Considerable mixing effects on total harvested biomass yield have recently been shown for  
482 the same sites as presented here (Finn *et al.*, 2013). Finn *et al.* (2013) found transgressive  
483 overyielding (mixtures outperformed highest yielding monocultures, Trenbath, 1974) of about

484 20% (excluding weed biomass), indicating that mixtures produced more biomass than either  
485 grass *or* legume monocultures. To maximise both total N output and total biomass yield,  
486 while at the same time minimising the risk of N losses to the environment (Loiseau *et al.*,  
487 2001; Nyfeler, 2009), we infer that an optimal range of legume proportion in mixtures should  
488 range between 30-50%.

489

490 *Legume proportion and N yield gain in mixtures are affected by environmental conditions*

491 We found evidence that legume proportion and the relative N yield gain in mixture were  
492 negatively correlated with minimum winter temperatures and low annual precipitation (Fig.  
493 5). Much of the evidence on restrictions of legumes' growth and symbiotic N<sub>2</sub> fixation comes  
494 from studies in growth chambers (e.g. Nesheim & Boller, 1991; Lynch & Smith, 1993; Zhang  
495 *et al.*, 1995; Serraj & Sinclair, 1996). Low temperature seems to hamper N<sub>2</sub> fixation more  
496 than plant growth (Lynch & Smith, 1993; Zhang *et al.*, 1995; Hartwig, 1998), and severe  
497 drought has been shown to disrupt nodule activity (Serraj & Sinclair, 1996; Serraj *et al.*,  
498 1999). Such results accord with our findings gained under field conditions. However, because  
499 we did not directly measure symbiotic activity of legumes but demonstrated impacts of  
500 climate on legume proportion, we cannot conclude whether harsh climatic conditions  
501 impacted more on symbiotic N<sub>2</sub> fixation or on legume plant growth.

502

503 A decrease in the legume proportion of mixed swards can be induced by application of N  
504 fertilizers. For example, in a related experiment (Nyfeler *et al.*, 2009), different levels of  
505 mineral N were applied to grass-clover mixtures (two grass and two clover species) managed  
506 for three consecutive years. Here, N fertilization significantly affected clover proportion:  
507 averaged across years and all mixtures receiving 50 kg N ha<sup>-1</sup> year<sup>-1</sup>, the two clover species  
508 (sum of both) achieved 41% proportional biomass; however, fertilized with 150 kg N ha<sup>-1</sup>

509 year<sup>-1</sup>, clover proportions were only 31% (Nyfeler *et al.*, 2009). Comparable results were  
510 found by Nassiri & Elgersma (2002), where in only one growing season the application of  
511 150 kg N ha<sup>-1</sup> year<sup>-1</sup> reduced the clover content in dry matter harvest of perennial ryegrass-  
512 white clover mixtures to 12% compared to 43% without N application. Such impacts of N  
513 fertilizers on legume persistence might be of less importance in temporary grassland as part of  
514 crop rotation systems, where swards are generally maintained for a seeding year and one or  
515 two production years before they are ploughed for growing cereal crops. Moreover, although  
516 legumes might decrease over time, their positive effect on mixture biomass yield can still be  
517 prevalent even at low proportion (Nyfeler *et al.*, 2009; Finn *et al.*, 2013), which can be  
518 explained by strong grass-legume interactions as outlined above or by legacy effects (e.g.  
519 release of fixed N from decaying roots or increased N pools in soil organic matter derived  
520 from N<sub>2</sub> fixation of preceding years).

