**Negative effects of allelopathic plant invasion intensify as the growth season progresses**

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**Data Availability**

All data, analysis scripts, and plot scripts are publicly available on Zenodo (DOI: [10.5281/13862911](https://doi.org/10.5281/zenodo.13862912)) or on GitHub at <https://github.com/eaperkowski/TT23_ms_data>

**Abstract**

A key trait associated with plant invasions is the production of allelopathic compounds that disrupt the plant-fungal symbioses of neighboring plants. Yet, we know remarkably little about the temporal effects of allelopathic invaders on the physiology of neighboring native species over the growth season as the tree canopy closes and soil resources deplete. Here, we collected gas exchange data during two measurement periods, reflecting an open or closed tree canopy, in two native understory species (*Trillium* spp. and *Maianthemum racemosum*) growing in a long-term *Alliaria petiolata* manipulation experiment where the invader was present at ambient levels or weeded. *Alliaria* is known to disrupt the AM fungal nutritional mutualism in these two species, with likely downstream effects on nutrient or water provisioning to photosynthesis. Overall, negative effects of this allelopathic invader on native plant photosynthetic traits became more pronounced as the growth season progressed. We also find differences among species, with the photosynthetic responses for one species indicating increased water stress over the growth season and the other indicating nutrient limitation. Interestingly, native species differ in their phenology, with the species with the longer leaf lifespan and growing period being the most water limited. Together, these results indicate that the disruption of AM fungal partner function due to *Alliaria* invasion will have a disproportionately large effect on understory species with long growth seasons and could scale up to have disparate effects on native plant community fitness and survivorship.

**Keywords**

*Alliaria petiolata*, AM fungi, photosynthesis, plant invasion, stomatal conductance water use efficiency, *V*cmax, *J*max

**Introduction**

Invasive plants often express unique traits that increase their likelihood of establishment in novel ecosystems. Allelopathy, defined as a secondary compound produced by a plant that negatively impacts neighboring plant species and/or soil microbial communities (Inderjit *et al.*, 2011), has emerged as a mechanism to explain the success of invasive plant species (Callaway & Ridenour, 2004; Callaway *et al.*, 2008). Allelopathy negatively affects native plant performance and soil microbial community composition (Hale *et al.*, 2011, 2016; Hale & Kalisz, 2012; Brouwer *et al.*, 2015; Bialic-Murphy *et al.*, 2020, 2021; Qu *et al.*, 2021; Roche *et al.*, 2021; Zhang *et al.*, 2021) and is estimated to occur in approximately 52% of invasive plant species (Kalisz *et al.*, 2021). Despite the prevalence of allelopathy among invasive species, our understanding of the temporal effects of allelopathic plant invasion on the physiology of coexisting native plants remains limited. This knowledge gap hinders our ability to understand how the disruptive impacts of allelopathic invasion on soil microbial communities scale to influence plant community dynamics.

Photosynthesis links ecosystem carbon, nutrient, and water cycles in terrestrial ecosystems (Hungate *et al.*, 2003). Through photosynthesis, plants convert carbon dioxide into simple sugars using enzymes such as Ribulose-1,5-bisphosphate carboxylase/oxygenase (“Rubisco”) that require large amounts of nutrients and energy to build and maintain (Evans & Seemann, 1989; Evans & Clarke, 2019). Aboveground conditions such as light availability, atmospheric CO2 concentration, vapor pressure deficit, and temperature regulate photosynthetic enzyme kinetics and substrate availability, rendering these factors key determinants of plant demand to acquire and allocate nutrients toward the construction and maintenance of photosynthetic enzymes and demand to acquire water needed to maintain a desired transpirational stream (Bernacchi *et al.*, 2001; Dong *et al.*, 2017, 2020, 2022; Smith *et al.*, 2019; Paillassa *et al.*, 2020; Peng *et al.*, 2021; Westerband *et al.*, 2023). Whether plants can satisfy this demand in a given environment depends on nutrient and water availability, as well as the uptake and allocation of these resources to photosynthetic tissues. For example, increased light availability often increases demand for soil nutrients and water to enhance photosynthetic capacity and stomatal conductance, respectively, to optimize light use (Walters, 2005; Smith *et al.*, 2019). When soil resources are abundant, plants can meet this increased demand by increasing nutrient and water uptake and allocating these resources to photosynthetic enzymes. However, plants cannot increase nutrient and water uptake to a similar extent when soil resources are limited. This scenario could cause individuals growing in resource-limited environments to display reduced physiological responses to increased light availability compared to individuals growing in resource-rich environments (Waring *et al.*, 2023) and could increase plant reliance on symbioses with soil microbial communities (e.g., mycorrhizal fungi) for soil resources (Treseder, 2004; van Diepen *et al.*, 2007).

Allelopathic compounds with antimicrobial properties can inhibit the growth and reproduction of soil microbial communities, such as mycorrhizal fungi, that are essential for provisioning nutrients and water resources to their host plants (Hale & Kalisz, 2012). Arbuscular mycorrhizal (AM) fungi form obligate symbioses with plants, exchanging mineral nutrients and water for photosynthate (Smith & Read, 2008). Antimicrobial compounds produced by allelopathic invaders can disrupt these symbioses by inhibiting AM fungal spore germination, fungal root colonization, and arbuscule formation, which can decrease AM fungal biomass, alter species richness, and modify AM fungal community composition (Burke, 2008; Callaway *et al.*, 2008; Burke *et al.*, 2011; Cantor *et al.*, 2011; Anthony *et al.*, 2019; Bialic-Murphy *et al.*, 2021). These disruptions can lead to decreased nutrient and water uptake in plants that rely on AM fungi, even when allelopathic invaders do not directly modify soil nutrient or water availability (Bialic-Murphy *et al.*, 2021). This is because disruptions in AM fungal community function may increase the plant carbon cost for acquiring nutrients and water, causing plants to spend more carbon for reduced resources provisioned by AM fungal partners (Kummel & Salant, 2006; Hale *et al.*, 2016). This pattern may scale to alter resource allocation to photosynthetic enzymes, as previous work indicates that increased costs of nutrient acquisition are associated with reduced resource allocation to photosynthetic enzymes (Perkowski *et al.*, 2021; Waring *et al.*, 2023). Thus, allelopathy-driven disruptions in AM fungal communities could cause native plants to be unable to satisfy the demand to build and maintain photosynthetic enzymes and/or maintain optimal stomatal conductance, which may explain why native species exhibit reduced net photosynthesis rates in response to allelopathic plant invasions (Hale *et al.*, 2011, 2016).

