Spring 5-17-2014

Investigations into Sensory Ecology and Gene Evolution of the pea aphid (*Acyrthosiphon pisum*)

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INVESTIGATIONS INTO SENSORY ECOLOGY AND GENE EVOLUTION OF THE PEA APHID (*Acyrthosiphon pisum*)

by

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A DOCTORAL DISSERTATION

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Major: Biological Sciences

(Ecology, Evolution, and Behavior)

Under the Supervision of Professors Brigitte Tenhumberg & Jennifer A. Brisson

Lincoln, Nebraska

May, 2014

INVESTIGATIONS INTO SENSORY ECOLOGY AND GENE EVOLUTION OF THE PEA APHID (*Acyrthosiphon pisum*)

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University of Nebraska, 2014

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Organisms use environmental cues to gather information required to perform activities that are essential for their survival and reproduction, such as searching for food, avoiding danger, and finding mates. They respond to the acquired information by changing their behavior or physiology, which may result in increased fitness. Due to the fundamental importance of information in an organism's life, it is important to understand its acquisition, processing, and the organism's response to it. In the work presented here, we used the pea aphid (*Acyrthosiphon pisum*), an insect that produces multiple phenotypes, or morphs, that are genetically identical, but differ in morphology, ecology, and behavior, as a model system for investigations into sensory ecology and gene evolution.

First, we examined the influence of aphid honeydew, a prey-associated cue, on the interactions between pea aphids and their ladybird predators. We found that the honeydew influenced the foraging behavior of predator larvae, but the larvae were not able to distinguish between the honeydew of high and low nutritional quality prey. Next, we compared the wing polyphenic response of pea aphids to two factors that are potential indicators of poor habitat - predator cues and crowding. The wing polyphenic response occurs when wingless pea aphids produce winged offspring in response to environmental

stress. We found that the intensity of the wing polyphenic response of pea aphids to crowding was much stronger than their response to predator cues, suggesting pea aphids acquire and process information from different cues to assess environment quality and differentially respond to it.

Thirdly, we compared chemosensory gene expression between different pea aphid morphs to investigate weather the chemosensory system changes with morph specialization. We found distinct chemosensory gene expression profiles of the pea aphid morphs that indicated intraspecific specialization of chemosensory systems. Finally, we compared the rates of evolution of morph-biased genes (genes highly expressed in one morph compared to the other morphs) with unbiased genes to explore the evolutionary consequences of phenotypic plasticity. Our results illustrated that morph-biased genes evolve faster than unbiased genes as a result of relaxed purifying selection.

DEDICATION

To my best friend and husband, Rahul, who inspires me to dream big.

ACKNOWLEDGEMENTS

First, I would like to thank my advisors Dr. Brigitte Tenhumberg and Dr. Jennifer Brisson for their continuous support. Dr. Tenhumberg gave me an opportunity to join the graduate school at UNL, and helped me learn mathematical modeling and experimental research. Dr. Brisson introduced me to the fascinating world of molecular biology, and helped me develop my skills in molecular genetics and genomics. I couldn't have completed the dissertation without their help and guidance.

My sincere thanks go to the supervisory committee members Dr. Diana Pilson, Dr. Jay Storz, Dr. Eileen Hebets, and Dr. Nicholas Miller for their encouragement and support. I greatly appreciate that their doors were open for me whenever I wanted to seek their advice. I would like to acknowledge my collaborators Dr. Ryan Bickel, Dr. Julie Jaquiery, and Dr. Claude Rispe. Working with them was an educative and enjoyable experience. I am thankful to Dr. Chad Brassil, Dr. Sabrina Russo, and Dr. Drew Tyre for their advice and encouragement.

I was very fortunate to have Travis Hinkelman as a lab mate. I value his friendship and help over the years. I miss our discussions about research and science. I would like to thank the members of Brisson lab. Neetha N.V. and Mary Chaffee helped me in learning molecular lab techniques. Tayler Schubauer, Kate Collars, Emma Erickson, Tiffany Schultz, and Kathy Williams helped with insect rearing, collecting, and processing samples. Emily Zhan helped in insect rearing and conducting experiments. Laramy Enders gave useful suggestions on my talks and manuscripts. I appreciate their

contributions in helping me improve my dissertation.

I would like to acknowledge the SBS faculty for the training and the SBS staff for their assistance. Special thanks to Dr. Stacey Smith, Dr. Etsuko Moriyama, and Dr. Lawrence Harshman for letting me audit their classes. I appreciate the comments and suggestions from fellow graduate students on my research projects during classes and R&D seminars.

I am thankful to my parents Usha and Kamalakar, my brother Parag, and my late uncle *Indulkar kaka* for their love and support. They have always felt very proud about my achievements however small those were. I am very fortunate to have in-laws, Ashok, Sulochana, Sudha, Atul, and Pradnya, who treat me like a favorite daughter in the family. My journey would not have been successful without their support. Many thanks to my caring friends Karen McGann and Rick Helweg for their help, when I needed it the most. My children are lucky to have another pair of parents in Lincoln.

Most importantly, I would like to thank my husband Rahul, for relentlessly encouraging and inspiring me to explore and pursue my passions, and always standing by me. He was the driving force behind my decision to pursue research career in biology. Thank you Rahul, I could not have done it without you. I am equally grateful to my children, Salil and Maitreyi, for their patience and love. The last few years were particularly stressful since the research was in the final stage. The children showed incredible understanding and maturity, and also provided right balance between work and personal life. Bruno, our English springer spaniel, often reminded me that long walks and fun games help maintain

good physical and mental health. This was a challenging journey for the whole family and I am very happy that together, we did it!

I have been grateful in my life for the opportunities that I received to explore nature and wilderness. The paths I traversed in the mountains and grasslands with a pair of binoculars eventually lead me to the exciting world of biological research. I feel that I owe it to the nature for the immense joy that I have received from my journey so far. Being a biologist gives me an edge to show my gratitude towards the nature by contributing to the understanding and conservation of the amazing diversity of life on earth. This dissertation is only the first step.

This research was supported by funding from University of Nebraska, School of Biological Sciences special funds, Center for Grassland Studies (Arthur William Sampson Fellowship) to Swapna R. Purandare and by University of Nebraska, School of Biological Sciences startup funds and award R00ES017367 from the National Institute of Environmental Health Sciences to Dr. Jennifer A. Brisson.

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Introduction

Organisms acquire information from their surroundings to deal with uncertainties associated with variable environment such as resource distribution, availability, and predation risk (Dall et al 2005). Environmental cues include various types of sensory cues, such as visual (vision), auditory (sound), tactile (touch), olfactory (smell), and gustatory (taste). Since the environment contains many different types of information, the organisms need to filter out unwanted information and acquire only relevant information, which then can be processed and utilized to maximize their fitness (Mappes and Stevens 2010). Therefore, sensory systems of organisms evolve to detect, discriminate and process relevant information (Stevens 2010). Organisms respond to the environmental cues by changing their behavior, physiology, or life history (reviewed in Whitman and Agrawal 2009). It is therefore important to ask questions, such as what information do organisms acquire from environmental cues? How do the sensory systems acquire it? How do the sensory systems evolve in different environments? What mechanisms are involved in processing the information? How do organisms respond to information, and does it result in increased fitness? Sensory ecology investigates the acquisition, processing, and response of the organisms to sensory information, as well as the function and evolution of sensory systems involved (Stevens 2013).

In the work presented here, we try to answer some of the above-mentioned questions using an insect as a model system. Pea aphids (*Acyrthosiphon pisum*) are small, soft-bodied, phloem feeding insects that make extensive use of environmental cues during

their life cycle. They use visual, tactile, and chemical cues for locating and discriminating host plants from other plants, sex pheromone to find mates, and alarm pheromone in conspecific communication, and predator avoidance (Pickett et al 1992).

Furthermore, pea aphids show a very strong response to biotic as well as abiotic environmental cues during their complex annual life cycle. They produce different seasonal morphs, such as winged and wingless asexual females, winged and wingless males, and sexual females in response to environmental cues. Presence or absence of wings in males has a genetic basis and is termed wing polymorphism. It is determined by a single locus on X chromosome. Presence or absence of wings in females (wing polyphenism), and the switch in the mode of reproduction (asexual to sexual), is environmentally determined. Thus, these female morphs are produced via polyphenism, which is an extreme form of phenotypic plasticity in which discrete phenotypes are produced from the same genotype in response to environmental variation. The morphs have the same or similar genotypes, but they differ in their morphology, ecology, and behavior, depending on the environment. Since the morphs are genetically identical, their alternate phenotypes are a result of differences in gene expression. Accordingly, the gene expression patterns differ between morphs. Some genes are highly expressed in one morph compared with another (morph biased genes) while some genes are expressed in only one morph (morph specific genes). These gene expression patterns are interesting targets for exploration of gene evolution. Pea aphids make extensive use of environmental cues and show a very strong polyphenic response and therefore they are an

ideal system to investigate sensory ecology as well as the influence of polyphenism on gene evolution.

In the work presented here, we describe our investigations into sensory ecology and gene evolution of the pea aphid. Initially, we investigated how environmental cues influence the interactions between pea aphids and their ladybird predators. In predator prey interactions, environmental cues, specifically cues associated with the prey, are known to improve predator foraging efficiency. Aphid honeydew, sugary excretion of aphids, is found in abundant amounts under plants infested with aphids and it is considered as a cue in prey location (Ide et al. 2007). We formulated and tested following hypotheses:

1. Aphid honeydew influences foraging behavior of the ladybird predators.
2. Predators assess nutritional quality of aphid prey from their honeydew and respond to it by avoiding honeydew of low quality prey.

To examine these hypotheses, we carried out laboratory experiments and video recorded the behavior of the ladybird larvae in presence of honeydew of two aphid species that differ in nutritional quality (high quality prey *A. pisum* and low quality prey *Aphis fabae*). We found that aphid honeydew influenced the foraging behavior of predatory larvae as they stayed longer in patches containing aphid honeydew but they did not engage in longer search bouts. Assessing nutritional quality of prey using cues may result in increased foraging efficiency for the predators. However, the predators were not able to distinguish between the honeydew of low quality and high quality prey, which suggests that they were not able to acquire information about nutritional quality of their prey from

aphid honeydew. We present the results of this investigation in Chapter 1. This work has been published in Ecological Entomology (2012), 37, 184-192.

Aphids display transgenerational wing polyphenism. In other words, in response to environmental stress, wingless females give birth to winged offspring that have an ability to disperse to new habitats. We explored whether the intensity of the wing polyphenic response of pea aphids varies with the type of the environmental cues that indicate stress. When ladybird predators forage in aphid colonies, they deposit a large number of cues (feces, tracks and eggs). These cues are indicators of predation risk in the environment, which may get translated into low probability of survival. Crowding condition is another indicator of poor habitat quality, which involves high density of feeding aphids and poor host plant quality. Pea aphids respond to these stress conditions by producing winged offspring. However, dispersal is risky, and pea aphids need to assess the costs and benefits of leaving the habitat. To examine if pea aphids can assess the risk associated with poor habitat quality based on the information obtained from the cues and respond to it, we compared the wing polyphenic response of the pea aphid to predator cues and crowding. We predicted that in response to crowding and cues from two predator species,

1. A higher proportion of aphids would produce winged offspring
2. The proportion of winged offspring would be higher compared to wingless offspring produced

In laboratory experiments, we exposed pea aphids to cues from two ladybird predators and to a crowding treatment and compared their response to pea aphids that were not

exposed to any treatment (control). We found that the intensity of the wing polyphenic response of pea aphids to crowding was much stronger than their response to predator cues. The response to cues of one predator species was weak and there was no response to the cues of the other predator species. This suggests that pea aphids acquire information from different cues and their response varies with the type of cue. The results of this study are presented in Chapter 2. This work has been published in Ecological Entomology (2014), 39, 263-266.

While trying to understand the mechanistic basis of pea aphid response to predator cues, we noted that the sensors used in detecting chemical cues are located on pea aphid antennae and vary in numbers and antennal segment location between different pea aphid morphs. These observations triggered the question: do the chemosensory systems differ between pea aphid morphs? Pea aphid morphs are specialists adapted to particular ecology and have a specialized chemosensory repertoire. For example, males use highly specific sex pheromone for finding females. Since pea aphid morphs are genetically similar or identical, the differences between them are mainly due to differences in gene expression. Therefore, we predicted that pea aphid morphs would have distinct chemosensory gene expression profiles based on their chemosensory needs.

We recorded differences in the antennal morphology of pea aphid morphs and used RNA-Seq data to compare chemosensory gene expression differences between the morphs. In addition, we used qRT-PCR to identify genes differentially expressed between head samples that would be involved in antennal chemosensation. Our results

illustrate that the chemosensory gene expression profiles vary between the different pea aphid morphs. We found that sexual dimorphism, polyphenism in mode of reproduction, as well as wing polyphenism and wing polymorphism contribute to the differences in chemosensory gene expression of pea aphid morphs. These results are presented in Chapter 3.

Discrete pea aphid morphs produced via polyphenism maximize their fitness during different seasons and in variable environments. Alternate gene expression underlies the alternate phenotypes. Some of the genes in these morphs are expressed at a higher level in one morph relative to other morphs (morph-biased expression) or exclusively in one morph (morph specific expression). As a consequence, genes underlying plastic traits are expressed only in a subset of a population. In theory, it is then expected that due to conditional expression of these genes, the selective constraints on them would be relaxed and deleterious mutations would build up, which in turn would cause accelerated rates of evolution of these genes (Van Dyken & Wade 2010). We tested this expectation by investigating the influence of pea aphid polyphenism on gene evolution. We hypothesized that morph biased genes will evolve faster than genes equally expressed in all morphs.

We analyzed RNA-Seq data collected from pea aphid morphs and compared the rates of evolution (dN/dS) of morph-biased genes with unbiased genes. We found that morph biased genes evolve faster than unbiased genes as a result of relaxed purifying selection and the rates of evolution of morph biased genes in rare morphs are higher than those in

the common morphs. We discuss and present these results in Chapter 4. This work has been accepted for publication in Molecular Biology and Evolution.

Sensory information plays a critical role in key biological processes of organisms. It is therefore, essential to study the mechanism involved in acquiring and processing sensory information. Our research on sensory ecology and gene evolution in the pea aphid contributes to advancing knowledge of how sensory information influences the life history and behavior of the organisms. Furthermore, it explores the evolutionary consequences of the organisms' plastic response to environmental variation, on gene evolution. With the availability of genomic data of multiple species, it will be possible to investigate molecular processes involved in detection and processing of sensory information across a broad range of species. Our work investigating the sensory ecology of pea aphid is a step forward in that direction. In the future, integration of various behavioral, molecular genetic, and genomic approaches is needed to understand the adaptive significance of sensory information and to unravel its role in evolutionary processes such as reproductive isolation and speciation.

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CHAPTER I

Influence of aphid honeydew on the foraging behavior of *Hippodamia convergens* larvae (Coleoptera: Coccinellidae)

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***This chapter has been published in Ecological Entomology (2012), 37, 184-192**

Abstract

Environmental cues associated with prey are known to increase predator foraging efficiency. Ladybird larvae are major predators of aphids. The sugary excretion of aphids (honeydew) has been proposed to serve as a prey-associated cue for ladybird larvae. Ladybird larvae are regularly found on the ground moving between plants or after falling off a plant. The use of prey associated cues would be particularly beneficial for ladybird larvae on the ground to decide what plant to climb because aphids are patchily distributed within a plant as well as among plants and as a result many plants are either not infested with aphids at all or do not host an aphid species of high nutritional value for ladybird larvae.

Laboratory experiments with larvae of *Hippodamia convergens* Guérin-Méneville were carried out to explore if honeydew accumulated on the ground is used as a foraging cue. If honeydew is a foraging cue, do larvae show differential responses to honeydew of high quality prey *Acyrthosiphon pisum* Harris compared to low quality prey *Aphis fabae* Scopoli? *H. convergens* larvae stayed longer in areas containing honeydew but they did not engage in longer search bouts. Furthermore, the larvae did not distinguish between honeydew from high and low quality aphid prey.

Introduction

Environmental cues often facilitate the location of prey and thus increase foraging efficiency. Such cues are particularly important if prey distribution is aggregated, because prey aggregation increases the variance in foraging success. Aphids (Homoptera: Aphididae) are aggregated both within host plants (not every leaf has aphids), and between plants (many plants have no aphids). Aphids feed on phloem that is rich in sugars but poor in amino acids. As a result aphids have to feed continuously to ingest phloem in large amounts and then excrete the excess sugars in form of honeydew (Dixon 1998). Natural enemies of aphids are known to use honeydew as part of their diet (Hogervorst et al. 2008; Lundgren 2009a) as well as a cue in host/prey location (Budenberg 1990; Romeis & Zebitz 1997; Ide et al. 2007) because honeydew typically accumulates in the vicinity of aphid aggregations. This knowledge has led to research on the effectiveness of spraying sugar solutions on crop fields to attract and retain natural enemies (Lundgren 2009b, Seagraves et al 2011). However, the benefit of providing sugar to increase the effectiveness of natural enemies is not clear because non prey food including sugars can also divert predators from predation (Spellman et al 2006). Ladybird beetles are major predators of aphids, and some species are commonly used as a biological control agent to control aphid populations. Both the adult and the larval stages of ladybird beetles consume aphids. In this paper we explored if the foraging behavior of predatory larvae of the ladybird species, *Hippodamia convergens* Guérin-Méneville (Colleoptera: Coccinellidae) is influenced by honeydew.

At a minimum, honeydew composition varies with the host plant, aphid species, aphid age, and the level of ant tending (Fischer & Shingleton 2001; Fischer et al. 2002). Thus, honeydew might not only provide information on the presence of prey but also information on prey suitability and vulnerability. Most of the research on the use of honeydew as an environmental cue has focused on parasitoids, which are more specialized foragers than ladybird beetles. Parasitoids are more reliant on environmental cues to find suitable aphid hosts. In the past researchers concluded that ladybird larvae search for prey randomly and are unable to detect prey prior to physical contact (Banks 1957; Dixon 1959). However, later evidence suggests that they respond to visual and olfactory cues (Stubbs 1980; Nakamuta 1984; Jamal & Brown 2001).

There are only few studies that evaluate the effect of honeydew on foraging behavior of ladybird larvae (Carter & Dixon 1984; Ide et al. 2007). Generally prey associated cues act as attractant stimuli resulting in foragers biasing their movement towards areas containing hosts or prey. Alternatively, they can act as an arrestant stimuli which reduce the movement rate (reducing the distance or area covered per unit time) and thereby increase the likelihood of prey encounter (Fellows et al. 2005). Carter & Dixon (1984) demonstrated that *Coccinella septempunctata* L. larvae were more likely to return to wheat ears that were covered with *Sitobion avenae* Fabricius honeydew compared to clean ones. Re-searching the ears with honeydew resulted in an increased number of aphids consumed compared to ears without honeydew.

Larvae move frequently from one plant to another via touching leaves of neighboring plants (Banks 1957). However, sometimes they must walk on the soil to reach a plant that is farther away or after being dislodged from plants due to wind, water, or by dropping in response to predators. It would be beneficial for ladybird larvae on the ground to recognize which plants are infested with aphids because aphids have a clumped distribution and, as a consequence, there may only be a few plants in a field that are infested with aphids. Experiments by Ide et al. (2007) suggest that honeydew accumulating on the ground beneath aphid-infested plants might be used by *C. septempunctata* larvae as a cue for locating aphid infested plants. Furthermore, plants can be infested with many different aphid species (either on the same plant or on different plant species) that vary in suitability for the ladybird larvae due to aphid abundance, size, escape ability, and nutritional quality (Dixon 2000). Thus, it would be an additional advantage if ladybird larvae could distinguish between honeydew from different aphid-plant systems to choose plants hosting the most profitable aphid species. Ide et al. (2007) showed that *C. septempunctata* larvae stayed longer in areas containing honeydew of prey that was easy to catch and so more profitable. We might expect a similar response from ladybird larvae if the difference in prey profitability is due to a difference in the nutritional value of aphids.

The objective of our study was to test the generality of Ide et al's (2007) findings by using a different ladybird species, and honeydew from two aphid species that differ in nutritional value. Specifically we asked: Does honeydew on the ground act as a prey associated cue? If so, does honeydew from low quality aphid species act as a deterrent?

Materials and Methods

The experiments were designed to increase our understanding of the behavior of ladybird larvae when searching on the ground for plants that are infested with aphids. We used honeydew of two aphid species, *Acyrthosiphon pisum* Harris (Homoptera: Aphididae) and *Aphis fabae* Scopoli (Homoptera: Aphididae) that vary considerably in nutritional value. Survival of *H. convergens* from first instar to the adult stage was reduced by 81%, developmental time increased by 55%, and adult mass decreased by 49% on an *A. fabae* diet as compared to an *A. pisum* diet (Hinkelman & Tenhumberg 2013). The experimental arena was similar to the one described in Ide et al. (2007). Each foraging arena contained two patches that differed in foraging cues (Figure 1). Each patch consisted of a Petri dish lid with a climbing structure in the middle (either a plant or a stick).

Hypothesis 1: Honeydew on the ground acts as a prey associated cue.

To test this hypothesis the foraging behavior of ladybird larvae on Petri dish lids with and without honeydew was compared (Experiments 1-3, Table 1). Ladybird larvae use visual and olfactory cues from plants and aphids to locate prey (Stubbs 1980; Jamal & Brown 2001). In order to isolate the effect of honeydew on foraging behavior aphids were not included in the trials and sticks (bamboo skewers, diameter 4mm, height 15 cm) were used as climbing structure instead of plants (Experiments 2, 3 and 4). To ensure that using a stick as climbing structure would not conceal the effect of honeydew, one experiment with three day old *Vicia faba* L. plants was conducted (Experiment 1). The

plants had two similar sized leaves, and were approximately 10 cm high. The aim of this experiment was to exclude the possibility that ladybird larvae recognize that sticks cannot contain aphids and thus do not respond to honeydew as a cue. If *H. convergens* larvae use honeydew as a cue we would predict that, compared to patches without honeydew, patches with honeydew attract a larger proportion of larvae, a higher proportion of the larvae would climb sticks, the patch residence time would be longer, the times until encountering a stick would be shorter, and larvae spend more time searching than resting or consuming honeydew.

Hypothesis 2: Honeydew from low quality aphids acts as a deterrent

To test this hypothesis we conducted experiment 4, where one foraging patch included honeydew of low quality *A. fabae*, while the other patch contained honeydew of high quality *A. pisum*. If larvae can distinguish between honeydew types then it is expected that the difference in response to *A. pisum* honeydew and *A. fabae* honeydew would be qualitatively similar to the difference in response to honeydew and no honeydew. Compared to Petri dish lids with *A. fabae* honeydew, patches with honeydew from high quality *A. pisum* are expected to attract a larger proportion of larvae, a higher proportion of the larvae would climb sticks, the patch residence time would be longer, the times until encountering a stick would be shorter, and larvae spend more time searching in favor of resting or consuming honeydew.

