RESEARCH PAPER

Combined experimental drought and nitrogen loading: the role of species-dependent leaf level control of carbon and water exchange in a temperate grassland

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ABSTRACT

- Nitrogen (N) loading and extreme drought strongly alter biomass production, species composition and carbon and water fluxes of temperate grasslands. Such changes at the community level are often attributed to species- and functional group-specific responses in phenology and/or physiology.
- In a multifactorial field experiment, we studied the responses of three abundant grassland species (forb *Centaurea jacea*, grasses *Arrhenatherum elatius* and *Dactylis glomerata*) to N loading and extreme drought, focusing on responses of carbon and water relations at the leaf level. We analysed (1) changes in bulk leaf N (uptake efficiency of additional N), (2) adaptation of plant water status (leaf water potential) and (3) impact on leaf carbon and water fluxes.
- We observed more efficient N utilization in the two grasses compared to *C. jacea*. Naturally occurring summer drought significantly impacted the plant water status of all species, while extreme drought treatment only further affected water status during and after summer drought. *C. jacea* was able to maintain much lower leaf water potentials compared to the grasses during drought. Despite these clear species-specific responses to N loading and drought, the species were able to maintain homeostasis of leaf carbon and water fluxes.
- Thus, strong declines in the (community) carbon sequestration observed at this site during the (natural) summer drought were not related to leaf physiological responses in assimilation, but were driven by phenological adaptions of the species community: the drought-sensitive grasses, even though exhibiting higher N uptake efficiency, responded with a shortened life cycle to severe summer drought.

INTRODUCTION

Semi-natural species-rich grasslands in Europe have been strongly declining in extent and biodiversity since the 1950s, despite ongoing conservation efforts. Two major threats to their stable functioning are nitrogen (N) loading and increasing drought events (Habel *et al.* 2013; Dengler *et al.* 2014; IPBES 2018; Török & Dengler 2018). The effects of N loading and drought stress on temperate grasslands have been widely studied separately in experimental approaches under controlled and natural field conditions, as well as at the single species and community level (Muller & Garnier 1990; Bobbink 1991; Volaire & Lelièvre 1997; Arp *et al.* 1998; Karsten & MacAdam 2001; Reich *et al.* 2003; Jacquemyn *et al.* 2003; Morecroft *et al.* 2004; Kahmen *et al.* 2005; Gilgen & Buchmann 2009; Jentsch *et al.* 2011). Generally, decreasing species richness/biodiversity was observed in N-limited grasslands upon N loading (Wedin & Tilman 1996; Stevens 2004; Stevens *et al.* 2006; Harpole & Tilman 2007; Hautier *et al.* 2009), while, at the same time, biodiversity was positively correlated with stable/resilient functioning of grasslands (Tilman & Downing 1994). Drought, in general, leads to decreased aboveground plant productivity and forage yield in grasslands. However, there is contrasting evidence as to the relationship between drought, decreased productivity and altered biodiversity/changes in the community composition (Kahmen *et al.* 2005; Grant *et al.* 2014).

With ongoing global change, N loading and extended drought will likely occur together more often and pose a multifactorial stress on plant communities. Nevertheless, comparatively few studies have examined the potential interacting effects of N loading and drought on grassland communities, either on the community (Hartmann & Niklaus 2012; Grant *et al.* 2014; Carlsson *et al.* 2017; Kübert *et al.* 2019) or species (Gonzalez-Dugo *et al.* 2005; Bloor & Bardgett 2012; Hofer *et al.* 2017) level. The focus of previous studies was predominantly on the impact on productivity, *i.e.* on the relationship between species diversity and biomass production of studied grassland communities and species.

Bloor & Bardgett (2012), for instance, found, at the species level, that higher soil N did not attenuate the negative impacts

of drought. No interactions were observed between soil N and drought on CO_2 fluxes or plant carbon storages pools. Hofer et al. (2017), on the other hand, discovered that N fertilization improved drought resistance of non-legume species. In contrast, Kübert et al. (2019) observed, at the community level, that N loading strongly enhanced the effect of drought stress on a grassland community. Drought-related reduction of the grassland's gross primary production and net ecosystem exchange was much stronger on plots with N loading, resulting in the ecosystem becoming a net carbon source. These authors related the interacting drought and N loading impact to significantly reduced aboveground cover and species richness of forb species. In summary, there has been a vast number of experiments worldwide studying the effect of drought and N loading at different scales. Nevertheless, there are still many open questions in our understanding of the combined impact of co-occurring drought and N loading. In particular, it is not clear how plant physiological parameters, such as carbon assimilation or water use efficiency (WUE), respond to the combined effects of N loading and drought and how leaf-/single plant-level physiological responses affect the overall response of plant communities.

Therefore, we investigated the plant physiological response of the abundant forb and grass species (Centaurea jacea L., Arrhenatherum elatius (L.) P.Beauv. ex J.Presl & C.Presl, Dactylis glomerata L.) in the same grassland studied by Kübert et al. (2019). We studied their response under natural, drought, N addition and combined drought and N addition conditions over the entire growing season. First, we quantified leaf N status, as this defines the use efficiency of the additional N and is a precondition for any potential response in assimilation due to the dependence of photosynthetic capacity on leaf N (Wright et al. 2004; Kattge et al. 2009). In particular, there is evidence that grasses globally benefit more from N addition compared to forbs, increasing their aboveground biomass due to N loading (You et al. 2017). Further, we assessed predawn and midday leaf water potentials (LWP) to quantify water use strategies and potential strategies to cope with drought (e.g. drought evasion versus drought tolerance). Grasses are, generally, found to be more sensitive to drought compared to forb species (Milbau et al. 2005; Leitinger et al. 2015; Stampfli et al. 2018), which can have both phenological (e.g. shortened life cycle) and physiological (reduced photosynthetic activity and biomass production) consequences. Finally, we quantified leaf-level assimilation rates, stomatal conductance and transpiration rates to see if differences in N uptake and drought adaptation affect leaf-level assimilation and WUE.

