Defense expression in the aphid *Myzocallis asclepiadis*

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Pierce Cedar Creek Institute
Final Report
October 3, 2008

Abstract

The aphid *Myzocallis asclepiadis* is a relatively unknown species that feeds on the common milkweed *Asclepias syriaca*. This aphid is very mobile and mobility appears to be the primary defense against predators. However, over the course of the summer, the aphid changes color, from a pale yellow-green to having orange spots. This aphid could be a model to answer the question of how aposematism evolves. In this study, a natural, unmanipulated population of *M. asclepiadis* was monitored, and experiments manipulating development and density to determine the nature of the color change were conducted. However, it does not appear that development and density are cues for color change. Photographic analysis of color and HPLC analysis are underway to determine the extent of color change and whether that change corresponds to an increase in cardenolide sequestration.

Introduction

The aphid *Myzocallis asclepiadis* is an extreme specialist on the common milkweed *Asclepias syriaca* (Apocynaceae). This aphid has all winged adults, and the nymphs are very mobile, exhibiting what Malcolm (1992) called a "run" defense, rather than the "fight" response of *Aphis nerii* or the "hide" response of *Aphis asclepiadis*, two other specialist, sympatric aphids on *A. syriaca* (Mooney et al 2007). However, *M. asclepiadis* may be evolving an aposematic strategy, or moving toward a fight response, because it changes color over the course of the season, from a pale green-yellow to having orange spots, and in some cases being entirely orange.

The milkweed *A. syriaca* is a highly modular plant, with one genet sending up many ramets, or clones, and produces toxic steroids known as cardenolides. Many species that feed on the milkweed sequester these cardenolides and are aposematically colored, such as the aphid *A. nerii*, the monarch butterfly, the red milkweed beetle *Tetraopes tetrophthalmus*, and the milkweed bugs *Lygaeus kalmii* and *Oncopeltus fasciatus*. Wohlford (2008) suggests *M. asclepiadis* may sequester high levels of cardenolides. This sequestration of cardenolides would be distasteful to potential predators, and combined with the novel coloration, may lead to the evolution of aposematism as a permanent defense strategy.

Järvi et al (1981b) define aposematism as the combination of distastefulness and easy recognition by potential predators. It is generally accepted that the ease of recognition is aided by bright or conspicuous coloration of prey (Harvey and Paxton 1981, Guilford 1990, 1992). The primary question about the evolution of aposematism is: how does it evolve? Harvey and Paxton (1981) propose two possibilities: distastefulness is acquired first followed by bright coloration later, or that distastefulness and bright coloration co-evolve. Because *M. asclepiadis* feeds on milkweed, a known producer of toxins which are readily sequestered by many species, it may be following both paths to aposematism, if it is indeed becoming aposematic. It may have become distasteful first, before any spots appeared, and now that it is distasteful and brightly colored, the distaste and coloration may be enhancing each other so that the brighter the color, the more distasteful the aphid. *M. asclepiadis*, like most aphids, reproduces parthogenetically, meaning all aphids are female, and all offspring are clones of the mother. This suggests that kin selection may be an important factor in the evolution of aposematism (Malcolm 1986, 1989). Kin selection is thought to be a major player in the evolution of aposematism, since it is more

likely for siblings to share the same genes (Järvi et al 1981a, Guilford 1985, Malcolm 1986). Malcolm (1990b) also suggests that in prey that are unlikely to survive attacks, such as soft-bodied insects like the aphid, kin selection is likely to play a larger role than individual selection. It is also suggested that individual selection can be the mechanism, if the prey are sufficiently noxious as to survive the first encounter with naïve predators (Engen 1986), which may play a role with this species because the adults can fly to several plants to reproduce, so aphids on a single leaf or ramet may be from several different parents.

Another aspect of the evolution of aposematism is gregariousness. Gregariousness is thought to enhance the effect of aposematic coloration, especially if the aposematic organism evolved by individual selection (Järvi et al 1981a). Sillén-Tullberg (1990) suggests that the evolution of gregariousness in aposematic species has to do with defense against predators, meaning that group survival is higher than individual survival. A potential advantage of bright coloration is that simply by being different than the search image a predator holds, the predator will avoid the conspicuous forms, or will at least learn to avoid them faster (Gittleman 1980). In *M. asclepiadis*, the possibility for the evolution of gregariousness exists, as seen in the decreasing nearest neighbor distances by the end of the summer, which also coincides with higher levels of orange aphids.

The goal of this research is to discover the nature and reasons for the shift in coloration as a potential mechanism that resolves the problem of how aposematism evolves. The hypotheses tested were: (1) High aphid density will not generate morphological change in the aphids. Alternatively, increasing aphid density will generate an increase in conspicuousness that may function as warning coloration. (2) *M. asclepiadis* will not change morphologically with development time. Alternatively, older individuals and later instars will have an increase in conspicuousness. (3) An increase in conspicuousness will not increase sequestration of cardenolides. Alternatively, an increase in conspicuousness will increase the amount of cardenolides sequestered by the aphids.

