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Predation Risk and Colony Structure in the Pea Aphid, Acyrthosiphon Pisum

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PREDATION RISK AND COLONY STRUCTURE IN THE PEA APHID,

ACYRTHOSIPHON PISUM

by

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(Under the Direction of Edward B. Mondor)

ABSTRACT

Many organisms live in transient or permanent aggregations to reduce individual predation risk. Hamilton's "Selfish Herd" theory states that an individual should assume a central position within a group to decrease individual predation risk relative to that of its neighbors (i.e., individuals should be selfish). This theory, however, cannot predict the spatial distribution of individuals within clonal aggregations, that is, when individuals are genetically identical (the "evolutionary self"). As aphids (small, herbivorous insects) are parthenogenetic, emit alarm signals, and have high levels of phenotypic plasticity to cope with environmental stressors like predation risk, they are a model organism for investigating the influence of predation risk on clonal aggregations. This research quantifies the proximate ecological factors that influence the feeding site choices of individual aphids within their natal colony and how these factors influence the spatial distribution of individuals after signals of increased predation risk. Here, I find no evidence for a foraging-predation risk tradeoff in feeding site selection within aphid colonies. Predation risk is greatest at particular feeding sites, and juvenile aphids gain no direct fitness benefits from feeding at these "dangerous" sites. When colonies detect an alarm signal, (E)-β-Farnesene (EBF), individuals drop off of the plant and disperse to "safer" feeding sites away from the natal leaf. When solitary pre-reproductive individuals detect EBF, their future

offspring occupy “safer” feeding sites within the natal colony or disperse from the natal leaf (i.e., transgenerational behavioral plasticity). These experiments suggest that selfish behaviors (i.e., which aid in individual survival) may also consequently increase survivorship of the clone. These results may help us better understand “Selfish Herd” theory in clonal aggregations.

INDEX WORDS: Alarm pheromone, Aphididae, Clonal organisms, Colony structure, Foraging-predation risk tradeoff, Phenotypic plasticity, Predation risk, Spatial structure, Transgenerational behavioral plasticity.

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Introduction

Many diverse animal taxa decrease individual predation risk through the formation of transient and continual aggregations. Widely recognized examples include migratory birds, grazing mammals, schools of fish, and migrating insects (locusts, monarch butterflies, etc.). Aggregations, both facultative and obligate, increase the fitness of participant individuals, through dilution effects (Foster and Treherne, 1981), as well as enhanced foraging (e.g., information center effect, Ward and Zahavi, 1973), vigilance (Cameron and Du Toit, 2005), and enhanced reproductive opportunities (Caro, 1994). Assemblages often appear to behave as a single unit; an emergent property of the sum of the behavior of individuals (Parrish and Edelstein-Keshet, 1999; Breder, 1976).

Traditionally, the spatial distribution of individuals within a group has been explained by "Selfish Herd" theory (Hamilton, 1971). This theory predicts that predation risk is lower in the center of a group, than at the periphery or as a solitary individual. Under the threat of predation, each individual in a group will attempt to attain a centralized position, thereby decreasing personal predation risk while raising that of their neighbors. Selfish Herd theory received notoriety for its ability to explain animal aggregations without invoking group selection (Wilson, 1975). Problems arise, however, when attempting to use Selfish Herd theory to explain aggregations of genetically identical (clonal) individuals (Hughes, 1987). Selection should not favor selfishness (i.e. decreasing individual predation risk while increasing your neighbor's risk) when nearby conspecifics are genetically identical (i.e. the "evolutionary individual", Janzen, 1977), unless reproductive value varies between individuals (Fisher, 1930).

Although members within clonal aggregations are genetically identical, individuals may serve ecologically distinct roles within the clone (e.g. defense, Stern and Foster, 1996; dispersal,

MacKay and Wellington, 1975; and reproduction, Francis, 1976). "Fitness Discounting" theory may help explain the colony structures observed in mixed-age clonal aggregations (Duff and Mondor, 2011). Fitness discounting suggests that immediate reproductive opportunities are more highly valued than are future events, which may never occur (Sozou and Seymour, 2003). Reproductive individuals have a greater reproductive value to the clone than do pre-reproductive individuals (Fisher, 1930). It follows that natural selection should favor prompt reproduction (while population size is increasing, Fisher, 1930), and in the case of clonal aggregations, reproductive individuals, those with the highest reproductive value, should assume centralized positions within the colony. In addition, pre-reproductive clone-mates should assume positions with higher predation risk (Mondor and Roitberg, 2004; Duff and Mondor, 2011).

The pea aphid, *Acyrthosiphon pisum*, is a gregarious, sedentary, phloem-feeding insect (Blackman and Eastop, 2000). This aphid is a suitable model organism for studies on spatial structuring in clonal aggregations for several reasons. During the spring and summer, adult females give birth to live nymphs (i.e. viviparity) through parthenogenesis (van Emden and Harrington, 2007). Maternal aphids reproduce continuously, each offspring passing through four juvenile instars before developing into a reproductive adult (5^{th} instar) 7-10 days after birth (Blackman and Eastop, 2000). Continued reproduction by the maternal aphid generates a colony of mixed-age nymphs that feed together but show instar-specific preferences in feeding-site selection (Duff and Mondor, 2011). For defense, these aphids have unique posterior anatomical structures called cornicles through which a droplet of the volatile alarm pheromone E-β-Farnesene (EBF) is emitted when an individual is attacked by a natural enemy (Nault et al., 1973). Reception of EBF elicits a suite of escape responses in nearby conspecifics, such as cessation of feeding, kicking, walking away, or dropping of the plant (Roitberg and Myers,

1978). This altruistic alarm signaling is thought to have evolved through inclusive fitness benefits (i.e. kin-selection, Hamilton, 1964; Mondor and Roitberg, 2004; Wu et al., 2010). Different instars emit varying amounts of EBF in a cornicle droplet (Mondor et al., 2000). Juvenile 2nd and 3rd instars, those that secrete the highest levels of EBF, choose feeding sites anterior to the maternal aphid, where predation risk is higher (Dixon, 1959; Duff and Mondor 2011; *Chapter 2*). Essentially, certain pre-reproductive instars with the greatest alarm signaling potential function as "sentinels" to warn reproductive clone-mates of increased predation risk.

This thesis describes a series of experiments carried out to address two fundamental ecological questions: (1) Is there a foraging-predation risk tradeoff in the feeding site selection of juvenile aphids?, and (2) Given the information in question 1, how does aphid colony structure change after reliable signals of increased predation risk? To determine if there is a foraging-predation risk tradeoff in feeding site selection, I measured the development and fecundity of individual aphids at two locations on a host-plant leaf (Experiment 1). I then conducted an experiment to quantify risk of attack by parasitoids and predators across the same leaf sites (Experiment 2). To investigate explicit colony structure changes after increased predation risk, I carried out an experiment where aphid colonies were exposed to an EBF emission and the proportions of aphids feeding in different areas relative to the maternal aphid were calculated (Experiment 3). I then conducted an experiment where individual 4th instar aphids were exposed to an EBF emission and I calculated the proportion of its future offspring which chose feeding sites in different areas relative to the maternal aphid (Experiment 4).

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Chapter 1

Aphid development and fecundity across leaf sites

Abstract

A key aspect of survival, for all animals, is the ability to locate suitable foraging sites. Particularly for small, relatively sessile organisms, micro-habitat feeding site selection may strongly influence development and performance As pea aphid, *Acyrthosiphon pisum*, juveniles develop, their feeding site selections within the natal colony change. It is unknown whether differential forage quality between feeding sites influences these feeding site changes. Although much is known about the within-plant distribution of aphid feeding sites, very few studies have identified how feeding at different sites on a single leaf may influence aphid life-history traits. I used three-chambered clip cages to isolate first instar aphids on two sections of a broad bean, *Vicia faba*, leaf: i.e., anterior and posterior to an adult aphid, with respect to the leaf petiole. We found that development time to adulthood did not differ across feeding sites, nor did fecundity within the first 24 hours of reproduction. Green aphids produced more offspring than did pink aphids at both feeding sites. These results suggest that there are genotypic differences in offspring production, but not development time, across feeding sites. Thus, nutritional quality of phloem sap at different feeding sites does not appear to be a significant factor for aphid colony structure, and is unlikely to influence any foraging-predation risk tradeoffs.

Introduction

Forage quality is a key factor influencing the location of foraging sites in many organisms, but especially for herbivores whose host plants may be spatially or temporally

variable (Templeton and Rothman, 1974). The relatively poor nutritional quality of plants may have evolutionarily and ecologically constrained phytophagy (e.g. "ecological stoichiometry", Sterner and Elser, 2002; Speight et al., 2008). Host-plant quality is believed to be a key factor in the performance in herbivorous insects, including aphids (Awmack and Leather, 2002).

Many studies have investigated within-plant feeding site preferences of phytophagous insects (e.g., Legrand and Barbosa, 2000; Gould et al., 2007). For example, various stem-borers of the saltmarsh grass *Spartina* occupy different regions of the stem (Strong et al., 1984) and caterpillar development on compound-leaved plants is diminished when feeding on basal leaflets as opposed to lateral or terminal leaflets (Gall, 1987).

Aphids (Homoptera: Aphididae) are moderately sessile, group-feeding, parthenogenetic, phloem-feeding insects (Dixon, 1985). Adult aphids select higher quality host-plants and younger leaves within a plant (Dixon, 1977). Pea aphids, *A. pisum*, feed on the abaxial (i.e., underside) surface of host plant leaves, facing towards the leaf petiole (Dixon, 1985; Hopkins and Dixon, 2000; C.N. Keiser, *unpublished data*). Aphid feeding is believed to increase nutrient availability for aphid aggregations by modifying "source-sink" dynamics of leaves; the infested area is induced to amass excess assimilates, which augments aphid development (Larson and Whitham, 1991). As a positive relationship exists between aphid colony size and fitness, the fitness benefits of group foraging are evident (Clark and Mangel, 1986; Michaud et al., 2006). Aphid feeding, however, has been shown to cause indirect interspecific competition for amino acid content in the phloem of a shared host (Petersen and Sandström, 2001). Salyk and Sullivan (1982), however, showed that pea aphids do not alter their feeding site selections when feeding alongside heterospecific bean aphids, *Aphis fabae*. Indeed, empirical data suggests that pea, *Pisum sativum*, phloem sap alone provides pea aphids with twice their necessary daily caloric

requirements (Barlow and Randolph, 1978). It is possible, though, that even in sessile phloem-feeding insects like aphids, which are birthed live onto a host-plant, micro-habitat feeding site selection may directly influence individual development and performance (Whitham, 1980).

Aphids select higher quality feeding sites within a host-plant, as access to necessary amino acids differs between leaves (Karley et al., 2002). Despite the information available about feeding site selection in phytophagous insects within host plants, very little is known about within-leaf preferences in feeding site selection. In gall-forming aphids, such as *Pemphigus betae*, leaf site selection is influenced by secondary compounds (Zucker, 1982). *Pemphigus betae* prefer to form galls nearer the base of the leaf, where the concentration of phenolic glycosides is lowest (Zucker, 1982). In fact, fitness is drastically reduced in galls that are distally located on leaves (Whitham, 1980). These aphids have also been shown to defend their "micro-territory" at preferable feeding sites (Whitham, 1979).

To our knowledge, only one experiment has explained the development time of non-gall forming aphids feeding at different sites on individual host-plant leaves. Groups of aphids (Black bean aphid, *Aphis fabae;* Foxglove aphid, *Aulacorthum solani;* and Ornate aphid, *Myzus ornatus*) feeding in clip cages attached to Broad Bean, *Vicia faba*, leaves at different locations relative to the leaf petiole showed differences in growth and honeydew production (Lowe, 1967). It was suggested that variation in nutritional quality, or access to phloem sap, at leaf sites augmented aphid performance at sites furthest from the leaf petiole (Lowe, 1967).

Juvenile aphids have been shown to exhibit instar-specific feeding site preferences within the natal colony relative to the position of the maternal aphid (Duff and Mondor, 2011). Juveniles may feed anterior to the maternal aphid (closer to the leaf petiole), posterior to the maternal aphid, or on other leaves. These feeding site preferences may result from the selective

pressures of predation risk on colony structures through the inclusive fitness benefits of alarm signaling (Mondor et al., 2000; Mondor and Roitberg, 2003). It is also possible, however, that there is a difference in forage quality at different leaf sites that influences feeding site selection. Forage quality has been shown to influence the likelihood that aphids will attempt to escape from predators (Dill et al., 1990).

The objective of this study was to empirically determine if differences in the development and fecundity of pea aphids exist when feeding at different distances from the leaf petiole on a single Broad bean leaf. Quantifying development rate and fecundity at different feeding sites for pea aphids will elucidate a potential foraging-predation risk tradeoff in feeding site selection for developing aphids. I hypothesize that juvenile development and fecundity will be augmented when feeding nearer the leaf petiole, as phloem enters the leaf via the petiole thereby providing unrestricted access to nutrients.

Methods

Study Organisms

Pea aphids, *A. pisum*, used in this study were from two asexual lineages (color morphs "pink" and "green") originally collected in Statesboro, GA on wild vetch, *Vicia sativa*, and maintained in the laboratory for approximately 4 years. Aphids were reared on Broad bean, *V. faba* (c.v. Broad Windsor), in mesh bags at 22.4 – 27.1 °C and 27 – 49% RH with a 16:8 L:D photoperiod under one "GE Ecolux Plant & Aquarium" 40W wide spectrum fluorescent bulb and one "GE Residential" 40W bulb (GE Lighting Inc., Cleveland, OH) and watered every 48 hours. Eight – ten days prior to experimentation, aphid colonies were "age structured" by allowing 25 –

30 adults to produce offspring for 24 hours on a single plant, and then removing the adults. All offspring produced in this time period were considered to be 1st instar.

Broad bean seeds of two cultivars (Broad Windsor and Aquadulce) were planted individually in Fafard 3B potting mix (Conrad Fafard Inc., Agawam, MA) in 1L round, black, plastic pots. Plants were kept in the laboratory at the same conditions as aphid rearing. Watered every 48 hours upon seedling emergence, plants were top-dressed with Osmocote 14-14-14 N-P-K slow-release fertilizer (Scotts-Sierra Horticultural Products, Marysville, OH) and transported to a greenhouse where they were ordered randomly and watered daily (16.6 – 48.6 °C, 20 - 69% relative humidity, ambient lighting). After 11-15 days, plants were transported to the laboratory for experiments.

Experimental Procedure

Experimentation took place in three consecutive trials. Prior to experimentation, aphid (green, n = 46; pink, n = 31) and plant (Broad Windsor, n = 44; Aquadulce, n = 33) combinations were randomly determined. A 3-chambered clip cage was attached length-wise to the underside of a fully developed 6 – 8cm leaf along the midrib using two metal all-purpose clips (Brentwood Beauty Labs International, Inc., Dallas, TX; Fig. 1.1). The upper leaf surface was covered with a plastic microscope slide (United Scientific Supplies Inc., Waukegan, IL) to ease attachment of the clip cage to the leaf. A 9-day old single adult aphid was placed in the center cage and a single first instar aphid was placed in each of the lateral cages (henceforth referred to as the "anterior", closest to petiole, and "posterior", furthest from petiole). This arrangement simulates natural conditions, where juvenile aphids feed at sites both anterior and posterior to the maternal aphid (Duff and Mondor, 2011). Once in the clip cage, the aphids were allowed to feed undisturbed for

24 hours, as it can take several hours for an aphid to find a suitable feeding site, and disturbance can result in low estimates of performance (van Emden and Harrington, 2007). After 24 hours, juvenile aphids were checked every 12 hours for evidence of molting (i.e., by the presence of exuviae and overall body size). Clip cages were checked by visual inspection through the mesh covering. Once juvenile aphids reached the adult stage, they were allowed to produce offspring for 24 hours, at which point the number of offspring in each cage were counted. Once the anterior and posterior aphids had finished the 24 hour reproductive period, the number of aphids feeding in the center cage (i.e., all offspring produced by the original adult aphid) were counted.

