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# **Cereal-legume mixtures increase net CO<sub>2</sub> uptake in a forage system of the Eastern Pyrenees**

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## 29 Abstract

30 Forage systems are the major land use, and provide essential resources for animal feeding. Assessing the  
31 influence of forage species on net ecosystem CO<sub>2</sub> exchange (NEE) is key to develop management  
32 strategies that can help to mitigate climate change, while optimizing productivity of these systems.  
33 However, little is known about the effect of forage species on CO<sub>2</sub> exchange fluxes and net biome  
34 production (NBP), considering: species ecophysiological responses; growth and fallow periods  
35 separately; and the management associated with the particular sown species. Our study assesses the  
36 influence of cereal monocultures vs. cereal-legume mixtures on (1) ecosystem-scale CO<sub>2</sub> fluxes, for the  
37 whole crop season and separately for the two periods of growth and fallow; (2) potential sensitivities of  
38 CO<sub>2</sub> exchange related to short-term variations in light, temperature and soil water content; and (3) NBP  
39 during the growth period; this being the first long term (seven years) ecosystem-scale CO<sub>2</sub> fluxes dataset  
40 of an intensively managed forage system in the Pyrenees region. Our results provide strong evidence that  
41 cereal-legume mixtures lead to higher net CO<sub>2</sub> uptake than cereal monocultures, as a result of higher gross  
42 CO<sub>2</sub> uptake, while respiratory fluxes did not significantly increase. Also, management associated with  
43 cereal-legume mixtures favoured vegetation voluntary regrowth during the fallow period, which was  
44 decisive for the cumulative net CO<sub>2</sub> uptake of the entire crop season. All cereal-legume mixtures and  
45 some cereal monocultures had a negative NBP (net gain of C) during the growth period, indicating C  
46 input to the system, besides the yield. Overall, cereal-legume mixtures enhanced net CO<sub>2</sub> sink capacity of  
47 the forage system, while ensuring productivity and forage quality.

48

49 **Key words:** ecosystem respiration ( $R_{eco}$ ), gross primary production (GPP), light response, management,  
50 monocultures, net ecosystem CO<sub>2</sub> exchange (NEE).



## 1. Introduction

Forage systems, including feed crops together with intensively and extensively managed pasturelands, are the major land use, covering about 30% of the world's terrestrial surface and 80% of agricultural land (Steinfeld and Wassenaar, 2007). Thus, assessing the role of forage species on the carbon (C) balance of these systems is essential to develop management strategies that can mitigate climate change, while optimizing productivity. To this regard, forage mixtures have been generally associated with higher productivity than monocultures (Brophy et al., 2017; Finn et al., 2013; Kirwan et al., 2007; Ribas et al., 2015), resulting from higher resource use efficiency, including light (Hofer et al., 2017; Milcu et al., 2014), water (Chapagain and Riseman, 2015; Liu et al., 2016), and nitrogen (Sturludóttir et al., 2013; Suter et al., 2015). Mixtures have also been described to present lower rates of weed invasion (Connolly et al., 2018; Frankow-Lindberg et al., 2009; Kirwan et al., 2007). However, the role of forage species in the net ecosystem CO<sub>2</sub> exchange (NEE), as well as on NEE components — gross primary production (GPP) and ecosystem respiration (R<sub>eco</sub>) — is less understood.

In addition, the interaction between local conditions and management practices result in high CO<sub>2</sub> exchange variability (Moors et al., 2010; Oertel et al., 2016). And, while information on the CO<sub>2</sub> budget of grasslands (Berninger et al., 2015; Imer et al., 2013; Schauffler et al., 2010) and forage crops (Ceschia et al., 2010; Kutsch et al., 2010; Vuichard et al., 2016) of central and northern Europe is rather abundant, such information is very scarce in the Mediterranean basin, even though it is a highly vulnerable region to climate change (FAO, 2010). Indeed, forage productivity in Mediterranean areas is among the lowest in Europe (Smit et al., 2008), due to important water constraints (Porqueddu et al., 2016), and more information is needed to establish management practices that may enhance C sequestration while ensuring productivity.

In addition, it is also crucial to understand the role of forage species in net biome production (NBP), accounting for all C inputs and exports ( $NBP = NEE - C_{input} + C_{export}$ ), to assess the final C budget, beyond the NEE. In fact, many grasslands and forage crops may be acting as net CO<sub>2</sub> sinks when only assessing NEE, but they become net CO<sub>2</sub> sources when accounting for the oxidation (via digestion by animals) of total exported biomass (Ceschia et al., 2010; Kutsch et al., 2010; Moors et al., 2010).

Our study presents in this regard the first long-term (seven years) dataset of ecosystem-scale CO<sub>2</sub> fluxes of an intensively managed forage system in the Pyrenees, which combines a crop rotation of cereal species grown in monocultures and cereal-legume mixtures, with direct grazing after the harvest (fallow period). Such practices have been traditionally conducted in Mediterranean mountain regions (Sebastià et al., 2011) to increase productivity and preserve soil fertility (Sánchez et al., 2013).

Thus, our objective is to assess differences between cereals grown in monoculture and cereal-legume mixtures in (1) ecosystem-scale CO<sub>2</sub> fluxes, for the whole crop season and separately for the two periods of growth and fallow; (2) potential sensitivities of CO<sub>2</sub> exchange related to short-term variations in light, temperature and soil water content; and (3) NBP during the growth period. Also, we hypothesize that cereal-legume mixtures in comparison to cereal monocultures: (1) will show more net CO<sub>2</sub> uptake (more negative NEE); (2) this increase in the net uptake will be due to increased GPP in combination with unchanged R<sub>eco</sub>; and (3) will show more negative NBP.



## 90 2. Material and methods

### 91 2.1 Study site and experimental design

92 The study site is a forage system located in the montane elevation belt of the Eastern Pyrenees, in Pla de  
 93 Riart (42° 03' 48" N, 1° 30' 48" E), at 1003 m a. s. l. Climate is sub-Mediterranean (Peel et al., 2007),  
 94 typical in mountain areas with Mediterranean influences, with a mean annual precipitation of 750 mm and  
 95 mean annual temperature of 11 °C (Ninyerola et al., 2000), including the summer drought period. The soil  
 96 is a petrocalcic calcixercept (Badía-Villas and del Moral, 2016).

97 All management events, including fertilizing, sowing and harvesting (Table 1) were reported by the  
 98 manager of the site and validated by in situ visits. The site was managed by a rotation of cereals grown in  
 99 monoculture and cereal-legume mixtures. Every year the yield was harvested, and during the fallow (from  
 100 harvest to next sowing), the voluntary regrowth of the vegetation was extensively grazed by around  
 101 30 cattle ( $\approx 0.91$  livestock units (LSU)  $\text{ha}^{-1}$ ) from late August to late October (Fig. 1).

102 Yield was estimated (Table 1) considering the productivity reported by the manager and in situ samplings  
 103 after oven drying plant material at 60 °C until constant weight. Plant material was analysed to determine C  
 104 content and forage quality indicators (Table S1). Analyses were performed by the Department of Animal  
 105 and Food Science, Autonomous University of Barcelona according to standard methods (Table S1).  
 106 Afterwards, C exported through yield (Table 1) was estimated, considering the yield, species proportions  
 107 (Fig. 1), and species C content (Table S1). C exported through yield was used to account for the NBP  
 108 (Sect. 2.5).

### 109 2.2 Eddy covariance measurements

110 The site is equipped with an eddy covariance flux station, running since August 2010, and our study  
 111 period included data from sowing of the first studied season (barley, sown 01/11/2010) until the end of  
 112 the fallow period of the last studied season (oat and vetch mixture, 01/11/2017, Fig. 1). The eddy  
 113 covariance flux station continuously measured the concentration of  $\text{CO}_2$  ( $\text{mmol m}^{-3}$ ) and  $\text{H}_2\text{O}$  ( $\text{mmol m}^{-3}$ )  
 114 using an open path  $\text{CO}_2$  and  $\text{H}_2\text{O}$  gas analyser (LI-7500, LI-COR Inc., Lincoln, NE, USA), and turbulent  
 115 flux components, including wind direction and speed using a 3D sonic anemometer (CSAT-3, Campbell  
 116 Scientific Inc, Logan, UT, USA) to calculate  $\text{CO}_2$ ,  $\text{H}_2\text{O}$ , and energy exchange at the ecosystem level.

