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Host tree impact on *Lymantria* species under CO₂ and temperature changes

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Introduction: Climate change has led to rising atmospheric CO₂ levels and temperatures, projected to double CO₂ concentrations and increase temperatures by 2–5°C by the end of the 21st century. These environmental changes influence plant primary and secondary metabolism, potentially altering plant-insect interactions. Herbivore performance depends on the nutritional quality of host plants, which may decline with elevated CO₂ due to an increased carbon-to-nitrogen (C:N) ratio. To explore these effects, the performance of spongy moth larvae (*Lymantria dispar*) was assessed on oak (*Quercus robur*) and spruce (*Picea abies*) seedlings grown under varying climatic conditions. This approach compares a preferred host with a non-preferred one in the case of *L. dispar*, providing insight into how host plant selection may be influenced under future climate scenarios. In addition, the nun moth (*Lymantria monacha*), a conifer-feeding species, was also studied on the experimental spruce seedlings to facilitate a comparison with a specialist herbivore.

Methods: Three-year-old oak and spruce seedlings were reared for 1 year under four climate scenarios combining two CO₂ levels (ambient: 410 ppm and elevated: 820 ppm) and two temperature regimes (20:15°C and 25:20°C). Seedlings were then processed into leaf powder diets for laboratory bioassays with larvae. Secondary metabolites in the seedlings were analyzed to assess climate-induced changes in tree composition and their effects on herbivores.

Results: Elevated CO₂ increased the C:N ratio in both tree species, with spruce showing a higher ratio than oak. Higher temperatures led to increased nitrogen content, particularly in oak seedlings. *L. dispar* performed better on oak despite higher secondary metabolite concentrations, while *L. monacha* exhibited minimal variation in performance on spruce across climate treatments.

Conclusion: The combined effects of elevated CO₂ levels and increased temperatures impacted plant quality; however, there were nearly no differences in the performance of *Lymantria* larvae. Despite the higher concentrations of secondary metabolites in the trees, the larvae were able to thrive effectively, demonstrating their resilience to environmental changes.

KEYWORDS

climate change, herbivory, plant chemistry, plant-insect interactions, secondary metabolites

1 Introduction

1.1 Human activity changes the atmospheric composition

Human activities have resulted in an increase in greenhouse gasses, particularly carbon dioxide (CO₂), which contributes significantly to global warming (IPPC, 2021; International Energy Agency [IEA], 2017; Xi-Liu and Gao, 2018; Yu et al., 2008). Despite established agreements and advancements in technology, there has been no meaningful reduction in CO₂ emissions and other harmful pollutants (Charfeddine, 2017). At the start of the Industrial Revolution, the concentration of atmospheric CO₂ was 280 ppm. Currently, it is approximately 400 ppm (Sodiq et al., 2023) and is projected to double by the end of the 21st century (IPCC, 2014). This increase in CO₂ levels is primarily due to the burning of fossil fuels and deforestation (IPCC, 2014; Begum et al., 2020; Uddin et al., 2021; Keeling, 1995). As a result, plants will need to adapt to the current conditions of CO₂ and temperature and future scenarios involving elevated CO₂ levels, higher temperatures, and potentially more frequent extreme weather events (Slot et al., 2021).

1.2 CO₂, temperature and plants

Increased levels of atmospheric CO₂ are resulting in higher global temperatures and more severe droughts (Diffenbaugh et al., 2017). Consequently, many trees have died (Hartmann et al., 2018). Forests are responsible for two-thirds of global photosynthesis (Giri, 2017) and cover over a third (43%) of the Earth's land surface (Bonan, 2008; Karnosky, 2003). While forests are directly exposed to the effects of climate change, they are also impacted by this phenomenon in other ways. Given forests crucial role in mitigating carbon emissions, it is important to understand how elevated CO₂ levels affect tree growth and ecosystem functions within forested areas (Psistaki et al., 2024).

Elevated levels of CO₂ and temperature can influence ecological processes in several ways: by directly affecting plant or insect herbivore species separately (Murray et al., 2013; Bale et al., 2002; Ellsworth et al., 2004), in combination (Zvereva and Kozlov, 2006), or indirectly through biotic interactions (Blois et al., 2013; Montoya and Raffaelli, 2010; Tylisanakis et al., 2008). The complex relationships among abiotic stress factors, host trees, and insect herbivores present significant challenges in predicting the overall impacts of climate change on forest ecosystems (Jactel et al., 2019).

1.2.1 CO₂ concentration

Numerous tree-ring studies have indicated that radial growth increases with rising atmospheric CO₂ concentration (Guo et al., 2022; Marchand et al., 2020; Zhang et al., 2003), but it seems that only for a short time period (Körner et al., 2005). Elevated levels of CO₂ in the atmosphere can change the chemistry of plants. This includes enhancements in photosynthesis and increases in carbohydrates (Zheng et al., 2019; Hartmann et al., 2018; Boullis et al., 2015; Zhang and Dang, 2007; Lindroth et al., 1993; Williams et al., 1998), as well as non-structural carbohydrates and carbon-based allelochemicals (Bae et al., 2019; Rajashekar, 2018). Additionally, the C:N ratio is affected (Sheng et al., 2021; Roy and

Mathur, 2021; Watanabe et al., 2021; Lotfiomran et al., 2016; Ziska, 2002). The C:N ratio reflects the plant's chemical composition, with an increase in carbohydrate content and a decrease in nitrogen concentration (by about 1/10–1/3) (Robinson et al., 2012; Lindroth et al., 2002; Ehleringer et al., 2002), a phenomenon referred to as the nitrogen dilution effect (Strain and Cure, 1985). However, these changes can vary among different tree species (Foss et al., 2013).

1.2.2 Air temperature

It is expected that the rise in atmospheric CO₂ levels will result in a global average surface temperature increase of at least 1–3°C within this century (Rapp, 2024; Adak et al., 2023; Ciaia et al., 2013; Solomon, 2007). Temperature is the primary factor limiting photosynthesis and plant growth (see Kaiser et al., 2015; Yamori et al., 2014; Kellomäki and Väisänen, 1988). Higher air temperatures are expected to enhance photosynthetic rate, which in turn will boost leaf biomass production and accelerate plant growth, further amplifying the effects of elevated CO₂ (Way and Oren, 2010; Farrar and Williams, 1991; Long, 1991). Notably, higher temperatures tend to promote growth in deciduous species more than in coniferous trees (Way and Oren, 2010). Additionally, the nutritional quality of host trees for herbivores is influenced by temperature (Holopainen et al., 2018). The temperature-induced changes in phytochemistry can vary significantly among different traits and tree species (Jactel et al., 2019). While increasing temperatures have a weak effect on nitrogen content or the C:N ratio, they significantly impact other characteristics, such as carbohydrates, phenols, and terpenoid content (Zvereva and Kozlov, 2006).

1.3 Plant-herbivore interaction

Herbivorous insects are closely connected to specific host plant species, which serve as their food source. The presence and success of herbivorous insects depend significantly on the quality of these host plants. The quality, in turn, is influenced by both primary and secondary metabolite composition and content (Schmitt et al., 2020). Organic carbon, bound in a wide range of plant-building compounds is the primary source of energy for insects, while their bodies are predominantly composed of proteins, containing also significant amounts of nitrogen. As a result, insects must consume not only a carbon-rich diet but also must contain substantial amounts of nitrogen to completely meet their nutritional needs (Zhou et al., 2015).

