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The impact of ozone exposure, temperature and CO_2 on the growth and yield of three spring wheat varieties.

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ABSTRACT

When assessing potentials for crop production under future climatic conditions, multiple environmental parameters need to be included. An increase in carbon dioxide [CO_2], higher temperatures, and regional changes in tropospheric ozone [O_3] will influence the growth responses of existing crop species and varieties. Ozone is phytotoxic and a plant stressor at current concentrations, reducing yields worldwide, but possible interactions with changes in other abiotic factors have been considered very little. In this study, we have used eight combinations: two levels of temperature, two levels of [CO_2], and three [O_3] exposure regimes to assess the impact of medium-to-high ozone concentrations (80-100 ppb) on wheat growth when other abiotic factors change. Two modern spring wheat varieties (KWS Bittern and Lennox) and a landrace variety (Lantvete) were grown to maturity in climate chambers. We examined plant performance during growth as development rate, rate of photosynthesis, stomatal conductance, water use, and at harvest as total aboveground dry matter and grain yield.

All three varieties lost yield in all treatments compared to the ambient treatment that had the following settings: Lowest temperature, ambient [CO_2], and very low [O_3]. For episodic ozone exposure in the ambient or high [CO_2] and high-level temperature treatment, the yield losses were 18 and 25%, respectively, for KWS Bittern; 44 and 34% for Lennox; and 16 and 37% for Lantvete. The yields of the modern varieties are significantly higher than the landrace variety in two out of eight treatments, although they are higher by weight in seven of the eight treatments. The landrace variety's fraction loss from its highest grain yield in the ambient treatment to the high-[CO_2]-and high-level-temperature-treatments was smaller than the modern varieties', showing a comparably higher degree of plasticity of performance. Current crop varieties might be more sensitive to ozone

than older varieties, emphasizing the need of future breeding programs to expand the gene pool to provide more climate robust crops.

Keywords

Climate change, air pollution, ecophysiology, multifactorial design, ozone episodes

1. Introduction

The changes in climate relating to the climate scenarios' emission of carbon dioxide include temperature increase, increased frequency of extreme weather events, regional aggravation of current challenges and addition of new challenges (IPCC, 2014) influencing plant growth in natural and agricultural ecosystems (Albert et al., 2011; Pleijel et al., 2018). These factors will not change individually and their impact on ecosystems is increasingly investigated in multifactorial settings (Ingvordsen et al., 2015; Langley and Hungate, 2014; Namazkar et al., 2016). When addressing responsiveness of existing crop species and varieties grown under future climatic conditions, multiple environmental parameters must be included (Frenck et al., 2013; Vázquez et al., 2017). Both temperature and CO_2 are well-known parameters influencing the growth and development of plants. Temperature influence amongst others enzyme activity and water relations of the plant and as a single factor changing, temperature can be both too low and too high to sustain optimal plant growth, the optimal temperature range depend on the genetics of the variety (Albert et al., 2011; Frenck et al., 2011; Gibson and Paulsen, 1999). Being the substrate for photosynthesis, CO_2 availability can be a limiting factor at ambient concentrations meaning that CO_2-increases enhance yields (AbdElgawad et al., 2015; Cure and Acock, 1986). However, simultaneous combinations of changes in climatic factors may not result in additive effects (Clausen et al., 2011; Dieleman et al., 2012; Shaw et al., 2002).

In addition, the air pollutant, ozone, which is causing yield loss under the current climatic conditions, may interact with the changing levels of other abiotic factors, and thus further impact temporal and spatial plant growth patterns and yield (Pleijel et al., 2018). Exposure to elevated ozone typically results in suppressed photosynthesis, accelerated senescence, decreased growth and lower yields (Booker et al., 2009). However, there are major knowledge gaps when assessing the threat that ozone plays (Ainsworth et al., 2012; Fuhrer and Booker, 2003).

Ozone is readily formed from available precursors in the presence of UV-radiation. The precursors are natural or anthropogenic volatile organic compounds (VOCs) and nitrogen oxides (NO_xs) (Finlayson-Pitts and Pitts, 1993). The precursors may be carried long distances and result in both peak ozone formations far from the precursor formation/emission and in a regional increase of background concentration (Monks et al., 2015). Efforts to reduce precursor emission contribute to regional control of ozone levels (Derwent et al., 2018). Precursors may also build up during periods of low radiation, i.e. in winter, and result in a peak ozone period in spring when radiation increases (Munir et al., 2013). In stable, high-pressure conditions, ozone concentration may build up to create a peak ozone episode, whilst the ozone molecules may disperse into adjacent areas in more windy conditions (Kleanthous et al., 2014).

While everyday ozone concentrations reflect regional and local activities and conditions, background ozone levels have been steadily rising with anthropogenic activity over the last century (Lamarque et al., 2010). The background ozone concentration, which is a result of the mixing of air and the emitted precursors, has also been projected to worsen in the future depending on possible climate scenarios (Vingarzan, 2004) due to emissions from industrialization and other human activities (Wild et al., 2012). Ozone concentration varies with the time of day and season, based on precursor availability and regional weather, and plants may therefore be exposed to periods of ozone peaks as well as the increase in background ozone.

The cost of the plant’s defense mechanism against ozone is that fewer carbon substances are available for allocation to build biomass, both above and below ground (Calatayud et al., 2011). Thus, ozone limits plant growth and agricultural yields, and Mills et al. (2018) estimate a global yield gap of 7.1% in wheat at the background levels and peak ozone incidents prevailing under current climatic conditions. Effects of changes in climatic factors and levels of ozone concentration can be synergistic or antagonistic interactions with the defense mechanism influencing plant growth and yield in unknown ways (Larsen et al., 2011).

In the present study, wheat (*Triticum aestivum*) is used as model crop selected for its global importance as staple food in many countries (Wrigley, 2009). Wheat yields have increased vastly with breeding, research, development, and intensification of production for the last 50 years, however, the rate of yield increase has diminished in the past 30 years (Brisson et al., 2010). The decline in soil carbon content may be one explanation (Brisson et al., 2010), and the combined increase of CO_2-concentration and temperature another (Dieleman et al., 2012). While an increase in [CO_2] has a growth promoting effect on many plants as the substrate of photosynthesis, and higher temperatures increase the rates of activity in many biochemical processes, the combination of CO_2-enrichment and temperature increase may have a less than additive effect on the plant biomass (Dieleman et al., 2012; Shaw et al., 2002). As ozone is known to reduce the yield of cereals (Broberg et al., 2015), the increase in tropospheric ozone with anthropogenic activity (Lamarque et al., 2010) suggests that the increasing ground-level ozone concentration cannot be ignored and might be a threat to future food production through interactions with other climate factors.

The aim of this study was to investigate eight climate treatments reflecting current conditions and future scenarios. From seed to maturity, the wheat varieties were regularly evaluated for a range of growth indicators to learn how the combinations of abiotic factors influenced the development and yield of the wheat plants. The following four hypotheses were tested: i) Yields of modern varieties are higher than yields of the old variety; ii) Episodic ozone exposure at Zadoks' growth stages (ZS)31-69 is as injurious to wheat yields as full-time ozone exposure; iii) The combined effect of elevated [CO_2] and elevated temperature, which stimulate plant growth less than expected by the elevation of each factor individually is further aggravated by exposure to elevated ozone concentrations; iv) Differences in yields are reflected in the way growth parameters respond to the treatments during growth.

2. Materials and methods

2.1. Climate chamber experiment

The study was done in climate chambers that provided a controlled environment and uniform conditions, thus eliminating other potentially interacting parameters. The RERAF phytotron (Risø Environmental Risk Assessment Facility, Technical University of Denmark, Risø, Denmark) consists of six gastight chambers sized 6 x 4 x 3 m, providing detailed control of temperature, [CO_2], air humidity, light, and ozone concentration and exposure duration. Chamber air was exchanged at a rate of 4 m^3 h^{-1}, resulting in a complete exchange of chamber air every 18 hours and a wind speed of less than 0.6 m s^{-1} (measured in earlier experiments). The lighting produced 313-389 µmol depending on chamber. The facility's computational system logged treatment parameters in high resolution. For detailed description of the physical facility see Frenck et al. (2011), Clausen et al. (2011) & Ingvordsen et al., (2015).

2.2. Common input

The studied spring wheat varieties included two modern varieties: *Lennox* (Saaten-Union) used in southern France and *KWS Bittern* (DanishAgro) used in Denmark, and one landrace variety: the Swedish *Lantvete* (Nordic Genetic Resource Center). All with a life span of approx. 3-4 months. Twelve seeds of the spring wheat varieties tested were sown in 11 L pots filled with 4 kg of sphagnum (Pindstrup Substrate No. 4, Pindstrup Mosebrug A/S, Ryomgaard, Denmark) and reduced to eight plants after germination, corresponding to ~165 plants m^{-2}. Each variety was represented in each treatment with five pots. No additional nutrients were added to the pots as the sphagnum was nutrient enriched. The watering water was tap water.

CO_2 was provided by AGA A/S (Linde Worldwide, Copenhagen, Denmark) and ozone was generated by the use of UV Pro 550 A ozone generators (Crystal Air Products and Services, Langley, BC, Canada). The UV-lamps in the ozone generators were at times partially shaded with cardboard to reduce the amount of ozone generated.

The experiment lasted from March 6th to June 25th 2016.

2.3. Growth conditions

2.3.1. Treatments

Eight treatments were tested in total. They were selected among possible combinations of two present and future temperature levels (19/12 °C or 24/17 °C, both levels simulating days (16h) that are warmer than nights (8h)), two concentrations of CO_2 (400 and 700 ppm), and one of three ozone regimes: 1) no ozone enrichment, 2) episodic ozone exposure (.EpO3), and full-time ozone exposure (.O3) (Fig. 1). The 'no ozone' ozone-enrichment treatments act as filtered air treatments, as ozone concentrations in the climate chambers 'background levels' (5.9 ±0.5 and 7.2 ±1.7 ppb, see Table 1) are lower than the outside average ozone concentration near the RERAF phytotron with an average of 40.4 ppb, and maximum ozone one-hour concentrations between 70,9-86,6 ppb (calculated with data from 2013-16 (May-July; 8am - 20pm), from station DK0012R, Danish Center for Environment and Energy, Aarhus University). For both the episodic and full-time ozone exposure, the ozone concentration target was 80-100 ppb during the day, thus resulting in 16 hours of daytime ozone exposure, at night the ozone concentration was as the chamber background measured in the 'no ozone enrichment' treatments. Unlike the full-time exposure treatments, which started at sowing, the ozone exposure of the .EpO3 treatments began when the Lennox variety reached Zadoks' developmental stage 31 (ZS31 - first node detectable) and ended when Lennox reached stage 69 (ZS69 - anthesis complete) (Zadoks and Board, 1974). Thus, the number of days of ozone exposure varied as plant development varied depending on climate. Relative humidity was kept at 70/55% (day/night) for all treatments.