In accordance with previous findings, we hypothesize that the two grass species, A. elatius and D. glomerata, can utilize the additional N more efficiently than the forb species, C. jacea. In contrast, we expect the deep-rooting C. jacea to tolerate drought significantly better than A. elatius and D. glomerata. Consequently, we assume that C. jacea will be less affected in leaf carbon assimilation (A) and WUE by drought compared to the grass species, while the grass species will show a stronger increase in A and WUE in response to N loading compared to C. jacea.

Kübert et al. (2019) observed a significant reduction of forb species richness and abundance under combined drought and N loading accompanied by a huge drop in ecosystem gross primary production during a naturally occurring summer drought on the grass-dominated plots. Based on their findings, we expect that a more efficient N use efficiency of A. elatius and D. glomerata compared to C. jacea can somewhat ameliorate their drought sensitivity, but that this is strongly dependent on drought intensity, timing and duration. In addition, we discuss whether the response at the community level to combined drought and N loading found by Kübert et al. (2019) and in recent literature can be attributed to species-specific physiological responses at the plant/leaf level or must be related to other responses, e.g. to the species phenology, i.e. to shifted phenological phases and, thereby, temporal 'loss' of the species' contribution to ecosystem productivity.

MATERIAL AND METHODS

Study site

Measurements took place in a perennial semi-natural temperate grassland. The study site is in the immediate vicinity of the technical faculty campus of Freiburg University (48°1′2″ N; 7°49′5″ E, 238 m a.s.l.). The area underwent denudation and landfill but was re-natured and allowed to recover for 4 years before measurements commenced. Until 2015, the area was extensively grazed by sheep once a year, from the end of June to the beginning of July. A semi-natural and ruderal grassland/succession system developed. The community consists of 59 species; characteristic species are Agrostis capillaris L., Festuca rubra L., Centaurea jacea L., Dactylis glomerata L., Festuca pratensis Huds., Achillea millefolium L., Carex hirta L. and Arrhenatherum elatius (L.) P.Beauv. ex J.Presl & C.Presl (species list provided in Table S1). The system is dominated by grass species and the forb species C. jacea. The long-term (1986–2015) average annual rainfall and temperature are 773.3 mm and 11.3 °C, with 178.4 mean days of rainfall per year (DWD, 2018; weather station approximately 700 m from study site). The soil can be characterized as an anthrosol; it contains lessivated brown earth as a top layer, while lower layers of crushed stone (LGRB L. für G. Rohstoffe und Bergbau 2017).

Experimental design

The study site is part of the international monitoring networks, drought-net and nutrient network (NutNet). This combination imposes four distinct treatments: (i) natural nutrient availability (control) and ambient precipitation (control) (CC), (ii) natural nutrient availability and reduced precipitation (CD), (iii) N addition with ambient precipitation (NC), and (iv) N addition with reduced precipitation (ND). Each treatment is replicated four times (control control CC, control drought CD, N control NC, N drought ND). Drought stress was experimentally manipulated using permanent rain-out shelters. These simulate an extreme drought, defined as a reduction in precipitation of 40% relative to the long-term (i.e. last 30 years: 1986–2015) annual precipitation amount. For N addition, a slow-release MultiCote 40% fertilizer (Haifa, Israel) was applied once a year in early spring (mid-March) by spreading 10 g·N·year^{-1} (1250 g·m^{-2} per plot of MultiCote). In 2015, pretreatment measurements were made to guarantee comparability between plots, including species composition and biomass (above- and belowground), soil C and N content, C:N ratio, soil properties

and water content profiles. No statistically significant differences between plots were found (T. Gebauer, personal communication). Treatments started in spring 2016. The data shown here were collected throughout the growing season of 2017. For more details on the setup and experimental design, see Kübert *et al.* (2019).

Environmental data

Precipitation (RG3-M HOBO rain gauge; Onset, Bourne, MA, USA), photosynthetic photon flux density (PPFD, S-LIA-M003; Onset), air temperature and air relative humidity (S-THB-M008; Onset) were measured above the canopy (1 m) every 30 min and stored in HOBO data loggers (HOBO H21-002 and U30; Onset). Soil temperature (T106, T108; Campbell Scientific, Logan, UT, USA) and soil volumetric water content (VWC, 10HS; Decagon, Pullman, WA, USA) were measured at 1, 5, 20 and 40 cm depth on experimental plots (n = 4 per treatment) and stored as 30-min averages in data loggers (CR1000, Campbell Scientific).

Measurement campaigns (April–September)

Measurements on the experimental plots were conducted from April to September 2017, covering the growing season of the grassland ecosystem. Leaf-level measurements were made for the most abundant forb species *Centaurea jacea* with year-round presence, the early abundant grass species *Arrhenatherum elatius* (L.) P.Beauv. ex J.Presl & C.Presl (period April–May) and the late abundant grass species *Dactylis glomerata* L. (period June–September). These two grass species were chosen because none of the studied grass species were present year-round on all plots. For all leaf measurements, mature, fully expanded and sun-exposed leaves were chosen randomly from also randomly chosen individuals on the 4 m × 3 m plots, *i.e.* measurements were taken from different individuals and leaves each time to minimize the repetition of individuals. However, a possible repetition of individuals cannot be completely excluded due to the limited available plant material on the experimental plots.

Elemental composition of bulk leaves
Once a month, parallel to gas exchange measurements, plant material was sampled for elemental composition to determine relative N of bulk leaves (N_{bulk}). Bulk leaf samples (n = 3 per species and treatment) were collected from close to the gas exchange measurements and kept frozen until analyses. Samples were analysed using an elemental analyser (EA, varioPYROcube; Elementar, Langenselbold, Germany) for bulk carbon/nitrogen (C:N) ratios. The measurement precision of the EA was ±0.87% and ±0.19% for C and N composition, respectively. The samples were measured against potassium nitrate (IAEA-NO-3), cellulose (IAEA-C3) and caffeine (IAEA-600) (International Atomic Energy Agency, Vienna, Austria) and two in-house standards, tyrosine and acetanilide.