*Alliaria petiolata* (M. Bieb) Cavara & Grande (Family: Brassicaceae; hereafter “*Alliaria*”) serves as a model species for investigating the impacts of allelopathic plant invasion. This biennial herb from Eurasia invades temperate forest understories in North America, releasing glucosinolates into soil environments through root exudation and leaf litter (Rodgers *et al.*, 2008). Glucosinolates produced by *Alliaria* hydrolyze into antimicrobial compounds such as allyl isothiocyanate, which inhibit AM spore germination, spore viability, root colonization, and arbuscule formation (Callaway *et al.*, 2008; Cantor *et al.*, 2011; Anthony *et al.*, 2019). Previous work has linked *Alliaria* invasion with decreased AM fungal biomass, increased AM species richness, and altered fungal community composition (Burke, 2008; Burke *et al.*, 2011; Cantor *et al.*, 2011; Bialic-Murphy *et al.*, 2021). Disrupted AM fungal communities due to *Alliaria* invasion have also been associated with negative impacts on native plant nutrient and water economics, population dynamics, and community composition (Hale *et al.*, 2016; Bialic-Murphy *et al.*, 2020, 2021; Roche *et al.*, 2021, 2023), with stronger negative impacts in native species that associate with AM fungi than those that do not (Callaway *et al.*, 2008; Roche *et al.*, 2021, 2023). These patterns occur despite evidence that *Alliaria* invasions do not affect soil nutrient or water availability, suggesting that the breakdown of the AM fungal mutualism is the mechanism that drives native plant community responses to *Alliaria* invasion (Bialic-Murphy *et al.*, 2021).

Previous work indicates that *Alliaria* reduces the net photosynthesis rates of coexisting native species (Hale *et al.*, 2011, 2016; Brouwer *et al.*, 2015). However, the mechanisms that govern these responses are not fully understood. Photosynthetic responses to *Alliaria* invasion could be driven by changes in photosynthetic capacity, indicating nutrient limitation, or by changes in stomatal conductance, indicating water limitation. Understanding whether the photosynthetic responses of coexisting native species to *Alliaria* invasion are driven by changes in photosynthetic capacity or stomatal conductance would provide valuable insight into understanding the mechanism that drives the negative effects of *Alliaria* invasion on native species’ photosynthesis and whether these responses are linked to AM fungal mutualism disruption.

Furthermore, existing field research has primarily quantified photosynthetic responses to *Alliaria* invasion at a single time point in the growth season, which provides limited insight into the impacts of allelopathic plant invasion across the growth season as understory light availability and soil resource availability decrease. Studies that investigate the mechanisms that explain the photosynthetic responses to allelopathic invaders at different time points in the growing season would be valuable for assessing how leaf-level physiological responses to allelopathic plant invasion compare to its finer-scale impacts on AM fungal community composition and broader-scale impacts on native plant productivity and survivorship.

Here, we investigated the effects of *Alliaria* invasion on leaf-level photosynthetic processes of two native understory species using a long-term *Alliaria* manipulation (present or weeded) field experiment. Gas exchange measurements were collected before and after tree canopy closure to assess seasonal variation in native species’ responses to *Alliaria* treatments. Specifically, we quantified net photosynthesis, stomatal conductance, stomatal limitation, apparent photosynthetic capacity, and relative chlorophyll content responses to *Alliaria* treatments in both species at each measurement time point. We used this experiment and measurements to test the following hypotheses:

1. Net photosynthesis in both native species will be reduced in *Alliaria*-ambient plots compared to *Alliaria*-weeded plots. This reduction will be driven by a reduction in apparent photosynthetic capacity, relative chlorophyll content, and stomatal conductance in *Alliaria*-ambient plots.
2. The negative effects of *Alliaria* treatment on leaf photosynthetic traits will vary based on the measurement time point
   1. Negative effects of *Alliaria* treatment on leaf photosynthetic traits will be strongest early in the growth season when soil resource demand is highest. Disrupted AM fungal symbioses will create resource stress that will make it more difficult for AM-associating plants to acquire nutrients and water needed to satisfy leaf demand to build and maintain photosynthetic enzymes
   2. Alternatively, the negative effects of *Alliaria* treatment on photosynthetic traits will become more pronounced as the growth season progresses. This may be driven by increased reliance on AM fungal partners for soil nutrients and water as soil resources deplete. However, declining light availability with canopy closure should decrease plant demand for soil resources, which may counterbalance any increase in reliance on AM fungal partners under reduced nutrient availability

**Methods**

*Study site and experimental design*

This study was conducted at Trillium Trail Nature Reserve in Fox Chapel, PA (40.520 °N, -79.901 °W). The mean annual precipitation and mean annual temperature of the study area was 1006 mm yr-1 and 11°C, respectively (2006-2020 U.S. Climate Normals; Palecki et al., 2021). Wire fences (2.5 m tall) were set up in 2002 at five 14 x 14 m experimental plots to exclude deer and other macroherbivores while allowing free movement of small mammals and birds. *Alliaria* was manually weeded at the beginning of each growth season from one half of each experimental plot since 2006, with *Alliaria* remaining at natural densities in the other half of each plot. Manual weeding has been an effective strategy for *Alliaria* removal (Roche *et al.*, 2021). This long-term split-plot experiment is located on 25-75% grade slopes and soils were classified as Gilpin-Upshur-Atkins soils with dominant shale, sandstone, and red clay shale bedrock components. *Alliaria* treatments were set up parallel to the slope to prevent the leaching of allelochemicals into the weeded side of the plot. Previous work conducted in this experiment has shown that AM fungal biomass and AM root colonization rates are reduced and AM fungal richness is increased in *Alliaria*-ambient plots compared to *Alliaria*-weeded plots (Burke, 2008; Burke *et al.*, 2011; Cantor *et al.*, 2011). These patterns have resulted in a change to the AM fungal community composition between *Alliaria* treatments (Bialic-Murphy *et al.*, 2021).

