Investigating Damage, Genetic Correlations, and Natural Selection to Understand

Multiple Plant Defenses in *Passiflora incarnata*

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy in the
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ABSTRACT

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# Abstract

Plants commonly produce multiple, seemingly redundant defenses, but the reasons for this are poorly understood. The specificity of defenses to particular herbivores could drive investment in multiple defenses. Alternatively, genetic correlations between defenses could lead to their joint expression, even if possessing both defenses is non-adaptive. Plants may produce multiple defenses if putative resistance traits do not reduce damage, forcing plants to rely on tolerance of damage instead. Furthermore, resource shortages caused by herbivore damage could lead to compensatory changes in expression and selection on non-defense traits, such as floral traits. Natural selection could favor producing multiple defenses if synergism between defenses increases the benefits or decrease the costs of producing multiple defenses. Non-linear relationships between the costs and benefits of defense trait investment could also favor multiple defenses.

*Passiflora incarnata* ('maypop') is a perennial vine native to the southeast United States that produces both direct, physical traits (leaf toughness and trichomes) and rewards thought to function in indirect defense (extrafloral nectar in a defense mutualism with ants), along with tolerance of herbivore damage. I performed two year-long common garden experiments with clonal replicates of plants originating from two populations. I measured plant fitness, herbivore damage, and defense traits. I ran a

genotypic selection analysis to determine if manipulating herbivore damage through a pesticide exclusion treatment presence mediated selection on floral traits, and if herbivore damage led to plastic changes in floral trait expression. To evaluate the role of selection in maintaining multiple defenses, I estimated fitness surfaces for pairwise combinations of defense traits and evaluated where the fitness optima were on each surface.

I found that resistance traits did not reduce herbivore damage, but plants demonstrated specific tolerance to different classes of herbivore damage. Tolerance was negatively correlated with resistance, raising the possibility that tolerance of herbivore damage instead of resistance may be the key defense in this plant, and that production of the two type of defense is constrained by underlying genetic architecture. Plants with higher levels of generalist beetle damage flowered earlier and produced proportionally more male flowers. I found linear selection for both earlier flowering and a lower proportion of male flowers in the herbivore exclusion treatment. I found that selection favored investment in multiple resistance traits. However, for two tolerance traits or one resistance and one tolerance trait, investment in only one trait was favored.

These results highlight the possibility of several mechanisms selecting for the expression of multiple traits, including non-defense traits. Resistance traits may have a non-defensive primary function in this plant, and tolerance may instead be a key

defense strategy. These results also emphasize the need to consider the type of trait—

resistance or tolerance—when making broad predictions about their joint expression.

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# 1. Introduction

## *1.1 Possible Reasons Plants Possess Multiple Defenses*

The occurrence of trade-offs between traits is a common prediction in evolutionary biology. Plant defense is one area where trade-offs are frequently hypothesized (Zangerl & Bazzaz 1992; Mole 1994). Plant defenses can require expensive resources such as nitrogen, and  may be expensive to produce (Simms & Rausher 1987). Plant resources are finite, and resources allocated to defense represent an allocation cost as they are not available for growth or reproduction (Strauss *et al.* 2002). Models have also supported this idea of trade-offs, under the framework that investment in multiple defenses will incur higher costs and lead to an optimal defense strategy of maximal investment in only one trait instead of a mixed strategy (van der Meijden *et al.* 1988).

Early studies appeared to substantiate these assumptions, as in work on ant-plants and acacias. For example, myrmecophytic acacias and ants, which participate in an obligate protection mutualism, were believed to demonstrate less chemical defense investment due to a trade-off among multiple defenses (Rehr *et al.* 1973). However, closer inspection has revealed that these trade-off assumptions are often incorrect (Heil *et al.* 2002), and that many plants instead invest in multiple, seemingly redundant defenses (Rudgers *et al.* 2004).

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Defense traits can be broken down into two categories. Resistance traits, which deter herbivore attack, include traits such as thorns and secondary chemicals (Ehrlich & Raven 1964). Tolerance occurs when herbivore damage triggers changes in plant growth or reproduction that mitigate the fitness effects of damage (Strauss & Agrawal 1999). Plants can invest in both of these defense strategies. Another possibility is that plants can demonstrate numerous traits within each category, such as by investing in multiple resistance traits.

Plants could feature multiple defenses for a variety of reasons. The specificity of plant defenses could lead to the production of multiple defenses. If plant defenses are tightly coevolved and tailored to a specific herbivore, multiple defenses may be required to defend against a suite of herbivores. As plants commonly face both specialist and generalist herbivores in the field, a combination of both narrowly-tailored defenses for well-adapted specialists and broader defenses for generalists may be required (Turley *et al.* 2013).

Another possible explanation for multiple defenses is that correlations between defenses may force their production, even if only producing one defense would be ideal. Genotypic correlations commonly occur between traits, and could constrain plants from reaching an ideal phenotype (Futuyma & Moreno 1988). Negative genetic correlations

could impede evolution of the expression of two traits together, while positive

correlations could facilitate the joint expression of traits, even if possessing only one

defense would be better (Mauricio *et al.* 1997).

In addition, if either the herbivores or defenses are unpredictable, multiple

defenses may be necessary. If herbivores vary greatly temporally or seasonally and are

unpredictable, a plant may be forced to provide a wide spectrum of defenses for a range

of possible threats (Rutter & Rausher 2004). Defenses may also be unreliable, favoring

multiple defenses as 'insurance'. In particular, facultative ant mutualisms may be

unreliable and often lead to variable outcomes (Bronstein 1994). Ant species vary in the

quality of defense they provide, and a plant may not be able to attract reliable ant

partners at all times (Rudgers & Strauss 2004). Even if two defenses are favored, the

balance between them may differ with biotic and abiotic context. The diffuse nature of

facultative ant-plant mutualisms may contribute to population-level variation in the

effectiveness of plant defenses, given variety in the species and abundances of both

herbivores and mutualist ants (Bronstein 1998). Numerous studies have demonstrated

local adaptation (Hereford 2009) and variation among populations in plant defense

production (Berenbaum & Zangerl 1998; Rios *et al.* 2008; Züst *et al.* 2012).

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Resistance can encompass compensatory changes in plant fitness or growth. For instance, if herbivore damage leads to changes in floral traits that then enable compensatory higher fitness, those traits could be broadly interpreted as resistance, and hence defense, traits. Floral sex ratios may shift in response to resource changes, such as those caused by herbivore damage (Spears Jr & May 1988). Another floral trait that herbivores may influence is flowering phenology, as plants may shift the timing of flowering later in the season if resource shortages occur due to herbivore damage (Brody 1997).

One way of determining how herbivores alter traits is to calculate if natural selection changes in response to herbivore presence. Investigating how selection acts on traits can also provide insight into hypotheses for the production of multiple defenses. Analyzing selection on traits can reveal if selection is acting to increase or decrease associations between traits to better understand how multiple traits evolve (Lande & Arnold 1983).

Non-linear cost or benefit functions or synergism could lead to selection favoring the possession of multiple defenses. The costs of producing defenses could accelerate with investment, making investment in two defenses cheaper than higher investment in one defense (Bergelson *et al.* 2001). The benefits of defense could also increase with

investment, with higher levels of two defenses providing proportionally more effective defense. Similarly, there could be synergistic benefits between defenses, such as from interactions between defenses making them more difficult to resist than each trait considered singly (Berenbaum & Zangerl 1996). In contrast, antagonism, where the interactions between defenses render them less effective or more costly, could oppose the expression of multiple defense traits.

Another possible explanation for possessing multiple defenses is that traits believed to function in defense may not actually reduce herbivore damage. In this scenario, tolerance of herbivore damage may be necessary, and plants may not actually possess multiple defenses. Tolerance could also be needed if some herbivores do not respond to resistance traits.

## *1.2 Broader Motivation for Studying Multiple Defenses*

Studying multiple defenses provides a more complete picture of how plants and herbivores interact in complex natural communities, where selection typically acts simultaneously on multiple traits (Phillips & Arnold 1989). While studying defense traits independently may be easier, doing so ignores the fact that defenses may interact in their effects on herbivores (Kursar & Coley 2003). The limitations of a narrow, single-

trait-based view of plant defenses have led to calls for plant defenses to be studied as multiple trait syndromes (Agrawal & Fishbein 2006; Carmona *et al.* 2011).

My research addresses gaps in the plant defense literature. While studies have investigated how multiple resistance traits interact, and how one resistance and one tolerance trait interact, the more complex possible range of interactions for multiple resistance and tolerance traits considered together has not been explored (Turley *et al.* 2013). My research also includes multiple study populations to more accurately reflect the natural diversity present in these plant traits and to capture the role of the local environment in determining the value of herbivore defenses.

## 1.3 Outline of the dissertation

In my dissertation research, I utilized a plant that both produces multiple resistance traits and exhibits tolerance of herbivore damage to investigate the potential advantages of producing multiple plant defenses.  I used two years of common garden experiments with vegetative clones of plants from two source populations. I manipulated herbivore presence and absence, and measured resistance and tolerance traits and plant fitness. I investigated three related questions on the evolutionary ecology of multiple defense trait production. The advantage of comparing all three

methods is that the hypothesis they consider are non-exclusive, and in many ways complement each other.

My second chapter covers three components. I calculated genetic correlations between resistance traits, between tolerance traits, and between resistance and tolerance to identify any underlying constraints on defense expression or linkages between defenses that could facilitate the joint expression of defenses. I found that the genetic correlations between resistance traits were zero or extremely weak, while there were positive correlations between tolerance of damage by different herbivores and negative correlations between resistance and tolerance. This could lead to selection favoring investment in resistance or tolerance, but opposing their joint expression. I investigated the level of resistance trait investment between two disparate populations, and found that plants from Florida source populations consistently invested more in resistance traits. I did not find population level differentiation in tolerance traits, which also appear to be effective against a broad range of herbivores. I evaluated the fitness implications of resistance trait investment. Higher investment in resistance traits did not decrease herbivore damage. Resistance traits may have a principal role other than defense, and tolerance may be the focal defense strategy in this plant.

In my third chapter, I considered how herbivore damage could shape the expression of traits not traditionally considered defense traits, such as floral traits. The resource shortages caused by herbivore damage could mediate changes in floral traits as a form of compensation, making these changes in trait expression a form of tolerance. I found that in response to generalist beetle damage, *P. incarnata* flowered earlier in the season and produced a higher ratio of male flowers. In plants under a pesticide application treatment, I found evidence of directional selection for earlier flowering and production of fewer male flowers. This suggests that herbivore damage can mediate changes in floral traits, and that plant traits not traditionally considered defenses may play a role in compensatory defense strategies (Pilson & Decker 2002). This analysis was a collaboration with Dr. Nina Sletvold, and hence the chapter is written in the second person.

In the fourth chapter, I investigated how natural selection may favor plants possessing multiple defenses. I used cubic splines to visualize fitness surfaces for the production of multiple defenses. By considering the pairwise interactions of different traits, I evaluated how natural selection may act on trait values and what combinations of trait investment natural selection may favor. I found that when I paired two resistance traits together, the fitness peak commonly favored investment in multiple traits. In

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contrast, when I paired two tolerance traits or one resistance and one tolerance trait, the fitness surface favored investment in only one trait and not a mixed investment in both. Some of these results, such as for selection favoring production of only one trait when resistance and tolerance traits were paired, coincided with the results in chapter 2 supporting multiple defenses. I also found that either non-linear shape parameters for traits or trait interactions could lead to the production of multiple defense traits. The contrasting results with the genetic correlations emphasize the need to consider multiple sources of evidence, such as both genetic correlations and selection surfaces, before drawing broad conclusions about how multiple defense traits may interact.

## 2. Deter and endure: understanding investment in both plant resistance and tolerance to herbivory

### *2.1 Introduction*

Many plants express multiple, seemingly redundant traits hypothesized to function as defenses, but why this occurs remains unresolved. Responses to herbivory are commonly categorized into resistance and tolerance (both of which I refer to here as "defenses"). Resistance traits, such as thorns or secondary chemicals, ward off herbivore attack (Ehrlich & Raven 1964). Tolerance occurs through re-growth or enhanced reproduction following herbivore damage (Strauss & Agrawal 1999). Resistance to and tolerance of herbivore damage were assumed to be mutually exclusive, but plants often possess both defenses (van der Meijden *et al.* 1988; Herms & Mattson 1992; Fineblum & Rausher 1995). This observation presents a paradox: if resistance traits reduce herbivore damage, why should plants demonstrate tolerance? Conversely, if putative resistance traits do not reduce herbivore damage, forcing plants to rely on tolerance, why do plants produce those traits? In this chapter, I address why a single plant may invest simultaneously in putative resistance traits to reduce attack and tolerance to endure herbivore damage.

Plants could produce both resistance and tolerance for several reasons. First, resistance traits may be specific to particular herbivores, and ineffective against others.

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When those other herbivores are present, a plant may rely on either general tolerance or tolerance that is specific to those herbivores not deterred by the resistance traits the plant possesses. Second, positive genetic correlations between resistance and tolerance could cause plants to express both defenses, even if only one would be optimal.  Finally, traits putatively assumed to function in herbivore resistance may not actually reduce herbivore damage. Instead, these traits may function primarily in non-defensive roles or even be completely lacking in function. Here, I assess the role of specificity, genetic correlations, and effectiveness in reducing damage as reasons for investment in resistance and tolerance in the vine *Passiflora incarnata*.

If resistance traits are highly specific to certain herbivores, the need for responses to a broad variety of herbivores could require a suite of defenses. Different herbivores— particularly specialists versus generalists—may respond in differing ways to specific resistance traits (Blüthgen & Metzner 2007; van Leur *et al.* 2008; Kim 2014). Tightly coevolved chemical defenses with differential impacts on generalists and specialists have been the focus of much past literature (Ehrlich & Raven 1964), yet herbivore types can differ in their response to non-chemical defenses as well. For example, generalist and specialist herbivores can be impacted differently by leaf toughness (Coley *et al.* 2006), trichomes (Mathur *et al.* 2011), and ant defenses (Dyer *et al.* 1999).  Tolerance could

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also be necessary if particular herbivores are not responsive to resistance traits. A

related—but frequently neglected question—is whether tolerance traits are specific to

damage caused by different herbivores (Stinchcombe & Rausher 2001).