Statistical Analyses

Aphid development data were analyzed using a 2-way Compound MANCOVA. Independent variables were: plant cultivar (Broad Windsor vs. Aquadulce), aphid genotype (Pink vs. green), and a cultivar × genotype interaction effect. Covariates were: the number of offspring produced by the adult aphid in the center cage ("colony size", 0-66 aphids), plant age (19-22 days), and trial number (1-3). The dependent variable was: the time, in hours, required to reach each developmental stage. A compound dependent variable was used in our model, as: factor 1 – instar (4 levels: 2, 3, 4, Adult) and factor 2 – feeding site (2 levels: Anterior, Posterior), are non-independent factors influencing the time to reach each developmental stage.

The numbers of offspring produced by each aphid in the anterior vs. posterior feeding sites in the first 24 hours of reproduction were analyzed using Repeated Measures MANCOVA with the same independent variables and covariates listed in the previous analysis.

Results

Juvenile aphids did not have different development times when feeding at different sites (Table 1.1). For instar, plant cultivar did not affect the time required for aphids to reach each developmental stage ($F_{3,29} = 0.88$, $p = 0.46$), nor did aphid genotype ($F_{3,29} = 1.92$, $p = 0.15$). The cultivar × genotype interaction was also not significant ($F_{3,29} = 0.16$, $p = 0.92$). No covariate had a significant effect on aphid development time (Colony size: $F_{3,29} = 0.70$, $p = 0.56$; Trial number: $F_{3,29} = 0.53$, $p = 0.67$; Plant age: $F_{3,29} = 1.72$, $p = 0.19$). For feeding site, neither plant cultivar ($F_{1,31} = 0.0006$, $p = 0.98$) nor aphid genotype ($F_{1,31} = 0.01$, $p = 0.92$) had a significant effect on aphid development time. The cultivar × genotype interaction was also not significant ($F_{1,31} = 0.007$, $p = 0.93$). Again, no covariate had a significant effect on development time (Colony size: $F_{1,31} = 0.27$, $p = 0.61$; Trial number: $F_{1,31} = 1.11$, $p = 0.30$; Plant age: $F_{1,31} = 3.03$, $p = 0.09$).

For offspring production overall, plant cultivar was not significant ($F_{1,30} = 1.75$, $p = 0.20$). Aphid genotype, however, was a significant factor in fecundity, as green aphids produced more offspring than pink aphids irrespective of feeding site ($F_{1,30} = 9.04$, $p = 0.0053$; Fig. 1.2). No covariates affected overall aphid fecundity (Colony size: $F_{1,30} = 1.68$, $p = 0.20$; Trial number: $F_{1,30} = 0.26$, $p = 0.61$; Plant age: $F_{1,30} = 0.93$, $p = 0.34$). Looking across feeding sites, there were no significant interactions between independent variables and feeding site (Feeding site × cultivar: $F_{1,30} = 0.27$, $p = 0.61$; Feeding site × genotype: $F_{1,30} = 1.88$, $p = 0.18$; Feeding site × cultivar × genotype: $F_{1,30} = 1.30$, $p = 0.26$), nor were there any significant interaction between covariates and feeding site (Colony size: $F_{1,30} = 1.82$, $p = 0.19$; Trial number: $F_{1,30} = 0.009$, $p = 0.92$; Plant age: $F_{1,30} = 0.02$, $p = 0.90$).

Discussion

The results of this experiment did not support my hypothesis; feeding site location on an individual leaf did not influence aphid development or fecundity. Although development time of juvenile aphids was consistent across feeding sites, green aphids produced more offspring than pink aphids across both feeding sites. Stacey et al. (2003) showed that pea aphid genotypes differ in fecundity, but not development time.

In addition to the direct fitness consequences of feeding site selection, choosing a "micro-habitat" must also be considered from the perspective of a predation risk landscape (Thomson et al., 2006). Predation on aphid colonies is exceptionally high and thought to be the key selective pressure on the evolutionary ecology of aphids (Frazer et al., 1981; Dennis and Wratten, 1991). Due to the foraging strategies of aphid predators (i.e. ladybird beetles), aphids feeding at sites nearest the petiole are at a higher risk of predation (Dixon, 1959; *Chapter 2*). Green pea aphid juveniles are more likely than pink juveniles to select feeding sites anterior to the maternal aphid (Duff and Mondor, 2011). Aphids feeding in these areas are considered "sentinels" for warning nearby colony members of pending predator attacks. When attacked by a natural enemy, an aphid will emit a volatile alarm pheromone, E-β-Farnesene (Dixon, 1958), allowing clone-mates an opportunity to escape predation (Roitberg and Myers, 1978; Mondor and Roitberg, 2004).

It is unknown if individual feeding site choices by juvenile aphids affect the overall fitness of the colony (i.e. if more aphids feeding anteriorly increases colony density over time), though one would expect that these individual choices should not influence colony success. Models of population dynamics suggest that if foraging site selection were preemptive (i.e., choosing a high quality site prevents others from access to that site), then reproductive success should decrease with group size (Pulliam and Danielson, 1991), which is not the case with

Macrosiphinae aphids (but see Morris and Foster, 2008 for the subfamilies Pemphiginae and Hormaphidinae). Manipulation experiments are needed to test the hypothesis that the spatial distribution of aphids across a leaf landscape can influence the overall fitness of the group.

This experiment is unique in that the habitats are not geographically isolated, but rather are only separated by a few centimeters. Also, it should be noted that pea aphids are capable of moving throughout the colony as they transition to new instars, so feeding site selection is not static but dynamic (Duff and Mondor, 2011). Further studies are required to determine if differential feeding site selections over time influences individual and colony fitness.

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Table 1.1: Mean development time from first instar to fifth instar (adulthood), and fecundity in the first 24 hours of adulthood, of Pea aphids at two feeding sites on two Broad bean cultivars.

Plant Cultivar	Aphid Genotype	Feeding Site	Development time (hours ± 1 SE)				Offspring in first 24 hours
			2^{nd} Instar	3^{rd} Instar	4^{th} Instar	Adult	
Broad Windsor	Green	Anterior	27.2 ± 2.9	60.0 ± 3.0	106.1 ± 6.1	144.0 ± 6.1	8.0 ± 0.9
		Posterior	30.0 ± 1.9	64.0 ± 2.6	108.5 ± 4.1	143.0 ± 2.9	6.9 ± 0.9
	Pink	Anterior	46.8 ± 7.3	89.6 ± 7.6	129.6 ± 7.6	159.6 ± 5.1	4.7 ± 1.1
		Posterior	33.3 ± 3.9	74.7 ± 5.6	122.8 ± 7.9	161.3 ± 7.0	6.7 ± 1.8
Aquadulce	Green	Anterior	29.1 ± 1.6	61.7 ± 3.0	100.3 ± 3.2	150.0 ± 3.5	6.7 ± 1.3
		Posterior	29.1 ± 1.9	63.8 ± 1.8	104.2 ± 3.1	147.2 ± 3.4	7.1 ± 1.0
	Pink	Anterior	31.5 ± 3.9	76.5 ± 5.0	120.0 ± 8.2	165.5 ± 6.6	4.1 ± 1.6
		Posterior	35.0 ± 3.5	73.0 ± 4.5	122.0 ± 7.2	163.0 ± 6.0	3.8 ± 0.7

Figure Legends

Figure 1.1. Clip cages used to restrict aphid foraging to a 2 cm diameter space in the anterior or posterior section on the underside of Broad bean leaves.

Figure 1.2. The average number of offspring produced by pea aphids of two genotypes in the first 24 hours of adulthood.

Figure 1.1.

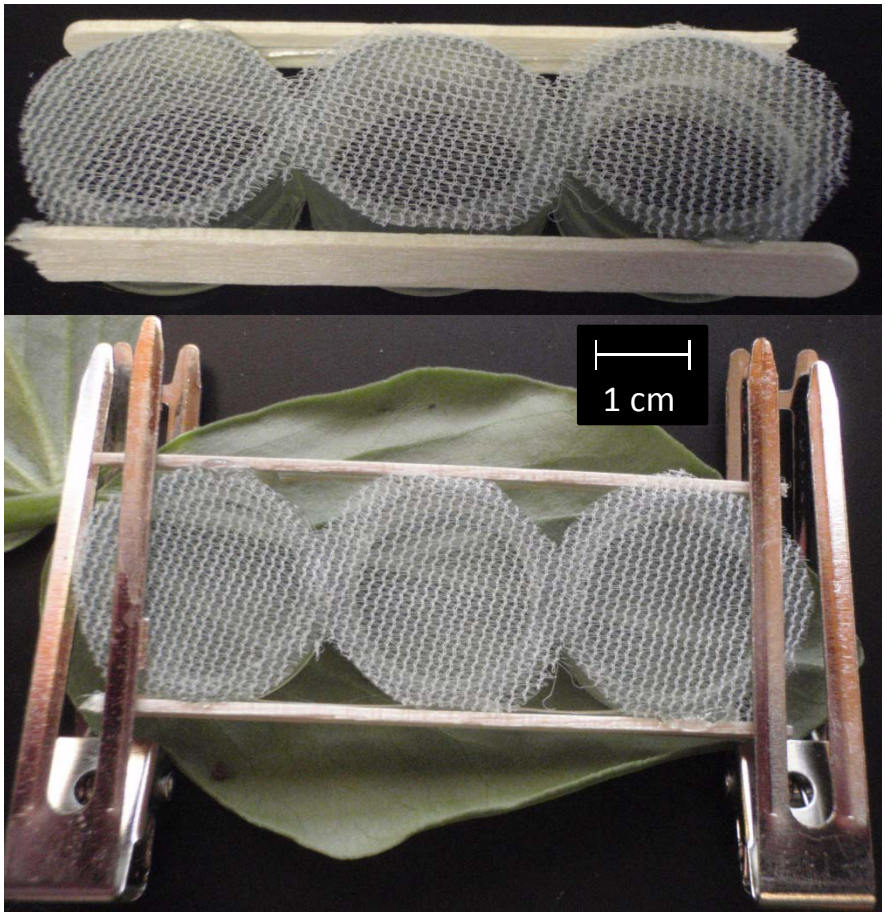
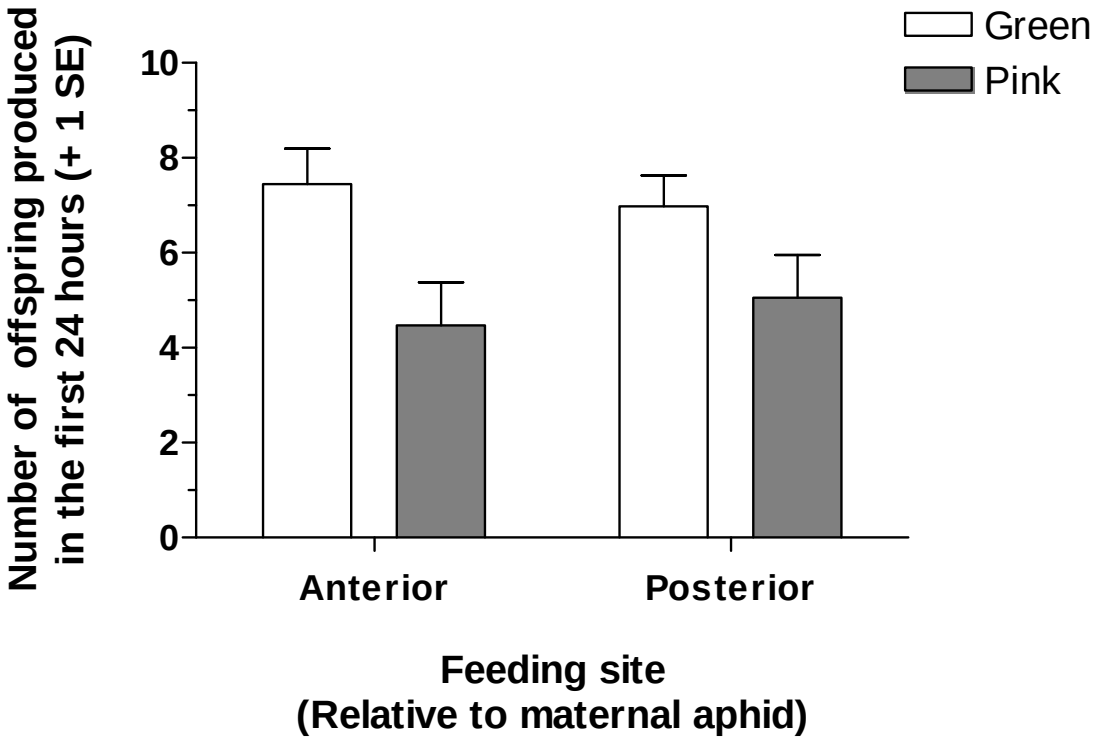


Figure 1.2.


Green
Pink
Number of offspring produced in the first 24 hours (+ 1 SE)
10
8
6
4
2
0
Anterior
Posterior
Feeding site
(Relative to maternal aphid)

Chapter 2

Parasitism and predation risk across leaf sites

Abstract

Predation risk is a key selective force on traits in aphid populations. No study, however, has yet quantified predation risk across feeding sites within a single aphid colony. Here, a simple experimental assay was used to record the frequency of natural enemy attacks (i.e. coccinellid predation and parasitoid wasp oviposition) on pea aphids, *Acyrthosiphon pisum*, adhered linearly to the underside of a broad bean, *Vicia faba*, leaf. For each trial, the location of attack at three leaf sites (Anterior, Middle, Posterior) was recorded and the time required for each natural enemy to find a prey item was assessed. This experiment showed that predation risk was greatest for aphids at feeding sites nearest the leaf petiole for foliar foraging predators, but not for parasitoids. No independent variables (aphid color morph, aphid age, or plant cultivar) affected foraging preferences for predators and parasitoids, nor did they affect the time until natural enemy attack. Leaf size, however, was positively correlated with foraging time for predators, but not parasitoids, which is consistent with the foraging styles of each natural enemy (i.e., predator, but not parasitoid, foraging is confined to the 2-dimensional plane of the leaf surface). These results suggest that, irrespective of aphid genotype, age, or host-plant variety, the risk of mortality by predation is greatest at feeding sites nearest the petiole. Thus, predation risk may be the key factor for aphid colony structure, strongly influencing feeding site selection.

Introduction

Predation is one of the key agents of natural selection on prey populations (Fisher, 1930; Lima, 2002; Calsbeek and Cox, 2010). Insect model systems have long been used to assess ecological paradigms in predator-prey dynamics (Dixon, 2000; Speight et al., 2008). Despite an extensive history of research, even well-understood systems like the foraging strategies of aphidophagous ladybird beetles can be better understood by contemporary research (e.g., Dixon, 2000). In fact, "Ecology of Aphidophaga" is an international symposium held every three years to discuss current discoveries on the ecology and behavior of aphid natural enemies.

