INTRODUCTION

Climate is a main driver of plant distributions, and differences in climatic conditions over many different spatial scales might be important for plant performance (Woodward and Williams 1987). Sexual plant reproduction is particularly sensitive to climatic variations (Hedhly et al. 2009), and several stages of the reproductive process, from flower development to pollination, fertilization and seed and fruit maturation, might be affected (Bykova et al. 2012). Experimental evidence also shows that the production of plant reproductive structures might depend on temperature and water availability. For example, warming can increase seed biomass (Klady et al. 2011), and drought may reduce seed set due to resource limitation (del Cacho et al. 2013). Understanding how climatic variation over different spatial scales influences the reproductive performance of plants is thus important to understand differences in performance among and within natural plant populations. While the effects of climatic variability on plant performance are relatively well-studied at moderate and large spatial scales (e.g. García et al. 2000, De Frenne et al. 2009, 2010, Dainese 2011), they have not been explored at smaller spatial scales, although it is well-known that organisms do not sample environmental data at such coarse scales (Geiger et al. 2009).

The influence of climatic variability on plant reproductive performance might also be indirect, and mediated by changes in plant traits, such as reproductive phenology (Inouye 2008), or changes in interactions with other species (Bale et al. 2002, Memmott et al. 2007). Changes in interactions are likely to play an important role, as they have been shown to strongly influence plant fitness (Agrawal et al. 2012, Ågren et al. 2013), and animals interacting with plants might be sensitive to the environmental context of the plant (Chamberlain et al. 2014). Moreover, indirect effects of climate on plant performance might often involve multiple steps. For example, climatic variation might induce changes in plant phenology, which in turn influences the intensity of mutualistic or antagonistic interactions (Elzinga et al. 2007, Valdés and Ehrlén 2017) and plant reproductive performance. Climate might also affect the context of plant-animal interactions, for example through effects on vegetation height (Valdés and Ehrlén 2018), canopy cover (Kolb and Ehrlén 2010), community composition (Biere and Tack 2013, Wynhoff and van Langevelde 2017), and density and quality of conspecific or heterospecific plant neighbors (Hambäck et al. 2014, Valdés and Ehrlén 2019). For example, small-scale variation in soil temperature or water availability may condition plant density, and differences in plant densities might in turn lead to differences in intensity of herbivory and in plant reproductive output (Shea et al. 2000). Taken together, this means that identifying the effects of variation in microclimate on reproductive performance and the complex indirect effects of climate is important for understanding within-population variation in plant performance.

In this study, we investigated the effects of microclimate on reproductive performance of the plant *Gentiana pneumonanthe* in presence of an antagonist, the specialized seed-predator butterfly *Phengaris alcon*. The caterpillars of these butterflies live inside the fruits of *G. pneumonanthe* during their first instars, and parasitize the nests of ants (*Myrmica* spp.) during later stages. It has previously been shown that the effects of plant phenology and ant presence on oviposition patterns are interdependent: butterflies oviposit preferentially on early-flowering plants where host ants are abundant (Valdés and Ehrlén 2019). Also the density and phenology of neighboring host plants influences oviposition patterns, with higher oviposition probability in plants with few late-flowering neighbors (Valdés and Ehrlén 2019). Here, we examine how microclimate may affect plant performance of *G. pneumonanthe*,through effects on butterfly oviposition, plant phenology, plant density, and the distribution of ant hosts. We addressed the following questions: 1) What are the direct effects of microclimate on plant reproductive performance?, 2) What are the indirect effects of microclimate on plant reproductive performance, mediated by changes in plant phenologyand predation by *P. alcon*?, and 3) What are the indirect effects of microclimate on plant reproductive performance, mediated in two steps by effects via plant phenology or the plant context, on predation by *P. alcon*?

