

**Resource overlap and dilution effects shape host plant use
in a myrmecophilous butterfly**

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Complete List of Authors:	Valdés, Alicia; Stockholm University, Department of Ecology, Environment and Plant Sciences; Stockholm University, Bolin Centre of Climate Research Ehrlén, Johan; Stockholm University, Ecology, Environment and Plant Sciences
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1 TITLE: Resource overlap and dilution effects shape host plant use in a myrmecophilous
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3 Valdés, Alicia^{*, a, b} and Ehrlén, Johan^{a, b}

4 ^a Department of Ecology, Environment and Plant Sciences, Stockholm University, SE-
5 106 91 Stockholm, Sweden

6 ^b Bolin Centre of Climate Research, Stockholm University, Stockholm, Sweden

7 *Corresponding author: aliciavaldes1501@gmail.com

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14

15 ABSTRACT

16 The effects of consumers on fitness of resource organisms are a complex function of the
17 spatio-temporal distribution of the resources, consumer functional responses and trait
18 preferences, and availability of other resources. Using the butterfly *Phengaris alcon* and
19 its two sequential hosts, the plant *Gentiana pneumonanthe* and *Myrmica* ants, we
20 investigated how butterfly egg-laying depended on focal host plant quality, density and
21 quality of neighboring host plants and host ant abundance. Butterflies preferred plants
22 that simultaneously maximized the availability of both larval resources, i.e., they chose
23 higher-quality early-flowering plants where host ants were abundant. Both the
24 probability of oviposition and the number of eggs were lower in plant individuals with a
25 high neighbor density than in more isolated plants, and this dilution effect was stronger
26 when neighbors were of high quality (i.e. flowered early). Our results show that plant-
27 herbivore interactions simultaneously depend on the spatio-temporal distribution of a
28 focal resource, and on the small-scale spatial variation in the abundance of other
29 herbivore resources. Given that consumers have negative effects on fitness and prefer
30 certain traits of the resource organisms, this implies that processes acting at the levels of
31 individuals, populations and communities simultaneously contribute to variation in
32 consumer-mediated natural selection.

33 Keywords: butterflies, flowering phenology, myrmecophily, plant-herbivore
34 interactions, resource use, spatial variation

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37 INTRODUCTION

38 Resource-consumer interactions often have important effects on the performance of
39 both partners and the distribution and abundance of consumers is expected to match the
40 spatio-temporal distribution of their resources. Resource use by insect herbivores is
41 often conditioned by oviposition site selection by females. As larval stages usually show
42 low mobility, insect oviposition preferences are predicted to match the spatial and
43 temporal distribution of host plants suitable for offspring development [1–3]. The
44 suitability of host plants, in turn, might depend on factors such as host plant nutritional
45 quality [4], size [5], microclimatic conditions [6] or enemy-free space [7]. Most
46 consumers depend on multiple resources throughout their life cycle, e.g. different
47 sources of food, shelter and resting sites [8,9], and the availability of one resource might
48 thus influence the use of other resources. For example, the availability of nectar
49 resources for adults [10] or secondary hosts for larvae [11] might determine host plant
50 choices in butterflies.

51 The quantitative relationship between the abundance of consumers and the
52 abundance of resource organisms, and thus resource exploitation, will depend both on
53 the birth and death processes and on the consumer behavior. Consumer behavior may
54 specifically cause consumer density and resource exploitation to increase (cf. resource
55 concentration hypothesis, e.g. [12–14]) or decrease (cf. resource dilution, e.g. [15]) with
56 increasing patch size and resource abundance. Observed differences in the relationships
57 between resource exploitation and patch size or patch density among different
58 consumers have been suggested to be the result of differences in how emigration and
59 immigration rates depend on patch size and patch density, and on the scale of consumer

60 selection [13,16]. For example, a strong negative relationship between consumer density
61 and patch area observed in visual searchers like butterflies might result from the fact
62 that immigration is diameter-dependent while emigration is perimeter-dependent. Not
63 only should the abundance of the resource organism but also its quality be important for
64 consumer responses. For example, in herbivores that depend on a specific
65 developmental stage of their host plant, the density of plants in suitable developmental
66 stages rather than the overall density is expected to influence host use. While many
67 studies have explored the effects of resource density on plant-insect interactions [17–
68 19], these studies have rarely considered also the role of resource quality and the effects
69 of the availability of other resources.