1.3.1 Nitrogen and carbon as important elements of life

Nitrogen is a crucial element for insect growth (Ren et al., 2022), and it plays a key role in plant metabolic processes, cellular structure, and genetic coding (Bala et al., 2018; Mattson, 1980). When nitrogen levels in leaves are low, the development of leaf-chewing insects is prolonged because they need to consume more plant tissue to obtain enough nitrogen-based nutrients (Stiling and Cornelissen, 2007; Johnson and McNicol, 2010; Johnson et al., 2014; Yin et al., 2010; Xie et al., 2015). In high CO₂ treatments, insect larvae can consume up to 80% more leaves (Lincoln et al., 1984). Additionally, insects that feed on plants grown in elevated CO₂

levels exhibit reduced efficiency in utilizing ingested food (Zhang et al., 2018; Teawkul and Hwang, 2019; Marks and Lincoln, 1996), which correlates with a reduced nitrogen content in their food source (White and Warrington, 1984). An increased feeding rate also leads to a higher intake of defensive compounds from host trees (Richardson et al., 2015).

Carbon provides the energy needed for metabolic processes (Bringaud et al., 2006). When the diet is too high in carbon compared to nitrogen, larvae may have plenty of energy but not enough protein for optimal growth. On the other hand, a diet high in nitrogen relative to carbon can enhance growth but may lack sufficient immediate energy. Although it is known that temperature changes can affect the plant nutritional value for many insect species, there is less understanding of how these temperature variations impact insect development (Stamp and Yang, 1996).

1.3.2 Secondary metabolites

Changes in the chemical composition of plants, particularly the levels of plant carbon-based secondary metabolites such as phenolic glycosides and tannins, can significantly affect the quality of plant foliage as food for insect herbivores (Boeckler et al., 2011; Singh et al., 2021; Moreira et al., 2017; Barbehenn and Constabel, 2011; Lindroth et al., 2001). Although secondary metabolites are not essential for the growth and development of organisms, they often serve other important ecological functions (Salam et al., 2023). These compounds can help plants adapt to environmental stresses and act as chemical defenses against various threats, including insects (Reddy and Guerrero, 2004; Chen et al., 2005). For example, phenolic compounds like tannin, lignin, and flavonoids play a role in defending against herbivores and pathogens. Condensed tannins can hinder the growth and development of herbivores by precipitating proteins (Croteau et al., 2000).

1.4 Hypothesis and goals

Previous studies have demonstrated that the effects of elevated CO₂ levels and temperature on plants vary among different tree species (Jactel et al., 2019; Way and Oren, 2010; Foss et al., 2013; Lindroth et al., 1993; Roth and Lindroth, 1994). Numerous studies have focused on the impact of elevated CO₂ on plants, trees and forests (Klein et al., 2016; Ceulemans and Mousseau, 1994; Curtis et al., 1996; Curtis and Wang, 1998; Saxe et al., 1998; Norby et al., 1999; Karnosky et al., 2001; Karnosky, 2003). Reviews focused on the combination of these factors report that the anticipated negative impacts of elevated CO₂ on herbivores may be mitigated by rising temperatures (Zvereva and Kozlov, 2006; Robinson et al., 2012).

Most studies on insect performance in elevated CO₂ atmospheres or varying air temperatures have primarily focused on a single host plant species, with herbivores raised directly on those plants, or considered only one climatic factor at a time (Henn and Schopf, 2001; Milanović et al., 2015; Xiaowei et al., 2006). Our research aim was to test the interactions between two climatic factors, temperature and CO₂ levels, on both broadleaf and coniferous trees, using two polyphagous herbivores that are significant forestry pests; one herbivore prefers conifers while the other prefers broadleaves. Furthermore, most of the studies cited in this article didn't analyze changes in plant chemical composition when evaluating larval performance.

In our study, we planted seedlings in four different combinations of climatic conditions, using two host tree species [*Quercus robur* (L.) and *Picea abies* (L.) Karst. and two closely related herbivores] [*Lymantria dispar* (L., 1758) and *Lymantria monacha* (L., 1758)]. We also prepared leaf powder diets from these seedlings to ensure precise scoring of herbivore performance. To analyze changes in plant chemistry, we conducted a chemical analysis of the leaves and needles, focusing on alterations in selected secondary metabolites. We expected that seedlings grown under less favorable conditions (higher temperature and CO₂ levels) would produce more defensive compounds, potentially leading to slower growth rates in the larvae.

The objectives of this study were: (I) to compare the effects of different combinations of CO₂ levels and temperatures on seedlings and the performance of two closely related herbivorous insects (II) to test how the secondary metabolic compounds in these seedlings are affected by varying environmental conditions.

2 Materials and methods

2.1 Study species

Our study examines two species of lepidopterans, the spongy moth (*Lymantria dispar*) and the nun moth (*Lymantria monacha*), along with two types of trees: the coniferous Norway spruce (*Picea abies*) and the broadleaf pedunculate oak (*Quercus robur*) from the nursery of the Tree Breeding Station in Kostelec nad Černými lesy in the Czech Republic.

The larvae of *L. dispar* originate from a laboratory rearing (the New Jersey Standard Strain) of USDA-APHIS (Animal and Plant Health Inspection Service, OTIS Methods Development Center, MA 02542). Nun moth larvae were reared from eggs collected around the town of Zgierz in Poland in February 2021.

Both larvae species belong to the family *Erebidae*. The spongy moth (*L. dispar*) is a common pest found in temperate forests across Europe. *L. dispar* typically feeds on broadleaves, especially oaks, rarely feeding on pines in natural environments (Castedo-Dorado et al., 2016). This study compared the response to a preferred broadleaf host with a non-preferred coniferous host. Its larvae are folivores, feeding on the leaves of over 300 tree species, including *Betula* sp. (birch), *Crataegus* sp. (hawthorn), *Larix* sp. (larch), *Populus* sp. (aspen), *Quercus* sp. (oak), *Salix* sp. (willow), and *Tilia* sp. (basswood) (Elkinton and Liebhold, 1990; Liebhold et al., 1995). The nun moth (*L. monacha*) primarily feeds on the needles of conifers, especially spruces and pines, as well as larch and fir (Nakládal and Brinkeová, 2015). In spring, the larvae of both species hatch from overwintering eggs and progress through five instars for males or six instars for females (Doane and McManus, 1981; Schwenke, 1978). The larvae feed on leaves from April to June, at which point they pupate. Adult moths have a short lifespan of less than 7 days, and females are unable to fly (Keena et al., 2007). Both species experience regular and cyclical population outbreaks (Hlásny et al., 2016), leading to extensive defoliation that has been documented since the Middle Ages (Komárek, 1931; Schwenke, 1978).

Norway spruce and pedunculate oak are common and economically important tree species in Europe. English oak

typically grows well in lowland areas, while Norway spruce is more prevalent at higher elevations. The economic significance of Norway spruce is highlighted by its extensive use in the forestry and timber industries (Jansson et al., 2013). However, its growth and resilience are increasingly threatened by climate change, especially at the trailing edge of its distribution (Honkaniemi et al., 2020).

2.2 Plant growing conditions

The experiment was conducted in four growth chambers (Step-In Fytoscope FS-SI 3400), which were set either to ambient CO₂ levels (410 ppm) or elevated CO₂ levels (820 ppm) at day and night temperatures to 20:15°C and 25:20°C under a light-dark cycle of 16:8 h. Relative humidity remained consistent at 70% in all chambers.

