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Elevated temperatures alter an ant-aphid mutualism

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Abstract

Ant-hemipteran mutualisms are keystone interactions that can be variously affected by warming: these mutualisms can be strengthened or weakened, or the species can transition to new mutualist partners. We examined the effects of elevated temperatures on an ant-aphid mutualism in the subalpine zone of the Rocky Mountains in Colorado, USA. In this system, inflorescences of the host plant, *Ligusticum porteri* Coult. & Rose (Apiaceae), are colonized by the ant-tended aphid *Aphis asclepiadis* Fitch or less frequently by the non-ant tended aphid *Cavariella aegopodii* (Scopoli) (both Hemiptera: Aphididae). Using an 8-year observational study, we tested for two key mechanisms by which ant-hemipteran mutualisms may be altered by climate change: shifts in species identity and phenological mismatch. Whereas the aphid species colonizing the host plant is not changing in response to year-to-year variation in temperature, we found evidence that a phenological mismatch between ants and aphids could occur. In warmer years, colonization of host plant inflorescences by ants is decreased, whereas for *A. asclepiadis* aphids, host plant colonization is mostly responsive to date of snowmelt. We also experimentally established *A. asclepiadis* colonies on replicate host plants at ambient and elevated temperatures. Ant abundance did not differ between aphid colonies at ambient vs. elevated temperatures, but ants were less likely to engage in tending behaviors on aphid colonies at elevated temperatures. Sugar composition of aphid honeydew was also altered by experimental warming. Despite reduced tending by ants, aphid colonies at elevated temperatures had fewer intraguild predators. Altogether, our results suggest that higher temperatures may disrupt this ant-aphid mutualism through both phenological mismatch and by altering benefits exchanged in the interaction.

Introduction

Ant-hemipteran mutualisms are keystone interactions with cascading effects on the larger ecological community (Ness et al., 2007; Styrsky & Eubanks, 2007; Ando et al., 2017). In these interactions, ants provide protection from predators in exchange for sugar-rich honeydew excreted by sap-feeding insects. Like other mutualisms, ant-hemipteran interactions are subject to the effects of elevated temperatures due to climate change (Barton & Ives, 2014; Sagata & Gibb, 2016; Zhou et al., 2017). Elevated temperature can alter the ant-hemipteran mutualism through several distinct mechanisms (Blanchard et al., 2019). Firstly, elevated temperatures can drive changes in the identity of the species involved. This has been experimentally demonstrated in ant-aphid interactions: Barton & Ives (2014) observed a shift in tending of aphid colonies from a more aggressive to a less aggressive ant species on warmed maize plants. Secondly, elevated temperatures could alter the temporal overlap between ants and hemipterans, that is, cause a phenological mismatch (Renner & Zohner, 2018). Earlier spring arrival of aphids on host plants is a key signal of warmer springs in temperate zones (Harrington et al., 2007; Bell et al., 2015). Elevated temperature could drive earlier arrival of aphids on host plants before ants are active. Studies of ant-plant mutualisms show the potential for such temperature driven

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mismatches (Warren & Bradford, 2014), although this has not been documented in ant-hemipteran mutualisms (Blanchard et al., 2019). For ant-tended aphid species, the outcome of advanced phenology may depend on how early arrival coincides with ant activity.

Elevated temperatures can also change the magnitude (e.g., weak to strong) or direction (e.g., + to 0) of the outcome of the interactions between ants and hemipterans (Chamberlain et al., 2014). The benefits exchanged in ant-hemipteran mutualisms have been well outlined by past decades of research (Stadler & Dixon, 2005; Styrsky & Eubanks, 2007). For aphids and other hemipterans, the benefit from the mutualism is chiefly protection from enemies, which results from first recruiting ants and then ant tending behaviors (Stadler & Dixon, 2005). Temperature could alter the outcome of this interaction by changing predation levels. For example, interactions with ants benefit the growth of low-elevation but not high-elevation aphid colonies because there are fewer predators at high elevation (Nelson et al., 2018). In the case of mealybugs feeding on potted cotton plants, elevated temperatures increased ant aggression toward predators (Zhou et al., 2017). Such behaviorally mediated responses are important mechanisms by which climate change alters species interactions (Harmon et al., 2009). Because ant recruitment is often driven by the numbers of hemipterans present, temperature may determine recruitment through its direct effects on hemipteran abundance, that is, if higher temperatures elicit larger hemipteran colonies or aggregations, these may recruit more protective ants. For ants, the benefit from engaging in interactions with hemipterans is energy gained from honeydew, which is also positively correlated with aphid population size (Stadler & Dixon, 2005). Elevated temperatures have the potential to affect honeydew composition or production by hemipterans as well as harvesting rates by ants. How temperature may affect the composition of honeydew is unknown, although temperature does determine composition of the plant phloem sap from which it is derived (Douglas, 2006). The other possible effects seem to be system specific. For example, Zhou et al. (2017) found elevated temperatures increased excretion rates of honeydew by mealybugs, whereas Sagata & Gibb (2016) found reductions in the amount of honeydew harvested by ants with elevated temperatures. Ant-tending itself can determine honeydew composition (Fischer & Shingleton, 2001). In this way, temperature may determine honeydew composition through its effects on ant recruitment or tending behavior. The extent to which any of these mechanisms underlie the effects of temperature across different ant-hemipteran mutualisms is as yet unknown.

The effect of climate change on the abundance of ant-tended hemipterans is often mediated through interactions with ants. For example, Marquis et al. (2014) found ant-tended aphid species to be less negatively affected by experimental warming than non-tended aphid species. Thus, understanding how elevated temperatures will affect mutualisms with ants is a key part of predicting hemipteran abundance as global temperatures increase. In our study system, mutualism with ants drives the abundance and distribution of aphids, *Aphis asclepiadis* Fitch (Hemiptera: Aphididae), on host plants, *Ligusticum porteri* L. (Apiaceae) (Mooney et al., 2016). However, elevated temperatures can override this dependency on ants, that is, when aphid colonies are warmed, aphids achieve similar population growth rates regardless of ant presence (Robinson et al., 2017). Despite the wealth of past work on ant-hemipteran mutualisms in general, very few ecological studies have addressed the impacts of elevated temperatures on these interactions in the field (Blanchard et al., 2019). In this study, we used both a long-term observational study and a warming experiment to examine the mechanisms by which elevated temperatures affect an ant-aphid mutualism.

Our first objective was to examine how year-to-year variation in temperature predicted the abundance and phenology of ants and aphids on host plants. We used observations of insect abundance from an 8-year field study to evaluate differential responses to temperature by ants and aphids. Using these data, we tested for two important mechanisms by which ant-hemipteran mutualisms may be altered by climate change: shifts in the species identity and phenological mismatch. Host plant flowering stalks are colonized most frequently by the ant-tended aphid *A. asclepiadis*, but colonies of the non-ant tended aphid *Cavariella aegopodii* (Scopoli) (both Hemiptera: Aphididae) also sometimes appear (Palmer, 1952; Addicott, 1981). Notably, elevated temperatures produce contrasting effects on the population dynamics of different aphid species (Blanchard et al., 2019). If the two aphid species in our study show such idiosyncratic responses, we would predict their abundance and phenology on host plants to respond differently to year-to-year temperature variation. Thus, the aphid species that ants encounter on host plants would change in response to increasing temperature. We included snowmelt date as the one additional climate cue driving these responses by ants and aphids. A model-selection approach had previously identified snowmelt date and temperature as key drivers of year-to-year variation in our system (Robinson et al., 2017). Snowmelt date governs many other plant and insect responses in the subalpine zone of our study sites (Boggs & Inouye, 2012; Iler et al., 2013).

