



Effects of Elevated CO₂ and Increased N Fertilization on Plant Secondary Metabolites and Chewing Insect Fitness

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Elevated atmospheric CO₂ (eCO₂) and increased nitrogen (N) fertilization significantly change the nutritional quality of plants and influence the growth and development of insects. However, little is known about plant metabolism and plant-insect interactions under eCO₂ and increased N fertilization, especially C₄ plants. Thus, the combined effects of eCO₂ and increased N fertilization on maize-*Ostrinia furnacalis* interactions were tested in this study. Our data demonstrated that both eCO₂ and increased N fertilization increased starch content, while increased N fertilization promoted the N content in maize. The combined effects of eCO₂ and increased N fertilization did not influence the total non-structural carbohydrates (TNC):N ratio in maize. The jasmonic acid level of maize was enhanced by increased N fertilization and *O. furnacalis* infestation. The total phenolics content and defensive enzyme activities of maize increased under eCO₂, increased N fertilization and *O. furnacalis* infestation. Protective enzyme activities were enhanced, while digestive enzyme activities, mean relative growth rate, body mass and efficiency of conversion of ingested food decreased for *O. furnacalis* feeding on maize grown under eCO₂ and increased N fertilization. Therefore, eCO₂ and increased N fertilization increased starch and N accumulation, and did not influence the TNC:N ratio, however, eCO₂ and N promoted the resistance-related secondary metabolites (with or without *O. furnacalis* induced) of maize, which ultimately decreased the fitness of *O. furnacalis* to the host. These results will help to better understand the metabolic mechanisms of plants and the plant-insect interaction under eCO₂ and increased N fertilization in the context of future climate change scenarios.

Keywords: climate change, elevated CO₂, N fertilization, plant-insect interaction, metabolites

INTRODUCTION

Industrialization has increased atmospheric CO₂ concentration from 280 ppm at the beginning of the Industrial Revolution to 400 ppm today and the value is expected to double by the end of the century (IPCC, 2014). Generally, carbohydrate content in plants increases with elevated atmospheric CO₂ (eCO₂), due to higher photosynthetic rates (Long et al., 2006; Reddy et al., 2010;

Kimball, 2016). Plants grown in eCO₂ are observed to have lower nitrogen (N) concentration compared to plants grown in ambient CO₂. However, the interpretations for lower N concentration are inconsistent across the literatures. There exist several reasons for this: the dilution effect due to enhanced production of carbohydrates (Novriyanti et al., 2012), the decrease in the specific uptake rates of N by roots under eCO₂ (Taub and Wang, 2008), or that eCO₂ directly inhibits plant nitrogen metabolism (Bloom et al., 2012). Therefore, more sophisticated approaches to nitrogen fertilization should be employed to enhance N concentration and improve plant quality under eCO₂ (Bloom et al., 2014).

Present and future eCO₂ is predicted to have a profound effect on plant-insect interactions due to changes in the C:N ratio and chemical compositions based on primary and secondary metabolites (Zavala et al., 2017). This change impacts the food quality of the host plant which subsequently influences insect growth and development. For example, eCO₂ increases the total non-structural carbohydrates (TNC) content in maize plants and decreases the N content, causing insects to consume more plant tissue to obtain enough N-based nutrients, and extends their development time (Yin et al., 2010; Xie et al., 2015). At the same time, eCO₂ also influences plant secondary metabolites that affect insect growth and development (Reddy et al., 2004; Chen et al., 2005b). For example, under eCO₂, the contents of tannin and gossypol are increased and Bt protein synthesis is decreased in cotton (Chen et al., 2005b), the content of total phenolics is increased in rape seed (Reddy et al., 2004), and the defensive enzyme activities against aphids are decreased in *Medicago truncatula* (Guo et al., 2014).

Increased N fertilization is expected to enhance the N content in plants grown under eCO₂. Previous studies have shown that increased N fertilization of wheat plants increases nitrate reductase activity, whereas eCO₂ reduces it, and the combined effects result in additional protein content (Pal et al., 2005; Xu et al., 2011). However, Sudderth et al. (2005) indicated that higher N availability in the presence of eCO₂ increased foliar N content in *Amaranthus viridis* (C₄ plant), but had no effect on *Solanum dulcamara* (C₃ plant). Therefore, we reason that the responses of C₃ and C₄ plants to increased N fertilization under eCO₂ are entirely different.

Several studies have indicated that the availability of N fertilization determines plant allocation of defensive compounds in response to eCO₂, which subsequently alters insect performance (Kinney et al., 1997; Hättenschwiler and Schafellner, 1999; Henn and Schopf, 2001; Saxon et al., 2004). However, changes in nutritional and defensive metabolisms of plants grown under eCO₂ and increased N fertilization, as well as insect fitness remain unclear.

Plants have evolved a series of defense systems against insects, one of which is the jasmonic acid (JA) signaling pathway (Kawazu et al., 2012). Several studies have examined the insect-induced plant defense response via JA under eCO₂, however, these responses have varied for different plant and insect species (Casteel et al., 2008; Zavala et al., 2008; Lu et al., 2018). In view of the above-mentioned facts, even if N fertilization

increases the protein content in plant tissue under eCO₂, it is not clear if this will be able to influence JA-mediated secondary metabolites.

Asian corn borer, *Ostrinia furnacalis* (order Lepidopteran), consumes C₄ plant-maize. One to seven generations of this insect occur per year in northern, cool temperate regions to southern tropical areas (Zhou et al., 1995). They cause a 10–30% yield loss in most maize production areas in China (Wang et al., 2000). Our previous study showed that the fitness of *O. furnacalis* in maize was adversely affected by an eCO₂-mediated decrease in maize nutritional quality (higher TNC:N ratio) during a field experiment (Xie et al., 2015). Based on this result and the evidence for different responses of C₃ and C₄ plants to increased N fertilization under eCO₂, as mentioned above, the interactions between maize and *O. furnacalis* may change due to the combined effects of eCO₂ and increased N fertilization.

The specific objectives of this study were to (1) quantify the effects of eCO₂ and increased N fertilization in maize on C and N primary metabolites and *O. furnacalis* induced defense-related secondary metabolites, (2) determine the successive performance of *O. furnacalis* to the host. Results from our study help to understand the metabolic mechanism

TABLE 1 | Calculations for indices for larval growth, development, and food digestibility.

