

Responses of plant phenology, growth, defense, and reproduction to interactive effects of warming and insect herbivory

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Abstract. Climate warming can modify plant reproductive fitness through direct and indirect pathways. Direct effects include temperature-driven impacts on growth, reproduction, and secondary metabolites. Indirect effects may manifest through altered species interactions, including herbivory, although studies comparing the interactive effects of warming and herbivory are few. We used experimental warming combined with herbivore exclusion cages to assess the interactive effects of climate warming and herbivory by *Popillia japonica*, the Japanese beetle, on flowering phenology, growth, defense, and lifetime reproduction of a biennial herb, *Oenothera biennis*. Regardless of temperature, herbivory delayed flowering phenology and, surprisingly, led to decreased levels of foliar defenses. At ambient temperatures, plants were able to compensate for herbivory by producing smaller seeds and increasing total seed production, leading to similar investment in seed biomass for plants exposed to and protected from herbivores. At elevated temperatures, plants had elevated total seed production, but herbivory had negligible impacts on flower and fruit production, and total lifetime seed biomass was highest in plants exposed to herbivores in warmed conditions. We speculate that warming induced a stress response in *O. biennis* resulting from low soil moisture, which in turn led to an increase in seed number at the expense of maternal investment in each seed. Plant-insect interactions might therefore shift appreciably under future climates, and ecologists must consider both temperature and herbivory when attempting to assess the ramifications of climate warming on plant populations.

Key words: climate change; compensation; fitness; herbivory; *Oenothera biennis*; *Popillia japonica*; reproduction; secondary chemistry.

INTRODUCTION

Numerous aspects of plant ecology and reproductive biology will be affected by rapidly rising temperatures associated with climate change (Walck et al. 2011). Warming enhances plant growth rates (Veteli et al. 2002) and affects fruit set, seed mass, seed number, and seed germination potential (Hovenden et al. 2008, Liu et al. 2012). Flowering phenology has also changed significantly for many species due to earlier snowmelt or prolonged growing seasons (Sherry et al. 2007, Wolko-vich et al. 2012). However, most studies on climate warming do not simultaneously manipulate herbivores (Volder et al. 2010, 2013), and many studies examining the effect of temperature on herbivory do not simultaneously expose plants to higher temperatures (Lemoine

et al. 2013, 2014a). Because plant fitness and evolution are often driven by insect herbivores (Agrawal et al. 2012), which are themselves sensitive to climate warming (Lemoine et al. 2014a), accurate assessments of the impact of climate warming on plant fitness should examine potential interactive effects of warming and herbivory on plant growth, defense, and reproduction.

Climate warming might alter plant–herbivore interactions by first changing plant physiology and nutritional quality, thereby affecting the extent to which herbivores feed and damage plant tissues. Foliar nitrogen and sugar content, both important components of herbivore nutrition and determinants of plant palatability (An et al. 2005, Zvereva and Kozlov 2006, Lemoine et al. 2013, 2014b), often decrease at high temperatures. Warming also affects plant expression of secondary defenses. Phenolic and terpenes, for example, decrease and increase with warming, respectively, which can in turn affect herbivore consumption and performance (Zvereva and Kozlov 2006). Warming can also indirectly affect plant susceptibility to herbivory by inducing stress responses to lower soil moisture content, a common consequence of increased temperatures (Melillo et al.

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2002). Water stress can increase foliar nitrogen content (Evans and Burke 2013) and reduce concentrations of secondary metabolites (Gutbrodt et al. 2011), making plants more susceptible to herbivores at higher temperatures. Thus, warming might exacerbate or negate the influence of herbivores on plant life history strategies by directly or indirectly changing the palatability and, as a result, damage inflicted by herbivores.

Warming might also disrupt plant–herbivore interactions by causing phenological mismatches between herbivores and plants. The timing of numerous plant ontogenetic stages changes with warming; rising temperatures advance plant germination, emergence, and flowering times (Post et al. 2008, Wolkovich et al. 2012). Such changes in plant phenology can be deleterious to plant reproduction. Flowering, for example, often advances more rapidly with earlier spring warming than does pollinator emergence, lowering pollination rates, and leading to reduced seed set and reproductive output (Kudo and Ida 2013). Plants also delay flowering in response to herbivory in order to avoid flower damage (Brody 1997). Warming, however, might advance flowering to match peak herbivore abundances, negating any advantage conferred by delayed flowering (Liu et al. 2011). Thus, changes in the timing of flowering can adversely affect plant reproduction by either reducing pollination rates or increasing herbivore damage.

Finally, herbivory rates will increase in the future as warming accelerates herbivore population growth rates (Savage et al. 2004), stimulates higher per capita consumption rates by herbivores (O'Connor 2009, Lemoine and Burkepile 2012), and might concentrate herbivory on a subset of particularly nutritious plant species (Lemoine et al. 2013). Additionally, rising temperatures can alter the efficacy of plant defenses against herbivores, which become either more or less susceptible to secondary metabolites at higher temperatures (Stamp and Yang 1996, Lemoine et al. 2013). To date, however, most studies quantifying the impact of warming on plant–herbivore interactions have focused on how warming affects foliar damage (Lemoine et al. 2013, 2014a) or growth rates of whole plants (Richardson et al. 2002, O'Connor 2009), and there is little information on whether climate warming will ultimately affect plant fitness, e.g., lifetime seed production, by altering plant–herbivore interactions.

In this study, we assessed how climate warming alters the impact of insect herbivores on flowering phenology, growth, secondary chemical defenses, and reproductive strategy in *Oenothera biennis* using a factorial experiment that manipulated both temperature and herbivore presence. In response to the treatments, we quantified (1) timing of flowering events, (2) plant size, (3) plant damage by herbivores, (4) defensive chemicals in leaves and flowers, (5) fruit mass and number, and (6) seed mass and number. Using these measurements, we calculated two metrics of lifetime fitness for *O. biennis*: total lifetime seed production and seed biomass. Total seed

production and biomass are differing metrics of maternal investment in offspring production, i.e., plants might maintain constant seed biomass production despite smaller seed sizes by increased total seed production. We expected that warming would significantly advance flowering time of *O. biennis* (Wolkovich et al. 2012) and would lead to taller plants (Walker et al. 2006). We hypothesized that herbivory would decrease fruit set, fruit size, and seed size, yielding lower total lifetime fitness of *O. biennis* (Poveda et al. 2003, Hovenden et al. 2008). We also expected that the effects of herbivores would be exacerbated in warmed conditions due to increased herbivore damage (Lemoine and Burkepile 2012, Lemoine et al. 2013, 2014a), resulting in even further suppression of lifetime fitness.