Oak and spruce seedlings were planted in trays (20 cm × 20 cm × 30 cm) filled with AGRO substrate (Agro CS a.s., Czech Republic) in the year 2016 in the nursery. On April 1st, 2019, nine spruce and nine oak seedlings were placed in each growth chamber. From November 10th to December 6th, 2019, the air temperature was gradually lowered from 20 and 25°C by 5 and 3°C per week until the temperature reached 5°C. On December 7th, 2019, the seedlings were placed outside for dormancy until February 17th, 2020, when they were returned to the chambers, and both the photoperiod and temperature were gradually increased again by 5 and 3°C each week until they finally reached 20 and 25°C. The collection of leaves was conducted in May 2020. The collected leaves were weighed, placed in paper bags, and frozen in liquid nitrogen. A freeze dryer (Alpha 2-4 LSC basic, Christ) was used for lyophilization, and the samples were then ground and homogenized using a mixer mill (MM 400, Retsch) and stored in a deep freezer at -80°C until diet preparation.

2.3 Chemical analysis

2.3.1 Elementary N and C analysis

For the analysis, 10–30 mg of the pure sample of the leaf powder was weighed into tin capsules designed for the instrument used. The capsules were then introduced into the combustion chamber of the Thermo Scientific Flash 2000 Elemental analyzer (Thermo Scientific, United States). The samples were combusted in a stream of pure oxygen at a temperature of 1,000°C. The resulting carbon and nitrogen oxides were passed through a copper reduction column and directed to a separation column, where moisture was removed. Helium was used as the carrier gas. The content of the separated oxides was determined using a thermal conductivity detector, and signal processing was performed with Eager Xperience software (Thermo Scientific).

2.3.2 Metabolite analysis

2.3.2.1 Extraction of carbohydrates

A total of 40 mg of freeze-dried samples (72 spruce and 36 oak samples) was extracted by 500 µL of methanol:chloroform: water in the ratio 12:5:3 and kept in the water bath at 60°C for 30 min. After 1,000 µL of water was added to the test tube. The solution was vortexed and then centrifugated for 3 min at 13,000 rpm. The

supernatant was filtered using a 0.22 µm PVDF filter prior to LC-MS-qTOF analysis.

2.3.2.2 Determination of total phenolic content

TCT was performed according to Makkar et al. (1993). Briefly, 20 µL of the extract was placed in the test tube and made up the volume to 0.5 mL of water. Then, 250 µL of the Folin Ciocalteu reagent was added. After 3 min, 1 mL of 20% sodium carbonate was added. The test tube was well mixed and kept in a dark place for 40 min. The absorbance was recorded at 725 nm using a spectrophotometer. The concentration of TPC was expressed in tannin acid equivalent.

2.3.2.3 Determination of total flavonoid content

The determination of TFC was carried out according to Zengin et al. (2016). Briefly, 150 µL of sample extract was placed into the 2 mL test tube and filled up to 1 mL with 70% of acetone. Then, a 2% solution of aluminum trichloride prepared in methanol was added. After 15 min, the absorbance was recorded at 415 nm, against blank. The concentration of flavonoid content was expressed in rutin equivalent (mg g⁻¹).

2.3.2.4 Determination of total condensed tannin content

The total flavonoid content was determined following the method described by Porter et al. (1985). 150 µL of sample and 150 µL of 70% acetone were placed in 2-mL test tube. Then, 1.5 mL of butal-HCL reagent was added [butanol-HCL reagent was prepared by mixing 95 mL of butanol and 5 mL of concentrated HCL (37%)]. After, 50 µL of ferric reagent was added (ferric reagent was prepared as 2% ferric ammonium sulfate in 2N HCL). The solution was well mixed and placed in the thermoshaker for 1 h at 95°C and 1,000 rpm. When the solution was cooled, the absorbance was recorded at 550 nm. The concentration of TCT was expressed in cyanidin equivalent (mg g⁻¹).

2.3.2.5 LC-MS-qTOF analysis of carbohydrates

LC-qTOF-MS/MS analysis was performed using Agilent 1290 Infinity II coupled with Agilent 6546 LC/MS QTOF system (Agilent, United States). Chromatographic separation was performed on Asahipak NH2P40 3E column 3.0 × 250 mm, 4 µm (Shodex, Japan) operated at 30°C. The mobile phase was composed of acetonitrile (A) and water (B). The isocratic elution was achieved with the started condition of mobile phase 70:30 (A:B) and increased up to 75:25 (A:B) for 30 min with a flow rate of 0.3 mL min⁻¹. The injection volume was set to 1 µL. The system was operated in negative ionization mode. The QTOF parameters were as follows: scan range 100–760 m/z; drying gas temperature, 280°C; sheath gas flow rate, 12.0 L/min; sheath gas temperature, 275°C; capillary voltage, 3.0 kV; fragmentor, 160 V; collision energy at 10, 20 and 40 eV. MS/MS data were acquired at a scan range was 50–760 m/z. During the analysis, two reference masses: 112.9855 m/z and 966.0007 m/z were continuously measured for mass correction. The data collection was carried out using Agilent Mass Hunter Acquisition software. The data analysis was performed using Agilent Mass Hunter Qualitative Analysis 10.0 and Q-TOF Quantitative analysis. Identification of monosaccharides and oligosaccharides was determined according to precursor ions, the fragment ions, and retention time compared with the standards of sucrose, glucose, fructose, and raffinose.

2.4 Leaf powder diet preparation

The larvae were fed a wheat control diet after Bell (1981) from emergence until the end of the second instar (the diet recipe can be found in Supplementary Table S1). This diet (later called “wheat control diet”) was also provided to control group larvae, which were fed this diet for the entire experimental duration.

We prepared leaf powder diets from leaves of spruce and oak experimental seedlings to feed the fourth instar larvae. The preparation process is similar to that of the wheat diet and includes the following steps: (1) Boil water and dissolve agar in it. (2) Allow the mixture to cool down to 50°C. (3) Add leaf powder, Sorbic acid, and Methyl Parabenzenes to the mixture. (4) Mix all the ingredients thoroughly. (5) Store the prepared mixture in Petri dishes or syringes in the refrigerator. All the ingredients can be found in Supplementary Table S2.

We used plastic syringes to maintain the freshness of the leaf powder diets, which made it easy to measure the amount of food served to the larvae. When opening the syringes, we cut off the tops and then covered them with parafilm before storing them in the refrigerator.

2.5 Experimental design

The eggs of both species (*L. dispar* and *L. monacha*) were placed separately in 250 mL plastic box containing a wheat control diet. They were kept at 20°C under a light:dark cycle of 16:8 h. Once the larvae emerged, they were allowed to feed on the wheat control diet until they reached the end of the second instar. Beginning in the third instar, the larvae were reared in groups of 15 within Petri dishes (ϕ6 cm) and provided with leaf powder diets. *L. dispar* larvae were fed both spruce and oak diets, while *L. monacha* larvae were fed only the spruce diet. We monitored the larvae hourly (besides night time (7:00 a.m.–10:00 p.m.)), to identify the premolt stage, which is indicated by the larvae being stuck with their heads moved forward. When in the premolt stage, the larvae were transferred to individual Petri dishes containing a small piece of leaf powder diet

(2 g). Immediately after molting into the fourth instar, we weighed both the larva and the piece of diet. During the fourth instar, the larvae were left to feed *ad libitum* for 5 days (the duration of the fourth instar at 20°C), with additional food provided if necessary. Once the larvae reached the premolt stage to the fifth instar, they were weighed, placed in small Eppendorf tubes, and frozen along with any remaining food and frass. The collected material was then dried in an incubator with blue desiccant and stored refrigerated at -20°C.

To ensure accurate measurements of evaporation-induced weight loss in artificial larval diets, we stored 10 samples of each diet type at 20°C for 5 days and determined the water loss thereafter. This information was then used to adjust our data analysis accordingly.

