Elevated CO$_2$ and O$_3$ alter the feeding efficiency of Acyrthosiphon pisum and Aphis craccivora via changes in foliar secondary metabolites

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Elevated CO$_2$ and O$_3$ can affect aphid performance via altering plant nutrients, however, little is known about the role of plant secondary metabolites in this process, especially for aphids feeding behaviors. We determined the effects of elevated CO$_2$ and O$_3$ on the growth and phenolics of alfalfa (*Medicago sativa*) and feeding behaviors of the pea aphids (*Acyrthosiphon pisum*) and cowpea aphids (*Aphis craccivora*). Elevated CO$_2$ improved plant growth, but could not completely offset the negative effects of elevated O$_3$. Elevated O$_3$ increased foliar genistin content at the vegetative stage, increased ferulic acid at the reproductive stage, and elevated CO$_2$ increased those at both stages. Simultaneously elevated CO$_2$ and O$_3$ increased foliar ferulic acid content at the reproductive stage and increased genistin content at both stages. For pea aphids, feeding efficiency was reduced under elevated CO$_2$ at the reproductive stage and decreased under elevated O$_3$ at the vegetative stage. For cowpea aphids, feeding efficiency was increased under elevated CO$_2$ at the vegetative stage and decreased under elevated O$_3$ at both stages. Simultaneously elevated CO$_2$ and O$_3$ decreased both aphids feeding efficiency. We concluded that CO$_2$ and O$_3$ independently or interactively had different effects on two aphids feeding behaviors through altering foliar ferulic acid and genistin contents.

Global atmospheric concentrations of greenhouse gases (e.g., CO$_2$ and O$_3$) have increased due to human activities since industrialization. The CO$_2$ concentration has increased from 280 μL/L to approximately 400 μL/L in 2017 (https://www.co2.earth/), and the tropospheric O$_3$ concentration has increased from 10 nL/L to 50 nL/L in 2009$^1$. Furthermore, the concentrations of CO$_2$ and O$_3$ are expected to continue to increase$^2$. Increases in CO$_2$ and O$_3$ concentrations have been anticipated to greatly influence agricultural and forest ecosystems$^3,4$; they can directly affect plant growth, primary and secondary metabolisms, and indirectly alter interactions between plants and herbivorous insects$^5–7$.

Elevated CO$_2$ typically stimulates plant growth, decreases plant nitrogen concentrations, and increases the carbon:nitrogen (C:N) ratio$^8,9$. Conversely, O$_3$, as an oxidizing agent, enters the leaf interior through the stomata and causes leaf tissue damage, thereby inhibiting plant growth$^9$; but the responses of foliar nutrients to elevated O$_3$ are species-specific and depend on the duration of O$_3$ exposure$^{10}$. Elevated CO$_2$ and O$_3$ generally increase plant secondary metabolites, such as phenolics, including total phenolics, condensed tannins, and flavonoids$^6,7,11$, and they also interactively affect plant metabolism that elevated CO$_2$ tends to offset the induction of phenolics by elevated O$_3$.$^6,12$ In addition, the plant chemical composition and concentrations often change with ontogenetic stage$^{11}$. For example, phenolic acids, i.e., trans-2-hydroxycinnamic, rosmarinic, vanillic, chlorogenic, gallic, and cinnamic acids, dominate during the early vegetative stage; whereas flavonoids, including amentoflavone, apigenin, quercetin, luteolin, coumarin, and rutin, predominate during the other growth stages in sweet marjoram (*Origanum majorana*)$^{14}$. Furthermore, the concentrations of glucosinolates in *Arabidopsis thaliana* and phenolic

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Elevated CO$_2$ increased plant aboveground biomass only at the vegetative stage (Fig. 1d) and increased plant CO$_2$ nor elevated O$_3$ affected plant flowering and podding time, though simultaneously elevated CO$_2$ and O$_3$ is lacking.

Many previous work has shown that elevated CO$_2$ or O$_3$ reduce the performance of most leaf-chewing insects due to decreased nitrogen concentrations in plants. On the other hand, the increased concentration of plant secondary metabolites may partially explain the reduced performance of leaf-chewing insects under elevated CO$_2$ and O$_3$, because the secondary compounds can induce or decline the detoxication activities of the digestion system, which thereby prolonging developmental time and reducing growth rates. For the piercing-sucking insects e.g., aphids, many studies have shown that elevated CO$_2$, or O$_3$, can increase aphid performance via promoting plant nitrogen based nutrition. However, Johnson et al. find that the fecundity of the pea aphid (Acyrthosiphon pisum) responds differently to elevated CO$_2$ when fed on five different alfalfa (Medicago sativa) cultures. Furthermore, the population abundance of Rhopalosiphum padi is reduced under elevated CO$_2$ when fed on tall fescue (Schedonorus arundinaceus), but is increased when fed on barley (Hordeum vulgare). These results presumably indicate that the same aphid species that fed on different host plants exhibits different responses to climate changes. On the other hand, different aphid species or even genotypes that fed on the same host plants may also perform differently under elevated CO$_2$ and O$_3$. For example, the specialist Brevicoryne brassicae are larger and accumulate more fat, while no changes are found in the generalist Myzus persicae reared on Brassica oleracea under elevated CO$_2$. Therefore, the responses of aphids to elevated CO$_2$ and O$_3$ seem to be species-specific, demonstrating decreased, increased, or unchanged population abundance, growth, and fecundity,

