BEHAVIORS MEDIATE APHID INTERACTIONS WITH NATURAL ENEMIES AND THE ENVIRONMENT

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Behaviors mediate aphid interactions with natural enemies and the environment

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ABSTRACT

Behavior is a crucial component of ecology that mediates how animals interact with one another and with the environment. Behaviors can allow animals to avoid the harmful effects of things like competition, predation, and extreme abiotic conditions. However, animals often have constraints that limit the potential benefits of their behaviors, so we addressed what factors contribute to these constraints in plant-aphid-wasp systems.

Parasitoids of aphids are tiny wasps that lay their eggs in aphids, where the larva feeds and develops. Each aphid can only sustain a single parasitoid, so parasitoids mark aphids when they lay an egg to discourage others from laying additional eggs. Not all parasitoids mark aphids the same way, and whether species with different marks can recognize one another's mark was unclear. We found that parasitoids with different marks fail to respond to one another's marks. Because these parasitoids laid eggs in aphids that were already parasitized, competition between them will occur through mortal combat between the larvae rather than egg-laying restraint in the adults.

Parasitoid attack induces aphid behaviors such as fighting, running away, or dropping off the host plant. These behaviors can deter or evade the attacker, but they cost the aphid an opportunity to feed. This feeding interruption decreases the number of offspring the aphids produce. When aphids run from a threat, they usually resettle on the newest leaves of a plant, where their reproduction is enhanced. However, when they drop from a plant to escape, they resettle randomly and do not benefit from the better location, indicating that more vigorous responses can lose benefits. We also showed that aphids exhibit similar movement and resettling behaviors when disturbed by heat shocks. However, this behavior did not affect aphid reproduction or survival when induced by a heat shock. While movement to new leaves appears to be a general response to several disturbances, its benefits to aphids only occur in specific situations.

We have shown that behaviors can be constrained by unfamiliar stimuli and the general context in which they are invoked. These constraints can dictate how insects interact with each other and the environment.

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INTRODUCTION

This dissertation focuses on the behavioral ecology of aphids (order Hemiptera, family Aphididae). The first two chapters address questions that fall in the general realm of trophic interactions between aphids and their natural enemies, while the final chapter focuses on interactions of aphids with their abiotic environment. Despite these parallels, the first chapter's focus is quite distinct from that of chapters two and three.

In chapter one, we focus on the behavioral interactions of two species of soybean aphid parasitoids with one another and with their host. Many species of parasitoids deposit marks on their hosts as they oviposit that deter subsequent ovipositions in the same host, either by the same individual parasitoid or by a different individual parasitoid (Chow and Mackauer 1991). Patterns of parasitoid responses to one another's marks suggest that host marking is an adaptive behavior, which suggests that recognizing and responding to heterospecific marks would require a coevolutionary history (Bai and Mackauer 1991). Using two parasitoid species that share a host but not an evolutionary history or a host marking mechanism, we tested whether the parasitoids responded to one another's host marks. We also considered that the host may provide cues to parasitoids that indicate whether or not it is parasitized in the form of defensive behavior, and tested whether the parasitoids induced changes in host behavior that deterred subsequent oviposition by another parasitoid. All together, these experiments addressed two questions: can parasitoids with different marking mechanism and no shared evolutionary history respond to one another's host marks, and can aphid defensive behaviors limit super-parasitism? While the common theme of "aphid behavior" did appear in this chapter, the research focused more on the parasitoid responses.

In chapters two and three, we investigated the behavioral responses of aphids to simulated predator attacks and heat shocks, respectively. Both of these disturbances can cause sublethal fitness costs in aphids, decreasing the number of offspring they produce following the disturbance (Nelson 2007, Russell and Moran 2006) We also investigated the short-term fitness response, in terms of reproduction, that the disturbances plus behavioral responses yielded for the aphids. In both cases, we focused on movement of the aphids within their host plant. In particular, we focused on the young actively growing

leaves of the host plant as a prospective superior feeding site based on lab observations that these leaves routinely hold the densest aggregations of aphids on the plant.

Chapter two was largely inspired by Nelson (2007), who showed that pea aphids are frequently disturbed by natural enemies in the field and that these disturbances cause fitness reductions even when the aphids evade consumption. We hypothesized that aphids dislodged from their feeding sites by foraging predators may resettle on superior feeding sites, thereby offsetting the cost of the disturbance. We used a fine paintbrush to stimulate predator attacks, prodding aphids until they either walked away from their feeding site or dropped off the plant entirely. Using these simulated attacks and varying the intensity to stimulate "walkers" (aphids that walk away from the feeding site) and "droppers" (aphids that drop off the plant altogether), we measured three aphid responses. These included the cost, in terms of lost feeding time, of aphids moving in response to the disturbance, where on the host plant the aphids resettled, and how many offspring the aphids produced over two days. We also compared short-term reproduction of aphids confined to actively growing leaves and aphids confined to older, mature leaves to see if feeding site contributed to aphid fitness. These experiments yielded a greater understanding of the costs and benefits of escaping predators beyond interrupted foraging and not being consumed.

Chapter three built off of the framework set in chapter two, but switched the type of disturbance from a non-lethal predator attack to a heat shock, or a short (one hour to a few hours) period of stressfully high temperatures. Pea aphids are small-bodied ectotherms that have been shown to be harmed by heat shocks (Russell and Moran 2006), but their behavioral responses to heat shocks were unclear. We hypothesized that they would stop feeding during a heat shock and move about their host plant in search of more moderate temperatures, which we expected to occur near the base of the plant when the soil is moist. To determine how the aphids responded to heat shocks, we exposed groups of aphids on host plants to experimental heat shocks and recorded the aphids' locations on the plants throughout the heat shock, as well as periodically surveying the aphids and recording how many aphids had stopped feeding and were moving about on the plant. We followed up our observations of behavior with an experiment to test the fitness consequences of those behaviors, controlling for where on the host plant the aphids settled by end of the heat shock and confining them to different plant parts after the heat shock. This experiment compared short-term aphid reproduction and survival between aphids based on their location

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during the heat shock, which may alter the heat shock's immediate effects, and their location after the heat shock, which may alter the aphids' recovery from the shock.

References

Bai, B., and M. Mackauer. 1991. Recognition of heterospecific parasitism – competition between aphidiid (*Aphidius ervi*) and aphelinid (*Aphelinus asychis*) parasitoids of aphids (Hymenoptera, Aphidiidae, Aphelinidae). Journal of Insect Behavior 4: 333-345.

Chow, A., and M. Mackauer. 1991. Patterns of host selection by 4 species of aphidiid (Hymenoptera) parasitoids – influence of host switching. Ecological Entomology 16: 403-410.

Nelson, E. H. 2007. Predator avoidance behavior in the pea aphid: costs, frequency, and population consequences. Oecologia 151: 22-32.

Russell, J. A., and N. A. Moran. 2006. Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. Proceedings of the Royal Society B-Biological Sciences 273: 603-610.

TO STING OR NOT TO STING: THE ROLE OF HOST MARKING MECHANISMS IN INTER-SPECIFIC MARK RECOGNITION BY PARASITOIDS[1]

Abstract

Insect parasitoids often avoid ovipositing in hosts that are already parasitized by responding to a mark, such as a pheromone, left by the preceding parasitoid. However, we do not know how effective this system will be when different species of parasitoids share hosts but do not share an evolutionary history and mark in different ways. *Lysiphlebus testaceipes* Cresson and *Binodoxys communis* Gahan are both parasitoids of the soybean aphid, *Aphis glycines* Matsumura. *L. testaceipes* marks hosts with a contact pheromone, while *B. communis* marks hosts by temporarily paralyzing them with a venomous sting. These parasitoids also originated on different continents. With these differences, the two species may fail to respond to one another's mark. However, both species induce similar defensive behaviors in aphids and trigger aphids to release alarm pheromones, which may also serve as cues that the aphid is parasitized. We devised a pair of experiments to elucidate whether these parasitoid species respond to one another's marks or to defensive aphid cues by measuring their foraging behaviors. We found that *L. testaceipes* rejects hosts more frequently when they are marked by conspecifics than when they were not previously exposed to a parasitoid or previously exposed to a *B. communis*, while *B. communis* did not reject aphids that were already parasitized, either by conspecifics or by *L. testaceipes*. Additionally, these parasitoids do not seem to use host-derived cues to assess the parasitism status of prospective hosts; neither wasp demonstrated evidence of using host defensive behaviors or alarm pheromones as cues to avoid ovipositing in already-parasitized aphids. These findings show that neither direct nor host-derived resource-claiming marks help to mediate competition between parasitoid species.

Keywords: competition, host discrimination, host mark, parasitoid, pheromone

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Introduction

To avoid competition, many animals, including insect parasitoids, claim resources such as hosts by marking (Chow and Mackauer 1999). In parasitoids, the resource-claiming mark may be directly left by the marker, or it may arise from behavioral or physiological changes to the host (Gardner et al. 1984, Outreman et al. 2001). Marks that are left directly, such as pheromones, tend to be species specific (Bai and Mackauer 1991). Alternatively, resource-derived marks, which arise as a side-effect of the resource being used, have a greater potential to be recognized and used by heterospecifics (Bai and Mackauer 1991). Thus, the type of mark used by the parasitoids may help determine whether it can be used by conspecifics or heterospecifics in foraging decisions (Bai and Mackauer 1991). However, at this point there are not enough examples to clearly predict whether the type of mark – species-specific or resource-derived – determines whether the mark will function beyond just self-recognition to the point of mediating conspecific and/or heterospecific interactions with competing parasitoids.

Host marking in parasitoids is often important to their foraging efficiency and potentially their competition. Host marking enables an individual parasitoid to avoid depositing surplus eggs in a single host, thereby limiting wasted time and eggs invested in hosts (Brodeur and Rosenheim 2000, Rosenheim and Mangel 1994). When conspecifics can recognize one another's host marks, the function of host marking can extend to mediating competition within a species (Vanbaaren et al. 1994). In some cases, heterospecifics can respond to one another's host marks. For example, in choice experiments *Anaphes* n. sp. preferentially parasitized unparasitized over parasitized eggs, and if unparasitized eggs were not available it preferentially parasitized conspecific-parasitized hosts over self-parasitized hosts or hosts parasitized by a closely related parasitoid (Vanbaaren et al. 1994). In these instances, the host marking can mediate competition between species as well as within species. The accuracy with which different species of parasitoids can host-discriminate varies broadly. Some species have imperfect host discrimination which results in some self-superparasitism or some hosts being missed in a patch (Rosenheim and Mangel 1994). Other species can discriminate and show preferences between hosts that are not parasitized, self-parasitized, conspecific-parasitized, or heterospecific-parasitized (Danyk and Mackauer 1993, Vanbaaren et al. 1994).

Parasitoids offer a rich opportunity to study the mechanisms of host marking and between-species mark recognition. Parasitoids deposit their eggs into other insects, which provide all of the nutrition necessary for the young parasitoids to develop to adulthood (Smith 1944). However, the host insect remains vulnerable to predators and other parasitoids after the initial parasitoid deposits eggs (Brodeur and Rosenheim 2000; Muller and Godfray 1999). Multiple solitary parasitoids deposited in a single host often fight to the death (Marris and Casperd 1996), so parasitoids can offer their eggs a greater chance of survival if they can prevent subsequent parasitoids from parasitizing their hosts and by preferentially parasitizing only hosts that are not yet parasitized (McBrien and Mackauer 1991). To this end, many parasitoids have been shown to avoid parasitizing hosts that they or conspecifics have already parasitized (Muller and Godfray 1999). However, parasitoids' ability to avoid hosts parasitized by heterospecifics has been far more mixed (Muller and Godfray 1999), which could result from a failure of the host marking of one species being detected or interpreted by another.

Knowing whether heterospecifics can recognize one another's marks can be important to understanding how they interact. When species respond well to marks, competition can largely occur via scramble competition, in which individuals race to claim as many resources as possible (Schellhorn at al. 2002). Conversely, if mark recognition is poor, competition is more likely to occur as contest competition where individuals physically fight over poorly claimed resources (Visser and Rosenheim 1998). Because some species are stronger scramble competitors and others are stronger contest competitors, the use of resource marking can affect the outcome of species interactions (Schellhorn et al. 2002).

Heterospecifics may come to recognize and use another species' marks. Another species may be able to detect and use the mark of a different species because the two species mark resources in similar ways, either because of close evolutionary relationships or through convergence (McBrien and Mackauer 1990). Additionally, even when species have different marking mechanisms, a long evolutionary history of competition may allow a species to adapt to the other's marks and respond to them (Bai and Mackauer 1991). Such responsiveness to marking may be related to the outcome of competitive interactions, with stronger contest competitors ignoring marks of weaker contest competitors (Muller and Godfray 1999). Therefore, understanding the mechanism of marking and the competitive abilities of the mark-interpreting

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species can allow predictions of whether other species are likely to detect or respond to one another's marks.

Aphid parasitoids can mark their hosts through a combination of several potential mechanisms. Most parasitoids in the Aphidiinae directly mark their hosts externally with a pheromone (Chow and Mackauer 1986, Danyk and Mackauer 1993, Medrzycki et al. 2002). Some are thought to also create internal cues within an aphid, which may be detected by the probing ovipositor of subsequent parasitoids (Bai and Mackauer 1991). However, many parasitoids in the tribe Trioxini use venom that temporarily paralyzes their host, and avoid ovipositing in such immobilized hosts (Desneux et al. 2009, Volkl and Mackauer 2000).

Alternatively, an attack by a parasitoid can trigger immediate host-derived cues such as the release of cornicle secretions and initiation of defensive behaviors by aphids (Gardner et al. 1984, Wyckhuys et al. 2008a). Aphid alarm pheromones, which are released in cornicle secretions, are highly volatile and are released when an aphid is attacked by a predator or parasitoid. They are also released continuously by crowded aggregations of aphids and, in some cases, by the aphids' host plant (Almohamad et al. 2008, Beale et al. 2006, Vandermoten et al. 2012). Aphid alarm pheromones are used as prey-finding kairomones by a large number of aphid predators and parasitoids (Hatano et al. 2008). Parasitoids have been shown to respond to aphid alarm pheromones in different ways, either following them to find colonies of aphids (Battaglia et al. 1993, Beale et al. 2006, Grasswitz and Paine 1992) or avoiding ovipositing in aphids that have recently emitted pheromone (Outreman et al. 2001). Furthermore, aphids that are attacked can use a variety of defensive behaviors that effectively deter parasitoids (Butler and O'Neil 2006, Weisser 1994, Wyckhuys et al. 2008). Because defensive behaviors are induced after a parasitoid attack and deter subsequent parasitoid attacks, they functionally serve as an indirect mark (Gardner et al. 1984). Active defensive behaviors or drying cornicle secretions on an aphid have been shown to decrease the likelihood of multiple ovipositions in some parasitoids (Gardner et al. 1984, Outreman et al. 2001). Parasitoids may use any combination of these cues when deciding whether or not to oviposit in a prospective host.

We devised a pair of experiments to determine whether parasitoids with different marking mechanisms change their foraging behaviors when presented with hosts that had previously been

parasitized. We used two different parasitoid species: *Lysiphlebus testeceipes* Cresson, which is native to North America and is thought to mark with a pheromone (Medrzycki et al. 2002), and *Binodoxys communis* Gahan, which was recently introduced to North America from Asia, which marks by paralyzing the host (Desneux et al. 2009). Because these two species use different types of marks and lack an extensive evolutionary history together, they provide a useful study system to determine if either species' marks are sufficiently general to cross species boundaries or if the parasitoids can rely on host-derived cues to discriminate between hosts. In our first experiment, we exposed colonies of aphids sequentially to two parasitoids and recorded both parasitoid foraging behavior and aphid behavior. We tested two hypotheses: 1) that the order in which the wasps foraged (e.g. first, following a conspecific, or following a heterospecific) would affect their foraging behavior and 2) that the wasps would induce changes in aphid behavior that would change the aphids' acceptability to subsequent wasps. We conducted a second experiment to determine whether the cues used by the parasitoids were specific to the aphids which were actually attacked, or affected the entire host patch. We hypothesized that the parasitoids would respond more strongly to the species-specific cues offered by previously parasitized hosts than to more general cues in the host patch.

Methods

Study System

We studied two parasitoids of the soybean aphid, *Aphis glycines* Matsumura (Hemiptera, Aphididae). *L. testaceipes* Cresson (Hymenoptera, Braconidae) is a North American parasitoid that parasitizes the soybean aphid (Desneux et al. 2009) and other aphids in the tribe Aphidini (Silva et al. 2008). *B. communis* (Hymenoptera, Braconidae) was introduced to North America from China to augment biological control of the soybean aphid (Wyckhuys et al. 2009). The host range of *B. communis* includes several species in the tribe Aphidini, with a preference for soybean aphid (Desneux et al. 2009). Thus, *L. testaceipes* and *B. communis* have no direct evolutionary history with one another, though other *Lysiphlebus* species have been found to share the native range of *B. communis* (Stary et al. 2010). *L. testaceipes* and *B. communis* could become competitors within the soybean agro-ecosystem in North America if *B. communis* establishes there, although *B. communis* has not been found to overwinter successfully in the field in North America (Gariepy et al. 2015).

The host marking systems in *L. testaceipes* and *B. communis* are different. Host discrimination in *L. testaceipes* has only been studied once. Medrzycki et al. (2002) claim *L. testaceipes* uses a contact pheromone as a species-specific cue to mark hosts when it probes with its ovipositor or deposits an egg. *B. communis*, on the other hand, delivers a paralyzing venom that immobilizes aphids and deters parasitism for up to 15 minutes in small aphids and an average of 4 minutes in larger aphids (Desneux et al. 2009), suggesting that the venom or immobilization may be a marking cue. However, it is unclear if this would work as a species-specific cue or a host-derived cue. Because of the differences in cues used to mark hosts in these two wasps and their lack of evolutionary history with one another, they are unlikely to recognize one another's direct marks. However, it is unclear whether these species originated alongside other species that use similar marks.

The soybean aphid may provide additional host-derived cues to parasitoids that can aid parasitoids in avoiding already-parasitized aphids. These cues can occur over short periods of time, including emission of alarm pheromone (Butler and O'Neil 2006) and induction of defensive behaviors (Wyckhuys et al. 2008). For example, we have observed both wasps inducing the emission of cornicle secretions from aphids they attack (unpublished data). These cornicle secretions contain and disseminate aphid alarm pheromone (Eichele et al. 2016). Furthermore, both wasps could induce temporary behavioral changes in aphids, either inducing defensive behaviors, or, in the case of *B. communis*, suppressing defensive behaviors with paralyzing venom.

It is unclear if either parasitoid will respond to host-derived cues or to the other species' species-specific cues. We do know that *L. testaceipes* responds to alarm pheromones from some, but not all, aphids (Grasswitz and Paine 1992). The specific responses of *B. communis* to aphid alarm pheromones have not, to our knowledge, been studied. However, aphid defensive behaviors can deter attacking *B. communis* (Wyckhuys et al. 2008). The ability of *L. testaceipes* and *B. communis* to avoid ovipositing in aphids parasitized by one another has not been empirically tested.

Rearing of Study Insects

We maintained colonies of soybean aphids, *L. testaceipes*, and *B. communis* in the laboratory. All colonies were reared with a soybean aphid-susceptible variety of soybean, *Glycine max* L. (RG607RR, NDSU Research Foundation, Fargo, ND, USA) at 22°C under 16:8 L:D cycle. Soybean aphids had been

maintained in the lab since 2008, with colony foundresses collected in soybean fields at the North Dakota Agricultural Experiment Station near Prosper, ND (47.00°N, -97.12°E). Colonies were periodically supplemented with additional wild-caught aphids from the same location to increase genetic diversity. *L. testaceipes* colonies were established in the fall of 2013 with wasps obtained from colonies maintained at the University of Minnesota. *B. communis* colonies were established in the winter of 2009 with wasps obtained from colonies maintained at the University of Minnesota. The first experiment was conducted during the fall and winter of 2013, while the second was conducted during the summer of 2015.

Soybean aphid colonies provided aphids for wasp rearing and experiments, and were reared using the same methods as Ballman et al. (2012). Aphid colonies were refreshed every 10-11 days by placing cut leaves from infested plants onto new soybean seedlings. Aphids dispersed from the cut leaves to the new plant. At the time of inoculating new plants with aphids, old plants were discarded and replaced with the new plants. Soybean aphids are normally a "heteroecious, holocyclic species (host-alternating with sexual reproduction during part of its life cycle)" (Ragsdale et al. 2004). Sexually-produced eggs overwinter on buckthorn (*Ramnus cathartica*), and the first two spring generations are wingless asexual females that remain on buckthorn (Ragsdale et al. 2004). Starting with the third generation, the aphids are winged asexual migrants that colonize soybean (Ragsdale et al. 2004). On soybean, they undergo many generations of asexual reproduction through the spring and summer, producing a mix of wingless morphs and winged morphs that facilitate dispersion (Ragsdale et al. 2004). In response to shortening photoperiod and decreasing temperature at the onset of autumn, winged morphs migrate from soybean back to buckthorn and produce the sexual generation, which then produces the overwintering eggs (Ragsdale et al. 2004). In our rearing environment, the constant temperature and long photoperiod of 16:8 L:D kept them in their asexual summer phase.

Colonies of *L. testaceipes* and *B. communis* were reared with the same methods. Each was kept in 40 cm X40 cm X 40 cm mesh cages with plants infested with soybean aphids. Every 10-11 days, old plants were cut and set aside in the cages for parasitized aphids to finish developing. Old plants were then replaced with new, infested plants. Wasps used for experiments were not more than three days old and had potential mates and aphids for oviposition continuously prior to being used for the experiments. We used potentially experienced wasps rather than naïve wasps because, in another system,

experienced wasps were better at discriminating between parasitized and non-parasitized hosts (Singh and Sinha 1981). Both species of wasps are solitary koinobiont parasitoids; only a single larva of either species can grow to maturity per host, and the host behaves more or less normally for most of the time that it is parasitized (Muller and Godfray 1999). The generation time of both species of wasps was comparable to the rearing rotation; when colonies were provisioned with fresh plants and aphids, very few or no adult wasps were still in the cages, and the wasp population consisted of larvae and pupae inside of parasitized aphids. Because of this strong generational synchrony with colony renewals, we could estimate the maximum ages of the wasps used in the experiments.

Sequential Exposure of Aphid Groups to Parasitoids

We devised the first experiment to test whether prior foraging by conspecifics or heterospecifics altered the foraging behavior of *L. testaceipes* and *B. communis.* Changes in foraging behaviors when following another parasitoid could indicate a response to some sort of mark. Secondarily, we investigated whether foraging may alter aphid defensive behaviors, which could then be used as a host-derived mark.

To do this, we exposed colonies of soybean aphids to one parasitoid for 15 min, then removed the first parasitoid and immediately exposed the same aphids to a second parasitoid randomly selected between the two species for an additional 15 min. This led to a combination of four treatments (Table 1) that combined each possible arrangement of each parasitoid species foraging first and second. These treatments enabled us to make six comparisons, the first four of which are the second wasps from each of the treatments listed in Table 1, and the last two are the first *L. testaceipes* and *B. communis*, respectively, that were used in each of those four treatments. Because the behavioral observations of the first wasps had no real differences whether the wasp was followed by a *L. testaceipes* or followed by a *B. communis*, in our analysis we combined the first *L. testaceipes* from the first two treatments and the first *B. communis* from the last two treatments. For each species, we observed and compared the foraging behavior of 3 groups: the first parasitoid (combined for each species between followed-by-conspecific and followed-by-heterospecific treatments), the second parasitoid after a conspecific, and the second parasitoid after a heterospecific.

Table 1. Treatments in the sequential exposure of whole aphid colonies experiment.

Treatments (sample sizes in parentheses)
L. testaceipes → *L. testaceipes* (*N* =9)
L. testaceipes → *B. communis* (*N* =9)
B. communis → *L. testaceipes* (*N* =9)
B. communis → *B. communis* (*N* =10)

We recorded the behaviors of both the first and second wasp in each replicate. Each treatment provided a unique second wasp for analysis, while the first wasps against which they were compared were combined across treatments (i.e. N=18 first *L. testaceipes* combined from the first two treatments and N=19 first *B. communis* combined from the second two treatments).

We set up arenas on soybean leaves under a microscope to observe the wasps' foraging and aphids' behaviors. On a 2nd trifoliate soybean plant, 10 1st-2nd instar aphids, 10 3rd-4th instar aphids, and 10 adult aphids were placed on the center leaf of the second trifoliate, similar to Medrzycki et al. (2002). The potted soybean plant was held tilted at approximately a 45° angle, with the aphid-infested leaf held on a foam stage, framed by a 50mm petri dish that had the bottom removed. The stage was set up under a dissecting microscope with an iPhone 4 (Apple Inc., Cupertino CA) mounted to the eyepiece to record the behaviors. The aphids were allowed to settle for 30 min, and then a wasp was introduced to the stage. The first wasp was observed for 15 min or until 5 min elapsed since its last encounter with an aphid, whichever happened first. We used 15 min observations because 15 min was the maximum duration of paralysis reported by Desneux et al. (2009). We also terminated observations for any parasitoid that failed to encounter an aphid for 5 minutes because, in cases where this occurred in preliminary observations, the parasitoid either stopped moving entirely or attempted to evacuate the arena for 20+ minutes. After we removed the first wasp, we randomly chose the species of the second wasp, which was then immediately introduced to the stage. The second wasp was also observed for 15 min or until 5 min elapsed since the last encounter with an aphid. Because the field of view was too narrow to observe wasp and aphid behaviors while observing the entire stage, the stage was moved under the microscope by hand so that the activity of the focal wasp and any adjacent aphids could be viewed and recorded.

Parasitoid Response to Parasitized or Non-parasitized Aphids

We then reviewed the video footage to determine treatment effects on parasitoid behavior. Evidence of responding to marks could manifest in several ways, enumerated in Table 2. Foraging among already-parasitized aphids could result in wasps abandoning the patch (Li et al. 1993, Li et al.

1997, Montovan et al. 2015, Rosenheim and Mangel 1994), so the actual duration of each foraging bout was recorded for each wasp. Additionally, wasps may modify the speed or search pattern in which they forage (Medrzycki et al. 2002), so we measured the number of aphid encounters performed by each wasp and corrected for foraging time by calculating the average number of encounters per min. Lastly, wasps responding to one another's marks are likely to reject hosts after examination during an encounter (Desneux et al. 2009), so the proportion of encounters that culminated in a sting was measured. Finally, the number of stings per min served as a composite response variable that factors in the foraging bout duration, the number of aphids encountered, and the proportion of encountered aphids that were actually stung (McBrien and Mackauer 1991).

