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1 The impact of ozone exposure, temperature and CO₂ on the growth and yield of three spring wheat
2 varieties.

3

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16

17 ABSTRACT

18 When assessing potentials for crop production under future climatic conditions, multiple
19 environmental parameters need to be included. An increase in carbon dioxide [CO₂], higher
20 temperatures, and regional changes in tropospheric ozone [O₃] will influence the growth responses
21 of existing crop species and varieties. Ozone is phytotoxic and a plant stressor at current
22 concentrations, reducing yields worldwide, but possible interactions with changes in other abiotic
23 factors have been considered very little. In this study, we have used eight combinations: two levels
24 of temperature, two levels of [CO₂], and three [O₃] exposure regimes to assess the impact of
25 medium-to-high ozone concentrations (80-100 ppb) on wheat growth when other abiotic factors
26 change. Two modern spring wheat varieties (KWS Bittern and Lennox) and a landrace variety
27 (Lantvete) were grown to maturity in climate chambers. We examined plant performance during
28 growth as development rate, rate of photosynthesis, stomatal conductance, water use, and at harvest
29 as total aboveground dry matter and grain yield.

30 All three varieties lost yield in all treatments compared to the ambient treatment that had the
31 following settings: Lowest temperature, ambient [CO₂], and very low [O₃]. For episodic ozone
32 exposure in the ambient or high [CO₂] and high-level temperature treatment, the yield losses were
33 18 and 25%, respectively, for KWS Bittern; 44 and 34% for Lennox; and 16 and 37% for Lantvete.
34 The yields of the modern varieties are significantly higher than the landrace variety in two out of
35 eight treatments, although they are higher by weight in seven of the eight treatments. The landrace
36 variety's fraction loss from its highest grain yield in the ambient treatment to the high-[CO₂]-and
37 high-level-temperature-treatments was smaller than the modern varieties', showing a comparably
38 higher degree of plasticity of performance. Current crop varieties might be more sensitive to ozone
39 than older varieties, emphasizing the need of future breeding programs to expand the gene pool to
40 provide more climate robust crops.

41

42 **Keywords**

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

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47 **1. Introduction**

48 The changes in climate relating to the climate scenarios' emission of carbon dioxide include
49 temperature increase, increased frequency of extreme weather events and regional aggravation of
50 current challenges and the addition of new (IPCC, 2014) influencing plant growth in natural and
51 agricultural ecosystems (Albert et al., 2011; Pleijel et al., 2018). These factors will not change
52 individually and their impact on ecosystems is increasingly investigated in multifactorial settings
53 (Ingvordsen et al., 2015; Langley and Hungate, 2014; Namazkar et al., 2016). When addressing
54 responsiveness of existing crop species and varieties grown under future climatic conditions,
55 multiple environmental parameters must be included (Frenck et al., 2013; Vázquez et al., 2017).
56 In addition, the air pollutant, ozone, which is causing yield loss under the current climatic
57 conditions, may interact with the changing levels of other abiotic factors, and thus further impact
58 temporal and spatial plant growth patterns and yield (Pleijel et al., 2018). Exposure to elevated
59 ozone typically results in suppressed photosynthesis, accelerated senescence, decreased growth and
60 lower yields (Booker et al., 2009). However, there are major knowledge gaps when assessing the
61 threat that ozone plays (Ainsworth et al., 2012; Fuhrer and Booker, 2003).
62 Ozone is readily formed from available precursors in the presence of UV-radiation. The precursors
63 are natural or anthropogenic volatile organic compounds (VOCs) and nitrogen oxides (NO_xs)
64 (Finlayson-Pitts and Pitts, 1993). The precursors may be carried long distances and result in both
65 peak ozone formations far from the precursor formation/emission and in a regional increase of
66 background concentration (Monks et al., 2015). Efforts to reduce precursor emission contribute to
67 regional control of ozone levels (Derwent et al., 2018). Precursors may also build up during periods
68 of low radiation, i.e. in winter, and result in a peak ozone period in spring when radiation increases
69 (Munir et al., 2013). In stable, high-pressure conditions, ozone concentration may build up to create

70 a peak ozone episode, whilst the ozone molecules may disperse into adjacent areas in more windy
71 conditions (Kleanthous et al., 2014).