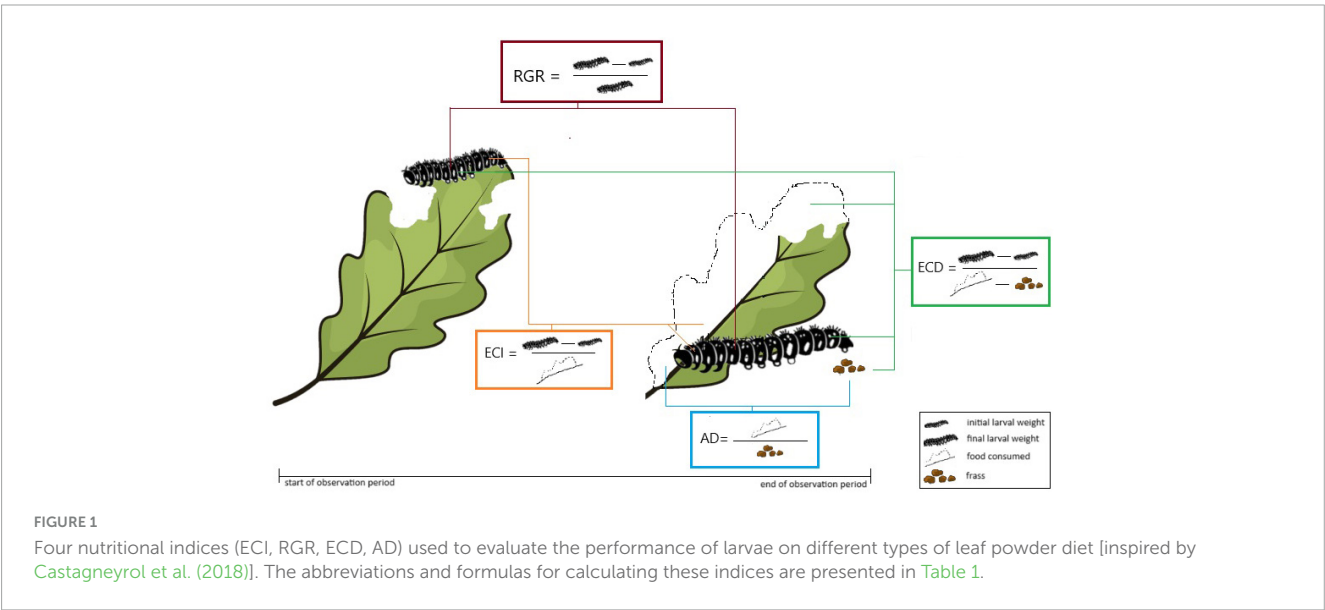
2.6 Evaluating larval performance

According to the gravimetric method of Waldbauer (1968), four nutritional indices were used to evaluate larval performance on the different diets (Table 1). These indices include AD (approximate digestibility), ECD (efficiency of conversion of digested food), ECI (conversion of ingested food), and RGR (relative growth rate) (Figure 1). All indices are calculated based on dry weights.

TABLE 1 Formulas of all nutritional indices used for evaluating larval performance, including their abbreviations.

Index	Description	Formula
RGR	Relative growth rate	$(\log W_2 - \log W_1) / t$
AD	Approximate digestibility (%)	$[(Q - F) / Q] \times 100$
ECI	Efficiency of conversion of ingested food (%)	$[(W_2 - W_1) / Q] \times 100$
ECD	Efficiency of conversion of digested food (%)	$[(W_2 - W_1) / (Q - F)] \times 100$

In the formulas, W represents the dry weight of larvae, Q the dry weight of consumed food, F the dry weight of frass, and t the time period.



Approximately 25 larvae were weighed immediately after molting from the third to the fourth instar (FW—Fresh weight). They were then frozen and dried (DW—Dry weight) to determine the FW/DW ratio. This ratio was used to estimate the dry weight of the larvae at the beginning of the fourth instar using the formula $WC = [(FW-DW) \times 100]/FW$. To determine the dry weight of the food at the start of the observed period, we applied this formula to 10 pieces (2 g) of each type of diet (as described in the previous chapter), and the average of these results was used as the initial diet weight.

The relative growth rate (RGR) explains the amount of larval gain within a specific unit of time. Approximate digestibility (AD) measures the efficiency of food conversion. The efficiency of conversion of ingested food (ECI) is an overall index of the insect's ability to utilize foliage for growth. The conversion efficiency of digested food (ECD) is an index of the insect's ability to assimilate and convert food into body substances (Waldbauer, 1968).

2.7 Statistical analysis

Statistical analyses were performed using R statistical software (R Core Team, 2021). Principal Component Analysis (PCA) was conducted using the *prcomp* function from base R.

To assess the effects of different diets on larval growth, estimated by four nutritional indices, an analysis of variance (ANOVA) was first performed using the *aov* function. This compared the means of the selected indices across diet groups for each moth species separately. Tukey's Honest Significant Difference (HSD) test was then applied to the ANOVA models to conduct pairwise comparisons between food groups, with the results summarized in plots using letter groupings (above each tasted treatment).

For further analysis of diet effects on nutritional indices, linear mixed-effects models were fitted for each growth index and moth species using the *lmer* function from the *lme4* package (Bates et al., 2015). The initial models included tree species, cultivation temperature, and CO₂ regimes as fixed effects, with individual food batches treated as random effects. Since only the tree species factor showed statistically significant differences, diets were categorized into three diet groups: control, oak-based and spruce-based. Subsequently, a simplified linear mixed-effects model was fitted with these diet categories as fixed effects and individual food batches as random effects. Then *emmeans* function from the *emmeans* package (Russell et al., 2021) was used to estimate marginal means and perform pairwise comparisons between diet categories. Results were visualized using letter groupings in summary plots (above each diet group).

To compare carbon (C), nitrogen (N), the carbon-to-nitrogen ratio (C:N), sugar compounds, and phenolic compounds among food batches, ANOVA models were employed. These included tree species, cultivation temperature, CO₂ regimes, and interactions of tree species with sampling date, cultivation temperature and CO₂ regimes as explanatory variables.

Effect sizes (η^2 values) were calculated using the *etaSquared* function from the *lsr* package (Navarro and Navarro, 2022) to evaluate the contribution of individual factors and interactions to variation in the response variables. The η^2 statistic quantifies

the proportion of the total variance in a response variable that is explained by each explanatory variable or interaction in the model. This provides a standardized measure of effect size, allowing for the comparison of the relative importance of different factors.

For all models, the normality of residuals was checked. Several outlying values, likely due to measurement errors, were omitted, and the data were reanalyzed to ensure robustness.

3 Results

3.1 Nitrogen, carbon, and C:N ratio in spruce and oak

The nitrogen concentration varied significantly among tree species, with lower levels observed in spruce. This difference was logically reflected in the C:N ratio, which was significantly higher in spruce. Seedlings growing in doubled CO₂ concentration had a higher C:N ratio. Seedlings growing at higher temperatures contained higher nitrogen content and didn't affect the C:N ratio (Table 2 and Figure 2).

3.2 Chemical analysis of oak leaves and spruce needles

The leaves of oak seedlings contained higher levels of phenolics and condensed tannins compared to spruce needles. Spruce had higher flavonoid content. However, there was no significant difference in non-tannins between oak and spruce seedlings (Tables 3, 4).