Materials and methods

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Natural populations at Pierce Cedar Creek Institute were monitored on 59 ramets, making up eight genets. In genets of more than ten ramets, only ten were monitored. Each monitored ramet was marked with blue painter's tape and a label. The genets were given a letter from A to H, and each ramet in the genet a number from one to ten. Genet A was made of 10 ramets, B had three ramets, C had 10 ramets, D had five ramets, E had two ramets, F had 10 ramets, G had 10 ramets, and H had nine ramets. Each marked ramet was closely examined for aphids every two weeks. The height of the ramet, nearest ramet neighbor, and number of leaf pairs, along with herbivore damage, were recorded. Any leaves that had aphids on them were measured lengthwise and widthwise, as well as the leaf pair number. For leaves with more than one aphid, the distance between the closest pair of aphids was measured. The instar of each aphid and whether or not it was colored were also noted. These ramets were not manipulated other than searching for and counting aphids.

Development and density

For development and density experiments, clip cages were used. These were made of two rounds of 2.9 cm diameter PVC pipe held together with a metal hair clip. The inside lips of the clip cages were lined with felt rings to protect the leaf, and the exterior surface was covered with a fine mesh to prevent aphid escape. All clip cages were washed in soap and hot water before use. Each experiment was run on ten separate genets of milkweed over a period of 20 days, with sampling occurring every four days, including a day 0 control: 0, 4, 8, 12, 16, 20 day samples. The development experiment was begun by placing one adult *M. asclepiadis* in each clip cage, while the density experiment was begun by placing five third and fourth instar nymphs into each clip cage. One clip cage from the development experiment and one from the density experiment were placed on opposite leaves of one ramet, so that each ramet held a single sampling day of development and density. The aphids were placed on the bottom side of the leaf, because they were always found on the bottom in natural populations. After collection of samples on their respective dates, they were brought back to Western Michigan University and photographed using a Nikon SMZ1500 binocular dissecting microscope fitted with a Nikon Coolpix 4500 camera. A Fostec AceI EKE fiberoptic lamp at 100% illumination was used when photographing the aphids. Representative aphids were photographed for each day, including pale aphids, orange-spotted aphids, and any other aphids that seemed very dark or differently colored. Because the aphids are so mobile, solid CO_2 pellets were used to knock them out long enough to take photographs. Color analysis will be completed using *Image J*® software to determine the differences between spotted and non-spotted aphids.

After the trials were completed, the samples were placed in a freezer until all other samples were collected. Leaves and aphids were placed into a Labconco Freeze Dryer 8™ freeze-drying apparatus and dried until brittle. Aphids were removed from the leaf and weighed for all samples. After being weighed, aphid trials were placed in glass test tubes, homogenized using a Brinkman™ homogenizer for 1 minute in 2 milliliters (mL) methanol, vortexed, and decanted into plastic 15 mL centrifuge tubes. The test tubes used for homogenization were washed with an additional 2 mL methanol, which was vortexed and added to the centrifuge tubes. These samples were vortexed, sonicated for 10 minutes in a Branson 2510® sonicator and hot water bath, centrifuged for 10 minutes in a Dynac™ centrifuge at high speed, and decanted into glass culture tubes. 2 mL methanol was added to resuspend the sample and the process was repeated and the supernatant was decanted into the original tubes.

Mass for all leaf samples was also recorded. Leaves were then homogenized using a mortar and pestle into a fine powder, discarding any pieces that failed to homogenize, such as stems. About 0.2 grams of the homogenized leaf sample were placed into a 15 mL plastic centrifuge tube. Leaf samples were then suspended in 4 mL methanol, vortexed, sonicated for 10 minutes in a hot water bath, and centrifuged for 10 minutes at high speed. The supernatant was collected and the pellets were resuspended in an additional 2 mL methanol, vortexed, sonicated for 10 minutes in a hot water bath, centrifuged for 10 minutes and the 2 mL supernatant was added to the original supernatant.

To remove methanol from vials, nitrogen gas was blown onto the test tubes while they were held in a hot water bath. After methanol removal, 1 mL HPLC grade acetonitrile was added to the test tubes and the tubes were vortexed and sonicated for 30 seconds. The solvated mixtures were

