Effects of elevated CO_2 and extreme climatic events on forage quality and *in vitro* rumen fermentation in permanent grassland

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Abstract. The aim of this study was to analyze changes in botanical and chemical composition, and *in vitro* rumen fermentation characteristics of an upland grassland exposed to climate changes in controlled CO_2 concentration, air temperature and precipitation conditions. Grassland was exposed to future climate scenario coupled with CO_2 treatments (390 and 520 ppm) from the beginning of spring. During summer, an extreme climatic event (two weeks of a +6 °C increase in temperature, together with severe drought, ECE) was applied and then followed by a recovery period. Three cutting dates were considered, i.e. in April, June and November. The results indicate that increases in greenness, nitrogen (N) content and changes in water-soluble carbohydrate profile in association with botanical composition changes for the November cut lead to higher *in vitro* dry matter degradability (IVDMD) in the rumen. Neutral detergent fiber:nitrogen (NDF:N) ratio appeared to be a key driver of forage quality, which was affected in opposite ways by elevated CO_2 and ECE, with a strong impact on rumen fermentation. Atmospheric CO_2 concentration in interaction with ECE tended to affect IVDMD, indicating that the effects of elevated CO_2 and ECE may partly offset each other. Our findings indicate that the various factors of climate change need to be considered together in order to properly characterize their effects on forage quality and use by ruminants.

1 Introduction

Global livestock production has increased rapidly and substantially in recent decades as a result of world population growth and a shift towards diets with a higher animal protein content in many countries (Tilman and Clark, 2014). The livestock sector is singled out as a significant contributor to global human-induced greenhouse gas (GHG) emissions (Gerber et al., 2013), particularly through energy and protein losses in the form of enteric methane (CH_4) and ammonia (NH_3), urea and nitrous oxide (N_2O) released during ruminant digestion. Using 100-year-timescale global warming potentials of 34 for CH_4 and 298 for N_2O (compared to CO_2) (IPCC, 2013), the livestock supply chains emit an estimated total of 7.1 gigatonnes CO_2-eq per year, with ruminants by far the largest contributors (Gerber et al., 2013). Recent studies show that atmospheric CH_4 levels have grown alarmingly rapidly in recent years (Nisbet et al., 2019) and that livestock CH_4 emissions may even have been underestimated (Wolf et al., 2017). Reducing GHG emissions is a crucial challenge for Earth system governance, and there is significant potential for mitigation in the ruminant sector (Herrero et al., 2016).

The bulk of ruminant diet consists of plant material in the form of forage. Globally, grasses comprised 48% (2.3 billion tons) of the total biomass used by livestock (4.7 billion tons) in 2000 (Herrero et al., 2013). Forage quality is a major actionable lever for decreasing GHG emissions, as variability in forage nutritive value has been shown to generate variability in the CH_4 emission levels from ruminants (Thornton and Herrero, 2010). Forage quality is closely linked to ingestibility and digestibility, both of which are largely dependent on the nature and concentrations of the major forage macronutrients, such as structural and non-structural carbohydrates or crude proteins (Fig. 1). Digestibility is the main driver of net dietary energy, rumen microbial synthesis, and production of volatile fatty acids (VFAs), which are the main sources of energy for the ruminant (INRA, 2018). On the other hand, digestible organic matter (OM) is the best indicator of CH_4 emissions, as it is produced by carbohydrate fermentation (Fig. 1, INRA, 2018). In the specific case of permanent grasslands, forage quality is mainly driven by botanical composition, i.e. a large diversity of self-seeded grasses and dicotyledons and broad variability in phenology and competitiveness, and is affected by agricultural practices (Rossignol et al., 2014; Andueza et al., 2016).

Forage quality can also be impacted by elevated temperatures and by the intensity and frequency of extreme climatic events (ECEs) such as droughts and heat waves, which are projected to increase (Planton et al., 2008). Lee et al. (2017) modeled the variation in nutritive value of forage species growing across a range of bioclimatic zones and showed that higher temperatures reduce forage nutritive value, likely due to changes in species identity, physiology and phenology. They also found that CH_4 production may increase by 0.9 % with a 1 °C temperature rise and by 4.5 % with a 5 °C rise. The effects of interactions between ECEs and elevated CO_2 on grassland ecology and forage quality are not well understood, especially for permanent grasslands (Dumont et al., 2015). There is a need to address the gap in knowledge on the drivers of forage quality under projected climatic conditions in order to adapt grass-based ruminant systems to the context of global climate change.

The aim of this study was to analyze changes in botanical and chemical composition of plants from an upland grassland when exposed to elevated atmospheric CO_2 level combined or not with an ECE (drought combined with heat wave) in controlled conditions. The impact of these changes on ruminant digestion was investigated by determining *in vitro* rumen fermentation

parameters. The hypothesis tested was that combined drivers of global change (elevated CO_2 and ECEs) may have different effects on forage characteristics and digestion by ruminants, with potential offsets.

2 Materials and methods

2.1 Experimental design

The experimental design was previously described in Roy et al. (2016) and Volaire et al. (2020). The present study tested the forage quality response of an upland-grassland plant community near Saint-Genès-Champanelle (central France) to future climate scenarios projected for the 2050s (Ciais et al., 2005). For the representative year 2045 given by the ARPEGEv4 atmosphere-ocean general circulation model under the A2-CO_2 emissions scenario (Nakicenovic et al., 2000) and using a multivariate statistical downscaling methodology (Boé et al., 2006) to generate projections over an 8 × 8 km grid, the projected annual means for air temperature and precipitation at Saint-Genès-Champanelle were 10.9 °C and 770 mm, respectively. These values correspond to +2.3 °C and -33 mm compared to the mean air temperature and precipitation measured over the 1990–2009 period. Here, we chose to apply less precipitation and higher temperatures than under the prevailing current climate from the start of the experiment and for all treatments (all monoliths were exposed to future climate scenario), in order to test and compare the effects of elevated CO_2 and increased ECE under these drier and warmer conditions. The baseline climate conditions of this experiment were therefore drier and warmer than at the origin site (Saint-Genès-Champanelle). Furthermore, we tested and compared the atmospheric CO_2 concentration forecasted for the 2050s (according to the A2 scenario), i.e. 520 ppm, against the CO_2 concentration of 390 ppm measured in 2010.

In June 2009, 1-m^2 monoliths (n = 48) formed by undisturbed soil and vegetation from an extensively managed upland semi-natural grassland, were excavated to 60 cm depth. The origin site (Redon, 45°43' N, 03°01' E, 800 m.a.s.l.) is located near Saint-Genès-Champanelle and is a long-term fertile (clover rich) grassland managed by a combination of grazing (three to five grazing periods) and cutting (one cut per year). One organic fertilization occurs at the end of the winter period. The average botanical composition of the plant communities was initially dominated by C_3 perennial grasses (60%), legumes (35%), and forbs (5%). At the start of the experiment, five species accounted for 70% of species composition: *Trifolium repens*, *Lolium perenne*, *Holcus lanatus*, *Agrostis tenuis*, and *Alopecurus pratensis*. The origin-site soil is a cambisol of 59.5% sand, 19.2% silt and 21.3% clay, with a pH of 5.9. Once excavated, the 48 monoliths were transferred to the INRAE research station (Clermont Ferrand, 45°46' N, 03°08' E, 350 m.a.s.l.) where the soil water content (SWC) was maintained at near 80% of original field capacity between natural precipitation and additional irrigation.