Aphids (Homoptera: Aphididae) are small, group-living, phloem-feeders that, during warmer months, reproduce through apomictic parthenogenesis forming large colonies of highly related individuals (Dixon, 1998). Aphid populations are attacked by predators (mainly larval and adult ladybirds - Coccinellidae, larval lacewings – Chrysopidae, and larval hoverflies – Syrphidae), parasitoid wasps (Braconidae – subfamily Aphidiinae, some Aphelinidae), and are at risk of mortality by some pathogens (Völkl et al., 2007). Ladybird beetles are thought to the main agent of natural selection on aphid populations (Frazer et al., 1981; Dennis and Wratten, 1991; Völkl et al., 2007), though both predators and parasitoids are efficient enough to function as biocontrol agents for certain aphid species (Dixon, 2000; van Emden and Harrington, 2007).

Pea aphids, *Acyrthosiphon pisum*, and their natural enemies have been used as an experimental model for understanding predator-prey dynamics. The pea aphid exhibits a stable genetic color polymorphism ("pink" and "green" phenotypes), where parthenogenetic individuals give birth to offspring of the same color (Markkula, 1963; Tomiuk and Wohrmann, 1982). Clonal aggregations of *A. pisum* exhibit age-specific spatial distributions, where juveniles choose feeding sites anterior and posterior to the maternal aphid (i.e., "anterior" and "posterior" means

in front of or behind the maternal aphid; Duff and Mondor, 2011). Aphids feed facing the leaf petiole; therefore juveniles feeding anteriorly to the maternal aphid are closer to the leaf petiole (Dixon, 1985; *Chapter 1*). When attacked by a natural enemy, pea aphids emit a droplet containing an alarm pheromone (E-β-Farnesene, Dixon, 1958; Nault et al., 1973), prompting defensive behaviors in clone-mates, such as walking away and/or dropping of the plant (Roitberg and Myers, 1978; Braendle and Weisser, 2001). The quantity of EBF in cornicle droplets differs between ontogenetic stages; juvenile instars that more readily choose "anterior" feeding sites (2^{nd} - 3^{rd} instars) produce more alarm pheromone than their younger kin (Mondor and Roitberg, 2002). Alarm signaling serves to warn nearby clone-mates of increased predation risk, thereby enhancing clonal survival (Mondor et al., 2000). The utility of this alarm signaling behavior may, however, largely depend on the natural enemy guild attacking the colony.

Coccinellids employ both visual and chemical cues in finding patches of prey (Nakamuta, 1984; Ninkovic et al., 2001). Larvae and adults display stereotyped foraging behaviors on host plants guided by a positive phototaxis and negative geotaxis (Dixon, 1959; 2000). This behavior results in foraging predators crawling onto leaf surfaces via the petiole (Frazer and McGregor, 1994), whereupon prey encounters induce an area-restricted "intensive" foraging characterized by positive klinotaxis and negative orthokinesis (Carter and Dixon, 1982).

Parasitoid foraging follows a hierarchical searching model (Hassell and Southwood, 1978; Völkl, 2000), where adults exhibit in-flight searching for prey habitats (i.e., "patches") using infochemicals from aphid-damaged plants to find their hosts (Wellings, 1993; Guerrieri et al., 2002). Once an infested plant is located, parasitoids further exploit host-derived compounds (e.g. honeydew, Budenberg, 1990; sex pheromones, Powell et al., 1993; and alarm pheromones, Battaglia et al., 1993) to find aphid colonies. Female parasitoids use tactile and contact chemical

cues to assess the suitability of individual aphids for oviposition (Pennacchio et al., 1994). Middle-instars Pea aphids are preferentially chosen (He and Wang, 2006), and green morphs are preferred over pink morphs (Li et al., 2002). In addition to host age and color, host defensive behaviors can influence the oviposition success of parasitoid wasps (Gerling et al., 1990). Some parasitoid species have equal success in attacking aphids feeding peripherally and centrally in colonies (Völkl, 1990).

Field studies have shown that natural enemy guilds impose differential rates of mortality, via predation and parasitism, on aphid colonies, and can have different effects on aphid population suppression (Costamanga et al., 2008; Raffel et al., 2008). The major difference between the foraging strategies of these natural enemy guilds is that predator foraging is limited to the "plane" of the leaf surface (i.e. two-dimensional foraging, Dixon, 2000) while parasitoid foraging is in three dimensions (Ayal, 1987) (Hamilton, 1971; Romey et al., 2008). Does this fundamental contrast, coupled with the foraging preferences exhibited by each guild, result in differential risk of mortality for individual aphids feeding at different sites on a leaf (i.e., at different locations within a colony)? If so, this differential risk between predation and parasitism may impose different selective forces on the evolution of feeding site selection and defensive behaviors in aphids. I hypothesize that that predators will most often attack prey at sites nearest the petiole while parasitoids will choose prey equally at all sites.

Methods

Study Organisms

Broad bean, *Vicia faba* (c.v. Broad Windsor, BW; Aquadulce, AD), seeds were planted individually in Fafard 3B potting mix (Conrad Fafard Inc., Agawam, MA) in 1L round, black,

plastic pots. Plants were kept in the laboratory at 23.2 – 26.7 °C with a 16:8 L:D photoperiod under one "GE Ecolux Plant & Aquarium" 40W wide spectrum fluorescent bulb and one "GE Residential" 40W bulb (GE Lighting Inc., Cleveland, OH) and watered every 48 hours. Upon seedling emergence, plants were top-dressed with Osmocote 14-14-14 N-P-K slow-release fertilizer (Scotts-Sierra Horticultural Products, Marysville, OH) and moved to the greenhouse where they were watered daily (17.9 – 38.0 °C, 20 – 77% RH, ambient lighting). After 9 – 10 days, plants were transported to the laboratory for experiments.

Pea aphids, *Acyrthosiphon pisum*, used in this study were of two genotypes ("pink" and "green" color morphs) originally collected at Eagle Creek (Georgia Southern University, Statesboro, GA) on *Vicia sativa* and maintained asexually in the laboratory for approximately 4 years. Aphids were reared on *V. faba* (c.v. Broad Windsor), in mesh bags at the laboratory conditions described above. Nine days prior to experimentation, aphid colonies were age structured by allowing 20 – 30 adults to produce offspring for 24 hours on a single plant, and then removing the adults in order to obtain large numbers of first-instar aphids.

Parasitoid wasps, *Aphidius ervi*, were reared in growth chambers (21.0 – 22.0°C, 23 – 50% RH, 16:8 L:D photoperiod) enclosed in mesh bags containing mixed-age Pea aphids feeding on Broad bean. Experimental wasps were collected as adults from the colonies, using an aspirator, and isolated in plastic specimen jars immediately prior to experimentation. Adult Convergent ladybird beetles, *Hippodamia convergens*, were purchased from a commercial supplier (Ladies in Red©, Bend, OR 97709). Beetles were separated in groups of ~30 in 10cm × 1.5cm plastic petri dishes, provided with a moist sponge and ~50 mixed-age aphids, and maintained under the same laboratory conditions described above. 48 hours before experimentation, beetles were placed in new petri dishes and starved.

Experimental Procedure

Experimental Broad bean plants were prepared by excising all but one leaflet to restrict natural enemy foraging. Plant height, leaf length, and leaf width were measured to the nearest 0.1cm. Three aphids, either juveniles (2^{nd} – 3^{rd} instar) or adults, were adhered linearly along the midrib of the underside of the leaflet using $2mm^2$ pieces of acid-free, double sided tape (Duck® Brand, Henkel Consumer Adhesives, Inc., Avon, OH 44011). Aphids were placed 2cm from each other, starting at the base of the leaf, where the leaflet meets the petiole (these ordinal sites are henceforth referred to as "anterior", "middle", and "posterior"). Predator and parasitoid trials were run separately. For predator trials, a single adult beetle was placed at the base of the plant. A 400 mL (110mm × 77mm) beaker was then placed over the plant to prevent the natural enemy from leaving the plant. For parasitoid trials, three adult parasitoids were shaken directly into a 400 mL beaker from the plastic specimen jars, and the beaker was placed over the experimental plant. Sex determination in *A. ervi* is difficult, so three wasps were applied to each replicate to increase the possibility that at least one of the individuals was a gravid female. A natural enemy was allowed to forage the plant and attack an aphid (i.e. eaten or oviposited in) for 30 minutes. The time until attack was recorded; if an attack did not occur within 30 minutes, the replicate was terminated. All trials were conducted in the laboratory at the same conditions described above.

Statistical Analyses

Frequencies of attacks at each location, for each natural enemy, were compared using chi-square analyses. Subsequently, two ordinal logistic regressions were performed to determine which factors better explain the pattern of attacks by each natural enemy guild. The independent

variables for these analyses were: plant cultivar (Broad Windsor vs. Aquadulce), aphid genotype (Pink vs. Green), and prey age (Juvenile vs. Adult). Covariates were leaf area (length × width) and plant height. The dependent variable was the location of attack (Anterior, Middle, and Posterior).

To analyze the time required for each natural enemy to find a prey item, a 3-factor ANCOVA was used, using the same independent variables as in the previous analysis. The dependent variable was natural enemy foraging time, transformed ($x' = \sqrt{x}$). Lastly, linear regressions were used to determine if a relationship existed between leaf size (length × width) and foraging time for each natural enemy guild.

Results

Predatory ladybird beetles attacked aphids at the anterior site more readily than at any other site ($\chi^2_2 = 62.30$, $p < 0.0001$, Fig. 2.1), while parasitoid wasps did not show a preference for prey sites during oviposition ($\chi^2_2 = 2.30$, $p = 0.32$, Fig. 2.1). In the ordinal logit analysis, no independent variable significantly predicted attack location for predators (Plant Cultivar: $\chi^2_{1,5} = 2.28$, $p = 0.13$; Genotype: $\chi^2_{1,5} = 1.85$, $p = 0.17$; Age: $\chi^2_{1,5} = 0.09$, $p = 0.76$; Table 2.1), nor were any covariates significant (Plant Height: $\chi^2_{1,5} = 0.26$, $p = 0.61$; Leaf Area: $\chi^2_{1,5} = 2.05$, $p = 0.15$; Table 2.1). In addition, no independent variables were significant predictors for attack location by parasitoids (Plant Cultivar: $\chi^2_{1,5} = 0.21$, $p = 0.64$; Genotype: $\chi^2_{1,5} = 0.03$, $p = 0.87$; Age: $\chi^2_{1,5} = 1.40$, $p = 0.24$; Table 2.1) and no covariates were significant (Plant Height: $\chi^2_{1,5} = 0.03$, $p = 0.87$; Leaf Area: $\chi^2_{1,5} = 0.05$, $p = 0.83$; Table 2.1).

The ANCOVA showed that no independent variables in this study significantly influenced the time until initial predator attack (Plant Cultivar: $F_{1,5} = 0.04$, $p = 0.83$; Genotype:

$F_{1,5} = 0.58$, $p = 0.45$; Age: $F_{1,5} = 0.01$, $p = 0.92$; Table 2.1), and plant height was not a significant predictor ($F_{1,5} = 0.0003$, $p = 0.99$). Leaf size, however, significantly affected predator foraging time ($F_{1,5} = 5.54$, $p = 0.02$). The time until parasitoid oviposition was not influenced by any independent variables (Cultivar: $F_{1,5} = 0.001$, $p = 0.97$; Genotype: $F_{1,5} = 0.03$, $p = 0.86$; Age: $F_{1,5} = 0.34$, $p = 0.57$; Table 2.1), and no covariates were significant predictors of parasitoid foraging time (Plant Height: $F_{1,5} = 0.14$, $p = 0.71$; Leaf Area: $F_{1,5} = 0.09$, $p = 0.76$; Table 2.1). Linear regression showed that it takes longer for predators to find prey on leaves of greater surface area ($F_{1,55} = 5.99$, $p = 0.02$, Fig. 2.2a). This trend was not observed for parasitoid foraging ($F_{1,55} = 0.02$, $p = 0.89$, Fig. 2.2b).

Discussion

These results support the hypothesis that the risk of predation by Coccinellid beetles is greatest for individual aphids feeding near the leaf base, compared to those at other feeding sites. This trend, however, is not conserved across different natural enemy guilds.

Alarm pheromone droplets are secreted though posterior anatomical structures called cornicles (Nault et al., 1973). These tube-like structures are ambulatory with regards to the angle at which they can be articulated (Strong, 1967), such that they may daub an alarm pheromone-containing secretion onto the cuticle of an attacking natural enemy (Mondor and Roitberg, 2004). A predator smeared with a cornicle secretion may have decreased foraging capabilities (Dixon, 1958), but the scent-marked predator also warns nearby aphids of increased predation risk (Mondor and Roitberg, 2004). The direct defensive benefits of cornicle secretions are not believed to be effective against parasitoid foraging (Goff and Nault, 1974).

The traits that make certain age classes of pea aphids better alarm signalers (i.e., cornicle length, proclivity of EBF emission, amount of EBF/cornicle droplet) are greatest in those instars who choose feeding sites near the petiole during colony formation (Duff and Mondor, 2011). The data presented here suggests that predation risk is greatest at these sites, so these traits may have arisen through differential predation risk and the inclusive fitness benefits of alarm signaling (Mondor and Roitberg, 2004). Parasitism risk, however, was not different across the leaf. The suite of defensive behaviors in *A. pisum* has evolved in response to a suite of natural enemies. The preferences of different natural enemy guilds are believed to have imposed balancing selection maintaining the color polymorphism in pea aphids (Losey et al., 1997). In addition, the effects of multiple predators on prey populations (i.e., "multiple predator effects") are often greater than simply the sum of the effect of each predator type (Sih et al., 1998).

Foraging time was not influenced by aphid color or cultivar. Although many Coccinellids use visual cues in close-proximity foraging and have preferences between different aphid color morphs, this trend is not observed in Convergent ladybird beetles (Harmon et al., 1998). These results may differ if other Coccinellid species were tested. That leaf area was positively correlated with foraging time for Convergent ladybird beetles, but not parasitoids, is consistent with the small-scale foraging styles of each natural enemy. Predators find prey through a systematic search of leaves within a plant (Dixon, 2000), while parasitoids may land directly on a leaf site containing hosts (Ayal, 1987). The correlation coefficients for both analyses were quite small, however, suggesting that leaf area may not be as important as other factors (e.g., prey feeding site).

Some laboratory-based experiments on natural enemy foraging may not be indicative of natural conditions and may be artifacts of experimental design (Dixon, 2000). Some studies have

addressed predation concurrently in field and laboratory experiments (e.g., Frazer and Gill, 1981); results are sometimes consistent and sometimes incongruent (Dixon, 2000). More detailed experiments are needed to further elucidate the differences in relative predation risk for aphids from predators and parasitoids. For example, this study used immobile, non-feeding aphids for both natural enemy treatments, though aphid movement influences foraging of the pine aphid parasitoid *Pauesia picta* (Völkl, 2000). Insect foraging is often context- or state-dependent and numerous physiological factors could influence the foraging proclivity of an individual natural enemy (Clark and Mangel, 2000; Roitberg et al., 2010). Several variables not measured in this study, such as female egg load (Minkenberg et al., 1992) could have influenced the tendency of individual wasps to oviposit. Irregardless, this experiment shows a generalized pattern of predation and parasitism across feeding sites within an aphid colony: predators preferentially attack prey nearest the petiole while parasitoids show no preference for host location.