While stings do not always result in the deposition of eggs (Rasekh et al. 2010), other aphidiine wasps have been shown to be capable of host discrimination with antennal examination alone and without need for probing with the ovipositor (Bai and Mackauer 1991). This suggests that behavioral responses to marks from other wasps are likely to manifest before a prospective host is probed with the ovipositor and are likely to be apparent from stinging behaviors, though we did not explicitly rule out last-second parasitoid decisions to lay an egg or not lay an egg during a sting. Host rejections feature stereotypical antennal inspection of the host but clearly no sting; the wasp may leave the host without bending its abdomen toward the host, bend its abdomen but not thrust toward the host, or thrust toward the host but clearly not contact it with the ovipositor. Stings, which we interpreted as host acceptances, were recorded whenever *B. communis* firmly grasped an aphid between her abdominal prongs and her ovipositor and whenever *L. testaceipes* made clear, direct contact with the tip of her abdomen against the head, thorax, or abdomen of the host aphid. The ratio of host acceptances and rejections can serve as a proxy for parasitoids detecting and responding to marks from other parasitoids or host-derived cues (Medrzycki et al. 2002).

Table 2. Variables analyzed in the sequential exposure of whole aphid colonies experiment.

Type of mark	Dependent variable	Independent variable(s)
Any marking mechanism	Aphid encounters per minute	Parasitoid order (i.e. first, following *L. testaceipes*, or following *B. communis*), analyzed separately for each species
Any marking mechanism	Proportion of encounters in which aphid was stung	Parasitoid order (i.e. first, following *L. testaceipes*, or following *B. communis*), analyzed separately for each species
Any marking mechanism	Stings per minute	Parasitoid order (i.e. first, following *L. testaceipes*, or following *B. communis*), analyzed separately for each species
Host-derived mark	Aphid defensive behavior at beginning of encounter	Parasitoid order (i.e. first, following conspecific, or following heterospecific)
Host-derived mark	Proportion of aphids stung	Aphid defensive behavior at beginning of encounter (analyzed only for first parasitoids)
Host-derived mark	Change in aphid behavior within each encounter	Parasitoid species (analyzed only for first parasitoids)

The first three dependent variables focus on wasp behavior and can indicate effects from either species-specific marks deposited by the parasitoids or host-derived cues expressed by the aphids. The last three dependent variables collectively connect parasitoid foraging behavior with aphid defensive behavior, and are informative more specifically about the role of host-derived cues.

Data analysis was conducted in R v. 3.2.1. To test for host discrimination, wasp foraging behaviors were compared using ANOVAs between leading wasps, wasps following conspecifics, and wasps following heterospecifics for both species. Significant results were then analyzed using Tukey's HSD contrasts to discern which treatments were driving the effects. To achieve normally distributed data for analysis, the number of stings per min were square-root transformed (Medrzycki et al. 2002).

Aphid Defensive Behaviors

To evaluate the role of induced defensive behaviors as a potential mechanism of host marking, we recorded aphid defensive behaviors at the beginning of each encounter and after each sting for each parasitoid observed in the first experiment described above. To determine if wasps induce or suppress defensive behaviors, for every aphid the parasitoids encountered, we recorded its behavior before and after it was stung. We loosely grouped behaviors into two categories so that we could compare behaviors of aphids before and after stings and compare between aphids in different treatments. Aphids that remained motionless, excreted honeydew, tensed their legs, or waved their antennae were considered non-defensive, whereas aphids that kicked their legs, waggled their abdomens, emitted defensive secretions from the cornicles, or fled were considered defensive.

We made two comparisons of aphid defensive behavior to determine whether wasps induced a change in their hosts' behavior. First, the defensive behaviors of aphids were compared between the

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beginning and end of individual encounters with wasps (i.e. was the behavior different after a sting than immediately before the sting?). This was informative about the very immediate, short-term behavioral consequence of an attack from a parasitoid. Then, defensive behaviors at the beginning of wasp encounters were compared between leading wasps and following wasps. This comparison allowed us to determine if any changes in aphid behavior persisted long enough to potentially affect the second wasp.

To determine whether the aphid defensive behavior was effective at deterring wasps, we compared the wasp stinging success to the aphid defensive behavior for leading wasps. The proportion of encounters that culminated in stings was compared for leading wasps of both species depending on aphid defenses before the sting. Only leading wasps were used for this analysis to avoid confounding the effects of other marks with the effects of aphid defenses.

Determining within-encounter changes in defensive behavior required calculations based on encoding aphid behaviors. For each aphid encounter by leading wasps, we calculated and averaged the change in defense category from the behaviors before the sting to the behaviors after the sting. To do this, each aphid behavior was encoded with a "1" for exhibiting defensive behaviors and a "0" for not exhibiting defensive behaviors. The numerical code for behaviors before the sting was then subtracted from the numerical code for behaviors after the sting, and these differences were averaged across all encounters that included a sting by each wasp. This calculation method yielded a score for each wasp that ranged from -1 to 1. Scores closer to -1 suggest that defensive behaviors of aphids encountered were generally stronger before the aphids were stung than after. Scores closer to 1 indicate that defensive behaviors of aphids were stronger after they were stung than before. Scores near 0 indicate little to no change in defensive behaviors of the aphids from immediately before a sting to immediately after a sting. These changes were averaged across all sting-yielding encounters for each leading wasp and compared by ANOVA. Because aphid defensive behaviors after a second wasp could be confounded by the leading wasp, this analysis only focused on leading wasps.

The first analysis determined whether or not wasps tend to induce defensive behavior, but not whether the defenses persist long enough to affect a subsequent wasp. To determine whether following wasps encountered more defensive aphids, the proportion of encountered aphids that were defensive before being stung was compared across the three treatments for each wasp by treatment, and

compared by ANOVA. This calculation did not consider the defensive behaviors of aphids during or after stings.

Lastly, wasp response to aphid defensive behavior was recorded and compared for leading wasps to determine whether aphid defenses were actually effective at deterring wasps. For both the leading *L. testaceipes* and *B. communis*, t-tests were used to compare the proportion of encounters that yielded stings when aphids were non-defensive to when they were defensive.

Exposure of Stung or Bystander Aphids to *L. testaceipes*

While our first experiment showed whether wasps altered their foraging in response to marks left by one another and explored whether induced defensive behaviors may be a major part of the mark, it did not completely delineate the roles of species-specific and host-derived marks. To determine whether *L. testaceipes* responded to stung aphids similarly to how it responded to aphids adjacent to those that were stung, a second sequential stinging experiment was set up. We hypothesized that host-derived cues could propagate beyond those aphids that were stung via alarm pheromones spreading through the arena and priming aphids to behave defensively. Conversely, contact pheromones deposited by *L. testaceipes*, as has been proposed by Medryzycki et al. (2002) would not be detected on aphids unless they were actually stung by an *L. testaceipes*.

In this second experiment, we presented a *L. testaceipes* with aphids that either were already stung by another *L. testaceipes* or with aphids that were not stung, but were present alongside aphids that were stung in a patch on which another *L. testaceipes* had already foraged. Adjacent aphids should be affected by non-contact cues such as alarm pheromones, but only those aphids that were stung would be affected by a contact pheromone or other contact mechanisms. Thus, the foraging behavior of an *L. testaceipes* that is presented with aphids that were stung or adjacent to stung would help reveal the type of cues that are used by *L. testaceipes.*

We set up two treatments for this experiment; *L. testaceipes* foraged for either aphids that had already been stung, or for "bystander" aphids that were in colonies that were exposed to wasps, but that were not actually stung themselves. To accomplish this, a single *L. testaceipes* was allowed to forage in a colony of twenty 3rd-4th instar aphids until half of them were stung. As the lead wasp foraged, we recorded which aphids it stung. When half of the aphids were stung by the first wasp, the wasp and either all of the

aphids that were stung were removed (the "bystander" treatment) or all of the aphids that were not stung were removed (the stung treatment). This left just the unstung or just the stung aphids in the arena when the second wasp was added.

This experiment was run in a similar, albeit smaller arena as the first experiment. This arena fit entirely within the field of view of the microscope (approximately 25mm X 25 mm). Aphids and wasps were small enough that this small space allowed room for twenty aphids to be placed at a density that still required wasps to search for aphids. Wasps were observed foraging in similar sized areas in the rearing colonies for 5-10 min before moving on (Kopco, personal observation). The 50 mm Petri dish that made up the arena had a section cut out of the bottom that closely matched the perimeter of the field of view of the microscope and was fitted with a grid of monofilament fishing line (Cabela's Pro Line, 0.008 inch diameter: Cabela's Inc., Sydney, NE) that provided landmarks for recognition of individual aphids. The cells of the grid were each approximately 5 mm x 5 mm.

Twenty 3rd-4th instar soybean aphids were placed on the center leaf of the first trifoliate of a V1 soybean plant, which was clamped in place under the arena, and aphids were then allowed to settle for 30 min. To minimize aggregating of aphids, they were placed not more than one per cell of the fishing line grid, though movement during the settling period sometimes resulted in small aggregations of 2-4 aphids in close proximity to one another. During the aphid settling period, some aphids left the leaf and climbed onto the underside of the lid of the arena. Any arena/aphid set-ups that lost more than 5 aphids were discarded, so all initial wasps foraged in colonies of 15-20 aphids. After aphids had settled, the lead *L. testaceipes* was introduced to the arena and allowed to sting half of the aphids, after which time the wasp and treatment-specified aphids were removed.

Immediately after removal of either the stung or bystander aphids, the second female *L. testaceipes* was introduced to the arena and observed as it foraged for 7.5 min, or until it left the Petri dish or stung all available aphids. The duration of the foraging bout, number of aphids stung by the lead and second wasps, and proportion of total aphids stung by the second wasp were recorded.

We compared foraging behavior of the second wasps as they foraged for stung or bystander aphids. The sting rate (number of stings divided by duration of foraging bout) was compared between the second wasps in each treatment using ANOVA. We also compared the proportion of aphids that were

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stung by the second wasp between the two treatments using ANOVA. Because related parasitoids exhibit

a functional response that produces fewer ovipositions per min at lower prey densities (Van Steenis and

El-Khawass 1995), the stings per min of the second wasps were not compared to that of the leading

wasps, which had twice as high prey density to forage among.

<div align="center">**Results**</div>

Sequential Exposure of Whole Aphid Groups to Parasitoids

Parasitoid Response to Parasitized or Non-parasitized Aphids

The order of whether a wasp led or followed another wasp influenced wasp foraging behavior, but

this varied among species and specific response variables. The number of stings per minute of *L.*

testaceipes was significantly affected by the order in which the wasp stung (ANOVA: $F_{2,33}$=4.45, P=0.019;

Fig 1A), but *B. communis* was not significantly affected (ANOVA: $F_{2,35}$=0.66, P=0.52; Fig 1B). *L.*

testaceipes administered significantly fewer stings per min when following another *L. testaceipes* than

when leading (Tukey's HSD: P=0.038) or when following a *B. communis* (Tukey's HSD: P=0.018).

The number of stings delivered per min is a function of the number of aphids encountered during

the foraging bout, the proportion of those aphids that were stung, and the duration of the foraging bout.

The proportion of aphids that were stung showed the same pattern as stings per minute according to

wasp species and order; for *L. testaceipes* (ANOVA: $F_{2,33}$=10.08, P=0.00038, Fig. 1C) the proportion of

encounters that yielded stings was significantly lower when following another *L. testaceipes* than when

leading (Tukey's HSD: P=0.00013) or following a *B. communis* (Tukey's HSD: P=0.0095). The order in

which *B. communis* foraged did not have a significant effect on the proportion of encounters that yielded

stings (ANOVA: $F_{2,35}$=0.81, P=0.45; Fig. 1D).

Unlike stings per min, the order in which wasps foraged had no effect on encounters per minute

for either *L. testaceipes* (ANOVA: $F_{2,33}$=0.72, P=0.49; Fig. 1E) or *B. communis* (ANOVA: $F_{2,35}$=0.17,

P=0.83; Fig. 1F). Wasps could also abandon the foraging arena early in response to marks from other

parasitoids. However, the duration of the foraging bouts of the wasps did not differ significantly between

different orders for *L. testaceipes* (ANOVA: $F_{2,33}$=0.84, P=0.44; Fig. 1G) or *B. communis* (ANOVA:

$F_{2,35}$=2.0061, P=0.1497; Fig. 1H).

<div align="center">18</div>
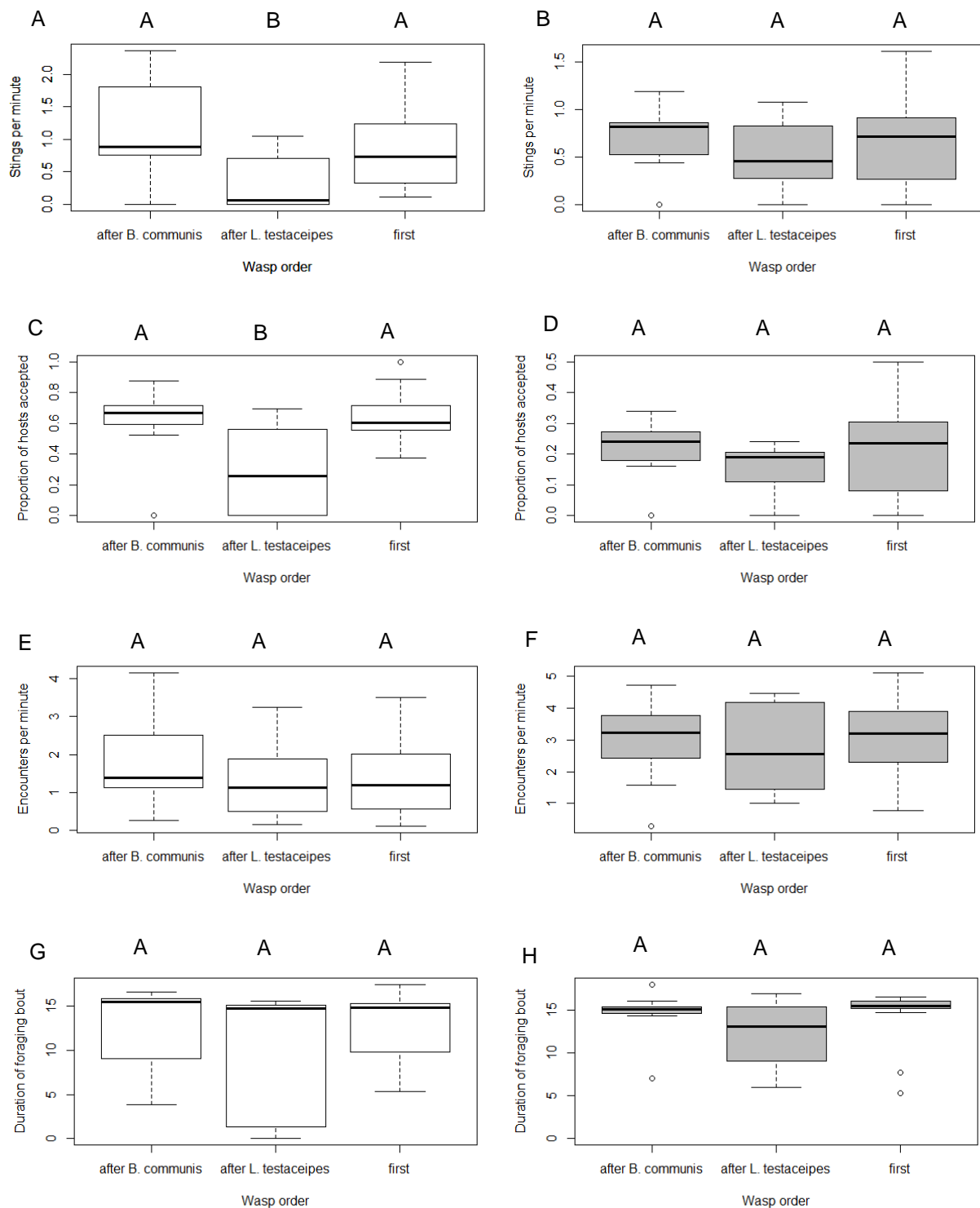
Figure 1. Comparisons between characteristics of foraging behaviors (± SEM) for *L. testaceipes* (A, C, E, and G, open bars) and *B. communis* (B, D, F, H, shaded bars). Characteristics of foraging behaviors include the number of stings per min (A, B), the proportion of wasp encounters that terminated in stings (C, D), the number of encounters per min (E, F), and the duration of the foraging bout (G, H). Significant differences are indicated by letters above the plots.

Aphid Defensive Behaviors

L. testaceipes induced short-term (few second), but not long term (~15 minute) increases in host defensive behavior. *L. testaceipes* elicited defensive behaviors within encounters by stinging aphids (t test: t_{17}=4.53, P=0.00029) (Fig. 2A), in that a larger proportion of aphids were defensive immediately after being stung by *L. testaceipes* than immediately before being stung. However, this did not translate to a significant increase of defensive behaviors encountered by wasps that followed *L. testaceipes* (ANOVA: $F_{5,67}$=1.04, P=0.43) (Fig. 2B). *L. testaceipes* was less likely to sting defensive aphids than non-defensive aphids (t test: t_{10}=7.40, P=0.000023) (Fig. 3). *L. testaceipes* was less than half as likely to sting defensive aphids than non-defensive aphids.

Unlike *L. testaceipes*, *B. communis* neither induced nor suppressed aphid defensive behaviors within encounters (t test: t_{16}=0.82, P=0.43) (Fig. 2A); there was no significant difference in the proportion of defensive aphids at the beginning of an encounter and the proportion of defensive aphids at the end of an encounter. Similarly, *B. communis* did not induce changes in defensive behavior between foraging bouts (ANOVA: $F_{5,67}$=1.04, P=0.43) (Fig. 2B); the proportion of aphids that were defensive at the beginning of encounters did not differ whether *B. communis* led, followed a conspecific, or followed *L. testaceipes*. These results indicate that the aphids do not have any short-term (<10 seconds) or long-term (~15 minute) behavioral responses to *B. communis* beyond the temporary paralysis observed in stung individuals. *B. communis* also stung defensive and idle aphids equally (t test: t_{12}=0.62, P=0.55) (Fig. 3). The proportion of aphids that *B. communis* stung did not differ whether aphids defended themselves at the beginning of the encounter or not.

Exposure of Stung or Bystander Aphids to L. testaceipes

L. testaceipes foraged differently for aphids that were already stung by another *L. testaceipes* than they did for aphids that had never been stung but were adjacent to aphids that were stung. *L. testaceipes* foraging for aphids that had been stung by a conspecific delivered fewer stings per min than those foraging for aphids that had not been stung (ANOVA: $F_{1,17}$=5.34, P=0.03) (Fig. 4A). This pattern matched that observed in the first experiment for lead *L. testaceipes* compared to *L. testaceipes* following a conspecific. The proportion of available aphids that were stung by the second foraging *L. testaceipes* was also significantly lower when wasps foraged for aphids that had already been stung (ANOVA:

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$F_{1,17}$=7.24, P=0.015) (Fig. 4B). However, there was no significant difference in the duration of the foraging

bout between wasps that stung already-stung aphids and wasps that stung naïve aphids (ANOVA:

$F_{1,17}$=0.50, P=0.48) (Fig. 4C).
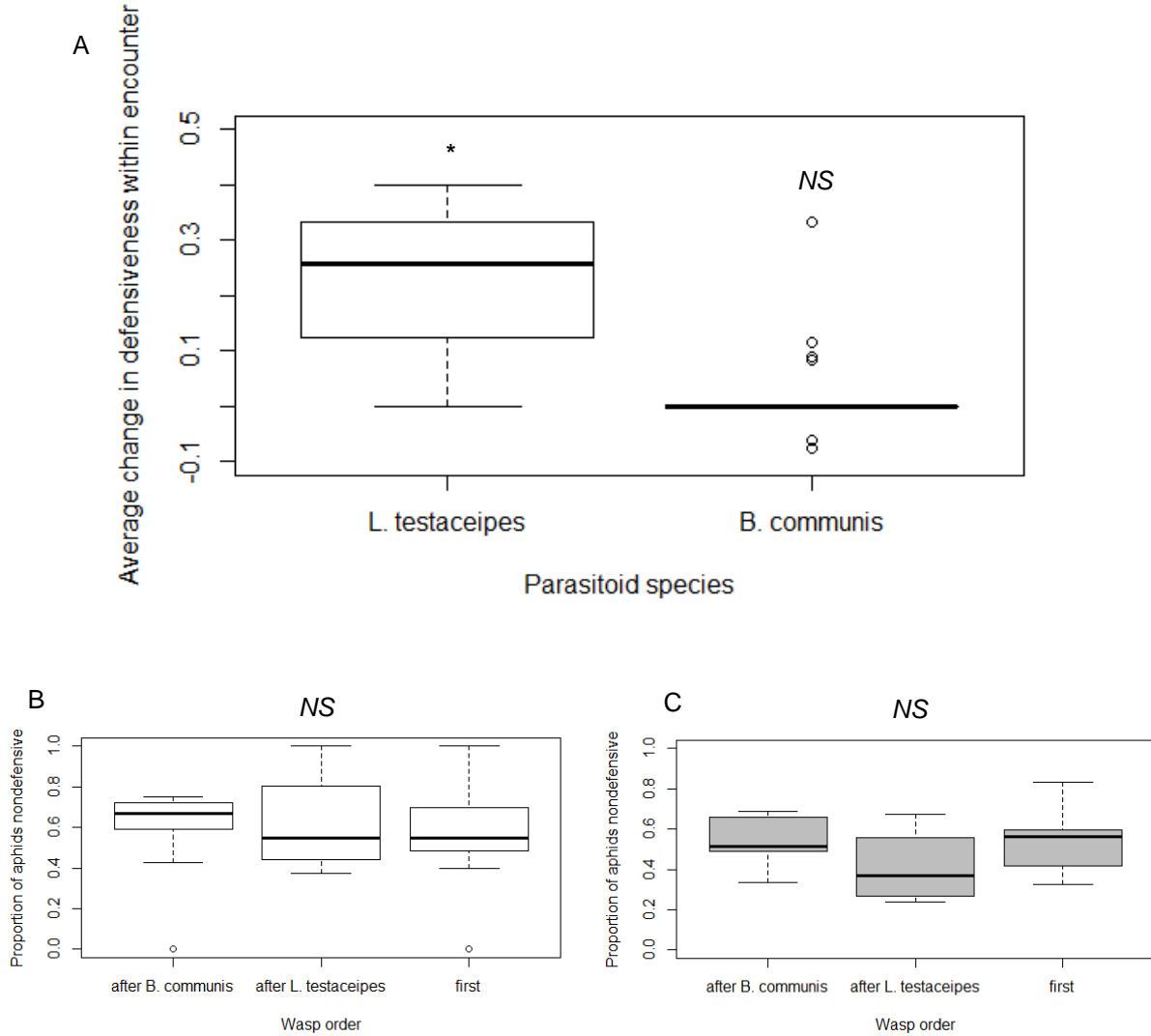
Figure 2. Aphid defensive response to wasps. **A.** Change in aphid defensiveness within an encounter (±
SEM). *L. testaceipes*, but not *B. communis*, had a significant change in defensiveness of host aphids over
the course of an encounter. To calculate the change in defensiveness, each aphid that was stung was
scored for defensive behavior before and after the sting, with aphids that kicked, waggled, emitted
cornicle secretions, or fled scoring "1" and aphids that did none of those things scoring "0." For each
aphid, the behavior score before the sting was subtracted from the behavior score after the sting, and all
stung aphids were averaged for each wasp. **B and C.** The proportion of encountered aphids that were
non-defensive before being stung (± SEM). **B** represents *L. testaceipes,* while **C** represent *B. communis*.
The proportion of aphids that the wasp encountered that were not defensive at the beginning of the
encounter did not differ depending on order. (*NS* non-significant difference, * P≤0.05).

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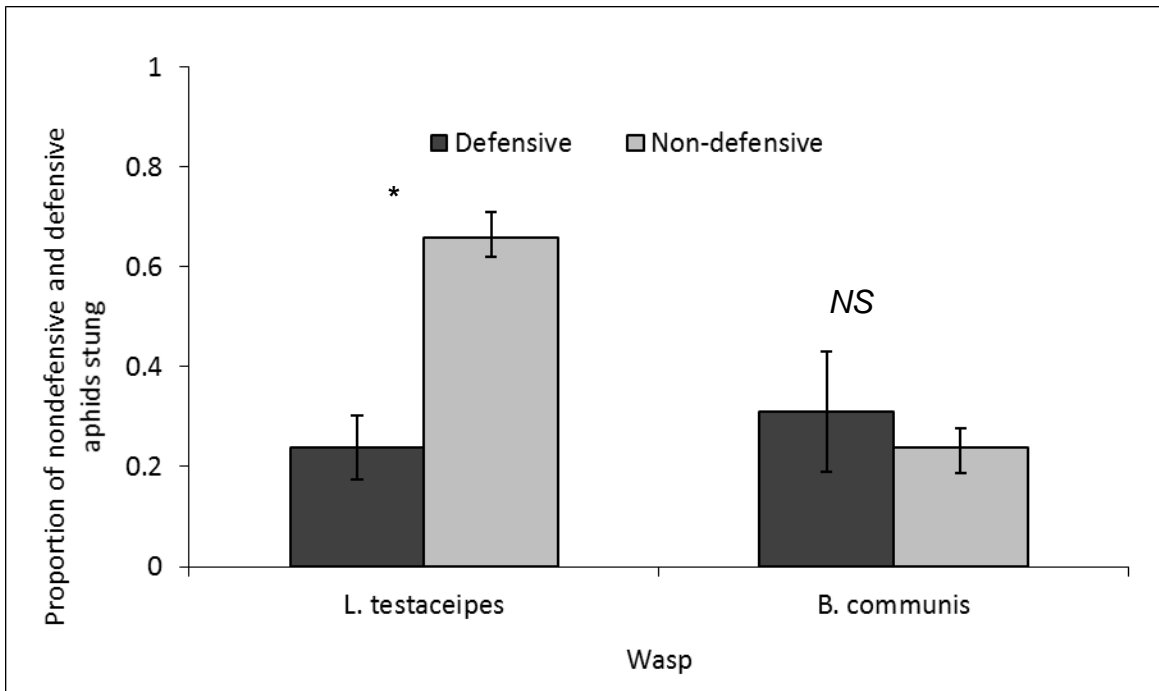
Figure 3. The proportion of defensive or non-defensive aphids that were stung. *L. testaceipes* stung a significantly lower proportion of aphids that were defensive than those that were idle. (*NS* non-significant difference, * P≤0.05).*B. communis*, on the other hand, stung comparable proportions of defensive and non-defensive aphids that it encountered. Error bars indicate the SEM.