Because year-to-year variation would be confounded by other factors in our observational study, we also used experimental manipulation of temperature on aphid colonies in the field. Thus, our second objective was to evaluate how experimentally elevated temperatures would alter the ant-aphid mutualism. We used a factorial experiment to determine how the effect of ant presence on aphid population dynamics depended on temperature. Given that this design replicated a previous study in this system (Robinson et al., 2017), we predicted that colonies at elevated temperatures would achieve high growth rates regardless of ant presence. To evaluate this result in terms of services exchanged in the ant-aphid mutualism, we measured a variety of response variables under both ambient and elevated temperature conditions. We predicted that energetic benefits to ants could be affected by temperature if honeydew composition or amount varied between ambient-temperature and elevated-temperature aphid colonies. Honeydew makeup and excretion rate depends on host plant physiology and ant presence (Völkl et al., 1999; Fischer & Shingleton, 2001; Fischer et al., 2005), both of which may be altered by elevated temperatures (Blanchard et al., 2019). In addition, we predicted that protection mutualism for aphids could be mediated by temperature if either ant attendance or enemy abundance were affected by temperature.

Materials and methods

Study site and organisms

We conducted the studies at sites surrounding the Rocky Mountain Biological Laboratory (RMBL; 39°01′38.77″N, 107°03′10.50″W) in Gunnison County, Colorado, USA. Langenheim (1962) describes the vegetation in the subalpine meadows and aspen understories at these sites. The aphid *A. asclepiadis* (syn. *Aphis helianthi*) colonizes the flowering stalks of the host plant *L. porteri* (Addicott, 1981; Lagos-Kutz et al., 2016). Several species of ants visit floral nectaries of the host beginning with anthesis in June, often prior to aphid colonization. Aphids colonize flowering stalks beginning in late June or early July (Addicott, 1979). Infrequently, the carrot-willow aphid (*C. aegopodii*) also colonizes flowering stalks (Palmer, 1952). Colonies of the aphid *A. asclepiadis* are tended by several species of ants including *Tapinoma sessile* (Say), *Formica* sp. (rufa group), *Formica fusca* L., and *Formica podzolica* Francoeur (Mooney et al., 2016). Mirid bug nymphs and adults also feed on host plant flowering stalks throughout summer; this group includes lygus bugs, *Lygus hesperis* (Knight), which are intraguild predators of aphids (Robinson et al., 2017). Various predators and parasites attack *A. helianthi* colonies including coccinellid beetle larvae and parasitoid wasps (Kummel et al., 2013). The wasp species that parasitize *A. helianthi* in this system are unknown, but several braconid and aphelinid wasp species parasitize *A. helianthi* when it occurs as a pest in sunflower fields (Rogers et al., 1972).

Responses to year-to-year temperature variation by ants and aphids

To assess the response of ants and aphids to year-to-year temperature variation, we used insect census data collected from 10 separate host plant populations near RMBL. In each year of study, we randomly selected 10 flowering host plants per population, and we then censused plants biweekly (2011) or weekly (2012–2018) in June through August. At each census, we counted the ants, aphids, and other arthropods on the focal host plants. Additional details about the host plant populations and census techniques can be found in Mooney et al. (2016) and Robinson et al. (2017).

Using these data, we determined how temperature and snowmelt date predicted host plant colonization by ants, *C. aegopodii* aphids, and *A. asclepiadis*. To test for shifts in species identity driven by temperature, we used an analysis of covariance (ANCOVA) to determine whether the response to temperature differed between the two aphid species, *C. aegopodii* and *A. asclepiadis*. We included snowmelt date as an additional explanatory variable because of its importance in this subalpine system (Boggs & Inouye, 2012). A permanent weather station located within 0.5 km of the study populations provided temperature data (weather station Billy Barr, http://wrcc.dri.edu/rmbl/), and yearly snowmelt was the date of first bare ground observed at this station (http://www.gothicwx.org/long-term-snow.html). To test for phenological mismatch between ants and aphids, we again used an ANCOVA to determine whether the response to temperature differed between ants and *A. asclepiadis* aphids. We also included snowmelt date as a covariate in this analysis.

We separately evaluated three response variables across study years 2011–2018: (1) number of colonized host plants during the census in the 3rd week of June (ordinal date: 173), (2) maximum number of colonized host plants, and (3) day of year of maximum colonization. The 3rd week of June coincides with both host plant anthesis and early aphid colonization (Addicott, 1978; Robinson et al., 2017). We have fewer observations of June abundance ($n = 7$) because no census was conducted at that time in 2011. For maximum colonization, this was the greatest number of host plants where an insect was counted during a census in a given year. For day of year of maximum colonization, this was the ordinal date of that maximum colonization. As an example, we observed a maximum of 24 host plants with *A. asclepiadis* aphids in 2017, and this

occurred on ordinal date 194. We created and tested all of the statistical models with the program R v.3.5.1 (R Development Core Team, 2018). We used different mean temperatures as explanatory variables depending on the response variable we were modeling. For June abundance, we used mean June temperature. For all other responses, we used mean June-July temperature. Because both colonization in June and maximum colonization were count variables, we created models using the 'glm()' function with Poisson-distributed errors (Qian, 2017). For those models, we used the 'Anova()' function from the *car* package to obtain test statistics (LR χ^2) and P-values for significance testing (Fox & Weisberg, 2011). To estimate overall model fit for the models with count variables as responses, we used pseudo-R^2 values calculated using the 'pR2()' function from the *pscl* package (Jackman, 2015). We obtained P-values for overall model fit using the 'pchisq()' function to compare the deviance of the full model to a null model containing only an intercept term. For the response of day of year at maximum abundance, we used the function 'lm()' in the *stats* package to create the statistical models.

Responses of ant-aphid mutualism to experimental warming

Experimental design and aphid population dynamics. To measure how warming affects the ant-aphid mutualism, we established experimental aphid colonies on 7 July 2017. Ten non-alate aphids were transferred from approximately 12 existing aphid colonies to the terminal umbels of 34 replicate host plants. Aphid colonies were established for 3 days while protected from predators by organza mesh bags and a stem guard, which consisted of a tape coated with insect barrier (Tree Tanglefoot; Contech Enterprises, Marysville, OH, USA). On 10 July 2017, we randomly assigned aphid colonies to one of four treatment combinations in a fully factorial design: ambient temperature + no ants, ambient temperature + ants, elevated temperature + no ants, and elevated temperature + ants. We removed organza bags, but left the stem guards intact for aphid colonies receiving the ant exclusion treatments. Temperature was elevated around flowering stalks using open-top warming chambers (OTCs) following the design of Robinson et al. (2017). The OTCs surrounded the tops of flowering stalks with a cylinder of clear polyethylene film while being open to flying insects at the top and the bottoms being open to ground and vegetation dwelling arthropods. We placed a data logger (HOBO; Onset Technology, Bourne, MA, USA) inside one OTC and at the same height near an ambient temperature aphid colony. We assessed aphid colony sizes after 1 day (11 July) and 4 days (14 July) in each treatment. We then calculated aphid colony relative growth rate (RGR) as $\ln(n_t)–\ln(n_0)/t$. Four aphid colonies either failed to establish and/or had their flowering stalks snapped, reducing the total sample size (n = 30). We used an ANOVA to test whether the effect of ant presence on RGR varied between temperature treatments (Qian, 2017). We created the statistical model using the 'lm()' function in R with RGR as the response variable and ant and temperature treatments as the explanatory variables (R Development Core Team, 2018). We also assessed per capita fecundity as a possible driver of temperature-dependent aphid colony growth rates. We counted embryos inside of dissected mature aphids (methods and analysis described below) to determine fecundity.