Pea aphid colonies consist of mixed-age juveniles feeding around a maternal adult, at varying distances from the leaf petiole (Duff and Mondor, 2011). Juveniles do not gain any life history benefits, such as decreased development time or increased fecundity, when feeding near the leaf petiole (*Chapter 1*), yet incur a greater predation risk. Therefore, there is no tradeoff between forage quality and predation risk at feeding sites within aphid colonies, suggesting that predation risk may be the primary factor influencing individual feeding site choices and colony structure.

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Table 2.1. (a) An ordinal logistic regression showing the significant influence of independent variables on the frequency of attack by natural enemies at three different feeding sites.

(b) Analysis of Variance showing the influence of independent variables on the foraging time until natural enemy attack.

(a) Attack Site Frequency	**Predator**		**Parasitoid**	
	$\chi^2_{1,5}$	**p-value**	$\chi^2_{1,5}$	**p-value**
Plant cultivar	2.28	0.13	0.21	0.64
Aphid genotype	1.85	0.17	0.03	0.87
Aphid age	0.09	0.76	1.40	0.24
Plant height	0.26	0.61	0.03	0.87
Leaf area	2.05	0.15	0.05	0.83

(b) Foraging Time	**Predator**		**Parasitoid**	
	$F_{1,5}$	**p-value**	$F_{1,5}$	**p-value**
Plant cultivar	0.04	0.83	0.001	0.97
Aphid genotype	0.58	0.45	0.03	0.86
Aphid age	0.01	0.91	0.34	0.57
Plant height	0.0003	0.98	0.14	0.71
Leaf area	5.53	0.02	0.09	0.76

Figure Legends

Figure 2.1. The proportion of Pea aphids attacked by predators (*Hippodamia convergens*) and parasitoids (*Aphidius ervi*) when aphids were adhered to anterior, middle, or posterior leaf sites.

Figure 2.2. The relationship between leaf area (length × width) and foraging time in predator and parasitoid foraging trials.

Figure 2.1.

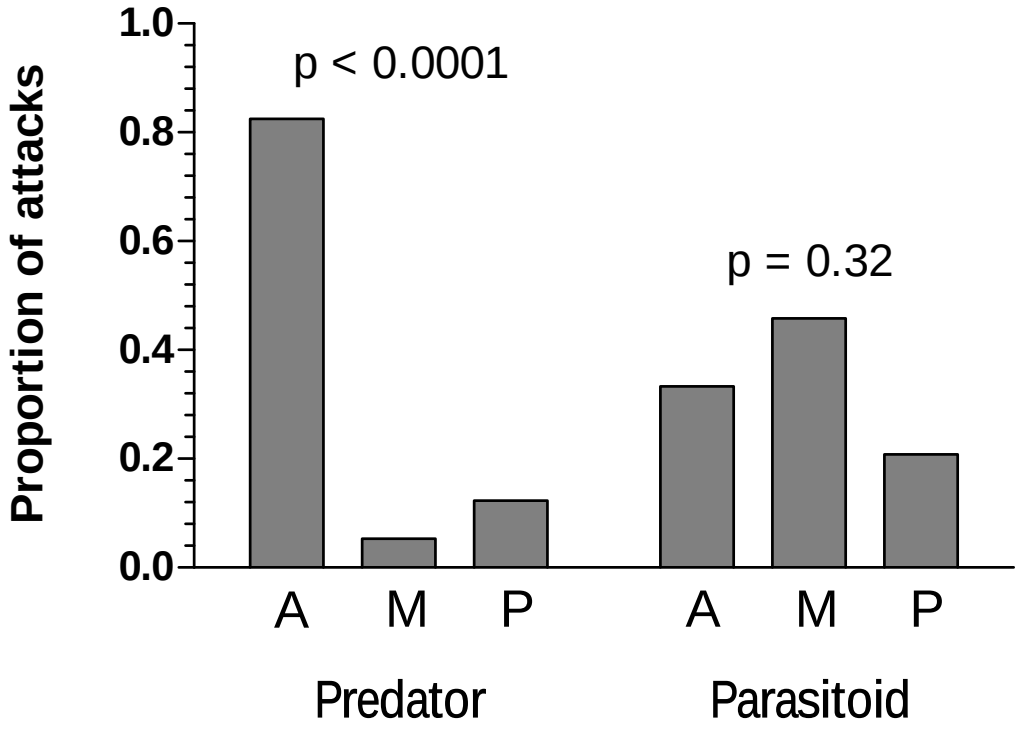
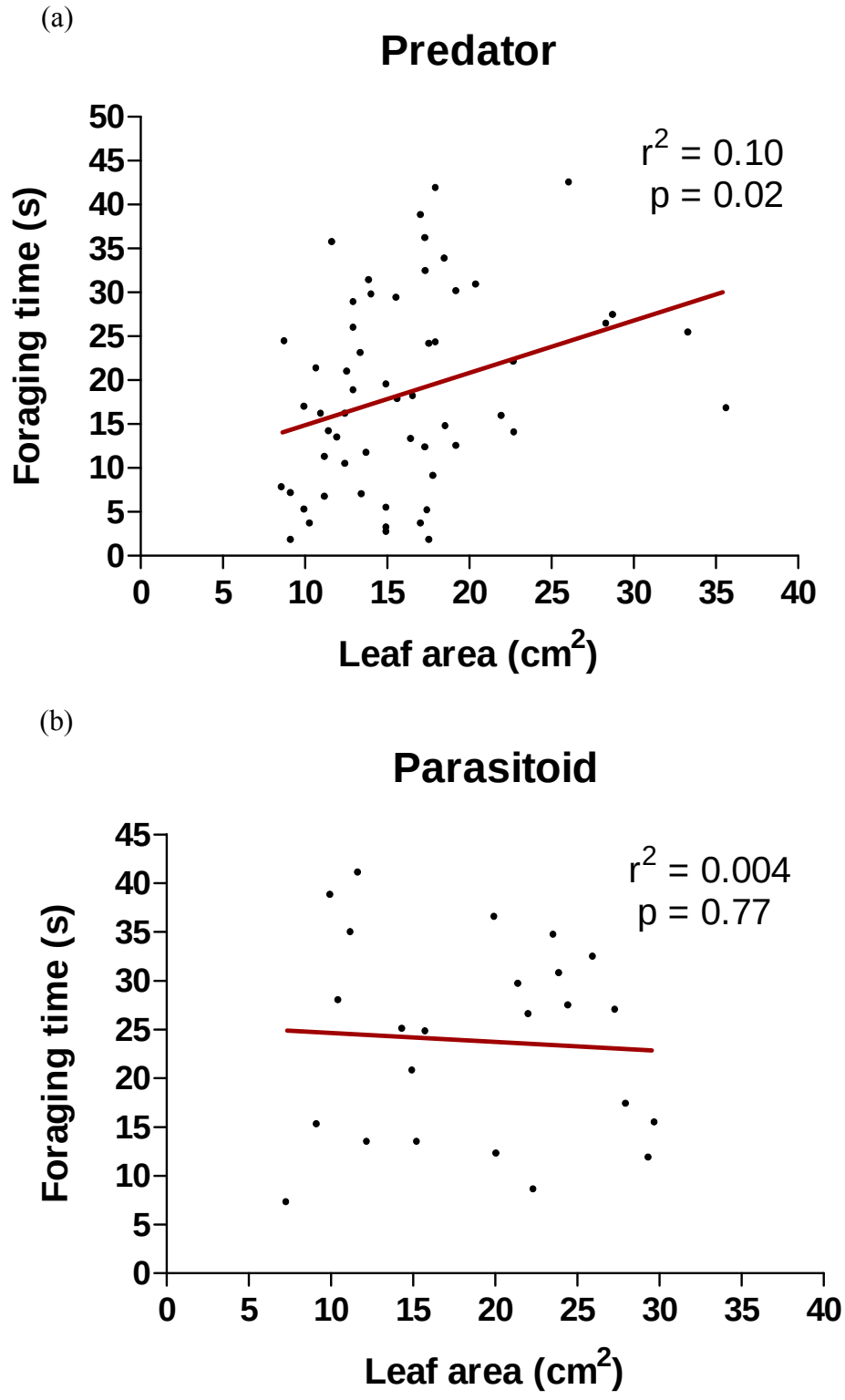

Proportion of attacks
1.0
0.8
0.6
0.4
0.2
0.0
p < 0.0001
p = 0.32
A
M
P
A
M
P
Predator
Parasitoid

Figure 2.2.


(a)
Predator
r^2 = 0.10
p = 0.02
Foraging time (s)
Leaf area (cm^2)
0
5
10
15
20
25
30
35
40
45
50
(b)
Parasitoid
r^2 = 0.004
p = 0.77
Foraging time (s)
Leaf area (cm^2)

Chapter 3

Effects of alarm pheromone on feeding sites in aphid colonies

Abstract

Organisms susceptible to attack by natural enemies often display some form of anti-predator behavior. Pea aphids, *Acyrthosiphon pisum*, which live in clonal aggregations, warn nearby conspecifics of pending attack by secreting a volatile alarm pheromone, (E)-β-Farnesene (EBF). This alarm pheromone allows clone-mates to evade predation by walking away or dropping off the host-plant. Here I show that a large proportion of juvenile aphids feeding around a single maternal aphid exposed to a brief burst of EBF disperse to other leaves on the plant. The proportion of 1st instar juveniles feeding anteriorly to the maternal aphid decreased after experimental treatment, though the proportion of 2nd – 4th instar aphids feeding in this sector remained constant between treatments. After exposure to EBF, maternal aphids settled at feeding sites at different distances from the leaf petiole. This response was genotype specific, as green maternal aphids fed further from the petiole while pink aphids fed closer. These data suggest that there are alternative strategies for individual survival (i.e., dispersal), within and between aphid clones. How these behavioral differences influence clonal survival has yet to be explored.

Introduction

Many animals live in transient or permanent aggregations to reduce individual predation risk (e.g., Dilution Effect, Foster and Treherne, 1981; Encounter Dilution Effect, Mooring and Hart, 1992; Increased Vigilance, Sirot and Pays, 2011). Hamilton's (1971) "Selfish Herd" theory explains how potentially unrelated individuals will form aggregations to decrease individual

predation risk, by increasing that of their neighbors. This theory suggests that individuals compete for safer, centralized sites within the group, thereby increasing the predation risk of others at more peripheral sites which are more vulnerable to attack by predators. Problems arise, however, when attempting to use Selfish Herd theory to explain the structure of aggregations of clonal organisms (i.e., assemblages of individuals produced through parthenogenesis; Hughes, 1987). It is not adaptive to behave selfishly, increasing your neighbors predation risk, when nearby kin are genetically identical to yourself (i.e. the "evolutionary individual", Janzen, 1977). Evolutionarily, a clonal aggregation is a single genetic entity even though it consists of, ecologically, distinct individuals (Hughes, 1987). Selection operates at the level of the individual, but its consequences are conserved across a clonal lineage (Janzen, 1977; Mondor and Messing, 2007; Folse, 2010).

Aphids are small, soft-bodied, group-living insects that feed on phloem (Dixon, 1998). During the summer months, aphids reproduce through parthenogenesis and vivipary, forming colonies of mixed-age clonal individuals (van Emden and Harrington, 2007; but see Lushai and Loxdale, 2002). In pea aphids, *Acyrthosiphon pisum*, juveniles (instars 1-4) can feed in one of three different "sectors": (1) anterior or (2) posterior to the maternal aphid or (3) on other leaves on the host-plant. Middle-instar juveniles are more likely to choose anterior feeding sites (Duff and Mondor, 2011). During early colony formation (i.e., a single reproductive aphid and her juvenile offspring), aphids are most vulnerable to attack by predators, especially coccinellid larvae and adults, and syrphid and chrysopid larvae (Völkl et al., 2007).

When an aphid is attacked by a natural enemy, it often emits a droplet of alarm pheromone (E-β-farnesene, EBF) from tubular, posterior anatomical structures called cornicles (Nault et al., 1973; Mondor and Roitberg, 2002). Alarm pheromone diffuses throughout the

colony, causing nearby aphids to stop feeding, walk/run away, and/or drop off the plant (Dixon, 1958; Montgomery and Nault, 1977). The presence of predators alone can cause apterous aphids to disperse to other plants (Roitberg et al., 1979; Nelson and Rosenheim, 2006). The ontogeny of cornicle length is believed to have evolved as a mechanism for inclusive fitness benefits from alarm signaling (Mondor and Roitberg, 2004). EBF reception sometimes initiates a transgenerational polyphenism whereby winged offspring are born (Podjasek et al., 2005). When these alates reach adulthood, they may disperse to relative enemy-free space on other plants or patches (Dixon and Agarwala, 1999; Sloggett and Weisser, 2002). Cornicles are also moveable, such that an aphid may daub the alarm pheromone droplet onto the cuticle of the predator (Mondor and Roitberg, 2004). As the predator continues to forage through the colony, it becomes a dynamic "messenger" for the alarm pheromone droplet (Mondor and Roitberg, 2004). Therefore, alarm pheromone reception is a reliable signal of immediate predation risk.

Alarm signaling potential (i.e., the proclivity to release a signal, amount of alarm pheromone per cornicle droplet, and cornicle length) varies between individuals at different life stages (Mondor et al., 2000; Mondor and Roitberg, 2002). Since aphids feed facing towards the leaf petiole (at rates >90%, C.N. Keiser, *unpubl. data*), juveniles feeding anterior to the mother are closer to the petiole where predators walk onto the leaf to find prey (Dixon, 1959). Aphids that feed nearest the petiole are at the greatest risk of attack by coccinellid beetles (*Chapter 2*). Despite this risk, 2nd and 3rd instar pea aphids often feed anterior to the maternal aphid, even when other feeding sites are available (Duff and Mondor, 2011). Juvenile aphids that feed at sites nearer the petiole gain no life history benefits (e.g., development time or fecundity; *Chapter 1*). It is hypothesized that individuals with greater alarm signaling potential may function as "sentinels" to warn clone-mates of oncoming predator attack (Mondor and Roitberg, 2002; Duff

and Mondor, 2011). Although the individual will usually die, alarm signaling during an attack is adaptive to the signaler through inclusive fitness benefits from the survival of clone-mates (Mondor and Roitberg, 2004). The surviving kin (including the maternal aphid) are likely to be closer to adulthood, and therefore may have a greater reproductive value to the clone (Fisher, 1930). I hypothesize that the colony structure (i.e. the proportions of juveniles feeding around the mother) will change after reception of EBF. A greater proportion of young juveniles will move anteriorly to the mother to better protect the maternal aphid, and older individuals will move to other plant leaves.

Methods

Study Organisms

Broad bean, *Vicia faba*, seeds were planted individually in Fafard 3B potting mix (Conrad Fafard Inc., Agawam, MA) in 1L black plastic pots. Pots were maintained under laboratory conditions (20.1 – 25.3°C, 28 – 78% RH) with a 16:8 L:D photoperiod under one 40W wide spectrum fluorescent bulb and one residential 40W bulb (GE Lighting Inc., Cleveland, OH) and watered every 48 hours. Upon germination, soil was top-dressed with Osmocote 14-14-14 N-P-K slow-release fertilizer (Scotts-Sierra Horticultural Products, Marysville, OH), moved to a greenhouse, ordered randomly, and watered daily (19 – 38 °C, 27 – 85% RH, ambient lighting). After 17 days, when each plant had at least one fully developed leaf pair, plants were transported to the laboratory for experimentation.