*Gas exchange measurements and calculations*

Gas exchange data were collected between April and June 2023 from fully expanded leaves of two perennial understory native species: *Trillium* spp. (*Trillium* *grandiflorum* (Michx.) Salisb and *Trillium erectum* L.) and *Maianthemum racemosum* L. Link. We use *Trillium* spp. to refer to *T. grandiflorum* and *T. erectum*, as these species are difficult to distinguish if they are not reproductive. *Trillium* spp. and *M. racemosum* are each understory perennial herbs with widespread distributions in temperate forests of North America (USDA NRCS, 2022), form rhizomes, and associate with AM fungi (Brundrett & Kendrick, 1987, 1990; Burke, 2008). Previous work indicates that the timing of aboveground phenology differs between the two species: *Trillium* spp. typically emerge in late April and senesce in July, while *M. racemosum* typically emerge in early May and senesce as late as November (Heberling *et al.*, 2019).

Gas exchange data were collected in three of the five experimental plots during two measurement periods: once early in the growth season when the tree canopy was open and tree canopy leaf out was occurring (April 19 through April 21 for *Trillium* spp. and May 5 through May 6 for *M. racemosum*) and once later in the growth season when the tree canopy was fully closed (June 12 through June 15 for both species). The first measurement period was conducted at different time points for *Trillium* spp. and *M. racemosum* because of phenological differences in the timing of full leaf expansion between species.

Net photosynthesis (*A*net; μmol m-2 s-1), stomatal conductance (*g*sw; mol m-2 s-1), and intercellular CO2 (*C*i; μmol mol-1) concentrations were measured across a range of atmospheric CO2 concentrations (i.e., an *A*net/*C*i curve) using the Dynamic Assimilation™ Technique (Saathoff & Welles, 2021), which allows for higher-throughput *A*net/*C*i curves that correspond well with traditional steady-state methods (Tejera-Nieves *et al.*, 2024). We generated all *A*net/*C*i curves along a reference CO2 ramp down from 420 µmol mol-1 CO2 to 20 µmol mol-1 CO2, followed by a ramp up from 420 µmol mol-1 CO2 to 1620 µmol mol-1 CO2 after a 90-second wait period at 420 µmol mol-1 CO2. The ramp rate for each curve was set to 200 μmol mol-1 min-1, logging every five seconds, which generated 96 data points per response curve. All *A*net/*C*i curves were initiated after *A*net and *g*sw stabilized in a LI-6800 cuvette set to a 500 mol s-1 flow rate, 10000 rpm mixing fan speed, 1.5 kPa vapor pressure deficit, 25°C leaf temperature, 2000 μmol m-2 s-1 incoming light radiation, and initial reference CO2 set to 420 µmol mol-1. We extracted snapshot *A*net and *g*sw measurements using the initial measurement of each *A*net/*C*i curve at 420 µmol mol-1 CO2.

*A/Ci curve fitting and parameter estimation*

We fit *A*net/*C*i curves using the ‘fitaci’ function in the ‘plantecophys’ R package (Duursma, 2015). This function estimates the maximum rate of Rubisco carboxylation (*V*cmax; µmol m-2 s-1) and maximum rate of electron transport for RuBP regeneration (*J*max; µmol m-2 s-1) using the Farquhar et al. (1980) biochemical model of C3 photosynthesis. Triose phosphate utilization (TPU) limitation was included as an additional rate-limiting step in all curve fits and the temperature standardization default in the function was turned off. Dark respiration was estimated in each curve fit as a fixed proportion of *V*cmax. Michaelis-Menten coefficients for Rubisco affinity to CO2 (*K*c; μmol mol-1) and O2 (*K*o; mmol mol-1), and the CO2 compensation point *(Γ*\*; μmol mol-1) were calculated using leaf temperature and equations derived in Bernacchi et al. (2001):

(1)

(2)

(3)

In all three equations, *T*k is the mean leaf temperature (in Kelvin) during each *A*net/*C*i curve, and R is the universal gas constant (8.314 J mol-1 K-1). All curves were visually inspected for goodness-of-fit before extracting *V*cmax and *J*max estimates.

For all *A*net/*C*i curve fits, *V*cmax and *J*max were standardized to25°C (referenced as *V*cmax25 and *J*max25 from this point forward) using a modified Arrhenius equation. This temperature standardization removed the influence of enzyme kinetics on *V*cmax and *J*max. The temperature-standardized rates, thus, reflect biochemical investment in the different underlying processes (Atkin & Tjoelker, 2003). Rate estimates were standardized to25°C using the formulation presented in Kattge & Knorr (2007)

(4)

where *k*25 represents the standardized *V*cmax or *J*max rate at 25°C, *k*obs represents the *V*cmax or *J*max estimate at the average leaf temperature measured inside the cuvette during the *A*net/*C*i curve. *H*a is the activation energy of *V*cmax (71,513 J mol-1; Kattge & Knorr, 2007) or *J*max (49,884 J mol-1; Kattge & Knorr, 2007). *H*d represents the deactivation energy of both *V*cmax and *J*max (200,000 J mol-1; Medlyn et al., 2002), and R represents the universal gas constant (8.314 J mol-1 K-1). *T*ref represents the standardized temperature of 298.15 K, and *T*obs represents the mean leaf temperature (K) during each *A*net/*C*i curve. ΔS is an entropy term (J mol-1°C-1) that Kattge & Knorr (2007) described as a linear relationship with acclimated growth temperature (*T*g, °C), where:

(5)

and:

(6)

We estimated *T*g as the mean temperature of the seven days leading up to each *A*net/*C*i curve, following the idea that photosynthetic acclimation typically occurs along this timescale (e.g., Smith & Dukes, 2018). Mean daily air temperature was estimated by calculating the average between the daily maximum and minimum temperature using data collected at a nearby weather station (station ID: USW000114762; coordinates: 40.35510° N, 79.92145° W) included in the Global Historical Climatology Network - Daily data product (Menne *et al.*, 2012).

Finally, *V*cmax25 and *J*max25 estimates were used to calculate the ratio of *J*max25 to *V*cmax25 (*J*max25:*V*cmax25; unitless).