521

522 In our experiment, positive effects of legumes on N<sub>tot</sub> were still evident in year two and three  
523 (Fig. 3) despite the successive decrease in legume proportion, and mixtures with only one  
524 third proportion of legumes provided a significant gain in N yield as compared to pure grass  
525 stands (Fig. 4). This proves our experiment with sown temporary grassland to be a good  
526 model system to study the relation between legume proportions realized in the sward and N  
527 yield gain in mixtures, and this relation holds also for permanent grassland as long as  
528 adequate proportions of legumes can be maintained. Yet, the persistence of legumes in  
529 permanent grassland is challenging (Guckert & Hay, 2001), and our study implies that  
530 research should focus on sward management strategies to stabilize legume proportion under  
531 varying climatic conditions. Evidence suggests that adjusting N fertilizer rates and defoliation  
532 frequency can increase the abundance of white clover (Schwank *et al.*, 1986; Hebeisen *et al.*,  
533 1997; Lüscher *et al.*, 2014), the most important legume species in permanent grassland of



534 temperate regions (Guckert & Hay, 2001). In our experiment, no specific actions were  
535 undertaken to counteract legume decrease. Also, realized legume proportion was not  
536 correlated to N application rates at sites ( $R = -0.219$ ,  $P = 0.434$ ), indicating that  
537 environmental conditions were more decisive for legume performance than N fertilizers at  
538 rates applied in our experiment.

539

540 Four sites merit further consideration. At the three continental sites Lithuania\_a, Poland\_a,  
541 and Poland\_b, legumes established well in the first year and reached proportions of up to  
542 96%; nevertheless, N gains in mixture were not evident (Fig. 4a) suggesting that symbiotic N<sub>2</sub>  
543 fixation of the clovers did not work properly. We suspect that in concert with low winter  
544 temperatures hampering nodulation (Hartwig, 1998), lack of *Rhizobia* species in soils of the  
545 agronomically improved grassland may have prevented legumes to form an efficient  
546 symbiotic relationship. The situation is different for Ireland, where legumes were present and  
547 mixtures realized high gains of total N yield in the first year, but legumes disappeared from  
548 the second year onward. With minimum temperatures of  $-1^{\circ}\text{C}$ , annual precipitation around  
549 the mean of the investigated range ( $932\text{ mm year}^{-1}$ ), and comparably high site productivity  
550 ( $14.1\text{ t DM ha}^{-1}\text{ year}^{-1}$ ), climatic conditions cannot be responsible for the strong legume  
551 decline. Because the Irish site had been a highly fertilized monoculture of *L. perenne*  
552 grassland for many years preceding the experiment, we speculate that the very high soil  
553 fertility and resulting high competition from the grasses during the experiment may have  
554 hampered the legumes' growth (Schwank *et al.*, 1986). Also, Ireland had a soil pH of 5.3,  
555 being the lowest among sites (Kirwan *et al.*, 2014), which may have hampered the growth of  
556 the two legume species.

557

558 Taken together, it can be concluded that climatic conditions and the natural presence of  
559 *Rhizobia* species define a boundary for the performance of legumes, in particular for the  
560 species used in this experiment. Our results indicate the need for specific research on how  
561 legumes can be maintained in mixed swards under varying and extreme climatic conditions.  
562 Besides the inoculation of soil with *Rhizobia* species and the optimization of sward  
563 management through adjusted N fertilizer inputs and defoliation frequencies, research should  
564 focus on breeding of adapted cultivars and selection of different legume species that would  
565 withstand low winter temperatures and/or severe drought.

566

567 *Wider implications: Legumes as a key contributor to sustainable intensification of grassland*  
568 *across largely differing productivity levels*

569 One important result of this study is that, although  $N_{\text{tot}}$  in mixture was strongly affected by  
570 site productivity, the relative N yield gain in mixture was not (Fig. 5f). This means that less  
571 productive sites as well as more productive sites can equally profit from grass-legume  
572 mixtures to increase N output. Adaptation of legumes to differing productivity levels can be  
573 explained by the concept of “N<sub>2</sub> fixation regulation by demand” (Hartwig, 1998; Soussana &  
574 Tallec, 2010). Following Hartwig (1998), the degree of symbiotic N<sub>2</sub> fixation of legumes is  
575 controlled by a series of eco-physiological triggers and N feedback mechanisms from the  
576 individual plant to the ecosystem level, with N<sub>2</sub> fixation of legumes being largely regulated by  
577 the N sink strength (N-demand) of the whole system (Hartwig, 1998; Soussana & Tallec,  
578 2010; Lüscher *et al.*, 2011). It has been shown repeatedly that, under low to medium N  
579 fertilizer supply ( $< 100 \text{ kg N ha}^{-1} \text{ year}^{-1}$ ), legumes in a balanced mixture with grasses acquire  
580 the large majority of their N nutrition through symbiotic N<sub>2</sub> fixation (Nesheim & Oyen, 1994;  
581 Høgh-Jensen & Schjoerring, 1997; Nyfeler *et al.*, 2011). In contrast, as amounts of N  
582 fertilizer increase, a decline of N acquired from symbiosis in legumes has been demonstrated