Discussion

We devised experiments to determine whether two parasitoids alter their foraging after hosts were parasitized by conspecifics or heterospecifics, and if they do, if that change correlates with potential marks that may have originated directly from the prior parasitoid or indirectly from the host. To do that, we measured the foraging behaviors of two species of parasitoids when they foraged for aphids that had never encounter parasitoids, aphids that had just been attacked by a conspecific parasitoid, or aphids that had just been attacked by a heterospecific parasitoid. *B. communis* showed no signs of responding to any cue of parasitism. It exhibited the same foraging behavior whether its hosts were non-parasitized or had just been attacked by another *B. communis* or a *L. testaceipes. L. testaceipes*, on the other hand, delivered significantly fewer stings when following another *L. testaceipes* than when leading or following *B. communis.* This suggests that the prior *L. testaceipes* directly or indirectly provided some cue to the subsequent *L. testaceipes* that the aphids were already parasitized.

Our first experiment also allowed us to test whether induced defensive behaviors by the aphids were potentially a cue used by *L. testaceipes* to avoid aphids that were already parasitized. To function
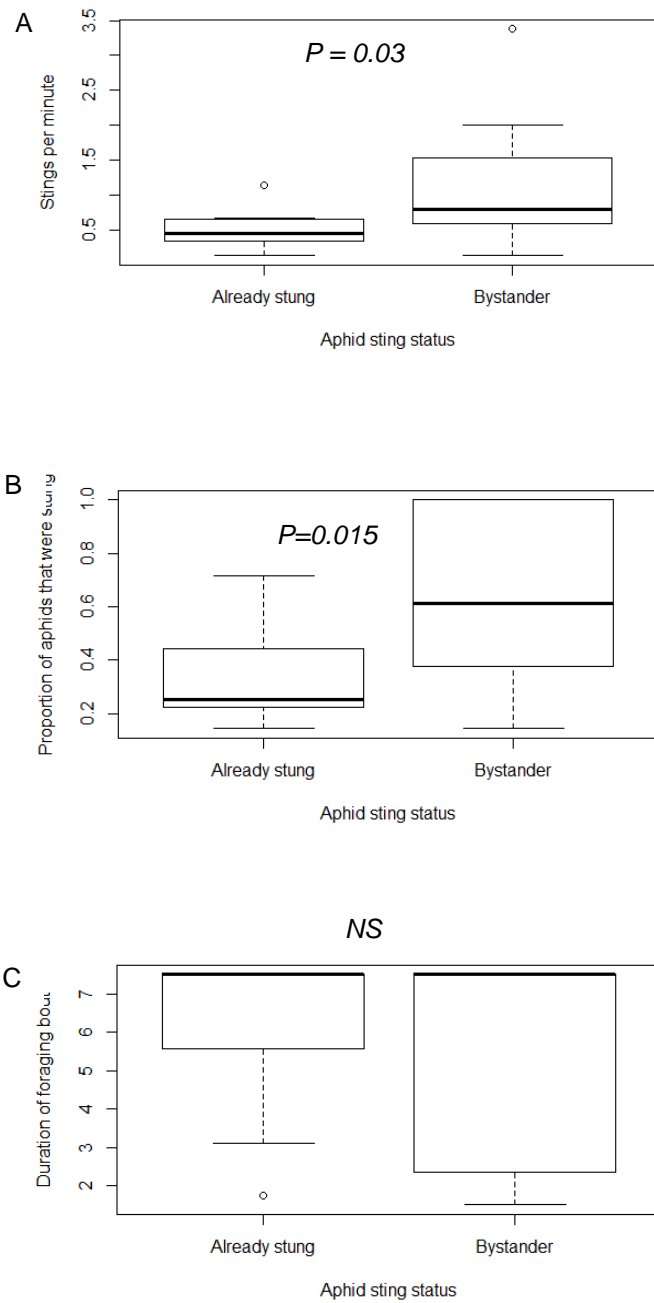
Figure 4. Comparisons between characteristics of foraging behaviors for *L. testaceipes* foraging for aphids that were already stung by a conspecific or for aphids that were never stung. Aspects of the foraging behavior included **A.** stings per min, **B.** the proportion of available aphids that were stung, and **C.** the duration of the foraging bout. (*NS* non-significant difference, * P≤0.05).

as a mark of parasitism, defensive behaviors would need to deter *L. testaceipes* from ovipositing and would need to be more likely to occur in parasitized aphids than in non-parasitized aphids. *L. testaceipes* was significantly less likely to oviposit in aphids that kicked, waggled, emitted defensive secretions, or fled compared to aphids that were idle, suggesting that the defensive behaviors could be an effective deterrent. Additionally, aphids increasingly exhibited these behaviors immediately after being encountered by an *L. testaceipes*. However, because our recording focused on following the parasitoid, when the parasitoid walked quickly after ovipositing her recently stung host was lost from the field of view within three or four seconds of the end of the encounter. Therefore, we were unable to record how long the aphids maintained their defensive behaviors after interacting with a parasitoid.

These defensive behaviors, while clearly a potential deterrent, could only provide information about the aphid's parasitism status to new foraging *L. testaceipes* if the behaviors change after the aphid is parasitized. However, the order of the foraging *L. testaceipes* (e.g. first parasitoid vs. following another *L. testaceipes*) did not affect the frequency of defensive behaviors at the beginning of each encounter. There are two possible explanations. First, the aphids may simply not continue their defensive behaviors for more than a few seconds or minutes, such that the aphids re-settle between the wasps' foraging bouts or during the beginning of the second wasp's foraging bout. Alternatively, *L. testaceipes* may be able to detect an aphid's defensive behavior from a further distance, which would allow them to avoid encounters with defensive aphids. Our data do not support this latter explanation, however, because the number of encounters for the second *L. testaceipes* was not reduced. Additionally, Medrzycki et al. (2002) found no evidence that *L. testaceipes* can discriminate between distant hosts, which suggests that the second *L. testaceipes* must closely inspect an aphid to assess its suitability. Because our results showed no difference in the number of aphids encountered by *L. testaceipes* depending on the order in which it foraged, it is consistent with the findings of Medrzycki et al. (2002) and supports their hypothesis that *L. testaceipes* relies on contact cues to evaluate hosts. Defensive behaviors may be a functional cue for *L. testaceipes* to avoid parasitizing hosts it has already parasitized, but our data do not suggest that they play a major role in avoiding hosts parasitized by other parasitoids.

We observed several instances of aphids emitting honeydew as they were antennated by the parasitoid. A total of two *L. testaceipes* and twelve *B. communis* induced a total of forty-nine aphids to

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emit honeydew as they were antennated. Ant mimicry, in the form of honeydew solicitation, has been reported for *L. fabarum* (Rasekh et al. 2010), and honeydew emission in response to parasitoids may have resulted from aphids mistaking wasps for ants. Honeydew solicitation could have arisen for two different reasons. Both *L. testaceipes* and *B. communis* have been reported feeding on aphid honeydew (Hopkinson et al. 2013, Wyckhuys et al 2008b) and one observation of *L. testaceipes* and three of the observations of *B. communis* antennations eliciting honeydew emission were followed by the parasitoids feeding on the honeydew fresh droplet. Alternatively, Rasekh et al. (2010) have proposed that honeydew solicitation may serve a role in reducing aphid defensive behaviors, though we did not explore this mechanism further. However, we also often observed emission of honeydew when *L. testaceipes* prodded aphids in three separate parasitoid-aphid encounters, and when *B. communis* grasped aphids between the abdominal prongs and ovipositor on twenty-two occasions. Therefore, honeydew emission may itself have been an alarm response of the aphids or a side-effect of physical pressure applied to the body of the aphid. Honeydew emission was a sufficiently infrequent response of aphids to parasitoids that it was unlikely to have a major effect on the overall results we observed for either wasp species.

In our second experiment, we tested whether the foraging behavior of *L. testaceipes* differs depending on whether the aphids were stung or were merely adjacent to aphids that were stung. Indirectly, this experiment should show if *L. testaceipes* is exclusively using contact pheromones deposited by conspecifics to assess individual hosts, or if species-specific or host-derived marks might apply on a larger patch scale. If *L. testaceipes* relied on just contact pheromones from the prior wasp, then those cues would be removed with any aphids that were stung. Therefore, for *L. testaceipes* that foraged for non-stung aphids, the second wasp would be expected to oviposit readily. Conversely, if *L. testaceipes* relied on cues that affect the patch, such as aphid alarm pheromone or primed aphid defensive behaviors, it should limit its oviposition whether stung or non-stung aphids remain. Because we did not conduct an unexposed-aphid control, we cannot completely rule out patch-wide cues. However, our methodology does allow us to compare whether patch-wide cues plus individual cues lead to a greater parasitoid response than patch-wide cues alone. Because *L. testaceipes* reduced its oviposition when foraging for stung aphids compared to non-stung aphids, patch-wide cues are unlikely to be a primary cue used by *L. testaceipes* in foraging. This result is consistent with the results of our first

experiment in that if *L. testaceipes* avoided ovipositing in aphids that are emitting alarm pheromone, it should have also reduced its stinging following *B. communis*, which also induces alarm pheromone.

Our experiments rely on oviposition behavior as a sign of response to a mark. Wasps are expected to mark hosts to avoid competition between their own offspring (Danyk and Mackauer 1993, Desneux et al. 2009) and the offspring of other parasitoids (Chow and Mackauer 1999, Danyk and Mackauer 1993). Depositing multiple eggs into the same host may waste eggs, as well as the time spent laying them, and expose wasps to the hazards of interacting with potentially defensive hosts (Bai and Mackauer 1990, Gardner et al. 1984, Wyckhuys et al. 2008a). Because depleting their egg supply can greatly reduce their overall fitness, parasitoids tend to behave in ways that maximize the fitness benefit they receive from every egg they lay (Heimpel and Rosenheim 1998, Rosenheim et al. 2000). However, depending on the context, parasitoids may detect marks on a host but still choose to oviposit. Depositing multiple eggs into a host may be a strategy to avoid subsequent parasitoids from taking over the host (Danyk and Mackauer 1993). The presence or absence of certain bacterial symbionts in the aphid can also alter a wasp's decision to reject the host, deposit a single egg, or deposit multiple eggs (Cheng et al. 2011, Oliver et al. 2012), though soybean aphids are not known to harbor facultative symbionts that affect wasp behaviors (Wulff et al. 2013). Additionally, offspring that win competition with other parasitoids and develop in multiply-parasitized hosts may be larger than their counterparts who develop alone in a host (Bai and Mackauer 1992). Therefore, parasitoids can actually benefit from depositing multiple eggs in a host, especially if hosts are limiting and the parasitoid has a large egg-load. This explains empirical observations of parasitoids depositing multiple eggs during single oviposition events on hosts or ignoring their own marks, despite the impossibility of more than one egg developing to maturity (Rosenheim and Hongkham 1996).

Parasitoid decisions to oviposit based on marks from conspecifics or heterospecifics can also depend heavily on timing. Eggs that are laid in hosts parasitized by other individuals often have some chance of defeating the resident parasitoid and developing successfully (Chow and Mackauer 1985, Chow and Mackauer 1986). The relative ages of the parasitoids are very important in predicting the victor of a fight between parasitoid larvae. Substantially older larvae usually defeat young larvae by a process called physiological suppression, in which resources in the host are too limited and waste products of the

first parasitoid too concentrated for a neonate larva to mature successfully (Fisher 1961, Fisher 1963, Fisher 1965, Marris and Casperd 1996). Because the parasitoids in this experiment were ovipositing within 30 minutes of one another (from the very beginning of the first parasitoid's foraging bout to the very end of the second parasitoid's foraging bout), age differences between eggs and larvae would be negligible. Therefore, none of the wasps had a substantial larval competitive advantage over the other due to a head start in larval development.

Additionally, the competitive abilities of parasitoid larvae of different species are not always equal. For instance, *Aphidius smithi* larvae are almost always killed by older or younger larvae of *Aphidius ervi* (McBrien and Mackauer 1990). *A. ervi*, in turn, is nearly always defeated by similar aged *Praon pequodorum* (Schellhorn et al. 2002). These patterns of larval competitive ability correspond with patterns of mark responses in the parasitoids. Continuing our previous example, *A. ervi* shows a preference for ovipositing in unparasitized aphids or aphids that were parasitized by *A. smithi*, an inferior competitor, than in aphids parasitized by itself or another *A. ervi* (McBrien and Mackauer 1991). As such, a parasitoid may fail to detect or detect and ignore marks of another parasitoid if its larva still has an appreciable chance of surviving in the host (McBrien and Mackauer 1991, Janssen et al. 1995a, Janssen et al. 1995b, Muller and Godfray 1999).

We are unaware of any clear advantage that either species of parasitoid used in these experiments would have over the other. With oviposition events occurring within 15 min of one another, the larvae of the first parasitoid do not have a considerable size or age advantage over the larvae of the second parasitoid. We also have not found any evidence that the larvae of either species are superior combatants to the other, either reported in the literature or from observing their interactions during this experiment. The only reference to larval competitive ability we could find for either of our study species was by Volkl and Stadler (1991), who compared larval competitive ability between *L. testaceipes* and *Aphidius colemani*. We also assume that the venom of *B. communis* does not affect the offspring of other parasitoids that were deposited at the same time; the effect of the venom lasts only a few minutes in the aphid host (Desneux et al. 2009), and is likely to be degraded by the time parasitoid eggs hatch. Even if one parasitoid has an advantage over the other, because they lack any evolutionary history with one

another, neither species would be expected to have evolved a behavioral pattern of ignoring the other's mark.

The evolutionary history of the competing species can strongly influence whether or not different species can recognize one another's marks. Species that are closely related may have little or no differentiation in their marks, such that the mark of a conspecific or a heterospecific is essentially indistinguishable to a foraging wasp (Van Baaren et al. 1994). While *B. communis* and *L. testaceipes* are both members of the same subfamily of insects, the Aphidiinae, they occupy different tribes within that subfamily (Smith 1944). *B. communis* is a member of the tribe Trioxini, which has been reported to consistently display different foraging behaviors, have distinct ovipositor morphology, and rely more heavily on venom than all other tribes within the Aphidiinae (Volkl and Mackauer 2000). Conversely, *L. testaceipes* belongs to the tribe Aphidiini (Smith 1944). The foraging behaviors, and presumably marking mechanism of externally applied contact pheromones, are widely conserved within the Aphidiini (Volkl and Mackauer 2000). Additionally, heterospecific recognition of marks is likely to develop in species with extensive evolutionary histories of competition with one another. This has been observed to have occurred between *Aphelinus asychis* and *Aphidius ervi*, which occupy different superfamilies, Chalcidoidea and Ichneumonoidea, respectively, and which mark differently, as a result of an extensive history of competition between these species for the same hosts (Bai and Mackauer 1991). Because of the extreme geographic distances between the endemic ranges of *L. testaceipes* and *B. communis*, these two species cannot share an evolutionary history with one another.

Our results for *L. testaceipes* are consistent with the assertion by Medrzycki et al. (2002) that the primary marking mechanism used by *L. testaceipes* is a directly deposited contact pheromone. *L. testaceipes* does not respond to parasitoid-derived marks of a drastically different nature than its own marks. Additionally, host derived cues such as alarm pheromones or induced defensive behaviors are unlikely to be the most important cues used by *L. testaceipes* in assessing prospective hosts. These cues may play a role in *L. testaceipes*'s avoidance of hosts parasitized by itself, but we found no evidence that they aid in avoidance of hosts parasitized by other parasitoids.

The paralysis induced by *B. communis* was not an effective deterrent to oviposition by *L. testaceipes*. Because *L. testaceipes* does not paralyze its hosts, it is likely that it lacks the behavioral

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repertoire to recognize and/or avoid paralyzed hosts. Furthermore, paralysis suppresses defensive behaviors, which we have demonstrated to be correlated with reduced likelihood of stinging for *L. testaceipes*. As such, aphids paralyzed by *B. communis* may have been more vulnerable to parasitism by *L. testaceipes* than were non-parasitized aphids. However, the paralysis caused by *B. communis* is short-lived, lasting only a few minutes (Desneux et al. 2009). As such, paralysis may serve as a cue for an individual *B. communis* to avoid depositing multiple of her own eggs into a host, but be too short-lived to be a meaningful cue to other parasitoids, conspecific or heterospecific, unless they encounter the same aphid at very nearly the exact same time.

Unexpectedly, *B. communis* showed no signs of altering its foraging behavior for already-parasitized aphids. Because we routinely observed ovipositing *B. communis* paralyzing their hosts and *B. communis* has been reported to avoid ovipositing in paralyzed aphids (Desneux et al. 2009), we expected to find reduced stinging of aphids when *B. communis* followed a conspecific. However, the marking role of transient host paralysis was proposed in a context of avoiding depositing multiple eggs in a single host rather than reducing competition with conspecifics. As such, it is possible that the paralysis was somewhat effective, but that much of the paralysis induced by the lead wasp had worn off by the time the second wasp was introduced. If we had conducted these experiments over a reduced time interval (e.g. five minute foraging bouts for each wasp instead of fifteen minutes) we may have seen a stronger reduction in oviposition by *B. communis* following a second *B. communis*. However, even with shorter foraging intervals, differences between the first and second *B. communis* could be obscured if self-parasitized aphids having a greater effect on foraging behavior than conspecific-parasitized aphids.

Parasitoids have also been described using vision in combination with scent to assess hosts (Michaud and Mackauer 1994, Losey et al. 1997), and aphid behaviors or paralysis status may be visually detectable to a hunting *B. communis* from a distance greater than an antenna's length. As such, *B. communis* may avoid even encountering paralyzed hosts. Because this study focused on the response of wasps to aphids after they approach near enough to make antennal contact, avoidance of hosts at a greater distance could confound our results. However, work focusing on parasitoid vision tended to focus more on aphid color than movement, and while some parasitoids show preferences for aphids of certain color morphs, they have not been shown to use vision to discriminate between parasitized and non-

parasitized hosts (Medrzycki et al. 2002). Furthermore, if *B. communis* were visually avoiding paralyzed hosts, we would expect to see a reduction in aphids encountered for *B. communis* following a conspecific compared to lead *B. communis*. However, this pattern was not observed, suggesting that *B. communis* does not effectively distinguish between hosts at a distance.

Our experiments focused on responses of parasitoids to potential marks left by other parasitoids, but they did not clearly delineate responses of parasitoids to their own marks. Especially in the first experiment, where stung aphids were not tracked, parasitoids surely encountered and, in some cases, stung the same aphids multiple times during a single foraging bout. As such, both species of parasitoids may have changed their foraging behavior over the course of a single foraging bout as they depleted the supply of hosts (Montovan et al. 2015). This may explain the lack of apparent results for *B. communis*; if paralysis serves primarily as a cue to avoid repeatedly stinging the same aphid, then each *B. communis* that was tested may have created its own oviposition-deterrent cue over the course of its foraging bout. Because all of the wasps had the potential to encounter aphids that they had personally already stung, our results may be conservative, as foraging restraint in response to their own marks would uniformly decrease host acceptance across all treatments and partially mask avoidance of aphids stung by prior wasps.

Conversely, in the first experiments, we did not ensure that all of the aphids were stung by the first wasp. It is likely that in most of the replicates, most or all of the aphids were stung by the initial wasp. However, there were likely to be at least a few aphids that the first wasp never stung. As illustrated by the second experiment, these non-stung aphids would not elicit as strong of an oviposition-deterrent effect on the second parasitoid as the stung aphids. The presence of non-stung aphids in the in the arena for the second wasps therefore makes our results more conservative. If we had ensured that the first parasitoid stung all of the aphids, we would expect a stronger response from the second wasp than we actually observed. However, large enough proportions of aphids were stung in each foraging bout, and *B. communis* showed sufficiently consistent results across all treatments, that it is unlikely that responses to marks for *B. communis* were hidden by non-stung aphids.

Despite the extensive body of literature exploring the ability of various parasitoids to respond to one another's marks or distinguish their own marks from those of other parasitoids, little effort has been

made to explain the large patterns that have emerged. We hypothesized that mark recognition is likely to occur if the marks used by different species are extremely similar (Van Baaren et al. 1994) or if those species have an extensive evolutionary history with one another (Bai and Mackauer 1991), but not if both of those conditions are not met. We further elaborated this hypothesis to include both marks left directly by a parasitoid, which are likely to be more species-specific, and cues offered by the host itself, which will tend to be more general. Neither of the parasitoids we tested relied on host-based cues (e.g. defensive behaviors or alarm pheromones) to discriminate between hosts parasitized by other parasitoids and non-parasitized hosts. Additionally, neither species responded to the other's host marking cues. Here we present evidence that early after oviposition, parasitoids rely more strongly on cues left by other parasitoids than on cues derived from the host. These cues are quite different between the parasitoids we studied, and the species lack a history of competition, so neither species had the opportunity to adapt and develop the ability to respond appropriately to the other's marks. Because the criteria of similar marks or evolutionary history are not met, cross-species recognition of marks could not be expected to occur.

Acknowledgments

References

Almohamad, R., Verheggen, F. J., Francis, F., Lognay, G., and Haubruge, E. 2008. Emission of alarm pheromone by non-preyed aphid colonies. Journal of Applied Entomology 132: 601-604.

Bai, B. and Mackauer, M. 1990. Host discrimination by the aphid parasitoid *Aphelinus asychis* (Hymenoptera, Aphelinidae) - when superparasitism is not adaptive. Canadian Entomologist 122: 363-372.

Bai, B. and Mackauer, M. 1991. Recognition of heterspecific parasitism – competition between aphidiid (*Aphidius ervi*) and aphelinid (*Aphelinus asychis*) parasitoids of aphids (Hymenoptera, Aphidiidae, Aphelinidae). Journal of Insect Behavior 4: 333-345.

Bai, B. and Mackauer, M. 1992. Influence of superparasitism on development rate and adult size in a

solitary parasitoid wasp, *Aphidius ervi*. Functional Ecology 6: 302-307.

Ballman, E. S., Ghising, K., Prischmann-Voldseth, D. A. and Harmon, J. P. 2012. Factors contributing to

the poor performance of a soybean aphid parasitoid *Binodoxys communis* (Hymenoptera:

Braconidae) on an herbivore resistant soybean cultivar. Environmental Entomology 41: 1417-

1425.

Battaglia, D., Pennacchio, F., Marincola, G. and Tranfaglia, A. 1993. Cornicle secretion of *Acyrthosiphon*

pisum (Homoptera, Aphididae) as a contact kairomone for the parasitoid *Aphidius ervi*

(Hymenoptera, Braconidae). European Journal of Entomology 90: 423-428.

Beale, M. H., Birkett, M. A., Bruce, T. J. A., Chamberlain, K., Field, L. M., Huttly, A. K., et al. 2006. Aphid

alarm pheromone produced by transgenic plants affects aphid and parasitoid behavior.

Proceedings of the National Academy of Sciences of the United States of America 103: 10509-

10513.

Brodeur, J. and Rosenheim, J. A. 2000. Intraguild interactions in aphid parasitoids. Entomologia

Experimentalis Et Applicata 97: 93-108.

Butler, C. D. and O'Neil, R. J. 2006. Defensive response of soybean aphid (Hemiptera : Aphididae) to

predation by insidious flower bug (Hemiptera : Anthocoridae). Annals of the Entomoogical Society

of America 99: 317-320.

Cheng, R. X., Meng, L., Mills, N. J. and Li, B. P. 2011. Host preference between symbiotic and

aposymbiotic *Aphis fabae*, by the aphid parasitoid, *Lysiphlebus ambiguus*. Journal of Insect

Science 11.

Chow, F. J. and Mackauer, M. 1985. Multiple parasitism of the pea aphid – stage of development of

parasite determines survival of *Aphidius smithi* and *Praon pequodorum* (Hymenoptera,

Aphidiidae).Canadian Entomologist 117: 133-134.

Chow, F. J. and Mackauer, M. 1986. Host discrimination and larval competition in the aphid parasite

Ephedrus californicus. Entomologia Experimentalis Et Applicata 41: 243-254.

Chow, A. and Mackauer, M. 1999. Marking the package or its contents: Host discrimination and

    acceptance in the ectoparasitoid *Dendrocerus carpenteri* (Hymenoptera : Megaspilidae).

    Canadian Entomologist 131: 495-505.

Danyk, T. P. and Mackauer, M. 1993. Discrimination between self-conspecific and conspecific-parasitized

    hosts in the aphid parasitoid *Praon pequodorum* Viereck (Hymenoptera, Aphidiidae). Canadian

    Entomologist 125: 957-964.

Desneux, N., Barta, R. J., Delebecque, C. J. and Heimpel, G. E. 2009. Transient host paralysis as a

    means of reducing self-superparasitism in koinobiont endoparasitoids. Journal of Insect

    Physiology 55: 321-327.

Fisher, R. C. 1961. A case study in insect multiparasitism II. The mechanism and control of competition

    for the host. Journal of Experimental Biology 38: 605-628.

Fisher, R. C. 1963. Oxygen requirements and the physiological sup- pression of supernumerary insect

    parasitoid. Nature 227: 191-192.

Fisher, R. C. 1965. The physiological suppression of insect parasitoids. Proceedings of the International

    Congress of Entomology 12: 413.

Gardner, S. M., Ward, S. A. and Dixon, A. F. G. 1984. Limitation of superparasitism by *Aphidius*

    rhopalosiphi – a consequence of aphid defensive behavior. Ecological Entomology 9: 149-155.

Grasswitz, T. R. and Paine, T. D. 1992.Kairomonal effect of an aphid cornicle secretion on *Lysiphlebus*

    testaceipes (Cresson) (Hymenoptera, Aphidiidae). Journal of Insect Behavior 5: 447-457.

Hatano, E., Kunert, G., Michaud, J. P. and Weisser, W. W. 2008. Chemical cues mediating aphid location

    by natural enemies. European Journal of Entomology 105: 797-806.