The following eight treatments were represented in this experiment: A, A.EpO3, A.O3, C.EpO3, CT, CT.EpO3, CT.O3, and T.EpO3 as shown in Fig. 1. A denotes 'ambient conditions' with temperatures that correspond to good growing conditions during the growth season of spring wheat in Denmark, and a CO_2-concentration corresponding to present average atmospheric concentrations; C denotes an elevated level of [CO_2], and T denotes a higher temperature.

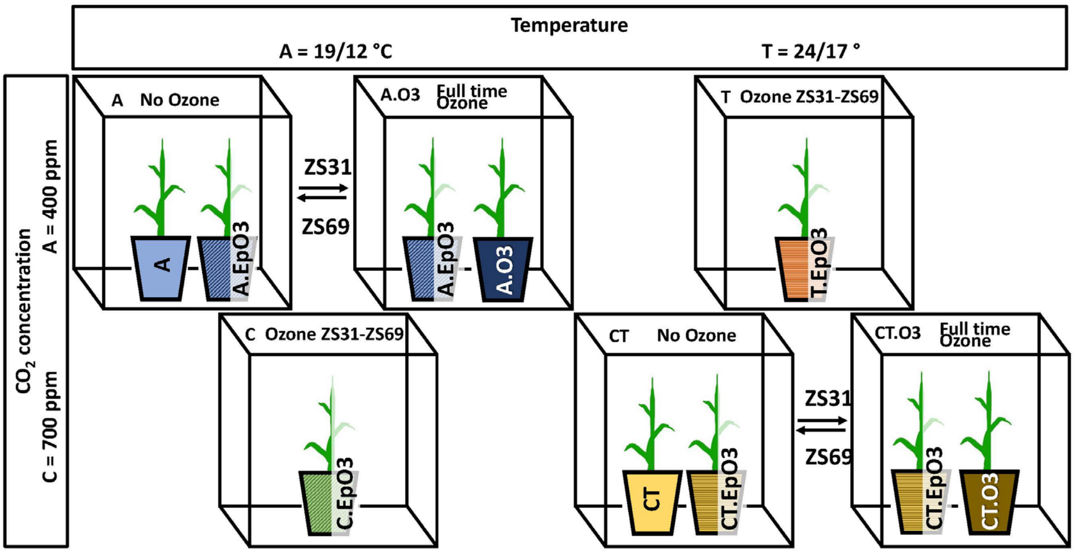

Fig. 1: The selected climate combinations are named A, A.EpO3, A.O3 (ambient [CO_2], lower temperature settings and no addition (), episodic addition (.EpO3) or chronic addition of ozone (.O3)) and CT, CT.EpO3. CT.O3 (high [CO_2] (C), higher temperature settings (T), and no addition (), episodic addition (.EpO3) or chronic addition of ozone (.O3)). Two treatments, C.EpO3 and T.EpO3 have episodic addition of ozone (EpO3) and either higher [CO_2] or higher temperature settings. The aim for concentration of ozone in the chambers was 80-100 ppb. The episodic exposure started at Zadoks' growth stage ZS31 and ended at stage ZS69. A.EpO3 and CT.EpO3 were transferred to the corresponding full-time ozone exposure chamber for ozone exposure and returned after exposure, while C.EpO3 and T.EpO3 received ozone exposure in their own chambers for the episode from ZS31-ZS69.

2.3.2. Watering regime

Plants were watered three times a week to provide a full supply of water; the warm treatment plants were, by design and by consumption, given more water than ambient treatment plants and all plants got increasingly more water as they grew. Pots were weighed before and after watering to ensure the same amount of water was accessible in the treatment regardless of the pot's previous consumption.

2.4. Potential chamber gradient effects

To minimize influences from potential gradients of treatment factors or chamber microclimates, the tables in each chamber were moved to different positions in the chamber following each watering. Also, once a week we moved the treatments between the chambers to reduce the risk of intrinsic traits of one chamber to manifest in any one treatment.

2.5. Process values and measurements of plant growth parameters

Process values of treatment parameters such as relative humidity, CO_2 concentration, and temperature were logged by a data collection system several times per minute. The ozone concentration was monitored sequentially, i.e. twice every hour. Photosynthesis, stomatal conductance, transpiration, and other parameters were measured once or twice a week throughout the experiment using two LI-6400 portable photosynthesis systems (LICOR, 2004). The LiCor light was set to 1500 µmol and the other settings settings followed the treatment parameters. The gas exchange measurements were done at the second leaf, i.e. at any time the youngest fully expanded leaf, representing throughout the life time of the wheat plants the most productive leaf, and hence the productivity of the plant as a whole as the most ozone-exposed leaves as well as senescence will cause main photosynthesis to take part in other leaves.

Water consumption was recorded by weighing the individual pots before and during watering; the plants' heights were measured; the development stage assessed according to Zadok's growth stages (Zadoks and Board, 1974) and noted. Ozone was not switched off during measurements and watering.

Leaf discoloration was assessed at four different dates following the initiation of ozone exposure in the episodic ozone exposure treatments (starting at 42 and 43 days after sowing). The ratio of green leaf area to total leaf area on the third leaf was assessed by image processing photos of the harvested leaf with the software ImageJ (Schneider et al., 2012). At sampling one leaf in each treatment was harvested.

Grain and aboveground biomass were weighed at the end of the experiment. Gluten index was determined by the ICC 155 procedure by the Nordic Seed Laboratory Services.

2.6. Ozone treatment recap, fluxes and plant ozone uptake.

The ozone flux depends on the ozone concentration in the air, the degree of stomatal opening (which, among other factors, depends on water status and CO_2 availability), and the ozone molecules' resistance to cross the boundary layer of the leaves. As the latter was not measured, the resistance part of the equation is left out and any assumptions would only reflect the similarity of wind conditions in the chambers. Thus, ozone flux is the product of the hourly ozone concentrations (in nmol m^{-3}) and the stomatal ozone conductance (H_2O mol m^{-2} s^{-1}). The stomatal conductance was measured approximately six times in each variety of every treatment, and the values for the days between measurements were found by linear interpolation. Ozone conductance was found by the ratio of molecule size O_3 to H_2O (0.66) and converted from mol m^{-2} s^{-1} into m s^{-1} (Monks et al., 2015). From the obtained values for fluxes of ozone into the plant, the accumulated plant uptake of ozone above a threshold of 6 was aggregated, giving a unit of mmol O_3 m^{-2} PLA (Projected Leaf Area). The threshold of 6 mmol O_3 reflects the sensitivity of wheat plants (ICP Vegetation, 2017). The data logging system occasionally failed to log ozone data, and thus data gap filling has been necessary for the summation of ozone into the plants. Gap filling for single or a few missing data points was done using the average of the 12 closest measurements. The average of daytime ozone concentration (ppb) in treatment and as background can be found in Table 1.

Table 1. The average daytime concentration of ozone in the eight different treatments with ozone levels in ppb and i) No ozone treatment equivalent to background concentration in the chambers, ii) daily average of episodic ozone exposure and iii) average of chronic ozone exposure. Averages based on ~32 recordings of ozone concentration per growing day. Further explanation of treatment abbreviations see Fig. 1.

Treatment	Episodic relocation		No ozone	Episodic ozone	Chronic ozone
	Base treatment	Exposure treatment			
A			6.4 ±2.1	-	
A.EpO3	A	A.O3	6.4 ±2.1	84.5 ±28.1	
A.O3					78.8 ±32.4
C.EpO3			6.8 ±1.3	82.7 ±35.2	
CT			5.9 ±0.5	-	
CT.EpO3	CT	CT.O3	5.9 ±0.5	80.1 ±35.9	
CT.O3				-	88.5 ±22.0
T.EpO3			7.2 ±1.7	98.9 ±22.5	

2.7. Data analysis

Data were statistically analyzed using the statistical software R or Microsoft Excel and tested with Breusch-Pagan test, pairwise t-tests and anovas. Levels of $p \leq 0.05$ were considered significant. Models that were tested followed the formula of e.g. Grain Yield = γ (Treatment$_i$ · Variety$_i$) +e$_i$. Different physiological responses, such as biomass or photosynthesis were tested for dependence on the climate treatments or elements thereof, i.e. ozone on/off, climates relating to the CO$_2$/temperature combinations (e.g. Tables 4 and 6).

3. Results

The results are consequently displayed 'per pot' to underline the phytotron experiment design. Nevertheless, the grain yield range of 30.8 ±1.0 to 55.7 ±1.1 g pot^{-1} (Fig. 2) converts to 5.0 ±0.2 to 12.1 ±0.2 tons ha^{-1}. Unless otherwise indicated, results are displayed with standard errors.