Ant recruitment and tending. To observe the effects of elevated temperatures on ant tending, we removed the ant barriers from all plants on 17 July. We measured two response variables related to ant tending: per aphid recruitment of ants to flowering stalks and behavior of ants. Assessment of ant recruitment followed the methods of Mooney & Agrawal (2008). We counted ants and aphids every 3-4 days from 20 through 31 July on both elevated (n = 13) and ambient temperature (n = 17) flowering stalks. During this time, *C. aegopodii* invaded eight experimental aphid colonies. To test for differences in recruitment, we used an ANCOVA with mean aphid colony size and temperature treatment as the explanatory variables and mean number of ants counted on flowering stalks as our response variable. Because we did not distinguish between ants actively tending aphids and those engaging in other behaviors, this response variable is recruitment to flowering stalks by aphid colonies. We excluded one aphid colony from the analysis because it was entirely *C. aegopodii* aphids, which are not tended by ants (Kindlmann et al., 2007). We created models using the 'glm()' function with Poisson-distributed errors to account for ants as a count variable (Qian, 2017). We used the 'Anova()' function from the 'car' package to obtain test statistics (LR χ^2) and P-values for significance testing (Fox & Weisberg, 2011).

We also made direct observations of ant behavior on five separate days. We tallied behaviors of all the ants that could be observed on a flowering stalk during a 1-min interval. We classified ants as tending aphids if they were in direct contact through either their antennae or legs. Other behaviors (ranked by frequency) included (1) moving around flowering stalk, (2) feeding on flowers, (3) interacting with other ants, (4) feeding on honeydew deposited on stem, or (5) grooming. As much as possible, we avoided double counting individual ants if they switched behaviors or moved during this observation period. We calculated the mean percentage of ants tending

aphids on each flowering stalk as the response variable (n = 5). We used ANCOVA to test whether tending varied between warmed and ambient temperate treatments using the mean aphid colony size as a covariate.

Sugars in honeydew and ant-collected honeydew. To test for differences in honeydew deposition or composition, we collected honeydew from aphid colonies using foil squares surrounding stems below colonies. The foils remained below the aphid colonies for 12 h on 1 August 2017. All these initial foils were lost in the field, so we replicated the warming experiment and collection procedures in 2018 using a smaller number of elevated (n = 7) and ambient (n = 6) temperature aphid colonies. We repeated the honeydew collection on 2 August 2018. We determined the density of honeydew droplets on foils using a stereo dissection microscope (EZ4 HD with LED, Leica Microsystems). We used ANCOVA to compare the counts of honeydew droplets on foils between ambient and elevated temperature aphid colonies. Because deposition amounts would vary as a function of the numbers of aphids, we used aphid colony size as a covariate. We created this statistical model using the 'glm ()' function in R with Poisson-distributed errors (Qian, 2017). We used the 'Anova()' function from the 'car' package to obtain test statistics (LR χ^2) and P-values for significance testing (Fox & Weisberg, 2011).

We removed the honeydew from foils by immersing them in ultrapure water (Resistivity>18.18 MΩ·cm) at 80 °C in a 50-ml centrifuge tube. We placed the tubes in a sonicator for 15 min at 60 °C in degas mode. We transferred 1 ml of each sample in an autosampler vial for direct analysis with high performance liquid chromatography (HPLC) tandem mass spectrometry (LC-MS/MS). We used a method developed in-house using a Waters Application Note as a springboard (Water Corporation, 2009). We used a Shimadzu LCMS-8030 system for chromatographic separation equipped with triple quadrupole mass spectrometer. The LC system consisted of a binary solvent delivery system and an autosampler. We performed hydrophilic interaction liquid chromatography (HILIC) on a Xbridge Amide column (3.0 × 100 mm, 3.5 µm; Waters, Milford, MA, USA). We used a combination of mobile phase A [acetonitrile and water (30/70, vol/vol), 0.1% ammonium hydroxide] and mobile phase B [acetonitrile and water (80/20, vol/vol), 0.1% ammonium hydroxide] with a gradient elution: 0 min (100% B) ramped to 50% B at 8 min, the column was returned to 100% B at 8.2 min and re-equilibrated for 10.8 min at initial conditions prior to injection of the next standard or sample. We set the flow rate of the mobile phase at 0.6 ml min^{-1}, the column temperature at 35 °C, and the injection volume to be 4 µl. We used electrospray ionization operated in negative ion mode with the mass spectrometer parameters set as follows: nebulizing gas flow, 1.5 l min^{-1}; DL temperature, 250 °C; heat block temperature, 400 °C; drying gas flow, 15 l min^{-1}. We performed selected ion monitoring (SIM) for detection of seven sugars (*m*/*z* 149.0 for xylose, *m*/*z* 179.0 for fructose and glucose, *m*/*z* 341.0 for glucose and trehalose, and *m*/*z* 503.0 for melezitose and raffinose). The dwell time was automatically calculated for each ion by the software to be 60 ms. All sugars were well resolved chromatographically.

Initially, we analyzed the samples for seven sugars commonly found in honeydew: xylose, fructose, glucose, sucrose, trehalose, melezitose, and raffinose (Fischer et al., 2002; Byrne et al., 2003; Pringle et al., 2014). Final analyses excluded xylose because it did not appear in any of the samples we analyzed. We used sugar standards to make eight calibration curve standards varying from 0.05 to 10 mg l^{-1}. For samples with concentration of sugars out of calibration curve, we also did 1:20 and 1:400 dilution of honeydew samples in a mixture of methanol and water (10/90, vol/vol). We used quadratic least squares fit for calibration curves with 1/x weighting to create calibration curves. The calibration curve of each sugar has a linear range of 0.05–5 mg l^{-1}, with a R^2 value of 0.9965 or higher. We used multiple analysis of variance (MANOVA) to simultaneously analyze variation in each of these sugars between temperature treatments. We used counts of honeydew droplets as a covariate in these analyses to account for variation in the amount of honeydew in each extract. We created the model using the 'manova()' function in R, and then performed univariate tests for individual sugar responses using the 'summary.aov()' function.

