INTRODUCTION

Tropospheric ozone ($O_3$) is a secondary air pollutant formed from the reaction of nitrogen oxides and volatile organic compounds in the presence of UV radiation. Many regions that currently experience high $O_3$ concentrations ($O_3$) overlap with the most productive crop-lands across the world (Ainsworth, 2017; Ramankutty et al., 2008). For example, from 1980 to 2011, $O_3$ concentrations ($O_3$) in the United States were estimated to have caused a yield loss of up to 10% for rain-fed maize, with an estimated economic cost of $7.2
billion per year (McGrath et al., 2015). While much is known about the metabolic and signaling responses within plant cells to elevated \( [O_3] \), major knowledge gaps remain with regard to: (a) the mechanisms underlying genetic variation in \( O_3 \) response, and (b) the nature of biochemical \( O_3 \) responses in the production environment of a farm field (Ainsworth, 2017). Additionally, our understanding of plant metabolic responses to \( O_3 \) stress largely comes from acute experiments in controlled environments (Carmody et al., 2016; Vainonen & Kangasjärvi, 2015), yet it is well known that the mechanisms of response to chronic \( O_3 \) exposure, generally defined as long-term exposure to concentrations of -100 nl L\(^{-1}\) or less, differ from acute responses to very high \( [O_3] \) (Grantz & Vu, 2012; Vahala et al., 2003). Increased emissions of precursor pollutants have primarily increased crop exposure to chronic \( O_3 \) stress, with tropospheric \( [O_3] \) increasing from \(-10\) nl L\(^{-1}\) in the late 1800’s to \(-40\) to \(50\) nl L\(^{-1}\) in recent years (Brauer et al., 2016; Monks et al., 2015). In many countries, \( [O_3] \) continue to increase (Brauer et al., 2016), further intensifying chronic \( O_3 \) exposure.

Ozone diffuses through the stomata into the intercellular air space where it rapidly reacts to form additional reactive oxygen species (ROS). It is also a powerful oxidizing agent capable of reacting with diverse molecules including lipids, proteins, nucleic acids, and carbohydrates. ROS formed from \( O_3 \) further react with apoplastic antioxidants and a number of proteins embedded in the plasma membrane (e.g., NADPH oxidases, aquaporins, receptor-like kinases, and G-proteins) and elicit an increase in cytosolic calcium (Short et al., 2012). Peroxidation and denaturation of membrane lipids can also occur with prolonged or acute \( O_3 \) exposure (Loreto & Velikova, 2001; Pell et al., 1997). The intracellular response to the influx of ROS depends upon the duration and intensity of \( O_3 \) exposure, with calcium, hormone signaling, and MAP kinase cascades all playing a role in regulation of transcriptional and biochemical changes (Vainonen & Kangasjärvi, 2015). Under chronic \( O_3 \) stress, transcriptional changes have been associated with decreased photosynthesis (Leitao et al., 2007; Li et al., 2019; Pell et al., 1997), increased rates of mitochondrial respiration and antioxidant production (Gillespie et al., 2012; Yendrek et al., 2015), increased hormone biosynthesis (jasmonates, ethylene, and salicylic acid) (Kangasjarvi et al., 2005; Vainonen & Kangasjärvi, 2015), and early activation of several senescence-associated genes (SAGs) (Miller et al., 1999; Fiscus et al., 2005; Kangasjarvi et al., 2005; Betzelberger et al., 2012; Gillespie et al., 2012; Yendrek et al., 2013). For example, LIGHT HARVESTING COMPLEX B6 (LHCB), encoding a minor subunit of the antennae complex responsible for transfer of light energy to photosystem II reaction centers, is an early marker of senescence (Breeze et al., 2011) and its expression is down-regulated early in plants exposed to elevated \( [O_3] \) (Yendrek et al., 2013). Other markers of senescence including LONG-CHAIN ACYL-COA SYNTHETASE 6 and AURORA2 were also accelerated in plants grown at elevated \( [O_3] \) (Yendrek et al., 2013). Shifts in metabolism from carbon assimilation to defense and detoxification in combination with early senescence are thought to be cumulative drivers of reduced plant productivity under elevated \( [O_3] \) (Choquette et al., 2019, 2020; Dizengremel, 2001; Feng et al., 2011; Morgan et al., 2006; Yendrek, Erice, et al., 2017).

A wide range of metabolites have been reported to change in response to elevated \( [O_3] \) in different species, many in common with plant defense responses (Iriti & Faoro, 2009; Munne-Bosch et al., 2013). Plant steroids, including phytosterols and brassinosteroids (BRs), increase plant tolerance to a wide range of abiotic and biotic stresses as well as control plant growth, flowering time, and senescence (Vriet et al., 2012). Changes in the ratios of membrane steroids during stress is also commonly reported following abiotic and biotic stress treatments (Rogowska & Szakiel, 2020). For example, the ratio of stigmasterol to \( \beta \)-sitosterol increased following exposure of Arabidopsis to pathogen-associated molecular patterns and ROS, which was thought to help maintain plasma membrane fluidity and permeability during stress (Griebel & Zeier, 2010). In the chloroplasts, \( \alpha \)-tocopherol is an important scavenger that protects photosynthetic machinery by quenching singlet oxygen or inhibiting the progression of lipid peroxidation (Havaux et al., 2005). Exposure to many abiotic stresses that increase ROS results in increased \( \alpha \)-tocopherol content in leaves (Munne-Bosch, 2005). As leaves age, \( \alpha \)-tocopherol content also increases (García-Plazaola & Becerril, 2001; Hornaetxe et al., 2005). It is unknown if these metabolites play a role in \( O_3 \) response under chronic exposure and field conditions. If so, breeding or biotechnology might be used to leverage the protective roles of phytosterols and non-enzymatic antioxidants to improve tolerance to \( O_3 \)-induced oxidative stress.