*Stomatal limitation*

The extent by which stomatal conductance limited net photosynthesis (unitless) was calculated following the approach described in Farquhar & Sharkey (1982), where:

(7)

where *A*net represents the measured net photosynthesis rate where atmospheric CO2 is 420 μmol mol-1 and *A*mod represents the theoretical photosynthetic rate where *C*i = *C*a = 420 μmol mol-1 (that is, no stomatal resistance to gas exchange), calculated as:

(8)

where *V*cmax is the non temperature-standardized maximum rate of Rubisco carboxylation, *C*i,mod is the intercellular CO2 concentration where *C*i = *C*a, set to 420 μmol mol-1, *Γ*\* (μmol mol-1) is the CO2 compensation point in the absence of dark respiration, *K*m is the Michaelis-Menten coefficient for Rubisco-limited photosynthesis (μmol mol-1), and *R*d is the dark respiration rate, estimated as a fixed proportion of *V*cmax. *K*m was calculated as:

(9)

where *K*c and *K*o were calculated following Eqns. 1 and 2, while *O*i refers to leaf intercellular O2 concentration, set to 210 μmol mol-1.

*Chlorophyll fluorescence measurements*

Relative chlorophyll content was measured after each *A*net/*C*i curve using a Soil Plant Analysis Development chlorophyll meter (SPAD, unitless) built into the MultispeQ V2.0 handheld device (PhotosynQ Inc., East Lansing, MI, USA).

*Soil characteristics*

To characterize plant-available nitrogen and phosphorus at the time of leaf gas exchange measurements, resin strips (Membranes International Inc., Ringwood, NJ, USA) were placed approximately 10 cm below the soil surface to quantify mobile ammonium (ppm), nitrate (ppm), and phosphate (ppm) concentrations in each plot. An initial batch of resin strips was incubated in the field between April 19 and June 1, 2023, followed by a second batch inserted in the same plot location between May 30 and June 29, 2023. A total of 36 strips, 12 for each nutrient, were placed in each plot to account for the high degree of spatial heterogeneity of soil nutrient availability in temperate forests (Akana *et al.*, 2023). Cation and anion concentrations were extracted from resin strips in 0.5 M potassium sulfate at a 1:5 dilution factor for ammonium, and nitrate, and 1M HCl for phosphate. Concentrations of each nutrient were determined through end products of standard colorimetric reactions (Weatherburn, 1967; Lajtha *et al.*, 1999; D’Angelo *et al.*, 2001; Doane & Horwáth, 2003). Soil inorganic nitrogen availability was estimated as the sum of the ammonium and nitrate concentrations. The soil inorganic nitrogen:phosphorus ratio was estimated as the ratio of soil inorganic nitrogen availability to soil phosphate availability.

Soil moisture data were collected using TOMST® TMS-4 data loggers (TOMST® s.r.o., Prague, Czech Republic). One data logger was placed in each *Alliaria* treatment of each plot (i.e., 2 data loggers per plot) on April 26, 2023 and was programmed to record soil moisture pulses every 15 minutes. Volumetric soil moisture content (%) was calculated using the calibration curves for a silt loam soil reported in Wild et al. (2019). We calculated the mean daily volumetric soil moisture content and used these values as the primary indicator of soil moisture throughout the measurement period.

*Data analysis*

We built a series of linear mixed-effects models to explore the effects of *Alliaria* treatment and measurement period on soil nutrient availability. Each model included *Alliaria* treatment (ambient levels, weeded) and measurement period (open, closed tree canopy) as categorical fixed effects, with an additional interaction term between *Alliaria* treatment and measurement period. Plot was included in the models as a random intercept term. We constructed separate models with this independent variable structure for soil nitrate availability, soil ammonium availability, soil inorganic nitrogen (nitrate + ammonium) availability, soil phosphate availability, and the soil nitrogen:phosphorus ratio. The models for soil inorganic nitrogen availability and the soil nitrogen:phosphorus ratio were fitted using dependent variables that were natural-log transformed, while the model for soil ammonium availability was fitted after soil ammonium availability was square root-transformed to normalize model residuals (Shapiro-Wilk: *p*>0.05 in all cases).

Next, we built a linear mixed-effects model to explore the effect of *Alliaria* treatment on volumetric soil moisture content across the measurement period. This model included *Alliaria* treatment (ambient levels, weeded) and day of year (continuous) as fixed effects, with an added interaction term between *Alliaria* treatment and day of year. Plot was included as a random intercept term.

Finally, we built a series of species-specific linear mixed-effects models to explore the effect of *Alliaria* treatment and measurement period on leaf physiological traits of *Trillium* spp. and *M. racemosum*. Species were not concatenated into a single linear mixed effect model for each trait because we did not seek to understand interspecies variability in measured traits. All models included *Alliaria* treatment (ambient levels, weeded) and measurement period (open, closed tree canopy) as categorical fixed effects, as well as an interaction term between *Alliaria* treatment and measurement period. Plot was included in each model as a random intercept term. We constructed separate models with this independent variable structure for each species for the following dependent variables: *A*net, *g*sw, stomatal limitation, *V*cmax25, *J*max25, *J*max25:*V*cmax25, and SPAD. Models for *A*net, *g*sw, *V*cmax25, and *J*max25 in *Trillium* spp. were fitted using dependent variables that were natural-log transformed to normalize model residuals, while models for stomatal limitation, *SPAD*, and *J*max25 in *M. racemosum* were fitted using dependent variables that were natural-log transformed to normalize model residuals (Shapiro-Wilk: *p*>0.05 in all cases).

Each model was fitted using the ‘lmer’ function in the ‘lme4’ R package (Bates *et al.*, 2015). Type II Wald’s χ2 and the significance (*α*=0.05) of each fixed effect coefficient was calculated using the ‘Anova’ function in the ‘car’ R package (Fox & Weisberg, 2019). We used the ‘emmeans’ R package (Lenth, 2019) to conduct post hoc comparisons using Tukey’s tests, where degrees of freedom were approximated using the Kenward-Roger approach (Kenward & Roger, 1997). All analyses and plots were conducted in R version 4.1.0 (R Core Team, 2021). Data, analysis scripts, and plot scripts are publicly available on Zenodo (DOI: [10.5281/13862911](https://doi.org/10.5281/zenodo.13862912)) or on GitHub at <https://github.com/eaperkowski/TT23_ms_data>.