584 studies and mathematical models (Soussana *et al.*, 2002; Soussana & Tallec, 2010). Such  
585 propensity to buffer N supply can also be an advantage for protecting water quality as long as  
586 N fertilizer application is not too high, and provided that the grass component in mixture is  
587 sufficiently large (> 30%) so that nitrate absorption can occur before the water leaches out of  
588 the root zone (Eriksen *et al.*, 2004). Indeed, with high application of N fertilizers (> 400 kg N  
589 ha<sup>-1</sup> year<sup>-1</sup>) or high legume proportion in swards (> 70%), considerable N leaching can be  
590 observed (Loiseau *et al.*, 2001; Ledgard *et al.*, 2009; Nyfeler, 2009). Thus, there are at least  
591 three reasons why N losses from legume-based grassland systems should be lower than from  
592 fertilized grass systems: (i) nitrogen is fixed symbiotically within the legume nodules and thus  
593 is not freely available in the soil in a reactive form, (ii) symbiotic N<sub>2</sub> fixation activity is down-  
594 regulated if the sink of N for plant growth is small, and (iii) in balanced grass-legume  
595 mixtures, the grass roots take up N derived from legumes and from mineralization of soil  
596 organic matter.

597

598 Sustainable intensification and food security are required for a wide range of agricultural  
599 systems (Godfray *et al.*, 2010) but the focus of action might differ among intensity levels of  
600 agricultural production. In less productive systems, such as those at the arctic sites in our  
601 study, savings in N fertilizer are probably of less importance due to low levels of N fertilizer  
602 use; however, our data show that for a given amount of N fertilizer input, higher N output  
603 ( $N_{\text{tot}}$  or forage protein per unit area) can be expected with grass-legume mixtures than with  
604 pure grass alone (more output for the same input). In systems that use high levels of N  
605 fertilizer to achieve high production levels, in contrast, the same N output can be achieved by  
606 mixed swards with less input of N fertilizer (the same output from less input), thereby

607 reducing energy use (Kitani *et al.*, 1999), nitrate losses (Jensen *et al.*, 2012), and GHG  
608 emissions (Davidson, 2009; Schils *et al.*, 2013; Schmeer *et al.*, 2014).  
609

610 Finally, the use of grass-legume mixtures fulfills recent demands for climate change  
611 mitigation (Smith *et al.*, 2013). The savings in application of N fertilizers that can potentially  
612 be achieved through the benefit of symbiotic N<sub>2</sub> fixation in grassland mixtures makes their  
613 use an important strategy for reduction of GHG emissions from agriculture (Smith *et al.*,  
614 2008; Canfield *et al.*, 2010). Therefore, the use of such mixtures should be actively supported  
615 as they also meet major targets of practical grassland management, such as robustness in  
616 gains of total biomass yield despite variation in legume proportions, and applicability across  
617 wide environmental gradients (Suter *et al.*, 2012; Finn *et al.*, 2013). Provided that cultural  
618 barriers can be overcome (e.g. lacking knowledge in establishment of mixtures and their  
619 management to improve legume persistence), grass-legume mixtures are also a mitigation  
620 measure that can be implemented in the near future (Smith & Gregory, 2013; Smith *et al.*,  
621 2013). This is an important feature to meet the urgent needs for reductions in GHG emissions  
622 from the agriculture, forestry and other land use sector, which are estimated to be around 25%  
623 of total anthropogenic GHG output (Bustamante *et al.*, 2014).  
624