METHODS

Study site and experimental design

To simulate climate warming, we installed experimental warming infrastructure at the Smithsonian Environmental Research Center in Edgewater, Maryland, USA. During the summer of 2013, we installed 16 2×2 m garden beds in an open, sunlit field. Plots were constructed of treated lumber, filled with commercial topsoil, and topped with a layer of compost. Garden bed soils were approximately 20 cm in depth and did not have a barrier on the bottom to prevent abnormally high water retention by allowing water to drain into natural soils beneath each garden bed. We initiated warming treatments by installing a single $165 \times 15 \times 15$ cm Kalglo MRM-1215 1500W (Kalglo Electronics Company, Bethlehem, Pennsylvania, USA) infrared heater ~ 1.5 m from the soil surface over eight randomly chosen plots. To control for shading by the heaters, we hung a “heater decoy” made out of aluminum sheeting of the same size and shape as the heaters over the ambient temperature plots ($n = 8$ per treatment). In August 2014, heaters were raised to a height of 2.5 m as the plants had outgrown the heaters. Heaters successfully raised leaf-surface temperatures by $\sim 3^\circ\text{C}$ during the day and $\sim 5^\circ\text{C}$ during the night. This increase mimics projected climate change by 2100 and is consistent with observed patterns of more rapid increases in nighttime temperatures than daytime temperatures (Easterling et al. 1997, IPCC 2014).

In each of the 16 plots, two perpendicular 1 m long aluminum metal sheets driven 10 cm into the soil divided the 2×2 m plot into four 1×1 m subplots. We only used two of the four sub-plots in this experiment. Within a plot, the two subplots were randomly assigned to “no herbivory” or “herbivory” treatments, so both treatments were present in every plot. No herbivory treatments were covered with translucent 1-mm mesh tucked into the soil around the garden edges to prevent herbivore access. Herbivory treatments consisted of the same net with all sides rolled up, mimicking shading effects of the net while still allowing herbivore access

($n = 16$ per herbivore treatment). This resulted in a split-plot experimental design, with warming as the whole plot factor and herbivory as the subplot factor.

Study species

We used *Oenothera biennis* as a model organism to assess the joint effects of warming and herbivory on plant fitness. *Oenothera biennis* is a biennial plant native to eastern North America that typically forms rosettes in its first year and bolts into a flowering stalk in its second year (Johnson 2011). Approximately 50 species of herbivorous insects feed on *O. biennis* (Johnson and Agrawal 2005), including Japanese beetles (*Popillia japonica*) and multiple arctiid and noctuid caterpillars. Although *O. biennis* hosts a number of insect and avian flower visitors, including bees, moths, butterflies, and hummingbirds, pollinators are not required for *O. biennis* fruit and seed production (Johnson 2011) due to its unique genetic system (permanent translocation heterozygosity) that effectively precludes outcrossing (Johnson 2011, Agrawal et al. 2012). Therefore, rearing *O. biennis* under nets excluding insect pollinators likely had little effect on its reproductive biology.

In July 2013, we purchased 0.45 kg (~0.5–1 million) of *O. biennis* seeds from a commercial supplier (Ernst Conservation Seeds, Meadville, Pennsylvania, USA). Seeds originated from multiple populations and were mixed thoroughly prior to planting. We sowed 0.5 g of seeds into each subplot, and allowed them to germinate and grow under each temperature condition. Garden beds were watered twice daily until germination, after which plants relied on natural precipitation for the remainder of the experiment. During the first year, nets were placed over all plots to prevent any effects of insect herbivores on germination and plant density. As a result, plant density was similar among treatments (4.1 ± 1.6 plants per plot overall [mean \pm SE]). In October 2013, after the growing season ended, heaters were turned off and *O. biennis* rosettes overwintered under ambient conditions. At the beginning of the 2014 growing season in May after the first emergence of leaves, heaters were turned on and nets placed over all plots. The sides of the nets were rolled up for the no herbivore plots to allow herbivore access.

Heaters were again turned off in October 2014 after leaves had begun to senesce. In Maryland, the last frost occurs in late April and first frost occurs in mid- or late October; a heating period of May–October therefore encompassed almost the entire growing season at our study site. We did not expose *O. biennis* to winter warming for several reasons. First, our warming period encompassed the entire duration of insect activity at our study site. Second, *O. biennis* flowers in the fall, and late-blooming species are phenologically insensitive to winter warming compared to species that flower in the spring (Wolkovich et al. 2012). Thus, it is unlikely that winter warming would substantially alter our results.

During the second week of August 2014, nets were removed from all plots as the predominant herbivore *P. japonica* was now largely absent. Indeed, few other herbivores were observed on the plants during our weekly flower counts, which required close inspection of each stem and fruit. Over the course of the growing season, we found one yellow bear caterpillar (*Spilosoma virginica*) on a plant stem and two woolly bear caterpillars (*Pyrrharctia isabella*) near or within the garden beds. Additionally, we found no evidence of seed predators, including *Schinia florida* and *Mompha* spp., over the course of this experiment.

Herbivore pressure

To estimate herbivore pressure in ambient and warmed conditions and to confirm that our herbivore exclusions successfully reduced damage, we quantified both the number of *P. japonica* in each plot and cumulative leaf damage. *Popillia japonica* is an invasive pest that consumes both leaves and flowers of its host plants. Therefore, at three points over the summer (July–August), we counted the total number of living *P. japonica* occurring in each plot and recorded whether they were on foliage or flowers. To estimate leaf damage that occurred during peak herbivore loads, we visually estimated damage on August 18, just after *P. japonica* numbers declined. We assigned a value of 0%, 25%, 50%, 75%, or 100% damage to 25 haphazardly chosen leaves in each subplot (approximately five leaves per stem). We averaged damage values over all leaves within each plot, yielding one damage estimate per plot.

Flowering phenology

We quantified how warming and herbivory influenced plant flowering phenology by counting the total number of flowers on every plant weekly for four months. *Oenothera biennis* flowered for the first time during the week of 14 July in 2014. Only fully open flowers were counted; we did not count buds or wilted flowers. Since *O. biennis* flowers only persist for 3–5 d (N. Lemoine and D. Doublet, *personal observation*), weekly flower counts likely did not result in recounting the same flowers. Indeed, flower counts changed dramatically on a week-to-week basis. Flower counts ceased on the week of October 6, which represented the last flowering event near the end of the growing season. We averaged the total number of flowers per plant in each subplot because subplots represented the experimental unit ($n = 8$ replicates for each treatment).