70 Myrmecophilous butterflies of the genus *Phengaris* need two sequential hosts to
71 complete their development: the caterpillars are predispersal seed predators during their
72 first instars and parasitize ant nests (*Myrmica* spp.) during later stages. Butterflies are
73 very specific regarding both host plant and host ant species and the developmental stage
74 of host plants [20–22]. Female butterflies oviposit preferentially on buds in early
75 phenological stages, probably because these constitute higher-quality food; the
76 developing seeds might become tougher, drier and more difficult to digest as buds
77 develop into flowers and fruits [23]. In some species, the presence of the preferred bud
78 developmental stages has been shown to overlap more with the period of butterfly
79 oviposition in early- than in late-flowering plants, and individuals flowering early in the
80 season receive more eggs [24]. Moreover, the mobility of caterpillars after leaving their
81 host plants is low [25], and further development is possible only if the foraging range of
82 suitable host ants is sufficiently close to the host plant individual (usually a few meters).

83 Butterflies are therefore expected to oviposit on host plants in suitable development
84 stages only if plants occur at micro-sites where ant hosts are present [26,27].

85 In this study, we investigated how the quality of the focal host plant *Gentiana*
86 *pneumonanthe* (in terms of the developmental stage of the reproductive structures), the
87 density and quality of neighboring conspecific plants, and the abundance of host ants
88 influence egg-laying by the butterfly *Phengaris alcon*. We used an extensive field data
89 set to test two hypotheses: (1) Effects of plant quality and ant abundance on egg
90 distribution patterns are interdependent: butterflies choose plants for oviposition that
91 simultaneously maximize the availability of both resources, i.e. they have a stronger
92 preference for high-quality early-flowering host plants where host ants are abundant,
93 and (2) The probability of a plant receiving at least one egg, as well as the number of
94 eggs in oviposited plants, decreases with increasing conspecific neighbor density (i. e.
95 dilution effect), and this decrease is stronger when neighbors are of high quality.

96 MATERIALS AND METHODS

97 **Study system**

98 The marsh gentian (*Gentiana pneumonanthe* L.) is a rare, long-lived perennial herb,
99 occurring in open habitats, such as wet heathlands and grasslands [41]. Plants can have
100 one to many, up to 45 cm high, shoots and produce deep blue flowers that are pollinated
101 by bumblebees. The species is self-compatible and flowers in July and August in the
102 study area (SW Sweden). Fruits are capsules containing a high number of minute, wind-
103 dispersed seeds (mean seed number per fruit \pm SD = 490 ± 243 , mean seed weight =
104 0.041 mg, authors' unpublished data). *Gentiana pneumonanthe* is the host plant of the

105 Alcon Blue butterfly (*Phengaris alcon*), a specialist predispersal seed predator which
106 oviposits on young flower buds in July and August [42]. During an oviposition event,
107 adult female butterflies usually lay more than one egg on the same plant and even on the
108 same bud, and there is no evidence of avoiding plants that already carry eggs [35]. The
109 caterpillars feed inside the capsule until they reach the fourth instar, when they drop to
110 the ground to be picked up by *Myrmica* ants. Caterpillars mimic the surface chemistry
111 of the ant brood [43] and the acoustic signals of queen ants [44], and these features
112 cause ants to carry them to their nest, where they spend the rest of their larval period as
113 brood parasites. Females of *Phengaris* detect host ant presence through plant chemicals
114 produced by ants or by plants in response to root tissue damage caused by ants
115 [27,33,36]. Contrary to the majority of *Phengaris* species, which prey on ant brood, *P.*
116 *alcon* is a “cuckoo” species [45], and caterpillars feed primarily on regurgitants from ant
117 workers, trophic eggs (i.e. nutritious, infertile eggs which are fed to the queens and ant
118 larvae) and prey items brought to the nest by ants. Only ants belonging to *Myrmica* sp.
119 can act as hosts of *P. alcon*. In Sweden, *M. rubra* is considered to be the most common
120 host ant species [46].