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

80 The cost of the plant's defense mechanism against ozone is that fewer carbon substances are
81 available for allocation to build biomass, both above and below ground (Calatayud et al., 2011).
82 Thus, ozone limits plant growth and agricultural yields, and Mills et al. (2018) estimate a global
83 yield gap of 7.1% in wheat at the background levels and peak ozone incidents prevailing under
84 current climatic conditions. Unknown synergistic effects between the defense mechanism and other
85 climate factors may act to reduce further plant growth. However, unidentified antagonistic effects
86 might act in the opposite direction (Larsen et al., 2011).

87 In the present study, wheat (*Triticum aestivum*) is used as model crop selected for its global
88 importance as staple food in many countries (Wrigley, 2009). Wheat yields have increased vastly
89 with breeding, research, development, and intensification of production for the last 50 years,
90 however, the rate of yield increase has diminished in the past 30 years (Brisson et al., 2010). The
91 decline in soil carbon content may be one explanation (Brisson et al., 2010), and the combined
92 increase of CO₂-concentration and temperature another (Dieleman et al., 2012). While an increase
93 in [CO₂] has a growth promoting effect on many plants as the substrate of photosynthesis, and

94 higher temperatures increase the rates of activity in many biochemical processes, the combination
95 of CO₂-enrichment and temperature increase may have a less than additive effect on the plant
96 biomass (Dieleman et al., 2012; Shaw et al., 2002). As ozone is known to reduce the yield of cereals
97 (Broberg et al., 2015), the increase in tropospheric ozone with anthropogenic activity (Lamarque et
98 al., 2010) suggests that the increasing ground-level ozone concentration cannot be ignored and
99 might be a threat to future food production through interactions with other climate factors.

100

101 The aim of this study was to investigate eight climate treatments reflecting current conditions and
102 future scenarios. From seed to maturity, the wheat varieties were regularly evaluated for a range of
103 growth indicators to learn how the combinations of abiotic factors influenced the development and
104 yield of the wheat plants. The following four hypotheses were tested: i) Yields of modern varieties
105 are higher than yields of the old variety; ii) Episodic ozone exposure at Zadoks' growth stages
106 (ZS)31-69 is as injurious to wheat yields as full-time ozone exposure; iii) The combined effect of
107 elevated [CO₂] and elevated temperature, which stimulate plant growth less than expected by the
108 elevation of each factor individually is further aggravated by exposure to elevated ozone
109 concentrations; iv) Differences in yields are reflected in the way growth parameters respond to the
110 treatments during growth.

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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, [CO₂], air humidity, light, and ozone concentration and exposure duration. 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 spring wheats tested 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). Twelve seeds of a variety 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. CO₂ 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.

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138 2.3. Growth conditions

139 2.3.1. Treatments

140 Eight treatments were tested in total. They were selected among possible combinations of two
141 present and future temperature levels (19/12 °C or 24/17 °C, both levels simulating a warmer day
142 (16h) and cooler night (8h) cycle), two concentrations of CO₂ (400 and 700 ppm), and one of three
143 ozone regimes: 1) no ozone enrichment (ozone residuals not reacting with surroundings in
144 ventilation system is recorded as ozone background level), 2) episodic ozone exposure (.EpO3), and
145 full-time ozone exposure (.O3) (Fig. 1). For both the episodic and full-time ozone exposure, the
146 ozone concentration target was 80-100 ppb during the day, thus resulting in 16 hours of daily ozone
147 exposure. Unlike the full-time exposure treatments, the ozone exposure of the .EpO3 treatments
148 began when the Lennox variety reached Zadoks' developmental stage 31 (ZS31 - first node
149 detectable) and ended when Lennox reached stage 69 (ZS69 - anthesis complete) (Zadoks and
150 Board, 1974). Thus, the number of days of ozone exposure varied as plant development varied
151 depending on climate. Relative humidity was kept at 70/55% (day/night) for all treatments.

152 The following eight treatments were represented in this experiment: A, A.EpO3, A.O3, C.EpO3,
 153 CT, CT.EpO3, CT.O3, and T.EpO3 as shown in Fig. 1. A denotes ‘ambient conditions’ with
 154 temperatures that correspond to good growing conditions during the growth season of spring wheat
 155 in Denmark, and a CO₂-concentration corresponding to present average atmospheric concentrations;
 156 C denotes an elevated level of [CO₂], and T denotes a higher temperature.