Aphids of two genotypes (green and pink color morphs) were reared separately on broad bean plants under the same laboratory conditions as described above. Nine days before experimentation, approximately 30 adult aphids of each genotype were transferred to two broad

bean plants and allowed to produce offspring for 24 hours. After this time, the adults were removed, permitting us to obtain a large number of 1st instar, equal-aged aphids for experimentation.

Experimental Procedure

Experimental replication occurred over two consecutive trials. Combinations of plant cultivar, aphid genotype, and experimental treatment were randomly generated. Two adult aphids of a single color morph were allowed to feed on a plant, separated from other experimental plants by a shallow water moat. The first aphid to begin reproducing was kept for the remainder of the experiment, and the other aphid was removed. The remaining aphid was allowed to produce offspring continually for 7 days, resulting in a colony of mixed-instar juveniles. Either 1 μL of hexane (control) or 1 μL of EBF diluted in hexane (50ng/μL; Bedoukian Research, Inc., Danbury, CT) was applied to a piece of filter paper with a micropipette and waved beside (within 1 cm) a colony (maternal aphids and juveniles on the same leaf) for 15 seconds. After this time, the treatment was removed. The locations of each aphid on each plant (i.e., in the three sectors: (1) anterior to the mother, (2) posterior to the mother, and (3) on other leaves) were photographed before treatment application and at 1, 3, 6, 12, and 24 hours after treatment using a digital camera (Olympus Stylus Tough-8000, Olympus America, Inc). The proportion of juvenile aphids feeding anterior and posterior to the maternal aphid, and those on other leaves were calculated for each time period.

Statistical Analyses

To better understand how the distribution of aphid feeding sites changed after the two treatments, proportions of each instar in different feeding sectors before treatment application were subtracted from each post-treatment time period to determine the proportional changes in behavior. Thus, a negative proportion represented aphids moving out of a feeding sector after treatment, a positive proportion represented aphids moving into a sector, and a zero represented no change. Proportional changes were analyzed with a Repeated Measures MANCOVA. Independent variables were: broad bean cultivar (Broad Windsor vs. Aquadulce), aphid genotype (Pink vs. Green), and experimental treatment (Hexane vs. EBF). Dependent variables were: the proportional changes in offspring feeding anterior and posterior to the maternal aphid, and those feeding on other leaves. Covariates were: the number of aphids feeding on the same leaf as the mother at each time period ("Colony Size": 1-29 aphids), the amount of hours the maternal aphid remained on the plant ("Longevity":), and the distance from the maternal aphid's feeding site to the leaf petiole ("Mother Distance": 0-60 mm). Colony size was added to the analysis as aphid behaviors can be density-dependent (Kunert et al., 2007). In addition, the distance between the maternal aphid and the petiole may influence the number of offspring that feed in that sector (i.e., it may be a space-limited factor). Post-hoc tests were performed within each sector with t-tests and Tukey's HSD tests, and t-test p-values were adjusted using a sequential Bonferroni adjustment to reduce the likelihood of a type-I error (Rice, 1989).

Results

After exposure to alarm pheromone, most of the aphids, including the maternal aphid, dropped off the host plant, but later crawled back up the same plant. Colonies of aphids treated

with EBF showed a significant increase in juveniles dispersing away from the natal leaf to other areas on the plant compared to control treatments ($F_{2,1022} = 21.42$, $p < 0.0001$; Fig. 3.1; Table 3.1). In addition, there was a significant sector × genotype × treatment interaction ($F_{2,1022} = 10.20$, $p < 0.0001$; Fig. 3.2), though post-hoc analyses showed that the significant differences in the proportions of offspring were only in the "other leaf" sector. Green aphids dispersed to other leaves more frequently after EBF exposure than when exposed to hexane (Tukey's HSD, $p < 0.0001$). It appears that pink aphids migrated to a greater degree under control treatments than experimental, though there is no significant difference in the anterior and posterior sectors. It may be that pink aphids did not migrate to other leaves, but simply walked off the experimental plant, thus removing them from analysis. There was a significant sector × cultivar × treatment interaction ($F_{2,1022} = 3.96$, $p < 0.0001$), though post-hoc analyses were not significant after sequential Bonferroni adjustment. The sector × treatment × instar interaction was significant; 1st instars dispersed to other leaves more than 2nd – 4th instars after EBF reception ($F_{6,2044} = 7.48$, $p < 0.0001$; Fig. 3.3).

All three covariates had significant effects on the data: with increased colony size, more juveniles fed posteriorly to the maternal aphid and less migrated to other leaves ($F_{2,1022} = 8.91$, $p = 0.0001$), more juveniles fed posteriorly to maternal aphids with increased longevity ($F_{2,1022} = 6.56$, $p = 0.0015$), and the proportion of juveniles in the anterior sector increased as the distance of the maternal aphid from the petiole increased ($F_{2,1022} = 17.95$, $p < 0.0001$) (Table 3.1). In addition, the distance at which maternal aphids fed from the leaf petiole differed between treatments and genotypes ($F_{1,7} = 15.15$, $p = 0.0001$; Fig. 3.4). Green maternal aphids moved further from the petiole after EBF reception, though they fed closer to the petiole than did pink aphids under either treatment.

Discussion

These results support the hypothesis that colony structure changes after EBF reception, but do not support the prediction that juvenile aphids will move anteriorly to the maternal aphid. After colony-wide alarm pheromone reception, fewer juvenile aphids aggregated on the same leaf as their mother and more dispersed throughout the plant. After reception of EBF, those that disperse occupy feeding sites individually or in groups on other plants or other leaves on the same plant (Wohlers, 1981). Dispersing to different feeding sites across the plant, after a predation event, can increase the survival of aphid colonies (Francke et al., 2008), though repeated disturbances can reduce the reproductive ability of individuals (Nelson, 2007). Other biotic and abiotic factors influence aphid dropping and subsequent within-plant redistributions (e.g., ambient temperature/humidity, Dill et al., 1990; predator species, Brodsky and Barlow, 1986; forage quality over time, Harrington and Taylor, 1990; the presence of bacterial endosymbionts, Dion et al., 2011).

Aphid colonies of different genotypes may respond differently to alarm pheromone reception, which is consistent with other experiments of genotypic differences in pea aphids (e.g., Braendle and Weisser, 2001; Kunert et al., 2010). There is evidence, however, that genotypic, as well as phenotypic, differences may exist within a single aphid clone (Lushai et al., 1997; Lushai and Loxdale, 2002; Schuett et al., 2011). Aphid responses to repeated predator disturbance are constant and repeatable at the individual level, but not at the level of the clone (Schuett et al., 2011). Therefore, individual behavioral plasticity exists and may account for some variation observed in analyses at the clone level.

The interaction between sector, alarm pheromone exposure, and instar should be investigated further. The results show that only first instars had a significant difference in the

change in proportion in the anterior sector (i.e. the sector of highest predation risk). The 2nd – 4th instar juveniles (i.e. those most likely to function as alarm signalers) showed no between-treatment differences. Stadler et al. (1994) combined empirical evidence with a mathematical model to test the likelihood of leaving a feeding site after predator disturbance depending on an individual's expected total reproductive success. Recently molted adults were more likely to drop off a high-quality feeding site than older adults who have already produced the majority of their offspring (Stadler, 1994). In this experiment, some of the older juveniles (4th instars) may stay in the anterior of the natal colony as they are closer to reproductive age and do not want to leave a quality feeding site, while the younger juveniles (2nd – 3rd instars) may remain in the natal colony to function as alarm signalers for future predation events. The 1st instar juveniles are neither valuable alarm signalers nor close to reproductive status and therefore it is less costly for them to leave the natal colony. In addition to an individual's reproductive value and alarm signaling potential, one's ability to disperse to potentially enemy-free space as an alate can provide a benefit to the survival of the clone (MacKay and Wellington, 1975). The responses of alates to EBF are more sensitive than apterae (Nault et al., 1973). Therefore, the propensity for an individual to decrease its own predation risk leave a feeding site in the natal colony (e.g., dropping and/or dispersal) is state-dependent and consequently may influence clonal survival.

It is surprising that maternal aphids of different genotypes, when surrounded only by their pre-reproductive offspring, settled at different distances from the leaf petiole after EBF reception. Aphids feeding at sites nearest the leaf petiole are at the greatest risk of attack by coccinellid beetles (*Chapter 2*). At this stage of colony growth, the maternal aphid is the only individual currently producing offspring, and therefore has the highest reproductive value (Fisher, 1930; Stadler et al., 1994). In addition, reproductive value is greatest at the age of first

reproduction, so adult aphids are the most valuable to the clone when populations are growing, and should assume safer feedings sites than pre-reproductive offspring (Fisher, 1930; Duff and Mondor, 2011). Some clones of the pink genotype show increased dropping behavior when exposed to artificial stimuli, but not in the presence of natural enemies (Braendle and Weisser, 2001). Although it has been suggested that pink aphids are more conspicuous to foraging predators (Losey et al., 1997), they may be able to obtain information about predation risk by moving closer to the petiole to sense plant vibration produced by predators on the plant stem (Clegg and Barlow, 1982).

These results support the notion that reception of alarm pheromone causes aphid colonies to drop off of the host-plant and subsequently redistribute throughout the host-plant. This study is the first to show, however, that a small proportion of juveniles remain on the natal leaf with the maternal aphid subsequent to EBF reception, and the change in proportion of alarm signaling instars anterior to the mother is not significantly different from control treatments. This suggests that there may be multiple defensive strategies for individual (i.e., dispersal) and clonal (i.e., alarm signaling) survival within a single aphid clone.

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Table 3.1. Independent variables included in the Repeated Measures MANOVA, where the dependent variables were the change in proportions of juvenile aphids feeding in three sectors: anterior to the mother, posterior to the mother, and on other leaves.

Independent Variables$_{\text{(degrees of freedom)}}$		
Model Effects	***F***	**p-value**
Variation Between:		
Instar$_{(3,1023)}$	4.98	**0.002**
Cultivar$_{(1,1023)}$	1.97	0.16
Genotype$_{(1,1023)}$	0.42	0.52
Cultivar × Genotype$_{(1,1023)}$	**4.63**	**0.03**
Treatment$_{(1,1023)}$	0.01	0.91
Treatment × Genotype$_{(1,1023)}$	24.36	**<0.0001**
Treatment × Cultivar$_{(1,1023)}$	0.48	0.49
Treatment × Genotype × Cultivar$_{(1,1023)}$	0.05	0.82
Instar × Treatment$_{(3,1023)}$	**5.87**	**0.0006**
Time$_{(4,1023)}$	0.23	0.92
Longevity$_{(1,1023)}$	2.57	0.11
Colony Size$_{(1,1023)}$	3.47	0.06
Mother Distance$_{(1,1023)}$	2.67	0.10
Variation Within:		
Sector$_{(2,1022)}$	1.36	0.26
Sector × Instar$_{(6,2044)}$	**3.38**	**0.0026**
Sector × Cultivar$_{(2,1022)}$	**24.41**	**<0.0001**
Sector × Genotype$_{(2,1022)}$	0.22	0.81
Sector × Cultivar × Genotype$_{(2,1022)}$	**9.82**	**<0.0001**
Sector × Treatment$_{(2,1022)}$	**21.42**	**<0.0001**
Sector × Treatment × Genotype$_{(2,1022)}$	**10.20**	**<0.0001**
Sector × Treatment × Cultivar$_{(2,1022)}$	**3.96**	**0.019**
Sector × Treatment × Genotype × Cultivar$_{(2,1025)}$	0.13	0.88
Sector × Instar × Treatment$_{(6,2044)}$	**7.48**	**<0.0001**
Sector × Time$_{(8,2044)}$	0.37	0.94
Sector × Longevity$_{(2,1022)}$	**6.56**	**0.0015**
Sector × Colony Size$_{(2,1022)}$	**8.91**	**0.0001**
Sector × Mother Distance$_{(2,1022)}$	**17.95**	**<0.0001**

Figure legends

Figure 3.1. Aphid colonies treated with alarm pheromone (EBF) (n= 30) showed an increased migration of juveniles away from the natal leaf onto other leaves on the plant compared to those treated with a hexane control (n = 27) (repeated measures MANOVA, $F_{2,1022} = 21.42$, $p < 0.0001$). A positive change in proportion represents a sector which aphids moved into after treatment. A negative change in proportion represents a sector which aphids moved away after treatment. Significant post-hoc differences (Sequential Bonferroni adjusted Student's t values) within each sector are indicated by different letters.

Figure 3.2. Change in proportions of offspring of two Pea aphid clones occupying feeding sites anterior and posterior to the maternal aphid, and those dispersing to other leaves after treatment with alarm pheromone or a hexane control. Significant post-hoc differences within each sector and within each treatment are indicated by different letters.

Figure 3.3. Change in proportions of four juvenile instars occupying feeding sites anterior and posterior to the maternal aphid, and those dispersing to other leaves after treatment with (a) alarm pheromone or (b) hexane. Significant post-hoc differences within each sector and within each treatment are indicated by different letters, while differences in instars between treatments are indicated with an asterisk.

Figure 3.4. The relationship between experimental treatment and the distance of maternal aphid feeding sites from the leaf petiole.

Figure 3.1.

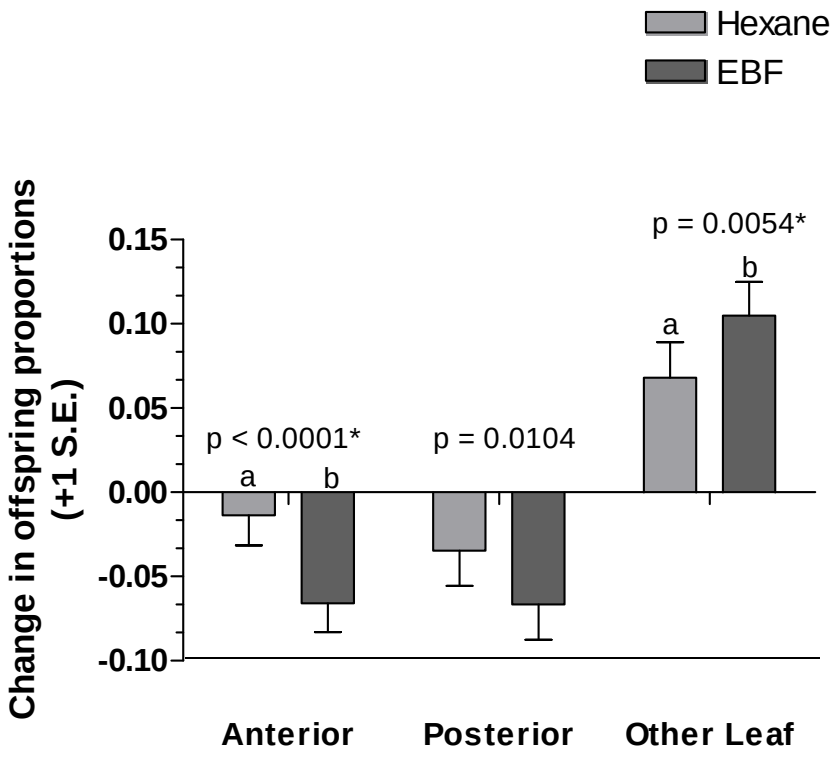

Hexane
EBF
Change in offspring proportions (+1 S.E.)
0.15
0.10
0.05
0.00
-0.05
-0.10
p < 0.0001*
a
b
p = 0.0104
p = 0.0054*
a
b
Anterior
Posterior
Other Leaf

Figure 3.2.