At the beginning of 2010, the monoliths were transported to the CNRS Ecotron near Montpellier (43°40′N, 03°52′E). Four monoliths were randomly allocated to each of the 12 Ecotron macrocosms. The macrocosms were exposed from April 2010 to early March 2011 to the future climate scenario forecasted for 2050, according to the ARPEGEv4 model, and to current CO_2 concentration. From mid-March 2011 to November 2012, six randomly-selected macrocosms were exposed to 520 ppm CO_2 and the other six to 390 ppm CO_2.

For each CO_2 concentration treatment, a first phase of the ECE treatment (reduction of 50% precipitation) was applied on three out of six monoliths from 25 June to 21 July. A second phase of the ECE treatment (no irrigation and a 3.4°C increase in air temperature per the year-2050-scenario) was applied from 22 July to 4 August. This temperature increase corresponded to 7.1 °C higher than the mean temperature from 2000-2009 at the same period, and was above the 14 consecutive hottest days of summer 2003. From 5 August to 31 August, irrigation was progressively applied in the treatment with ECE to allow the same cumulative precipitation as in the non-ECE-treated monoliths. From the end of August until the beginning of November, all macrocosms were exposed to the 2050 climate conditions. From April 2010 to November 2011, the Ecotron climate-regulation system monitored hourly means air temperature and humidity, daily precipitation and CO_2 concentration. Each of the four experimental treatments, combining both CO_2 and ECE treatments was replicated three times. Further details on the experimental conditions can be found in Roy et al. (2016). In each of the 48 monoliths, SWC was continuously measured at soil depths of 7, 20 and 50 cm using Time Domain Reflectometry (TDR) probes (IMKO, Ettlingen, Germany) and averaged across soil depths and monoliths in order to get one value per macrocosm. In addition, to match with data on forage quality, SWC was averaged across regrowth periods before the cuts, i.e. from 1 April to 26 April, from 27 April to 9 June, and from 22 September to 3 November.

2.2 Plant materials, cutting and botanical composition

In 2011, on three dates (26 April, 9 June, 3 November), above-ground biomass was harvested on a fixed center-square (0.5 × 0.5 m^2) in each monolith using a precision mower (6 cm cutting height). The April cut was the expression of winter and spring growth, and no treatments were applied except for one month of CO_2 treatment, whereas the June cut was the expression of spring and early summer growth and CO_2 treatment, and the November cut was the expression of summer and fall growth and included the CO_2 treatment, the ECE and the recovery phase.

The cut material was weighed to determine fresh above-ground biomass and then separated into three subsamples: the first subsample was oven-dried at 60°C for 72 h and used for dry matter (DM) determination and chemical analyses; the second subsample was freeze-dried and used for the *in vitro* rumen fermentation assay; the third subsample was used to sort out green, dead and flower biomass and determine the botanical composition of the green material. Species were sorted by hand, oven-dried (60°C, 72 h) and weighed separately, in order to calculate relative abundance for each species and then define the functional groups, i.e. grasses, legumes and forbs (shown as relative abundance).

2.3 In vitro rumen fermentation assay

All experimental procedures were performed according to the European Union Directive 2010/63/EU, reviewed by the local institutional review board (C2E2A, “Comité d’Ethique pour l’Expérimentation Animale en Auvergne”), and approved under French Ministry for Research authorization No. CE 69-12.

For each macrocosm and each cutting date, a representative sample was reconstituted with cut freeze-dried material from the four monoliths, weighted according to the values of above-ground biomass measured on each monolith (thereby pooling the four monoliths, n = 12). The rumen fermentation assay was performed on the 12 samples taken on each cutting date, three times over a period of two weeks.

Freeze-dried plant material (600 ± 0.5 mg) was transferred in 120-mL serum bottles, pre-warmed at 39 ± 0.5 °C, and flushed with N_2 to eliminate the oxygen. The serum bottle was then added with 40 mL buffered rumen fluid, and the bottle was hermetically sealed with a butyl rubber stopper and aluminum crimp seals. The buffered rumen fluid was prepared as follows: rumen contents were collected before the morning feeding from three cannulated sheep fed daily with 1200 g of a diet composed of 80% permanent grassland hay and 20% concentrate mix. Rumen contents from the three sheep were mixed in the same proportions in a container and squeezed through two layers of cheesecloth (800-µm mesh size) to obtain the fluid used as inoculum for the *in vitro* rumen fermentation assay. Strained rumen fluid was diluted in an anaerobic buffer solution (phosphate:carbonate, 1:2 v/v) as described by Goering and Van Soest (1970) and modified by Niderkorn et al. (2011). Initial pH of the buffered rumen fluid was 7.03 ± 0.02. All bottles were incubated in a shaking water bath at 39 ± 0.5 °C, and blanks without any plant substrate (only buffered rumen fluid) were included. At t = 0, samples of buffered rumen fluid were taken to determine the VFAs and NH_3 concentrations in the medium before incubation.

After 24 h of incubation, the volume of gas produced in the headspace of the serum bottles was determined using a pressure transducer (Theodorou et al., 1994) and gas samples were taken for determination of CH_4 and CO_2 concentrations. The entire contents of the bottle were then transferred into a pre-weighed 50-mL Falcon tube, and the pH was immediately measured. Tubes were centrifuged at 3,400 × *g* for 10 min at 4 °C, and samples of supernatant were taken for determination of VFA and NH_3 concentrations (Niderkorn et al., 2011). To recover all the non-degraded particles, the bottle was washed twice with distilled water, and the washing water was transferred into the Falcon tube. Tubes were again centrifuged at 3,400 × *g* for 10 min at 4 °C, and after removal of the supernatant, the residue was used for DM determination.

2.4 Analytical procedures

Plant substrates and leftovers after fermentation (residues) were analyzed to determine DM by oven-drying at 60 °C for 72 h and OM by ashing at 550 °C for 6 h in a muffle furnace. In-plant neutral detergent fiber (NDF) content was determined according to the method described by Van Soest et al. (1991) using an Ankom fiber analyzer (Ankom Technology Corporation, Fairport, NY). The leaf carbon (C) and nitrogen (N) contents were determined at the INRAE-Nancy isotopic analysis platform using a stable isotope-ratio mass-spectrometer (IsoPrime 100, IsoPrime, Manchester, UK). Water-soluble carbohydrates (WSC) were successively extracted from dry powder with 80% ethanol and water, according to Benot et al. (2019). Supernatants were pooled and evaporated under vacuum to eliminate ethanol and water and, thus concentrate the samples. The residue was dissolved in water and passed through ion exchange resins to remove charged compounds before HPLC analysis. WSC were separated on a cation exchange column (Sugar-PAK I, 300×6.5 mm, Millipore Waters Milford, MA) and detected using a refractometer (see Benot et al., 2019 for more details). Condensed tannins (CT) content was determined using the

colorimetric HCl-butanol method (Grabber et al., 2013). Pepsin-cellulase OM digestibility was evaluated according to the method described by Aufrère and Michalet-Doreau (1988). *In vitro* DM degradability (IVDMD) was determined as the difference between the DM of plant material before the fermentation and the DM of fermentation residue after 24 h of fermentation. The CH_4 and CO_2 concentrations in gas samples were determined by gas chromatography using a MicroGC 3000A system (Agilent Technologies, France). Total and individual VFA (acetate, propionate, butyrate, valerate, caproate, isobutyrate, isovalerate) in the supernatant were measured by gas chromatography and NH_3 was determined using the Berthelot reaction (Park et al., 2009).