More broadly, metabolomics provides a tool to explore biochemical signatures that may be predictive of environmental stress effects on primary productivity, even in field conditions. Studies have examined the relationship between leaf metabolites and physiological traits in the field under various abiotic stress conditions in maize (Obata et al., 2015; Riedelsheimer et al., 2012), rice (Melandri et al., 2020), and Guinea grass (Wedow et al., 2019). Additionally, metabolomics has been used to identify markers associated with greater yield potential in maize (Cañas et al., 2017). But, metabolomic responses of maize to elevated \( [O_3] \) have not yet been widely characterized despite that maize is one of the world’s most widely grown crops (USDA FAS, 2020). Maize is also a model species for the \( C_4 \) plant functional group that includes many other important crops used to produce food, fuel, forage, and fiber. Therefore, we examined the metabolomic profile of maize inbred (B73 and Mo17) and hybrid (B73 × Mo17) lines grown at a Free Air Concentration Enrichment (FACE) facility at the University of Illinois, Urbana-Champaign. The elevated \( [O_3] \) treatment was roughly 2.5 times the current average summer concentration in central Illinois, and consistent with current \( [O_3] \) in polluted regions of Asia (Hong et al., 2019).

In this study, leaf metabolomic profiles were investigated at three time points from early to mid-stages of leaf senescence as characterized by chlorophyll content. Metabolite content was then linked to leaf mass per unit area and grain yield to identify potential biochemical markers for \( O_3 \) response in maize. Ozone tolerance at the leaf level has been linked to dry mass per unit leaf area (LMA) (Feng et al., 2018), which is correlated with seasonal changes in photosynthetic capacity in maize.
Photosynthetic capacity is also highly sensitive to O$_3$ and correlated with yield in maize (Choquette et al., 2020). In this study, we investigated B73 and Mo17, which are two classic elite inbred maize lines (Stuber et al., 1992), and serve as the parents for widely used mapping populations to study the genetic architecture of numerous traits (Balint-Kurti et al., 2007; Mickelson et al., 2002; Pressoir et al., 2009; Sorgini et al., 2019; Wassom et al., 2008). We aimed to test the hypotheses that (a) maize metabolomic signatures would be altered under elevated [O$_3$], with more pronounced effects as the leaves aged under elevated [O$_3$] resulting in greater cumulative uptake; (b) an increase in metabolites associated with oxidative stress and/or membrane stabilization would occur in sensitive maize lines; and (c) key metabolites would be correlated with variation in yield loss under elevated [O$_3$] providing potential molecular markers of O$_3$ response in maize.

2 | MATERIALS AND METHODS

2.1 | Field site and experimental conditions

In 2015, maize (Zea mays) inbred (B73 and Mo17) and hybrid (B73 × Mo17) lines were studied at the FACE research facility in Savoy, IL (https://soyface.illinois.edu/). The facility is a 32-ha farm where maize and soybean (Glycine max) are grown in annual rotation. Inbred and hybrid lines were planted with a precision planter in rows spaced 0.76 m apart and 3.35 m in length at a density of 8 plants m$^{-2}$. There were four pairs of ambient [O$_3$] (~40 nl L$^{-1}$) and elevated [O$_3$] (100 nl L$^{-1}$) plots (n = 4) in the experiment (Figure S1). Inbred and hybrid lines were grown in separate plots to avoid the taller hybrids altering the light environment or interfering with fumigation of shorter inbred lines, which resulted in the use of 16 octagonal, 20-m-diameter plots. One replicate pair of ambient and elevated [O$_3$] plots with the hybrid line was dropped from the analysis due to water logging. Each genotype was planted in five different locations within each plot (Figure S1). Additional site information and field conditions were described in Yendrek, Erice, et al. (2017).

Air enriched with O$_3$ was delivered to the experimental rings with FACE technology as described in Yendrek, Tomaz, et al. (2017). The target elevated [O$_3$] was 100 nl L$^{-1}$ and the O$_3$ treatment was administered from 10:00 to 18:00 throughout the growing season when it was not raining and the wind speed was greater than 0.5 m/s. Based on 1 min average [O$_3$] collected in each ring, the fumigation was within 10% of the 100 nl L$^{-1}$ target for 56% of the time and within 20% of the target for 79% of the time in 2015. Weather conditions were measured with an on-site weather station as reported previously in Yendrek, Erice, et al. (2017).

2.2 | Sampling protocol and tissue handling

Leaf material for metabolomic profiling of B73, Mo17, and B73 × Mo17 was sampled on three dates corresponding to similar leaf physiological maturity and early- and mid-senescence stages between the hybrid and inbreds. Samples were taken in the inbred experiment on: 4 August 2015, 13 August 2015, and 24 August 2015 (day of year DOY: 216, 225, and 236) and in the hybrid experiment on: 27 July 2015, 7 August 2015, and 17 August 2015 [(DOY): 208, 219, and 229]. Different plants within the replicate rows were sampled on each date. All material was collected from the plots between 12:00 and 14:00. Approximately 100 cm$^2$ of leaf material was cut from the middle of the leaf subtending the ear with scissors, wrapped in tinfoil, and immediately placed in liquid nitrogen, before being stored at ~80°C. Five samples per inbred and hybrid line (1 per sub-block) were taken from each ambient and elevated [O$_3$] plot.