Heimpel, G. E. and Rosenheim, J. A. 1998. Egg limitation in parasitoids: A review of the evidence and a

    case study. Biological Control 11: 160-168.

Hopkinson, J. E., Zalucki, M. P. and Murray, D. A. H. 2013. Honeydew as a source of nutrition for

    Lysiphlebus testaceipes (Cresson) (Hymenoptera: Braconidae): effect of adult diet on lifespan

    and egg load. Australian Journal of Entomology 52: 14-19.

Janssen, A., Vanalphen, J. J. M., Sabelis, M. W. and Bakker, K. 1995a. Odor-mediated avoidance of

    competition in *Drosophila* parasitoids – the ghost of competition. Oikos 73: 356-366.

Janssen, A., Vanalphen, J. J. M., Sabelis, M. W. and Bakker, K. 1995b. Specificity of odor mediated avoidance of competition in *Drosophila* parasitoids. Behavioral Ecology and Sociobiology 36: 229-235.

Li, C., Roitberg, B. D. and Mackauer, M. 1993. Patch residence time and parasitism of *Aphelinus asychis* – a simulation model. Ecological Modelling 69: 227-241.

Li, C., Roitberg, B. D. and Mackauer, M. 1997. Effects of contact kairomone and experience on initial giving-up time. Entomologia Experimentalis Et Applicata 84: 101-104.

Losey, J. E., Ives, A. R., Harmon, J., Ballantyne, F. and Brown, C. 1997. A polymorphism maintained by opposite patterns of parasitism and predation. Nature 388: 269-272.

Marris, G. C. and Casperd, J. 1996. The relationship between conspecific superparasitism and the outcome of in vitro contests staged between different larval instars of the solitary endoparasitoid *Venturia canescens*. Behavioral Ecology and Sociobiology 39: 61-69.

McBrien, H. and Mackauer, M. 1990. Heterospecific larval competition and host discrimination in 2 species of aphid parasitoids – *Aphidius ervi* and *Aphidius smithi*. Entomologia Experimentalis Et Applicata 56: 145-153.

McBrien, H. and Mackauer, M. 1991. Decision to superparasitize based on larval survival – competition between aphid parasitoids *Aphidius ervi* and *Aphidius smithi*. Entomologia Experimentalis Et Applicata 59: 145-150.

Medrzycki, P., Cesari, M. and Maini, S. 2002. *Lysiphlebus testaceipes* on *Aphis gossypii*: studies on remote host discrimination. Bulletin of Insectoogy 1: 29-33.

Michaud, J. P. and Mackauer, M. 1994. The use of visual cues in host evaluation by aphidiid wasps 1. Comparison between 3 *Aphidius* parasitoids of the pea aphid. Entomologia Experimentalis Et Applicata 70: 273-283.

Montovan, K. J., Couchoux, C., Jones, L. E., Reeve, H. K. and van Nouhuys, S. 2015. The Puzzle of Partial Resource Use by a Parasitoid Wasp. American Naturalist 185: 538-550.

Muller, C. B. and Godfray, H. C. J. 1999. Indirect interactions in aphid-parasitoid communities. Researches on Population Ecology 41: 93-106.

Oliver, K. M., Noge, K., Huang, E. M., Campos, J. M., Becerra, J. X. and Hunter, M. S. 2012. Parasitic wasp responses to symbiont-based defense in aphids. BMC Biology 10.

Outreman, Y., Le Ralec, A., Plantegenest, M., Chaubet, B. and Pierre, J. S. 2001. Superparasitism limitation in an aphid parasitoid: cornicle secretion avoidance and host discrimation ability. Journal of Insect Physiology 47: 339-348.

Rasekh, A., Michaud, J. P., Kharazi-Pakdel, A. and Allahyari, H. 2010. Ant mimicry by an aphid parasitoid, *Lysiphlebus fabarum*. Journal of Insect Science 10.

Rosenheim, J. A., Heimpel, G. E. and Mangel, M. 2000. Egg maturation, egg resorption and the costliness of transient egg limitation in insects. Proceedings of the Royal Society B – Biological Science 267: 1565-1573.

Rosenheim, J. A. and Hongkham, D. 1996. Clutch size in an obligately siblicidal parasitoid wasp. Animal Behavior 51: 841-852.

Rosenheim, J. A. and Mangel, M. 1994. Patch-leaving rules for parasitoids with imperfect host discrimination. Ecological Entomology 19: 374-380.

Schellhorn, N. A., Kuhman, T. R., Olson, A. C. and Ives, A. R. 2002. Competition between native and introduced parasitoids of aphids: Nontarget effects and biological control. Ecology 83: 2745-2757.

Singh, R. and Sinha, T. B. 1982. Factors responsible for the super-parasitic ability of the parasitoid wasp *Trioxys indicus* (Hymenoptera, Aphidiidae). Entomologia Generalis 7: 295-300.

Smith, C. 1944. *The Aphidiinae of North America (Braconidae: Hymenoptera)*. Columbus, Ohio: The Ohio State University.

Vandermoten, S., Mescher, M. C., Francis, F., Haubruge, E. and Verheggen, F. J. 2012. Aphid alarm pheromone: An overview of current knowledge on biosynthesis and functions. Insect Biochemistry and Molecular Biology 42: 155-163.

Visser, M. E. and Rosenheim, J. A. 1998. The influence of competition between foragers on clutch size decisions in insect parasitoids. Biological Control 11: 169-174.

Volkl, W. and Mackauer, M. 2000. Oviposition behaviour of aphidiine wasps (Hymenoptera : Braconidae, Aphidiinae): Morphological adaptations and evolutionary trends. Canadian Entomologist 132: 197-212.

Volkl, W. and Stadler, B. 1991. Interspecific larval competition between *Lysiphlebus testaceipes* and *Aphidius colemani* (Hym, Aphidiidae). Journal of Applied Entomology 111: 63-71.

Weisser, W. W. 1994. Age-dependent foraging behavior and host-instar preference of aphid parasitoid *Lysiphlebus cardui*. Entomologia Experimentalis Et Applicata 70:1-10.

Wulff, J. A., Buckman, K. A., Wu, K. M., Heimpel, G. E. and White, J. A. 2013. The endosymbiont *Arsenophonus* is widespread in soybean aphid, *Aphis glycines*, but does not provide protection from parasitoids or a fungal pathogen. PLoS One 8: 7.

Wyckhuys, K. A. G., Stone, L., Desneux, N., Hoelmer, K. A., Hopper, K. R. and Heimpel, G. E. 2008a. Parasitism of the soybean aphid, *Aphis glycines* by *Binodoxys communis*: the role of aphid defensive behaviour and parasitoid reproductive performance. Bulletin of Entomological Research 98: 361-370.

Wyckhuys, K. A. G., Strange-George, J. E., Kulhanek, C. A., Wäckers, F. L. and Heimpel, G. E. 2008b. Sugar feeding by the aphid parasitoid *Binodoxys communis*: how does honeydew compare with other sugar sources? Journal of Insect Physiology 54: 481-491.

Wyckhuys, K. A. G., Koch, R. L., Kula, R. R. and Heimpel, G. E. 2009. Potential exposure of a classical biological control agent of the soybean aphid, *Aphis glycines*, on non-target aphids in North America. Biological Invasions 11: 857-871.

DISTURBANCES ALTER WITHIN-PLANT DISTRIBUTION TO THE POTENTIAL BENEFIT OF HERBIVOROUS INSECTS[2]

Abstract

Non-consumptive disturbances by predators can induce prey behaviors to mitigate the risk of consumption, but at a cost. The costs of these behaviors are a major component of predator effects on prey populations. For example, aphids are typically sedentary until they are disturbed by predators because movement incurs costs to feeding. As aphids are repeatedly disturbed, the feeding costs of fleeing can accumulate to reduce their fitness. However, the benefits of these behavioral responses beyond evading consumption are unclear. Using pea aphids, we studied the fleeing behavior and resettling locations after disturbances in two sets of experiments where we induced aphids to either walk away or drop from the host plant in response to a disturbance. "Walker" aphids accumulate on plant new growth with more disturbances, where they can achieve higher reproduction. However, the number of disturbances did not affect "dropper" distribution over the plant. Walkers produced more offspring with more disturbances, probably because they settled on better plant parts. However, for droppers reproduction and the number of disturbances were not correlated. These results show that behavioral responses to predators can have both costs and benefits. Depending on the type of behavior induced, the behaviors interfere with feeding for some period of time, but also allow aphids to more rapidly move to the best locations on the plant. The benefit of resettling in a better location can offset or outweigh the cost of behavior and affect the prey's fitness.

Key words: Aphid, benefit, cost, escape response, tradeoff

Introduction

Predators disturb their prey even when they do not successfully capture and eat them (Preisser and Bolnick 2008, Clinchy et al. 2013). Prey are not passive in the face of predator attack (Brown et al.

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[2] The material in this chapter was co-authored by James Kopco, Tyler J. Follman, Ned A. Dochtermann, and Jason P. Harmon. James Kopco and Jason P. Harmon conceived and designed the experiments. James Kopco and Tyler J. Follman performed the experiments. James Kopco, Ned A. Dochtermann, and Jason P. Harmon analyzed the data. James Kopco wrote the manuscript; other authors provided editorial advice.

1999, Preisser and Bolnick 2008). Defensive behaviors reduce the risk of being eaten, but there are tradeoffs; the prey can lose feeding opportunities that reduce its fitness (Brown et al. 1999, Preisser and Bolnick 2008). Such behavioral responses usually have a fitness cost even though they benefit the prey by helping them avoid being immediately consumed (Nelson et al. 2004, Clinchy et al. 2013). These behavior costs can lead to population- scale consequences (Nelson and Rosenheim 2006). Therefore, they constitute a major component of predator-prey interactions (Dicke and Grostal 2001, Peckarski et al. 2008, Michaud et al. 2016, Sitvarin et al. 2016). While the costs of avoiding predation have been measured in a wide range of systems, we have a weaker understanding of the potential benefits these prey behaviors could have outside of helping prey to avoid being consumed in that moment (Peckarski et al. 2008). A better understanding of prey's costs and benefits from predator-induced behaviors is necessary to strengthen our knowledge of predator-prey interactions.

Fleeing is one widely utilized prey response that brings a unique set of consequences for the prey. It can be effective in preventing being consumed, but incurs costs of lost foraging opportunities (Brown et al. 1999, Preisser and Bolnick 2008). The interruptions in foraging can have significant fitness consequences, especially for animals that spend a great deal of time feeding (Nelson 2007). Moreover, fleeing may create additional costs when prey move to new locations that may be dangerous or lack necessary resources (Roitberg and Myers 1979, Losey and Denno 1998, Courbin et al. 2016).

After prey flee from a predator, they must find a new location to settle (Courbin et al. 2016). They could return to where they fled from, or they could search for a new location (Cooper and Perez-Mellado 2004, Harding and Sheibling 2015). Re-settling prey are likely to settle in the best location they can find (Mueller et al. 2013, Harding and Scheibling 2015). The final settling location could be superior to the initial location, especially if location quality diminishes over time or if the displaced prey can assess several locations and choose the best (Charnov 1976, Cooper and Perez-Mellado 2004). If fleeing prey are likely to resettle in higher-quality locations (Harding and Sheibling 2015), then there could be a benefit to fleeing in addition to avoiding consumption.

The balance between such costs and benefits from fleeing could depend on several factors, including the frequency and severity of predator disturbances. Predator disturbance frequencies vary widely in most predator-prey systems (Peckarski et al. 2008, Cassus et al. 2016). The costs of fleeing for

an individual prey, such as lost foraging opportunity or energy expenditure, accumulate with more

predator encounters (Nelson 2007, Twardek et al. 2017). As a result, prey that flee from predators more

frequently incur more costs than do prey that flee less frequently (Nelson 2007, Courbin et al. 2015,

Twardek et al. 2017). Additionally, the frequency with which prey must flee their predators may affect

where prey resettle, especially if repeated disturbances cause prey to prioritize safer, rather than

resource-rich, resettling locations (Gal et al. 2015). These frequency-dependent changes to the behavior

can result in changes to the costs and benefits of the behavior (Cooper et al. 2004, Nelson 2007,

Twardek et al. 2017).

Furthermore, not all predator encounters are equal from the prey's perspective (Preisser and

Bolnick 2008, Michaud et al. 2016). A variety of extrinsic factors, including the species and behavior of

the predator, can affect exactly how the prey flees (Fill et al. 2012, Cooper and Sherbrooke 2016).

Because the details of how prey flees (e.g. direction, speed, distance, and time spent fleeing) can affect

the costs and benefits of the predator encounter (Nelson 2007, Peckarsky et al. 2008), different predator

encounters should cause different fitness outcomes for fleeing prey.

Aphids have a diverse repertoire of defensive behaviors to counter a variety of predator threats.

Aphids experience considerable variation in the frequency with which they encounter predators (Nelson

2007). Furthermore, aphid predators are a very diverse group of arthropods that can elicit different

behavioral responses from their aphid prey (Brodsky and Barlow 1982, Bell et al. 2008). Depending on

the predator cues an aphid receives, it may respond to predators by fighting back, running away, or

dropping from its host plant (Phelan et al. 1976, Wohlers 1981, Clegg and Barlow 1982, Brodsky and

Barlow 1986, Gish et al. 2010). These different cues can also differentially affect an aphid's resettling

behavior. For example, different cues can induce aphids to drop and then quickly seek out new plants or

to drop and then disperse across the ground before seeking a new host plant (Phelan et al. 1976,

Wohlers 1981). It is not clear if evading predators and resettling has any effects on the within-plant

distribution of aphids or if the within-plant distribution has any effects on the aphids' fitness.

Pea aphids exhibit a wide range of defensive behaviors in response to various disturbances.

They may flee from threats, withdrawing their mouthparts and either dropping from their host plant or

walking away (Nelson 2007), or they may stand their ground and fight back by kicking their legs, lashing

their antennae, waggling the abdomen, or secreting sticky defensive secretions from their abdominal cornicles (Edwards 1966, Gerling et al. 1990). This defensive secretion also contains an alarm pheromone, which makes nearby aphids more likely to defend themselves or flee (Bowers et al. 1972). Pea aphid behaviors are often effective at evading predators, so the consequences of behavioral responses to predators can be more important to the population dynamics of pea aphids than are direct consumptive effects of predators (Nelson and Rosenheim 2006).

Aphids' fleeing responses to predators have well-demonstrated costs (Roitberg et al. 1979, Dill et al. 1990, Nelson et al. 2004). The behaviors interrupt aphid feeding, which results in decreased reproductive output (Nelson et al. 2004). Dropping also can expose aphids to dangerously high soil temperatures (Roitberg et al. 1979) or to ground-foraging predators (Losey and Denno 1998). Despite these important costs, there have been few examples of potential benefits from these behaviors beyond escaping predators.

We conducted a series of laboratory experiments to determine how aphids resettle after a simulated predator attack and the fitness consequences of the resettling behaviors. We used a fine paint brush to simulate predator attacks, prodding aphids gently to induce them to walk away or rarely drop from the host plant, or prodding them more forcefully to induce them to drop from their host plant. We started by quantifying the costs of disturbance as the amount of time aphids took to resettle after experiencing a simulated predator attack. By varying the intensity of the attack, we induced "walker" and "dropper" aphids to leave their initial locations by either walking or dropping. Because we have seen aphids resettle on different locations within plants, and different plant parts vary in quality for aphids (Gould et al. 2007, Whalen and Harmon 2012), we also measured whether colonizing different locations on the plant has fitness effects for aphids. Finally, we put aphids into a variety of disturbance regimes, in which they were induced to either walk or drop at differing frequencies over two days, and measured where they settled, their mortality, and their reproductive output. Together, these experiments revealed the costs and benefits of walking or dropping away from disturbances and resettling in new locations for aphids.

Methods

Study System

Aphids

We conducted these experiments using pea aphids, *Acyrthosiphon pisum* Harris, reared on fava bean plants, *Vicia faba* L., var. Broad Windsor. All aphids used in these experiments came from a single clone (line 82B-AB) of pink-morph aphids that harbored no known facultative symbionts, which could alter their physiology and behavior in a variety of ways (Dion et al. 2011, Oliver et al. 2010). This aphid line was obtained from researchers at the University of Georgia in summer of 2013, who had previously collected the aphids in Georgia. Pea aphids all harbor an obligate symbiont, *Buchnera aphidicola*, which provides essential nutritional supplementation. However, this species may also harbor a variety of different secondary symbionts, which can have a wide range of effects (Oliver et al. 2010). These effects include changes to overall aphid fitness (Oliver et al. 2006), and for at least one secondary symbiont, reductions in aphid defensive behavior (Dion et al. 2011). In addition to symbiont effects, aphid clone has been shown to have significant effects on a variety of aphid characteristics, including color, susceptibility to parasitoids, and behavior (Braendle and Weisser 2001, Martinez et al. 2014).

Aphids' behavioral responses to predators have been shown to have strong effects on the reproductive capacity of aphids. Pea aphid reproduction is an effective and convenient measure of fitness and vigor, because they can generally produce 6-14 offspring per day (Gwynn et al. 2005) and reproduce clonally, such that populations can be established in which all individuals are female, and are genetically identical to one another. Aphid reproduction can vary depending on how crowded the aphid colony is, with heavily crowded aphids reproducing less than less crowded aphids. To maximize the expected effect sizes, we used non-crowded aphids in these experiments, with not more than four adult aphids and their progeny produced over two days on each host plant (Nelson 2007).

Plants

We used fava beans as the host plants for rearing our aphids and in all of our experiments. In lab colonies, we have observed that the greatest densities of aphids tend to occur on the newest growth of the plant, especially on the actively growing leaves. Likewise, pea aphids collected in the field off of alfalfa were most abundant near the top of the canopy, which corresponds with where the majority of young,

actively growing shoots are found (personal observation). These observations led us to hypothesize that different parts of the plant may vary in their value to pea aphids.

The architecture of a fava bean plant var. Broad Windsor grown in the lab generally consists of a single stem arising from the seed, with pairs of leaves arising at intermittent nodes along the stem. We defined the actively growing leaves as the expanding leaves that are not yet full size, and are at least somewhat folded along the longitudinal midline, with the dorsal surfaces of each side of the leaf opposing one another (Figure 5). Conversely, in mature leaves, the leaves are either flat or slightly curled with the ventral surface, rather than the dorsal surface, on the interior of the curl. At the plant growth stage used in our experiments, all plants had actively growing leaves at the tips of a main central stem. In addition, some of the plants had up to two lateral branches arising from near the base of the central stem that also bear actively growing leaves at the tips. We hypothesized that the age and growth stage of the leaves were more important to the aphids than their location on central or lateral stems, so we did not distinguish between the locations of the leaves in recording the settling locations of aphids.

Experiments

We conducted three experiments to measure the costs, behaviors, secondary effects, and net fitness consequences of disturbances. In our first experiment, we administered artificial disturbances to individual aphids and recorded how long each aphid took to resettle following the disturbances. Because the costs of escape behavior following disturbances are thought to primarily arise through lost feeding time, this should serve as a proxy for the cost of the disturbance (Nelson 2007). In the second experiment, we tested whether relocation following a disturbance can have fitness consequences by confining aphids to different plant parts and comparing their fecundity over two days. In the third experiment, we quantified the relocation behavior of walkers or droppers and the net fitness consequence of disturbances of different frequencies. All together, these experiments quantify a cost of the disturbances, a potential benefit of the disturbances, and the net outcome of disturbances on pea aphids.

Time to Resettlement After Disturbance

We ran behavioral observation experiments to determine the latency to resume feeding after disturbances. Because defensive behaviors incur costs by interrupting feeding (Nelson 2007), we

presumed that measuring the time to resettlement after a disturbance would help compare the cost of

disturbance.
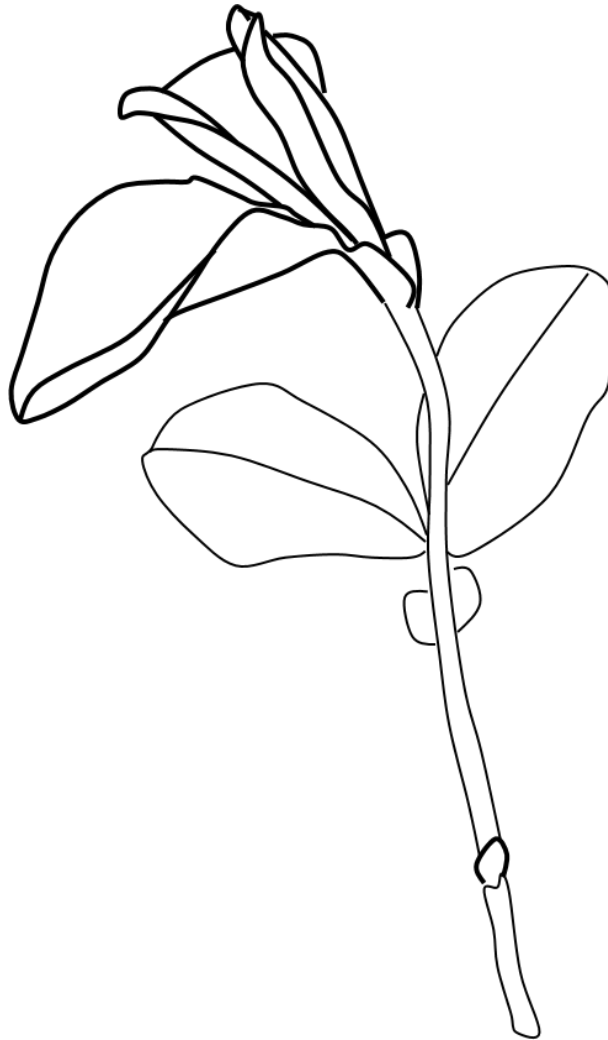
Figure 5. Diagram of a fava bean plant. The actively growing leaves as we've defined them for these experiments are the curled, developing leaves at the tip of each stem, illustrated with **bold** lines. This plant specimen does not have any lateral branches, but the node from which lateral branches can arise is visible on all plants (node in bold, just above the base of the central stem). On plants that do develop lateral branches, the lateral branches are very similar in structure to the central stem. Secondary actively growing leaves are variable between plants; in some plants, they never develop, while in others they are well-developed. We treated actively growing leaves on central stems and lateral branches as being equivalent; aphids that settled on either were considered "on actively growing leaves."

Two researchers observed 62 aphids over 13 days, divided between two treatments based on the

behaviors we induced, walking (n=35) or dropping (n=27). We infested plants with a single aphid, and

allowed 6 hours for the aphids to settle. We applied disturbances with home-made brushes consisting of

only 3 bristles, which were fine enough to facilitate disturbing aphids without forcibly pushing them. To induce walking, we continuously prodded aphids gently with the brush until they withdrew their stylets and walked at least one body length from their initial location. To induce dropping, we continuously prodded aphids, but slightly more forcefully than to induce walking, until the aphid dropped from the plant. Both walkers and droppers often did not respond to the first prod, so we continued prodding them until we elicited the desired behavior. Brodsky and Barlow (1986) showed that pea aphids tend to walk when approached by predatory syrphid larvae but drop when approached adult coccinellids, so the behaviors we induced in these treatments correspond with aphid behavioral responses to predators.

We continuously recorded aphid behaviors until they spent 20 minutes with their mouthparts in continuous contact with the plant, as described by Nelson (2007) confirming that the aphid had indeed resettled. The 20 minutes of feeding used to establish that aphids had resettled was not included in the total time to resettle. Latency to resettlement was compared between aphids induced to drop and those induced to walk by ANOVA, which also checked for block effects for observations that occurred on separate days and by different observers. We did not record resettling location in this experiment.

Fitness of Aphids on Actively Growing Leaves vs. Old Leaves

We compared the relative fitness of aphids on the actively growing leaves versus on older leaves of a plant. Because aphids respond to disturbances by moving away from them, and then recolonizing the plant, this experiment was intended to discover whether different resettling locations are correlated with superior aphid fitness over other resettling locations.

To perform the experiment, adult apterous aphids were collected from mixed-age colonies and were immediately placed, one each, on the lowest leaf of the plant and on the newest actively growing leaf of the same plant, and they were restricted by fine mesh bags fastened around the leaf petioles with twist-ties. The aphids were contained on their respective leaves for two days, after which the mesh bags were removed and the number of nymphs they produced were counted. All plants on which one or both adult aphids died were not included in data analysis, leaving a final sample size of 54 plants from the initial 74, each with two aphids, divided between four experimental blocks that were run on separate days from 7 July 2016 to 27 July 2016. Because the two aphids on each plant were linked by the plant rather

than fully independent, the number of nymphs produced on actively growing leaves and older leaves were compared by a paired *T*-test. We also checked for block effects with ANOVA.

Disturbance Frequency Experiments

We conducted a pair of experiments to assess the behavioral responses of small populations of aphids to disturbance and the net fitness consequences of disturbance. To do this, we varied the frequency with which aphids experienced disturbances over a two day period, and recorded where on the plants the adult aphids settled, the number of surviving adults, and the number of nymphs they produced. These two experiments were nearly identical in methodology, except that in the first experiment we induced aphids to walk and in the second we induced aphids to drop. In both experiments, we induced the target behaviors using the same methodology as in the time to resettlement after disturbance experiment. Between the disturbance frequency treatments within each experiment and the separate walker and dropper experiments, we explored the consequences of disturbances over both different disturbance frequencies and different disturbance severity.

We hypothesized that the settling aphids' settling locations on the plant after a disturbance would affect their fitness. These experiments allowed us to measure both responses; we recorded the within plant distribution of the aphids and the total reproduction of all aphids on the plant. All plants used in this experiment initially had two to three pairs of fully expanded leaves, plus the actively growing leaves at the tip of the main stem and, in some cases, one or two short, young lateral branches tipped by actively growing leaves. We initially contained all adult aphids on the bottom-most mature leaf pair until they were settled, then recorded the number of adult aphids and their offspring on each leaf or stem segment between leaf nodes. When we analyzed the data, we lumped the mature leaves and stems together as a broad category of not actively growing plant tissue, and lumped together the actively growing leaves at the tips of the main stem and lateral branches.

The timeline of disturbances and data collected are presented in Table 3. Initially, four adult apterous aphids were placed on the lowest leaf of each plant and confined within a 25 mm clip cage that held them on the leaf. They were allowed 9 hours to settle, at which point the clip cages were removed and replaced with tube cages that kept aphids confined to their respective plants, but allowed them to move freely on the plant. Disturbances were applied, according to treatment, at 9 a.m., 3 p.m., and 9 p.m.