Higher temperatures affected phenolic and flavonoid concentrations. Phenolic concentrations increased at higher temperatures, except in oak under ambient CO₂ concentration. Flavonoid concentrations lowered at higher temperatures except

TABLE 2 Influence of tree species (spruce and oak), elevated temperature (20°C vs. 25°C), and doubled air CO₂ concentration (410 ppm vs. 820 ppm) on nitrogen, carbon, and the C:N ratio in leaves.

N	F-value	p-value	Eta ²	Eta ² part
Tree species	411.570	<0.001	0.954	0.991
Temperature	17.509	0.014	0.039	0.814
CO ₂	5.369	0.081	0.012	0.573
C	F-value	p-value	Eta ²	Eta ² part
Tree species	1.295	0.319	0.061	0.168
Temperature	1.791	0.252	0.135	0.309
CO ₂	3.040	0.156	0.228	0.432
C:N	F-value	p-value	Eta ²	Eta ² part
Tree species	224.088	<0.001	0.915	0.983
Temperature	7.460	0.052	0.030	0.651
CO ₂	11.233	0.029	0.044	0.737

Bold numbers represent significant impact.

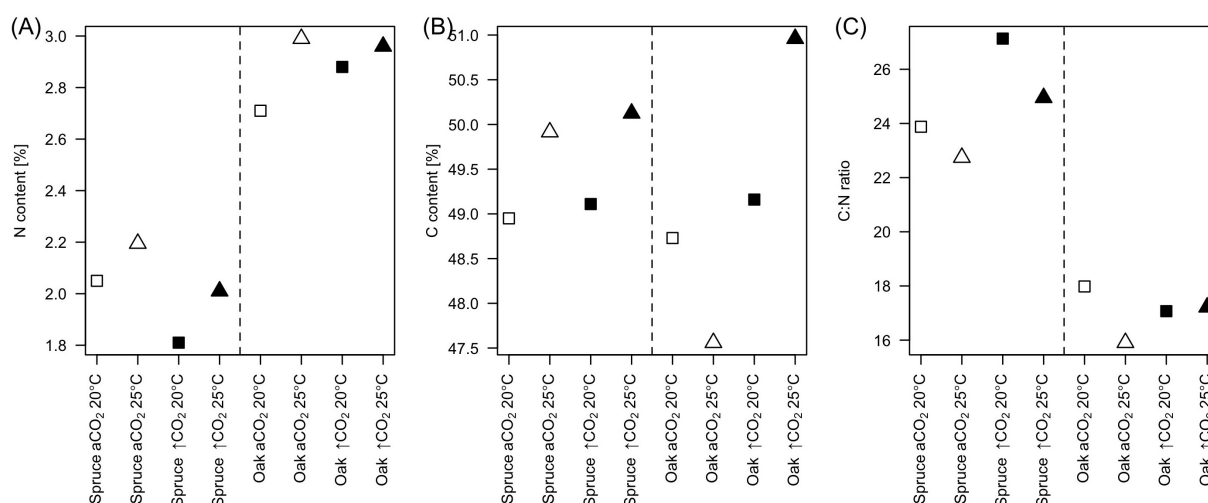


FIGURE 2

Influence of CO₂ concentrations and temperature on the nitrogen (A), carbon (B) content and the C:N ratio (C) in leaf powder diets prepared from spruce and oak seedlings. In all cases, 36 samples were analyzed.

in spruce under doubled CO₂ concentration (Tables 3, 4). The CO₂ treatments didn't affect any secondary metabolites we studied (Table 4).

Sucrose and raffinose concentrations were significantly higher in oak compared to spruce. The levels of all sugar types in diets from seedlings grown under both temperature regimes and in both ambient and doubled CO₂ concentrations were similar (Tables 5, 6).

3.3 The performance *Lymantria* species on different leaf powder diets prepared from oak (*L. dispar*) and spruce (*L. dispar* and *L. monacha*)

For *L. dispar*, the nutritional indices, except for the ECD, were significantly higher in larvae reared on oak diets compared to those reared on spruce diets. Except for the ECD index, the oak diet showed a significant difference when compared to the wheat control diet. Except for the ECD index, larvae reared on spruce diets performed worst.

Only a small influence of climatic conditions on *L. dispar* larval performance was found, but significant differences were found between different climatic conditions in three cases. AD index was higher for larvae reared on spruce needles grown at 20°C than at 25°C when growing in doubled CO₂ concentration. ECI index was higher for larvae reared on spruce needles grown at 20°C and doubled CO₂ concentration than other spruce variants. ECD index was lower for larvae reared on spruce needles grown at ambient CO₂ and 20°C compared to all other spruce variants (Figure 3).

For *Lymantria monacha*, the RGR, AD, and ECI indices were significantly higher in the wheat control diet than in the spruce needle powder diet.

Only a small influence of climatic conditions on *L. monacha* larval performance was found. Still, significant differences were found between different climatic conditions in three cases. RGR

index was higher for larvae reared on spruce grown at ambient CO₂ and 20°C than fed on spruce grown in doubled CO₂ concentrations and both temperatures. AD index was higher for larvae reared on spruce grown in doubled CO₂ when comparing both CO₂ treatments at 20°C. ECD index was higher for larvae reared on spruce grown at ambient CO₂ when comparing both CO₂ treatments at 25°C (Figure 4).

4 Discussion

In our experiment, seedlings of two tree species, spruce and oak, were reared under four different climatic conditions for 1 year. The study aimed to examine how these factors interact and their potential effects on the food quality for herbivorous insects. We compared a preferred host tree (oak) with a non-preferred host tree (spruce) and the response of Spongy moth (*L. dispar*) larval performance and conifer-preferring nun moth (*L. monacha*) on spruce, providing insight into how host plant selection may be influenced under future climate scenarios.

4.1 Effects of doubled CO₂ concentration and higher temperature on C:N ratio and water soluble carbohydrates in seedlings

Our findings support the hypothesis that the plant C:N ratio in plants increases with elevated air CO₂ concentrations, as observed in previous studies (Vencl et al., 2024; Zheng et al., 2019; Lotfiomran et al., 2016; Du et al., 2019; Hikosaka et al., 2006; Luo et al., 2006). The extent of this impact can vary based on plant species (Kubiske et al., 2015), growth conditions, and other environmental factors (Gifford et al., 2000).

Understanding how climate warming affects ecosystem function and stability is crucial for predicting the impacts of global climate change (Liu et al., 2021). Our results showed

TABLE 3 Contents of secondary metabolites in leaves of oak ($n = 36$) and spruce ($n = 72$) seedlings grown under lower (20°C) and higher (25°C) temperatures, as well as ambient (410 ppm) and doubled (\uparrow , 820 ppm) CO₂ concentrations.

Diet	Total phenolic and tannins content (mean, mgTA/g)	Total phenolics (SD)	Total non-tannins (mean, mgTA/g)	Total non-tannins (SD)	Total flavonoids (mean, mgRU/g)	Total flavonoids (SD)	Total condensed tannins (mean, mgCD/g)	Total condensed tannins (SD)
aCO ₂ , 20°C spruce	49.87	3.69	0.42	0.01	34.30	0.40	6.59	0.01
aCO ₂ , 25°C spruce	57.53	0.83	0.41	0.05	32.44	0.61	1.29	0.03
\uparrow CO ₂ , 20°C spruce	51.84	0.27	0.35	0.02	27.81	0.21	2.25	0.03
\uparrow CO ₂ , 25°C spruce	52.29	4.37	0.38	0.01	30.42	0.76	3.56	0.05
aCO ₂ , 20°C oak	91.67	2.12	0.47	0.03	36.29	0.65	6.21	0.10
aCO ₂ , 25°C oak	52.74	1.75	0.30	<0.01	25.93	0.42	3.28	0.01
\uparrow CO ₂ , 20°C oak	74.01	2.90	0.43	<0.01	32.52	0.33	5.66	0.01
\uparrow CO ₂ , 25°C oak	77.35	0.86	0.46	0.02	26.54	1.39	6.21	0.03

TABLE 4 Comparison of the contents of individual groups of secondary metabolites in leaves of oak ($n = 36$) and spruce ($n = 72$) seedlings grown under lower (20°C) and higher (25°C) temperatures, as well as ambient (410 ppm) and doubled (820 ppm) CO₂ concentrations.