Plant secondary metabolites may also contribute to the idiosyncratic responses of aphids to climate changes, though few studies have focused on it. For the aphids that feed exclusively on the phloem sap, plant secondary metabolites mainly affect aphid feeding behavior rather than the digestion system because the secondary metabolites rarely distribute in the phloem sap. Many plant secondary compounds, including alkaloid, luteolin, genistein, apigenin, and saponin etc., can negatively affect the penetration pathway stage of aphid feeding. For example, caffeic, ferulic, and sinapic acids disfavor the grain aphid (Sitobion avenue) feeding by prolonging the early pathway phases of probing, increasing the number of probing, and reducing salivation into sieve elements and ingestion of phloem sap. Furthermore, the same secondary metabolites seem to have different effects on feeding activities of different aphid species. For example, the total time of probing of Aphis fabae, Aphis craccivora, A. pism, and M. persicae increases with a reduced alkaloid content in narrow-leaved lupins (Lupinus angustifolius), whereas the alkaloid content has no influence on Macrosiphum albifrons; furthermore, when fed on the alkaloid-rich cultivar Azuro, a reduced occurrence of phloem phases is observed, especially for A. pism and A. Fabae, whereas M. albifrons shows the longest phloem phase. However, it is also reported that secondary compounds may act as probing stimulants of aphids. Therefore, the increases in plant secondary metabolites may increase or decrease epidermis and mesophyll resistance against aphids during pathay and probing feeding stages under elevated CO$_2$ or O$_3$, and the differential feeding responses of aphid species to plant secondary metabolites might contribute to their idiosyncratic responses to elevated CO$_2$ or O$_3$. However, the evidence about how climate changes, especially for elevated O$_3$, alter the aphid feeding activities via plant secondary metabolites is lacking.

The current study aimed to investigate how elevated CO$_2$ and O$_3$, alone or in combination, alter plant secondary metabolites at different ontogenetic stages and produce cascading effects on aphid feeding behaviors. By using 12 field open-top chambers with four treatments (control, elevated CO$_2$, elevated O$_3$, and elevated CO$_2$ and O$_3$), we measured plant growth traits, secondary metabolites, and feeding behaviors of the pea aphid (Acyrthosiphon pisum Harris) and the cowpea aphid (Aphis craccivora Koch) on alfalfa (Medicago sativa). Our specific objectives were to determine: (1) the effects of elevated CO$_2$ and O$_3$ on plant growth traits and secondary metabolites phenolics of alfalfa, and (2) the feeding behaviors of the two species of aphids when fed on alfalfa grown under different treatments.

**Results**

**Responses of plant growth traits to elevated CO$_2$ and O$_3$.** Elevated CO$_2$, O$_3$, plant developmental stage, and their interactions significantly influenced alfalfa growth (Table 1). Elevated CO$_2$ did not significantly alter the plant chlorophyll content but did increase the plant net photosynthetic rate at the vegetative stage, while decreased them at the reproductive stage (significant CO$_2 \times$ developmental stage interaction, Table 1, Fig. 1a,b). Elevated CO$_2$ increased plant aboveground biomass only at the vegetative stage (Fig. 1d) and increased plant belowground biomass only at the reproductive stage (Fig. 1e). Elevated CO$_2$ also increased the numbers of flowers and pods (Fig. 1g,h). Contrary to elevated CO$_2$, O$_3$ fumigation decreased the plant chlorophyll content, net photosynthetic rate, and aboveground biomass at both developmental stages, and decreased plant belowground biomass at the vegetative stage, regardless of CO$_2$ levels (Table 1, Fig. 1a,b,d and e). Furthermore, O$_3$ fumigation alone or in combination with CO$_2$, also decreased the numbers of flowers and pods (Fig. 1g,h). Neither elevated CO$_2$, nor elevated O$_3$ affected plant flowering and podding time, though simultaneously elevated CO$_2$ and O$_3$, significantly delayed both (Table 1, Fig. 1f). The plant height was only significantly influenced by developmental stage, with significantly higher plant height at the reproductive stage than at the vegetative stage (Table 1, Fig. 1c).