625 In conclusion, this study demonstrates that the N output of forage harvest is maximized in  
626 mixtures over a wide range of production levels. In the face of high economic and  
627 environmental costs of industrial N fertilizers (Kitani *et al.*, 1999; Gruber & Galloway, 2008;  
628 Canfield *et al.*, 2010), the contribution of symbiotic N<sub>2</sub> fixation by legumes to grassland N  
629 supply appears to be a key strategy to maintain and increase current levels of production and  
630 protein self-sufficiency in a more sustainable way than achieved so far.

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**820 Supporting information**

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

822

823 *Site information and details on the experimental design*

824 **Table S1** Site information on the sixteen sites including experimental details

825 **Table S2** Sowing proportions of the two grass and legume species in experimental stands

826

827 *Supporting text and information on the methods and analyses*

**828 Appendix A**

829

830 *Supplementary data*

831 **Table S3** Predicted total nitrogen yield of monocultures and mixing effects of the equi-

832 proportional mixture

833 **Table S4** Site values of environmental variables, total nitrogen yield ( $N_{\text{tot}}$ ) and N yield gain

834 in mixture ( $N_{\text{gainmix}}$ )

835 **Table S5** Correlation matrix of the five environmental variables recorded in the experiment

836 **Table S6** Regression estimates and their standard errors from the linear mixed model

837 **Table S7** Summary of regressions relating realized legume proportion, total nitrogen yield

838 ( $N_{\text{tot}}$ ), and the relative N yield gain in mixture ( $N_{\text{gainmix}}/N_{\text{tot}}$ ) to site productivity and four

839 climatic variables

840 **Fig. S1** Fitted lines to total N yield of individual sites as affected by sown legume proportion

841

842 **Tables**

843

844 **Table 1** Identity and functional types of species selected for experimentation in different regions of Europe. The species reflected a agronomic  
 845 importance and the adaptation to pedo-climatic conditions and were selected on the advice of local experts.

Species group	# Sites	Grass species		Legume species	
		GI <sup>‡</sup>	G2 <sup>§</sup>	LI <sup>‡</sup>	L2 <sup>§</sup>
Mid European	(ME)	12	<i>Lolium perenne</i> L.	<i>Dactylis glomerata</i> L.	<i>Trifolium pratense</i> L. <i>Trifolium repens</i> L.
Northern European	(NE)	3	<i>Phleum pratense</i> L.	<i>Poa pratensis</i> L.	<i>Trifolium pratense</i> L. <i>Trifolium repens</i> L.
Other	(O)	1	<i>Lolium perenne</i> L.	<i>Phleum pratense</i> L.	<i>Trifolium pratense</i> L. <i>Trifolium repens</i> L.

846 ‡ \_ Fast establishing species: fast germination and fast establishment, thereby providing adequate cover of soil in the sowing year and high biomass  
 847 yields in the first and second years after sowing. These species often lack persistency.

848 § Temporally persistent species: slower in germination and growth rate during establishment but highly competitive in the long run, therefore  
 849 increasing in cover and biomass yields over initial years and constituting the majority of yield from the third year onwards.

850

851 **Table 2** Summary of analysis of total N yield ( $N_{\text{tot}}$ ) regressed on legume proportion  
 852 (Legume) in the multisite grassland experiment across Europe. *F*-tests refer to the fixed  
 853 effects of the linear mixed model. See Material and Methods for detailed information on the  
 854 model.