121 **Data collection**

122 The study was carried out in a population of *G. pneumonanthe*, where the
123 butterfly *P. alcon* was present, located in Tånga Hed nature reserve in the county of
124 Västra Götaland, SW Sweden (58°01'40.0"N 12°49'47.0"E). Field work was conducted
125 between 9 July and 3 September 2016. The plant population occupies a fenced meadow
126 that is grazed by cattle at the end of the summer every year. A study plot measuring 69
127 m × 45 m was established in the meadow. This plot was further divided into a grid

128 comprising 345 3 m × 3 m subplots with corners permanently marked by wooden poles
129 (figure 1A). In 154 of the subplots where *G. pneumonanthe* was present, all
130 reproductive shoots of *G. pneumonanthe* were mapped (figure 1B), and their phenology
131 and the number of eggs of *P. alcon* were recorded between 29 July and 5 August (18
132 additional occupied subplots were not possible to fully survey due to time constraints).
133 Shoots were mapped by recording their coordinates within subplots. We mapped shoots
134 rather than plant individuals (that may have one or several shoots) because we judged
135 that individual shoots are the relevant unit for the butterfly to assess the food resource
136 quality and because individual shoots act as the unit of attraction for butterfly females.
137 For each shoot, phenology was recorded as the developmental stage of the most
138 advanced bud, according to six ordinal categories: (1) the sepals covering the bud
139 completely, (2) bud becoming visible, (3) bud growing over the sepals, (4) bud turning
140 blue, (5) flower opening, and (6) flower showing signs of wilting [22]. Each stage
141 corresponds to a time interval of flower development, with higher values indicating a
142 more advanced floral development at the day of recording, i.e. an earlier flowering
143 phenology. The number of eggs was counted on the whole shoot.

144 The ant community was sampled at each corner of the occupied subplots (a total
145 of 254 points) at two occasions, 14 July and 3 September (figure 1B). Baits consisted of
146 a 50-ml plastic tube in which we placed a sugar cube. The tubes were left open on the
147 ground in the evening, and collected the next morning. Ants collected were transferred
148 to 70° ethanol and the number of ants of different *Myrmica* species were counted in the
149 lab (using [47] for identification). Ants belonging to other genera were not identified
150 to species, but counted as “other ants”.

151 **Calculation of neighborhood measures**

152 Field maps were digitized using a Geographic Information System (GIS) created
153 with ArcGIS 10.3.1, assigning relative coordinates to each recorded reproductive shoot
154 of *G. pneumonanthe* (figure 1B). For each shoot, we defined a buffer zone with a 3-m
155 radius, centered at the focal shoot (see example in figure 1B), and calculated the number
156 of reproductive shoots of *G. pneumonanthe* per m² within this buffer zone (hereafter,
157 “neighbor density”) as well as the mean phenology of these shoots (hereafter, “neighbor
158 phenology”), excluding in each case the focal shoot.

159 **Interpolations of ant abundances**

160 We also incorporated the locations of the 254 points where the ant community was
161 sampled into the GIS. We used inverse distance weighted interpolation [48] based on
162 the values at the sampling points to generate values of the abundance of ant species over
163 the surface of all occupied subplots. A maximum distance of 3 m was used because it
164 represents the ground foraging distance of most investigated *Myrmica* species (Elmes *et*
165 *al.* 1998). From these interpolated surfaces (see electronic supplementary material
166 figures S1-5), we extracted values of abundance of the different ant species for each of
167 the mapped shoots.

168 **Statistical analyses**

169 The effects of shoot phenology, host ant abundance, neighbor density and neighbor
170 phenology on *P. alcon* egg distribution were examined using two Generalized Linear
171 Models (GLMs). Effects on the probability of a shoot having at least one egg were
172 evaluated using a binomial GLM, and effects on the number of eggs in shoots with at

173 least one egg were evaluated using a negative binomial GLM. Both models included
174 also the effects of three interactions. First, we included the interaction between shoot
175 phenology and ant abundance in order to test if the effects of the two resources on egg
176 distribution patterns are interdependent. Second, we included the interaction between
177 neighbor density and neighbor phenology in order to test if the effect of host plant
178 density depends on phenology of neighboring host plants. Third, we included the
179 interaction between shoot phenology and neighbor phenology to account for the fact
180 that the relationship between egg distribution and phenology of the focal plant might
181 depend on the phenology of neighboring host plants. As a measure of host ant
182 abundance, we used the summed abundances of *M. rubra* and *M. schencki* (hereafter,
183 “ant abundance”). *M. rubra* is considered to be the most common host ant species of *P.*
184 *alcon* in Sweden [46], and *M. schencki* has been cited as host in several places in
185 Europe [49]. Our data agreed with this previous knowledge, as the combined abundance
186 of *M. rubra* and *M. schencki* had the strongest positive effect on the probability of host
187 plants receiving eggs (electronic supplementary material table S1).