117 In addition, the station recorded ancillary meteorological variables, including incoming and outgoing  
 118 shortwave and longwave radiation (NR01, Hukseflux, Delft, the Netherlands); air temperature ( $T_a$ , CS215,  
 119 Campbell Scientific Inc, Logan, UT, USA); average soil temperature 1-20 cm ( $T_s$ , TCAV, Campbell  
 120 Scientific Inc, Logan, UT, USA); volumetric soil water content (SWC, CS616, Campbell Scientific Inc,  
 121 Logan, UT, USA); photosynthetically active radiation (PAR, SKP215, Skye Instruments Ltd, Powys, UK);  
 122 and normalized difference vegetation index, calculated as  $\text{NDVI} = (\text{NIR} - \text{Red}) / (\text{NIR} + \text{Red})$ , where  
 123 “Red” and “NIR” are the spectral reflectance measurements acquired in the red and near infrared regions,  
 124 respectively.

125 Raw data provided by the sensors were processed and  $\text{CO}_2$  fluxes were calculated at 30-minute averages  
 126 using the EddyPro software (LI-COR Inc, Lincoln, NE, USA). Negative values refer to the flux from the



127 atmosphere to the biosphere and positive values correspond to the flux from the biosphere to the  
 128 atmosphere (micrometeorological sign convention).

129 We applied frequency response corrections (Moncrieff et al., 2004, 1997), density fluctuation corrections  
 130 (Webb et al., 1980), and determination of data quality using the Foken et al., (2004) approach. The  
 131 Foken et al. (2004) approach suggests a quality scale ranging from 1 (highest data quality) to 9 (poorest  
 132 data quality), and records with quality 7 or higher were excluded (Papale, 2012). Also, CO<sub>2</sub> fluxes outside  
 133 a physically realistic range ( $\pm 50 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) were rejected.

134 We inspected night-time ( $\text{PAR} < 5 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) CO<sub>2</sub> fluxes, as they tend to be underestimated  
 135 under low turbulence (Aubinet et al., 2012), conditions that can be frequent at night. We carefully  
 136 examined the possibility of a low turbulence effect assessing the existence of an  $u_*$  threshold at all  
 137 recorded  $T_s$  classes (Reichstein et al., 2005), ranging from  $-3$  to  $34$  °C in  $1$  °C intervals. Relevant  $u_*$   
 138 thresholds were not detected. In addition, we inspected night-time CO<sub>2</sub> fluxes in order to detect possible  
 139 outliers and calculated the 0.025, 0.25, 0.5, 0.75 and 0.975 quantiles for each  $T_s$  class. Data below the  
 140 lowest (0.025) or the highest (0.975) quantile were excluded from further analysis.

141 Data were filtered according to the footprint, based on the Kljun model (Kljun et al., 2004), including all  
 142 the fluxes in which more than 80% of the contribution came from the study field (Göckede et al., 2008).  
 143 After all data cleaning and filtering, retained data for further analysis were a 65% of all the available data,  
 144 ranging between 81% and 53% depending on year (Table S2).

145 Afterwards, we gap-filled NEE data using the sMDSGapFill function (Reichstein et al., 2005) of the  
 146 REdDyProc package (Wutzler et al., 2018) for R software (R core Team, 2017). The goodness of the  
 147 gap-filling was also inspected comparing observed NEE data with their theoretically predicted data by  
 148 gap-filling (see an example in Fig. S1). Gap-filled NEE data were also partitioned into GPP and  $R_{\text{eco}}$ ,  
 149 using the night-time based partitioning approximation, SMRFLuxPartition equation, also of the  
 150 REdDyProc package.

151 In line with our first objective, we described NEE, GPP and  $R_{\text{eco}}$  dynamics, and performed budgets  
 152 (expressed in  $\text{g C m}^{-2}$ ) for each: (a) crop season — from sowing to sowing —, (b) growth period — from  
 153 sowing to harvesting —, and (c) fallow period — from harvesting to sowing. Note that in 2014 systematic  
 154 data gaps occurred due to energy supply problems, for which NEE, GPP and  $R_{\text{eco}}$  budgets could not be  
 155 calculated. However, 2014 gap-filled data were used to describe CO<sub>2</sub> exchange dynamics, and 2014 real  
 156 recorded data were included in all the modelling.

### 157 2.3 Net ecosystem CO<sub>2</sub> exchange modelling: diversity-interaction model

158 Species can drive ecosystem functions via species identity effects, but also via species interactions and  
 159 complementarity effects (Kirwan et al., 2007; Orwin et al., 2014; Wolfgang et al., 2017). Thus, also in  
 160 line with our first objective we disaggregated the influence of cereal monocultures from cereal-legume  
 161 mixtures on NEE using a diversity-interaction approach (Kirwan et al., 2007, 2009). The approach  
 162 compares a null model, in which a change in the diversity has no effect on the response variable, with  
 163 models that address the diversity influence at different levels.

164 In our study we compared the null model Eq. (1), in which NEE ( $\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$ ) depended only on  
 165 environmental variables, including  $T_a$  (°C), net radiation ( $R_{\text{net}}$ ,  $\text{W m}^{-2}$ ), SWC (fraction), vapour pressure



deficit (VPD, hPa), and time — considering time as crop season — with a diversity-interaction model, which included species identity and species interaction effects Eq. (2).

168

$$NEE = \beta_{T_a} T_a + \beta_{R_{net}} R_{net} + \beta_{SWC} SWC + \beta_{VPD} VPD + \beta_{time} time + \varepsilon$$

169

(Equation 1. Null model)

170

$$NEE = \text{Null model} + \beta_B P_B + \beta_T P_T + \beta_W P_W + \beta_{OV} P_{OV} + \beta_{TOV} P_{TOV} + \varepsilon$$

171

(Equation 2. Diversity-interaction model)

172

Here  $P$  indicates species proportions and the sub-index  $B$  indicates barley,  $T$  triticale,  $W$  wheat,  $O$  oat and  $V$  vetch respectively. The models were run without intercept in order to test the effect of all the species proportions at the same time.

A preliminary modelling showed that SWC and time could be excluded from the null model Eq. (1), since the inclusion of these variables did not provide a better fitting. Then, the null model Eq. (1) and the diversity-interaction model Eq. (2) were compared by an analysis of variance (ANOVA) to account for the most parsimonious and explanatory model. The diversity-interaction model was significantly different from the null model ( $F = 7.65$ ,  $p < 0.001$ ); therefore, the final model was the diversity-interaction model, which included the proportion of each forage species and its interactions, in addition to environmental variables ( $T_a$ ,  $R_{net}$ , VPD).

The approach was run on all observed data (30-minute average); on daily-averaged data; and on weekly-averaged data. The model performed the best fitting (best adjusted  $R^2$ ) when using weekly-averaged data, probably due to a considerable day-to-day variability of the environmental variables and  $CO_2$  fluxes. Also, considering that the main goal of this analysis was to assess the influence of forage species on NEE, whose influence is probably more noticeable at a seasonal scale, we present the model run on the weekly-averaged data, as it was able to reduce noise and extract the influence of forage species with greater reliability.

## 190 2.4 $CO_2$ exchange response to light, temperature and soil water content

In line with our second objective, we explored differences between cereal monocultures and cereal-legume mixtures from a mechanistic perspective, modelling separately light response of observed  $CO_2$  fluxes during daytime (termed as  $NEE_{day}$  in what follows), and  $T_s$  and SWC response of night time fluxes (termed as  $R_{eco,night}$  in what follows) as explained below.

