Interspecific plant competition mediates impact of climate change on the

ecophysiology of a plant-herbivore interaction.

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Abstract

Biotic interactions shape community evolution, but we lack mechanistic insights into how

physiological and ecological processes under climate change are altered by biotic

interactions.

We used a two-trophic model community consisting of the aphid *Dysaphis plantaginea*

feeding on the forb *Plantago lanceolata,* and a grass competitor *Lolium perenne* that does not

experience herbivory by the aphid. Monocultures and mixtures were exposed to three

relevant simulated climate scenarios.

Elevated CO_2 reduced the nitrogen content quality of *P. lanceolata*, while simultaneous

increases of CO_2 and temperature modified the plant metabolomic component in different

directions. Elevated CO_2 enhanced defense systems in *P. lanceolata*, but these effects were

not altered by warming. Interspecific plant competition did, however, neutralise these

responses. There were no indications for indirect effects of climate on aphid performance as

mediated by changes in plant defense, nutritional quality and biomass.

We thus demonstrate interactions between abiotic and biotic processes on plant metabolite

profiles, but more importantly, that climate change effect on a selection of the metabolic

pathways are altered by herbivory and intraspecific competition. Our experiment under semi-

natural conditions thus demonstrates the non-additive and often neutralizing effects of biotic

interactions on plant physiology and ecology under climate change.

Introduction

Interactions between insect herbivores and host plants are influenced by primary and

secondary metabolites. The concentrations and types of these may, in turn, be affected by

climate change (Bidart-Bouzat & Imeh-Nathaniel, 2008; Zvereva & Kozlov, 2006). Plants

exposed to a CO_2-enriched environment show higher concentrations of carbohydrates

(including starch and soluble sugars), lower concentrations of nitrogen (either from dilution

by increased carbohydrates or reallocation) and thus a higher C:N ratio (Bezemer & Jones,

1998; Lincoln *et al.*, 1993; Robinson *et al.*, 2012; Stiling & Cornelissen, 2007). Lower

nitrogen concentration implies lower levels of leaf protein and amino acids and, as a result,

reduced nutritive value for herbivores (Lincoln *et al.*, 1986). Consequently, in general, the

performance of insects reduces under high CO_2 (Bezemer & Jones, 1998; Hunter, 2001;

Lincoln *et al.*, 1993). Contrary to elevated CO_2, warming has a direct positive effect on insect

herbivore performance by reducing their development time (Bale *et al.*, 2002; Van De Velde

et al., 2016) and increasing fecundity (Meisner *et al.*, 2014) up to a certain threshold.

Therefore, warming may indirectly compromise insect herbivores via their host plant quality

(Bauerfeind & Fischer, 2013; Jamieson *et al.*, 2015). Nevertheless, such indirect effects are

unlikely to fully counterbalance the direct positive effects, and the overall herbivore response

to warming will be positive (Bauerfeind & Fischer, 2013; Zvereva & Kozlov, 2006).

Secondary metabolites (e.g. lignin, tannins, phenolics and terpenoids), affect tissue quality by

altering the nutritive value, palatability, digestibility and/or toxicity of foliage. Compared to

primary metabolites, the response of secondary metabolites to elevated CO_2 and warming is

highly variable and less understood (Bidart-Bouzat & Imeh-Nathaniel, 2008; Robinson *et al.*,

2012). According to the carbon-nutrient balance hypothesis (Bryant *et al.*, 1983), elevated

CO_2 is expected to increase carbon-based secondary compounds as a result of the 'excess'

carbon (fertilizing effect) and to decrease nitrogen-based compounds a result of the nitrogen

scarcity (Robinson *et al.*, 2012). On the other hand, the growth-differentiation balance

hypothesis (Herms & Mattson, 1992) proposes that warming-accelerated photosynthesis

should contribute to growth rather than defense if resources (e.g. soil moisture and nutrients)

are not limited. However, so far, studies suggest that the effects of warming and elevated CO_2

on the different groups of chemical defenses are idiosyncratic (Bidart-Bouzat & Imeh-

Nathaniel, 2008; Zavala *et al.*, 2013; Zvereva & Kozlov, 2006).

Additional complexity arises from competition with neighboring plants. For example,

competition for limited nutrients enhances the availability of carbon in a focal plant relative

to its demand. This, in turn, can increase carbon-based defenses and thus reduce herbivory

compared with control plants that are less constrained by competitors (Bryant *et al.*, 1983).

Also the identity of a neighboring plant may affect the resistance of a focal plant by

influencing the outcome of plant-plant competition (Barton & Bowers, 2006; Broz *et al.*,

2010). Conspecific competition, in particular, may result in stronger decline of plant growth

and increased defense compared to heterospecific competition. Indeed, Broz *et al.* (2010) for

instance showed that focal plants with conspecific neighbors allocated more resources

towards production of carbon-based defense molecules, whereas those grown with

heterospecific neighbors allocated more resources towards growth. Hence, elevated CO_2 as

well as warming is anticipated to indirectly affect the defense of a focal plant, through species

specific stimulation of plant growth.