Leaf water potentials
Leaf water potentials (LWP, predawn/midday) were measured once a month, in May twice and July three times (n = 4 per species and treatment). LWP (Pressure Chamber 1505D; PMS Instrument, Albany, OR, USA) was measured during midday (12:00–14:00 h) and predawn (starting 2 h before sunrise; n = 3 per species and treatment).

Gas exchange measurements and water use efficiencies
Carbon and water fluxes at the leaf level (n = 4 per species and treatment) were measured with a gas exchange system (GFS-3000 with a 4 cm^2 leaf cuvette; Walz, Effeltrich, Germany) with ambient relative air humidity and air temperature but at constant CO_2 concentration (400 ppm) and PPFD (1200 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Readings were taken after at least 15 min of equilibration between 10:30 h and 16:30 h; narrowing the time window in the early and later season to adjust for changes in peak activity. Net assimilation (*A*), stomatal conductance (g_s) and transpiration (*E*) were calculated after von Caemmerer & Farquhar (1981). Instantaneous (WUE = *A/E*) and intrinsic (WUE$_{i}$ = *A/g_s*) water use efficiencies were calculated from measured gas exchange parameters, with the assumption of a complete use of water and CO_2 in the photosynthesis process (Farquhar & Sharkey 1982; Ehleringer *et al.* 1993). Gas exchange measurements took place every 3–4 weeks and were intensified in July, with weekly measurements.

Statistical analyses

Analyses focused on the responses of the studied species to the treatments N loading, *N*, and experimental extreme drought, *D* (single and combined effect of *N* and *D*; control treatment *C*: *CC, NC, CD, ND*), in the growing season 2017. All statistical analyses were conducted by comparing the single data of all plots and campaigns over the entire growing season, treating the grasses *A. elatius* and *D. glomerata* as one group.

During the growing season 2017, a natural summer drought occurred (see more details in Results: Growing season 2017). Therefore, collected data were split into three periods: before, during and after the naturally occurring summer drought (*i.e.* before drought comprises the months April and May, during drought June till mid-July, and after drought mid-July till September); these periods were analysed accordingly. *A. elatius* was sampled from April till May, *i.e.* analyses before drought were retrieved from *A. elatius*. *D. glomerata* was sampled from June to September, *i.e.* analyses during and after the summer drought included data on *D. glomerata*.

We performed linear mixed-effect models with ANOVAs to test for significant differences between treatments (*N*: N addition, *D*: experimental extreme drought) and species (*S*) including interaction effects of *N*, *D* and *S* (indicated with ✕). We further added campaign/date and plot as random factors to account for repeated measurements and plot design. Possible repetition of individuals was not considered since random sampling minimized repetition of individuals.

Diagnostic plots based on the models, *i.e.* residual *versus* fitted plots and quantile–quantile plots, were plotted to validate the assumptions of homogenous variance and normal distribution of residuals of ANOVA. *A*, *E* and WUE were transformed with square root to meet criteria of ANOVA; predawn LWP, midday LWP and g_s were log10 transformed (except *A* in the period after drought).

The relationships between physiological parameters and ambient parameters were assessed with linear mixed-effect models, with campaign/date as random effect. Here, the

treatment means over the entire growing season at different time points (date) were compared. Critical values, probability values and correlations of determinations were derived based on the models, with .<0.1, *<0.05, **<0.01 and ***0.001.

All analyses were conducted with *R* (version 3.6.0; R Foundation for Statistical Computing, Vienna, Austria). Additional software packages used for models were *lme4* (Bates *et al.*, 2019), *MuMIn* (Bartoń 2019) and *glmmTMB* (Magnusson *et al.*, 2019).

RESULTS

Growing season 2017

The grassland's growing season in 2017 showed the typical seasonal dynamics of a cool temperate climate, with spring starting in April, midsummer in June and the start of autumn in September. With an average air temperature of 16.7 °C and precipitation of 446.6 mm, the growing season 2017 (April–September) was slightly cooler and drier compared to the long-term mean (17.0 °C and 527.2 mm, 1987–2016). Experimental extreme drought plots received approximately 283 mm precipitation, which is 12% below the driest growing season on record (April–September 2003: 321.1 mm; DWD, 2018). Consequently, experimental extreme drought significantly decreased VWC ($P < 0.05$), by $20.0 \pm 0.3\%$ for the drought (*CD*) *versus* control treatment (*CC*), and even stronger in the N addition treatments, with a decrease of $38.0 \pm 0.5\%$ (*ND versus NC*; mean $\pm$ 1 SE; Fig. 1).

The grassland's growing season was strongly affected by a naturally occurring summer drought in mid-June, with a dry period of 2 weeks (<3.5 mm) and mean air temperatures >20 °C (for more details see Kübert *et al.* 2019). During this time, mean VWC dropped considerably below 0.1 m^3·m^{-3} for more than 30 days for all treatments except *NC* (Fig. 1). Also, VWC on *NC* plots was significantly lower (−35%) during this period in comparison to the previous period. This summer drought exerted a significant impact on the carbon and water relations of the studied grassland species. Therefore, we will further group our results into the three distinct growing periods: (1) spring/summer growing peak, (2) summer die-back and (3) autumn growing peak, investigating the impact of N

addition and experimental extreme drought (1) before, (2) during and (3) after the summer drought.