195



#### 196 2.4.1 NEE<sub>day</sub> light response

197 NEE<sub>day</sub> (PAR > 5 μmol photons m<sup>-2</sup> s<sup>-1</sup>) light response was modelled using a logistic sigmoid response  
 198 function (Moffat, 2012), which models NEE<sub>day</sub> (μmol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup>) as function of PAR Eq. (3).  
 199

$$NEE_{day} = -2 \cdot GPP_{sat} \cdot \left( -0.5 + \frac{1}{1 + e^{\frac{-2 \cdot \alpha \cdot PAR}{GPP_{sat}}}} \right) + R_{eco,day}$$

200 (Equation 3)

201

202 Here GPP<sub>sat</sub> (μmol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup>) is the asymptotic gross primary production, α (dimensionless) is the  
 203 apparent initial quantum yield, defined as the initial slope of the light-response curve, and R<sub>eco,day</sub>  
 204 (μmol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup>) the average daytime ecosystem respiration. Light response parameters (GPP<sub>sat</sub>, α and  
 205 R<sub>eco,day</sub>) were calculated for each day and crop season, using the nlsList function of the nlme package  
 206 (Pinheiro et al., 2015). Parameters whose estimates were not significantly different from zero (p ≥ 0.05)  
 207 were discarded from further analysis.

208 Afterwards, we described light response dynamics and assessed differences on the light response parameters  
 209 between cereal monocultures and cereal-legume mixtures for each period (growth and fallow). For that purpose  
 210 we ran an ANOVAs and tukey post-hoc tests, using the HSD.test function of the agricolae package  
 211 (Mendiburu, 2017), with the given parameter (GPP<sub>sat</sub>, α and R<sub>eco,day</sub>) as a function of forage type (cereal  
 212 monoculture and cereal-legume mixture) in interaction with period (growth and fallow).

#### 213 2.4.2 R<sub>eco,night</sub> response to temperature and soil water content

214 A preliminary overview of R<sub>eco,night</sub> (PAR < 5 μmol photons m<sup>-2</sup> s<sup>-1</sup>) suggested that R<sub>eco,night</sub> increased with  
 215 T<sub>s</sub> at T<sub>s</sub> < 20°C, but decreased above this threshold. Therefore, we modelled R<sub>eco,night</sub> (μmol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup>)  
 216 as a function of T<sub>s</sub> (°C) and SWC (fraction) using the equations proposed by Reichstein et al. (2002),  
 217 which consider changes in the temperature sensitivity depending on soil moisture Eq. (4-6).  
 218

$$R_{eco,night} = R_{eco,ref} \cdot f(T_s, SWC) \cdot g(SWC)$$

219 (Equation 4)

220

$$f(T_s, SWC) = e^{E_0(SWC) \cdot \left( \frac{1}{T_{ref} - T_0} - \frac{1}{T_s - T_0} \right)}$$

221 (Equation 5)

222

$$g(SWC) = \frac{SWC - SWC_0}{(SWC_{1/2} - SWC_0) + (SWC - SWC_0)}$$

223 (Equation 6)

224

225 Here the activation energy, E<sub>0</sub> (°C<sup>-1</sup>), is a linear function of SWC (E<sub>0</sub> = a+b·SWC); T<sub>ref</sub> is the reference  
 226 temperature, set as the mean temperature of a given period, here set as the mean T<sub>s</sub> of the entire





measuring period ( $T_{ref} = 12.12\text{ }^{\circ}\text{C}$ );  $T_0$  the lower limit for  $R_{eco,night}$ , here set at  $-46.02\text{ }^{\circ}\text{C}$ , as in the original model by Lloyd and Taylor (1994);  $SWC_0$  (fraction) the soil water content below which  $R_{eco,night}$  ceases;  $SWC_{1/2}$  (fraction) the soil water content at which maximal  $R_{eco,night}$  halves; and  $R_{eco,ref}$  ( $\mu\text{mol CO}_2\text{ m}^{-2}\text{ s}^{-1}$ ) the reference ecosystem respiration at standard conditions ( $T_{ref}$ ) and non-limiting SWC (Reichstein et al., 2002).  $R_{eco,night}$  response parameters ( $R_{eco,ref}$ ,  $E_0$ ,  $SWC_0$ ,  $SWC_{1/2}$ ) were calculated considering all seasons together (2011–2017) and for each crop season, using the nlsList function.

Similarly as in the diversity-interaction model (Sect. 2.3), we performed the  $R_{eco,night}$  modelling on all observed data (30-minute average), on daily-averaged data and on weekly-averaged data. Afterwards, we calculated  $R^2$  as the linear relationship between modelled and measured observations. The model performed best (highest  $R^2$ ) when using weekly-averaged data, probably due to the high day-to-day variability of  $R_{eco,night}$  and  $T_s$ .

## 2.5 Net biome production (NBP)

Finally, in line with our third objective, we estimated the NBP during the growth period. NBP can be estimated knowing the NEE; C exports, including harvest/grazing and other gas emissions such as methane or volatile organic compounds; and C imports, including organic C fertilizers and sowing. In our study, C exports through methane were expected not to be very significant, because methane effluxes require water saturated soils, typically with standing water (Oertel et al., 2016), which was never the case; and volatile organic compounds were expected to be negligible (Soussana et al., 2010). C inputs through sowing and fertilizers (mostly inorganic nitrogen fertilizers, Table 1) could also be neglected as they only represent a very small C amount. Thus, we estimated the NBP during the growth period as the sum of the NEE budget of that period and C exported through the yield Eq. (7).

$$NBP = NEE + Yield$$

(Equation 7)

## 3. Results

### 3.1 Forage species influence on CO<sub>2</sub> exchange dynamics and budgets

Seasonal CO<sub>2</sub> flux dynamics evolved according to environmental conditions, forage growth and management events (Fig. 2). Maximum net CO<sub>2</sub> uptake was achieved during spring, when temperatures were mild, SWC increased, and the forage development reached its peak biomass (Fig. 2). CO<sub>2</sub> exchange capacity of the system decreased with harvesting (Fig. 2.a), also showed by the drastic decrease of the NDVI (Fig. 2.d).

The field acted as a net CO<sub>2</sub> sink throughout all the studied crop seasons (negative NEE, Fig. 3.a). NEE of cereal-legume mixtures was more negative and less variable ( $-363\text{ g C m}^{-2}$ , year 2013, and  $-383\text{ g C m}^{-2}$  year 2017, Fig. 3.a) than that of cereal monocultures (ranging from  $-70$  to  $-226\text{ g C m}^{-2}$ , Fig. 3.a).



During the growth period, cereal-legume mixtures showed the highest net CO<sub>2</sub> uptake, with a NEE of −359 and −429 g C m<sup>−2</sup> in 2013 and 2017 respectively (Fig. 3.b). On the other hand, cereal monocultures had a NEE that ranged from −128 to −348 g C m<sup>−2</sup> (Fig. 3.b), with triticale being the cereal monoculture with the highest net uptake (−348 g C m<sup>−2</sup>, Fig. 3.b).

During the fallow period R<sub>eco</sub> was the dominant flux in all cases (Fig. 3.c), although there were some differences in the CO<sub>2</sub> exchange dynamics between cereal monocultures and cereal-legume mixtures (Fig. 2.a), which were decisive for the cumulative net CO<sub>2</sub> uptake of the whole crop season. During the fallow of grass-legume mixtures there was a more marked voluntary regrowth of the vegetation (Fig. 2.d) that promoted a period of net CO<sub>2</sub> uptake after the harvest, especially strong in the triticale, oat and vetch mixture (year 2013), and the oat and vetch mixture (year 2014, Fig. 2.a). Note that although gap-filled 2014 data were not used to account for CO<sub>2</sub> exchange budgets (Fig. 3) due to systematic gaps; 2014 gap-filled data could be used to describe CO<sub>2</sub> exchange dynamics and allowed us to identify this rebound in the net CO<sub>2</sub> uptake during the fallow period of that year.

On the contrary, cereal monocultures generally did not show this voluntary regrowth during the fallow period (Fig. 2.d), and gross and net CO<sub>2</sub> uptake capacity of the system decreased drastically (Fig. 2.a). The exception was the wheat monoculture in 2015, when there was vegetation voluntary regrowth after the harvest that resulted in net CO<sub>2</sub> uptake during the fallow period.