Although atmospheric CO_2 and temperature increase concurrently, empirical studies

investigating their combined effect on multi-trophic communities are surprisingly scarce and

insufficient to effectively guide theory or synthesis (Cornelissen, 2011; Zvereva & Kozlov,

2006). The current study investigates individual effects of elevated CO_2 and combined effects

of elevated CO_2 and warming on a simple model community consisting of three species: rosy

apple aphid *Dysaphis plantaginea* Passerini (*Hemiptera: Aphididae*) feeding on plantain,

Plantago lanceolata L*.,* and a heterospecific neighboring plant species, perennial ryegrass,

Lolium perenne L*.* The aphid does not feed on *L. perenne.* The experimental design consisted

of monocultures of *P. lanceolata* and mixtures of *L. perenne* and *P. lanceolata* exposed to

three simulated climate scenarios. We hypothesized that (1) elevated CO_2 and warming alter

foliar nutrients and defense molecules, (2) altered host quality and plant resistance affect

insect herbivore performance, (3) elevated CO_2 and warming indirectly influence host quality

and plant resistance via effects on neighboring plants.

Materials and methods

Experimental set-up

The study was performed at the Drie Eiken Campus, University of Antwerp, Wilrijk, Belgium (51° 09' N, 04° 24'E) in 12 sunlit, south-facing, climate-controlled chambers (details in Naudts *et al.* 2014). Three climate scenarios (four chambers per scenario) were simulated in an additive design: (1) current atmospheric CO_2 concentration and temperature (C); (2) future atmospheric CO_2 and current temperature (CO_2); and (3) future atmospheric CO_2 and temperature (TCO_2). Climate scenarios with elevated CO_2 had a target CO_2 concentration of 620 µmol mol^{-1} and future temperature chambers simulated a continuous 3 °C warming above fluctuating ambient temperatures. Climate manipulations were based on the IPCC-SRES B2-scenario prediction of moderate change for the year 2100 (IPCC, 2001).

The CO_2 concentration in each chamber was continuously measured and maintained at the target concentration with a CO_2 control group with an infrared analyzer (WMA-4, PPSystems, Hitchin, UK). Air temperature and relative humidity were monitored every 0.5 h with a combined humidity–temperature sensor (Siemens QFA66, Erlangen, Germany), by averaging instantaneous readings in half-hour mean values. During the experiment, the CO_2 concentration was 382 ± 55 µmol mol^{-1} (SD) in the current climate, while it was 615 ± 70 µmol mol^{-1} (SD) in the climate scenarios with future CO_2 concentration (CO_2 and TCO_2). The monthly average air temperature in the C and CO_2 chambers was 16.2, 17.2 and 18.7 °C in June, July and August, respectively. TCO_2 chambers were 2.9 ± 1.0 °C (SD) warmer than current temperature chambers. Average vapor pressure deficit was 0.60 ± 0.34 and 0.64 ± 0.52 kPa (SD) in the climate treatments with ambient and warmed air, respectively. Irrigation mimicked the rainfall outside (Naudts et al. 2014), with monthly totals equalling 64.4, 85.1 and 80.2 mm in June, July and August, respectively. Water freely drained while capillary rise

was prevented by a drainage system placed below the chambers. Future climate chambers

(TCO$_2$) received the same amount of water as current climate chambers, so that any increase

in water consumption would result in (aggravated) soil drought.

Plant and insect communities

We used two common co-occurring grassland species, *L. perenne* and *P. lanceolata*. *P.*

lanceolata is characterized by the presence of the iridoid glycosides aucubin and catalpol.

These compounds stimulate feeding and oviposition by specialist insects and can act as

defense against generalist herbivores (Bowers & Puttick, 1988; Puttick & Bowers, 1988).

Both plant species were sown at the end of March in a non-climate controlled greenhouse

with a time lag of one week to prevent size differences at the start of the experiment (Cotrufo

& Gorissen, 1997), and were watered twice a week. Four or five-week-old seedlings were

transplanted into PVC containers (24 cm inner diameter and 40 cm height), filled with sandy

soil (93.9% sand, 4.1% silt, 2.0% clay; pH 7.5; Kjeldahl-N 0.125 g kg^{-1}; 2.1% C in humus).

Each of the 12 chambers received 20 containers with two different compositions: (1) 10

monocultures of *P. lanceolata*, and (2) 10 mixtures of both plant species in a 50:50 ratio.

Each community contained 18 individuals planted in a hexagonal grid with 4.5 cm interspace.

