GENETIC AND BIOCHEMICAL MECHANISMS OF CUCURBITA PEPO RESISTANCE TO STRIPED CUCUMBER BEETLE

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GENETIC AND BIOCHEMICAL MECHANISMS OF CUCURBITA PEPO RESISTANCE TO STRIPED CUCUMBER BEETLE

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Pest-resistant crops are a cornerstone of integrated pest management and are especially vital for crop-pest systems where effective pesticides are not available or have undesirable externalities. However, breeding progress for pest resistance can be constrained by low heritability and a limited understanding of resistance mechanisms. Here I present integrative research to breed for resistance to the Cucurbitaceae-specialized *Acalymma vittatum* (striped cucumber beetle) in *Cucurbita pepo* (zucchini, summer squash) that draws on fundamental chemical ecology principles and plant genomics to directly address these challenges.

First, it was established that *Acalymma vittatum* preference is defined by *C. pepo* population structure, where *C. p. pepo* is preferred over *C. p. ovifera*. I then characterized resistance mechanisms on the subspecies level. I found differences in defense induction within and between subspecies, and identified variation in foliar volatiles that may affect *A. vittatum* acceptance of host plants. In addition, inter-subspecific populations were developed to dissect the genetic and mechanistic basis of traits confounded by subspecies. I tested how cucurbitacins, bitter triterpenoids, affect *A. vittatum* preference in *C. pepo* agricultural systems. The key results were that cucurbitacin accumulation in specific tissues did not have plant-wide effects on *A.*

vittatum preference, and cucurbitacin biosynthesis is tightly linked with developmental stage. In addition, inter-subspecific populations were used for phenotypic and genomic selection for non-preference, where genomic selection had moderate predictive ability.

A central theme in this research is a critical understanding of which traits to phenotype in the context of a breeding program. For breeding goals that center on biotic interactions, this relies on weighing the value of resource-intensive mechanism discovery and application, and mechanism-blind applications that take advantage of genomic tools. The fulcrum of balancing these efforts is the predicted difference between the response to selection on biotic resistance traits directly or correlated response to selection on chemical traits. Overall, these tandem and complementary approaches are crucial in developing pest-resistant crops.

BIOGRAPHICAL SKETCH

Lauren Jane Brzozowski was born on March 1, 1991 to Jane and Jeff Brzozowski in Park Ridge, IL, USA. Along with her younger sister, Sara, she grew up near Chicago, London and then Milwaukee, and graduated from Brookfield East High School.

She completed her undergraduate degrees at University of Wisconsin – Madison, and her interests were driven by her goal to enhance environmental sustainability in her career. She completed her B.Sc. in Electrical and Computer Engineering before focusing on working to reduce chemical inputs in agriculture. While completing her B.Sc. in Horticulture, Lauren worked in the lab of Dr. Irwin Goldman by assisting carrot and beet breeding projects. She credits that experience and his mentorship for choosing to pursue graduate work. During this time, she was involved in teaching as a calculus and physics tutor for the College of Engineering.

She began as a graduate student in Plant Breeding and Genetics at Cornell University in 2014, and rotated in the Mazourek, Sorrells and Agrawal labs, before beginning in the Mazourek lab. Her research built upon existing projects on resistance to biotic stresses in vegetable crops, and ultimately focused on mechanisms of resistance to the striped cucumber beetle, *Acalymma vittatum*, in *Cucurbita pepo*. Lauren was a teaching assistant in plant genetics courses and developed lectures on the intersection of chemical ecology and plant breeding. Lauren served as co-President of the plant breeding graduate student group, Synapsis, and was involved the academic community through planning symposia and participating in review panels.

This dissertation is dedicated to my parents, Jane and Jeff Brzozowski, and sister, Sara Brzozowski

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CHAPTER 1: INTRODUCTION

Insect pests can decrease yield and quality of crop harvests, but damage can be mitigated by breeding and growing plants resistant to insect pests. Use of resistant plants is a desirable solution across management methods, and even more vital in crops where pesticides have negative externalities or are unavailable [1]. Resistance to insect pests can be described in many ways – from traits that lead to greater insect mortality to traits that affect host plant choice [2] – and through many means, including physical and chemical defense [1] as well as recruitment of antagonists of pests [3]. Plant breeders have sought to augment these diverse traits in a variety of crops, but challenges remain in making agriculturally-relevant progress.