Plant size

We measured the tallest stem of each plant (there were no single stemmed plants) within each subplot at the end of the second growing season. We then averaged this height within each subplot, yielding one estimate of maximum plant height per subplot.

Plant chemistry

To assess plant defenses, we collected 10 green leaves and 10 flowers from each subplot on a single day at the end of the second growing season (early October). Collections were randomly distributed among all plants occurring within a subplot. Flowers and leaves were immediately frozen at -80°C and then freeze-dried. Freeze-dried material was ground into a powder, and all samples from within a single subplot were combined and homogenized to yield one sample per subplot. 10 mg of plant powder were extracted quantitatively with $3 \times 1,400 \mu\text{L}$ acetone/water (80:20, v:v). Acetone was evaporated with an Eppendorf concentrator, water-phase was freeze-dried and then re-dissolved in 5 mL Milli-Q purified water. The extract was filtered with 0.20 μm PTFE filters and analysed by UPLC-DAD-MS as explained in Johnson et al. (2014). We quantified concentrations of oenothien B (the ellagitannin [ET] dimer), oenothien A (the ET trimer), oxidized oenothien A, ET tetramers, and ET pentamers at 280 nm as oenothien B equivalents. All these compounds have been characterized earlier in *O. biennis* (Johnson et al. 2009, Karonen et al. 2010, McArt et al. 2013) and even purified recently (Baert et al. 2015, 2016, 2017), since they are not commercially available. However, since these ETs all share similar UV spectra, it was possible to purify only the smallest one (oenothien B, 1568 g/mol) to be used as the quantitation standard for these five ETs. The sum of these ETs was treated as total ETs, and the ratio of oenothien A to oenothien B was taken as an additional parameter, since this ratio has been shown to be important determinant of chemical differences between *O. biennis* genotypes (Johnson et al. 2009, Agrawal et al. 2012). In addition, we quantified caffeoyl tartaric acid at 315 nm as chlorogenic acid equivalents, since this compound has the same UV chromophore, i.e., the caffeoyl moiety as found in the commercially available chlorogenic acid. These phenolics make the majority of phenolics in *O. biennis* (Johnson et al. 2009, 2014, Parker et al. 2012) and many of them have been shown to be stimulated by herbivore damage in *O. biennis* (McArt et al. 2013).

Plant reproduction

To quantify the joint influence of climate warming and herbivory on *O. biennis* fitness, we measured five reproductive traits during the second growing season. At the end of the second growing season in October, we counted the number of fully developed fruits on each plant. Aborted fruits were rare; therefore we did not quantify the number of undeveloped fruits. We summed fruit counts for each plant within a subplot ($n = 32$, eight per treatment) and then averaged them at the subplot level to yield a single estimate of fruit production per plant in each subplot. We also calculated the number of fruits produced per meter of plant height to determine if plants exposed to warming or herbivory generated more dense fruiting bodies.

Next, we randomly harvested 10 fruits across all stems bearing fruit in each subplot and weighed them immediately to estimate average fresh fruit mass. We were careful to only choose fruits of similar age, as evidenced by slightly brown tips on the fruit wall of each fruit. We did not choose fruits that exhibited more than one-half of the husk brown or were entirely green, which necessarily limited the number of fruits available on each plant and within each subplot, because mature fruits split open and eject seeds once ripe. We then counted the number of seeds per fruit (for two fruits per subplot; McArt et al. 2013). Finally, we weighed the entire group of seeds from each of the 10 fruits to the nearest milligram and then divided by the total number of seeds to estimate individual seed mass. Fruit mass, seed count, and seed mass were averaged in each subplot to yield a single estimate of each metric per subplot.

Data analysis

We tested for treatment effects on plant size using a Bayesian split-plot ANOVA to determine whether plant final height differed among treatments. We used identical models to determine if abundances of *P. japonica* and percent leaf damage differed among treatments. For phenology, we calculated the week of peak flower production for each plot as the integer week with the maximum number of flowers (with the week of 14 July being week 1). We modeled week of peak flower production as a Poisson-distributed response variable with a log link function with a similar split-plot design.

We used a Bayesian split-plot MANOVA to test for differences in foliar and floral oenothien A, oxidized oenothien A, oenothien B, the ratio of oenothien A and oenothien B, and total ellagitannins (Total ETs) among treatments (foliar and floral chemistry data were analyzed in separate MANOVAs). We assessed treatment effects on reproduction using a Bayesian split-plot MANOVA that analyzed total fruit number, fruits per m plant height, fruit mass, the number of seeds per fruit, and seed mass. Finally, we calculated total lifetime seed numbers (total number of fruits \times fruit mass \times seeds per g fruit) and lifetime seed biomass (total seed numbers \times individual seed mass) as measures of cumulative lifetime fitness. Differences in lifetime seed numbers and lifetime seed biomass among treatments were characterized using a Bayesian split-plot MANOVA. All split-plot models included temperature as the whole-plot factor with herbivory and the herbivory \times warming interaction as sub-plot factors. All response variables were standardized to a mean of 0 and variance of 1 prior to analysis. Due to small sample size ($n = 8$ per treatment), we consider any effect with $\text{Pr} \geq 0.90$ as marginally significant (e.g., Johnson et al. 2009) and any effect with $\text{Pr} \geq 0.95$ as significant.

We conducted all analyses using Bayesian methods to avoid overestimating effect sizes. Bayesian parameter estimates converge to maximum likelihood estimates in

the absence of informative priors. Unfortunately, small sample sizes like the ones in our experiment can lead to overestimates of the true effect sizes when using traditional analytical methods and have been a persistent problem in biological research (Button et al. 2013, Lemoine et al. 2016). In contrast, Bayesian methods allowed us to place weakly informative priors with a mean of 0 and standard deviation of 2 [$N(0, 2)$] on all parameters, which shrinks parameter estimates toward 0 and helps prevent overestimation errors (i.e., Type M errors) during the analysis of small sample sizes (Lemoine et al. 2016). Given that our priors are symmetric around 0, they will not change the sign of an effect, simply restrict its magnitude to more believable values. Thus, our estimates of effect sizes are conservative but potentially more accurate.