Aphid antagonists. To determine how temperature treatment may affect aphid predators and parasites, we counted the aphid antagonists – intraguild predators, other predators, and parasitoid wasps – observed in aphid colonies from 20 through 31 July 2017. Intraguild predators were adult and nymph lygus bugs (Hemiptera: Miridae), and the other predators included adult and larval (1) coccinellid beetles, (2) lacewings (Neuroptera: Chrysopidae), and (3) syrphid flies. Lygus bug abundance far exceeded the abundance of all other predators combined; we observed a mean (± SE) of 35 (± 6.6) lygus bugs per stalk and 0.9 (± 0.20) of all other predators per stalk. To test for differences in lygus abundance, we used ANCOVA with warming treatment as the explanatory variable and mean aphid colony size as a covariate. To test for differences in the abundance of other predators, we repeated this analysis using a combined response variable that summed counts of coccinellids, lacewings, and

syrphids. Because abundances were count variables, we used Poisson-distributed errors for model testing (Qian, 2017). In the case of lygus abundance, we used a quasi-Poisson distribution to account for overdispersion. We created these statistical models in R using the 'glm()' function, and we used the 'Anova()' function from the 'car' package to obtain test statistics (LR χ^2) and P-values (Fox & Weisberg, 2011).

We observed very few parasitoid wasps (n = 7) on only three aphid colonies in the field, so we assessed parasitism by dissecting individual aphids. On 2 August 2017, we used a soft-bristled paintbrush to collect mature aphids. At this time, 21 plants had large enough aphid colonies to take samples of at least eight mature aphids (n = 12 and 9 colonies for elevated and ambient temperature, respectively). We placed the samples in vials containing insect preservative solution (5% glycerin in 70% EtOH). Seven of the collected aphid colonies included both *C. aegopodii* and *A. asclepiadis*. We dissected the mature aphids following the methods of Kaiser & Heimpel (2016) and then counted the parasitoid larvae. To test for differences in parasitism of aphids (y/n) between temperature treatments, we used a multilevel model with temperature treatment as a fixed effect and aphid colony as a random effect (Qian, 2017). We used the function 'glmer' from the 'lme4' package to create a general linear mixed model (Bates et al., 2015). We used the 'Anova()' function from the 'car' package to obtain P-values for significance testing (Fox & Weisberg, 2011). This model was used for all of the mature aphids dissected (n = 192) and for the subset of *A. asclepiadis* (n = 138).

Results

Responses to year-to-year temperature variation by ants and aphids

Ants and *A. asclepiadis* aphids showed different responses to year-to-year variation in temperature and snowmelt date across the seven study years (2012–2018). For ants, colonization ranged from three (2012) to 23 (2013) of the 100 host plants we censused in June. For *A. asclepiadis* aphids, colonization at this time ranged from zero (2015) to 24 (2017) of 100 host plants censused. We observed a significant three-way interaction (temperature*snowmelt date*species; Table 1), indicating that how temperature and snowmelt date predicted host plant colonization in June differed between ants and *A. asclepiadis* aphids. Moderate temperatures in June combined with later snowmelt dates increased ant abundance at this time, but temperature was a poor predictor of aphid abundance in June (Figure 1). A similar pattern occurred when the maximum colonization achieved by ants and *A. asclepiadis* aphids is compared across study years. Maximum colonization of

Table 1 Regression results for insect abundance and phenology response to year-to-year variation in temperature and snowmelt date. Estimates for regression coefficients include standard errors (SE) in parentheses

	Estimate (SE)	z-value	P
June colonization by ants and *Aphis asclepiadis* aphids (overall model: pseudo-R^2 = 0.45, P<0.01)			
Intercept	76.71 (28.88)	2.66	<0.01
Temperature	−6.64 (2.56)	−2.59	<0.01
Snowmelt date	−0.52 (0.21)	−2.52	0.01
Species	−354.84 (101.46)	−3.50	<0.01
Temperature* snowmelt date	0.046 (0.02)	2.53	0.01
Temperature*species	29.32 (8.64)	3.40	<0.01
Snowmelt date*species	2.37 (0.70)	3.39	<0.01
Temperature*snowmelt date*species	−0.19 (0.06)	−3.27	<0.01
Maximum colonization by ants and *A. asclepiadis* aphids (overall model: pseudo-R^2 = 0.45, P<0.01)			
Intercept	49.80 (19.48)	2.56	0.01
Temperature	−4.05 (1.72)	−2.36	0.02
Snowmelt date	−0.31 (0.15)	−2.06	0.04
Species	−63.0 (25.43)	−2.48	0.01
Temperature* snowmelt date	0.03 (0.01)	2.020	0.04
Temperature*species	4.74 (2.23)	2.13	0.03
Snowmelt date*species	0.38 (0.19)	2.01	0.05
Temperature*snowmelt date*species	−0.03 (0.02)	−1.65	0.10
Day of year at maximum colonization by ants and *A. asclepiadis* aphids (overall model: R^2 = 0.66, P = 0.02)			
Intercept	−1209.42 (495.09)	−2.44	0.04
Temperature	121.55 (42.80)	2.84	0.02
Snowmelt date	11.11 (3.80)	2.92	0.02
Species	1061.67 (700.16)	1.52	0.17
Temperature*snowmelt date	−0.97 (0.33)	−2.93	0.02
Temperature*species	−89.82 (60.53)	−1.48	0.18
Snowmelt date*species	−7.37 (5.38)	−1.37	0.21
Temperature*snowmelt date*species	0.63 (0.47)	1.35	0.21
Maximum colonization by *Cavariella aegopodii* and *A. asclepiadis* aphids (overall model: pseudo-R^2 = 0.66, P<0.01)			
Intercept	−13.19 (16.35)	−0.81	0.42
Temperature	0.69 (1.42)	0.49	0.63
Snowmelt date	0.07 (0.12)	0.62	0.54
Species	78.13 (68.80)	1.14	0.26
Temperature* snowmelt date	−0.01 (0.01)	−0.10	0.92
Temperature*species	−6.35 (5.71)	−1.11	0.27
Snowmelt date*species	−0.69 (0.51)	−1.36	0.17
Temperature*snowmelt date*species	0.06 (0.042)	1.31	0.19

