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TITLE: Butterfly seed predators mediate shifts in selection on flowering phenology in their host plant

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ABSTRACT

Variation in selection among populations and years has important implications for evolutionary trajectories of populations. Yet, the agents of selection causing this variation have rarely been identified. Selection on timing reproduction within a season in plants might differ both among populations and among years, and selection can be mediated by both mutualists and antagonists. We investigated if differences in the direction of phenotypic selection on flowering phenology among 20 populations of *Gentiana pneumonanthe* during two years were related to the presence of the butterfly seed predator *Phengaris alcon*, and if butterfly incidence was associated with the abundance of the butterfly’s second host, *Myrmica* ants. In plant populations without the butterfly, phenotypic selection favored earlier flowering. In populations where the butterfly was present, it preferentially attacked early-flowering individuals, shifting the direction of selection to favoring later flowering. Butterfly incidence in plant populations increased with ant abundance. Our results demonstrate that antagonistic interactions can shift the direction of selection on flowering phenology, and suggest that such shifts might be associated with differences in the community context.

Keywords: phenotypic selection, timing of reproduction, plant phenology, plant-animal interactions, predispersal seed predation, community context, *Gentiana pneumonanthe*, *Phengaris alcon*, *Myrmica*, spatial variation, myrmecophily

INTRODUCTION

Timing of reproduction within a season is a key trait influencing interactions both with the physical environment and with other organisms. For plants in temperate regions, selection on flowering phenology is mediated by abiotic conditions (Franks et al., 2007, Giménez-Benavides et al., 2011) and by species interactions (Elzinga et al., 2007, Sletvold et al., 2015). Mutualistic pollinators might select for both earlier (e.g. Munguía-Rosas et al., 2011a, Chapurlat et al., 2015), and later flowering (e.g. Sandring & Ågren, 2009). Likewise, antagonistic interactions with herbivores or pre-dispersal seed predators might favor both later (e.g. Parachnowitsch & Caruso, 2008, König et al., 2015) and earlier flowering (e.g. Fukano et al., 2013). Moreover, plants can simultaneously experience selection for earlier and later flowering mediated by different agents. For example, flowering early might be advantageous for plants at northern latitudes because it increases pollinator availability and the time available for seed development (Munguía-Rosas et al., 2011b), but at the same time costly if early flowering increases the exposure to antagonists. In these cases, net selection depends on the relative strengths of these interactions (e.g. Ehrlén and Münzbergová 2009, Sletvold et al. 2015).

Spatial variation in selection mediated by species interactions has been documented in many systems (Thompson 2005, Siepielski et al. 2013), and may result in local adaptation and adaptive population divergence. Variation in selection strength may potentially be driven by differences in interaction intensities or trait preferences of the interactors among populations. For plant-animal interactions, such as pre-dispersal seed predation, studies have demonstrated that differences in selection on plant traits among populations or years can be associated with differences in both interaction intensities (Benkman 2013, Benkman et al., 2013, Vanhoenacker et al., 2013), and trait preferences (Rey et al*.*, 2006; Kolb et al*.*, 2007). Interaction intensities and animal preferences for plant traits might, in turn, depend on the environmental context in terms of the abiotic conditions (e.g. light availability, Arvanitis et al., 2007; Kolb & Ehrlén, 2010, soil moisture, von Euler et al*.*, 2014). The community context, in terms of natural enemies, competitors or alternative hosts, has also been shown to influence the outcome of plant-animal interactions (Strauss & Irwin, 2004; Siepielski & Benkman, 2007; Chamberlain et al*.*, 2014). In spite of an increasing awareness of the ubiquitous spatial and temporal variation in selection, the biotic selective agents causing this variation as well as how the environmental context influences the impact of a given selective agent have been little explored.

Large Blue butterflies of the genus *Phengaris* Doherty, 1891 (the senior synonym of *Maculinea* van Eecke, 1915) are specialist predispersal seed predators during their first larval instars, and often show strong preferences for particular plant developmental stages (Thomas & Elmes, 2001; Van Dyck & Regniers, 2010). This means that plant individuals for which the developmental stage preferred for oviposition coincides with the peak of butterfly activity suffer most from seed predator attacks. *Phengaris* larvae need also a second host to complete their development, and most species are parasites of ant nests (*Myrmica* spp.) during later instars (Als et al*.*, 2004). Butterfly-mediated selection on plant traits in this system might thus be influenced by the community context, if the incidence and intensity of predation are related to host ant abundance. In this study, we examined if among-population variation in phenotypic selection on flowering phenology in the perennial herb *Gentiana pneumonanthe* is the result of differences in the intensity of interactions with its specialist predispersal seed predator, the butterfly *Phengaris alcon*, and if variation in the incidence of the butterfly in plant populations is associated with the community context in terms of the abundance of the second host, *Myrmica* ants. As developmental stages preferred for oviposition in early-flowering plants usually coincide with the oviposition period of the butterfly (A. Valdés and J. Ehrlén, *pers. obs.*), we hypothesized that: (1) The presence of the butterfly seed predator in plant populations shifts the direction of phenotypic selection on flowering phenology in *G. pneumonanthe*, from favoring early flowering to favoring late flowering, and (2) Community context, in terms of host ant abundance, is related to the probability of butterfly presence within plant populations. To test these hypotheses, we assessed selection gradients for flowering phenology, seed predation intensities and ant abundances in 11 *G. pneumonanthe* populations where the butterfly was present and 9 where it was absent.

METHODS

**Study system**

The marsh gentian (*Gentiana pneumonanthe* L.) is a rare, long-lived perennial herb (10-20 years, Oostermeijer et al. 1992), occurring in open habitats, such as wet heathlands and grasslands (Simmonds, 1946). In natural populations, individuals do not flower until their third year (Simmonds, 1946). Plants can have one to many, up to 45 cm high, shoots and produce deep blue flowers that are pollinated by bumblebees. The species is self-compatible and flowers in July and August in SW Sweden. Fruits are capsules containing a high number (usually 300-700, Appelqvist et al*.*, 2007) of minute (mean seed weight = 0.044 mg, Simmonds, 1946), wind-dispersed seeds. *Gentiana pneumonanthe* is the primary host of the Alcon Blue butterfly (*Phengaris alcon*), a specialist predispersal seed predator which oviposits on young buds in July and August (Appelqvist et al*.*, 2007). The caterpillars feed inside the capsule until they reach the fourth-instar, when then they drop to the ground to be picked up by *Myrmica* ants (Mouquet et al*.*, 2005). Caterpillars mimic the surface chemistry of the ant brood (Nash et al*.*, 2008) and the acoustic signals of queen ants (Sala et al. 2014), and this makes ants carry them to their nest, where they spend the rest of their larval period as parasites (Mouquet et al*.*, 2005). Contrary to the majority of *Phengaris* species, which prey on ant brood, *P. alcon* is a “cuckoo” species (Als et al*.*, 2004), and larvae feed primarily on regurgitations from ant workers, trophic eggs (i.e. nutritious, infertile eggs which are fed to the queens and larvae) and prey items brought to the nest by ants. Only ants belonging to the genus *Myrmica* can act as second hosts of *P. alcon* (Mouquet et al*.*, 2005). In our study area, *M. ruginodis* is thought to be the most commonly used host ant species (Appelqvist et al*.*, 2007).