Variable	Df <sub>num</sub>	Df <sub>den</sub>	<i>F</i> -value	<i>P</i>
Legume linear (Legume)	1	14.9	19.4	< 0.001
Legume quadratic (Legume <sup>2</sup> )	1	15.0	19.4	< 0.001
Legume cubic (Legume <sup>3</sup> )	1	14.9	12.0	0.003
Year	2	12.8	9.4	0.003
DeltaBiomass <sup>‡</sup>	1	749.4	1664.4	< 0.001
Legume x Year	2	10.4	5.6	0.022
Legume x DeltaBiomass	1	730.0	4.7	0.030
DeltaBiomass x Year	2	715.1	7.8	< 0.001

855 ‡ DeltaBiomass is an effect on  $N_{\text{tot}}$  that is uncorrelated to the other predictors (see  
 856 Appendix A, Supporting Information, for details). Seed density was not significant and  
 857 was omitted. For regression estimates and their standard errors see Table S6, Supporting  
 858 Information.

859 Df<sub>num</sub>: degrees of freedom of term; Df<sub>den</sub>: degrees of freedom of error (which can be  
 860 fractional in restricted maximum likelihood analysis)

861 **Table 3** Total nitrogen yield ( $N_{\text{tot}}$ ) in monocultures and grass-legume mixtures and N yield  
 862 gain ( $N_{\text{gain}}$ ) in stands containing legumes across all sites of the continental-scale grassland  
 863 experiment. Values are the fixed estimates ( $\pm 1$  standard error) derived from the linear mixed  
 864 model corresponding to Table 2 (eqn. S1, Appendix A, Supporting Information, and Table  
 865 S6). N yield gain in legume pure stands ( $N_{\text{gainleg}}$ ) and mixtures ( $N_{\text{gainmix}}$ ) was calculated as the  
 866 difference between  $N_{\text{tot}}$  of a stand and  $N_{\text{tot}}$  of the pure grass stand. Values of  $N_{\text{tot}}$  in mixture  
 867 ( $N_{\text{totmix}}$ ),  $N_{\text{gainmix}}$ , and  $N_{\text{gainmix}}/N_{\text{totmix}}$  are predicted for a stand with one third proportion of  
 868 legumes (L) and two thirds of grasses (G).

	a) $N_{\text{tot}}$ ( $\text{kg ha}^{-1} \text{ year}^{-1}$ )			Minimum legume proportion to attain 95% of maximum $N_{\text{tot}}$
	Pure stands Grass	Legume <sup>‡</sup>	Mixture 1/3:2/3 L:G	
Year 1	178 ( $\pm 25.8$ )	299 ( $\pm 37.8$ )	286 ( $\pm 31.7$ )	35
Year 2	173 ( $\pm 26.2$ )	211 ( $\pm 36.4$ )	253 ( $\pm 32.6$ )	24
Year 3	119 ( $\pm 16.4$ )	162 ( $\pm 23.1$ )	195 ( $\pm 24.6$ )	25

	b) $N_{\text{gain}}$ ( $\text{kg ha}^{-1} \text{ year}^{-1}$ )			
	Grass	Legume <sup>‡</sup> $N_{\text{gainleg}}$	1/3:2/3 L:G $N_{\text{gainmix}}$	$N_{\text{gainmix}}/N_{\text{totmix}}$
Year 1	-	121 ( $\pm 29.7$ )	108 ( $\pm 22.7$ )	0.38 ( $\pm 0.090$ ) <sup>§</sup>
Year 2	-	38 ( $\pm 24.6$ )	80 ( $\pm 18.7$ )	0.32 ( $\pm 0.085$ )
Year 3	-	43 ( $\pm 12.1$ )	76 ( $\pm 16.2$ )	0.39 ( $\pm 0.097$ )

869 ‡ Values for pure legume stands (sown as pure legume seeds) are at maximal realized legume  
 870 proportion across sites, which were 100%, 97%, and 80% in years 1, 2, and 3, respectively  
 871 (compare Fig. 4).

872 § Approximate standard error of ratio following Lee & Forthofer (2006), p. 37.