**Responses of plant secondary metabolites to elevated CO$_2$ and O$_3$.** Elevated CO$_2$ significantly influenced foliar rutin, genistein, ferulic acid, and genistin concentrations (Table 2). Elevated O$_3$ significantly affected foliar kaempferol, apigenin, genistein, p-coumaric acid, ferulic acid, and genistin contents (Table 2).
Table 1. Effects of CO2, O3, plant developmental stage, and their interactions on alfalfa growth traits. F and P values from MANOVAs are shown. P values < 0.05 are bolded.

| Plant growth traits | CO2 stage    | O3 stage    | Developmental stage | CO2 × O3 stage | O3 × developmental stage | CO2 × O3 × developmental stage |
|---------------------|--------------|-------------|---------------------|----------------|--------------------------|---------------------------------|
|                     | F            | P           | F                   | P              | F                        | F                               |
| Photosynthetic rate | 12.416       | 0.001       | 122.083             | < 0.001        | 807.924                  | < 0.001                         |
| Chlorophyll content | 0.070        | 0.792       | 19.599              | < 0.001        | 25.991                   | < 0.001                         |
| Plant height        | 0.913        | 0.341       | 1.006               | 0.317          | 218.391 < 0.001          | 11.098 0.001                    |
| Aboveground biomass | 0.001        | 0.975       | 50.332              | < 0.001        | 1477.290 < 0.001         | 3.541 0.062                     |
| Belowground biomass | 8.674        | 0.004       | 7.223               | 0.008          | 296.733 < 0.001          | 0.664 0.417                     |
| Flowering time      | 2.301        | 0.180       | 4.279               | 0.084          | 8.387 0.027              |                                 |
| Podding time        | 0.521        | 0.497       | 0.646               | 0.452          | 1.819 0.226              |                                 |
| Number of flowers per plant | 4.126 | 0.045 | 50.080 < 0.001 |                                 |
| Number of pods per plant | 4.109 | 0.047 | 168.686 < 0.001 | 13.533 < 0.001 |
Discussion
Elevated CO₂ and O₃ affect the performance of herbivorous insects mainly by altering host plant primary and secondary metabolites. Phenolics are important secondary metabolites for plants in defence against herbivory. In the current study, elevated CO₂ and O₃ altered alfalfa growth traits and concentrations of phenolics such as genistein, ferulic acid, p-coumaric acid, and apigenin, which were antifeedants or feeding stimuli to aphids according to our exogenous application bioassay. Thus, aphid feeding efficiency was differentially altered by these changes in secondary metabolites. Furthermore, the responses of plant secondary metabolites and corresponding aphid feeding activities to elevated CO₂ and O₃ varied between plant developmental stages.
The positive effects of elevated CO₂ on the plant photosynthesis, growth, and seed yield in legume alfalfa are consistent with the results of a previous work that has studied soybean (Glycine max)⁴⁵. However, elevated O₃ negatively affected alfalfa growth, and these reductions in plant photosynthesis and growth under elevated O₃ may be due to the generation of ROS damage to photosynthetic processes such as the synthesis of rubisco⁴⁶. Our finding also showed that elevated CO₂ did not offset the negative effects of elevated O₃ on the plant growth. This finding is in contrast to other studies in which elevated CO₂ has been shown to ameliorate the negative effects of elevated O₃⁴⁷,⁴⁸. These contradictory results may be due to the interactions between CO₂ and O₃ depending on plant species⁴⁹ and plant developmental stages⁵⁰.

In addition to having effects on alfalfa growth traits, elevated CO₂ and O₃ altered the concentrations of the foliar secondary metabolites phenolics, such as rutin, ferulic acid, genistin, apigenin, and p-coumaric acid. For example, ferulic acid and genistin contents were significantly increased at both plant developmental stages under elevated CO₂. Plant phenolics are formed from phenylalanine via the shikimic acid-phenylpropanoid pathway⁵⁰, and the biosynthesis of phenylpropanoids requires the efficient flow of carbon into phenylalanine biosynthetic⁵¹. This process can be regulated by phytohormones, such as JA (jasmionic acid), SA (salicylic acid), and ET (ethylene), which can be affected by elevated CO₂⁵²,⁵³. For example, elevated CO₂ increases the concentration of SA-regulated phenolics, such as flavonoids (e.g., quercetin, kaempferol, and fisetin) in Malaysian young ginger (Zingiber officinale Roscoe)⁵⁴, but reduces the concentration of JA-regulated isoflavonoids, such as genistein in soybean (G. max)⁵⁵. Although our results are not absolutely consistent with these studies mentioned above, we all indicate that individual phenolic compounds differentially respond to elevated CO₂. In addition, plant secondary metabolites, such as alkaloids and phenolics, often change dramatically as plants develop⁵⁶,⁵⁷. Furthermore, plant developmental stages interact with atmospheric changes to influence the secondary metabolism⁵⁸. However, most studies have investigated only one developmental stage. For example, six of the nine sympatric British grassland species studied exhibits a significant increase in one or more secondary metabolites throughout seedling ontogeny⁵⁹. Few studies have focused on multiple ontogenetic stages, such as the seedling stage, vegetative juvenile stage, and mature stage⁶⁰. Our study included two plant developmental stages, the vegetative and reproductive stages. We found that the responses of foliar ferulic acid and genistin contents to elevated CO₂ and O₃ were influenced by the plant developmental stage. For example, genistin content was increased only at the vegetative stage, ferulic acid content was increased only at the reproductive stage under elevated O₃, and the foliar apigenin, kaempferol, and genistin contents were much higher at the vegetative stage than those at the reproductive stage. The heterogeneous defence chemical composition between vegetative and reproductive stages might be explained by that the direction of changes in defensive compounds during the transition from juvenile to mature stage depends on the types of compounds in herbs⁶¹. An increase in phenolic content under O₃ fumigation is also commonly reported⁶², though elevated O₃ has been shown to damage plant photosynthesis⁶³. These changes in phenolics under elevated O₃ may be due to the increase in the activities of phenylalanine-ammonium lyase (PAL) and chalcone synthase enzymes (CHS), which are key enzymes in the biosynthesis of phenolics⁶⁴. The increased phenolics may also act as antioxidants against oxidative stress caused by O₃⁶⁵.