Both genetic and environmental factors influence trait values, including defense

traits. Plants may produce multiple defense traits due to low heritability: even if the

traits do not currently provide much benefit as defenses, natural selection would be less

effective at eliminating traits with low heritability. Plants may also be forced to produce

multiple defenses due to positive genetic correlations between them, even if producing

only one defense would be ideal. Conversely, negative correlations could limit the

production of several defenses by forcing trade-offs in their production. Genotypic

correlations commonly occur between traits, and could constrain plants from reaching

an optimal phenotype (Futuyma & Moreno 1988).

*Passiflora incarnata* ('may-pop') produces physical resistance traits (leaf trichomes

and toughness) and participates in an indirect ant defense mutualism mediated by

extrafloral nectar. The plant also produces chemical defenses (such as cyanogenic

glycosides), but here I focus on the physical and ant-mediated defenses. The plant is

attacked by both specialists (caterpillars of the gulf fritillary butterfly *Agraulis vanilla*e)

and a range of generalists (including thrips and flea beetles) (McGuire 1999). In Florida,

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the specialist caterpillars are present for much of the year, but the seasonal migration of the butterflies means that caterpillars occur in North Carolina for only a brief period each year (Walker 2001). This seasonal specialist migration may lead to differing herbivore pressures between Florida and North Carolina.

Finally, traits believed to function in resistance may actually not decrease herbivore attack. Many putative resistance traits may provide no benefit (Carmona *et al.* 2011) or even increase attack from particular herbivores (Bidart-Bouzat & Kliebenstein 2008). These limitations on the effectiveness of resistance traits could lead to investment in tolerance as an alternative response to herbivory.

I used common garden experiments to investigate possible reasons for investment in both resistance and tolerance to herbivore damage in *P. incarnata*. Specifically, I grew plant clonal replicates to assess genotypic variation and covariation in resistance and tolerance to herbivory. I measured three putative resistance traits in addition to calculating tolerance of different types of herbivore damage. I used the experimental data to ask:

-Do different putative resistance traits reduce damage by specific types of herbivores?

-Does tolerance to one type of herbivore damage also convey tolerance of other types?

-Are plant resistance traits heritable? Do genotypes differ in their tolerance of herbivory?

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-Are there genetic covariances between resistance traits and tolerance of herbivory that may force or limit their joint expression?

## 2.2 Methods

### 2.2.1 Field Experiment

*Passiflora incarnata* is a perennial vine native to the southeast U.S. that grows in disturbed habitats such as roadsides and field margins. Genotypes were collected from roadsides near the town of Lakeland in central Florida and near the town of Wilmington in southeast North Carolina. In both sites, populations spanned an area with a radius of 160 km. I conducted common garden experiments in 2012 and 2013 at field sites in Durham, North Carolina.

For the Year One experiment, the plants were grown in a shared greenhouse environment for one year to minimize the impact of prior growing conditions on trait values. At the end of the year, each plant was divided into vegetative clonal ramets. The clonal replicates were grown in pots in local soil for one week and then planted into a disked field in three blocks in May 2012. Plants were grown approximately 1 meter apart and were not staked. There were 32 genotypes for North Carolina and 25 for Florida, with 2 to 6 clonal replicates of each genotype planted across the three blocks.

Although I had intended to continue the experiment for an additional year, the common garden was accidentally plowed under at the beginning of the second year, necessitating the rapid replacement of all genotypes. Hence, in Year Two, plants were not grown in a shared environment prior to the experiment. Genotypes were again collected from Florida and North Carolina in May from sites nearby but not identical to the first year's collection. This second, independent collection enhanced the diversity of genotypes considered in the experiment. Genotypes were again divided into vegetative clones and grown in pots in local soil for one week before planting into a disked field in two blocks. A different field (approximately .8 km away from the first year's site) was used for the Year Two experiment. There were 76 genotypes for North Carolina, and 80 for Florida, with again between 2 and 6 vegetative clones of each genotype.

Both fields contained the heavy clay soil found in the North Carolina piedmont (White Store sandy loam, USDA soil survey). Each field had lain fallow for at least one year prior to the experiment. Year One was characterized by hot, dry conditions (average monthly temperature from May to October growing season 22.5 C, 67 cm total precipitation, National Climatic Data Center, National Oceanic and Atmospheric Administration), and plants were grown in a matrix of grass and morning glories (*Ipomoea* spp.). Year Two was a very wet, cool summer, with higher than average rainfall

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totals (average monthly temperature from May to October 21.6 C, 85 cm total

precipitation, National Climatic Data Center, National Oceanic and Atmospheric

Administration) and plants grew in a matrix of grass.

In both years, clones within a genotype were randomly allocated to either an

herbivore exclusion or control treatment to calculate the costs and benefits of producing

resistance traits. This chapter discusses trait values; the cost/benefit data will be

discussed in chapter 4. In Year One, the herbivore exclusion treatment involved

treatment with Sevin Dust (Solaris Group, Scotts, San Ramon, CA). In year two, a

Spinosad insecticide (Monterey Garden Insect Spray, Lawn and Garden Products, Inc,

Fresno, CA) was used in an unsuccessful attempt to better control thrips damage.

Pesticide treatments were re-applied every two weeks or following rainfall. The

pesticide treatment did not alter trait values in either year (ANOVA, $P \geq .1$ for all traits in

both years) so the treatment and control plants were pooled for all analyses. Pesticide

treatment also did not alter ant visitation in either year (ANOVA, all $P \geq .15$). All plants

were fertilized twice during the growing season with a slow release fertilizer (Multicote

4 NPK Pro 14-14-16, Haifa Chemical Inc, Allamonte Springs, FL) according to package

directions.  In Year Two I manipulated specialist presence by placing one 1st instar *A.*

*vanillae* larvae from a commercial farm (Shady Oak Butterfly Farm, Brooker, FL) on each

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control plant to investigate any larval response to defense traits and if resistance traits reduce specialist herbivory.

Trichome density, nectar volume, and leaf toughness measurements were made in August through September on mature, fully expanded leaves. Trichome density was measured by taking a hole punch sample from three leaves per plant, counting trichomes on the adaxial leaf surface using a dissecting microscope, and averaging the counts.

Nectar volume was measured by blotting the visible nectar on extrafloral nectaries onto a sheet of quantitative filter paper (grade 43, Whatman PLC, Kent, UK) for three leaves per plant (Baker 1979). The diameter of the nectar blot formed was then measured with digital calipers and used to calculate the nectar volume from the surface area of the blot. Nectar sampling was performed early in the morning before the initiation of visible ant, wasp, or bee foraging.

Leaf toughness for three leaves per plant was measured with a penetrometer based on the design of Feeny (1970). Leaf midribs were avoided during sample collection. Ant visitation was recorded a minimum of once a week from July until October. Ants were identified to genus.

I calculated total leaf area by measuring the area of three leaves per plant and multiplying the mean by the total number of leaves per plant. I classified herbivore damage into three categories based on the agent creating it—specialist caterpillars, thrips, or generalist beetles. Caterpillars left jagged leaf margins, while thrips created leaf stippling, and beetles made scattered leaf bites. For each herbivore class, I assigned a classification level of percentage leaf area damaged based on a visual inspection and I counted the number of damaged leaves per plant, which I then scaled by the total number of leaves to arrive at a percentage damage for the entire plant. Damage was arcsin square root-transformed prior to analysis to improve normality.

## 2.2.2 Data Analysis

Trait data were analyzed using random effects analysis of variance with restricted maximum likelihood (REML) using the lme4 and MASS packages in R (R Core Team, 2014). Block and genotype were treated as random effects. Broad sense heritability was calculated by dividing the total genetic variance (additive and non-additive) by the total phenotypic variance (genetic and environmental). Bias-adjusted confidence intervals for heritabilities were obtained from a parametric bootstrap with 1,000 replicates. The coefficient of genetic variation expresses the ability of a trait to respond to selection (Houle 1992) and was calculated as the square root of the total

genetic variance divided by the trait mean. Pearson correlation coefficients and genetic covariances of family mean values were calculated to investigate the genetic associations among traits. The genetic covariance was calculated as $r_g(G_{11}G_{22})^{0.5}$ where $r_g$ is the Pearson correlation coefficient for the traits $i$ and $j$, and $G_{ii}$ is the genetic variance of trait $i$ from the REML model.

Differences in trait values between source populations were evaluated by F-tests from a mixed model ANOVA with block and genotype as random factors. To investigate how trait values impacted the amount of damage inflicted by particular herbivores, I used a linear mixed model with amount of damage as the response variable, plant defense traits, population of origin (North Carolina or Florida), and herbivore exclusion treatment as fixed effects, and blocks and genotype as random effects.

I used the log response ratio to better understand the difference in overall defense level in plants originating from Florida versus North Carolina (Hedges *et al.* 1999). The response ratio calculates the effect of source population on defense as a whole (i.e. nectar volume, leaf toughness, trichome density, and ant visitation combined). The differences in mean trait values for Florida versus North Carolina were divided by the pooled within-state standard deviation. The log response ratio was utilized for statistical testing, but I report the means and confidence intervals for the response ratio itself.

I looked for genotypic variation in tolerance by testing for a significant impact on plant fitness (leaf area) of the interaction of genotype (a random effect) and herbivore damage (a fixed effect) in a linear mixed model (Simms 2000). Essentially, this analysis asks whether plant performance declines more steeply with increasing herbivore damage for some genotypes than for others (Simms 2000). I ran this analysis separately for all three types of herbivore damage to determine any specificity of tolerance. I then tested if there were significant differences in tolerance between states with likelihood ratio $X^2$ tests. I also calculated Pearson correlations between tolerance and defense traits. Plant leaf area is an appropriate measure of fitness in a clonal, perennial plant that needs to devote resources to vegetative growth to ensure survival to the next year (Winkler & Fischer 1999).

## 2.3 Results

Resistance trait investment did not reduce damage by specific herbivores in either year (table 1 and 2).

**Table 1: Year 1 damage by state. How resistance traits and source population impacted damage by different herbivore classes. Regressions were run with the amount of damage as the response variable and defense traits, state, and treatment as predictor values, with random effects for field plot and plant genotype.**

| trait | state | thrip damage | | | beetle and generalist damage | | |
|---|---|---|---|---|---|---|---|
| | | df | F | p-value | df | F | p-value |
| treatment | NC | 1 | 1.175 | .310 | 1 | .703 | .340 |
| | FL | 1 | .034 | .851 | 1 | .003 | .956 |
| toughness | NC | 1 | 1.605 | .143 | 1 | .115 | .725 |
| | FL | 1 | 4.199 | .066 | 1 | .617 | .475 |
| trichomes | NC | 1 | .0204 | .829 | 1 | .0001 | .964 |
| | FL | 1 | .410 | .511 | 1 | 1.613 | .167 |
| nectar volume | NC | 1 | .056 | .813 | 1 | .449 | .503 |
| | FL | 1 | .004 | .948 | 1 | 1.895 | .169 |
| ant visitation | NC | 1 | 18.394 | .00014* | 1 | 2.007 | .308 |
| | FL | 1 | 1.181 | .394 | 1 | .338 | .686 |

**Table 2: Year 2 damage by state. Table 2 displays how plant defense production, state of origin, and pesticide treatment influence the amount of herbivore damage. Regressions were run with the amount of damage as the response variable and defense traits, state, and treatment as predictor values, with random effects for field plot and plant genotype.**

| trait | state | thrips damage | | | beetle, generalist damage | | | caterpillar damage | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | df | F | p-value | df | F | p-value | df | F | p-value |
| treatment | NC | 1 | 0.133 | 0.716 | 1 | 0.890 | 0.328 | 1 | 39.3 | 3.38 e -10* |
| | FL | 1 | 5.32 | 0.006* | 1 | 0.411 | 0.661 | 1 | 20.7 | 1.30 e -9* |
| toughness | NC | 1 | 0.124 | 0.726 | 1 | 0.232 | 0.625 | 1 | 0.377 | 0.494 |
| | FL | 1 | 0.018 | 0.895 | 1 | 1.35 | 0.459 | 1 | 0.177 | 0.689 |
| trichomes | NC | 1 | 0.956 | 0.324 | 1 | 0.006 | 0.827 | 1 | 0.049 | 0.881 |
| | FL | 1 | 6.55 | 0.011* | 1 | 1.03 | 0.283 | 1 | 1.07 | 0.432 |
| nectar volume | NC | 1 | 0.500 | 0.481 | 1 | 1.41 | 0.236 | 1 | 3.61 | 0.057 |
| | FL | 1 | 0.251 | 0.617 | 1 | 0.800 | 0.371 | 1 | 0.939 | 0.334 |
| ant visitation | NC | 1 | 0.585 | 0.446 | 1 | 2.70 | 0.168 | 1 | 0.003 | 0.753 |
| | FL | 1 | 0.341 | 0.560 | 1 | 20.8 | 6.051 e -6* | 1 | 0.0001 | 0.901 |

I measured population differences in defense trait production to assess the specificity of resistance traits, and if source population impacted this specificity. While the exact values of resistance traits differed between years, there was a strikingly consistent difference between plants from the two source populations. In both years, plants from Florida produced leaves that were significantly tougher than plants from North Carolina (fig. 1a, 1b), produced a greater volume of extrafloral nectar (fig. 1c, 1d), and received higher average ant visitation (fig. 1e, 1f). The variable nature of ant visitation is reflected in the wider error bars for this trait. Year Two featured higher ant visitation, even though nectar volumes were lower in that year (fig. 1c, 1d). Ant visitation was not correlated with plant size (ANOVA, $P>.2$ for both years and states). In both years, plants from Florida had a higher density of trichomes compared to plants from North Carolina (fig. 1g, 1h).
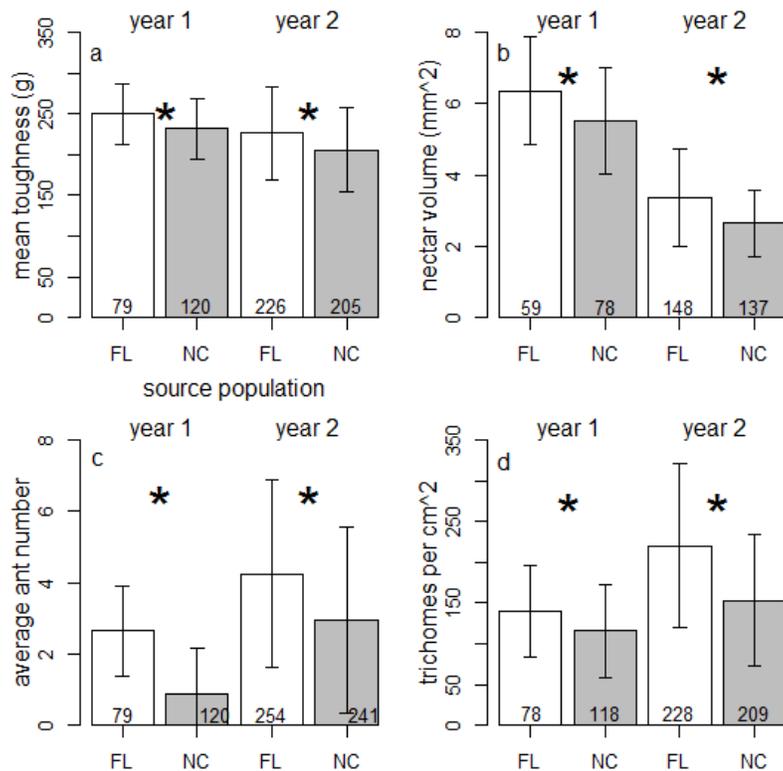
**Figure 1: Differences in defense trait values between plants from Florida (white bars) and North Carolina (gray bars) in the two common garden experiments; \*P<0.05. (a) leaf toughness (mean ± SD) in terms of the force in grams required to puncture a leaf in year one (P=0.0017) and year two ( P<0.0001). (b) The volume of nectar (mean ± SD) produced by plants from each source population for year one (P = 0.0002) and year two (P<0.0001). (c) Ant visitation (mean ± SD) for all ant species binned together across all ant observation dates in year one (P<0.0001 ) and year two ( P=0.0056). (d) Foliar trichome density (mean ± SD) for plants from each state in year one (P=0.0022) and year two (P<0.0001). Sample sizes are shown inside bars.**

In aggregate, Florida plants had higher resistance trait values as measured by the

response ratio. In both years, the response ratio was 28% higher across all resistance

traits for plants from Florida compared to plants from North Carolina (Year One log

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response ratio mean 1.282, 95% confidence interval 1.09-1.51; Year Two mean 1.283, 95% confidence interval 1.18-1.47.)