Leaf mass per unit area (LMA) was measured from leaf disks taken at the same time as samples for metabolomic profiling. Three leaf disks (0.02 m dia) per row were cut with cork borers, placed into coin envelopes, and dried in an oven at ~60°C for 1 week. Samples were then weighed and data from the five rows within each plot were averaged for a plot-level estimation of LMA (g/m$^2$).

2.3 | Leaf chlorophyll content

Chlorophyll content was estimated from measurements collected with a SPAD meter (Konica-Minolta SPAD-502 Chlorophyll meter). Measurements were collected from the leaf subtending the ear of three plants per genotype at five locations per ring. Six readings were taken from the middle third of each leaf and the average value was recorded. Measurements were taken approximately every 3 days starting at anthesis, continuing through leaf senescence (DOY 204–255). The equation: chlorophyll content (µg/cm$^2$) = (99 × SPAD) / (144 – SPAD) was used to convert SPAD values into chlorophyll content (Cerovic et al., 2012).

2.4 | Seed yield

At maturity, ears were harvested from 8 plants in each of the 5 rows per genotype per plot, dried for ~1 week in an oven at ~60°C, then shelled, and weighed to estimate yield (g plant$^{-1}$).

2.5 | Metabolomic profiling by GC-MS

Untargeted metabolomic profiling was performed with 15 mg of lyophilized leaf tissue according to the protocol described in Ulanov and Widholm (2010). A total of 40 samples per time point for each inbred, B73 and Mo17, and 30 samples per time point for B73 × Mo17 were processed for metabolomic profiling. Samples were extracted with methanol:acetone: H$_2$O (1:2:1, v/v/v) at ambient temperature. For quality control (QC), 10 µl of leaf extract was taken from each sample and pooled, then run and analyzed after every nine biological samples. Samples and QC were dried under vacuum and derivatized with 75 µl methoxyamine hydrochloride
The analysis of chlorophyll loss over time was tested by fitting a single quadratic model where \( x \) equals day of year. To test for differences in chlorophyll loss over time in ambient and elevated \([O_3]\), a single quadratic model was first fit to the data for each genotype (PROC NLIN, SAS 9.4, SAS Institute), and then models were fit to each genotype and treatment combination. An F statistic was used to test if the model with genotype and treatment produced a significantly better fit to the data, that is, if there were significant differences in the response of chlorophyll over time in ambient and elevated \([O_3]\), following the approach of Yendrek, Erice, et al. (2017). The area under each curve was estimated by integrating the area under the curve from the date of the first measurement to the date of the last measurement using the integrating curve function in Sigma Plot 14.5 (Systat Software Inc.).

LMA and seed yield were analyzed using analysis of variance. For the inbred experiment, the model included fixed-effect terms for inbred line and treatment, and a random term for block. The model for the hybrid experiment included treatment as a fixed effect and block as a random term in the model. Significant differences between treatments were determined by Tukey tests with a threshold of \( p < .05 \).

The inbred and hybrid metabolite data were analyzed separately because they were grown in different field plots and harvested on different dates (Figure S1). For each dataset, metabolite data were log\(_{10}\) transformed and processed with univariate analysis to identify and remove outliers (studentized residual \( > 4 \)). The total number of removed outlier points within the inbred datasets was between 3 and 8 and between 0 and 2 for the hybrid datasets. The five observations for a genotype within each FACE or control plot were averaged for analysis and time points were analyzed separately. Each metabolite was tested independently using a two-way ANOVA (Kirpich et al., 2018) for the inbred experiment with treatment and genotype as fixed effects, and a one-way analysis of variance model for the hybrid experiment (Figure S2). Statistical differences in least squared mean estimates between ambient and elevated \([O_3]\) for each time point were determined by Tukey tests with a threshold of \( p < .05 \) (Kuehl, 2000). The statistical analysis was done using SAS software (SAS, Version 9.4).

Multivariate statistics were performed using R (version 3.5.1; The R Foundation for Statistical Computing). Multivariate clustering analysis was done with the log\(_{10}\)-transformed and Pareto scaling normalized data, with identified outlier observations removed. Missing values were estimated prior to multivariate analysis using k-nearest neighbor (KNN) in the MetaboAnalystR package (Chong & Xia, 2018). The total number of missing values within the inbred datasets was between 4.2% and 6.4% of all observations and between 4.8% and 6.4% for the hybrid datasets. Principal component analysis (PCA) was performed using the prcomp function (R stat package) for the inbred and hybrid experiments independently for each time point. A singular value decomposition data matrix was applied to each normalized dataset. When the unsupervised PCA identified a clear treatment separation, a supervised partial least square - discriminant analysis (PLS-DA) was performed, with the mixOmics package (Rohart et al., 2017). The number of latent variables included in the model was selected by testing the predictability value (\( Q^2 \)) using an increasing number of latent variables from 1 to 10. The relative importance of the metabolites in the models was summarized using PLS-DA loadings, with significance considered when the contribution was greater than 0.1.