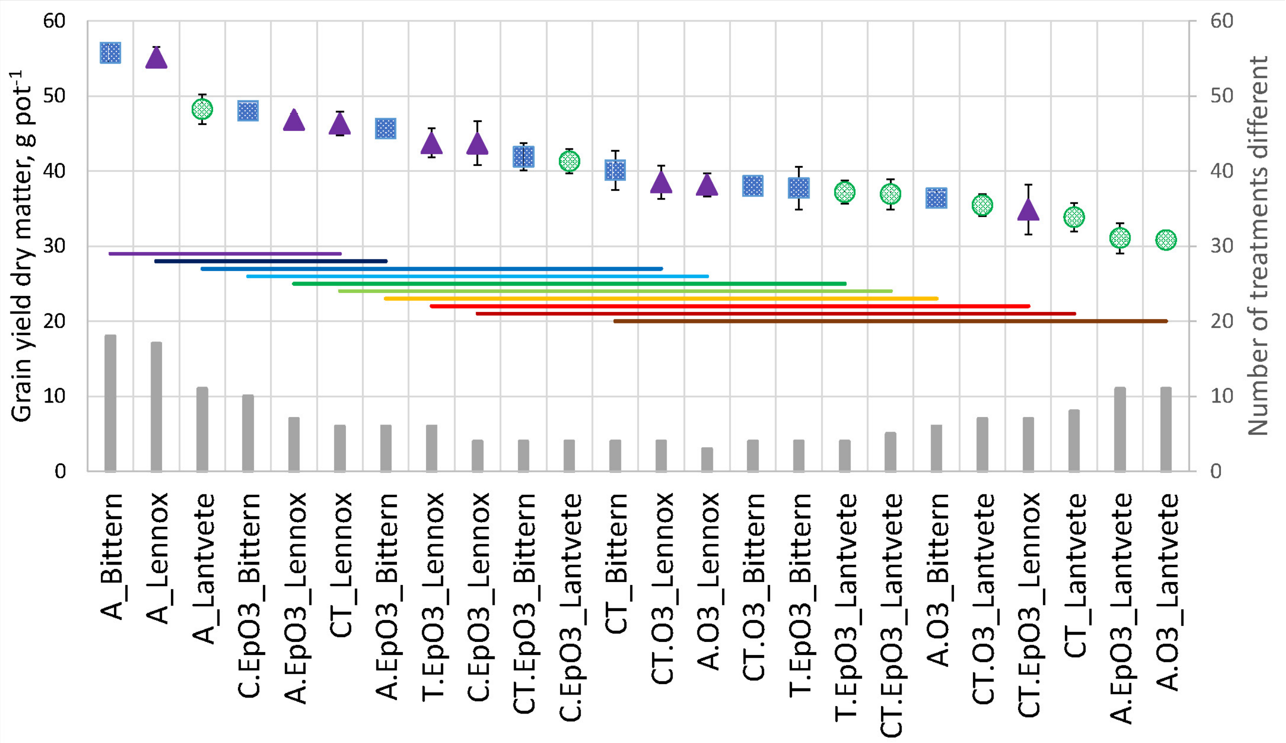

Fig. 2. Grain yields with standard errors, sorted from the highest to the lowest; blue squares, purple triangles, and green circles indicate if a variety is KWS Bittern, Lennox or Lantvete, respectively. Colored bars indicate groups of no significant difference. Grey columns indicate how many other treatment*varieties this treatment*variety is significantly different from ($p \leq 0.05$). n=5. Further explanation of treatment abbreviations see Fig. 1.

3.1. Experimental levels of treatment parameters.

Treatment parameters are reported with standard deviations, TP = X ±SD, each average is based on 10-15 recordings per growing day or night, the data is not shown. The average temperatures in the low level temperature treatments (set points 19/12 °C) were 19.3 ±1.1 and 12.1 ±0.7 °C, and 24.0 ±0.3 and 17.1 ±0.9 °C (for set points 24/17 °C) in the high level temperature treatments. The average CO_2 concentration in the low level CO_2 treatments (set point 400 ppm) were 540 ±37, and 724 ±57 ppm in the high level CO_2 treatments (set point 700 ppm). The average relative humidity with set points of 55/70% day/night, were 54 ±3 and 69 ±4 % respectively.

The ambient treatment pots were initially watered to a total pot weight of 5200 g to 7000 g when they consumed the most, and the warmer treatments were watered from 5400 g to 7200 g accordingly. When irrigated based on water consumption, this corresponded to a daily average in all the growth period of 8 ±1 mm in cold treatments and 9 ±1 mm in warm treatments. The growth dependent water consumption distribution of the treatment*varieties consuming most (Lantvete T.EpO3) and least (Lennox C.EpO3) can be seen as their daily average in Fig. 3.

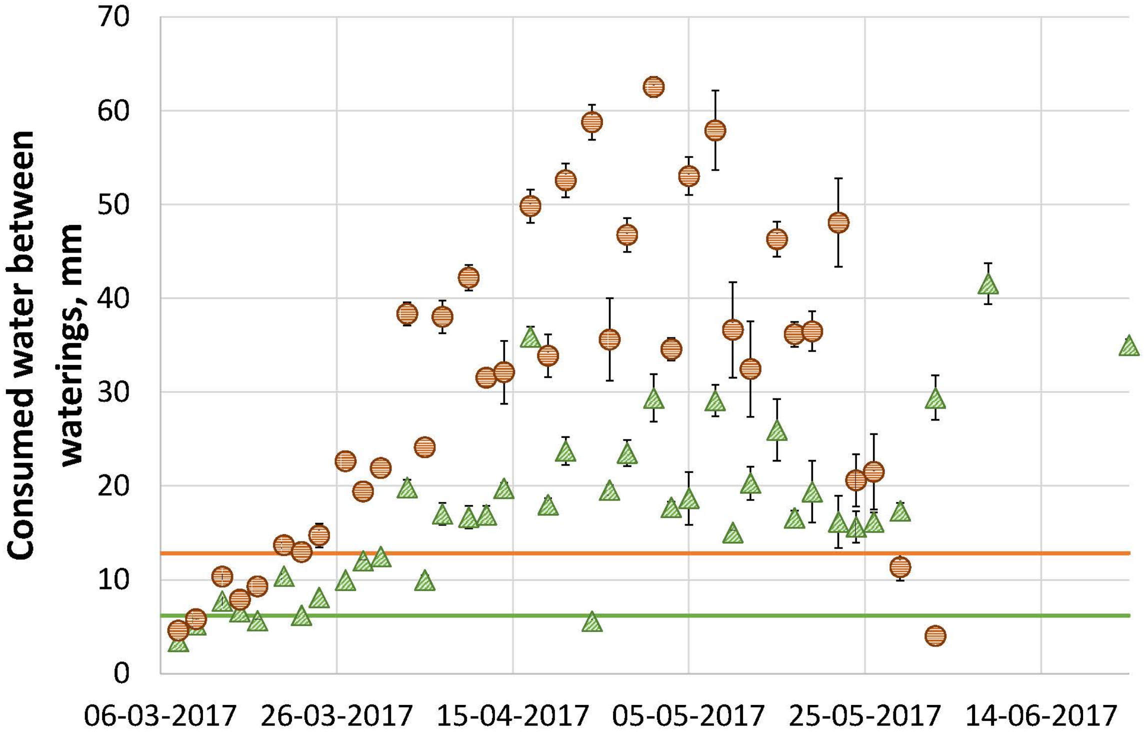

Fig. 3. Water consumption in mm in the treatments consuming most and the least water, Lantvete T.EpO3 (orange circles) and Lennox C.EpO3 (green triangles). n=5, with standard errors. The horizontal bars indicate the daily average for each treatment.

3.2. Grain yields

Grain yields were significantly influenced by the main effects of treatment, variety and interactions between them (p-values of resp. 2.2E-16, 1.11E-11, and 9.55E-05). Elevated temperatures outcompete other effects (Table 2), whereas at ambient temperatures i) ozone significantly reduces yield (except for Lennox A.EpO3) and ii) elevated CO_2 mitigates effects of episodic ozone exposure EpO3, only in the Lantvete variety. On average, across all treatments, yields of the landrace variety, Lantvete, were significantly lower than the modern varieties, $p = 0.0013$ (KWS Bittern) and $p = 0.00012$ (Lennox). Similarly, across the varieties, treatment A had significantly higher yields than all other treatments ($p < 0.01$), and treatment C.EpO3 had significantly higher yields than treatment A.O3 ($p = 0.0004$) and CT.O3 ($p = 0.02$). There were interactions between the varieties and treatments which led to significant differences between some treatment*variety-combinations, e.g. A Lennox, CT.EpO3 Bittern and A.O3 Lantvete (Fig.2). Within a treatment, the varieties only showed a significant difference in A.EpO3, where Lantvete was different from both Lennox and KWS Bittern, and in CT, where Lantvete was different from Lennox but not from KWS Bittern. All three varieties lost grain yield relative to treatment A, but the losses were differently distributed between the treatments without any patterns between losses of grain yield and biomass to explain loss or gain of harvest index (Table 2). A high harvest index (ratio grain yield mass to total aboveground biomass (grain and vegetative biomass)) indicates a high-yielding variety (Peltonen-Sainio et al., 2008). Modern varieties have been bred to be high-yielding and as such have high harvest indexes, but the production of non-reproductive biomass may serve other purposes, e.g. an increased resilience capacity towards biotic and abiotic stresses (Dolferus, 2014). Lantvete had the highest biomass production, which led to lower harvest indexes than the modern varieties (Table 2). In the colder treatments, Lantvete's harvest index decreased with ozone exposure. In warmer treatments, Lantvete's harvest indexes increased compared to the colder treatments, but ozone exposure diminished that increase. For Lennox and KWS Bittern, the harvest index decreased with full-time ozone in the colder treatments and in general in the warmer treatments (Table 2). Lennox lost the same percentage of biomass in the colder treatments regardless of ozone exposure and elevation of [CO_2]. In the warm T.EpO3, Lennox lost the most biomass of all treatments. However, it lead to an increase in harvest index. KWS Bittern lost the most biomass in the warmer CO_2-enriched treatment with no ozone, CT; however the grain yield loss in that treatment was not the most severe and there was an increase in harvest index.

Table 2. Grain yield change, Aboveground DM (grain and vegetative dry matter) and change, Harvest Index and change relative to the best yielding treatment A. For grain yield, see Fig. 2. Harvest Index (HI) is grain yield divided by Aboveground DM. Comparison within same variety, n=5. Star symbol indicate if a change relative to treatment A is statistically significant. Further explanation of treatment abbreviations see Fig. 1.