decanted into a 3 mL syringe equipped with a Millipore™ filter. The solvents were pushed through the filter into 1mL HPLC vials for HPLC analysis. Between samples 1 mL acetonitrile was passed through the syringe and filter twice to remove any remaining cardenolides. The syringe and filter were blotted with a Kimwipe™ to remove remaining acetonitrile. Each Millipore™ filter was used 10 times or until physical resistance developed, and when switching between aphid and plant samples. Sample analyses were performed using the method of Wiegrebe and Wichtl (1993) on a Waters gradient HPLC system with WISP 717 autosampler, 600E pump, 996 diode array detector and Millennium32™ chromatography software. The reverse-phase elution gradient was acetonitrile:water at 1.2 ml·min-1 at 40°C, with 20% acetonitrile at start, to 32% after 35 min., 40% after 45 min., 50% after 55 min., then back to 20% at 61 min., and 20% at 65 min., on a 250-4 LiChroCART® RP-18 column packed with LiChrospher® 100, 5µm (E. Merck). Sample injections were 20 µl and were separated by 10 minute equilibration at 20% acetonitrile. Cardenolides were detected at 218.5 nm and identified by their symmetrical spectra between 205 and 235 nm and a λ_{max} of between 214 and 224 nm. Cardenolide concentration for each peak (µg/0.1g sample DW) was calculated from a calibration curve with the cardenolide standard digitoxin (Sigma, St Louis, Missouri). Only cardenolide peaks reported by Millennium software as consistently pure were considered for analysis. The HPLC analysis is ongoing.

Results

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Aphids in the natural population at Pierce Cedar Creek Institute (PCCI) were most likely to be found on the bottom half of the plant (Figure 1). This is a significant distribution when compared to an expected even distribution over the entire ramet (χ^2 test, $p<0.005$, df=10 for all dates). The distribution was also significant when individual ramets were examined, with the p-value generally much less than 0.005, with four ramets having p-values less than 0.05, although genet B did not have a significant distribution ($p>0.05$) on August 5. As the season progressed, aphid populations increased to an apparent asymptote, with the total number of orange aphids following a similar trend (Figure 2). The initial aphid count was 295 aphids, and the final aphid count was 876 aphids over all ramets. Between the initial and final counts, eight ramets had died, one from genet A, one from B, four from C, and two from D. The proportion of orange aphids increased linearly ($R^2=0.997$) from the beginning of the season, where no orange aphids were seen, to the end where nearly half of the aphids were orange to the naked eye (Figure 3). As expected, the near aphid neighbor distances decreased with increasing density for all genets with the exception of genet F, on which near aphid distances stayed approximately the same during the course of monitoring (Figure 4).

Development and Density

Both development and density increased the proportion of orange aphids throughout the experiment (Figure 5). However, only the density treatment had a good fitting trend line ($R^2=0.986$), while development was widely scattered and did not fit the trend line well ($R^2=0.052$). Both treatments had an apparently exponential growth rate (development: $R^2=0.868$, density: $R^2=0.935$), as shown in Figure 6, with density being higher because those

trials had more aphids to start with. Cardenolide analysis is ongoing, and the expected results are an increase in cardenolide sequestration with increasing conspicuous coloration. Color changes will be analyzed with *ImageJ*® software, and we expect the analysis to show a significant difference in coloration of non-spotted aphids and spotted aphids. This should also correlate to a larger difference between the aphid and the background of the milkweed leaf, meaning an increase in conspicuousness. Initial analysis has shown that there is a large difference in Lab space between a spotted aphid and a non-spotted aphid.

Discussion

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In occupying the lower part of the ramet, *M. asclepiadis* may be creating a predator-free zone. The top of the plant contains the new growth, which is generally the most nutritious for herbivores, meaning that many herbivores will be found at the top of the plant, such as the other two aphid species, so predators may focus their attention toward the top instead of expending the energy to search all leaves on a ramet. This suggests that *M. asclepiadis* may be adapted to feed on the less nutritious phloem found in the lower leaves, which may also be a reason for their mobility: if the leaf they are feeding on is not profitable, they need to be able to move to another leaf easily. All instars of this aphid are very mobile, with adults being able to fly. Older instars are more readily mobile, so adults will move at the slightest disturbance – many adults moved when the leaf was turned over for inspection – while first and second instars require more prodding. It is more difficult for first and second instars to move through the trichomes on the underside of the milkweed leaf because they are so small, requiring more effort to move away from their initial location than later instars.

While the other two specialist aphid herbivores, *A. nerii* and *A. asclepiadis*, are aggregated in colonies, *M. asclepiadis* is not known to aggregate. This observation was confirmed in monitoring the natural populations. Only in six cases were two aphids touching, but no more than two aphids touched on any leaf, and the average near aphid was between 12 mm and 23 mm on each of the monitoring days. With a null hypothesis of even distribution at the ramet level, meaning the same number of aphids on all ramets, a Chi-squared analysis was performed, and the likelihood of an even distribution was nearly zero for all days ($p<0.005$). The distribution of aphids over a single ramet was also significant compared to a null hypothesis of even distribution. An alternative hypothesis of random distribution is more likely both at the ramet level and at the leaf level. At the leaf level in particular, a random distribution is supported by the large distance between aphids, which could not be seen in an aggregated distribution.