**Results**

*Soil characteristics*

Soil inorganic nitrogen availability was reduced by 75% and soil phosphate availability by 26% after tree canopy closure (*p*<0.001 in all cases; Table S1; Fig. 1a-b), leading to 63% decrease in the soil nitrogen:phosphorus ratio (*p*<0.001; Table S1; Fig. 1c). Soil nitrate availability decreased by 71% after the tree canopy closed (*p*<0.001; Table S1; Fig. S1), whereas soil ammonium availability did not change between the two measurement periods (*p*>0.05; Table S1; Fig. S1).

*Alliaria* treatment had no effect on soil inorganic nitrogen availability, soil phosphate availability, soil ammonium availability, or soil nitrate availability (*p*>0.05 in all cases; Table S1; Fig. 1a-b; Fig. S1). However, the soil nitrogen:phosphorus ratio was 51% greater in the *Alliaria*-ambient treatment compared to the *Alliaria*-weeded treatment (*p*<0.05; Table S1; Fig. 1c). This pattern was due to an insignificant 16% increase in soil inorganic nitrogen availability (*p*=0.371; Table S1) and an insignificant 15% decrease in soil phosphate availability (*p*=0.107; Table S1; Fig. 1b) in the *Alliaria*-ambient treatment compared to the *Alliaria*-weeded treatment.

Soil moisture decreased as the growth season progressed (*p*<0.001; Table S2; Fig. 2). Soil moisture was lower in the *Alliaria*-ambient treatment than the *Alliaria*-weeded treatment (*p*<0.001; Table S1). This *Alliaria* treatment effect on soil moisture was similar across the growth season (*Alliaria* treatment- by-day of year interaction: *p*>0.05; Table S2).

**Figure 1**

**A chart of different types of soil

Description automatically generated**

**Figure 1** Effects of tree canopy and *Alliaria* treatment on soil inorganic nitrogen availability (a), soil phosphate availability (b), and the soil nitrogen: phosphorus ratio (c). Tree canopy status is on the x-axis. Teal points and boxplots indicate measurements collected in plots where *Alliaria* was weeded and gold points and boxplots indicate measurements collected in subplots where *Alliaria* was present at ambient levels. Boxes represent the upper (75% percentile) and lower (25% percentile) quartiles, and whiskers represent 1.5 times the upper and lower quartile values. Lettering above each treatment group indicates statistically different groups where Tukey: *p*<0.05.

**Figure 2**

A graph showing the growth of a number of days

Description automatically generated with medium confidence

**Figure 2** Effects of *Alliaria* treatment on mean daily volumetric soil moisture content across the 2023 growth season. Date is on the x-axis. Points reference daily volumetric soil water content averaged across the three plots used to collect gas exchange measurements. The teal points and trendline indicate measurements collected in plots where *Alliaria* was weeded and gold points and trendline indicate measurements collected in plots where *Alliaria* was present at ambient levels. Error ribbons represent the trendline standard error.

*Gas exchange*

For *Trillium* spp., net photosynthesis decreased by 64% after tree canopy closure, a pattern that was associated with a 22% reduction in stomatal conductance and 55% reduction in stomatal limitation compared to measurements collected before the tree canopy closed (*p*<0.001 in all cases; Table 1). Net photosynthesis rates were reduced in the *Alliaria*-ambient treatment compared to the *Alliaria*-weeded treatment (*p*<0.05; Table 1). However, this net photosynthesis response to *Alliaria* treatment was only observed after tree canopy closure (*Alliaria* treatment-by-canopy status interaction: *p*<0.05; Table 1; Fig. 3a). *Alliaria* treatment had no effect on stomatal conductance or stomatal limitation in *Trillium* spp. (*p*>0.05 in both cases; Table 1) regardless of measurement period (*Alliaria* treatment-by-canopy status interaction: *p*>0.05; Table 1; Fig. 3b-c).

For *M. racemosum*, net photosynthesis decreased by 59% after tree canopy closure, a pattern that was associated with a 62% reduction in stomatal conductance and a 13% increase in stomatal limitation compared to measurements collected before the tree canopy closed (*p*<0.05 in all cases; Table 1; Fig. 3b, 3d, 3f). Net photosynthesis decreased by 18% and stomatal conductance decreased by 27%, while stomatal limitation increased by 28% in the *Alliaria*-ambient treatment compared to the *Alliaria*-weeded treatment (*p*<0.001 in all cases; Table 1). Net photosynthesis and stomatal conductance responses to *Alliaria* treatment were observed regardless of measurement period (*Alliaria* treatment-by-canopy status interaction: *p*>0.05 in both cases; Table 1), while stomatal limitation responses to *Alliaria* treatment were only observed after tree canopy closure (*Alliaria* treatment-by-canopy status interaction: *p*<0.05; Table 1; Fig. 3f).

*Relative chlorophyll content*

SPAD values were 26% greater in *Trillium* spp. and 51% greater in *M. racemosum* after tree canopy closure (*p*<0.001 in both cases; Table 1; Fig. S2). *Alliaria* treatment had no effect on SPAD in either species (*p*>0.05; Table 1) regardless of measurement period (*Alliaria* treatment-by-canopy status interaction: *p*>0.05 in both cases; Table 1; Fig. S2).

**Table 1** Analysis of variance results exploring the role of *Alliaria* treatment and measurement period on leaf gas exchange\*

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | ***A*net** | | ***g*sw** | | **Stomatal**  **limitation** | | ***SPAD*** | |
|  |  | *χ*2 | *p* | *χ*2 | *p* | *χ*2 | *p* | *χ*2 | *p* |
| ***Trillium* spp.** | |  |  |  |  |  |  |  |  |
|  | *Alliaria* treatment (A) | 5.830 | **0.016** | 0.148 | 0.701 | 0.498 | 0.481 | 0.300 | 0.584 |
|  | Canopy status (C) | 1163.336 | **<0.001** | 23.864 | **<0.001** | 289.318 | **<0.001** | 73.833 | **<0.001** |
|  | A\*C | 4.833 | **0.028** | 0.622 | 0.430 | 0.132 | 0.717 | 0.496 | 0.481 |
| ***M. racemosum*** | |  |  |  |  |  |  |  |  |
|  | *Alliaria* treatment (A) | 19.547 | **<0.001** | 20.507 | **<0.001** | 15.684 | **<0.001** | 1.792 | 0.181 |
|  | Canopy status (C) | 336.988 | **<0.001** | 157.676 | **<0.001** | 8.300 | **0.004** | 285.711 | **<0.001** |
|  | A\*C | 0.012 | 0.913 | 0.046 | 0.831 | 5.094 | **0.024** | 0.853 | 0.356 |