**Data collection**

The study was carried out in 20 populations of *G. pneumonanthe* located in the county of Västra Götaland in SW Sweden (see Appendix S1 in Supporting Information for details). The populations are mainly located in moist heathlands. *P. alcon* was present in 11 of the study populations and absent from 9. Predation intensity varied considerably among the populations where *P. alcon* was present (Appendix S1).

We collected data on plant reproductive traits, interaction intensity and plant fitness in 100 marked individuals in each of the 20 study populations during 2010 and 2011. The number of shoots per individual ranged from 1 to 44 (mean = 3). For recordings, we selected one shoot of median length in each individual. In these focal shoots, we recorded shoot height (in cm), number of flowers, and developmental stage of each bud and flower. Buds and flowers were assigned to one of six developmental stages: (1) the 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 showing signs of wilting. Each of these stages corresponded to a time interval of flower development, and the average duration of stages 2-5 was about one week during this study (A. Valdés, *pers. obs.*). All plant traits were measured once per year, at the end of July-beginning of August. Phenology of plant individuals was estimated based on the reproductive development stage of flowers at the day of recording. We calculated two different phenology measures: (1) the mean development stage of all flowers and buds within the focal shoot (a continuous variable ranging from 1 to 6), and (2) the stage of the most advanced bud within the focal shoot (an ordinal variable ranging from 1 to 6). In both cases, higher values indicate a more advanced floral development at the day of recording, i.e. an earlier flowering. According to the observed average duration of the stages, a one-unit increase in these measures roughly corresponds to one week earlier development.

Interaction intensity was estimated by the maximum number of *P. alcon* eggs observed on the focal shoot during 2-6 visits to each population. Populations were visited once at the end of July-beginning of August (when data on reproductive traits was collected) and from 1 to 5 times from the end of August until all fruits had matured in mid-October (the number of visits depending on the time needed for fruit maturation).

Plant fitness was estimated by the maximum observed number of intact (i.e. not damaged by the butterfly) mature fruits on the focal shoot (assessed from counts of fruits on 1 to 5 visits to each population, see above). Although some seeds might remain in attacked fruits, the larvae consume a large proportion of seeds in the capsules (A. Valdés *pers. obs.*), and the total number of mature seeds in intact and attacked fruits is strongly correlated with the number of intact fruits (r = 0.85, N = 1136 individuals in 2010). In 2010, information on fruit production was collected in all 20 populations but in 2011 we were only able to get information from 16 of the populations.

Ant abundance was estimated once per study year along transects in each of the 20 study populations. In 2010, 20-m transects were established, and in 2011 we used a 40-m transect in each population. Sugar cubes were placed at one-meter intervals along the transects, and the number of *Myrmica* sp. ants at each of the sugar cubes was counted 30 minutes after presenting them to ants. Assessments were only carried out under dry (no precipitation) and warm conditions. To further reduce variation due to varying weather conditions during ant counts, we used the highest yearly value of the average number of ants per sugar cube in each population as a measure of ant abundance.

**Statistical analyses**

To assess phenotypic selection on flowering phenology, we performed selection gradient analyses in each of the two years by regressing relative fitness on standardized estimates of phenology, flower number and shoot height (Lande & Arnold, 1983). Fitness, in terms of the number of intact fruits, was relativized within populations by dividing individual values by population mean values. Traits were standardized by subtracting the population mean and dividing by the population standard deviation. To test for differences in phenotypic selection gradients among populations, we included the interaction between standardized reproductive traits and population in the models. The main effect of population was not included as fitness was relativized within populations prior to analysis. We fitted a linear model with Type II sums of squares. In addition to tests of directional (linear) selection, we also tested for non-linear (quadratic and correlational) selection. Quadratic selection is defined as the covariance between relative fitness and the squared deviations of trait values from the mean, while correlational selection is the covariance between relative fitness and the product of trait deviations (Lande & Arnold, 1983; Arnold, 1986). Non-linear selection was assessed by examining the effects of quadratic and interaction terms in a model including also the linear terms. Results for models using mean and most advanced flower developmental stages within shoots as estimates of phenology were very similar in all cases. Below, we present only results for the stage of the most advanced bud (results for selection gradient analyses using the mean flower developmental stage are shown in Appendix S2 in Supporting Information).

To test if linear selection on reproductive traits differed among populations with vs. without *P. alcon*, and to avoid basing contrasts on estimated selection coefficients, we created a variable “Predation”, coded as 0 in populations without *P. alcon* and as 1 in populations with *P. alcon*, and constructed a linear hierarchical mixed model (Type II sums of squares) for each year. Predictors included standardized reproductive traits and their interactions with population as random effects, and predation as a fixed effect. Effects of trait × population and trait × predation interactions were only included in these models if the trait × population interaction was significant. We did not include an individual intercept by population because fitness was relativized within populations prior to analysis.

In populations where the butterfly was present, we investigated predator preferences, by regressing the probability (0 or 1) and intensity (number of eggs in all individuals) of attack by *P. alcon* on reproductive traits, population and their interactions in each of the two years. We excluded population D (Appendix S1) in 2010 from these analyses because only one plant individual was attacked.

We also used path analyses to examine the relative importance of direct and indirect effects of reproductive traits on fitness (Grace, 2006), indirect effects being mediated by either the probability or the intensity of predator attack (i.e., two different saturated models were considered, see Appendix S3 in Supporting Information for details). To test for differences among populations, we used multigroup analysis (Grace, 2006). Because populations differed significantly, we fitted separate models for each population and year. We excluded population D in 2010 also from these analyses.