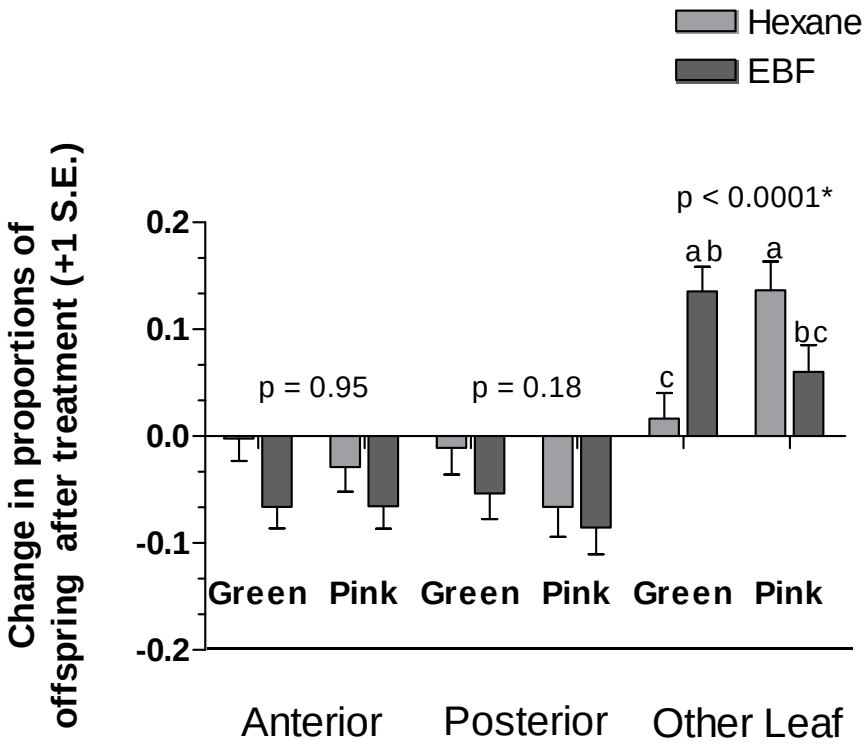

Hexane
EBF
Change in proportions of offspring after treatment (+1 S.E.)
0.2
0.1
0.0
-0.1
-0.2
p = 0.95
p = 0.18
p < 0.0001*
a b
a
b c
c
Green
Pink
Green
Pink
Green
Pink
Anterior
Posterior
Other Leaf

Figure 3.3.

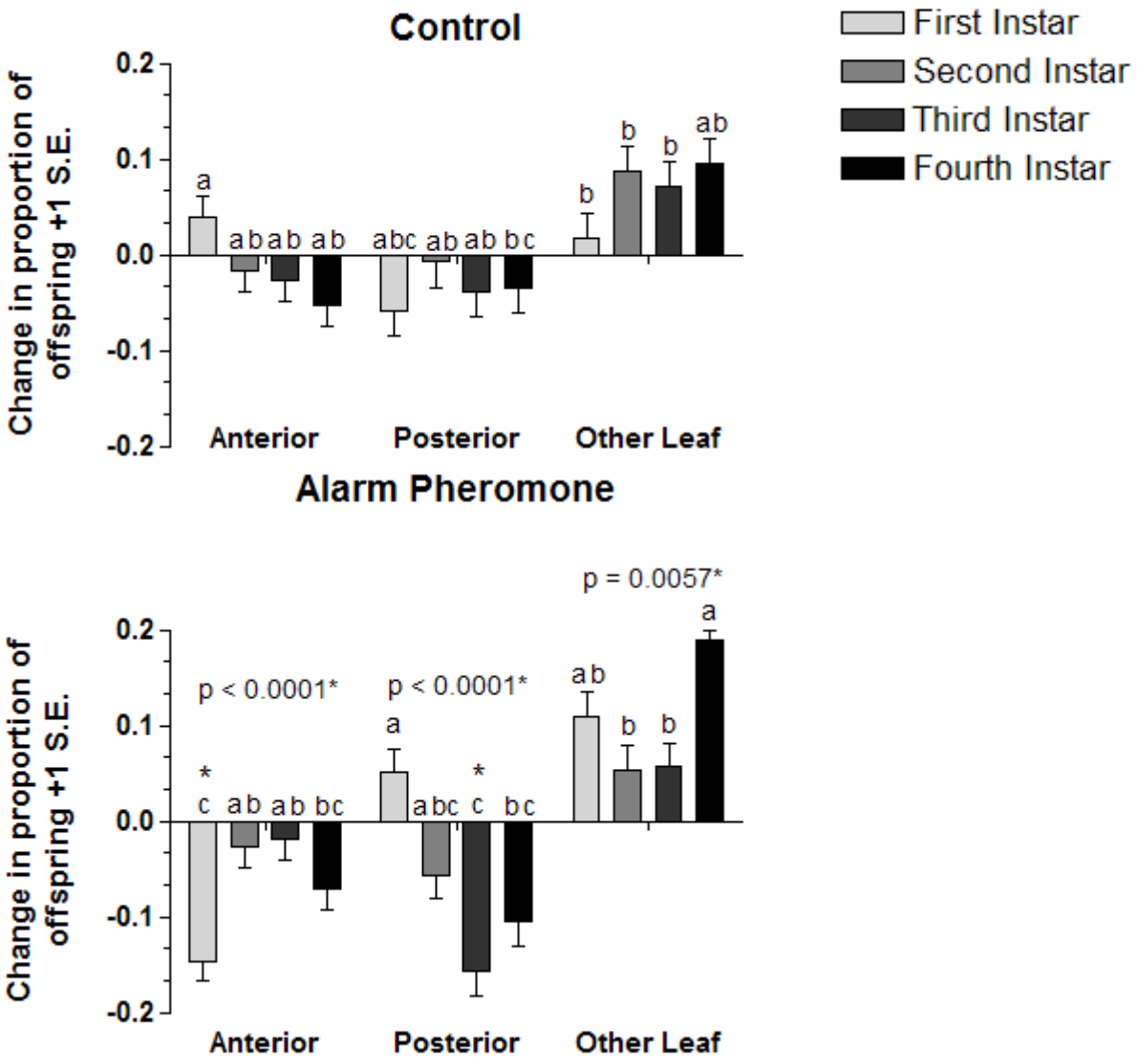
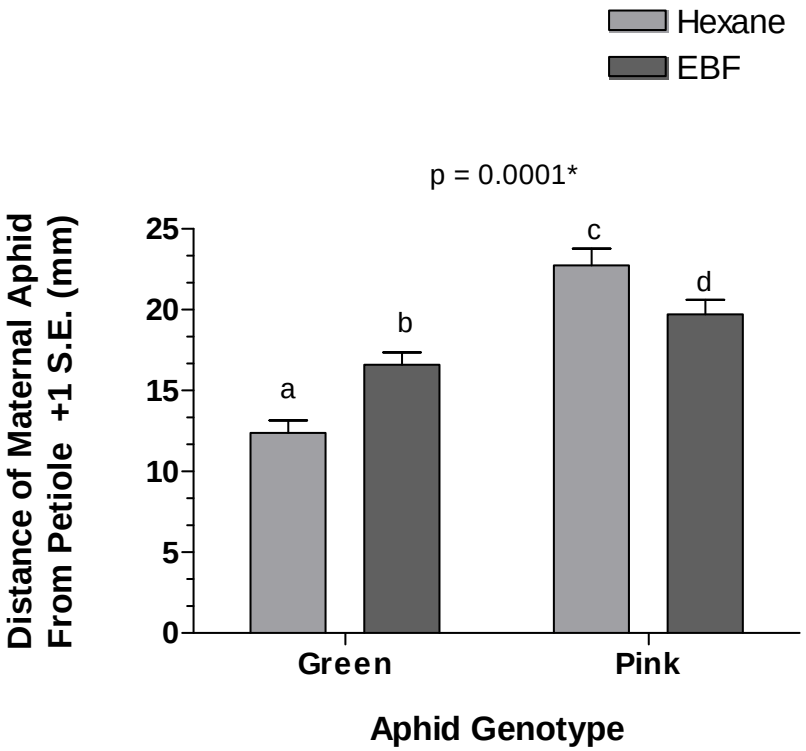

Control
First Instar
Second Instar
Third Instar
Fourth Instar
Change in proportion of offspring +1 S.E.
0.2
0.1
0.0
-0.1
-0.2
a
a b a b a b
a b c
a b
a b
b c
b
b
b
a b
Anterior
Posterior
Other Leaf
Alarm Pheromone
p = 0.0057*
p < 0.0001*
p < 0.0001*
*
*
c
a b
a b
b c
a
a b c
c
b c
a b
b
b
a
Anterior
Posterior
Other Leaf

Figure 3.4.


Hexane
EBF
p = 0.0001*
Distance of Maternal Aphid From Petiole +1 S.E. (mm)
25
20
15
10
5
0
a
b
c
d
Green
Pink
Aphid Genotype

Chapter 4

Effects of alarm pheromone on transgenerational behavioral plasticity in feeding sites in aphid colonies

Abstract

Reliable cues of increased predation risk can induce phenotypic changes in an organism's offspring (i.e. transgenerational phenotypic plasticity). While induction of defensive morphologies in naïve offspring in response to maternal predation risk has frequently been observed, relatively little is known about transgenerational changes in offspring behavior. Here I provide evidence for transgenerational behavioral plasticity in the pea aphid, *Acyrthosiphon pisum*. When pre-reproductive individuals of two (pink and green) "clones" were exposed to the alarm pheromone (E)-β-Farnesene (EBF), a reliable cue of increased predation risk, next-generation offspring altered their feeding site choices relative to the location of the maternal aphids. Offspring of maternal aphids exposed to a single alarm pheromone emission occupied "safer" feeding sites. Offspring of the two clones also behaved differently; green offspring occupied "safer" feeding sites in the natal colony, while pink offspring were more likely to disperse to occupy "safer" feeding sites on neighboring plant leaves. Offspring also behaved differently on two broad bean, *Vicia faba,* cultivars, indicating an effect of host-plant quality on aphid defensive behavior. Further studies are needed to clarify the association between the transgenerational induction of morphological and behavioral defenses, and how transgenerational behavioral plasticity helps to ensure the survival of the clone.

Introduction

Organisms must adapt to a multitude of environmental stressors, like predation risk, that differ both spatially and temporally (Lima and Dill, 1990). Rapid phenotypic responses to a changing environment, such as physiological acclimation, morphological plasticity, and anti-predator behavior increase the likelihood of an organism surviving in a stochastic environment (Bock, 1980; Gotthard and Nylin, 1995). In addition to direct responses, it is clear that maternal environmental stressors can influence the phenotypic expression of individuals in subsequent generations (Rasanen and Kruuk, 2007; Wolf and Wade, 2009; Shimada et al., 2010). These transgenerational, or "maternal", effects have been observed in a broad array of organisms, including prokaryotes (e.g. Wakamoto and Yasuda, 2006), animals (e.g. insects, Andersen et al. 2005; reptiles, Shine and Downes, 1999; and birds, Coslovsky and Richner 2011; etc.), and plants (e.g. Agrawal et al. 1999).

Transgenerational phenotypic plasticity requires maternal reception of a reliable cue of a changing environment and subsequent proximate alterations to her internal (prenatal) environment, which influence the development and expression of offspring phenotypes (Mousseau and Fox, 1998.). In many cases, reliable cues of increased predation risk have been shown to alter offspring morphologies, such as the development of defensive crests in *Daphnia* (Agrawal et al., 1999) and wing dimorphism in aphids (Dixon and Agarwala, 1999; Weisser et al., 1999; Podjasek et al., 2005). Although individuals exhibiting the inducible trait often have decreased fecundity (Mackay and Wellington, 1975), the induction of offspring morphologies modified for defense and dispersal has been posited to be adaptive by helping to ensure the survival of the genetic lineage, i.e., inclusive fitness (Price et al., 2003; Marshall and Uller, 2007; Storm and Lima, 2010).

If organisms have the ability to reliably perceive cues of increased future predation risk, transgenerational phenotypic plasticity can also enhance anti-predator behavior in the offspring. For example, Storm and Lima (2010) demonstrated that field cricket, *Gryllus pennsylvanicus,* offspring of mothers exposed to predatory spiders behaved differently than offspring of non-exposed mothers. Predator-exposed mothers gave birth to offspring with decreased mobility and increased vigilance, and these behaviors improved offspring survival when exposed to lethal predators. Transgenerational effects have also been observed for a number of behaviors in various taxa, including neophobia in mice, *Mus musculus* (Curley et al., 2008), shoaling in three-spined sticklebacks, *Gasterosteus aculeatus* (Giesing et al., 2011), and range expansion in western bluebirds, *Sialia mexicana* (Duckworth, 2009). As previously stated, predator-induced transgenerational morphological plasticity can be costly when future predation risk is low, but some induced phenotypic traits are reversible (Relyea, 2003). Indeed, a benefit of inducing defensive behaviors across generations is that they are not fixed, as in some morphological traits.

Aphids (Homoptera: Aphididae) have often been used as model organisms to study predator-induced transgenerational effects on offspring morphologies (e.g. Dixon and Agrawala, 1999; Weisser et al. 1999; Podjasek et al., 2005). Aphids give birth to live nymphs (viviparity) through apomictic parthenogenesis, and each offspring is genetically identical to the maternal aphid and to siblings (van Emden and Harrington, 2007; but see Lushai and Loxdale, 2002). Using asexual lineages, hereafter referred to as "clones", as models reduces the possibility of misinterpreting genetic variability between offspring as maternal effects. Pea aphids, *Acyrthosiphon pisum*, produce young over 10 – 15 days, each offspring reaching the adult stage (5^{th} instar) 7-10 days after birth (Dixon, 1998; Mondor and Roitberg, 2003). Continual reproduction by maternal aphids produces colonies of mixed-age clone-mates that feed together

(i.e. in the natal colony) but show instar-specific preferences in feeding-site selection (Duff and Mondor, 2011).

When attacked by a natural enemy, aphids emit a droplet of fluid containing a volatile alarm pheromone, E-β-Farnesene (EBF) in pea aphids, through structures called cornicles (Dixon, 1958). Reception of EBF can cause behavioral responses in colony-members, such as cessation of feeding, "agitation", walking away, and/or dropping from the plant (Roitberg and Myers, 1978; Braendle and Weisser, 2001). As aphids do not have good visual acuity, EBF reception is a reliable indicator of a nearby predation event and therefore increased predation risk (Döring and Chittka, 2007). Predator exclusion experiments have indicated that predation risk on aphid colonies is extremely high (Frazer et al., 1981; Dennis and Wratten, 1991) and alarm signaling increases survival of the clone through inclusive fitness benefits (Mondor and Roitberg, 2004).

