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How host plants and predators influence pea aphid (*Acyrthosiphon pisum* Harris) populations in a complex habitat

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Abstract. The predation rate of pea aphids, *Acyrthosiphon pisum* Harris by ladybird-beetles, spiders, and parasitism by hymenopterous parasitoids (braconids) within complex habitats was tested. Altogether 20 pea, 20 alfalfa and 20 red clover plots were established in a sequential block design, whereupon the aphids along with their predators and parasitoids were assessed both in the middle and at the field margins. The performance of aphids and predation/parasitism were also tested in *meso*-environments (i.e. enclosed field cages) with two different host plant species per environment. The data shows that spider density on pea is generally high, but it is lower on plants in the vicinity of clover and alfalfa. Braconid parasitoids, *Aphidius ervi* Haliday parasitized pea aphids on all host plants and similar parasitoid pressure was observed on all host plants tested. *Meso*-environment experiments further explained the low predation of ladybirds when foraging for prey on pea plants. Using five polymorphic microsatellite markers, it was found that whilst there was no clear trend in terms of the pattern of population genetic variation detected, the fact that some pea aphid populations on certain sample plots showed marginally statistically significant differences (divergence) in terms of interpopulation *F*st values, may well reflect predator choice in relation to plant host. Overall, aphids appear to be under various selective pressures from both higher and lower trophic levels.

Key words: Adaptation, evolutionary consequences, hymenopterous parasitoids, predation rate, selection.

Introduction

For insect communities associated with a particular host plant or plants, there has been much discussion as to whether the host plant or predators control population dynamics within the community (Walker & Jones 2001). This understanding derives largely from researches assessing variation in host plant-herbivore-predator interactions (Schmitz 2010, Mooney et al. 2010, Burghardt & Schmitz 2015). Insect species (i.e. aphids) often form so called 'host races', considered as a first step towards sympatric speciation (Kunert et al. 2010, Peccoud & Simon 2010). These host races form when individuals shift their use from a common or universal host plant to specialize on alternative host plant species, potentially leading to divergent selection via reproductive isolation (Ferrari et al. 2006, Peccoud & Simon 2010). It has been proposed for phytophagous insects that change behaviourally under new environmental conditions (e.g., a new host plant/s), selection acts differentially within the old and new environments (Futuyma & Moreno 1988).

Shifting to and accepting a new host is however difficult. The pea aphid *Acyrthosiphon pisum* Harris *sensu lato*, in effect a host plant adapted complex (see below), is a plant parasite that feeds by penetrating the host's leaves or stem with its stylets to feed on vascular fluids (phloem) (Caillaud & Via 2000). The chemical and/or physical characteristics of host plant tissues and phloem make aphids reluctant to accept a new plant. This is because new host selection includes a trade-off between preference for feeding with phloem and the necessity to deal with new secondary plant chemicals, especially toxic ones (Caillaud & Niemeyer 1996).

Another crucial determinant of both behaviour and diversifying selection in phytophagous insects is the presence of natural enemies (Nosil & Crespi 2006). Continuous predator pressure can evoke population-wide behavioural, morphological and/or physiological adaptation of prey that leads to differential performance and fitness in different environmental contexts (Relyea 2001, Hawlena et al. 2011). When predation pressure on phytophagous insects changes temporarily, spatially (e.g., on a new host plant), or qualitatively, those prey individuals that are capable of responding flexibly to predation risk have a higher likelihood (probability) of survival and subsequent fitness benefits (Frommen et al. 2010). These complex relations are mediated not only by variations in prey density but also by changes in behavioural responses and other traits, consequently influencing sympatric speciation of prey species (Veen et al. 2006, Bukovinszky et al. 2008).

In light of the aforementioned, the following hypotheses were tested experimentally in the field via a series of plot-based trials and also in the case of the aphids, using high resolution molecular markers (microsatellites): 1) pea aphids host plant (pea, alfalfa and red clover) vicinity (various host plant habitats) affects the spatial distribution of pea aphid predators and parasitoids; 2) different predator density on different host plants may influence the prey (pea aphids in this case) density, i.e. a polyphagous predator (spider) dominance may allow higher aphid density on a particular host plant/s; and 3) the spatial distributions of aphids and predators on different host plants may also be viewed as shifts in the pattern of genetic variability between pea aphid populations inhabiting different host plants in this complex habitat and tested accordingly following application of microsatellite markers. These aspects are interesting and important, not only for a better understanding of 'top-down' and/or 'bottom-up' effects on prey species, but also more generally for understanding modes of sympatric speciation in insects.

Material and Methods

Ethics Statement

For the collection of aphids and their arthropod predators, no permits were necessary since the area where the animals were collected did not belong to any protected region. All arthropod assessment and laboratory work was conducted according to relevant national and international rules and guidelines (European Commission, Ethics for Researchers 2013).

Study system

Pea aphids are known to have complex life cycles. This includes all-female, asexual (parthenogenetic) generations that alternate with sexual generations. Each female aphid may give birth to up to 50-100 living young at the rate of 6 to 12 a day. Therefore, up to 14 or more asexual generations may be produced per year. Because pea aphids have repeatedly generated host-specialized populations that become adapted to both crops and wild legumes, they provide one of the best studied model species of host-plant adaptation (Via 1999, Chaneton & Bonsall 2000, Ferrari et al. 2001). The species is well known for having two principal colour morphs – green and red – related to host preference, itself related to the type of symbiotic bacteria present (Oliver et al. 2010, Tsuchida et al. 2010), the proportion of these morphs varying between host plants within natural field populations (Balog & Schmitz 2013a).

Sympatric specialization and host plant differentiation of *A. pisum sensu lato* has been most closely studied in populations infesting red clover and alfalfa (Via 1999, Caillaud & Via 2000, Ferrari et al. 2006), whilst a number of recent publications have involved detailed investigations of host plant specialization in this aphid across many species of host plant (Peccoud et al. 2009a, Ferrari et al. 2012). Related perennial and annual leguminous plants (clover, alfalfa and pea), are typically planted under open field conditions in close vicinity to one another, thereby forming host plant mosaics of different sizes. Clover is generally 20–80 cm tall, has a high nutritional value of nitrogen, N, as well as being one of the richest sources of calcium, chromium, magnesium, phosphorus, potassium, isoflavones, and the vitamins thiamine and niacin (Rosso & Pagano 2005). Alfalfa can grow to 1m tall, also has a high nutritional N value, is a good source of protein, and usually has the highest feeding value of all common fodder crops (Yuegao & Cash 2009). Pea can likewise grow to 1–2 m high, has a lower nutrient value compared with clover and alfalfa, but is rich in fibre, protein and vitamins (Zohary et al. 2012).