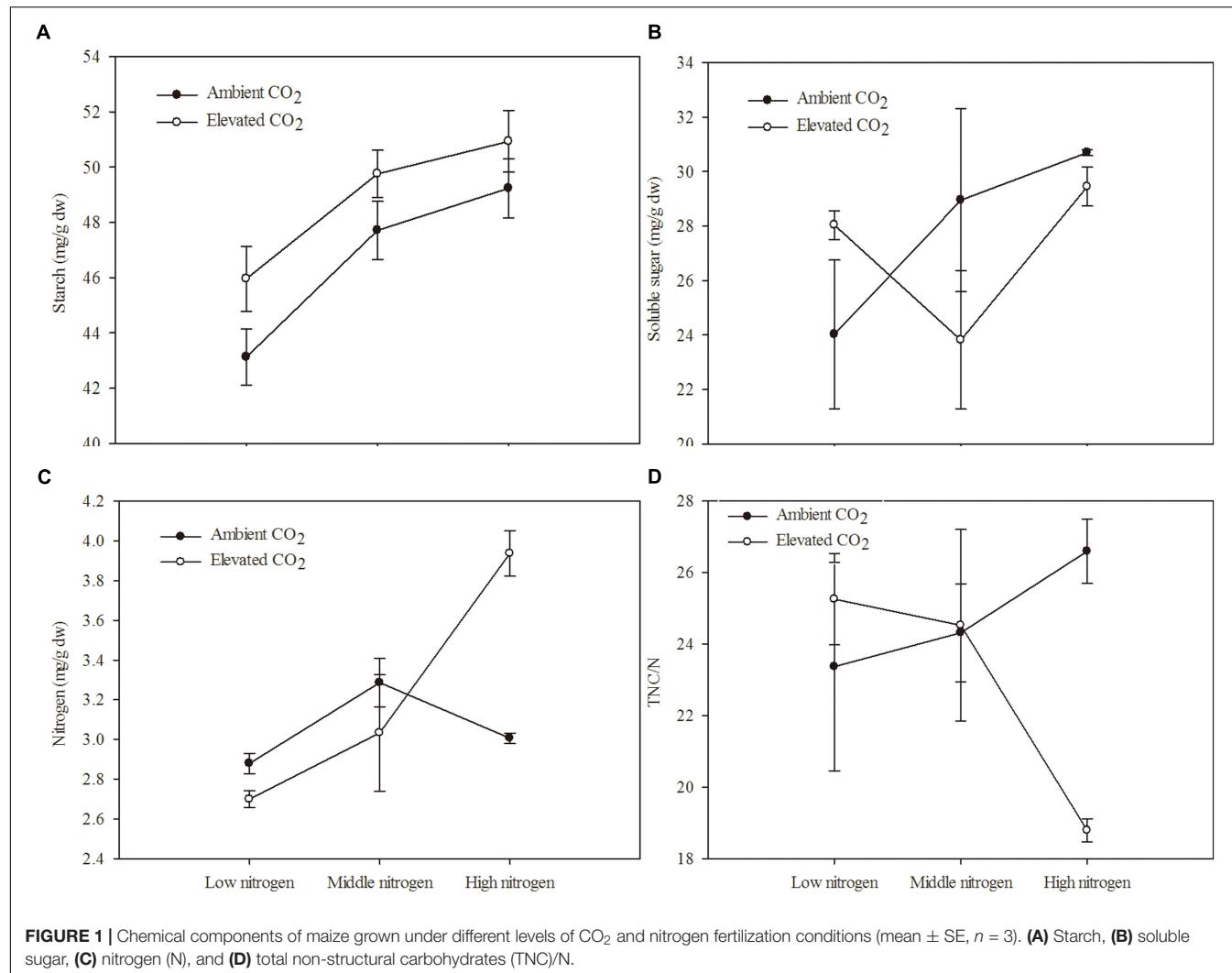
Measuring indices	Formulation
Mean relative growth rate, MRGR	MRGR = $(\log W_2 - \log W_1)/t$
Approximate digestibility (%), AD	AD = $[(Q-F)/Q]*100$
Efficiency of conversion of ingested food (%), ECI	ECI = $[(W_2 - W_1)/Q]*100$
Efficiency of conversion of digested food (%), ECD	ECD = $[(W_2 - W_1)/(Q-F)]*100$

*Q, the diet consumption (mg); F, the frass excretion (mg); W₁, the initial larval wet mass (mg), W₂, the final larval wet body mass (mg); t, days from the initial to the final day (d).

TABLE 2 | Summary of ANOVA results for effects of elevated CO₂ and increased nitrogen fertilization on chemical components of maize.

Measurement	Treatment	df	F	P
Starch	CO ₂	1	10	0.01
	N	2	23.2	<0.01
	CO ₂ *N	2	0.24	0.80
Soluble sugar	CO ₂	1	0.16	0.70
	N	2	1.71	0.24
	CO ₂ *N	2	1.8	0.23
Nitrogen	CO ₂	1	3.15	0.11
	N	2	17.83	<0.01
	CO ₂ *N	2	16.83	<0.01
TNC/N	CO ₂	1	0.11	0.75
	N	2	1.47	0.29
	CO ₂ *N	2	10.45	<0.01

*TNC, total non-structural carbohydrates; N, low N fertilization level (100 mg N/kg soil mixture), middle N fertilization level (200 mg N/kg soil mixture) and high N fertilization level (300 mg N/kg soil mixture); CO₂, ambient CO₂ concentrations (~380 ppm) and elevated CO₂ concentrations (~750 ± 15 ppm); n = 3.



of the plant and the plant-insect interactions under eCO₂ and increased N fertilization, which will subsequently aid in adjusting pest control strategies under the context of future climate change.

MATERIALS AND METHODS

Plant Preparation and Treatments With CO₂ and Nitrogen

The experiment was carried out in six environmental chambers with three environmental chambers maintained at ambient CO₂ concentrations (~ 380 ppm) and three environmental chambers maintained at elevated CO₂ concentrations ($\sim 750 \pm 15$ ppm). The ambient CO₂ was the surrounding air entering the environmental chamber facilities, whereas the elevated CO₂ was supplied from direct gas tanks. Details of the automatic control system for CO₂ levels were described by Chen et al. (2005a). The environmental chambers were maintained at $28 \pm 1^\circ\text{C}$ and 60–70% RH, with a photoperiod of 16:8 h (L:D) and illumination

($90 \mu\text{mol m}^{-2} \text{s}^{-1}$) provided by fluorescent lamp. Maize seeds were planted in trays (26.5 cm \times 18.5 cm \times 7.5 cm) filled with a sand, peat and vermiculite mixture (1:1:1). Three N fertilization levels were established, low N (100 mg N/kg soil mixture); middle N (200 mg N/kg soil mixture), and high N (300 mg N/kg soil mixture) under each CO₂ level (i.e., in each environmental chamber). In preliminary experiments, low N produced adequate growth of maize plants, while high N significantly enhanced growth. Each tray was planted with 20 seedlings, three trays were used in each N fertilization level. Of these three trays, maize plants from two trays were used for *O. furnacalis* rearing experiment, and from the remaining tray was used for metabolic analysis. After collecting tissue for metabolic analysis, the tray with maize plants was removed from the environmental chamber to avoid the wounding signals transmission among the plants. All trays were provided the same amount of water (100 mL) each day. To account for possible spatial variability from micro environmental factors, the environmental chambers were arranged in three blocks of two adjacent chambers per block.