Plant secondary metabolites can affect the behavior, growth, and development of herbivorous insects⁶⁶,⁶⁷. According to our bioassay, ferulic acid and p-coumaric acid acted as feeding stimulants of the cowpea aphids, while ferulic acid did not affect the pea aphids, and p-coumaric acid acted as an antifeedant of the pea aphids. These results suggest that a single compound can have different and even opposite effects on these two species of aphids. Similar results have been reported for p-coumaric acid and ferulic acid, which are phagostimulants for the stem borer (Chilo partellus Swinhoe)⁶⁸, but feeding inhibitors for maize weevil (Sitophilus zeamais Motschulsky)⁶⁹. However, studies have concluded that p-coumaric acid and ferulic acid have negative effects on the performance of the grain aphid (S. avenae)⁶⁶. The discrepancy among these studies might be due to the feeding guilds or food habits of herbivores, such as specialist and generalist responding differently to plant secondary chemistry⁷⁰,⁷¹, reflecting different coevolution between insects and plant defence. In this study, genistin acted as an antifeedant of both the pea aphids and cowpea aphids, which had been demonstrated to negatively affect stinkbugs (Nezara viridula and Piezodorus guildinii) and whitefly (Bemisia tabaci)⁷²,⁷³. They all seem to imply that genistin may be important secondary compounds conferring resistance to many insects that belong to different feeding guilds. Our study also indicated that p-coumaric acid did not play a key role in responses of the cowpea aphids feeding

### Table 2. Effects of CO₂, O₃, plant developmental stage, and their interactions on alfalfa foliar secondary metabolite contents. F and P values from MANOVAs are shown. P values < 0.05 are bolded.

| Secondary metabolites | CO₂×O₃ | CO₂×developmental stage | O₃×developmental stage | CO₂×O₃×developmental stage |
|-----------------------|--------|-------------------------|-----------------------|-----------------------------|
|                       | F      | P                       | F                     | P                           |
| Kaempferol            | 1.666  | 0.218                   | 8.618                 | 0.011                       |
|                       | F      | P                       | 40.404                | <0.001                      |
|                       | F      | P                       | 0.162                 | 0.693                       |
|                       | F      | P                       | 2.099                 | 0.169                       |
|                       | F      | P                       | 0.003                 | 0.954                       |
|                       | F      | P                       | 1.393                 | 0.257                       |
| Rutin                 | 7.512  | 0.016                   | 4.154                 | 0.061                       |
|                       | F      | P                       | 1.049                 | 0.323                       |
|                       | F      | P                       | 49.999                | <0.001                      |
|                       | F      | P                       | 6.010                 | 0.028                       |
|                       | F      | P                       | 31.700                | <0.001                      |
|                       | F      | P                       | 3.883                 | 0.069                       |
| Apigenin              | 2.688  | 0.123                   | 56.624                | <0.001                      |
|                       | F      | P                       | 170.928               | <0.001                      |
|                       | F      | P                       | 3.268                 | 0.092                       |
|                       | F      | P                       | 0.016                 | 0.901                       |
|                       | F      | P                       | 26.736                | <0.001                      |
|                       | F      | P                       | 0.002                 | 0.967                       |
| Luteolin              | 1.302  | 0.273                   | 44.409                | <0.001                      |
|                       | F      | P                       | 1.362                 | 0.263                       |
|                       | F      | P                       | 1.288                 | 0.276                       |
|                       | F      | P                       | 1.662                 | 0.218                       |
|                       | F      | P                       | 1.230                 | 0.286                       |
| Quercetin             | 3.257  | 0.091                   | 0.475                 | 0.502                       |
|                       | F      | P                       | 0.567                 | 0.460                       |
|                       | F      | P                       | 0.986                 | 0.338                       |
|                       | F      | P                       | 0.221                 | 0.646                       |
|                       | F      | P                       | 1.627                 | 0.223                       |
|                       | F      | P                       | 1.043                 | 0.325                       |
| p-Coumaric acid       | 4.403  | 0.054                   | 16.082                | <0.001                      |
|                       | F      | P                       | 19.018                | <0.001                      |
|                       | F      | P                       | 4.544                 | 0.051                       |
|                       | F      | P                       | 0.516                 | 0.485                       |
|                       | F      | P                       | 2.102                 | 0.169                       |
|                       | F      | P                       | 6.218                 | 0.026                       |
| Ferulic acid          | 50.654 | <0.001                  | 4.673                 | 0.048                       |
|                       | F      | P                       | 1.053                 | 0.322                       |
|                       | F      | P                       | 3.631                 | 0.077                       |
|                       | F      | P                       | 9.938                 | 0.007                       |
|                       | F      | P                       | 6.503                 | 0.023                       |
|                       | F      | P                       | 1.288                 | 0.275                       |
| Genistin              | 18.965 | 0.001                   | 5.042                 | 0.041                       |
|                       | F      | P                       | 1.519                 | 0.238                       |
|                       | F      | P                       | 0.713                 | 0.413                       |
|                       | F      | P                       | 3.763                 | 0.017                       |
|                       | F      | P                       | 1.916                 | 0.188                       |
|                       | F      | P                       | 0.035                 | 0.854                       |