Such host plants vicinity may well have a direct effect on pea aphid predation rate (Lushai et al. 2002). These animals are often attacked by several predators, including ladybird beetles, especially the 7-spot ladybird beetle, *Coccinella septempunctata* L. and the 2-spot ladybird beetle, *Adalia bipunctata* L. (Coleoptera: Coccinelidae), the nursery web spider, *Pisaura mirablilis* (Arachnida: Araneae), syrphid larvae (Diptera: Syrphidae), and also by endoparasitic wasps, i.e. parasitoids (Hymenoptera: Ichneumonoidea: Bracondidae). These predator species are present in such host plants mosaics, feeding within the pea aphid colonies. Their predation rate might vary between pea aphid colonies on different host plants and thus may create the conditions necessary to promote, and ultimately cause, differential aphid reproduction rates among the host-associated aphid colonies.

Field experiment I - assessment of aphids, their predators and parasitoids

For this experiment, populations of pea aphid and its predator and parasitoid density were assessed when host plants were planted in close proximity to each other. In the spring of 2012, 20 pea, alfalfa and red clover plots (units of replications, 60 total) each of 50 m^2 were established in a sequential block design at the Sapientia University experimental field site, such that each plot was bordered on each side by one other host plant field (Fig. 1). Replication units were separated from each other by a distance of approximately 40-50 cm with no other vegetation. The entire site was surrounded by a pea

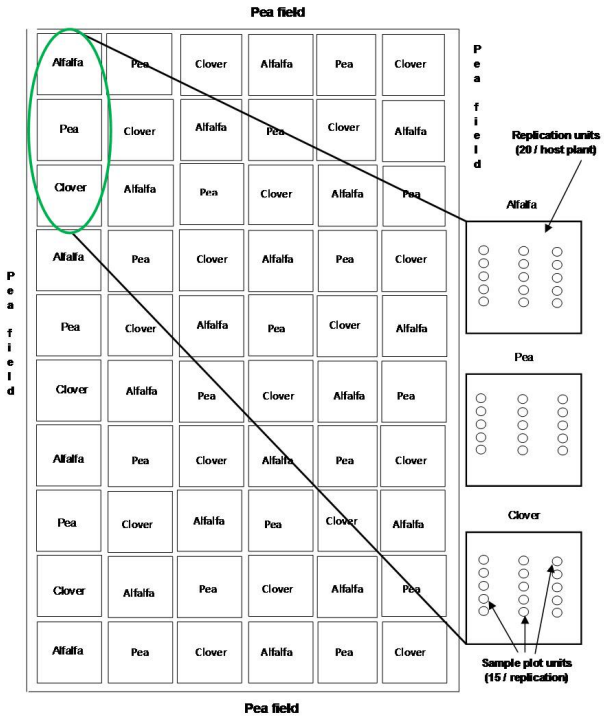


Figure 1. Experimental design of 20 pea, alfalfa and red clover plots (replication units; 60 total), each of 50 m2 (0.25 ha). Pea aphids and predator samples were taken from both field margins (five + five) and from the middle (five) of the plots (15 sample point units / one replication unit).

crop (Figure 1). Host plants were seeded per normal agricultural practice in March. No fertiliser or pesticide applications were used during the entire growing season. Within each replication unit, 15 sample plot units each of about 4 m^2 were established (Fig. 1). Of these, five were placed along the right border of the replication unit immediately neighbouring the other host plant (at about 2 m distance from the border), five in the middle of the replication unit, and five placed along the left border of the replication unit next to the third host plant (Fig. 1).

The sampling procedure for aphids and their arthropod predators was begun in May, the day following the first observation of the aphids at a density of at least four adults per sample plot (randomly checked in five sample plots per replication unit). This was done in order to determine the establishment of aphids on plants and reduce the possibility of having several sample plots without aphids. During this period all host plant species were relatively young and the architecture of the various trial plots similar. The assessment started inside the six marginal replication units and followed inside the next six until all 60 replicates had been intensively surveyed once.

Because aphids are known to vary hugely in terms of population size and predation and parasitism pressures across the growing season, the first data assessment of the 60 replication units was performed within five days (12 replication units sampled per day). Ten randomly selected plants with aphids per sample plot were selected by enclosing the infested plant in a polythene bag and then cutting this free with scissors or a knife. On return to the laboratory (speed was of the essence, since some predation might occur in the collecting bags themselves), the entire content was stored at -20C° and after the five day collection period, was carefully assessed for the arthropods present and their respective numbers under a stereo microscope. In this way, all insects from the enclosed plants, including those with low mobility (e.g. Syrphid larvae), were captured and counted. Pea aphids and their predators were also counted, with the adults and nymphs of green and red aphid morphs and their associated predators (ladybird beetles, spiders, Syrphid larvae) evaluated

separately.

Pea aphid colour morphs were counted separately for each host plant in order to assess the appropriate proportion of the two morphs for the second field experiment testing predator choice according to Losey et al. (1997), green-red colour polymorphism as found in pea aphids is maintained by differential vulnerability to natural enemies). From each sample plot parasitized aphid mummies were also assessed. After counting all arthropods in the laboratory, first instar aphid nymphs were collected separately from each of the host plant sample plot units (clover middle, alfalfa middle, pea middle, alfalfa-pea margins, alfalfa-clover margins and clover-pea margins) and placed in absolute ethanol for subsequent DNA analysis. By this means, pure DNA representing the genotype of each individual asexual aphid only was obtained (Monti et al. 2012, Loxdale et al. 2013).

The five day sampling procedure on the 60 replication units was repeated in the same way after ten days, whilst the assessment of arthropods was made as during the first sampling. After another ten days, the third assessment was performed, again in the 60 replication units following the same procedures. By this time, some pea plants had become too old and woody and hence unsuitable for pea aphids to further survive on.