All researchers wore dust masks during disturbances to avoid inadvertently disturbing aphids with their breath, because mammal breath has been shown to induce rapid dropping in pea aphids (Gish et al. 2010).

Treatments for this experiment consisted of 0 disturbances per day, ½ disturbance per day (only half of the aphids were disturbed once per day, which served as an imperfect intermediate treatment between 0 disturbances and one disturbance per day and a half), one disturbance every day and a half, one disturbance per day, two disturbances per day, and three disturbances per day. These disturbance frequencies are consistent with predator encounter frequencies in the field (Nelson 2007). The specific timeline of disturbances is presented in Table 1. Each experiment was conducted over three separate blocks, which each consisted of 7-8 replicates of each treatment per block and with each replicate consisting of one plant and its cohort of adult aphids and their progeny. In total, experiments with walkers were replicated 135 times, while those with droppers were replicated 144 times.

Disturbance Frequency Experiment Analysis

We measured and analyzed three responses to the number of disturbances: settling location, mortality, and reproduction. Based on our results from the fitness of aphids on actively growing leaves vs. older leaves experiment, settling location served as a proxy for benefits of fleeing the disturbances. Mortality and reproduction were considered as components of aphid fitness that would indicate net effects of costs and benefits of the behaviors.

Settling location and mortality were analyzed as binary responses (aphids were either on actively growing leaves or not and either alive or dead) using logistic mixed models (Siers et al. 2016) on a per-aphid basis. The number of disturbances aphids received was the primary fixed effect of interest. We also included experimental block and data recorder as fixed effect covariates. Because aphids were allocated four per plant and subsequently confined to their respective plants, we included plant as a random effect in the mixed models to control for plant-based effects on aphid mortality or settling location. Models were run using the "glmer" function from the R package "lme4" on R v. 3.2.1 (Siers et al. 2016).

Table 3. Specific timeline of disturbances.

Time (Hours from start of experiment)	Treatment					
	0	½	1/1.5	1	2	3
0	put aphids on lower leaf of plant, confined to clip cage					
9	remove clip cage	remove clip cage	remove clip cage	remove clip cage	remove clip cage, disturb	remove clip cage, disturb
21					disturb	disturb
27	record data	record data, disturb 2/4 of the aphids	record data	Record data, disturb	record data	record data, disturb
33			disturb		disturb	disturb
45					disturb	disturb
51	record data					
Total Disturbances	0	0.5	1	1	4	5

While treatments were defined by the number of disturbances the aphids received per day, data were analyzed for both day 1 and day 2 using the actual total number of disturbances the aphids had received, which is enumerated in the last two rows. The "Remove clip cage" at 9 hours is when the clip cages that confined aphids to the bottom-most leaf was removed, and aphids were able to relocate over the plant. At 27 hours, data were recorded before disturbances were applied, to avoid biasing recordings of aphid locations.

Mixed models were run a total of four times to separately analyze two response variables, mortality and settling location at the end of two days. Two were run to analyze mortality of walkers and droppers, respectively, at the end of each two-day duration experiment. The other two were run to analyze settling locations of walkers and droppers, respectively, at the end of each experiment.

To test for significance of the number of disturbances yielded by the logistic mixed models, we used likelihood ratio tests. To do this, we repeated each model, but excluded the number of disturbances from the second iteration of the model. We then did pairwise comparisons of the original models against the models excluding the number of disturbances using the lmerTest package in R. Because the only difference between the models we compared was the inclusion or omission of the total disturbances, significant differences between the models would reflect significant effects of total disturbances (Siers et al. 2016).

Because the mothers of specific nymphs could not be determined, reproduction was treated as a collective sum for all aphids on a given plant, corrected for the number of surviving adults on that plant. The advantages of the logistic mixed models were their ability to handle binary response variables and random factors (Siers et al. 2016). Therefore, logistic mixed models were inappropriate for analyzing reproduction data because it was not binary and had to be summed over the random factor (plant) such

that each random factor could have only one observation. To correct for the number of surviving adults on the plant producing nymphs, the response variable considered was the total number of nymphs counted, divided by the number of adult aphids still alive on the plant.

Results

Time to Resettlement After Disturbance

We measured the amount of time aphids took to resettle after disturbances as a measure of the severity of the disturbances. Disturbances that require longer to resettle would be assumed to incur greater costs, since they have a greater opportunity cost on feeding (Nelson 2007). Walkers took 10.20 ± 2.05 minutes to resettle and droppers took 28.41 ± 4.64 minutes to resettle (Figure 6). The resettlement time was significantly longer for droppers than for walkers ($F_{1,31} = 11.8158$, $P = 0.0017$).

Fitness of Aphids on Actively Growing Leaves vs. Older Leaves

We measured the reproductive output of aphids confined to actively growing leaves or the oldest leaves of a plant to determine if within-plant location affects aphid fitness. Aphids confined to the actively growing leaves produced significantly more nymphs than their counterparts on older leaves of the same plants ($t_{51} = 4.21$, $P = 0.00010$) (Figure 7).
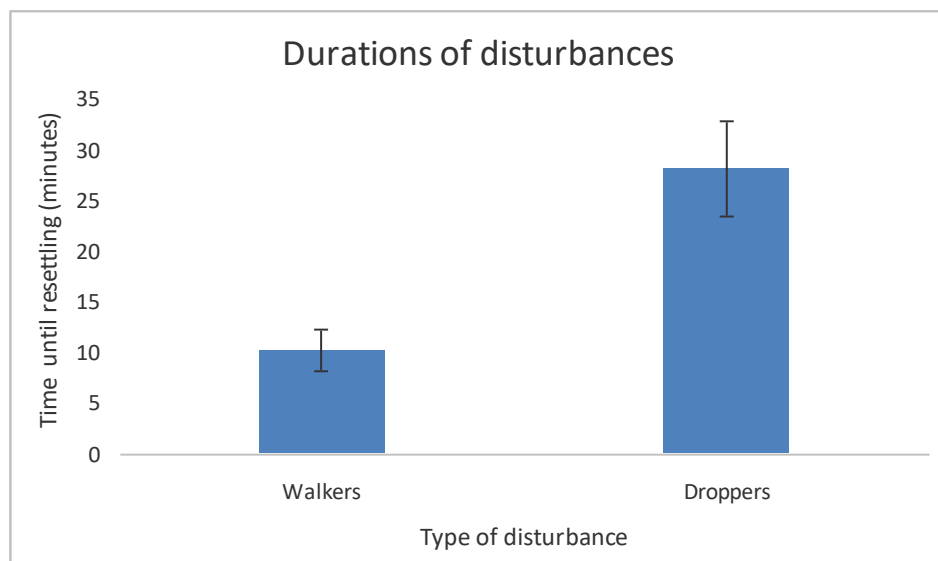

Figure 6. Average time to resettle of walkers and droppers. Droppers took significantly longer to resettle than walkers. Error bars indicate the SEM.

Disturbance Frequency

The disturbance frequency experiments yielded data on the aphids' settling behaviors following disturbance as well as the fitness consequences of these disturbances, both as mortality and reproduction. Because aphids reproduce more on actively growing leaves than on older leaves, a tendency to colonize actively growing leaves after disturbances could provide a benefit. For walkers' actively growing leaf colonization, the coefficient for total disturbances was +0.27 ($P = 0.00077$) (Table 4), showing that disturbances were positively correlated with aphids being on actively growing leaves. Therefore, the proportions of walkers that occupied the actively growing leaves were higher with more disturbances (Figure 8A). For droppers, the coefficient calculation was -0.0027 ($P = 0.97$) (Table 4). This shows that actively growing leaf colonization was not correlated with total disturbances for droppers (Figure 8B).

Because disturbances incur metabolic costs to aphids, we tested whether mortality over the two day period varied with disturbance treatment. Coefficient estimates were -0.16 ($P = 0.057$) for walkers and -0.045 ($P = 0.43$) for droppers (Table 2). In each case, coefficients for mortality did not significantly differ from 0, indicating that disturbances were not correlated with mortality.

The nymphs produced by the adult aphids should approximate the net effects of disturbances, after costs and indirect benefits affect the aphids. For walkers, nymph production was positively correlated with disturbances ($y = 13.27 + 0.34x$, $r^2 = 0.03$, $F_{1,133} = 4.678$, $P = 0.03$) (Figure 8C). Unlike the walkers, nymph production of droppers was not correlated with disturbances ($y = 15.99 + 0.05x$, $r^2 < 0.01$, $F_{1,142} = 0.09$, $P = 0.75$) (Figure 8D).

Nymph production on actively growing leaves and older leaves
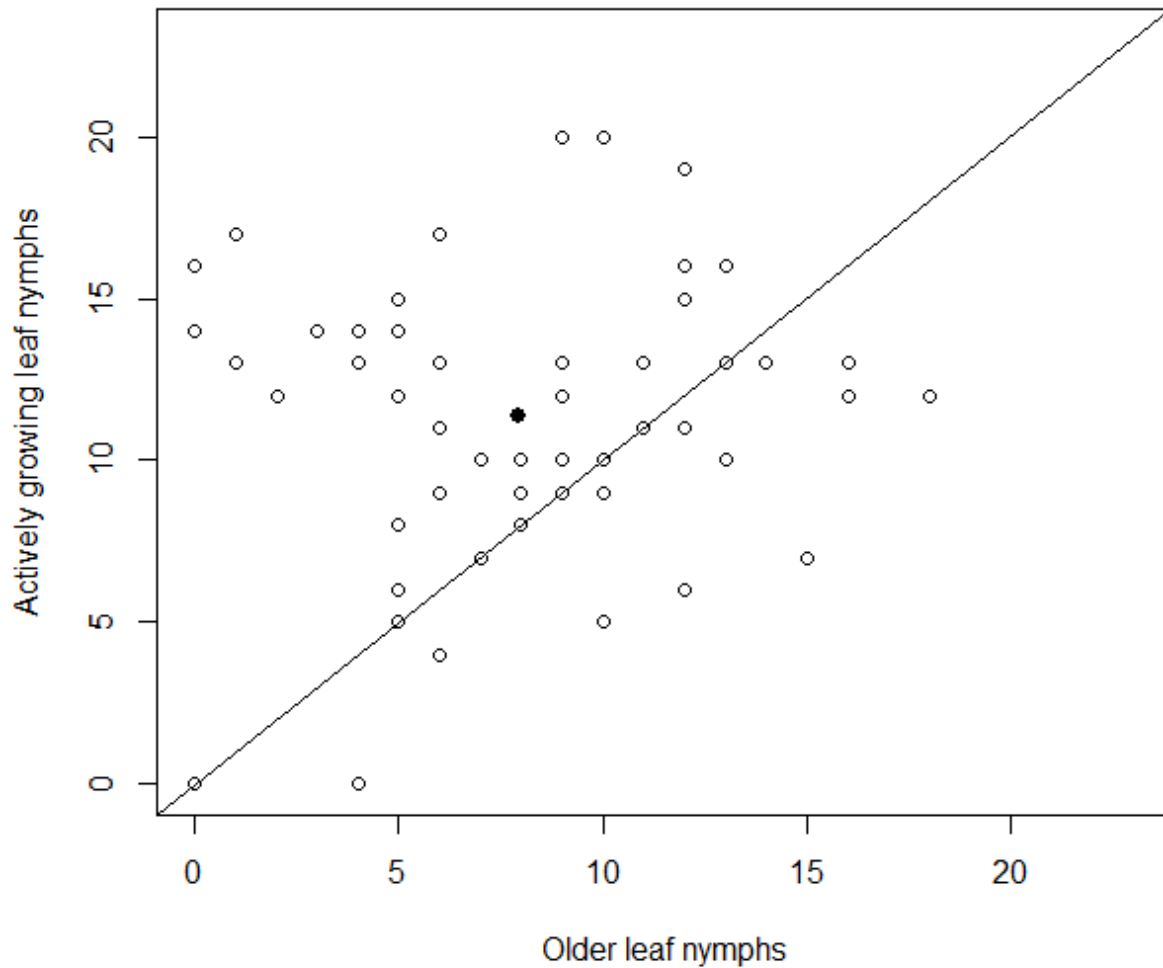
Figure 7. Reproduction of aphids confined to actively growing leaves or older leaves. Each open circle represents the number of nymphs produced by two aphids on a single plant, with the nymph production by the aphid on the older leaves dictating the position on the x-axis and the nymph production by the aphid on the actively growing leaf dictating the position on the y-axis. The closed circle indicates the average nymph production of all aphids. The diagonal line represents a 1:1 ratio of reproduction between aphids on actively growing leaves and older leaves; data points above the line represent plants on which actively growing leaf aphids produced more offspring than older leaf aphids on the same plant, while the reverse is true for points below the line. Aphids confined to the actively growing leaves produced significantly more nymphs than their counterparts on older leaves.

Table 4. Coefficient estimates for effects of disturbance on actively growing leaves colonization.

	Walkers	Droppers
Actively growing leaf colonization, day two	+0.27 ± 0.16, $P = 0.00077$	-0.0027 ± 0.11, $P = 0.97$
Mortality	-0.16 ± 0.17, $P = 0.057$	-0.045 ± 0.11, $P = 0.43$

Ranges indicate 95% confidence intervals.

Discussion

These experiments illustrated that behavioral responses to disturbances have benefits for pea aphids beyond evading the disturbance that can offset their costs. In our first experiment, we showed that we could simulate disturbances of varying severity to elicit different defense responses (walking vs. dropping) and that these two defenses incur different costs in terms of time to resettle on the host plant. In our second experiment, we showed that aphids on actively growing leaves produce significantly more offspring over a two-day period than aphids on older leaves. These results led us to hypothesize that aphids that are disturbed while feeding on older leaves may be able to offset the cost of moving by resettling on a superior plant part. We ran our final experiment twice – first with walkers, then with droppers – to test whether the frequency of disturbances effected the within-plant distribution of the aphids and/or the short-term reproductive output of the aphids. Walkers exhibited a significant positive correlation between disturbance frequency and the number of aphids that occupied the actively growing leaves. They also exhibited a weak, but statistically significant, positive correlation between disturbance frequency and reproduction. Droppers did not exhibit any significant correlations between disturbance frequency and the number of aphids occupying actively growing leaves or reproduction.

Our artificial disturbances were able to consistently induce different behaviors from the aphids. By varying the force with which we prodded aphids with brushes, we reliably induced them to either walk or drop from the host plant. We showed that the initial behavior was not the only difference; droppers took nearly three times as long to resettle as walkers. Because the primary cost of these behaviors in our controlled system is likely to be lost feeding time (Nelson et al. 2004), we would expect droppers to incur greater costs than walkers. In the field, dropping could incur additional costs by exposing aphids to

dangerously hot or dry ground conditions (Roitberg et al. 1979) or ground-foraging predators (Losey and

Denno 1998).
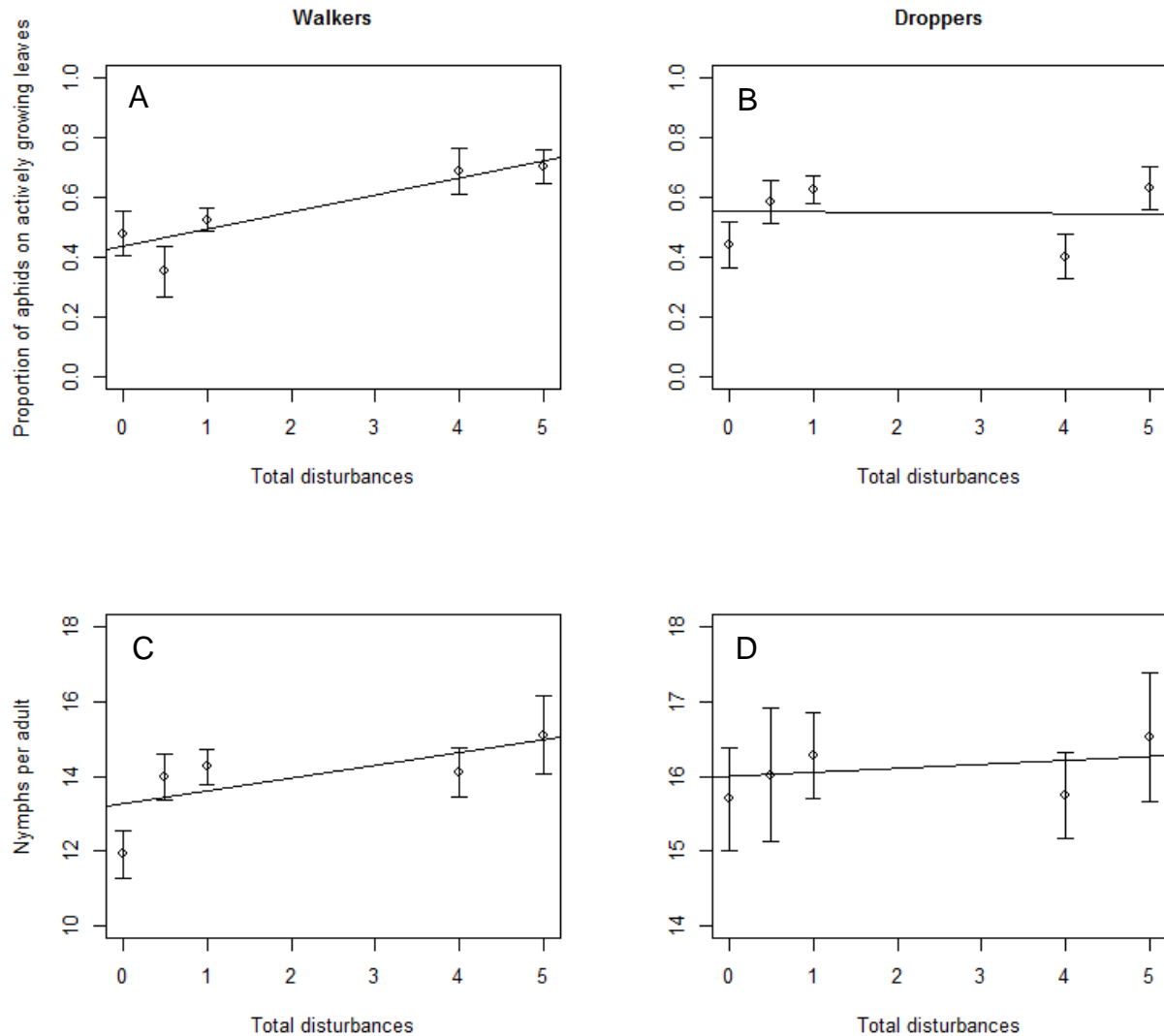
Figure 8. Results from the disturbance frequency experiment. These figures emphasize final settling location (top row) and total nymph production per adult (bottom row). Panels in the left column show data from walkers, whereas panels in the right column show data from droppers. Panels in the top row show the average proportion of aphids on each plant that were on the actively growing leaves ± SE. Panels in the bottom row show total per capita nymph production by the end of two days, with data points indicating the average ± standard error at each disturbance level. For walkers, the number of total disturbances was positively correlated with increased movement to the actively growing leaves, while no such relationship was found for droppers. Similarly, nymph production was positively correlated for walkers, but was not correlated for droppers.

Where aphids settle on a plant had significant effects on their fitness. We showed that pea aphids

that settle on actively growing fava bean leaves had greater fecundity than those on older leaves of the

same plant. This affect may be due to differences in the aphids' ability to feed on actively growing leaves compared to older leaves (Whalen and Harmon 2012). The actively growing leaves may be easier for the aphids to pierce with their mouthparts or the phloem could contain a different composition of amino acids (Gould et al. 2007). Settling on actively growing leaves may have additional effects on the aphids that our experimental methodology would not capture. For example, the positioning and complex structure of actively growing leaves may be more or less exposed to sunlight, wind, or predators than older, flatter leaves, which could affect the fitness of aphids that settle there (Wyckhuys et al. 2007, Keiser et al. 2013, Barton 2014, Burdick et al. 2015).

The aphids' settling location varied with their response to the disturbances. Walkers that experienced more disturbances were more likely to be on actively growing leaves. Because aphids on actively growing leaves reproduce more than those on older leaves, colonizing actively growing leaves more rapidly following disturbances could yield benefits to the aphids. In the disturbance frequency experiment, aphid reproduction served as a measure of the combined effects of costs and benefits. If benefits exceed costs, then reproduction would increase with more disturbances. Otherwise, reproduction would decrease with more disturbances. Reproduction of walkers was positively correlated with the number of disturbances. This positive correlation indicated that the benefits of colonizing actively growing leaves outweighed the costs of interrupting feeding and continued to accrue with additional disturbances. This effect was small, but the direction of the effect (positive rather than negative) was the opposite of what we had expected based on the results of Nelson (2007).

Droppers exhibited different response patterns than walkers. Droppers' settling on actively growing leaves was not correlated with the number of disturbances. Therefore, droppers did not benefit by moving onto actively growing leaves. Additionally, reproduction of droppers was not correlated with the number of disturbances. The absence of correlation between number of disturbances and reproduction for droppers was unexpected. Because droppers were not more likely to occupy actively growing leaves when they experienced more disturbances, we would not expect them to experience any location-based benefit from their disturbances, but to incur the cost of interrupted feeding. As a result, we expected disturbance frequency to be negatively correlated with reproduction. However, our assumption that the costs accumulate equally with each subsequent disturbance may not be valid. If droppers can decrease

the time needed to resettle with subsequent disturbances, they could reduce costs of later disturbances. Because the time droppers took to resettle after an initial disturbance was highly variable, repeated disturbances inducing shorter feeding interruptions may induce costs that are obscured by the variation in aphid responses. Droppers also may exhibit a delay in expressing the costs of their disturbances as cumulative feeding interruptions interfere with the production of new embryos.

Our findings that aphids on actively growing leaves produce more offspring than those on older leaves raises a major question: why would aphids ever occupy any plant parts other than actively growing leaves? Because actively growing leaves eventually senesce, they provide a constantly moving ideal location for aphids. Some species of aphids have been found to track the development of new leaves on their host plant, periodically moving from older leaves to actively growing leaves (Gould et al. 2007). Moving may be inherently risky for aphids, and undisturbed aphids may adopt a more risk-averse strategy and remain at their location until the quality of that location deteriorates a great deal. Aphids must contend with a wide range of predators (Bell et al. 2008) and typically encounter predators at least every day or two (Nelson 2007). They also may be induced to move by other factors such as browsing mammalian herbivores (Gish et al. 2010), host plant resistance (Whalen and Harmon 2012), or host plant-produced alarm pheromone (Beale et al. 2006). Because they are likely disturbed frequently in the field, it is plausible that pea aphids may rely on external disturbances rather than tracking gradual changes in feeding site quality to stimulate movement. Additionally, movement to new plant parts may incur costs that our experiments were unable to detect. Pea aphids have been shown to mediate the amino acid composition of the phloem upon which they feed (Leroy et al. 2011), and it is unclear whether this effect is systemic on the entire plant or localized to where the aphid(s) were feeding. If the effect is localized, then a well-established aphid infestation on a particular leaf, even if the leaf is older, may be a superior resource than the young, unmanipulated actively growing leaves. A feeding site may also accrue value to an aphid by accumulating that aphid's offspring; as more nymphs accumulate around a reproductive adult, they form a living, defensive secretion oozing barrier that may decrease the predation risk of the aphid (Mondor and Roitberg 2002, Hartbauer 2010, Duff and Mondor 2012). Additionally, Mutti et al. (2008) showed with electrical penetration graphs that pea aphids took nearly 150 minutes from

when they started probing to reach the phloem. Because moving even a short distance incurs the lengthy re-insertion cost, moving too frequently to try to track plant growth may incur greater costs than benefits.

The costs of behaviors induced by predators have been studied in a wide range of systems (Peckarsky et al. 2008, Preisser and Bolnick 2008), but the benefits beyond escaping consumption are less clear. This study shows that the behavioral responses of prey to their predators can have benefits beyond avoiding consumption. Between the short duration of the experiments presented here and the focus on short-term reproduction, this study is just one piece of a much broader understanding of sub-lethal disturbance effects. These benefits depend on the behavioral response to the disturbance, and on how often disturbances are experienced. These benefits of induced behaviors can offset, or even outweigh the costs. Gaining a deeper understanding of the costs and benefits of predator induced behaviors will provide a more complete and accurate picture of predator effects on prey.

References

Barton, B. T. 2014. Reduced wind strengthens top-down control of an insect herbivore. Ecology 95: 2375-2381.

Beale, M. H., M. A. Birkett, T. J. A. Bruce, K. Chamberlain, L. M. Field, A. K. Huttly, J. L. Martin, R. Parker, A. L. Phillips, J. A. Pickett, I. M. Prosser, P. R. Shewry, L. E. Smart, L. J. Wadhams, C. M. Woodcock, and Y. H. Zhang. 2006. Aphid alarm pheromone produced by transgenic plants affects aphid and parasitoid behavior. Proceedings of the National Academy of Sciences of the United States of America 103: 10509-10513.

Bell, J. R., A. Mead, D. J. Skirvin, K. D. Sunderland, J. S. Fenlon, and W. O. C. Symondson. 2008. Do functional traits improve prediction of predation rates for a disparate group of aphid predators? Bulletin of Entomological Research 98: 587-597.

Bowers, W. S., R. E. Webb, L. R. Nault, and S. R. Dutky. 1972. Aphid alarm pheromone – isolation, identification, synthesis. Science 177: 1121-&.

Braendle, C., and W. W. Weisser. 2001. Variation in escape behavior of red and green clones of the pea aphid. Journal of Insect Behavior 14: 497-509.

Brodsky, L. M., and C. A. Barlow. 1986. Escape responses of the pea aphid, *Acyrthosiphon pisum* (Harris) (Homoptera, Aphididae) – Influence of predator type and temperature. Canadian Journal of Zoology-Revue Canadienne De Zoologie 64: 937-939.

Brown, J. S., J. W. Laundre, and M. Gurung. 1999. The ecology of fear: optimal foraging, game theory, and trophic interactions. Journal of Mammalogy 80: 385-399.

Burdick, S. C., D. A. Prischmann-Voldseth, and J. P. Harmon. 2015. Density and distribution of soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae) in response to UV radiation. Population Ecology 57: 457-466.

Charnov, E. L. 1976. Optimal foraging, marginal value theorem. Theoretical Population Biology 9: 129-136.