188 All predictors were standardized by subtracting the mean and dividing by the
189 standard deviation before the analyses. We checked for spatial autocorrelation in the
190 residuals of models by plotting spatial correlograms and by calculating global Moran's I
191 with a permutation test (1000 random permutations). Moran's I was calculated based on
192 a connectivity matrix of pairwise Euclidean distances among the shoots up to a distance
193 of 30 m. Residual spatial autocorrelation could lead to biased model estimates and
194 invalid statistical inference [50]. A significant spatial autocorrelation was found in the
195 residuals of the binomial GLM, but not for the negative binomial GLM (global Moran's
196 $I = 0.0150$, $p < 0.001$ and global Moran's $I = 0.0023$, $p = 0.613$, respectively, electronic

197 supplementary material figures S6 and S7). We therefore applied Moran's eigenvector
198 mapping (MEM), which translates the spatial arrangement of data points into
199 explanatory variables (eigenvectors) that capture spatial effects, to the binomial model
200 examining effects on the probability of a shoot having at least one egg [51,52]. The
201 model was refitted including two eigenvectors issued from MEM as spatial predictors,
202 and this strongly reduced the residual spatial autocorrelation (global Moran's $I = 0.0003$,
203 $p = 0.03297$, electronic supplementary material figure S6). Although residual spatial
204 autocorrelation was still significant, the low value of Moran's I and visual inspection of
205 the spatial correlogram (electronic supplementary material figure S6) indicated that
206 almost all of the residual spatial autocorrelation was removed by the spatial predictors.
207 The results of this model were similar to the results of the binomial GLM not including
208 the spatial predictors (electronic supplementary material table S2). Statistical analyses
209 were carried out in R 3.4.0 [53].

210 RESULTS

211 Eggs of *P. alcon* were found on 731 (8.3%) of 8848 reproductive shoots of *G.*
212 *pneumonanthe* recorded in 154 subplots. On shoots with at least one egg, the mean
213 number of eggs was 5.03 ± 0.20 SE.

214 The effects of shoot phenology and ant abundance on *P. alcon* oviposition were
215 interdependent. The probability of a shoot of *G. pneumonanthe* having at least one egg
216 increased with an earlier phenology (i.e. with a more advanced floral development at the
217 day of recording, table 1, figure 2A). This increase was stronger in shoots where host
218 ants were more abundant (significant effect of the interaction shoot phenology \times ant
219 abundance, table 1, figure 2A). The number of *P. alcon* eggs on individuals with at least

one egg increased with an earlier shoot phenology and with a higher host ant abundance, but there was no significant effect of the interaction between phenology and ant abundance (table 1, figure 2B and C).

High densities of host plant shoots resulted in a dilution effect, and the strength of this effect depended on the quality of neighboring plants. Both the probability of having at least one egg and the number of eggs on shoots with at least one egg decreased with the overall density of neighboring host plant shoots (table 1, figure 3). In both cases, the decrease was stronger when neighboring host plants were of higher quality, i.e. had an earlier phenology (significant effect of the interaction neighbor density \times neighbor phenology, table 1, figure 3).

DISCUSSION

The results of this study show that resource use in the myrmecophilous butterfly *P. alcon* is shaped by both the spatial overlap of the two key larval resources and by a dilution effect. Specifically, we found that butterflies prefer to oviposit on early-flowering plants that have a high abundance of host ants, that plants surrounded by many conspecific neighbors receive fewer eggs, and that the dilution effect is stronger when neighbors are of a high quality.