Elemental composition of bulk leaf

Nitrogen addition significantly increased relative bulk leaf N (N_{bulk}) of all species over the entire growing season, which confirms the uptake of the N fertilizer by all species (Fig. 2, F = 116.0***). Nevertheless, *C. jacea* differed strongly in N uptake behaviour from the two grasses (*NxS*: F = 9.3**). Higher increases of N_{bulk} for *A. elatius/D. glomerata* than for *C. jacea* indicate that the grass species took up more of the N fertilizer: N_{bulk} increased by 66% for *NC* (*i.e. NC versus CC*) and 53% for *ND* (*i.e. ND versus CD*) for *A. elatius/D. glomerata*, relative to an increase of 18% for *NC* and 32% for *ND* for *C. jacea*. On control plots *CC/CD* without N addition, N_{bulk} was, generally, higher for *C. jacea*. (growing season: F = 3.4.). No single significant effect of drought on N_{bulk} could be found over the growing season, but there were significant interactions between drought and species (*DxS*: F = 5.0*).

Leaf water potential

Before the summer drought the leaf water potential (LWP) reflected ample water supply and did not differ significantly between treatments, ranging from −0.2 ± 0.1 to −0.7 ± 0.4 MPa (Fig. 3). Only small differences were found between species (predawn: F = 4.0*; midday: F = 2.8.) However, species responded considerably differently in LWP during and after summer drought. While all species exhibited significantly lower midday LWP, *C. jacea* decreased its LWP strongly in response to low VWC, reaching values as low as −3.9 ± 1.1 MPa. At the same time, midday LWP of *D. glomerata* did not decrease below −1.9 ± 0.2 MPa. Furthermore, predawn and midday LWP of both *C. jacea* and *D. glomerata* were significantly lower in drought-treated plots *CD/ND* during summer drought (predawn: F = 13.0***; midday: F = 3.3.) This effect of drought on LWP was still visible after the summer drought (Fig. 3, right panel).

The drought treatment, generally, affected LWP throughout the growing season (predawn: F = 8.2**; midday: F = 5.1*). Moreover, no significant N or interacting treatment effects
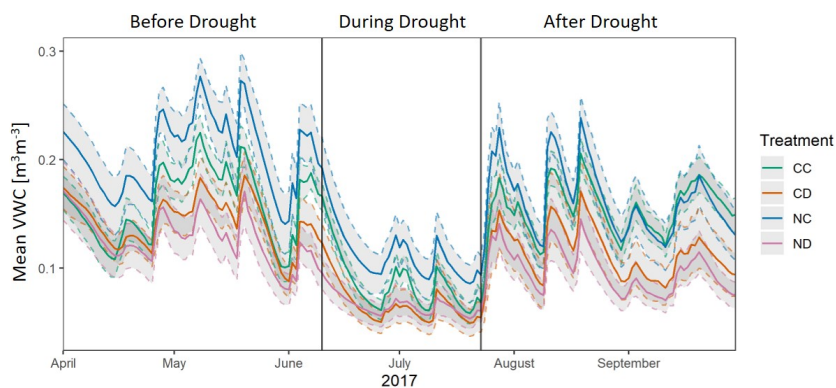

Fig. 1. Volumetric water content (VWC, m^3·m^{-3}) during the growing season 2017, averaged over soil depth (1, 5, 20, 40 cm), per treatment before, during and after summer drought (grey lines). Treatments: *CC* control (green), *CD* experimental extreme drought (orange), *NC* nitrogen addition (blue), and *ND* nitrogen addition and experimental extreme drought (rose). Mean ± 1 SE, n = 4.
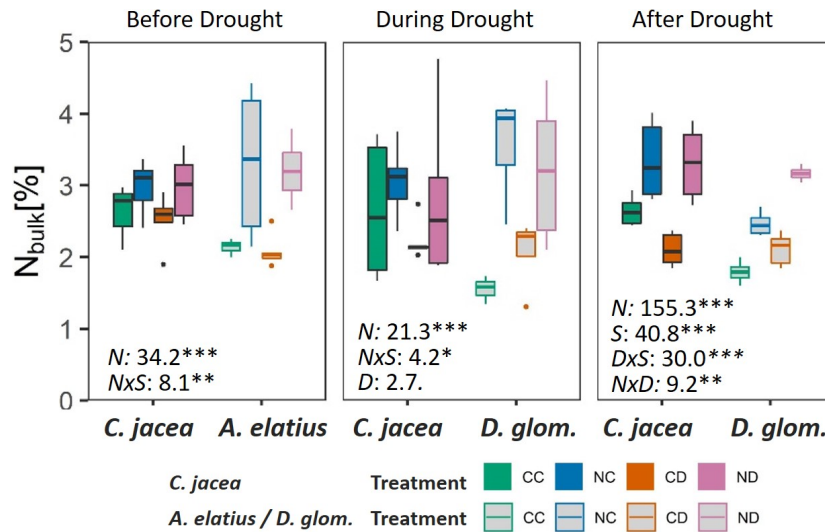
Fig. 2. Bulk leaf relative N content (N_{bulk}, %) of *C. jacea* (filled bars), *A. elatius* and *D. glomerata* (both open bars) per treatment before, during and after summer drought in the growing season 2017. Treatments: *CC* control (green), *NC* nitrogen addition (blue), *CD* experimental extreme drought (orange), *ND* nitrogen addition and experimental extreme drought (rose). Boxplot of median (solid black line), upper and lower quartiles (box), 1.5 interquartile range (whiskers) and outliers (circles), n = 4. F-values and asterisks indicate significant differences between species (*S*) and treatments (*N*: nitrogen addition, *D*: experimental extreme drought) and their interactions (×). Significance levels: .<0.1, *<0.05, **<0.01, ***<0.001.
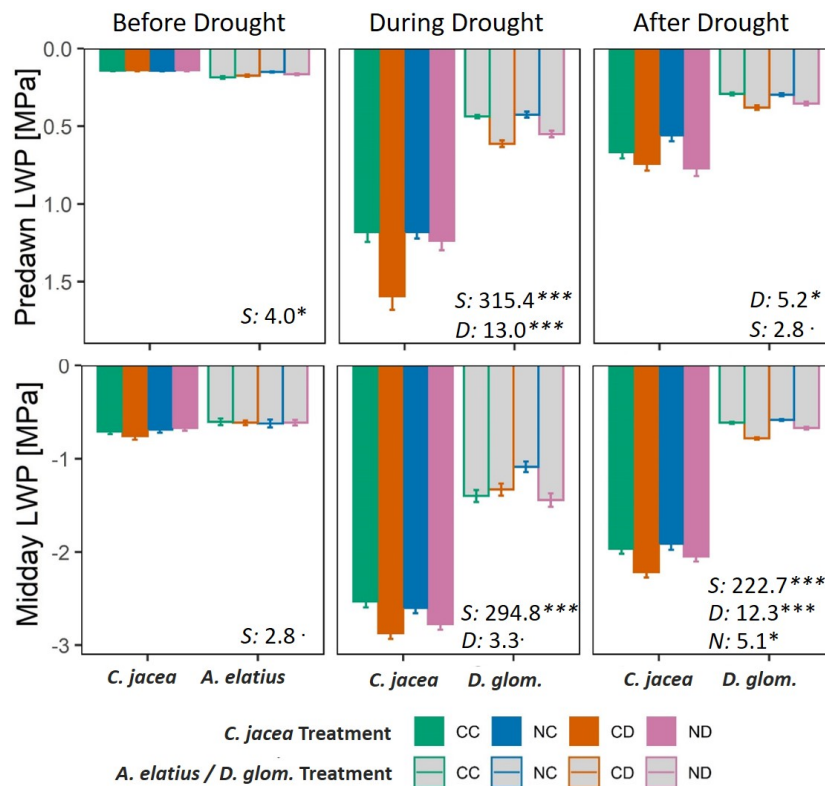