157 Fig. 1: The selected climate combinations are named A, A.EpO3, A.O3 (ambient [CO₂], lower
 158 temperature settings and no addition ()), episodic addition (.EpO3) or chronic addition of ozone
 159 (.O3)) and CT, CT.EpO3, CT.O3 (high [CO₂] (C), higher temperature settings (T), and no addition
 160 ()), episodic addition (.EpO3) or chronic addition of ozone (.O3)). Two treatments, C.EpO3 and
 161 T.EpO3 have episodic addition of ozone (EpO3) and either higher [CO₂] or higher temperature
 162 settings. The aim for concentration of ozone in the chambers was 80-100 ppb. The episodic
 163 exposure started at Zadoks' growth stage ZS31 and ended at stage ZS69. A.EpO3 and CT.EpO3
 164 were transferred to the corresponding full-time ozone exposure chamber for ozone exposure and
 165 returned after exposure, while C.EpO3 and T.EpO3 received ozone exposure in their own chambers
 166 for the episode from ZS31-ZS69.
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169 2.3.2. Watering regime

170 Plants were watered three times a week to provide a full supply of water; the warm treatment plants
 171 were, by design and by consumption, given more water than ambient treatment plants and all plants
 172 got increasingly more water as they grew. Pots were weighed before and after watering to ensure

173 the same amount of water was accessible in the treatment regardless of the pot's previous
174 consumption.

175

176 2.4. Potential chamber gradient effects

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

181

182 2.5. Process values and measurements of plant growth parameters

183 Process values of treatment parameters such as relative humidity, CO₂ concentration, and
184 temperature were logged by a data collection system several times per minute. The ozone
185 concentration was monitored sequentially, i.e. twice every hour. Photosynthesis, stomatal
186 conductance, transpiration, and other parameters were measured regularly throughout the
187 experiment using two LI-6400 portable photosynthesis systems (LICOR, 1998).
188 Water consumption was recorded by weighing the individual pots before and during watering; the
189 plants' heights were measured; the development stage assessed according to Zadok's growth stages
190 (Zadoks and Board, 1974) and noted. Grain and aboveground biomass were weighed at the end of
191 the experiment.

192

193 2.6. Ozone treatment recap, fluxes and phytotoxic ozone dose

194 The ozone flux depends on the ozone concentration in the air, the degree of stomatal opening
195 (which, among other factors, depends on water status and CO₂ availability), and the ozone
196 molecules' resistance to cross the boundary layer of the leaves. As the latter was not measured, the

197 resistance part of the equation is left out and any assumptions would only reflect the similarity of
 198 conditions in the chambers. Thus, ozone flux is the product of the hourly ozone concentrations (in
 199 nmol m^{-3}) and the stomatal ozone conductance ($\text{H}_2\text{O mol m}^{-2} \text{s}^{-1}$). The stomatal conductance was
 200 measured approximately six times in each variety of every treatment, and the values for the days
 201 between measurements were found by linear interpolation. Ozone conductance was found by the
 202 ratio of molecule size O_3 to H_2O (0.66) and converted from $\text{mol m}^{-2} \text{s}^{-1}$ into m s^{-1} (Monks et al.,
 203 2015). From the obtained values for fluxes of ozone into the plant, the accumulated plant uptake of
 204 phytotoxic ozone above the threshold of 6 for phytotoxicity of ozone to wheat (POD_6) was
 205 aggregated, giving a POD_6 unit of $\text{mmol O}_3 \text{m}^{-2} \text{PLA}$ (Projected Leaf Area) (ICP Vegetation, 2017).
 206 The data logging system occasionally failed to log ozone data, and thus data gap filling has been
 207 necessary for the POD_6 summation. Gap filling for single or a few missing data points was done
 208 using the average of the 12 closest measurements. When an entire day or days were missing, the
 209 average measurement of the overall treatment was used to fill gaps. The average of daily ozone
 210 concentration (ppb) in treatment and as background can be found in Table 1.

211
 212
 213 Table 1. The average daily concentration of ozone in the eight different treatments with ozone
 214 levels in ppm and i) No ozone treatment equivalent to background concentration in the chambers, ii)
 215 daily average of episodic ozone exposure and iii) average of chronic ozone exposure. Averages
 216 based on ~32 recordings of ozone concentration per growing day. Further explanation of treatment
 217 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	

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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 $p < 0.05$ were considered significant, but p-values < 0.1 are sometimes included here and termed ‘trending’.

Models that were tested followed the formula: $Y_i = \gamma (\text{Treatment}_i \cdot \text{Variety}_i) + e_i$. Different physiological responses, such as yield, biomass, and photosynthesis were tested for dependence on the climate treatments or elements thereof, i.e. ozone on/off, climates relating to the CO₂/temperature combinations (e.g. Tables 4 and 6).