In the multivariate analysis, the correlation matrix of the response variables was given an uninformative inverse Wishart prior with five degrees of freedom. All models were run for 25,000 burn-in iterations. Posterior distributions of each parameter were constructed using 25,000 additional samples from each chain. Chain convergence was assessed using traceplots and density plots of posterior samples. Furthermore, $\hat{R} = 1$ for all parameters, indicating model convergence. Traceplots did not indicate any autocorrelation of posterior estimates, so no thinning occurred. The posterior distribution for each parameter therefore contained 100,000 samples. We estimated treatment effects by adding posterior distributions calculating relevant contrasts for each treatment or effect (e.g., ambient: no-herbivore – ambient: herbivore).

All statistical analyses were conducted in Python v3.5 using the numpy, scipy, and pandas modules (Jones et al. 2001, McKinney 2010, van der Walt et al. 2011). Bayesian models were run using STAN v2.9 accessed via PyStan (Stan Development Team 2015). Results are reported as mean \pm SE. For fitness metrics, effects and effect sizes are reported from posterior distributions of contrasts, rather than of the raw data, as posterior effects correct for variation among plots. Descriptions of the Bayesian split-plot model, analytical code for reproductive traits, goodness of fit plots, and posterior contrasts can be found in Appendix S1.

RESULTS

Herbivore pressure

Herbivore exclusion nets successfully inhibited herbivory while warming treatments had little influence on herbivore pressure. In the no herbivores treatment, foliar damage averaged $1.13\% \pm 0.62\%$, compared to the herbivores treatment where foliar damage averaged $32.38\% \pm 1.72\%$. Based on direct observations of feeding and widespread leaf skeletonization of exposed plants, the majority of damage was caused by Japanese beetles, *P. japonica*. Adult beetles emerged in early June

and remained at high abundances throughout July (72 ± 23 beetles per plant, mean \pm SD). By early August, *P. japonica* numbers declined to <10 individuals per plant. *Popillia japonica* preferred flowers to leaves, with $63\% \pm 4\%$ occurring on flowers compared to leaves. The proportion of beetles occurring on flowers did not differ between temperature treatments (Pr(warmed > ambient) = 0.84). Warmed plots contained marginally fewer *P. japonica* over the course of the growing season (Pr(ambient > warmed) = 0.90), but this effect was relatively small as ambient plots contained, on average, only 14 ± 12 more adult beetles. Although there were slightly more beetles in ambient plots, there was little evidence that there was more foliar damage in ambient treatments (Pr(ambient > warmed) = 0.86) with plants at ambient temperatures showing only $2.5\% \pm 2.3\%$ more damage leaf damage than plants in warmed conditions. Although other herbivores were observed on *O. biennis* after removal of the nets (e.g., yellow wooly bear caterpillars *Spilosoma virginica*), they occurred infrequently and we observed no obvious change in foliar damage after net removal.

Flowering phenology

Early in the growing season a large rainfall event stimulated flower production (Fig. 1). However, because *O. biennis* mainly blooms in early to mid fall, we restricted our analyses to the second bloom event by analyzing data after 18 August. Warming had little effect on these peak flowering dates (Pr(warming effect) = 0.72, Fig. 1). Exposure to herbivores, however, delayed flowering by approximately 2 weeks (Pr(herbivores > no herbivores) = 0.90, Fig. 1) under both ambient and warmed environments (Pr(interaction) = 0.62).

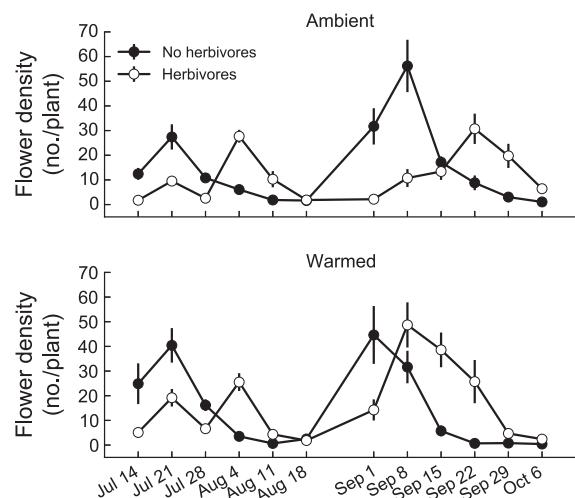


FIG. 1. Number of flowers per plant over the course of the growing season for *Oenothera biennis*. Points and error bars denote means \pm 1 SE ($n = 8$ per treatment).

Plant size

The effects of insect herbivores on plant height strongly depended on temperature ($\text{Pr}(\text{interaction}) = 0.99$, Fig. 2). At ambient temperatures, herbivores reduced plant height by $11\% \pm 3\%$ ($\text{Pr}(\text{no herbivores} > \text{herbivores} \mid \text{ambient}) = 1.00$), whereas herbivores did not affect plant height in warmed plots ($\text{Pr}(\text{herbivores} > \text{no herbivores} \mid \text{warmed}) = 0.60$). Warmed plants were $8\% \pm 5\%$ shorter than ambient plants grown without herbivores ($\text{Pr}(\text{warmed} < \text{no herbivores ambient}) = 0.95$).

Plant chemistry

Among the secondary metabolites examined here, only oenothien A and total ETs exhibited strong correlations ($r = -0.4$) between leaf and floral tissues. The negative correlation of total ETs likely arose because oenothien A comprised the bulk of ETs in *O. biennis* individuals in our study. Plants exposed to *P. japonica* had consistently lower expression of secondary chemicals in leaves relative to plants protected from herbivores (Fig. 3). Herbivory moderately or significantly reduced concentrations of oenothien A, oxidized oenothien A, the oenothien A/B ratio, and total ETs (Fig. 3), and this pattern was consistent among most measured chemical compounds. Neither herbivory nor warming affected concentrations of foliar oenothien B (Fig. 3c), although warming did moderately reduce concentrations of total ETs ($\text{Pr}(\text{ambient} > \text{warmed}) = 0.90$, Fig. 3e).

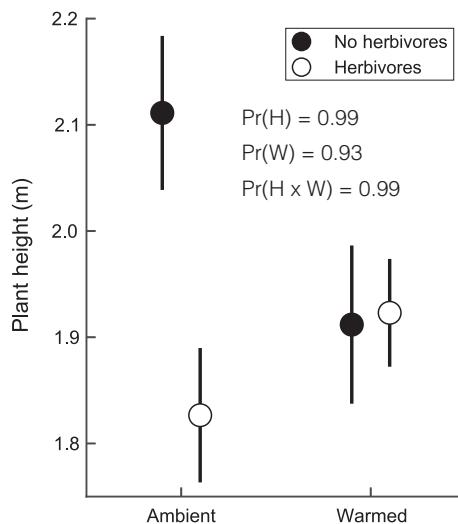


FIG. 2. Final height of *Oenothera biennis* under each warming-herbivore combination. Points and error bars denote means \pm SE. Probabilities denote probability that each effect was important, ignoring sign. That is, we report $\max(\text{Pr}(> 0), \text{Pr}(< 0))$ for each effect. Higher values indicate a higher likelihood that the given effect was important ($0.5 =$ effect just as likely to be positive or negative, $1.0 =$ effect was entirely positive or negative). $\text{P}(\text{H})$ refers to the probability of an herbivore effect, $\text{P}(\text{W})$ refers to the probability of a warming effect, and $\text{P}(\text{H} \times \text{W})$ refers to the probability of an interaction ($n = 8$ per treatment).