The diversity-interaction model (Table 2) confirmed the influence of forage species on NEE. The model estimates indicated less net CO<sub>2</sub> uptake in cereal monocultures than in cereal-legume mixtures (Table 2, negative sign in the estimate means uptake), again with a high variability within cereal monocultures. Barley was the cereal monoculture with the lowest net uptake ( $-1.0 \pm 0.3 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ,  $t = -3.39$ ,  $p < 0.001$ , Table 2) and triticale was the cereal monoculture with the highest net uptake among the monocultures ( $-1.6 \pm 0.4 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ,  $t = -4.40$ ,  $p < 0.001$ , Table 2). Cereal-legume mixtures, however, showed higher net CO<sub>2</sub> uptake rates (oat x vetch  $-2.0 \pm 0.3 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ,  $t = -7.44$ ,  $p < 0.001$ , Table 2) than all cereal species in monoculture. The addition of triticale in the mixture did not have a significant effect on NEE (Table 2).

### 3.2 Cereal monocultures vs. cereal-legume mixtures: NEE<sub>day</sub> light response

All three light response parameters exhibited pronounced seasonality, as result of phenological changes and management events (Fig. 4). During the growth period, cereal-legume mixtures exhibited on average slightly higher values of GPP<sub>sat</sub> than cereal monocultures, while R<sub>eco,day</sub> did not increase (Fig. 5). During the fallow period, cereal-legume mixtures presented on average significantly higher GPP<sub>sat</sub> and  $\alpha$  values than cereal monocultures (Fig. 5), due to the voluntary regrowth of the vegetation (Fig. 2.d), which also caused a rebound on GPP<sub>sat</sub> and  $\alpha$  (Fig. 5).

### 3.3 Cereal monocultures vs. cereal-legume mixtures: R<sub>eco,night</sub> response to temperature and soil water content

R<sub>eco,night</sub> models, based on the equations proposed by Reichstein et al. (2002, our Eq. 4–6), presented a satisfactory fitting, with R<sup>2</sup> ranging from 0.19 to 0.75 across seasons (Table 3). When assessing all seasons together, T<sub>s</sub> and SWC drove R<sub>eco,night</sub> (Fig. 6); with an activation energy (E<sub>0</sub>) significantly dependent on



SWC ( $E_0 \sim a + b \cdot \text{SWC}$ ,  $a = 76 \pm 40$  and  $b = 483 \pm 259 \text{ }^\circ\text{C}^{-1}$ , Table 3), indicating that temperature sensitivity was dependent on SWC Eq. (5). Also, soil water content at which maximal  $R_{\text{eco},\text{night}}$  halves ( $\text{SWC}_{1/2}$ ) was significant ( $0.06 \pm 0.01$ , Table 3), indicating that  $R_{\text{eco},\text{night}}$  decreased to half-maximum or lower at  $\text{SWC} \leq 6 \pm 1 \%$ . However, some estimates of the  $R_{\text{eco},\text{night}}$  response parameters were not significantly different from zero ( $p \geq 0.05$ , see significant estimates in bold, Table 3); and when assessing differences between forage types, non-significant estimates were not considered for comparison. Yet,  $E_0$  of barley, in year 2011 ( $b = 3668 \pm 1645 \text{ }^\circ\text{C}^{-1}$ , Table 3), and of wheat, in year 2015 ( $b = 850 \pm 627 \text{ }^\circ\text{C}^{-1}$ , Table 3), were significantly dependent on SWC, both values being much higher than the average of all crop seasons ( $b = 483 \pm 259 \text{ }^\circ\text{C}^{-1}$ , Table 3). Also, the reference ecosystem respiration ( $R_{\text{eco},\text{ref}}$ ) of triticale in year 2012, was significantly different from zero ( $4 \pm 2 \text{ } \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ , Table 3), exceeding  $R_{\text{eco},\text{ref}}$  of all seasons together ( $2.8 \pm 0.3 \text{ } \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ , Table 3). Finally, soil water content below which  $R_{\text{eco},\text{night}}$  ceases ( $\text{SWC}_0$ ) and  $\text{SWC}_{1/2}$  had a significant influence on  $R_{\text{eco},\text{night}}$  in the triticale, oat and vetch mixture (year 2013), the oat and vetch mixture (year 2014), and in the wheat monoculture (year 2015, Table 3). Both cereal-legume mixtures (year 2013 and 2014), had a  $\text{SWC}_{1/2}$  that was very close to  $\text{SWC}_0$ , indicating that SWC could reach very low values before  $R_{\text{eco},\text{ref}}$  halved, although this SWC value was already very close to the limit at which  $R_{\text{eco},\text{ref}}$  ceases ( $\text{SWC}_0$ ). On the contrary, during the wheat monoculture of 2015,  $\text{SWC}_{1/2}$  ( $0.08 \pm 0.03$ , Table 3) doubled  $\text{SWC}_0$  ( $0.04 \pm 0.03$ , Table 3).

#### 3.4 Cereal monocultures vs. cereal-legume mixtures: Net biome production (NBP)

Finally, NBP during the growth period indicated net C input into the system (negative NBP), except during the cereal monocultures of triticale (year 2012), and barley (year 2011, Fig. 7). The most negative NBP was detected in the wheat monoculture in 2015 ( $\text{NBP} \approx -108 \text{ g C m}^{-2}$ , Fig. 7), followed by the oat and vetch mixture in 2017 ( $\text{NBP} \approx -67 \text{ g C m}^{-2}$ , Fig. 7).

## 4. Discussion

Forage species drove  $\text{CO}_2$  exchange responses consistently throughout the assessed years and different environmental conditions in the studied forage system of the Eastern Pyrenees. Cereal-legume mixtures had more negative NEE, during the whole crop season (Fig. 3.a) and during the growth period (Fig. 3.b) than cereal monocultures. Also, cereal-legume mixtures had lower NEE inter-annual variability ( $-363 \text{ g C m}^{-2}$ , year 2013, and  $-383 \text{ g C m}^{-2}$  year 2017, Sect. 3.1) than cereal monocultures (ranging from  $-70$  to  $-226 \text{ g C m}^{-2}$ , Sect. 3.1), suggesting a consistent diversity effect on NEE along different forage mixtures and proportions of species in the mixtures.

Moreover, the diversity-interaction model (Table 2) confirmed the capacity of cereal-legume mixtures to take up more  $\text{CO}_2$ , oat and vetch being the mixture with the highest net  $\text{CO}_2$  uptake (Table 2). The inclusion of legumes was key for promoting this diversity effect, since the oat and vetch mixture had a significant effect on NEE, while the triticale addition in the mixture did not significantly increase the net  $\text{CO}_2$  uptake (Table 2).



337 These results agree with our first hypothesis: cereal-legume mixtures enhance the net CO<sub>2</sub> uptake in  
 338 comparison to cereal monocultures (barley, wheat and triticale). Those differences in CO<sub>2</sub> fluxes between  
 339 cereal-legume mixtures and cereal monocultures could be explained by plant species complementarity,  
 340 together with mechanisms related to ecophysiological responses, including CO<sub>2</sub> uptake and respiration  
 341 (Sect. 4.1), as well as management (Sect. 4.2).

#### 342 4.1 Forage species influence on gross CO<sub>2</sub> uptake and respiration

343 From a mechanistic perspective, cereal-legume mixtures had higher light use efficiency than cereal  
 344 monocultures, as indicated by the slightly higher values of GPP<sub>sat</sub> achieved during the growth period, and  
 345 the marked  $\alpha$  and GPP<sub>sat</sub> rebound during the fallow period (Figs. 4-5). Accordingly, cereal legume  
 346 mixtures have been reported to increase gross CO<sub>2</sub> uptake, not only via the increased photosynthesis of  
 347 legumes (Reich et al., 1997, 2003), but also increasing photosynthesis of the overall community via  
 348 nitrogen transfer from the legume to the cereal in the mixture. Interestingly, our results showed that this  
 349 increase in the gross CO<sub>2</sub> uptake and the photosynthetic activity was not accompanied by a significant  
 350 increase of daytime respiration rates (R<sub>eco,day</sub>, Figs. 4-5).