Phenolics	<i>F</i> -value	<i>p</i> -value	Eta ²	Eta ² part
Tree species	224.545	<0.001	0.720	0.894
Temperature	12.394	0.002	0.044	0.341
CO ₂	3.729	0.065	0.013	0.135
Non-tannins	<i>F</i> -value	<i>p</i> -value	Eta ²	Eta ² part
Tree species	0.455	0.507	0.015	0.017
Temperature	0.011	0.917	<0.001	0.001
CO ₂	0.002	0.961	<0.001	<0.001
Flavonoids	<i>F</i> -value	<i>p</i> -value	Eta ²	Eta ² part
Tree species	68.339	<0.001	0.474	0.705
Temperature	12.091	0.002	0.100	0.335
CO ₂	0.133	0.718	0.001	0.006
Condensed tannins	<i>F</i> -value	<i>p</i> -value	Eta ²	Eta ² part
Tree species	69.563	<0.001	0.740	0.747
Temperature	1.436	0.243	0.015	0.056
CO ₂	0.048	0.829	<0.001	0.002

that seedlings grown at higher temperatures had increased nitrogen concentrations in their leaves and needles. This finding aligns with research by [Yuan et al. \(2021\)](#), which reported an almost 8% decrease in the C:N ratio at elevated temperatures in *Quercus mongolica* Fisch. ex Turcz. and *Fraxinus mandschurica* Rupr. Similarly, [Zhang et al. \(2011\)](#) indicated that increased nitrogen concentrations in leaves at higher temperatures are linked to enhanced photosynthesis and a greater capacity for nitrogen uptake. This mechanism likely explains our findings, where doubled CO₂ levels did not result in a significant increase in carbon content in the seedlings.

The response of the C:N ratio varied between tree species. Spruce seedlings exhibited a higher C:N ratio compared to oak trees, which had correspondingly higher nitrogen concentrations in their leaves. The low C:N ratio in oak indicates a greater investment in proteins essential for photosynthesis ([Zhang et al., 2020](#)). Additionally, oak leaves contained a higher content of certain sugars, particularly sucrose and raffinose. These sugars are critical products of photosynthesis, providing carbon and energy necessary for synthesizing secondary metabolites ([Rolland et al., 2006](#)). Compounds such as phenols, flavonoids, and condensed tannins are synthesized via the shikimate pathway, which relies on intermediates derived from sugar metabolism, including phosphoenolpyruvate and erythrose-4-phosphate as primary substrates ([Marchiosi et al., 2020](#)). This relationship may explain why oak, with its higher sugar content, also exhibited elevated levels of these carbon-based secondary metabolites.

TABLE 5 Sugar contents in oak ($n = 36$) and spruce ($n = 72$) seedlings grown under lower and higher temperatures (20°C and 25°C), as well as ambient (410 ppm) and doubled (820 ppm) CO₂ concentrations.

Diet	Sucrose content (mean, mg/g)	Sucrose content (SD)	Fructose content (mean, mg/g)	Fructose content (SD)	Glucose content (mean, mg/g)	Glucose content (SD)	Raffinose content (mean, mg/g)	Raffinose content (SD)
aCO ₂ , 20°C spruce	23.35	0.68	2.61	0.15	1.93	0.05	0.09	0.01
aCO ₂ , 25°C spruce	20.76	0.70	2.67	0.15	1.85	0.22	0.13	0.03
↑CO ₂ , 20°C spruce	23.47	0.80	3.03	0.06	2.33	0.09	0.22	0.01
↑CO ₂ , 25°C spruce	22.46	0.94	2.55	0.09	1.88	0.19	0.11	0.03
aCO ₂ , 20°C oak	27.50	0.11	3.14	0.11	2.74	0.04	0.22	0.11
aCO ₂ , 25°C oak	26.87	0.13	2.75	0.31	2.56	0.12	0.24	0.03
↑CO ₂ , 20°C oak	25.68	0.84	2.82	0.07	2.29	0.27	0.25	0.01
↑CO ₂ , 25°C oak	23.89	0.36	3.78	0.07	3.90	0.35	0.29	0.01

TABLE 6 Comparison of the sugar contents in oak ($n = 36$) and spruce ($n = 72$) seedlings grown under lower and higher temperatures (20°C and 25°C), as well as ambient (410 ppm) and doubled (820 ppm) CO₂ concentrations.

Sucrose	<i>F</i> -value	<i>p</i> -value	Eta ²	Eta ² part
Tree species	20.676	0.010	0.477	0.806
Temperature	5.861	0.073	0.168	0.594
CO ₂	0.737	0.439	0.021	0.156
Glucose	<i>F</i> -value	<i>p</i> -value	Eta ²	Eta ² part
Tree species	5.164	0.086	0.360	0.546
Temperature	0.084	0.787	0.006	0.020
CO ₂	0.002	0.963	<0.001	<0.001
Fructose	<i>F</i> -value	<i>p</i> -value	Eta ²	Eta ² part
Tree species	2.017	0.229	0.209	0.309
Temperature	0.148	0.720	0.017	0.036
CO ₂	0.017	0.902	0.002	0.004
Raffinose	<i>F</i> -value	<i>p</i> -value	Eta ²	Eta ² part
Tree species	20.576	0.011	0.631	0.824
Temperature	0.843	0.411	0.029	0.174
CO ₂	0.496	0.520	0.017	0.110

Bold numbers represent significant impact.

4.2 Effects of doubled CO₂ concentration and higher temperature on plant secondary metabolites

4.2.1 Phenolics

Our research shows that higher temperatures in both tree species, along with doubled CO₂ in oak, lead to an increase in phenolic concentrations. Only one opposite finding was in oak at ambient CO₂ with rising temperature, which aligns with a meta-analysis by Zvereva and Kozlov (2006) and a review by Julkunen-Tiitto et al. (2015), which stated that phenolic concentrations tend to decrease as temperatures rise under ambient CO₂ conditions. In contrast, spruce needles exhibited a different response under ambient conditions, with no significant differences observed in seedlings exposed to elevated CO₂ levels, as reported in previous studies (Koricheva et al., 1998; Zvereva and Kozlov, 2006; Lindroth, 2012; Julkunen-Tiitto et al., 2015; Robinson et al., 2012). Moreover, our findings indicate that the interaction between CO₂ and temperature can interfere with each other's effects, suggesting that the impact of these factors varies depending on the specific context (Julkunen-Tiitto et al., 2015; Zvereva and Kozlov, 2006; Veteli et al., 2007).