2.5 Statistical analysis

All variables related to the chemical composition of plant communities and *in vitro* rumen fermentation parameters were analyzed using a mixed model (MIXED procedure, SAS Enterprise Guide 5.1, SAS Institute Inc., Cary, NC). Each macrocosm was considered as an experimental unit. For the April and June cuts, CO_2 concentration was used as a fixed effect, and the effect of ECE treatment and CO_2 × ECE interaction were added as fixed effects for the November cut with the macrocosm used as a random factor. For each variable analysed, data were subjected to three covariance structures: compound symmetry, autoregressive order 1, and unstructured covariance. The covariance that resulted in the smallest Akaike's Information Criterion was retained for analysis. Fractions (relative abundances) were transformed by the arcsine of the square root before the analysis of variance. Significance was set at $p \leq 0.05$ and trend was set at $0.05 < p < 0.10$. Relationships between above-ground biomass characteristics, chemical composition and *in vitro* rumen fermentation parameters were analyzed with non-parametric Spearman correlation tests.

3 Results

The measured values of temperature and atmospheric CO_2 concentration throughout the experiment for the different treatments were reported in Roy et al. (2016). When the ECE was imposed, mean daily air temperature peaked at 25 °C. As expected, the ECE strongly affected SWC during the stress period (Table 1, $p < 0.001$). There was a significant effect of CO_2 × ECE interaction during the period preceding the November cut (Table 1, $p = 0.014$), reflecting a higher SWC for the control at 520 ppm CO_2 compared to the other treatments.

3.1 Above-ground biomass characteristics and chemical composition

Above-ground biomass did not differ significantly among treatments. The November cut had more green material ($p = 0.013$) and less dead material ($p = 0.018$) with the ECE than in the control (Tables 2 and S1, and seasonal pattern shown in Fig. 2). There were very few differences among treatments in terms of relative abundances of functional groups or species (Tables 2, S1 and S2, Fig. 4) due to large variability among the macrocosms, except for *Holcus lanatus* which decreased dramatically after the ECE ($p = 0.001$). There was a seasonality-driven cutting date effect on above-ground biomass, fractions of green and

dead materials and flowers ($p < 0.001$), and relative abundances of several species, but without a significant cutting date × CO_2 effect (Table S2).

The N content in the above-ground biomass was significantly lower at 520 ppm CO_2 concentration compared to 390 ppm (Tables 3 and 4). This was shown in cuts from every season: April (-11%, $p < 0.001$), June (-9%, $p = 0.003$) and November (-21%, $p = 0.007$). Increasing the CO_2 concentration caused an increase in OM content in April (+1%, $p = 0.033$) and a decrease in NDF content in June (-3%, $p = 0.002$), and increased C:N ratio and NDF:N ratio at the three cutting dates ($p < 0.05$). After ECE recovery in November, there were strong increases in N content (+54%, $p < 0.001$), sucrose content (+31%, $p = 0.022$) and fructose content (+23%, $p = 0.031$). The ECE significantly decreased NDF content (-7%, $p = 0.027$), C:N ratio (-34%, $p < 0.001$) and NDF:N ratio (-39%, $p < 0.001$) and increased pepsin-cellulase OM digestibility (+14%, $p = 0.005$). There was no effect of CO_2 × ECE interaction on chemical composition of the above-ground biomass ($p > 0.05$). There was a significant cutting date effect on all the chemical composition parameters except C content, with a significant cutting date × CO_2 effect on N content ($p < 0.001$), C:N ratio ($p < 0.001$) and NDF:N ratio ($p < 0.001$) (Table S3). By taking all data into account (36 observations), many significant relationships were observed between above-ground biomass characteristics and chemical composition of forage (Table S4). Chemical composition was strongly driven by green material percentage as well as plant species abundances, especially those of *Alopecurus pratensis*, *Holcus lanatus* and *Lolium perenne* (Figure S1).

3.2 *In vitro* rumen fermentation characteristics

Estimated IVDMD was significantly lower (-3%, $p = 0.041$) in plants exposed to 520 ppm CO_2 concentration compared to 390 ppm for the April cut and tended to be lower for the November cut ($p = 0.075$) (Tables 3 and 4). Increasing the level of CO_2 drastically decreased the NH_3 concentration in the incubation medium for all cuts (-21%, $p = 0.014$; -31%, $p = 0.001$ and -34%, $p = 0.005$, respectively), decreased the proportion of valerate for the cuts of April and June (-7%, $p = 0.016$ and $p = 0.017$, respectively) and increased the acidification for the November cut (+11%, $p = 0.007$). Increasing the level of CO_2 also tended to increase total VFA concentration ($p = 0.056$) and decrease the proportion of iso-valerate for the April cut ($p = 0.062$), and decrease the proportion of iso-butyrate for the November cut ($p = 0.063$).The ECE treatment very significantly increased NH_3 concentration in the incubation medium (+90%, $p < 0.001$), IVDMD (+10%, $p = 0.001$), the proportions of propionate, valerate and iso-valerate (+4%, $p = 0.008$; +21%, $p = 0.004$ and +25%, $p = 0.006$, respectively), and tended to decrease the proportion of acetate ($p = 0.064$), whereas acetate:propionate ratio, which is related to the fermentation pathways in the rumen, decreased (-5%, $p = 0.013$). The CO_2 × ECE interaction tended to have effects on IVDMD ($p = 0.053$) and iso-valerate concentration ($p = 0.067$). There was a cutting date effect (seasonality) on all *in vitro* rumen fermentation parameters except iso-valerate concentration and CO_2:CH_4 ratio, with a significant cutting date × CO_2 effect on NH_3, valerate and iso-valerate concentrations (Table S3).

By taking all data into account, many strong significant relationships were observed between *in vitro* rumen fermentation characteristics and chemical composition of forage (Table S4). For example, IVDMD was negatively correlated with NDF and NDF:N ratio and positively correlated to fructan content. NH_3 emission was positively correlated with above-ground biomass

N content (Table S4, Figure S1). As expected due to the link between chemical composition and above-ground biomass characteristics, significant correlations were observed between *in vitro* rumen fermentation characteristics and plant species abundances, especially those of *Lolium perenne*, *Holcus lanatus* and *Alopecurus pratensis* (Table S4, Figure S1). Acidification and CH_4 emission appeared to be strongly positively driven by the abundance of *Lolium perenne* (Table S4) while NH_3 emission was driven by that of *Alopecurus pratensis* (Figure S1). The strong negative relationship between *Holcus lanatus* abundance and iso-valerate production (Figure S1) suggests that the increase of isovalerate production following the ECE was due to the decline of *Holcus lanatus*.

4 Discussion

4.1 Elevated CO_2 and ECE modify above-ground biomass and botanical composition

In this experiment, above-ground biomass was not affected by elevated CO_2 or an ECE, probably because the control was actually exposed to drier and warmer conditions compared to the current ones (referred to the mean of the 1990-2009 period). These conditions were applied to allow us to compare the effect of elevated CO_2 and ECE under the projected year-2050 climate scenario. The fact that the control was under little stress may have limited biomass growth and may explain why no difference was observed between treatments with and without an ECE, as expected. Although there was no overall effect of elevated CO_2 on the plant fractions, we nevertheless observed a significant increase of green mass for the control in November. This can be related to increases of leaf area index and canopy photosynthesis linked with higher SWC under elevated CO_2 as indicated by additional measurements made in the same experiment (Roy et al., 2016).