TABLE 3 | Summary of ANOVA results for effects of elevated CO₂ and increased nitrogen fertilization on chemical defenses of maize.

Measurement	Treatment	df	F	P
JA	CO ₂	1	0.82	0.38
	N	2	48.27	<0.01
	Infestation	1	29.86	<0.01
	CO ₂ *N	2	2.94	0.07
	CO ₂ *infestation	1	0.86	0.36
	N*infestation	2	1.72	0.20
Total phenolics	CO ₂	1	4.4	0.05
	N	2	9.49	<0.01
	Infestation	1	12.46	<0.01
	CO ₂ *N	2	1.66	0.21
	CO ₂ *infestation	1	5.23	0.03
	N*infestation	2	3.73	0.04

*JA, jasmonic acid; N, low N fertilization level (100 mg N/kg soil mixture), middle N fertilization level (200 mg N/kg soil mixture), and high N fertilization level (300 mg N/kg soil mixture); CO₂, ambient CO₂ concentrations (~380 ppm) and elevated CO₂ concentrations (~750 ± 15 ppm); n = 3.

Insect Rearing

The *O. furnacalis* neonates used in this study were obtained from a laboratory colony that originated from a field population and had been maintained on a regular artificial diet (Zhou et al., 1995) for 5–6 generations in the lab. The centrifuge tubes (50 mL) were used as *O. furnacalis* larval rearing containers in the environment chamber as above description. Centrifugal tube orifice was covered with three layers of gauze. A piece of maize leaf (1 cm × 4 cm) from the same environment chamber was placed in a centrifuge tube and fed by a neonate larva (~12 h). When larva developed to the 4th instar, a short stem (5 cm long) was supplied to it instead of leaf tissue

(Zhou et al., 1995). Twenty larvae were reared in each treatment, with three replications per treatment (total 60 larvae per treatment). The leaf or stem issues were replaced with fresh leaves or stems every other day until larvae pupated. Meanwhile the unconsumed leaf- or stem-tissues and frass were collected and weighed after oven drying at 80°C for 72 h. The water content was calculated by daily drying of the fresh leaf or stem to obtain the dry weight of larval food consumption. The neonate (~12 h) and mature larvae (before pupation) fresh weight were measured and the duration (in days) of larval and pupal stages were also recorded. The conventional, ratio-based nutritional indices, including mean relative growth rate (MRGR), efficiency of conversion of ingested food (ECI) and of digested diet (ECD), and approximate digestibility (AD) were determined gravimetrically following the methods of Rayapuram and Baldwin (2006) and Chen et al. (2007). The amount of food consumption, frass produced, larval body weight, and weight gain were all calculated as dry weights. Formulas for calculation of the indices measured are shown in Table 1.

The *O. furnacalis* feeding maize plant from each treatment were used to test the activity of digestive and protective enzymes. The digestive enzymes included trypsin, total protease, lipase and amylase, the protective enzymes included catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD). 10 larvae (4–5th instar) from each treatment were selected and were grinded in physiological saline (0.9%). Homogenates were centrifuged at 10,000 × g for 10 min, and the supernatants were subjected to enzyme activity analysis according to the kit instructions (MM-126401 for Trypsin, MM-3439701 for total protease, MM-3442501 for lipase, MM-3439801 for amylase, MM-3440701 for CAT, MM-3440801 for POD, MM-3439601 for SOD, Jiangsu Kete Biological Technology Co., Ltd., China).