MATERIAL AND METHODS

**Study system**

The marsh gentian (*Gentiana pneumonanthe* L.) is a rare, long-lived perennial herb which occurs in open habitats as wet heathlands and grasslands (Simmonds 1946). Plants have one to many shoots measuring up to 45 cm. The species is self-compatible and flowers in July and August in the study area (SW Sweden), producing deep blue flowers that are pollinated by bumblebees. Fruits are capsules containing many small, wind-dispersed seeds (mean seed number per fruit ± SD = 490 ± 243, mean seed weight = 0.041 mg, authors’ unpublished data). *G. pneumonanthe* is the host plant of the Alcon Blue butterfly (*Phengaris alcon*), a specialist predispersal seed predator. Adult female butterflies oviposit on young flower buds in July and August (Appelqvist et al. 2007), and usually lay more than one egg on the same plant or even on the same bud. Females have not been shown to avoid plants that already carry eggs (Van Dyck and Regniers 2010). The caterpillars feed inside the fruit until their fourth instar, when they drop to the ground and are picked up by *Myrmica* ants. Due to caterpillars mimicking the surface chemistry of the ant brood (Nash et al. 2008) and the acoustic signals of queen ants (Sala et al. 2014), ants carry them to their nest, where they live as brood parasites for the rest of their larval period. *Phengaris* females detect host ants through plant chemicals produced by ants or by plants in response to root tissue damage caused by ants (Van Dyck et al. 2000, Patricelli et al. 2015, Wynhoff and van Langevelde 2017). *P. alcon* is a “cuckoo” species and does not prey on ant brood (Als et al. 2004), but instead caterpillars consume regurgitations from ant workers, trophic eggs (i.e. nutritious, infertile eggs which are fed to ant queens and larvae) and prey items brought to the nest by ants. *Myrmica* sp. ants are the only hosts of *P. alcon*, and *M. rubra* is considered to be the most common host ant species in Sweden (Elmes et al. 1994).

**Data collection**

Field work was carried out between 9 July and 3 September 2016 in a population of *G. pneumonanthe*, where the butterfly *P. alcon* was present, located in Tånga Hed nature reserve (Västra Götaland, SW Sweden, 58°01'40.0"N 12°49'47.0"E). The study population occupies a fenced meadow grazed by cattle late in the summer every year. A study plot of 69 m × 45 m was established in the meadow. This plot was subdivided into a grid of 345 3 m × 3 m subplots with corners marked by wooden poles (see Valdés and Ehrlén 2019, Fig. 1). In 154 of the subplots that were occupied by *G. pneumonanthe*, all reproductive shoots were mapped by recording their coordinates within subplots, and their phenology and the number of eggs of *P. alcon* were recorded between 29 July and 5 August (18 additional occupied subplots were not surveyed due to time constraints). Shoots were mapped instead of plant individuals (having one or several shoots) because we judged that individual shoots are the most relevant unit for the butterfly female to assess the food resource quality, and act as the unit of attraction for butterfly females. Phenology of each shoot was recorded as the developmental stage of the most advanced bud, according to six ordinal categories: (1) sepals covering the bud completely, (2) bud becoming visible, (3) bud growing over the sepals, (4) bud turning blue, (5) flower opening, and (6) flower wilting (Valdés and Ehrlén 2017, 2018, 2019). Higher values of this phenology measure indicate a more advanced floral development at the day of recording and thus an earlier flowering phenology, with a one-unit increase roughly corresponding to one week earlier development (Valdés and Ehrlén 2017, 2018, 2019). Eggs were counted on the whole shoot.

Up to five *G. pneumonanthe* shoots belonging to five different plants were permanently marked into each subplot to measure plant reproductive performance. Less shoots (1-4) were marked if less than five plants were available in the subplot. In the marked shoots, we recorded number of flowers between 29 July and 5 August, and counted the number of intact (i.e. not damaged by the butterfly) and preyed fruits between 30 August and 1 September. We also took a sample of one intact and preyed fruit (when available) per shoot. The number of developed seeds in these fruits was then counted in the lab. For each of the marked shoots, we calculated the number of seeds per flower as a measure of plant reproductive performance, as:

The ant community was sampled using sugar baits at each corner of all occupied subplots (a total of 254 points, (Valdés and Ehrlén 2019, Fig. 1) at two occasions, 14 July and 3 September. Baits consisted of a sugar cube placed into a 50-ml plastic tube which was left open on the ground in the evening, and collected the next morning. Ants collected were transferred to 70º ethanol and the number of ants of different *Myrmica* species were then counted in the lab (using Douwes et al. 2012 for identification).