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to elevated O₃, though p-coumaric acid acted as their feeding stimulant. This may be because many compounds coexist in plant leaves and interactions among them may alter the effects of a single chemical.

In contrast to studies suggesting that elevated CO₂ favours aphid phloem feeding, such as pea aphid fed on *Medicago truncatula* and green peach aphid fed on *Nicotiana attenuata* (72–74), our research demonstrated that elevated CO₂ discouraged the pea aphids feeding at the reproductive stage, though favoured the cowpea aphids feeding at the vegetative stage. These results imply that the responses of aphids or even aphid-plant to elevated CO₂ are species-specific. The heterogeneous responses have been widely reported (75), however, the underlying mechanism is very complex. Our study suggests that the differential responses of two species of aphids to various compounds (genistin, ferulic acid, and p-coumaric acid) may be one reason. Indeed, studies have also shown that the aphid species or genotypes differentially respond to the same secondary compounds, such as thymol,

**Figure 2.** Foliar secondary metabolites contents in alfalfa grown under ambient or elevated CO₂ and O₃. (a) Genistin, (b) Apigenin, (c) Ferulic acid, (d) p-coumaric acid, (e) Kaempferol, (f) Rutin, (g) Genistein, (h) Luteolin, and (i) Quercetin. Each value represents the average (±SE) of three replicates. Different lowercase letters indicate significant differences among the CO₂ and O₃ treatments within the same plant developmental stage. Different uppercase letters indicate significant differences between developmental stages within the same CO₂ and O₃ concentrations.
Table 3. Effects of CO₂, O₃, plant developmental stage, and their interactions on aphid feeding activities. F and P values from MANOVAs are shown. P values < 0.05 are bolded. np (nonpenetration), stylets are outside the plants; C (pathway), mostly intramural probing activities between mesophyll or parenchyma cells; E (phloem feeding), aphids are injecting watery saliva into the sieve element and ingesting the phloem sap; pd (potential drops), aphids briefly puncture cells during plant penetration.

| Aphids feeding activities | CO₂ | O₃ | Developmental stage | CO₂ × O₃ | CO₂ × developmental stage | O₃ × developmental stage | CO₂ × O₃ × developmental stage |
|--------------------------|-----|----|---------------------|---------|--------------------------|------------------------|-------------------------------|
|                          | F   | P  | F      | F      | F           | F         | F                | F                        |
| Pea aphid                |     |    |        |        |             |           |                  |                           |
| C                        | 0.250 | 0.87 | 0.213   | 0.645  | 7.913        | 0.006     | 11.749            | 0.001                     |
| E                        | 12.509 | 0.001 | 2.149   | 0.147  | 3.335        | 0.072     | 8.202             | 0.005                     |
| pd                       | 5.386 | 0.022 | 20.220  | <0.001 | 29.310       | <0.001    | 4.199             | 0.043                     |
| Time to first pd         | 9.641 | 0.002 | 13.297  | <0.001 | 112.430      | <0.001    | 4.104             | 0.045                     |
| Total number of pd       | 7.001 | 0.009 | 0.029   | 0.864  | 15.048       | <0.001    | 22.365            | <0.001                    |

| Cowpea aphid             |     |    |        |        |             |           |                  |                           |
| np                       | 0.057 | 0.812 | 12.406  | 0.001  | 24.891      | <0.001    | 0.385             | 0.536                     |
| C                        | 1.696 | 0.195 | 5.275   | 0.023  | 5.207       | 0.024     | 5.395             | 0.022                     |
| E                        | 3.498 | 0.065 | 64.482  | 0.000  | 157.132     | <0.001    | 3.424             | 0.067                     |
| pd                       | 4.510 | 0.035 | 0.002   | 0.963  | 16.261      | <0.001    | 1.373             | 0.243                     |
| Time to first pd         | 4.199 | 0.043 | 53.929  | <0.001 | 18.124      | <0.001    | 0.014             | 0.907                     |
| Total number of pd       | 2.531 | 0.114 | 19.853  | <0.001 | 3.129       | 0.080     | 1.036             | 0.311                     |

hydroquinone, and alkaloid34,76, but more efforts are needed to explain the heterogeneous responses of aphids to atmospheric changes.