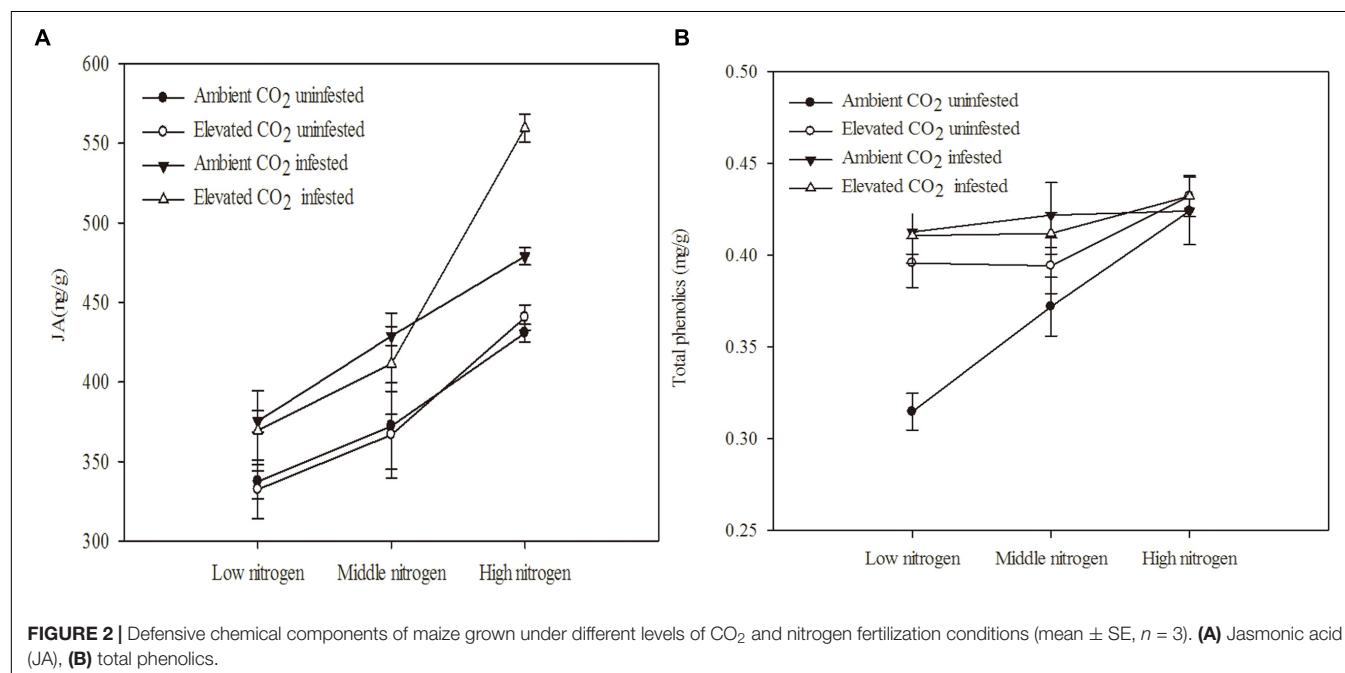


FIGURE 2 | Defensive chemical components of maize grown under different levels of CO₂ and nitrogen fertilization conditions (mean ± SE, n = 3). **(A)** Jasmonic acid (JA), **(B)** total phenolics.

Primary and Secondary Metabolites Analysis of Maize

For primary metabolic analysis, one leaf was collected at random from each maize plant on the 20th day after sowing, a total of 5 leaves were collected in each tray and mixed for leaf samples. The TNC (primarily soluble sugars and starch) were analyzed using the method of Tissue and Wright (1995). The nitrogen content was determined according to the Official Methods of Analysis of AOAC International (2009), using a Kjeltec N analyzer (Model KDY-9830; Foss automated Kjeltec instruments, Beijing, China).

For analysis of secondary defense metabolism, 2nd to 3rd instar larvae of *O. furnacalis* were placed on each maize leave. Damaged areas from approximately 1 cm around the feeding sites were taken from the leaves using a knife. Leaf samples were collected 2 h after infestation (Guo et al., 2018). One leaf was collected at random from each maize plant on the 20th day after sowing, a total of 5 leaves were harvested in each tray and mixed for leaf samples. After harvesting, leaf samples were immediately frozen in liquid nitrogen and then stored at -80°C until use. Uninfested leaves were used as controls and treated as described above. For JA analysis, frozen leaves (0.1 g) were ground in 1:10 [leaf: extracting solution (w/v) ratio]. The extracting solution contained methyl alcohol, formic acid, and pure water (15/1/4, v/v/v). Homogenates were centrifuged at 8,000 × g for 20 min. The supernatants were subjected to JA content analysis according to the kit instructions (MM-0887001, Keteshengwu, Nanjing, China). For the remaining analysis, frozen leaves (0.1 g) were ground in buffer (1:10 w/v ratio; pH 7.0–7.4). Homogenates were centrifuged at 10,000 × g for 10 min. The supernatants were then analyzed for total phenolics content, the activities of polyphenol oxidase (PPO), POD, phenylalanine ammonia-lyase (PAL), CAT, and proteinase inhibitors (PIs) according to the kit instructions (MM-123301 for total phenolics, MM-3269901 for PPO, MM-3596001 for POD, MM-089901 for PAL, MM-079901 for CAT, MM-063301 for PIs, Jiangsu Kete Biological Technology Co., Ltd., China).

Statistical Analyses

The main effects of CO₂, N, infestation and block (pairs of adjacent environmental chambers) on plant secondary defense metabolism (JA and total phenolics content; and PPO, POD, CAT, PAL, and PIs activities) were tested by four-factor ANOVA according to a model (Sudderth et al., 2005). A split-split plot design with CO₂ and block was used as the main effects, N was used as the subplot effect, and *O. furnacalis* presence or absence was used as the sub-subplot effect (SAS Institute, 2006). The effects of CO₂, N and block on plant primary metabolism (starch, soluble sugar, and N content and TNC:N ratio) and *O. furnacalis* performance (trypsin, total protease, amylase, lipase, catalase, POD, and SOD activities; and larvae, pupal stage duration, food consumption, MRGR, ECI, ECD, AD metrics) were tested by three-factor ANOVA using a split-plot design with CO₂ and block as the main effects and N as the subplot effect.

RESULTS

C and N Primary Metabolism in Maize

The C and N primary metabolism of maize was influenced by eCO₂ and increased N fertilization (Table 2 and Figures 1A–D). Relative to the ambient CO₂ treatment, the starch content of maize was significantly increased by 4.69% in plants grown under eCO₂ (Table 2 and Figure 1A). Relative to the low N fertilization treatment, plants grown with middle and high N fertilizations showed a significant increase in the starch content (9.38 and 12.41%, respectively; Table 2 and Figure 1A), and N content (9.38 and 12.42%, respectively; Table 2 and Figure 1C). However, eCO₂ and increased N fertilization did not influence the soluble sugar content and TNC:N ratio of maize, while the interaction

TABLE 4 | Summary of ANOVA results for effects of elevated CO₂ and increased nitrogen fertilization on defensive response of maize.

Measurement	Treatment	df	F	P
POD	CO ₂	1	7.89	0.01
	N	2	13.41	<0.01
	Infestation	1	7.16	0.02
	CO ₂ *N	2	0.8	0.46
	CO ₂ *infestation	1	2.04	0.17
	N*infestation	2	1.85	0.18
PPO	CO ₂	1	18.53	<0.01
	N	2	11.53	<0.01
	Infestation	1	13.6	<0.01
	CO ₂ *N	2	1.38	0.27
	CO ₂ *infestation	1	0.32	0.58
	N*infestation	2	0.17	0.84
CAT	CO ₂	1	0.04	0.84
	N	2	5.82	0.03
	Infestation	1	1.92	0.18
	CO ₂ *N	2	0.99	0.39
	CO ₂ *infestation	1	5.82	0.03
	N*infestation	2	6.54	<0.01
PAL	CO ₂	1	9.48	<0.01
	N	2	3.42	0.05
	Infestation	1	17.83	<0.01
	CO ₂ *N	2	0.87	0.43
	CO ₂ *infestation	1	1.55	0.22
	N*infestation	2	0.64	0.54
PIs	CO ₂	1	1.03	0.32
	N	2	11.47	<0.01
	Infestation	1	7.82	0.01
	CO ₂ *N	2	0.35	0.71
	CO ₂ *infestation	1	0.8	0.38
	N*infestation	2	10.84	<0.01

*PPO, polyphenol oxidase; POD, peroxidase; CAT, catalase; PAL, phenylalanine ammonia-lyase, PIs, proteinase inhibitors; N, low N fertilization level (100 mg N/kg soil mixture), middle N fertilization level (200 mg N/kg soil mixture), and high N fertilization level (300 mg N/kg soil mixture); CO₂, ambient CO₂ concentrations (~380 ppm) and elevated CO₂ concentrations (~750 ± 15 ppm); n = 3.