Two microclimatic variables (soil temperature and soil moisture) were recorded at the same 254 points. At each point, an iButton data logger was buried in the soil at 5 cm depth in mid-July 2016. Data loggers recorded temperature every third hour from 14 July to 2 September. We used this information to calculate the average of daily mean temperatures over the recording period (hereafter, soil temperature). At the same points, we measured soil moisture (%) with a Theta Probe sensor connected to a HH2 moisture meter (Delta-T Devices, Cambridge, UK) on one occasion (30 July). We chose a date when the conditions were semi-dry (i.e. 5 days after the last rain), in order to get maximum resolution in moisture measurements. Although moisture values might vary a lot from one day to another, the relative differences should be similar, independently of the date. We took three moisture measurements at each point, and calculated the average of the three values (hereafter, soil moisture).

**Data preparation**

Field maps were digitized using a Geographic Information System (GIS) in ArcGIS 10.3.1, where we assigned relative coordinates to each recorded shoot of *G. pneumonanthe*. We defined a buffer area with a 3-m radius, centered at each shoot (see Valdés and Ehrlén 2019, Fig. 1), and calculated the number of reproductive shoots of *G. pneumonanthe* per m2 within this buffer area (hereafter, “neighbor density”) as well as the mean phenology of these shoots (hereafter, “neighbor phenology”), excluding in each case the focal shoot.

The locations of the 254 points where the ant community and microclimatic variables were sampled were also incorporated into the GIS. We used inverse distance weighted interpolation (Pebesma 2004) based on the values at the sampling points with a maximum distance of 3 m to generate values of *Myrmica rubra* abundance over the surface of all occupied subplots (see Valdés and Ehrlén 2019 for more details on the choice of ant species and maximum distance). From this interpolated surface (see Supporting Information Figure S1 in Valdés and Ehrlén 2019), we extracted values of *M. rubra* abundance for each of the mapped shoots (hereafter, “ant abundance”).

We used an ordinary kriging interpolation (autoKrige function of the R package automap, Hiemstra et al. 2009) based on the values of soil temperature and soil moisture at the sampling points to generate values of these two microclimatic variables over the surface of all occupied subplots. This method first produces a variogram to describe the spatial autocorrelation between every pair of points. Then, an interpolation model is fitted to this variogram to assign values (with standard errors) over the interpolated surface based on the distance between sampling points and the location to be predicted. From these interpolated surfaces, we extracted values of soil temperature and soil moisture for each of the mapped shoots.

**Statistical analyses**

First, in order to evaluate the overall relationship between reproductive performance of *G. pneumonanthe* (measured as the number of seeds per flower) and microclimate, we performed univariate linear regressions of number of seeds per flower against soil temperature and soil moisture.

Second, in order to decompose the overall relationship into different pathways, we applied piecewise structural equation modeling (piecewise SEM) to assess the relationships between microclimate, plant phenology and context (in terms of host ant presence, density and phenology of neighboring host plants), occurrence of seed predation by *P. alcon*, and the number of seeds per flower. We chose a piecewise approach, rather than the traditional variance-covariance based SEM, because it allows to fit multiple separate linear models with non-normal distributions. In our models, we considered both direct effects of microclimate on number of seeds per flower, indirect effects mediated by seed predation or plant phenology, and indirect effects mediated by effects of changes in plant phenology and plant context, on seed predation. Before performing the SEMs, we checked for a possible correlation between soil temperature and soil moisture. As the correlation was relatively high (Pearson's r = -0.40, p < 0.001), we performed a principal component analysis (PCA, Table A1) on these two variables. The first and second axes of this PCA explained, respectively, 68.5% and 31.5% of the total variance. The first axis (hereafter, “PC1”) was positively correlated with soil temperature and negatively correlated with soil moisture, while the second axis (hereafter, “PC2”) was positively correlated with both soil temperature and soil moisture. PC1 and PC2 were then used as explanatory variables in the SEM.