Field experiment II – Predator choice assessment on different host plant pairs

The second field experiment was conducted using the same typical agriculture row-crop planting of host plants. The experiment was performed in the same field the following year and concerned evaluation of the preferences of predators (ladybirds and spiders) and parasitism of braconid parasitoids of pea aphid colonies on different host plants. The effect of predators on pea aphid host races under open field conditions could be mediated by the presence of a second pea aphid host race. In other words, the predation rate could be influenced by nearby host plants. We therefore evaluated the performance of pea aphid populations and their associated predation rate, including percentage hymenopterous parasitism, on host plant combinations as pea with alfalfa, pea with clover, and clover with alfalfa, respectively. The experiment comprised use of 60 cages (so called *meso*-environments) of 80 cm wide x 120 cm high placed on the border between two replication units, each comprising paired host plants in the following combinations:

1. Red clover + Alfalfa (10 control and 10 with predators)
2. Red clover + Pea (10 control and 10 with predators)
3. Alfalfa + Pea (10 control and 10 with predators)

The mesh size was small enough to keep arthropod predators within the cages but allowed for colonisation of the aphids by hymenopterous parasitoids. Host-plant pairs received ten wingless, fourth instar pea aphid nymphs each (so that reproduction would begin at the same time for all asexual females) in similar colour combinations for each host plant, as previously observed in experiment I (zero red and ten green for pea, three red and seven green for alfalfa, and one red and nine green for clover). Unparasitized aphids of the appropriate colour morph were seeded on each host plant. Aphids were allowed to reproduce without any disturbance for three days, having about 160-180 offspring in each *meso*-environment by the end of this time. Thereafter, in ten cages considered as treatments, two adult 7-spotted ladybirds beetle and two adult nursery web spiders were placed (one predator individual to one host plants, at the top of the plants) and the cages again closed. Ten other cages from each host plant combinations were considered as control and kept free of predators. After one day, all *meso*-environments were carefully checked; the positions of each predator were noted on three occasions (i.e. morning, noon and afternoon at the same time) by checking each host plant pair inside the treatment cages. For example, in the pea + alfalfa combination, if the two spiders were detected on pea in the morning, we noted '2-0', where zero denotes their absence on alfalfa. The numbers of parasitized pea aphid mummies were also counted in control cages on each host plant and carefully collected and kept until hatching of the adult parasitoid wasps from these to enable their subsequent microscopic identification. All *meso*-environments were carefully controlled and assessed every second day until the end of the 22nd day of the experiment, since at that time, the 7-spotted ladybird adults started laying eggs and the young larvae would bias the predator pressure. Aphid population density (adults and offspring separately from each colour morphs and host plant) was recorded by separately assessing adults and their offspring on the whole host plant pairs at the end of the experiment. The collection method as described for the first field experiment was used.

Genetic analyses of Pea aphids on different host plants

DNA isolation from green pea aphid morphs (red morphs were not included in the analysis because of their low number on pea) collected during the first field experiment was performed using the Wizard Genomic DNA Isolation Kit (Promega), and with genetic variability assessed using five previously published microsatellite primer pairs known to provide high levels of genetic polymorphism (AIB07M, AIB08M, AIB012M, ApH08M, ApH10M; (Caillaud et al. 2004). Green pea aphids from the subplots (Fig. 1) were compared for levels of polymorphism as detailed below (Data analysis). PCR (polymerase chain reaction) amplification was performed in Corbett Palm-Cycler thermocyclers (Corbett CG1-96) using the following protocol: an initial denaturation step at 94°C for 3 min., followed by 32 cycles at 94°C for 30 sec; an annealing step at 56°C for 30 sec; extension at 72°C for 45 sec; a final extension step at 72°C for 7 min; and a subsequent incubation at 4°C. The amplified products were separated on 10 cm, 7% polyacrylamide gels, stained with RedSafe and visualized under UV light using the GelDoc System from Bio-Rad.

Data analysis

In experiment I, arthropod abundances were analysed between collection dates by constructing principal response curves (PRS) (Brink et al. 2008) to compare population dynamics in each of the three data sets collected throughout the season. Mean number of arthropods per sample plot unit (sample plot units at the same location, i.e. same host plant plot side and middle) per host plant per sampling date was considered. However, whilst a small increase in aphid abundance was observed, no significant differences were apparent. Therefore, average values of three data collection periods were used in all subsequent analyses. To avoid pseudo replications, the average arthropod data of five sample plot units from the same margin of host plant pairs and/or five from the middle of the host plant plot were used (outer row data from plots not bordering any other plot were not used in the analyses).

In the first modelling approach, generalized linear model (GLM) with quasi-Poisson error distribution was used to compare arthropod densities (aphids colour morphs, predators and parasitized mummies) on different host plants without edge effects, i.e. average data of aphids, predators and aphids mummies only from the five middle sample units of each replication units [for better separation from the other model used (see below) this first approach was marked on Table 1 as GLM-I.1-3]. All the data obtained from the 20 host plant replicates was used. Firstly, arthropods average densities in different host plant plots were compared using block and host plants as independent variables and arthropod densities as dependent variables (GLM-I.1 in Table 1). The second model (GLM-I.2 in Table 1) compared pea aphids colour morphs (green and red separately, except pea fields where the low red morph abundance made such a comparison impossible and only green densities were used) with predators (ladybird beetles and spiders) and parasitoids, using host plants and aphid colour morphs as independent variables and predators and parasitized mummies densities as dependent variables. The third model (GLM-I.3 in Table 1) compared predators and parasitoids in different host plants using host plants and predators (i.e. ladybird beetles) as independent variables and the other predator (i.e. spider) and parasitized mummies densities as dependent

variables.

In the second approach the generalized linear model (GLM) with quasi-Poisson error distribution was also used to evaluate the effect/s of host plant vicinity on pea aphid abundance and on aphid predation and parasitism, respectively. Here we considered blocks and host plants pairs as independent variables and arthropod densities as dependent variables. The first model (grey bars Fig. 2A-C) compared pea aphid densities at field margins considering mean abundances of the five sample plot units from margins (five from the left and rights sides, respectively, of each replication units) per sample effect, i.e. same host plant sides (ex. alfalfa near pea and pea near alfalfa separately, and similarly with clover). Thereafter, the second model compared ladybird beetles densities from different host plants from the same margins (black line Fig. 2A). The third model compared spider densities from different host plants from the same margins (black line Fig. 2B), the fourth model parasitized mummy densities between host plants from the same field margins (black line Fig. 2C). Because the adjacent pea field might have significant effects on marginal replication units, data from these were not considered in these analyses. Aphid, predator and parasitoid data were again used as average data for the five sample plot units of the 17 replication unit pairs, except for the combinations Alfalfa(Clover) and Clover(Alfalfa), where only average data from 16 replication units were tested within the experimental design (Fig. 1). The data is graphically presented together as Figure 2A-C.