873 **Figure legends**

874

875 **Fig. 1.** Sites of the coordinated field experiment to analyze total N yield in grass-legume  
 876 mixtures. Site numbers refer to Kirwan *et al.* (2014) and are as follows: 1: Belgium,  
 877 10: Germany\_a, 11: Germany\_b, 13: Iceland\_a, 14: Iceland\_b, 15: Ireland, 18: Lithuania\_a,  
 878 20: Lithuania\_c, 22: Norway\_a, 23: Norway\_b, 24: Norway\_c, 26: Poland\_a, 27: Poland\_b,  
 879 34: Switzerland, 35: Wales\_a, 36: Wales\_b (see Table S1, Supporting Information, for further  
 880 information on sites). Symbols refer to the set of plant species sown (see Table 1).

881

882 **Fig. 2.** Illustrative example of potential fractions of total nitrogen (N) yield in grass-legume  
 883 mixtures and the respective monocultures. In grass monocultures (legume proportion = 0), the  
 884 total N yield accumulated by the sward derives solely from the soil and from fertilizer N  
 885 (quantity A). In legume monocultures (legume proportion = 1), the total N yield is greater (A  
 886 + B), as legumes also have access to atmospheric N through symbiotic N<sub>2</sub> fixation. Mixing  
 887 grasses with legumes should lead at first to a linear increase of accumulated N by the stand  
 888 with increasing legume proportion (continuous white line) through a constant amount of N  
 889 added to the system per unit of legumes. In addition, positive grass-legume interactions (e.g.  
 890 stimulation of symbiotic N<sub>2</sub> fixation, N transfer) can result in a nonlinear surplus of N yield in  
 891 mixtures (quantity C). The area of B+C defines the N yield gain ( $N_{\text{gain},i}$ , see text) for varying  
 892 legume proportions in mixtures as compared to grass monocultures.

893

894 **Fig. 3.** Total nitrogen yield ( $N_{\text{tot}}$ ) of monocultures (Grass, Legume) and mixtures at three  
 895 years for sixteen sites across Europe.  $N_{\text{tot}}$  is averaged across seed density and additionally  
 896 across monocultures of the two grass and legume species, respectively. Sites are arranged in  
 897 order of decreasing means of monoculture  $N_{\text{tot}}$  averaged over the first two experimental years,  
 898 with horizontal bars denoting the annual mean  $N_{\text{tot}}$  of mixtures. Inference on differences



899 between mixtures and *grass* monocultures is indicated on top of each panel (Wilcoxon rank  
 900 sum test on the unpooled data). Missing symbols in b) for site 15 and in c) for sites 15 and 20  
 901 reflect the absence of legumes. Site numbers follow the codes used in Fig. 1, and missing site-  
 902 years indicate no analysis of N concentration.

903 \*\*\*  $P \leq 0.001$ , \*\*  $P \leq 0.01$ , \*  $P \leq 0.05$ , ns = not significant