Interspecific interactions were maximized by avoiding clumping. All communities were

fertilized with 10 g m^{-2} NH$_4$NO$_3$, 5 g m^{-2} P$_2$O$_5$, 10 g m^{-2} K$_2$O and micro-elements (Fe, Mn,

Zn, Cu, B, Mo), given dissolved in water in two equal amounts.

When the plants were three months old, *P. lanceolata* was involuntarily infested with

powdery mildew *Podosphaera plantaginis*. *P. plantaginis* is a biotrophic fungal pathogen

which means that it feeds on living plant tissue, but does not kill the infected host. Biotrophic

parasites extract nutrients from living cells and have extended periods of physiological

interaction with their hosts (Agrios, 2005). The powdery mildew was found in all climate

scenarios and plant compositions but not all the containers were equally affected. Elevated

CO_2 and warming have been shown to affect the severity of pathogen infections (Mikkelsen

et al., 2015, Thomas & Blanford, 2003). We took advantage of this unplanned infestation to

study the impact of elevated CO_2 and warming on mildew infestation and the putative

additive effects of mildew infestation on plant-insect herbivore interaction.

The rosy apple aphid *D. plantaginea* was used as an insect herbivore. It overwinters as eggs

on apple trees, the primary host plant, and migrates in spring to the obligate alternate hosts, *P.*

major L. and *P. lanceolata* (Alford, 2014). On *Plantago* spp., they give birth to apterous

(wingless) morphs that reproduce by parthenogenesis (Blommers *et al.*, 2004). *L. perenne* is

not a host plant for *D. plantaginea*. The aphids were reared in small cages on *P. lanceolata*

under laboratory conditions of 22 ± 1 °C. They were introduced on *P. lanceolata* when the

plants were 20 weeks old. At this time, two adult, apterous aphids were placed with a dry

paintbrush on the apex of each *P. lanceolata* plant in monocultures and mixtures.

Consequently, at the start of the infestation each container contained 36 (monocultures) or 18

(mixtures) aphids. In each chamber, four monocultures of *P. lanceolata* and four mixtures

were randomly chosen for aphid infestation. Containers that did not receive aphids acted as

controls. During infestation, all containers (both control and herbivory treatments) were

individually enclosed with an 85-cm-tall cylinder of lightweight netting to ensure aphids did

not migrate between pots. The infrastructure did not physically limit plant growth and did not

cause photosynthetic stress effects (F_v/F_m, the intrinsic efficiency of PSII in controls = 0.84

which is an optimal value (Johnson *et al.*, 1993)).

Data collection

In the fourth week after the aphid introduction we determined the mildew infestation. The

degree of powdery mildew on *P. lanceolata* was categorized by a rating system: 1) healthy

(no visible lesions); 2) 1% - 25% of the leaves damaged; 3) 26% - 50% of the leaves

damaged; 4) 51% - 75% of the leaves damaged; 5) more than 75% of the leaves damaged. At

the same time, aphid populations of four replicate communities per plant composition in each

chamber were collected (totaling 4 replicate communities × 2 plant compositions × 12

chambers). Aphids were brushed directly into 70% ethanol with a dry paintbrush. It was not

possible to collect all the aphids because their number per container was either too high or too

low (hard to find), therefore a subsample of the population per container was collected: we

searched for and collected aphids for 30 minutes. The total number of aphids per community

was divided by the number of *P. lanceolata* individuals in that community. At the same time,

we also harvested the plants of two replicate communities per treatment in each chamber

(totaling 2 replicate communities × 4 treatments × 12 chambers). Alive aboveground plant

biomass in each community was separated from dead biomass by species, dried at 70 °C for

48 h and weighed. These biomass values per species were likewise divided by the number of

the species' individuals in the community, providing primary data for the statistical analysis.

The alive biomass samples of *P. lanceolata* plants of the two harvested communities per

treatment in each chamber (2 replicate communities × 4 treatments × 12 chambers) were

ground in a mill. Three subsamples per community were analyzed for nitrogen and carbon

content using a NC element analyzer (NC-2100 element analyzer, Carlo Erba Instruments,

Milano, Italy), which were averaged prior to data analysis.

A separate subsample of the milled live aboveground biomass of *P. lanceolata* of the two

harvested communities per treatment in each chamber (2 replicate communities × 4

treatments × 12 chambers) was taken to quantify several biochemical parameters: fructose,

sucrose, glucose, total soluble sugars, starch, total proteins, lipids, phosphorus, cellulose,

tannin, lignin, catalpol, aucubin, total phenols, tocopherols, carotenoids, total antioxidant

capacity (TAC), jasmonic acid, salicylic acid, malondialdehyde (MDA) and proline

(Appendix S1).

Data analysis

We focused on *P. lanceolata* because this is the host plant for the aphid and powdery mildew.