Variety	Treatment	Grain yield	Aboveground DM		Harvest Index	
		Change (%)	g pot^{-1}	Change (%)	HI	Change (%)
KWS Bittern	A	0 ±1	76.4 ±1.9	0 ±2	0.41 ±0.006	0 ±2
	A.EpO3	-18 ±1*	62.8 ±1.7	-18 ±2	0.44 ±0.005	0 ±2
	A.O3	-35 ±2*	66.0 ±1.9	-14 ±3	0.35 ±0.005	-16 ±2
	C.EpO3	-14 ±1	65.8 ±1.5	-14 ±2	0.44 ±0.004	0 ±2
	CT	-28 ±5*	52.6 ±5.3	-31 ±7*	0.44 ±0.013	3 ±3
	CT.EpO3	-25 ±3*	62.1 ±6.0	-19 ±8	0.42 ±0.014	-4 ±4
	CT.O3	-32 ±2*	55.3 ±2.0	-28 ±3*	0.4 ±0.008	-3 ±2
	T.EpO3	-32 ±5*	61.6 ±8.1	-19 ±11	0.42 ±0.039	-8 ±9
Lantvete	A	0 ±2	104.1 ±3.7	0 ±4	0.3 ±0.005	0 ±2
	A.EpO3	-44 ±4*	84.6 ±2.6	-19 ±3	0.27 ±0.015	-15 ±5
	A.O3	-45 ±2*	82.5 ±2.9	-21 ±3*	0.27 ±0.003	-14 ±2
	C.EpO3	-26 ±3	92.2 ±4.1	-29 ±18	0.31 ±0.013	-3 ±4
	CT	-39 ±3*	66.6 ±1.6	-36 ±2*	0.34 ±0.011	6 ±4
	CT.EpO3	-34 ±4*	72.9 ±1.4	-30 ±1*	0.34 ±0.011	6 ±4
	CT.O3	-36 ±3*	79.0 ±2.8	-24 ±3*	0.31 ±0.013	-2 ±4
	T.EpO3	-33 ±3*	70.7 ±1.9	-32 ±2*	0.34 ±0.009	9 ±3
Lennox	A	0 ±1	71.8 ±2.7	0 ±4	0.44 ±0.004	0 ±1
	A.EpO3	-16 ±1	60.0 ±0.8	-16 ±1	0.44 ±0.005	1 ±2
	A.O3	-31 ±3*	61.0 ±0.7	-15 ±1	0.39 ±0.01	-12 ±2
	C.EpO3	-22 ±5*	60.5 ±5.1	-16 ±7	0.42 ±0.035	-3 ±8
	CT	-17 ±3	63.1 ±2.7	-12 ±4	0.42 ±0.006	-3 ±2
	CT.EpO3	-37 ±6*	73.6 ±3.4	2 ±5	0.32 ±0.029	-26 ±7*
	CT.O3	-31 ±4*	63.3 ±6.9	-12 ±10	0.39 ±0.034	-11 ±8
	T.EpO3	-22 ±3*	56.9 ±3.8	-21 ±5	0.44 ±0.025	0 ±6

3.3. Ozone uptake and grain yield.

The A and CT treatments contained all three regimes of ozone exposure (Fig. 4). For the A-treatments, the correlation between grain yield and ozone dose was between R^2 0.57 and 0.95, indicating that the increase in ozone dose contributed considerately to the explanation of yield loss. Furthermore, as indicated by the regression equations in Table 3, yield change due to an increased ozone dose was higher in the A-treatments than in the CT-treatments, where the increase in ozone uptake explained only 11 to 55% of the change in yield, due to the detrimental effect of elevated temperature in CT treatments. In both A and CT-treatments, it was the landrace variety's yield change that was least correlated with the increase in ozone dose (R^2-values of 0.57 (A-settings) and

0.11 (CT settings) versus 0.95 and 0.93 in A for KWS Bittern and Lennox resp., and 0.55 and 0.40 in CT). But it may be worth noticing that the Lantvete's $R^2 = 0.11$ in CT-settings covered a yield-increase in the same order of magnitude as the KWS Bittern's yield loss in those settings. The anova in Table 4 specifies that in addition to ozone dose (p= 1.71E-09), the main effects of variety and climate as well as interactions between the factors significantly influence grain yield.

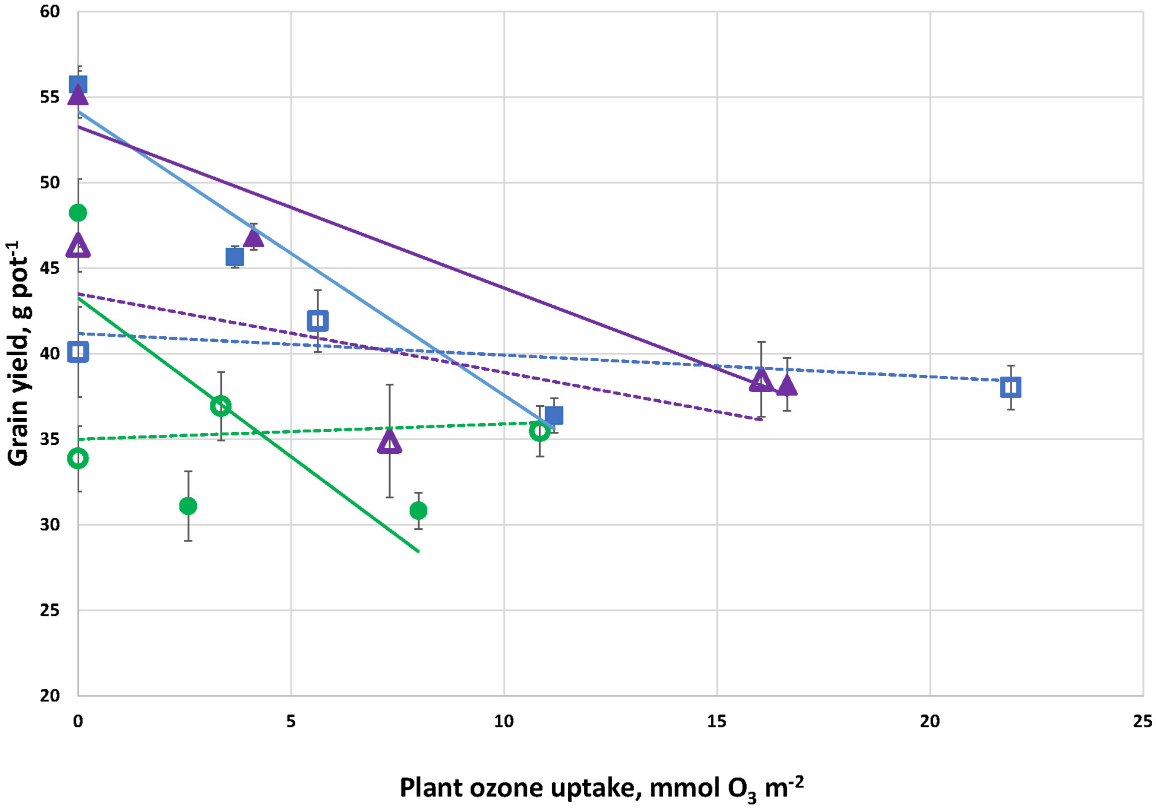

Fig. 4. Grain yield relating to different amounts of ozone uptake in the two general climate settings; A (ambient [CO2] + low temperature) and CT (high [CO2] + high temperature). The varieties are presented by color and shape; KWS Bittern (blue squares), Lennox (purple triangles), and Lantvete (green circles). Solid lines and filled data marks are A treatments and dashed lines with open data marks are CT treatments. n = 5.

Table 2. Regression equations and correlation coefficients of relation between the wheat varieties' grain yield and plant ozone uptake in ambient temperature and ambient CO_2 treatments (A) and combined elevated CO_2 and elevated temperature treatments (CT).

	Variety	Regression equation	R^2
A	KWS Bittern	-1.7 + 54.1	0.95
	Lennox	-1.0 + 53.3	0.93
	Lantvete	-1.9 + 43.2	0.57
CT	KWS Bittern	-0.1 + 41.2	0.55
	Lennox	-0.5 + 43.5	0.40
	Lantvete	0.1 + 35.0	0.11

All three varieties had large uptakes of ozone in the T.EpO3 treatment compared to the uptake in the other episodic exposure treatments (not shown). For the episodically exposed treatments with elevated temperature, T.EpO3 and CT.EpO3, the uptake of ozone in the CT.EpO3 treatment appear lower compared to the T.EpO3 for all varieties (Fig. 5). In the colder treatments, the effect of elevated [CO_2] was not as clear and it depended on variety: In the Lennox variety, the ozone uptake was lower in the C.EpO3 treatment than in the A.EpO3, but in the KWS Bittern and Lantvete varieties, the A.EpO3 ozone uptake was the lowest.

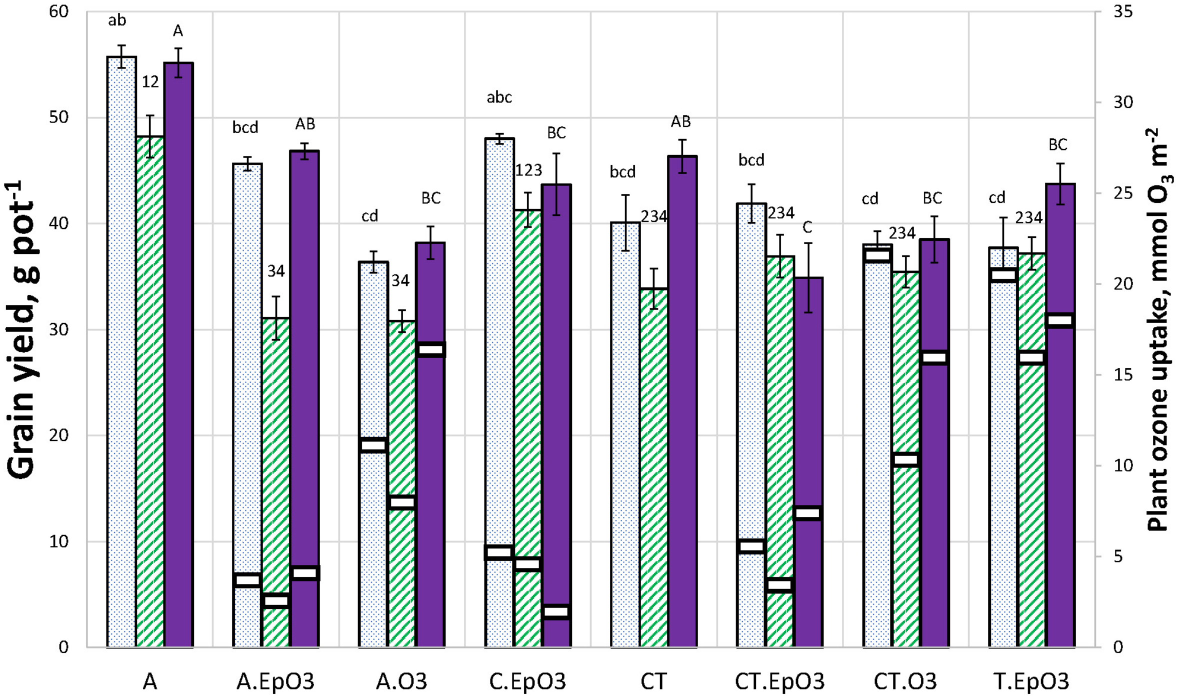

Fig. 5. Grain yield of the varieties sorted in treatments. KWS Bittern, Lantvete and Lennox are blue, green and purple, respectively. The plant ozone uptake values of varieties in ozone-exposed treatments are marked with white interruptions on the relevant bar; read at secondary axis. n=5. The marks of no significant difference between the treatments relate to the individual variety (letters = KWS Bittern, numbers = Lantvete, capital letters = Lennox).