Fig. 3. Predawn (top) and midday (bottom) leaf water potentials (LWP, MPa) of *C. jacea* (filled bars), *A. elatius* and *D. glomerata* (both open bars), per treatment, before, during and after summer drought, in the growing season 2017. Treatments: *CC* control (green), *CD* (experimental extreme drought (orange), *NC* nitrogen addition (blue), *ND* nitrogen addition and experimental extreme drought (rose). Mean ± 1 SE, n = 4. F-values and asterisks indicate significant differences between species (*S*) and treatments (*N*: nitrogen addition, *D*: experimental extreme drought) and their interactions (×). Significance levels: .<0.1, *<0.05, ***<0.001.

(<0.05) on LWP of any species could be observed over the growing season (Fig. 3).

The species-specific response in LWP to a decrease in VWC is also clearly visible in Fig. 4 and was maintained even after the summer drought period (Fig. 3). All species started to significantly decrease LWP at VWC of $0.1\ m^3 \cdot m^{-3}$ or below. Despite clear differences in VWC between treatments, VWC only decreased below $0.1\ m^3 \cdot m^{-3}$ during the summer drought. During this dry period, however, all treatments reached values of $0.1\ m^3 \cdot m^{-3}$ or below, which explains why the long-term extreme drought treatment had such a small effect on LWP of all species (Figs 3 and 4).

Gas exchange and water use

Centaurea jacea and the two grass species, *A. elatius/D. glomerata,* differed considerably in midday assimilation (*A*) and stomatal conductance (*g_s*) over the entire growing season (*A:* F = 33.5**, *g_s*: F = 8.9*), particularly before and during summer drought (Fig. 5). However, treatments individually and in combination did not have a significant impact on midday gas exchange (*A*, *E* and *g_s*; Fig. 5). During the spring/summer growing period, both *C. jacea* and *A. elatius* had relatively high rates of *A* and *g_s* for all treatments (9.4–14.0 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 122.4–238.4 $mmol \cdot m^{-2} \cdot s^{-1}$, respectively). The naturally occurring summer drought had a strong negative impact on *A* and *g_s*. Under conditions of high air temperature and very low VWC, both species strongly downregulated *A* and *g_s* (2.7–5.6 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 36.6–78.0 $mmol \cdot m^{-2} \cdot s^{-1}$, respectively). Moreover, *g_s* decreased more strongly than *A* for *C. jacea* in response to summer drought, leading to higher values of WUE_i for *C. jacea* compared to *D. glomerata* during this period (Fig. 6). Experimental drought affected *g_s* sigificantly (*D:* F = 5.0*) over the growing season. This effect was most visible after drought with lower *g_s* values for *C. jacea*, resulting in higher values of WUE_i (*D:* F = 3.4.) Neither N addition nor N addition combined with drought had a significant (<0.1) influence on *A*, *T*, *g_s*, WUE or WUE_i (growing season).

DISCUSSION

Increasing drought events and nutrient loading are major threats to the sustainable functioning of grassland communities (Habel *et al.* 2013; IPBES 2018) and important drivers of plant physiology, *e.g.* by enhancing/limiting plant productivity or even causing plant mortality (Tilman & Downing 1994; Bobbink *et al.* 1998; Kahmen *et al.* 2005; Hautier *et al.* 2009; You *et al.* 2017; Stampfli *et al.* 2018). Each species has specific strategies to cope with and adapt to an altered environment. Consequently, the community's response to an altered environment is largely shaped by individual species' responses, *i.e.* regarding their potential physiological or phenological adaptation. As expected, we found strong differences between species in their N uptake efficiency for both N addition treatments. While all three species clearly took up N fertilizer, the two grass species increased their bulk leaf N content significantly more, outweighing, in comparison to *C. jacea*, the lower N content under non-fertilized conditions. Moreover, we found two distinct water use strategies as a response to the naturally occurring summer drought, with *C. jacea* able to tolerate very low leaf water potentials (LWP) and *D. glomerata* strongly controlling LWP. However, in contrast to our expectations, these species-specific responses to low soil water content and N loading did not transmit to respective responses in their leaf-level assimilation and transpiration rates. This shows strong homeostasis of leaf-level carbon and water relations, despite strongly altered N and water availability. In the following, we discuss our findings with respect to the literature-based recent insights into functional group-specific responses to N loading and extreme drought, as well as their combined impact at the leaf/plant level. Finally, we discuss the potential role of leaf/plant physiological responses to single and combined N loading and extreme drought in the context of recent findings of strong interacting effects of N loading and extreme drought on carbon and water fluxes at the community level (see *e.g.* Kübert *et al.* 2019).