Clegg, J. M., and C. A. Barlow. 1982. Escape behavior of the pea aphid *Acyrthosiphon pisum* (Harris) in response to alarm pheromone and vibration. Canadian Journal of Zoology-Revue Canadienne De Zoologie 60: 2245-2252.

Clinchy, M., M. J. Sheriff, and L. Y. Zanette. 2013. Predator-induced stress and the ecology of fear. Functional Ecology 27: 56-65.

Cooper, W. E., and V. Perez-Mellado. 2004. Tradeoffs between escape behavior and foraging opportunity by the Balearic lizard (Podarcis lilfordi). Herpetologica 60: 321-324.

Cooper, W. E., V. Perez-Mellado, and L. J. Vitt. 2004. Ease and effectiveness of costly autotomy vary with predation intensity among lizard populations. Journal of Zoology 262: 243-255.

Cooper, W. E., and W. C. Sherbrooke. 2016. Strategic escape direction: orientation, turning, and escape trajectories of zebra-tailed lizards (*Callisaurus draconoides*). Ethology 122: 542-551.

Courbin, N., A. J. Loveridge, D. W. Macdonald, H. Fritz, M. Valeix, E. T. Makuwe, and S. Chamaille-Jammes. 2016. Reactive responses of zebras to lion encounters shape their predator-prey space game at large scale. Oikos 125: 829-838.

Dicke, M., and P. Grostal. 2001. Chemical detection of natural enemies by arthropods: An ecological

    perspective. Annual Review of Ecology and Systematics 32: 1-23.

Dill, L. M., A. H. G. Fraser, and B. D. Roitberg. 1990. The economics of escape behavior in the pea aphid,

    Acyrthosiphon pisum. Oecologia 83: 473-478.

Dion, E., S. E. Polin, J. C. Simon, and Y. Outreman. 2011. Symbiont infection affects aphid defensive

    behaviours. Biology Letters 7: 743-746.

Duff, K. M., and E. B. Mondor. 2012. All Clone-mates are not Created Equal: Fitness Discounting Theory

    Predicts Pea Aphid Colony Structure. Journal of Insect Behavior 25: 48-59.

Edwards, J. S. 1966. Defence by smear – supercooling in cornicle wax of aphids. Nature 211: 73-&.

Fill, A., E. Y. Long, and D. L. Finke. 2012. Non-consumptive effects of a natural enemy on a non-prey

    herbivore population. Ecological Entomology 37: 43-50.

Gal, S., S. Alpern, and J. Casas. 2015. Prey should hide more randomly when a predator attacks more

    persistently. Journal of the Royal Society Interface 12: 9.

Gerling, D., B. D. Roitberg, and M. Mackauer. 1990. Instar-specific defense of the pea aphid,

    Acyrthosiphon pisum, influence on oviposition success of the parasite *Aphelinus asychis*

    (Hymenoptera, Aphelinidae). Journal of Insect Behavior 3: 501-514.

Gish, M., A. Dafni, and M. Inbar. 2010. Mammalian herbivore breath alerts aphids to flee host plant.

    Current Biology 20: R628-R629.

Gould, G. G., C. G. Jones, P. Rifleman, A. Perez, and J. S. Coleman. 2007. Variation in eastern

    cottonwood (*Populus deltoides* Bartr.) phloem sap content caused by leaf development may

    affect feeding site selection behavior of the aphid, *Chaitophorous populicola* Thomas

    (Homoptera: Aphididae). Environmental Entomology 36: 1212-1225.

Gwynn, D. M., A. Callaghan, J. Gorham, K. F. A. Walters, and M. D. E. Fellowes. 2005. Resistance is

    costly: trade-offs between immunity, fecundity and survival in the pea aphid. Proceedings of the

    Royal Society B 272: 1803-1808.

Harding, A. P. C., and R. E. Scheibling. 2015. Feed or flee: Effect of a predation-risk cue on sea urchin

    foraging activity. Journal of Experimental Marine Biology and Ecology 466: 59-69.

Hartbauer, M. 2010. Collective defense of Aphid nerii and Uroleucon hypochoeridis (Homoptera, Aphididae) against natural enemies. PLoS ONE 5: e10417.

Keiser, C. N., L. E. Sheeks, and E. B. Mondor. 2013. The effect of microhabitat feeding site selection on aphid foraging and predation risk. Arthropod-Plant Interactions 7: 633-641.

Leroy, P. D., B. Wathelet, A. Sabri, F. Francis, F. J. Verheggen, Q. Capella, P. Thonart, and E. Haubruge. 2011. Aphid-host plant interactions: does aphid honeydew exactly reflect the host plant amino acid composition? Arthropod-Plant Interactions 5: 193-199.

Losey, J. E., and R. F. Denno. 1998. Positive predator-predator interactions: Enhanced predation rates and synergistic suppression of aphid populations. Ecology 79: 2143-2152.

Martinez, A. J., S. G. Ritter, M. R. Doremus, J. A. Russell, and K. M. Oliver. 2014. Aphid-encoded variability in susceptibility to a parasitoid. Bmc Evolutionary Biology 14: 10.

Michaud, J. P., P. R. R. Barbosa, C. L. Bain, and J. B. Torres. 2016. Extending the "ecology of fear" beyond prey: reciprocal nonconsumptive effects among competing aphid predators. Environmental Entomology 45.

Mondor, E. B., and B. D. Roitberg. 2002. Pea aphid, Acyrthosiphon pisum, cornicle ontogeny as an adaptation to differential predation risk. Canadian Journal of Zoology-Revue Canadienne De Zoologie 80: 2131-2136.

Mueller, J. P., D. Laloi, C. Yepremian, C. Bernard, and F. D. Hulot. 2013. To flee or not to flee: detection, avoidance of and attraction to food resources by Daphnia magna studied with an olfactometer. Journal of Limnology 72: 464-472.

Mutti, N. S., J. Louis, L. K. Pappan, K. Pappan, K. Begum, M.-S. Chen, Y. Park, N. Dittmer, J. Marshall, J. C. Reese, and G. R. Reeck. 2008. A protein from the salivary glands of the pea aphid, Acyrthosiphon pisum, is essential in feeding on a host plant. Proceedings of the National Academy of Sciences of the United States of America 105: 9965-9969.

Nelson, E. H. 2007. Predator avoidance behavior in the pea aphid: costs, frequency, and population consequences. Oecologia 151: 22-32.

Nelson, E. H., and J. A. Rosenheim. 2006. Encounters between aphids and their predators: the relative

   frequencies of disturbance and consumption. Entomologia Experimentalis et Applicata 118: 211-

   219.

Nelson, E. H., C. E. Matthews, and J. A. Rosenheim. 2004. Predators reduce prey population growth by

   inducing changes in prey behavior. Ecology 85: 1853-1858.

Oliver, K. M., N. A. Moran, and M. S. Hunter. 2006. Costs and benefits of a superinfection of facultative

   symbionts in aphids. Proceedings of the Royal Society B-Biological Sciences 273: 1273-1280.

Oliver, K. M., P. H. Degnan, G. R. Burke, and N. A. Moran. 2010. Facultative symbionts in aphids and the

   horizontal transfer of ecologically important traits. Annual Review of Entomology 55: 247-266.

Peckarsky, B. L., P. A. Abrams, D. I. Bolnick, L. M. Dill, J. H. Grabowski, B. Luttbeg, J. L. Orrock, S. D.

   Peacor, E. L. Preisser, O. J. Schmitz, and G. C. Trussell. 2008. Revisiting the classics:

   Considering nonconsumptive effects in textbook examples of predator-prey interactions. Ecology

   89: 2416-2425.

Phelan, P. L., M. E. Montgomery, and L. R. Nault. 1976. Orientation and locomotion of apterous aphids

   dislodged from their hosts by alarm pheromone. Annals of the Entomological Society of America

   69: 1153-1156.

Preisser, E. L., and D. I. Bolnick. 2008. The many faces of fear: Comparing the pathways and impacts of

   nonconsumptive predator effects on prey populations. PLoS One 3: 8.

Roitberg, B. D., and J. H. Myers. 1979. Behavioral and physiological adaptations of pea aphids

   (Homoptera, Aphididae) to high ground temperatures and predator disturbance. Canadian

   Entomologist 111: 515-519.

Roitberg, B. D., J. H. Myers, and B. D. Frazer. 1979. Influence of predators on the movement of apterous

   pea aphids between plants. Journal of Animal Ecology 48: 111-122.

Siers, S. R., R. N. Reed, and J. A. Savidge. 2016. To cross or not to cross: modeling wildlife road

   crossings as a binary response variable with contextual predictors. Ecosphere 7: 19.

Sitvarin, M. I., A. L. Rypstra, and J. D. Harwood. 2016. Linking the green and brown worlds through

   nonconsumptive predator effects. Oikos 125: 1057-1068.

Whalen, R., and J. P. Harmon. 2012. Rag1 aphid resistant soybeans alter the movement and distribution of soybean aphid (Hemiptera: Aphididae). Environmental Entomology 41: 1426-1434.

Wohlers, P. 1981. Effects of the alarm pheromone (E)-β-farnesene on dispersal behaviour of the pea aphid *Acyrthosiphon pisum*. Entomologia Experimentalis et Applicata 29: 117-124.

Wyckhuys, K. A. G., R. L. Koch, and G. E. Heimpel. 2007. Physical and ant-mediated refuges from parasitism: Implications for non-target effects in biological control. Biological Control 40: 306-313.

APHID BEHAVIORAL RESPONSES TO HEAT SHOCKS DO NOT AFFECT SHORT-TERM APHID FITNESS[3]

Abstract

Disturbances such as heat shocks induce animals to change their behaviors. Often, these behaviors lessen the effects of the disturbance and benefit the animal. However, in many cases the benefits of the behavior are assumed rather than explicitly tested. Many insects respond to disturbances from heat shocks, or brief periods of extremely high temperatures, with a variety of behaviors. However, the benefits of those behaviors are not always clear. We recorded the behaviors of pea aphids in response to heat shocks and tested whether these behaviors have short-term fitness effects for the aphids. Most heat shocked aphids became more active and moved to the actively growing leaves of their host plants. However, this movement provided no clear immediate benefits to the aphids; those that moved to the actively growing leaves had the same survival and produced the same number of offspring as those that remained on older leaves or stems. It is possible that the behaviors we observed are a general response to any disturbance, but that benefits are only realized with certain types of disturbances. These findings show that behaviors induced by disturbances do not always provide immediate benefits to the animal.

Key words: aphid, behavior, heat shock, movement, thermoregulation

Introduction

Animals respond to a wide range of extreme abiotic conditions and disturbances by changing their behavior. Heat shocks, which are few-hour periods of stressfully high temperature, are a major type of abiotic disturbance (Williams et al. 2016). Climate change predictions indicate that heat shocks will increase in frequency, severity, and duration (Williams et al. 2016). These climate change effects will increase the overall ecological effects from heat shocks. Heat shocks can push animals beyond the limits of their temperature tolerance (Williams et al. 2016). Additionally, the rapid temperature changes of heat

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shocks induce major physiological stress (Liang et al. 2017). As a result, heat shocks can cause major

reductions in fitness and even death (Williams et al. 2016). These effects are not limited to individuals;

they also ripple through food webs (Barton and Schmitz 2009, Harmon et al. 2009). Developing a more

comprehensive understanding of these behavioral and ecological effects of heat shocks is important to

predict the effects of climate change.

The behavioral changes stimulated by heat shocks can affect an animal's fitness, either

negatively through their costs or positively by mitigating the harmful effects of the disturbance (Nelson et

al. 2004, Nelson 2007, reviewed in Harmon and Barton 2013). In many cases, ecologists have

demonstrated the behavior's beneficial effects (Semtner et al. 1971, Snucins and Gunn 1995). However,

the behaviors are sometimes assumed to be beneficial without clear testing (Kreuger and Potter 2001,

Ma and Ma 2012 a, b). Such induced behaviors may be general responses to a wide range of

disturbances (Ikegawa et al. 2014). However, these non-specific behaviors may be generally

advantageous when considered over all disturbances that can stimulate them, but provide no benefits

with specific disturbances (Ikegawa et al. 2014). Therefore, assuming a general response is beneficial in

the context of a single specific disturbance may be inappropriate, and may lead to misinterpretations of

an animal's ability to tolerate a specific disturbance. Making the full connection from disturbance to

behavior to fitness effects of the behavior is essential to understanding the function of the behavior as

either a general or a specific response.

The life history of a given animal may constrain what behaviors can be used to evade heat

shocks. Intertidal species, such as limpets, shrimp, and sea urchins, are subjected to extreme increases

in temperature when tides recede and they are left exposed to the sun, and many rely on physiological

rather than behavioral mechanisms to tolerate the heat shock (Monaco et al. 2016, Ravaux et al. 2016,

Drake et al. 2017, Vergara-Amado et al. 2017). Similarly, marine and aquatic organisms that undertake

diel vertical migrations are subjected to extreme swings in temperature as they migrate through a

thermocline from cold deep water to warm surface water or vice versa (Elder and Seibel 2015). In these

species, the behavior – diel vertical migration – is the cause of the abrupt thermal stress, but they have

physiological mechanisms for tolerating such rapid and extreme swings in temperature (Faria et al. 2017).

Conversely, a species may use behaviors to moderate the temperatures it actually experiences. For

example, perching dragonflies assume an obelisk posture to reduce their exposure to sunlight, while flying dragonflies can increase hemolymph circulation through the abdomen to dissipate heat or switch to a gliding-dominated flight to reduce metabolic heat production (May 1976). Similarly, female mosquitoes can dissipate heat from hot blood meals by evaporative cooling of droplets of fluid emitted from the anus (Lahondere and Lazzari 2012). There is limited evidence that certain aphids may dissipate heat in a similar manner by means of "honeydew panting" (Paul 1975), but otherwise their sedentary lifestyle and continuous feeding behavior contrast with behaviors that mitigate the effects of heat shocks. These examples illustrate that an animal's life history may limit its ability to minimize exposure to heat shocks, and a combination of physiological and behavioral responses are often employed.

Many ectotherms, including a variety of insects, rely on behaviors to modulate exposure to unsuitable temperatures (May 1979). Moving to cooler microhabitats is a widespread mechanism to avoid extreme heat. This behavior has been documented in several species of insects, lizards, fish, spiders, and ticks (May 1979, reviewed in Harmon and Barton 2013). For example, grain aphids become more active and drop from their host plants during heat shocks (Ma and Ma 2012 b). Ma and Ma (2012 b) speculated that a temperature difference between the top of the plant and the base of the plant was sufficient that dropping allows aphids to evade the extreme temperatures of the heat shock. Conversely, Roitberg and Myers (1979) drew attention to the huge risks of dropping in hot conditions for pea aphids, especially if the ground is hot and dry, because aphids may succumb to heat and/or dehydration before they find a new host plant. Additionally, aphids must balance their tendency to drop with the costs from interrupted feeding that can negatively affect their fitness (Nelson 2007, Gish et al. 2012). While dropping pea aphids can reliably land on their feet (Ribak et al. 2013), we are unaware of any evidence that they can direct the trajectory of their fall to increase the probability of landing on a lower leaf or stem. If aphids do experience more moderate temperatures by dropping, then dropping may limit the harmful effects of the heat shock and provide fitness benefits. Therefore, more explicitly measuring the fitness effects of heat escape behavior will provide a greater understanding of the ecological effects of heat shocks on these insects.

We investigated the behavioral responses of the pea aphid, *Acyrthosiphon pisum* Harris, to heat shocks in a series of laboratory experiments. We compared the behaviors of aphids in heat shocks to the

behaviors of aphids under constant, mild, ambient temperatures. In particular, we hypothesized that aphids would move down toward the soil in a manner similar to that observed in grain aphids (Ma and Ma 2012 b). Therefore, we examined where on the plants the aphids were located and how many aphids were walking at several time points during and 15 minutes after heat shocks. We also measured how these behaviors affected aphid fecundity and survival following heat shock as an indicator of the behaviors' effectiveness at mitigating the harm of the heat shock. Based on the results of the behavioral observations, we conducted a second experiment to measure the effects of settling location during and after heat shocks on the short-term fecundity of the aphids.

Methods

Study System

We conducted these experiments using pea aphids, *Acyrthosiphon pisum* Harris (Hemiptera, Aphididae), on fava bean plants, *Vicia faba* L., var. Broad Windsor. Heat shocks have been shown to have serious detrimental effects on pea aphids (Russell and Moran 2006). These effects include increases in development time and mortality, and strongly reducing fecundity, either sterilizing aphids or reducing their reproductive output (Russell and Moran 2006). We used adult aphids because they show stronger behavioral responses than nymphs to several types of predator disturbances, presumably because they incur relatively lower costs by dropping or fleeing from a disturbance (Gerling et al. 1990, Gish et al. 2012).

Pea aphid susceptibility to heat shocks can vary with heritable characteristics of the aphids, which we controlled for by using a single aphid clonal line with uniform symbiont composition. A common mutation in a gene for heat shock proteins in *Buchnera aphidicola*, an obligate endosymbiont of pea aphids, makes the aphid more sensitive to heat shocks (Burke 2010). Additionally, infection with vertically-transmitted facultative symbionts, especially *Serratia symbiotica*, makes aphids more tolerant of heat shocks (Montllorr et al. 2002, Russell and Moran 2006, reviewed in Oliver et al. 2010). Because endosymbionts are reliably transmitted from mother to daughter aphids, they are analogous to heritable traits that are subject to the forces of natural selection (Moran 2007). The prevalence of *S. symbiotica* in pea aphids varies over time and space, with greater proportions of aphids harboring *S. symbiotica* after hot summers (Montlorr et al. 2002). Aphids from regions with different local climates also exhibit different

sensitivities to heat (Roitberg and Myers 1979). These variations in aphids' tolerance of heat shock suggest that climate is a major evolutionary driver for pea aphids. Because pea aphids are strongly affected by heat shocks and show evolutionary responses to these effects, they provide a strong study system for investigating the role of behavior in mediating heat shock effects.

All aphids used in these experiments were reared in the laboratory at $25\pm1^{\circ}$C. They were housed communally in 40 cm X 40 cm X 40 cm mesh cages under L16:D8 photoperiod. All aphids belonged to a single clone (line 82B-AB) of genetically identical pink-morph aphids that harbored no known facultative symbionts and primary symbionts that lacked the heat susceptibility mutation. Pea aphid behaviors and sensitivity to heat shocks can vary with genotype and symbiont infections (Braendle and Weisser 2001, Montllor et al. 2002, Russell and Moran 2006, Dion et al. 2011). Therefore, using a single aphid line without known secondary symbionts minimized the sources of variation of behavior and heat shock effects within our experimental treatments. This aphid line was obtained from researchers at the University of Georgia in summer of 2013, and were sustained in culture in our laboratory for ~100 generations before conducting these experiments.

We used features of the aphids' host plant, fava bean, as landmarks for measuring aphid location. Each plant typically consists of a central stem that arises from the seed, with pairs of leaves arising from nodes along the stem. The actively growing leaves occur at the tips of stems and often hold the densest aggregations of aphids in our colonies. We defined these actively growing leaves to include the most distal one or two pairs of leaves. Actively growing leaves are still not full-sized, are oriented vertically rather than horizontally, and are folded lengthwise at the mid-vein so that the dorsal surfaces of the leaf oppose each other. Mature leaves that we did not consider to be part of the actively growing leaves had a more horizontal orientation, were either flat or slightly rolled with the ventral side of the leaf inside the roll, and had achieved their maximum size (Figure 9). In a companion paper, we showed that aphids are more commonly found on actively growing leaves after certain simulated predator attacks (Chapter 2).
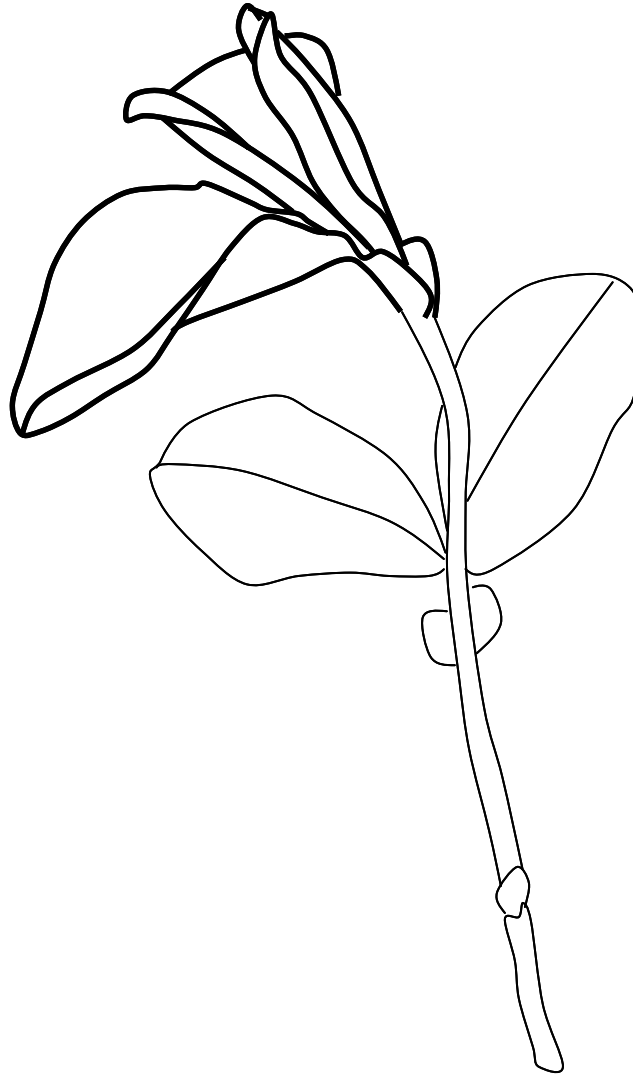
Figure 9. Diagram of a fava bean plant. The actively growing leaves as we've defined them for these experiments are the curled, developing leaves at the tip of each stem, illustrated with **bold** lines.

Behavioral Responses to Heat Shocks

We measured the behavior of pea aphids on fava bean plants during artificial heat shocks and

compared these behaviors between heat shocked aphids and control aphids that remained in ambient

laboratory temperatures. We focused on movement behaviors, measuring locations of aphids and aphid

walking activity.

Fifteen adult apterous aphids were distributed over all leaves of a potted fava bean plant fourteen

days after the seed was planted and allowed to settle for one hour at 25.3°C under fluorescent grow

lights. Plants harboring control aphids remained in the laboratory where they settled during the

observations, while plants infested with heat shock aphids were moved into a heated growth chamber to

apply the heat shock for 75 minutes. 4 hours prior to placing aphids, the potted plants were distributed between 8 potting trays (four for heat shock, four for control), and pots full of potting soil, but without plants, were added to fill the empty space in each tray. All pots were watered generously by filling the trays with water and allowing it to soak through the pot drain holes into the soil, so that by the time the experiments were run the soil surface was damp and evaporative cooling from the soil surface would create a vertical temperature gradient. We did not measure relative humidity, but would expect it to vary according to distance to soil surface and distance from the plants, which were likely transpiring moisture. Every fifteen minutes, the locations of all aphids on each plant were recorded. We recorded the number of aphids at each cm interval along the vertical length of the plant and the number of aphids on and off the actively growing leaves, which are readily colonized by manually disturbed aphids (Chapter 2). Finally, we recorded the number of aphids that were walking on each plant at each fifteen minute interval. These experiments were conducted over four experimental blocks, each run on separate days, which each included 8 aphid-infested plants that experienced heat shocks of 35.7 ˚C and 8 aphid-infested control plants that remained in the lab at 25.2 ˚C.

To confirm the temperatures that heat shock and control aphids experienced, we recorded temperatures during the heat shocks. Temperatures were recorded at 5 cm, 10 cm, and 15 cm above the soil surface every five minutes with Onset HOBO pendant temperature loggers (Onset, Bourne MA) strung on a bamboo stake placed within 5 cm of a plant. The highest of these measurements occurred even with or just below the apical meristems of the plants, while the lowest corresponded with the lowest leaf node of the plants. Temperatures remained constant for control aphids at 25.2 ˚C, whereas heat shocked aphids experienced a temperature increase from 25.2˚C to 35.7˚C (averaged over heights and experimental blocks) over a period of 30-35 minutes after being moved to the heat shock chamber. After this time, temperatures stabilized in the heat shocks at 35.7˚C. Moreso in the heat shock treatment than in the control treatment, we observed a vertical temperature gradient, with higher temperatures near the top of the plant than near the base of the plant (Table 5). During each experimental block, we used three temperature loggers at each height in each treatment; as a result, our temperature measurements lack the replication that would be needed to determine if there are significant differences between

temperatures at different heights. Our heat shock temperatures are consistent with temperatures that have been shown to harm pea aphids (Russell and Moran 2006, Harmon et al. 2009).

Table 5. Temperatures at each height in behavioral responses to heat shocks experiment.

Height above soil surface	5 cm	10 cm	15 cm
Control Temperature	25.08°C	25.11°C	25.40°C
Heat Shock Temperature	35.14°C	35.78°C	36.33°C

After temperature stabilized, temperature differences between 15 cm and 5 cm ranged from 0.6-1.7°C for heat shocked aphids and from 0.1-0.7°C for control aphids. These ranges span all blocks and all time periods after 35 minutes. Ma and Ma (2012a) showed that aphids moving along temperature gradients of 26°C-43°C oriented toward cooler temperatures at >40°C when acclimated at 25°C. If pea aphids exhibited a similar behavior, we would expect them to stay below a certain maximum temperature in a thermal gradient. The maximum temperature difference over the length of our plants was considerably smaller less than that shown by Ma and Ma (2012a), and the peak temperature we used was less than that which aphids would not cross in their thermal gradient experiment. Therefore, we would not expect our aphids to be excluded from any plant parts by a distinct thermal barrier.

All researchers took precautions to avoid inadvertently disturbing the aphids as they were observed. To reduce the risk that observer breath affected the aphid behavior (Gish et al. 2010), all researchers wore dust masks to block their breath and reduce the risk of accidentally inducing dropping by breathing on the aphids as we observed them. Additionally, vibrations moving through the plant can stimulate aphids to move or drop (Clegg and Barlow 1982), so researchers were careful to not touch plants. To facilitate this, small mirrors were used to count aphids in difficult to view plant parts, such as under the lower leaves.