4.2.2 Flavonoids

We observed that flavonoid levels significantly decreased at higher temperatures in oak leaves under both ambient and elevated CO₂ conditions. This finding is consistent with the research conducted by Julkunen-Tiitto et al. (2015). In contrast, we found

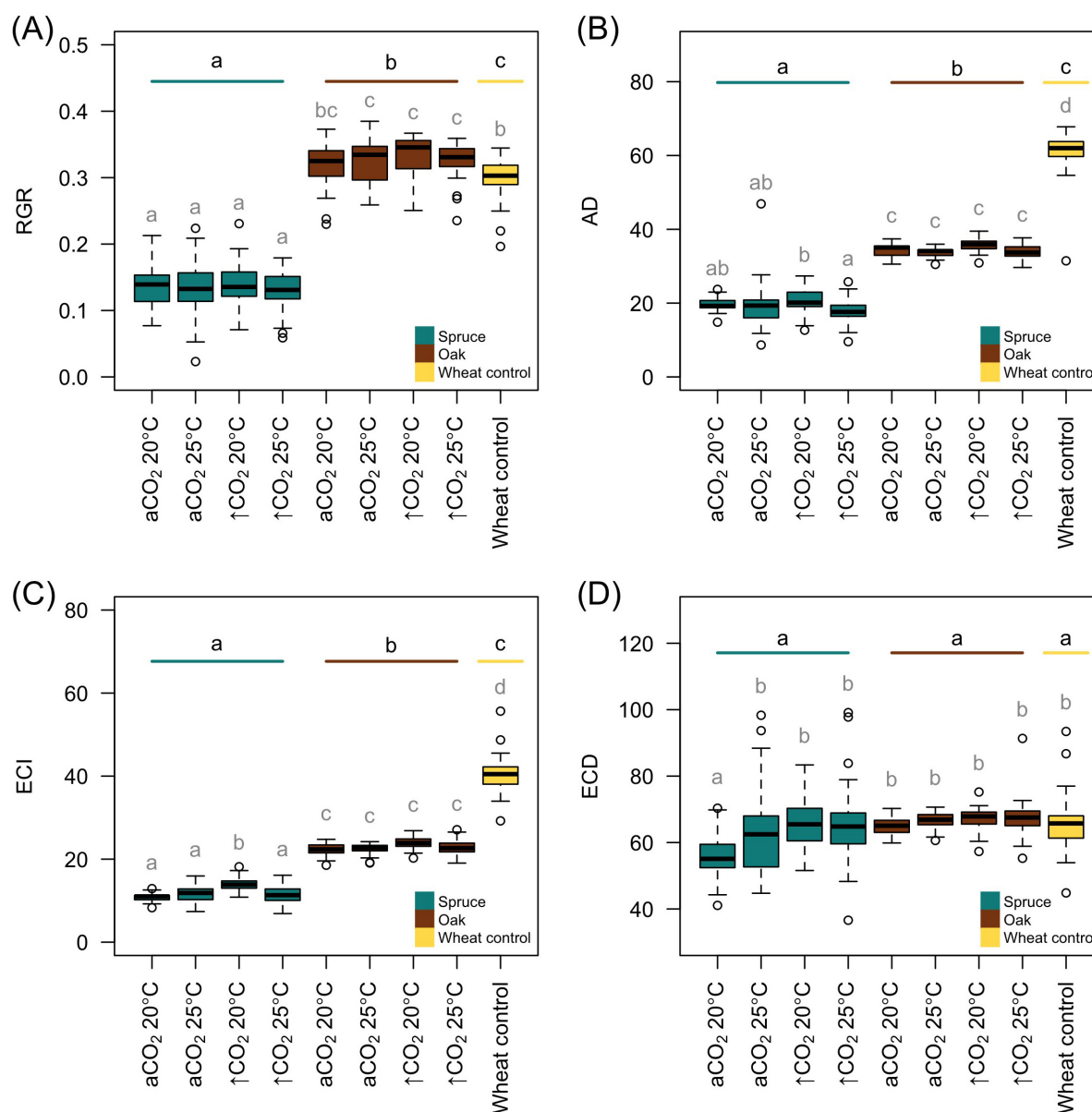


FIGURE 3

The influence of spruce and oak leaf powder diet from trees grown under two temperatures (20°C and 25°C) and two CO₂ concentrations (410 ppm and 820 ppm) on the growth rate (RGR) (A), approximate digestibility (AD) (B), efficiency of conversion of ingested food to body substances (ECI) (C), and efficiency of conversion of digested food to body substances (ECD) (D) of *L. dispar* compared to the growth on wheat germ diet (controls) (after Bell, 1981). Colorful lines with different letters in the upper part of graphs summarize the significant differences between spruce, oak, and wheat control diet groups. Different gray letters above boxplots indicate significant differences between the tested treatments. For further details on the indices, refer to Table 1 and Figure 1.

an opposing result in spruce needles grown under doubled CO₂ concentration, where the flavonoid content was higher at 25°C compared to 20°C. This trend was also reported in studies by Li et al. (2011) and Shamloo et al. (2017). Additionally, Holopainen et al. (2018) mentioned that elevated CO₂ levels should increase flavonoid concentrations, although this effect is weaker than that of temperature changes. This discrepancy may help explain the differences we observed, but a similar trend was not found in oak trees. Some studies, including those by Holopainen et al. (2018) and Julkunen-Tiitto et al. (2015), indicated that the combination of temperature and CO₂ had no significant impact on flavonoid levels.

In summary, our findings indicate that the effects of climate change factors, such as elevated temperatures and increased CO₂ concentrations, on secondary metabolites are highly variable and depend on the specific context. The responses of phenolics and flavonoids differ, suggesting that the interaction between CO₂ levels and temperature can either enhance or reduce the impacts on plant metabolites. Further studies with a longer exposure period of the trees under the corresponding temperature and CO₂ conditions could possibly provide a clearer picture of the factors influencing the changes in leaf constituents and contribute to a better understanding of the