228

229 **3. Results**

230 The results are consequently displayed ‘per pot’ to underline the phytotron experiment design.

231 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

232 12.1 ± 0.2 tons ha⁻¹. Unless otherwise indicated, results are displayed with standard errors.

233

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

239

240

241 3.1. Experimental levels of treatment parameters.

242 Treatment parameters are reported with standard deviations, $TP = X \pm SD$, each average is based on
243 10-15 recordings per growing day or night, the data is not shown. The average temperatures in the
244 low level temperature treatments (set points 19/12 °C) were 19.3 ± 1.1 and 12.1 ± 0.7 °C, and 24.0
245 ± 0.3 and 17.1 ± 0.9 °C (for set points 24/17 °C) in the high level temperature treatments. The
246 average CO₂ concentration in the low level CO₂ treatments (set point 400 ppm) were 540 ± 37 , and
247 724 ± 57 ppm in the high level CO₂ treatments (set point 700 ppm). The average relative humidity
248 with set points of 55/70% day/night, were 54 ± 3 and 69 ± 4 % respectively.

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

255

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

259

260 3.2. Grain yields

261 Grain yields were significantly influenced by the main effects of treatment ($p < 2.2E-16$), variety (p
 262 $< 1.11E-11$) and interaction therebetween ($p < 9.55E-05$). On average, across all treatments, yields
 263 of the landrace variety, Lantvete, were significantly lower than the modern varieties, $p = 0.0013$
 264 (KWS Bittern) and $p = 0.00012$ (Lennox). Similarly, across the varieties, treatment A had
 265 significantly higher yields than all other treatments ($p < 0.01$), and treatment C.EpO3 had
 266 significantly higher yields than treatment A.O3 ($p = 0.0004$) and CT.O3 ($p = 0.02$). The treatments
 267 C.EpO3 and A.EpO3 trended a difference to yields of CT.EpO3 and A.O3 respectively with p -
 268 values of 0.06 and 0.10. There were interactions between the varieties and treatments which led to

269 significant differences between some treatment*variety-combinations, e.g. A Lennox, CT.EpO3
270 Bittern and A.O3 Lantvete (Fig.2). Within a treatment, the varieties only showed a significant
271 difference in A.EpO3, where Lantvete was different from both Lennox and KWS Bittern, and in
272 CT, where Lantvete was different from Lennox but not from KWS Bittern. All three varieties lost
273 grain yield relative to treatment A, but the losses were differently distributed on the treatments
274 without any patterns between losses of grain yield and biomass to explain loss or gain of harvest
275 index (Table 2). Lantvete had the highest biomass production, which led to lower harvest indexes
276 than the modern varieties (Table 2). In the colder treatments, Lantvete's harvest index decreased
277 with ozone exposure. In warmer treatments, Lantvete's harvest indexes increased compared to the
278 colder treatments, but ozone exposure diminished that increase. For Lennox and KWS Bittern, the
279 harvest index decreased with full-time ozone in the colder treatments and in general in the warmer
280 treatments (Table 2). Lennox lost the same percentage of biomass in the colder treatments
281 regardless of ozone exposure and elevation of [CO₂]. In the warm T.EpO3, Lennox lost the most
282 biomass of all treatments. However, it lead to an increase in harvest index. KWS Bittern lost the
283 most biomass in the warmer CO₂-enriched treatment with no ozone, CT, which also led to an
284 increase in harvest index, while a grain yield loss may be observed as well.

285 Table 2. Grain yield change, Aboveground DM (grain and vegetative dry matter) and change,
286 Harvest Index and change relative to the best yielding treatment A. For grain yield, see Fig. 2.
287 Harvest Index (HI) is grain yield divided by Aboveground DM. Comparison within same variety,
288 n=5. Star symbol indicate if a change relative to treatment A is statistically significant. Further
289 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 dose 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 considerably to the explanation of yield. 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 11 to 55% of the change in yield. 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.,

301 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
 302 covered a yield-increase in the same order of magnitude as the KWS Bittern's yield loss in those
 303 settings. The anova in Table 4 specifies that in addition to ozone dose ($p= 1.71E-09$), the main
 304 effects of variety and climate as well as interactions between the factors significantly influence
 305 grain yield.