General experimental procedure*:*

An overview of the different experiments is listed in Table 1. The arena was uniformly covered with white desert sand such that the sand was flush with the edges of the Petri dish lids. The sand in the experimental arena was rinsed five times with water after each experimental trial; all plants, sticks and larvae were used only once. In all experiments honeydew quantity was recorded to detect any bias resulting from differences in honeydew quantity. The experiments were carried out at 25 °C under a fluorescent light (27 W) on a laboratory table. To acclimatize the ladybird larvae to the experimental arena a single individual was placed under a Petri dish lid in the center of the experimental arena equidistant from both Petri dish lids. After 3 minutes the Petri dish lid was carefully removed and the behavior of the released ladybird larva was videotaped using two cameras (Sony Model HDR-SR11 and SR5).The cameras were positioned to cover behavior on Petri dishes and both sides of the plant or stick. As a consequence the resolution of the video recordings was insufficient to distinguish between detailed behavioral categories like being still and consuming honeydew. Thus we merged both behaviors into the "resting/feeding" category. The trial was terminated after the larva left the first encountered Petri dish lid or after 30 minutes. If a larva did not start searching within the first 10 minutes after removing the Petri dish lid, the trial was discarded. The videos were re-played and the behavior of ladybird larvae was scored using an event recorder (Jwatcher software, version 1.0 for Windows XP Blumstein & Daniel. 2007). All behavioral categories are listed in the Appendix, Table A1; an example of a behavioral sequence is shown in the Appendix, Figure A1.

Honeydew collection: Honeydew was collected on 4 cm Petri dish lids placed inside clip

cages (diameter 6cm, depth 3cm). Thirty adult aphids were transferred to each clip cage using a paint brush. Then the clip cages were fastened to leaves of intact *V. faba* plants. The clip cages restricted the aphids to feed in a confined area and to drop honeydew on Petri dish lids in the bottom of the clip cages. The aphids were allowed to feed and deposit honeydew for 24 hours. Each Petri dish lid was weighed before and after honeydew collection to measure the quantity of honeydew deposited (Ohaus, Adventurer Pro AV64C, read=0.0001g). For the experiments with plants and the experiments investigating the influence of honeydew presence, equal numbers of aphids were used per clip cage (30 *A. pisum* and 30 *A. fabae* respectively). The amount of honeydew produced this way varied between the two aphid species (*A. pisum*: mean 13.04 mg, range 6.10-22.60 mg; *A. fabae*: mean 5.26 mg, range 2.03- 12.37 mg). So, for the last experiment 12 *A. pisum* and 30 *A. fabae* were used per clip cage to get approximately equal amounts of honeydew (Table 1).

Providing experience: Prior to our experiments *H. convergens* larvae were fed exclusively *A. pisum* rather than a mixed diet because of the extremely low survival on an *A. fabae* diet (13% on *A. fabae*; and 70% on *A. pisum*, Hinkelman & Tenhumberg 2013). To make sure that a single aphid diet would not bias our results in any way (e.g. learning has been demonstrated in ladybird larvae; Boivin et al. 2010) we provided all experimental larvae with an opportunity to make an association between honeydew type and aphid species. Specifically, prior to the experimental trials all ladybird larvae were provided two hours experience with each aphid species and its honeydew, e.g. they were allowed to forage for *A. pisum* aphids in the presence of *A. pisum* honeydew and *A. fabae*

aphids in the presence of *A. fabae* honeydew. The sequence of honeydew type experience was randomized. To ensure that the ladybird larvae made the association between honeydew type and aphid species only larvae that consumed at least one aphid of each species were used for the trials. *V. faba* leaves covered with honeydew were cut from aphid infested plants and all aphid exuviae were removed. Then each leaf was placed on the bottom of a clip cage with four first instar aphids of the species that produced the honeydew. First instars were used because their size was sufficiently small to not satiate the ladybird larvae and thus not affect their motivation to search for food. A single fourth instar ladybird larva was transferred to the honeydew covered leaf in the clip cage and then the clip cage was fastened to an intact leaf of a *V. faba* plant.

Insect rearing: *A. pisum*, *A. fabae* and *H. convergens* cultures were reared in growth chambers at approximately 27°C on a 16:8 (L: D) cycle. Both aphid species had been maintained in the laboratory for three years using *V. faba* as the host plant. The aphids and their host plants were kept in Dacron chiffon netted aluminum cages (31 x 31 x 61 cm; Bioquip Products). Adult *H. convergens* were purchased from commercial suppliers (Carolina Biological Supply Co.) and reared on an *A. pisum* diet in larger chiffon netted aluminium cages (44 x 51 x 61 cm). Clay pots were provided as oviposition substrate, and once eggs were found the pots were transferred to a hatching cage to avoid egg cannibalism by the adults. Within 24 hours of hatching individual larvae were transferred to glass vials (diameter 2.5cm, length 9.5cm) with foam stoppers. The larvae were fed an excess amount of fresh *A. pisum* daily until they reached the fourth instar stage. Fourth-instar ladybird larvae (within 24 hours of molting from the third instar) were used for all

experiments as this is the most voracious juvenile stage (Dixon 2000).

***Statistical Analysis**:*

All analyses were performed using R (v. 2.10.0, R Development Core Team 2009).

Binomial tests were used to analyze if the proportion of larvae arriving at a Petri dish lid is influenced by the presence and type of honeydew (Experiments 1-4). Tests of equal or given proportions were used to test the null hypothesis that the proportion of larvae climbing a stick or plant is independent of the presence or type of honeydew (Experiments 1-4). The patch residence times (Experiments 1-4), the times until encountering the stick (Experiments 2-4) and bout duration data (Experiments 2-4) were analyzed with Cox's proportional hazards models using the presence or type of honeydew, and honeydew quantity as covariate. If a larva did not stop a particular behavior until the end of the trial (30 minutes) the observation was censored. Transition rates were calculated as follows: For example, let "searching on a Petri dish" be state A, "resting/feeding on a Petri dish" be state B, "searching on sand (off Petri dish)" be state C, and climbing a stick be state D, then the rate of transitioning from searching to resting/feeding (a_{AB}) was calculated as

$$a_{AB} = N_{AB}/(N_{AB} + N_{AC} + N_{AD})\ \bar{x} \qquad \text{(Eq 1)}$$

Where N indicated the total number of observed transitions between two states, and $\bar{x}$ was the mean search duration (Haccou & Meelis 2002, p20).

Results

In all experiments the covariate honeydew quantity had no significant effect on the patch residence time, the time until ladybird larvae encountered a stick, and the search and rest bout durations (results not shown). Thus only the results of Cox proportional hazard models that included a single covariate z_1, i.e. the presence (experiments 1 – 3) or type (experiment 4) of honeydew, were shown.

Hypothesis 1: Honeydew on the ground acts as a prey associated cue.

Arrival at Petri dish lid: *H. convergens* larvae were equally likely to arrive at Petri dish lids with or without honeydew (Appendix Table A2; Experiments 1, 2 and 3; in all three binomial tests $p >> 0.05$), suggesting that the arrival of ladybird larvae at a Petri dish lid is independent of the presence of honeydew.

Climbing response: The presence of honeydew had no effect on the proportion of larvae climbing plants or sticks at least once during a patch visit (Appendix Table A3; Experiments 1, 2, and 3; in all three tests of equal or given proportions $p >> 0.05$).

Patch residence time: Ladybird larvae stayed significantly longer on Petri dish lids containing *A. pisum* or *A. fabae* honeydew compared to Petri dish lids without honeydew (Table 2, Experiments 1, 2, and 3). Figure 2 illustrates how the proportion of ladybird

larvae remaining in the patch changed over time (Experiment 2). In the presence of *A. pisum* honeydew this proportion changed more slowly compared to the absence of honeydew. The time when 50% of the ladybird larvae have left a patch (T50) can be interpreted as the average patch residence time. Without honeydew 50% of the larvae left after only 30 seconds (T50, Table 2), while ladybird larvae stayed on average 443 seconds in patches with *A. pisum* honeydew. Using plants as climbing structure produced qualitatively similar results, i.e. the presence of *A. pisum* honeydew significantly increased patch residence time (Experiment 1, Table 2). However, the average patch residence time in both treatments was longer when plants were the climbing structure.

The β -value of the Cox's proportional hazards model quantifies how much the leaving tendency of the baseline hazard changes as a result of covariates. In Experiments 1, 2, and 3, the baseline hazard was the model without honeydew, and in Experiment 4 the baseline hazard was the leaving tendency on Petri dish lids with *A. fabae* honeydew. The β -value of -0.99 in Experiment 1indicated that in the presence of *A. pisum* honeydew the leaving tendency was about 1/3rd of the leaving tendency in the absence of honeydew ($e^{-0.99}$ =0.37). A low leaving tendency produced long patch residence times, thus the more negative the β -value the longer was the average patch residence time (Table 2, No honeydew: T50 =223 sec; *A. pisum* honeydew: T50 =712 sec).

Time till first stick encounter: After arriving at a Petri dish lid, ladybird larvae took significantly longer to encounter the stick when *A. pisum* or *A. fabae* honeydew was present as compared to when honeydew was absent (negative β -values, Table 3).

Behavioral Pattern: The duration of individual rest bouts was significantly influenced by the presence of honeydew (Table 4). If there was honeydew on a Petri dish lid *H. convergens* larvae rested for a longer amount of time (negative β-values in Table 4) compared to larvae on clean Petri dish lids. The presence of *A. fabae* honeydew significantly decreased search bouts duration compared to the absence of honeydew (positive β -values in Table 5), but the effect was very small (T50 in the absence of honeydew was 6 seconds compared to 4 seconds in the presence of *A. fabae* honeydew). The difference was no longer significant (p=0.117) when removing three unusual data points that had unusually long search bouts in the absence of *A. fabae* honeydew. In contrast, the presence of *A. pisum* honeydew had no effect on search bout duration. However, when honeydew was present ladybird larvae were more likely to transition from searching to resting/feeding than to any other behavior (Figure 3A). In contrast, in the absence of honeydew the transition from searching to resting/feeding was smallest and *H. convergens* larvae were more likely to transition to climbing or leaving the Petri dish lid (Figure 3B). High transition rates to resting/feeding and long resting/feeding bouts resulted in longer patch residence times in the presence of honeydew compared to its absence (Table 2, Note, that the resting/feeding category included consuming honeydew). *A. fabae* honeydew had similar effects on behavioral transitions as *A. pisum* honeydew (Appendix, Figure A2).

Hypothesis 2: Honeydew from low quality aphids acts as a deterrent

The foraging behavior of ladybird larvae was not influenced by the type of honeydew. There was no difference in the proportion of larvae arriving at Petri dish lids with *A. pisum* honeydew compared to Petri dish lids with *A. fabae* honeydew (Binomial Test, $p >> 0.05$) consistent with the hypothesis that the arrival of ladybird larvae at a Petri dish lid is independent of the type of honeydew. After arriving on a Petri dish lid the tendency to leave was not influenced by the type of honeydew (Table 2). The time until encountering a stick was not influenced by the type of honeydew (Table 3), and honeydew type had no effect on the proportion of larvae climbing sticks (Test of equal or given proportions, $p >> 0.05$). Finally, the duration of rest and search bouts were not influenced by honeydew type (Table 4 and 5), and the behavioral transitions were similar on Petri dish lids containing *A. pisum* and *A. fabae* honeydew (Appendix, Figure A3).

Discussion

Foraging cues from the environment generally increase the foraging efficiency of insect natural enemies. In this paper we explored if honeydew on the ground acts as a foraging cue for *H. convergens* larvae. If honeydew acts as an attractant stimulus, we would expect that the proportion of *H. convergens* larvae arriving at Petri dishes containing honeydew would be higher compared to Petri dish lids with no honeydew. In contrast, in our experiment *H. convergens* larvae arrived at each Petri dish lid in equal proportions. This is consistent with the findings of Ide et al. (2007), although they used larvae of a different ladybird species. The experiments of this study and the ones by Ide et al. were not conducted in a wind tunnel because the main purpose was to examine a change in behavior after encountering honeydew. Thus, it is possible that the experimental design

was not suitable for detecting responses to olfactory cues. There is evidence that *H. convergens* larvae respond to olfactory volatile chemicals associated with the aphid *Myzus nicotianae* Blackman. Jamal & Brown (2001) found that in a wind tunnel *H. convergens* larvae responded to aphids feeding on leaves as well as to the leaves that were previously exposed to aphids. However, these authors did not isolate the effect of *M. nicotianae* honeydew in their experiments.

It has been suggested that honeydew encounter elicits an intensive search mode in mealybug predators (ladybird species *Cryptolaemus montrouzieri* Mulsant, Heidari & Copland 1993) and psyllid parasitoids (*Psyllaephagus pistaciae* Ferrière, Mehrnejad & Copland 2006), and may explain that *C. septempunctata* larvae consumed a higher number of aphids on wheat ears that were covered with honeydew compared to clean wheat ears that were only recently colonized with aphids (Carter & Dixon 1984). However, the ladybird larvae also spent a much longer time on honeydew covered ears, so that the number of aphids consumed per minute (consumption rate) was actually smaller. In our experiments, *H. convergens* larvae responded to honeydew presence by staying longer in patches containing honeydew as compared to clean patches. However, our experimental design did not allow us to examine aphid encounter rate as we did not include aphids in the trials.

If aphid honeydew encounter would elicit an intensive search mode in *H. convergens* larvae, then larvae searching on Petri dish lids with honeydew would encounter a stick (and climb it) more frequently compared to clean Petri dish lids. In contrast, in our

experiments the time until encountering a stick was longer on Petri dish lids with honeydew compared to Petri dish lids without honeydew. Almost all stick encounters were followed by climbing the stick as a consequence of negative geotaxis. The slow rate of stick encounter in the presence of honeydew was related to *H. convergens* larvae resting/feeding frequently (they were more likely to transition from searching to resting/feeding than to any other behavior) and remaining in a resting/feeding state for a very long time (Table 4, Note, that the resting category included consuming honeydew). In contrast, Ide et al. (2007) found that the proportion of ladybird larvae (*C. septempunctata*) climbing a stick was higher if honeydew was present at the base of Petri dish lids compared to clean Petri dish lids. Ide et al. (2007) also suggested that the climbing response of the larvae is influenced by the vulnerability of the aphid species excreting the honeydew. They reported that more larvae climbed the sticks in the presence of *Aphis craccivora* Koch honeydew as compared to honeydew from the difficult to catch aphid species *A. pisum*. In our experiments, *A. fabae* is a lower quality prey than *A. pisum,* but we did not find any effect of honeydew type on the foraging behavior of *H. convergens* larvae (Experiment 4).

There are several potential reasons for the aforementioned discrepancies between our results and the results of Ide et al (2007). First, Ide et al. used *C. septempunctata* larvae, while we used *H. convergens* larvae. It is possible that different coccinellid species respond differently to honeydew suggesting that findings from one species cannot be generalized to all other aphidophagous coccinellid species.

Second, the honeydew of the aphid species in the study by Ide et al. (2007) differed in honeydew quantity and distribution (*A. craccivora* produced more honeydew and excreted it closer to the plant stem than *A. pisum*). *C. septempunctata* larvae returned and re-searched areas with honeydew more frequently compared to areas without honeydew (Carter & Dixon 1984) and it is possible that this response to honeydew is stronger if honeydew occurs in larger amounts. This response to honeydew might have resulted in a higher probability of stick encounter in trials with either more honeydew or where most of the honeydew was located closer to the stick independent of the aphid species that produced the honeydew. Thus, the differences in the distribution and quantity of honeydew of *A. pisum* and *A. craccivora,* rather than differences in honeydew composition might explain differences in the climbing response of *C. septempunctata* larvae. In contrast, we used clip cages to collect honeydew, which allowed us to control honeydew quantity by using different numbers of aphids (30 *A. fabae*, 12 *A. pisum*) per clip cage. Furthermore, because the clip cages were fastened to the leaves and the collection area was small, we did not see any obvious differences in the honeydew distribution between the two aphid species. Third, in Ide et al. (2007) the predatory larvae had a longer experience with both prey species compared to our study. *C. septempunctata* were reared on approximately equal amounts of *A. craccivora* and *A. pisum* , while in our study the *H. convergens* larvae were exclusively reared on *A. pisum* because of the poor survival on *A. fabae*; thus their experience with *A. fabae* was restricted to a two hour exposure prior to the experiment. However insufficient experience with *A. fabae* would have biased *H. convergens* larvae to respond more strongly to the more familiar prey (Ettifouri and Ferran 1993). In contrast, *H. convergens* larvae were impartial to the two

honeydew types, and the likelihood of larvae climbing a stick or plant was not influenced by prior *A. pisum* honeydew encounter. This suggests the short experience was sufficient for *H. convergens* larvae to become familiar with *A. fabae* and its honeydew. The results of the present study are also consistent with the observation that *H. convergens* larvae did not discriminate against *A. fabae* when reared on a mixed diet of *A. fabae* and *A. pisum* (in glass vials in the lab, Hinkelman and Tenhumberg 2013)

Why did H. convergens larvae not use A. pisum or A. fabae honeydew on the ground as a foraging cue indicating the presence of aphids on the plants above?

It is possible that the cue use may vary between different predator and prey systems. For instance, *H. convergens* larvae may not climb a plant or stick in response to honeydew of *A. fabae* or *A. pisum* because of a relatively short evolutionary history between those species. Both aphid species have originated in Europe and were introduced to North America before 1880 (Foottit et al. 2006), whereas *H. convergens* is native to North America. It is possible that predators in Ide et al. (2007) used honeydew as a cue because the authors used predator and prey species that have a longer shared evolutionary history. Ladybird beetles are generalist predators that feed on a large number of different aphid species (Hodek & Honek 1996), resulting in a relatively low encounter rate with the introduced aphid species, which would slow the selection pressure for recognizing cues from introduced prey species.

Furthermore, honeydew on the ground might not be a reliable indicator of aphid density on surrounding plants, and the usefulness of cues to increase the foraging efficiency

depends on how reliable cues are (Vet et al. 1991). If the benefit of using honeydew as cue is small it is possible that not all aphidophagous predator species have evolved a response to honeydew. It is possible that under field conditions, honeydew evaporates quickly, washes away through rain, or the volatile components of honeydew loose kairomonal activity in a short period of time. For instance, it has been reported that the kairomonal activity of *Brevicoryne brassicae* L. (Aphididae) honeydew decreased over time and was lost completely after 72 hours (Shaltiel & Ayal 1998). Furthermore, when ladybird larvae encounter honeydew on the ground the aphids that produced the honeydew may no longer be present because in the meantime they may have been preyed upon or dispersed (Li et al. 1997).

Conclusions: Our experiments illustrate that *H. convergens* larvae are not more likely to climb a stick or plant in the presence of *A. pisum* or *A. fabae* honeydew nor do they seem to distinguish between the honeydew of aphids that differ in profitability. We speculate that our results could be explained by the short shared evolutionary history between predator and prey.

Acknowledgments

Funding for the research was provided by the Arthur William Sampson Fellowship (SRP), School of Biological Sciences Special Funds (SRP), and Walker Fellowship (SRP). Susan Greni and Matt Koziol helped in aphid honeydew collection. John Reese (Kansas State University) and Stephen Kaffka (UC-Davis) provided *A. pisum* and *A. fabae* aphids to start our laboratory cultures. Special thanks to Travis Hinkelman for

useful discussions, and comments on earlier versions of the manuscript. Comments by Diana Pilson, Sabrina Russo, Natalie West, Ben Nolting, and Drew Tyre also improved the manuscript.

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Tables and Figures

Table 1: Overview of experiments. The treatment indicates whether one or both Petri dishes contained honeydew (*A. pisum* or *A. fabae*), and if one of the Petri dishes was clean (None). CT specifies the climbing structure; n is sample size

Experiment				**No of Aphids used for honeydew collection**	
No	**Treatment**	**CT**	**n**	***A. pisum***	***A. fabae***
1	*A. pisum/None*	Plants	31	30	-
2	*A. pisum/None*	Sticks	63	30	-
3	*A. fabae/None*	Sticks	68	-	30
4	*A. pisum /A. fabae*	Sticks	66	12	30

Table 2: Results of the Cox's proportional hazards analysis of patch residence time. The treatment indicates whether one or both Petri dishes contained honeydew (*A. pisum* or *A. fabae*), and if one of the Petri dishes was clean (None). CT specifies the climbing structure; T50 refers to the time until 50% of the ladybird larvae have left the patch, and CT is the climbing structure

Experiment		**CT**	***β***	**SE**	**Pr(>|*z*|)**	**T50 (seconds)**		
No	**Treatment**					**None**	***A. pisum***	***A. fabae***
1	*A. pisum/None*	plant	-0.99	0.42	0.017	223	712	-
2	*A. pisum/None*	stick	-1.34	0.29	< 0.001	30	443	-
3	*A. fabae/None*	stick	-0.87	0.27	0.002	10	-	160
4	*A. pisum /A. fabae*	stick	-0.16	0.25	0.53	-	252	355

Table 3: Results of the Cox's proportional hazards analysis of Time-Till-First-Stick-Encounter. The treatment indicates whether one or both Petri dishes contained honeydew (*A. pisum* or *A. fabae*), and if one of the Petri dishes was clean (None). CT specifies the climbing structure; T50 refers to the time until 50% of the ladybird larvae have encountered a stick.

Experiment		**CT**	***β***	**SE**	**Pr(>|z|)**	**T50 (seconds)**		
No	**Treatment**					**None**	***A. pisum***	***A. fabae***
2	*A. pisum/None*	stick	-1.45	0.54	0.007	25	405	-
3	*A. fabae/None*	stick	-1.42	0.47	0.002	25	-	368
4	*A. pisum/A. fabae*	stick	0.15	0.30	0.61	-	265	208

Table 4: Results of the Cox's proportional hazards analysis of rest bout duration. The treatment indicates whether one or both Petri dishes contained honeydew (*A. pisum* or *A. fabae*), and if one of the Petri dishes was clean (None). CT specifies the climbing structure; T50 refers to the time until 50% of the ladybird larvae have transitioned to another behavior.