We found that both the probability of *P. alcon* butterflies laying at least one egg on a host plant as well as the number of eggs increased with an earlier phenology of its host plant *G. pneumonanthe*. This agrees with previous studies showing that insects usually prefer plants with a particular phenology [28], and more specifically, that antagonists preferentially attack early-flowering plants [22,24,29,30]. Interestingly, in

our study the strength of this preference for an early plant phenology did depend on the abundance of the antagonist's second resource, i.e. the two host ant species *Myrmica rubra* and *M. schencki*. The preference of *P. alcon* for early-flowering host plants was stronger at locations where the host ants were more abundant. For example, the probability of a shoot with the earliest possible phenology receiving at least one egg was 0.07 at sites where host ants were absent, and 0.40 at sites with the maximum ant abundance observed ($n = 34$ ants). This indicates that the butterflies are able to simultaneously track the distribution of the two resources, and to preferentially oviposit on plants where both the availability of high-quality food for the caterpillars and the probability of adoption by the right species of *Myrmica* are higher. When suitable host ants were absent or very scarce, host plant phenology did not seem to affect egg-laying, and the probability that early-flowering plants should receive eggs was very low. Spatial overlap between the distribution of host plants and host ant nests at larger scales has previously been shown to increase the density and population persistence of the congeneric species *P. arion* [31,32]. Other studies with *Phengaris* butterflies have demonstrated effects of host plant phenology [21,22,24], host ant presence [26,27,33] or both of these factors [34] for oviposition site selection. Our study shows that the spatial overlap between these two resources also at the scale of the host plant individual is associated with butterfly oviposition preference, and that the effects of plant phenology and ant abundance are interactive. A general implication of our findings is that differences in availability of a resource over very small spatial scales can influence the use of other resource by consumers that depend on multiple resources.

Our results show both that there is a dilution effect of local host plant density on butterfly resource use, and that the strength of this effect depends on the quality of

266 neighboring plants. Higher densities of neighboring host plants were associated with
267 both a decreasing probability of a focal plant receiving at least one egg and a lower
268 number of eggs in plants with eggs. Based on our results we are not able to say whether
269 the lower number of eggs was the result of fewer females visiting the plant, or of each
270 visiting female laying fewer eggs. The observed negative relationship between
271 consumer density and overall resource abundance was stronger when the neighbors
272 represented high-quality resources during the period of butterfly flight, i.e. when
273 surrounding plants flowered early in the season. In other words, at a given conspecific
274 density more eggs were found in *G. pneumonanthe* plants surrounded by non-attractive,
275 low-quality neighbors (i.e. late-flowering plants). In our study, plant phenology thus
276 influences butterfly oviposition not only because the phenology of a focal plant interacts
277 with host ant availability to determine plant suitability for oviposition, but also because
278 the phenology of neighbors modulates the strength of host plant density effects on
279 oviposition. Our results suggest that oviposition decisions in myrmecophilous
280 butterflies are taken at two levels. When flying over an area populated with host plants,
281 females might primarily use visual cues [35] to evaluate host plant availability (i.e.
282 density) and identify high-quality (i.e. early-flowering) host plants. When butterflies
283 approach host plants, or after alighting on a plant, females may use both visual cues to
284 evaluate plant quality and chemical cues to detect ant presence [11,27,36], thereby
285 evaluating the spatial overlap of the two resources needed by their offspring. If females
286 use plant developmental stage as a cue indicating plant quality, host plants surrounded
287 by low-quality neighbors would be more attractive to butterflies than host plants
288 surrounded by high-quality neighbors. Negative density-dependence in antagonists has
289 been documented also in other systems, where egg clumping on plants with few

290 conspecific neighbors was explained by a disproportionate attraction of insect
291 herbivores to more isolated host plants [37,38]. For herbivores feeding on several plant
292 species, associational effects linked to the surrounding plant community have been
293 documented more frequently (e.g. [39,40]). Our study shows that associational effects
294 are present also for a specialized herbivore using a single host species, and that the
295 strength of these density-dependent effects depends on the quality of conspecific
296 neighboring host plants.