306
 307 Fig. 4. Grain yield relating to different amounts of ozone taken up (Phytotoxic Ozone Dose 6,
 308 POD6) in the two general climate settings; A (ambient [CO₂] + low temperature) and CT (high
 309 [CO₂] + high temperature). The data marks represent the temperature treatments by being
 310 horizontally hatched (CT-treatments) or obliquely hatched (A-treatments). The varieties are
 311 presented by color and shape; KWS Bittern (blue squares), Lennox (purple triangles), and Lantvete
 312 (green circles). The grain yield/ POD6-relation of the three varieties from the C.EpO3 and T.EpO3
 313 treatments are added as stand-alone data marks in the variety's shape but following colors; black
 314 outline (C.EpO3) and orange (T.EpO3). n = 5.
 315

316 Table 2. Regression equations and correlation coefficients of relation between the wheat varieties'
 317 grain yield and ozone uptake (POD6) in ambient temperature and ambient CO₂ treatments (A) and
 318 combined elevated CO₂ and elevated temperature treatments (CT).
 319

	Variety	Regression equation	R ²
A	KWS Bittern	-1.7 + 54.1	0.95
	Lennox	-1.0 + 53.3	0.93
	Lantvete	-1.9 + 43.1	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

320

321

322 All three varieties had large uptakes of ozone in the T.EpO3 treatment compared to the uptake in

323 the other episodic exposure treatments; (Fig. 5, Table 4). For the episodically exposed treatments

324 with elevated temperature, T.EpO3 and CT.EpO3, the uptake of ozone in the CT.EpO3 treatment

325 appear lower compared to the T.EpO3 for all varieties. In the colder treatments, the effect of

326 elevated [CO₂] was not as clear and it depended on variety: In the Lennox variety, the ozone uptake

327 was lower in the C.EpO3 treatment than in the A.EpO3, but in the KWS Bittern and Lantvete

328 varieties, the A.EpO3 ozone uptake was the lowest.

329

330 Fig. 5. Grain yield of the varieties sorted in treatments. KWS Bittern, Lantvete and Lennox are blue

331 dotted, green hatched and solid purple, respectively. The POD₆s of varieties in ozone-exposed

332 treatments are marked with black interruptions on the relevant bar; read at secondary axis. n=5. The

333 marks of no significant difference between the treatments relate to the individual variety (letters =

334 KWS Bittern, numbers = Lantvete, capital letters = Lennox).

335 Table 3. Anova results for the test of Grain yield as a function of main effects and interactions of
 336 Phytotoxic Ozone Dose (threshold 6), POD₆, in the climate treatments of either ambient [CO₂] +
 337 low temperature (A-), or high [CO₂] + high temperature (CT-) and Variety.

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

338

339

340 3.4. Plant performance

341 3.4.1. Photosynthesis versus intercellular carbon dioxide.

342 The amount of photosynthesis taking place at different availabilities of carbon dioxide is illustrated
 343 in Fig. 6 for each variety. If the photosynthetic apparatus is well-functioning, it responds to an
 344 increase in CO₂ with an increase in photosynthesis. In the figures, the CO₂-component of the
 345 treatments divides the possible increase in photosynthesis depending on treatment. As expected, the
 346 CO₂-enrichments resulted in measurements of high rates of photosynthesis, but also measurements
 347 of low rates of photosynthesis at high levels of carbon dioxide. The CO₂-concentration in the plant
 348 cells of the non-CO₂-enriched treatments was less varied in distribution, and photosynthesis never
 349 peaked as high as in the C-treatments but were more often in the lower range (more PS<10 than in
 350 C-treatments).

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

357 Table 4. Anova result of the test for Rate of photosynthesis as a function of main effects of variety
 358 and main effects and interactions of intercellular carbon, ozone (on/off), (high/low) temperature
 359 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	**

360

361

362 3.4.2. 1000-grain weight, water consumption, protein content, and gluten index.

363 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

364 Bittern, Lennox and Lantvete, respectively. There was statistically significant interaction between

365 treatments and varieties (Table 6), although the data for individual treatments are not shown here.

366 Gluten index was on average across the treatments 28.7 ±1.9, 29.4 ±2.5 and 30.8 ±0.8 (with SD) for

367 KWS Bittern, Lennox and Lantvete, respectively. There was significant interaction between

368 treatment and variety. The individual treatment*varieties are illustrated with grain yield in Fig. 8.

369 The 1000-grain weight was also significantly influenced by interactions between treatment and

370 variety. The total water consumption reflected the different water efficiencies between the landrace

371 and the modern varieties, with the modern varieties consuming less water than the landrace (Fig. 8).