Finally, we examined if the abundance of the host ant of *P. alcon* was associated with an increased probability of predator presence or an increased interaction intensity. First, we performed a logistic regression of butterfly presence on log-transformed ant abundance, using the 20 study populations. Second, we regressed the mean number of eggs per plant and the proportion of plants with *P. alcon* eggs in each of the two years on log-transformed ant-abundance, using the 11 populations where the butterfly was present.

Path analyses were carried out in Amos 16.0 (Arbukle, 2007). All other analyses were conducted in R 3.1.2 (R Development Core Team, 2014).

RESULTS

Directional phenotypic selection on flowering phenology varied among populations, and was statistically significant in 5 populations in 2010, and in 3 populations in 2011 (Table 1A, Appendix S4 in Supporting Information). Among-population variation in selection on flowering phenology was strongly associated with the incidence of the predator in plant populations (Table 2, Appendix S4). In populations where the predator was absent, there was selection for earlier flowering (mean ± 95% CI of selection gradients, 2010: 0.22 ± 0.15, 2011: 0.30 ± 0.17), while in populations where the predator was present there was selection for later flowering (mean ± 95% CI of selection gradients, 2010: - 0.19 ± 0.15, 2011: - 0.10 ± 0.11, Fig. 1). The intensity of selection varied also among populations with the butterfly (Appendix S4). Differences in selection gradients for phenology among populations with the seed predator were not significantly related to predation intensity, in terms of the mean number of eggs per individual (2010: b = -0.01, p = 0.808; 2011: b = -0.03, p = 0.080).

We found evidence of directional selection also on flower number and shoot height in both study years, and again selection varied among populations (except for selection on shoot height in 2010, Table 1A). We found evidence of non-linear selection, in terms of quadratic effects of flower number and correlational effects of flower number × phenology, and these effects varied among populations (Table 1B).

In both study years, early development of floral structures increased the probability of being attacked by the predator within all populations where the predator was present (Table 3). The probability of being attacked was higher in plants with higher number of flowers in 2011, while the effect of flower number differed among populations in 2010. Shoot height did not influence probability of attack in 2010, and in 2011 the effect differed among populations. Also the number of eggs per plant was correlated with phenology and flower number within populations, but relationships varied among populations in both study years (Table 3, Appendix S5 in Supporting Information). Multigroup path-analyses revealed significant among-population differences in trait-fitness relationships (Appendix S6 in Supporting Information). Nevertheless, models fitted for each population and year consistently identified effects of phenology on fitness mediated by seed predator preference for early-flowering plants, effects being significant or marginally significant in 40% of the cases (considering models with probability and intensity of predator attack for two years; Appendices S7-9 in Supporting Information). Direct effects of traits on fitness were less consistent, effects being significant or marginally significant in 14% of the cases.

The probability of *P. alcon* presence increased significantly with increasing abundance of *Myrmica* ants in the population (Fig. 2). Although there were populations with high ant abundance where *P. alcon* was absent, ant abundance was rarely low in populations where it was present. In populations where the butterfly was present, neither the proportion of plants with *P. alcon* eggs (2010: b = -3.74, p = 0.571; 2011: b = -5.31, p = 0.246) nor the mean number of eggs per plant (2010: b = -0.30, p = 0.552; 2011: b = -0.61, p = 0.575) were significantly related to ant abundance.

DISCUSSION

In this study, we have shown that the butterfly pre-dispersal seed predator *P. alcon* shifts the direction of phenotypic selection on flowering phenology in its host plant *G. pneumonanthe*. In the absence of the seed predator, phenotypic selection favored earlier flowering. Because *P. alcon* preferentially attacked earlier-flowering individuals within populations, selection favored later flowering in populations where the predator was present. Moreover, the incidence of the predator in host plant populations was higher in populations with a high abundance of host ants, suggesting that community context in terms of the second host of the seed predator influences selection on flowering phenology in the host plant.

In populations where the seed predator was absent, phenotypic selection favored early flowering in *G. pneumonanthe*. In late-flowering plant species like *G. pneumonanthe*, earlier flowering might be beneficial at northern latitudes, where the growing season is short, because it increases the time and resources available for seed maturation. It is also possible that the availability of pollinators is higher or that the competition with other plants is less intense earlier during the season. The pattern of early-flowering plants having higher fitness in the absence of seed predators found in *G. pneumonanthe* is consistent with the general trend suggested by Munguía-Rosas et al*.* (2011b). It is important, however, to bear in mind that higher fitness in earlier-flowering plants could result from early flowering directly increasing fitness, or from other plant traits correlated with early flowering having a positive effect on fitness. Positive correlations between early flowering and high fitness may also be the result of environmental covariance, i.e., both early flowering and fitness are correlated with favorable microsite conditions and high resource availability (Rausher, 1992; Ehrlén, 2015). In our study, we tried to alleviate problems by incorporating traits that we considered likely to be correlated with flowering phenology, as well as traits likely to be correlated with plant resource state, as covariates in our models.

Irrespective of the selective agents responsible for the observed selection for earlier flowering in the absence of antagonists, our results clearly show that this selection is reversed to selection for later flowering when antagonists are present. In our study system, the direction of selection on phenology differed markedly between *Gentiana* populations with vs. without the butterfly pre-dispersal seed predator. This happened because *P. alcon* consistently preferred early-flowering plants for oviposition within populations, thus increasing the relative fitness of late-flowering plants. This means that the predator mediates shifts in selection from favoring early to favoring late flowering. The relationship between selection on flowering time and butterfly presence in this study was based on observational data, meaning that we did not prove causation in a strict sense. However, the facts that we know from direct observations that butterfly attack reduces fitness by larval feeding on seeds, and that butterflies preferentially attack early-flowering individuals, strongly suggest that the observed relationship is indeed a causal one. Previous studies with this system have examined butterfly preferences and shown that females of *Phengaris* sp. prefer to oviposit on buds that are not fully developed (corresponding to stages 1-3 in our classification), thereby increasing time available for brood feeding and development (Thomas & Elmes, 2001; Patricelli et al*.*, 2011). Our study instead focused on the response of the predator to the timing of bud development of individuals, and showed that given the butterfly preference for a given developmental stage, plants starting bud development early in the season are more prone to be attacked by the butterfly than plants flowering late. This is most likely because the presence of suitable floral developmental stages overlaps more with the oviposition period of *P. alcon* in early-flowering plants than in late-flowering. Our findings with *G. pneumonanthe* agree with studies in other systems demonstrating predator-mediated selection for late flowering (Pilson, 2000; Kolb et al*.*, 2007; Parachnowitsch & Caruso, 2008). However, selection for early flowering mediated by pre-dispersal seed predators has also been reported (Kolb et al*.*, 2007). Although most plant populations where the seed predator was present experienced selection for later flowering in our study, the intensity of this selection varied. The fact that these differences in intensity of selection were not related to predation intensity within populations suggests that also other, unidentified, selective agents (e.g. abiotic factors) contributed to net selection on flowering phenology. In conclusion, our results with *G. pneumonanthe* demonstrate that the presence of antagonists in plant populations can shift the direction of selection, and that the distribution of antagonists among plant populations is strongly related to the observed patterns of among-population variation in the direction of selection.