Table 3. Anova results for the test of Grain yield as a function of main effects and interactions of Ozone Uptake (OU), in the climate treatments of either ambient [CO_2] + low temperature (A-), or high [CO_2] + high temperature (CT-) and Variety.

Grain Yield	Pr(>F)	
Climate	1.01E-03	**
OU	1.71E-09	***
Variety	8.14E-11	***
Climate : OU	1.06E-07	***
Climate : Variety	2.80E-03	**
OU : Variety	0.915516	
Climate : OU : Variety	0.007374	**

3.4. Plant performance

3.4.1. Photosynthesis versus intercellular carbon dioxide.

The amount of photosynthesis taking place at different availabilities of carbon dioxide is illustrated in Fig. 6 for each variety. If the photosynthetic apparatus is well-functioning, it responds to an increase in CO_2 with an increase in photosynthesis. In the figures, the CO_2-component of the treatments divides the possible increase in photosynthesis depending on treatment. As expected, the CO_2-enrichments resulted in measurements of high rates of photosynthesis, but also measurements of low rates of photosynthesis at high levels of carbon dioxide. The CO_2-concentration in the plant cells of the non-CO_2-enriched treatments was less variated in distribution, and photosynthesis never peaked as high as in the C-treatments but were more often in the lower range (more PS<10 than in C-treatments).

The T.EpO3 Lennox, CT.O3 Lantvete, and A.O3 Lantvete, all had negative relations between intercellular carbon dioxide and photosynthesis (Fig. 7). The majority of the other treatments*varieties have regression slopes with values of 0.05 or less, only six have higher values, among these are all A.EpO3-varieties, modern varieties of A, and Lantvete from the CT-treatment. Table 5 shows the variance analysis for the photosynthesis' dependence on ozone, treatment, intercellular carbon, and variety.

Table 4. Anova result of the test for Rate of photosynthesis as a function of main effects of variety and main effects and interactions of intercellular carbon, ozone (on/off), (high/low) temperature setting.

Photosynthesis ~ Variety + Ci * ozone * Temp	Pr(>F)	
Variety	2.46E-07	***
Ci	< 2.2e-16	***
Ozone	1.37E-02	*
Temp	9.63E-07	***
Ci : Ozone	0.001023	**
Ci : Temp	6.11E-06	***
Ozone : Temp	1.94E-13	***
Ci : Ozone : Temp	2.24E-03	**

3.4.2. 1000-grain weight, water consumption, protein content, and gluten index.

The protein mass was, on average across the treatments, 5.8 ±0.1, 5.8 ±0.2 and 5.2 ±0.1 g for KWS Bittern, Lennox and Lantvete, respectively. There was statistically significant interaction between treatments and varieties (Table 6), although the data for individual treatments are not shown here. Gluten index was on average across the treatments 28.7 ±1.9, 29.4 ±2.5 and 30.8 ±0.8 (with SD) for KWS Bittern, Lennox and Lantvete, respectively. There was significant interaction between treatment and variety. The individual treatment*varieties are illustrated with grain yield in Fig. 8. The 1000-grain weight was also significantly influenced by interactions between treatment and variety. The total water consumption reflected the different water use efficiencies between the landrace and the modern varieties, with the modern varieties consuming less water than the landrace (Fig. 8).

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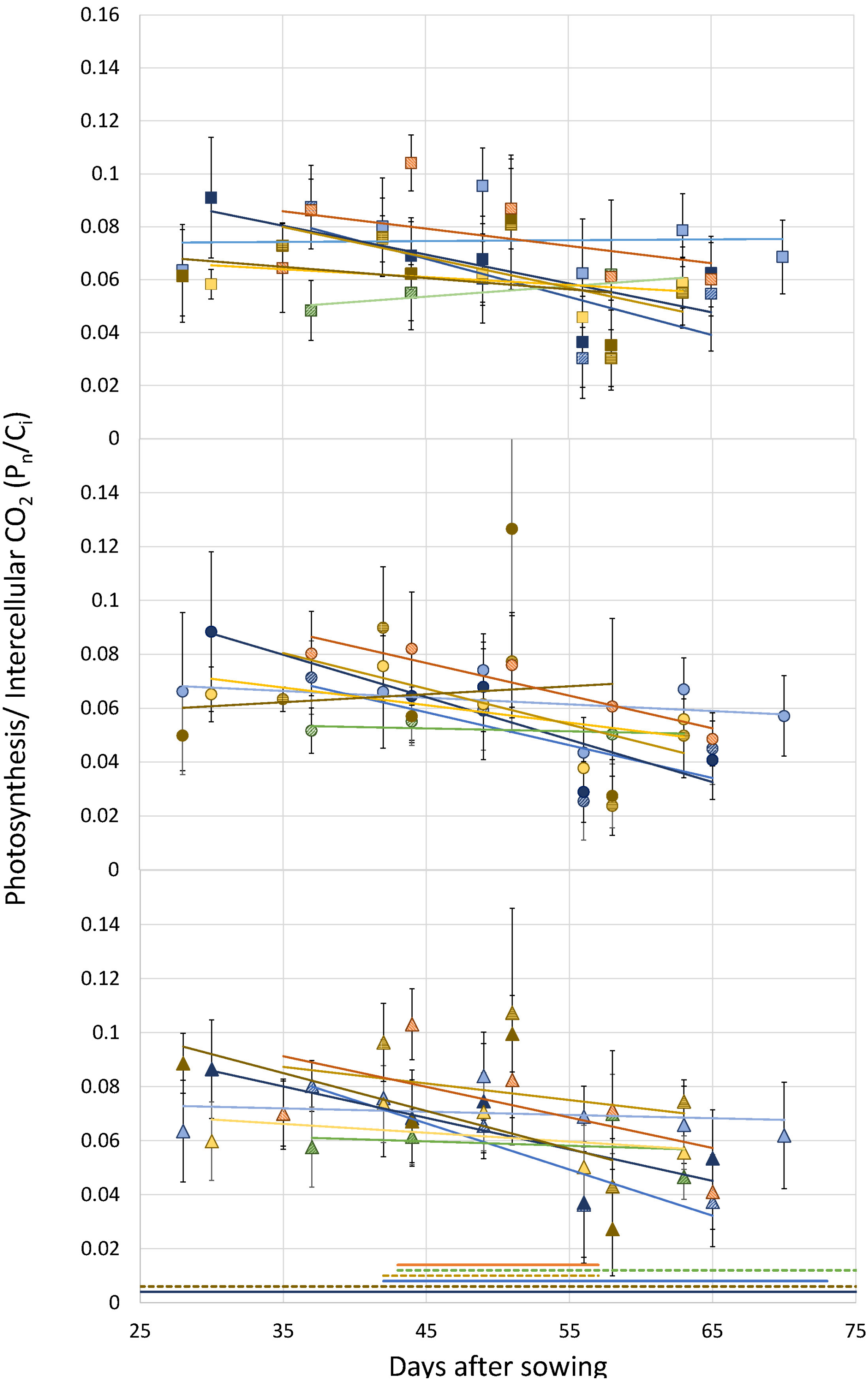

407 Fig. 6. Obtained net photosynthesis/ intercellular CO$_2$-concentration over time. The varieties are
408 referred to by the data mark shapes; squares, circles, triangles are KWS Bittern, Lantvete, and

Lennox, respectively. The colors refer to Fig. 1 of the treatments: lightest blue (A), medium blue (A.EpO3), dark blue (A.O3,) orange (T.EpO3), yellow with black outline (CT), tan (CT.EpO3), dark tan (CT.O3), and green (C.EpO3). Above the horizontal axis is marked the duration of ozone exposure in the colors of the treatments (dashed lines represent carbon enrichment). See Fig. 1 for further explanation of treatment abbreviations.

Table 6. Anova results of test of protein content (g pot^{-1}), gluten index (ICC 155), 1000-grain weight (g pot^{-1}) and vegetative dry matter (g pot^{-1}) as functions of main effects and interactions of treatment and variety.