The piecewise SEM consisted of: 1) a linear model (LM) with plant phenology as the response variable and PC1 and PC2 as predictor variables; 2) a generalized linear model (GLM) with a negative binomial error distribution with ant abundance as the response variable and PC1 and PC2 as predictor variables; 3) a LM with neighbor density as the response variable and PC1 and PC2 as predictor variables; 4) a LM with neighbor phenology as the response variable and PC1 and PC2 as predictor variables; 5) a GLM with a binomial error distribution with a binomial variable “egg occurrence” (being 1 when the plant had at least one egg, and 0 otherwise) as the response variable and plant phenology, ant abundance, the interaction plant phenology  ant abundance, and the interaction neighbor density  neighbor phenology as predictor variables; and 6) a LM with the number of seeds per flower as the response variable and plant phenology, egg occurrence, PC1 and PC2 as predictor variables. We first constructed a global model containing all possible paths (Fig. A1). We then simplified this model by backward stepwise removing paths based on Akaike information criterion corrected for small sample sizes (AICc). For this, we constructed alternative models by removing one path at a time, and considered that alternative models improved the model fit to the data if the AICc was more than two units lower than the AICc of the original model (models with ΔAICc < 2 are considered to fit the data equally well, Burnham and Anderson 2002). All alternative models showed a ΔAICc < 2, and we therefore retained the global model. Overall fit of the model was assessed using Shipley’s test of direct separation (d-separation, Shipley 2009), which evaluates the probability that none of the paths missing from the hypothesized causal network contain useful information. This test yields the Fisher’s C statistic that is χ2-distributed. A χ2-value of Fisher’s C below the significance level (p < 0.05) indicates that the model is inconsistent with the data, and should be rejected. The test of direct separation suggested several missing paths between variables. We modeled these paths as correlated errors, as no causal relationships between them appeared meaningful (see Table A2). Finally, obtaining standardized coefficients for model 2) was not possible because of transformation via the link function of the GLM. Therefore, we repeated the piecewise SEM using a LM for model 2) in order to obtain standardized coefficients for this model (although their significance was assessed using the previously fitted GLM).

Statistical analyses were carried out in R ver. 3.6.2 (R Core Team 2020) using (apart from the above mentioned) the packages MASS (Venables and Ripley 2002) and piecewiseSEM (Lefcheck 2016).

RESULTS

There was considerable variation in microclimate among *G. pneumonanthe* plants: soil temperature ranged from 14.7 to 17.2 ºC with a mean of 16.2 ºC, and soil moisture ranged from 35.4 to 80.4 % with a mean of 66.1 %. Plant reproductive performance, measured as the number of seeds per flower, decreased with soil temperature (Fig. 1A,  = -66.41, p = 0.002, adjusted R2 = 0.02) and showed a tendency to increase with soil moisture (Fig. 1B,  = 1.53, p = 0.059, adjusted R2 = 0.01).

The piecewise SEM model had a reasonable fit to the data (Fisher’s C = 13,61, p = 0.093). Reproductive performance decreased with increasing soil temperature, especially in combination with increasing soil moisture (negative effects of PC1 and strong negative effects of PC2 on number of seeds per flower). However, there was no evidence of significant direct effects of microclimate on plant reproductive performance. Instead, microclimate affected reproductive performance indirectly through multiple pathways (Fig. 2, Table 1). Predation by *P. alcon* was more likely to occur in plants located at microsites with a high temperature (significant positive effects of PC1 and PC2 on egg occurrence), and this increased occurrence of predation in warmer microsites led to a reduced number of seeds per flower. There were no significant indirect effects of microclimate on reproductive performance mediated only by changes in plant phenology, as plant phenology did not have a significant direct effect on reproductive performance.