Experiment		**CT**	***β***	**SE**	**Pr(>|z|)**	**T50 (seconds)**		
No	**Treatment**					**None**	***A. pisum***	***A. fabae***
2	*A. pisum/None*	stick	-2.12	0.48	< 0.001	3	24	-
3	*A. fabae/None*	stick	-0.91	0.29	< 0.001	6	-	16
4	*A. pisum/A. fabae*	stick	-0.10	0.14	0.44	-	27	24

Table 5: Results of the Cox's proportional hazards analysis of search bout duration. The treatment indicates whether one or both Petri dishes contained honeydew (*A. pisum* or *A. fabae*), and if one of the Petri dishes was clean (None). CT specifies the climbing structure; T50 refers to the time until 50% of the ladybird larvae have transitioned from searching to another behavior

Experiment		**CT**	***β***	**SE**	**Pr(>|*z*|)**	**T50 (seconds)**		
No	**Treatment**					**None**	***A. pisum***	***A. fabae***
2	*A. pisum/None*	stick	0.15	0.14	0.31	7	6	-
3	*A. fabae/None*	stick	0.44	0.14	0.003	6	-	4
4	*A. pisum/A. fabae*	stick	-0.06	0.11	0.58	-	5	5

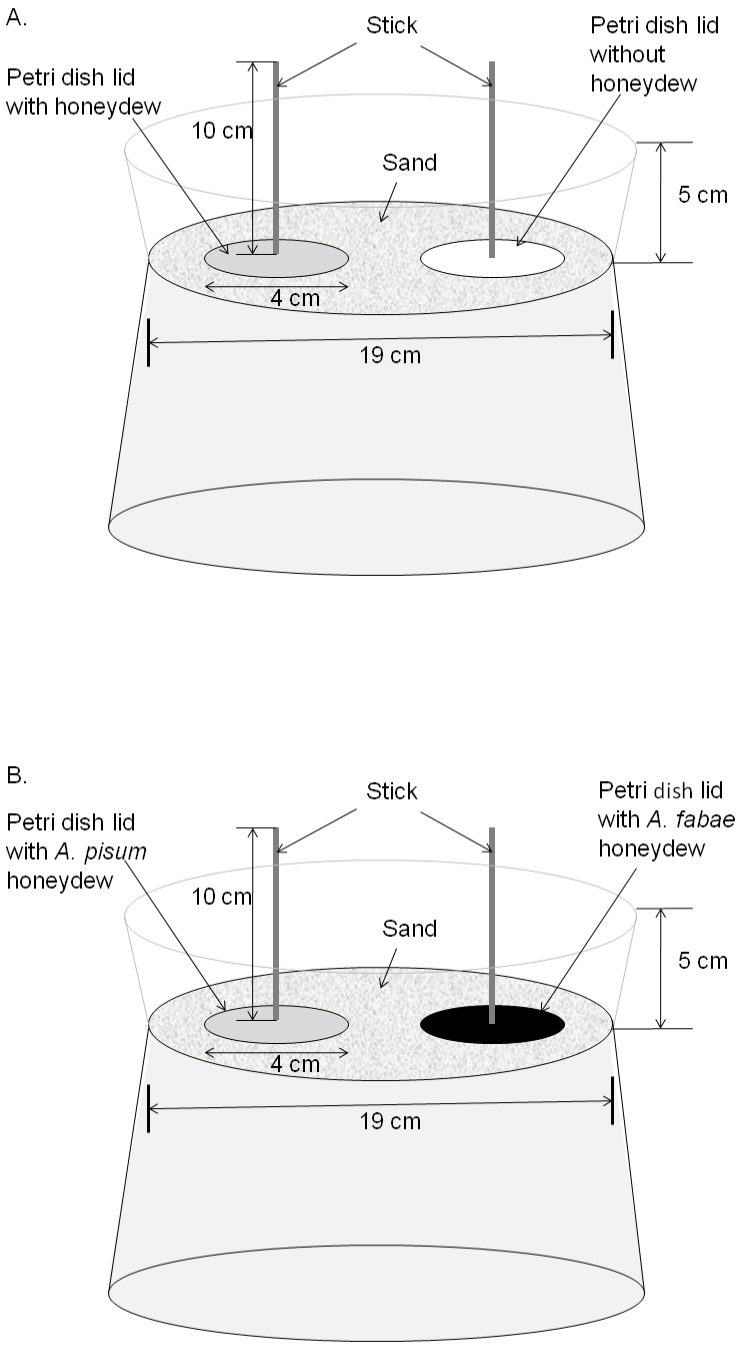

Figure 1: Experimental arena. (A) Setup for experiments No 2 and No 3; in experiment No 1 the sticks were replaced with small *Vicia faba* plants. (B) Setup for experiment No 4.

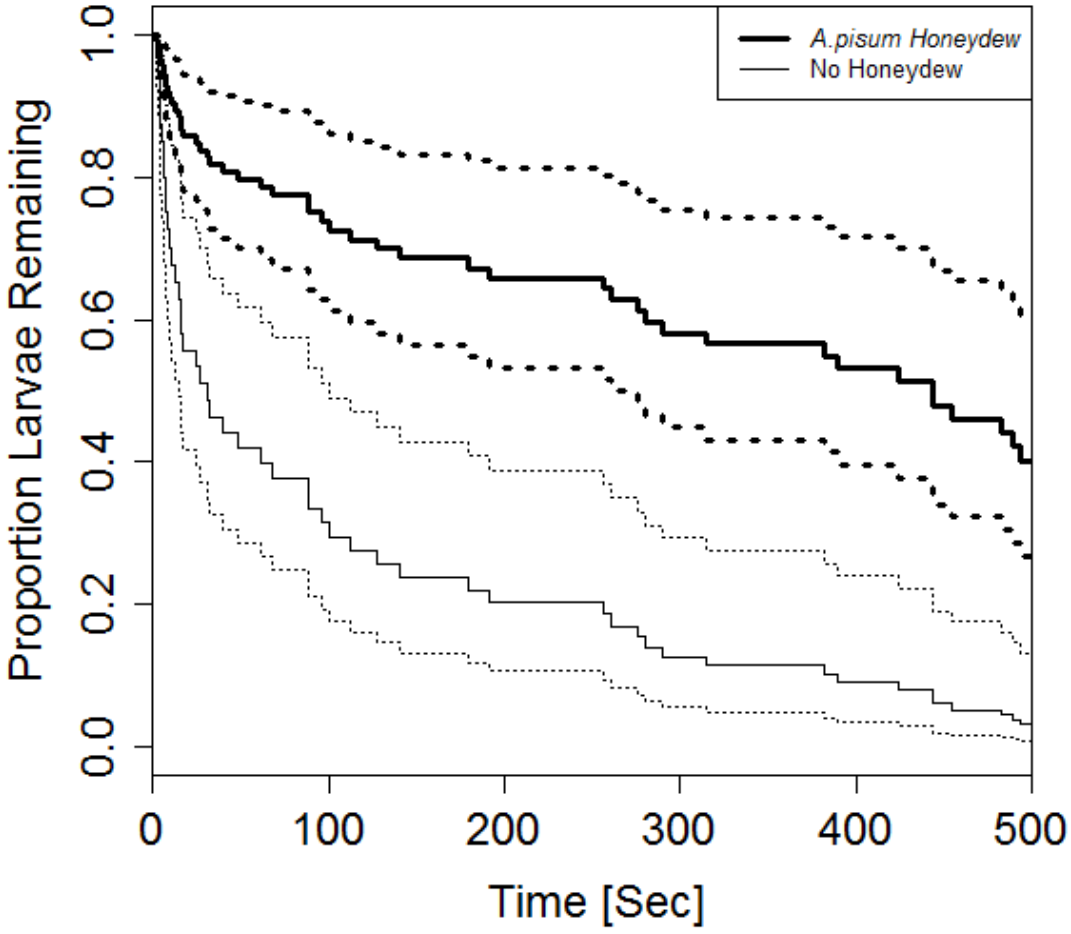

Figure 2: Effect of honeydew presence on patch residence time. The solid lines represent how the proportion of ladybird larvae remaining in the patch changes over time, and the dotted lines are the 95% confidence intervals.

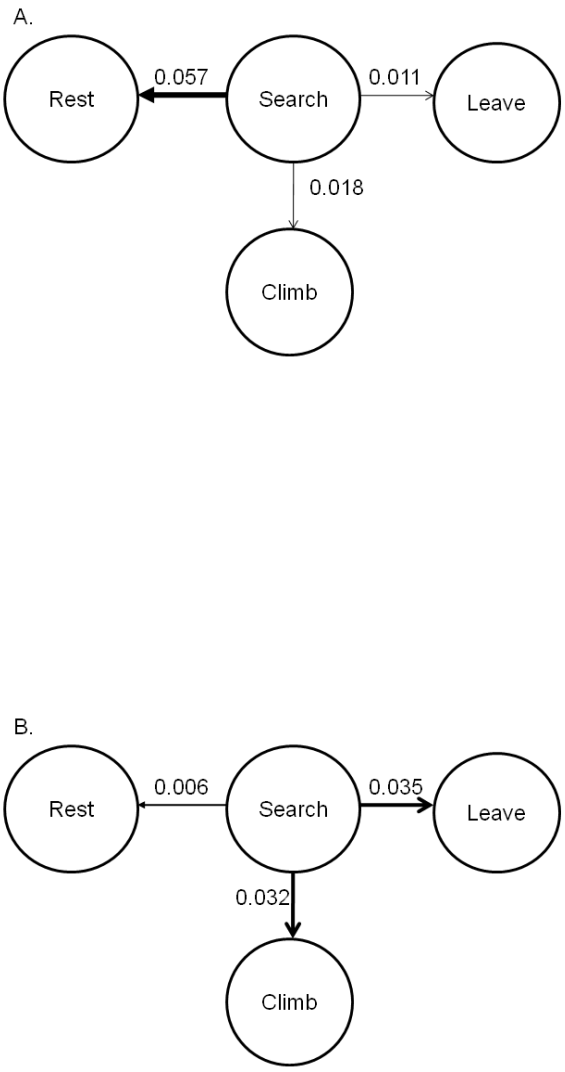

Figure 3: Rates at which *H. convergens* larvae transition from searching on Petri dish lids to resting/feeding, climbing or leaving a patch in Experiment No 2. The thickness of the arrow is proportional to the transition rates. (A) Presence of *A. pisum honeydew*; (B) Absence of honeydew

Supplementary Tables and Figures

Table A1: Description of behavioral categories (see Fig A1 for an example)

Behavior	**Description**
Arrive at Petri dish lid	The larva touched the Petri dish lid of either patch.
Searching	Movement of the entire body on the Petri dish lid
Resting/Feeding	Legs of the larva stopped moving on Petri dish or stick. Rest on Petri dish included the time spent feeding on honeydew. Sometimes larvae were found resting with some part of the body on the stick and with the head and front part of the body on the Petri dish lid. This behavior was also scored as resting/feeding on Petri dish.
Stick encounter	Front part of the larva was within 1mm of the base of the stick
Climbing stick	The larva placed the front part of the body on the stick after stick encounter
Leave Petri dish lid	All body parts are off the Petri dish lid

Table A2: Arrival at Petri dish lids. The treatment indicates whether one or both Petri dishes contained honeydew (*A. pisum* or *A. fabae*), and if one of the Petri dishes was clean (None). CT specifies the climbing structure. The binomial test evaluated if the proportion of larvae arriving at a Petri dish (number of successes) is influenced by the presence of honeydew. If the confidence intervals include 0.5 the presence of honeydew does not significantly influence the arrival response.

Experiment		**CT**	**p-value**	**No of successes**	**No of trials**	**Proportion of larvae arriving**	**95 % Confidence interval**	
No	**Treatment**							
1	*A.pisum/None*	plant	1	15	31	0.48	0.30	0.67
2	*A.pisum/None*	stick	1	32	63	0.51	0.38	0.64
3	*A. fabae/None*	stick	0.90	35	68	0.51	0.39	0.64
4	*A.pisum /A.fabae*	stick	0.90	32	66	0.48	0.36	0.61

Table A3: Climbing Response. The treatment indicates whether one or both Petri dishes contained honeydew (*A. pisum* or *A. fabae*), and if one of the Petri dishes was clean (None). CT specifies the climbing structure. The test of equal or given proportions evaluated if the proportion of larvae climbing a plant or a stick (number of successes) is influenced by the presence of honeydew. If the confidence intervals include 0.5 the presence of honeydew does not significantly influence the climbing response.

Experiment		**CT**	**p-value**	**No of successes**	**No of trials**	**Proportion of larvae climbing**	**95 % Confidence interval**	
No	**Treatment**							
1	*A.pisum/None*	plant	0.12	11	15	0.73	0.45	0.91
2	*A.pisum/None*	stick	0.11	21	32	0.66	0.47	0.81
3	*A. fabae/None*	stick	1	17	35	0.49	0.32	0.66
4	*A.pisum /A.fabae*	stick	0.12	22	34	0.65	0.46	0.80

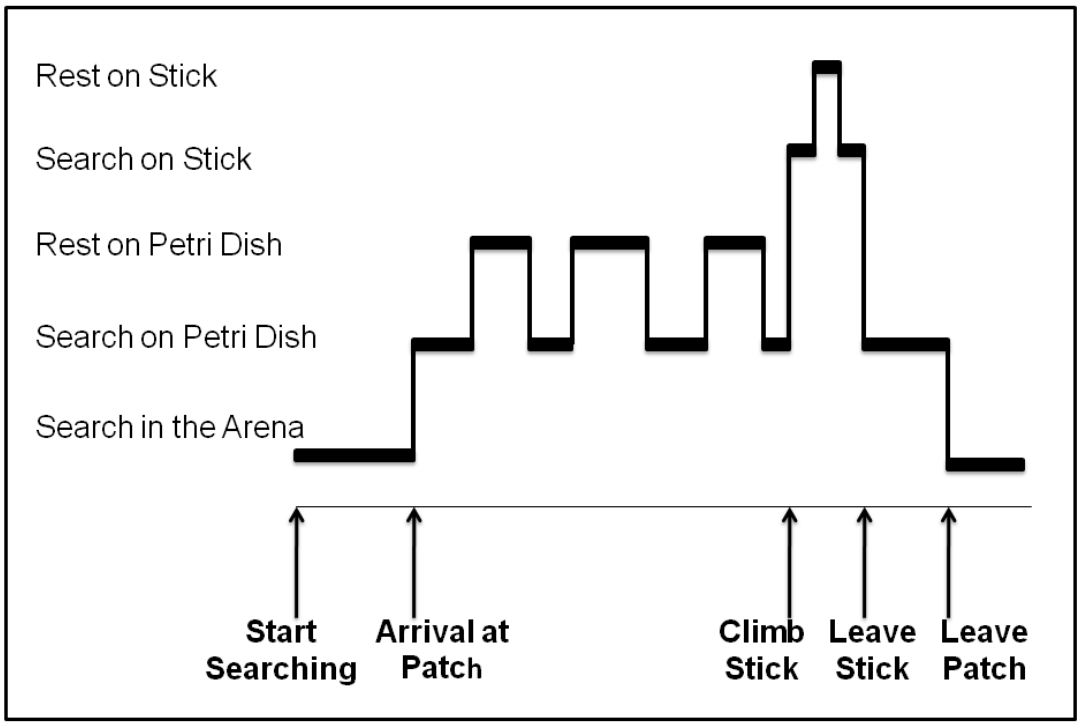

Figure A1: Bar plot of behaviors scored during one example visit of a Petri dish lid.

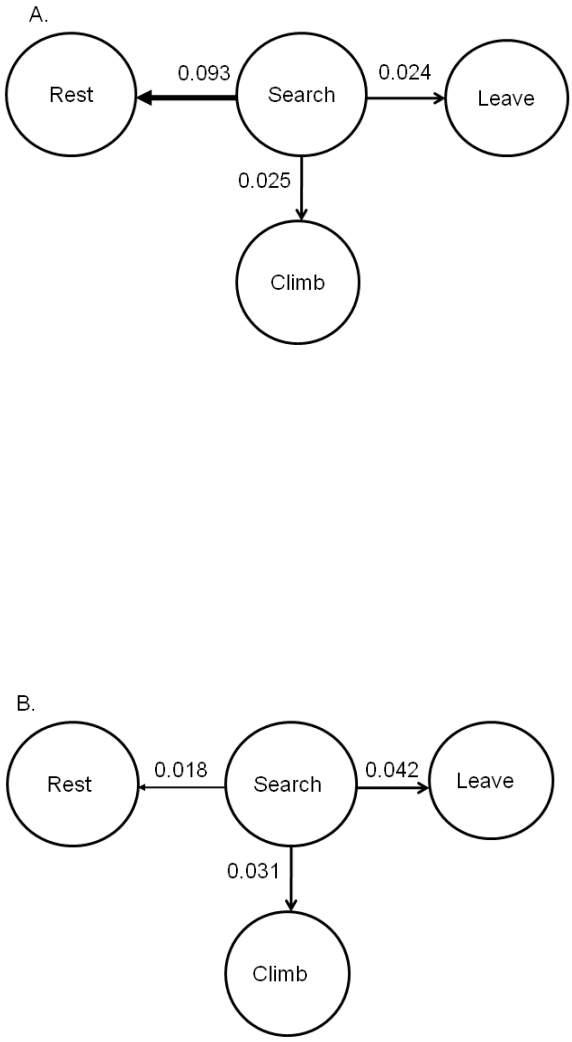

Figure A2: Rates at which *H. convergens* larvae transition from searching on the Petri dish to resting, climbing or leaving a patch in the presence and absence of honeydew in Experiment 3. The thickness of the arrow is proportional to the transition rates. (A) Presence of *A. fabae* honeydew; (B) Absence of honeydew

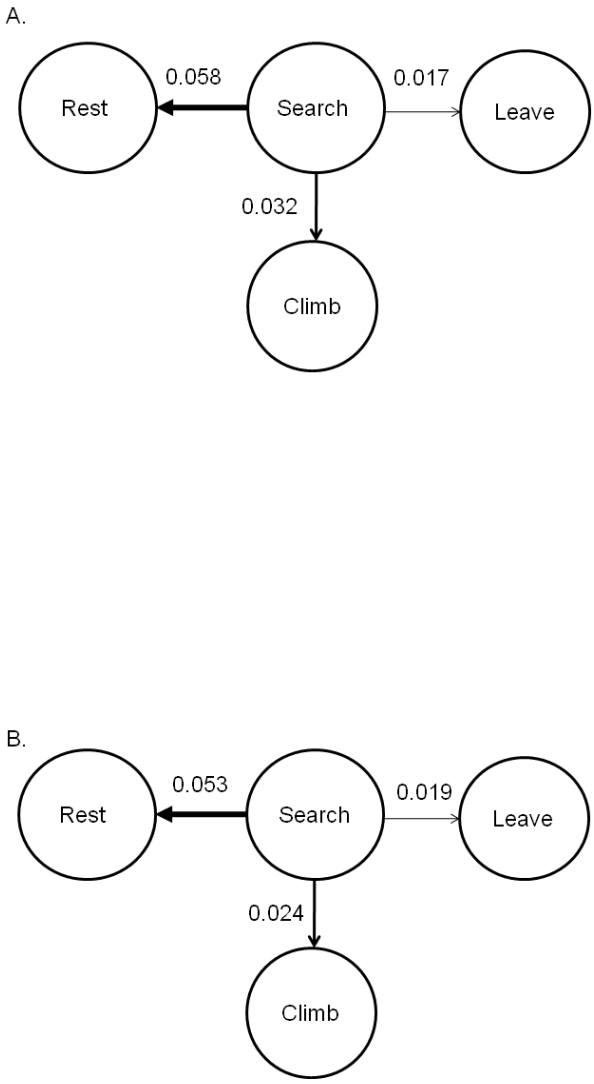

Figure A3: Rates at which *H. convergens* larvae transition from searching on the Petri dish lid to resting, climbing or leaving a patch (Experiment No 4). The thickness of the arrow is proportional to the transition rates. (A) Presence of *A. fabae* honeydew; (B) Presence of *A. pisum* honeydew

CHAPTER II

Comparison of the wing polyphenic response of pea aphids (*Acyrthosiphon pisum*) to crowding and predator cues

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***This chapter has been published in Ecological Entomology (2014), 39, 263-266**

Abstract

Pea aphids (*Acyrthosiphon pisum* Harris; Hemiptera: Aphididae) exhibit transgenerational wing polyphenism, in which unwinged females produce genetically identical winged offspring in response to environmental cues such as overcrowding and predation risk that indicate poor habitat quality. Laboratory experiments were carried out to explore the intensity of the wing polyphenic response of pea aphids exposed to cues from ladybird predators and crowding, and their response was compared to pea aphids that were not exposed to any cues (control).

The study used cues from two different ladybird species: *Coccinella septempunctata* L. (Coleoptera: Coccinellidae) and *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae) to investigate whether the wing polyphenic response of pea aphids to predator cues can be generalized The intensity of the wing polyphenic response of pea aphids to crowding was found to be much stronger than their response to predator cues. There was no response to *H. convergens* cues and the response to *C. septempunctata* cues was mixed.

Introduction

Polyphenism is an extreme form of phenotypic plasticity in which alternate, discrete phenotypes are produced from the same genotype as a response to environmental variation (Nijhout 1999). The pea aphid (*Acyrthosiphon pisum*) exhibits a transgenerational wing polyphenism, in which unwinged asexual females produce genetically identical winged offspring. Winged morphs engage in long-range dispersal and thus can escape declining habitat quality, but long-range dispersal is risky because it mainly occurs passively through wind (Dieckmann *et al.* 1999, Compton 2002) and consequently a large proportion of dispersers die before reaching a new host plant (Ward *et al.* 1998). Furthermore, the costs for producing winged morphs include an extended development time and reduced fecundity (Dixon 1998).

Crowding conditions induce the production of winged offspring in aphids because host plant quality deteriorates when the number of feeding aphids is high (Sutherland 1969a; Sutherland 1969b). Aphids also experience elevated mortality risk if the density of natural enemies and/or pathogens is high and hence, transgenerational wing polyphenism in aphids can be induced by the presence of parasitoids (Sloggett and Weisser 2002), pathogens (Hatano *et al.* 2012) and aphid predators (Weisser *et al.* 1999, Kunert & Weisser 2003). For wing induction via predation, it is sufficient for aphids to encounter cues associated with high predation risk like predator tracks (Dixon and Agarwala 1999, Mondor *et al.* 2005).

Aphid predators are mobile and may leave an area soon after depositing cues. Thus, the presence of predatory cues may not predict poor habitat quality with the same certainty as crowding in which case we would expect a weaker intensity of the wing polyphenic response. In order to understand the costs and benefits of dispersal it is important to evaluate the intensity of wing polyphenism associated with different habitat quality indicators. We compared the intensity of the wing polyphenic response of pea aphids to crowding and cues of two different ladybird species *C. septempunctata* and *H. convergens*. Our results provide insight into understanding the interactions between two fundamental ecological processes, predation and dispersal.

Materials and Methods

Insect collection and rearing:

Predators: Adult *C. septempunctata* were collected from an alfalfa field in Lincoln, NE, in July 2011, while adult *H. convergens* were purchased from commercial suppliers (Hirt's Gardens) in May 2011. Both coccinellid species were reared in chiffon netted aluminum cages (44 x 51 x 61 cm) in growth chambers at approximately 25°C on a 16:8 (L: D) photoperiod on *Vicia faba* L. plants infested with pea aphids.

Aphids: Three pea aphid clones were used to increase the generalization of our results because different aphid clones vary in their sensitivity to environmental cues and in their wing polyphenic response (Sutherland 1969a; Weisser and Braendle 2001). To prevent mixing of clones, all aphids were caged by enclosing the host plant *V. faba* using

Plexiglass tubes (21.5 cm high and 6.5 cm diameter) with a mesh on top. The aphids were maintained in the laboratory at 17±1.5 °C, 35-45% relative humidity on a 16:8 (L: D) photoperiod.

Experimental procedure:

The experiments were carried out in growth chambers at 17±1.5 °C, 35-45% relative humidity and a 16:8 (L: D) photoperiod. Pea aphids were maintained on *V. faba* plants at low density (six individuals per plant) until they started reproducing. Aphids reared on the same plant were divided randomly between the treatments to avoid differences in the maternal environment that could influence their offspring phenotype. Pea aphids are viviparous and therefore adult asexual females have embryos in their ovaries. Since the winged/unwinged morph determination in pea aphids is prenatal (Sutherland 1969a; Sutherland 1969b), all experimental treatments were applied to adult asexual females within the first three days of the beginning of their reproductive period.

The experimental arena consisted of a plastic Petri plate (60mm x 15 mm, Fisher) with two *Medicago arborea* leaves inserted in 3 ml of 2 % bactoagar mixed with MiracleGro (plate). Leaves, rather than plants, were used in order to restrict the predator cues to a small area, which increased the probability that aphids encountered the cues during the experiment. Adult unwinged asexual female pea aphids reared on the same plant were divided randomly between the following treatments.