In a first step we examined covariation in the metabolites responses to the three treatments

(aphid infestation, climate scenario and plant composition) by using a hierarchical clustering

analysis. Metabolites were normalized by Z-transformation and subjected to a hierarchical

clustering analysis with an Euclidean distance metric, and visualized as a heat map using

Multi Experiment Viewer (Mev) version 4.8 (Saeed *et al.*, 2003). A distance cut off of 0.3

was applied. The clusters were used in the structural equation models (see below).

In a second step, we fitted two piecewise Structural Equation Models (SEM), which combine

information from multiple separate linear models into a single causal network (Shipley,

2009). The first SEM investigated the effect of aphid infestation, climate scenario, plant

composition and mildew infestation on the metabolites and alive aboveground biomass of *P.*

lanceolata. In a second SEM, we separated the metabolites of *P. lanceolata* in control pots

from those with aphids. This allowed us to test the effects of the metabolites on the aphid

population, as well as to quantify effects of the aphid population on these metabolites. The

response of the aphid population was measured as their number at the end of the infestation.

The four clusters obtained by the clustering analysis were used to divide the metabolites in

separate groups. We standardized the metabolites, the alive aboveground biomass of *P.*

lanceolata and the number of aphids by converting to Z-scores to equalize variances (see

above). To reduce the number of mildew categories, we rearranged degree of mildew in two

categories: (1) no or mild mildew infection (category 1 - 2) (2) severe mildew infection

(category 3 - 5).

Traditional SEM estimation methods assume that all variables follow a normal distribution

and all observations are independent (Grace, 2006). In our analyses, we used piecewise SEM

that allows fitting general linear mixed effect models that can incorporate random effects.

Each mixed effects model was fitted using the "lme" function in the "nlme" package (version

3.1-128) in R. For each model, we fitted a random effect of chamber. The overall path model

(the SEM) was fitted using the "piecewiseSEM" package (version 1.2.1) in R (Lefcheck,

2016). Goodness of fit was estimated using Shipley's test of d-separation, which yields a

Fisher's C statistic that is Chi-squared distributed (Shipley, 2009). If the resulting P-value >

0.05, then the SEM can be said to adequately reproduce the hypothesized causal network.

In a final step, all data were analyzed with General Linear Mixed models (GLM) in SAS

(version 9.2, SAS Institute Inc., Cary, NC) (Littell *et al.*, 1996) with chamber as a random

factor nested within climate scenario. Climate scenario, plant composition, aphid infestation

and two-way and three-way interactions between these predictors were included as fixed

factors. Because mildew infestation did not have a significant effect on biochemical plant

responses, this treatment was excluded from the GLM analysis. Non-significant factors were

backwards-excluded from the model. In case of significant effects, *a posteriori* means

comparisons using the Tukey test, corrected for multiple comparisons, were made. Effects

were considered significant at $P \leq 0.05$.

Results

Hierarchical clustering analysis of metabolites

The variation in primary and secondary metabolites of *P. lanceolata* was subjected to a

hierarchical clustering analysis in order to understand their grouping according to their

covariation in response to the imposed herbivory, climate and competition treatments. We

obtained four major clusters of metabolites (1 - 4 in Fig. 1).

The first cluster containing metabolites consistently upregulated by the presence of aphids

contains the phenol antioxidants, the lipophilic antioxidant tocopherol, the C:N ratio, tannins,

and the non-structural carbohydrates soluble sugars (including glucose, fructose) and starch

(Fig. 1). The second cluster contains another group of antioxidant molecules that are

downregulated by elevated CO_2 and more so by aphids in mono-cultures, the carotenoids, as

well as the growth regulator, jasmonic acid, total proteins, phosphorous and lipids (Fig. 1).

The nutrient N shows a unique pattern, not shared by other metabolites. Carbon

concentration, the C:P and N:P ratio, and lignin:N were classified in the fourth cluster that is

specifically upregulated by aphids in monocultures under elevated CO_2 (Fig. 1). Cluster 4

also contains metabolites that play a role in plant defenses against herbivores, such as

salicylic acid and the iridoid glycosides (catalpol and aucubin), and also contains TAC,

lignin, cellulose, proline and MDA. Notably in this cluster, metabolites are strongly induced

in CO2 and TCO2.

Causal changes as inferred by structural equation models

SEM quantify the strength of both the direct and indirect interactions between the climate

change scenarios, herbivory and plant phenotype. The first SEM presents the effect of aphid

infestation (qualitative effect), climate scenario, plant composition and mildew

infestation on the chemical composition and the live aboveground biomass of *P.*

lanceolata (Fig. 2A). In this analysis, the metabolites of *P. lanceolata* were subdivided

into the four groups obtained from the hierarchical clustering. The hypothesized

structural relationship as outlined at the end of the introduction adequately fits the data

(χ^2 = 42.53, df = 32, p = 0.101). Fig. 2A and Table S2.1 (appendix S2) provide us with

three insights. Firstly, TCO_2 increased the mildew infestation compared to C, but the

mildew infestation, in turn, did not affect the metabolites and the alive aboveground

biomass of *P. lanceolata*. Secondly, neither climate scenario, nor aphid infestation or

plant composition had a significant impact on the live aboveground biomass of *P.*

lanceolata. Thirdly, climate scenario, aphid infestation and plant composition impacted the

distinguished clusters in a different manner. (see previous section and Fig. 1). We noticed a

significant interaction between climate scenario and aphid infestation for metabolites

associated to this cluster. Also the metabolites in cluster 2 (see previous section and Fig.