There was no effect of the ECE on above-ground biomass for the November cut, although it had more green tissue and less dead tissue compared to the control. This could be attributed to a strong increase in the shoot N pool driven primarily by an effect of the ECE on the below-ground compartment (Roy et al., 2016). These authors showed that ECEs strongly increased the root N pool, thereby increasing N availability. The CO_2 × ECE interaction suggests that SWC before the November cut in the 520 ppm CO_2 treatment was reduced with the ECE and was enhanced in the control compared to the other treatments. This could be explained by enhanced leaf area index, photosynthesis, greenness and C sequestration leading to higher water extraction from the soil after an ECE, corresponding to higher recovery under elevated CO_2 (Roy et al., 2016).

The lack of significant differences in relative abundances of functional groups and species can be attributed to the huge variability in ecosystem responses to elevated CO_2 and ECE. We did, however, observe a notable change due to the high vulnerability to the ECE of *Holcus lanatus* that mostly disappeared. Volaire et al. (2020) recently showed that soluble carbohydrate metabolism, particularly fructans and sucrose, plays a role in the lack of *Holcus lanatus* recovery to the ECE.

4.2 Elevated CO_2 and ECE modify the chemical composition of above-ground biomass

We observed contrasted effects of elevated CO_2 and ECE on the chemical composition of above-ground biomass. The lower N concentration under elevated CO_2 compared to control was shown for all the cutting dates and is consistent with findings from meta-analyses addressing climate change effects on grassland (Dumont et al., 2015; Dellar et al., 2018). Note that this lower N concentration was not accompanied by significant changes in legume proportions, which could have been an explanatory factor due to their high N content. The reduction in N content may therefore reflect a combination of increased growth and changes in photosynthetic N use efficiency (Leakey et al., 2009). Even though NDF concentration was only affected for the June cut, the NDF:N ratio increased at elevated CO_2 concentration for all cutting dates, but without negative impact on pepsin cellulase OM digestibility. This parameter could have been affected, as a high concentration of partially digestible fiber (NDF) and a limiting N concentration can be detrimental for the microbial ecosystem in the rumen (Sinclair et al., 1995). These results are globally well in line with those described in a recent study on mixed grass prairie (Augustine et al., 2018) and a meta-analysis on the effects of climate change on pasture quality in Europe (Dellar et al., 2018).

We observed a clear increase in N concentration in above-ground biomass from the November cut. ECE-driven dehydration of plant material may have resulted in the asynchrony between plants and soil microbial community functioning. High plant litter and microbial detritus during the ECE are both sources of energy for microbial recovery in soil during rehydration, which is faster than in plants (Hofer et al., 2017). Microbial mineralization of the OM produces inorganic N that plants can only uptake if they have recovered. In addition, it has been shown that the maintenance of root exudates during drought may be one of the factors that enable above-ground recovery, since root functionality ensures increased N availability (Karlowsky et al., 2018). The flush of N can explain the increase of N and sucrose content in the above-ground biomass due to high photosynthesis and transpiration indicated by lower SWC (Roy et al., 2016). An increase in fructose content, as observed here after the ECE, is usually linked to hydrolysis of fructans (Simpson and Bonnett, 1993), but we did not find any significant decrease in fructan content in this experiment. These ECE-driven changes led to a much lower (~ 50%) NDF:N ratio than in the control. A low NDF:N ratio, in addition to effects on some sugars, is particularly beneficial in terms of forage quality, as confirmed by higher pepsin cellulase OM digestibility due to an increase in readily-degradable nutrients providing increased amounts of energy and nitrogen for rumen microbial synthesis (Nocek and Russell, 1988).

4.3 Changes in chemical composition affect rumen fermentation parameters

The changes in chemical composition in above-ground biomass following the ECE strongly affected rumen fermentation parameters. The lower NDF content led naturally to increased IVDMD as some fibers especially lignin are known to be indigestible (Jung and Allen, 1995). This is consistent with the increase in pepsin cellulase OM digestibility, a parameter that is closely correlated with *in vivo* digestibility (Aufrère et al., 1988). Interestingly, despite an increase in IVDMD following the ECE, we did not find any of the usually observed increased total gas production, including CH_4 (Getachew et al., 2004). This could mean that a potential increase in energy available for the animal was not accompanied by more energy losses and pollutant emissions. This could be due to the changes observed in VFA profiles (acetate, propionate and valerate) indicating

changes in fermentation pathways. In particular, we observed a decrease in acetate:propionate ratio, which is known to be related to hydrogen availability and CH_4 production in the rumen (Russell, 1998). The increase in N concentration after the ECE, which may be partly linked to the increase of *Alopecurus pratensis* abundance, also led to an increase in ruminal NH_3, which is a main end-product of protein degradability through amino acid deamination. The increase in isovalerate with the ECE, which appears to be related to the decrease of *Holcus lanatus* abundance, also indicates increased protein degradation as this branched-chain VFA results from deamination of branched-chain amino acids such as leucine (Menahan and Schultz, 1964). Part of the NH_3 produced is incorporated into the rumen microbial biomass, but the surplus is transformed into urea, which gets excreted into the environment and thus drives N loss and pollutant emission. Indeed, the fraction of urinary N not used by soil microbes and plants is transformed into N_2O, a potent GHG, during microbial processes of nitrification and denitrification (Firestone et al., 1980).

The changes in chemical composition under elevated CO_2 affected rumen fermentation parameters in a different manner compared to the ECE. Interestingly, we observed contrasted effects according to cutting date. Elevated CO_2 decreased IVDMD for the April cut but then increased IVDMD in November following the ECE, resulting in a trend towards a CO_2 × ECE interaction. This could mean that the ECE counteracts the negative effect of elevated CO_2 on IVDMD, likely due to the decrease in NDF concentration. Note, however, that we never observed any significant change in VFA production, which is one of the main drivers of energetic value for the animal as VFAs provide more than 70% of ruminant energy supply (Bergman, 1990). For all cutting dates, the decrease of N content in above-ground biomass under elevated CO_2 led to lower NH_3 concentrations of in the rumen, for the reasons given above.

Analysis of the correlation matrix (data not shown) showed a positive correlation between CT content in above-ground biomass and CO_2:CH_4 ratio in fermentation gas ($r = 0.51$, $p = 0.002$) and negative correlations between CT content and IVDMD ($r = -0.38$, $p = 0.024$) and total VFA production ($r = -0.40$, $p = 0.015$). The antimethanogenic effect of CT and the reduction of IVDMD are consistent with the literature (meta-analysis by Jayanegara et al., 2012). However, in this experiment, mean CT content values were higher following the ECE compared to control, but the difference was not significant.

Figure 4 provides a schematic illustrative overview of the results obtained in this experiment, showing the impact of the ECE and elevated CO_2 on ruminant digestive degradation of plant macronutrients (carbohydrates and protein). Our findings suggest that the ECE, by increasing aboveground biomass N content, increased N metabolism in the rumen, probably with a positive effect on rumen microbial synthesis. This, along with the lower in-plant NDF content and higher sucrose and fructose contents, may explain the observed increase in IVDMD. In contrast, the elevated atmospheric CO_2 concentration reduced aboveground biomass N content and ruminal N metabolism, thereby resulting in a negative impact on IVDMD.