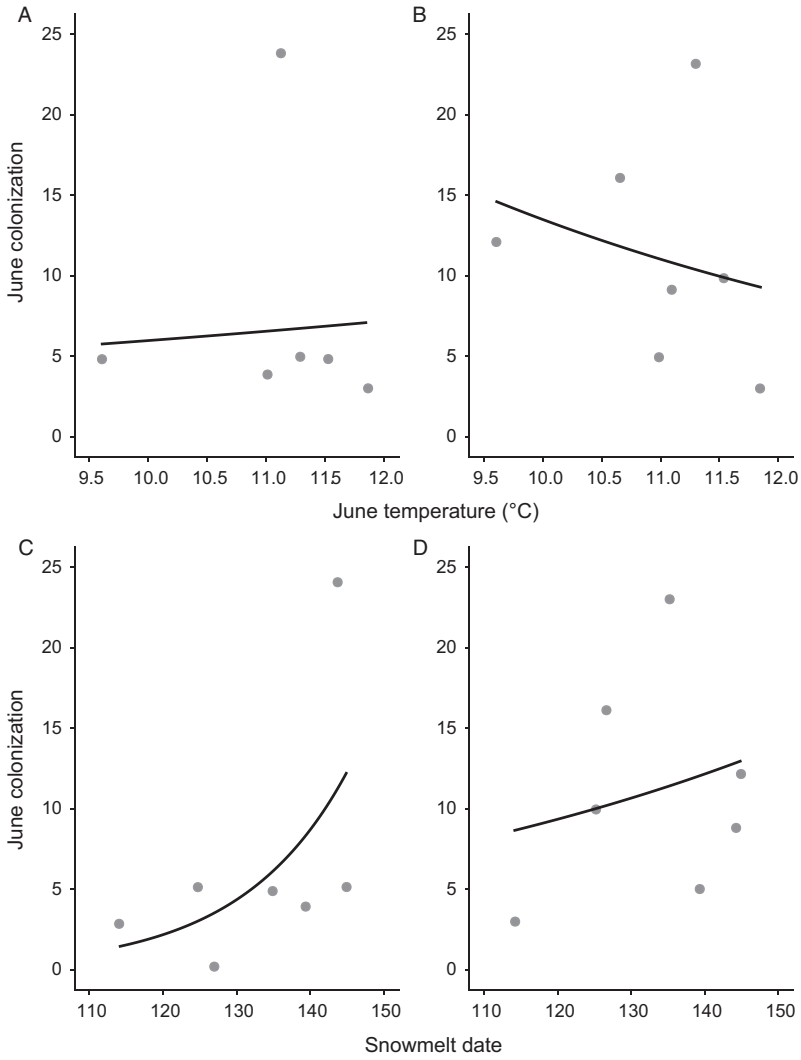


Figure 1 Year-to-year variation in June colonization of host plants by (A,C) *Aphis asclepiadis* aphids and (B,D) ants, associated with (A,B) mean June temperature and (C,D) snowmelt date. Individual data points show the number of host plants colonized during the 3rd week of June for a particular study year. Curves represent the results of a Poisson regression.

host plants by ants in a given year ranged from seven (2012) to 33 (2015) and from one (2015) to 60 (2016) host plants for *A. asclepiadis* aphids. We observed a significant two-way interaction (temperature*species) indicating that how temperature predicted maximum host plant colonization differed between ants and *A. asclepiadis* aphids (Table 1). Mean June-July temperature tended to be a poor predictor of colonization by *A. asclepiadis* aphids, whereas for ants, higher temperatures consistently reduced maximum colonization achieved across study years (Figure 2). As with June colonization, snowmelt date was a more consistent predictor of maximum aphid colonization than temperature, although we did not observe a significant three-way interaction. For both ants and *A. asclepiadis* aphids, the effect of snowmelt date on the day of the year at maximum abundance depended on mean June-July temperature (temperature*snowmelt date: Table 1). However, the response was consistent across these two species groups, as indicated by the lack of a significant three-way interactive effect (temperature*snowmelt date*species: $P>0.05$).

Over the eight study years, fewer plants were colonized by *C. aegopodii* than by *A. asclepiadis*. The greatest colonization level was achieved in 2017 with 12 of 100 host plants colonized by *C. aegopodii*. Although higher temperatures tended to increase maximum colonization achieved by *C. aegopodii*, none of the effects in this model were significant (Table 1). Likewise, we found that no model terms significantly predicted either June abundance or day of year at maximum abundance for *C. aegopodii*.

Responses to experimental warming of ant-aphid mutualism

Aphid population dynamics. The open-top warming chambers elevated daytime (09:00–17:00 hours)

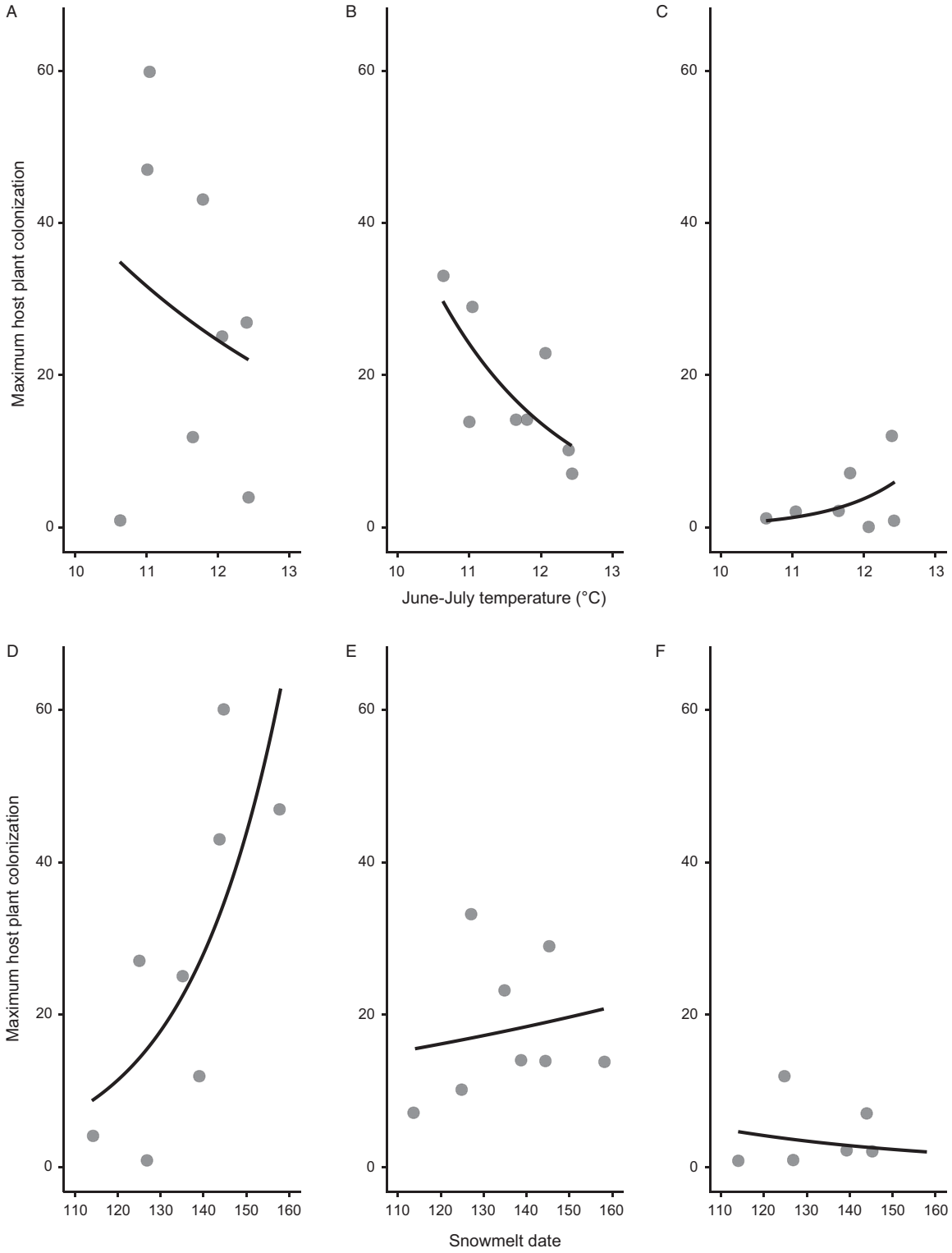


Figure 2 Year-to-year variation in maximum colonization of host plants by (A,D) *Aphis asclepiadis* aphids, (B,E) ants, and (C,F) *Cavariella aegopodii* aphids, associated with (A–C) mean June-July temperature and (D–F) snowmelt date. Individual data points show the highest number of host plants colonized in a particular study year. Trend lines represent the results of a Poisson regression.