1) differed according to an interaction between Climate scenario and aphid infestation

interactions also affected the cluster 2 metabolite values . However, the three

treatments did not affect the metabolites in cluster 3 (see previous section and Fig. 1).

Aphid infestation, CO_2 and TCO_2 increased the metabolites in cluster 4 (see previous

section and Fig. 1). Besides these single factor treatments effects, climate scenario,

aphid infestation and plant composition also interacted with each other in affecting the

metabolites in cluster 4.

The second SEM presents the effect of climate scenario, plant composition and chemical

composition of *P. lanceolata* on the aphid population and whether the aphid population, in

turn, altered the chemical composition of *P. lanceolata* (quantitative effect). The mildew

infestation was left out of this analysis since it is a parasite of the plants, not of the aphids.

The results of this SEM model show the following goodness of fit statistics: $\chi^2 = 77.52$, df =

80 and p = 0.558 (Table S1.1, S1.2 and Fig 2) provide us with two insights. Firstly, climate

scenario, plant composition and the chemical composition of *P. lanceolata* did not affect the

number of aphids. Secondly, the aphid population size in turn, did not change the chemical

composition of *P. lanceolata*. Bringing together the findings from both SEM models, we

conclude that the treatment aphid infestation altered the chemical composition of *P.*

lanceolata but the number of aphids had no effect on it.

Detailed analyses of the separate paths by General Linear Mixed models

Climate scenario, but not plant composition or aphid infestation altered the live aboveground

biomass of *P. lanceolata* ($F_{2,9} = 6.34$, p = 0.019; $F_{1,82} = 0.84$, p = 0.362; $F_{1,82} = 0.98$, p=

0.325, respectively, Fig. 3A). The live aboveground biomass tended to be higher in CO_2 than

C and was significantly lower in TCO_2 than CO_2, which combined lead to similar values in

TCO_2 and C. Plant composition, but not climate scenario or aphid infestation altered the dead

aboveground biomass of *P. lanceolata* ($F_{1,75} = 72.82$, p = <0.001; $F_{2,9} = 0.93$, p = 0.428; $F_{1,75}$

= 0.71, p = 0.401, respectively, Fig. 3B). Competition from *L. perenne* reduced the dead

aboveground biomass relative to growing with conspecific neighbors. Furthermore, climate

scenario and plant composition did not alter relative herbivory effects ($F_{2,42} = 0.02$, p = 0.981;

$F_{1,42} = 2.37$, p = 0.131, respectively). In addition, climate scenario and plant composition did

not alter the number of aphids per plant ($F_{2,9} = 0.45$, p = 0.650; $F_{1,78} = 0.87$, p = 0.353,

respectively).

An overview of all GLM summary statistics for the metabolomics endpoints and figures of

the mean (+SE) values are provided in supplementary material 2 (Tables S2.1-4). We here

synthesize the most prominent results, and visualize a selection of relevant metabolites in Fig.

4 .

Cluster 1 metabolites (Fig S2.1)

As a baseline, the aphid infestation increased the concentrations of the metabolites in cluster

1 irrespective of climate scenario and plant composition (except for C:N ratio) (Fig. 4A).

However, the effect of aphid infestation on the concentration of tannin depended on a

significant interaction with plant composition. Aphid infestation increased the concentration

of tannin in monocultures but not in mixtures. The concentrations of glucose and fructose

increased with aphid infestation only in C, while the concentration of total phenol increased

with aphid infestation in C and TCO_2 but not in CO_2 (Fig. 4B). Moreover, TCO_2 strengthened

the effect of aphid infestation on the concentration of total phenol. In addition, CO_2,

compared to C, increased the concentration of starch and the C:N ratio (Fig. A). TCO_2, in

turn, reduced the concentration of starch (except for mixtures with aphids) and the C:N ratio

compared to CO_2. The concentrations of glucose and soluble sugars increased at elevated

CO_2 but only in monocultures, while TCO_2 did not alter the concentrations of glucose relative

to the CO2 treatment but decreased the concentrations of soluble sugars in monocultures (Fig.

4C). In general CO_2, compared to C, increased the concentration of tannin while TCO_2 did not

impose further differences. Pairwise comparison revealed that in C the concentration of

tannin was higher in mixtures compared to monocultures while the opposite was true in CO_2.