Analysis of Behavioral Responses to Heat Shocks

We used ANOVA to investigate differences in aphid height above the soil surface in two ways. First, we calculated the average height of all aphids for each plant at the beginning of the heat shock and at the end of the heat shock. Despite our efforts to minimize plant variation – planting three times as many plants as we used at a time and selecting the most uniform plants, planting all plants for each experimental block at the same time and soil depth, and growing all plants in the same greenhouse

conditions, the plants ranged in total height from 16 cm to 27 cm, with an average of 21.5 cm ± 0.4 cm (SEM). To correct for variation in plant height, these average aphid heights were divided by plant heights to convert all measurements into proportions. To turn these location measurements into movement measurements, we then subtracted the proportional average height at the beginning of the heat shock from the proportional average height at the end of the heat shock. These differences in proportional average heights were normally distributed, so these differences were used in the ANOVA without transformation. Factors included in the ANOVA included treatment (heat shock or control) and block. Significant results were further resolved using Tukey's HSD.

We conducted a follow-up analysis that offered a more detailed view of vertical movement of aphids. We divided each plant vertically into quarters and summed the number of aphids on each plant quarter at the beginning and end of the heat shock. We subtracted the beginning plant fourth sums from the end plant fourth sums to calculate the change over the course of the heat shock. We then entered these numbers into a MANOVA and four separate ANOVAs to distinguish separate levels; one each for the first (bottom) fourth of the plant, second fourth of the plant, etc. to test for treatment and block effects (Hunt et al. 2004).

In addition to height, we considered changes in the proportion of aphids occupying the actively growing leaves as a response to heat shocks. Aphids tend to aggregate on these plant parts, which provide a different nutritional resource for aphids than older leaves (Gould et al. 2007, Chapter 2). Therefore, actively growing leaves may attract or retain aphids that are disturbed by the heat shock. For each plant, we calculated the proportion of aphids that were on the actively growing leaves at the beginning and at the end of the heat shock. We then subtracted the number at the beginning from the number at the end to determine net changes in the proportion of aphids on the actively growing leaves. We used ANOVA to compare the changes in aphids on actively growing leaves between the control and heat shocks and check for block effects. Differences in proportions of aphids on actively growing leaves from the beginning to end of the experiment included some negative values that precluded square root, arcsine square root, and log transformations. However, residuals with raw data did not strongly differ from a normal distribution, so we did not transform the data to run the ANOVA. We used Tukey's HSD to contrast significant results.

Both of our measures of aphid location can indicate net aphid movement on the plant, but may not fully capture aphid activity. To better account for overall activity, we counted the number of aphids that were walking on each plant every 15 minutes during the heat shock period. For each plant, we added together the number of aphids we observed moving over each of the six observations, and tested for differences between heat shock and control aphids and block effects with ANOVA. We used Tukey's HSD to further examine significant results. All analyses were conducted in R v. 3.2.0.

Fitness Effects

Based on the behaviors we observed in the first experiment, we conducted a second experiment to determine the fitness consequences of movement onto actively growing leaves during and after heat shocks. If this behavior provides benefits to aphids, we would expect aphids on actively growing leaves during heat shocks to have greater survival and/or reproduction than aphids off s. Alternatively, aggregation on actively growing leaves may occur spontaneously (Gould et al. 2007), and simply be accelerated by the heat shock inducing aphids to move. If movement to actively growing leaves during heat shocks is a general response to disturbances (Gould et al. 2007, Chapter 2), it may or may not benefit aphids during a heat shock.

Determining whether movement to actively growing leaves during heat shocks has fitness effects for aphids was accomplished by haphazardly selecting aphids from actively growing leaves and from older leaves during heat shocks and tracking their fecundity and survival. Nutritional differences of the actively growing leaves may help aphids to withstand the stress of a heat shock (Gould et al. 2007), or the partially closed leaves and more complex architecture may create a sheltered microhabitat. For aphids to benefit from these hypothetical effects, the aphid would need to be on the actively growing leaves during the heat shock.

We devised an experiment to assess whether movement to actively growing leaves during heat shocks benefits aphids, and whether benefits depend on being on actively growing leaves during the heat shock or settling there afterward. We used a 2X2 factorial design; aphids on or off actively growing leaves during a heat shock, and aphids restrained on or off actively growing leaves for two days after a heat shock (Table 6).

Table 6. Treatments and sample sizes from the fitness effects experiment.

Factors	On actively growing leaves during heat shock	Off actively growing leaves during heat shock
On actively growing leaves after heat shock	n=34	n=32
On first leaf pair after heat shock	n=33	n=35

Depending on whether aphids moved to actively growing leaves during the heat shock and where they were confined after the heat shock, aphids belonged to one of four treatments.

To accomplish the first factor of the experiment, we conducted a heat shock using the same methods as in the behavioral responses experiment and collected aphids from actively growing leaves and older plant parts at the end of the heat shock. This design allowed aphids to sort themselves into the treatments of the first factor of the experiment; we did not manipulate where aphids were on plants during the heat shock. Therefore, differences observed between aphids on or off actively growing leaves during the heat shock may reflect innate differences between individual aphids rather than differences due to location. Conversely, the plant parts where aphids were confined after the heat shock were alternately determined by the researchers. Therefore, any differences inherent to the aphids should be equally represented in aphids on and off actively growing leaves after the heat shocks. This design helps to distinguish the effects of being on actively growing leaves during heat shocks from the effects of being on actively growing leaves in general.

This experiment was conducted over three experimental blocks run on separate days. Each block included eight plants infested with fifteen aphids each in the heat shock. After the heat shock, 22-23 of the aphids were collected from on actively growing leaves and from off actively growing leaves. These were transferred to new plants, 1 aphid per plant, and contained either on or off actively growing leaves with mesh bags. More aphids were exposed to heat shocks than were included in the full study because we wanted to balance our treatments, and equal ratios of aphids on and off actively growing leaves during heat shocks were not assured. By heat shocking surplus aphids, we ensured that all treatments could be filled in each block of the experiment.

Analysis of Fitness Effects

We used a paired T-test to compare the proportions of aphids on actively growing leaves at the beginning and end of the heat shock. In the behavioral response to heat shocks experiment, we found that the proportion of the aphids that were on actively growing leaves increased over the 75 minute

experimental period more in heat shocks than controls. We confirmed that aphids in this experiment repeated the pattern of movement to actively growing leaves by recording the number of aphids on and off actively growing leaves just before and at the end of each heat shock. We compared the proportions of aphids on actively growing leaves before and at the end of the heat shocks with a paired t-test, and used ANOVA to check for block effects. Because we did not include control aphids that did not experience heat shocks in this experiment, we could not compare movement of heat shock aphids to control aphids. Instead, we used a paired T-test to test whether or not there was a net change in location by comparing the proportion of aphids on actively growing leaves at the beginning of the heat shock and at the end of the heat shock.

We recorded two measures of aphid fitness: numbers of nymphs produced and mortality. We analyzed reproduction with ANOVA. We included experimental block, location during the heat shock, and location after the heat shock as fixed effects. Because aphids experienced heat shocks in batches of fifteen aphids per plant, and plant effects could be shared by these aphids, we included the heat shocked plant as a random effect to account for this grouping.

We analyzed location effects on mortality with a logistic mixed model. Because mortality was a binary response (aphids are either dead or alive), it could not be analyzed with ANOVA like reproduction. In our model, we included location during heat shock, location after heat shock, and experimental block as fixed effects and heat shock plant was a random effect (Siers et al. 2016). We tested for significance of the coefficients produced by the model for effects of location during and after heat shocks by repeating the model with those factors excluded, and comparing these iterative models using likelihood ratio tests.

<div align="center">

Results

</div>

Behavioral Responses to Heat Shocks

Because fava bean plants provide aphids with a tall, narrow habitat, the vertical position of aphids at multiple time points allowed us to measure their movement. Aphids responses varied between experimental blocks, but overall heat shocked aphids were more active than control aphids and generally aggregated on actively growing leaves and about three fourths of the way up their host plants. For each plant, we took the average height of all aphids, and standardized it for plant variation by dividing by the plant height. Therefore, aphid height is measured in units of plant height. For example, if a 20 cm plant

had an aphid at 2, 5, 6, 8, 12, and 16 cm, we would calculate the average aphid height as

((2+5+6+8+12+16)/6)/20 = 8.167/20 = 0.408. This average aphid height would indicate that on average, the aphids are 40.8% of the way up the plant from its base. Net vertical movement of aphids during heat shocks was inconsistent between experimental blocks, and we found a significant treatment*block interaction ($F_{3,55}$=0.0023, P=0.0023). This effect was driven by heat shocked aphids in the first block, whose average height increased by 0.24 ± 0.045 times the plant height over the heat shock period. Treatments had no significant effects in any of the other three blocks. Changes in average heights ranged from descending by 0.040 ± 0.036 times the plant height to ascending by 0.078 ± 0.044 times the plant height in blocks two, three, and four.

When we divided the plant into quarters we developed a more detailed analysis of aphids' changes in vertical height. We conducted a MANOVA and four follow-up ANOVAs, one for each quarter of the plant, to analyze the difference in number of aphids between the beginning and end of the heat shock. Movement patterns between heat shocked and control aphids were considerably different, but also varied over block (Table 7, Table 8) (multivariate analysis of variance (MANOVA); block, Pillai's trace=0.455, $F_{3,55}$=2.42, P=0.0066; treatment, Pillai's trace=0.76, $F_{1,55}$=41.25, P=<0.00001; interaction, Pillai's trace=0.379, $F_{3,55}$=1.95, P=0.032). The number of aphids in the bottom quarter increased for control aphids but stayed nearly constant for heat shocked aphids. The number of aphids in the second quarter increased slightly for control aphids, but dropped for heat shocked aphids. The number of aphids in the third quarter decreased for control aphids, but increased for heat shocked aphids. Changes in the numbers of aphids in the top quarter had significant block effects that affected the magnitude of the changes, but aphids decreased from the top fourth in both heat shocked and control aphids across all treatments.

Aphids naturally track growth of actively growing leaves (Gould et al. 2007), so these plant parts may attract or retain moving aphids. Treatment and block had a significant interaction for movement to the actively growing leaves ($F_{3,55}$=5.07, P=0.0036). Despite variation between blocks for both heat shock and control aphids, the same within-block pattern arose in three of the four blocks. Heat shock aphids aggregated on actively growing leaves more than control aphids in all except the third block where there was no significant difference (Figure 10).

Table 7. Results of MANOVA analysis of the change in aphids in each fourth of the plants.

MANOVA				
	Pillai's trace	F	d.f.	P
block	0.455	2.42	3	0.0066
treatment	0.76	41.25	1	<0.00001
block*treatment	0.379	1.95	3	0.032
residuals			55	

	Block 1	Block 2	Block 3	Block 4
Control				
Bottom	3.5 ± 0.91^{bc}	4.25 ± 0.80^{bc}	3.125 ± 0.57^{c}	2.75 ± 0.96^{abc}
Second	-0.125 ± 1.13^{bc}	0.125 ± 0.99^{bc}	1.625 ± 0.77^{c}	1.875 ± 1.08^{c}
Third	0.25 ± 0.82^{bc}	-3.5 ± 0.63^{ab}	-6 ± 0.99^{a}	-0.25 ± 1.46^{bc}
Top	-4 ± 0.60^{a}	-0.75 ± 0.31^{a}	-0.875 ± 0.14^{a}	-3.25 ± 0.90^{a}
Heat Shock				
Bottom	-0.125 ± 0.30^{a}	-0.25 ± 0.49^{a}	1 ± 0.95^{ab}	1 ± 0.71^{abc}
Second	-5.875 ± 1.32^{a}	-3.625 ± 0.46^{ab}	-2 ± 0.85^{abc}	-3.25 ± 0.92^{ab}
Third	3.625 ± 1.36^{c}	1.5 ± 1.13^{c}	-1.25 ± 1.08^{bc}	0 ± 1.13^{bc}
Top	-0.375 ± 0.96^{a}	-2.125 ± 0.93^{a}	-1.75 ± 1.11^{a}	-1.375 ± 1.12^{a}

Treatments and block had a significant interaction term. Provided values are means ± standard error. Coefficients were derived from post-hoc Tukey's HSD tests on ANOVAs. Because each ANOVA was run on each plant fourth, letters are binned according to plant fourths.

More heat shock aphids than control aphids walked over the plants. As with changes in average aphid height and actively growing leaves colonization, movement exhibited a significant treatment*block interaction ($F_{3,55}$=4.56, P=0.0064). In all blocks except block three, significantly more heat shocked aphids than control aphids walked on the plants (Figure 11). The standard error and means from block three shown in Figure 4 suggest that heat shock and control may be different. However, the heat shock number was biased by a high outlier (8 moving aphids on one plant) that pulled the average up, while the remainder of the data clustered averaged only 2 moving aphids per plant.

Fitness Effects

Aphids behaved similarly during the fitness effects experiment as during the behavioral responses experiment, but we found no effect of aphid location during or after a heat shock on short-term reproduction or survival. The number of aphids on the actively growing leaves increased over the course of the heat shock experiment. The proportion of aphids on the actively growing leaves at the end of the

heat shocks were significantly greater than at the beginning of the heat shocks (t_{23}=2.90, P=0.0081).

There were no significant block effects on the movement of aphids to the actively growing leaves

($F_{2,21}$=0.16, P=0.86). On average 30% of aphids were on actively growing leaves at the beginning of the

heat shock. Aphids on actively growing leaves increased to 45% by the end of the heat shock. This

response matched the aphid response in the behavioral responses to heat shocks experiment.

Table 8. Results of the ANOVAs testing changes in the number of aphids in each quarter of the plant.

Plant quarter	Factor	DF	MS	F	P
Bottom quarter	Block	3	1.034	0.23	0.87
	Treatment	1	152.29	34.46	<0.00001
	Block*Treatment	3	17.27	1.3	0.28
	Error	55	243.09		
Second quarter	Block	3	84.21	3.82	0.015
	Treatment	1	341.24	46.49	<0.00001
	Block*Treatment	3	9.9	0.45	0.72
	Error	55	403.73		
Third quarter	Block	3	78.663	8.11	0.00015
	Treatment	1	160.691	16.57	0.00015
	Block*Treatment	3	18.389	1.9	0.14
	Error	55	9.695		
Top quarter	Block	3	5.87	1.04	0.38
	Treatment	1	7.51	1.32	0.25
	Block*Treatment	3	25.61	4.51	0.0067
	Error	55	5.67		

No significant interaction was found between aphid locations during and after heat shock affecting

nymph production ($F_{1,13}$=0.022, P=0.88). Individually, aphid location during heat shock had no significant

effect on nymph production ($F_{1,13}$=1.63, P=0.22). Similarly, location after heat shock did not affect

reproduction ($F_{1,13}$=0.85, P=0.37) (Figure 12A). Aphids that were on actively growing leaves during heat

shocks produced 6.2±0.87 nymphs if on actively growing leaves after the heat shock and 5.4±0.96

nymphs if off actively growing leaves after the heat shock. Aphids that were off actively growing leaves

during heat shocks produced 5.0±1.0 nymphs if on actively growing leaves after heat shock and 4.4±0.72

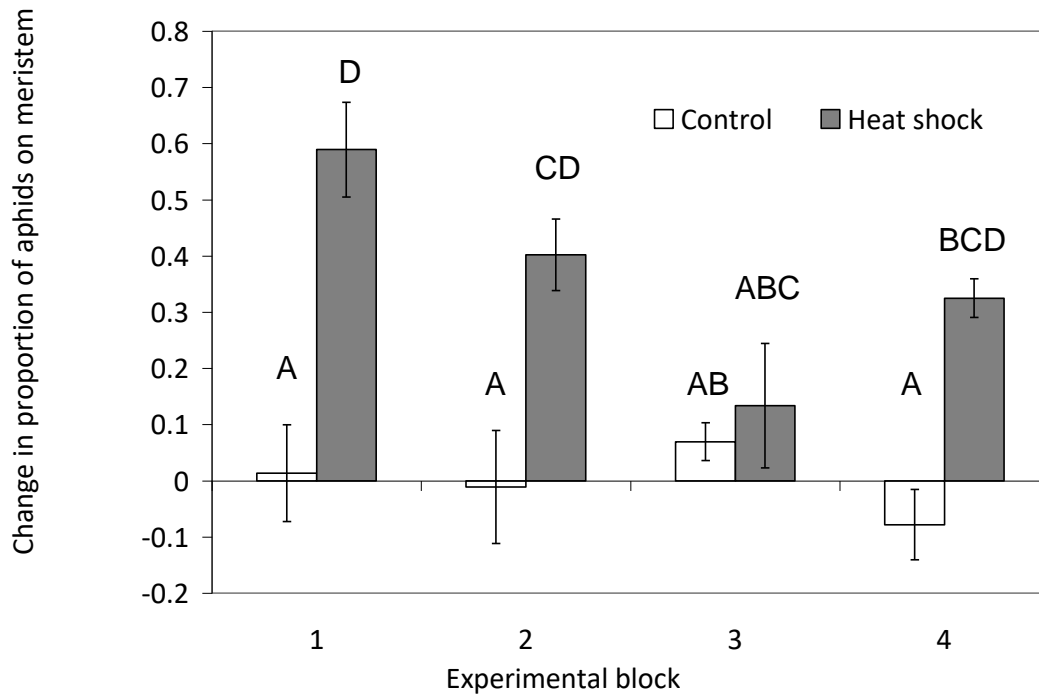if off actively growing leaves after heat shock.

Figure 10. Change in the proportion of aphids on actively growing leaves over the heat shock period. Error bars indicate the SEM. From the beginning to the end of the heat shock, the proportion of aphids on the actively growing leaves of the plant increased significantly more for heat shock aphids than for control aphids in three of the four experimental blocks. Aphids exhibited a net movement onto actively growing leaves during heat shocks. Capital letters indicate significant differences.

If heat shocks were sufficiently severe to increase mortality, then behaviors during heat shocks that mitigate the effect should decrease mortality. However, aphid location during and after heat shocks had no effect on aphid survival (Figure 12B). The logistic mixed model estimated coefficients to account for variation due to locations during and after heat shocks, while accounting for block as a fixed effect and plants that grouped aphids during the heat shock as a random effect. The coefficients for location during heat shock and location after heat shock did not significantly differ from 0 (during: P=0.41; after: P=0.77). Across all treatments, only 42% of aphids were still alive by the end of the experiment.
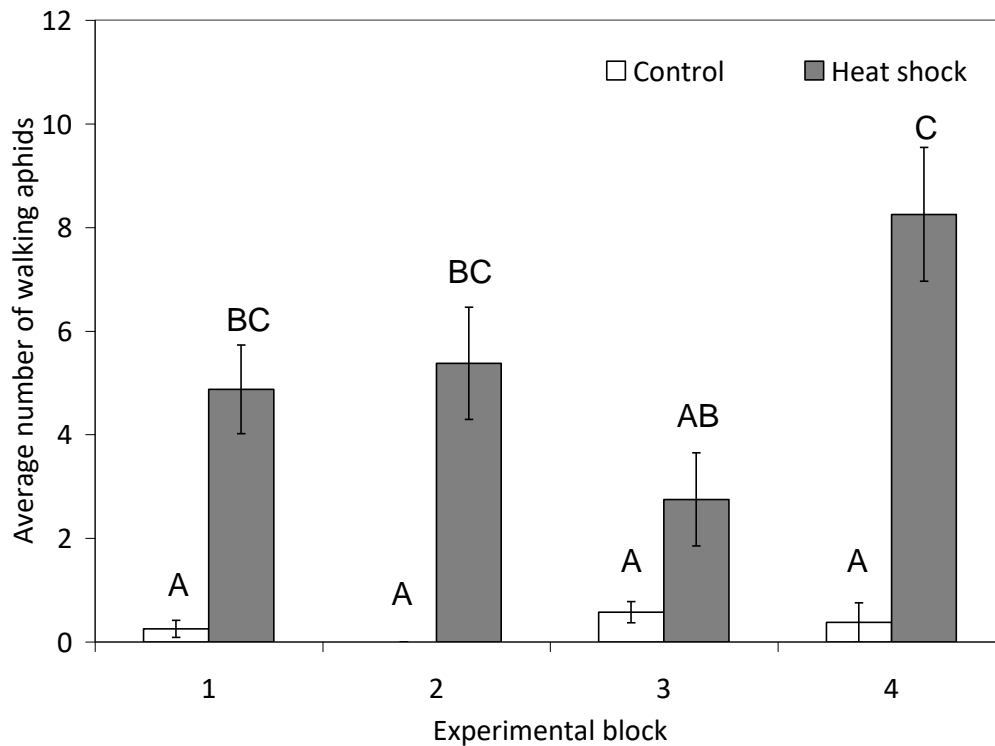
Figure 11. Walking aphids during the heat shock period. More heat shock aphids walked than control aphids. Bar heights indicate the average ± standard error of the total number of aphids observed walking on each plant, with walking aphids counted six times during each heat shock period. Significant differences are indicated with capital letters.

Discussion

Heat shocks had strong effects on aphid behavior, but the behavior did not appear to benefit the aphids' short-term reproduction or survival. Heat shocks induced a large proportion of the aphids on each plant to move and aggregate on the actively growing leaves of their host plants. If movement to the actively growing leaves mitigated the harmful effects of the heat shock, then the aphids that moved to the actively growing leaves would exhibit greater fitness than those that remained off the actively growing leaves throughout the heat shock. However, we found no evidence that moving to the actively growing leaves during a heat shock enhances fitness, either by allowing aphids to reproduce more following the heat shock or by increasing aphid survival after the heat shock. This result held true regardless of where the aphid was located after the heat shock occurred. Furthermore, we found no evidence that occupying actively growing leaves after heat shocks confers any short-term benefits to aphids.

There are several reasons why aphids might move to actively growing leaves during heat shocks. Heat shocks may stimulate a non-directional walking response in pea aphids. The actively growing leaves may then accumulate aphids for a number of reasons. Their tight arrangement of folded leaves and location at tips of plants may create a natural roadblock that causes moving aphids to accumulate. Alternatively, aphids that wander onto actively growing leaves by chance may stop walking upon reaching the actively growing leaves, while wandering aphids continue walking. This pattern would eventually lead to an accumulation of aphids on the actively growing leaves. Aphids may also be innately attracted to actively growing leaves, and orient toward actively growing leaves to resettle. In each of these potential scenarios, any benefits of occupying actively growing leaves could easily be independent of heat shocks, or may even be negated by heat shocks.

We found considerable variation between blocks in the behavioral responses experiment for each of our response variables. This variation may represent subtle differences in the health of the aphids or the structure of the fava bean plants. Adult aphids used in these experiments were pulled from colonies as adults. Any differences in the colonies, such as the overall crowding of the colony, could affect the initial condition of the aphids in the experiment (Tabadkani et al. 2013, Purandare et al. 2014). We also did not explicitly control for aphid size or age, though all aphids were adults. Variation in the plants also may have contributed some variation to the aphids' behavior (Buchman and Cuddington 2009). Plants were grown in a greenhouse, which allows them to be affected by ambient outdoor conditions such as sunlight intensity. Variation in the weather during plant rearing may have affected nutritionally relevant traits in the plant, such as the amino acid content of the phloem sap (Riga 2014). Even external temperature could affect the plants, both by causing minor perturbations in temperature in the greenhouse and by causing the cooling fans to run more and create windier conditions (Bennell and Verbyla 2008).
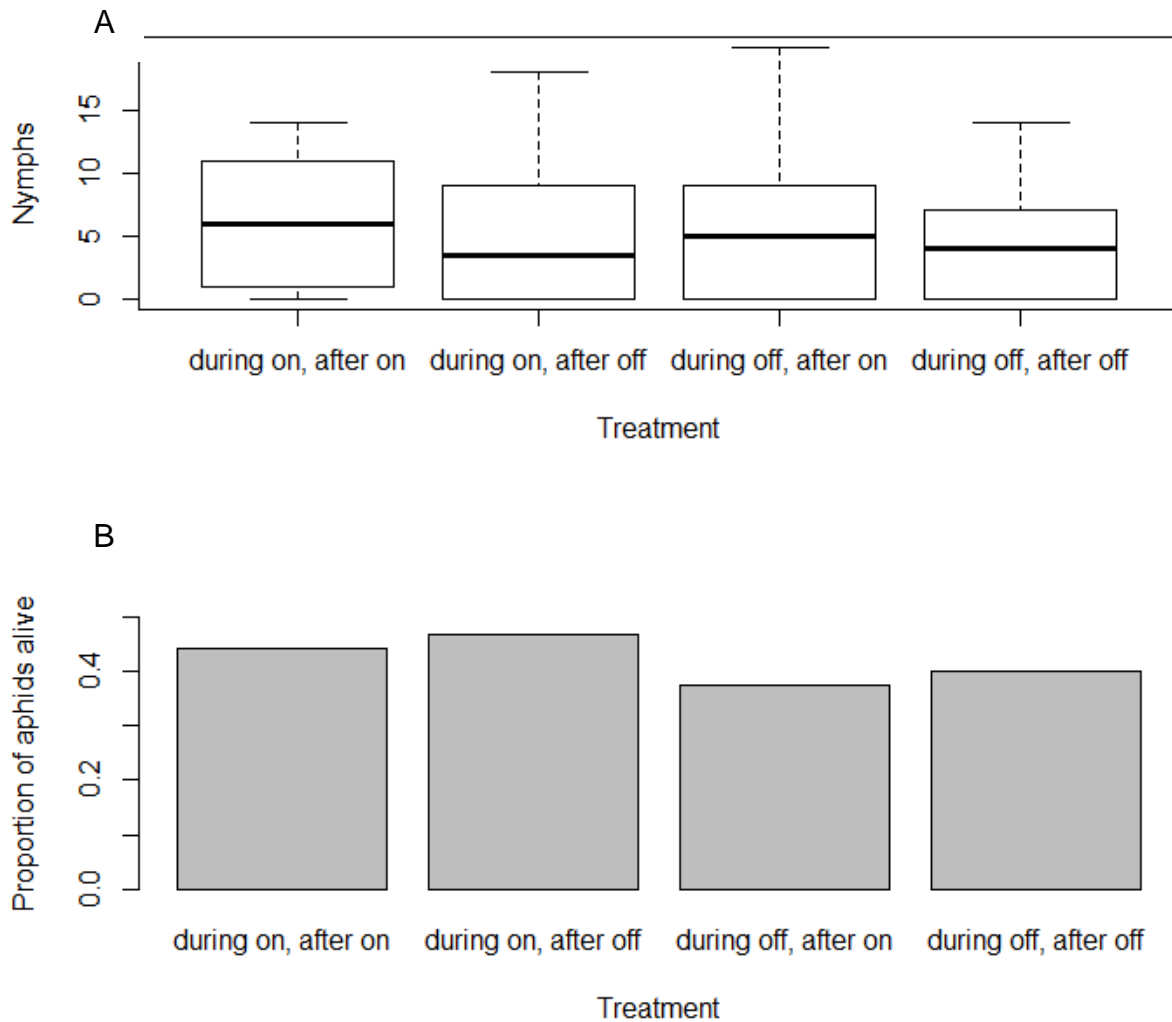
Figure 12. Reproduction and mortality of aphids in different locations during and after heat shocks. **A.** Aphid location during or after heat shocks had no effect on the number of nymphs produced over two days. **B.** The proportion of aphids that survived the full duration of the experiment was also unaffected by aphid location during or after heat shocks.