Three analyses for experiment II were computed. These included the relative fitness (mean number of aphid nymphs surviving at the end of the experiment relative to the initial number of adults) of red and green aphid morphs at the end of experiment II compared between host plants separately for control and predator treatments. The average number of aphids per host plant per cage was considered using ANOVA to compare the variables. This approach was done in order to test whether pea aphid red and green morph fitness varied under different predator pressures on different host plants. The same test was next used to analyse the parasitoid effects on pea aphids, involving the average number of aphid mummies recorded relative to the total number of aphids per host plant at the end of the experiment. Because parasitoids had equal access to both control and predator treatment cages, only data from control cages were used for this last analysis, since within predator cages several parasitized aphids may have been consumed by predators. Furthermore, because the colours of the parasitized mummies are often difficult to assess accurately in terms of species identification, data for all parasitized mummies considered were lumped together. Results are presented in Figure 3A-C. The final analysis tested the choice of predators (ladybirds and spiders) feeding upon pea aphids on particular host plants. For this, the average predator choice per replicates per treatments between host plants across sampling dates was considered and a repeated measurements approach involving multivariate analysis of variance (MANOVA) employed to compare variables during the observations within host plants. Results are presented in Figure 4.

For studying the genetic variability of pea aphid colonies, analysis of length polymorphism of the microsatellite sequence of the green morphs only (representing the largest samples available) between host plants was performed using DnaSP software (Rozas et al. 2010). Comparisons were made for all five loci and variation between aphids collected in the same data assessment compared with those from Clover middle, Alfalfa middle, Pea middle, Alfalfa(Pea) margins (aphid samples from alfalfa with pea margins. The same was performed for the other samples tested, i.e. Pea(Alfalfa) margins, Alfalfa(Clover) margins, Clover(Alfalfa) margins, Clover(Pea) and Pea(Clover) margins, respectively. The allele number per locus, levels of observed (*Ho*) and expected heterozygosity (*He*) over all loci, the latter assuming populations to be in Hardy-Weinberg equilibrium (HWE) consequent upon random mating of individuals, and *F*-statistics (*F*st, a measure of population differentiation due to genetic structuring) to compare levels of variability was calculated using GENEPOP (available online at: http://genepop.curtin.edu.au/). Results are presented in Table 2A, B.

Results

Field experiment I

– Arthropod abundances without edge effects

a.) Arthropod densities on different host plant

No significant temporal differences between sampling data per host plant per arthropod species were detected. In total, 10,727 pea aphids were counted during the first field experiment from which 6,001 were assessed in the middle of the host plants replication units and used for the analyses. No varietal block effects were detected on pea aphid green and red morph densities or on predators and parasitoids (Table 1). Pea aphid colour morphs varied on all host plants, green morphs clearly dominating (an average of 36.74±2 per sampling point in pea, 31.04±3 in alfalfa, and 29.55±1.9 in red clover), whilst red aphid morphs were almost absent from pea crops (0.8±0.1 per sampling point in pea, 7.28±0.4 in alfalfa, and 2.58±0.5 in red clover). Highly statistically significant differences were observed between green and red aphid morph abundances on all host plants (Table 1, GLM-I.1). A total of 1,176 predators were counted and used for analyses. The most important predators found in the middle of the host plant plots were 7-spot ladybirds (an average of 2.1±0.2 per sampling point in pea, 7±0.6 in alfalfa, and 16.2±0.7 in red clover) and the nursery web spider (average 19.8±2.2 per sampling point in pea, 6.7±0.8 in alfalfa, and 5±0.5 in red clover). Ladybird beetles density was low in pea and high in red clover, while the opposite was detected for the nursery web spider. No differences in alfalfa between predators were detected. Also no effect of host plants on parasitism were observed (Table 1).

b.) Aphids colour morphs predators and parasitoid densities

Feeding on aphids was observed for both predator species. Upon comparing pea aphid colour morph densities on different host plants with predator densities, a decrease of ladybirds on pea was detected, while the population density of this insect increased on alfalfa (for both green and red aphid morphs) and on clover infested with green pea aphids. An opposite effect on spiders was detected, their population density increasing on pea and decreasing significantly on alfalfa and clover, except when clover hosted red pea aphid morphs. The parasitized aphid mummy density was rather similar on all host plants whether aphids were green or red (Table 1-GLM-I.2). Very few Syrphid larvae were found on any of the host plants sampled from (3.7±0.4 per sampling point in pea, 1.2±0.2 in alfalfa, and 1±0.2 in red clover) and hence no statistical analyses were possible here.

c.) Differences between predator species and between predators and parasitoids

Differences between predators were significant. While ladybird abundances were low on pea, that of nursery web spiders were contrastingly high; the opposite trend was detected in red clover, where ladybird abundances were significantly higher than nursery web spider abundances. No differences on alfalfa were detected. An average of 4.0±0.5 parasitized aphid mummies per sampling point on pea, 3±0.3 on alfalfa, and 2.8±0.3 on red clover, were found. There were no observable effect of predators on parasitism in the

Table 1. Comparisons between arthropods (aphids colour morphs, predators and parasitoids) on different host plants without edge effects (GLM with quasi-Poisson error distribution), using average data from the five middle sample units of each replication units.