Aphid infestation increased the concentration of tocopherol in monoculture and mixture in C,

whereas in CO_2 and TCO_2 the increase under aphid infestation was more noticeable in

mixture. Furthermore, the concentrations of tocopherol in monocultures with aphids declined

at CO_2 compared to C. Despite this, the concentrations were higher in mixtures without

aphids at TCO_2 compared to CO_2 and C. Furthermore, interspecific interactions between *P.*

lanceolata and *L. perenne* increased the concentrations of total phenols, starch and the C:N

ratio, but did not modify the concentration of fructose (Fig S2.1).

Cluster 2 metabolites (Fig S2.2)

The concentration of jasmonic acid in C increased with aphid infestation in monocultures and

mixtures but aphid infestation in CO_2 and TCO_2 decreased it in monocultures (Fig. 4D).

Interspecific interactions between *P. lanceolata* and *L. perenne* mitigated the negative effect

of aphid infestation in these climate scenarios. Furthermore, aphid infestation decreased the

concentration of proteins in mixtures in C but did not alter the concentrations in monocultures

and mixtures in CO_2 and TCO_2 (Fig. 4E). In general, CO_2 compared to C decreased the

concentration of proteins irrespective of the plant composition while aphid infestation and

TCO_2, compared to CO_2, did not alter it. The concentration of lipids decreased with aphid

infestation in monocultures and mixtures in all climate scenarios (except for mixtures in C).

Also, CO_2, compared to C, decreased the concentration of lipids in monocultures without

aphids and mixtures with aphids, but in TCO_2, compared to CO_2, this concentration increased

again to similar levels as C in monocultures without aphids. For carotenoids, there was a

significant interaction between aphid infestation and climate scenario (Table 2). Aphid

infestation in C did not alter the concentration of carotenoids while aphid infestation in TCO_2

reduced it. This was mainly due to an increase in the concentration of carotenoids in TCO_2 in

controls, compared to C and CO_2. Aphid infestation reduced the concentration of P in

monocultures, but did not alter it in mixtures (Fig. 4D). Furthermore, also CO_2, compared to

C, reduced the concentration of P and the concentration was further reduced in TCO_2.

Cluster 3 metabolites

In cluster 3, aphid infestation and interspecific plant interactions slightly reduced the leaf

nitrogen content (Fig. 4G).

Cluster 4 metabolites (Fig S2.3 and Fig S2.4)

Aphid infestation, irrespective of the climate scenario considerably increased the

concentration of the metabolites of cluster 4 (except carbon) but more in monocultures than

in mixtures Table S2.4). In other words, interspecific plant interactions between *P. lanceolata*

and *L. perenne* mitigated the effect of aphid infestation. However, the concentration of

aucubin increased with aphid infestation in both monocultures and mixtures. Furthermore, the

metabolites of cluster 4 (except cellulose, catalpol, aucubin, carbon and MDA) varied also

according to a significant interaction between plant composition and climate scenario.

Elevated CO_2, compared to CO_2, increased the metabolites of cluster 4, but this positive

effect of a future climate was mitigated by interspecific plant interaction (Table S2.4). TCO_2,

compared to CO_2, increased further the N:P and the C:P ratio in monocultures but did not

alter the concentration of lignin, sucrose, salicylic acid, proline, TAC, and the ratio lignin:N.

Climate scenario did not alter the concentration of catalpol and carbon.

CO_2 and TCO_2 strengthened (salicylic acid and lignin) or induced (proline) an effect of aphid

infestation but only in monocultures (Fig. 4H). The concentrations of lignin and proline did

not differ between CO_2 and TCO_2 while the concentration of salicylic acid increased in

monocultures with aphids in TCO_2 compared to CO_2. For cellulose, the three-way interaction

between climate scenario, plant composition and aphid infestation was mainly due to a

significant increase in the concentration of cellulose in monocultures with aphids in C

compared to other treatments. In addition, CO_2 and TCO_2 also strengthened the effect of

aphid infestation on the concentration of aucubin but irrespective of the plant composition.

Furthermore, TCO_2 increased the concentration of MDA compared to the other climate

scenarios and this was most clear in monocultures with aphids.

Discussion

Our semi-natural experimental approach demonstrated that aphid herbivory induced

resistance against oxidative stress and herbivory in *P. lanceolata*, thereby reducing biomass

loss. Elevated CO_2 increased specific defense molecules and enhanced the effect of aphid

herbivory on these molecules. However, warming did not alter these effects of CO_2.

Furthermore, elevated CO_2 reduced the leaf quality for insect herbivores irrespective of the

plant composition while aphid infestation and TCO_2, compared to CO_2, did not alter it.