351 On the other hand, R<sub>eco,night</sub> was clearly driven by T<sub>s</sub> and SWC (Albergel et al., 2010; Davidson and  
 352 Janssens, 2006; Yvon-Durocher et al., 2012), although it was limited at the highest T<sub>s</sub> and lowest SWC  
 353 (Fig. 6). In agreement, some authors have identified a temperature threshold at which temperature  
 354 sensitivity changes, decreasing respiration (Carey et al., 2016; Hernandez and Picon-Cochard, 2016;  
 355 Reichstein et al., 2002). This change in respiration-temperature sensitivity has been explained by  
 356 (a) changes in microbial activity (Balser and Wixon, 2009), decreasing the heterotrophic component of  
 357 R<sub>eco</sub>; and (b) an indirect effect through limitations on GPP, resulting in limitations on the autotrophic  
 358 component of R<sub>eco</sub>, particularly affected by the combination of high temperatures with low SWC (Niu et  
 359 al., 2012; Reichstein et al., 2002). In our study, we did not partition R<sub>eco</sub> into autotrophic and  
 360 heterotrophic respiration, but this shift in respiration-temperature at the highest temperatures and the  
 361 lowest SWC mostly happened after harvest (Fig. 2), which irretrievably decreased GPP and  
 362 photosynthesis, and most likely lowered the autotrophic component of R<sub>eco</sub> (Larsen et al., 2007).

363 Moreover, R<sub>eco,night</sub> responded similarly to T<sub>s</sub> and SWC in both cereal monocultures and cereal-legumes  
 364 mixtures, since differences in CO<sub>2</sub> respiration response to T<sub>s</sub> and/or SWC were not detected (inconsistent  
 365 differences between response parameters: R<sub>eco,ref</sub>, SWC<sub>0</sub>, SWC<sub>1/2</sub> and E<sub>0</sub>; see Table 3). This may well be  
 366 because although generally legumes have higher autotrophic respiration rates, with both higher leaf (Li et  
 367 al., 2016) and root respiration rates (Warembourg et al., 2003) than cereals, and there is a strong nitrogen  
 368 content – respiration relationship (Reich et al., 2008), this increase in respiration is largely driven by  
 369 higher GPP and photosynthetic activity (Larsen et al., 2007). Thus, although there had been differences in  
 370 the autotrophic respiration resulting from differences in photosynthetic rates, this does not necessarily  
 371 mean that night-time fluxes (R<sub>eco,night</sub>) of cereal-legume mixtures had higher temperature and/or SWC  
 372 sensitivity than cereal monocultures. In addition, even if there had been differences between legume and  
 373 cereal species in their R<sub>eco,night</sub> sensitivity to T<sub>s</sub> and SWC, these differences were not noticeable at the  
 374 community scale (Table 3).



375 Interestingly, this is in line with the previously discussed NEE light response results, since the increase in  
 376 the CO<sub>2</sub> input, favoured by the presence of legumes in the community, overcompensated CO<sub>2</sub> respiration  
 377 losses, both during day ( $R_{eco,day}$ ) and night ( $R_{eco,night}$ ) time. This is in agreement with our second  
 378 hypothesis, cereal-legume mixtures having more negative NEE (Table 2) due to higher photosynthetic  
 379 rates, but not higher respiration rates. Chen et al. (2017) found a similar result, with legumes increasing  
 380 gross CO<sub>2</sub> uptake (higher GPP), but not enhancing CO<sub>2</sub> release, resulting in more negative NEE. Most  
 381 likely, increased total nitrogen availability, mediated by legumes, increased photosynthetic activity of the  
 382 overall community at a higher rate than respiration losses (Chen et al., 2017).

#### 383 4.2 Management associated with forage types: influence on NEE and NBP

384 Management associated to each forage type had inherent particularities. Cereal monocultures were  
 385 harvested once the yield was sufficiently dry and grains were mature; while cereal-legume mixtures were  
 386 harvested when the vegetation was still fresh (before boot stage) for silage; the latter being a conventional  
 387 practice to improve forage nutritional value, and favour the voluntary regrowth after the harvest  
 388 (Canevari, 2000).

389 In our study, these differences in harvesting time resulted in clear differences in vegetation regrowth  
 390 dynamics (Fig. 2.d), which were decisive for the cumulative net CO<sub>2</sub> uptake of the whole crop season.  
 391 Thus, cereal-legume mixtures markedly regrew after the harvest, in May or early June, because the  
 392 vegetation was still in an earlier stage of phenological development, and environmental conditions were  
 393 also favourable during that time of the season. On the contrary, cereal monocultures had completed their  
 394 development cycle, and this usually left no room for voluntary regrowth after harvest (Fig. 2.d), and  
 395 hence no net CO<sub>2</sub> uptake during the fallow period (Fig. 2.a). Also, seeds that remained in the field after  
 396 the harvest did not encounter the environmental conditions required to germinate, since temperatures  
 397 were too high and SWC was too low at that time of the season, July-August.

398 On the other hand, all cereal-legume mixtures had a NBP that was negative during the growth period  
 399 (Fig. 7), indicating that there was C input into the system beyond the yield. In this sense, it is worth  
 400 estimating the optimum amount of biomass that can be harvested and left in the field, in order to achieve  
 401 the maximum NBP of the system, without compromising the yield. Yet, our third hypothesis had to be  
 402 rejected: cereal-legume mixtures did not clearly increase NBP as compared with cereal monocultures  
 403 during the growth period, since some cereal monocultures (wheat, year 2015, and barley, year 2016) had  
 404 a similar NBP during the growth period (Fig. 7).

405 However, we do still believe that cereal-legume mixtures could have shown an increase in NBP  
 406 magnitude (more negative NBP) compared with cereal monocultures, had we assessed the entire crop  
 407 season (growth and fallow). The particularly pronounced voluntary regrowth of the vegetation during the  
 408 fallow period of cereal-legume mixtures (Fig. 2.d), provided a profitable resource for livestock, besides  
 409 providing an important litter input into the system. This, combined with the moderate grazing intensity  
 410 ( $\approx 0.91$  LSU ha<sup>-1</sup>), left an important part of the vegetation in the field, thereby increasing NBP, and partly  
 411 offsetting C losses due to harvesting. Thus, for future studies, we recommend to estimate C exports  
 412 through grazing during the fallow period (in addition to determine soil C content), to more accurately



413 estimate C inputs and exports, and consequently NBP during the whole crop season in the studied forage  
414 system.

415 Finally, legumes present in cereal-legume mixtures had higher crude protein, lower neutral detergent  
416 fibre, and higher nitrogen content than all cereals (Table S1), and vegetation remaining in the field could  
417 also be increasing soil nitrogen. Soil nitrogen determination would also be recommendable in further  
418 studies to fully assess the effect of forage species on soil fertility.

419

## 420 **Conclusions**

421 Based on the findings of seven years of continuous NEE measurements in an intensively managed forage  
422 system in the Pyrenees, we found strong evidence that cereal-legume mixtures increased net CO<sub>2</sub> uptake  
423 compared with cereal monocultures. Cereal-legume mixtures enhanced photosynthetic activity and gross  
424 CO<sub>2</sub> uptake compared with cereal monocultures, without significantly increasing respiration, therefore  
425 increasing net CO<sub>2</sub> uptake. Also, management practices associated with cereal-legume mixtures,  
426 particularly an earlier harvesting time, allowed higher voluntary regrowth of the vegetation during the  
427 fallow period. This provided additional feed for the livestock, and enhanced net CO<sub>2</sub> uptake during that  
428 period, which was decisive for the net CO<sub>2</sub> budget of the whole crop season. Cereal-legume mixtures  
429 enhance net CO<sub>2</sub> uptake capacity of forage systems compared with cereal monocultures, while ensuring  
430 productivity and forage quality.

431

## 432 **Data availability**

433 Data are not public as are currently being used for other research projects. Please contact the  
434 corresponding author by e-mail for queries concerning the data used in this study.

435

## 436 **Author contribution**

437 MI performed research, analysed data and wrote the paper; NA conceived and designed the study,  
438 performed research and revised the paper; AR conceived and designed the study and revised the paper;  
439 WE analysed data and revised the paper; MTS conceived and designed the study and revised the paper.