- *Predator cues:* One well-fed adult of either *H. convergens* or *C. septempunctata* was released in the experimental arena. The predators were allowed to search and deposit cues (eggs, feces and tracks) at 22 °C on a 16:8 (L: D) photoperiod under fluorescent light for 24 hours. Since the response of aphids to ladybird tracks can depend on the predator sex (Ninkovic *et al.* 2013), we included both sexes in our experiments and randomly distributed them between the treatments. After removal of the predator, one adult, unwinged aphid female was placed in each plate.
- *Crowding:* Ten adult unwinged aphid females were placed in a small empty plastic Petri dish (32.5mm x 15 mm) for 24 hours and then each individual was transferred to its own plate. In our statistical analysis we included only one randomly chosen aphid per Petri dish.
- *Control:* A single adult, unwinged aphid female was placed in a plate; predator cues were absent.

The response of aphids to predator tracks is known to decrease with the age of the tracks (Ninkovic *et al.* 2013). Therefore, female aphids were removed from all plates after 24 hours, and the offspring produced during that duration were reared until they reached adulthood (14±2 days), and the phenotype of the offspring after reaching maturity was recorded. We recorded the proportion of pea aphid females producing at least one winged offspring ("induced aphids") and the proportion of winged offspring produced by induced pea aphid females. The experiments were replicated for each of the three aphid clones (Hf-alf-07: 7 replicates, Roc-1: 6 replicates, HF-74: 4 replicates).

All analyses were performed using R (v. 2.15.0, R Development Core Team 2012). Backwards model selection was performed and likelihood ratio tests were used to decide which model fit the data best. A generalized linear mixed model (GLMM) with a binomial error distribution was used to analyze the proportion of induced aphids, treating clone and treatment as fixed effects and starting date as a random effect. The same statistical model was used to analyze the proportion of winged offspring produced by each induced aphid.

Results

Aphid clone had no significant effect on the proportion of aphids induced (Hf-alf-07 p=0.17; Roc-1 p= 0.33, Table S1) and proportion of winged offspring produced by the induced aphids (Hf-alf-07 p=0.30; Roc-1 p= 0.92, Table S2). Crowding produced a strong wing polyphenic response. In comparison to the control treatment (no cues), the proportion of induced aphids (p=0.001, Fig.1) and the proportion of winged offspring produced by induced aphids (p=0.0001, Fig. 2,) were significantly higher than in the control. Exposure to *C. septempunctata* cues elicited a weak but significant response. Compared to the control the proportion of induced aphids increased significantly (p=0.003), but the proportion of winged offspring produced by induced aphids decreased (p=0.03). In contrast, exposure to *H. convergens* cues had no effect on the proportion of induced aphids (p=0.14) and winged offspring (p=0.87).

Discussion

We found a strong wing polyphenic response of pea aphids to crowding (roughly twice as high as the control, Fig 1), which is consistent with previous studies (Sutherland 1969a, Sutherland 1969b). We found no response of pea aphids to *H. convergens* cues and a mixed response to *C. septempunctata* cues. Even though the proportion of induced aphids ($P_{induced}$) was significantly higher in aphids exposed to *C. septempunctata* cues, the proportion of winged offspring ($P_{offspring}$) was lower than the control aphids suggesting that the total dispersal response to predator cues is negligible ($P_{induced}P_{offspring}$= 12% (Control), 13% (*H. Convergens*), and 14% (*C. septempunctata*). We suggest two potential reasons for the weak response to predator cues. Firstly, the wing polyphenic response is transgenerational in pea aphids and the delay between the time the females are induced and the offspring dispersal response is substantial. Thus, the predatory ladybird that induces the production of winged offspring in an aphid colony is unlikely to be present when the winged offspring mature (Minerotti & Weisser 2000). The evolution of delayed predator induced dispersal is only adaptive under special circumstances. According to a model by Poethke *et al.* (2010), a substantial proportion (more than 80%) of the population should disperse only if predation risk is high (≥ 0.8), predators revisit patches (return probability ≥ 0.8), and predator-induced mortality increases after the first visit of a predator. These conditions may not apply to ladybirds foraging for aphids. Secondly, it is possible that our predator treatment did not provide a sufficient number of cues to induce a strong wing polyphenic response. In our predator treatment, we used isolated aphids to separate the effect of predator cues on wing induction from cues such as tactile cues associated with crowding (Sutherland 1969a). Only a weak response by isolated pea

aphids to predator tracks has been observed in a different aphid-coccinellid system (pea aphids-*Adalia bipunctata*, Dixon and Agarwala 1999).

Acknowledgements

We would like to thank two anonymous reviewers for their helpful comments, Emily Zahn for her help with aphid rearing and conducting the experiments and, Travis Hinkelman for the useful discussions. This work was supported by the University of Nebraska-Lincoln, School of Biological Sciences Startup Funds and NIEHS award 4R00ES017367 (JAB) and University of Nebraska-Lincoln, School of Biological Sciences Special Funds (SRP).

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Tables and Figures

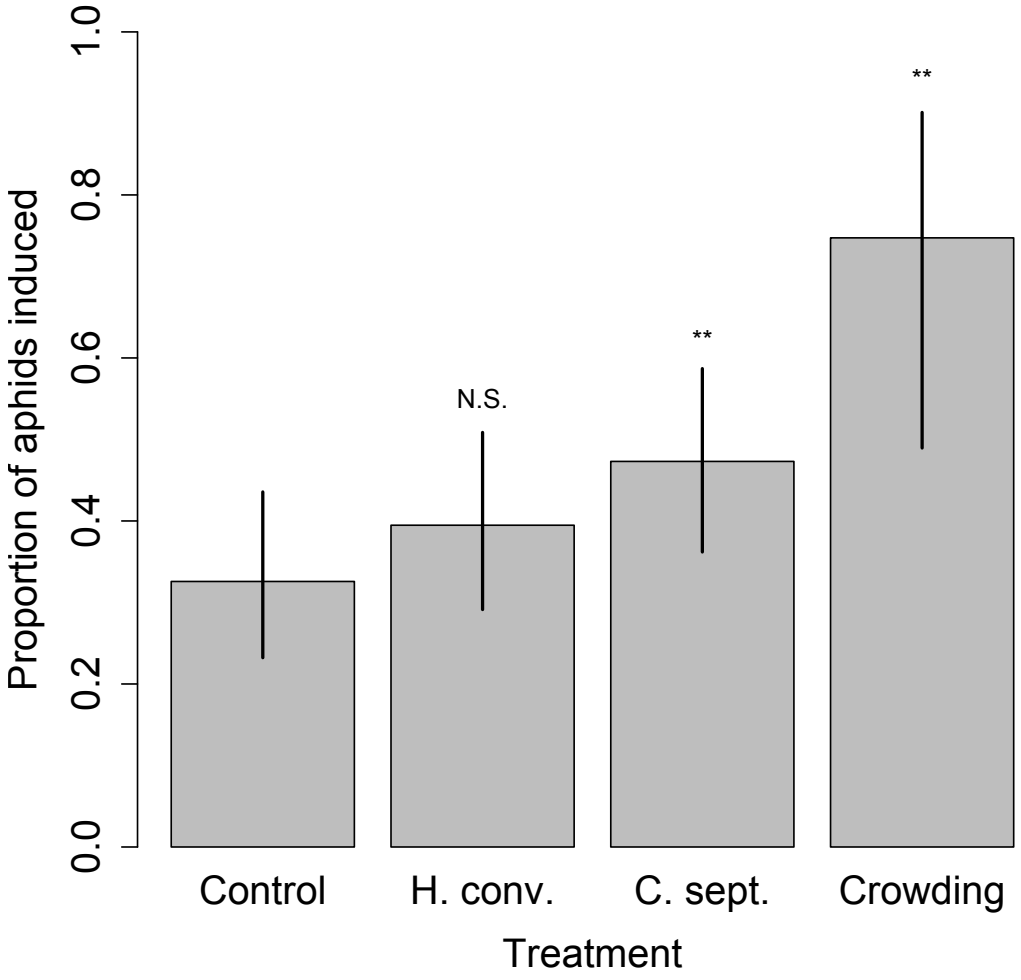

Figure 1. Proportion of induced pea aphids for each treatment. The bars indicate the mean values, and the lines show the 95% confidence interval. Control = No cues, H. conv. = exposure to *H. convergens* cues, C. sept = exposure to *C. septempunctata* cues, Crowding = exposure to crowding. All comparisons are made with respect to the control.

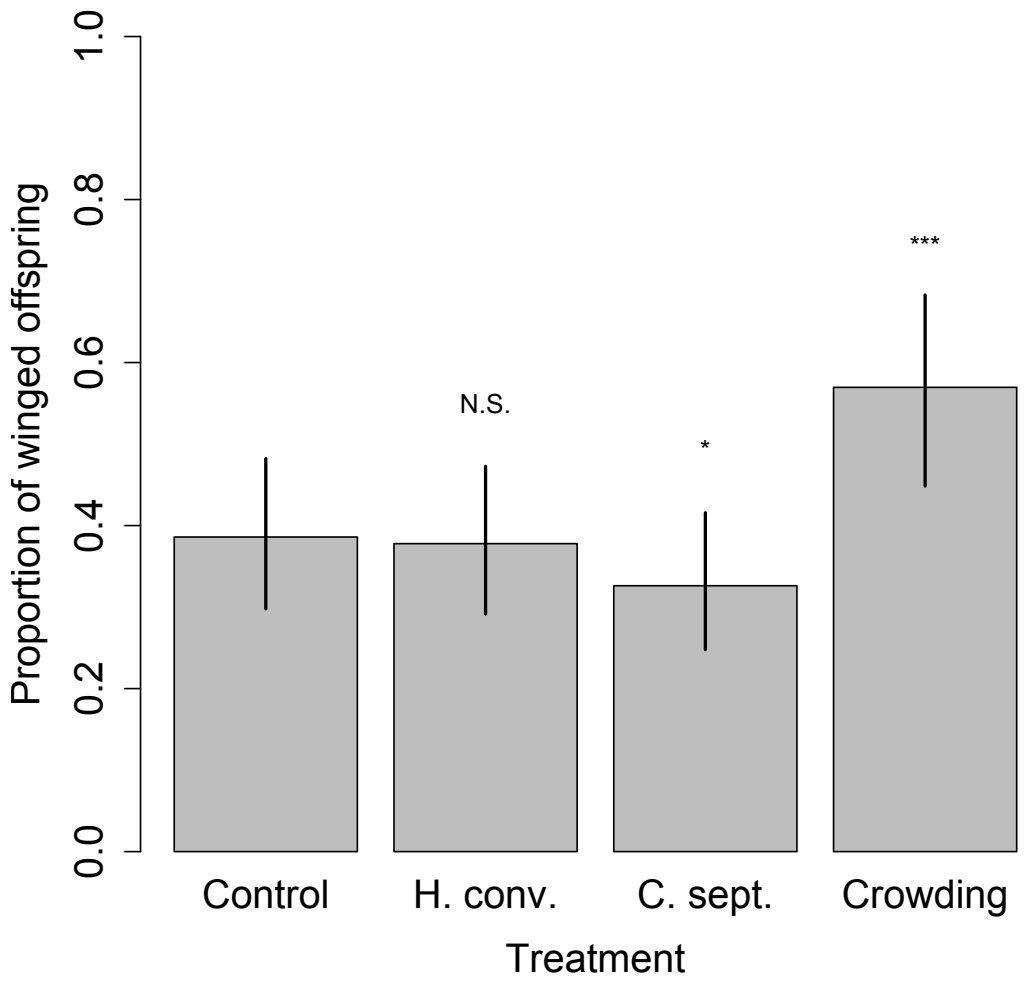

Figure 2. Proportion of winged offspring produced by induced pea aphids for each treatment. The bars indicate the means, and the lines show the 95% confidence interval. Control = No cues, H. conv. = exposure to *H. convergens* cues, C. sept = exposure to *C. septempunctata* cues, Crowding = exposure to crowding. All comparisons are made with respect to the control.

Supplementary Tables and Figures

Table S1: Proportion of pea aphids induced. The predicted proportion of induced aphids for each of the treatment is depicted in Figure 1.

SE = standard error.

Fixed Effects	**Estimate**	**SE**	**P Value**
Control and Clone HF-74 (Intercept)	-0.72	0.38	0.054
Predator Treatment-C7	0.66	0.21	0.0017**
Predator Treatment-HC	0.31	0.21	0.14
Crowding Treatment-CR	1.81	0.56	0.001**
Clone HF-alf-07	-0.61	0.45	0.17
Clone Roc-1	0.4	0.44	0.33

Table S2: Proportion of winged offspring produced by induced pea aphids. The predicted proportion of induced aphids for each of the treatment is depicted in Figure 2. SE = standard error.

Fixed Effects	**Estimate**	**SE**	**P Value**
Control and Clone HF-74 (Intercept)	-0.55	0.31	0.0795
Predator Treatment-C7	-0.26	0.12	0.03 *
Predator Treatment-HC	-0.02	0.13	0.87
Crowding Treatment-CR	0.78	0.19	4.97e-05 ***
Clone HF-alf-07	0.39	0.38	0.30
Clone Roc-1	-0.04	0.37	0.92

CHAPTER III

Molecular genetics of chemosensory specialization in pea aphid morphs

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Abstract

Chemosensation, the ability to detect and respond to chemical cues in the environment, is important for many organisms. It plays a critical role in insect life history. Insects use chemical cues for finding hosts, mates, and in avoiding predators. Due to the fundamental importance of chemicals in insect life history, insect sensory systems have evolved to sense and discriminate among a large number of chemicals.

The pea aphid (*Acyrthosiphon pisum* Harris, Hemiptera: Aphididae) is an insect that produces multiple morphs (winged and wingless asexual females, sexual females, winged and wingless males) during its life cycle. These morphs have the same or similar genotypes but differ in morphology, ecology, and behavior. Therefore, it can be expected that the morphs would have specialized chemosensory repertoire and their chemosensory systems would have evolved to suit their ecology.

We used RNA sequencing data collected from whole bodies of the pea aphid morphs to compare their chemosensory gene expression profiles. Our analysis revealed that many of the chemosensory genes were differentially expressed between the pea aphid morphs based on sex, mode of reproduction and presence or absence of wings. Our findings unravel intraspecific patterns of chemosensory gene expression, which can be further used to understand diversification between species.

Introduction

Chemosensation involves detection, discrimination and response to chemical cues and is fundamental to the life history of most organisms. In insects, chemosensation is essential for foraging, communicating with conspecifics, and avoiding predators. It also plays an important role in host plant specialization, mate location, and may contribute to reproductive isolation and speciation (Smadja et al. 2009; Vieira & Rozas 2011). Unraveling the molecular genetic mechanisms involved in chemosensation is important to improve our understanding of these ecological and evolutionary processes. In addition, an insight into the molecular processes involved in insect chemosensation can be further used for better management of insect pests and disease vectors by developing strategies that can be used to manipulate the insect chemosensory system.

Insects sense and discriminate volatile odorants using hair like structures called sensilla. Typically insect olfactory sensilla are located on their antennae or on the labial or maxillary palp (de Bruyne et al. 1999). The odorants are detected by olfactory receptor neurons in these sensilla and the odorant information is sent to the brain, which transforms the chemical stimuli and invokes a behavioral response (reviewed in de Bruyne & Baker 2008). The odorant molecules enter through the pores in the sensilla. Odorant binding proteins (OBPs) and chemosensory proteins (CSPs) present in the sensillum lymph dissolve the odorants, bind and carry them to the neuron dendrites where the odorants are then bound by olfactory receptor proteins (ORs) (reviewed in A Sa´nchez-Gracia et al. 2009). However, OBPs and CSPs are also expressed in other body parts of insects and may have other physiological functions such as storing and releasing

pheromones (reviewed in Pelosi et al. 2006). Recent evidence suggests that ORs are also expressed in tissues other than olfactory tissues and may have other physiological functions (Zhang et al. 2013). In addition to olfaction, insects use taste organs called gustatory sensilla for contact chemosensation. Their gustatory sensilla are distributed on multiple body parts such as proboscis, legs, wings and genitalia (reviewed in Vosshall & Stocker 2007). The gustatory sensilla have a single pore at the tip through which the chemicals enter when they come in physical contact with these sensilla. Two to four gustatory receptor cells (GRs), one mechanosensory neuron and several types of accessory cells are present in the sensilla (reviewed in Montell 2009). GRs translate and transmit extracellular chemical signals into intracellular electrical signals, similar to that of ORs, by working at the interface between the insect and the environment (A Sa´nchez-Gracia et al. 2009, Fan et al. 2011).

Chemosensory genes belong to multi-gene families that are highly diverse, fast evolving, and adaptive (Vogt 2002, Vosshall & Stocker 2007, Zhou et al. 2010). Differential expression of OR genes has previously been implicated in host plant specialization in *Drosophila* species. Specifically, inactivation of some OR genes and accelerated evolution of many OBP and OR genes in the specialist species *Drosophila sechellia* implied an increase or decrease in sensitivity to ecologically relevant odorants (Kopp et al. 2008). These findings suggest a connection between differential expression of chemosensory genes and insect ecology. Smadja et al. 2012 used a large scale candidate gene approach to illustrate a high level of genetic differentiation in the OR and the GR gene families among three host races of pea aphid that are closely related but highly

specialized on different host plants, suggesting that these genes could play a key role in local adaptation and reproductive isolation in the pea aphid (Smadja et al. 2012).

Analogous to host plant specialization, different morphs expressed via polymorphism and polyphenism are specialists adapted to particular ecological conditions. In polyphenism, the morphs are genetically identical with their alternate phenotypes being determined environmentally, whereas in polymorphism the alternate phenotypes are genetically determined. Sexual dimorphism is an example of polymorphism in which males and females have different phenotypes. Chemosensory specialization between sexes has been reported in insects such as *Menduca sexta* (Grosse-Wilde 2010) and, *Anopheles gambiae* (Justice et al. 2003, Pitts et al. 2011). Similar to polymorphism, chemosensory specialization in wing polyphenic morphs of *A. gossypii* has been recently reported (Gu et al. 2013). Polymorphic and polyphenic morphs provide an excellent opportunity to investigate the influence of genes and environment on intraspecific specialization of chemosensory systems.

Pea aphids (*Acyrthosiphon pisum*) are small, soft-bodied phloem feeding insects that produce winged and wingless, sexual and asexual morphs during their complex annual life cycle (Fig. 1). During summer, wingless asexual females produce wingless or winged asexual females depending on the environment (wing polyphenism). At the onset of winter, the wingless asexual female morphs produce sexual female morphs (polyphenism in the mode of reproduction) and males that mate and lay overwintering eggs. In pea aphids, males could be winged or wingless. The presence and absence of wings is

genetically determined (wing polymorphism) by a single locus on the X chromosome (Caillaud et al. 2002). The only genetic difference between males and females is that males carry only one of the X chromosomes of the females. Females are thus XX and males XO. Thus, the pea aphid morphs have a same or similar genotype but differ in their morphology, ecology and behavior. They display both polyphenism and polymorphism and therefore, they are ideal candidates to study the influence of the genes and the environment and to investigate how intraspecific chemosensory specialization occurs with ecological specialization.

Chemosensation plays an important role in pea aphid ecology and behavior. Pea aphids use chemical cues for locating host plants and mates, in conspecific communication, and in predator avoidance (Pickett et al. 1992). During dispersal, the winged morphs use volatile cues from plants to locate and distinguish host plants from other plants (reviewed in Pickett et al. 1992). Thus, winged morphs need a highly developed chemosensory system. Sexual females attract males by means of a volatile sex pheromone (Pettersson 1971). The specificity of sex pheromone is known to play a crucial role in locating conspecific females by male aphids (Dawson et al. 1990). In addition, sexual females need to locate suitable sites to oviposit eggs before the onset of winter. Thus, the five pea aphid morphs (wingless and winged asexual female, wingless male, winged male and sexual female) have diverse ecology and specialized chemosensory needs. Therefore, we expect their chemosensory system to be specialized to suit their ecology.

Morphological differences in the olfactory system of aphids have been documented. Aphids respond to volatile compounds using receptor neurons in olfactory sensilla

located on the antennae called distal primary rhinaria, proximal primary rhinaria and secondary rhinaria (Bromley et al. 1979, Dawson et al. 1990, Hardie et al. 1994). Primary rhinaria are present in all aphid species, morphs and life stages (Shambaugh et al. 1978). However, differences in the abundance and location of the secondary rhinaria have been documented between species, sexes, winged and wingless morphs as well as between sexual and asexual morphs, indicating that the aphid olfactory receptor system has evolved specific to species and morphs (Park & Hardie 2002). Pea aphids have primary rhinaria present on the fifth and sixth segment of the antennae and the secondary rhinaria are found on the third, fourth and fifth antennal segment in varying numbers (Shambaugh et al. 1978, De Biasio et al. 2014) (Fig. 2). In addition, the presence of two types of trichoid sensilla that may be involved in mechanorecption or contact chemosensation has been documented on pea aphid antenna (Shambaugh et al. 1978, De Biasio et al. 2014).

In this study, we examined the antennal morphology to quantify the differences in the number of secondary rhinaria and characterized transcriptional differences in the chemosensory genes of five pea aphid morphs to explore if they have distinct chemosensory profiles. Specifically, we used RNA sequencing data collected from the whole bodies of five pea aphid morphs to assay the expression differences in four categories of chemosensory genes: OBP, CSP, OR, and GR. In addition, we collected OBP gene expression data from pea aphid head samples using qRT-PCR, to generate hypotheses about potential role of these genes in antennal chemosensation.

Overall, we expected that if the pea aphid morphs had chemosensory systems specialized

for their ecology they would have distinct chemosensory gene expression profiles. We expected differential chemosensory gene expression based on polymorphism and polyphenism. Our first hypothesis was that chemosensory gene expression would differ between males and females (sexual dimorphism) because of highly specific chemosensory needs of males, e.g., use of highly specific sex pheromone for locating sexual females. Secondly, we hypothesized that the chemosensory genes would be differentially expressed based on the mode of reproduction because asexual and sexual females are present during different seasons and experience different environments, which may lead to differences in their chemosensory needs. In addition, we anticipated that the differences in their reproductive biology would result in different gene expression profiles. For example, asexual females are viviparous and reproduce parthenogenetically, while sexual females aggregate, emit sex pheromone to attract males, mate, and lay overwintering eggs. They might need specialized chemosensory abilities for finding suitable oviposition sites, which may lead to some genes being highly expressed in sexual females. Thirdly, we predicted that chemosensory genes would be differentially expressed between winged and wingless morphs because the winged morphs disperse and require greater chemosensory ability as compared to wingless morphs.

Chemically mediated behavioral isolation and/or host plant specialization may result in speciation and therefore, the identification of genes underlying interspecific differences in chemosensory repertoire is essential (Smadja and Butlin 2009). In this study, we identify patterns of intraspecific chemosensory specialization and try to relate the gene expression patterns to the ecology of the pea aphid morphs. The identification and

analysis of these within species patterns can be used to form hypotheses about the basis of diversification between species. Furthermore, our findings help to unravel the role of some of the OBP, CSP, OR and, GR genes in aphid chemical ecology by generating hypotheses about their functions which is important to establish a link between the molecular genetic basis of chemosensation and ecology.