Pearson linear correlation analysis was done to investigate correlations between metabolite content and physiological traits (chlorophyll and LMA) based on the class separation observed in the multivariate clustering. Correlations with an adjusted \( p \) value (False Discovery rate, (Benjamini & Hochberg, 1995)) of .05 or less, and a correlation coefficient of absolute value \( > .55 \) or greater were considered significant and biologically meaningful in this analysis. These correlations were performed to identify any possible interactions of interest that would not be expected based on current literature or from the multivariate statistical results performed.

Statistical analyses of sterol:chlorophyll (relative concentration/100 mg DW: \( \mu g/cm^2 \)) and day of year (DOY) were performed using linear regression (Proc Reg; SAS). An F statistic was used to
test if treatments had significantly different slopes in the regression, with each metabolite analyzed independently. Differences in slopes between ambient and elevated \([\text{O}_3]\) were considered significant when \(p < .05\).

## 3 | RESULTS

### 3.1 | Maize leaf metabolomic profiles in ambient and elevated \([\text{O}_3]\)

Metabolomic profiles of the leaf subtending the ear from two inbred lines (B73 and Mo17) and the hybrid cross (B73 × Mo17) were measured in ambient and elevated \([\text{O}_3]\) on three dates in 2015. These three time points captured the initial and middle stages of leaf senescence based on leaf chlorophyll content (Figure 1a–c). Both inbred lines and the hybrid showed acceleration of senescence, but it was more pronounced in the hybrid, which lost 25% more chlorophyll over time in elevated \([\text{O}_3]\) compared to ambient \([\text{O}_3]\) (Figure 1a). A total of 41, 45, and 46 annotated metabolites were detected in the inbred lines at the three time points (Table S1), and 51, 49, and 56 metabolites at each time point in B73 × Mo17 (Table S2).

Based on clustering and principle component analysis, the leaf metabolite profile of the inbred lines did not show a significant response to elevated \([\text{O}_3]\) in any of the time points (Figure 2a–c). This was true whether both inbred lines were analyzed together (Figure 2a–c) or independently (data not shown). The principle component analysis showed that there was a strong genotype separation between B73 and Mo17 along the first principle component in all time points. Similarly, statistical tests (ANOVA) identified 22, 37, and 24 metabolites in time point A, B, and C, with significant differences in content between genotypes (Table S3). In contrast, the content of only three metabolites differed between leaves grown in ambient and elevated \([\text{O}_3]\) in the inbred lines (Table S3).

Multivariate clustering of the leaf metabolite profile of B73 × Mo17 across time points showed a clear shift in leaf metabolism under elevated \([\text{O}_3]\) as the leaves aged (Figure 3). When the PCA plots revealed strong discrimination between ambient and elevated \([\text{O}_3]\), partial least square-discriminant analysis (PLS-DA) was performed to identify maximum separation among treatments and to rank individual metabolite contributions to separation of ambient and elevated \([\text{O}_3]\) (Figure S3). On the first date of sampling when the flag leaf was relatively young (time point A, DOY 208), the metabolite pools in B73 × Mo17 did not differ in ambient and elevated \([\text{O}_3]\) (Figure 3a). As leaves aged in elevated \([\text{O}_3]\), both PCA and PLS-DA revealed differences in the metabolite profiles of leaves grown in ambient and elevated \([\text{O}_3]\) (Figure 3b,c). Statistical tests (one-way ANOVA) identified 20 metabolites with significant differences in content between ambient and elevated \([\text{O}_3]\) in time point B, and 12 metabolites with significant differences in time point C (Table S4). Secondary metabolites quinic acid, stigmasterol, and \(\alpha\)-tocopherol were greater in elevated \([\text{O}_3]\) in both time points (Figure 4b,c), while sitosterol was greater in ambient \([\text{O}_3]\) (Figure 4b,c). Fatty acids palmitate and linoleate were also greater in ambient \([\text{O}_3]\) (Figure 4e,f). The sugar, benzyl glucopyranoside, and two TCA cycle compounds, citric acid and malic acid, were greater in elevated \([\text{O}_3]\) in both time points. Pyruvate, which is converted into acetyl-CoA in the initial step of the citric acid cycle, was greater in ambient \([\text{O}_3]\) (Figure 4e,f). Similarly, threonine, glycerol-3-P, and glycophosphoglycerol were greater in ambient \([\text{O}_3]\) in time points B and C (Table S2).

The ratios of \(\alpha\)-tocopherol, campesterol, stigmasterol, and sitosterol to chlorophyll content are used as indices of senescence (Li et al., 2017) and were investigated in the maize inbred and hybrid lines. Alpha-tocopherol, campesterol, and stigmasterol increased over time in B73 × Mo17, especially in elevated \([\text{O}_3]\) (Figure 5a–c). These trends were due to both the decline in chlorophyll (Figure 1) and an increase in \(\alpha\)-tocopherol, campesterol, and stigmasterol content in aging leaves, especially in elevated \([\text{O}_3]\) (Figure 4). A significant difference between ambient and elevated \([\text{O}_3]\) in the accumulation of \(\alpha\)-tocopherol relative to chlorophyll (Figure 5a) was observed, along with trends toward greater accumulation of stigmasterol and campesterol relative to chlorophyll (Figure 5b,c), indicating accelerated senescence in elevated \([\text{O}_3]\). The reverse trend was seen in the sitosterol:chlorophyll ratio with decreasing ratios in the elevated \(\text{O}_3\) condition (Figure 5d). The inbred lines showed an age-dependent increase in \(\alpha\)-tocopherol:chlorophyll ratio, but no effect of elevated \([\text{O}_3]\) (Figure 6).