	Protein content	Gluten index	1000-grain weight	Vegetative DM
Treatment	< 2.2E-16 ***	< 2.2E-16 ***	< 2.2E-16 ***	9.50E-12 ***
Variety	0.2898	< 2.2E-16 ***	< 2.2E-16 ***	< 2.2E-16 ***
Treatment : Variety	7.59E-12 ***	< 2.2E-16 ***	0.000188 ***	0.000244 ***

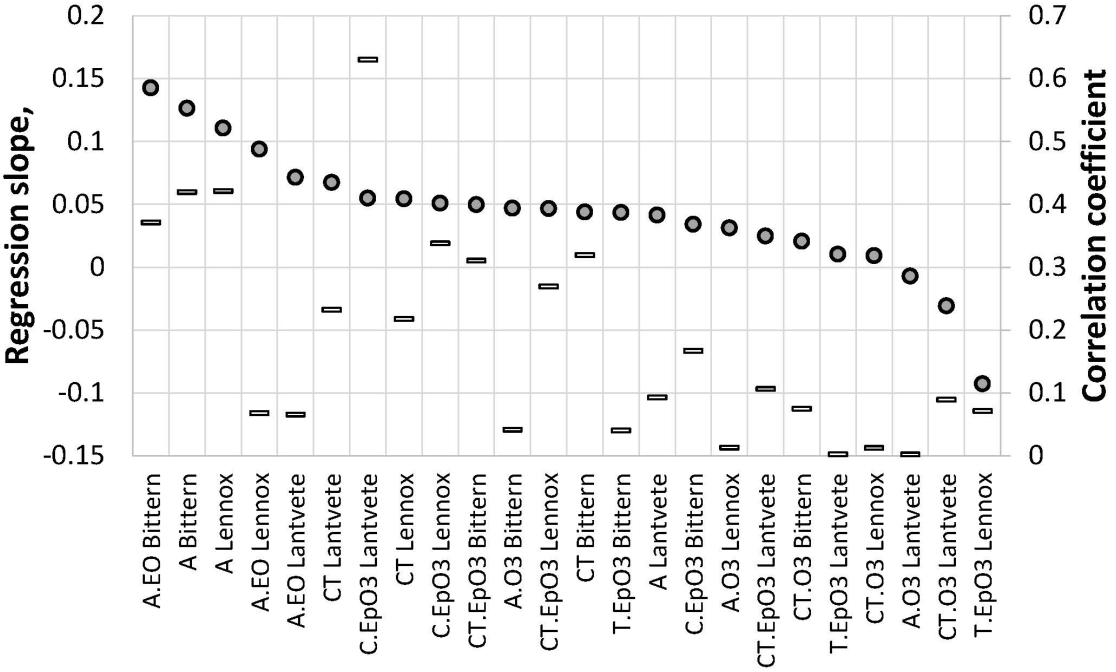

Fig. 7. In descending order, the regression slope values (circles) from Fig. 6 Rate of photosynthesis versus intercellular CO_2-concentration. Correlation coefficient, R^2 (dashes).

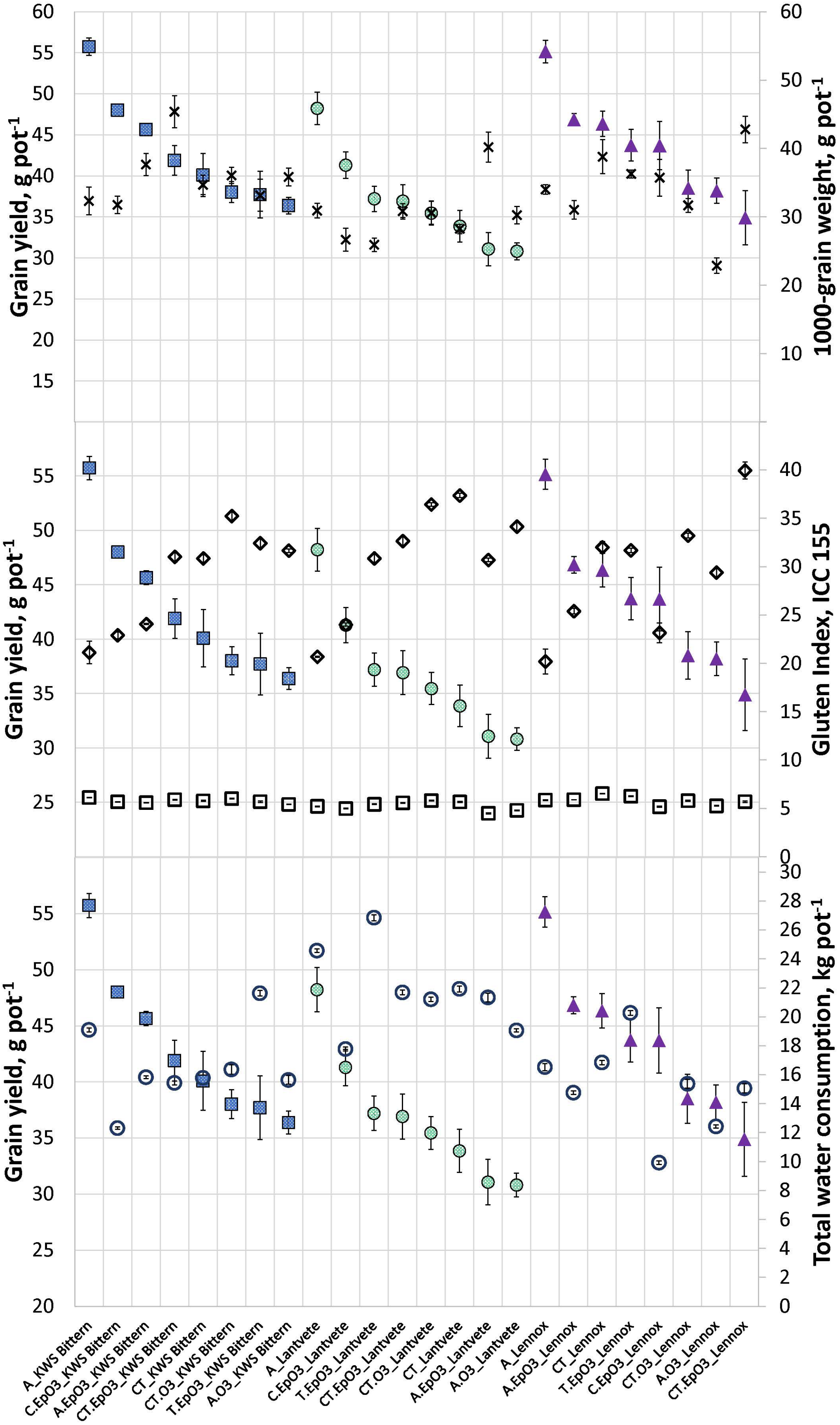
Grain yield, g pot^{-1}
1000-grain weight, g pot^{-1}
Grain yield, g pot^{-1}
Gluten Index, ICC 155
Grain yield, g pot^{-1}
Total water consumption, kg pot^{-1}
60
55
50
45
40
35
30
25
20
15
10
5
0
A_KWS Bittern
C.EpO3_KWS Bittern
A.EpO3_KWS Bittern
CT.EpO3_KWS Bittern
CT_KWS Bittern
CT.O3_KWS Bittern
T.EpO3_KWS Bittern
A.O3_KWS Bittern
A_Lantvete
C.EpO3_Lantvete
T.EpO3_Lantvete
CT.EpO3_Lantvete
CT.O3_Lantvete
CT_Lantvete
A.EpO3_Lantvete
A.O3_Lantvete
A_Lennox
A.EpO3_Lennox
CT_Lennox
T.EpO3_Lennox
C.EpO3_Lennox
CT.O3_Lennox
A.O3_Lennox
CT.EpO3_Lennox

Fig. 8. The three diagrams show the grain yield of the treatments presented in descending order but clustered in variety (please note that the treatments do not show in the same order for all varieties). Alongside the yield is the 1000-grain weight, the gluten index and the total water consumption. The data marks are as follow: x (1000 grain weights), ◊ (gluten index) and o (total water consumption). See Fig. 1 for explanation of treatment abbreviations.

3.4.3. The plant development and leaf discoloration.

The wheat plant varieties developed at a similar pace with differences induced by treatments (Fig. 9). The majority of the differences could be attributed to differences in temperature; however, periods of 5-7 days of delay of development from CO_2-enrichment and ozone exposure could be detected at different times during growth, mostly in the lower temperature treatments. Similar influence from temperature first and ozone second can be seen in the development of leaf discoloration as in Fig. 10 showing also *some* inter-varietal differences. In the Lantvete variety the different treatments induce the largest differences and in the KWS Bittern the smallest.