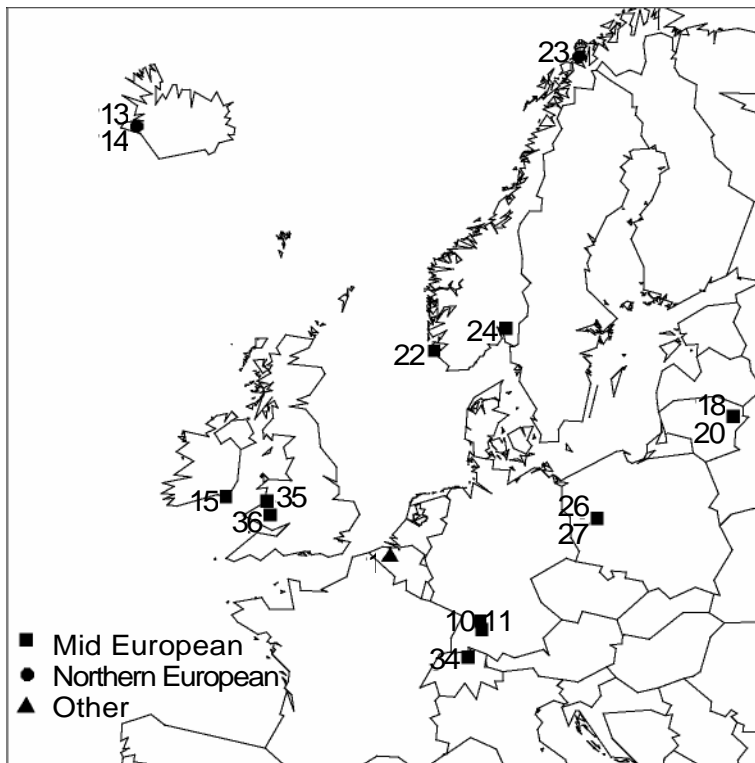
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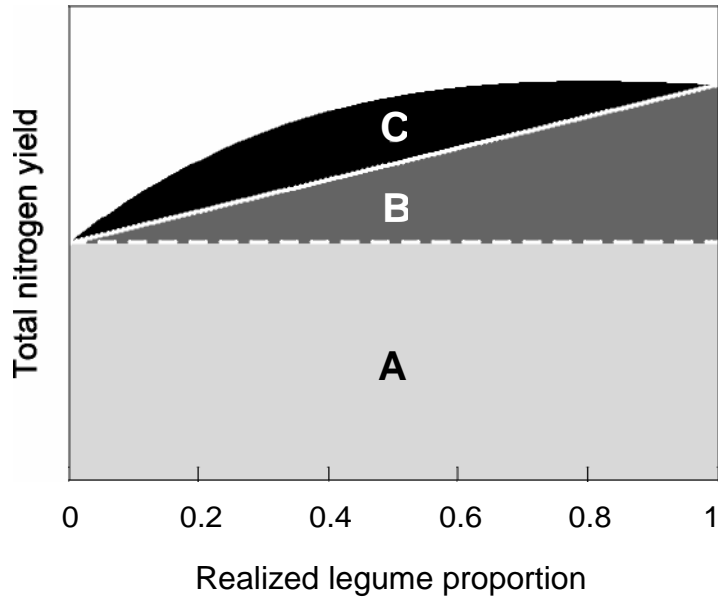
905 **Fig. 4** Total nitrogen yield ( $N_{\text{tot}}$ ) as affected by legume proportion in swards at three years of  
 906 the multisite grassland experiment. The bold black line displays the predicted  $N_{\text{tot}}$  across all  
 907 sites (fixed mean from linear mixed regression corresponding to Table 2), and coloured lines  
 908 display predicted  $N_{\text{tot}}$  for individual sites (the variation around the fixed mean) over the range  
 909 of legume proportion realized in the respective year. Numbers on lines refer to sites and are  
 910 located at the mean legume proportion realized across all stands per site. The shaded area  
 911 indicates N yield gain in mixture as compared to grass monoculture across sites (compare Fig.  
 912 2). The horizontal bold line at the bottom of the graph indicates the range of legume  
 913 proportion for which  $N_{\text{tot}}$  across all sites was significantly smaller ( $P \leq 0.05$ ) than at  
 914 maximum (■); consequently, the dotted line displays the non-significant range. No line could  
 915 be drawn for site 15 in year two and for sites 15 and 20 in year three because legumes were  
 916 absent. Other missing site-years indicate no analysis of N concentration.