		Dependent variables				
GLM		Population density				
Model	Independent variables	aphid green morphs	aphid red morphs	ladybird beetles	spider	parasitized mummies
GLM-I.1	Block	$F_{1,60}$=0.20^{ns}	$F_{1,60}$=1.1^{ns}	$F_{1,60}$=1.2^{ns}	$F_{1,60}$=1.6 ^{ns}	$F_{1,60}$=0.9^{ns}
	Block+Pea	**$F_{1,60}$=8.2**↑**	**$F_{1,60}$=5.1*↓**	**$F_{1,60}$=4*↓**	**$F_{1,60}$=7.3**↑**	$F_{1,60}$=0.2^{ns}
	Block+Afalfa	**$F_{1,60}$=6.7**↑**	**$F_{1,60}$=4.2*↓**	$F_{1,60}$=2.2^{ns}	$F_{1,60}$=2.8^{ns}	$F_{1,60}$=0.3^{ns}
	Block+Clover	**$F_{1,60}$=5.4**↑**	**$F_{1,60}$=4.6*↓**	**$F_{1,60}$=4.5*↑**	**$F_{1,60}$=6.2**↓**	$F_{1,60}$=0.2^{ns}
GLM-I.2	Pea+green morphs	-	-	**$F_{1,20}$=5.7**↓**	**$F_{1,20}$=5.6**↑**	$F_{1,20}$=1.2^{ns}
	Alfalfa+green morphs	-	-	**$F_{1,20}$=5.5**↑**	**$F_{1,20}$=5.6**↓**	$F_{1,20}$=1.5^{ns}
	Alfalfa+red morphs	-	-	**$F_{1,20}$=4.4*↑**	**$F_{1,20}$=4.5*↓**	$F_{1,20}$=0.7^{ns}
	Clover+green morphs	-	-	**$F_{1,20}$=5.4**↑**	**$F_{1,20}$=5.6**↓**	$F_{1,20}$=0.3^{ns}
	Clover+red morphs	-	-	$F_{1,20}$=0.3^{ns}	$F_{1,20}$=0.88^{ns}	$F_{1,20}$=0.6^{ns}
GLM-I.3	Pea+ladybird	-	-	-	**$F_{1,20}$=5.4**↑**	$F_{1,20}$=2.1^{ns}
	Pea+spiders	-	-	**$F_{1,20}$=4.3*↓**	-	$F_{1,20}$=2.7^{ns}
	Alfalfa+ladybird	-	-	-	$F_{1,20}$=1.3^{ns}	$F_{1,20}$=1^{ns}
	Alfalfa+spiders	-	-	$F_{1,20}$=1.2^{ns}	-	$F_{1,20}$=1.7^{ns}
	Clover+ladybird	-	-	-	**$F_{1,20}$=4.7*↓**	$F_{1,20}$=0.3^{ns}
	Clover+spiders	-	-	**$F_{1,20}$=4.5*↑**	-	$F_{1,20}$=0.5^{ns}

Minimum adequate model results from generalized linear models with quasi-Poisson error distribution. Significant values are bolded. Arrows after value show the direction of the main effects: upwards arrow indicates a positive relationship and downwards arrow indicates a negative relationship. * $p < 0.01$, ** $p < 0.001$; ns- not significant.

middle of the host plant plots (Table 1, GLM-I.3).

Field experiment I

– Arthropod densities with the effect of adjacent host plant

To better understand the aphid and predator density variations between host plant margins, results of the various analyses performed are presented together in Figure 2A-C, aphid density as grey bars, predators as black lines (Fig. 2A, B), while parasitized mummies densities are shown as black lines (Fig. 2C). Considering the effect of host plant vicinity on pea aphids, significantly higher aphid abundances were observed on all pea plots, including field margins with alfalfa (11.9±1 per sampling point, *df*=16, *F*=15, $p \leq 0.001$) and clover (11.8±1.1 per sampling point, *df*=16, *F*=9, $p \leq 0.001$) (Fig. 2A, B, C), whereas no differences were detected in terms of aphid abundances between alfalfa-pea (6.4±1.1 per sampling point, *df*=16, *F*=1.4, *p*=0.98) and alfalfa-clover margins (6.5±1 per sampling point, *df*=15, *F*=1.3, *p*=0.99). Differences in aphid abundance were observed between clover-pea (3.6±0.2 per sampling point, *df*=16, *F*=3.9, $p \leq 0.01$) and clover-alfalfa margins (4.2±0.3 per sampling point, *df*=15 *F*=4.4, $p \leq 0.01$). There was also a difference in predator abundance. Considering pea fields with other host plant margins (pea-alfalfa and pea-clover), ladybird beetles dominated both alfalfa-pea (*df*=16, *F*=3, *p*=0.05) and alfalfa-clover margins (d*f*=15, *F*=3.2, *p*=0.05). A significantly higher ladybird density was also detected in clover-pea (*df*=16, *F*=5, *p*=0.03) and clover-alfalfa margins (*df*=15, *F*=3, *p*=0.05), respectively (Fig. 2A black line). Spiders clearly dominated both pea-alfalfa (*df*=16, *F*=6.3, $p \leq 0.001$) and pea-clover margins (*df*=16, *F*=6, *p*=0.002) upon comparison with alfalfa-clover and alfalfa-pea field margins. The same trend with clover-pea (*df*=16, *F*=3.1, *p*=0.05) and clover-alfalfa (*df*=15, *F*=3.3, *p*=0.05) was also detected (Fig. 2B black line). The parasitized mummy densities at field margins were only significantly different between pea-alfalfa and clover-alfalfa margins (*df*=10, F=4.2, p=0.01) (Fig. 2C, black line).