Indirect effects of microclimate on plant reproductive performance also acted through pathways involving multiple steps (Fig. 2, Table 1). Microclimate was related to plant phenology and to the plant context, and these factors influenced predation and reproductive performance. Plants flowered earlier in microsites with higher temperature in combination with lower moisture (high values of PC1), and later in microsites with higher temperature in combination with higher moisture (high values of PC2). Early-flowering plants, in turn, had a higher occurrence of predation than late-flowering plants and therefore produced less seeds per flower. The increase in egg occurrence with an earlier phenology was stronger in plants where host ants were abundant, as evidenced by the significant effect of the interaction plant phenology  ant abundance. Ant abundance, in turn, was higher in microsites with higher temperature in combination with lower moisture (high values of PC1). Moreover, pathways involving conspecific neighbors also mediated effects of microclimate on plant performance. Neighbor density was lower and neighbors flowered earlier in microsites with higher temperature in combination with lower moisture (high values of PC1), while the opposite was true in microsites with higher temperature in combination with higher moisture (high values of PC2). Plants surrounded by few late-flowering neighbors showed a higher egg occurrence, as evidenced by the significant effect of the interaction neighbor density  neighbor phenology, and therefore produced less seeds per flower.

DISCUSSION

Our results show that the effects of microclimate on reproductive performance of the plant *G. pneumonanthe* were mainly indirect. Microclimate, in terms of soil temperature and moisture, was related to seed predation by the butterfly *P. alcon*, which had a strong negative effect on plant performance. Microclimate also influenced plant phenology, host ant abundance and density and phenology of neighboring host plants, which in turn affected seed predation and plant reproductive output.

We found no direct effects of microclimate on reproductive performance of *G. pneumonanthe*. Direct effects of climatic variation might be more important at larger spatial scales than the microclimatic differences among plants within a population that we studied. Still, the role of direct effects in previous larger-scale studies reporting climatic influences on plant reproduction is difficult to assess because these studies do not separate direct and indirect effects (García et al. 2000, De Frenne et al. 2009, 2010, Dainese 2011). Experimental studies with other species have found effects of small-scale warming and drought on seed production (Klady et al. 2011, del Cacho et al. 2013). These studies also did not discriminate between direct and indirect effects on reproductive performance, but suggested that the observed responses to experimental treatments might partly be due to effects of warming on plant phenology (Klady et al. 2011), or due to effects of drought on plant size (del Cacho et al. 2013). Although the range of variation, at least in soil temperature, was larger in our study than in these previous studies, we did not detect direct effects of microclimate on plant performance.

In contrast to the lack of direct effects, we detected several indirect effects of microclimate on reproductive performance of *G. pneumonanthe*. The overall impact of these indirect effects was that the number of seeds per flower was lower in warm microsites, particularly when soil moisture was high. The incidence of predation by *P. alcon* was higher in warm microsites, irrespective of soil moisture. Microclimatic effects on the intensity of plant-animal interactions, such as seed predation, might be due to effects on larval growth and survival (Bonebrake et al. 2010). Our results agree with previous studies showing an increased incidence of butterfly oviposition in warmer microclimates, and that this effect is particularly pronounced near the margins of their geographical range (Eilers et al. 2013, Fourcade and Öckinger 2016). The increased incidence of eggs in *G. pneumonanthe* individuals in warmer microsites was associated with a reduced reproductive output. Our results therefore show that antagonistic interactions play an important role in mediating effects of microclimate on local-scale environmental variation on plant performance.

Microclimate was also related to incidence of predation and performance of *G. pneumonanthe* individuals through effects on plant phenology, host ant abundance, and neighboring conspecific plants. Plants flowered earlier in warm microsites if the soil moisture content was low, but later if moisture was high. The few studies that have documented variations in plant phenology at small scales in relation to soil temperature (Dahlgren et al. 2007, Toftegaard 2016), found that plants flowered earlier in warmer microsites. Earlier flowering has also been related to higher nutrient levels (Dahlgren et al. 2007), and one possibility is that the later flowering of *G. pneumonanthe* observed in warm and moist microsites is due to that moister microsites are also poorer in nutrients. In accordance with previous findings (Valdés and Ehrlén 2018), variation in plant phenology only affected reproductive performance indirectly, through its effects on the antagonistic interaction with *P. alcon*.

There was also a higher abundance of host ants (i.e. *Myrmica rubra*) in warm and dry microsites. *Myrmica* ants occupy different ecological niches with respect to vegetation and soil characteristics (Elmes et al. 1998). Specifically, *M. rubra* shows preferences for intermediate soil temperature and moisture (Elmes et al. 1998), and might thus avoid moist microsites. Lastly, the fact that neighbor density was higher in warm and moist microsites, might be the result of that a higher water availability in the soil can support higher plant densities.