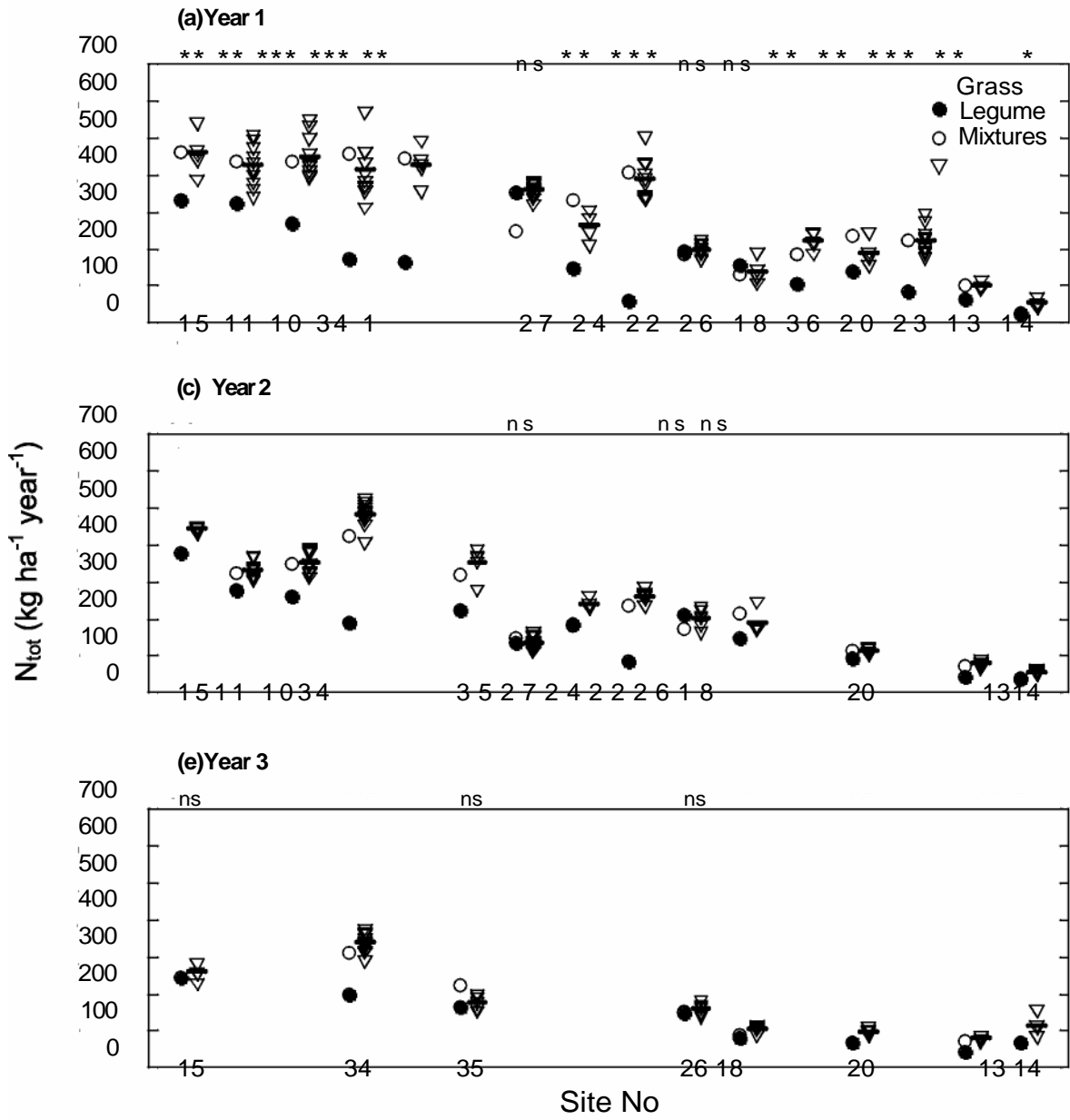
917

918 **Fig. 5.** Correlation between legume proportion in the sward and the climatic variables  
 919 minimum site temperature (a) and annual precipitation (b), and correlation between the  
 920 relative N yield gain in mixture ( $N_{\text{gainmix}}/N_{\text{totmix}}$ ) and the same climatic variables (c, d),  
 921 realized legume proportion (e), and site productivity (f). Data are site means across the first  
 922 two experimental years, with  $N_{\text{gainmix}}$  and  $N_{\text{totmix}}$  being estimated for a mixture with one third  
 923 proportion of legumes and two thirds of grasses (following eqn. S2, Appendix A, Supporting  
 924 Information). Pearson's  $R$ , its significance, and the trend line following least square

925 regression refer to filled symbols (see Table S7, Supporting Information, for regressions on  
926 all climatic variables). Site 15 (Ireland, open symbol) was omitted from these analyses  
927 because legumes were absent from the second year onward. For site names to labels see Fig.  
928 1.









(c) Year 3