The performance of aphids can be decreased, increased, or unchanged under elevated O₃ depending on the duration and concentration of O₃ exposure and the age of the exposed plants32,77. However, our results showed that elevated O₃ decreased the feeding efficiency of aphids at both plant developmental stages, except for no impact on phloem feeding of the pea aphids at the reproductive stage. Furthermore, the feeding efficiency of two species of aphids was inhibited under simultaneously elevated CO₂ and O₃, indicating that elevated CO₂ did not completely offset the negative effects of elevated O₃. This finding is in contrast to previous studies that elevated CO₂ ameliorates the negative impact of elevated O₃ on herbivores19,78. A possible explanation is that those studies use leaf-chewing herbivores and use trees as the host plants, while we use phloem-feeding insects and use herbage as the host plants. The SoyFACE experiments have demonstrated that elevated O₃ has no impacts on soybean aphid (Aphis glycines) numbers, and that the effects of simultaneously elevated CO₂ and O₃ on aphid are similar to that of elevated CO₂ alone29. Thus, the responses of plant growth forms and feeding guilds to atmospheric changes seem to be heterogeneous.

The decreased time the pea aphids and cowpea aphids spent on phloem feeding under simultaneously elevated CO₂ and O₃ may result in reduced direct damage to the plants. In addition, the aphid feeding activities can also be used to predict indirect damage caused by transmitting plant virus. The piercing-sucking mouthparts of aphids facilitate the delivery of virions into plant cells without causing irrevocable damage40. Among a series of aphid feeding activities, the potential drops (pd) and phloem feeding (E) are relevant to virus transmission31. Elevated CO₂ and O₃ significantly influenced the time aphids spent on potential drops (pd) and the total number of potential drops (pd), which in turn altered the transmission of stylet-borne viruses by aphids. However, more evidence is needed to demonstrate how the loss of plant productivity caused by virus transmission will be altered under future atmospheric changes.

In summary, elevated CO₂ and O₃ have the potential to affect aphid feeding behaviors via the alteration of plant secondary metabolites, and the responses of aphids to climate changes depend on aphid species and plant developmental stage. This study has generated several significant findings. First, it provides evidence that the heterogeneous responses of aphids to atmospheric changes may result from the differential responses of aphids to the chemicals. Second, direct damage and population outbreaks of aphids may be decreased under future atmospheric conditions due to the reduced efficiency of phloem ingestion under simultaneously elevated CO₂ and O₃. Finally, and perhaps most importantly, the responses of aphids to elevated CO₂ and O₃ alone or in combination are different and vary with plant developmental stage, suggesting that multi-factor and long-term research is needed. More research is needed to further elucidate the mechanisms underlying the effects of elevated CO₂ and O₃ on herbivores and the role of plant secondary metabolites in the adaption of aphids to future atmospheric environments.
Figure 3. The time pea aphids and cowpea aphids spent in various feeding activities on alfalfa grown under ambient or elevated CO$_2$ and O$_3$. (a–g) Various feeding activities of pea aphids, (h–n) Various feeding activities of cowpea aphids. ‘Phloem feeding’ indicates that aphids are injecting watery saliva into the sieve element and ingesting the phloem sap; ‘potential drops’ indicates that aphids briefly puncture cells during plant penetration; ‘nonpenetration’ indicates that stylets are outside the plants; ‘pathway’ indicates that mostly intramural probing activities between mesophyll or parenchyma cells. Values are the means (±SE) of 21 biological replicates. Different lowercase letters indicate significant differences among the CO$_2$ and O$_3$ treatments within the same plant developmental stage. Different uppercase letters indicate significant differences between developmental stages within the same CO$_2$ and O$_3$ concentrations.
samples were stored in a −80 °C. The extract was centrifuged at 12,000 rpm for 15 min, and the supernatant was filtered with a 0.22 μm filter. The shoots and roots of each plant were collected, oven-dried (50 °C) for 48 h, and weighed. (six for the vegetative stage, six for the reproductive stage, and 144 plants in total) were harvested for measurement observed every day for flowering and podding after they had grown for 2 months. The remaining 12 plants per OTC Telaire Company, Goleta, CA, USA) and O3 analyser (Aeroqual, series 200, New Zealand), respectively, once every minute to maintain relatively stable CO2 and O3 concentrations. The OTCs were ventilated with air daily from 8:00 am to 5:30 pm.