The population of *M. asclepiadis* appeared to grow exponentially to an asymptote (Figure 2) which could indicate carrying capacity. The number of orange aphids appeared to follow the same curve as the total number of aphids; however the proportion of orange aphids increased linearly over time. This shows that the color shift is real and it appears to be a naturally occurring phenomenon. As the population grew, the nearest aphid neighbor distance decreased, as shown in Figure 4, which was not surprising. With the increasing number of aphids, and when the milkweeds started senescing and dropping leaves, some aphids migrated to feed on the seed pods where the plant would be focusing the nutrients, while the leaves become less nutritious.

Development and density

Neither development nor density appeared to be the stimulus for the aphids to change color. While density had a more linear increase in the proportion of orange aphids, development had a higher overall proportion of orange aphids, as indicated by the trend line in Figure 5. Instead, changing color may be associated with changing day length, specifically as the rate of decrease in daylight reaches its steepest slope, since the amount of daylight decreases from the time the aphids appear in mid-June.

Conclusions

The aphids are randomly distributed among the ramets, spreading to other ramets as the population grows. Hypothesis 1 was supported because, while there was increasing color with density, the color change does not appear to be reliant upon density as a stimulus for morphological change. Hypothesis 2 was not supported in the experiments, rather, the alternative was. The aphids did change color with time, and at the end of the summer, nearly all adults were orange, while nearly all first instars were still pale yellow-green. However, this may not be due explicitly to development, but rather may be due to seasonal changes, such as the decreasing daylight, which are manifested as time passes. Hypothesis 3 has yet to be supported or contradicted because HPLC analysis has not been completed, however it is expected that an increase in orange coloration will also signify an increase in the sequestration of cardenolides. Thus far, it appears *M. asclepiadis* is a possible model for the evolution of aposematism.

Future questions

Now that it is known that *M. asclepiadis* does in fact change color over the course of the summer, there are several questions that can be explored further in future work. First, a palatability test should be conducted to see how predators, such as the seven-spot ladybird *Coccinella septempunctata*, react to the changing color, and whether color is an indicator of distastefulness. This would be a very useful experiment, because it would help to answer the question of how the evolution of aposematism occurs. If the aphids are indeed distasteful, and more aphids become conspicuous, then this species may be evolving aposematism as a primary defense rather than a "run" response to predators. Second, the mechanism of the color change needs to be investigated further. Because there is not a clear association between the color shift and either density or development time, other signals must be at work. One possible cue is the decrease in daylight. This could be investigated in environment chambers with programmed lighting, to mimic natural day shortening as a control and manipulate day length experimentally, either by changing the rate of decrease, maintaining day length, or increasing day length.

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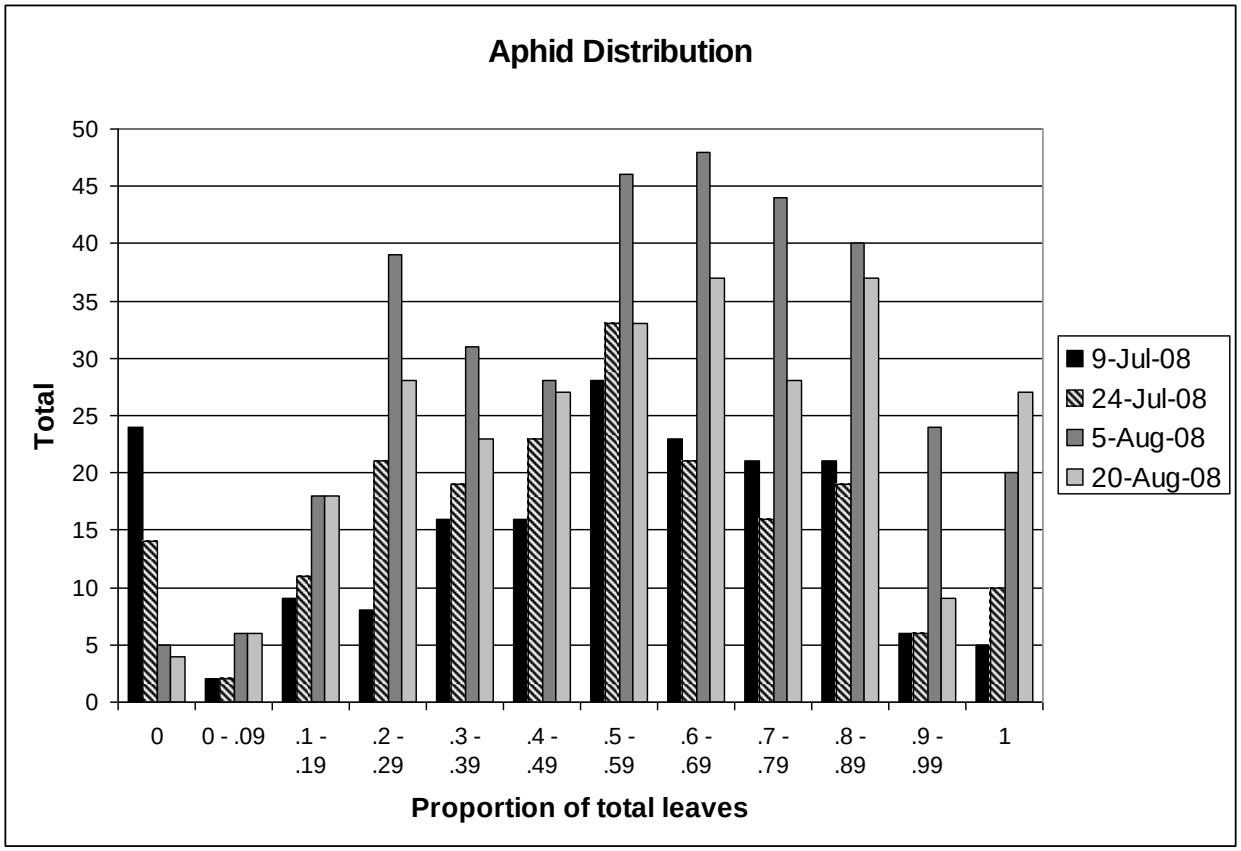