Butterflies did not only prefer early-flowering individuals but also oviposited more often on taller shoots, which are more conspicuous and represent a visually attractive target (Nowicki et al. 2005). Taller shoots possibly also constitute safer oviposition sites for the female butterflies, allowing them to escape from predators dwelling in the vegetation, such as lizards or spiders (Van Dyck & Regniers, 2010). Moreover, eggs laid on taller shoots might experience higher temperatures and a more suitable microclimate for larval growth (Alonso, 1997). Still, the preference for taller shoots was only observed in some populations and years, suggesting that the positive effect of higher shoots depends on environmental context, e.g. in terms of the height of the surrounding vegetation.

Given that our results link among-population differences in the direction of selection on flowering phenology to the incidence of the butterfly seed predator, the next step in understanding how environmental variation influences selection is to identify the factors influencing butterfly distribution and abundance. In our study system, *P. alcon* was more probable to be present in host plant populations with a high abundance of *Myrmica* ants. This finding agrees with what is known about the biology of the butterfly, which is unable to complete its life cycle without ants. In order to be able to reach their final size, pupate, and eclose as adults, caterpillars need to be fed in the ant nest (Mouquet et al. 2005). Although we did not demonstrate a causal relationship between ant abundance and butterfly presence, our correlative results together with the dependence of the butterfly on ant presence suggest that the observed among-population variation in selection on plant flowering phenology mediated by the butterfly seed predator is influenced by the abundance of its second host, and the environmental factors determining ant abundance. Several other studies showing that selection on plant traits by mutualists and antagonists is altered by interactions with other community members (Biere & Tack, 2013, Fedriani & Delibes, 2013, Arceo-Gómez & Ashman, 2014), also suggest that the community context has important effects on the outcome of plant-animal interactions.

In this study we examined how seed predators influenced selection on flowering time by estimating effects on one fitness component, fruit production. To assess the likelihood of that observed differences in the direction of selection on flowering time translate into population divergence, we need to know also how well our fitness component correlates with lifetime fitness, how consistent differences in selection (i.e., persistence of butterfly populations) are over time, and to what extent there is genetic variation in flowering time. While it is true that selective agents acting on other components of fitness might influence net selection on flowering time, our study should still provide a reliable picture of the part of selection on flowering time that is mediated by pre-dispersal seed-predators. Regarding genetic variation, we know through common garden experiments that there are significant, genetically based differences among populations in flowering phenology (A. Valdés and J. Ehrlén, unpublished data). However, we still lack data from a sufficient number of populations to confirm if these differences are related to the presence of the predator in the population of origin.

In recent years, the ubiquitous variation in selection among populations and years and its important implications for evolutionary trajectories of populations have been increasingly acknowledged (e.g. Thompson, 2005; Siepielski et al. 2013). Yet, the environmental factors causing this variation have been rarely identified (Siepielski et al., 2013). In this study, we have shown how an antagonistic interactor mediates selection on timing of reproduction in its host plant, and how the presence of this interaction is related to among-population variation in the direction of selection. We have also shown that the presence of the antagonist is associated with the abundance of a second host, suggesting that the community context might contribute to among-population variation in selection. These results illustrate that in order to link variation in the environment to variation in natural selection, we need to both assess the effects of species interactions on fitness of different phenotypes, and examine how the physical environment and the community context influence the incidence and abundance of the interacting species.

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TABLES

Table 1: Selection gradient analyses for three traits, flowering phenology, flower number, and shoot height, in *G. pneumonanthe* in 2010 (N = 2000 plants in 20 populations) and 2011 (N = 1598 plants in 16 populations). Results are from linear models with Type II sums of squares, including: A) only linear effects, B) linear, quadratic and interaction effects. All models included effects of population × trait interactions. Estimates from a model without interaction terms are given for significant main effects where the population × trait interaction is not significant. Fitness was estimated by the number of intact fruits. Traits were standardized and fitness relativized before analyses.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Source of variation | |  | 2010 |  |  |  | 2011 |
| df | F | Estimate |  | df | F |
| A) Linear terms | |  |  |  |  |  |  |
|  | Phenology (early flowering) | 1 | 0.02 |  |  | 1 | 0.50 |
|  | Flower number | 1 | 183.73\*\*\* |  |  | 1 | 50.81\*\*\* |
|  | Shoot height | 1 | 7.18 \*\* | 0.128 |  | 1 | 0.47 |
|  | Population × Phenology | 19 | 3.61\*\*\* |  |  | 15 | 1.98\* |
|  | Population × Flower number | 19 | 3.88\*\*\* |  |  | 15 | 2.90\*\*\* |
|  | Population × Shoot height | 19 | 1.19 |  |  | 15 | 2.35\*\* |
|  |  |  |  |  |  |  |  |
| B) Non-linear terms | |  |  |  |  |  |  |
|  | Phenology 2 | 1 | 3.51 |  |  | 1 | 0.32 |
|  | Flower number 2 | 1 | 0.95 |  |  | 1 | 0.02 |
|  | Shoot height 2 | 1 | 0.19 |  |  | 1 | 0.00 |
|  | Population × Phenology 2 | 19 | 0.64 |  |  | 15 | 1.31 |
|  | Population × Flower number 2 | 19 | 1.88\* |  |  | 15 | 2.82 \*\*\* |
|  | Population × Shoot height 2 | 19 | 1.00 |  |  | 15 | 0.73 |
|  | Phenology × Flower number | 1 | 2.09 |  |  | 1 | 0.06 |
|  | Phenology × Shoot height | 1 | 0.45 |  |  | 1 | 2.16 |
|  | Flower number × Shoot height | 1 | 1.33 |  |  | 1 | 0.84 |
|  | Population × Phenology × Flower number | 19 | 2.39 \*\*\* |  |  | 15 | 2.42\*\* |
|  | Population × Phenology × Shoot height | 19 | 0.63 |  |  | 15 | 0.76 |
|  | Population × Flower number × Shoot height | 19 | 1.06 |  |  | 15 | 2.14 \*\* |