Duff and Mondor (2011) demonstrated that pea aphid colonies have explicit spatial structures; younger juveniles occupied more "dangerous" feeding sites, i.e. anteriorly to the maternal aphid and at the periphery of the colony, relative to older instars. Because aphids feed facing towards the leaf petiole, any feeding position anterior to the maternal aphid is closer to the petiole (Dixon, 1985), a region of higher predation risk (Dixon, 1959; Keiser et al., *in prep., Chapter 2*). Under conditions of low predation risk, maturing juvenile aphids change feeding site preferences within the colony as they develop (Duff and Mondor, 2011). Pre-reproductive juveniles assume positions with higher predation risk and take increasingly safer positions as they advanced toward reproductive maturity and their reproductive value becomes greater than that of their younger siblings (Fisher, 1930). However, when treated with EBF emissions, the proportions of juveniles feeding around the maternal aphid will change, and these changes are

both genotype- and instar-specific (Keiser and Mondor, *in prep., Chapter 3*). How the reception of a cue of increased predation risk alters next-generation juvenile feeding site selection has not yet been investigated.

I hypothesize that when pre-reproductive 4th instar aphids are exposed to a single alarm pheromone emission, future offspring will occupy "safer" feeding sites compared to control colonies.

Methods

Study Organisms

Two pea aphid, *Acyrthosiphon pisum*, asexual lineages (pink and green "clones"), collected at Eagle Creek (Georgia Southern University, Statesboro, GA) on wild vetch, *Vicia sativa*, were used in this experiment. They have been continuously reared in the laboratory for approximately 4 years. Aphids were reared on broad bean, *Vicia faba* (c.v. Broad Windsor), in mesh bags at 21.5 – 26.4°C and 25 – 50% RH with a 16:8 L:D photoperiod under one "GE Ecolux Plant & Aquarium" 40W wide spectrum fluorescent bulb and one "GE Residential" 40W bulb (GE Lighting Inc., Cleveland, OH) and watered every 48 hours. Prior to experimentation, aphid colonies were age structured by allowing 25 – 30 adults to produce offspring for 24 hours on a single plant, and then removing the adults. All offspring produced in this time period were considered to be 1st instars, ensuring that subsequent life-stages could be reliably determined.

Broad bean, *V. faba*, seeds were planted individually in Fafard 3B potting mix (Conrad Fafard Inc., Agawam, MA) in 1L round, black, plastic pots. Plants were top-dressed with Osmocote 14-14-14 N-P-K slow-release fertilizer (Scotts-Sierra Horticultural Products, Marysville, OH) prior to seedling emergence and watered daily in the greenhouse (16.6 –

48.6°C, 20 - 69% RH, ambient lighting). Two broad bean cultivars, Broad Windsor (BW) and Stereo (St), were used to determine if host plant type influenced aphid behavior. After 14 days, plants were transported to the laboratory for experiments.

Experimental Protocol

Prior to experimentation, plants were randomly assigned a treatment and aphid genotype, and the order of replicates within the greenhouse was randomized with JMP 8.0 (SAS Institute Inc. 2007). Individual fourth instar aphids of two clones (green, n = 44; pink, n = 20) were inspected for wing pads (to ensure the adult individuals would be wingless apterae) and placed at the base of a host plant (plants were segregated from each other by a water moat). Aphids were allowed to climb the plant, select a feeding site, and feed undisturbed. After 8 hours, treatments were administered: 1µl of (E)-β-Farnesene solution (50 ng EBF/1µl Hexane (experimental), or 1µl Hexane (control) was applied to a piece of filter paper with a micropipette, waved beside (< 1cm) a feeding aphid for 15 seconds, and then removed. There was no physical contact with the aphids or the plant. EBF was obtained from Bedoukian Research, Inc. (Danbury, CT). Aphids began producing offspring 48-72 hours after treatments. Experiments were conducted in two consecutive trials.

Replicates were monitored for one week; aphid colonies were photographed every 24 hours with a digital camera (Olympus Stylus Tough-8000, Olympus America, Inc.). Photographs were compiled and scored blind for treatment and cultivar to eliminate observer bias. For each sampling period (48, 72, 96, 120, 144, and 168 hours), the number of first, second, third, and fourth (penultimate) instar aphids anterior and posterior to the maternal aphid were counted, as well as the number of individuals that migrated to another leaf on the host-plant

(Duff and Mondor 2011). A replicate was terminated when the maternal aphid dispersed from the natal colony to another location on the plant or off the plant. Although the number of offspring reaching the 4th instar was low in this study (n = 17), we included them in our analyses. Experiments were carried out in two consecutive trials.

A finer-scale analysis of juvenile feeding site distribution within the natal colony was conducted by measuring the average distance (to the nearest 0.5mm) and direction (to the nearest 5°) of each individual from the feeding site of the maternal aphid. Mean values were calculated for each instar at each time period. Individuals were assigned an absolute direction from 0° - 180° regardless of whether the individual was to the left or right of the maternal aphid (i.e. 0° is directly in front of the aphid and 180° is directly behind; Duff and Mondor 2011). Distance and direction data were collected from photographs using the program MB-Ruler (Markus Bader MB-Softwaresolutions, Iffezheim, Germany).

Statistical Analyses

Feeding site distribution had four nominal independent variables: host plant cultivar (Broad Windsor vs. Stereo), aphid genotype (Pink vs. Green), experimental treatment (Hexane vs. EBF), and time (24 – 192 hours) and one ordinal independent variable: aphid instar (1st – 4th instar). The following were included as interaction terms: cultivar × genotype, cultivar × treatment, genotype × treatment, and cultivar × genotype × treatment (Table 4.1). A full factorial model was not performed as the degrees of freedom could not support such an analysis. Also, some interactions would have been largely unbalanced, due to the biology of the organism. The dependent variables were: the proportions of offspring of each instar at feeding sites anterior to the maternal aphid, posterior to the maternal aphid, and those feeding on another leaf. Proportion

data were transformed (x' = arcsine$\sqrt{x}$) and analyzed using Repeated Measures MANCOVA. Covariates were: Colony size (1-31 aphids), trial (1-2), the time a maternal aphid spent in the natal colony ("Longevity": 48-192 hours) and distance from the maternal aphid to the leaf petiole (0-56 mm). Post-hoc analyses were carried out using student's t and Tukey's HSD tests. For t-test post-hocs, p-values were corrected using a sequential Bonferroni test, so as to not inflate the experiment-wise error rate and increase statistical power (Rice, 1989).

The analysis of juvenile distance and direction included the same independent variables as in the previous analysis, including the interaction terms and covariates (Table 4.2). The two dependent variables were: the average distance (mm) and direction (degrees) of each instar at each time period, for each replicate. Data were analyzed using two, five-factor ANCOVAs. Post-hoc analyses were performed with Tukey's HSD tests.

Results

Fourth instar pea aphids, of two clones, exposed to EBF produced offspring that chose "safer" feeding sites, compared to mothers exposed to hexane controls ($F_{2,318} = 17.0$, $p < 0.0001$; Figs. 4.1 and 4.2). Offspring of green aphids that were treated with EBF had an increased preference for feeding sites posterior to the maternal aphid ($F_{2,318} = 18.0$, $p < 0.0001$). Offspring of pink EBF-treated aphids, meanwhile, frequently dispersed to other leaves on the host plant ($F_{2,318} = 18.0$, $p < 0.0001$). Therefore, offspring of EBF-treated aphids of either lineage were less likely to occupy feeding sites anterior to the maternal aphid, i.e., the "dangerous" feeding sites (maternal aphids in this study settled facing towards the petiole at a rate of 91%). Juvenile aphids also responded differently on the two host plants ($F_{2,318} = 4.8$, $p = 0.0089$; Fig. 4.2), as a larger proportion of juveniles dispersed from the natal colony on Broad Windsor.

Juveniles of the green genotype settled at feeding sites farther away from the maternal aphid than did pink individuals ($F_{1,19} = 50$, $p < 0.0001$). Offspring of either genotype did not occupy feeding sites at different distances from the maternal aphid following experimental treatments ($F_{1,19} = 0.42$, $p = 0.52$). In addition, host-plant cultivar did not significantly influence offspring distance ($F_{1,19} = 2.3$, $p = 0.13$), although there was a cultivar × treatment interaction ($F_{1,19} = 4.2$, $p = 0.042$): juveniles decreased their distance from EBF-treated mothers on Broad Windsor, while they increased their distance from EBF-treated mothers on Stereo.

With regards to direction, neither host-plant cultivar ($F_{1,19} = 0.1$, $p = 0.76$) nor aphid genotype ($F_{1,19} = 0.6$, $p = 0.44$) had a significant effect on juvenile feeding site relative to the maternal aphid, although a cultivar × genotype interaction term was significant ($F_{1,19} = 14.0$, $p = 0.0002$): pink offspring increased their direction (i.e. moved more anteriorly) from the maternal aphid only on Stereo. Maternal treatment with EBF decreased the average direction of offspring in the natal colony, i.e. offspring showed a preference for feeding sites behind the mother ($F_{1,19} = 11.0$, $p = 0.0011$), which coincides with the results of the previous analysis. Juveniles increased their distance ($F_{3,19} = 14.0$, $p < 0.0001$; Fig. 4.3a) and decreased their direction, i.e. moved more anteriorly, relative to the maternal aphid as they developed to new instars ($F_{3,19} = 5.0$, $p = 0.0021$; Fig. 4.3b), though they remained largely in the posterior section (i.e. the average direction for all instars was > 90°).

Discussion

The data support the hypothesis that exposure to an EBF emission at the 4th instar pre-reproductive stage causes aphids to have offspring which choose “safer” feeding sites. As there is virtually no genetic variability between clone-mates in a colony and there is no persistence of

alarm pheromone in the juvenile environment (i.e., the active period of EBF, under natural conditions, decreases exponentially for 60-70 minutes after emission, Schwartzberg et al. 2008), the results presented here clearly demonstrate a maternal effect on offspring behavior (i.e., transgenerational behavioral plasticity). The maternal aphids used in this experiment were exposed to alarm pheromone during the 4th instar, a critical period in which wing induction in offspring occurs (Podjasek et al., 2005). As aphids began producing offspring for 48-72 hours after treatments, it is clear that reception of EBF by the maternal aphids caused them to produce offspring that differed behaviorally from offspring produced under conditions of low predation risk.

Offspring behavior also differed between host-plant types, which have been shown to affect fitness and performance of pea aphids (Morgan et al., 2001). Differences in foraging quality might influence the behavior of juveniles directly, or it may have been a transgenerational effect from the foraging decisions of the mother, as food quality can influence trait plasticity across generations (Rotem et al., 2003).

Although fitness discounting may select for particular aphid colony structures (Duff and Mondor, 2011), under conditions of increased maternal predation risk (i.e. exposure to EBF), juveniles seek safer feeding sites in the colony, or disperse to other leaves on the host plant. This behavioral response is likely linked with an alarm pheromone-induced transgenerational wing induction (Podjasek et al., 2005; Weisser et al., 1999). In fact, a correlation between morphological and behavioral traits has been identified in the influence of population density on transgenerational phenotypic plasticity in the desert locus, *Schistocerca gregaria* (Maeno and Tanaka, 2009). As EBF is well known to induce wing formation in offspring, these dispersers should occupy safer feeding positions, as alates have a longer development time than wingless

morphs (Dixon and Howard, 1986), and dispersal is advantageous under situations of increased predation risk (Mackay and Wellington, 1975).

The relationship between wing induction and colony structure should be further investigated by exposing aphids to other factors that induce wing induction, but do not alter predation risk (e.g. overcrowding, poor host-plant quality, etc.; Müller, et al. 2001). This would allow one to tease apart the effects of wing induction on feeding site preference, with and without increased predation risk, and better understand how these behavioral and morphological changes are interconnected. For example, in our experiment there was a large amount of variation among juveniles, even though there were overall behavioral differences between treatments. It would be valuable to know if only winged dispersers choose the "safer" feeding positions within the colony (or away from the colony), and if the apterae choose the "more dangerous" feeding positions. Feeding site choices are not constrained by forage quality, as aphid development time is not affected by feeding at different distances from the petiole (Keiser and Mondor, *in prep., Chapter 1*).

The proximate physiological mechanisms mediating transgenerational behavioral plasticity are largely unknown. Storm and Lima (2010) question if, upon maternal reception of an environmental cue of predation risk, the stress hormone octopamine is released into field cricket, *G. pennsylvanicus*, eggs, thereby influencing future behaviors of unborn offspring. Molecular studies have suggested that phenotypic plasticity is controlled by conditional gene expression (Gerhart and Kirshner, 1997). Gene expression differs between apterae and alates in *A. pisum* (Brisson et al. 2010); 15% of maternal genes show significant changes in expression after exposure to alarm pheromone (de Vos et al. 2010). It has been warned, however, that

differences in gene expression may be a result of the behavioral modification of interest, and not the cause (Shimada et al., 2010).

It is important to consider maternal effects when studying trait variation across environmental gradients (Bernardo, 1996). Transgenerational effects can influence life history variation, and therefore community/population dynamics, but investigating this requires study at the level of the individual rather than the population. Maternal effects have the potential to influence broad ecological, evolutionary, and behavioral phenomena, such as transgenerational medication use in Lepidoptera (Lefèvre et al., 2010), social environment on grand-offspring emotional state and reproduction (Curley et al., 2009), and sex-specific prenatal epigenetics (Dunn et al., 2011). Transgenerational effects on behavior may be much more prevalent than currently realized in environments where predation risk is reliably predictable across generations.

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Table 4.1. Effects of host-plant cultivar, aphid genotype, and experimental treatment on the distribution of juvenile aphid feeding sites relative to the maternal aphid.

Independent Variables$_{\text{(degrees of freedom)}}$		
Model Effects	***F***	**p-value**
Variability Between:		
Cultivar$_{(1,319)}$	0.06	0.80
Time$_{(6,319)}$	1.25	0.28
Genotype$_{(1,319)}$	0.0001	0.99
Cultivar × Genotype$_{(1,319)}$	0.09	0.77
Treatment$_{(1,319)}$	0.42	0.52
Treatment × Genotype$_{(1,319)}$	4.30	**0.04**
Treatment × Cultivar$_{(1,319)}$	0.05	0.83
Treatment × Genotype x Cultivar$_{(1,319)}$	0.47	0.49
Variability Within:		
Sector$_{(2,318)}$	48.0	**< 0.0001**
Sector × Time$_{(12,636)}$	2.10	**0.02**
Sector × Cultivar$_{(2,318)}$	4.79	**0.009**
Sector × Genotype$_{(2,318)}$	4.72	**0.01**
Sector × Cultivar x Genotype$_{(2,318)}$	3.32	**0.037**
Sector × Treatment$_{(2,318)}$	16.92	**< 0.0001**
Sector × Treatment × Genotype$_{(2,318)}$	0.37	0.69
Sector × Treatment × Cultivar$_{(2,318)}$	18.22	**< 0.0001**
Sector × Treatment × Genotype x Cultivar$_{(2,318)}$	6.71	**0.001**

Table 4.2. Effects of host-plant cultivar, aphid genotype, experimental treatment, and aphid instar on the distance and direction of juvenile aphid feeding site location within the natal colony relative to the maternal aphid.