Materials and Methods

Insect rearing and sample collection:

We reared three pea aphid clones F1, BK11 and I18 in small cages using *Vicia faba* L. as a host plant at 18°C on a 16:8 (L: D) cycle.

Asexual winged and wingless female morph collection:

We reared asexual female aphids at low density (less than six individuals per cage) and collected adult wingless asexual females when they were two day old. To produce winged asexual females, we crowded the aphids by placing 12 asexual adult females in a small Petri plate for 24 hours. We then transferred the crowded females to caged plants and reared the offspring to maturity and collected the winged asexual females when they were two days old.

Sexual winged and wingless male and sexual female morph collection:

We transferred adult asexual female aphids to an incubator that mimics fall photoperiod (L: D cycle = 13:11) and colder temperature (15°C) in order to induce sexual morph production. After five to six weeks the aphids started producing sexual morphs. We

collected third and fourth instar aphids as potential sexual morphs and placed them on a caged *V. faba* plant and observed them everyday until they molted into an adult morph. We transferred the newly molted adult morphs individually to a new caged plant and collected two-day old adult morphs by flash freezing them in liquid nitrogen. All individuals were collected between noon and 2 PM to control for circadian changes in gene expression. Sexual females were hard to identify when they were two-day old. We waited for four more days to collect them to make sure that they are not asexual females and did not produce live nymphs or until they laid eggs.

We collected whole bodies of 30 adult aphids of each of the five morphs and three clones (total of 15 samples) by flash freezing them in liquid nitrogen. We stored these samples at -80°C for subsequent RNA extractions. In addition, we dissected 50 aphid heads (with intact antennae) of each of the five morphs and three clones. Dissected samples were frozen in Trizol at -80°C (Invitrogen, Carlsbad, CA).

Examination of antennal morphology:

We dissected twenty antennae of each of the five morphs to examine the antennal morphology using light microscopy (Leica model DM5000B). We counted the number of secondary rhinaria present on each antennal segment of the five aphid morphs.

RNA extraction and cDNA Synthesis:

Total RNA was extracted using a phenol/chloroform extraction. We used 15-33 whole bodies and 27-50 heads of each aphid morph for RNA-extraction. First-strand cDNAs

were synthesized from the head RNA using SuperScript III Reverse Transcriptase (Invitrogen) and an oligo (dT) primer, following manufacturer's instructions. We treated each sample with rDNase I (Ambion, Austin, TX, USA) at 37 °C for 30 min. Two cDNA conversions were done per RNA sample. The heads produced ~5 µg of cDNA which was sufficient to do all of the qRT-PCR reactions for this study.

RNA Sequencing:

The quality of RNA extracted from the whole body adult morphs was checked using Bioanalyzer (Agilent) and quantified using a Qubit fluorometer (Invitrogen). RNA sequencing libraries were constructed per manufacturer instructions (Illumina) and sequenced on an Illumina HiSeq 2000 sequence analyzer at the University of Nebraska Medical Center using 50 or 100 base single end sequencing.

RNA-Seq data mapping and analysis:

We mapped sequencing reads to 36,961 gene predictions of v2.1 annotation of the pea aphid genome (available at http://www.aphidbase.com/aphidbase/) using Arraystar Software (DNAStar Inc.). Reads were aligned to the reference transcriptome if 97% of the bases matched within each read. Reads aligning to more than one transcript equally were excluded. The numbers of mapped reads per library ranged from 32 to 78 million. The DESeq2 R package (Anders & Huber 2010) was used to normalize the libraries and to identify differentially expressed genes between the different pea aphid morphs. The comparison between sexes was done between male and sexual female morphs while for the mode of reproduction we compared asexual females and sexual females. Other factors

such as clone and winged/wingless condition were controlled during these comparisons. Winged and wingless asexual female morphs and winged and wingless male morphs were compared separately by controlling for clone. For each of the 36,961 genes, two generalized linear models (GLMs) were compared using likelihood ratio tests implemented in DESeq2. A clone effect (with three levels, corresponding to the three clonal lines) and wing phenotype effect (with two levels, winged or wingless) were also included in both models to control for the structure of the data. If the inclusion of the condition as a factor significantly improved the model fit for a particular gene ($P < 0.05$ after correcting for multiple testing using the Benjamini-Hochberg method), it was concluded that the expression of that gene significantly differs between the morphs. For example, likelihood ratio test was used to compare a full model with the condition to be tested and a reduced model without the condition. e.g. a full model used to test if the gene expression differs by the presence of wing included (~Clone + Wing) and the reduced model included (~Clone). A significant p-value indicated that the full model is significantly better than the reduced model suggesting that the gene expression differs by the condition presence/absence of wings.

Thirteen classic OBP (having six highly conserved cysteines), 2 plus C OBPs (with eight conserved cysteines), 13 CSP, 79 OR and 77 GR genes have been putatively identified and annotated in the pea aphid genome (Zhou et al. 2010, Smadja et al. 2009). We selected 12 OBP genes, 11 CSP genes and 30 OR and, 18 GR genes that had matching gene identifiers on AphidBase (annotation v2.1) (http://www.aphidbase.com/). The genes that had significant differences in the expression ($P < 0.05$) between the morphs were

categorized as differentially expresses genes (DEG). We used Principle Component Analysis (PCA) to investigate how the five pea aphid morphs were clustered in each of the chemosensory (OBP, CSP, OR and GR) gene expression phenotypic space. All analyses were performed using R (v. 2.15.0, R Development Core Team 2012).

Primer design and qRT-PCR:

We compared the chemosensory gene expression differences in pea aphid head samples to examine their role in antennal chemosensation and to generate hypotheses about their functions. We used RNA extracted from pea aphid heads (with intact antennae) to perform qRT-PCR and quantified the relative transcript accumulation of 11 OBP, four OR, two CSP, and two GR genes between the five morphs. We designed gene-specific primers (forward and reverse) that span introns for these genes using Primer3 (Rozen & Skaletsky 2000). We used In silico PCR amplification (http://insilico.ehu.es/) to ensure that the designed primers do not amplify multiple genes. qRT-PCR reactions were performed using 20 ng of cDNA template, 0.2 μM of each primer, and SybrGreen PCR master mix on a 7500 Fast Real Time PCR system (Applied Biosystems). We used GAPDH as our control gene. Therefore, each gene of interest was quantified in each sample relative to the expression level of GAPDH. Gene expression levels were quantified as cycle thresholds (CT) – the number of cycles required to reach exponential amplification. CT value is a negative logarithmic function of transcript abundance: higher initial concentration allows PCR to reach exponential stage after fewer cycles. Lower values indicated stronger expression.

Results

We examined antennal morphology and characterized differences in chemosensory gene expression of five pea aphid morphs to determine if they differ between ecologically specialized morphs. In addition, we used the chemosensory gene expression data from the head samples to generate hypotheses about potential olfactory functions of some of these genes.

Variability in the numbers and location of olfactory receptors on pea aphid antenna

We quantified differences in the location and number of secondary rhinaria between the pea aphid morphs (Fig. 3). Our results show statistically significant differences in the number of secondary rhinaria on the third and fifth antennal segment of the five pea aphid morphs (Kruskal-Wallis rank sum tests, $p < 0.001$). We found a larger number of secondary rhinaria in male pea aphids as compared to females (Fig. 3). Secondary rhinaria on the fifth segment were present only in males, but did not significantly differ between winged and wingless males across the clones (Kruskal-Wallis rank sum test, $p=0.14$). A single secondary rhinarium was found on the fourth antennal segment in only one wingless male. Winged asexual females had a significantly larger number of secondary rhinaria on the third antennal segment as compared to wingless asexual females (Kruskal-Wallis rank sum test, $p < 0.001$). A maximum of two secondary rhinaria were found in sexual females on any segment.

Differential expression of chemosensory genes in RNA-Seq Data

Analysis of RNA-Seq data revealed that many of the chemosensory genes were

differentially expressed among the pea aphid morphs based on sex, mode of reproduction and presence or absence of wings.

Influence of sexual dimorphism

We anticipated that chemosensory gene expression would differ due to differences in the chemosensory repertoires of male and female morphs. As expected, we found that eight out of 12 OBP genes, eight out of 11 CSP genes, 14 out of 30 OR genes and, nine out of 18 GR genes, were differentially expressed between male and female morphs using a criteria of FDR corrected p value < 0.05. We also found that a higher proportion of genes were highly expressed in males as compared to females (Table 1, Tables 2a, 2b, 2c, 2d). .

Polyphenism in the mode of reproduction

Pea aphids display polyphenism in the mode of reproduction. In other words, asexual females produce genetically identical asexually reproducing females or sexually reproducing females in response to environmental cues. Consequently, the differences in the gene expression between sexual and asexual females are entirely due to phenotypic plasticity. In our second hypothesis, we predicted that chemosensory genes would differ in their expression profiles between asexual and sexual females due to the differences in their reproductive biology. Furthermore, asexual females are present during all seasons while sexual females are present only during the fall. Thus, due to seasonal variation, the two morphs may have different chemosensory repertoire. Despite the lack of genotypic differences between asexual and sexual females, we found that six OBP, seven CSP, three GR and four OR genes were differentially expressed between them (Table 1, Tables

2a, 2b, 2c, 2d) suggesting that there is a great deal of plasticity in the pea aphid chemosensory gene expression based on mode of reproduction.

Wing polyphenism and polymorphism

In pea aphids, the asexual females are wing polyphenic and males are wing polymorphic. In other words, the female phenotype (winged or wingless) is environmentally induced while in males it is genetically determined. In our third hypothesis we expected that chemosensory genes would be differentially expressed between winged and wingless morphs due to the differences in their chemosensory needs. This anticipation was based on the fact that the winged morphs disperse and require higher chemosensory abilities for locating new host plants as compared to wingless morphs, which are more sedentary. We found four OBP genes and five CSP, one GR gene with significant differences in the expression between the winged and wingless female morphs and four OBP, five CSP, and four OR genes were differentially expressed between winged and wingless male morphs (Table 1, Tables 2a, 2b, 2c, 2d). We also anticipated that many genes would be highly expressed in winged morphs as compared to wingless morphs (winged morph biased genes). As expected, we found one OBP, one CSP and four OR genes were highly expressed in winged male morphs and only one OBP gene was highly expressed in wingless male morphs (Tables 2a, 2b, 2c, 2d). However, we found only one CSP and one GR gene that was highly expressed in winged females. In contrast, we found three OBP and three CSP genes that were highly expressed in wingless female morphs (Tables 2a, 2b, 2c, 2d). Our results indicate that both wing polyphenism and polymorphism

contribute to the chemosensory genes expression differences between the pea aphid morphs.

Chemosensory gene expression profiles

Principal component analysis (PCA) of each chemosensory gene category showed how the five morphs are distributed with respect to the two major PCA axes of variation. OBP gene expression differed for each of the morphs. For OBP genes, PC1 primarily separated males and females while PC2 separated sexual females as well as winged and wingless females (Fig. 4a). CSP expression differed between sexes with PC1 primarily showing separation between males and females and PC2 separating sexual females from other morphs to some extent (Figure 4b). However, no differentiation was found between winged and wingless morphs. OR expression distinctly differed for males, asexual females and sexual females on PC1 but did not between winged and wingless morphs (Figure 4c). GR gene expression was not separated by morph (Figure 4d) on PC1 or PC2.

Differential expression of chemosensory genes in head samples

We analyzed qRT-PCR data collected from pea aphid heads to examine the differences in the expression of 11 classic OBP genes, two CSP genes, four OR genes, and, two GR genes and, to generate hypotheses about potential functions of these genes in antennal chemosensation. We found that three out of 11 OBP and two out of four OR genes were differentially expressed between males and sexual females (ANOVA, FDR corrected p value < 0.05). One OR and one GR gene was differentially expressed between the winged and wingless females and one OBP gene showed differential expression between winged

and wingless males (Table 3). We found eight chemosensory genes that were differentially expressed between the head samples, suggesting these genes could play an important role in antennal chemosensation. OBP9, OBP10, OR1, and OR21 genes were differentially expressed between sexes in head samples as well as in whole bodies. OR1, GR3 were differentially expressed between winged and wingless females and OBP4 was differentially expressed between winged and wingless males in whole body and head samples.

Discussion

Our results illustrate that pea aphid morphs are differentiated in chemosensory gene expression due to sexual dimorphism, polyphenism in mode of reproduction as well as due to wing polyphenism and wing polymorphism. In addition, we identified chemosensory genes differentially expressed in pea aphid heads and generated hypotheses about their potential functions in antennal chemosensation.

Striking sexual dimorphism in chemosensory gene expression

Chemosensory gene expression analysis of RNA-seq data revealed that there were significant differences between male and female morphs of the pea aphid. Similarly, our principal component analysis showed a clear divide in OBP, CSP, and OR expression between males and sexual females. We observed that a higher proportion of genes from all chemosensory categories were highly expressed in males. This could be indicative of a male-specific chemosensory repertoire. Sexual dimorphism in the expression of chemosensory genes has been reported in fruit flies (Zhou et al. 2009), moths (Grosse-

Wilde 2010), mosquitoes (Pitts et al. 2011), and ants (Zhou et al. 2012). Some of the recent studies on other insect species have reported a potential role of chemoreceptors, and in particular ORs, in the reception of sex pheromones and thus mate choice (Ostrinia nubilalis: Lassance et al. 2011; Heliothis: Vasquez et al. 2011). Our results showing striking sexual dimorphism in the expression of chemosensory genes are consistent with the findings of these studies and with a recent study by Gu et al. 2013 that revealed higher expression of CSP transcripts in male adult libraries of pea aphid. In pea aphids, the only genetic difference between males and females is that males carry only one of the X chromosomes of the females. This makes our results showing striking sexual dimorphism in chemosensory gene expression even more interesting because all the gene expression differences that are not related to X chromosome are due to plasticity.

We found two of the OBP genes that were highly expressed in whole bodies of males were also differentially expressed in pea aphid heads, suggesting their role in antennal chemosensation. In an electroantennogram study, Hardie et al. (1994) showed that antennae of male of aphid *Aphis fabae* were more sensitive to sex pheromone as compared to asexual females and suggested that the secondary rhinaria in males may play a role in sex pheromone detection. We also observed a large number of secondary rhinaria on the fifth antennal segment only in male morphs. Therefore, the two OBP genes: OBP9 and OBP10 that were differentially expressed in pea aphid heads might be involved in male specific olfaction such as detection of sex pheromone. It would be interesting to investigate if these genes are involved in detection and discrimination of sex-pheromone in pea aphid males.

Mode of reproduction influences chemosensory gene expression

Genes from all chemosensory categories showed significant differences in their expression based on mode of reproduction. It is important to note that the asexual females and sexual females used in this comparison are genetically identical and therefore, all the differences that we observe are due to phenotypic plasticity. In PCA, we found that sexual and asexual female are clearly distinct for the expression of OBP and OR genes; and to a lesser extent for CSP and GR genes. We also observed significant differences in the number of rhinaria between asexual and sexual females. We found many OBP and CSP genes overexpressed in sexual females as compared to asexual females indicating that OBP and CSP genes might have an important role in sexual reproduction. Examination of the qRT-PCR data for the expression of 11 OBP, four OR, two CSP, and two GR genes in pea aphid heads did not result in any genes with significant differential expression based on the mode of reproduction. This suggests that the sexual females and asexual females are not differentiated for the expression of genes in the antennal and head tissues. In addition to GRs that are found on multiple body parts of insects, ORs, OBPs and CSPs are also expressed in body parts other than insect heads and are thought to have other physiological functions (Sun et al. 2012, Zang et al. 2013, De Biasio et al. 2014). OBPs expressed in other body parts of insects are thought be involved in functions such as releasing pheromones to the environment (Sun et a. 2012, De Biasio et al. 2014). It is therefore possible that the differential expression of OBP genes observed in our RNA-Seq data is due to their expression in other tissues and they might be involved in functions such as emission of sex pheromone.

In insects, CSPs are known to be involved in functions related to female survival and reproduction (*Spodoptera exigua* ; Gong et al. 2012), and in embryo development (*Apis mellifera;* Maleszka et al. 2007). Our RNA-seq data were collected from whole bodies of pea aphids and included both gonadal and non-gonadal tissues. There is a possibility that the gene expression differences observed between the sexual females and asexual females are due to the expression of OBP and CSP genes in the gonadal tissues. Sexual females have eggs in their ovaries while asexual females have embryos in them, which may result in differential expression of these genes. Expression of chemosensory genes in tissue specific manner needs to be examined to determine their exact function.

Differential gene expression due to wing polyphenism and wing polymorphism

Many OBP and CSP genes and a few OR and GR genes were differentially expressed between the winged and wingless morphs (Table 1, Tables 2a, 2b, 2c, 2d). In pea aphids, winged and wingless females are produced by polyphenism whereas winged and wingless males are produced by polymorphism. So the differences in winged and wingless asexual females are due to plasticity whereas the differences in males are genetically determined. We expected that the chemosensory genes would be differentially expressed as a result of wing polyphenism as well as wing polymorphism. Our PCA results indicated that winged and wingless asexual females were distinctly separated for OBP expression and somewhat for CSP expression. However, winged and wingless males were not separated for any category of chemosensory genes.

We expected to find a higher proportion of genes highly expressed in winged morphs as compared to wingless morphs because the winged morphs disperse and may require greater chemosensory capabilities. As expected, we found more genes highly expressed in winged males as compared to wingless males. However, to our surprise, we found more genes highly expressed in wingless females than winged females. These finding are contradictory to our expectations that winged females, which have a larger number of secondary rhinaria would also have a higher proportion of genes overexpressed. These genes may not be involved in chemosensation related to dispersal such as detection of host plant volatiles but may have other functions specific to wingless females such as detection of cues that result in offspring wing induction response.

We found some genes differentially expressed between winged and wingless morphs in the pea aphid heads. For example, GR3, which was highly expressed in winged females, was also differentially expressed in the heads of winged and unwinged females. It would be interesting to examine if GR3 is involved in host plant selection. OBP4, which was highly expressed in bodies of wingless males and females, was also differentially expressed in the heads of wingless males suggesting it is involved in antennal chemosensation of unwinged morphs.

In summary, all categories of chemosensory genes were differentially expressed between pea aphid morphs suggesting that the pea aphid morphs have distinct chemosensory gene expression profiles. Our findings reveal how the chemosensory repertoire of the polyphenic morphs is shaped by their ecological specialization. The understanding of

these within species patterns can be used to form hypotheses about the basis of diversification between species. Furthermore, characterization of transcriptional differences in chemosensory genes of pea aphids will help in improving our understanding of the molecular genetic mechanisms involved in chemosensation, which in turn will help to unravel the role of chemosensation in evolutionary processes such as reproductive isolation and speciation.

Acknowledgments

We would like to thank Travis Hinkelman and Julie Jaquiery for the statistical help. This work was supported by startup funds from the School of Biological Sciences at the University of Nebraska-Lincoln and by award R00ES017367 from the National Institute of Environmental Health Sciences to JAB and University of Nebraska-Lincoln, School of Biological Sciences Special Funds (SRP).

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Tables and Figures

Table 1: Summary of total number of genes analyzed and number of chemosensory genes differentially expressed (DEG). Comparisons were done between pea aphid morphs: males and sexual females (M/SF), asexual and sexual females (SF/AF), winged and wingless asexual females (WF/WLF), and winged and wingless males (WM/WLM).

Gene	No of genes analyzed	No of DEG (M/SF)	No of DEG (SF/AF)	No of DEG (WF/WLF)	No of DEG (WM/WLM)
OBP	12	8	6	4	4
CSP	11	8	7	5	5
OR	30	14	4	0	4
GR	18	9	3	1	0

Table 2a: OBP genes showing differential expression (DEG) and fold change between different pea aphid morphs: males and sexual females (M/SF), asexual and sexual females (SF/AF), winged and wingless asexual females (WF/WLF), winged and wingless males (WM/WLM). Genes with a fold change > 5 are highlighted.

Gene Family	**Sexual dimorphism (M/SF)**			**Mode of reproduction (SF/AF)**			**Wing polyphenism (WF/WLF)**			**Wing polymorphism (WM/WLM)**		
OBP	**DEG**	**Fold change**		**DEG**	**Fold Change**		**DEG**	**Fold change**		**DEG**	**Fold change**	
	M/SF	**M**	**SF**	**SF/AF**	**SF**	**AF**	**WF/WLF**	**WF**	**WLF**	**WM/WLM**	**WM**	**WLM**
	OBP2	22		**OBP2**	14		**OBP1**		6	**OBP4**		11
	OBP6	8		OBP4	2		OBP2		2	OBP6	2	
	OBP7	24		**OBP5**	35		**OBP4**		5	OBP10	2	
	OBP8	424		**OBP7**	6		OBP12		3	OBP12		1
	OBP9	36		OBP10	3							
	OBP10	162		OBP12	3							
	OBP11	28										
	OBP12		2									

Table 2b: CSP genes showing differential expression (DEG) and fold change between different pea aphid morphs: males and sexual females (M/SF), asexual and sexual females (AF/SF), winged and wingless asexual females (WF/WLF), winged and wingless males (WM/WLM). Genes with a fold change > 5 are highlighted.

Gene Family	**Sexual dimorphism (M/SF)**			**Mode of reproduction (SF/AF)**			**Wing polyphenism (WF/WLF)**			**Wing polymorphism (WM/WLM)**		
CSP	**DEG**	**Fold change**		**DEG**	**Fold Change**		**DEG**	**Fold change**		**DEG**	**Fold change**	
	M/SF	**M**	**SF**	**SF/AF**	**SF**	**AF**	**WF/WLF**	**WF**	**WLF**	**WM/WLM**	**WM**	**WLM**
	CSP1	11		CSP1	3		CSP2		2	CSP1	2	
	CSP2	5		**CSP2**	5		**CSP3**		5	CSP6	2	
	CSP3	6		**CSP3**	12		CSP7		2	CSP8		2
	CSP4	15		**CSP6**	9		CSP9		1	CSP9	2	
	CSP5	3		**CSP7**	20		**CSP11**	5		CSP11	4	
	CSP6	34		CSP8	1							
	CSP8		14	**CSP9**	5							
	CSP10	18										

Table 2c: OR genes showing differential expression (DEG) and fold change between different pea aphid morphs: males and sexual females (M/SF), asexual and sexual females (AF/SF), winged and wingless asexual females (WF/WLF), winged and wingless males (WM/WLM). Genes with a fold change > 5 are highlighted.

Gene Family	**Sexual dimorphism (M/SF)**			**Mode of reproduction (SF/AF)**			**Wing polyphenism (WF/WLF)**			**Wing polymorphism (WM/WLM)**		
OR	**DEG**	**Fold change**		**DEG**	**Fold Change**		**DEG**	**Fold change**		**DEG**	**Fold change**	
	M/SF	**M**	**SF**	**SF/AF**	**SF**	**AF**	**WF/WLF**	**WF**	**WLF**	**WM/WLM**	**WM**	**WLM**
	OR1	16		**OR2**	8		None	None	None	OR1	3	
	OR7		8	**OR13**		5				OR52	2	
	OR17	47		**OR30**		45				OR70	3	
	OR21	17		OR50C		3				OR62C	3	
	OR22	164										
	OR30		16									
	OR43		7									
	OR52	5										
	OR62C	11										
	OR65P	7										
	OR67	6										
	OR69	4										
	OR70	8										
	OR76C	6										

Table 2d: GR genes showing differential expression (DEG) and fold change between different pea aphid morphs: males and sexual females (M/SF), asexual and sexual females (AF/SF), winged and wingless asexual females (WF/WLF), winged and wingless males (WM/WLM). Genes with a fold change > 5 are highlighted.