297 Our results are important to understand the factors causing variation in the
298 intensity of interactions between consumers and their resources. Given that consumers
299 often have important effects on fitness in the consumed organisms and that they show
300 preferences for traits of the resource organisms, identifying the factors that influence the
301 intensity of interactions is also important to understand the mechanisms underlying
302 variation in natural selection. In our study system, the butterfly seed predator has strong
303 negative effects on plant fitness and shows a preference for plants with an early
304 flowering phenology, leading to butterfly-mediated selection for later flowering [22].
305 The results of this study show that the strength of butterfly preferences for early-
306 flowering plants is influenced by both population context, in terms of population density
307 and the trait distribution with the population, and by community context in terms of the
308 abundance of the ant species that function as the second host of butterfly caterpillars.
309 Taken together, these findings illustrate that observed spatial variation in intensities of
310 interactions and consumer-mediated selection on traits of the resource organisms can be
311 the result of processes acting at the levels of individuals, populations and communities.

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Table 1: Results of the GLMs of the effects on the probability of a flowering shoot of *Gentiana pneumonanthe* receiving at least one egg from the butterfly *Phengaris alcon* (binomial GLM) and the number of eggs in shoots with at least one egg (negative binomial GLM), of shoot phenology, ant abundance, neighbor density and neighbor phenology, as well as three two-way interactions of these predictors. In the binomial GLM, two eigenvectors issued from Moran's eigenvector mapping (MEM) are also included as predictors, in order to reduce residual spatial autocorrelation (see details in the text).

	Probability of having at least one egg (n = 8848, R ² = 0.45)		Number of eggs (n = 731, R ² = 0.22)	
	Estimate	SE	Estimate	SE
Shoot phenology	1.462 ***	0.079	0.257 ***	0.036
Ant abundance	-0.017	0.052	0.089 **	0.031
Neighbor density	-1.691 ***	0.102	-0.368 ***	0.067
Neighbor phenology	-1.150 ***	0.124	-0.121 **	0.041
Shoot phenology × Ant abundance	0.132 *	0.062	0.004	0.031
Shoot phenology × Neighbor phenology	0.068	0.074	0.014	0.035
Neighbor density × Neighbor phenology	-0.500 ***	0.087	-0.100 *	0.042
MEM Vector 1	0.289 ***	0.030		
MEM Vector 2	0.324 ***	0.052		

***P < 0.001; **P < 0.01; *P < 0.05

FIGURE LEGENDS

Figure 1: Sampling design. A) Study plot ($69 \times 45 \text{ m}^2$), comprising 345 3 m^2 subplots. B) Detail of several occupied subplots with reproductive shoots of *Gentiana pneumonanthe* and sampling points for ants. Shoots are represented by points, and colored according to shoot phenology. Numbers indicate the number of *Phengaris alcon* eggs found on the shoot. An example of a 3-m buffer used for neighbor measures on a focal shoot (represented by a star) is shown.

Figure 2: Effects of shoot phenology and host ant abundance on the distribution of *Phengaris alcon* eggs on its host plant *Gentiana pneumonanthe*. A) Effects on the probability of a shoot having at least one egg, the relationship between the probability of having at least one egg and shoot phenology shown for different ant abundances. B) Effects of shoot phenology on the number of eggs in shoots with at least one egg. Higher values of shoot phenology indicate earlier flowering. C) Effects of ant abundance on the number of eggs in shoots with at least one egg. In A), binomial GLM fit lines are represented with a color gradient for a continuous increment in ant abundance within the range of observed values. In B) and C), negative binomial GLM fit lines and confidence intervals are shown.

Figure 3: Effects of the interaction between neighbor density and neighbor phenology on the probability of a flowering shoot of *Gentiana pneumonanthe* having at least one egg of the butterfly *Phengaris alcon* (A) and the number of eggs in shoots with at least one egg (B). The relationships with neighbor density are shown for different values of neighbor phenology. Binomial (A) or negative binomial (B) GLM fit lines are

represented with a color gradient for a continuous increment in neighbor phenology within the range of observed values. Higher values of neighbor phenology indicate earlier flowering.