5 Conclusions

This study shows that different drivers of climate change, i.e. elevated atmospheric CO_2 concentration and ECEs (drought and heat wave), have contrasted impacts on forage quality through their effects on plant characteristics. An ECE was followed by

an increase in greenness and in N and water-soluble carbohydrate contents in the above-ground biomass produced during the regrowth stage, resulting in higher OM digestibility. Taken together, our results point to NDF:N ratio as a major driver of forage quality, which is highly likely to be affected differently by elevated CO_2 and ECE, both of which will have strong impacts on rumen fermentation. In addition, our data on the CO_2 × ECE interaction indicate that elevated CO_2 may limit the ECE-driven gain of IVDMD.

Author contributions

CPC designed the study, AMB, ALM, AA, MLD, VN and CPC contributed to the acquisition, analysis and interpretation of data. VN wrote the manuscript with contributions from all co-authors.

Competing interests

No competing interests to disclose.

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Tables

Table 1. Factor effects (*p*-values) on soil volumetric water content (SWC) at two levels of CO_2 concentration (390 and 520 ppm) with or without an extreme climatic event (ECE).

	1-26 April	27 April – 9 June	21 July – 3 August (stress period)			22 September – 3 November		
	CO_2 effect	CO_2 effect	CO_2 effect	ECE effect	CO_2 × ECE effect	CO_2 effect	ECE effect	CO_2 × ECE effect
SWC	0.736	0.430	0.775	**<0.001**	0.312	0.111	0.366	**0.014**
d.f (num/den)	1/10	1/10	1/8	1/8	1/8	1/8	1/8	1/8

d.f (num/den), degrees of freedom (numerator and denominator). In bold: $p < 0.05$; Underlined: $0.05 < p < 0.1$

Table 2. Factor effects (*p*-values) on: above-ground mass, fractions of green material, flower and dead material, relative abundance of functional groups, relative abundance of the ten most abundant species and other species (< 15%) in plant communities exposed to two levels of CO_2 concentration (390 and 520 ppm) with or without an extreme climatic event (ECE) at three different cutting dates

	Cut April	Cut June	Cut November		
	CO_2 effect	CO_2 effect	CO_2 effect	ECE effect	CO_2 × ECE effect
Above-ground mass	0.315	0.301	0.467	0.405	0.818
Fractions					
Green material	0.396	0.332	0.222	**0.013**	0.355
Flower	0.076	0.329	0.406	0.066	0.650
Dead material	0.267	0.718	0.278	**0.018**	0.417
d.f (num/den)	1/10	1/10	1/8	1/8	1/8
Relative abundance of functional groups					
Grasses	0.845	0.822	0.335	0.632	0.129
Legumes	0.451	0.986	0.647	0.339	0.310
Forbs	0.918	0.968	0.091	0.939	0.176
d.f (num/den)	1/10	1/10	1/8	1/8	1/8
Relative abundance of species					
Agrostis spp	0.305	0.989	0.987	0.070	0.126
Arrhenaterum elatius	0.429	0.802	0.811	0.544	0.414
Alopecurus pratensis	0.251	0.212	0.130	0.452	**0.026**
Dactylis glomerata	0.834	0.668	0.696	0.721	0.643
Holcus lanatus	0.693	0.999	0.475	**0.001**	0.379
Lolium perenne	0.497	0.777	0.736	0.732	**0.037**
Poa angustifolia	0.809	0.788	0.415	0.754	0.644
Poa pratensis	0.967	0.952	0.957	0.988	0.846
Trisetum flavescens	0.856	0.475	0.469	0.519	0.192
Ranunculus acris	0.299	**0.017**	0.584	**0.028**	0.166
Other species	0.377	0.443	0.426	0.201	0.324
d.f (num/den)	1/10	1/10	1/8	1/8	1/8

d.f (num/den), degrees of freedom (numerator and denominator). In bold: $p < 0.05$; Underlined: $0.05 < p < 0.1$

Table 3. Chemical composition and *in vitro* rumen fermentation parameters of plant communities exposed to two levels of CO_2 concentration (390 and 520 ppm) with or without an extreme climatic event (ECE) at three different cutting dates