## 440 **Competing interests**

441 The authors declare that they have no conflict of interest.



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## 688 Tables

689 **Table 1.** Sward management: Forage type, species, fertilizer type (NPK 9-23-30: nitrogen 9%, phosphorus  
 690 23%, potassium 30%; urea; and NAC 27: calcium ammonium nitrate 27% nitrogen) and rate, sowing date  
 691 and rate, harvesting date, yield and C exported through yield.

Forage type	Species	Fertilizer (kg ha <sup>-1</sup> )	Sowing date	Sowing rate (kg ha <sup>-1</sup> )	Harvesting date	Yield (dry weight) (kg ha <sup>-1</sup> ) (g C m <sup>-2</sup> )	
Cereal monoculture	Barley	NPK 9-23-30, 250	01/11/2010	221	07/07/2011	3000	138
Cereal monoculture	Triticale	Urea, 140	01/11/2011	221	01/07/2012	13133	607
Cereal-legume mixture	Triticale, oat, vetch	Not applied	01/11/2012	225	19/06/2013	7500	339
Cereal-legume mixture	Oat, vetch	Urea, 130	01/11/2013	239	01/07/2014	6720	304
Cereal monoculture	Wheat	NPK 9-23-30, 250	01/11/2014	212	01/08/2015	2580	118
		Urea, 120					
Cereal monoculture	Barley	NAC 27, 100	01/11/2015	221	01/09/2016	4500	208
Cereal-legume mixture	Oat, vetch	Not applied	01/11/2016	235	01/06/2017	7200	326

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694 **Table 2. Diversity-interaction model results. Net ecosystem exchange (NEE) as function of air temperature**  
 695 **( $T_a$ ), net radiation ( $R_{net}$ ), vapour pressure deficit (VPD), and species proportions: barley, triticale, wheat, oat**  
 696 **and vetch (see forage species proportions in Fig. 1). Model performed on weekly-averaged values of all the**  
 697 **variables. Estimates (Est.) of the explanatory variables, standard error (SE), t and p-value.**

698

	NEE ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ )			
	Est.	SE	t	p
$T_a$ ( $^{\circ}\text{C}$ )	0.19	0.04	5.06	< 0.001
$R_{net}$ ( $\text{W m}^{-2}$ )	-0.030	0.002	-12.61	< 0.001
VPD (hPa)	0.17	0.05	3.56	< 0.001
Barley (fraction)	-1.0	0.3	-3.39	< 0.001
Triticale (fraction)	-1.6	0.4	-4.40	< 0.001
Wheat (fraction)	-1.5	0.3	-4.42	< 0.001
Oat x vetch (fraction)	-2.0	0.3	-7.44	< 0.001
Triticale x oat x vetch (fraction)	1	2	0.58	0.6
$R^2_{Adj}$	0.45			< 0.001

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700



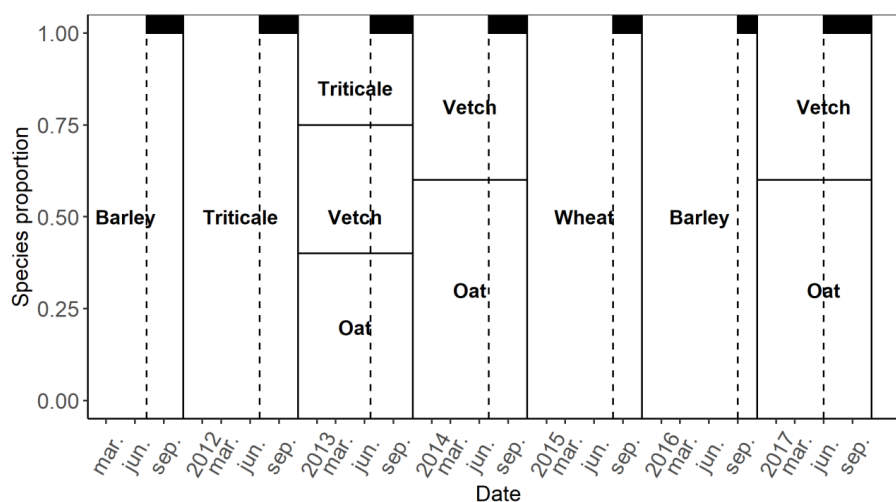
Table 3.  $R_{\text{eco,night}}$  soil temperature and soil water content response parameters based on the equations proposed by Reichstein et al. (2002, Eq. 4-6): reference ecosystem respiration ( $R_{\text{eco,ref}}$ ); soil water content below which  $R_{\text{eco}}$  ceases ( $\text{SWC}_0$ ); soil water content at which maximal  $R_{\text{eco,night}}$  halves ( $\text{SWC}_{1/2}$ ); and a and b parameters of the activation energy linear function ( $E_0 = a + b \cdot \text{SWC}$ ). Model performed on weekly averaged values of all the variables. Estimates (Est.) and standard error (SE) of the parameters. Estimates in bold are significantly different from zero ( $p < 0.05$ ).

Parameters	2011 Barley		2012 Triticale		2013 Triticale, oat, vetch		2014 Oat, vetch		2015 Wheat		2016 Barley		2017 Oat, vetch		All seasons	
	Est.	SE	Est.	SE	Est.	SE	Est.	SE	Est.	SE	Est.	SE	Est.	SE	Est.	SE
$R_{\text{eco,ref}}$ ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ )	1	2	4	2	2.9	0.3	2.3	0.2	2.7	0.6	9	15	3	2	2.8	0.3
$\text{SWC}_0$ (fraction)	0.3	0.6	0.01	0.02	<b>0.048</b>	<b>0.005</b>	<b>0.05</b>	<b>0.002</b>	<b>0.04</b>	<b>0.03</b>	0.03	0.06	0	0.2	0.01	0.01
$\text{SWC}_{1/2}$ (fraction)	0.4	0.9	0.1	0.1	<b>0.054</b>	<b>0.003</b>	<b>0.052</b>	<b>0.002</b>	<b>0.08</b>	<b>0.03</b>	0	1	0.1	0.07	<b>0.06</b>	<b>0.01</b>
a ( $^{\circ}\text{C}^{-1}$ )	-263	221	136	135	<b>215</b>	<b>94</b>	162	138	64	118	83	140	18	126	<b>76</b>	<b>40</b>
b ( $^{\circ}\text{C}^{-1}$ )	<b>3688</b>	<b>1645</b>	596	1251	-603	744	547	987	<b>850</b>	<b>627</b>	-37	833	451	694	<b>483</b>	<b>259</b>
$R^2$	0.59		0.61		0.49		0.69		0.75		0.36		0.19		0.35	