### 3.2 | LMA and yield correlations with leaf metabolite content

LMA has been linked to variation in leaf physiology, plant growth strategy, resource investment, and \(\text{O}_3\) tolerance across diverse species (Feng et al., 2018; Shipley et al., 2006). LMA was lower in the hybrid line compared to the inbred lines (Table 1). LMA was not significantly affected by elevated \([\text{O}_3]\) in either inbred line at any time point, and was only significantly lower in elevated \([\text{O}_3]\) in the hybrid at the last sampling time (Table 1). Seed yield was not significantly affected by elevated \([\text{O}_3]\) in either inbred line, but was nearly 30% lower in elevated \([\text{O}_3]\) in the hybrid (Table 1).

To assess the predictive potential for metabolite concentrations to reveal changes in physiology or yield, correlations between metabolite content and LMA or yield were tested separately for inbred and hybrid maize lines. The linear correlations for B73 and Mo17 were performed independently due to the genotypic separation identified with PCA (Figure 2). There was no treatment separation at any time point and so correlations were done across \(\text{O}_3\) treatments. In the inbred lines, itaconic acid measured at time point A was positively correlated with yield (Table S5). Stigmasterol and LMA were negatively correlated at time point B in B73, while raffinose and LMA showed a positive correlation at time point C. The strongest relationships within Mo17 were between malic acid and LMA in time point A, and sucrose and LMA in time point C (Table S5).
FIGURE 1 Chlorophyll content estimated from SPAD measurements of the leaf subtending the ear from leaf maturity through senescence for hybrid line B73 × Mo17 (a) and inbred lines B73 (b) and Mo17 (c), measured at ambient (blue symbols) and elevated [O_{3}] (orange symbols). The area under the curve was integrated to calculate the percentage change in chlorophyll content over the lifetime of the leaf, and is shown in the top right of each plot, along with significance values. Vertical lines indicate the measurement dates for leaf metabolite content. Each point indicates the median value within each plot at each sampling date.
In B73 × Mo17, there was no treatment separation between ambient and elevated [O₃] at time point A, so correlations were done across O₃ treatments (Table S6). Stigmasterol, campesterol, and ethanolamine contents measured in recently mature leaves (time point A) were negatively correlated with yield, while alanine was positively correlated with yield (Table 2). Multivariate clustering for time points B and C showed significant differences in ambient and elevated [O₃] in B73 × Mo17, so correlations were done separately for each O₃ treatment (Table 3). Benzyl glucopyranoside, campesterol, glycerohexose, and malic acid content were negatively correlated with yield in elevated [O₃], but not in ambient [O₃] (Table 3). Scylo inositol was positively correlated with yield in ambient [O₃], and negatively correlated with yield in elevated [O₃] (Table 3). Ferulic acid, itaconic acid, and citric acid measured at time point C (when leaves were senescing) were positively correlated with yield in ambient [O₃], but not in elevated [O₃] (Table 4).

Sitosterol and LMA showed opposite correlations in ambient and elevated [O₃] when measured at time point B (Table 3) for B73 × Mo17. Benzyl glucopyranoside and LMA were negatively correlated in time point B in ambient [O₃] (Table 3). In time point C, LMA and α-tocopherol were positively correlated in elevated [O₃], but not in ambient [O₃] (Table 4). Two significant linear correlations were identified in the ambient [O₃] treatment with LMA, α-coumaric acid and malonic acid (Table 4). Itaconic acid, pyruvic acid, ribose, alanine, and pyroglutamic acid were all negatively correlated with LMA in time point C in elevated [O₃] (Table 4).

4 | DISCUSSION

Field metabolomics can be a powerful approach for profiling the metabolite changes in plants in response to environmental stress (Melandri et al., 2020; Wedow et al., 2019). The physiological response of crops to O₃ pollution has been well studied, with elevated [O₃] decreasing carbon assimilation, accelerating senescence and cell death, and reducing economic yield (Ainsworth, 2017). However, the metabolite profile underpinning the response to O₃ in maize has not been investigated and could provide information for targets to

![FIGURE 2 Principle component analysis plot of the metabolomic profile of inbred lines B73 (filled symbols) and Mo17 (open symbols) grown at ambient (blue symbols) and elevated [O₃] (orange symbols) sampled at time points (a) DOY: 216, (b) DOY: 225, and (c) DOY: 236. For each time point and treatment, n = 20](image-url)
improve O$_3$ tolerance in maize and C$_4$ crops. We found that maize metabolomics signatures were altered by elevated [O$_3$] in a hybrid line (Figure 2), but not in two inbred lines (Figure 3). As predicted, the differences in metabolite signatures between ambient and elevated [O$_3$] in the hybrid line emerged as leaves aged (Figure 2). Notably, there was a significant increase in $\alpha$-tocopherol and phytosterol content in elevated [O$_3$], supporting the prediction that metabolites that quench oxidative stress and stabilize membranes are key biochemical responses to O$_3$ stress.