Figure 1. Distribution of aphids as a proportion of total leaves. 0 signifies plants that did not have any aphids, 0-0.9 shows aphids on the top 9% of the plant, and 1 shows aphids on the bottommost leaves. The y-axis shows the total number of plants at each proportion.

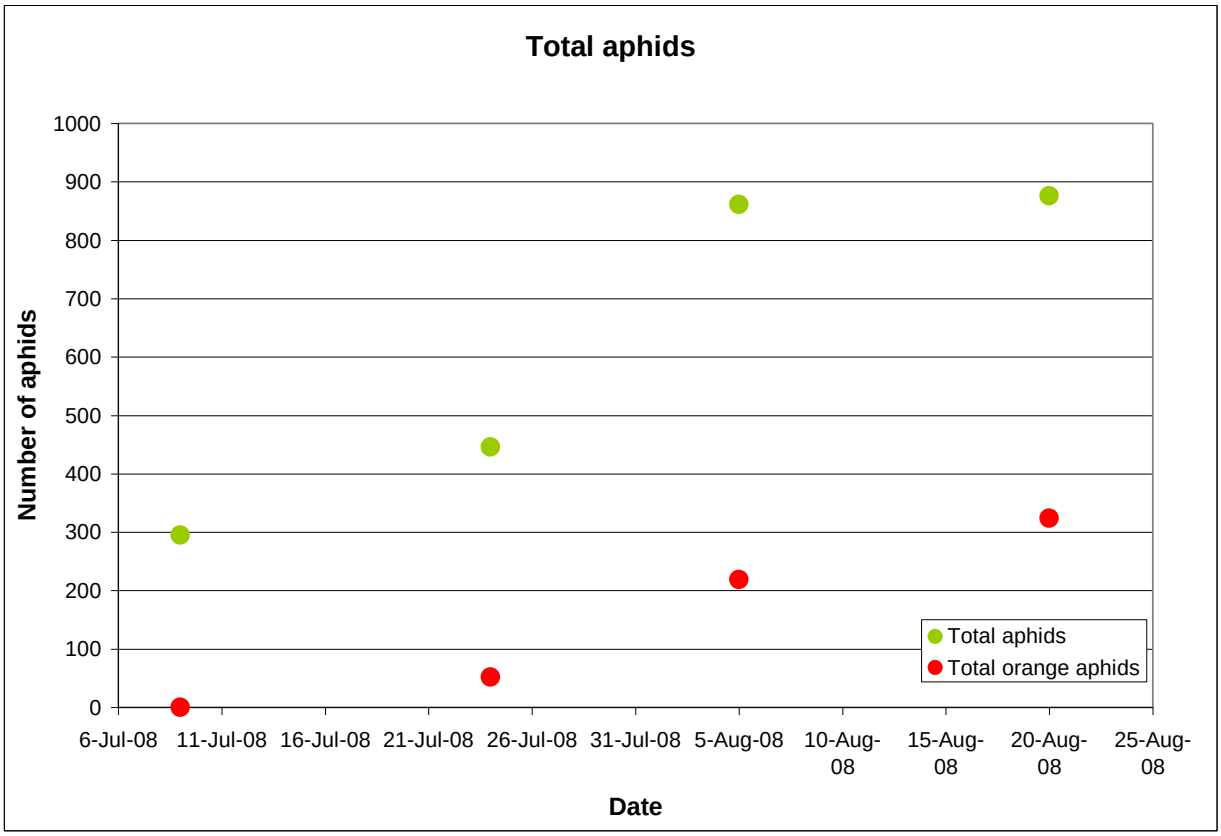

Figure 2. Total number of aphids and orange aphids counted per sampling day at PCCI.