\* p < 0.05; \*\* p < 0.01; \*\*\* p < 0.001

Table 2: Selection gradient analyses for three traits, flowering phenology, flower number, and shoot height, in *G. pneumonanthe* in 2010 (N = 2000 plants in 20 populations) and 2011 (N = 1598 plants in 16 populations). The presence of butterfly seed predators (Predation), coded as 0 in populations without the predator and as 1 in populations with the predator, was included in all models. Results are from linear hierarchical mixed models with Type II sums of squares (Wald 2 values are shown). Effects of population × trait (random effects, not shown) and predation × trait interactions were only included in the models if the population × trait interaction was significant. Fitness was estimated by the number of intact fruits. Traits were standardized and fitness relativized before analyses.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Source of variation | 2010 | |  | 2011 | |
| df | 2 |  | df | 2 |
| Phenology (early flowering) | 1 | 0.00 |  | 1 | 0.86 |
| Flower number | 1 | 53.85\*\*\* |  | 1 | 27.82\*\*\* |
| Shoot height | 1 | 7.82\*\* |  | 1 | 0.21 |
| Predation | 1 | 0.11 |  | 1 | 0.00 |
| Predation × Phenology | 1 | 14.72\*\*\* |  | 1 | 14.95\*\*\* |
| Predation × Flower number | 1 | 0.01 |  | 1 | 3.69 |
| Predation × Shoot height | 1 | - |  | 1 | 5.69\* |

\* p < 0.05; \*\* p < 0.01; \*\*\* p < 0.001

Table 3: Effects of population, flowering phenology, flower number and shoot height on the probability (0 or 1) and intensity (number of eggs in all individuals) of attack by *P. alcon* in 10 populations of the plant *G. pneumonanthe* in 2010 (N = 1000 plants) and 11 populations in 2011 (N = 1099 plants) where the predator was present. 2-values are shown for logistic regressions, and F values for linear regressions. Estimates from a model without interaction terms are given for significant main effects where the interaction with population is not significant

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Source of variation | Response: probability of attack | | | | | | |  | Response: intensity of attack | | | | |
| 2010 | | |  | 2011 | | |  | 2010 | |  | 2011 | |
|  | df | 2 | Estimate |  | df | 2 | Estimate |  | df | F |  | df | F |
| Phenology (early flowering) | 1 | 39.32\*\*\* | 0.420 |  | 1 | 39.00\*\*\* | 0.545 |  | 1 | 79.57\*\*\* |  | 1 | 46.87\*\*\* |
| Flower number | 1 | 27.69\*\*\* |  |  | 1 | 81.15\*\*\* | 0.794 |  | 1 | 75.43\*\*\* |  | 1 | 240.98\*\*\* |
| Shoot height | 1 | 1.93 |  |  | 1 | 0.04 |  |  | 1 | 0.01 |  | 1 | 0.03 |
| Population | 9 | 181.74\*\*\* |  |  | 10 | 138.77\*\*\* |  |  | 9 | 29.09\*\*\* |  | 10 | 46.00\*\*\* |
| Population × Phenology | 9 | 8.62 |  |  | 10 | 16.65 |  |  | 9 | 9.91\*\*\* |  | 10 | 5.55\*\*\* |
| Population × Flower number | 9 | 29.67\*\*\* |  |  | 10 | 14.11 |  |  | 9 | 8.77\*\*\* |  | 10 | 33.61\*\*\* |
| Population × Shoot height | 9 | 9.98 |  |  | 10 | 18.38\* |  |  | 9 | 0.52 |  | 10 | 0.72 |

\* p < 0.05; \*\* p < 0.01; \*\*\* p < 0.001

FIGURE LEGENDS

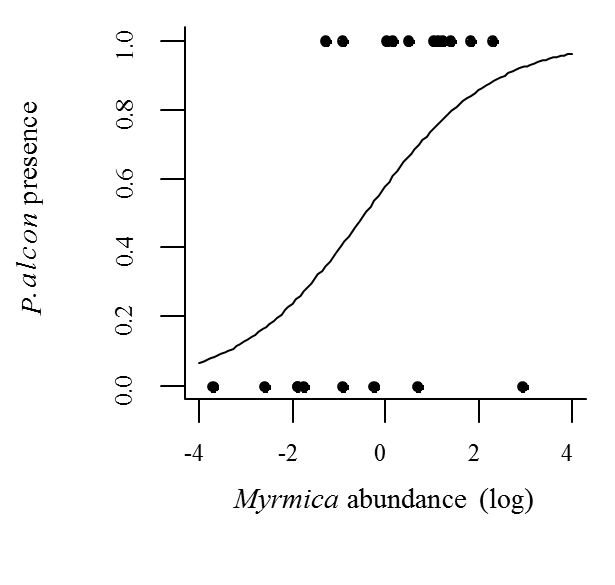
Figure 1: Differences in linear selection gradients for flowering phenology between populations where the predator was present (*P. alcon* =1, N = 11) and absent (*P. alcon* = 0, N = 9) in 2010 and 2011. Filed circles represent means, and bars standard errors (P < 0.001 in both years, one-way ANOVAs).

Figure 2: Relationship between abundance of *Myrmica* host ants (log mean number of observed individuals), and presence/absence of *P. alcon* in populations of *G. pneumonanthe* (fitted curve is a logistic regression, N = 20 populations, p = 0.045).

Figure 1



Figure 2



APPENDICES

Appendix S1: Description of the study populations. The colum “*Pa*” indicates if *P. alcon* is present (1) or absent (0). The columns “Mean n eggs” and “Prop. attacked” indicate, respectively, the mean number of eggs per plant and the proportion of plants attacked, in each population where the predator is present. X and Y coordinates are given for the centroid of the population in Swedish grid (RT 90) coordinate system.