\*Significance determined using Type II Wald χ2 tests (α=0.05). *P*-values less than 0.05 are in bold. Key: *A*net = light-saturated net photosynthesis rate (μmol m-2 s-1), *g*sw = stomatal conductance (mol m-2 s-1), SPAD = relative chlorophyll content (unitless)

**Figure 3**

**A collage of graphs and charts

Description automatically generated**

**Figure 3** Effects of *Alliaria* treatment and tree canopy status on net photosynthesis (a-b), stomatal conductance (c-d), and stomatal limitation of net photosynthesis (e-f). The left column shows *Trillium* spp. responses, while the right column shows *M. racemosum* responses. Tree canopy status is on the x-axis. Teal points and boxplots indicate measurements collected in plots where *Alliaria* was weeded and gold points and boxplots indicate measurements collected in plots where *Alliaria* abundance was not manipulated. Boxes represent the upper (75% percentile) and lower (25% percentile) quartiles, and whiskers represent 1.5 times the upper and lower quartile values. Lettering above each treatment group indicates statistically different groups where Tukey: *p*<0.05.

*Photosynthetic capacity*

In *Trillium* spp., *V*cmax25 and *J*max25 decreased by 76% and 75%, respectively, after tree canopy closure, resulting in a 4% increase in *J*max25:*V*cmax25 (*p*<0.05 in all cases; Table 2; Fig. 4a, 4c, 4e). *Alliaria* treatment had no effect on *V*cmax25 (*p*>0.05; Table 2), although *J*max25 was 8% lower in the *Alliaria*-ambient treatment compared to the *Alliaria*-weeded treatment (*p*<0.05; Table 2). *Alliaria* treatment effects on *J*max25 were only observed after tree canopy closure (*Alliaria* treatment- by-canopy status interaction: *p*<0.05; Table 1; Fig. 4c). *Alliaria* treatment had no effect on *J*max25:*V*cmax25 (*p*>0.05; Table 2; Fig. 4e).

For *M. racemosum*, *V*cmax25 and *J*max25 each decreased by 57% after tree canopy closure, while *J*max25:*V*cmax25 decreased by 5% (*p*<0.001 in all cases; Table 2; Figs. 4b, 4d, 4f). The decrease in *J*max25:*V*cmax25 due to tree canopy closure was only observed in the *Alliaria*-weeded treatment (*Alliaria* treatment-by-canopy status interaction: *p*<0.05; Table 2). *Alliaria* treatment had no effect on *V*cmax25, *J*max25, or *J*max25:*V*cmax25 (*p*>0.05 in both cases; Table 2).

**Table 3** Analysis of variance results exploring the role of *Alliaria* treatment and measurement period on apparent photosynthetic capacity\*

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | ***V*cmax25** | | ***J*max25** | | ***J*max25:*V*cmax25** | |
|  |  | *χ*2 | *p* | *χ*2 | *p* | *χ*2 | *p* |
| ***Trillium* spp.** | |  |  |  |  |  |  |
|  | *Alliaria* treatment (A) | 1.090 | 0.296 | 4.008 | **0.045** | 2.622 | 0.105 |
|  | Canopy status (C) | 1585.012 | **<0.001** | 2001.653 | **<0.001** | 7.314 | **0.007** |
|  | A\*C | 1.973 | 0.160 | 5.417 | **0.020** | 0.753 | 0.386 |
| ***M. racemosum*** | |  |  |  |  |  |  |
|  | *Alliaria* treatment (A) | 0.162 | 0.688 | 0.370 | 0.543 | 2.510 | 0.113 |
|  | Canopy status (C) | 284.148 | **<0.001** | 391.314 | **<0.001** | 8.456 | **0.004** |
|  | A\*C | 0.219 | 0.640 | 0.045 | 0.832 | 3.221 | *0.073* |

\*Significance determined using Type II Wald χ2 tests (α=0.05). *P*-values less than 0.05 are in bold, while values where 0.05<*p*<0.1 are italicized. Key: *V*cmax25 = maximum rate of Rubisco carboxylation at 25°C (μmol m-2 s-1), *J*max25 = maximum rate of electron transport for RuBP regeneration at 25°C (μmol m-2 s-1), *J*max25:*V*cmax25 = ratio of *J*max25 to *V*cmax25 (unitless)

**Figure 4**

**A collage of graphs and charts

Description automatically generated**

**Figure 4** Effects of *Alliaria* treatment and tree canopy status on the temperature-standardized maximum rate of Rubisco carboxylation (a-b), the temperature-standardized maximum rate of electron transport for RuBP regeneration (c-d), and the ratio of the temperature-standardized maximum rate of Rubisco carboxylation to the temperature-standardized maximum rate of electron transport for RuBP regeneration (e-f). The left column shows *Trillium* spp. responses, while the right column shows *M. racemosum* responses. Tree canopy status is on the x-axis. Teal points and boxplots indicate measurements collected in subplots where *Alliaria* was weeded, and gold points and boxplots indicate measurements collected in subplots where *Alliaria* abundance was not manipulated. Boxes represent the upper (75% percentile) and lower (25% percentile) quartiles, and whiskers represent 1.5 times the upper and lower quartile values. Lettering above each treatment group indicates statistically different groups where Tukey: *p*<0.05.

**Discussion**

Both native species growing under ambient levels of *Alliaria* exhibited significantly reduced net photosynthesis rates compared to those growing in the *Alliaria*-weeded treatment, supporting our first hypothesis. In *Trillium* spp., this *Alliaria* treatment response was associated with a reduction in apparent photosynthetic capacity coupled with no change in stomatal conductance and stomatal limitation. Conversely, this response to *Alliaria* treatment in *M. racemosum* was associated with a reduction in stomatal conductance that increased stomatal limitation and was not affected by a change in apparent photosynthetic capacity. Building on results reported in Bialic-Murphy et al. (2021), these observations suggest that *Alliaria* invasion modifies net photosynthesis rates by altering nutrient uptake and allocation to photosynthetic enzymes in *Trillium* spp. and by altering water uptake and use in photosynthesis in *M. racemosum*.