As a consequence of the earlier phenology, higher abundance of host ants and lower neighbor density, the incidence of *P. alcon* attacks increased, and reproductive performance of *G. pneumonanthe* decreased, in warm and dry microsites. However, these effects of microclimate were partly counteracted by that individuals in warm and dry microsites also had neighbors with an earlier phenology, and this acted to decrease the incidence of attacks and increase plant reproductive performance. In a previous study with this species it was shown that incidence of *P. alcon* eggs increased with an earlier plant phenology, and the preference for early-flowering host plants was stronger where host ants were abundant (Valdés and Ehrlén 2019). Moreover, plants with neighbors that were less preferred by *P. alcon* for oviposition had a higher probability of receiving eggs. As a result of these interactive effects, plants that flowered earlier and where host ants were more abundant, and plants surrounded by fewer and later-flowering neighbors produced fewer seeds per flower because they had a high occurrence of predation. The fact that both plant phenology, host ant abundance and density and phenology of neighboring conspecific plants were related to microclimate, implies that microclimate indirectly affects plant reproduction not only by directly influencing the intensity of plant-animal interactions, but also by modifying plant phenology and the plant context, which in turn influence interactions. Although indirect effects of climate on plant reproduction through altered species interactions have been investigated before (e.g. Lemoine et al. 2017, Richman et al. 2020), the type of complex indirect effects of microclimate on within-population differences in plant reproductive performance found in this study have not previously been explored.

Our results illustrate the complexity of the relationship between local-scale environmental variation and plant performance, and highlight that species interactions can play an important role in mediating effects of climate. We simultaneously assessed the effects of microclimatic variation via multiple pathways, including direct effects of microclimate on plant performance, effects via plant-animal interactions, as well as effects of microclimate on plant traits and the environmental context that influence these interactions. This enabled us to provide a more comprehensive understanding of the many and complex ways in which among-individual variation in small-scale climatic conditions within populations can affect individual plant performance. Approaches considering multiple pathways, such as the one used in this study, provide a powerful way to identify the environmental causes of differences in performance of individuals over small spatial scales, which is necessary to understand the ecological and evolutionary dynamics of plant populations.

DATA ACCESSIBILITY STATEMENT

Should the manuscript be accepted, the data supporting the results will be archived in an appropriate public repository and the data DOI will be included at the end of the article.

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Table 1: Effects of microclimate (first and second axes of a PCA performed on soil temperature and soil moisture) on plant reproductive performance (number of seeds per flower) calculated from the piecewise SEM (Fig. 1). Total, direct and indirect effects are shown, and indirect effects are divided into different pathways.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Type of effect on number of seeds per flower | | | | Microclimatic variable | |
| PC1  (high temperature, low moisture) | PC2  (high temperature, high moisture) |
| Total effect | | | | -0.033 | -0.169 |
|  | Direct effect | | | -0.032 | -0.040 |
|  | Indirect effect | | | -0.001 | -0.129 |
|  |  |  | |  |  |
|  | | | Through changes in predation | -0.052 | -0.073 |
|  | | | Through changes in phenology | 0.025 | -0.026 |
|  |  |  | |  |  |
|  | | | Through effects of changes in plant phenology and ant abundance on predation | -0.050 | 0.055 |
|  | | | Through effects of changes in neighbour density and phenology on predation | 0.076 | -0.084 |

Figure 1: Relationships among the number of seeds per flower in *Gentiana pneumonanthe* and A) soil temperature and B) soil moisture. Fit lines and confidence intervals for linear models (n = 454 plants where seed data was available) are shown in each case.



Figure 2: Results of a piecewise structural equation model (piecewise SEM) examining the relationships among microclimate (first and second axes of a PCA performed on soil temperature and soil moisture), *G. pneumonanthe* phenology and context (host ant abundance, density and phenology of neighboring host plants), occurrence of seed predation by *P. alcon*, and *G. pneumonanthe* reproductive performance (number of seeds per flower). Values shown are standardized coefficients. Non-significant relationships and correlated errors have been omitted from the diagram for clarity (see Table A2 for the full model output).