Table 4. Relationships between secondary metabolite contents in the leaves of alfalfa and the time aphids spent on E (phloem feeding) and pd (potential drops). Correlation coefficient r and P values are shown. Values in bold indicate a significant correlation. E (phloem feeding) indicates that aphids are injecting watery saliva into the sieve element and ingesting the phloem sap; pd (potential drops) indicates that aphids briefly puncture cells during plant penetration.

| Secondary metabolites | Pea aphid | Cowpea aphid |
|-----------------------|----------|--------------|
|                       | E        | pd           | E           | pd           |
|                       | N | r | P | N | r | P | N | r | P | N | r | P |
| Rutin                 | 8 | 0.167 | 0.693 | 8 | −0.356 | 0.387 | 8 | −0.121 | 0.776 | 8 | −0.352 | 0.392 |
| Apigenin              | 8 | −0.054 | 0.900 | 6 | −0.819 | 0.046 | 8 | −0.510 | 0.197 | 8 | 0.623 | 0.099 |
| Genistein             | 8 | 0.108 | 0.800 | 8 | −0.050 | 0.905 | 8 | −0.552 | 0.156 | 8 | 0.531 | 0.176 |
| p-Coumaric acid       | 8 | −0.232 | 0.580 | 7 | −0.867 | 0.012 | 8 | −0.781 | 0.022 | 8 | 0.388 | 0.342 |
| Ferulic acid          | 8 | −0.772 | 0.025 | 8 | −0.584 | 0.128 | 8 | −0.134 | 0.752 | 8 | 0.785 | 0.037 |
| Genistin              | 8 | −0.837 | 0.010 | 8 | −0.118 | 0.780 | 8 | 0.066 | 0.877 | 8 | 0.105 | 0.805 |

Methods
Treatments under different CO2 and O3 concentrations. The study was performed from March to July 2015 in 12 octagonal open-top chambers (OTCs) at the Observation Station of the Global Change Biology Group, Institute of Zoology, Chinese Academy of Sciences in Xiaotangshan County, Beijing, China (40°11′N, 116°24′E). The atmospheric CO2 and O3 concentration treatments were as follows: current atmospheric CO2 and O3 concentrations (CK, 400 μL/L CO2 and 35 nL/L O3), elevated CO2 concentration (ECO2, 750 μL/L CO2), elevated O3 concentration (EO3, 70 nL/L O3), and simultaneously elevated CO2 and O3 concentrations (ECO2, 750 μL/L CO2 and 70 nL/L O3). Three blocks were used for CO2 and O3 treatments, and each block contained four OTCs, one OTC with ambient atmospheric CO2 and O3 concentrations, one OTC with an elevated CO2 concentration, one OTC with an elevated O3 concentration, and one OTC with elevated CO2 and O3 concentrations. CO2 and O3 concentrations in each OTC were monitored and adjusted with an infrared CO2 analyser (Ventostat 8102; Telaire Company, Goleta, CA, USA) and O3 analyser (Aeroqual, series 200, New Zealand), respectively, once every minute to maintain relatively stable CO2 and O3 concentrations. The OTCs were ventilated with air daily from 8:00 am to 5:30 pm.

Aphids and host plants. The pink pea aphid (A. pismum) and the cowpea aphid (A. craccivora) were collected from alfalfa (M. sativa). The nymphal instars from the same parthenogenetic aphid female were reared on alfalfa with 14 h light (25 °C); 10 h dark (22 °C) in photoclimate chambers (Safe PRX-450C, Ningbo, China).

The alfalfa cultivar ‘Algonquin’ was purchased from the Chinese Academy of Agricultural Sciences. The ‘Algonquin’ seeds were sown in sterilized soil and watered every 4 days. After the seedlings had grown in sterilized soil for 2 weeks, they were transplanted into plastic pots (17 cm diameter and 24 cm height) and placed in the OTCs. Pot placement was re-randomized within each OTC once per week. No chemical fertilizers or insecticides were used. After the plants were fumigated for 3 weeks (vegetative stage) or 8 weeks (reproductive stage), they were used for the assays described in the following sections.

Plant growth traits. Six plants per OTC (18 plants for each treatment and 72 plants in total) were randomly selected for measurement of growth traits. The leaf chlorophyll content was determined with a Minolta SPAD-502 plus (Konica Minolta Sensing Inc., Osaka, Japan). The leaf net photosynthetic rate was determined with a Li-Cor 6400 gas exchange system (Li-Cor Inc., Lincoln, NE, USA) between 9:00 hours and 12:00 hours. These plants were plus (Konica Minolta Sensing Inc., Osaka, Japan). The leaf net photosynthetic rate was determined with a Li-Cor 6400 gas exchange system (Li-Cor Inc., Lincoln, NE, USA) between 9:00 hours and 12:00 hours. These plants were observed every day for flowering and podding after they had grown for 2 months. The remaining 12 plants per OTC (six for the vegetative stage, six for the reproductive stage, and 144 plants in total) were harvested for measurement of the biomass. The shoots and roots of each plant were collected, oven-dried (50 °C) for 48 h, and weighed.

Aphid feeding behaviors. Seven plants per OTC (21 plants for each treatment and 84 plants for each aphid) were randomly selected as host plants at the vegetative stage to evaluate aphid feeding behaviors using the electrical penetration graph (EPG) technique. Another 84 plants were also randomly selected at the reproductive stage. The principle of EPG was introduced by Tjallingii and Hogen-Esch82. Eight plants were placed in a Faraday cage to avoid noise and interference. Each plant was infested with one apterous adult aphid, and its feeding behavior was recorded for 8 h. The aphids were starved for 10 h before the test. Two eight-channel amplifiers simultaneously recorded 16 individual aphids on separate plants (four plants per treatment). Twenty-one biological replicates were included for each treatment. The feeding waveforms in this study were scored according to Tjallingii and Hogen-Esch82; nonpenetration (np), styles are outside the plants; pathway (C), mostly intramural probing activities between mesophyll or parenchyma cells; phloem feeding (E), aphids are injecting watery saliva into the sieve element and ingesting the phloem sap; potential drops (pd), aphids briefly puncture cells during plant penetration.