Impact of N loading

Generally, N addition has been found to increase photosynthetic capacity (Lee *et al.* 2001) and WUE (Singh *et al.* 1975; Taylor *et al.* 1991) of grassland species. The longer time that the species adapts to N addition (here: N fertilization started in 2016, data collected from 2017) the smaller is the response in the long term, although there will be some continued enhancement to species' photosynthesis and WUE (Evans 1989; Lee
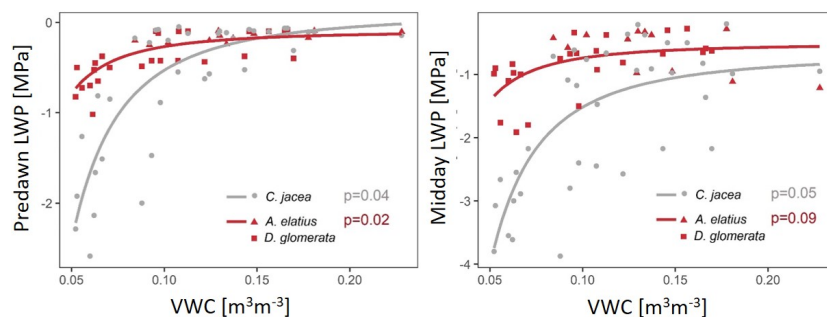

Fig. 4. Relationship between leaf water potentials (predawn and midday LWP, MPa) and volumetric water content (VWC, $m^3 \cdot m^{-3}$) during the growing season 2017 for *C. jacea* (grey, dot) and *A. elatius/D. glomerata* (both red, triangle/square). Mean VWC (0–40 cm) plotted against mean LWP at the same time point of measurements (treatment means). Left: Predawn LWP. Right: Midday LWP. *P*-values indicate a (significant) relationship between VWC and LWP. Lines depict linear regression lines based on linear mixed-effect models.
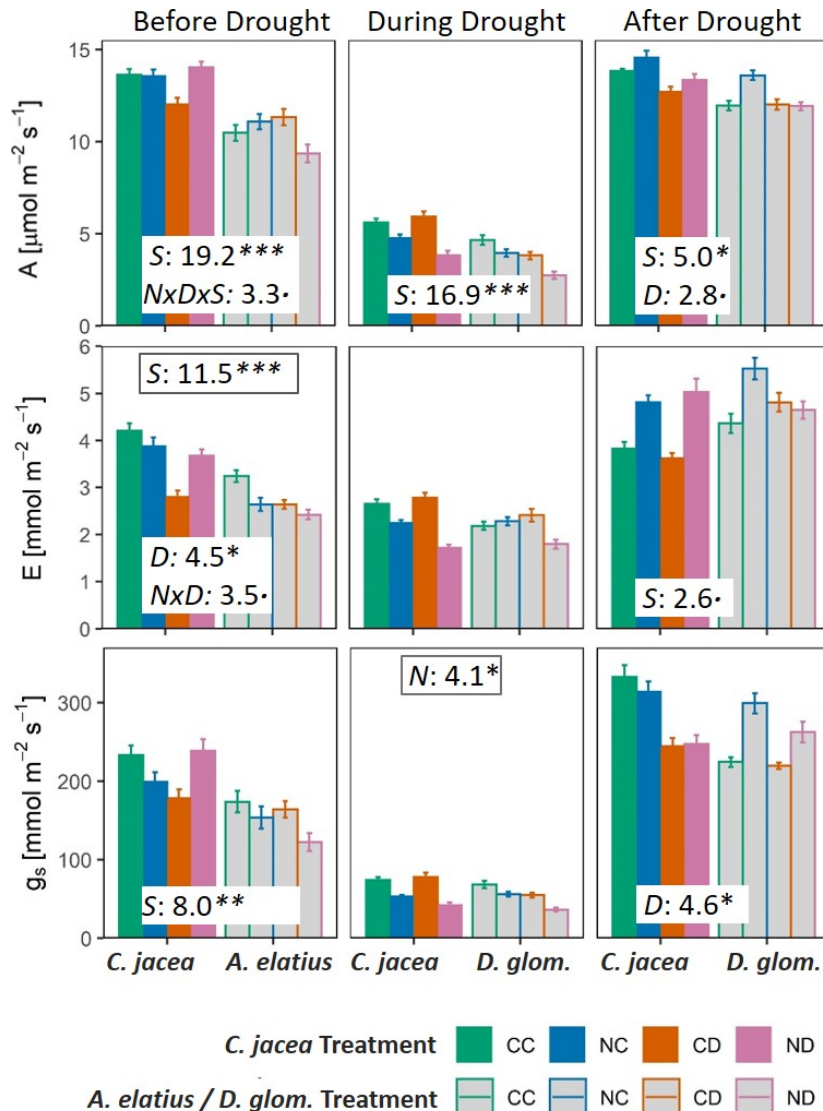
Fig. 5. Mean midday gas exchange rates of *C. jacea* (filled bars), *A. elatius* and *D. glomerata* (both open bars) per treatment before, during and after summer drought, in the growing season 2017. Top: Assimilation rate [*A*, μmol·m^{-2}·s^{-1}]. Middle: Transpiration rate [*E*, mmol·m^{-2}·s^{-1}]. Bottom: Stomatal conductance [*g$_s$*, mmol·m^{-2}·s^{-1}]. Treatments: *CC* control (green), *NC* nitrogen addition (blue), *CD* experimental extreme drought (orange), *ND* nitrogen addition and experimental extreme drought (rose). Mean ± 1 SE, n = 3–4. F-values and asterisks indicate significant differences between species (*S*) and treatments (*N*: nitrogen addition, *D*: experimental extreme drought) and their interactions (×). Significance levels: .<0.1, *<0.05, **<0.01, ***<0.001.

et al. 2001). In that regard, our findings are in accordance with other studies which could not find long-term positive effects of higher soil N availability on carbon flux (Bloor & Bardgett 2012; Mackie *et al.* 2019).