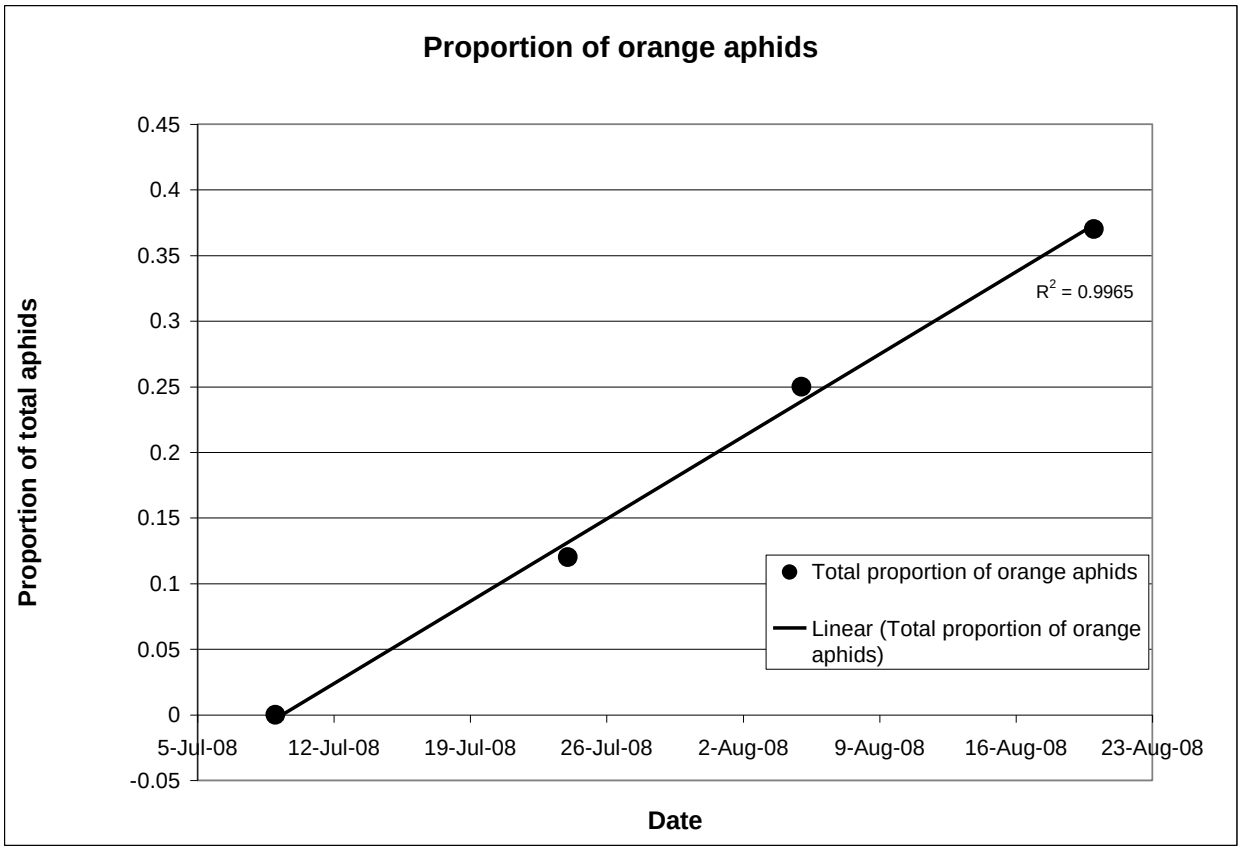

Figure 3. Proportion of orange aphids in natural populations at PCCI.