373 Fig. 6. Rate of photosynthesis vs. intercellular CO₂-concentration. 6a, b and c refer to the variety by
 374 the data mark shapes; squares, circles, triangles are KWS Bittern, Lantvete, and Lennox,
 375 respectively. The colors refer to Fig. 1 of the treatments: light blue (A), obliquely hatched blue
 376 (A.EpO3), dark blue (A.O3,) horizontally hatched orange (T.EpO3), yellow (CT), horizontally
 377 hatched tan (CT.EpO3), dark tan (CT.O3), and obliquely hatched green (C.EpO3). See Fig. 1 for
 378 further explanation of treatment abbreviations.
 379

380 Table 6. Anova results of test of protein content (g pot⁻¹), gluten index (ICC 155), 1000-grain
 381 weight (g pot⁻¹) and vegetative dry matter (g pot⁻¹) as functions of main effects and interactions of
 382 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 ***

383

384

387 Fig. 7. In descending order, the regression slope values (circles) from Fig. 6 Rate of photosynthesis
388 versus intercellular CO₂-concentration. Correlation coefficient, R² (dashes).

390 Fig. 8. The three diagrams show the grain yield of the treatments presented in descending order but
 391 clustered by variety (please note that the treatments do not show in the same order for all varieties).
 392 Alongside the yield is the 1000-grain weight, the gluten index and the total water consumption. The
 393 data marks are as follow: x (1000 grain weights), $\diamond$ (gluten index), $\square$ (protein mass) and o (total
 394 water consumption). See Fig. 1 for explanation of treatment abbreviations.
 395

396 3.4.3. The plant development

397 The wheat plant varieties developed at a similar pace with differences induced by treatments
 398 exemplified by the development of the Lantvete variety (Fig. 9). The majority of the differences
 399 could be attributed to differences in temperature; however, periods of 5-7 days of delay of
 400 development from CO₂-enrichment and ozone exposure could be detected at different times during
 401 growth, mostly in the lower temperature treatments.

402
 403 Fig. 9. The plant development after sowing, scored as Zadoks' growth stages 2-3 times a week,
 404 exemplified by the Lantvete variety. The colors refer to Fig. 1 of the treatments: lightest blue (A),
 405 medium blue (A.EpO3), dark blue (A.O3,) orange (T.EpO3), yellow (CT), tan (CT.EpO3), dark tan
 406 (CT.O3), and green (C.EpO3). Carbon-enriched treatments (C-) are shown as dashed lines. Further
 407 explanation of treatment abbreviations in Fig. 1.
 408

409

410 **4. Discussion**

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

413 4.1. Realized climates

414 Apart from the level of CO₂ in treatments with settings of ambient CO₂, the logging of variables by
415 the RERAF facilities show that the experimental values in general were in accordance with the set
416 points of variables (see 3.1). An unplanned increase in CO₂ level in a facility as RERAF, that
417 cannot remove excess CO₂, may arise from several sources. The use of a growth medium with a
418 high content of organic matter may result in microbial decomposition causing a measurable impact
419 on CO₂-concentration as in Ingvordsen et al. (2018). An increase at nighttime when no
420 photosynthesis occur is similar to natural ecosystems where increases in [CO₂] are seen at low wind
421 speeds (Mikkelsen et al., 2008), and also suggested by Ingvordsen et al. (2018) as a possible
422 explanation for their high average [CO₂]. In the present case, however, the set point was exceeded
423 by approximately 150 ppm, approx 100 ppm higher than Ingvordsen et al. (2018) reported. One key
424 difference to the Ingvordsen et al. experiment, was the manual watering we did to obtain knowledge
425 on the water consumption throughout the plants' growth. Human activities from measuring and
426 maintaining the plants in the chambers resulted in peaks of [CO₂] of 6-900 ppm depending on the
427 activity, and consequently the impact of human respiration on the CO₂-concentration in the
428 chambers were not adequately accounted for in the experiment set-up. For future trials of a similar
429 kind, exchanging the chamber air at a higher rate than 4 m³ day⁻¹ could be explored for better
430 neutralizing such peak CO₂-enrichments. An extract of the carbon dioxide measurements from the
431 chambers of treatment A is shown in Fig. 10, where the nighttime respiration accumulation as well
432 as the attribution of human activity to chamber [CO₂] can be seen.

Fig. 10. CO₂ concentration of treatment A, CO₂-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 Table 1, the background ozone in ambient treatments is stated to be between 5.9 ± 0.5 and 7.2 ± 1.7 ppb. That is lower than the average field level in May-July of app. 40 ppb ozone with 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).