Gene Family	**Sexual dimorphism (M/SF)**			**Mode of reproduction (SF/AF)**			**Wing polyphenism (WF/WLF)**			**Wing polymorphism (WM/WLM)**		
GR	**DEG**	**Fold change**		**DEG**	**Fold Change**		**DEG**	**Fold change**		**DEG**	**Fold change**	
	M/SF	**M**	**SF**	**SF/AF**	**SF**	**AF**	**WF/WLF**	**WF**	**WLF**	**WM/WLM**	**WM**	**WLM**
	GR1	4		**GR1**	5		GR3	4		None	None	None
	GR2	6		**GR6**		8						
	GR3	13		**GR19**	9							
	GR4	6										
	GR5N		8									
	GR6		27									
	GR19	39										
	GR26C	4										
	GR49	4										

Table 3: Differentially expressed chemosensory genes in pea aphid head samples

Sexual dimorphism (M/SF)	**Mode of reproduction (SF/AF)**	**Wing polyphenism (WF/WLF)**	**Wing polymorphism (WM/WLM)**
OBP1	None	OR1	OBP4
OBP9		GR3	
OBP10			
OR1			
OR21			

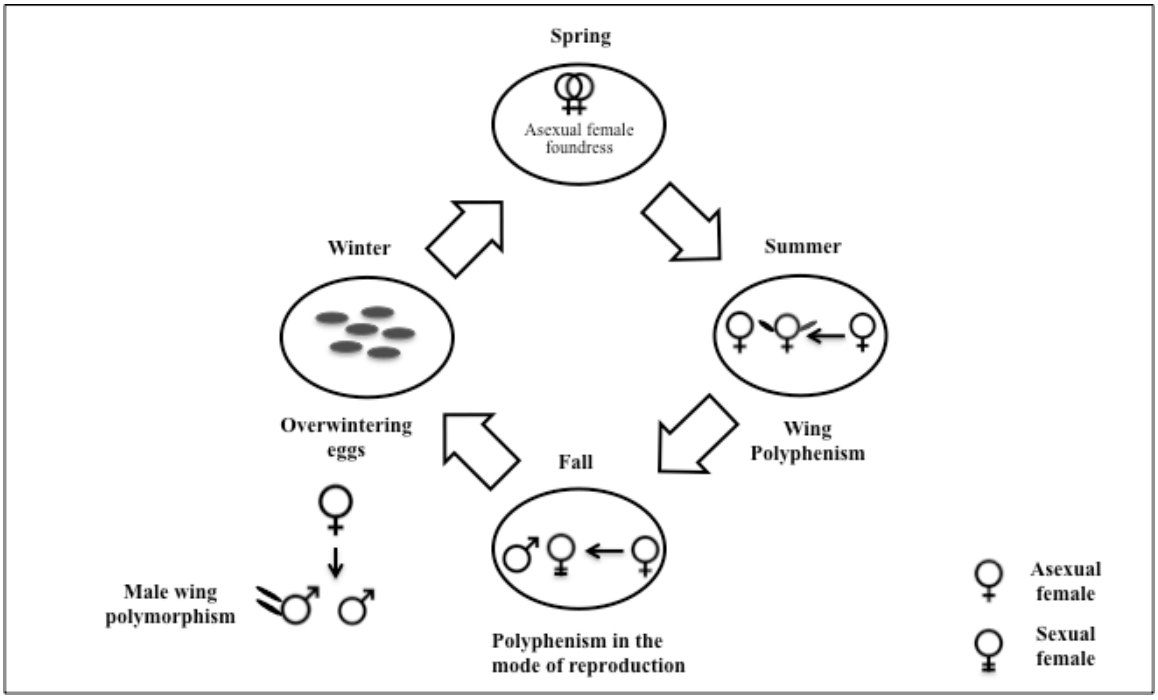

Figure 1: Annual life cycle showing seasonal morphs and polyphenism, and polymorphism in the pea aphid. Pea aphid displays wing polyphenism in asexual females, wing polymorphism in males and polyphenism in the mode of reproduction (asexual and sexual reproduction).

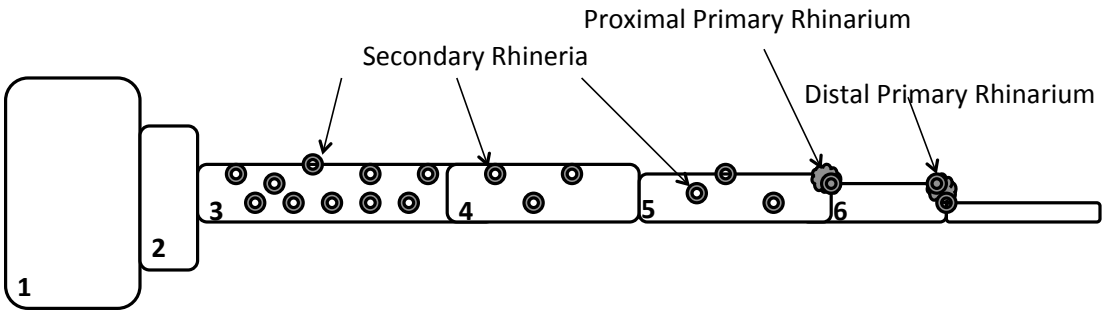

Figure 2: Schematic diagram of pea aphid antenna (adapted from Park and Hardie 2002) showing location of primary and secondary rhinaria on the pea aphid antennal segments. Numbers 1 to 6 indicate the number of antennal segment.

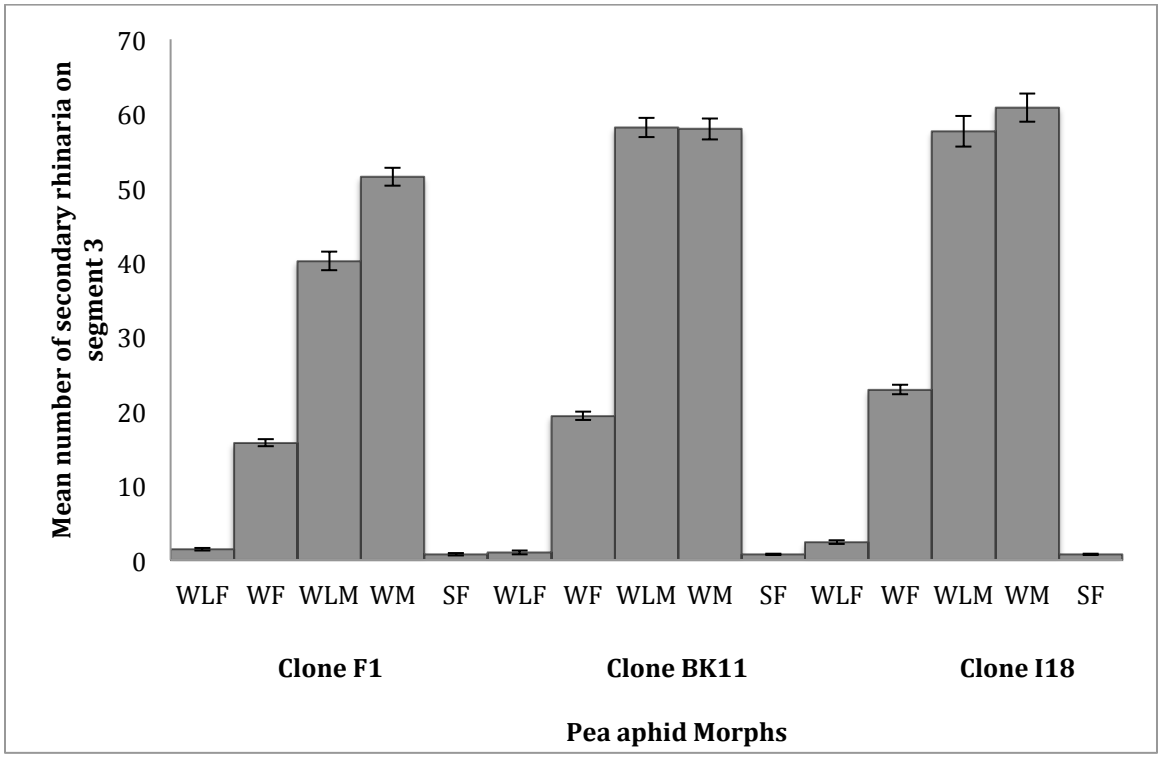

Figure 3: Comparison of the mean number of secondary rhinaria on the third antennal segment of five pea aphid morphs (WLF: wingless female, WF: winged female, WLM: wingless male, WM: winged male, SF: sexual female) from three aphid clones F1, BK11 and I18. Error bars indicate standard error.

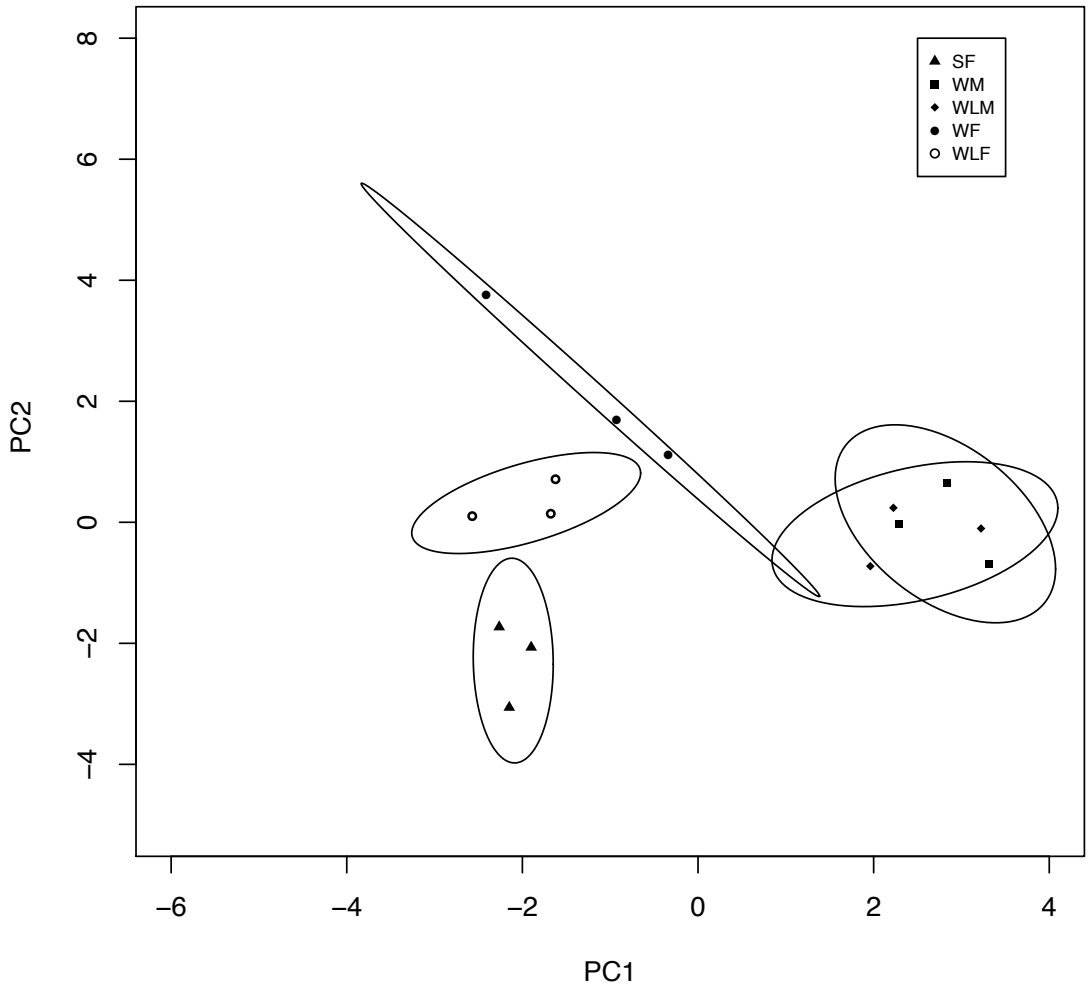

Figure 4a: Principal component analysis of OBP genes showing the distribution of pea aphid morphs with respect to the two major PC axes of variation. PC1 accounts for 45% of the variation and PC2 accounts for 22% of the variation.

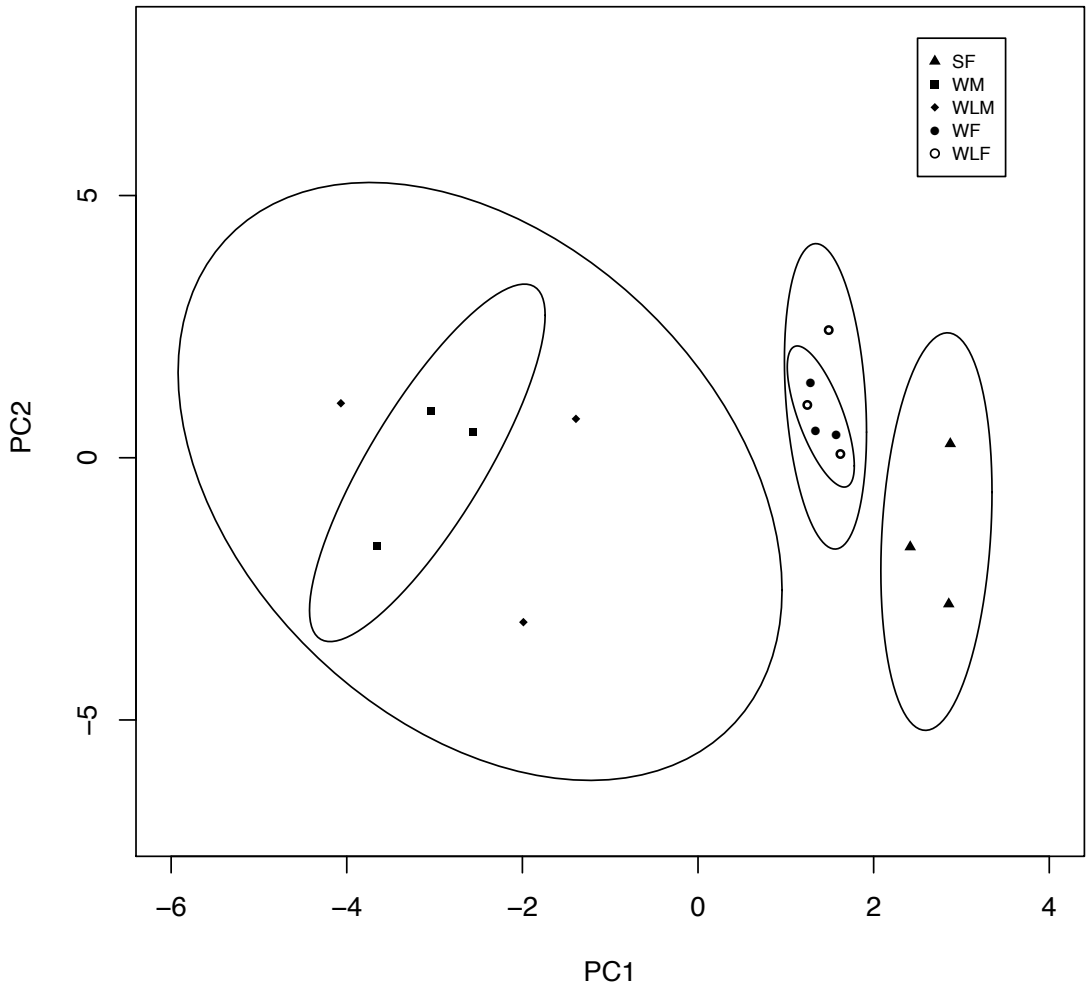

Figure 4b: Principal component analysis of CSP genes showing the distribution of the pea aphid morphs with respect to the two major PC axes of variation. PC1 accounts for 47% of the variation and PC2 accounts for 25% of the variation.

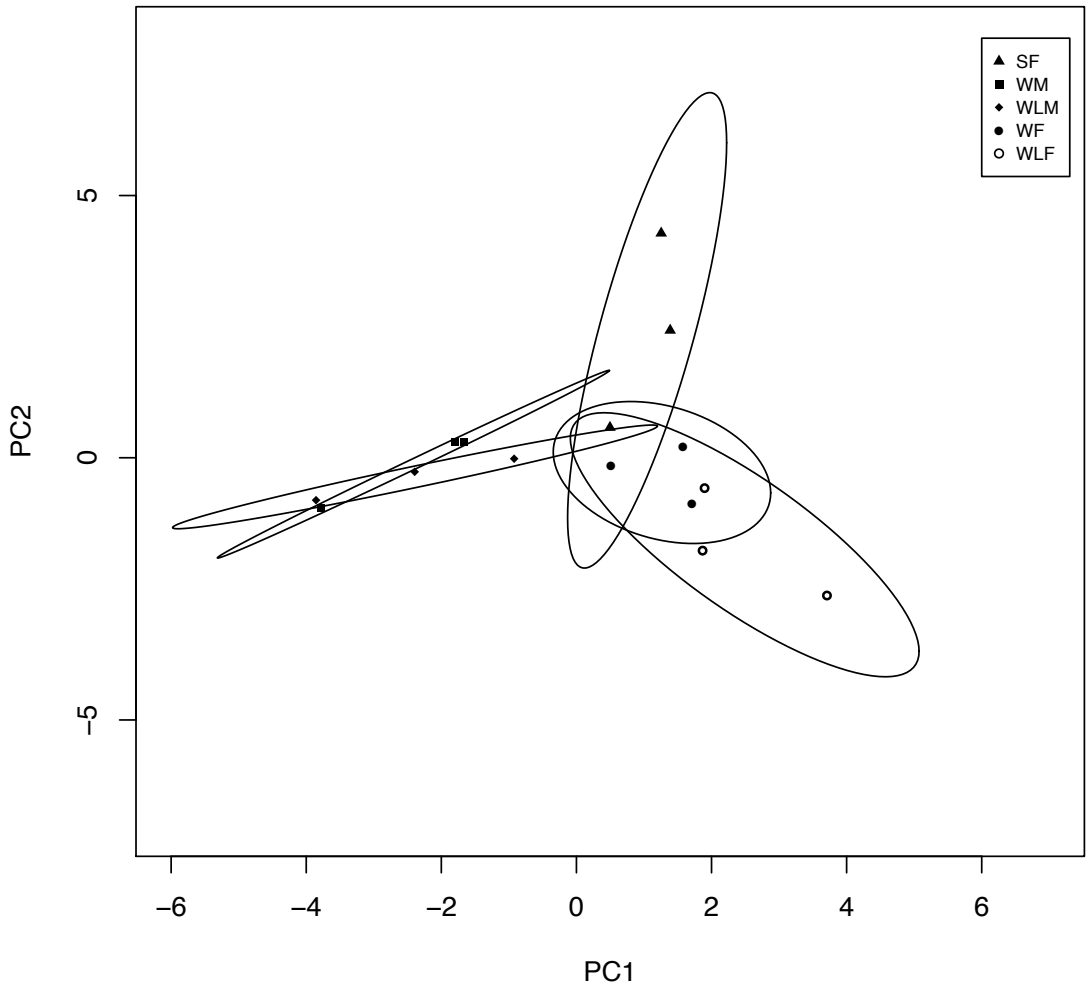

Figure 4c: Principal component analysis of OR genes showing the distribution of the five pea aphid morphs with respect to the two major PC axes of variation. PC1 accounts for 41% of the variation and PC2 accounts for 17% of the variation, and PC3 contributes to 10% of the variation.

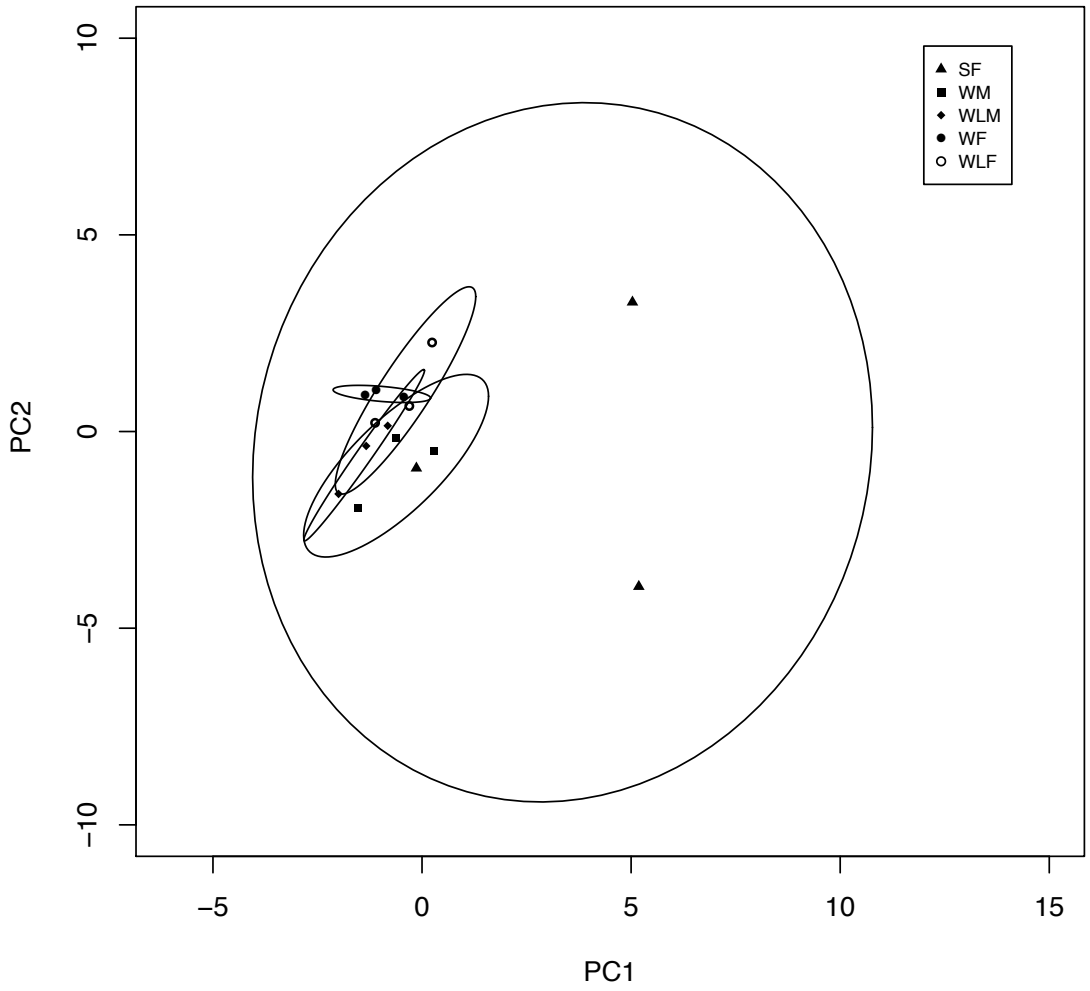

Figure 4d: Principal component analysis of GR genes showing the distribution of five pea aphid morphs with respect to the two major PC axes of variation. PC1 accounts for 27% of the variation and PC2 accounts for 17% of the variation, and PC3 contributes to 13% of the variation.