While the mechanisms that drove photosynthetic responses to *Alliaria* treatment were species-dependent, responses in both species were generally more pronounced after tree canopy closure. This pattern supported our second hypothesis that the negative effects of allelopathic plant invasion would intensify as the growth season progressed. Stronger late-season photosynthetic responses to ambient levels of *Alliaria* coincided with a reduction in soil nutrient availability and soil moisture as the growth season progressed. Seasonal reductions in soil resource availability may have increased reliance on AM fungal partners for soil resources, which likely increased the cost of acquiring soil resources and decreased plant resource uptake and allocation toward photosynthetic tissues when native species grew under ambient levels of *Alliaria*. These patterns may have been exacerbated by the reduction in soil moisture in the *Alliaria*-ambient treatment compared to the *Alliaria*-weeded treatment, which may have increased late-season reliance on disrupted AM fungal partners for soil resources in the *Alliaria*-ambient treatment.

*Photosynthetic responses to Alliaria presence intensify as the growth season progresses*

We hypothesized that the effects of *Alliaria* treatment on leaf-level photosynthesis would be more apparent early in the growth season when understory demand for maintaining photosynthetic enzymes and a desired transpiration stream is greatest (Heberling *et al.*, 2019). Contrary to this hypothesis, there was no effect of *Alliaria* treatment on any photosynthetic trait in *Trillium* spp. before tree canopy closure. However, net photosynthesis and stomatal conductance in *M. racemosum* were decreased in the *Alliaria*-ambient treatment compared to the *Alliaria*-weeding treatment prior to tree canopy closure. This response was associated with a reduction in soil moisture in the *Alliaria*-ambient treatment compared to the *Alliaria*-weeded treatment, which may have caused *M. racemosum* individuals to close their stomata as a water-savings mechanism or rely more heavily on disrupted AM fungal partners for water. While treatment responses in *M. racemosum* were observed at both measurement timepoints, net photosynthesis and stomatal conductance responses to *Alliaria* treatment was strongest after tree canopy closure, contrasting this hypothesis.

Limited early-season photosynthetic responses to *Alliaria* treatment may be attributed to resource optimization that caused individuals to favor investment toward direct uptake regardless of *Alliaria* treatment. Resource optimization theory predicts that, given multiple potential acquisition strategies (e.g., direct uptake, mycorrhizal symbioses, etc.), plants should prioritize investment toward the resource uptake strategy that minimizes the cost and maximizes the uptake efficiency of acquiring soil resources (Bloom *et al.*, 1985; Rastetter *et al.*, 2001; Kummel & Salant, 2006). Thus, plants should invest more strongly in direct uptake pathways early in the growth season when soil resources are more abundant, as costs to acquire soil resources through direct uptake pathways are often reduced under high resource availability (Perkowski *et al.*, 2021, 2024; Lu *et al.*, 2022). Therefore, limited photosynthetic responses to *Alliaria* treatment early in the growth season may have been due to investment toward direct uptake that allowed individuals to satisfy demand to build and maintain photosynthetic enzymes and maintain a desired transpiration stream while minimizing any negative consequence of relying on AM fungal partners for resources.

Alternatively, we hypothesized that the effects of *Alliaria* treatment on leaf-level photosynthesis would intensify as the growth season progressed. Our findings supported this hypothesis, as both native species exhibited stronger reductions in net photosynthesis rates when individuals grew under ambient levels of *Alliaria* after tree canopy closure than before tree canopy closure. This pattern was associated with decreased nitrogen availability, phosphorus availability, and volumetric soil moisture following tree canopy closure. Late-season photosynthetic responses were observed despite *Alliaria* treatment having no direct effect on nitrogen or phosphorus availability, although soil moisture was reduced in the *Alliaria*-ambient treatment compared to the *Alliaria*-weeding treatment. These patterns suggest that late-season photosynthetic responses to *Alliaria* treatment may have been due to increased reliance on disrupted AM fungal partners as the cost to acquire resources through direct uptake increased (Perkowski *et al.*, 2021, 2024), which may have been further exacerbated by stronger soil moisture reductions in subplots where *Alliaria* was present. Future work involving isotopic tracers (e.g., Hodge & Fitter, 2010) or soil resource manipulation experiments that cross AM fungal community compositions (e.g., Gustafson & Casper, 2004) would be a useful next step for linking soil microbial community, soil resource availability, and photosynthetic responses to *Alliaria* invasion.

Overall, these findings highlight the necessity of quantifying the temporal effects of plant invasion on coexisting native plant populations. Ecophysiological studies have traditionally focused on assessing the impacts of plant invasion on the physiological processes of coexisting native species at single timepoints, mainly due to logistical constraints. While data from these studies are very useful for understanding snapshot effects of plant invasion on native population physiology, they may risk providing misleading results when using these responses to understand consequences of plant invasion on native population and community dynamics, especially in dynamic systems where tree canopies close and soil resource availability changes across the growth season. Experiments that assess the impacts of plant invasion across multiple timepoints, as shown here, provide important insight into understanding the temporal nuances that underpin the effects of plant invasion on native populations and provide important empirical data that will allow us to more reliably predict the impacts of plant invasion on plant population and community dynamics. Furthermore, soil microbial and plant communities operate on largely different spatiotemporal scales, a big challenge when scaling soil microbial dynamics up to plant community dynamics. Quantifying the temporal effects of plant invasion on coexisting native plant populations may allow us to better integrate and scale the effects of plant invasions on belowground soil microbial and plant community dynamics.

*Photosynthetic responses to Alliaria presence are linked to altered nutrient and water economics*

The two native species used in this study were expected to occupy similar niches in the plant community. Both species grew under the same aboveground and belowground environments and experienced the same *Alliaria* manipulations. They each form rhizomes and reproduce clonally, acquire nutrients and water through direct uptake pathways or symbioses with AM fungi, and have similar emergence times (Brundrett & Kendrick, 1987, 1990; Heberling *et al.*, 2019). As expected, net photosynthesis rates in both species were reduced in the *Alliaria*-ambient treatment, supporting previous work (Brouwer *et al.*, 2015; Hale *et al.*, 2016). However, the mechanisms that determined these responses differed between species.