453

454 4.2. Older versus modern wheat varieties.

455 The modern varieties, KWS Bittern and Lennox, which we used in the experiment, were bred to
456 yield well and are used in Denmark and Southern France respectively, and the harvest index (Table
457 2) reveals that their higher yields are based on less biomass than the landrace variety's. Breeding of
458 varieties for modern production take place under current ozone conditions, and as so, modern
459 varieties are supposedly naturally adapted to the current increases in ozone concentration, but ozone
460 sensitivity is also a matter of breeding method (Biswas et al., 2013). Studies examining the effects
461 of filtered air as well as unfiltered air on the growth and yields of wheat show significant yield loss
462 at current concentrations of ozone, indicating that breeding is not successful in obtaining full ozone
463 resistance (Pleijel et al., 2018, 2006). On the other hand, the landrace variety in this study yielded as
464 poorly when temporarily exposed to ozone as when constantly exposed, where the yield of the
465 modern variety were insignificantly different from no ozone to temporary exposure. This indicates
466 perhaps that the modern varieties are resistant to some degree of ozone at the lower temperature
467 settings compared to the resistance of the landrace.

468 The landrace variety used in this study has no commercial use at present, which points to its lack of
469 ability to compete on yields or sales. The Lantvete yield is on the other hand only significantly
470 lower than the modern varieties in two treatments. Its larger biomass may be responsible for its
471 resilience towards the warmer CO₂-enriched treatments (Lopes et al., 2015). The changes in harvest
472 indexes between the treatments reflect that the biomass allocation is a possible actor in the way the
473 varieties deal with the changes in growth related climatic factors (Table 2).

474 As Lennox is a common variety grown in the south of France, it could be expected to resist warmer
475 temperatures (in CT and T.EpO₃ treatments in particular) better than KWS Bittern and Lantvete.

476 This seems to be the case: the yield loss in CT treatment is approx. 17% for Lennox, while it's 28%

477 and 39% for KWS Bittern and Lantvete. That is the only non-significant loss ($p = 0.19$) from the A
478 treatment to a warm treatment within the same variety, it is also significantly higher than the yield
479 of CT_Lantvete (Fig. 2). The yield loss in T.EpO₃ treatment is approx. 22% for Lennox and
480 approx. 32% and 33% for KWS Bittern and Lantvete respectively (Table 2), although the loss is
481 significant in all three ($p < 0.05$).

482

483 4.3. Episodic versus full time ozone exposure and effects on wheat yield

484 It was expected that the plants experiencing ozone as a longer-stretched episode at a critical timing
485 lose as much in yields as plants that have adjusted to ozone from growing in a continuously
486 medium-high ozone environment. The Lantvete variety had similar losses in the colder treatment,
487 but the two modern varieties lost more in the A.O₃ than in the A.EpO₃ treatment (Fig. 4). It could
488 be assumed that intraspecific variations in the ozone acclimation of growth physiological processes
489 exist among wheat cultivars. Such results were shown on thermal acclimation of photosynthesis
490 among alfalfa cultivars (Zaka et al., 2016). However, it could also refer to the contradictory
491 conditions between ambient air ozone concentration and chamber background concentration of
492 ozone. Treatment A had in fact conditions resembling filtered air treatments, resulting in A
493 reflecting the natural selection for ozone adaptation in the modern varieties' yield. This aligns with
494 literature on ozone diminishing agricultural yields at present conditions (Pleijel et al., 2018).
495 In the warmer treatment, the correlation between the dose of phytotoxic ozone and grain yield was
496 not as strong as in the cold treatments (Table 3). In these wheat varieties, an adaptation to ozone to
497 reduce the impact of continuous ozone to the level of episodic ozone exposure was not found.
498 Linear correlations between grain yield and Phytotoxic Ozone Dose 6 (POD₆) were also reported by
499 Harmens et al. (2018). The weaker correlations between grain yield and POD₆ in the warmer CO₂-

500 enriched treatments indicate that the climate components influence the yields more than the ozone
501 dose.

502

503 4.4. Multiple factors and additional ozone exposure

504 Increases in [CO₂] have been expected to induce a fertilizing effect on plant growth and the effect
505 of temperature follows a bell curve indicating that it may be too low as well as too high for
506 optimum plant functioning (Norby and Luo, 2004). The best yields in this study were attained in the
507 ambient [CO₂]/ low temperature treatment (Table 2), indicating that the additional parameters,
508 increase in [CO₂] and temperature, alone or combined all skewed the plant growth out of its
509 optimum. However, the less than additive positive effect of the combination of changes in climate
510 factors have been documented previously (Frenck et al., 2013; Ingvordsen et al., 2015; Shaw et al.,
511 2002). Broberg et al. (2015) observe in their review that both negative and positive effects are to be
512 expected from ozone exposure.