Notwithstanding the effects of a future climate on leaf quality and defense molecules, we

found no indications for indirect effects on aphid performance and herbivory. More

importantly, we shod that interspecific plant competition neutralises the positive effects of

elevated CO_2 on the production of defense molecules in *P. lanceolata* were neutralized.

Induced effects of climate change can thus be altered and even equalised by biotic

interactions. We first discuss the general herbivore-induced responses followed by an in-

depth discussion on their dependency on the imposed climate and competition treatments.

Herbivore-induced responses

Our experiment, as conducted under unique semi-natural conditions confirmed in first

instance findings that aphid infestation is reducing N, P, total proteins and lipids in *P.*

lanceolata leaves thereby impairing nutritional requirements of insect

herbivores(Schoonhoven *et al.*, 2005) .In contrast to previous research (Walling, 2000), aphid

infestation led to an increase in salicylic acid and jasmonic acid pathway (Kuśnierczyk *et al.*,

2011; Morkunas & Gabryś, 2011) as parallelized by the increased production of catalpol,

aucubin, tannin, lignin and cellulose.

Aphid herbivory increased levels of ROS in plants as well (Wu & Baldwin, 2010). We

assessed the induction of oxidative stress by measuring MDA, which aphid herbivory

increased considerably. A common defense response of plants against oxidative stress entails

changes in ROS scavenging antioxidant metabolites. Indeed, TAC and the well-known

antioxidant molecules phenols and tocopherols increased due to aphid herbivory. Moreover,

P. lanceolata responded to herbivory by increasing non-structural carbohydrates including

starch and soluble sugars. At higher concentrations, also sugars can act as ROS scavengers or

they can have a signaling function in regulating stress and defense responses (Gómez-Ariza

et al., 2007, Peshev *et al.*, 2013). Herbivory and the induced resistance against oxidative

stress appeared sufficiently effective to constrain biomass loss.

Climate dependency of the plant

Elevated CO_2 and temperature did not change the impact of herbivory effects on *P.*

lanceolate biomass. This implies that, warming and CO_2 did not affect the net interaction

strength between plants and herbivores under semi-natural conditions. In contrast to other

studies (Van De Velde *et al.*, 2016), our experiment allows a profound analysis on the

underlying physiological processes.

First, we found elevated CO_2 increased to increase starch, soluble sugars (in monocultures)

and lowered leaf proteins, hereby increasing the C:N ratio. In addition, elevated CO_2 reduced

P and consequently increased the C:P ratio (in monocultures), thereby reducing the plant's

nutritive value to herbivores (Huberty & Denno, 2006; Lincoln *et al.*, 1986; Mattson, 1980;

Schoonhoven *et al.*, 2005).We demonstrate here that these effects van be countered when

elevated CO_2 is accompanied with a temperature rise. This contradicts findings based on a

limited set of primary metabolites (Murray *et al.* 2013), but urges to apply broader phenomic

approaches to fully understand plant responses to climate change.

Second, elevated CO_2 increased the defense molecules lignin and tannin, but the level of

induction after aphid infestation. The induction of plant chemical defenses (Bidart-Bouzat &

Imeh-Nathaniel, 2008; Bidart-Bouzat *et al.*, 2005), is thus not consistent for all defense

molecules, and are thus anticipated to render prediction on future plant-insect herbivore

interaction highly complex (Agrawal, 1999). In our study, the higher C:N ratio reflected

higher lignin and tannin levels. While there is a consensus that the carbon-nutrient-balance

hypothesis fails as predictive hypothesis (Hamilton *et al.*, 2001; Lindroth, 2012), alternative

hypotheses have been proposed that resource utilization for chemical defense is linked with

photosynthesis, hormone regulation and the control of gene expression (Zavala *et al.*, 2017).

We here provide evidence for this alternative mechanism as we found CO_2 to alter

phytohormones (i.e., salicylic acid and jasmonic acid) that play an important role in

promoting compounds responsible for herbivore defense (Wu & Baldwin, 2010). Since no

further quantitative changes were observed under higher temperature, we can confidently

conclude that climate-change induced changes in metabolic profiles are more due to

enhanced CO_2 rather than the in parallel acting temperature increases.

Competition: a biotic interaction mediating climate-induced effects

The presence and identity of neighboring plants can influence the quality of the host plant by

altering primary and secondary chemistry (Barton & Bowers, 2006; Broz *et al.*, 2010,

Lankau, 2012; Thorpe *et al.*, 2011). We provide evidence that these biotic interactions are

additionally able to alter climate-induced phenomic changes, and by that further impacting

ecological interactions under climate change. Energetic growth-defense-trade-offs have

usually been attributed to these competition effects (Herms & Mattson, 1992), but recent

studies demonstrated the role of light limitation for plant defense downregulation (Ballaré,