CHAPTER IV

Accelerated evolution of morph-biased genes in pea aphids

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***This chapter has been accepted for publication in Molecular Biology and Evolution**

Abstract

Phenotypic plasticity, the production of alternative phenotypes (or morphs) from the same genotype due to environmental factors, results in some genes being expressed in a morph-biased manner. Theoretically, these morph-biased genes experience relaxed selection, the consequence of which is the buildup of slightly deleterious mutations at these genes. Over time, this is expected to result in increased protein divergence at these genes between species and a signature of relaxed purifying selection within species. Here we test these theoretical expectations using morph-biased genes in the pea aphid, a species that produces multiple morphs via polyphenism.

We find that morph-biased genes exhibit faster rates of evolution (in terms of dN/dS) relative to unbiased genes and that divergence generally increases with increasing morph bias. Further, genes with expression biased towards rarer morphs (sexual females and males) show faster rates of evolution than genes expressed in the more common morph (asexual females), demonstrating that the amount of time a gene spends being expressed in a morph is associated with its rate of evolution. And finally, we show that genes expressed in the rarer morphs experience decreased purifying selection relative to unbiased genes, suggesting that it is a relaxation of purifying selection that contributes to their faster rates of evolution. Our results provide an important empirical look at the impact of phenotypic plasticity on gene evolution.

Introduction

The production of alternative phenotypes by the same genotype in response to environmental stimuli, or polyphenism, is prevalent in the animal kingdom (West-Eberhard 2003). Well-known polyphenisms include caste polyphenisms of social insects (Wilson 1971), the omnivorous and carnivorous morphs of spadefoot toads (Pfennig 1992), and horn dimorphic dung beetles (Hunt & Simmons 1997; Moczek & Emlen 1999). Alternative phenotypes are usually composed of a suite of traits, with natural selection acting to match individual character variation to appropriate environments (e.g., Kingsolver 1995). As such, polyphenism allows a single genotype to maximize fitness via multiple phenotypes when the environments that it experiences are heterogeneous in time or space (Scheiner 1993).

Gene expression profiling has definitively shown that alternative gene expression profiles underlie alternative phenotypes, indicating that different subsets of a genome's gene content contribute to each phenotype (e.g., Brisson *et al.* 2007; Grozinger *et al.* 2007; Smith *et al.* 2008; Snell-Rood *et al.* 2011; Pointer *et al.* 2013). Because of this, a particular gene may or may not be expressed in all morphs produced by a single genotype. Genes are considered morph-specific if they are only expressed in one morph, they are considered morph-biased if they have higher expression in one morph relative to another, or they are considered unbiased if they are expressed at comparable levels in all morphs. The morph-bias or morph-specificity of gene expression in alternative phenotypes of polyphenism is considered a necessary condition for the evolution of

plasticity; by expressing genes in only one morph, that gene can be fine-tuned by natural selection to optimize function of that morph independent of other morphs (West-Eberhard 1989; Nijhout 2003).

Morph-specificity can impact the rate of evolution of morph-biased genes. A handful of studies have shown that the amino acid sequences of morph-biased genes evolve at a faster pace than unbiased genes (Brisson & Nuzhdin 2008; Hunt *et al.* 2010; Hunt *et al.* 2011; Snell-Rood *et al.* 2011; Leichty *et al.* 2012). These faster rates of evolution are consistent with two, nonmutually exclusive hypotheses. The first hypothesis states that morph-biased gene expression causes faster gene evolution due to relaxed purifying selection. If gene products experience the effects of selection in only a subset of the population, they have relaxed selective constraint compared to genes expressed in multiple morphs. In these morph-specific genes, relaxed selection results in the buildup of slightly deleterious mutations and increases polymorphism within populations (Kawecki 1994; Kawecki *et al.* 1997; Barker *et al.* 2005; Van Dyken & Wade 2010). In the long-term, increased levels of genetic variation could act as a reservoir for future adaptation if rare environments become more common (Van Dyken & Wade 2010). Similarly, morph-biased genes could be viewed in the context of pleiotropy, with these biased genes exhibiting less pleiotropy precisely because they are expressed highly in only one morph. Less pleiotropy results in accelerated evolution, as has previously been demonstrated with sex-specific and tissue-specific gene expression (Duret & Mouchiroud 2000; Ellegren & Parsch 2007).

An alternative hypothesis for the pattern of faster rate of evolution of morph-biased genes is that rapidly evolving genes may be more likely to be recruited into morph-biased or morph-specific roles. Rapidly evolving genes tend to display low levels of pleiotropy and high levels of dispensability (Hirsh & Fraser 2001), and therefore could be recruited from old to new roles without negatively affecting organismal fitness. Consistent with this latter hypothesis, two studies have found that morph-biased genes identified in a phenotypically plastic species are also fast evolving in non-plastic relatives (Hunt *et al.* 2011; Leichty *et al.* 2012). These two hypotheses, however, are not mutually exclusive; fast-evolving genes may be preferentially co-opted into morph expression, but they are still expected to experience relaxed purifying selection due to their less frequent expression once co-opted.

We examined the rate of molecular evolution of morph-biased genes in pea aphids (*Acyrthosiphon pisum*). Aphids have long been models for studying the causes and consequences of phenotypic plasticity (Lees 1966; Blackman 1974; Dixon 1997). Pea aphids, like most aphids, have a complex life cycle that alternates between asexual and sexual forms of reproduction depending on the season (reviewed in Moran 1992). During the spring and summer months, pea aphids reproduce parthenogenetically, producing genetically identical daughters for as many as 10 to 20 successive generations. These asexual females can be winged or wingless depending on environmental conditions (Sutherland 1969; Blackman 1987; Hales *et al.* 2002). A single generation of sexual morphs (sexual females and males) are born from parthenogenetic females in the fall and are induced by a combination of cold temperatures and short photoperiods (MacKay

1989; Via 1992). Males, which are XO and thus have a single X chromosome, are produced genetically by the random loss of one X (Orlando 1974; Blackman 1987; Wilson *et al.* 1997). No recombination occurs in this process, so the X chromosome of a male and his mother are identical. The presence of the winged or wingless morph in males is due to a single locus on the X chromosome (Caillaud *et al.* 2002).

This complex life cycle results in a variety of adult aphid morphs, including asexual and sexual as well as winged and wingless phenotypes. Asexual females dominate the life cycle in terms of the total number of aphids and number of generations, while sexual females and males are far less prevalent because they are present for only a short period of time in the fall. Both winged and wingless morphs can be found in most populations for asexual females and males in the United States, whereas population have been found to differ by host plant for the prevalence of male winged versus wingless morphs in Europe (Frantz *et al.* 2010).

We previously showed that a small set of male-biased genes identified via microarray analysis evolve faster in pea aphids due to a relaxation of purifying selection (Brisson & Nuzhdin 2008). Here we significantly extend this analysis. We used RNA-Seq to identify morph-biased genes in cDNA derived from adult, whole bodies of winged asexual females, wingless asexual females, sexual females, winged males, and wingless males to identify the gene expression basis of morph differences. Winged asexual females, wingless asexual females, and sexual females are genetically identical, while males are identical to the females except they only have one of her X chromosomes. We used these

data to identify morph-biased and morph-specific genes. We sampled all five morphs from three different aphid genotypes, allowing us to identify gene expression differences that are truly biased towards particular morphs rather than specific to a particular morph within a particular genotype. Our goal was to investigate patterns of intraspecific polymorphism and interspecific divergence in morph-biased genes relative to unbiased genes. Our results provide significant information regarding how phenotypic plasticity impacts the evolution of morph-specific genes.

Materials and Methods

Insect rearing and sample collection: We reared three pea aphid clonal lines, F1, BK11 and Ithaca18, in small cages using *Vicia faba* L. as a host plant at 18°C on a 16:8 (L: D) cycle. BK11 and Ithaca18 were collected in Massachusettes and New York, respectively. The F1 line resulted from a cross between two New York lines (Braendle *et al.* 2005).

We reared asexual female aphids at low density (less than six individuals per cage) to produce wingless asexual females. To produce winged asexual females, we crowded 12 asexual adult females by placing them in a small Petri plate for 24 hours (crowding induces the production of winged offspring; Sutherland 1969). We then transferred females to caged plants and reared their offspring to maturity. We collected winged and wingless asexual adult females on the second day after their adult molt. All individuals (including the ones described below) were collected between noon and 2 PM to control for circadian changes in gene expression.

We transferred adult asexual female aphids to an incubator that mimics fall photoperiod (L: D cycle = 13:11) with a colder temperature (15°C) to induce sexual morph production. After five to six weeks the aphids began producing sexual morphs. We collected third and fourth instar nymphs and placed them on a caged *V. faba* plant. We observed them every day until they molted into adulthood. We transferred the newly molted adult morphs individually on a new, caged plant and collected two-day old adults. Sexual females were difficult to identify as early adults. We therefore sometimes waited for four more days to collect them to make sure that they were not asexual females.

We collected whole bodies of 30 adult aphids of each of the five morphs and three clones (total of 15 samples) by flash freezing them in liquid nitrogen. Samples were stored at -80°C. We used three different clones as biological replicates for downstream analyses rather than three replicates of the same clone in order to identify genes that were systematically expressed in a particular morph, not just a particular morph of a particular clone.

RNA extraction and sequencing: Total RNA was extracted using a phenol/chloroform extraction. RNA was quantified by Qubit and the quality was checked by Bioanalyzer (Agilent). RNA sequencing libraries were constructed per manufacturer instructions with sample-specific tags (Illumina) and sequenced on an Illumina HiSeq 2000 sequence analyzer at the University of Nebraska Medical Center using 50 or 100 base single end sequencing. Two samples were pooled per lane for a total of 7.5 lanes of sequencing.

RNA-Seq samples have been deposited to NCBI's GEO archive under accession numbers GSE56830.

RNA-Seq data mapping and analysis: We mapped sequencing reads to the 36,961 gene predictions of v2.1 annotation of the pea aphid genome (available at aphidbase.com) using the Arraystar Software (DNAStar Inc.). Reads were aligned to the reference transcriptome if 97% of the bases matched within each read. Reads aligning to more than one transcript equally were excluded. The numbers of mapped reads per library ranged from 32 to 78 millions (average is 52.5 million). Genes with a total raw read count of less than 100 for all 15 libraries were filtered out to produce a final set of 20,657 genes.

The DESeq2 R package (Anders & Huber 2010) was used to normalize the libraries and to identify the significantly differentially expressed genes among the three types of morphs (asexual females, sexual females, and males). For each of the 20,657 genes, two generalized linear models (GLMs) were compared using likelihood ratio tests implemented in DESeq2, one with morph type as a factor (this factor having three levels: male, sexual female and asexual female), and one without it. A clone effect (with three levels, corresponding to the three clonal lines) and wing phenotype effect (with two levels, winged or wingless) were also included in both models to control for the structure of the data. If the inclusion of the morph type as a factor significantly improved the model fit for a particular gene ($P < 0.05$ after correcting for multiple testing using the Benjamini-Hochberg method), it was concluded that the expression of that gene significantly differs between the three morphs. All analyses were performed using R (v.

2.15.0 R Development Team 2012).

Identification of morph-biased, unbiased genes, and morph-specific genes: Morph-biased genes were identified as having an adjusted $P < 0.05$ for morph effect in the GLMs implemented in DESeq2 and two-fold higher expression in one morph relative to the other morphs. The unbiased gene set consisted of genes with adjusted $P > 0.05$ from the differential gene expression analysis of three-morphs (DESeq2) or fold change less than two relative to other morphs. To look at increasing levels of morph-bias, we used a conservative measure of bias that defined the bias by the lowest fold change of the focal morph relative to each of the two other morphs as in Jaquiéry et al. (2013). We used categories of 2-5x, 5-10x, and greater than 10x. For example, a gene that was expressed 3x higher in asexual females than males and 10x higher in asexual females than sexual females would be placed in the 2-5x category because the lowest fold change of the two (3x and 10x) was 3x.

Morph-specificity was measured for each gene as $\tau = \sum_{i=1}^{N} \frac{1 - \frac{x_i}{\max(x)}}{N-1}$ (Yanai *et al.* 2005), where x_i stands for the average normalized expression level of the focal gene in morph i (i = asexual female, sexual female or male). This results in a specificity term that ranges from 0 to 1, with 1 being the most specific.

We identified genes biased towards winged and wingless morphs that were a subset of male- and asexual female-biased genes to examine the effect of increased morph bias on

dN/dS. To do this, we first used additional GLMs in DESeq2 to identify genes significantly differentially expressed among the five pea aphid morphs. We compared the model in which we included a morph effect (this factor having here five levels: winged male, wingless male, sexual female, winged asexual female and wingless asexual female) and a clone effect. The effect of morph on a particular gene was considered as significant if the full model (including morph effect) was significantly better than the model without this factor based on likelihood ratio tests (after adjusting for multiple testing, Benjamini-Hochberg method). Morph-biased genes were defined as having $P < 0.05$ and a 2x fold change greater in the focal morph relative to all four other morphs. From this data set, we then selected winged and wingless asexual female-biased genes that were a subset of asexual female-biased genes and winged and wingless male-biased genes that were a subset of male-biased genes to examine the effect of increased morph bias on dN/dS. Wing specificity was calculated using the above equation, with i = winged or wingless morph, independently calculating this value for males and asexual females.

We defined morph-specific genes as those having average read counts less than 4 for all morphs but one, with the remaining morph having an average read count greater than 49. A read count of less than 4 for our data corresponds to a -4 $\log_2$RPKM given our average of 49 million mapped reads per sample, and a read count greater than 49 corresponds to a positive $\log_2$RPKM. Expression levels below -4 $\log_2$RPKM are likely to be nonfunctional transcripts (Hebenstreit *et al.* 2011) and thus we consider these genes as not expressed.

Reconstruction of protein coding genes from expressed sequence tag sequences: Expressed sequence tags from *A. gossypii* were analyzed through blastx (Altschul *et al.* 1990) against Uniprot (cut-off e-value of $10e^{-10}$) as described in Ollivier et al. (2010). This information helped to identify potential homology and was used in the CDS detection process. We then ran FrameD with the frameshift detection and correction option (Schiex *et al.* 2003) to predict CDSs from unique transcripts. Frameshifts were detected and corrected. We retained only CDSs of at least 150bp.

Identification of *A. gossypii* orthologs and calculation of divergence values: The RBH method was used to identify putative orthologs between A. pisum and A. gossypii (Hirsh & Fraser 2001; Jordan et al. 2002) as described in Ollivier et al. (2010). For all RBH pairs, translated sequences were aligned using T-coffee (Notredame et al. 2000), with nucleotide sequences being aligned using the protein alignment as a guide. Alignments were trimmed using Gblocks (Castresana 2000) to retain only the parts that aligned well. The following options were used: "codons" (trimming only entire codons), -b3 = 6 (maximum number of contiguous nonconserved positions), and –b4 = 9 (minimum length of a block). The minimum alignment length after trimming was 60bp. We then estimated maximum likelihood pairwise synonymous (dS) and non-synonymous (dN) evolutionary rates, using a codon-based model [Codeml from PAML; (Yang 1997)]. PAML was parameterized for pairwise rates estimations (runmode = -2) and nucleotide frequencies at three codon positions were used as free parameters (CodonFreq = 3).

Population genetic statistics: As previously described (Bickel *et al.* 2013), we calculated Tajima's D (Tajima 1989) to identify genes not evolving neutrally. As detailed in Bickel et al. (2013), this statistics was calculated from low coverage genome resequencing of 21 pea aphid genotypes.

Synonymous codon usage analysis: We estimated codon usage bias using the effective number of codons (Wright 1990) using the CodonW program (http://bioweb.pasteur.fr/seqanal/interfaces/codonw.html). This measure is lower for genes with stronger synonymous codon usage bias.

Results

Gene expression profiles of different pea aphid morphs

We found that the five pea aphid morphs are differentiated at the gene expression level. We performed a principle component analysis (PCA) on the total expressed genes. Figure 1a shows how the five morphs are distributed with respect to the two major principle component (PC) analysis axes of variation generated from 20,657 genes. PC1, which accounts for 33% of the variance in the transcriptional data, primarily separates sexual females, asexual females, and males. PC2, which accounts for 23% of the variance, further separates the asexual females into winged and wingless. Hierarchical clustering of the gene expression profiles of each morph demonstrates that the largest divide is between females and males (Figure 1b). Females and males are not genetically identical; they differ from each other in that males have only one copy of the female's X chromosome (females are XX and males XO). In contrast, sexual and asexual females

have no genetic differences despite their unique gene expression patterns. These females are phenotypically differentiated at the whole-body level most noticeably in their ovaries. Asexual females contain developing embryos in their ovaries while sexual females contain developing eggs. Many of the gene expression differences observed between them may arise from these ovarian differences.

We identified a large number of morph-biased genes: 3,374 sexual female-biased, 1,856 asexual female-biased, and 4,938 male-biased genes, using the criteria of an FDR-corrected $P < 0.05$ for differential expression as determined in DESeq2 and greater than twofold higher expression relative to the other morphs (winged and wingless morphs were not used separately for this analysis). Sexual female-biased genes were significantly enriched (Fisher's Exact Test, FDR-corrected $P < 0.05$) for gene ontology (GO) terms related to the cell cycle, chromatin organization, and gene expression. Asexual female-biased genes were primarily enriched for terms related to development and cellular differentiation, likely reflecting the presence of developing embryos in their ovaries. Male-biased genes were more varied in their identity, with only a handful of GO terms related to ion channel, hormone, and hydrolase activity being significant.

Morph-biased and morph-specific gene evolution

To determine the impact of morph-biased gene expression on rates of gene evolution, we first identified putative orthologs of pea aphid genes in the cotton aphid (*Aphis gossypii*) using expressed sequence tag (EST) data from that species. We then examined the relationship between morph bias and the rate of nucleotide substitution. It is well known

that a number of factors can influence the rate of change in genes such as presence on the sex chromosomes, overall expression level, and gene length. Thus to control for these other factors, we used a linear model to look at the effects of morph specificity (see Methods for how this measure was calculated), chromosome (X versus autosome as determined in Bickel *et al.* 2013), normalized expression level, and coding sequence (CDS) length on log transformed dN/dS values. This model used information from all genes for which we had dN/dS data (n = 5,844).

We found that morph-specificity does indeed have a highly significant effect on dN/dS (Table 1). This confirmed our expectations that higher morph specificity would correspond to faster rates of evolution between species. CDS length was also highly significant, but negatively correlated such that shorter coding regions were associated with higher dN/dS. This relationship was not due to short genes causing spurious alignments with putative orthologs, inflating dN/dS; we noted that alignments of less than 300bp actually exhibited a positive relationship between gene length and dN/dS (n = 319, Spearman R = 0.19, $P = 0.001$). Thus, globally, it appears as though short genes evolve faster, with the exception of very short genes. This global relationship is the opposite of the one observed in *Drosophila*, where longer proteins were observed to evolve more rapidly (Lemos *et al.* 2005). Asexual and sexual female expression levels also significantly negatively impacted dN/dS, indicating that weakly expressed genes evolve faster than genes with higher transcript levels as observed in other species (Duret & Mouchiroud 2000; Pal *et al.* 2001; Rocha & Danchin 2004). Overall, morph specificity, CDS length, and expression levels all significantly affected the rate of gene evolution.

Morph specificity also positively impacted dN values and dS values (Supplemental Table 1, 2). Typically, synonymous sites are thought of as neutrally evolving and thus dS should be the same for all gene classes. But, we do observe a difference in dS values across gene categories. We postulate that this may be due to differences in selection on codon usage. We find that the effective number of codons, a commonly used measure of codon bias representing how more codons are used in less constrained genes, is significantly positively associated with morph specificity (Spearman correlation = 0.035, $P = 0.007$). This indicates that more morph-biased genes may exhibit less selection for codon usage at synonymous sites, as was first shown in *Drosophila melanogaster* male-biased genes (Hambuch & Parsch 2005).

We also considered the effect of increasing morph bias on rates of evolution compared to unbiased genes. We defined unbiased genes as those having expression values that were not significantly different among morphs (using the DESeq2 package with an FDR-adjusted $P > 0.05$) or had a fold change of less than two for each morph relative to all other morphs. For morph-biased genes, we divided them into three levels of bias: 2-5x, 5-10x, and >10x fold change relative to other morphs, and examined their divergence via dN/dS. This analysis resulted in three main outcomes. First, we hypothesized that all three classes of morph-biased genes would exhibit faster rates of evolution than unbiased genes, given that unbiased genes are expressed equally in all morphs and thus always expressed in the population. Consistent with this expectation, we observed that morph-biased genes do evolve faster than unbiased genes, although for asexual females this is

only true in highly morph-biased genes (5x and greater, Figure 2a). Second, we expected and found that increasing levels of bias result in increasing rates of evolution (Figure 2a). And finally, we hypothesized that sexual female- and male-biased genes would evolve faster than asexual female-biased genes. We anticipated this because sexual females and males are the least common morph and are only present in pea aphid populations for a few weeks in the fall (one generation). In contrast, the asexual females are present for 15-20 generations in the summer. We therefore expected that sexual female- and male-biased genes would have elevated nonsynonymous substitutions relative to asexual female-biased genes because of a relaxation of purifying selection. Our results indicate that sexual female- and male-biased genes do generally evolve faster than asexual female-biased genes. This is true for the 2-5x biased gene categories as well as the highly (>10x) morph-biased genes (Mann-Whitney U tests: males versus asexual females $P = 3.4\text{x}10^{-13}$ for 2-5x, $P = 0.029$ for > 10x; sexual females versus asexual females $P = 5.3\text{x}10^{-8}$ for 2-5x, $P = 0.024$ for > 10x). Similar plots for dN and dS can be found in Supplemental Figure 1.

We further identified genes that are biased to the winged and wingless morphs produced within asexual females and males. We hypothesized that winged or wingless-biased genes within a morph would experience less purifying selection than genes expressed regardless of the wing phenotype, and thus these biased genes would have elevated levels of nonsynonymous nucleotide substitutions. We identified genes as winged or wingless male-biased in a similar manner as the previous groups: they were differentially expressed (FDR-corrected $P < 0.05$) across the normalized expression values of all five

morphs, they had greater than 2x fold change relative to all other morphs, and they were a subset of the male-biased genes. Winged and wingless asexual female-biased genes were identified in the same manner. We found 69 winged asexual female-biased, 838 wingless asexual female-biased, 326 winged male-biased, and 1149 wingless male-biased genes. Very few of these genes had orthologs in *A. gossypii*, and thus examination of dN/dS values involved small samples sizes (Figure 3a). Further, analysis of dN/dS values for these genes did not yield the anticipated result: neither winged nor wingless asexual female-biased genes exhibited evidence of evolving faster than asexual female-biased genes. The same pattern was seen in males. Because sample sizes were low in this analysis, we also pursued a second approach to evaluating the rate of evolution of increasingly morph-biased genes. For male- or female-biased genes, we calculated their winged or wingless specificity on a scale of zero to one (see Methods for the calculations). We used a linear model to examine the influence of wing-morph specificity on the rate of evolution. Our results suggest that the rate of evolution does not increase with higher morph specificity for either males (n = 476, $P = 0.34$) or asexual females (n = 602, $P = 0.89$). Future analyses that examine this increasing morph-specificity will have to await greater dN/dS sample sizes brought about by whole genome sequencing of other aphid species.