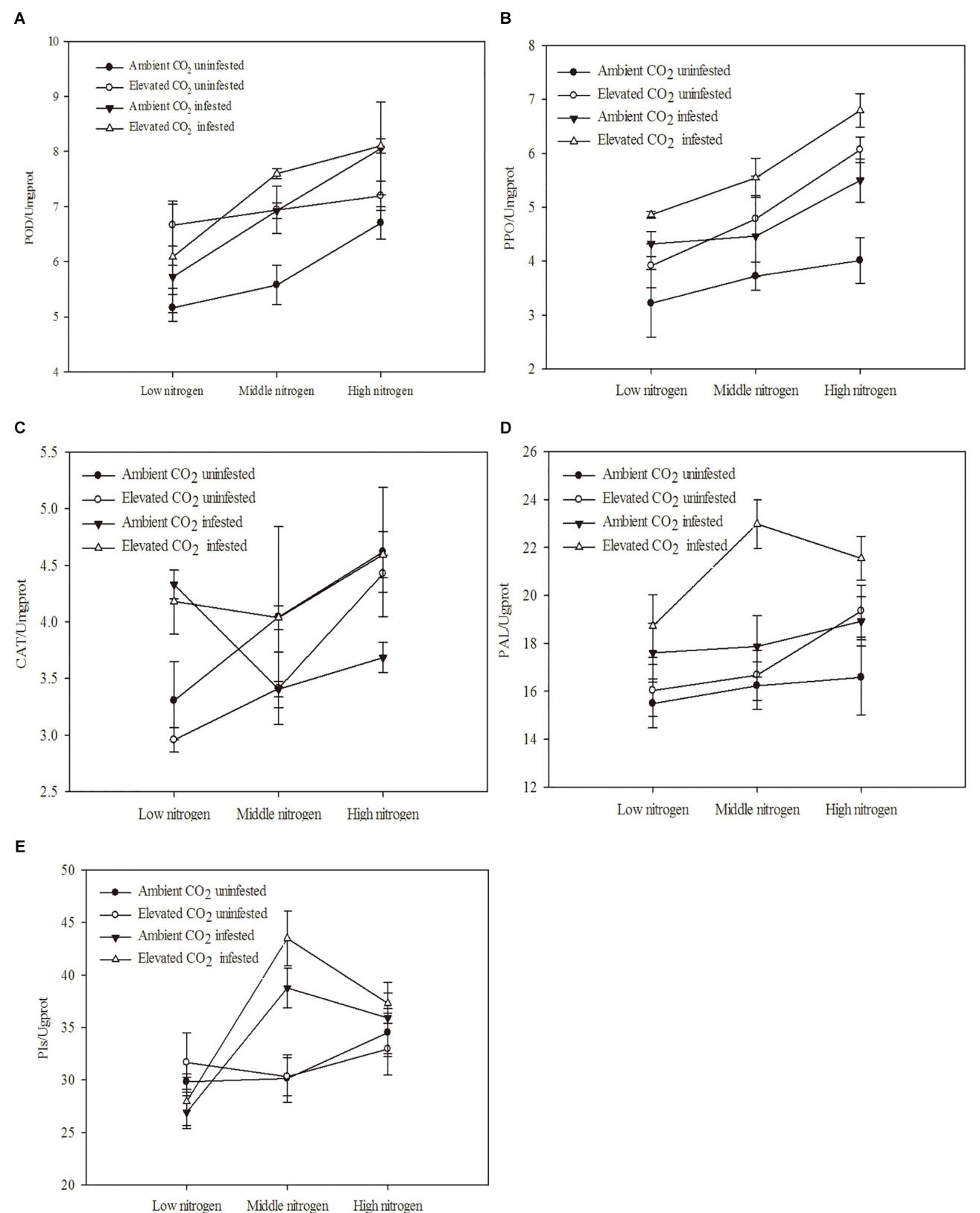


FIGURE 3 | Defensive response of maize grown under different levels of CO₂ and nitrogen fertilization conditions (mean \pm SE, $n = 3$). **(A)** Peroxidase (POD) activity, **(B)** polyphenol oxidase (PPO) activity, **(C)** catalase (CAT) activity, **(D)** phenylalanine ammonia-lyase (PAL) activity, and **(E)** proteinase inhibitors (Pls).

between them was significant on N content and TNC:N ratio (Table 2 and Figures 1B,D).

***O. furnacalis* Induced Defense-Related Secondary Metabolites in Maize**

The chemical defense of maize was significantly influenced by eCO₂, increased N fertilization and *O. furnacalis* infestation (Table 3 and Figures 2A,B). Relative to ambient CO₂, the total phenolics content of maize was increased by 5.13% under eCO₂ (Table 3 and Figure 2B). Relative to low N fertilization, the JA content of maize increased by 11.64 and 34.99% in middle and high N fertilization treatments, while the total phenolics content increased by 11.75% in the high N fertilization (Table 3 and Figure 2A). *O. furnacalis* infestation increased the JA and total phenolics content of maize by 15.06 and 7.69%, respectively, compared with uninfested tissues (Table 3 and Figures 2A,B). Furthermore, the interactions between CO₂ and infestation and between N and infestation on total phenolics content were significant (Table 3 and Figure 2B).

The defensive enzyme activities of maize were significantly influenced by eCO₂, increased N fertilization and *O. furnacalis* infestation (Table 4 and Figures 3A–E). Relative to ambient CO₂, the POD, PPO and PAL activities of the maize were increased by eCO₂ (Table 4 and Figures 3A,B,D). Relative to low N fertilization, the POD, PAL, CAT, and PIs activities were increased in the middle and high N fertilization treatments, whereas the PPO activities were increased in only the high N fertilization (Table 4 and Figures 3A–E). *O. furnacalis* infestation increased the POD, PPO, PAL, and PIs activities of maize, compared with the uninfested tissues (Table 4 and Figures 3A–C,E). The interactions between CO₂ and infestation and between N and infestation on CAT activity, and between N and infestation on PIs were significant (Table 4 and Figures 3C,E).