Paradoxically, Florida plants in the pesticide treatment suffered more thrips damage than did plants in the control group in the second year experiment. Florida plants with more trichomes also received more thrips damage (fig. 2).
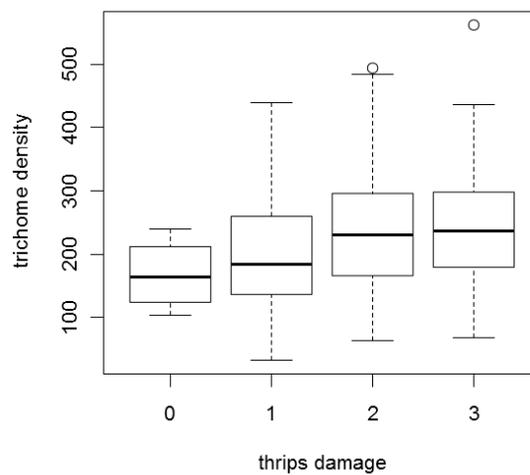


**Figure 2: Foliar trichome density versus thrips damage in plants from Florida in the second experimental year (n=302 plants)**

Plants from Florida with higher ant visitation received less generalist beetle

damage (fig. 3).
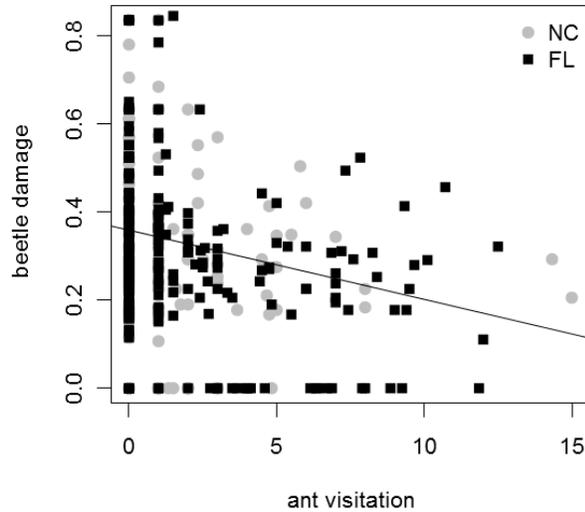


**Figure 3: Amount of generalist beetle damage received by plants from North Carolina and Florida in year 2 plotted versus mean ant visitation; the regression line refers to Florida plants (r2 = 0.067, P= 0.0019, Florida plants n= 303, North Carolina plants n= 307)**

In the first year, North Carolina plants with higher ant visitation featured,

unexpectedly, higher levels of thrips damage (fig. 4).
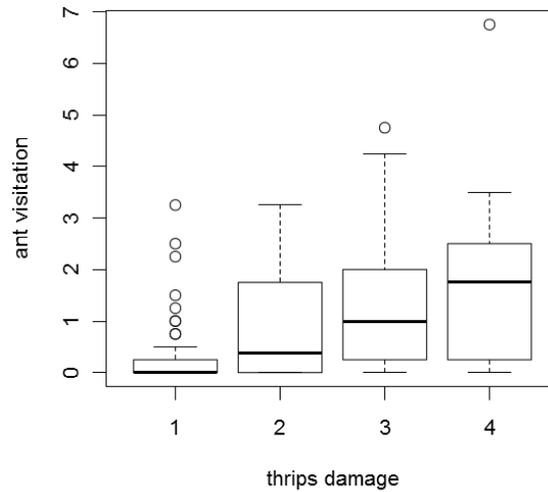


**Figure 4: Mean ant visitation versus thrips damage on plants from North Carolina in year 1 (n=120 plants)**

Otherwise, there were no differences in the damage caused by generalist beetles

and thrips in response to plant resistance traits. Generalist beetles did not appear to

respond to plant investment in resistance or to the pesticide treatment, and there were

no differences in the amount of damage between the two source populations.

In Year One, genotypes differed in tolerance of thrips damage, but source

populations did not (table 3).

**Table 3: Results of a linear mixed model testing for differences between source populations and genotypes in tolerance of different types of herbivore damage. Fitness (leaf area) is the response variable. Likelihood ratio X2 tests were used to evaluate the interaction of herbivore damage (a fixed effect), state source population (a fixed effect) and plant genotype (a random effect).**

| year | terms | thrips damage | | | beetle and generalist | | | caterpillar damage | | |
|------|-------|----|-----|------|----|-----|------|----|-----|------|
| | | df | $X^2$ | p-value | df | $X^2$ | p-value | df | $X^2$ | p-value |
| year 1 | damage x genotype | 1 | 45.7 | 1.36 e -11* | 1 | 19.7 | 9.24 e -6* | NA | NA | NA |
| | state x genotype | 1 | 2.51 | 0.113 | 1 | 3.34 | 0.068 | NA | NA | NA |
| year 2 | damage x genotype | 1 | 4.35 | 0.037* | 1 | 0.177 | 0.674 | 1 | 17.5 | 2.95 e -5* |
| | state x genotype | 1 | 5.31 | 0.021* | 1 | 1.75 | 0.186 | 1 | 0.241 | 0.623 |

Genotypes varied in tolerance of beetle damage but again source populations did not. In Year Two, there were genotypic differences in tolerance of thrips. There were also genotypic differences between the source populations. Tolerance of generalist beetle damage did not differ between genotypes. Tolerance of caterpillar damage differed between genotypes, but there were no differences in tolerance between source populations.

The heritability for most resistance traits was low, indicating a low degree of genetic determination and high amount of environmental influence (table 4).

**Table 4: Broad sense heritabilities (H2) and coefficient of genetic variation (CV%) for putative defense traits in year one and year two. 95% confidence intervals are a parametric bootstrap with 1,000 replicates (bias adjusted).**

| trait | state | Year 1 | | | Year 2 | | |
|---|---|---|---|---|---|---|---|
| | | heritability | confidence interval | CV % | heritability | confidence interval | CV % |
| trichomes | NC | .712 | .564 - .887 | 43.2 | .364 | .256 - .646 | 34.4 |
| | FL | .43 | .124 - .727 | 21.7 | .166 | -.021 - .278 | 14.2 |
| nectar volume | NC | .085 | -.098 - .236 | 9.3 | 0 | -.146 - .075 | 0 |
| | FL | .136 | -.148 - .328 | 8.04 | 0 | -.177 - .078 | 0 |
| toughness | NC | 0 | -.175 - .089 | 0 | 0 | -.119 - .065 | 0 |
| | FL | 0 | -.196 - .354 | 3.2 | 0 | -.050 - .192 | 0 |

The heritability estimates tended to be higher for Year One, which may reflect the shared greenhouse growth environment reducing environmentally-induced

variation prior to the experiment. In both years, trichome density had the highest heritability.

Due to the low heritability values, the coefficient of genetic variation was low or zero for many resistance traits (table 4). The values for Year One were again higher than the values for Year Two. In both years, trichomes again featured the highest coefficient of genetic variation and hence potential to change under selection.

The genetic correlations between defense traits were weak and non-significant for both years (table 5), as expected for traits with low heritability. There were no correlations between resistance traits and tolerance of thrips damage across both experimental years and source populations (table 5). In Year Two, both states featured negative correlations between caterpillar tolerance and the resistance traits of nectar volume and leaf toughness, respectively. North Carolina plants showed negative correlations between tolerance of generalist damage and both trichome density and leaf toughness in Year One, and nectar volume in Year Two. For both years and states, there were significant positive correlations between tolerances of different types of herbivore damage.

**Table 5: Genetic covariances and Pearson correlation coefficients between traits. Pearson correlation coefficients are above and genetic covariances are below the diagonal. Correlations that are significantly non-zero (P <0. 05) are marked with \*.**

**Year 1 North Carolina**

|  | trichomes | nectar | toughness | tolerance generalist | tolerance thrips |
|---|---|---|---|---|---|
| trichomes |  | -.0078 | .345 | -.266 | -.0975 |
| nectar | -.00014 |  | -.339 | .230 | .212 |
| toughness | .00024 | -.01143 |  | -.179 | -.229 |
| tolerance generalist | -5.92 e -5 | .00268 | -7.06 e -5 |  | .674* |
| tolerance thrips | -5.84 e -5 | -.00273 | -.000113 | .000128 |  |

**Year 1 Florida**

|  | trichomes | nectar | toughness | tolerance generalist | tolerance thrips |
|---|---|---|---|---|---|
| trichomes |  | -.244 | -.118 | .293 | -.273 |
| nectar | -.0067 |  | -.235 | .153 | .102 |
| toughness | -.00012 | -.009 |  | .113 | .0374 |
| tolerance generalist | .00030 | .0524 | -.0035 |  | .8006* |
| tolerance thrips | 4.32 e -6 | .00391 | -.000142 | .0030 |  |

## Year 2 North Carolina

|  | trichomes | nectar | toughness | tolerance generalist | tolerance thrips | tolerance caterpillar |
|---|---|---|---|---|---|---|
| trichomes |  | .0697 | .083 | -.333 | -.380 | -.327 |
| nectar | .0016 |  | -.015 | -.413* | -.325 | -.285* |
| toughness | 3.46 e -5 | -.0067 |  | -.0098 | .0724 | -.447* |
| tolerance generalist | -.0029 | -.0378* | .000114 |  | .962* | .780* |
| tolerance thrips | -.0038 | -.557 | .00219 | .0410* |  | .8003* |
| tolerance caterpillar | -.00437 | -.383* | -.00999* | .0256* | .451* |  |

## Year 2 Florida

|  | trichomes | nectar | toughness | tolerance generalist | tolerance thrips | tolerance caterpillar |
|---|---|---|---|---|---|---|
| trichomes |  | .0078 | -.0192 | -.0315 | .0324 | -.151 |
| nectar | .0011 |  | 0.0482 | -.086 | -.143 | -.321* |
| toughness | -6.23 e -5 | .0011 |  | -.220 | -.124 | -.397* |
| tolerance generalist | 5.95 e -5 | -.0194 | -.000826 |  | .981* | .908* |
| tolerance thrips | .000155 | -.0459 | -.00203 | .00787* |  | .882* |
| tolerance caterpillar | .000112 | -.04168 | -.00235* | .00516* | .0128* |  |

## *2.4 Discussion*

In this study, putative resistance traits did not decrease herbivore damage, even though these traits differed significantly between source populations. However, plants did possess tolerance of herbivore damage, and there were negative genetic correlations between resistance traits and tolerance of damage. Moreover, tolerance to different types of herbivore damage was generally positively correlated across genotypes. In the following paragraphs, I discuss how these results inform our knowledge of herbivore specificity of resistance and tolerance, the role of genetic correlations between defenses, and the implications of defense heritability and genotypic variation.

I quantified differences in resistance trait expression between source populations to determine if possible source population differences in herbivore damage drove differential trait production. I could then investigate if differences in resistance traits reduced herbivory by specific herbivores. In both years, differences in resistance trait expression between the two populations did not alter the amount of herbivore damage plants accumulated (tables 1 and 2). In particular, Florida plants produced higher levels of putative resistance traits but did not receive less herbivore damage (fig. 1).

One possibility for the lack of benefits from resistance traits is that the value of resistance may vary based on the year and the insect attacking. For instance, in year two,

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higher ant visitation reduced beetle damage in Florida plants, although it had no effect in year one (fig. 3). Ant defense mutualisms rely on the partner ant species providing a benefit (defense from herbivores) in exchange for a reward (nectar). Invasive or non-aggressive ants may not be reliable partners in this mutualism and may provide few defense benefits (Bronstein 2001). Indeed, a previous study on ant defense in *P. incarnata* found that the attending ant species strongly impacted the value of defense provided (McLain 1983).

Furthermore, some putative resistance traits may not be effective against all herbivores. For instance, in both years, thrips were not deterred by increased resistance trait expression (fig. 2, fig. 4). Tiny thrips may be too inconspicuous for ants to attack, yet ant visitation may reduce visitation by other insects, leaving thrips with less competition or predation. One possibility is that very small insects such as thrips may not respond to defenses such as trichomes, or that trichomes may prevent other insects from competing with or consuming thrips. A recent review found that trichomes in many plant species have negative impacts on beneficial, predatory insects such as ants (Riddick & Simmons 2014). In addition, Carmona et al. (2011) found that all resistance traits had a very low ability to predict damage by piercing-sucking herbivores such as thrips.

Another possibility for the low benefits of resistance trait production is that these traits may have primary functions not related to defense. For instance, plant trichomes may participate in water balance, heat retention, and UV protection for trichomes in addition to defense (Levin 1973). Leaf toughness can deter herbivore attack, but this trait is also influenced by changes in nutrient availability, leaf age, growth rates, and light exposure (Westbrook *et al.* 2011). Extrafloral nectar may serve to divert ants away from flowers to keep them from disturbing pollinators (Wagner & Kay 2002; Ness 2006). Thus, each of the putative defense traits investigated here may primarily function in other roles.