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Pop. ID | County | Population name | Habitat type | *Pa* | Mean n eggs | |  | Prop. attacked | | X | Y | Area (Ha) |
| 2010 | 2011 |  | 2010 | 2011 |
| A | Ale | Bockemossen | Bog | 1 | 0.51 | 2.17 |  | 0.19 | 0.46 | 1277444 | 6420758 | 10.16 |
| B | Göteborg | Högsjön, Vättlefjäll, pop. 1 | Lake shore | 1 | 0.32 | 1.88 |  | 0.15 | 0.51 | 1284183 | 6418711 | 0.20 |
| C | Göteborg | Högsjön, Vättlefjäll, pop. 2 | Lake shore | 1 | 0.02 | 0.92 |  | 0.01 | 0.33 | 1283548 | 6418770 | 0.20 |
| D | Göteborg | Orremossen, Vättlefjäll | Bog | 1 | 0.27 | 0.32 |  | 0.09 | 0.12 | 1278600 | 6416407 | 10.30 |
| E | Herrljunga | Remmene skjutfält, pop. 1 | Bog | 1 | 0.94 | 2.16 |  | 0.35 | 0.44 | 1332333 | 6439185 | 0.90 |
| F | Herrljunga | Remmene skjutfält, pop. 2 | Bog | 1 | 2.68 | 3.32 |  | 0.46 | 0.47 | 1332388 | 6438949 | 0.95 |
| G | Herrljunga | Remmene skjutfält, pop. 3 | Bog | 1 | 0.87 | 1.72 |  | 0.27 | 0.31 | 1332460 | 6439135 | 0.49 |
| H | Lerum | Vite mosse | Bog | 1 | 3.22 | 5.4 |  | 0.61 | 0.58 | 1286925 | 6421701 | 2.71 |
| I | Partille | Maderna-Haketjärn | Lake shore, bog | 1 | 4.6 | 12.47 |  | 0.64 | 0.61 | 1282891 | 6404125 | 5.80 |
| J | Vårgårda | Lida | Pasture | 1 | 3.99 | 7.7 |  | 0.57 | 0.67 | 1322251 | 6423501 | 0.92 |
| K | Vårgårda | Tånga hed | Pasture | 1 | 1.18 | 3.24 |  | 0.34 | 0.45 | 1324202 | 6437602 | 5.35 |
| L | Ale | Stora Kroksjön | Lake shore | 0 | - | - |  | - | - | 1291750 | 6432023 | 0.68 |
| M | Borås | Näsudden i Frisjön | Lake shore | 0 | - | - |  | - | - | 1328934 | 6388474 | 0.07 |
| N | Mark | Härsnäs, Härsjön | Lake shore | 0 | - | - |  | - | - | 1300990 | 6387807 | 0.02 |
| O | Svenljunga | Hjortås | Lake shore, meadow | 0 | - | - |  | - | - | 1335103 | 6348447 | 0.13 |
| P | Svenljunga | Tovhult, Kalvsjön | Lake shore, meadow | 0 | - | - |  | - | - | 1333221 | 6344766 | 0.43 |
| Q | Svenljunga | Kalv Camping | Lake shore | 0 | - | - |  | - | - | 1334890 | 6349681 | 0.01 |
| R | Svenljunga | Olshult | Lake shore, meadow | 0 | - | - |  | - | - | 1342387 | 6395122 | 0.65 |
| S | Tranemo | Blåbo, Marjebosjön | Lake shore | 0 | - | - |  | - | - | 1361234 | 6371331 | 2.51 |
| T | Tranemo | Algutstorpasjön | Lake shore | 0 | - | - |  | - | - | 1357106 | 6379112 | 0.13 |

Appendix S2: Selection gradient analyses for three traits, flowering phenology, flower number, and shoot height, of the plant *G. pneumonanthe* in 2010 (N = 2000 plants in 20 populations) and 2011 (N = 1598 plants in 16 populations), using the mean flower developmental stage (rather than the stage of the most advanced flower) as the estimate of phenology. Results are from linear models with Type II sums of squares, including: A) only linear effects, B) linear, quadratic and interaction effects. All models included effects of population × trait interactions. Estimates from a model without interaction terms are given for significant main effects where the population × trait interaction is not significant. Fitness was estimated by the number of intact fruits. Traits were standardized and fitness relativized before analyses.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Source of variation | |  | 2010 |  |  |  | 2011 |
| df | F | Estim |  | df | F |
| A) Linear terms | |  |  |  |  |  |  |
|  | Phenology (early flowering) | 1 | 0.42 |  |  | 1 | 2.45 |
|  | Flower number | 1 | 207.08\*\*\* |  |  | 1 | 69.87\*\*\* |
|  | Shoot height | 1 | 6.25 \* | 0.122 |  | 1 | 0.41 |
|  | Population × Phenology | 19 | 3.10\*\*\* |  |  | 15 | 1.63\* |
|  | Population × Flower number | 19 | 4.78\*\*\* |  |  | 15 | 2.56\*\*\* |
|  | Population × Shoot height | 19 | 1.12 |  |  | 15 | 2.63\*\* |
|  |  |  |  |  |  |  |  |
| B) Non-linear terms | |  |  |  |  |  |  |
|  | Phenology 2 | 1 | 0.56 |  |  | 1 | 0.52 |
|  | Flower number 2 | 1 | 1.58 |  |  | 1 | 0.83 |
|  | Shoot height 2 | 1 | 0.57 |  |  | 1 | 0.01 |
|  | Pop. × Phenology 2 | 19 | 0.63 |  |  | 15 | 0.92 |
|  | Pop. × Flower number 2 | 19 | 1.81\* |  |  | 15 | 2.09 \*\*\* |
|  | Pop. × Shoot height 2 | 19 | 1.06 |  |  | 15 | 0.36 |
|  | Phenology × Flower number | 1 | 1.50 |  |  | 1 | 0.30 |
|  | Phenology × Shoot height | 1 | 1.54 |  |  | 1 | 1.18 |
|  | Flower number × Shoot height | 1 | 1.36 |  |  | 1 | 0.66 |
|  | Pop. × Phenology × Flower number | 19 | 2.38 \*\*\* |  |  | 15 | 2.41\*\* |
|  | Pop. × Phenology × Shoot height | 19 | 0.80 |  |  | 15 | 0.57 |
|  | Pop. × Flower number × Shoot height | 19 | 1.03 |  |  | 15 | 2.21 \*\* |