In general, the effects of warming and herbivores on floral chemistry were more variable than those of foliar chemistry (Fig. S3). Neither warming nor herbivory significantly altered oenothien A or total ET concentrations (Fig. 4). However, there was a significant interaction between warming and herbivory on oxidized oenothien A in flowers ($\text{Pr}(\text{interaction}) = 0.97$, Fig. 4b). At ambient temperatures, oxidized oenothien A was marginally lower in flowers of plants exposed to herbivores ($\text{Pr}(\text{no herbivores} > \text{herbivores} \mid \text{ambient}) = 0.92$). This pattern reversed under warming, wherein flowers exposed to herbivores produced slightly more oxidized oenothien A ($\text{Pr}(\text{herbivores} > \text{no herbivores} \mid \text{warmed}) = 0.87$; Fig. 4b). In addition, there was a marginally significant interaction of herbivory and warming on concentrations of oenothien B ($\text{Pr}(\text{interaction}) = 0.90$, Fig. 4c). Herbivores did not affect oenothien B at ambient temperatures ($\text{Pr}(\text{no herbivores} > \text{herbivores} \mid \text{ambient}) = 0.67$, Fig. 4c), whereas herbivores significantly suppressed oenothien B under warmed conditions ($\text{Pr}(\text{no herbivores} > \text{herbivores} \mid \text{warmed}) = 0.98$, Fig. 4c). Finally, warming reduced the oenothien A/B ratio ($\text{Pr}(\text{ambient} > \text{warmed}) = 0.960$), but herbivory had no effect on this measure of floral defense (Fig. 4d).

Plant reproduction

Neither warming nor herbivory significantly affected total fruit production (Fig. 5a). Fruits per meter plant height, however, increased by 195 ± 96 fruits in warmed plants ($\text{Pr}(\text{warmed} > \text{ambient}) = 0.90$, Fig. 5b), but did not depend on herbivory nor the interaction between warming and herbivory (Fig. 5b). The interaction between herbivory and warming was significant for fruit mass (Fig. 5c). Fruits on plants exposed to herbivory were 0.08 ± 0.03 g larger than those on plants protected from herbivores at ambient temperatures ($\text{Pr}(\text{herbivores} > \text{no herbivores} \mid \text{ambient}) = 1.00$). Under warmed conditions, fruit mass was no longer affected by the presence of herbivores ($\text{Pr}(\text{herbivores} > \text{no herbivores} \mid \text{warmed}) = 0.88$, Fig. 5c). Herbivory also moderately stimulated seed production, as plants suffering herbivory produced 16 ± 15 more seeds per fruit than did plant protected from herbivores, and warming did not alter this pattern (Fig. 5d). Warming did, however, affect the relationship between herbivores and individual seed mass (Fig. 5e). Seeds were smaller in the presence of herbivores at ambient temperatures ($\text{Pr}(\text{herbivores} < \text{no herbivores} \mid \text{ambient}) = 0.94$), whereas herbivory had no effect on individual seed mass under warmed conditions ($\text{Pr}(\text{herbivores} < \text{no herbivores}) = 0.53$).

Plant total lifetime fitness

Neither warming nor herbivory increased total seed numbers ($\text{Pr} \leq 0.89$ for both effects) (Fig. 6a). In contrast to seed numbers, warming and herbivory interactively increased lifetime seed biomass ($\text{Pr}(\text{Interaction}) = 0.93$,