The metabolomic profiles of inbred and hybrid leaves reflected differences in acceleration of senescence under elevated [O$_3$]. In inbred lines and when chlorophyll content was similar in ambient and elevated [O$_3$] in B73 × Mo17, the metabolomic profile was not different in ambient and elevated [O$_3$] (Figures 3 and 2a). However, as chlorophyll was lost more rapidly at elevated [O$_3$] during senescence in the hybrid, a clear separation in metabolite profiles between ambient and elevated [O$_3$] was apparent (Figure 2b,c). The inbred genotypes had no discernible difference in their metabolomic profiles in ambient and elevated [O$_3$] at any of the time points, although B73 was very different from Mo17 (Figure 3). These results agree with our prediction that a metabolomic signature develops with the accumulation of O$_3$ damage in aging leaves and reflects altered biochemistry. Ozone-induced acceleration of senescence has been previously reported to impact grain yield losses due to a decrease in photosynthetic capacity and shortened leaf lifespan (Emberson et al., 2018). A reduction in photosynthetic carbon assimilation in maize ear leaves during grain filling was greater in hybrid maize compared to inbred lines (Yendrek, Erice, et al., 2017). Additionally, seed yield loss under elevated [O$_3$] was 30.4% for B73 × Mo17, only 5.0% for Mo17, and 2.1% for B73. Thus, the hybrid maize line showed greater response to elevated [O$_3$] from the biochemical to the agronomic scale, which could be related to whole-plant O$_3$ dose since the hybrid lines had greater total leaf area and biomass. This is despite the observation that stomatal conductance was not significantly altered by elevated [O$_3$] in either B73 × Mo17 or the two inbred lines (Yendrek, Erice, et al., 2017), suggesting that differential stomatal sensitivity to O$_3$ was not a primary driver of differences between the hybrid and inbred lines.

The metabolites contributing to the O$_3$ treatment differences during leaf senescence in B73 × Mo17 included TCA cycle
intermediates, and specialized metabolites such as phytosterols and fatty acids (Figure 4). In the latter two time points, citric acid and malic acid contents were greater in elevated \([O_3]\). This trend is consistent with increased flux through the TCA cycle and greater rates of mitochondrial respiration in plants exposed to O_3 stress (Betzelberger et al., 2012; Dizengremel et al., 2012; Yendrek et al., 2015). The shift in metabolism from photosynthetic carbon assimilation to the TCA cycle and mitochondrial respiration supplies energy for repair and detoxification of the plant cells against oxidative damage (Dizengremel, 2001). The additional influence of specialized secondary metabolites including \(\alpha\)-tocopherol, benzyl glucopyranoside, stigmasterol, and quinic acid is consistent with a shift to defense and repair under elevated O_3 stress (Figure 4). Steryl esters (SEs), palmitate and linoleate, which are commonly found to function as membrane lipids were greater in ambient \([O_3]\), possibly reflecting differences in membrane fluidity or other membrane properties between ambient and elevated \([O_3]\).

We found that \(\alpha\)-tocopherol increased to a greater extent as leaves aged in elevated \([O_3]\) in the hybrid B73 × Mo17 (Figure 5a–c), but not in either inbred line (Figure 6). The protective function of tocopherols to preserve cell membrane integrity during the final stages of leaf development has been well documented (Falk & Munne-Bosch, 2010; Fryer, 1992; Lira et al., 2017). Leaf \(\alpha\)-tocopherol content increases in response to abiotic stresses, such
as high light (Krieger-Liszkay & Trebst, 2006; Lizarazo et al., 2010), drought (Akhami et al., 2019; Munne-Bosch & Alegre, 2000) and high temperature stress (Spicher et al., 2017). In Mediterranean species, α-tocopherol showed greater environmental sensitivity to drought and high temperature stress compared to small antioxidant molecules (ascorbate, glutathione) and xanthophyll-cycle

![Figure 5](image1)

**Figure 5** Ratio of leaf metabolite (relative concentration/100 mg DW) content to chlorophyll content (µg/cm²) over time for hybrid B73 × Mo17. (a) α-tocopherol/chlorophyll ratio; (b) campesterol/chlorophyll ratio; (c) stigmasterol/chlorophyll ratio; (d) sitosterol/chlorophyll ratio. Solid lines indicate statistically significant relationships, while dashed lines are not significant. The linear regression equations, r², and p-values for ambient (blue symbols, grey lines) and elevated [O₃] (orange symbols, black lines) are denoted. p-value in the top left indicates the statistical difference between slopes in the linear regressions. Each point indicates the median ratio of all ambient or elevated samples (n = 15 per treatment)

![Figure 6](image2)