	Cut April		Cut June		Cut November			
					Control		ECE	
CO_2 (ppm)	390	520	390	520	390	520	390	520
Chemical composition								
OM (g.kg^{-1} DM)	918 ± 6	926 ± 5	918 ± 4	921 ± 4	909 ± 10	918 ± 6	918 ± 2	909 ± 13
C (g.kg^{-1} DM)	448 ± 4	447 ± 2	448 ± 2	446 ± 3	444 ± 6	444 ± 3	449 ± 3	442 ± 8
N (g.kg^{-1} DM)	23.1 ± 1.1	20.5 ± 0.8	18.2 ± 0.5	16.5 ± 0.9	17.7 ± 1.5^{bc}	14.3 ± 0.6^{c}	27.6 ± 4.0^{a}	21.6 ± 1.6^{b}
C:N ratio	19.5 ± 0.9	21.8 ± 0.8	24.6 ± 0.7	27.1 ± 1.4	25.2 ± 2.3^{b}	31.0 ± 1.3^{a}	16.4 ± 2.2^{c}	20.5 ± 1.1^{bc}
NDF (g.kg^{-1} DM)	515 ± 23	513 ± 22	586 ± 11	568 ± 11	550 ± 8^{a}	546 ± 37^{ab}	534 ± 6^{ab}	490 ± 26^{b}
NDF:N ratio	22.3 ± 0.9	25.0 ± 1.2	32.2 ± 1.1	34.5 ± 2.1	31.2 ± 2.5^{b}	38.0 ± 1.3^{a}	19.5 ± 2.5^{c}	22.7 ± 0.5^{c}
WSC (g.kg^{-1} DM)	263 ± 36	241 ± 36	187 ± 27	192 ± 33	146 ± 39	194 ± 50	167 ± 36	195 ± 4
Glucose (g.kg^{-1} DM)	19.8 ± 4.2	17.7 ± 2.7	20.2 ± 3.7	16.8 ± 4.2	10.3 ± 2.7	7.8 ± 1.3	10.8 ± 1.7	10.6 ± 1.2
Sucrose (g.kg^{-1} DM)	39.7 ± 6.2	41.4 ± 9.1	29.4 ± 4.9	30.5 ± 6.5	18.7 ± 2.9	20.0 ± 3.4	23.0 ± 5.2	27.6 ± 2.5
Fructose (g.kg^{-1} DM)	21.6 ± 4.2	19.0 ± 3.6	20.5 ± 3.1	17.9 ± 4.5	13.0 ± 2.2	12.7 ± 2.1	15.1 ± 2.3	16.6 ± 1.0
Fructans (g.kg^{-1} DM)	182 ± 29	163 ± 22	117 ± 21	127 ± 22	104 ± 40	153 ± 47	118 ± 27	140 5
CT (g.kg^{-1} DM)	20 ± 7	16 ± 3	30 ± 14	31 ± 15	19 ± 3	17 ± 4	25 ± 13	21 ± 4
Pepsin-cellulase OM digestibility (g.kg^{-1})	775 ± 24	765 ± 28	644 ± 14	649 ± 47	610 ± 37^{b}	645 ± 56^{ab}	696 ± 42^{ab}	737 ± 2^{a}
In vitro rumen fermentation parameters								
Acidification (dpH)	0.91 ± 0.05	0.94 ± 0.05	0.82 ± 0.04	0.84 ± 0.03	0.69 ± 0.04^{ab}	0.77 ± 0.03^{a}	0.66 ± 0.03^{b}	0.73 ± 0.03^{ab}
IVDMD (g.kg^{-1})	602 ± 13	584 ± 14	525 ± 24	516 ± 19	515 ± 17^{b}	512 ± 17^{b}	541 ± 15^{ab}	584 ± 20^{a}
NH_3 (mmol.l^{-1})	9.60 ± 1.18	7.54 ± 1.20	7.10 ± 0.89	4.92 ± 0.67	7.99 ± 1.72^{bc}	4.18 ± 1.38^{c}	13.32 ± 2.23^{a}	9.80 ± 0.85^{ab}
Total VFA (mmol.g^{-1} DM)	8.10 ± 0.17	8.37 ± 0.25	7.58 ± 0.45	7.45 ± 0.27	7.14 ± 0.39	7.37 ± 0.05	7.42 ± 0.33	7.72 ± 0.55
Acetate (%)	63.5 ± 0.9	64.2 ± 1.1	64.5 ± 0.6	65.0 ± 1.3	65.2 ± 0.9	65.8 ± 0.5	64.8 ± 0.3	63.9 ± 1.5
Propionate (%)	23.7 ± 0.5	23.1 ± 0.7	23.0 ± 0.3	22.7 ± 0.5	22.2 ± 0.2^{b}	22.6 ± 0.4^{ab}	23.0 ± 0.06^{ab}	23.4 ± 0.7^{a}
Butyrate (%)	9.19 ± 0.42	9.27 ± 0.41	9.10 ± 0.32	9.12 ± 0.83	8.90 ± 0.35	8.67 ± 0.25	8.21 ± 0.12	8.79 ± 0.67
Valerate (%)	1.19 ± 0.04	1.11 ± 0.05	1.12 ± 0.04	1.04 ± 0.06	1.15 ± 0.12^{ab}	0.99 ± 0.07^{b}	1.31 ± 0.03^{a}	1.27 ± 0.12^{a}
Iso-butyrate (%)	0.93 ± 0.08	0.87 ± 0.10	0.85 ± 0.07	0.83 ± 0.16	0.97 ± 0.18	0.79 ± 0.09	1.07 ± 0.16	0.88 ± 0.02
Iso-valerate (%)	1.41 ± 0.10	1.27 ± 0.12	1.34 ± 0.07	1.25 ± 0.12	1.47 ± 0.22^{ab}	1.12 ± 0.11^{b}	1.61 ± 0.11^{a}	1.64 ± 0.15^{a}
Acetate:propionate ratio	2.69 ± 0.09	2.79 ± 0.12	2.82 ± 0.06	2.89 ± 0.11	2.95 ± 0.06^{a}	2.93 ± 0.07^{ab}	2.84 ± 0.02^{ab}	2.75 ± 0.13^{b}
Total gas (mmol.g^{-1} DM)	7.07 ± 0.23	7.14 ± 0.16	6.43 ± 0.41	6.41 ± 0.21	6.06 ± 0.16	6.13 ± 0.39	6.25 ± 0.28	6.50 ± 0.13
CH_4 (mmol.g^{-1} DM)	1.25 ± 0.03	1.26 ± 0.03	1.10 ± 0.08	1.11 ± 0.06	1.06 ± 0.05	1.08 ± 0.08	1.13 ± 0.05	1.12 ± 0.05
CO_2 (mmol.g^{-1} DM)	5.83 ± 0.22	5.86 ± 0.13	5.28 ± 0.29	5.31 ± 0.16	5.00 ± 0.14	5.06 ± 0.31	5.12 ± 0.22	5.38 ± 0.11
CO_2:CH_4 ratio	4.67 ± 0.16	4.66 ± 0.08	4.80 ± 0.08	4.81 ± 0.19	4.72 ± 0.20	4.71 ± 0.11	4.53 ± 0.04	4.80 ± 0.18

Data shown are means ± standard deviation. ^{a,b,c} For the November cut, means in a given row with different letters are significantly different ($p < 0.05$).

OM, organic matter; C, carbon; N, nitrogen; NDF, neutral detergent fiber; WSC: water-soluble carbohydrates; CT, condensed tannins; IVDMD, *in vitro* dry matter degradability, DM, dry matter; VFAs, volatile fatty acids

Table 4. Factor effects (*p*-values) on chemical composition and *in vitro* rumen fermentation parameters of plant communities exposed to two levels of CO_2 concentration (390 and 520 ppm) with or without an extreme climatic event (ECE) at three different cutting dates

	Cut April	Cut June	Cut November		
	CO_2 effect	CO_2 effect	CO_2 effect	ECE effect	CO_2 × ECE effect
Chemical composition					
OM	**0.033**	0.158	0.973	0.989	0.123
C	0.547	0.333	0.299	0.603	0.304
N	**<0.001**	**0.003**	**0.007**	**<0.001**	0.346
C:N ratio	**<0.001**	**0.003**	**0.002**	**<0.001**	0.425
NDF	0.883	**0.018**	0.108	**0.027**	0.175
NDF:N ratio	**0.001**	**0.038**	**0.002**	**<0.001**	0.128
WSC	0.337	0.784	0.112	0.616	0.662
Glucose	0.379	0.170	0.227	0.147	0.303
Sucrose	0.712	0.744	0.198	**0.022**	0.452
Fructose	0.306	0.262	0.619	**0.031**	0.473
Fructans	0.261	0.445	0.106	0.986	0.515
Condensed tannins	0.252	0.887	0.495	0.295	0.788
Pepsin-cellulase OM digestibility	0.518	0.808	0.139	**0.005**	0.903
d.f (num/den)	1/10	1/10	1/8	1/8	1/8
In vitro rumen fermentation parameters					
Acidification (dpH)	0.201	0.560	**0.007**	0.112	0.734
IVDMD	**0.041**	0.496	0.075	**0.001**	0.053
NH_3	**0.014**	**0.001**	**0.005**	**<0.001**	0.882
Total VFA	0.056	0.577	0.256	0.186	0.871
Acetate	0.243	0.427	0.779	0.064	0.237
Propionate	0.107	0.210	0.110	**0.008**	0.865
Butyrate	0.749	0.956	0.464	0.251	0.121
Valerate	**0.016**	**0.017**	0.111	**0.004**	0.317
Iso-butyrate	0.308	0.755	0.063	0.289	0.999
Iso-valerate	0.062	0.134	0.111	**0.006**	0.067
Acetate:propionate ratio	0.151	0.223	0.261	**0.013**	0.484
Total gas	0.545	0.916	0.308	0.103	0.554
CH_4	0.567	0.876	0.908	0.122	0.750
CO_2	0.788	0.828	0.225	0.105	0.413
CO_2:CH_4 ratio	0.841	0.960	0.181	0.566	0.137
d.f (num/den)	1/10	1/10	1/8	1/8	1/8

OM, organic matter; C, carbon; N, nitrogen; NDF, neutral detergent fiber; WSC: water-soluble carbohydrates; CT, condensed tannins; IVDMD, *in vitro* dry matter degradability, VFAs, volatile fatty acids; d.f (num/den), degrees of freedom (numerator and denominator). In bold: $p < 0.05$; Underlined: $0.05 < p < 0.1$