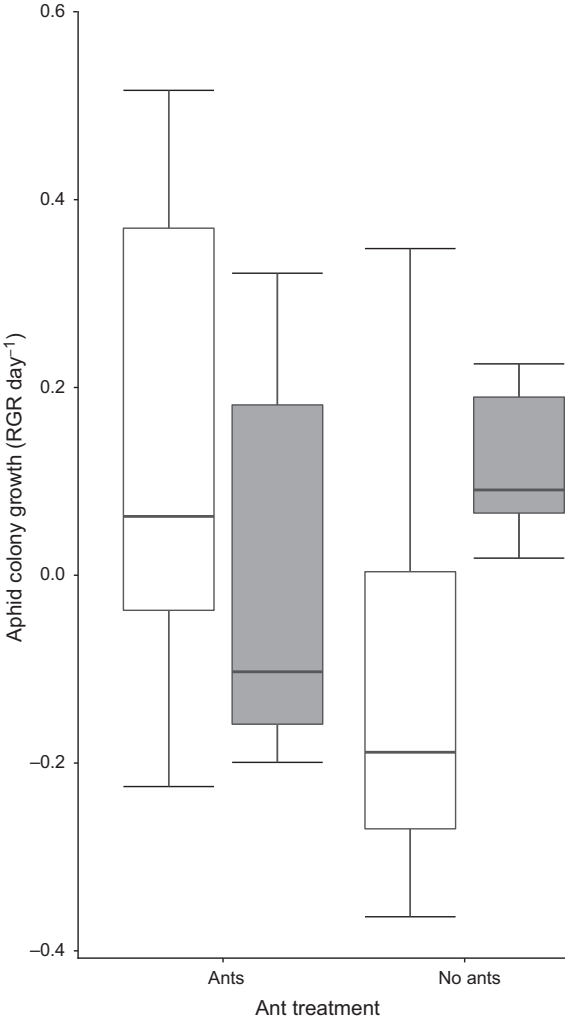


Figure 3 Relative growth rates (RGR) of aphid colonies at ambient (white bars) and elevated (grey bars) temperatures with (Y) and without (N) tending by mutualist ants. RGR measures colony growth from counts of individual aphids across 4 days. Boxplots show underlying distributions of data: thick lines represent the median and boxes represent the interquartile range (IQR). Whiskers represent the minimum (1st quartile − 1.5*IQR) and maximum (3rd quartile + 1.5*IQR).

temperature around *L. porteri* flowering stalks by an average of 4.6 °C relative to ambient temperature flowering stalks. The presence of tending ants increased aphid population growth rate at ambient temperatures but not at elevated temperatures (ant*temperature interaction: $F_{1,29} = 4.27$, $P = 0.049$). In fact, the effect of tending ants on aphid population growth rate at ambient temperatures was similar to the effect of elevated temperature in the absence of ants (Figure 3). Neither the main effect of temperature nor the main effect of ant presence was significant. Among mature aphids of both

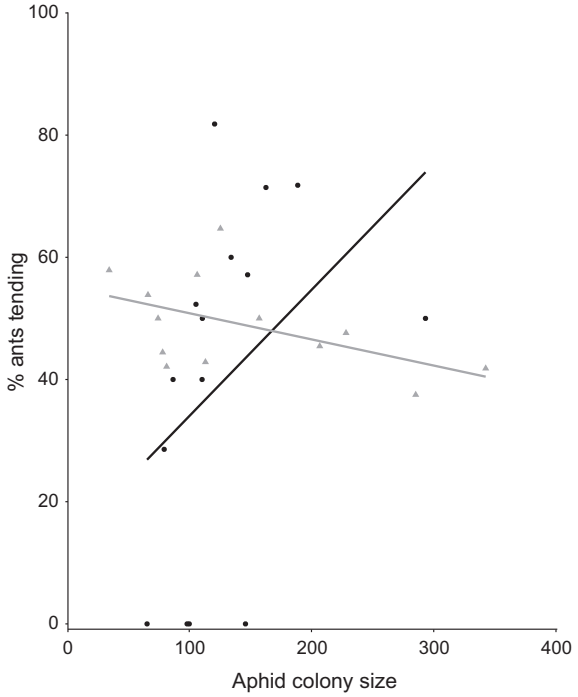


Figure 4 Mean percentage of ants engaged in tending of aphid colonies as a function of aphid colony size at ambient (black symbols) and elevated (grey symbols) temperature. Mean percentage tending represents an average across five focal observations of ant behavior on flowering stalks. Trend lines represent linear regression results for ant tending of aphid colonies at ambient (black line) and elevated (grey line) temperature.

species, elevated temperatures did not affect the number of embryos they contained ($\chi^2 = 0.005$, $P = 0.95$).

Ant recruitment and tending. Larger aphid colonies recruited more ants to flowering stalks (LR $\chi^2 = 8.16$, $P = 0.004$), but elevated temperatures had no effect on this pattern (LR $\chi^2 = 2.26$, $P = 0.13$). Likewise, elevated temperatures had no effect on the overall number of ants observed (LR $\chi^2 = 0.06$, $P = 0.81$). However, elevated temperatures reduced the tending behavior exhibited by ants in aphid colonies. At ambient temperature, larger aphid colonies had more ants tending than did small colonies; this relationship was not apparent at elevated temperatures (Figure 4; $F_{1,28} = 4.60$, $P = 0.04$). The level of ant tending behavior was not affected by aphid colony size ($F_{1,28} = 0.34$, $P = 0.57$) or the main effect of temperature ($F_{1,28} = 1.26$, $P = 0.27$).

Sugars in honeydew and ant-collected honeydew. Whereas the amount of honeydew droplets on foils tended to increase with aphid colony size ($z = 1.90$, $P = 0.06$), this

relationship did not vary with temperature treatment ($z = -0.43$, $P = 0.69$), and temperature did not have an overall effect ($z = 0.37$, $P = 0.71$). Across all six sugars, elevated temperatures increased concentrations of sugars extracted from foil samples of honeydew (Table 2). As the density of honeydew droplets on the foil sample increased, so did the sugar concentration in the resulting extracts. However, this relationship was most pronounced for honeydew foils collected from elevated-temperature aphid colonies. The overall response was largely driven by increases in the concentrations of two sugars – sucrose and trehalose – in honeydew collected from elevated-temperature aphid colonies (Figure 5). Although larger gasters tended to contain more sugar, elevated temperatures had no effect on the concentration of any of the five sugars inside of ant gasters (Table 2).