**Figure 6** Ratio of α-tocopherol (relative concentration/100 mg DW) to chlorophyll (µg/cm²) content measured over time in (a) B73 and (b) Mo17 grown at ambient (blue symbols) and elevated [O₃] (orange symbols). p-value in the top left indicates the test of differences in slope of the regression line between ambient and elevated [O₃]. Each point indicates the median ratio of all ambient or elevated samples (n = 20 per treatment)
where highly reactive singlet oxygen (\(^1\text{O}_2\)) is formed, \(\alpha\)-tocopherol is an essential scavenger protecting the thylakoid membranes against lipid peroxidation (Piller et al., 2014). In high light conditions, the rapid turnover of \(\alpha\)-tocopherol and plastoquinone have been correlated with the increased turnover rate of the D1 protein within PSII, thereby protecting the photosynthetic process (Krieger-Liszkay & Trebst, 2006). Experimental studies exposing plants to oxidative stresses have reported conflicting results regarding \(\alpha\)-tocopherol levels in leaf extracts; beech leaves in full sun showed a significant increase over shade leaves, along with an acceleration in leaf senescence (Garcia-Plazaola & Becerril, 2001). Snap bean showed a significant increase in \(\alpha\)-tocopherol concentrations following exposure to elevated \([\text{O}_3]\); however, the total concentration of \(\alpha\)-tocopherol was not correlated with differences in \(\text{O}_3\) sensitivity between cultivars (Burkey et al., 2001). In contrast, spinach leaves exposed to elevated \([\text{O}_3]\) were observed to have a decrease or no change in \(\alpha\)-tocopherol in leaf extracts (Calatayud et al., 2003, 2004). In this study, the response of leaf \(\alpha\)-tocopherol content to elevated \([\text{O}_3]\) varied with maize genotype and leaf age (Figures 5a and 6). The conflicting results from previous studies regarding effects of elevated \([\text{O}_3]\) on leaf \(\alpha\)-tocopherol content may be attributed to the timing of sampling and the progression of leaf senescence. We would not have identified a relationship between \(\alpha\)-tocopherol and \(\text{O}_3\) in B73 × Mo17 had samples only been taking at the initial time point (Figure 4a), but clearly the content increased as leaves aged in elevated \([\text{O}_3]\). Elevated \(\alpha\)-tocopherol concentrations in leaves exposed to elevated \([\text{O}_3]\) are potentially quenching ROS that form during the disassembly of the photosynthetic membranes during senescence and/or inhibiting lipid peroxidation (Rogers & Munne-Bosch, 2016). Manipulation of \(\alpha\)-tocopherol content in leaves has been proposed as an efficient trait to improve dynamic responses to abiotic stress (Lizarazo et al., 2010). It would be interesting to test if transgenic approaches to increase \(\alpha\)-tocopherol in aging leaves would specifically improve \(\text{O}_3\) tolerance.

Senescing hybrid leaves exposed to elevated \([\text{O}_3]\) showed a trend toward accumulating campesterol and stigmastanol (Figure 5b,c) at the expense of sitosterol (Figure 5d). Phytosterols are proposed to modulate membrane integrity to improve abiotic stress tolerance (Dufourc, 2008; Kuczynska et al., 2019). In stress conditions stigmasterol and sitosterol interact with phospholipids to maintain the permeability and fluidity of the plasma membrane (Dalal et al., 2016; Griebel & Zeier, 2010). Heat stressed hard fescues up-regulated ethyl sterol content, including stigmastanol and sitosterol, and the more heat tolerant variety showed a significantly greater increase in stigmasterol compared to a heat sensitive variety (Wang et al., 2017). In the current study, we found greater concentrations of stigmasterol and campesterol in hybrid leaves exposed to elevated \([\text{O}_3]\), but not in inbred lines (Figure 5; Table S5 and Table S6). Genotypic variation in phytosterol concentrations has been documented in wheat (Nurmi et al., 2010), rice (Kumar et al., 2018), and potato (Hancock et al., 2014), and genetic variation in the concentrations of phytosterols during drought was associated with differences in stress tolerance (Kumar et al., 2015). It is interesting that in elevated \([\text{O}_3]\), increased phytosterol content only occurred in the hybrid genotype that also showed accelerated senescence and significant yield loss, not in the inbred lines. This suggests that increased phytosterol content was not associated with \(\text{O}_3\) tolerance, instead with changes to membranes in aging leaves. Furthermore, campesterol is a precursor of brassinosteroids (Fujikoa & Yokota, 2003), and brassinosteroid signaling mutants display a delayed senescence phenotype (Clouse & Sasse, 1998). Thus, the accumulation of campesterol in aging leaves exposed to elevated \([\text{O}_3]\) in B73 × Mo17 could further promote leaf senescence. Such fluctuations in the levels of one phytosterol at the expense of another are proposed to alter plant responses to environmental stimuli (Aboobucker & Suza, 2019). Our work also supports

### TABLE 1
Leaf mass per unit area (LMA, g/m²) measured at three time points (A, B, C) on the leaf subtending the ear. Inbred leaves were sampled on DOY: 216 (A), DOY: 225 (B), and DOY: 236 (C). Hybrids were sampled on DOY: 208 (A), DOY: 219 (B), and DOY: 229 (C). Yield (kernel mass per plant, g plant⁻¹) was measured at maturity in ambient (Amb) and elevated [O₃] (Ele). Bold font indicates significant differences in ambient and elevated [O₃] (p < .05).

| Trait, Time Point | B73               | Mo17               | B73 × Mo17          |
|-------------------|-------------------|--------------------|---------------------|
|                   | Amb [O₃]         | Ele [O₃]           | Amb [O₃]           | Ele [O₃]           | Amb [O₃] | Ele [O₃] |
| LMA, A            | 39.1 ± 4.6        | 39.0 ± 3.6         | 36.0 ± 4.0          | 34.7 ± 2.8         | 30.4 ± 3.0 | 30.2 ± 3.0 |
| LMA, B            | 44.5 ± 5.6        | 42.4 ± 4.4         | 35.2 ± 3.6          | 35.0 ± 3.7         | 32.7 ± 3.0 | 32.8 ± 2.0 |
| LMA, C            | 43.8 ± 5.9        | 43.0 ± 4.4         | 37.0 ± 3.8          | 37.1 ± 4.3         | 33.9 ± 3.2 | 31.5 ± 2.8 |
| Yield             | 100.2 ± 3.4       | 96.9 ± 4.2         | 82.2 ± 4.1          | 78.4 ± 2.8         | 175.9 ± 4.9 | 126.8 ± 6.0 |

### TABLE 2
Significant Pearson linear correlations (p < .05) between yield and metabolite content for hybrid line, B73 × Mo17. Linear correlation statistics for time point A (DOY: 208) contain all samples from both ambient and elevated [O₃].

| Time Point | Trait | Metabolite | All (r) |
|------------|-------|------------|---------|
| A          | Yield | Stigmasterol | -.72    |
|            | Yield | Campesterol | -.68    |
|            | Yield | Alanine     | .57     |
|            | Yield | Ethanolamine | -.55    |
the hypothesis that the balance of various phytosterols may be a key signaling response for additional cellular defenses (Griebel & Zeier, 2010; Schaller, 2003).