Figures

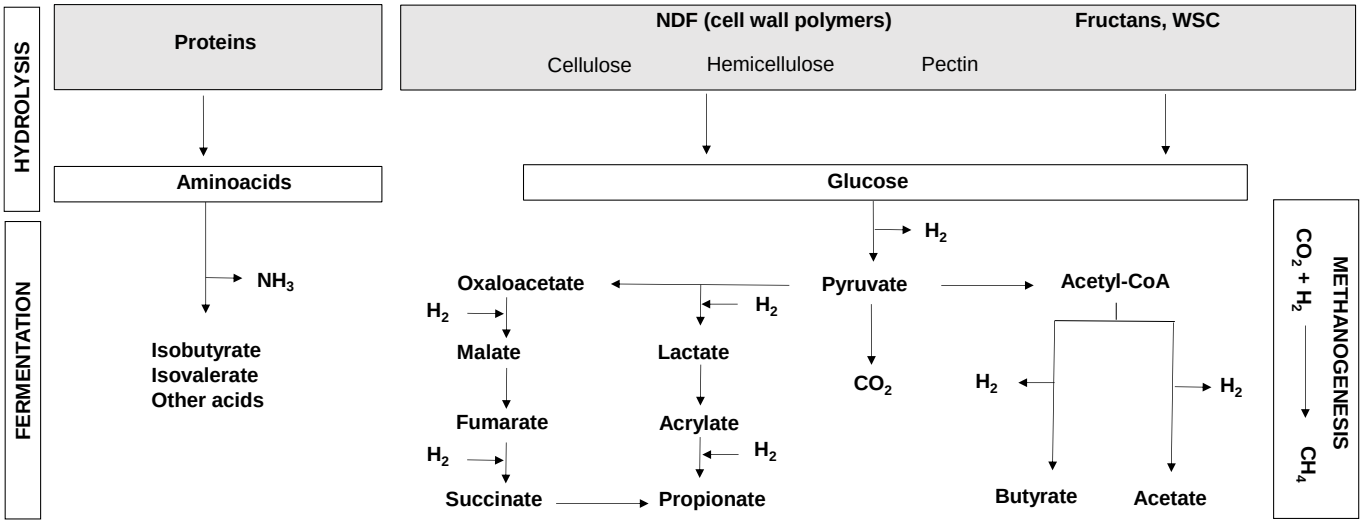

Figure 1. Overview of protein and carbohydrate metabolism in the rumen. WSC: water-soluble carbohydrates; NDF: neutral detergent fiber (cell wall carbohydrates); CH_4: methane; CO_2: carbon dioxide; NH_3: ammonia; H_2: hydrogen.

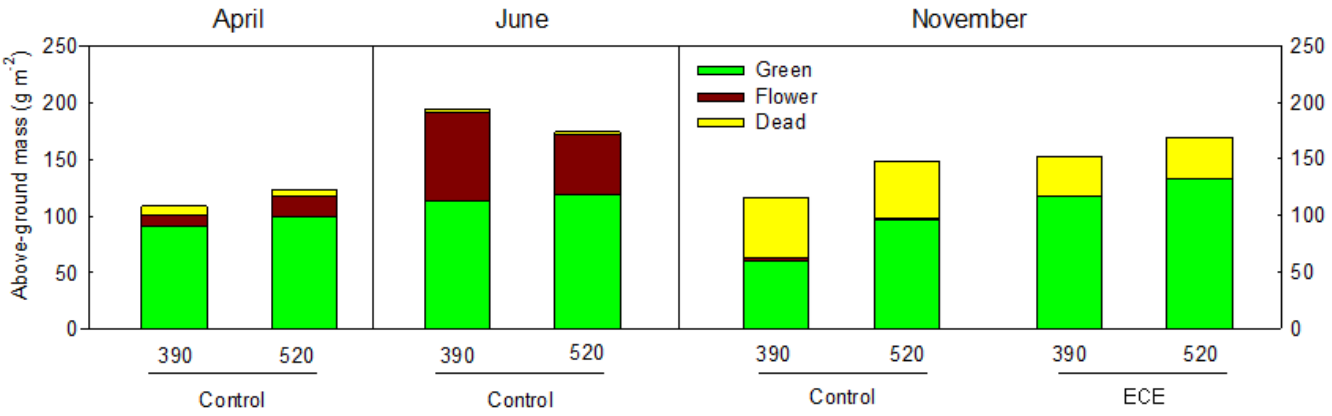

Figure 2. Above-ground biomass of green material, dead material and flowers measured before (April, June) and after the extreme climatic event (ECE, November) exposed to four climate scenarios: 390 or 520 ppm atmospheric CO_2 concentration without (Control) or combined with the ECE. n = 3.

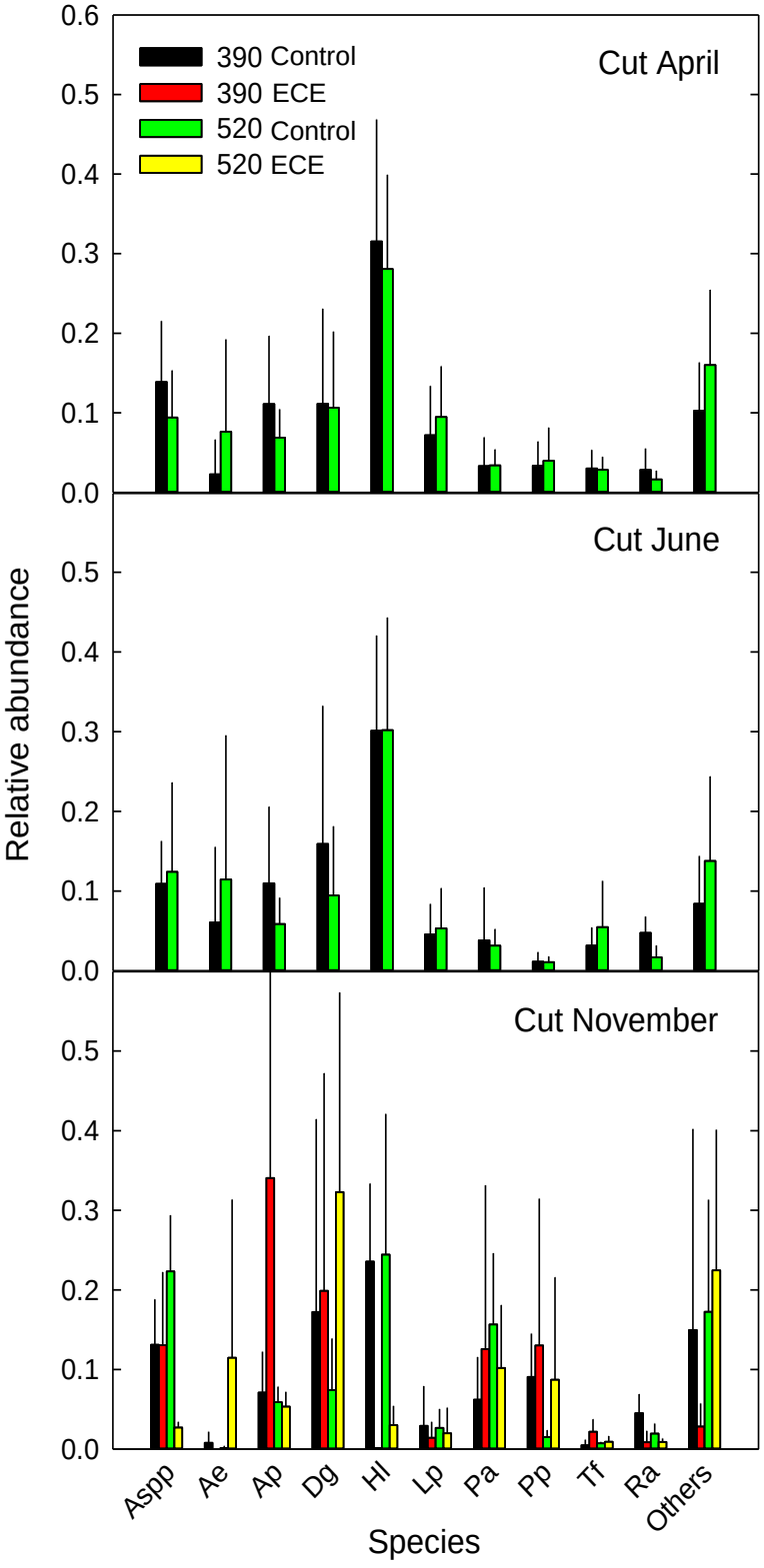
390 Control
390 ECE
520 Control
520 ECE
Cut April
Cut June
Cut November
Relative abundance
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Aspp
Ae
Ap
Dg
Hl
Lp
Pa
Pp
Tf
Ra
Others
Species