The Growth and Development of *O. furnacalis*

The digestive and protective system enzyme activities varied for *O. furnacalis* feeding on maize grown under different levels of CO₂ and N fertilization (Table 5 and Figures 4A–G). Compared with ambient CO₂, amylase activity decreased and SOD activity increased for *O. furnacalis* feeding on maize grown under eCO₂ (Table 5 and Figures 4C,G). Compared with low N fertilization, the trypsin, total protease and amylase activities decreased for *O. furnacalis* feeding on maize grown in the middle and high N fertilization treatments, whereas the CAT and SOD activities increased for *O. furnacalis* feeding on maize grown at high N fertilization and two increasing N fertilization treatments, respectively (Table 5 and Figures 4A–C,E).

The development and digestibility of *O. furnacalis* were changed after feeding on maize grown under different levels of CO₂ and N fertilization (Table 6 and Figures 5A–H). Relative to ambient CO₂, eCO₂ extended the duration of the larval and pupal stage by 3.99 and 7.13%, respectively; reduced larval body mass by 5.64%; and decreased MRGR, ECI, and ECD by 4.15, 18.89, and 19.23%, respectively (Table 6 and Figures 5A–C,E,F). Relative to low N fertilization, the middle and high N fertilization

TABLE 5 | Summary of ANOVA results for effects of elevated CO₂ and increased nitrogen fertilization on digestive and protective system enzymes of *Ostrinia furnacalis*.

Measurement	Treatment	df	F	P
Trypsin	CO ₂	1	2.78	0.13
	N	2	13.86	<0.01
	CO ₂ *N	2	0.95	0.43
Total protease	CO ₂	1	4.09	0.08
	N	2	10.48	<0.01
	CO ₂ *N	2	0.85	0.46
Amylase	CO ₂	1	7.55	0.02
	N	2	10.88	<0.01
	CO ₂ *N	2	0.55	0.6
Lipase	CO ₂	1	0.67	0.43
	N	2	2.02	0.19
	CO ₂ *N	2	1.23	0.34
CAT	CO ₂	1	0.1	0.76
	N	2	7.05	0.02
	CO ₂ *N	2	0.04	0.96
POD	CO ₂	1	4.23	0.07
	N	2	1.02	0.40
	CO ₂ *N	2	1.07	0.39
SOD	CO ₂	1	6.86	0.03
	N	2	6.19	0.02
	CO ₂ *N	2	0.81	0.48

*CAT, catalase; POD, peroxidase; SOD, superoxide dismutase, N, low N fertilization level (100 mg N/kg soil mixture), middle N fertilization level (200 mg N/kg soil mixture), and high N fertilization level (300 mg N/kg soil mixture); CO₂, ambient CO₂ concentrations (~380 ppm) and elevated CO₂ concentrations (~750 ± 15 ppm); n = 3.

treatments extended the larval stage duration by 4.75 and 8.32%, and decreased MRGR by 4.32 and 9.52%, respectively; in the high N fertilization, the larval body mass and ECI were decreased by 7.31 and 26.12%, respectively (Table 6 and Figures 5A,C,E,F).

DISCUSSION

Both eCO₂ and increased N fertilization increased the starch content of the maize in this experiment. A previous study found that the C₄ photosynthesis in maize was enhanced by increasing N nutrition under eCO₂ (Cousins and Bloom, 2003), thus the accumulation of photosynthetic products (carbohydrate) might increase in C₄ plants. The eCO₂ did not reduce the N content. However, increased N fertilization enhanced the N content in this experiment. Thus, eCO₂ and increased N fertilization increased C and N primary metabolites, although the combined effects of eCO₂ and increased N fertilization did not influence the TNC:N ratio. However, our previous results from a different study indicate that eCO₂ decreases the N content and increases the TNC:N ratio in maize. In this experiment, the altered response of N content to eCO₂ may be due to the close interaction between CO₂ and N fertilization (P < 0.05, Pal et al., 2005). In other words, increased N fertilization could offset the decreasing N content of maize grown under eCO₂.