\* p < 0.05; \*\* p < 0.01; \*\*\* p < 0.001

Appendix S3: Appendix S5: Description of the path analyses performed to assess the causal effects of phenology and other reproductive traits on fitness

We used path analysis (Grace, 2006) to examine the direct and indirect (predator-mediated) causal effects of phenology and other reproductive traits on fitness in the subset of populations with the predator (excluding population D in 2010). We built two saturated models. The first (model\_attack) included direct effects of phenology, flower number and shoot height on number of intact fruits, effects of these traits on the probability of attack, and the effect of *P. alcon* attack on number of intact fruits. The second model (model\_eggs) had the same structure, but interaction intensity (number of eggs) was used instead of probability of attack. Correlations between the reproductive traits were included in both models. The adequacy of the hypotheses of our causal models to our data (given by a non-significant 2 value) was not a major issue, as our aim was to compare the relationships depicted in these models among populations (cf. Rey *et al.*, 2006). To test for among-population variation in selection on flowering phenology, we used multigroup analysis (Grace, 2006). This analysis evaluates if the parameters in the model differ between groups (i.e. populations in our case). We first evaluated the most restrictive hypothesis of equality of all path coefficients between populations, and then we imposed equality constraints on individual paths and examined the effect of these constraints on overall model fit (Appendix S6). Those constraints for which the imposition of the equality assumption causes a significant decrease in the chi-square value indicate path coefficients that are significantly different between groups. We then fitted the path models in each population separately (Appendices S7-8) because the multigroup analysis showed that they differed statistically. As data departed from multivariate normality (results not shown), we calculated bootstrap parameter estimates and test statistics for patch coefficients, based on 5000 bootstrap samples. As we were specifically interested in spatial variation in selective scenarios for flowering phenology, path coefficients for direct and indirect effects of phenology on fitness are shown in Appendix S9.

Appendix S4: Linear selection gradients (’s from multiple regressions of relative fitness on standardized reproductive traits) for each population in 2010 and 2011.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Population ID | |  | 2010 | | |  | 2011 | | |
| Phenology | Flower  number | Shoot  height |  | Phenology | Flower  number | Shoot  height |
| With *P. alcon* | |  |  |  |  |  |  |  |  |
|  | A |  | -0.173 | 0.747\*\*\* | -0.101 |  | -0.129 | 0.766\*\*\* | -0.346\*\* |
|  | B |  | -0.580\* | 0.950\*\*\* | 0.186 |  | 0.047 | 0.411\* | 0.015 |
|  | C |  | 0.057 | 0.872\*\*\* | -0.068 |  | -0.003 | 0.263 | 0.305 |
|  | D |  | 0.004 | 0.354\* | 0.222 |  | -0.001 | 0.474 | -0.078 |
|  | E |  | -0.205 | 0.656\*\* | 0.125 |  | -0.271 | 1.076\*\*\* | -0.157 |
|  | F |  | -0.235 | 0.541\* | -0.040 |  | -0.287 | 0.525 | 0.001 |
|  | G |  | -0.355\*\* | 0.436\*\* | -0.011 |  | 0.215 | 0.222 | -0.467\* |
|  | H |  | -0.192 | 0.479\* | 0.119 |  | -0.154 | 0.668\*\*\* | -0.095 |
|  | I |  | -0.469\*\* | -0.095 | 0.250 |  | -0.473\* | 0.000 | -0.418 |
|  | J |  | 0.131 | 0.163 | 0.360\* |  | 0.073 | 0.137 | 0.114 |
|  | K |  | -0.034 | 0.651\*\*\* | -0.005 |  | -0.077 | 1.380\*\*\* | -0.374 |
| Without *P. alcon* | |  |  |  |  |  |  |  |  |
|  | L |  | 0.115 | 0.096 | 0.357\* |  | 0.368\*\* | 0.247\* | 0.156 |
|  | M |  | 0.197\* | -0.005 | 0.258\*\* |  | 0.234\*\* | 0.247\*\* | 0.121 |
|  | N |  | 0.520\*\*\* | 0.830\*\*\* | 0.203 |  | - | - | - |
|  | O |  | 0.059 | 0.573\*\*\* | 0.041 |  | - | - | - |
|  | P |  | 0.197 | 0.482\*\* | -0.278 |  | - | - | - |
|  | Q |  | 0.079 | 0.558\*\*\* | 0.058 |  | - | - | - |
|  | R |  | 0.581 | 0.344 | 0.120 |  | 0.440 | -0.019 | -0.244 |
|  | S |  | 0.155 | 0.952\*\*\* | 0.104 |  | 0.315 | 0.053 | 0.723\* |
|  | T |  | 0.038 | 0.827\*\*\* | -0.150\* |  | 0.133 | 0.482\*\*\* | 0.200\* |

\* p < 0.05; \*\* p < 0.01; \*\*\* p < 0.001

Appendix S5: Among-population variation on the effect of *G. pneumonanthe* traits on itensity of predation by *P. alcon*. Partial linear regression plots showing among-population variation on the effect of *G. pneumonanthe* flowering phenology on the number of eggs of *P. alcon* in 2010 and 2011. Populations are represented by different colours (N = 10 in 2010 and N = 11 in 2011).



Appendix S6: Results of the multigroup analyses for the model with probability of attack (model\_attack, A) and with number of eggs (model\_eggs, B) in both years. 2 and P for the models with different paths constrained to be equal are shown. A significant 2 (P<0.05) indicates that the path coefficient differs significantly among populations.