Finally, we identified morph-specific genes (in contrast to morph-biased genes) as genes that were expressed exclusively in one morph. We suspected that these genes would exhibit especially high rates of evolution. We identified 543 morph-specific genes. Male-specific genes dominated this list, with 515 male-specific, 16 asexual female-specific,

and 12 sexual female-specific. It was impossible to test if these morph-specific genes are evolving particularly fast given that we only obtained four *A. gossypii* orthologs for them. When we further divided these morph-specific genes into winged and wingless, this resulted in a paucity of genes, with only 78 wingless male-specific genes, 2 winged male-specific genes, 1 winged asexual female-specific gene, and 0 wingless asexual female-specific genes.

Intraspecific variation in morph-biased genes

We used Tajima's D values (Tajima 1989) to assess whether the different classes of morph-biased genes were experiencing different selection intensities. Note that the Tajima's D values for pea aphid coding regions as previously calculated (Bickel *et al.* 2013) are skewed negative. This is likely due to a demographic factor of these sampled lines: they were collected in North America where pea aphids are introduced (e.g., Thomas 1878) and have experienced range expansion. This rapid expansion can cause negative Tajima's D values even in presumably neutrally evolving regions, such as synonymous sites. Further negative skew likely resulted from sequencing errors (Achaz 2008) given that these values were calculated from low coverage genome sequencing (Bickel *et al.* 2013). [Note that using Tajima's D values in our study was preferable to using McDonald-Kreitman tests (McDonald & Kreitman 1991) due to the limited number of A. gossypii orthologs available.] Thus, comparisons of Tajima's D values between groups of genes is more relevant here than the absolute value of Tajima's D.

If morph-biased genes evolve under relaxed purifying selection, they should exhibit higher values of Tajima's D relative to unbiased genes. We observed this expected result with sexual female- and male-biased genes (Figure 2b). We therefore conclude that relaxed selection underlies the higher rates of evolution seen at sexual female- and male-biased genes. We did not observe this expected result with asexual female-biased genes. Asexual female-biased genes at 2x and 5x levels of bias had Tajima's D values significantly lower than unbiased genes (Figure 2b). This result suggests that the higher rates of evolution seen in asexual female-biased genes may be due to adaptive evolution. Interestingly, winged asexual female-biased genes exhibited higher Tajima's D values (Figure 3b) than genes that were asexual female-biased. This observation supports the idea that at least some asexual female morph-biased genes experience less purifying selection.

Chromosomal location of biased genes

We found that male-biased genes are more likely to be found on the X chromosome. Of the 4,254 male-biased genes, 539 are on the X chromosome (gene location determined in Bickel *et al.* 2013). This observed 12.7% is a significant enrichment of male-biased genes on the X chromosome compared to 9.4% of all genes (Fisher's exact text, $P = 8.7 \times 10^{-10}$) detected in our study. Female-biased genes are not significantly over or underrepresented on the X chromosome (asexual female-biased = 9.5% and sexual female-biased = 8.6%).

Discussion

The existence of phenotypic plasticity results in some genes exhibiting morph-biased or morph-specific expression, which in turn can theoretically affect their rate of evolution (Van Dyken & Wade 2010). Pea aphids produce different morphs that are present in the population for different lengths of time. Here we have used these different morphs as a natural system for examining how morph-biased gene expression affects gene evolution.

Rare pea aphid morphs evolve quickly and experience relaxed purifying selection

Several lines of evidence presented here suggest that genes that only have a role in less common morphs will diverge in sequence faster than genes that are expressed ubiquitously. Here, we have used biased gene expression as a proxy for function within a morph. Although the exact relationship between morph bias and morph fitness is not well-understood, *Drosophila* genes with highly sex-biased expression (greater than 4-fold differential expression) have the most demonstrable effects on sex-specific fitness (Connallon & Clark 2011). Thus we assume that some morph-biased genes with high levels of differential expression may be functionally important for that morph.

First, our results show that rates of evolution, as measured by dN/dS, are generally elevated in morph-biased genes relative to unbiased genes, which are expressed in all morphs at all times and therefore spend the most time being expressed. This pattern of higher rates of evolution of morph-biased genes was particularly noticeable when differential expression rose to 5-fold or higher. Therefore, these highly morph-biased genes may be functionally relevant for the morphs yet simultaneously be rapidly evolving

in a potentially nonadaptive manner. Second, sexual female- and male-biased genes evolve faster than asexual female-biased genes for highly morph-biased genes (>10x). Sexual females and males are of special interest in this study because they are far less common in the pea aphid life cycle. These two morphs are produced for a single sexual generation in the fall; in contrast, asexual females are present throughout the spring and summer months. Because of their rarity, we expected sexual female- and male-biased genes to experience relaxed selection and, consequently, exhibit higher rates of evolution. And third, increasing morph-bias, regardless of the morph (asexual female, sexual female, or males) results in higher rates of evolution. This is likely due to the fact that increasing morph-bias means that other morphs exhibit less expression of this gene.

We further showed that relaxed purifying selection was the driver of these increased rates of evolution in sexual female- and male-biased genes. When a gene is expressed at high levels in only one morph, it experiences purifying selection, the most common mode of selection, in only that morph. A morph-biased gene is therefore less often under selection than an unbiased gene, making the impact of drift larger at that gene. The increased importance of drift in the drift/selection balance of biased genes likely leads to an increase in the number of slightly deleterious mutations at these genes (Van Dyken & Wade 2010). We expected and observed this pattern to be strongest in sexual female- and male-biased genes given their low occurrence and thus smaller relative impact of purifying selection. We also expected this pattern to be much weaker in asexual females because their numbers dominate the life cycle, although asexual female-biased genes would still be exposed to selection less than unbiased genes. Interestingly, we observed

lower values of Tajima's D for asexual female-biased genes compared to unbiased genes despite the higher divergence of these same genes between species. This pattern indicates that adaptive evolution may be acting on these genes. In the future, it would be intriguing to investigate whether morph-biased genes retain their morph-biased status across species, given the strong relative effect of genetic drift on some of these genes. We expect that asexual female-biased genes remain relatively constant across species, while sexual female- and male-biased genes exhibit more turnover.

The presence of winged and wingless morphs allowed us to examine even less common morphs. We did not observe significantly higher rates of divergence between species for winged or wingless biased genes of asexual females and males relative to genes that are not biased to either wing morph, although sample sizes were too low to convincingly test this. We did, however, observe significantly higher Tajima's D values within pea aphids for winged asexual female-biased and winlgess male-biased genes, indicating that less purifying selection may be currently acting on these genes in pea aphid populations.

Another way of considering morph-biased genes is by recognizing different sexes as different morphs, although most species harbor genetic differences between sexes. Sex-biased genes, especially male-biased genes but also sometimes sexual female-biased genes, are known to evolve more quickly than unbiased genes (Swanson & Vacquier 2002; Ellegren & Parsch 2007; Parsch & Ellegren 2013). In many cases, this has been attributable to positive selection and thus adaptive evolution of genes coding for proteins expressed specifically in reproductive tissues (e.g., Aguade 1998; Swanson et al. 2001;

Swanson & Vacquier 2002; Swanson et al. 2004; Zhang et al. 2004; Proschel et al. 2006). Alternatively, the faster rates of evolution of genes expressed in reproductive tissues could be due to relaxed selective constraint, given that these genes are often limited in expression breadth and thus exhibit low levels of pleiotropy (Meisel 2011).

Reproductive tissue-specific expression could contribute to the molecular evolution of morph-biased gene evolution in pea aphids. Our pea aphid samples, which were whole bodies, contained both reproductive and non-reproductive tissues. We observed a pattern of relaxed selection driving faster rates of evolution in sexual female and male pea aphids. This could be due to the majority of the sexual-female and male-biased genes experiencing relaxed purifying selection, while simultaneously a subset of the genes encoding key reproductive proteins could be evolving quickly due to positive selection. In other words, our results do not preclude adaptive evolution occurring at sex-biased genes. Further, the lower levels of pleiotropy exhibited by largely reproductive tissue-limited expression could be significantly contributing to our observed patterns of relaxed selective constraint. Even more generally, any kind of tissue-specific expression would result in lower pleiotropy and thus result in relaxation of purifying selection. To investigate these issues, in the future it will be critical to perform expression profiling of specific tissues, such as gonads, separately.

Sex-biased genes are often nonrandomly distributed among autosomes and sex chromosomes (Parisi *et al.* 2003; Ranz *et al.* 2003; Ellegren & Parsch 2007). A previous study in the pea aphid found that male-biased genes are found on the X chromosome at

levels higher than chance (Jaquiery *et al.* 2013). Here we also observed a masculinization of the X chromosome. Our results extend the validity of these previous results, since our data set included three different aphid genotypes and therefore increased the likelihood of identifying truly morph-biased (and not just biased in one genotype), while the previous study only considered replicates of a single genotype. Unlike the previous study (Jaquiery *et al.* 2013), we did not observe a significant enrichment of asexual female-biased genes on autosomes, nor a significantly higher rate of evolution for X-linked genes. This is potentially explained by the different methods of assigning genes to the X versus autosomes used by the two studies.

A scarcity of morph-specific genes in the pea aphid

One of the novel aspects of this study compared to analysis of morph-biased genes in other systems (Hunt *et al.* 2010; Hunt *et al.* 2011; Snell-Rood *et al.* 2011; Leichty *et al.* 2012) is that we used RNA-Seq data to assay gene expression levels rather than microarray data. Low expression cannot be distinguished from a lack of expression with microarrays; RNA-Seq is more accurate at assaying expression levels of lowly expressed and unexpressed genes (Marioni *et al.* 2008; Wilhelm *et al.* 2008; Fu *et al.* 2009). This highly sensitive approach, combined with high sequence coverage (an average of 49 million mapped reads per sample), allowed us to identify genes that were putatively not expressed.

When we considered gene expression across all five pea aphid morphs, we found very few genes that were expressed in only a single morph. We conclude that truly morph-

specific genes are uncommon. There are many other developmental stages (*e.g.,* eggs, nymphs, older adults) and even other morphs (*e.g.*, fundatrices, sexuparae) that could be examined in this species. If these were added to the data set, it is likely that the list of morph-specific genes would shorten. This is an important conclusion because models that address how relaxed selection affects gene evolution are often built upon the assumption of morph-specific genes (Van Dyken & Wade 2010) even though conditional-specific gene expression is common (reviewed in Snell-Rood *et al.* 2010). Our data suggest that future theory efforts should focus more on the effect of morph-biased, rather than morph-specific, expression.

However, it may also be true that some genes with low expression are functionally not important. Their low expression may just be a consequence of leaky transcription that results in no protein product or a protein product at levels too low to contribute to cellular processes. In other words, some of the morph-biased genes that we've identified may functionally be morph-specific. In this case, a much larger number of genes may fit the mentioned models based on morph-specificity. At this time it is impossible to determine which morph-biased genes may be in this category.

Conclusion

Phenotypic plasticity is ubiquitous, yet we are only beginning to understand the impact of plasticity on molecular evolution. Our results join a growing body of work in showing that morph-biased genes evolve more quickly than ubiquitously expressed genes, and that these faster rates of evolution are due primarily to relaxed purifying selection. We extend

previous analyses by showing that the rarer the morph, the more quickly genes biased to these rarer morphs evolve. Our results thus support the hypothesis that relaxed purifying selection leads to faster rates of gene evolution. Unresolved by our approach, however, is the nonmutually exclusive hypothesis of alternative morphs co-opting rapidly evolving genes. Addressing this hypothesis will have to await the availability of an appropriate aphid outgroup to test whether rapid evolution predated morph bias.

Acknowledgments

We would like to thank Travis Hinkelman and Drew Tyre for the statistical help provided. We thank two reviewers for thoughtful comments that improved this manuscript. This work was supported by University of Nebraska-Lincoln, School of Biological Sciences Special Funds to SRP, startup funds from the School of Biological Sciences at the University of Nebraska-Lincoln and by award R00ES017367 from the National Institute of Environmental Health Sciences to JAB. INRA SPE supported a two month stay in INRA Rennes for JAB. JJ acknowledges support by *Agence Nationale de la Recherché,* grant ANR-11-BSV7-005-01.

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Tables and Figures

Table 1: Results from a linear model examining the effect of the following factors on log-transformed dN/dS.

Factors	Estimate	Standard Error	t -value	P-value
(Intercept)	-2.94	0.053	-55.29	$< 2x10^{-16}$ ***
Sexual female Expression	$-1.02x10^{-05}$	$2.52x10^{-06}$	-4.06	$4.9x10^{-05}$ ***
Male Expression	$1.68x10^{-06}$	$7.94x10^{-07}$	2.12	0.034 *
Asexual female Expression	$-7.88x10^{-06}$	$1.32x10^{-06}$	-5.96	$2.73x10^{-09}$ ***
Morph specificity	0.475	0.076	6.23	$4.90x10^{-10}$ ***
Chromosome	-0.015	0.065	-0.23	0.82
CDS length	$-4.16x10^{-05}$	$7.52x10^{-06}$	-5.53	$3.37x10^{-08}$ ***

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

Figures

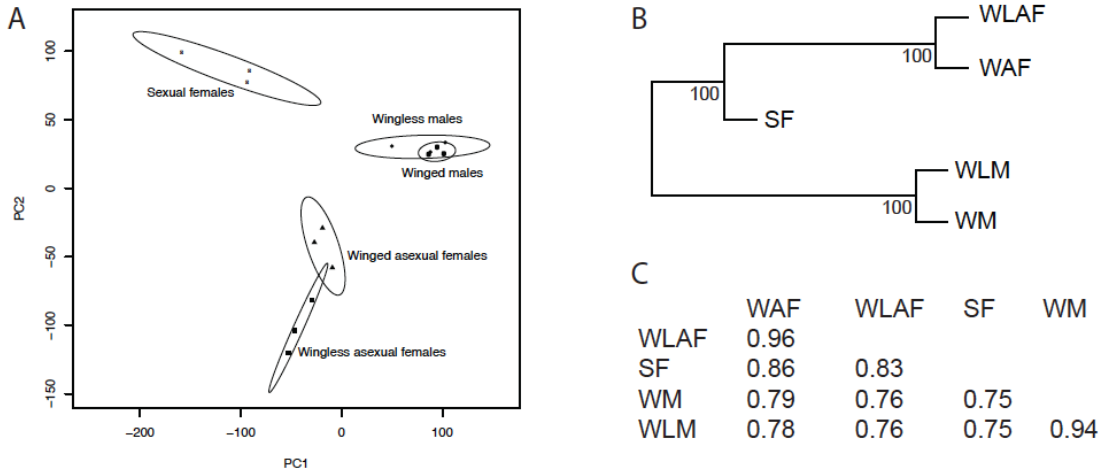


Figure 1: Differences in gene expression values among the five pea aphid morphs. A) The first two PCA axes, which account for 33% and 23% of the total variance in gene expression, differentiate most of the morphs. Ellipses indicate the 95% standard deviation confidence limit. B) Hierarchical clustering of the normalized gene expression values for each morph. The biological replicates (three distinct genotypes) were averaged and data were log +1 transformed prior to clustering. The pvclust package in R was used for clustering via the function hclust and for assessing uncertainty in the hierarchical cluster analysis using default parameter values. P values on nodes (in %) were generated via multiscale bootstrap resampling using 1000 replicates. C) Pearson correlation coefficients for each pairwise comparison on log +1 transformed data. WLAF = wingless asexual female; WAF = winged asexual female; SF = sexual female; WLM = wingless male; WM = winged male.

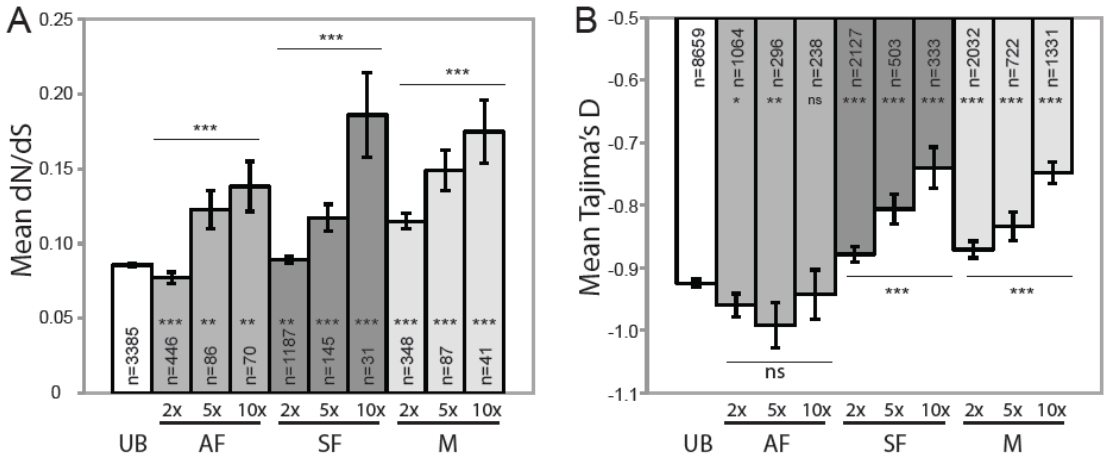


Figure 2: Measures of divergence and selection for unbiased (UB), asexual female (AF), sexual female (SF), and male (M) biased genes. Panel (A) shows mean dN/dS values and panel (B) shows mean Tajima's D values. Samples sizes are indicated within each bar. Error bars show standard error. Fold change categories are indicated by 2x, 5x, or 10x (see text for details on how these were defined). Significance of a Kruskal-Wallis test within morphs is shown as asterisks above or below the bars and significance of a Mann-Whitney test comparing each bar to the unbiased gene set is indicated by asterisks within the bar. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns $P > 0.05$.

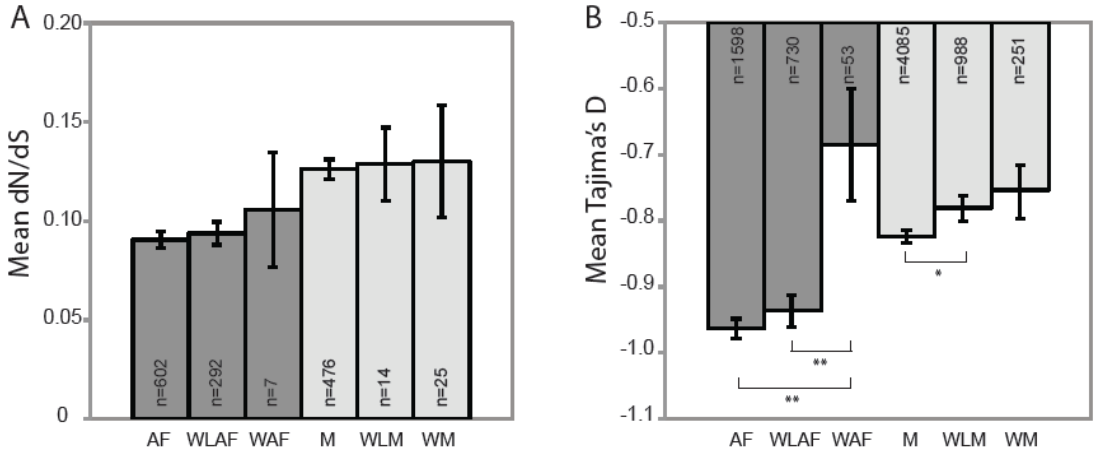


Figure 3: Measures of divergence and selection for winged and wingless-biased genes. Panel (A) shows mean dN/dS values and panel (B) shows mean Tajima's D values. Samples sizes are indicated within each bar. Error bars show standard error. Significance of a Mann-Whitney test comparing each winged (W) or wingless (WL) bar to the asexual female (AF) or male (M) gene set is indicated by asterisks below the bar. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns $P > 0.05$.

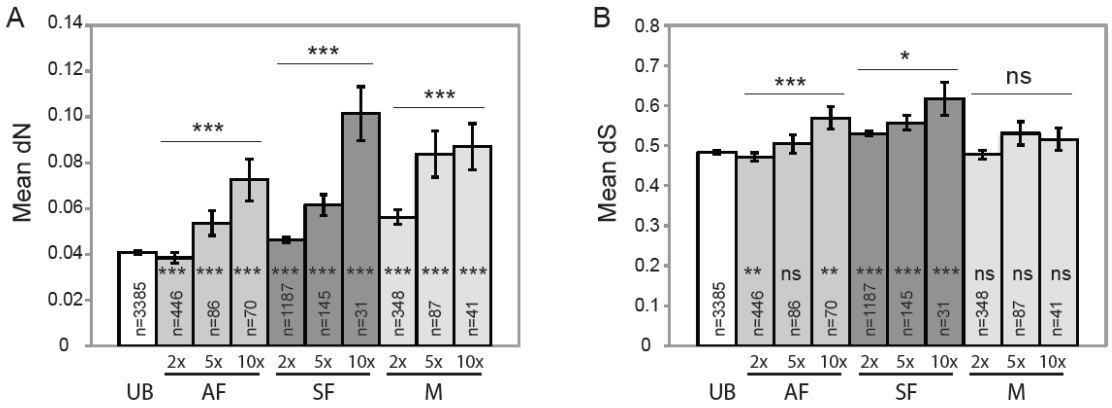
Supplementary Tables and Figures

Supplementary Table 1: Results from a linear model examining the effect of the below factors on log-transformed dN.

	Estimate	Standard Error	t-value	P-value
(Intercept)	-3.78	0.057	-67.11	$< 2x10^{-16}$ ***
Sexual female Expression	$-9.17x10^{-06}$	$2.68x10^{-06}$	-3.42	$6.3x10^{-04}$ ***
Male Expression	$1.08x10^{-06}$	$8.46x10^{-07}$	1.27	0.203
Asexual female Expression	$-1.09x10^{-05}$	$1.49x10^{-06}$	-7.71	$1.43x10^{-14}$ ***
Morph specificity	0.657	0.081	8.09	$< 2x10^{-16}$ ***
Chromosome	0.014	0.069	0.21	0.834
CDS length	$-4.47x10^{-05}$	$8.01x10^{-06}$	-5.57	$2.65x10^{-08}$ ***

Supplementary Table 2: Results from a linear model examining the effect of the below factors on dS.

	Estimate	Standard Error	t-value	P-value
(Intercept)	0.45	0.009	48.7	$< 2x10^{-16}$ ***
Sexual female Expression	$7.26x10^{-08}$	$4.48x10^{-07}$	-0.17	0.868
Male Expression	$-2.47x10^{-08}$	$1.38x10^{-07}$	-0.18	0.858
Asexual female Expression	$-1.16x10^{-06}$	$2.30x10^{-07}$	-5.03	$4.99x10^{-07}$ ***
Morph specificity	0.102	0.013	7.73	$1.26x10^{-14}$ ***
Chromosome	0.011	0.013	0.98	0.328
CDS length	$-1.62x10^{-06}$	$1.31x10^{-06}$	-1.24	0.215



Supplementary Figure 1: Measures of dN and dS for unbiased (UB), asexual female (AF), sexual female (SF), and male (M) biased genes. Panel (A) shows mean dN values and Panel (B) shows mean dS values. Sample sizes are indicated within each bar. Error bars show standard error. Fold change categories are indicated by 2x,5x,10x(see text for details on how these were defined). Significance of Kruskal-Wallis test within morphs is shown as astricks above or below the bars and significance of Mann-whitney test comparing each bar to the unbiased gene set is indicated by astricks within the bar. ***P < 0.001, **p <0.01, *P <0.05, ns P>0.05.