Aphid antagonists. The abundance of lygus bugs generally increased with aphid colony size (LR $\chi^2 = 8.17$, $P = 0.004$), but this relationship was most pronounced on ambient temperature aphid colonies (Figure 6; LR $\chi^2 = 4.27$, $P = 0.04$). There was a trend for elevated temperatures to reduce the numbers of lygus bugs overall (LR $\chi^2 = 2.96$, $P = 0.09$). The abundances of other predators (coccinellid beetles, lacewings, and syrphid flies) did not show these same responses. The abundance of other predators did not increase with aphid colony size (LR $\chi^2 = 1.37$, $P = 0.37$) and was not affected by temperature treatment (LR $\chi^2 = 0.97$, $P = 0.33$). Likewise, there was no evidence that the relationship between aphid colony size and predator abundance varied between the temperature treatments (LR $\chi^2 = 1.53$, $P = 0.22$). We observed only 12 parasitoid larvae among the 192 mature aphids we dissected; these 12 parasitoid larvae were only found in *A. asclepiadis*. Elevated temperatures had no effect on the incidences of endoparasitism in the sampled aphid colonies ($P>0.05$).

Discussion

Based on 8 years of host plant censuses, aphids and their mutualist ants appeared to respond differently to year-to-year variation in temperature. For ants, elevated temperatures reduce both colonization of host plant inflorescences in June and maximum colonization achieved in a given year. For aphids, both colonization in June and maximum colonization achieved in a given year is better predicted by snowmelt date than by temperature. Higher June temperatures suggest low levels of ant abundance on host plant flowering stalks, but not necessarily low levels of aphid colonization at this time. Further, our experimental results show warming can increase aphid colony growth rate

Table 2 MANOVA results of six sugars analyzed from honeydew and ant gasters collected from ambient-temperature and elevated-temperature aphid colonies

			Overall response			Fructose			Glucose			Sucrose			Trehalose			Melezitose			Raffinose		
Sugar source	Factor	Pillai's trace	F	d.f.	P	F	d.f.	P	F	d.f.	P	F	d.f.	P	F	d.f.	P	F	d.f.	P	F	d.f.	P
Honeydew	Density	0.98	33.61	6,4	0.002	69.96	1,9	0.001	11.89	1,9	0.007	29.59	1,9	0.001	10.46	1,9	0.01	24.03	1,9	0.001	1.95	1,9	0.20
	Temperature	0.99	52.22	6,4	0.001	2.79	1,9	0.13	0.79	1,9	0.40	2.12	1,9	0.18	1.84	1,9	0.21	2.93	1,9	0.12	0.34	1,9	0.57
	Density* temperature	0.99	44.19	6,4	0.001	0.32	1,9	0.58	0.002	1,9	0.96	4.85	1,9	0.06	4.74	1,9	0.06	2.52	1,9	0.15	0.98	1,9	0.35
Ant gasters	Weight	0.37	2.03	5,17	0.13	12.19	1,21	0.002	8.95	1,21	0.007	2.33	1,21	0.14	2.95	1,21	0.10	1.71	1,21	0.20	–	–	–
	Temperature	0.17	0.72	5,17	0.62	0.05	1,21	0.82	0.46	1,21	0.50	1.52	1,21	0.23	1.11	1,21	0.30	1.54	1,21	0.22	-	–	–
	Weight* temperature	0.17	0.72	5,17	0.62	0.90	1,21	0.35	0.04	1,21	0.85	0.25	1,21	0.62	0.009	1,21	0.93	0.13	1,21	0.71	–	–	–

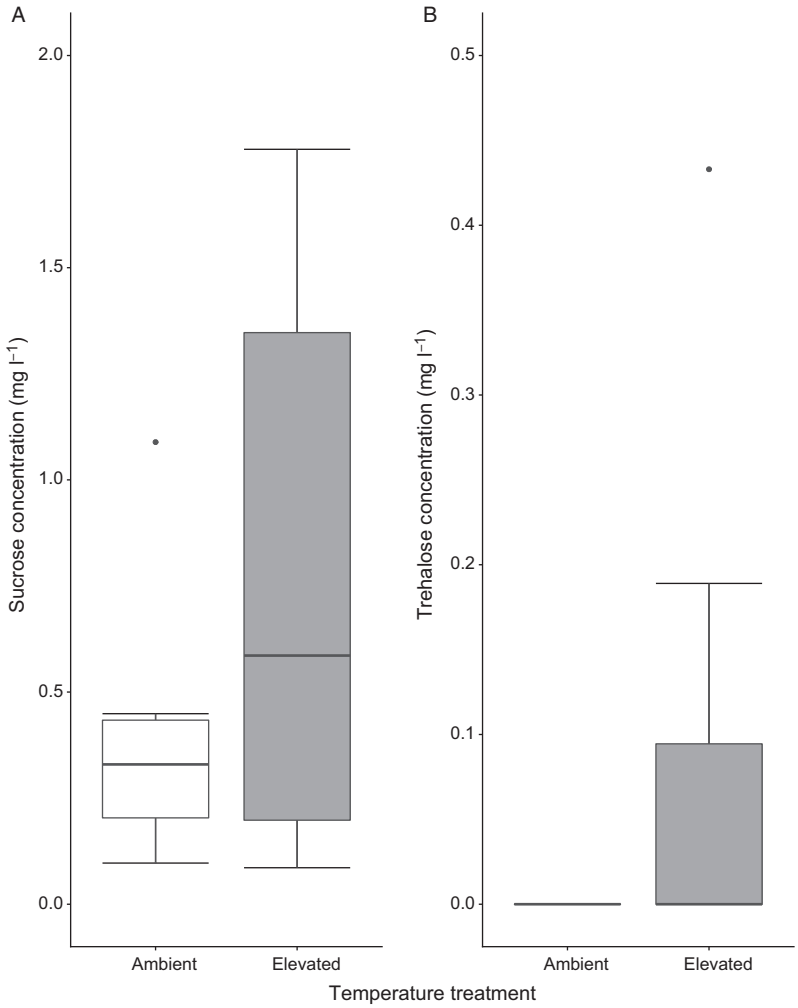


Figure 5 Concentration (mg l^{-1}) of (A) sucrose and (B) trehalose in honeydew collected from aphid colonies at ambient and elevated temperatures. Boxplots show underlying distributions of data: thick lines represent the median and boxes represent the interquartile range (IQR). Whiskers represent the minimum (1st quartile − 1.5*IQR) and maximum (3rd quartile + 1.5*IQR), with dots representing outliers.

independent of ant attendance. This response seems to be largely due to reduced intraguild predator abundance with higher temperatures, rather than changes in fecundity, predation by other guilds, or endoparasitism. So, although ant abundance and ant-tending may be suppressed in warm years, aphids still could benefit from elevated temperatures if these higher temperatures were accompanied by a later snowmelt date.