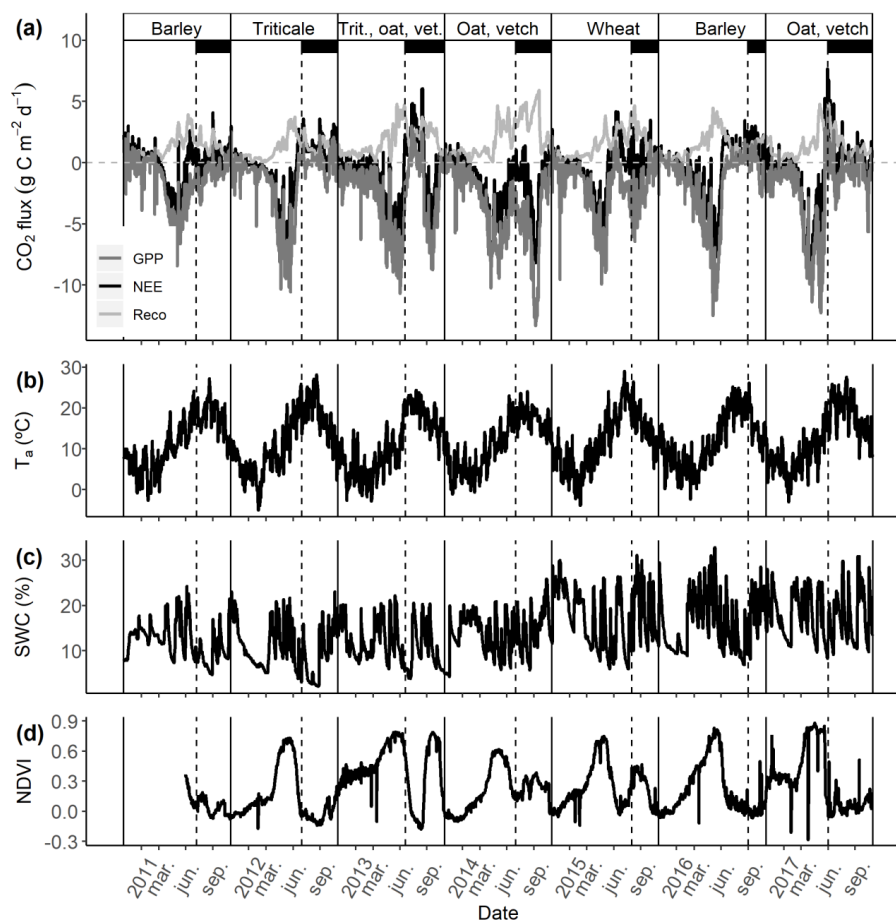


# 708 **Figures**



709 **Figure 1. Crop rotation timeline, species proportions and management events: black dashed lines indicate**  
 710 **harvesting and solid black lines indicate sowing. Top black bands indicate fallow periods in which there was**  
 711 **grazing.**

712



**Figure 2.** Daily averaged (a) CO<sub>2</sub> fluxes: net ecosystem exchange (NEE), gross primary production (GPP) and ecosystem respiration (R<sub>eco</sub>); (b) air temperature (T<sub>a</sub>); (c) volumetric soil water content (SWC); and (d) normalized difference vegetation index (NDVI). Titles in the top panel indicate forage species. Black dashed lines indicate harvest events and solid black lines indicate sowing events. Top black bands indicate fallow periods in which there was grazing.

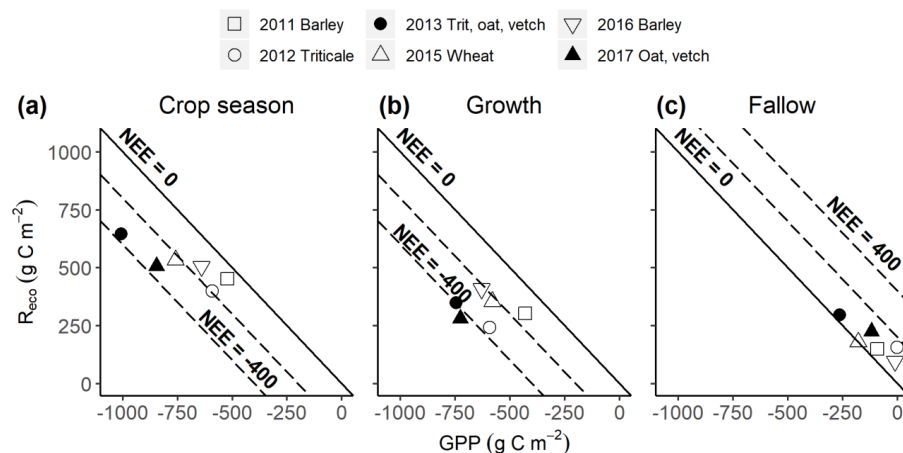


Figure 3. Net ecosystem exchange (NEE), gross primary production (GPP) and ecosystem respiration ( $R_{eco}$ ) budgets after gap-filling per: (a) Crop season, defined as the time from sowing to next sowing; (b) growth period, defined as the time from sowing to harvest; and (c) fallow period, defined as the time from harvest to next sowing. Solid diagonal line indicates  $NEE = 0\ g\ C\ m^{-2}$ , dashed diagonal lines indicate  $\pm 200\ g\ C\ m^{-2}$  NEE intervals. Open symbols indicate cereal monocultures and solid symbols cereal-legume mixtures.

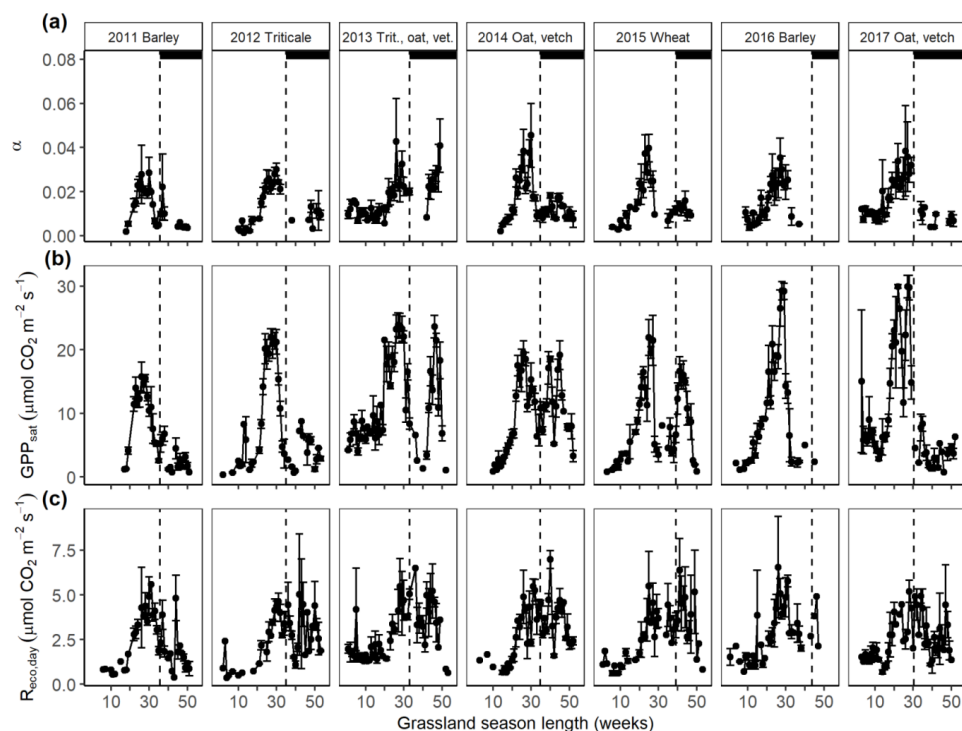
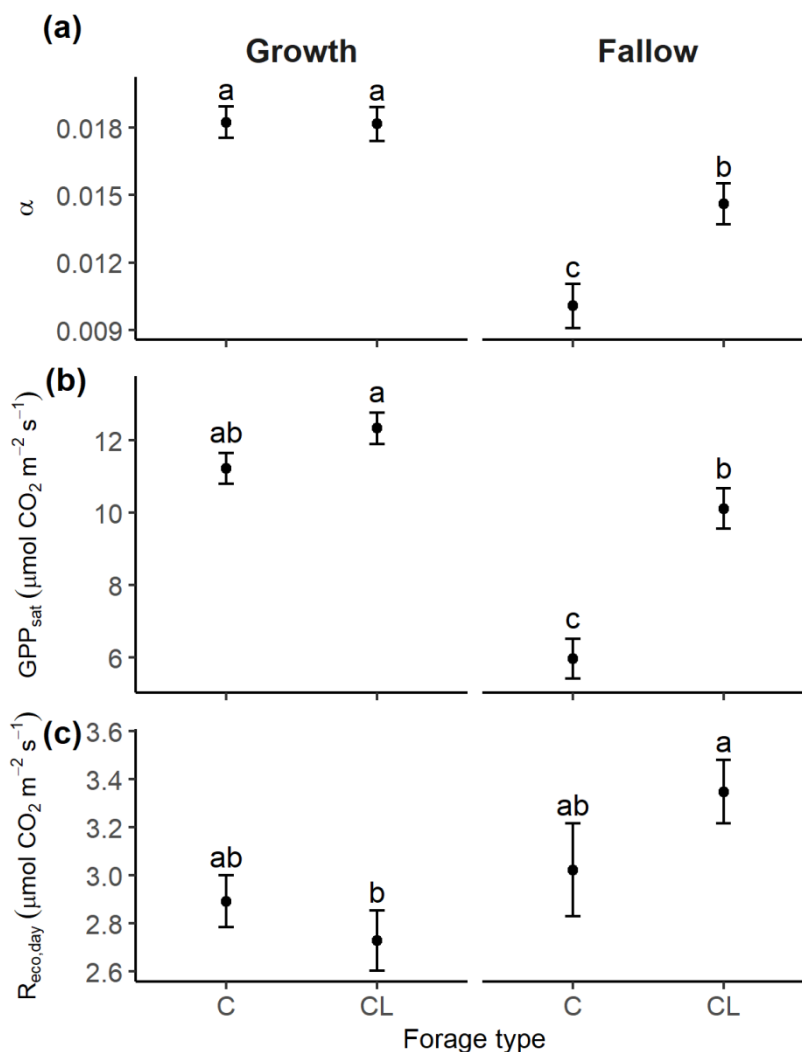
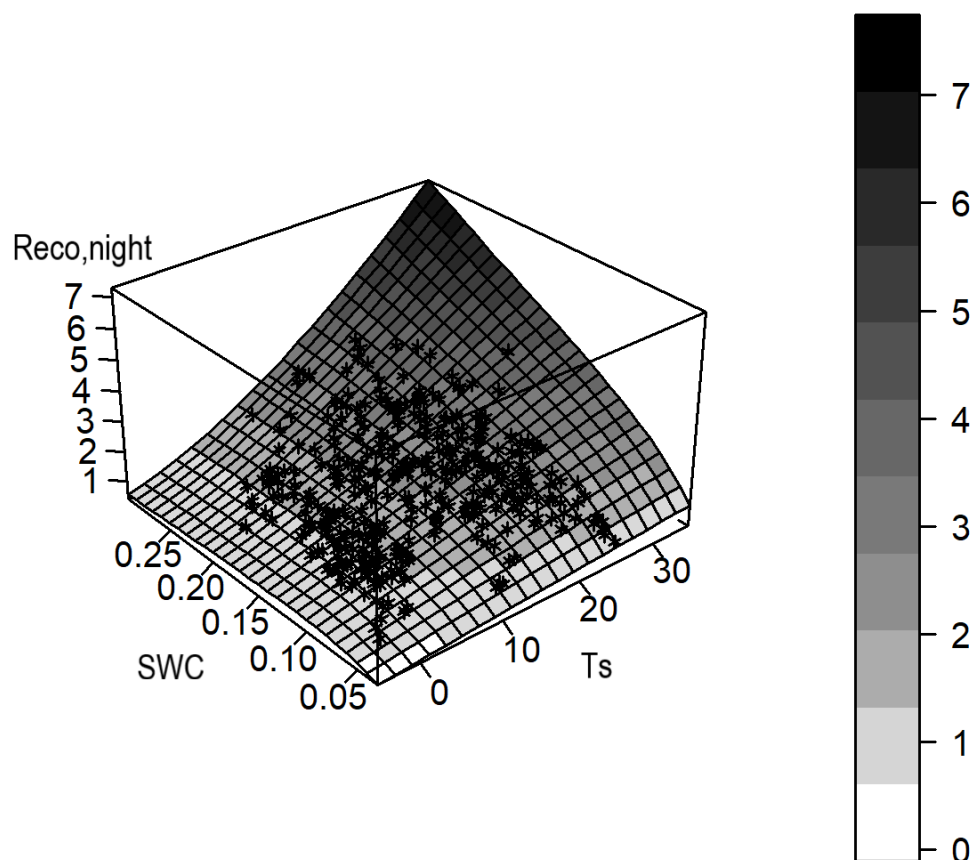