| Paths constrained to be equal | 2010 | |  | 2011 | |
| --- | --- | --- | --- | --- | --- |
| 2 | P |  | 2 | P |
| A) model\_attack |  |  |  |  |  |
| All | 348.82 | <0.001 |  | 335.92 | <0.001 |
| Attack → Number of intact fruits | 18.10 | 0.034 |  | 62.39 | <0.001 |
| Phenology → Number of intact fruits | 10.93 | 0.280 |  | 5.23 | 0.875 |
| Phenology → Attack | 14.10 | 0.119 |  | 20.45 | 0.025 |
| Shoot height → Attack | 7.94 | 0.540 |  | 23.90 | 0.008 |
| Shoot height → Number of intact fruits | 8.39 | 0.495 |  | 20.88 | 0.022 |
| Flower number → Attack | 43.83 | <0.001 |  | 20.01 | 0.029 |
| Flower number → Number of intact fruits | 110.35 | <0.001 |  | 86.85 | <0.001 |
| Phenology ↔ Flower number | 36.60 | <0.001 |  | 35.85 | <0.001 |
| Phenology ↔ Shoot height | 16.24 | 0.062 |  | 33.44 | <0.001 |
| Shoot height ↔ Flower number | 34.58 | <0.001 |  | 31.05 | 0.001 |
|  |  |  |  |  |  |
| B) model\_eggs |  |  |  |  |  |
| All | 480.19 | <0.001 |  | 558.46 | <0.001 |
| Number of eggs → Number of intact fruits | 51.54 | <0.001 |  | 105.35 | <0.001 |
| Phenology → Number of intact fruits | 10.03 | 0.348 |  | 8.38 | 0.592 |
| Phenology → Number of eggs | 53.40 | <0.001 |  | 28.68 | 0.001 |
| Shoot height → Number of eggs | 9.56 | 0.388 |  | 9.37 | 0.497 |
| Shoot height → Number of intact fruits | 9.66 | 0.378 |  | 17.37 | 0.067 |
| Flower number → Number of eggs | 85.05 | <0.001 |  | 134.70 | <0.001 |
| Flower number → Number of intact fruits | 110.19 | <0.001 |  | 80.19 | <0.001 |
| Phenology ↔ Flower number | 36.60 | <0.001 |  | 35.85 | <0.001 |
| Phenology ↔ Shoot height | 16.24 | 0.062 |  | 33.44 | <0.001 |
| Shoot height ↔ Flower number | 34.58 | <0.001 |  | 31.05 | 0.001 |

Appendix S7: Path models for model\_attack fitted for each population and year. Solid lines indicate significant (P<0.05, black lines) or marginally significant (P<0.1, grey lines) paths, based on 5000 bootstrap samples. Dashed lines indicate non-significant paths. Standardized coefficients are shown.

|  |  |  |
| --- | --- | --- |
| Pop. | 2010 | 2011 |
| A |  |  |
| B |  |  |
| C |  |  |
| D |  |  |
| E |  |  |
| F |  |  |
| G |  |  |
| H |  |  |
| I |  |  |
| J |  |  |
| K |  |  |

Appendix S8:: Path models for model\_eggs fitted for each population and year. Solid lines indicate significant (P<0.05, black lines) or marginally significant (P<0.1, grey lines) paths, based on 5000 bootstrap samples. Dashed lines indicate non-significant paths. Standardized coefficients are shown.

|  |  |  |
| --- | --- | --- |
| Pop. | 2010 | 2011 |
| A |  |  |
| B |  |  |
| C |  |  |
| D |  |  |
| E |  |  |
| F |  |  |
| G |  |  |
| H |  |  |
| I |  |  |
| J |  |  |
| K |  |  |

Appendix S9: Standardized path coefficients for direct and indirect effects of phenology on fitness, obtained from fitting the saturated models (model\_attack and model\_eggs) to each population. Phen🡪Fruits: effect of phenology on number of intact fruits, Phen🡪Attack: effect of phenology on probability of attack, Attack🡪Fruits: effect of probability of attack on number of intact fruits, Phen🡪Eggs: effect of phenology on number of eggs, Eggs🡪Fruits: effect of number of eggs on number of intact fruits.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Pop. | model\_attack | | | | | | | |  | model\_eggs | | | | | | | |
| Phen🡪Fruits | |  | Phen🡪Attack | |  | Attack🡪Fruits | |  | Phen🡪Fruits | |  | Phen🡪Eggs | |  | Eggs🡪Fruits | |
| 2010 | 2011 |  | 2010 | 2011 |  | 2010 | 2011 |  | 2010 | 2011 |  | 2010 | 2011 |  | 2010 | 2011 |
| A | -0.13 | -0.06 |  | 0.08 | 0.39\*\*\* |  | -0.17 | -0.20 • |  | -0.12 | -0.05 |  | 0.15 | 0.31\*\* |  | -0.21\* | -0.30\*\* |
| B | -0.24\* | 0.12 |  | 0.02 | 0.16 |  | -0.29\*\* | -0.52\*\*\* |  | -0.23\* | 0.10 |  | 0.06 | 0.16 |  | -0.27\*\* | -0.40\*\*\* |
| C | 0.07 | 0.01 |  | 0.25\* | 0.12 |  | -0.10 | -0.08 |  | 0.08 | 0.01 |  | 0.28\* | 0.17 |  | -0.10 | -0.10 |
| D | - | -0.06 |  | - | 0.22 • |  | - | 0.26\* |  | - | -0.02 |  | - | 0.17 |  | - | 0.13 |
| E | -0.15 | -0.10 |  | 0.30\* | 0.18 • |  | -0.01 | -0.16 |  | -0.13 | -0.09 |  | 0.10 | 0.18 • |  | -0.15 • | -0.22\* |
| F | -0.11 | -0.09 |  | 0.21\* | 0.19 |  | -0.21 • | -0.26\* |  | -0.05 | -0.10 |  | 0.23\* | 0.17 • |  | -0.49\*\*\* | -0.23 • |
| G | -0.33\*\* | 0.14 |  | 0.08 | 0.00 |  | -0.02 | -0.25\* |  | -0.31\*\* | 0.15 |  | 0.12 | 0.07 |  | -0.14 | -0.22 • |
| H | -0.09 | 0.01 |  | 0.15 | 0.24\* |  | -0.22\* | -0.55\*\*\* |  | -0.08 | 0.10 |  | 0.23\* | 0.38\*\*\* |  | -0.20 • | -0.58\*\*\* |
| I | -0.25\* | 0.06 |  | 0.34\*\*\* | 0.49\*\*\* |  | -0.18 • | -0.62\*\*\* |  | -0.26\* | -0.17 |  | 0.46\*\*\* | 0.25\*\*\* |  | -0.12 | -0.30\* |
| J | 0.07 | 0.05 |  | 0.29\*\* | 0.23\* |  | 0.13 | 0.02 |  | 0.03 | 0.07 |  | 0.40\*\*\* | 0.13 • |  | 0.19 • | -0.15 |
| K | 0.02 | 0.06 |  | 0.39\*\* | 0.27\* |  | -0.18\*\* | -0.37\*\*\* |  | 0.02 | 0.06 |  | 0.26\* | 0.14 |  | -0.28\*\*\* | -0.73\*\*\* |

• p<0.1;\* p < 0.05; \*\* p < 0.01; \*\*\* p < 0.001