Responses to year-to-year temperature variation by ants and aphids

Across study years, higher June and summer temperatures reduced the abundance of ants on host plant inflorescences, whereas colonization by *A. asclepiadis* aphids was largely driven by snowmelt date. Ant thermal tolerances may underlie their reduced abundance on host plant inflorescences in warmer years. Foraging at higher temperatures imposes greater metabolic costs (Traniello et al., 1984), and some ant species will alter foraging timing to avoid high temperatures (Jayatilaka et al., 2011; Stuble et al., 2013). Fewer ants on flowering stalks in warmer years could occur if ants shift foraging to times of day outside of our observations. However, experimental warming did not reduce ant abundance on flowering stalks in this study. This may be an artefact of the OTC design, which only warmed flowering stalks not ant colonies themselves or the larger environment through which ants forage. Larger-scale warming experiments are warranted, although the few examples of simultaneous ant and aphid colony warming highlight the practical challenges of this experimental approach (Marquis et al., 2014; Blanchard et al., 2019). Besides ant thermal tolerances, aphid and plant-mediated effects may also be responsible for this pattern of reduced ant abundance. In our system, ants forage on host plant flowering stalks for floral nectaries and aphid honeydew, both of which could be altered by temperature. Our experimental results show changes in honeydew sugar composition with elevated temperatures. For nectary sugars, elevated temperatures reduced nectar volume across a

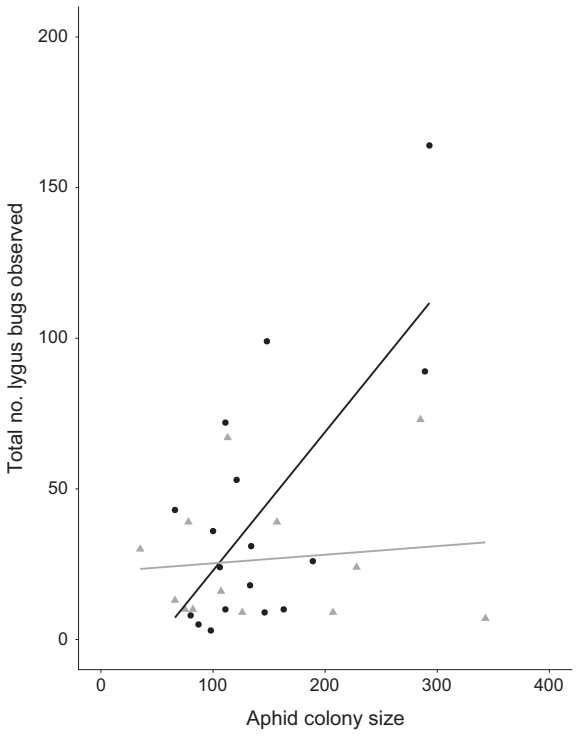


Figure 6 Total number of lygus bugs observed on flowering stalks as function of aphid colony size at ambient (black symbols) and elevated (grey symbols) temperature. Total number of lygus bugs is the sum of all adults and nymphs observed on a flowering stalk. Trend lines represent results from Poisson regression for lygus bugs on aphid colonies at ambient (black line) and elevated (grey line) temperature.

range of plant species and altered sugar content in some plant species (Takkis et al., 2018). Changes in aphid and floral resources on host plant flowering stalks could intensify the impact of high temperatures on ant foraging patterns in very warm years.

Our year-to-year results for aphid abundance seem to contrast with those of our field experiment, that is, temperature is a poor predictor of aphid colonization across years yet enhances experimental aphid colony growth. However, selection and colonization of host plants by aphids is driven by a series of chemical and physiological cues (Powell et al., 2006), each of which could respond differently than aphid colony growth rate to higher temperatures. Rather than temperature, we found aphid colonization in June to be most associated with snowmelt date. This contrasts with predictions that increased temperature will drive increases in aphid abundance (e.g., Bale et al., 2002). In the subalpine zone of our study system, later snowmelt dates both delay plant phenology and increase soil moisture in June (Iler et al., 2013). Aphid colonization of host plant flowering stalks is likely sensitive to both of these effects. For example, climate change effects on host plant phenology alters herbivore performance in a variety of systems (Schwartzberg et al., 2014; Doiron et al., 2015). Likewise, drought stress can sometimes increase host plant quality for aphids (Huberty & Denno, 2004), but this is not a consistent response across host plant or aphid species (Pritchard et al., 2007). Future experimental work could separate these effects, but our observations suggest that advanced plant phenology and drought stress reduce aphid colonization.

Responses to experimental warming of ant-aphid mutualism

Warming reduces the tending behavior of ants to aphid colonies in this experiment. We found that on ambient-temperature plants, larger aphid colonies had significantly higher percentages of ants tending aphids than did small colonies; this relationship was not apparent on elevated-temperature plants. Behavior of ants in other mutualisms is responsive to environmental temperatures. In an ant-plant mutualism, ants without broad thermal tolerance abandoned their plants during the hottest parts of the day (Fitzpatrick et al., 2014). In our experimental results, the overall number of ants was not reduced by warming, indicating that this is a behavioral shift by ants. The main reward for ants – aphid honeydew – is altered by experimental warming: both sucrose and trehalose are increased in the honeydew collected from elevated-temperature aphid colonies. Few previous studies have examined the effect of temperature on honeydew composition, but Yao & Akimoto (2001) also found increased proportions of sucrose and trehalose in ant-tended relative to non-tended aphid honeydew. Trehalose is an important storage carbohydrate for insects, but is largely ignored as a sugar source by foraging ants (Yao & Akimoto, 2001; Fischer & Shingleton, 2001; Detrain et al., 2010). Melezitose is the most effective honeydew sugar for attracting ants (Völkl et al., 1999; Fischer & Shingleton, 2001), so ants could be changing their behavior in response to a relative decrease in melezitose in honeydew as more sucrose and trehalose are excreted by aphids at elevated temperatures.

Despite reduced tending from ants, aphid colonies at elevated temperatures had fewer intraguild mirid predators. In this way, elevated temperatures are taking the place of the protection mutualism with ants. Other predators – coccinellid beetles, lacewings, and syrphid flies – were not affected by temperature in the same way as the mirids. Because they are an important agricultural pest, the thermal tolerance of lygus bugs has been documented in laboratory trials (Cooper & Spurgeon, 2015; Spurgeon & Brent, 2016). Developmental abnormalities appear at fluctuating temperatures near 35 °C (Spurgeon & Brent,

2016), which approaches the temperature ranges achieved by the OTC in this experiment. Reduced mirid abundance may be the product of elevated temperatures disrupting the lygus bug life cycle. As intraguild predators, fewer lygus bugs would reduce predation as well as competition in aphid colonies at elevated temperatures. Follow-up studies could separate the effects of predation and competition by lygus bugs on aphid colonies. However, a recent meta-analysis confirms that top-down control is the primary driver of herbivore abundance in a wide range of systems (Vidal & Murphy, 2018).

Conclusions

Aphids are among the key groups of insect herbivores demonstrating the global fingerprint of climate change. Long-term data from suction trap networks show poleward distribution shifts and advanced phenology with earlier, warmer springs (Harrington et al., 2007; Bell et al., 2015). In a recent review, Renner & Zohner (2018) summarize two key elements of phenological mismatches: interacting species will (1) show different year-to-year patterns in traits driven by climate cues, and (2) respond differently to these cues. Given the differential response to temperature and snowmelt date, our results provide some evidence that a phenological mismatch between ants and aphids could occur. In our system, yearly variation in aphid abundance is primarily driven by snowmelt date, whereas ant abundance on host plants is largely associated with temperature. Through its direct effects on aphid life cycles, increased temperature is considered a key driver of aphid outbreaks in natural and managed systems (Lima et al., 2008; Ciss et al., 2014). Our experimental results suggest that suppression of top-down control with elevated temperatures would contribute to increases in aphid abundance. For aphid species whose abundances and distributions rely on ant tending, elevated temperatures may override this dependency. If this is the case, any phenological mismatch between ants and aphids might have little impact on aphid abundance.

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