Tolerance was effective against a variety of herbivores (table 3). In contrast to the pattern for resistance traits, populations did not differ in tolerance. Tolerance appears to be a broadly generalized defense, as indicated by the positive correlations between tolerance of different classes of herbivore damage, and by the lack of variation between source populations. The mechanisms that comprise tolerance—whether through compensatory growth or reproduction—are likely widely applicable to a range of herbivore classes and not specific.

This broad effectiveness may make tolerance a more effective strategy in a range of environments and herbivore-attack conditions. It could also explain the lack of

differentiation in this trait between source populations.  Damage by specific herbivores may vary between populations, yet the overall magnitude of damage need not necessarily vary. If tolerance is a broad response triggered regardless of herbivore identity, there would thus be no differential selective pressure to alter the amount of tolerance between source populations.

The broad-sense heritability of resistance traits was zero or nearly so (table 4). Previous studies have reported broad-sense heritabilities for defense traits higher than those found in this study (Stevens & Lindroth 2005; Johnson *et al.* 2009).  One possible explanation for the low heritability values is that selection on defense traits has eliminated much of the genetic variation in these traits within populations. These traits could still differ between populations due to past differences in selection, whether by herbivores or other agents. Thus, even if heritability is low or non-existent in the present population, past selection could have led to trait differences between populations. An alternative interpretation is that resistance traits may still be produced if their low heritability impedes natural selection acting against their production. Drift or founder effects could also lead to the source population trait differentiation.  Another possibility is that these traits do function in resistance, but that it is only possible to detect this benefit at higher levels of herbivore attack.

In sharp contrast to the low heritability values for resistance traits, tolerance of herbivore damage differed between genotypes (table 3). This genotypic variation could facilitate the evolution of tolerance instead of resistance traits, especially if plants receive little benefit from resistance trait investment. In particular, the genotypic variation in tolerance of thrips damage for both experimental years was intriguing, especially considering how little thrips responded to defense traits and the low heritability of those traits. This raises the possibility that the broad effectiveness of tolerance acts to reduce the fitness impacts of damage caused by specific herbivores not impacted by resistance traits.

The genetic correlations between resistance traits were zero or nearly so (table 5). The low genetic correlation shows little indication of genetic constraints between resistance traits limiting changes in trait values. The low genetic variance for resistance traits would make it more difficult to detect correlation between resistance traits. Previous studies have found low (Agrawal *et al.* 2002), high (Andrew *et al.* 2005), and a mixture of low and high (Johnson *et al.* 2009) correlations between resistance traits.

In contrast to the values for resistance traits, there were consistent positive correlations between tolerance of different types of herbivore damage across years and study source populations (table 5). Few studies have considered connections between

tolerance of different classes of herbivore damage (Kotanen & Rosenthal 2000; Stinchcombe & Rausher 2001; Manzaneda *et al.* 2010). In *Boechera stricta*, tolerance to different types of herbivores was also positively correlated (Manzaneda *et al.* 2010). These positive genetic correlations between tolerance classes could again support the evolution of tolerance as a broad mechanism effective against several herbivore classes. This broad applicability of tolerance against different type of herbivore attack could be an advantage, especially if the specific herbivores attacking are unpredictable or highly variable. The disturbed, marginal spaces that *P. incarnata* colonizes may host uncertain, fluctuating herbivores and hence make the inclusive defense tolerance provides worthwhile.

Contrary to the low correlations for resistance traits, genotypic correlations between tolerance of herbivore damage and putative resistance traits were consistently negative (table 5). Plants like *P. incarnata* that grow in disturbed habitats are often expected to invest less in resistance and more in tolerance (Coley 1988; Simms & Triplett 1994). These negative correlations suggest that genetic constraints could lead to trade-offs between expression of tolerance and resistance traits. The combination of positive correlations between tolerance of different types of herbivore damage and negative correlations between resistance and tolerance could facilitate the evolution of broad

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tolerance against a range of herbivores and constrain simultaneous investment in resistance traits. One possibility for the continued production of both tolerance and non-beneficial resistance traits is that the low heritability of resistance traits makes selection against these traits difficult. Alternatively, if resistance traits function in non-defensive roles, there could still be advantages to plants possessing both resistance and tolerance despite the genetic constraints.

The negative genetic correlations between resistance and tolerance in *P. incarnata* argue against the production of resistance traits simply due to underlying genetic constraints. Negative correlations between tolerance and resistance traits are frequently predicted, although zero or positive correlations have also been found (Mauricio *et al.* 1997; Tiffin & Rausher 1999; Fornoni *et al.* 2003). The occurrence of negative correlations that limit the joint production of resistance and tolerance may be less common than originally thought (Leimu & Koricheva 2006).

In summary, increased investment in resistance traits did not lead to decreases in damage by specific herbivores. The lack of benefits from resistance could indicate that these traits have a primarily non-defensive role. Production of resistance traits differed between populations. In contrast to the resistance results, there was genotypic variation in tolerance of herbivore damage and there were negative correlations between tolerance

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and resistance.  This could potentially lead to trade-offs between expression of tolerance

and resistance and facilitate tolerance to multiple herbivores.

# 3. Does herbivore damage impact expression of and selection on floral traits?

## *3.1 Introduction*

Flowers are highly visible structures plants can use to interact with pollinators and exchange genetic material. Pollinator preferences can shape floral traits such as flower shape, color, and nectar rewards (Galen 1989; Schemske & Bradshaw 1999). For example, hummingbird floral preferences have led to the evolution of long, tubular flowers that tend to be red, orange, or yellow, with high nectar volumes (Meléndez-Ackerman *et al.* 1997). Yet, interactions with organisms other than pollinators may also shape floral traits. Herbivores may influence selection on floral traits through resource shortages caused by damage. We investigated whether herbivore damage alters expression of two floral traits—flowering phenology and floral sex ratio—and how natural selection acts on these traits.

Plants demonstrate a wide range of mating systems, including andromonoecy, in which both perfect (hermaphroditic) and staminate (male-only) flowers occur on the same plant. Approximately 2% of flowering plants are andromonoecious (Miller and Diggle 2003). Herbivore damage that leads to resource shortages could influence floral sex ratios (Bertin 1982). Male flowers can require fewer resources, as pollen is cheaper to produce than seeds and fruit (Vallejo-Marín & Rausher 2007). Resources shortages may

spur increased relative production of male flowers as a resource-saving measure and thus alter the floral sex ratio (Quesada-Aguilar *et al.* 2008). Hence, incorporating an analysis of changes in sex ratio in response to herbivory can provide a more complete picture of the full suite of plant traits—both defensive and compensatory—on which herbivores may exert selective pressure.

While numerous studies have looked at pollinator influences on floral traits, few have also investigated whether herbivores influence mating system evolution (Marshall & Ganders 2001; Wise & Hébert 2010). Furthermore, fitness measurements usually only address female fitness by recording seed size and number, yet selection may also act on floral traits that determine male fitness (Strauss 1997). In an andromonoecious species, male flower investment could lead to higher siring success due to increased pollen production.

The timing of flowering can also alter plant fitness by changing susceptibility to herbivores or creating more time to flower and mature seeds. Flowering phenology can enable plants to escape herbivore attack and hence impact fitness (English-Loeb & Karban 1992; Mahoro 2003; Kawagoe & Kudoh 2010). While studies have traditionally focused on how resistance traits ward off herbivore attack, phenology could be a strategy for herbivore escape and hence impact fitness. If herbivore damage leads to

resource shortages that impair floral investment and hence decrease reproductive success, strategies such as shifting flowering phenology to mitigate these fitness costs could constitute an effective defense strategy.

Tolerance is an herbivore defense strategy in which herbivore damage triggers changes in plant growth or reproduction (Strauss & Agrawal 1999). If herbivore damage leads to changes in floral trait expression, these changes could be interpreted as compensatory measures to the resource costs incurred by herbivore damage, that is, as a form of tolerance.

Selection analysis is a method to reveal how natural selection acts on trait values (Lande & Arnold 1983). Through a series of regressions, it partitions selection into directional (linear), stabilizing or disruptive (quadratic), and correlational (i.e., selection on trait combinations) components. By performing selection analysis on subjects in different treatment groups, one can determine if an experimental manipulation alters how natural selection acts on traits. We investigated if herbivores drive changes in selection by comparing results for plants in herbivore exclusion and control treatments to identify the role of herbivores in shaping floral traits. Numerous studies have demonstrated that floral herbivores can drive selection on floral traits (Louda & Potvin 1995; Brody 1997; Pilson 2000; Parachnowitsch & Caruso 2008). However, the majority

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of studies have considered herbivory by seed predators, which directly impact flowers, and not attack by herbivores that consume other plant parts (Fukano *et al.* 2013).

Our research centers on *Passiflora incarnata* (may-pop), a perennial vine native to the southeast U.S. *P. incarnata* is functionally andromonoecious: it produces both hermaphroditic and male flowers which do not set fruit (May & Spears Jr 1988). Previous studies have investigated the production of male flowers in this species, and found that foliage damage near a developing bud makes it more likely that the bud will mature into a functionally male flower (Spears Jr & May 1988; Dai & Galloway 2012).

We utilized plants collected from one North Carolina population and one Florida population. The herbivore pressures faced by the plant may vary across its range due to the migration pattern of its specialist herbivore, the caterpillars of the gulf fritillary butterfly *Agraulis vanillae* (McGuire 1999). These caterpillars are present for much of the year in more southern populations but migrate north for only a brief period (Walker 2001). We performed a common garden field experiment in North Carolina using vegetative clones of plants from the two source populations. We measured herbivore damage, floral traits, and a proxy for fitness to investigate the following questions:
1. Are there population or genotypic differences in flowering phenology or the floral sex ratio?

2. Does herbivore damage alter the timing of flowering or the floral sex ratio?

3. Does herbivore presence or absence change natural selection acting on flowering phenology or floral sex ratio?

## *3.2 Methods*

### 3.2.1 Field Experiment

Here, we provide a brief description of the relevant field experiment. A more detailed explanation of the field methods is available in chapter 2.

*Passiflora incarnata* is a perennial vine native to the southeast United States. It grows in disturbed habitats such as roadsides and field margins. A range of herbivores attack *P. incarnata*: a specialist herbivore (*Agraulis vanillae* gulf fritillary butterfly caterpillars), generalist thrips (Thysanoptera), generalist flea beetles (Alcitini), and others. Genotypes used in the study were collected from roadsides in central Florida (near the town of Lakeland) and southeast North Carolina (near the town of Wilmington).

The experimental year (summer 2013) had higher than average rainfall totals and lower than average temperature, which was likely responsible for the low fruit and seed set we observed in our experimental plants. Consequently, we used total leaf area at the end of the growing season in October as a measure of fitness. Leaf area can be an

appropriate measure of fitness in a perennial plant that also reproduces clonally

(Winkler & Fischer 1999). Larger plants are more likely to survive through the next

season and experience greater future reproductive success. (Ollerton & Lack 1998).

Plants were allocated to herbivore exclusion (through pesticide application) or control

treatments.

The number of hermaphroditic and male-only flowers were recorded on each

plant every three days. Male flowers can be identified by their permanently upright,

unflexed stigma which does not come into contact with pollinators (figure 5).



**Figure 5: A male (left) and hermaphroditic (right) flower on P. incarnata. Image
by U.S. Fish and Wildlife Service Southeast Region, Creative Commons 2.0 license.**

Flowering time was measured as the number of days from the initial census date

in July to the date the first bud was observed. Floral sex ratio was calculated as the

number of male flowers divided by the total number of flowers for each plant. We

quantified pollen production in both male and hermaphroditic flowers by collecting

anthers and storing them in ethanol. For 10 plants from each population, we sampled

the anthers for one hermaphroditic flower and one male flower from the same plant, for

a total of 40 flowers. We counted the number of pollen grains number under a dissecting

microscope for three sub-samples for each plant, with the final value averaged.

At the end of the growing season in October, total leaf area of each plant was

calculated by measuring the area of three representative leaves per plant and

multiplying average area per leaf by the total number of leaves on the plant.  Damage

was attributed to three herbivore classes--specialist caterpillars, thrips, or generalist

beetles—based on the type of damage observed (see Ch. 2).  The degree of damage was

assigned a classification level based upon visual inspection and the number of damaged

leaves per plant was recorded.  I scaled this amount of damage by the total number of

leaves to calculate a percentage damage for the entire plant. Damage was arc-sine

square-root transformed to improve normality.

## 3.2.2 Data Analysis

We completed all analysis in R (R Core Team, 2015). Broad sense heritability was

calculated with REML by dividing the total estimated genetic variance (additive and

non-additive) by the total phenotypic variance (genetic and environmental). Bias-adjusted confidence intervals were obtained from a parametric bootstrap with 1,000 replicates. To investigate how herbivore damage impacts sex ratio, Wald $X^2$ tests were performed using a binomial distribution in the lme4 package in R. For flowering date, the flowering dates were binned into three classes (early, middle and late flowering) and Wald $X^2$ tests were used on a multinomial regression using the function 'glmer'. For both models, the floral trait was the response variable, with the amount of damage by each herbivore class and source population as fixed effects, with field block and genotype as random effects. We tested if genotypes vary in how floral traits change in response to herbivore damage. We used a linear mixed model with the floral trait as the response, and tested for an interaction of herbivore damage (a fixed effect) and genotype (a random effect), with f block also as a random effect. We looked for differences in pollen production between male and hermaphroditic flowers, and if this varied between source populations. We fit a linear mixed model with pollen number as a response variable, floral sex and source population as fixed effects, and plant and field block as random effects.

We calculated linear, quadratic, and correlational selection gradients using the method described by Lande and Arnold (1983). We applied a series of regressions of

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fitness (leaf area) against the trait values to estimate model coefficients that describe the components of selection. As we utilized genotypic values in the analysis, this represents a genotypic rather than a phenotypic selection analysis, and should be less vulnerable to bias from environmental correlations between fitness and traits (Rausher 1992).

## *3.3 Results*

There were no differences between the source populations in bud date or male flower ratio (likelihood ratio test, all P>0.6). Hence, for all other analysis, the data from the two source populations were combined.