Figure 1

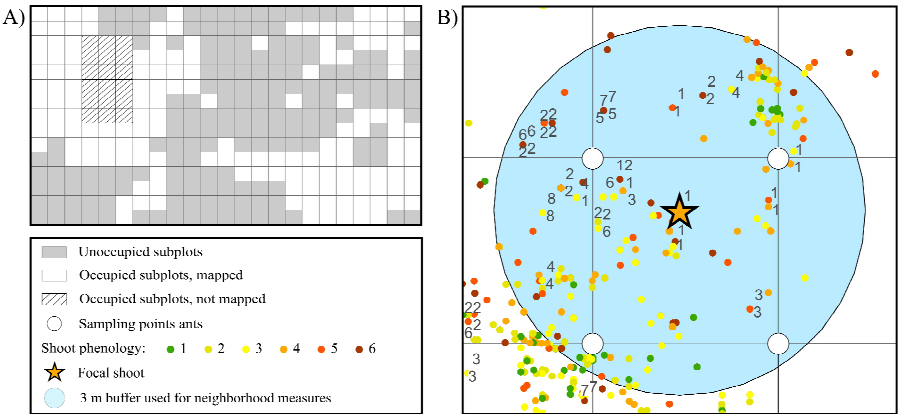


Figure 2

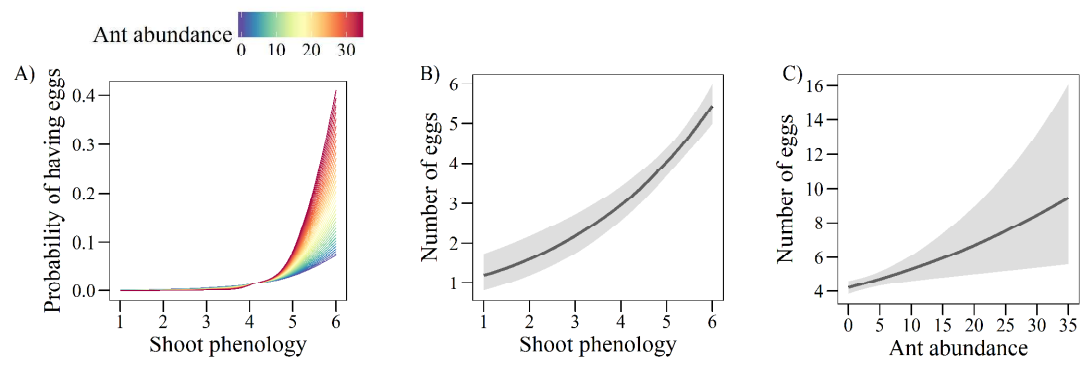
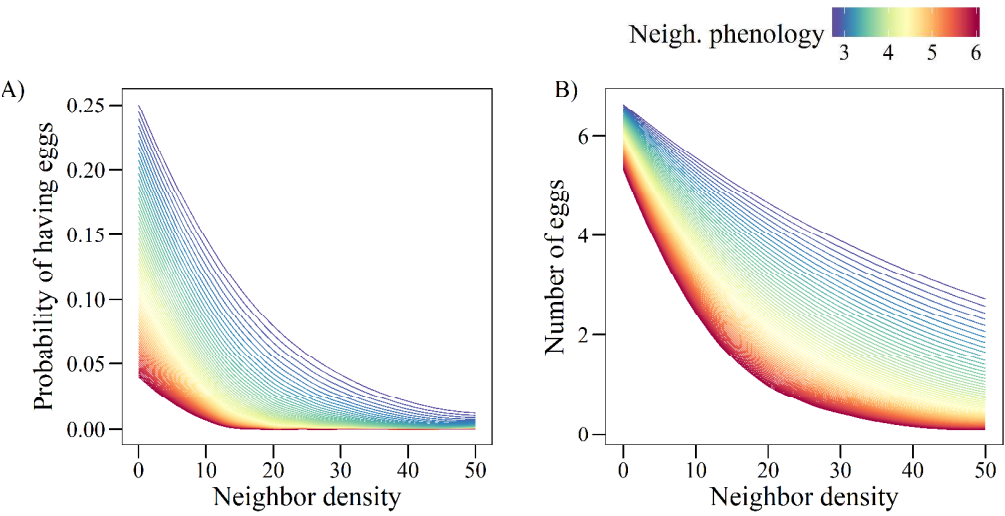