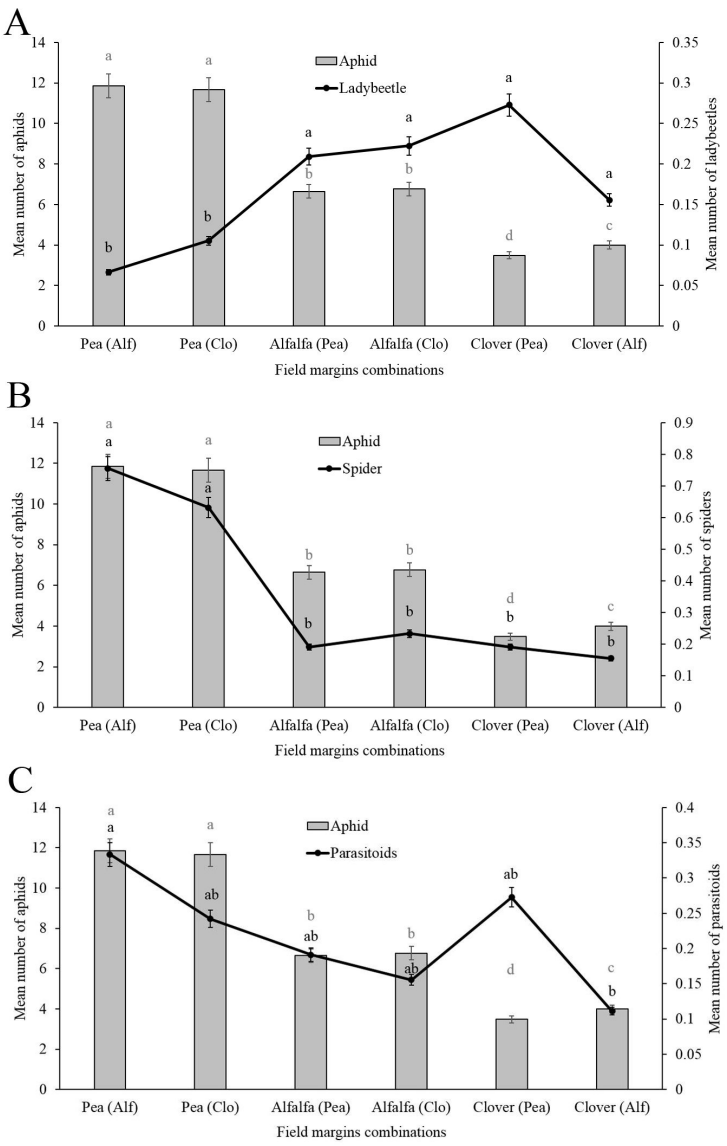
Field experiment II

- Reproduction success of pea aphids' red and green morphs

Statistical analysis revealed a higher reproduction of red pea aphid morphs than green on both alfalfa ($\bar{x}$=9.0±05, *df*=9, *F*=5, *p*=0.03) and clover ($\bar{x}$=8.7±1, *df*=9, *F*=3.08, *p*=0.03) on control plots lacking predators. The number of surviving offspring between red and green aphid morphs differed significantly under predator pressures. Lower number of red nymphs survived on both alfalfa ($\bar{x}$=1.3±0.2, *df*=9, *F*=9, *p*=0.001) and clover ($\bar{x}$=0.4±0.1, *df*=9, *F*=5.1, *p*=0.03). Furthermore, low numbers of green nymphs survived on pea (Fig. 3B). Overall, most parasitism of pea aphids (98.5%) was due to one parasitoid species, *Aphidius ervi* Haliday, the remaining 1.5% by a braconid of another genus, i.e. *Praon* spp. (Hymenoptera: Braconidae: Aphidiinae). A mean of 1.56±0.2 parasitoids was detected in pea crops, 1.18±0.1 in alfalfa, and 1.2±0.2 in clover. No differences in parasitoid pressure were observed between host plants (pea-alfalfa *df*=9, *F*=1.3, *p*=0.10; pea-clover *df*=9, *F*=1.57, *p*=0.19; clover-alfalfa *df*=9, *F*=1.01, *p*=0.95) (Fig. 3C). Statistical analysis revealed that predator preferences when feeding on pea aphids were highly different on pea, where most hunting by spiders was observed (*F*=4.14, *p*=0.03) (Fig. 4). In contrast, no differences were detected between predator preferences on clover (*F*=0.81, *p*=0.45) and alfalfa (*F*=1.56, *p*=0.68) (Fig. 4).

Microsatellite polymorphisms between pea aphids on different host plants

In terms of microsatellite polymorphisms for pea aphid subpopulations within and among host plant sample plots (Table 2a, b), the mean number of alleles per locus varied from 8-15 ($\bar{x}$=11.5±SE 0.83), with pea aphid populations showing the highest number of alleles on pea plants (Table 2a). There was no clear trend between plots sampled in terms of allele number. Observed (*Ho*) and expected heterozygosity (*He*), the latter assuming populations to be in HWE proportions, ranged from 0.550-0.982 ($\bar{x}$=0.731±SE 0.067) and 0.639-0.881



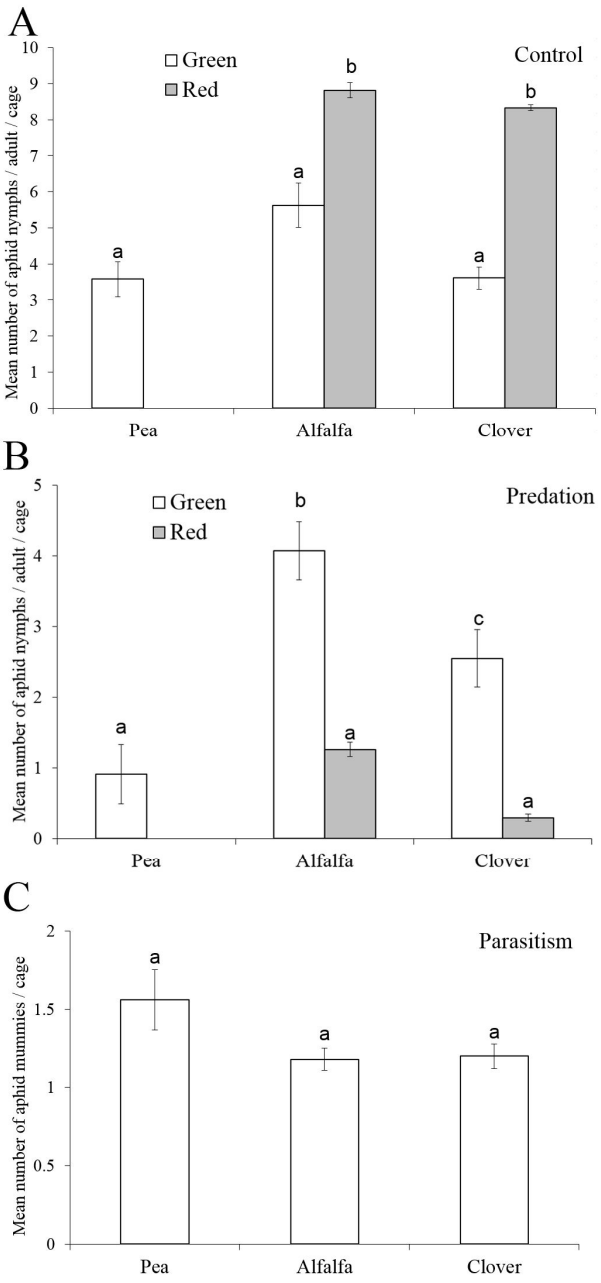
Figures 2. A, B, C. The effect of host plant vicinity on pea aphids (bars) and their dominant arthropod predator (lines); ladybirds (A), spiders (B) and parasitized mummies (C). Y axes represent the mean number of aphids / sample plot unit / sampling data. The first model (grey bars figure 2A-C) compared pea aphid densities, the second model compared predators' densities from different host plants from the same margins (black line figure 2A, B). The third model compared parasitized mummy densities between host plants from the same field margins (black line figure 2C). All field margin combinations on the X axis are presented as follows: the host plants in which samples were collected are without brackets, their adjacent host plants are shown bracketed. Bars in figures represent mean values of aphids from the average data of sample plot units / sampling data of the 17 replication units except for the combinations Alfalfa(Clover) and Clover(Alfalfa), where only average data from 16 replication units existed to allow for the experimental design. Lines represents predators and parasitized mummy densities from the same replication units averaged their sample plot units as above.