We found differences between the control and pesticide treatments in selection gradients for floral traits, indicating the role of herbivore damage in altering selection on these traits. We did not find any evidence of linear, correlational, or quadratic selection acting on bud date or male flower ratio for plants in the control treatment (table 6). In contrast, there was selection for later flowering date for plants in the pesticide treatment and disruptive selection on this trait. There was linear selection for a lower male flower ratio (and hence, proportionally more hermaphroditic flowers) for ants in the pesticide treatment. The linear selection gradients for both flowering date and male flower ratio between the control and pesticide treatments were significantly different (fig.6). There was significant correlational selection acting on bud date and floral ratio for the

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treatment plants. There was no correlational selection on control plants, and no

significant difference in correlational selection due to the treatment.

**Table 6: Linear, quadratic, and correlational selection gradients for flowering date and male flower ratio, along with differences in selection due to treatment. $\gamma_{ii}$ are doubled from regression values.**

| trait | control | | | | treatment | | | |
|---|---|---|---|---|---|---|---|---|
| | $\beta$ | p-value | $\gamma_{ii}$ | p-value | $\beta$ | p-value | $\gamma_{ii}$ | p-value |
| flowering date | -0.160 | 0.294 | -0.110 | .366 | 0.206 | 0.020* | 0.208 | 0.012* |
| male flower ratio | -0.223 | 0.159 | -0.248 | .365 | -0.303 | 0.007* | 0.027 | 0.891 |

| trait | change due to treatment | | | |
|---|---|---|---|---|
| | $\Delta\beta$ | $\Delta\beta$ p-value | $\Delta\gamma_{ii}$ | $\Delta\gamma_{ii}$ p-value |
| flowering date | -0.366 | 0.020* | -0.318 | 0.0051* |
| male flower ratio | 0.08 | <.0005* | -0.275 | 0.202 |

| trait | control | | treatment | | change due to treatment | |
|---|---|---|---|---|---|---|
| | $\gamma_{ij}$ | $\gamma_{ij}$ p-value | $\gamma_{ij}$ | $\gamma_{ij}$ p-value | $\Delta \gamma_{ij}$ | $\Delta\gamma_{ij}$ p-value |
| flowering date: male flower ratio | 0.089 | 0.605 | 0.160 | 0.047* | 0.041 | 0.1754 |
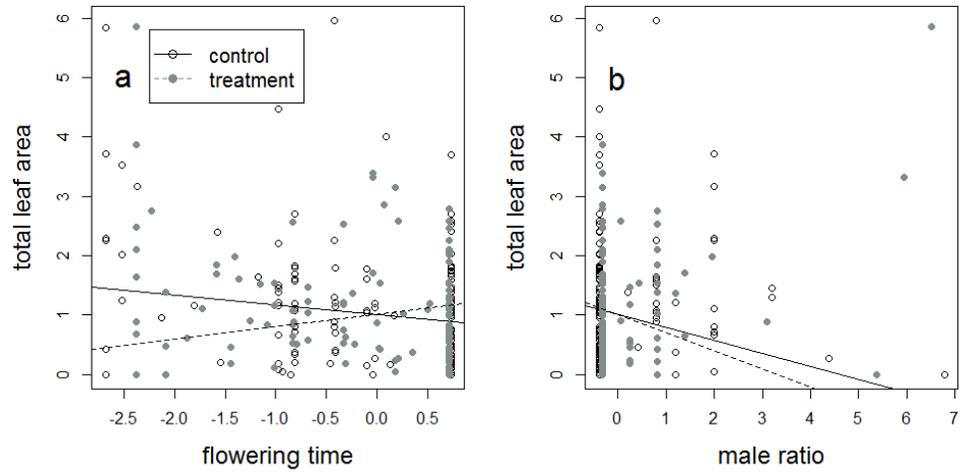
**Figure 6: Differences in linear selection gradients between control (open circles, solid line) and treatment (grey closed circles, dashed line) plants for the floral traits of a) flowering time and b) male ratio. The values on the x and y axis represent the scaled and standardized genotype mean values utilized in the regression analysis.**

The broad-sense heritability of floral sex ratio was zero (table 7). In contrast, the heritability of flowering date was higher and the confidence interval did not overlap zero. Flowering date was significantly impacted by generalist beetle damage, although not by caterpillar or thrips damage (table 8).

**Table 7: Broad sense heritability (H2) for floral traits. 95% confidence intervals are a parametric bootstrap with 1,000 replicates.**

| Trait | Heritability | Confidence interval |
|---|---|---|
| male flower ratio | 0 | -0.068- 0.0307 |
| flowering date | 0.189 | 0.074- 0.253 |

**Table 8: Impact of herbivore damage on flowering date. Wald X2 tests were used to investigate the impact of damage by different herbivore classes on flowering date.**

| flowering date | | early | | middle | | late | |
|---|---|---|---|---|---|---|---|
| damage type | df | Wald $X^2$ | p-value | Wald $X^2$ | p-value | Wald $X^2$ | p-value |
| generalist beetle damage | 1 | 0.801 | 0.423 | 0.552 | 0.581 | 3.859 | 0.0001* |
| thrips damage | 1 | 0.131 | 0.895 | 1.676 | 0.094 | 0.954 | 0.340 |
| caterpillar damage | 1 | 1.250 | 0.211 | 1.306 | 0.192 | 0.345 | 0.730 |

Plants that suffered more beetle damage flowered earlier in the season than those with less beetle damage (fig. 7).
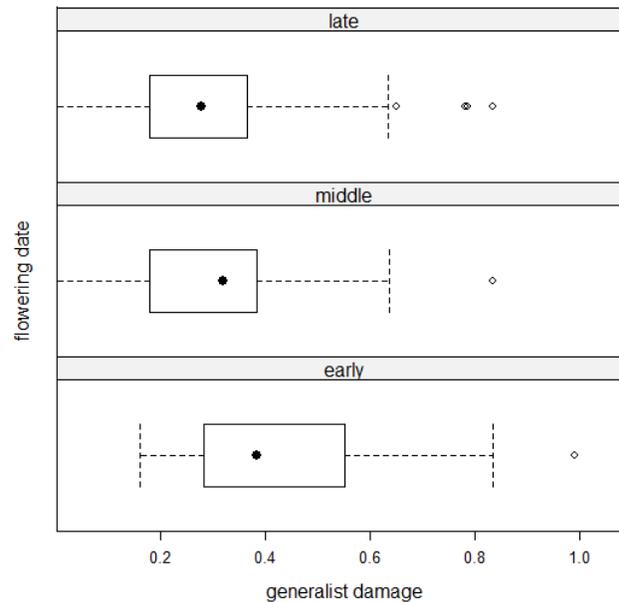


**Figure 7: The relationship between generalist beetle damage and flowering date. We divided flowering date into three categories ('early', 'middle' and 'late' in the season).**

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Floral sex ratio was significantly altered by the amount of generalist beetle damage plants received, but not by thrips damage or specialist caterpillar damage (table 9).

**Table 9: Impact of herbivore damage on floral sex ratio. Wald X2 tests were used to investigate how herbivore damage impacted the sex ratio of male to hermaphroditic flowers produced by a plant.**

| Damage type | df | Wald $X^2$ | p-value |
|---|---|---|---|
| Generalist beetle damage | 1 | 2.648 | 0.0081* |
| Thrips damage | 1 | -0.570 | 0.569 |
| Caterpillar damage | 1 | -0.760 | 0.447 |

Plants with more beetle damage expressed a higher ratio of male to hermaphroditic flowers (fig 8).
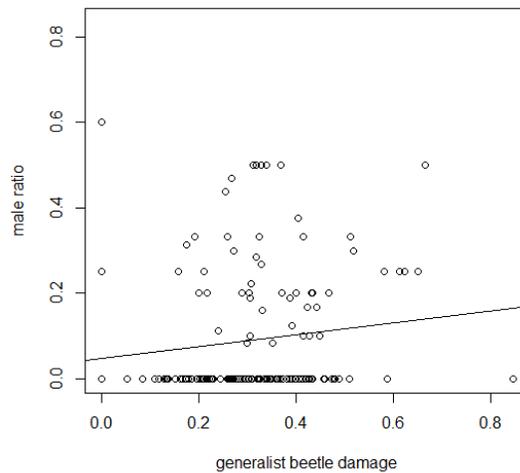


**Figure 8: The relationship between generalist beetle damage and male ratio, with each point representing a genotype mean.**

There were no differences between genotypes in changes in floral traits in response to damage—that is, all genotypes tended to respond in the same way (bud date: $X^2$ test, $X = 0.337$, df=1, P=0.562, floral ratio: $X = 0.228$, df=1, P= 0.633). There were no differences in the pollen production between male and hermaphroditic flowers (mean ± sd hermaphroditic flowers 82.6 ±52.2, male flowers 84.2 ± 51.05, paired t-test P=0.94), or between source populations (mean ± sd Florida 69.6 ± 50.4, North Carolina 93.8 ± 49.7, paired t-test P=0.24).

## 3.4 Discussion

Briefly, we did not find differences in floral trait expression between the two source populations. Selection on floral traits differed between the control and treatment groups, with reduced herbivory leading to selection for later flowering and a decrease in relative male flower production. The broad-sense heritability for the male flower ratio was essentially zero, although we did find measurable heritability for flowering date. Plants with higher levels of generalist damage produced more male flowers and flowered earlier in the season. Here, we place the results in context with our other results for this study system, and we discuss the possible implications of changes in both trait expression and selection due to herbivore damage.

The selection gradients favoring later flowering in the absence of herbivores and a higher proportion of hermaphroditic flowers, along with the significant treatment effect, suggest that herbivore damage alters selection on these traits (table 6, fig. 6). The selection on flowering date could be due to trade-offs in resource allocation between growth and flowering. Later flowering could allow plants to devote resources early in the season to growth before later allocations to reproduction. Resources devoted to growth and survival beyond one field season could be especially key in perennial plants such as *P. incarnata.* Plants that delay flowering may also have reached a larger size by the time they initiate flowering, and be able to produce higher flower numbers. Plants in the pesticide treatment also experienced selection for a higher proportion of hermaphroditic flowers, suggesting that resource shortages caused by herbivore damage constrain investment in hermaphroditic flowers.  The higher proportion of male flowers produced by plants in the control treatment could thus be making the best of a poor situation—if damaged plants lack insufficient resources to mature fruit, male flowers could still provide outcrossing success.

We did not find evidence of population differences in flowering phenology or in floral sex ratio. In contrast, plants from the southern Florida populations invest more in resistance traits than do plants from North Carolina (Ch.2). We had expected to find

differences in flowering date between the populations, due to the earlier warming and

start to the growing season predicted in Florida compared to North Carolina. However,

if flowering date is heavily influenced by environmental cues, there may be little

differentiation between plants from different source populations when grown in a

common environment. Indeed, the heritability for flowering date was limited, so

environmental cues would play a relatively strong role in influencing expression of this

trait (table 7). Although we found evidence of heritability for this trait, there were no

differences between genotypes in how flowering date changed in response to generalist

herbivore damage. This lack of differentiation between genotypes would indicate that

they would respond similarly to selective pressures acting on this trait.

The male flower ratio also did not vary between populations, but in addition it

did not demonstrate heritability. This trait may thus be more responsive to local

environmental conditions than flowering date. Previous studies have reported variation

both within populations (between families) and between populations in male flower

ratios in *Solanum carolinense* (Elle 1998). In *Solanum carolinense*, local resource shortages

appear to drive changes in floral sex ratio. Both floral sex ratios and flowering date may

demonstrate high levels of plasticity due to the impact of environmental cues on

expression of these traits. Consistent with the lack of heritability for male flower ratio,

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there were no differences between genotypes in how male flower ratio changed in response to generalist herbivore damage.

Generalist beetle damage impacted both flowering time and the floral sex ratio, with higher levels of damage leading to earlier flowering and higher male flower production (table 8 and 9). These plastic changes in trait expression may be an attempt to compensate for the resource costs of herbivore damage.

Early flowering could be viewed as a tolerance trait. Plants growing in high-disturbance environments may face more pressure to rapidly flower and set fruit than to invest in defense traits, particularly if the rewards from investing in defense are few or unpredictable (de Jong and van der Meijden 2000). *P. incarnata* grows in weedy, disturbed habitats, which may influence how plants respond to herbivore damage. If damage—whether from herbivores or field mowing—is frequent, unpredictable, and at a high level—tolerance of damage may be a more effective strategy than resistance (Bazzaz et al 1987). Plasticity in flowering date could be advantageous if damage is variable year to year. In years with lower herbivory, plants that wait to flower until later in the season may be larger and able to devote more resources to reproduction (Cheplick 1995). Juenger and Bergelson reported earlier flowering in damaged *Ipomopsis aggregata*,

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and suggested that shifts in flowering phenology may function as a tolerance trait along with compensatory growth (Juenger & Bergelson 2000).

One possible explanation for increased male flower production following damage is that male flowers could still contribute to outcrossing success if high female success is no longer possible following damage. Other studies have investigated the possibility of higher pollen production in male flowers, as resources not devoted to fruit set could be re-allocated to increased pollen production to increase male fitness (Podolsky 1993). We did not find any support for higher pollen production in male flowers. It may still be possible for male-only flower production to increase out-crossing success. One possible advantage to male-only flowers is that they may experience higher visitation than hermaphroditic flowers by producing higher pollen or nectar levels, although this does not appear to occur in *P. incarnata* (Vaughton & Ramsey 1998; Dai & Galloway 2012). Pollen discounting, where self-deposition of pollen decreases outcrossing success, could also explain male flower production (Harder & Wilson 1998). *P. incarnata* is self-incompatible, yet pollen deposition from a flower's own anthers could still physically interfere with successful fertilization by pollen from other plants. Producing male-only flowers could reduce this risk of autogamy and promote out-crossing success (Dai & Galloway 2011).

Herbivore damage may have both short and long term effects on floral traits in *P. incarnata.* Herbivore damage led to plastic changes in trait expression within a field season. The changes in selection due to herbivore presence and absence also identify herbivores as selective agents on these traits. These selection pressures could then alter trait values beyond the ephemeral plasticity changes found in one season.

Our results link herbivore damage to changes in floral traits and to shifts in how selection acts on these traits. This emphasizes that herbivores can shape floral traits not normally connected explicitly to defense and that these traits may function in a defensive capability (Pilson & Decker 2002). Shifts in flowering time can be shaped by herbivory, and flowering early versus late could be a strategy to avoid or compensate for herbivore damage (Elzinga et al. 2007, Juenger and Bergelson 2000). As discussed in chapter 2, we found no fitness benefits or signs of decreased herbivore damage from increased investment in resistance traits, although broad tolerance appears to be present in *P. incarnata*. This broaches the possibility that tolerance is a key defense strategy in the plant, instead of investment in resistance traits. Changes in flowering and hence reproductive phenology that mitigate the fitness costs of herbivore damage could then constitute an overlooked component of tolerance (Tiffin 2000).