*Trillium* spp. responses to *Alliaria* treatment suggest that the allelopathic invader induced a form of nutrient limitation, modifying net photosynthesis by reducing apparent photosynthetic capacity through a shift in nutrient allocation to photosynthetic enzymes. The null effect of *Alliaria* treatment on relative chlorophyll content and the temperature-standardized maximum rate of Rubisco carboxylation coupled with a reduction in the temperature- standardized maximum rate of electron transport for RuBP regeneration in the *Alliaria*-ambient treatment implies that any reduction in nutrient provisioning toward photosynthetic enzymes may have been due to a reduction in the fraction of leaf nutrients allocated to bioenergetics (Niinemets & Tenhunen, 1997; Niinemets *et al.*, 1998; Waring *et al.*, 2023). Null effects of *Alliaria* treatment on stomatal conductance and stomatal limitation indicate that *Alliaria* invasions do not impact the water economics of *Trillium* spp., suggesting that physiological responses to the allelopathic invader are driven entirely by shifts in nutrient economics.

In contrast, *M. racemosum* responses to *Alliaria* treatment may have been due to increased water limitation, as reduced net photosynthesis rates in the *Alliaria*-ambient treatment were driven by a reduction in stomatal conductance that increased late-season stomatal limitation. While these effects could have been due to direct phytotoxic effects of *Alliaria* on *M. racemosum* through reductions in soil moisture, similar net photosynthesis and stomatal conductance patterns were observed in a controlled greenhouse experiment under well-watered conditions (Hale et al., 2016). These patterns corresponded with null effects of *Alliaria* treatment on apparent photosynthetic capacity, supporting previous work suggesting that physiological responses of *M. racemosum* to *Alliaria* invasion are associated with changes in water economics, not nutrient economics (Hale *et al.*, 2011, 2016).

Our results suggest that the effects of plant invasion may vary among species with different leaf economic strategies. Although both species were expected to occupy similar niches and displayed similar directional photosynthetic responses to the *Alliaria* treatments, differences in leaf lifespan between species may have positioned them at distinct positions along the leaf economics spectrum (Wright *et al.*, 2004; Reich, 2014). In *Trillium* spp., shorter leaf lifespans may foster rapid nutrient and water uptake to allow for fast growth and reproduction, leading to high leaf nutrient demand to build and maintain photosynthetic enzymes. In contrast, longer leaf lifespans in *M. racemosum* may have fostered conservative resource uptake strategies that favor long-term investment in photosynthetic tissues with reduced leaf nutrient demand to build and maintain photosynthetic enzymes. These patterns were observed here, as *Trillium* spp. had greater temperature-standardized maximum rates of Rubisco carboxylation on average than *M. racemosum* (*V*cmax25 mean ± SD: 76.4 ± 40.9 μmol m-2 s-1 in *Trillium* spp. compared to 48.4 ± 19.6 μmol m-2 s-1 in *M. racemosum*).

The greater demand for photosynthetic enzyme production and maintenance in *Trillium* spp. may explain why its photosynthetic capacity was reduced in the *Alliaria*-ambient treatment, especially if individuals relied more heavily on disrupted AM fungi for nutrient uptake. In other words, disruptions in AM fungi due to *Alliaria* may have made it more difficult for *Trillium* spp. individuals to satisfy demand to build and maintain photosynthetic enzymes, inducing nutrient stress and reducing net photosynthesis rates despite *Alliaria* treatments having no effect on soil nutrient availability. In contrast, resource conservative strategies in *M. racemosum* may have allowed individuals to satisfy nutrient demand to build and maintain photosynthetic enzymes irrespective of whether individuals associated with disrupted AM fungal partners. Isotopic tracer studies may be useful for confirming these conjectures.

*Using leaf physiology to assess linkages between aboveground and belowground responses to allelopathic plant invasion*

Native species’ physiological responses to *Alliaria* treatments have direct implications for understanding the integrated negative effects of *Alliaria* invasion on the belowground soil microbial community and aboveground plant community form and function. *Alliaria* disrupts the belowground AM fungal community composition by reducing AM fungal biomass and root colonization rates while also increasing AM fungal richness (Burke, 2008; Burke *et al.*, 2011; Cantor *et al.*, 2011; Anthony *et al.*, 2019; Roche *et al.*, 2021; Bialic-Murphy *et al.*, 2021). This allelopathic invader also negatively affects the abundance and survivorship of AM native plants that coexist with *Alliaria* in its non-native range (Callaway *et al.*, 2008; Bialic-Murphy *et al.*, 2020; Roche *et al.*, 2021, 2023). Our results indicate that photosynthetic responses to *Alliaria* are directionally similar to its impacts on AM fungal community and plant community dynamics, suggesting that the effects of *Alliaria* invasion across these levels of organization may be inherently linked and scalable through its impacts on native plant physiology. In other words, disruptions in AM fungal community composition due to *Alliaria* invasion modify nutrient and water provisioning and uptake in native plant species, which decreases net carbon assimilation and, over time, has negative consequences for plant community survivorship and fitness.

*Conclusions*

The *Alliaria*-ambient treatment negatively affected leaf-level photosynthetic processes in two native AM fungal-associating understory perennial species, and these effects were generally strongest after tree canopy closure and soil resource availability decreased. The mechanisms underlying these responses to *Alliaria* differed between species: photosynthetic responses to *Alliaria* treatment in *Trillium* spp. may have been due to reduced nitrogen uptake and nitrogen allocation to photosynthetic leaf tissue, while responses to *Alliaria* treatment in *M. racemosum* may have been due to reduced water use that increased stomatal limitation. These results provide important insight into understanding the mechanisms that drive photosynthetic responses to allelopathic plant invasion and are a critical piece of empirical data needed to link the effects of allelopathic plant invasion on belowground soil microbial communities with its effects on plant population and community dynamics. Furthermore, our findings indicate that understanding the temporal effects of invasion on coexisting native species is critical for predicting the effects of invasion and other anthropogenic drivers of environmental change on population and community dynamics.

**Author Contributions**

The sampling approach was the product of discussions between EAP, JM, LB-M, SNK, SK, and NGS. EAP conducted field work in collaboration with KC, JM, SC, and NGS. EAP conducted the analyses and wrote the first draft of the manuscript. Manuscript feedback was provided by all co-authors. The experiment has been maintained by SK since its inception. All authors support submission of this manuscript to XX.

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**Conflicts of Interest**

None.

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