Independent Variables$_{\textbf{(degrees of freedom)}}$	**Distance**		**Direction**	
	F	**p-value**	***F***	**p-value**
Cultivar$_{(1,314)}$	2.30	0.13	0.09	0.76
Genotype$_{(1,314)}$	50.0	< **0.0001**	0.60	0.44
Cultivar × Genotype	0.34	0.56	14.63	**0.0002**
Treatment$_{(1,314)}$	0.42	0.52	10.81	**0.001**
Treatment × Cultivar $_{(1,314)}$	4.20	**0.042**	1.00	0.32
Treatment × Genotype $_{(1,314)}$	0.77	0.38	1.90	0.17
Treatment × Genotype × Cultivar$_{(1,314)}$	0.61	0.44	9.53	**0.002**
Instar$_{(3,314)}$	14.0	< **0.0001**	5.02	**0.002**

Figure Legend

Figure 4.1. Proportions of offspring of two Pea aphid clones occupying feeding sites anterior and posterior to the maternal aphid, and those dispersing to other leaves after maternal treatment with (E)-β-Farnesene or Hexane. Significant post-hoc differences within each sector are indicated by different letters.

Figure 4.2. Proportions of offspring of two Pea aphid clones occupying feeding sites anterior and posterior to the maternal aphid, and those dispersing to other leaves on two Broad bean cultivars after treatment with EBF or Hexane. Significant post-hoc differences within each sector are indicated by different letters.

Figure 4.3. Average (a) distance and (b) direction of juveniles of different instars occupying feeding sites in the natal colony relative to the location of the maternal aphid. Significant post-hoc differences within each sector are indicated by different letters.

Figure 4.1.

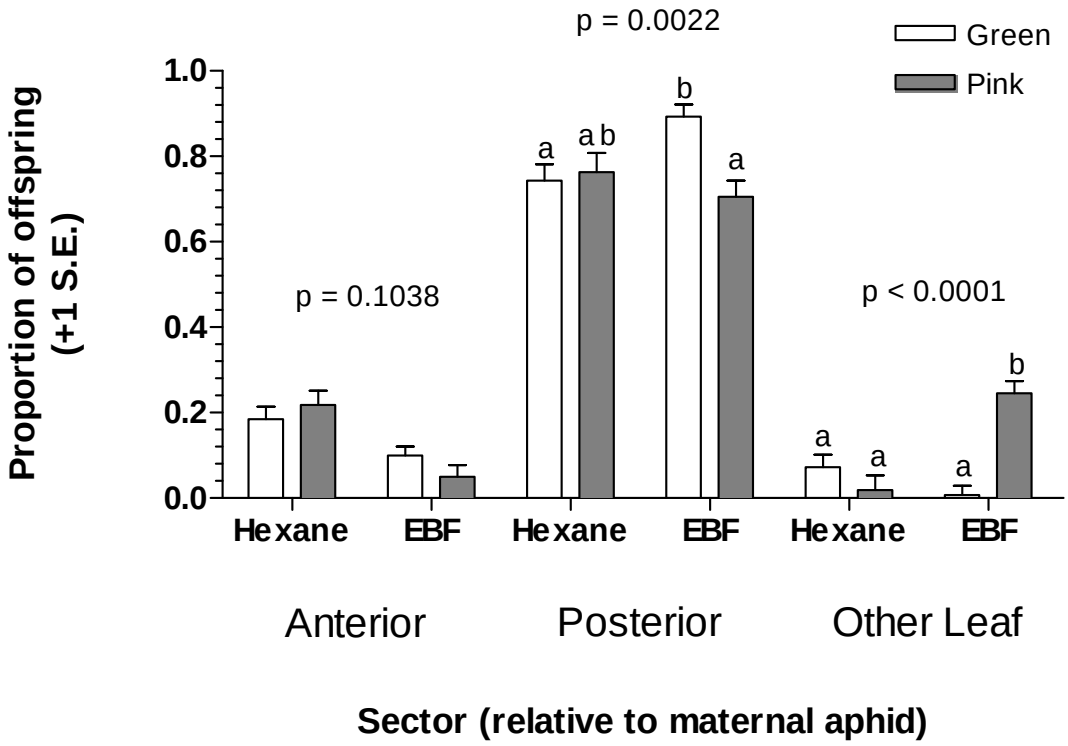

p = 0.0022
Green
Pink
Proportion of offspring (+1 S.E.)
1.0
0.8
0.6
0.4
0.2
0.0
p = 0.1038
p < 0.0001
a
a b
b
a
a
a
a
b
Hexane
EBF
Hexane
EBF
Hexane
EBF
Anterior
Posterior
Other Leaf
Sector (relative to maternal aphid)

Figure 4.2.

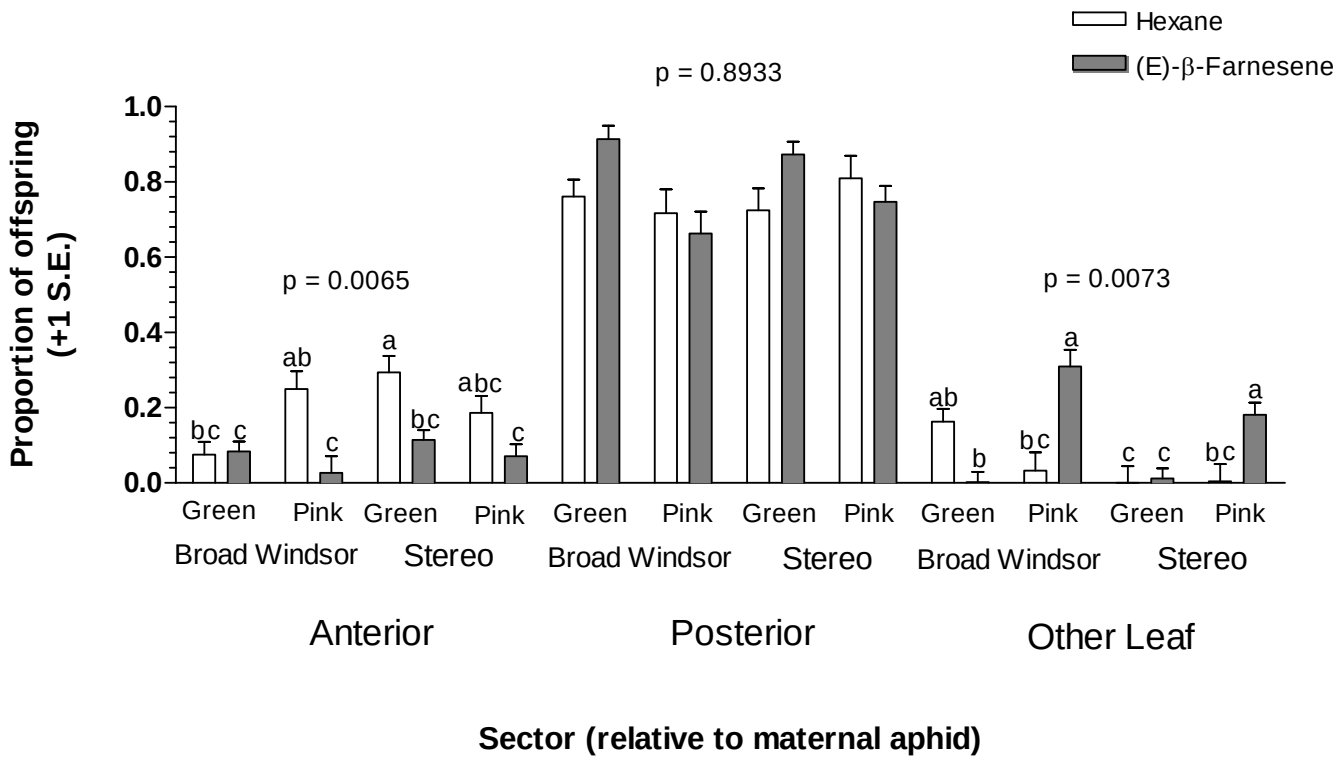
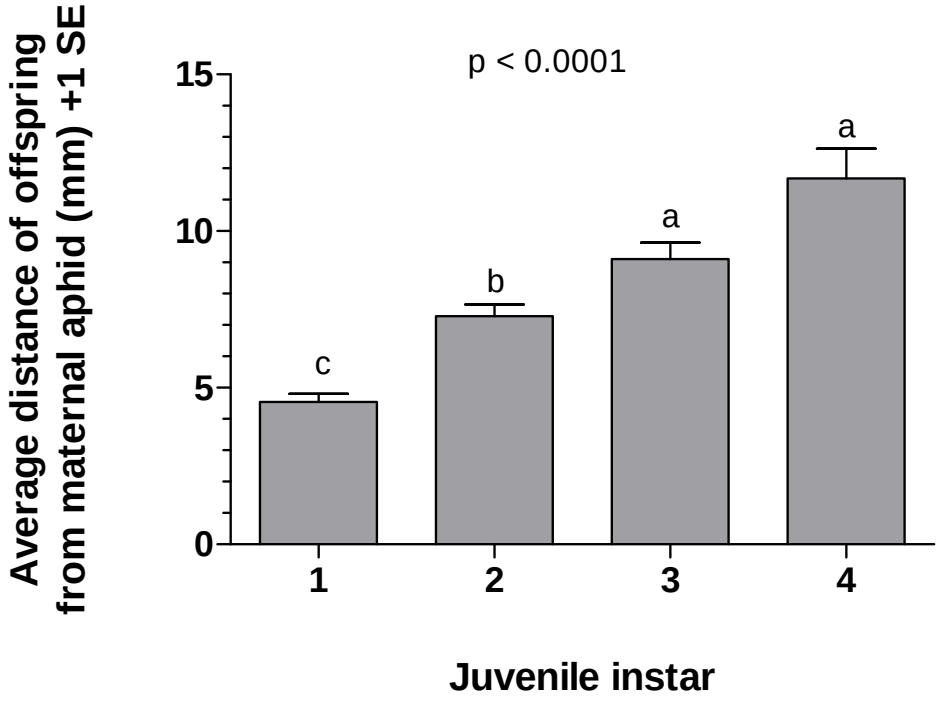
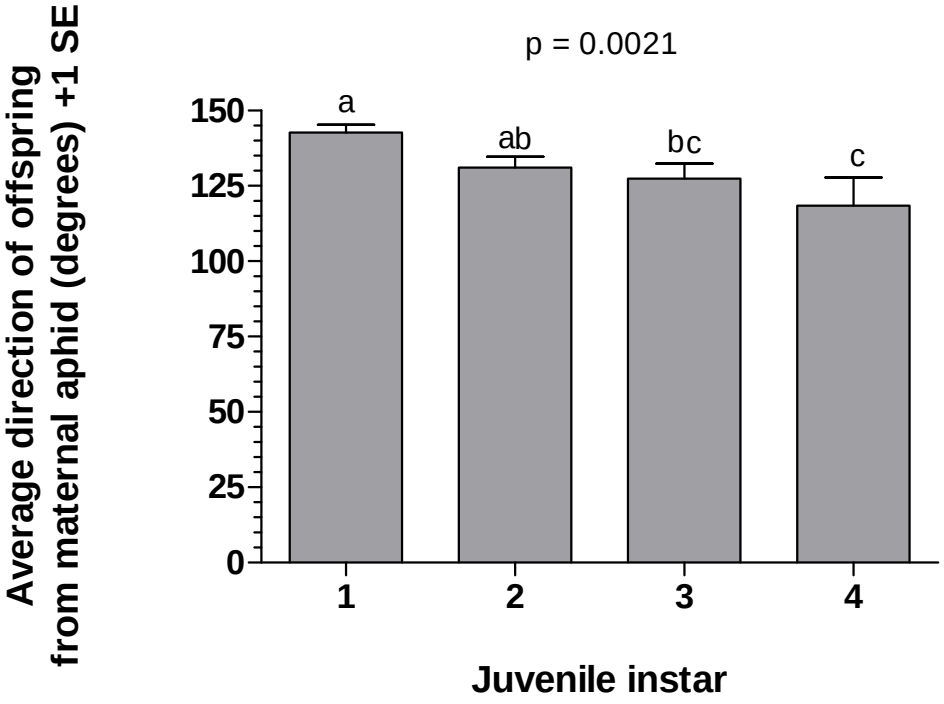

Hexane
(E)-β-Farnesene
p = 0.8933
p = 0.0065
p = 0.0073
Proportion of offspring (+1 S.E.)
1.0
0.8
0.6
0.4
0.2
0.0
bc c
ab c
a bc
ab b
bc a
c c
bc a
Green Pink Green Pink Green Pink Green Pink Green Pink Green Pink
Broad Windsor Stereo Broad Windsor Stereo Broad Windsor Stereo
Anterior
Posterior
Other Leaf
Sector (relative to maternal aphid)

Figure 4.3.

(a)


Average distance of offspring from maternal aphid (mm) +1 SE
p < 0.0001
15
10
5
0
a
a
b
c
1
2
3
4
Juvenile instar

(b)


Average direction of offspring from maternal aphid (degrees) +1 SE
p = 0.0021
150
125
100
75
50
25
0
a
ab
bc
c
1
2
3
4
Juvenile instar

Conclusions

Investigating the ecological characteristics of the "micro-territories" where juvenile aphids feed within the natal colony (Chapters 1 and 2) provided a better understanding of the proximate factors that influence the feeding site choices of individuals, and thus the structure of aphid colonies. In addition, examining changes in colony structure after predation risk increases (Chapters 3 and 4) provided insight into how individual feeding site choices influence inclusive fitness and clonal survival.

In Chapter 1, I showed that when feeding either anteriorly or posteriorly to the maternal aphid, there is no difference in individual development time, or fecundity in the first 24 hours of adulthood. A juvenile aphid gains no life-history benefits from feeding at one site over the other. In Chapter 2, the data indicate that predation risk is greatest at the site nearest the petiole for foraging coccinellid beetles, though not for foraging parasitoid wasps. Combining the results of these experiments, it suggests that there is no tradeoff between forage quality and predation risk among "anterior" and "posterior" leaf sites for juvenile aphids. Juveniles gain no benefits by feeding anteriorly to the maternal aphid, and by doing so are at the greatest risk of attack by predators. Therefore, at the level of the individual, no advantage is gained, though the individual may serve as an alarm signaling "sentinel" and confer survival benefits at the level of the clone.

In Chapter 3, I showed that after reception of alarm pheromone (a reliable cue of immediate predation risk), aphid colonies consisting of a single reproductive aphid and her mixed-age offspring dispersed throughout the host-plant, though there was no difference in the change in proportions of 2^{nd} – 4^{th} instar juveniles anterior to the mother, between experimental and control treatments. A proportion of offspring (2^{nd} – 4^{th} instars) remain in the anterior as "sentinels" after predation risk increases, while some of the offspring (1^{st} instars) will disperse to

potentially enemy-free space. Therefore, within a single aphid clone, different strategies for individual and clonal survival exist (i.e., dispersal and alarm signaling). In Chapter 4, I showed that pre-reproductive individuals exposed to alarm pheromone produce offspring that assume "safer" feeding sites: either posterior to the maternal aphid or on other leaves. This shows that, in addition to immediate responses to increased predation risk, a transgenerational behavioral response may also augment clonal survival.

This series of experiments represent the first thorough investigation into spatial group structuring in a clonal animal. It suggests that behaviors which are selfish at the level of the individual (e.g. feeding posterior to the maternal aphid, dispersal, etc.), those which help individual survival, may consequently increase survivorship of the clonal genotype. These results have implications for the study of inclusive fitness and the evolution of variable survival strategies within clonal aggregations.