In summary, we found differences in selection between plants in control and herbivore exclusion treatments, with herbivore exclusion leading to selection for later flowering and a decreased ratio of male flowers. These differences in selection due to herbivore manipulation indicate that herbivores act as selective agents on these floral traits. Plants with higher levels of generalist herbivore damage demonstrated earlier flowering and the produced a higher ratio of male flowers. These results point to the influence of herbivores on floral traits, and the possibility of changes in floral traits as a compensatory form of tolerance following herbivore damage. This highlights the need to consider the role of selective agents other than pollinators in influencing plant floral traits, and the flexibility of plants in responding to herbivore damage through both plasticity and evolution.

# 4. Does natural selection favor multiple, simultaneous plant defenses against herbivores?

## *4.1 Introduction*

Plants commonly produce multiple defense traits as protection against herbivore damage. This mixed investment in multiple traits has still not been explained: why do plants not produce maximum levels of the single most effective trait? Models have predicted the conditions and situations that could lead to the expression of intermediate levels of two defenses instead of higher investment in only one defense, but few studies have tested experimentally for mechanisms that could facilitate selection for multiple defenses (Mauricio 2000).

Here, I describe theoretical surfaces drawn from a modification of existing cost/ benefit models for plant defense production. I then evaluate fitness surfaces fitted to data from an experiment in which I manipulated herbivore presence and absence to calculate the costs and benefits of defense production. Finally, I interpret these empirical fitness surfaces in the context of the results from the theoretical surfaces.

Defenses can be categorized as either resistance traits, such as secondary chemicals or physical traits that ward off herbivore attack, or tolerance traits, such as changes in growth or reproduction following herbivore damage that mitigate its negative effects (Strauss & Agrawal 1999). Interactions between these defense strategies

are possible, as plants may invest in both resistance and tolerance, or in several resistance traits simultaneously. Here, I consider both resistance and tolerance traits.

At least three, non-exclusive hypotheses could explain the production of multiple defense traits. If defenses are highly specific and only effective against certain herbivores, the need for responses to a broad variety of herbivores could require a suite of defenses. Genetic correlations between defenses could also lead to the production of multiple defenses, even if only one defense functions in defense.

Alternatively, synergism could explain multiple defenses, and could occur through various mechanisms. The costs of producing defenses could accelerate with investment, making investment in each of two defenses cheaper than higher investment in one defense (Bergelson *et al.* 2001). Similarly, selection could favor the production of multiple defenses if the benefits of defense investment increase non-linearly from increased production of a defense providing proportionally more benefit as the trait level increases (Berenbaum & Zangerl 1996). Possessing multiple defenses could also be beneficial by forcing an herbivore to overcome two defenses, making them more difficult to resist in combination than they would be singly.

One possible method of determining if synergism occurs between defenses is to plot fitness surfaces from different trait combinations to examine how traits interact.

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Furthermore, the manipulation of herbivore presence can elucidate the costs and benefits and highlight the role that herbivores play in determining the optimal fitness surface. I now discuss how theoretical surfaces can guide interpretation of empirical selection surfaces, and the limitations of distinguishing between trait interactions and non-linear trait functions based solely on the fitness surface.

## *4.2 Theory*

Models of plant resistance and tolerance have been used to investigate non-linear costs and benefits and trait interactions (Fornoni and Rausher 2004). Here, I describe a range of possible theoretical fitness plots to aid in interpretation of the empirical fitness surfaces. I review how synergism between the costs or benefits of multiple defenses would be expected to change the shape of the fitness surface in the absence versus presence of herbivores.

A simple model based on Fornoni et al. (2004) for plant fitness as a function of the levels of two defenses is:

$$W = W_0 - C_{max} [(D_1)^a + (D_2)^a + j(D_1)^a (D_2)^a)] + B_{max} [(D_1)^b + (D_2)^b + k(D_1)^b (D_2^b)].$$

Fitness ($W$) is a function of $W_0$ (a constant, plant fitness with no defense) and the costs $C_{max}(D_1)^a + (D_2)^a + j(D_1)^a (D_2)^a$ and benefits $B_{max}(D_1)^a + (D_2)^a + k(D_1)^a (D_2)^a$ of different levels of investment in two defenses ($D_1$ and $D_2$). The a and b exponents are shape parameters

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to allow the possibility of non-linear functions for costs and benefits.  As a simplifying

assumption, $C_{max}$, $B_{max}$, and the shape parameters are here assumed to be the same for the

two defenses (i.e, a=b). The terms $j(D_1)^a (D_2)^a$ and $k (D_1)^b (D_2)^b$ represent interactions in

costs $j (D)_1{}^a (D_2)^a$ or benefits $k (D_1)^b (D_2{}^b)$, respectively, for the two defenses. I have added

the interaction term $j(D_1)^a (D_2)^a$ to the original Fornoni model to evaluate the possibility

of interactions for the costs of producing multiple defenses.

To investigate synergism of costs, first consider the possible shapes of the fitness

surface without any benefit term in the model (i.e., Bmax=0), as for plants in an

herbivore exclusion treatment. If there is no synergism of costs (parameters are 1), the

costs and benefits increase linearly. In this case, the cost of investing in one defense

would be independent of the cost of producing another defense (j=0, fig. 9a). If costs for

two defenses are more than additive, forming an antagonistic interaction, it would be

more costly to produce two defenses than to only invest in one defense (j>0, fig 9b).  In

this scenario, investment in only one trait would yield higher fitness than the mixed

investment. Alternatively, if there is synergism of costs, smaller amounts of investment

in two defenses would be less costly than higher investment in only one defense (j<0, fig.

9c). If costs accelerate non-linearly with the amount of defense produced, this could

create a surface visually indistinguishable from that for synergistic costs, but in the

absence of any actual trait interaction. An equivalent investment in two defenses would

thus be less expensive than higher investment in each individual defense (a>1, fig 9d).

Thus, more than one underlying mechanism could create the same final surface.
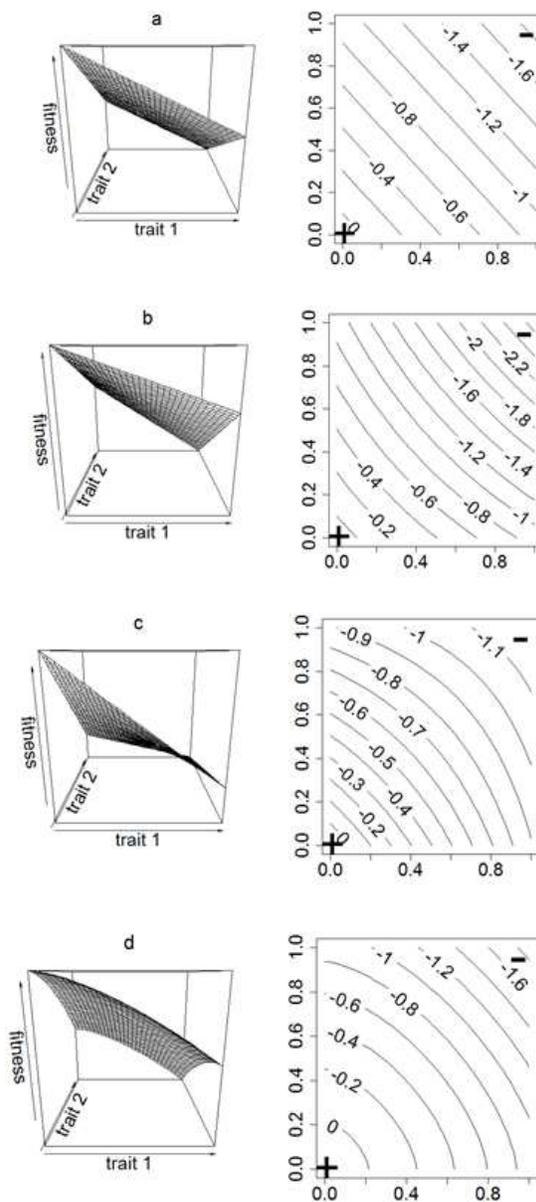
**Figure 9: Theoretical cost surfaces from investment in two traits. For all surfaces W0= 0.1, Cmax = 0.9, Bmax = 0, a = b = 1 unless otherwise noted. The fitness and corresponding contour plot depict the same surface. a) j=0, no interaction b) j=0.8, antagonistic costs c) j = -0.8, synergistic costs d) j=0, a=1.5, no interaction**

To investigate synergism of benefits, one can examine fitness surfaces with both

costs and benefits included, as in the presence of herbivores. If there are no interactions

in benefits from two defenses, the fitness surface would resemble fig. 10a. In this surface,

each trait acts independently in terms of benefit—investing in only one defense would

yield the same benefit as an equivalent investment in two defenses (k=0). If multiple

defenses interact antagonistically, investing in only one defense would yield higher

fitness, with higher investment in two traits required to gain the same fitness as lower

investment in only one trait (k<0, fig. 10b). If multiple defenses act synergistically to

improve benefits, lower investment in two traits could yield higher fitness than only

investing in one defense (k>0, fig. 10c). However, a non-linear function for the benefits of

increased investment in each defense could yield a similar surface. This could lead to the

false interpretation of a synergistic interaction between benefit terms in the absence of

any true trait interaction (b<1, fig 10d).

**Figure 10: Theoretical net benefit surfaces. For all surfaces W0= 0.1, Cma x = 0.9, Bmax = 1, a = b = 1 unless otherwise noted. a) k=0, no interaction b) k= -0.15, antagonistic benefit c) k= 0.5, synergistic benefit d) k=0, a=.1, b=.12, no interaction**

If the differences between the shape parameters a and b are increased, additional possible shapes occur. An intermediate fitness peak can occur when the benefit function increases more rapidly than the cost function (fig. 11a). For the reverse combination, with a more rapidly increasing cost function, an intermediate fitness valley occurs (fig. 11b). If maximum costs are higher than maximum benefits, with an antagonistic interaction term and non-linear shape function, a saddle-shaped fitness surface can occur (fig. 11c).

There can be strong visual similarities between surfaces created from trait interactions and those lacking trait interactions but with non-linear shape functions included. Thus, definitive classification of the presence of an interaction between traits cannot be made from interpretation of the fitness surfaces and contour plots alone (ie, compare fig. 9c and d, fig. 10c and d). Information about the underlying shape function used to generate the model surface is required to clarify the interpretation of the theoretical models. However, the theoretical surfaces still remain useful as a guide for later visual interpretation of the experimental surfaces.

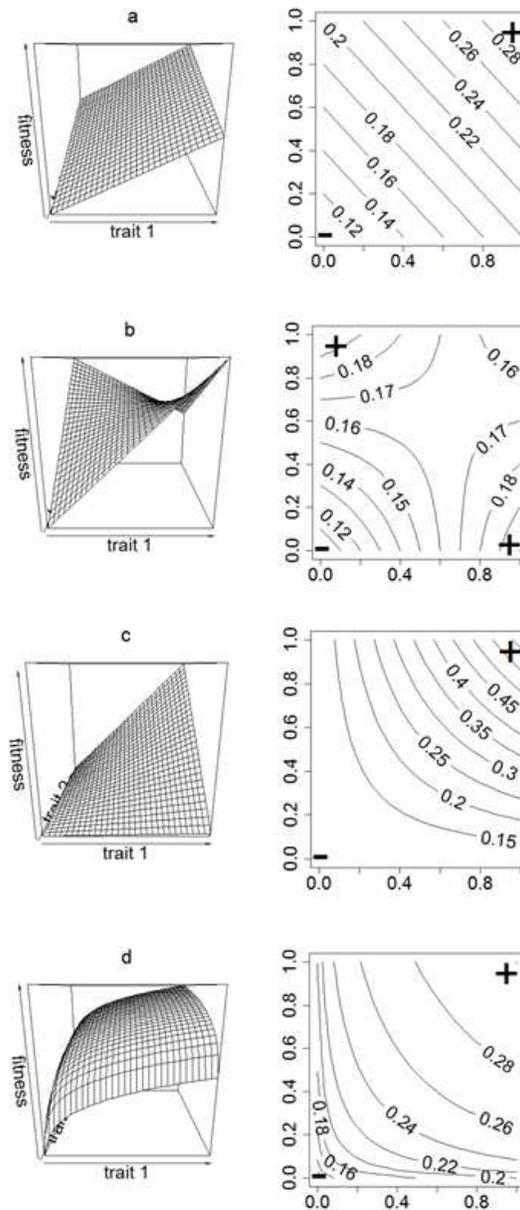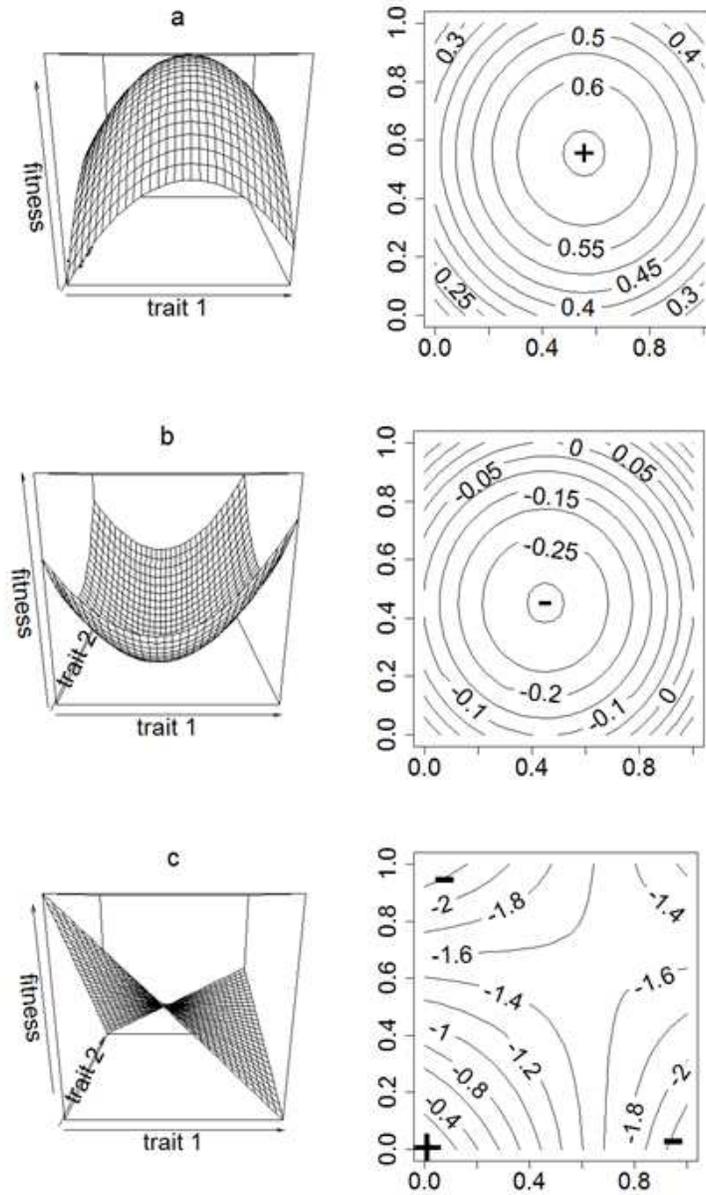**Figure 11: Theoretical net benefit surfaces. For all surfaces W0= 0.1, Cmax = 0.9, Bmax = 1 unless otherwise noted. a) a=2, b=1, j=k=0, no interaction b) a=1, b=2, j=k=0, no interaction  c) j= -1.5, Cmax = 2.5, a=1.01, b= 1, antagonistic cost**

## 4.3 Methods

I conducted a common garden experiment with *Passiflora incarnata* as the study system. It faces attack by a range of generalist and specialist herbivores, and it produces both direct physical defenses (leaf toughness and trichomes) and participates in an indirect ant-protection mutualism mediate by production of extrafloral nectar. *P. incarnata* also exhibits tolerance of herbivore damage (Ch. 2). This combination of resistance and tolerance traits make it an ideal study system to investigate possible reasons for the production of multiple defense traits.