($\bar{x}$=0.822±SE 0.026), respectively. Thus overall, subpopulations were found to be slightly more homozygous, i.e. inbred, than expected, although again there was no obvious trend. From the *F*st analysis (Table 2b), the main finding was of a generally moderate level of genetic variation in terms of values calculated among and between various pea aphid populations for the different host plant combinations as outlined in Figure 1 (range 0.01- 0.18; overall $\bar{x}$=0.047±SE 0.006 over all populations and loci; total 36 pairwise comparisons). Of these comparisons, 20/36 (55.6%) were non-significantly different, 13/36 (36.1%) showed significance at p<0.05, and 3/36 (8.3%) at p<0.01. Hence, some pairwise comparisons were marginally significant and showed a slightly increased divergence between aphids infesting certain trial plots. For example, whilst there is no clear overall trend, there was found to be some slightly higher degree of subpopulation divergence between alfalfa (mid.) and 3/5 (60%) of the field margin comparisons (Table 2b, column 1), clover (mid) and pea (mid.) directly, and clover with 3/5 (60%) of the field margin comparisons (column 2). Of the 15 direct field margin comparisons (columns 4-8), 6/15 (40%) were marginally significantly different (p<0.05).

Discussion

The results show that pea aphids are under various selective pressure arising from both higher (predators) and lower (host plant) trophic levels. The data in relation to green and red aphid morphs was found to vary considerably between host plants, with red morphs almost absent in pea crops. In the case of different host plants attacked by one pest aphid species, and when these crops are planted in close proximity to one another forming a complex habitat, a strong host plant influence on aphid predation was seen. Both the number of aphids and the number of predators are much higher on the central plants (e.g. 38 aphids on pea, 38 aphids on alfalfa, 32 aphids on clover) than on the edges (e.g. 12 aphids on pea, 7 aphids on alfalfa and 4 aphids on clover). For all pea plots, spider density was higher in close proximity with both alfalfa and clover crops (Fig. 2B). Such high spider density reflects their preference for aphids as prey in pea, in turn impacting upon green pea aphid morph abundance.

In previous studies (Balog 2013, Balog & Schmitz 2013b), the presence of natural enemies and host plant are together seen to clearly influence pea aphid population growth rate in



Figures 3A, B, C. The relative fitness of aphids during the second field experiment (the survived offspring number at the end of the experiment and reported to the initial number of adults) in control cages with no predators (A) and with predators (B), and parasitized aphids (C), respectively, at the end of the experiment. Parasitoids had equal access to both control and predator cages, but only control cages were considered for the parasitoid analyses.

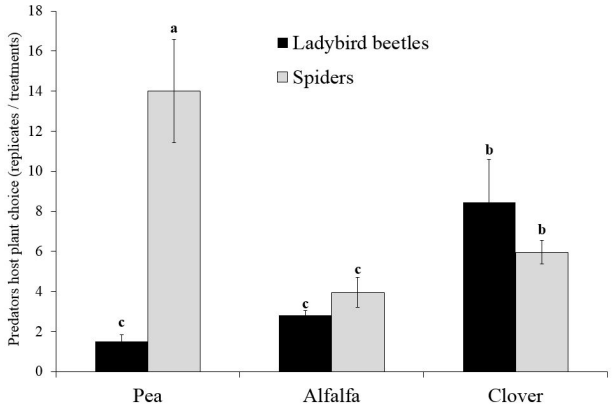


Figure 4. Predator preferences (ladybirds and spiders) for pea aphids on the different host plants tested. Bars in figures represent the average predator choice per replicates per treatments between host plants from 30 cages (predators inside) involving 33 observations / cage.

terms of the relative abundance of the two colour morphs. Several complex factors, however, influence pea aphid predation on different host plants, regardless of aphid morph colour. The population density of pea aphids was higher on pea compared with clover and alfalfa (Fig. 2), and a higher aphid reproduction rate on pea seemingly has an influence on predator density. Plants have a number of different defences against aphids and the ability of a aphid to feed well and grow fast depends on its ability recognize a plant as a potential host, to tolerate the chemical defence of plant and to manipulate the plant in a way to avoid the activation and induction of further defences. Different plant species have different sizes and therefore different levels of resources, a supposition requiring further study. Predators may also be differentially attracted by certain plant structures and quality. Reasons for the observed asymmetry between predator densities include: (i) variations in habitat complexity (i.e. leafiness) of host plants; and/or (ii) difference in trait-mediated effects. The higher leaf complexity (leaf density) of red clover and/or alfalfa plants compared with pea plants may influence arthropod predator behaviour through an indirect trait-mediated effect. To search for prey and to locate proper habitat patches species of arthropod predator (ladybird beetles) use chemical cues emanating from the host plant.

The strength and the quality of the cue released by host plants can vary between plant species and their arthropod pests; together these could indicate to predators the presence of qualitatively higher prey (Chaneton & Bonsall 2000). This may be the case of the asymmetry in predator abundance, and possible higher predation rate by ladybird beetles on both clover and alfalfa. Previous studies have revealed that specialist predators (here ladybird beetles) feeding on aphids infesting legumes (clover and alfalfa) have to consume a low amount of prey individuals to fulfil their physiological demands (Diehl et al. 2013). The higher ladybird density on clover and alfalfa may relate to the predators acquiring their nitrogen requirements for growth and reproduction more readily by eating pea aphids on these plants. Another factor influencing differential predator density may be the feeding habits and amounts of aphids that the different predators consume. Since ladybirds are specialist aphid predators (but also use visual signals for predation), they probably usually consume more aphids (especially red morphs) in a given amount of time than spiders do. Spiders are, in contrast, polyphagous predators, so that *intra*-guild predation is likely to occur at the same time as consumption of aphids by ladybirds. According to Bond (2007), it may be difficult for predators to simultaneously search for two cryptic prey species than to search for one only. Thus, visual predators tend to focus on more visible colour morphs (i.e. red colour pea aphids in this case) and neglect the more cryptically con-

Table 2.A,B. (a) Pea aphid green morph genetic analyses (samples from the first field experiment) and (b) genetic differentiation according to F_{ST} pairwise values (over all five polymorphic microsatellite loci) between all nine host-population samples tested, i.e. pea, alfalfa and clover field sample plot middles and margins. Alf(Pea) means aphid samples from alfalfa with pea margins. Same for the other samples.