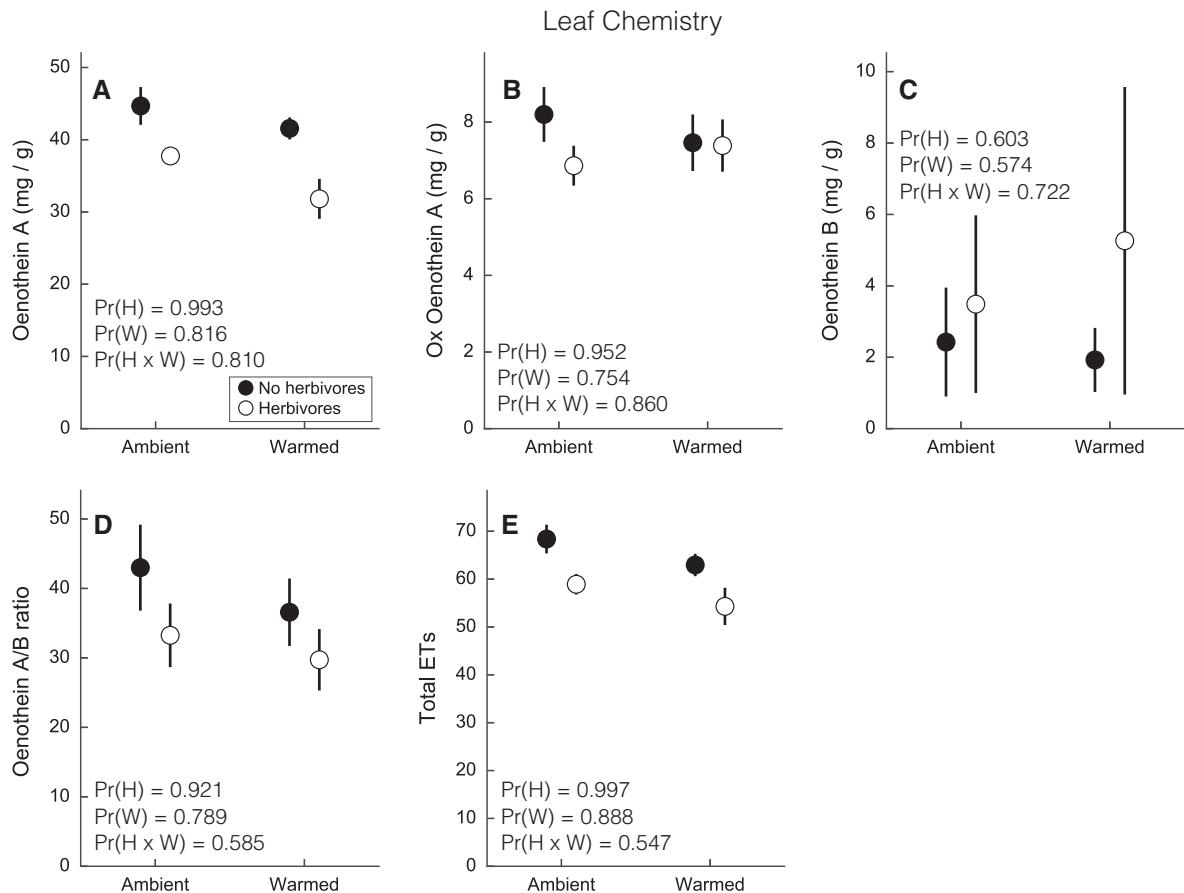


FIG. 3. Leaf chemistry for *Oenothera biennis* under each warming–herbivore combination: (A) oenothien A; (B) oxidized Oenothien A; (C) oenothien B; (D) the ratio of oenothien A/oenothien B; and (E) total ellagitannins (ETs). Points and error bars denote mean \pm SE. P(H) refers to the probability of an herbivore effect, P(W) refers to the probability of a temperature effect, and P(H \times W) refers to the probability of an interaction ($n = 8$ per treatment). Note the different scales on each y-axis.

Fig. 6b). At ambient temperatures, herbivores had no effect on total seed biomass (Pr(no herbivores > herbivores | ambient) = 0.83), whereas herbivory marginally increased lifetime seed biomass in warmed plots by $27\% \pm 23\%$ (Pr(herbivores > no herbivores | warmed) = 0.90, Fig. 6b).

DISCUSSION

Although the direct effects of warming and herbivory on plant fitness have been relatively well studied, little information exists regarding the combined effects of both warming and herbivores on plant lifetime fitness and no studies to date have considered multiple metrics of plant fitness that include phenology, chemical defenses, and lifetime seed production. Here, we demonstrate that herbivory affected the reproductive strategy of *O. biennis*, but did so differently depending on temperature. Under ambient temperatures, intense herbivory by *P. japonica* resulted in heavier fruits filled with numerous small seeds, offsetting the negative impacts of reduced fruit numbers on lifetime seed production and total seed biomass. When warmed, however, plants were

shorter but produced numerous, small fruits regardless of herbivore presence, resulting in more total seed biomass under warmed conditions. Ultimately, warming thus shifted the impact of herbivory on plant fitness from little overall impact at ambient temperatures to a surprising increase in fitness under warming.

Interestingly, leaf damage from *P. japonica* was similar in both ambient and warmed conditions, despite that fact that warming often increases insect damage to plants (Lemoine and Burkepille 2012, Lemoine et al. 2014a). However, *P. japonica* thermal response curves differ widely depending on host plant; warming can increase, reduce, or have no effect on consumption rates, depending on plant nutritional content and secondary metabolites (Lemoine et al. 2013, 2014a). Additionally, *P. japonica* is a highly mobile herbivore (Potter and Held 2002) and was able to move freely between ambient and warmed plots. Such behavioral thermoregulation potentially enables *P. japonica* to offset higher metabolic demands caused by high temperatures (Kearney et al. 2009). Despite similar levels of damage across temperature treatments, we still found differences in foliar

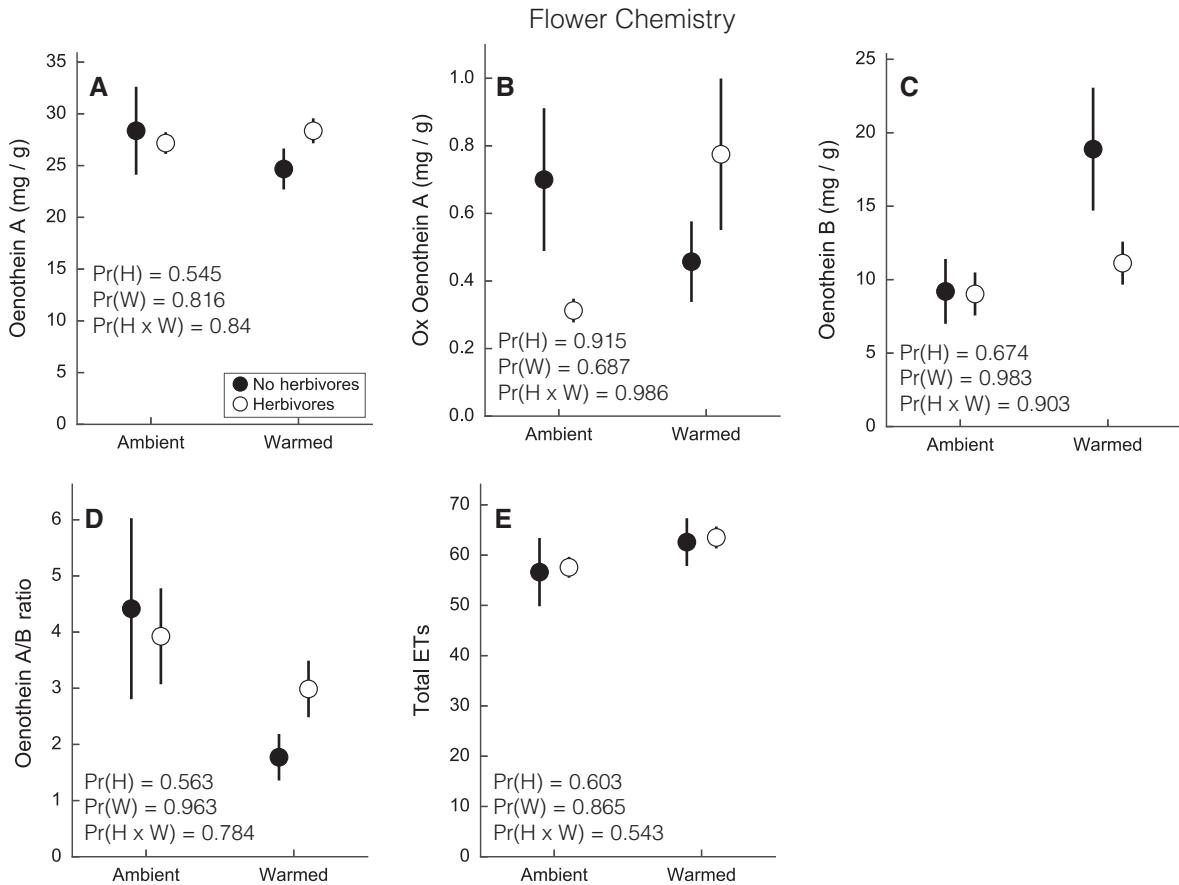


FIG. 4. Flower chemistry for *Oenothera biennis* under each warming-herbivore combination: (A) oenothien A; (B) oxidized oenothien A; (C) oenothien B; (D) the ratio of oenothien A/oenothien B; (E) total ETs. Points and error bars denote mean \pm SE. P (H) refers to the probability of an herbivore effect, P(W) refers to the probability of a temperature effect, and P(H \times W) refers to the probability of an interaction ($n = 8$ per treatment). Note the different scales on each y-axis.

chemistry and reproductive strategy between temperature treatments.