My work in this system has found limited evidence for resistance traits decreasing generalist or specialist herbivory (Ch. 2).  I also found that genetic correlations between resistance traits were weak or zero, while there were trends for negative genetic correlations between the production of resistance and tolerance traits and significant positive genetic correlations between tolerance of different types of herbivore damage.

I grew vegetative clones of plants collected from two source populations in a common garden experiment for two years.  I manipulated herbivore presence through pesticide application to calculate the costs and benefits of defense trait production

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against herbivore damage through comparison with untreated control plants. I measured resistance and tolerance trait investment and plant fitness.

### 4.3.1 Field experiment

Here, I provide a brief overview of the field experiment. A more detailed description of the methods is available in Chapter 2.

*Passiflora incarnata* is a perennial vine that grows in disturbed areas like roadsides and field margins. I studied plants from both northern (North Carolina) and southern (Florida) populations. Genotypes were collected from two broad populations near the town of Lakeland, in central Florida, and the other near the town of Wilmington, in southeast North Carolina. These genotypes were then planted into common garden field plots in an experimental plot in Durham, North Carolina.

The first year study featured 57 genotypes split between plants from the two populations. The second year experiment comprised 156 genotypes, with roughly half from each population. Each genotype was represented with vegetative clones.

I randomly allocated clones within a genotype to an herbivore exclusion treatment to calculate costs of defense production in the absence of a benefit from herbivore damage deterrence. Comparison of the results between the control and pesticide treatments then allows calculation of the benefits of resistance trait investment.

The pesticide treatment did not alter trait values in either year (ANOVA, $P \geq .1$ for all traits in both years). In addition, in year two I manipulated specialist herbivore presence by placing an individual *Agraulis vanillae* butterfly caterpillar larvae on control plants to investigate the response of specialist herbivores to resistance traits.

I measured the resistance traits of trichome density, extrafloral nectar volume, and leaf toughness in August and September, and calculated tolerance to damage by generalist beetles, thrips, and caterpillars. I calculated total leaf area for each plant by measuring the area of three representative leaves per plant and multiplying the mean area by the total number of leaves.

I completed all analysis in R (R Core Team, 2015). Fitness measurements of genotype mean leaf area were calculated separately for each treatment.

I initially tried fitting the modified Fornoni model to the experimental data, but model selection with AIC supported a cubic spline approach as a better representation of the data. Cubic splines have been previously used to visualize selection surfaces, and allow more complex surfaces than a polynomial regression (Schluter & Nychka 1994; Brodie *et al.* 1995). However, the spline provides limited information about the underlying fit parameters, which can limit the scope of interpretation.

To create fitness surfaces to visualize trait interaction, I used a general additive

model to fit a cubic spline model to the data (Schluter & Nychka 1994). This method

produces a flexible surface without many of the assumptions inherent in traditional

quadratic fitness models. I used the R package 'mgcv' to fit the cubic spline model.

Smooth parameters were selected to minimize the generalized cross-validation score,

which aims to maximize the predictive ability of the model. Leaf area was used as the

fitness measure. I used a tensor product interaction to fit the main effect of each trait and

the interaction of the pair of traits. I plotted fitness surfaces with the functions 'persp'

and 'contour' in R.

For each surface and contour plot, I plotted genotype mean trait values along the

x and y axis, and fitness (leaf area) on the z axis. Leaf area can be an appropriate fitness

measure in a clonal, perennial plant that must devote resources to vegetative growth to

ensure survival to the next year (Winkler & Fischer 1999). I used leaf area in the

pesticide treatment to measure the cost of producing defenses in the absence of

herbivores and hence the absence of benefits. I used leaf area in the control treatment to

measure both the costs and benefits of defenses in the presence of herbivores. I fit the

fitness model separately for the control and pesticide treatments, and then subtracted

the predicted fitness values of the 'cost' treatment from the 'cost and benefit' treatment

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to obtain the benefit of defense traits. This produced three final fitness surfaces for comparison: the herbivore exclusion (cost surface), the herbivore presence (cost and benefit) surface, and the inferred benefit surface obtained from subtraction.

I then examined each surface to determine if selection favored investment in no defense traits, one trait, or both traits based on the location of the fitness optimum.  I also considered if trait combinations—as two resistance traits, two tolerance traits, or one of each—impacted  the frequency of occurrence of fitness optima favoring one trait or multiple traits.  For each surface, I considered the underlying shape of each trait's smooth parameter to determine if each trait was linear or nonlinear (appendix A). I also evaluated if the addition of an interaction term improved the cubic spline model fit with an ANOVA (Appendix A). Although I can determine statistically if the presence or absence of an interaction term improves the model fit, I cannot interpret the fit parameters statistically to determine if the interaction demonstrates synergism or antagonism.  Thus, I determined if the interaction term was synergistic or antagonistic based on visual interpretation of the fitness contours.

There are limitations to the conclusions about trait interactions which I can draw from interpretation of the fitness surfaces. Due to the flexibility of the spline model, surfaces do not necessarily feature the same shape of trait interaction over the entire

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surface. In addition, some surfaces are simply too complex and diverge too far from the theoretical surfaces to permit reliable interpretation of the trait interactions. Hence, not every surface with an interaction term present has a corresponding interpretation of that interaction: in the absence of complete confidence of interpretation, these surfaces were not included in summary statistics regarding the frequency of synergism and antagonism. However, in these cases, I could still determine the location of the fitness optimum. Thus, all surfaces are included in the determination of trait combinations favored by selection.

## 4.3.2 Empirical fitness surfaces

Here, I present two representative examples of estimated fitness surfaces (fig 12 and 13). The herbivore exclusion (cost) surface for generalist tolerance and thrips tolerance led to a minimum in fitness for production of both traits (fig. 12a, compare with fig. 11b). In this case, production of minimum values of each trait would maximize fitness, as in the absence of herbivory defense production increases costs with no corresponding fitness benefit. The non-linear smooth parameter for both traits determines the location of the fitness minimum. For the herbivore presence surface (fig. 12b) the fitness peak again occurs with minimal trait production, and the surface represents an antagonistic interaction (compare with fig. 9b). In contrast, in the benefit

surface, the expression of the two traits leads to an intermediate fitness peak (fig. 12c,

compare with fig. 11a), with non-linear functions for each trait.
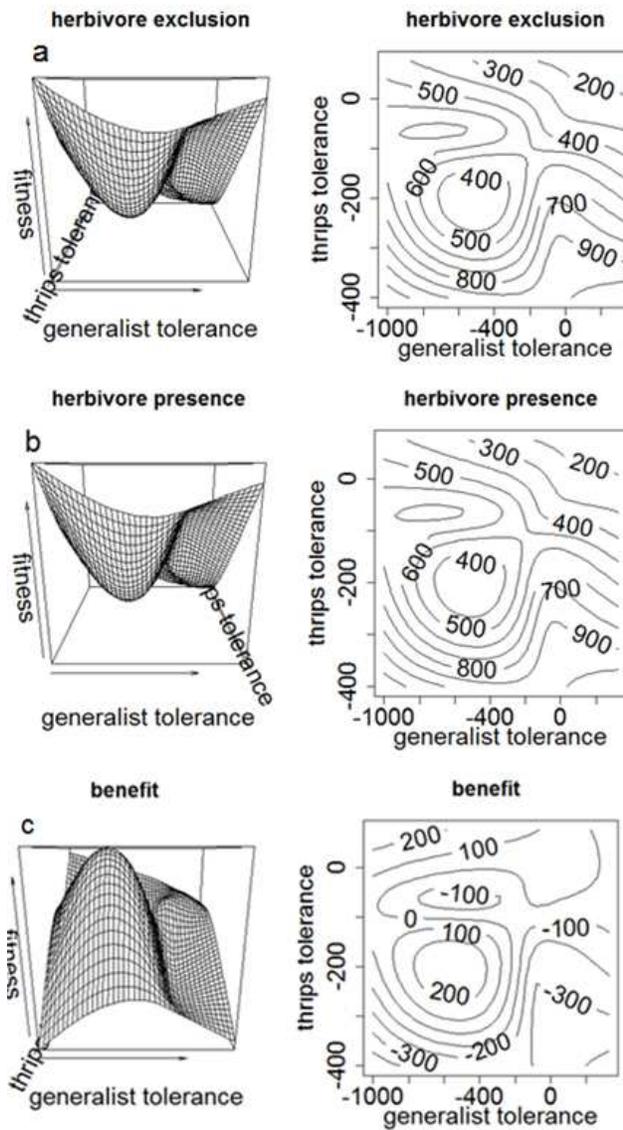
**Figure 12: Fitness surfaces and contour plots for generalist tolerance and thrips tolerance in year 1. The x and y axis represent the genotype mean values of defense traits, and the z axis represents fitness (leaf area). The sequential paired plots represent: fitness surfaces for herbivore exclusion, surfaces for herbivore presence, benefit surface from subtraction of herbivore exclusion surface (cost) from herbivore presence surface (benefit and cost).**

For the herbivore exclusion (costs) surface for leaf toughness and caterpillar tolerance, the maximum fitness peak again occurred at zero investment for both traits (fig. 13a). Here, any trait investment lowers fitness, with a linear smooth parameter for leaf toughness and a nonlinear smooth parameter for tolerance of caterpillar damage, in addition to a synergistic interaction between the two traits (compare fitness contours with fig. 9c). For the surface in the presence of herbivores (costs and benefits), maximum investment in only one trait—here, leaf toughness—leads to a fitness peak (fig. 13b). Similarly, the benefit surface points to investment in only leaf toughness as the fitness optima (fig. 13c). An antagonistic interaction occurs.

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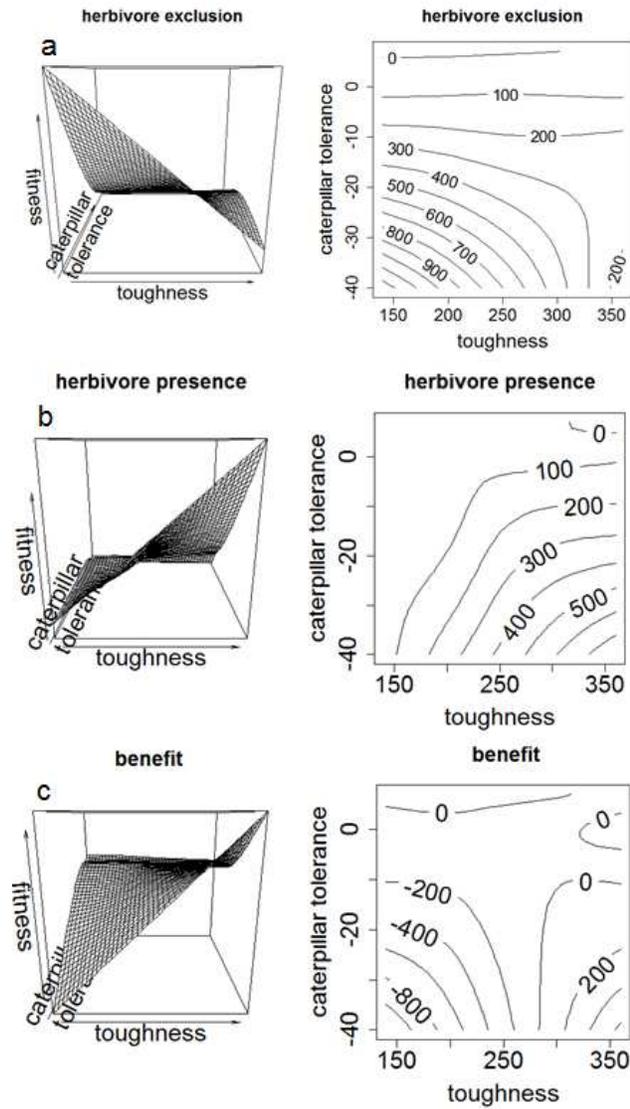
**Figure 13: Fitness surfaces and contour plots for leaf toughness and tolerance of caterpillar damage in year 2. The x and y axis represent the genotype mean values of defense traits, and the z axis depicts fitness (leaf area). The sequential paired plots represent: fitness surfaces for herbivore exclusion, surfaces for herbivore presence, benefit surface from subtraction of herbivore exclusion surface (cost) from herbivore presence surface (benefit and cost).**