On a global scale, N loading has been reported to increase the aboveground biomass of grasses, whereas the biomass of forbs remained unchanged (You *et al.* 2017). Underlying mechanisms, such as the height advantage of grasses compared to forbs in the competition for light (Hautier *et al.* 2009), or higher tolerance of grasses to soil acidification (Tian *et al.* 2016). Another possible driver could be a higher root density in the upper soil layers where N addition took place (Huang *et al.* 2008). Indeed, we observed stronger uptake of N by the grass species (*A. elatius*/*D. glomerata*; Fig. 2), but this did not

directly translate into higher assimilation rates (Fig. 5). A higher bulk leaf N content could indicate a higher content of the CO_2 fixing enzyme RuBisCo, and thus higher assimilation capacity. Although a higher RuBisCo content does not necessarily lead to increased assimilation since other factors, such as CO_2 or light availability, can constrain it (Lawlor 2002). In particular, under longer-lasting extreme drought, the efficiency of N addition and its mitigating effect on assimilation was found to decline (Gonzalez-Dugo *et al.* 2005; Hofer *et al.* 2017). Moreover, instead of increasing photosynthetic capacity, the studied grass species could also have used the additional N source to increase leaf area, which is in accordance with measurements of grass biomass/leaf area index from this site (Kübert *et al.,* 2019).
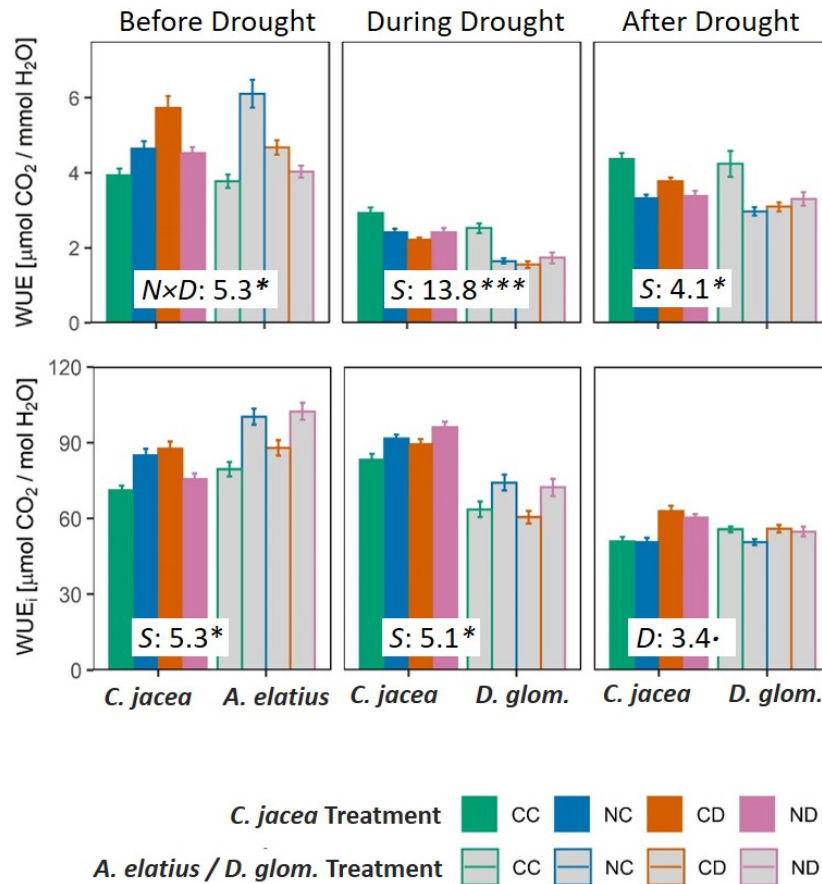
Fig. 6. Mean water use efficiencies of *C. jacea* (filled bars), *A. elatius* and *D. glomerata* (both open bars) per treatment before, during and after summer drought, in the growing season 2017. Top: Instantaneous water use efficiency (WUE, μmol CO_2/mmol H_2O). Bottom: Intrinsic water use efficiency (WUE$_i$, μmol CO_2/mol H_2O). Treatments: *CC* control (green), *NC* nitrogen addition (blue), *CD* experimental extreme drought (orange), *ND* nitrogen addition and experimental extreme drought (rose). Mean ± 1 SE, n = 3–4. F-values and asterisks indicate significant differences between species (*S*) and treatments (*N*: nitrogen addition, *D*: experimental extreme drought) and their interactions (×). Significance levels: .<0.1, *<0.05, ***<0.001.

Impact of experimental extreme drought and naturally occurring summer drought

Mackie *et al.* (2019) observed that drought resistance of grass-land biomass was positively related to the drought resistance of the forbs. Forbs usually have deeper roots and thus could access less dry soil layers while grass species often have much shallower root systems (Leitinger *et al.* 2015; Stampfli *et al.* 2018). Moreover, forbs might contain larger plant water reserves than grasses due to a higher total leaf area, which can delay desiccation of the meristem during drought stress (Milbau *et al.* 2005). Naturally occurring summer drought significantly affected LWP of both *D. glomerata* and *C. jacea*, albeit with significant differences in magnitude (Figs 3 and 4). Remarkably, the midday LWP of *C. jacea* declined to −3.9 ± 1.1 MPa during summer drought stress, suggesting an anisohydric strategy, *i.e.* tolerance to very low LWP prevented the loss of hydraulic connection to the soil and maintained gas exchange (Sperry *et al.* 2002). Similarly, Guderle *et al.* (2018) found that (primarily) tall herbs tolerated lower LWP during drought and, thereby, maintained high rates of root water uptake and transpiration. In contrast, *D. glomerata* only decreased its midday LWP −1.9 ± 0.2 MPa and maintained an ample predawn LWP compared to *C. jacea* (Fig. 3). Similarly,

leaf gas exchange rates also significantly decreased in all three species during the summer drought. A slight but significantly higher WUE$_i$ of *C. jacea* compared to *D. glomerata* during summer confirms that *C. jacea* could more efficiently tolerate drought than the studied grass species.