Despite the variation between blocks, two fairly robust behavior patterns emerge. Heat shocked aphids move to actively growing leaves more than control aphids. This result was consistent in three of four blocks of the behavioral responses experiment and in all blocks of the fitness effects experiment. In the one block that did not have significantly more movement to actively growing leaves in heat shock aphids, the trend was still strongly toward more heat shock aphids moving to the actively growing leaves. Heat shocked aphids also were observed walking more than control aphids. As with the movement to

actively growing leaves described above, a single block did not have a significant difference between heat shocked and control aphids, but the trend was consistent with that pattern. The relative numbers of aphids walking, despite the walking being a mix of downward and upward walking, and aphids on actively growing leaves showed nearly identical patterns, which might indicate that much of the walking we observed contributed to the aphids aggregating on the actively growing leaves.

The patterns of movement to actively growing leaves and changes in vertical height seem contradictory. The number of heat shock aphids on the actively growing leaves increased over the course of the heat shock. With the exception of seven plants, which had lateral branches with actively growing leaves arising from the base of the stem, actively growing leaves were all at the tops of the plants. This is contrary to our measurements of aphid heights, which showed movement out of the top fourth of the plants for both treatments across all blocks. However, the actively growing leaves are not the only available leaf surface at the top of the plant; the leaves just beneath the actively growing leaves also extended into the top fourth of the plant on most plants. Aphids in this region of the plant concentrated on the actively growing leaves or moved to lower parts of the plant.

Despite aphids' typical behavior of tracking actively growing leaves as their host plants grow (Gould et al. 2007), the movement onto actively growing leaves during the heat shock was surprising. In the heat shock, temperatures 15 cm above the soil were slightly warmer than temperatures near the soil. Therefore, movement to actively growing leaves may expose aphids to higher temperatures. Furthermore, another species of aphid, *Sitobion avenae*, drops from its host plant to escape high temperatures (Ma and Ma 2012 b). Therefore, we expected the aphids to drop or walk down the plant.

There are several reasons why the aphids may not have behaved as we expected. We measured temperature using data loggers attached to a stake that was placed in a pot with a plant, but was as far as possible from the plant to avoid obstructing viewing the aphids. Because of the distance between the data logger and the plant, we would be unable to detect any fine-scale variations in temperature or cool boundary layers within a few millimeters of the plant. Because the actively growing leaves are tightly clustered and folded, the aphids on the actively growing leaves can be somewhat enclosed by leaves. Being surrounded by foliage may shelter the aphids from the high temperatures or provide shade from radiant heat. This is especially likely if fava beans have a mechanism for thermoregulating themselves,

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because the plants might create cooler areas (Crawford et al. 2012). For this reason, we cannot ensure that the aphids experienced the exact same temperatures that we measured. Aphids benefited from being on actively growing leaves when they were artificially confined there or moved there in response to mechanical disturbance (Chapter 2). This result may be due to the actively growing leaves having a greater nutritional value to the aphids than older leaves (Gould et al. 2007). However, the aphid's ability to utilize that nutrition may depend on the aphid having a healthy complement of *B. aphidicola*, which provisions aphids with essential amino acids (Moran and Degnan 2006). However, *B. aphidicola* is sensitive to heat shocks, and cells harboring the symbiont are reduced by heat shocks (Montllor et al. 2002). Damage to *B. aphidicola* limiting the aphids' ability to utilize the better resources at the actively growing leaves may explain why aphids on actively growing leaves, either during or after heat shocks, do not benefit from moving to actively growing leaves.

The aphids in our experiment may have failed to respond to the temperature gradient because they were not able to detect subtle temperature differences. Disturbed grain aphids and bird-cherry oat aphids walk along a strong temperature gradient, and do not turn away from the heat until they reach temperatures over 40°C (Ma and Ma 2012 a). If pea aphids behave similarly, then these experiments never exposed them to temperatures high enough to elicit a strong negative thermotaxis response. We also had a far weaker temperature gradient. It is also possible that the temperatures everywhere on the plant were high enough to induce a non-directional walking response, but not high enough to provide a directional stimulus (Ma and Ma 2012 a). While changing the intensity of the heat shock may yield different behavioral responses, we still used temperatures that were sufficiently high to yield strong behavioral responses and consistent with temperatures that have been shown to harm pea aphids (Russell and Moran 2006, Harmon et al. 2009). Alternatively, the plant may have increased its evaporative cooling at the actively growing leaves, creating a cooler microclimate within a boundary layer too narrow for us to measure (Crawford et al. 2012).

This study shows that aphids' behavioral responses to heat shocks do not provide immediate, direct fitness benefits. However, behavior is not the only mechanism by which pea aphids can cope with heat shocks. Pea aphids exhibit local adaptation to climate, with pea aphids from hotter regions tolerating higher temperatures than aphids from cooler regions (Roitberg and Myers 1979, Hazell et al. 2010). This

indicates that pea aphids can evolve to have a greater physiological tolerance for extreme heat. This adaptation may be largely dependent on symbionts; pea aphids infected with *S. symbiotica* have greater tolerance to high temperatures (Montllor et al. 2002, Russell and Moran 2006). Because *S. symbiotica* is vertically transmitted, its effects behave as an extension of the pea aphid genotype, and changes in its abundance in a pea aphid population can arise through natural selection (Moran 2007). Therefore, evolutionary forces affecting both pea aphids and their symbionts that affect their tolerance to heat shocks may be more important than their behaviors to avoid heat shocks (Buckley and Huey 2016).

Climate change is predicted to increase the frequency, severity, and duration of heat shocks in the future (IPCC 2007). Insects that can move to sufficiently cooler microhabitats may be able to mitigate these heat shocks (May 1979). While pea aphids did move in response to heat shocks, they did not move to cooler microhabitats near the soil. Therefore, we have no evidence that their movement evaded the high temperatures of the heat shock. Because pea aphid movement during heat shocks did not benefit them, behaviors are unlikely to help pea aphids escape heat shocks in the field. Instead, pea aphids will rely primarily on evolution of their physiology and their symbionts to tolerate extreme temperatures. Heat tolerance that arises through evolution has finite limitations, both in terms of how rapidly it can occur and how extreme it can become (Chown 2001, Hazell et al. 2010). Therefore, pea aphids may be particularly vulnerable to extreme heat due to climate change.

Behavioral responses to abiotic disturbances have been studied in many systems (reviewed in Harmon and Barton 2013). In addition to characterizing the behavioral response itself, some studies investigated costs of the behavior or ways that the behavior affects other ecological interactions (Fellers 1989, Barton 2010). However, the behavior's ability to mitigate the negative effect of the disturbance has often been assumed rather than explicitly tested (Kreuger and Potter 2001, Ma and Ma 2012 a, b). This study, however, provides an example of a strong behavioral response to a disturbance that does not provide immediate benefits. In Chapter 2, we showed that the severity of a disturbance can affect the aphids' behavioral response and the fitness consequences of that behavior. Here, we expand these findings to show that the overall type of disturbance (heat shock vs. mechanical prodding) also determines aphids' behavioral responses and their fitness effects. Therefore, explicitly studying the

fitness effects of an induced behavior is essential to properly understand its roll in responding to disturbances.

Acknowledgements

References

Barton, B. T. 2010. Climate warming and predation risk during herbivore ontogeny. Ecology 91: 2811-2818.

Barton, B. T., and O. J. Schmitz. 2009. Experimental warming transforms multiple predator effects in a grassland food web. Ecology Letters 12: 1317-1325.

Bennell, M. R., and A. P. Verbyla. 2008. Quantifying the response of crops to shelter in the agricultural regions of South Australia. Australian Journal of Agricultural Research 59: 950-957.

Braendle, C., and W. W. Weisser. 2001. Variation in escape behavior of red and green clones of the pea aphid. Journal of Insect Behavior 14: 497-509.

Buchman, N., and K. Cuddington. 2009. Influences of Pea Morphology and Interacting Factors on Pea Aphid (Homoptera: Aphididae) Reproduction. Environmental Entomology 38: 962-970.

Buckley, L. B., and R. B. Huey. 2016. How extreme temperatures impact organisms and the evolution of their thermal tolerance. Integrative and Comparative Biology 56: 98-109.

Chown, S. L. 2001. Physiological variation in insects: hierarchical levels and implications. Journal of Insect Physiology 47: 649-660.

Clegg, J. M., and C. A. Barlow. 1982. Escape behavior of the pea aphid *Acyrthosiphon pisum* (Harris) in response to alarm pheromone and vibration. Canadian Journal of Zoology-Revue Canadienne De Zoologie 60: 2245-2252.

Crawford, A. J., D. H. McLachlan, A. M. Hetherington, and K. A. Franklin. 2012. High temperature exposure increases plant cooling capacity. Current Biology 22: R396-R397.

Dion, E., S. E. Polin, J. C. Simon, and Y. Outreman. 2011. Symbiont infection affects aphid defensive behaviours. Biology Letters 7: 743-746.

Drake, M. J., N. A. Miller, and A. E. Todgham. 2017. The role of stochastic thermal environments in modulating the thermal physiology of an intertidal limpet, *Lottia digitalis*. Journal of Experimental Biology 220: 3072-3083.

Elder, L. E., and B. A. Seibel. 2015. The thermal stress response to diel vertical migration in the hyperiid amphipod *Phronima sedentaria*. Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology 187: 20-26.

Faria, S. C., R. O. Faleiros, F. A. Brayner, L. C. Alves, A. Bianchini, C. Romero, R. C. Buranelli, F. L. Mantelatto, and J. C. McNamara. 2017. Macroevolution of thermal tolerance in intertidal crabs from Neotropical provinces: A phylogenetic comparative evaluation of critical limits. Ecology and Evolution 7: 3167-3176.

Fellers, J. H. 1989. Daily and seasonal activity in woodland ants. Oecologia 78: 69-76.

Gerling, D., B. D. Roitberg, and M. Mackauer. 1990. Instar-specific defense of the pea aphid, *Acyrthosiphon pisum* – influence on oviposition success of the parasite *Aphelinus asychis* (Hymenoptera, Aphelinidae). Journal of Insect Behavior 3: 501-514.

Gish, M., A. Dafni, and M. Inbar. 2010. Mammalian herbivore breath alerts aphids to flee host plant. Current Biology 20: R628-R629.

Gish, M., A. Dafni, and M. Inbar. 2012. Young aphids avoid erroneous dropping when evading mammalian herbivores by combining input from two sensory modalities. PLoS One 7: 7.

Gould, G. G., C. G. Jones, P. Rifleman, A. Perez, and J. S. Coleman. 2007. Variation in eastern cottonwood (Populus deltoides Bartr.) phloem sap content caused by leaf development may affect feeding site selection behavior of the aphid, *Chaitophorous populicola* Thomas (Homoptera: Aphididae). Environmental Entomology 36: 1212-1225.

Harmon, J. P., and B. T. Barton. 2013. On their best behavior: how animal behavior can help determine the combined effects of species interactions and climate change, pp. 139-147. *In* A. L. Angert, S. L. LaDeau and R. S. Ostfeld (eds.), Climate Change and Species Interactions: Ways Forward, vol. 1297. Blackwell Science Publ, Oxford.

Harmon, J. P., N. A. Moran, and A. R. Ives. 2009. Species response to environmental change: impacts of food web interactions and evolution. Science 323: 1347-1350.

Hazell, S. P., B. P. Neve, C. Groutides, A. E. Douglas, T. M. Blackburn, and J. S. Bale. 2010. Hyperthermic aphids: insights into behaviour and mortality. Journal of Insect Physiology 56: 123-131.

Hunt, J., R. Brooks, M. D. Jennions, M. J. Smith, C. L. Bentsen, and L. F. Bussiere. 2004. High-quality male field crickets invest heavily in sexual display but die young. Nature 432: 1024-1027.

Ikegawa, Y., H. Ezoe, T. Namba, and M. Tuda. 2014. Effects of nonspecific adaptive defense by pests on efficiency of biological control by multiple natural enemies. Journal of the Faculty of Agriculture Kyushu University 59: 305-311.

IPCC. 2007. Climate change 2007: the physical science basis. Climate Change 2007: the Physical Science Basis: 1-996.

Kreuger, B., and D. A. Potter. 2001. Diel feeding activity and thermoregulation by Japanese beetles (Coleoptera: Scarabaeidae) within host plant canopies. Environmental Entomology 30: 172-180.

Lahondere, C., and C. R. Lazzari. 2012. Mosquitoes cool down during blood feeding to avoid overheating. Current Biology 22: 40-45.

Liang, J., Y. Shaulov, C. Savage-Dunn, S. Boissinot, and T. Hoque. 2017. Chloride intracellular channel proteins respond to heat stress in *Caenorhabditis elegans*. PLoS One 12: 15.

Ma, G., and C. S. Ma. 2012a. Effect of acclimation on heat-escape temperatures of two aphid species: Implications for estimating behavioral response of insects to climate warming. Journal of Insect Physiology 58: 303-309.

Ma, G., and C.-S. Ma. 2012b. Climate warming may increase aphids' dropping probabilities in response to high temperatures. Journal of Insect Physiology 58: 1456-1462.

May, M. L. 1976. Thermoregulation and adaptation to temperature in dragonflies (Odonata-Anisoptera). Ecological Monographs 46: 1-32.

May, M. L. 1979. Insect thermoregulation. Annual Review of Entomology 24: 313-349.

Monaco, C. J., D. S. Wethey, and B. Helmuth. 2016. Thermal sensitivity and the role of behavior in driving an intertidal predator-prey interaction. Ecological Monographs 86: 429-447.

Montllor, C. B., A. Maxmen, and A. H. Purcell. 2002. Facultative bacterial endosymbionts benefit pea

aphids *Acyrthosiphon pisum* under heat stress. Ecological Entomology 27: 189-195.

Moran, N. A. 2007. Symbiosis as an adaptive process and source of phenotypic complexity. Proceedings

of the National Academy of Sciences of the United States of America 104: 8627-8633.

Moran, N. A., and P. H. Degnan. 2006. Functional genomics of Buchnera and the ecology of aphid hosts.

Molecular Ecology 15: 1251-1261.

Nelson, E. H. 2007. Predator avoidance behavior in the pea aphid: costs, frequency, and population

consequences. Oecologia 151: 22-32.

Nelson, E. H., C. E. Matthews, and J. A. Rosenheim. 2004. Predators reduce prey population growth by

inducing changes in prey behavior. Ecology 85: 1853-1858.

Oliver, K. M., P. H. Degnan, G. R. Burke, and N. A. Moran. 2010. Facultative symbionts in aphids and the

horizontal transfer of ecologically important traits. Annual Review of Entomology 55: 247-266.

Paul, R. G. 1975. Honeydew panting in *Tuberolachnus salignus* (Gmelin) (Hemiptera: Aphididae).

Entomologist's Gazette 26: 70.

Purandare, S. R., B. Tenhumberg, and J. A. Brisson. 2014. Comparison of the wing polyphenic response

of pea aphids (Acyrthosiphon pisum) to crowding and predator cues. Ecological Entomology 39:

263-266.

Ravaux, J., N. Leger, N. Rabet, C. Fourgous, G. Voland, M. Zbinden, and B. Shillito. 2016. Plasticity and

acquisition of the thermal tolerance (upper thermal limit and heat shock response) in the intertidal

species *Palaemon elegans*. Journal of Experimental Marine Biology and Ecology 484: 39-45.

Ribak, G., M. Gish, D. Weihs, and M. Inbar. 2013. Adaptive aerial righting during the escape of dropping

wingless pea aphids. Current Biology 23: 102-103.

Riga, P. 2014. Flower abscission in pepper plants grown under different regimes of nitrogen fertilization

and photosynthetically active radiation. Journal of Plant Nutrition 37: 907-927.

Roitberg, B. D., and J. H. Myers. 1979. Behavioral and physiological adaptations of pea aphids

(Homoptera Aphididae) to high ground temperatures and predator disturbance. Canadian

Entomologist 111: 515-519.

Russell, J. A., and N. A. Moran. 2006. Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. Proceedings of the Royal Society B-Biological Sciences 273: 603-610.

Semtner, P. J., R. W. Barker, and J. A. Hair. 1971. Ecology and behavior of lone star tick (Acarina-Ixodidae) .2. Activity and survival in different ecological habitats. Journal of Medical Entomology 8: 719-&.

Siers, S. R., R. N. Reed, and J. A. Savidge. 2016. To cross or not to cross: modeling wildlife road crossings as a binary response variable with contextual predictors. Ecosphere 7: 19.

Snucins, E. J., and J. M. Gunn. 1995. Coping with a warm environment – behavioral thermoregulation by lake trout. Transactions of the American Fisheries Society 124: 118-123.

Tabadkani, S. M., S. M. Ahsaei, V. Hosseininaveh, and J. Nozari. 2013. Food stress prompts dispersal behavior in apterous pea aphids: Do activated aphids incur energy loss? Physiology & Behavior 110: 221-225.

Vergara-Amado, J., A. X. Silva, C. Manzi, R. F. Nespolo, and L. Cardenas. 2017. Differential expression of stress candidate genes for thermal tolerance in the sea urchin *Loxechinus albus*. Journal of Thermal Biology 68: 104-109.

Williams, C. M., L. B. Buckley, K. S. Sheldon, M. Vickers, H. O. Portner, W. W. Dowd, A. R. Gunderson, K. E. Marshall, and J. H. Stillman. 2016. Biological impacts of thermal extremes: mechanisms and costs of functional responses matter. Integrative and Comparative Biology 56: 73-84.

CONCLUSIONS AND FUTURE DIRECTIONS

This manuscript explores the roles of behavior in the ecology of competition between aphid parasitoids, host-parasitoid interactions, and sublethal biotic and abiotic disturbances of aphids. The laboratory experiments presented here build on pre-existing bodies of knowledge, but an appropriate next step for each of the studies conducted here would be to repeat them in a field setting to see if the results are repeatable or relevant in a natural environment. Despite the laboratory-only limitations of these studies, they test novel hypotheses that add to the nuance and detail of these broader ecological topics.

In chapter one, we showed that *Lysiphlebus testaceipes* and *Binodoxys communis*, two parasitoids of the soybean aphid, are unable to distinguish between unparasitized soybean aphids and aphids parasitized by the other species. Furthermore, we were unable to show that *B. communis* even responds to conspecific parasitism cues or that either parasitoid uses soybean aphid defensive behavior as an indicator of parasitism status. We also provide evidence that suggests that *L. testaceipes* relies more heavily on cues deposited directly on a parasitized aphid than on cues that affect an entire foraging patch to guide its foraging behavior.

We hypothesized that a prerequisite for different parasitoid species to respond to one another's parasitism marks is that the marks are either essentially the same between the two species, or the species share an evolutionary history that would allow them to coevolve their marks and foraging behaviors. This hypothesis helps to frame our explanation of our findings, but we have not tested it directly. If *B. communis* establishes in North America or if the two were reared in association with one another for several years, it would be interesting to repeat the first experiment conducted here to see if the parasitoids coevolve and develop an ability to respond to one another's marks.

Still another question that remains unanswered by chapter one is the role that larval competitiveness plays in determining an adult parasitoid's responses to host marks left by other species of parasitoids. We do not know if *L. testaceipes* or *B. communis* larvae have any competitive advantage over one another in multi-parasitized hosts, but in some other parasitoid systems (e.g. *Aphidius ervi* and *Praon pequodorum*), one parasitoid species has larvae that are strongly dominant over similar-aged larvae of the other species (Schellhorn et al. 2002). From our literature review, we have noticed a trend that parasitoids with strongly competitive larvae tend to be unresponsive to marks of parasitoids with less

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competitive larvae. Therefore, another intriguing direction this research could take is to compare

parasitoid larval competitiveness, especially in a system with one host that is shared by many species of

parasitoids, and comparing outcomes of larval competition with adult oviposition decisions. From these

comparisons, we could build a more robust theory on whether parasitoid larval competitiveness and adult

discriminatory behavior are correlated.

In chapter two, we examined where on a plant pea aphids resettle after a simulated predator

attack and how that new location affects their short-term fitness. Because escape responses are

metabolically costly, even a non-lethal predator encounter can be costly to the aphids (Nelson 2007). We

varied the attacks to run the experiments twice, once with walker aphids and once with dropper aphids.

The more we disturbed walkers, the more of them settled on actively growing leaves at the tips of the

plants. These leaves offer the aphids short-term fitness benefits, as aphids that occupy them produce

more offspring than aphids on older leaves. Despite our expectations that more disturbances would have

detrimental effects on the aphids' fitness, walkers produced slightly more offspring with more

disturbances. This effect was very small, but in the opposite direction of what we expected. We argue that

this result arose because the increased frequency of occupying high-quality microhabitat (actively

growing leaves) offset the costs of the disturbance. On the other hand, droppers did not significantly vary

their frequency of occupying actively growing leaves or the number of offspring they produced with the

number of disturbances.

Perhaps the greatest weakness of this chapter was the short duration of the experiments. We

measured aphid reproduction over only two days, and did not account for fitness effects that may have

arisen later in life. Our experiments accounted for reproductive decisions of the aphid (e.g. lay a nymph or

retain it), but not for physiological or nutritional consequences that may have impaired or enhanced the

aphids' ability to produce embryos over time. Adjusting the experimental methodology to make it

logistically feasible to continue the experiments over longer periods of time could capture a larger picture

of the aphids' responses to these disturbances.

Another major question that this study raises is why aphids do not more closely track the growth

of their host plant if the actively growing leaves really are the best place for them to feed. We can propose

several hypotheses that may account for aphids' undisturbed behavior. One is that the aphids may

manipulate the plant to increase the nutritional value of the leaf on which they feed. As they feed, they modulate the amino acid concentration and composition of the plant's phloem sap (Leroy et al. 2011), and moving to a new leaf may forsake that beneficial plant manipulation. Therefore, testing in more detail whether pea aphids' host plant manipulation is systemic or localized to where the aphids feed could explain why they do not more closely track plant growth. Another hypothesis that may explain why pea aphids fail to tightly track plant growth is that the aggregation of offspring that accumulates around a sedentary mother aphid may offer her benefits, such as protection from predators (Duff and Mondor 2012) or helping to overwhelm plant defenses and manipulate the host plant (Leroy et al. 2011). If adult aphids moved more frequently to track plant growth, they may leave their less mobile offspring behind and live in relative isolation.

Finally, in chapter three we explored essentially the same questions as in chapter two, but replaced the simulated predator attack with a heat shock. While these disturbances are quite dissimilar, they both can have serious consequences for an aphid: predators can eat them, while heat shocks can kill them or reduce their ability to reproduce. We showed that adult pea aphids in heat shocks become much more active, leaving their original feeding sites and walking on their host plants, and that these walking aphids accumulate on the actively growing leaves. However, we did not detect any short-term benefits for aphids moving to actively growing leaves either during the heat shock or immediately afterward. Offspring production and survival did not vary whether aphids were collected from actively growing leaves or older leaves at the end of a heat shock or whether they were confined to actively growing leaves or older leaves for two days after the heat shock.

One question that arises from these findings is why the aphids move during the heat shock if the movement does not offer them any apparent benefits. It is possible that our fitness experiment did not span a long enough time period to fully capture fitness effects after the heat shock. However, the high degree of mortality (55-65%) that aphids exhibited over two days after the heat shock suggests that all of the aphids were severely stressed by the shock despite their behaviors. Another possibility is that the wandering aphids were searching for a refuge from the heat, and upon failing to find one they aggregated on the most attractive plant parts. If the experiment were repeated with a stronger thermal gradient or with

a dense aggregation of plants that collectively form a shading and insulating canopy, the aphids may find a refuge from the heat and settle in the cooler location.

Another direction that this study could take is to investigate how heat shocks interact with wind or ambient humidity. The actively growing leaves on which aphids aggregated in both chapters two and three occupy the highest points of the plant, and would likely be the most strongly affected by wind, especially if plants grew tightly together so that wind below the canopy was largely blocked by neighboring plants. Under those conditions, evaporative cooling from the leaves coupled with the wind may change the quality of the top of the plant for small insects seeking shelter from heat shocks. Similarly, relative humidity may affect an aphids' response to extreme heat. While we did not explicitly measure humidity, our heat shocks did occur in a fairly high relative humidity chamber, facilitated by the damp soil surface and all researchers sweating profusely in the confines of the heated rearing room. However, aphids may have exhibited different responses under a lower humidity environment (Roitberg and Myers 1979), especially if the combination of heat and dry air imposed osmotic stress, as well as heat stress, in non-feeding aphids.

References

Duff, K. M., and E. B. Mondor. 2012. All clone-mates are not created equal: fitness discounting theory predicts pea aphid colony structure. Journal of Insect Behavior 25: 48-59.

Leroy, P. D., B. Wathelet, A. Sabri, F. Francis, F. J. Verheggen, Q. Capella, P. Thonart, and E. Haubruge. 2011. Aphid-host plant interactions: does aphid honeydew exactly reflect the host plant amino acid composition? Arthropod-Plant Interactions 5: 193-199.

Nelson, E. H. 2007. Predator avoidance behavior in the pea aphid: costs, frequency, and population consequences. Oecologia 151: 22-32.

Roitberg, B. D., and J. H. Myers. 1979. Behavioral and physiological adaptations of pea aphids (Homoptera Aphididae) to high ground temperatures and predator disturbance. Canadian Entomologist 111: 515-519.

Schellhorn, N. A., T. R. Kuhman, A. C. Olson, and A. R. Ives. 2002. Competition between native and introduced parasitoids of aphids: nontarget effects and biological control. Ecology 83: 2745-2757.