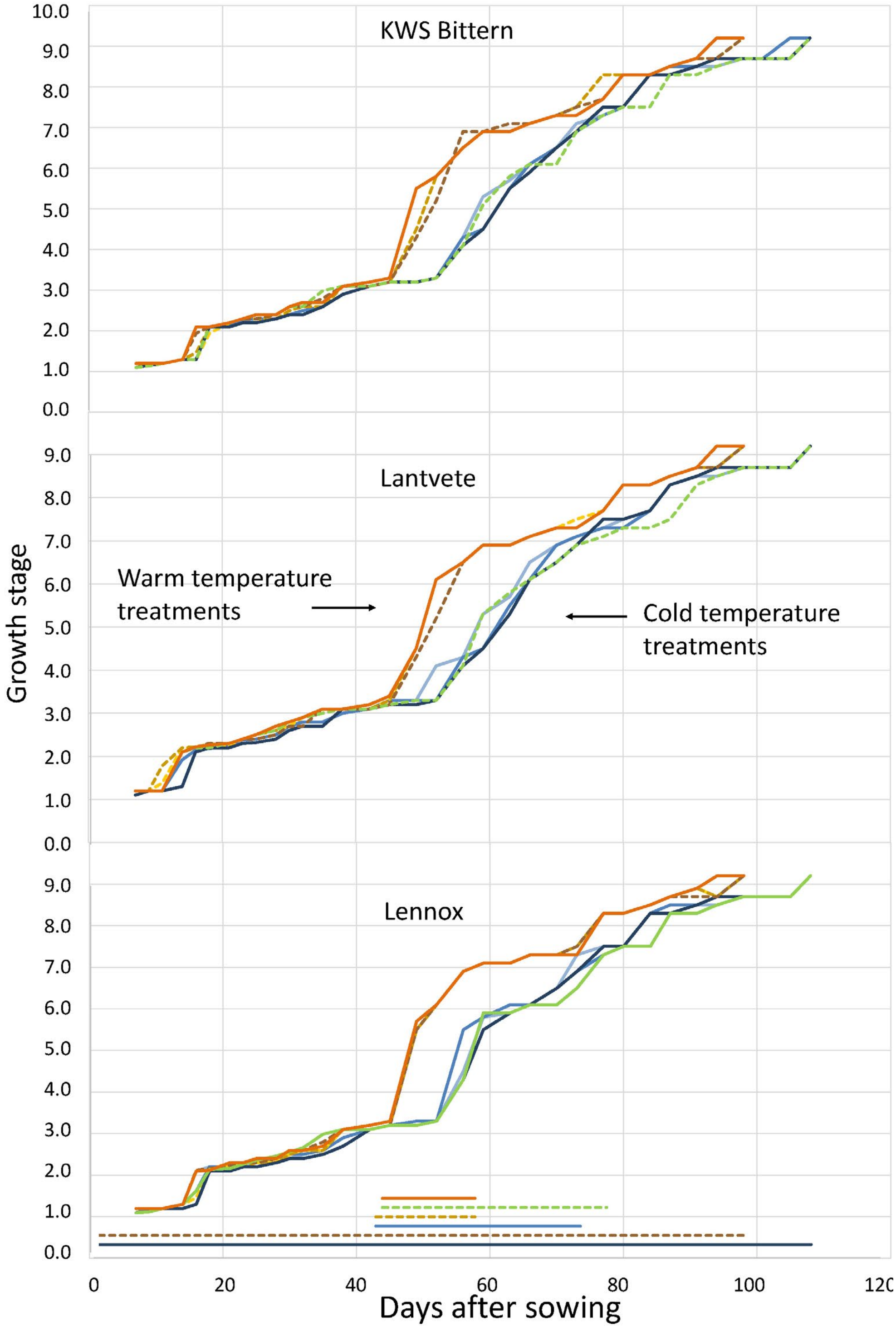

Fig. 9. The plant development after sowing, scored as Zadoks' growth stages 2-3 times a week, exemplified by the Lantvete variety. The colors refer to Fig. 1 of the treatments: lightest blue (A), medium blue (A.EpO3), dark blue (A.O3,) orange (T.EpO3), yellow (CT), tan (CT.EpO3), dark tan (CT.O3), and green (C.EpO3). Carbon-enriched treatments (C-) are shown as dashed lines. Above the horizontal axis is marked the duration of ozone exposure in the colors of the treatments (dashed lines represent carbon enrichment). Further explanation of treatment abbreviations in Fig. 1.

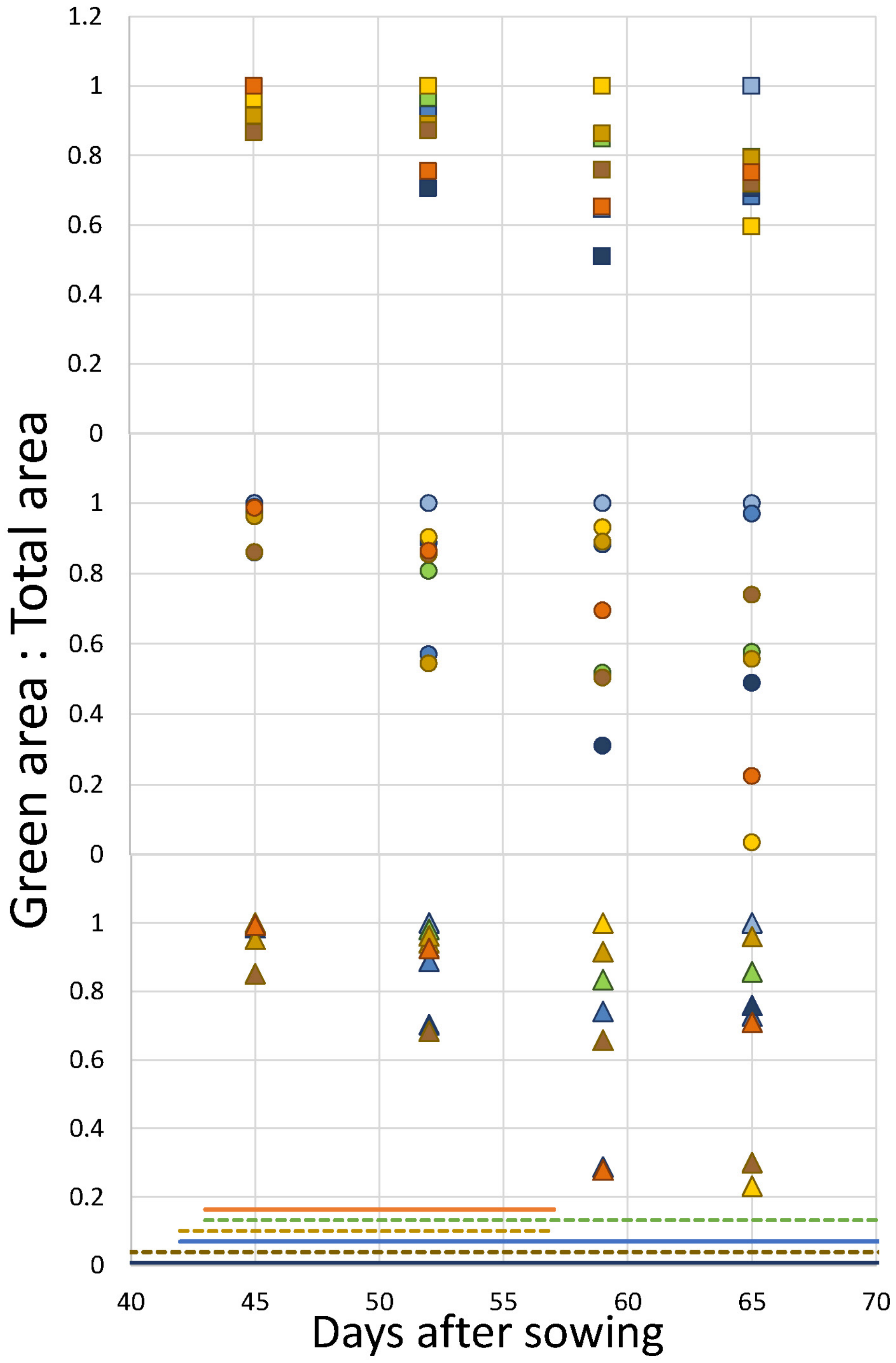

Fig. 10 Discoloration of second leaf at four dates in the three varieties in the eight treatments. The discoloration is shown as part of total leaf area being green. Square, circle and triangle data marks are KWS Bittern, Lantvete and Lennox respectively. The discoloration is assessed while all treatments were subjected to ozone exposure following the initiation of ozone exposure for the episodic ozone treatments. Further explanation of treatment abbreviations in Fig. 1.

4. Discussion

The discussion includes the performance of facilities and settings to realize the chosen climate treatments, after which the discussion considers the results relating to the hypotheses.

4.1. Realized climates

Apart from the level of CO_2 in treatments with settings of ambient CO_2, the logging of variables by the RERAF facilities show that the experimental values in general were in accordance with the set points of variables (see 3.1). The elevated temperature treatment could possibly have been so high as to induce a moderate chronic heat stress to the wheat plants (Sandhu et al., 2018), contributing to an explanation of the dominating impact of temperature relative to the ozone stress.

An unplanned increase in CO_2 level in a facility as RERAF, which cannot remove excess CO_2, may arise from several sources. The use of a growth medium with a high content of organic matter may result in microbial decomposition causing a measurable impact on CO_2-concentration as in Ingvordsen et al. (2018). An increase at nighttime when no photosynthesis occur is similar to natural ecosystems where increases in [CO_2] are seen at low wind speeds (Mikkelsen et al., 2008), and also suggested by Ingvordsen et al. (2018) as a possible explanation for their high average [CO_2]. In the present case, however, the set point was exceeded by approximately 150 ppm, approx 100 ppm higher than Ingvordsen et al. (2018) reported. One key difference to the Ingvordsen et al. experiment, was that we did manual watering to obtain knowledge on the water consumption throughout the plants' growth which increased human presence in the chambers. Human activities from measuring and maintaining the plants in the chambers resulted in peaks of [CO_2] of 6-900 ppm depending on the activity, and consequently the impact of human respiration on the CO_2-concentration in the chambers were not adequately accounted for in the experiment set-up. The increase in average chamber CO_2 compared to atmospheric CO_2-concentration minimize the detectable effects of the CO_2-enriched treatments as the difference in plant functioning will be more influenced by the first increase of [CO_2] compared to the influence arising from the increase assigned the CO_2-enrichment-treatments. For future trials of a similar kind, exchanging the chamber air at a higher rate than 4 m^3 h^{-1} could be explored for better neutralizing such peak CO_2-enrichments. An extract of measurements of carbon dioxide concentration from the chambers of treatment A is shown in Fig. 11, where the nighttime respiration accumulation as well as the attribution of human activity to chamber [CO_2] can be seen.

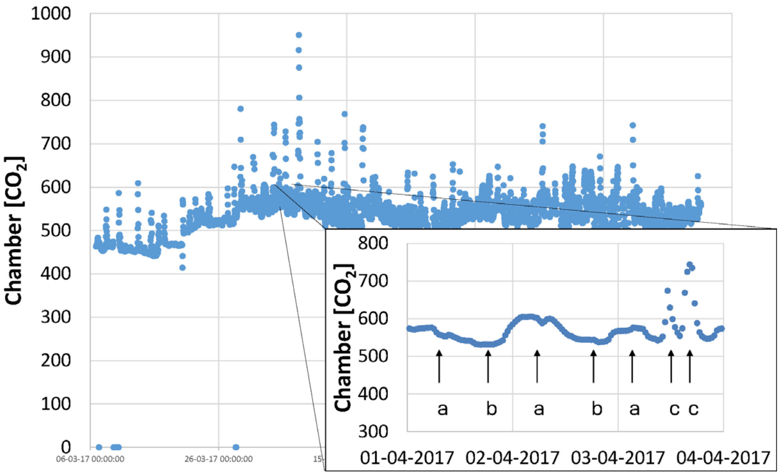

Fig. 11. CO_2 concentration of treatment A, CO_2-setting 400 ppm. Highlighted two days of no human interference and one with. Arrows 'a' indicate the decrease in daytime when photosynthesis is activated, 'b' indicate nighttime increase due to plant and soil respiration, and ‘c’ denote incidents of human presence in the chamber for a shorter or longer period for watering or measuring reasons.

When growing agricultural crops in pots, there are many conditions that are different from field conditions. In this study, there were fewer plants m^{-2} than would be normal in the field, and the boundary conditions of the potted plants would be far from those of field-grown plants. Albeit grown in sphagnum in pots and while inside climate chambers, the plants in this study grew to maturity and produced yields in a range from 5-12 ton ha^{-1} (see 3. Results), which is better than, but including, the Danish spring wheat yield average from 2017 of 5.2 ton ha^{-1} (Lundø, 2017). A higher yield in climate chambers than in fields is not rare, as many biotic and abiotic yield-reducing factors are eliminated in climate chambers (Poorter et al., 2016). In the ambient treatments in this study, ozone as a yield-reducing factor is eliminated due to the molecules' reactivity with surfaces through the ventilation system. In the ozone treatments high ozone concentrations were measured in the chambers, but due to low wind speed in the chambers (<0.6 m s^{-1}) the boundary layer resistance can be assumed to have been considerable and that the plants therefore were less exposed to toxic levels of ozone than what the concentration itself provided. The light supply can be claimed to be insufficient compared to what can be measured in the field, however as it was constant we believe the drawbacks from a suboptimal lighting are evened by the consistency in the supply.