2014; Campos *et al.*, 2016). More specifically, a reduced red to far-red ratio leads to a

negative regulation of defenses by a simultaneous inhibition of the jasmonic acid and the

salicylic acid pathway (Wit *et al.*, 2013). The lower levels of defense molecules and salicylic

acid in *P. lanceolata* support this hypothesis.. Competition did, however, also countered the

CO_2 –induced changes in primary and secondary metabolites, as well as those induced by

herbivores, rendering an understanding of plant defenses far from trivial (de Vries *et al.*,

2017). It is well-understood that bottom-up effects from plant quality to herbivore abundance

cannot be generalized across the feeding guilds (Bezemer & Jones, 1998; Robinson *et al.*,

2012; Stiling & Cornelissen, 2007), but phloem feeders as aphids were at least expected to be

facilitated. Despite the complex, high-dimensional changes in plant metabolomes under

competition and climate change, the overall effects on aphid population sizes were small to

non-existent.

We here thus demonstrate that the joint action of climate change and biotic interactions is

able to induce pronounced changes in plant metabolomes and biomass, but that translation of

these physiological changes towards ecological responses appears non-trivial. Climate-

change imposed changes are shown to be completely neutralized when common biotic

interactions are taken into consideration. Plant-plant interactions thus add a layer of

complexity in mechanistic studies of climate change effects on plant-enemy ecological

interaction.

Supplementary data:

Supplementary material 1: Metabolite quantification methods and SEM statistics

Supplementary material 2: results from the univariate GLM analyses (Statistics and

figures)

Acknowledgements

H. Van De Velde is a Research Assistant of the Fund for Scientific Research-Flanders

(FWO). D. Bonte was funded by the FWO research network W0.003.16N "An eco-

evolutionary network of biotic interactions". We thank the Earth and Life Institute

(Université Catholique de Louvain) for providing *Dysaphis plantaginea*, N. Calluy and M.

Wellens for technical assistance, colleagues for field assistance and Martijn Bezemer and

Martijn Vandegehuchte for helpful comments on an earlier version of this manuscript.

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Figure legends

Fig. 1 Heat map showing the metabolite levels in the leaves of *P. lanceolata*, normalized to

Z-score for each metabolite (blue-white-red heat map). Red and blue colours indicate a low

and high metabolite level, respectively. Clustering was based on the Euclidean distance for

metabolites. Labels 1-4 and colors indicate the four prominent clusters. Labels C, CO_2 and

TCO_2 indicate current climate, elevated CO_2 and combined warming and elevated CO_2,

respectively. Plant communities consist of monocultures of *P. lanceolata* (mono) and

mixtures of *Lolium perenne* and *P. lanceolata* (mix) with (A) and without aphids (C).

Fig. 2 (A) Structural equation model showing how climate scenario (CO_2 and TCO_2), mildew

infestation, plant composition and aphid infestation affect the chemical composition and the

live aboveground biomass of *P. lanceolata.* (B) Structural equation model showing how

climate scenario, plant composition and chemical composition of *P. lanceolata* affect aphid

population and how aphid population, in turn, affects the chemical composition of *P.*

lanceolata. The four clusters refer to those obtained by the hierarchical clustering analysis

(see Fig. 1). Solid black, green and orange arrows represent significant relationships (P

≤0.05) and dashed grey lines significant interactions. Blue lines stand for significant effects

of both CO_2 and TCO_2, green lines for significant effects of CO_2 and orange lines for

significant effects of TCO_2. Light grey arrows represent non-significant relationships.

Standardized path coefficients are shown next to pathways. For the effect of CO_2 and TCO_2,

the average path coefficients are shown. The individual path coefficients of CO_2 and TCO_2

can be seen in Table S1 and Table S2 (see supplementary material section 2). Metabolites

levels, live aboveground biomass of *P. lanceolata* and number of aphids were scaled before

analysis.

Fig. 3 Effect of climate scenario (C, CO_2 and TCO_2), aphid infestation and plant composition

on the live (A) and dead (B) aboveground biomass of *Plantago lanceolata*. Bars represent

means ± SE. Plant communities consist of monocultures of *P. lanceolata* and mixtures of

Lolium perenne and *P. lanceolata*.

Fig. 4 Effect of climate scenario (C, CO_2 and TCO_2), aphid infestation and plant composition

on the on a selection of relevant metaboloites from the four distinguished clusters. Bars

represent means ± SE. Plant communities consist of monocultures of *P. lanceolata* and

mixtures of *Lolium perenne* and *P. lanceolata*.

Cluster 1: A: C:N ratio; B: total phenols; C: soluble sugars

Cluster 2: D: Jasmonic acid; E: phosphorous, F: total proteins.

Cluster 3: G leaf nitrogen fractions

Cluster   4:   H: salicylic   acide;   I: TAC;   J: N:P   ratio;   K: C:P   ratio;

Fig. 1

Fig. 2

Fig. 3

701   **Fig. 4**