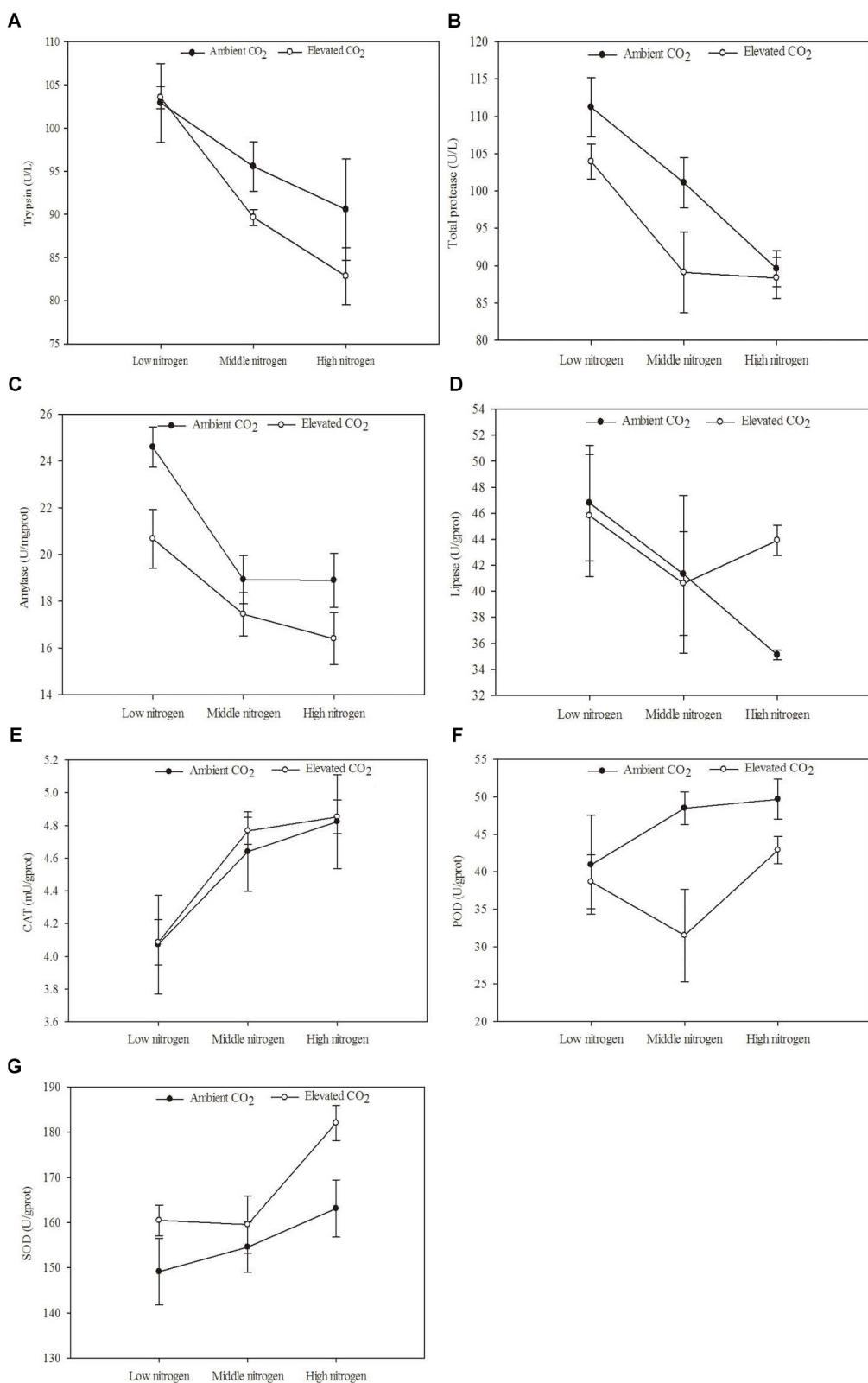


FIGURE 4 | Defense chemical components of *Ostrinia furnacalis* feeding on maize grown under different levels of CO₂ and nitrogen fertilization conditions (mean \pm SE, $n = 3$). **(A)** Trypsin activity, **(B)** total protease activity, **(C)** amylase activity, **(D)** lipase activity, **(E)** catalase (CAT) activity, **(F)** peroxidase (POD) activity, and **(G)** superoxide dismutase (SOD) activity.

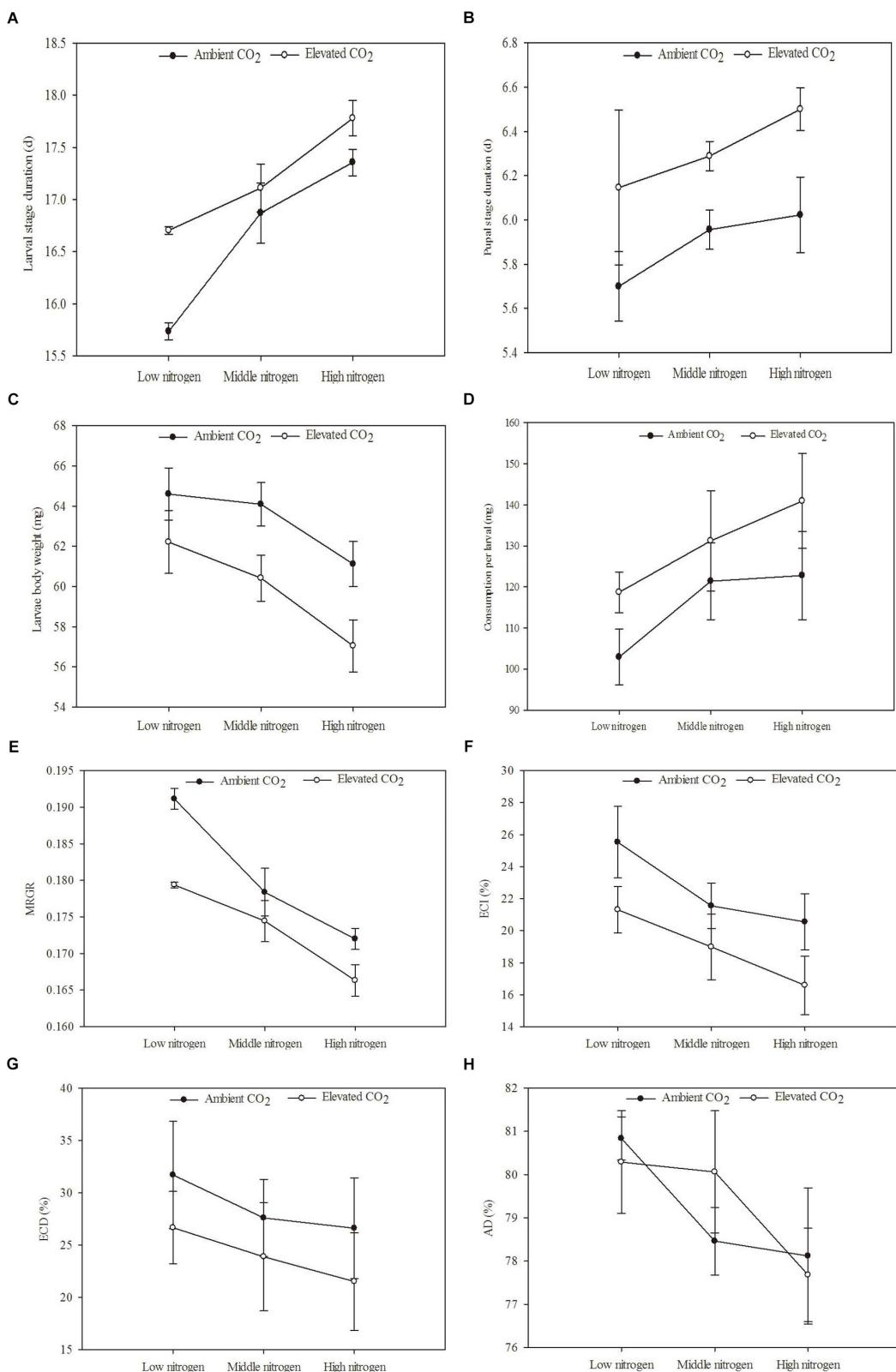


FIGURE 5 | Development and digestibility of *O. furnacalis* feeding on mazie grown under different levels of CO₂ and nitrogen fertilization conditions (mean \pm SE, $n = 3$). **(A)** Larvae stage duration, **(B)** pupal stage duration, **(C)** larvae body mass, **(D)** food consumption, **(E)** mean relative growth rate (MRGR), **(F)** efficiency of conversion of ingested food (ECI), **(G)** efficiency of conversion of digested food (ECD), and **(H)** approximate digestibility (AD).

TABLE 6 | Summary of ANOVA results for effects of elevated CO₂ and increased nitrogen fertilization on development and digestibility of *O. furnacalis*.