Temperature also influenced plant height in response to insect herbivory. At ambient temperatures, herbivory reduced *O. biennis* flowering stalk height by $\sim 11\%$. Shorter stature is a common consequence of insect herbivory (Poveda et al. 2003, Norgauer and Newbery 2013), caused by either reduced photosynthesis due to leaf tissue removal (Morrison and Reekie 1995), or by reallocation of resources away from growth toward other functions (Strauss et al. 2002, Züst et al. 2015). However, herbivore suppression of plant growth was absent at warmer temperatures. Climate warming often increases height or biomass of graminoids (Wan et al. 2005, Walker et al. 2006, Na et al. 2011), and forbs (Liu et al. 2012), although effects vary markedly among species (de Valpine and Harte 2001). Here, reduced plant height under warming despite the presence or absence of herbivores may have been caused by lower soil moisture content within warmed plots, a common consequence of warming experiments (Melillo et al. 2002). Indeed, warming decreased soil moisture by $\sim 10\%$ in our plots

(J. D. Parker, *unpublished data*), indicating that our warming treatments are having the expected effects on soil moisture as seen in other experiments (Harte and Shaw 1995), and thus many warming impacts on herbivory might be indirect and driven by changes to the abiotic conditions.

Reduced size was not, however, generally accompanied by increased investment in chemical defenses in the presence of herbivores. In fact, herbivory resulted in lower concentrations of nearly all measured foliar secondary metabolites regardless of temperature treatment. In contrast to foliar defenses, neither warming nor herbivory affected *O. biennis* production of most floral defenses. Concentration of floral oxidized oenothien A was lower in plants exposed to herbivory at ambient temperatures, whereas herbivory in warmed conditions stimulated production of floral oxidized oenothien A, and herbivory reduced the production of floral oenothien B only under warmed conditions. Increased floral oxidized oenothien A concentration in response to herbivory has previously been documented for *O. biennis* as an effort to protect flowers, fruits, and seeds from

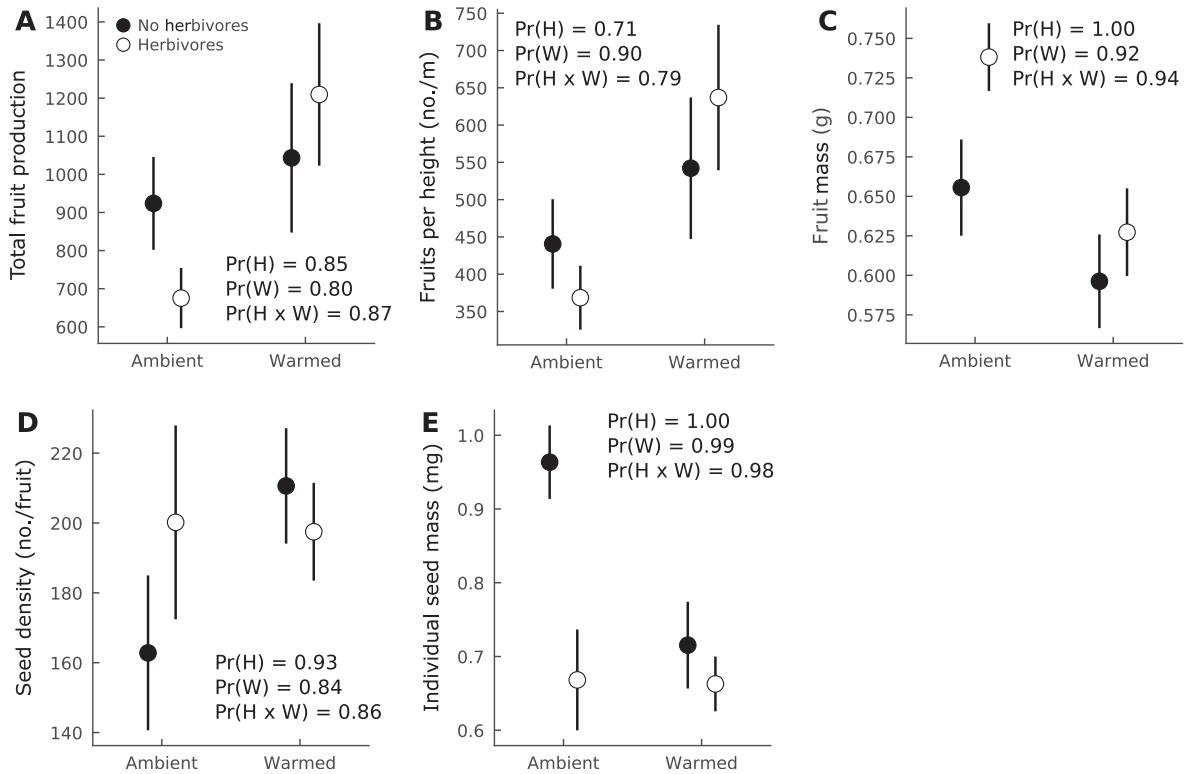


FIG. 5. Fitness metrics for *Oenothera biennis* under each warming–herbivore combination. (A) Total number of fruits observed over the course of the growing season. (B) Number of fruits produced per meter plant height over the course of the growing season. (C) Average fruit mass per plant. (D) Number of seeds per fruit. (E) Average mass of an individual seed contained within fruits. Points and error bars denote mean \pm SE. P(H) refers to the probability of an herbivore effect, P(W) refers to the probability of a warming effect, and P(H \times W) refers to the probability of an interaction between temperature and warming ($n = 8$ per treatment).

subsequent predation (McArt et al. 2013). We show that a similar mechanism operated in our study for oxidized oenotherin A and that induced responses to herbivory might be temperature dependent. However, herbivory

decreased floral oenotherin B under warmed conditions and also decreased almost all foliar defense compounds.