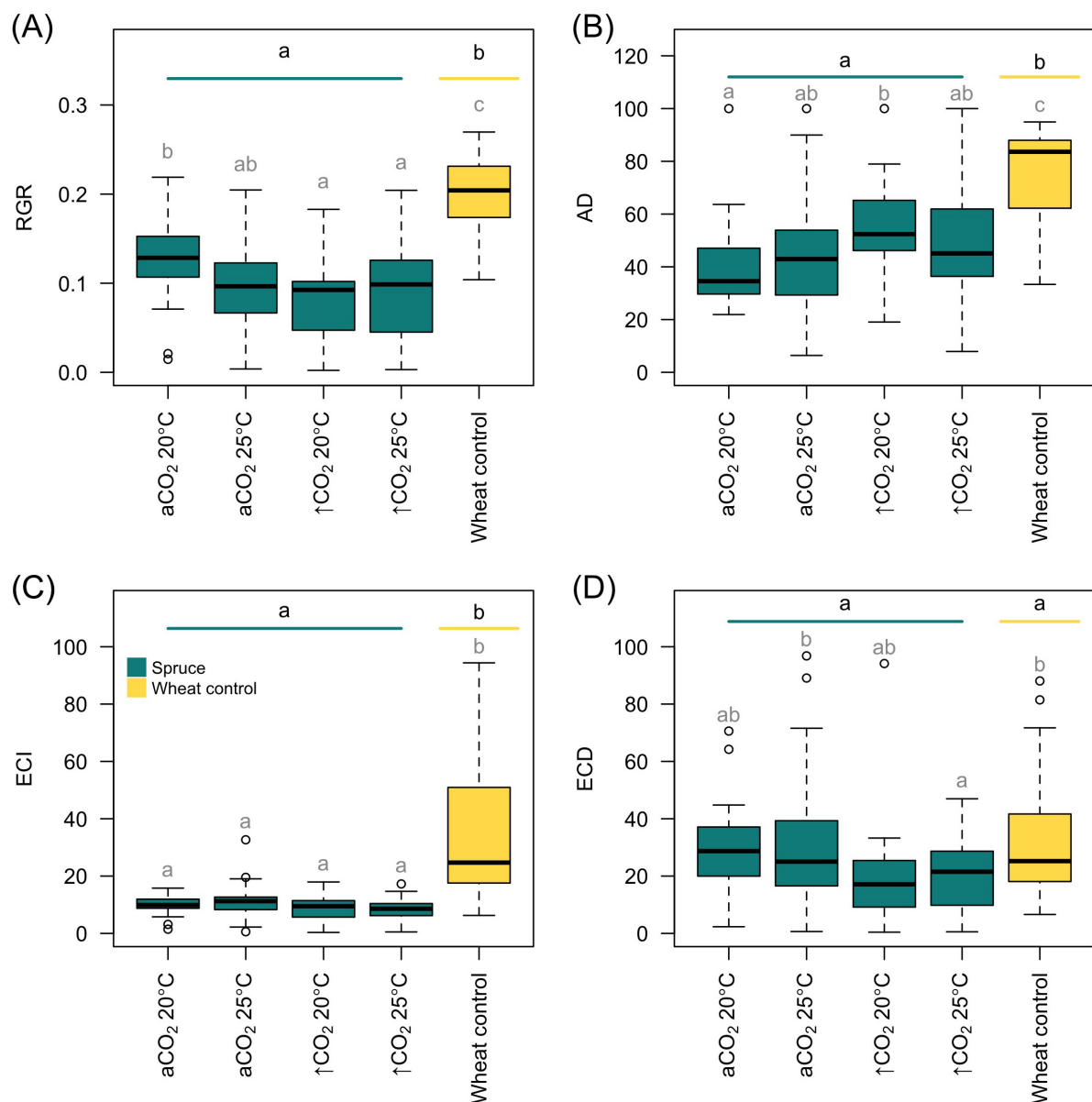


FIGURE 4

Influence of spruce leaf powder diet from trees grown under two temperatures (20°C and 25°C) and two CO₂ concentrations (410 ppm and 820 ppm) on the growth rate (RGR) (A), approximate digestibility (AD) (B), efficiency of conversion of ingested food to body substances (ECI) (C), and efficiency of conversion of digested food to body substances (ECD) (D) of *L. monacha* compared to the growth on wheat germ diet (controls) (after Bell, 1981). Colorful lines with different letters in the upper part of graphs summarize the significant differences between spruce, oak, and wheat control diet groups. Different gray letters above boxplots indicate significant differences between the tested treatments. For further details on the indices, refer to Table 1 and Figure 1.

complex interactions between climate factors and their effects on plant chemistry.

4.3 Effects of doubled CO₂ concentration and higher temperature on larval performance

Both *Lymantria* species examined are polyphagous insects, with *L. dispar* preferring deciduous trees and *L. monacha* preferring coniferous species as their host trees. The larvae of *L. dispar*

have been reared in a laboratory for decades on the wheat germ diet, which was used as a control diet in our study, while the nun moth larvae were collected from the field. Therefore, it was not surprising that in the gravimetric analyses, the spongy moth larvae showed the highest AD and ECI values on the artificial wheat control diet, which lacked the secondary metabolites found in their natural food sources. Interestingly, development on the high wheat germ diet showed similar RGR values to those on the oak powder diet; however, both diets produced RGR values that were more than twice as high as those on the spruce powder diet. The effects of different temperatures and CO₂ exposure on

the trees were only evident with the less suitable spruce needle diet.

The reason why the effects were evident only in spruce can be explained by Stowe and Osborn (1980), as they reported that the toxicity of phenolic compounds is closely linked to nutrient concentrations. Specifically, phenolic acids have been shown to inhibit growth only at low nutrient levels significantly. This helps clarify why *L. dispar* performs better on oak, despite its higher levels of phenolics, and condensed tannins (see Barbehenn et al., 2013; Milanović et al., 2015). Additionally, in another study, *L. dispar* demonstrated significantly better performance on white alder (*Alnus rhombifolia* Nutt.) compared to Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] (Joseph et al., 1991). Interestingly, the alder had higher levels of phenolic compounds and greater nitrogen content, contributing to its overall nutritional value (Joseph et al., 1991; also see Hättenschwiler and Schaffellner, 1999).

For *L. dispar*, the effects of increased CO₂ on ECI and ECD were more pronounced at the lower temperature (20°C) compared to the higher temperature (25°C). This finding is supported by higher flavonoid levels in spruce trees at higher temperatures with doubled CO₂ levels. Conversely, flavonoid concentrations decreased at lower temperatures, which allowed the larvae to perform better when exposed to doubled CO₂ concentration in spruce. Additionally, for *L. monacha*, the lower temperature (20°C) seems to positively impact larval performance at ambient CO₂ concentrations, resulting in higher RGR and ECD. This effect can be attributed to the higher phenol concentrations in the needles from trees at higher temperatures, thereby underscoring the role of secondary metabolites in influencing larval performance.

The diet of larvae from oak trees also contained higher levels of sugars, particularly sucrose and raffinose. Glucose seemed to be higher in oak in most cases, especially when temperatures were higher. Still, the result is not significant, probably because the numbers are similar to those of spruce, and the amount of oak is much higher in ambient CO₂ and lower temperature treatment. This likely explains why *L. dispar* larvae performed better on oak than on spruce. Sugars serve as a primary energy source for insects (see Skowronek et al., 2021). Simple sugars like glucose and fructose are quickly metabolized, providing immediate energy necessary for essential activities such as growth, movement, and reproduction (Sheng et al., 2019; Mayack et al., 2020).

The overall quality of a plant as a food source is influenced by several factors, including the balance of nutrients, the presence of secondary metabolites, and the plant's digestibility (Scogings et al., 2013; Wink, 2018). The artificial diet was prepared by powdering lyophilized leaves, a standardized method for the chemical analysis of leaves (Šulc et al., 2021). However, this method overlooks physiological factors that are also crucial in plant-herbivore interactions. For instance, leaf hardness, which can be increased by higher levels of structural carbohydrates resulting from elevated CO₂ concentrations (Prior et al., 2004; Gely et al., 2020; Münzbergová and Skuhrovec, 2020), cannot be taken into account when using dried leaves.

Escalating temperatures lead to the combined effects of drought and heat waves (Sato et al., 2024; Cook et al., 2018).

These factors have become significant direct consequences that severely impact ecosystems (Bailey-Serres et al., 2019). Including drought as an additional variable in studies like ours would provide more complex insights into the effects of climate change on forest ecosystems (Clark et al., 2016). Our study focused on elevated CO₂ concentrations in the atmosphere and their direct consequence: higher air temperatures. The results indicate that trees respond to changes in climate conditions, which in turn affects their chemical composition. However, herbivorous insects can still thrive on these trees despite variations in nutritional quality, suggesting that climate change does not significantly impact their performance via this indirect effect.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical approval was not required for the studies involving animals in accordance with the local legislation and institutional requirements because the partner who sent us the eggs of larvae breeds them especially for this kind of experiments. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

BD: Conceptualization, Funding acquisition, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. JHo: Conceptualization, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. DM: Investigation, Methodology, Writing – review & editing. AK: Formal Analysis, Validation, Writing – review & editing. JHr: Formal Analysis, Validation, Writing – review & editing. JČ: Data curation, Formal Analysis, Visualization, Writing – review & editing. AS: Conceptualization, Methodology, Resources, Supervision, Validation, Writing – review & editing.

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Conflict of interest

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.

Generative AI statement

The authors declare that no Generative AI was used in the creation of this manuscript.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/ffgc.2025.1564011/full#supplementary-material>

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