Figure 4. Seasonal dynamics of  $NEE_{day}$  light response parameters Eq. (3): (a) apparent initial quantum yield ( $\alpha$ ); (b) asymptotic gross primary production ( $GPP_{sat}$ ); and (c) daytime ecosystem respiration ( $R_{eco,day}$ ). Weekly averaged values and corresponding standard error bars. Titles in the top panels indicate forage species. Black dashed lines indicate harvesting events. Top black bands indicate fallow periods in which there was grazing. Gaps are due to missing data or not significant estimates ( $p \geq 0.05$ ), which have been discarded.

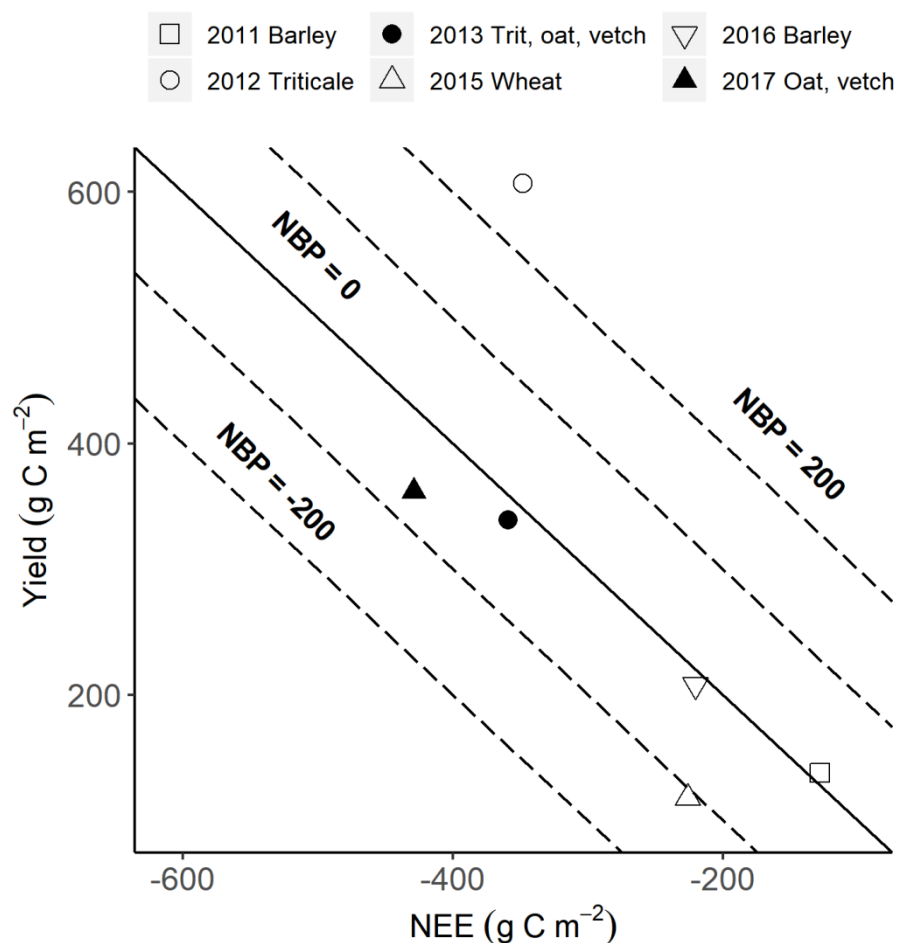


730 **Figure 5.** Light response parameters Eq. (3): (a) apparent initial quantum yield ( $\alpha$ ); (b) asymptotic gross  
 731 primary production ( $GPP_{sat}$ ); and (c) average daytime ecosystem respiration ( $R_{eco,day}$ ) mean  $\pm$  standard error,  
 732 and Tukey post-hoc test per forage type (C: cereal monoculture, CL: cereal-legume mixture) and period  
 733 (growth and fallow). Letters indicate significant differences among groups ( $p < 0.05$ ). See ANOVAs results in  
 734 Table S3.



735 **Figure 6.**  $R_{\text{eco},\text{night}}$  trend surface as a function of soil temperature ( $T_s$ ) and soil water content (SWC), by the  
 736 equations proposed by Reichstein et al. (2002, Eq. 4-6). Model performed on weekly averaged data of all the  
 737 variables. The grid shows the trend surface and dots are observed data.

738



739 **Figure 7.** Net biome production (NBP), net ecosystem exchange (NEE) and yield during the growth period,  
 740 defined as the time from sowing to harvest. Solid diagonal line indicates  $\text{NBP} = 0 \text{ g C m}^{-2}$ , dashed diagonal  
 741 lines indicate  $\pm 100 \text{ g C m}^{-2}$  NBP intervals. Open symbols indicate cereal monocultures and solid symbols  
 742 cereal-legume mixtures.