Plant secondary metabolites. The chemical analysis was determined according to Oleszek and Stochmal83 and Nour et al.84 with some modification. Freeze-dried leaves were ground into a fine powder. For a typical extraction, approximately 50 mg samples were soaked with 1.5 mL of 70% aqueous MeOH for 1 h in a 60 °C water bath. The extract was centrifuged at 12,000 rpm for 15 min, and the supernatant was filtered with a 0.22 μm filter. The samples were stored in a −20 °C freezer until chemical analysis. Using high-performance liquid chromatography
(HPLC), we analysed 12 phenolic compounds: phenolic acids, including chlorogenic acid, caffeic acid, cinnamic acid, \( p \)-coumaric acid, and ferulic acid; flavonoids, including rutin, luteolin, apigenin, kaempferol, and quercetin; and isoflavones, including genistein and genistin. Among these compounds, chlorogenic acid, caffeic acid, and

**Figure 4.** The time pea aphids and cowpea aphids spent in various feeding activities on treated (+genistin, +apigenin, +ferulic acid, and +\( p \)-coumaric acid) and control plants. (a–g) Various feeding activities of pea aphids. (h–n) Various feeding activities of cowpea aphids. ‘Phloem feeding’ indicates that aphids are injecting watery saliva into the sieve element and ingesting the phloem sap; ‘potential drops’ indicates that aphids briefly puncture cells during plant penetration; ‘nonpenetration’ indicates that stylets are outside the plants; ‘pathway’ indicates that mostly intramural probing activities between mesophyll or parenchyma cells. Values are the means (±SE) of 15–20 biological replicates. Significant differences: *there was significant difference between the treatment and control at \( P < 0.05 \).
cinnamic acid were not detected in the alfalfa leaves. Determination of compounds was performed on a Waters system with a diode array detector. Chromatograms were registered and integrated at 280, 350, and 254 nm for phenolic acid, flavonoids, and isolavone, respectively. The mobile phase consisted of 1% H₃PO₄–AcN (a linear gradient of 15–100% AcN) with a flow rate of 1 mL/min for 60 min. Compounds were identified by comparing retention times to those of authentic standards.

**Bioassays with pure compounds.** According to the chemical analysis, elevated CO₂ and O₃ had significant impacts on foliar rutin, genistin, ferulic acid, p-coumaric acid, genistein, and apigenin contents of alfalfa (Table 2). As aphid feeding behaviors were altered under elevated CO₂ and O₃, and as aphid feeding activities were significantly related to genistin, ferulic acid, p-coumaric acid, and apigenin (Table 4), we performed bioassays to test whether the four secondary compounds affected the aphid feeding. All test compounds were commercially available products.

For the bioassay, differing from the in vitro detached leaves and artificial diets⁷⁷,⁸⁵, we applied the pure compounds to the living plant leaves to provide aphids with the most realistic feeding conditions. All the bioassays were performed on alfalfa plants in a greenhouse. Twenty plants were randomly selected for the experiment. The leaves in the same location were separately treated with 50 μL of genistin, ferulic acid, p-coumaric acid, and apigenin solution. Five biological replicates were included for each treatment. Another five plants were selected as controls. The treated and adjacent systemic control leaves were collected to measure the concentrations of the four compounds at 24 h, 48 h, and 72 h after treatment. Contents of genistin, ferulic acid, p-coumaric acid, and apigenin were much higher in the treated leaves compared to the control leaves. The variation tendency remained similar at 24 h, 48 h, and 72 h (see Supplementary Table S1 online). Thus, we selected 24 h to assess the effects of compounds on aphid feeding behaviors using EPG as above.

**Statistical analysis.** We analysed the univariate responses of the growth traits, secondary metabolite contents and aphid feeding activities with a split-plot design using the following model: \( Y_{ijkl} = b_i + C_j + O_k + CO_{jk} + e_{ijkl} \). In this model, \( b \) represents block, \( C \) represents CO₂ level, \( O \) represents O₃ level, \( CO_{jk} \) represents the sub-plot error. \( Y_{ijkl} \) represents the average response of block \( i \), CO₂ level \( j \), O₃ level \( k \) and developmental stage \( l \) (SAS 9.2, USA). Effects were considered significant when \( P < 0.05 \). LSD multiple range tests were used to separate means when ANOVAs were significant.

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Author Contributions
H.Y., Y.S. and F.G. formulated the original idea and developed methodology; H.Y. performed experimental processing and statistical analysis; E.Y. helped to analyze chemicals; H.Y. interpreted the data; H.Y. wrote the manuscript with editorial advice from F.G., Y.S. and H.G. All authors gave final approval for publication.

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