Figure 3. Relative abundance of the 10 most abundant species present in the community measured before (April, June) and after the extreme climatic event (ECE, November) grown under four climate scenarios: 390 or 520 ppm atmospheric CO_2 concentration without (Control) or combined with the ECE. Aspp: *Agrostis* spp.; Ae: *Arrhenatherum elatius* L.; Ap: *Alopecurus pratensis* L.; Dg: *Dactylis glomerata* L.; Hl: *Holcus lanatus* L.; Lp: *Lolium perenne* L.; Pa: *Poa angustifolia* L.; Pp: *Poa pratensis* L.; Tf: *Trisetum flavescens* L.; Ra: *Rumex acetosa* L.; Others: <15% of species. n = 3.

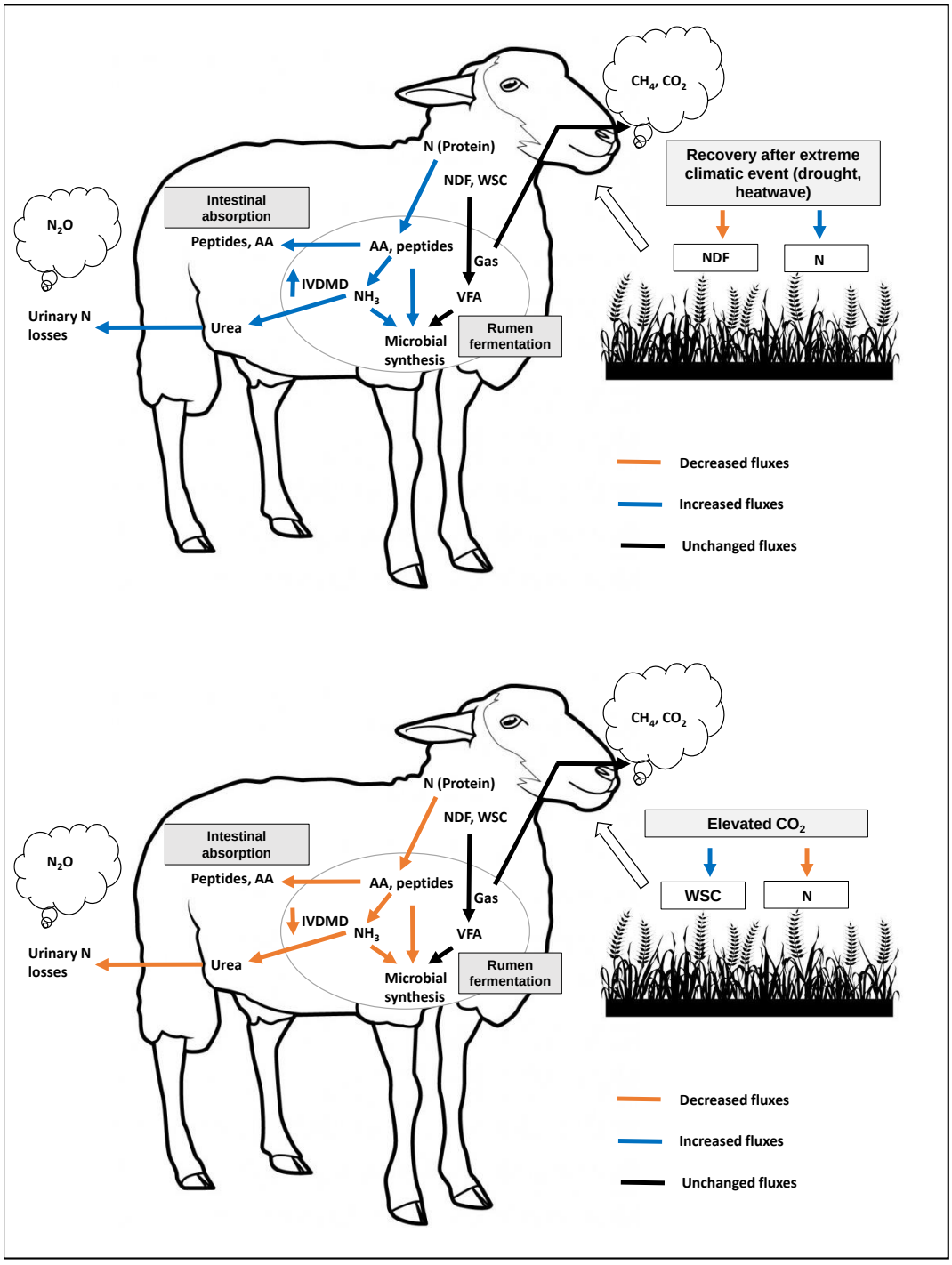
CH_4, CO_2
N (Protein)
NDF, WSC
Intestinal absorption
N_2O
Peptides, AA
AA, peptides
Gas
IVDMD
NH_3
VFA
Urinary N losses
Urea
Microbial synthesis
Rumen fermentation
Recovery after extreme climatic event (drought, heatwave)
NDF
N
Decreased fluxes
Increased fluxes
Unchanged fluxes
CH_4, CO_2
N (Protein)
NDF, WSC
Intestinal absorption
N_2O
Peptides, AA
AA, peptides
Gas
IVDMD
NH_3
VFA
Urinary N losses
Urea
Microbial synthesis
Rumen fermentation
Elevated CO_2
WSC
N
Decreased fluxes
Increased fluxes
Unchanged fluxes

Figure 4. Schematic overview of treatment effects (recovery after an extreme climatic event (ECE) and elevated atmospheric CO_2 concentration) on forage quality, digestive use of macronutrients by the ruminant and atmospheric greenhouse gas emissions. WSC: water-soluble carbohydrates; N: nitrogen; NDF: neutral detergent fiber (cell wall carbohydrates); AA: amino acids; VFA: volatile fatty acids; CH_4: methane; CO_2: carbon dioxide; NH_3: ammonia; N_2O: nitrous oxide; IVDMD, *in vitro* dry matter degradability