Measurement	Treatment	df	F	P
Larvae stage duration	CO ₂	1	15.14	<0.01
	N	2	30.97	<0.01
	CO ₂ *N	2	2.43	0.15
Pupal stage duration	CO ₂	1	8.02	0.02
	N	2	1.78	0.23
	CO ₂ *N	2	0.09	0.91
Body mass	CO ₂	1	7.44	0.03
	N	2	4.36	0.05
	CO ₂ *N	2	0.17	0.85
Food consumption	CO ₂	1	5	0.06
	N	2	3.73	0.07
	CO ₂ *N	2	0.14	0.87
MRGR	CO ₂	1	16.21	<0.01
	N	2	27.64	<0.01
	CO ₂ *N	2	1.8	0.23
ECI	CO ₂	1	6.89	0.03
	N	2	4.33	0.05
	CO ₂ *N	2	0.14	0.87
ECD	CO ₂	1	5.81	0.04
	N	2	2.48	0.15
	CO ₂ *N	2	0.05	0.95
AD	CO ₂	1	0.06	0.81
	N	2	3.56	0.08
	CO ₂ *N	2	0.74	0.51

*MRGR, mean relative growth rate; ECI, efficiency of conversion of ingested food; ECD, efficiency of conversion of digested food; AD, approximate digestibility; N, low N fertilization level (100 mg N/kg soil mixture), middle N fertilization level (200 mg N/kg soil mixture), and high N fertilization level (300 mg N/kg soil mixture); CO₂, ambient CO₂ concentrations (~380 ppm) and elevated CO₂ concentrations (~750 ± 15 ppm); n = 3.

Numerous experiments have shown that defensive metabolisms of plants are influenced by eCO₂, increased N fertilization, or insect infestation (Lou and Baldwin, 2004; Guo et al., 2012; Zavala et al., 2013; Guo et al., 2018; Lu et al., 2018). However, relatively little is known about their combined effects on the defensive metabolism of plants. In this study, defensive metabolisms of maize were enhanced by eCO₂, increased N fertilization and insect infestation. We observed that the eCO₂, increased N fertilization or insect infestation effects were additive, but there were few interactions between factors. The accumulation of JA level and activation of antioxidative enzymes (POD, PPO, and CAT) and PIs play an important role in regulating chewing-induced resistance of plants to insects (Scott et al., 2010; Nahar et al., 2011; Kawazu et al., 2012; Kerchev et al., 2012; Wang and Wu, 2013; War et al., 2015). In this study, both eCO₂ and increased N fertilization improved defensive enzymes (POD, PPO, and PAL) activities, and increased N fertilization also improved JA level, CAT, and PIs activities. Similarly, a previous study found that the defensive enzyme activities in maize were increased under eCO₂ or increased N fertilization (Zhang et al., 2007; Zhao et al., 2008). Lou and Baldwin (2004)

indicated that JA levels decreased in plants grown with low N fertilization, which was due to the decreased expression levels of JA-related genes in plants grown in low N fertilization. Our observations may be explained by sufficient CO₂ and N driving the maize metabolites, which enhanced the JA level and defensive enzymes activities at the same time (Nunes-Nesi et al., 2010). Like other chewing insects, *O. furnacalis* infestation can also induce the defensive metabolism of maize (Guo et al., 2018). In the present study, *O. furnacalis* infestation increased JA level, total phenolic content and defensive enzyme (POD, PPO, PAL, and PIs) activities even under eCO₂ and increased N fertilization conditions, i.e., the maize defensive response still exist under eCO₂ and increased N fertilization. The activity of PAL, which is a principle enzyme involved in a rate-limiting step in phenolic biosynthesis, could be induced by some environmental factors (Hartley et al., 2000; Zhao et al., 2009). In this study, PAL activity was enhanced by increased N fertilization and *O. furnacalis* infestation, which subsequently raised the total phenolic content in these treatments. At the same time, there is a positive interaction between *O. furnacalis* infestation and CO₂ or N fertilization on the total phenolics content in maize plants. We conclude that the defensive metabolism of maize plants grown under elevated CO₂ and higher N fertilization could be enhanced, especially during *O. furnacalis* infestation.

Plant proteinase inhibitors (PIs) are able to reduce the feeding fitness of chewing insects by suppressing insect gut proteases (Govind et al., 2010). The results in this experiment also indicated that increased N fertilization increased PIs activity in maize, and accordingly, increased N fertilization decreased the activities of *O. furnacalis* digestive enzymes (trypsin, total protease, and amylase). The eCO₂ also decreased amylase activity, while both eCO₂ and high N fertilization increased protective enzyme activities. Thus, *O. furnacalis* digestive ability was decreased and the defensive response was enhanced during feeding on maize plants grown under eCO₂ and increased N fertilization.

Both eCO₂ and increased N fertilization extended the larvae development time; and decreased the MRGR, body mass and ECI of *O. furnacalis*. These observations could be explained by the results above, due to the fact that TNC:N ratio did not change, but the defensive metabolism of maize plants was increased by eCO₂ and increased N fertilization. Thus, the increased defensive metabolism subsequently may increase *O. furnacalis* defensive response, slowed growth and decreased food digestibility and utilization (Xie et al., 2013; Guo et al., 2018; Lu et al., 2018). Henn and Schopf (2001) demonstrated increased consumption and decreased ECI for *Lymantria dispar* fed on trees grown under ambient CO₂ and low N fertilization. Considering our results, the different effects of eCO₂ and increased N fertilization on ECI may be due to the N availability and the level of defensive metabolism for host species.

CONCLUSION

Our data demonstrate that eCO₂ and increased N fertilization increased C and N primary metabolites. The combined effects of eCO₂ and increased N fertilization did not influence the TNC:N

ratio, because increased N fertilization could offset the decreasing N content of maize grown under eCO₂. The resistance-related secondary metabolites (with or without *O. furnacalis* induced) in maize were enhanced by eCO₂ and increased N fertilization, which increased the *O. furnacalis* defensive response, slowed its growth; and decreased its food digestibility and utilization. Thus, increased N fertilization will increase starch and N accumulation, do not influence the TNC:N ratio. But increased N fertilization promote resistance of maize to *O. furnacalis* feeding, which may decrease the fitness of *O. furnacalis* to its host in future eCO₂ scenarios.

Furthermore, the metabolic mechanisms of plants and insects may vary with plant and insect species, the insect fitness over multiple generations to host plant and insect enemy behavior may also change under eCO₂ and increased N fertilization. The optimal N application rates should be employed to improve plant growth and insect population control under eCO₂. Thus, more research will be needed to elucidate the effects of eCO₂ and

increased N fertilization on interaction of plant-insect, which will help to predict the plant damage in agroecosystem.

AUTHOR CONTRIBUTIONS

HuX wrote the manuscript. HaX designed the experiments. HaX and SW performed the experiments. ZW and KH provided the insect, reagents, and materials.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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