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

517 The ozone taken up influenced the varieties' yields differently, for example, very different ozone
518 amounts were taken up in in T.EpO3, but with no comparative yield decline, stressing the
519 interactions of climate and variety on the grain yield (Fig. 5, Table 4). For the episodically exposed
520 treatments with elevated temperature, T.EpO3 and CT.EpO3, a possible effect of the stomatal
521 regulative effect of elevated [CO₂] is visible qua the lower uptake of ozone in the CT.EpO3
522 treatment compared to the T.EpO3.

523

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

528 From Fig. 4, the impact of ozone is presumably greater in colder temperature treatments, and the
529 addition of ozone to a treatment with elevated [CO₂] and elevated temperature does not significantly
530 increase injury to yields already affected by the higher temperature. However, Tai and Martin
531 (2017) suggest an adaptation of crops to regional ozone and water constraints to induce a stronger
532 ozone tolerance. Elevated [CO₂] may also influence the water relation. In this study, it was decided
533 to keep the plants well-watered. However, had the plants lacked water at times, a better stoma
534 regulation might have reduced the ozone uptake.

535

536 4.5. Growth parameters and responses during growth

537 The photosynthesis vs. intercellular CO₂ depicted in Fig. 6 a, b, c, show that the photosynthetic
538 apparatus of the plants worked as expected on altered input in all varieties and in most treatments.
539 From the sorting of regression slopes in Fig. 7, it seems that the photosynthetic apparatus was
540 affected by the amendment of ozone, and especially full-time ozone treatments resulted in negative
541 regression slopes. This is in accordance with the general perception that ozone has a direct negative
542 influence on photosynthesis (Mikkelsen, 1995; Mikkelsen and Ro-Poulsen, 1994).

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

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

549 As expected, the phenological development of the plants in the treatments (i.e. Lantvete in Fig. 9)
550 revealed a stronger dependence on temperature than on any other treatment parameter. However,
551 there was a 5-degree difference between colder and warmer treatments, and the strong reflection of
552 temperature influence could be caused by this biologically large leap. The impact of [CO₂]-
553 enrichment could be detected on close observation of the development data (not shown here) and
554 this impact may also relate to the absolute difference between the treatment concentrations.
555

556

557 **5. Conclusion**

558 The various combinations of two levels of temperature, two levels of [CO₂], and three ozone
559 exposure regimes affected both growth and yield in spring wheat, while interacting with the studied
560 spring wheat varieties. On average, across all treatments, the landrace variety Lantvete had
561 significantly lower yields than the modern varieties Lennox and KWS Bittern. However, the yields
562 were significantly lower within only two treatments; in the treatment with ambient levels of
563 temperature and [CO₂] and episodically exposure to ozone (A.EpO3), and in the treatment with an
564 elevated level of temperature and [CO₂] (CT). Within the other six treatments, there was no
565 significant difference between the yields of the three tested varieties.

566 The effect on yield with episodic ozone exposure relative to treatment A depended on variety. Yield
567 losses relative to treatment A were significant in all CT.EpO3 and T.EpO3, in A.EpO3 of KWS
568 Bittern and Lantvete, and in C.EpO3 of Lennox. Yield losses due to chronic ozone exposure were
569 significant in all varieties. Increased [CO₂] and higher temperature without ozone exposure induced
570 significant loss of yield in Lantvete and KWS Bittern. Full-time exposure to ozone reduced yields
571 more than ozone exposure during limited time. The combination of climate factors and ozone has
572 shown that the effect of changes in climate factors on wheat yield is beyond the effect of ozone.

573 While an increase in the ozone dose induced yield loss in the low temperature and ambient [CO₂]
574 treatment, the effect in the warm treatments was less clear and yields were lower even at low ozone
575 doses. The different reactions of the varieties to higher ozone doses in the warmer CO₂-enriched
576 treatments compared to the reactions in the treatments with lower temperature and lower [CO₂],
577 lead to the assumption that responses to the combined effects of climate factor changes
578 (temperature, [CO₂], ozone) are due to more or less additive intraspecific variations and adaptations.

579

580

581 Declaration of interest

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

583

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