Figure 3



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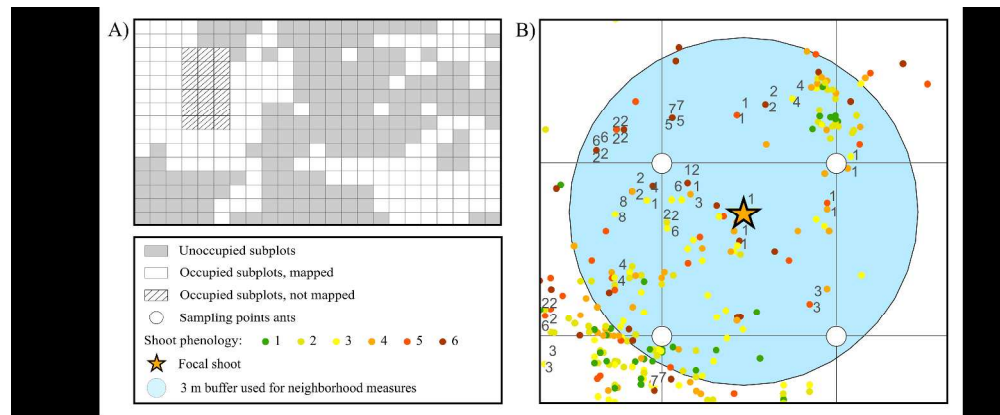


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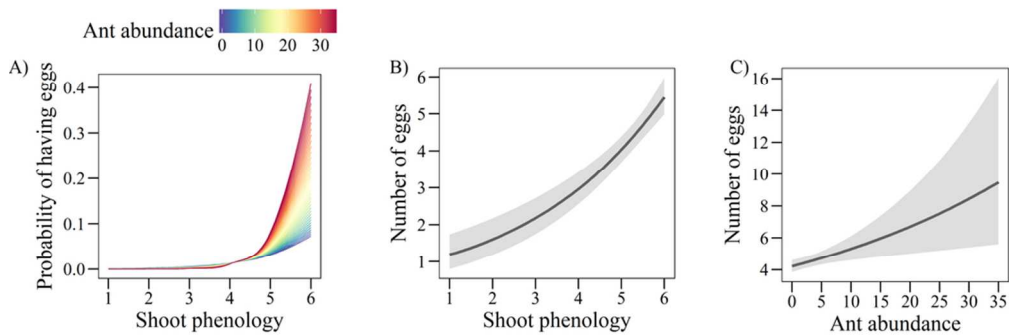


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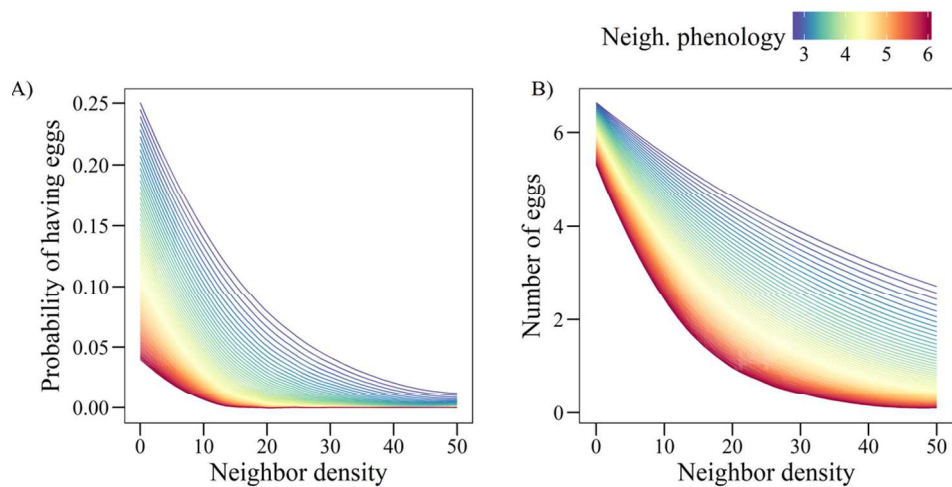


Figure 3: Effects of the interaction between neighbor density and neighbor phenology on the probability of a flowering shoot of *Gentiana pneumonanthe* having at least one egg of the butterfly *Phengaris alcon* (A) and the number of eggs in shoots with at least one egg (B). The relationships with neighbor density are shown for different values of neighbor phenology. Binomial (A) or negative binomial (B) GLM fit lines are represented with a color gradient for a continuous increment in neighbor phenology within the range of observed values. Higher values of neighbor phenology indicate earlier flowering.

109x55mm (300 x 300 DPI)