Our results stand in contrast to the increased production of *O. biennis* defensive compounds in response to

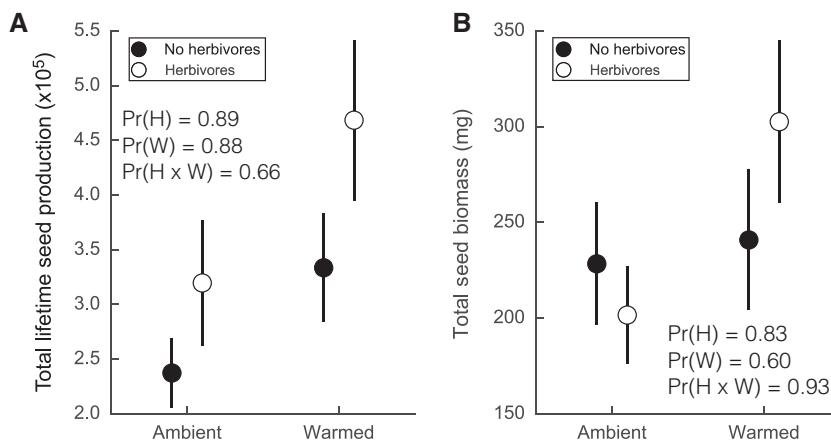


FIG. 6. Estimates of (A) total lifetime seed numbers and (B) total lifetime seed biomass per plant for *Oenothera biennis* under each of the four treatments. We calculated total seed numbers by multiplying seeds per g fruit by average fruit mass by the number of fruits per plant in each plot. Total seed biomass was calculated by multiplying total seed numbers by the average individual seed mass for each plot. Points and error bars denote mean \pm SE. P(H) refers to the probability of an herbivore effect, P(W) refers to the probability of a warming effect, and P(H \times W) refers to the probability of an interaction between temperature and warming ($n = 8$ per treatment).

herbivory reported by previous studies (Agrawal et al. 2012, McArt et al. 2013). We note, however, that comparing our experiment to previous work is difficult due to high herbivore damage rates and the absence of seed predators of our experiment. Although our results differ from Agrawal et al. (2012) and McArt et al. (2013), reduced investment in chemical defenses in response to herbivory (i.e., induced susceptibility) can be a common occurrence (Karban and Kittelson 1999, Marti et al. 2013). For example, plants that share no evolutionary history with particular insect species often fail to produce the requisite defensive compounds in response to herbivory (Desurmont et al. 2011), and *O. biennis* might have failed to produce extra defenses because *P. japonica* is an introduced species with only a ~100 yr shared history with *O. biennis*. Alternatively, herbivores might manipulate chemical signaling pathways such that plants do not recognize herbivores as such (Chung et al. 2013), and *P. japonica* can effectively manipulate defense pathways in other plant species (Hammons et al. 2009). Lastly, it is possible that plants exposed to herbivores produced fewer defenses over the course of our experiment because the relatively high levels of foliar loss ($32.38 \pm 1.72\%$) surpassed some threshold after which producing defenses would have been ineffective.

Ultimately, we expected that herbivory would change *O. biennis* reproductive strategies. In support of our hypothesis, herbivory drove very different patterns in *O. biennis* reproductive allocation at ambient vs. warmed temperatures. At ambient temperatures, *O. biennis* exposed to herbivores produced larger fruits with more, albeit smaller, seeds. Seed mass and fruit set often decline in response to herbivory (Ruohomäki et al. 1997, Maron 1998, Warner and Cushman 2002, Poveda et al. 2003). However, instead of exacerbating the effects of herbivores on reproductive allocation, warming appeared to negate many of the effects of herbivores on *O. biennis*. Warming had weak effects on fruit production but reduced both fruit and individual seed mass of *O. biennis*, similar to responses of arctic and alpine plants (Totland 1999, Kudernatsch et al. 2008, Liu et al. 2012), and did so regardless of herbivore treatment. The marginal increase in seed numbers in response to herbivory, coupled with no herbivore effect on fruit abundance, fruit mass, or seed mass, enabled *O. biennis* exposed to herbivores to produce more total seed biomass than their counterparts without herbivores.

Timing of peak flower production in *O. biennis* was delayed by up to three weeks by herbivory but was unaffected by warming. Climate warming generally advances flowering phenology because increased temperatures stimulate plant germination, growth, and development (Wolkovich et al. 2012). However, phenological responses to warming vary considerably among species (Sherry et al. 2007). Plants that bloom in the fall, like *O. biennis*, may delay or protract flowering periods as warming extends the length of the growing season (Sherry et al. 2007). Yet the fall flowering event of *O. biennis* did not

change under warmed conditions, potentially due to heavy rainfall during the preceding months. Although our results contradict our hypotheses that timing of flowering would advance under warmed conditions, warming does not affect phenology of many plant species (Hoffmann et al. 2010). Herbivory, on the other hand, often forces plants to shunt resources away from reproduction toward regrowth of lost tissue, which in turn delays flowering (Brody 1997, Freeman et al. 2003, Poveda et al. 2003). In our study, *O. biennis* delayed flowering well after the dominant herbivore had disappeared from the site, indicating that early-season herbivory can significantly impact late-season reproductive phenology. However, given that protection from herbivores did not increase fruit production, delayed phenology did not successfully defend flowers and fruits from consumption by *P. japonica*. However, climate warming might adversely affect other important floral interactions, like plant–pollinator interactions (Hoover et al. 2012). Unfortunately, we were unable to explore this potentially important dynamic given *O. biennis*' rare reproductive strategy.

Our study represents a novel investigation of the interactive effects of warming and herbivory on plant fitness. Both temperature and herbivory affect plant reproductive biology and population dynamics (Poveda et al. 2003, Roy et al. 2011, Walck et al. 2011), but most previous work on the temperature dependence of herbivory only considered how temperature affects consumption of plant tissue (O'Connor 2009, Lemoine et al. 2013, 2014a). No study, to our knowledge, has tracked the joint effects of warming and herbivory on plant fitness. Here, we demonstrate that warming alters plant strategies for mitigating foliar damage, whereby plants exposed to herbivores invested significantly more resources into seed production. As a result, increased seed production might translate into higher population growth rates or faster evolution (Agrawal et al. 2012). Plant–insect interactions might therefore shift appreciably under future climates, and ecologists must consider both temperature and herbivory when attempting to assess the ramifications of climate warming on plant populations.

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