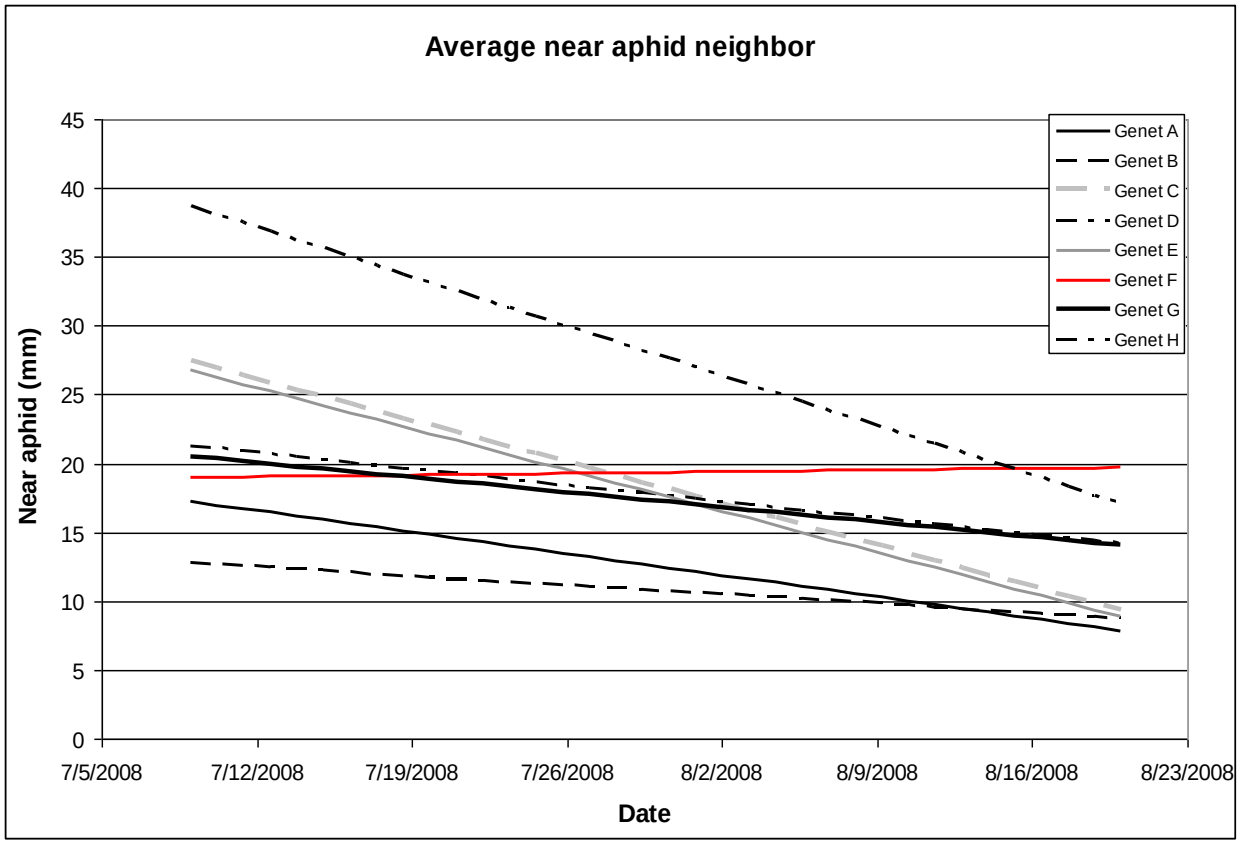

Figure 4. Near aphid neighbors. Distances decrease over the summer, as populations increase.