(A)

Samples host plant	*N*	Average no. alleles/locus	*Ho*	*He*
Alfalfa mid.	60	10	0.630	0.881
Clover mid.	60	11	0.550	0.840
Pea mid.	60	15	0.970	0.861
Alf(Pea) margins	51	10	0.833	0.757
Pea(Alf) margins	51	15	0.982	0.856
Alf(Clov) margins	48	8	0.568	0.639
Clov(Alf) margins	48	11	0.552	0.841
Clov(Pea) margins	51	10	0.533	0.866
Pea(Clov) margins	51	14	0.961	0.861

(B)

Samples/ Host plants	Alfalfa mid.	Clover mid.	Pea mid.	Alf(Pea) margins	Pea(Alf.) margins	Alf(Clov.) margins	Clov(Alf) margins	Clov(Pea) margins
Alfalfa mid.	0.02^{ns}	-						
Clover mid.	**0.09****	**0.07***	-					
Pea mid.	0.01^{ns}	**0.07***	0.02^{ns}	-				
Alf(Pea) margins	**0.06***	0.02^{ns}	0.03^{ns}	0.02^{ns}	-			
Pea(Alf) margins	0.01^{ns}	0.02^{ns}	**0.10****	0.01^{ns}	0.02^{ns}	-		
Alf(Clov) margins	0.02^{ns}	0.01^{ns}	**0.11****	**0.06***	**0.06***	0.02^{ns}	-	
Clov(Alf) margins	**0.06***	**0.07***	0.03^{ns}	0.03^{ns}	0.04^{ns}	**0.06***	**0.06***	-
Clov(Pea) margins	**0.06***	**0.07***	0.02^{ns}	0.02^{ns}	0.03^{ns}	**0.06***	**0.06***	0.02^{ns}

Explanations: ns – non significant, * - $p<0.05$, ** - $p<0.01$, (F-statistics), N - sample sizes for each subpopulation tested; *Ho* – frequency of observed heterozygosity; *He* – frequency of expected heterozygosity, assuming population to be in HWE.

cealed morphs (green pea aphids). In this way, predators induce frequency-dependent selection on pea aphid colour morphs and stabilize the colour polymorphism.

In our study, red pea aphid morphs also differed significantly in terms of reproduction rate, as shown by experiment II. This also has ecological-evolutionary consequences in terms of host plant adaptation and predator avoidance. Red morphs maximize their fitness under enemy-free conditions (Fig. 3A), since ladybirds, as visual predators, mostly predate red rather than green individuals, thereby adding to the predator pressure against red morphs in mixed aphid colour populations (Fig. 3B) (as also demonstrated by (Losey et al. 1997). Alate (winged) morph production of red morphs *vs.* green is also known to be generally higher; on average red aphids produce a higher proportion of winged morphs than green ones under the same environmental-ecological conditions (Weisser & Braendle 2001). Winged individuals are produced under predator pressure (Balog 2013), thereby reducing the predator pressure following colonisation of new, potentially enemy-free host plants (Weisser & Braendle 2001, Kunert et al. 2010). When in the present study no red aphid morphs were found on pea, the survival of green morphs was also found to be low, suggesting that ladybird efficiency is lower on green morphs. If this is so, this further explains the low predation of ladybirds when foraging for aphid prey on pea, and is also a further indication of strong selective pressure on pea aphids from the upper trophic level. Braconid parasitoids, which use chemical cues (plant and aphid) to detect their prey, probably had similar effects on pea aphids on all host plants tested (Fig. 2C, 3C).

Overall, our study reveals that the host plant influence may have important evolutionary significances for traits that influence pea aphid tolerances to different selective pressures. Pea aphids collected from pea or clover and alfalfa neighbouring pea were genetically rather similar (Table 2A,B), as were those collected from pea, or clover and alfalfa neighbouring pea and there was no clear relationship between the average allele number per locus, observed and expected heterozygosity and overall *F*st values among and between host plant sample plots. There was, however, a slightly higher level of sub-population divergence between aphids inhabiting alfalfa and the field margin comparisons (3/5 or 60%) as similarly also observed between aphids inhabiting clover and aphids from plots neighbouring pea crops (Table 2b). Such genetic differences, albeit small, probably reflect the choice that predators make in terms of aphid prey preference in relation to plant host, both intraspecifically and *inter*specifically. Whilst the microsatellite markers are largely selectively neutral (Li et al. 2002), and the microsatellite genotypes as such are thus also largely neutral, they nevertheless undoubtedly relate to genetically distinct aphids, distinct at some point/s in the large genome (~ 530 Mbp) of the pea aphid (Loxdale et al. 2015), perhaps associated with bacterial endosymbiont co-evolution (Oliver et al. 2010, Tsuchida et al. 2010). In our cases differences in abundance of the different aphid genotypes may be also caused by colonisation from adjacent different legume species that harbour genetically distinct aphid populations. Moreover, the general pattern (many spiders on pea, many ladybirds on clover and similar numbers on alfalfa could be found both on central plants and on the edges, but with much lower numbers of predators on the edges, which suggests that predators may not be the main drivers of the observed differences in microsatellite markers. Previous studies have confirmed that both the alfalfa and clover races of the pea aphid can also use pea as a host, but to a lesser extent, whereas the pea and clover races cannot survive on alfalfa (Via 1999, Via et al. 2000, Peccoud et al. 2009b, Peccoud & Simon 2010), a phenomenon related to the nature of the bacterial endosymbiont dependence of aphids when attack-

ing this particular plant species, which tends to induce greater specialism (Ferrari et al. 2012). Therefore, some of the host adapted races are quite limited in their ability to use hosts other than their preferred ones. During our study, mixtures of pea aphid host races were most probably compared on pea plants.

Conclusions

In this study, we focused on understanding 'top-down' and 'bottom-up' effects on natural pea aphid populations in an agro-ecological complex system. Results from several experimental approaches and associated statistical analyses revealed that different host plants, which are suitable for one aphid species, here pea aphid, have a significant influence on predator spatial distribution that differentially influencing prey abundances. The presence of variable host plant complexity was seen to influence the choice of prey of arthropod predators on different host plants, a situation that potentially has important ecological-evolutionary consequences. Such information as here described is needed not only to acquire a better understanding of top-down or bottom-up effects on prey species, but also for the general understanding of modes of sympatric speciation in phytophagous insects general.

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