Previous experiments have identified metabolites associated with maize grain yield under drought and heat stress (Obata et al., 2015), and here we investigated potential metabolite markers associated with yield under elevated [O$_3$] stress. In the B73 × Mo17 genotype, stigmasterol and campesterol showed negative correlations to yield at the first time point, before any treatment effect could be identified (Table 2). In the second time point, campesterol content was negatively correlated with yield in the elevated [O$_3$] treatment, but not in ambient [O$_3$] (Table 3). There were no metabolites associated with yield in both ambient and elevated [O$_3$] in either time point B or C (Tables 3 and 4), when PCA and PLS-DA analysis revealed strong treatment effects on the leaf metabolite profile. This supports previous work that identified different metabolite correlations with yield under drought stress compared to heat stress (Obata et al., 2015). LMA and $\alpha$-tocopherol were positively correlated under elevated [O$_3$] (Table 4). A reduction in LMA is generally linked to decreasing leaf nitrogen content and leaf area under elevated O$_3$ conditions (Oikawa & Ainsworth, 2016). The positive relationship identified suggests a potential protective role of $\alpha$-tocopherol in stabilizing thylakoid membranes and preventing excessive degradation of chlorophyll under elevated [O$_3$]. The strong relationships identified in this study provide potential metabolic signatures for improving O$_3$ tolerance, and provide metabolite markers that can now be screened across a diverse genetic background.

Field experiments can be more variable than controlled environment experiments (Lovell et al., 2016), and metabolites respond rapidly to environmental changes (Caldana et al., 2011), both of which present challenges for field metabolomic studies. Recent criticism of metabolomics data includes the low repeatability and strong effects of environmental perturbations on plant metabolism (Tucker et al., 2020). We found that environmental conditions influenced the metabolomic profiles; yet there were clear patterns of response in hybrid maize. Moreover, we discovered that maize inbred lines have significantly different metabolite profiles from one another, but lacked a response to elevated [O$_3$]. Our study emphasized that metabolomic profiling is a vital tool that can be used alongside additional ’omic’ and standard physiological measurements to improve understanding of plant metabolic responses to stress. Despite the dynamic and variable nature of field-based metabolomics, we

| Time Point | Trait | Metabolite                  | Ambient ($r$) | Elevated ($r$) |
|------------|-------|-----------------------------|---------------|----------------|
| B          | Yield | Benzyl glucopyranoside      | .15           | −.60           |
|            |       | Campesterol                 | .01           | −.58           |
|            |       | Glycerohexose               | −.26          | −.56           |
|            |       | Malic acid                  | .14           | −.55           |
|            |       | Inositol, scylo             | .56           | −.54           |
| B          | LMA   | Sitosterol                  | −.58          | .55            |
|            |       | Threonic acid               | −.50          | −.57           |
|            |       | Benzyl glucopyranoside      | −.57          | −.32           |

**Table 3** Significant Pearson linear correlations ($p < .05$) of yield and leaf mass per unit area (LMA) with metabolite content sampled at time point B (DOY: 219) for hybrid line, B73 × Mo17 in ambient and elevated [O$_3$]

| Time Point | Trait | Metabolite                  | Ambient ($r$) | Elevated ($r$) |
|------------|-------|-----------------------------|---------------|----------------|
| C          | Yield | Ferulic acid                | .81           | .02            |
|            |       | Itaconic acid               | .68           | −.04           |
|            |       | Citric acid                 | .57           | −.29           |
| C          | LMA   | $\alpha$-tocopherol         | .14           | .60            |
|            |       | Maltose                     | −.25          | .60            |
|            |       | Malonic acid                | −.59          | .05            |
|            |       | $\omega$-coumaric acid      | −.67          | .03            |
|            |       | Itaconic acid               | −.05          | −.64           |
|            |       | Pyruvic acid                | .21           | −.63           |
|            |       | Ribose                      | .11           | −.60           |
|            |       | Alanine                     | −.12          | −.58           |
|            |       | Pyroglutamic acid           | −.40          | −.57           |

**Table 4** Significant Pearson linear correlations ($p < .05$) of yield and leaf mass per unit area (LMA) with metabolite content sampled at time point C (DOY: 229) for hybrid line, B73 × Mo17 in ambient and elevated [O$_3$]
identified novel markers of O₃ response in hybrid maize, which can be broadly tested across diverse germplasm.

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CONFLICT OF INTEREST
The authors declare no conflict of interest associated with the work described in this Manuscript.

AUTHOR CONTRIBUTIONS
JMW and EAA conceptualized the study. JMW performed metabolomic and statistical analysis. CHB collected and analyzed leaf chlorophyll data with EAA. LRA and ADBL collected yield data. ADBL and EAA designed the field experiment. JMW and EAA wrote the manuscript with input from all authors.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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