The frequency with which the fitness optimum was achieved through greater-than-minimal investment in only one trait versus two traits differed between trait groupings and for herbivore exclusion (costs), herbivore presence (cost and benefits), and benefits (fig. 14). For the herbivore exclusion (cost) surfaces, there was little differentiation between trait combinations for the frequency with which investment in only one trait represented the fitness optimum (fig. 14a). Minimal investment in defense was frequently favored, suggesting that in the absence of herbivory pressure, the costs of defense investment lower fitness. In comparison, for the herbivore exclusion (cost and benefit) surfaces, when two resistance traits were considered, none of these six trait pairs favored investment in only one trait (fig. 14b). When two tolerance traits or one tolerance and one resistance trait were compared, investment in only one trait was optimal for a substantial proportion of the surfaces. For the benefit surface, there was little differentiation between the trait groupings, with all three combinations leading to between 66-75% of the evaluated pairs favoring investment in one trait (fig. 14c).
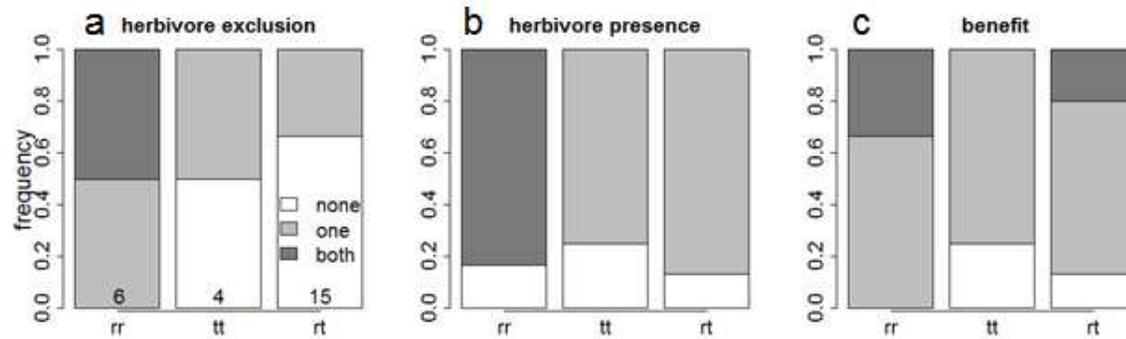
**Figure 14: The frequency with which the fitness peak corresponds to investment in no traits, one trait, or both traits for the fitness surfaces of a) herbivore exclusion, b) herbivore presence, and c) benefit. The trait groupings represent two resistance traits ("rr"), two tolerance traits ("tt"), and one of each ("rt").**

The frequency of occurrence of a mixed strategy of investment in two traits differed between trait pairings and for the herbivore exclusion (cost), herbivore presence (cost and benefits), and benefit surfaces. For the herbivore exclusion (cost) surfaces, when two resistance trait were evaluated, 3/6 of the surfaces favored a mixed investment (fig. 14a). In contrast, for two tolerance traits or for a combination of resistance and tolerance traits, none of the surfaces favored a mixed investment. Similarly, for the herbivore presence surfaces, when two resistance traits were compared, a mixed strategy was favored in 5/6 trait pairs (fig. 14b). A mixed strategy was never optimal for two tolerance traits or for one tolerance and one resistance trait. Yet, these differences between trait groupings disappear when the benefits surface is evaluated (fig. 14c). For the benefits surfaces, very similar low levels of a mixed strategy occurred across each trait pairing. Herbivore addition changed the fitness peak in 19 of 25 comparisons. The most common pattern reflected a shift from supporting no investment in the absence of herbivores to investment in only one trait in the presence of herbivores (11/19 pairs).

In most cases, the fitness surfaces for year 1 did not feature a significant interaction term, while significant interaction terms occurred in the majority of pairs for year 2. Low sample sizes in year 1 may limit the ability to detect the occurrence of trait interactions.

For surfaces with a significant interaction between the two traits, I determined if synergism or antagonism was present based on visual inspection of the surface. I found evidence of synergism in trait interactions on the herbivore exclusion (cost) surfaces, but not for the benefit or net benefit surfaces. There was antagonism in trait interactions for the benefit and net benefit surfaces, and in the majority of cases this led to the fitness surface favoring investment in only one trait (antagonism in 9/11 pairs).

## 4.4 Discussion

I did not find synergistic benefits in fitness surfaces, although I found synergistic interactions on the herbivore exclusion (costs) surfaces and antagonistic interactions on both the herbivore presence and benefit surfaces. I found differences between trait groupings in the location of the fitness optimum. Mixed investment in multiple defenses was commonly favored for two resistance traits, but not for the other trait pairs. Here, I discuss these implications for how natural selection may act on the production of multiple defenses, and place these results in context with my previous work investigating genetic correlations between defense traits and herbivore responses to defense traits.

I found limited support for synergistic trait interactions, with synergism occurring in the herbivore exclusion (cost) surfaces but not for the herbivore presence or

benefit surfaces. Indeed, many of the trait pairs that favored a mixed defense investment did not include a significant interaction term, indicating that non-linearities in the trait smooth parameters were responsible for the mixed trait optima. However, many of these fitness surfaces are visually indistinguishable from those depicting synergism between trait interactions. Thus, either mechanism could lead to the same fitness surface and experience similar selective pressures. Yet, as the outcome is the same—selection favoring multiple defenses—the implications for defense trait expression would be identical, regardless of the responsible mechanism.

Theoretical models have posited the conditions that could lead to different combinations of defense traits. Many models predict maximum investment in either resistance or tolerance, based on the hypothesis that higher investment in two defenses will always be more expensive than maximal investment in one defense (van der Meijden *et al.* 1988; Herms & Mattson 1992; Belsky *et al.* 1993). Similarly, other models posit that negative genetic correlations would lead to disruptive selection and maximal investment in one or the other defense strategy (Fineblum & Rausher 1995). My results for the combination of resistance and tolerance traits are consistent with these predictions, as I found negative genetic correlations between these traits and fitness surfaces predicting selection for investment in only one trait.

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In many models, the shape of the cost and benefit curves is key to determining the predicted fitness optimum. Some models hold that additive costs and antagonistic benefits would lead to the evolution of either trait extreme (Mauricio *et al.* 1997). Other models predict intermediate investment in multiple defenses, based on the costs and benefits of the traits being independent and unequal (Tiffin 2000). The Fornoni model predicted that investment in one trait or the other would primarily occur with antagonistic benefits (Fornoni *et al.* 2004). My results were consistent with many of these predictions. Antagonistic interactions did lead to favoring investment in only one trait. Yet, in the absence of such an interaction, non-linear trait parameters could also lead to a fitness optimum for investment in only one trait.

Few studies have tested these theoretical model predictions experimentally. Mauricio and Rausher (1997) found that herbivores led to selection for a mixed optimum of multiple resistance traits in *Arabidopsis thaliana*. Other studies have found evidence for mixed resistance and tolerance in *Datura stramonium* (Carmona & Fornoni 2013), and similar patterns in *Arabidopsis lyrata* (Puentes & Ågren 2013). However, each of these studies only investigated a single pair of traits—one resistance trait and one tolerance trait, or two resistance traits. What these previous studies lack is a comparison of how both multiple resistance and tolerance traits interact in one species.

In my previous work in this system, I found weak genetic correlations between resistance traits, positive correlations between tolerance traits, and negative correlations between resistance and tolerance traits (Ch. 2). Only some of the selection surfaces align with these results.  The results for the combination of resistance and tolerance are consistent with expectations from the genetic correlations.  Negative genetic correlations between traits could constrain selection acting on the joint expression of both traits and instead facilitate expression of one trait or the other. Indeed, investment in only one of the two traits was the most common prediction in the fitness surfaces (fig. 14).  The results in Chapter 2 suggest that tolerance is a key component of defense in these plants, with no defense benefits from resistance trait production.  Plants may thus require tolerance due to the failure of resistance traits to reduce damage, leading to selection for tolerance instead of resistance.

However, the results for tolerance were unexpected. The selection surfaces favored investment in only one or the other tolerance trait in most cases. This contrasts with the positive genetic correlations between these traits, which had led me to expect a mixed investment in multiple traits. Furthermore, tolerance appeared to be effective against a range of herbivores (Ch. 2).  In this situation, the positive genetic correlations

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could impede selection acting against investment for tolerance of damage by multiple herbivores.

Finally, for two resistance traits, there were weak or zero genetic correlations, with no reduction in herbivory from investment in these traits, which had led me to expect little selection on their expression. Yet, I found that when costs and benefits were considered, a mixed investment in these traits was frequently favored. Selection for multiple resistance traits in the absence of benefits from their production appears paradoxical.

Selection may favor possessing multiple resistance traits if selection is acting on another function of these traits. The lack of change in herbivory damage between the control and treatment plants suggests that herbivores are not the responsible selective agent and that selection is not acting on the role of resistance traits in reducing damage. Plant traits commonly fill multiple roles, so it is possible that another function of resistance traits—such as UV protection—could provide a fitness benefit and selection for possessing multiple resistance traits (Levin 1973).

However, other roles for resistance traits would not explain the difference in selection found between the herbivore exclusion and herbivore presence treatments, unless application of the pesticide treatment itself impacted plant fitness. An alternative

explanation for the difference between treatment groups is that the pesticide treatment may have reduced pathogen transmission by an insect vector in the treatment plants. This may have increased fitness for these plants, leading to the change in selection measured between treatment groups. Damage by an unobserved herbivore that responded to the pesticide treatment could also lead to the difference in selection.

In summary, consideration of the costs, benefits, and trait interactions from investment in multiple traits led to different predicted trait investment and selection optima. The type of defense pairs considered—whether resistance, tolerance, or a combination of the two—altered the occurrence of different defense combinations. When two resistance traits were considered, a mixed investment in two traits was frequently optimal. However, this did not occur for two tolerance traits or for the combination of resistance and tolerance traits, where investment in only one trait represented the fitness maxima. These results were partially consistent with the predictions from genetic correlations. I found evidence of synergistic interactions in costs, although non-linear functions for trait parameters could also generate mixed defense trait optima that resemble synergism. Both of these mechanisms could lead to the same outcome favoring selection for multiple defenses. I found antagonistic interactions between traits for the herbivore presence and benefit surfaces, which predominantly led to selection for

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maximum investment in only one trait. These results suggest the necessity of

considering the type of defense trait when making predictions about what trait

combinations natural selection may favor.

# Appendix A

Smooth parameters and interaction terms for cubic spline models in year one and year two. The cubic spline regression has leaf area as a response, with two defense traits and their interaction as predictor terms. The trait 1 and trait 2 columns list the smooth parameters used for each trait, with a value of 1 indicating a linear parameter. The significance of the interaction term was assessed with an ANOVA.

## Year 1

| traits | herbivore treatment | trait 1 | trait 2 | interaction F | p-value |
|---|---|---|---|---|---|
| toughness,trichomes | presence | 1.00 | 1.00 | 0.027 | 0.871 |
| | exclusion | 6.167 | 1.00 | 0.693 | 0.410 |
| toughness, nectar | presence | 1 | 1 | 0.870 | 0.357 |
| | exclusion | 6.812 | 1 | 1.012 | 0.383 |
| toughness, | presence | 1 | 2.141 | 1.617 | 0.200 |
| generalist tolerance | exclusion | 1 | 6.69 | 1.031 | 0.428 |
| toughness, thrips | presence | 3.584 | 1 | 3.938 | 0.008* |
| tolerance | exclusion | 1 | 1 | 2.966 | 0.014* |
| trichomes, nectar | presence | 1 | 1 | 0.172 | 0.680 |
| | exclusion | 1 | 3.451 | 2.584 | 0.050* |
| trichome, generalist | presence | 1 | 2.15 | 0.289 | 0.594 |
| tolerance | exclusion | 1 | 4.392 | 1.492 | 0.198 |
| trichome, thrips | presence | 1 | 1.376 | 0.029 | 0.866 |
| tolerance | exclusion | 1 | 1 | 1.819 | 0.102 |
| nectar, generalist | presence | 1 | 2.385 | 0.584 | 0.448 |
| tolerance | exclusion | 1 | 4.162 | 1.528 | 0.189 |
| nectar, thrips | presence | 1 | 1.831 | 1.827 | 0.148 |
| tolerance | exclusion | 1.965 | 1 | 1.314 | 0.282 |
| generalist tolerance, | presence | 1 | 1 | 3.691 | 0.050* |
| thrips tolerance | exclusion | 1 | 1 | 2.438 | 0.053 |

**Year 2**

| Traits | treatment | trait 1 | trait 2 | interaction F | p-value |
|---|---|---|---|---|---|
| toughness,trichomes | presence | 1 | 7.139 | 1.493 | 0.183 |
| | exclusion | 1 | 1.443 | .035 | 0.852 |
| toughness, caterpillar | presence | 1 | 3.628 | 6.578 | <0.005* |
| tolerance | exclusion | 1 | 2.441 | 3.571 | 0.003* |
| toughness, nectar | presence | 1 | 2.084 | 1.713 | 0.135 |
| | exclusion | 1 | 1.825 | .574 | 0.620 |
| toughness, generalist | presence | 1 | 0.998 | 11.833 | <0.005* |
| tolerance | exclusion | 1 | 3.228 | 3.722 | 0.010* |
| toughness, thrips tolerance | presence | 1.131 | 1 | 6.348 | <.005* |
| | exclusion | 1 | 2.776 | 2.294 | 0.0257* |
| trichomes, caterpillar | presence | 7.106 | 1 | 1.742 | 0.076* |
| tolerance | exclusion | 3.642 | 1 | 4.272 | <0.005* |
| trichomes, nectar | presence | 7.361 | 2.141 | .008 | 0.929 |
| | exclusion | 3.468 | 1 | 3.656 | 0.026* |
| trichome, generalist | presence | 3.076 | 1 | 2.037 | 0.052 |
| tolerance | exclusion | 3.943 | 1 | 3.308 | <0.005* |
| trichome, thrips tolerance | presence | 2.921 | 2.047 | 2.655 | 0.043* |
| | exclusion | 2.787 | 1.955 | 1.839 | 0.0652 |
| nectar, caterpillar tolerance | presence | 1.907 | 1 | 2.943 | 0.0435* |
| | exclusion | 1.493 | 2.461 | 4.075 | 0.001* |
| nectar, generalist tolerance | presence | 1.525 | 1.401 | .694 | 0.571 |
| | exclusion | 4.000 | 2.565 | 3.652 | <0.005* |
| nectar, thrips tolerance | presence | 1.770 | 1.691 | .835 | 0.490 |
| | exclusion | 1.445 | 2.465 | 4.975 | <0.005* |
| generalist tolerance, | presence | 2.452 | 3.059 | 1.465 | 0.228 |
| caterpillar tolerance | exclusion | 1 | 3.061 | 7.1 | <0.005* |
| generalist tolerance, thrips | presence | 1 | 1.584 | .484 | 0.124 |
| tolerance | exclusion | 0.998 | 0.995 | 5.167 | 0.011* |
| thrips tolerance, caterpillar | presence | 1 | 2.942 | 3.703 | 0.020* |
| tolerance | exclusion | 1 | 3.108 | 6.263 | <0.005* |

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