Although we observed a strong decrease in soil water availability in the upper 40 cm (on average −29%; Fig. 1) in both drought treatments, the long-term experimental drought only had a significant effect on LWP when VWC fell below 0.1 m^3·m^{-3} (during and after the summer drought). Moreover, experimental drought did not affect any of the leaf-level gas exchange parameters, except for small/transient effects on g_s and A after drought (Fig. 5). This might point towards a phenotypic response of the observed species in response to long-term experimental drought. For example, grasses were found to avoid dehydration during drought by reducing their growth and through senescence of the aboveground biomass (Bollig & Feller 2014; Wellstein *et al.* 2017). In which case leaf-level assimilation and transpiration of the (few) remaining leaves can be maintained relatively compared to control conditions. Similarly, Kübert *et al.* (2019) observed early heading of grasses on drought-treated (*CD/ND*) plots. Consequently, many grass species could complete their drought-sensitive life cycle before the dry summer period and thereby escape the risk of

dehydration. Early heading was also found to be used as a drought-escape strategy by *D. glomerata* in the study of Volaire & Lelièvre (1997).

Impact of species-specific response to combined extreme drought and N loading at the leaf physiological level on observed community effects

One of our main objectives was to determine and quantify interacting effects of N loading and extreme drought on leaf-level physiological control. Contrasting results have been observed in recent literature in regard to potential additive or mitigating effects of combined N loading and extreme drought in grassland plant species and communities (Bloor & Bardgett 2012; Carlsson *et al.* 2017; Hofer *et al.* 2017; Kübert *et al.* 2019; Bharath *et al.* 2020). While there are some indications that N loading might improve drought resistance of some grassland species and communities (Carlsson *et al.* 2017; Hofer *et al.* 2017), others studies have shown that N loading can strongly enhance the stress impact of drought (Kübert *et al.* 2019; Bharath *et al.* 2020). The study of Kübert *et al.* (2019) showed the ecosystem response to combined drought and N loading at our study site, where the grassland ecosystem net ecosystem exchange (NEE) and ecosystem water use efficiency (WUE$_{eco}$) were drastically reduced (about twice the effect of drought alone) on additional N loading, turning the ecosystem into a net carbon source. Changes in NEE and WUE$_{eco}$ at the community level could be obtained by a combination of many changes at different organizational levels. For example, changes at the community level could be attributed to a N loading-dependent shift in species/functional group abundance to species/functional groups that suffer stronger drought stress or generally have lower NEE and WUE, to changes in phenology of the abundant species or to potential additive or mitigating effects of N loading on the drought response of A and E of abundant species at the leaf level.

However, the species in this study and their observed physiological responses cannot explain recent findings of Kübert *et al.* (2019) at the community level on the same study site and occurring at the same time. We could not find any significant responses of the forb *C. jacea* or the grasses *A. elatius* and *D. glomerata* to drought, N loading or combined drought and N loading that were reflected in the species' carbon and water relations at the leaf level. In particular, we did not find additive or mitigating responses of leaf gas exchange parameters to combined experimental extreme drought and N loading. This indicates that the negative effects of combined N loading and drought on gross primary production (GPP) and NEE found by Kübert *et al.* (2019) were not driven by a single abundant species. Instead, N loading and experimental extreme drought combined strongly changed the functional group abundance and diversity, considerably altering the grassland's species composition. The decisive factors controlling community-scale changes in carbon and water relations were, therefore, a drought-related decline in the abundance of forbs and a N-related decline in the number of forb species. The resulting dominance of grasses during the naturally occurring summer drought under combined N loading and drought stress resulted in a phenologically severely shortened growing season and, thus, enormous reductions in the WUE and carbon sink potential of the system (see Kübert *et al.* 2019). Nevertheless, our results indicate significant differences in water and N use efficiency for a dominant forb species compared to two abundant grass species, which explain the observed abundance shifts and seasonal changes in community structure. The more efficient N uptake of the grasses relates well to their significantly increased abundance on N addition plots, and the strong control of their water status clearly emphasizes grass drought sensitivity and related summer die-back. Therefore, while species-specific differences in water use and N uptake did not directly translate into leaf differences for photosynthetic parameters, they are still crucial in order to understand how the grassland community as a whole responded to combined drought and N loading.

Long-term response of the grassland community to extreme drought and N loading

Our results suggest a relatively high capacity of leaf physiological control to maintain physiological activity even under 2 years of experimental extreme drought. The observed leaf physiological response to N loading was low. Consequently, the strong decline in community-scale water and carbon fluxes under combined long-term N loading and extreme drought observed in Kübert *et al.* (2019) was not reflected in the physiological responses to N and drought at the leaf level of the most abundant species in this grassland. Instead, long-term extreme drought and N loading led to an altered species composition, which, in turn, caused the observed responses in the grassland's carbon fluxes and water use. Future research should study both above- and below-ground plant carbon and water processes and related plant traits in order to fully understand species-specific strategies in response to an altered environment.

AUTHOR CONTRIBUTIONS

AK, CW, YR and MD planned and designed the experiment. AK, CW, DD and MD set up the experiment. AK collected and analysed the data, AK and MD wrote the manuscript. EK and MG helped in collecting and analysing the data. DD and ME processed the elemental analyser data. All authors reviewed the manuscript.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any personal, commercial or financial relationships that could be construed as a potential conflicts of interest.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. List of species present at Freiburg study site in 2015.

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