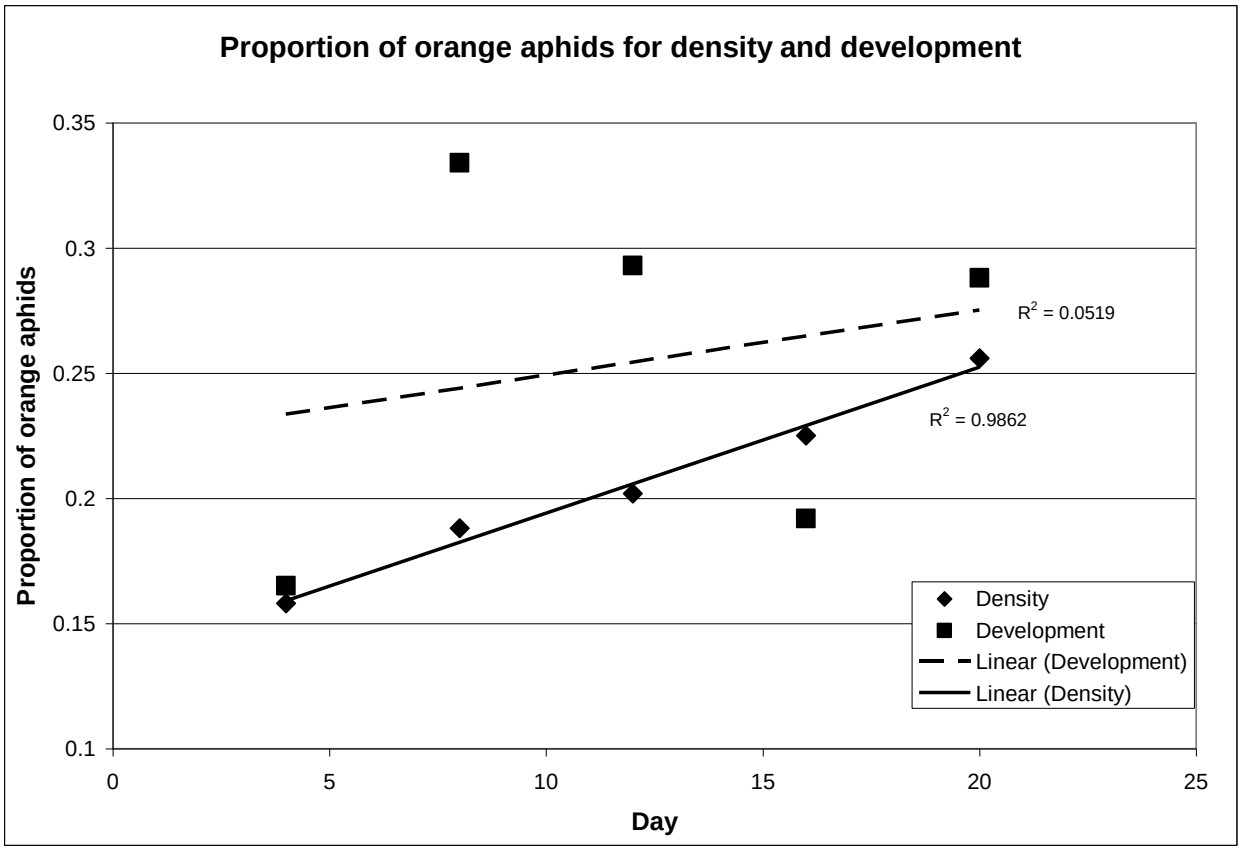

Figure 5. Proportion of total orange aphids in density and development experiments. Density has a goodness of fit R^2=0.986, while development is much more scattered R^2=0.052.

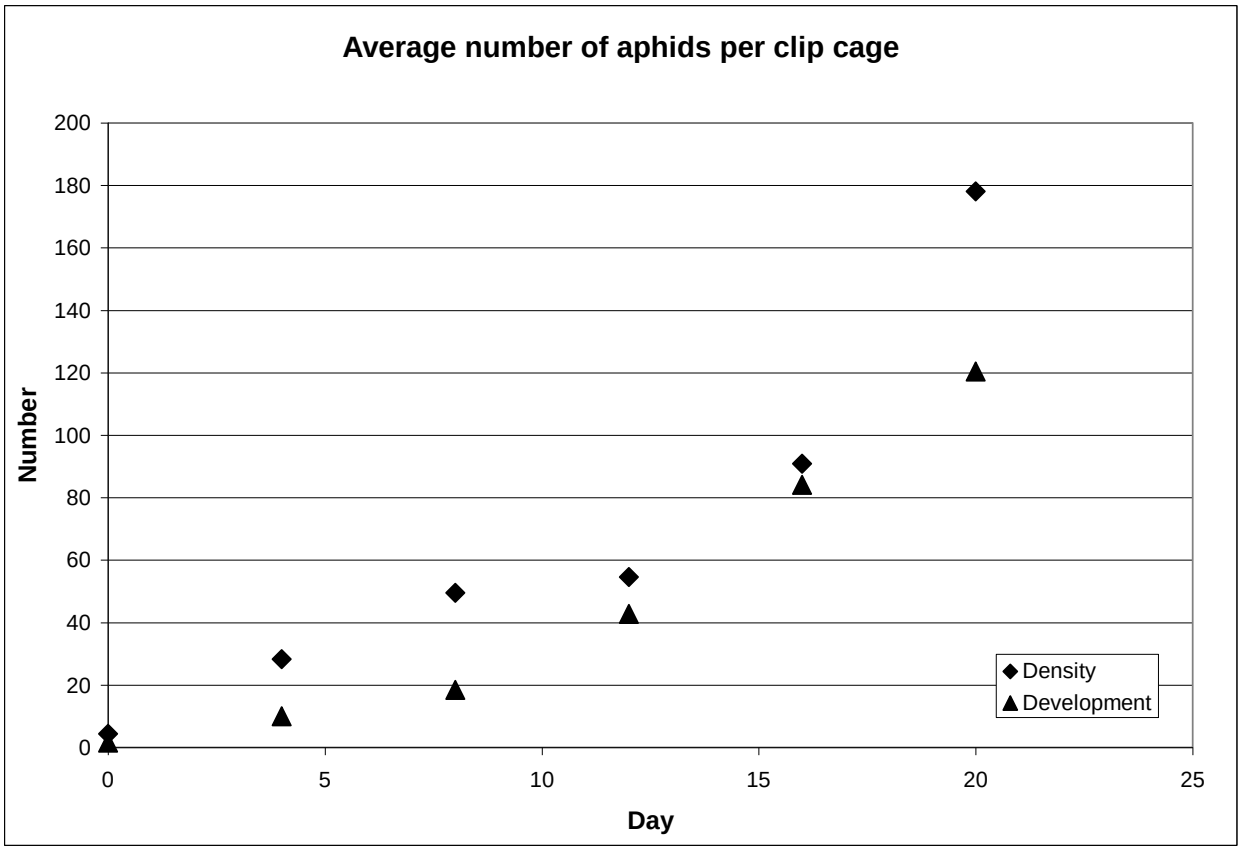

Figure 6. Average number of aphids per clip cage in the ten replicates per sampling day.