4.2. Older versus modern wheat varieties.

The modern varieties, KWS Bittern and Lennox, which we used in the experiment, were bred to yield well and are used in Denmark and Southern France respectively, and the harvest index (Table 2) reveals that their higher yields are based on less biomass than the landrace variety's. Breeding of varieties for modern production take place under current ozone conditions, and as so, modern varieties are supposedly naturally adapted to the current increases in ozone concentration, but the modern wheat varieties tend to be more sensitive to ozone (Biswas et al., 2008; Pleijel et al., 2006). Studies examining the effects of filtered air as well as unfiltered air on the growth and yields of wheat show significant yield loss at current concentrations of ozone, indicating that breeding is not successful in obtaining full ozone resistance (Pleijel et al., 2018, 2006). On the other hand, the landrace variety in this study yielded as poorly when temporarily exposed to ozone as when constantly exposed, where the yield of the modern variety were not significantly different from no ozone to temporary exposure. This indicates perhaps that the modern varieties are resistant to some degree of ozone at the lower temperature settings compared to the resistance of the landrace.
The landrace variety used in this study has no commercial use at present, which points to its lack of ability to compete on yields or sales. The Lantvete yield is on the other hand only significantly

lower than the modern varieties in two treatments. Its larger biomass may be responsible for its resilience towards the warmer CO_2-enriched treatments (Lopes et al., 2015). The changes in harvest indexes between the treatments reflect that the biomass allocation is a possible actor in the way the varieties deal with the changes in growth related climatic factors (Table 2).

As Lennox is a common variety grown in the south of France, it could be expected to resist warmer temperatures (in CT and T.EpO3 treatments in particular) better than KWS Bittern and Lantvete. This seems to be the case: the yield loss in CT treatment is approx. 17% for Lennox, while it's 28% and 39% for KWS Bittern and Lantvete. That is the only non-significant loss ($p = 0.19$) from the A treatment to a warm treatment within the same variety, it is also significantly higher than the yield of CT Lantvete (Fig. 2).

The yield losses in T.EpO3 treatment compared to the A.EpO3 are approx. 10% for Lennox and approx. 26% for KWS Bittern, while Lantvete gains 20% from A.EpO3 to T.EpO3. The differences in loss as a temperature response align with the geographic purpose of the two modern varieties, where Lennox is used in the south of Europe and KWS Bitten in more northern regions. The Lantvete gain is probably more related to the severe grain yield loss in A.EpO3 than to a proper acclimatization to elevated temperatures (table 2).

4.3. Episodic versus full time ozone exposure and effects on wheat yield

It was expected that the plants experiencing ozone as a longer-stretched episode at a critical timing lose as much in yields as plants that have adjusted to ozone from growing in a continuously medium-high ozone environment. The Lantvete variety had similar losses in the colder treatment, but the two modern varieties lost more in the A.O3 than in the A.EpO3 treatment (Fig. 4). It could be assumed that intraspecific variations in the ozone acclimation of growth physiological processes exist among wheat cultivars. Such results were shown on thermal acclimation of photosynthesis among alfalfa cultivars (Zaka et al., 2016). However, it could also refer to the contradictory conditions between ambient air ozone concentration and chamber background concentration of ozone. Treatment A had in fact conditions resembling filtered air treatments, resulting in A reflecting the natural selection for ozone adaptation in the modern varieties' yield. This aligns with literature on ozone diminishing agricultural yields at present conditions (Pleijel et al., 2018). In the warmer treatment, the correlation between the dose of phytotoxic ozone and grain yield was not as strong as in the cold treatments (Table 3). In these wheat varieties, an adaptation to ozone to reduce the impact of continuous ozone to the level of episodic ozone exposure was not found. Linear correlations between grain yield and plant ozone uptake (reported as POD_6) were also reported by Harmens et al. (2018) and Grünhage et al (2012). The weaker correlations between grain yield and ozone uptake in the warmer CO_2-enriched treatments indicate that the climate components influence the yields more than the ozone dose.

4.4. Multiple factors and additional ozone exposure

Increases in [CO_2] have been expected to induce a fertilizing effect on plant growth and the effect of temperature follows a bell curve indicating that it may be too low as well as too high for optimum plant functioning (Norby and Luo, 2004). The best yields in this study were attained in the ambient [CO_2]/ low temperature treatment (Table 2), indicating that the additional parameters, increase in [CO_2] and temperature, alone or combined all skewed the plant growth out of its optimum. However, the less than additive positive effect of the combination of changes in climate factors have been documented previously (Frenck et al., 2013; Ingvordsen et al., 2015; Shaw et al., 2002). Broberg et al. (2015) observe in their review that both negative and positive effects are to be expected from ozone exposure.

The yields of CT and CT-ozone-treatments were not significantly different. If ozone uptake was the main reason of yield reduction, increased ozone exposure should diminish the yields accordingly. However, the uptake of ozone cannot alone explain the yield decrease of the CT-treatments, and the climate treatment must be contributing.

The ozone taken up influenced the varieties’ yields differently, for example, very different ozone amounts were taken up in in T.EpO3, but with no comparative yield decline, stressing the interactions of climate and variety on the grain yield (Fig. 5, Table 4). For the episodically exposed treatments with elevated temperature, T.EpO3 and CT.EpO3, a possible effect of the stomatal regulative effect of elevated [CO_2] is visible qua the lower uptake of ozone in the CT.EpO3 treatment compared to the T.EpO3.

Although not significant, we observe that the distribution of Lantvete yields suggests a plasticity of the grain yield from A.EpO3 to CT.EpO3, indicating a trend of not losing additional yields with the future climate scenario. The modern varieties lose yield from A.EpO3 to CT.EpO3; Lennox significantly, KWS Bittern not significantly.

From Fig. 4, the impact of ozone is presumably greater in colder temperature treatments, and the addition of ozone to a treatment with elevated [CO_2] and elevated temperature does not significantly increase injury to yields already affected by the higher temperature. However, Tai and Martin (2017) suggest an adaptation of crops to regional ozone and water constraints to induce a stronger ozone tolerance. Elevated [CO_2] may also influence the water relation. In this study, it was decided to keep the plants well-watered. However, had the plants lacked water at times, a better stoma regulation might have reduced the ozone uptake.

4.5. Growth parameters and responses during growth

The photosynthesis vs. intercellular CO_2 depicted in Fig. 6 a, b, c, show that the photosynthetic apparatus of the plants worked as expected on altered input in all varieties and in most treatments. From the sorting of regression slopes in Fig. 7, it seems that the photosynthetic apparatus was affected by the amendment of ozone, and especially full-time ozone treatments resulted in negative regression slopes. This is in accordance with the general perception that ozone has a direct negative influence on photosynthesis (Mikkelsen, 1995; Mikkelsen and Ro-Poulsen, 2013).

Protein production could be depending on soil nutrient availability rather than treatment in this case (Thomsen et al., 2008). The obtained amount of protein mass did not vary as much as grain yields, but the baking quality in form of gluten index indicates that the quality and quantity was not tightly linked in this case. Rather, the lower yields represented a better baking quality.

The higher total water consumption (Fig. 8c) of the landrace variety is in accordance with the higher biomass of the Lantvete (Table 2) and is a trait of a less efficient variety.

As expected, the phenological development of the plants in the treatments (Fig. 9) revealed a stronger dependence on temperature than on any other treatment parameter. However, there was a 5-degree difference between colder and warmer treatments, and the strong reflection of temperature influence could be caused by this biologically large leap. The impact of $[CO_2]$-enrichment could be detected on close observation of the development data (not shown here) and this impact may also relate to the absolute difference between the treatment concentrations.

Due to the nature of the sampling of only one leaf in a treatment at a time the results of the development of discoloration of leaves are indicative rather than significant, however, as seen in Fig. 10, the varieties respond differently to the treatments, with the Lantvete variety showing more variation than the modern varieties, and the modern expressing some difference in their response to temperature.

5. Conclusion

The various combinations of two levels of temperature, two levels of [CO_2], and three ozone exposure regimes affected both growth and yield in spring wheat, while interacting with the studied spring wheat varieties. On average, across all treatments, the landrace variety Lantvete had significantly lower yields than the modern varieties Lennox and KWS Bittern. However, the yields were significantly lower within only two treatments; in the treatment with ambient levels of temperature and [CO_2] and episodically exposure to ozone (A.EpO3), and in the treatment with an elevated level of temperature and [CO_2] (CT). Within the other six treatments, there was no significant difference between the yields of the three tested varieties.

The effect on yield with episodic ozone exposure relative to treatment A depended on variety. Yield losses relative to treatment A were significant in all CT.EpO3 and T.EpO3, in A.EpO3 of KWS Bittern and Lantvete, and in C.EpO3 of Lennox. Yield losses due to chronic ozone exposure were significant in all varieties. Increased [CO_2] and higher temperature without ozone exposure induced significant loss of yield in Lantvete and KWS Bittern. Full-time exposure to ozone reduced yields more than ozone exposure during limited time. The combination of climate factors and ozone has shown that the effect of changes in climate factors on wheat yield influence the effect of ozone, and that the influence of temperature can be detrimental regardless of ozone exposure. While an increase in the ozone dose induced yield loss in the low temperature and ambient [CO_2] treatment, the effect in the warm treatments was less clear and yields were lower even at low ozone doses. The different reactions of the varieties to higher ozone doses in the warmer CO_2-enriched treatments compared to the reactions in the treatments with lower temperature and lower [CO_2], lead to the assumption that responses to the combined effects of climate factor changes (temperature, [CO_2], ozone) are due to either more or less additive intraspecific variations and adaptations or one of the factors (e.g. elevated temperature) having an overriding influence on the impact of other factors.

Declaration of interest

The authors declare that they have no conflict of interest.

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