Major successes in breeding for insect resistance are united by characteristics like oligo-genetic architecture, high heritability of resistance trait, and systems where an incremental reduction of damage translates to quantitative gains in yield [4]. For instance, resistance to some Hemipteran insects is mediated by one to a few genes that reduce damage, and has been applied in agronomic and horticultural crop systems [5]–[8]. Resistance in systems where there is low heritability or more complex genetic architecture have not progressed as rapidly [9], and will rely on further integrating techniques from chemical and insect ecology to make comparable gains [10], [11].

Thus, a framework for breeding in such systems relies on germplasm selection, knowledge of mechanisms of resistance, and understanding the genetic architecture of traits to inform breeding strategies. Like other traits, breeding for insect resistance requires first establishing if economically meaningful phenotypic variance exists for the trait, and choosing appropriate germplasm to establish breeding populations. Then,

dissecting the mechanistic and behavioral components of insect resistance is important because components of resistance may have different genetic control, heritability or ease of phenotyping [12], [13], and mechanistic knowledge and particular screening methods would inform how a resistance trait would perform at field scale [14]–[16]. Finally, understanding the genetic architecture and heritability of the trait as a whole or component traits can dictate the best breeding approach.

In this dissertation, I follow this framework by surveying variation, characterizing mechanisms and testing selection methods that reduce damage to squash (*Cucurbita pepo*) by the specialized pest, the striped cucumber beetle (*Acalymma vittatum*, Coleoptera: Chrysomelidae). These species share an evolutionary history in the Americas, where *A. vittatum* has evolved to sequester cucurbitacins, defensive triterpenoids of the Cucurbitaceae, for its own defense against predators [17]. In more recent history, two cultivated *C. pepo* subspecies, *C. p. pepo* (e.g. zucchini and pumpkin) and *C. p. ovifera* (syn. *texana*; e.g. summer squash, acorn squash), arose from independent domestication events [18], [19]. Breeding for pest resistance in this system has been a longstanding goal of plant breeders [20], [21], and I sought to reduce damage by *A. vittatum* to *C. pepo* leaves and cotyledons.

I assessed phenotypic variation for leaf resistance in *C. pepo* to *A. vittatum*, as loss of leaf tissue reduces yield in *C. pepo* [22]. Intraspecific differences in degree of *A. vittatum* herbivory have been reported in *C. pepo* for cotyledons [23] and leaves [24], and this work builds upon those efforts by structuring within *C. pepo* subspecies.

I then used techniques from chemical ecology to evaluate the mechanistic bases of

the split in degree of *A. vittatum* herbivory between subspecies. Previous work in the Cucurbitaceae established cucurbitacins, non-volatile triterpenoids, to be kairomones for *A. vittatum* [17]. In *C. pepo*, cotyledon cucurbitacins correlate with cotyledon preference at the cultivar level [23], but there is mixed information about cucurbitacins in leaves – cucurbitacins are generally low [25], [26], but may be inducible [27]. In this dissertation, I present four studies on mechanisms that affect herbivory: characterizing constitutive and induced leaf resistances between subspecies, profiling the effect of cotyledon cucurbitacins on *A. vittatum* herbivory of leaves and cotyledons, dissecting components of *A. vittatum* host choice, and describing plant and beetle metabolic response during their interaction.

Finally, I connected work on mechanisms to applications for breeding progress. Building directly the mechanistic work, I tested two indirect selection schemes for reducing *A. vittatum* damage: selection on cotyledon cucurbitacins, and selection on performance of another proxy insect. Finally, I tested a genomic selection scheme to reduce *A. vittatum* damage without mechanistic knowledge.

In summary, this dissertation explores avenues to elucidate mechanisms to mitigate *A. vittatum* damage in *C. pepo*, as well as strategies to select without understanding of mechanisms. It builds a model where *C. p. ovifera* has lost cotyledon susceptibility by lacking cucurbitacins and has leaf resistance likely due to deterrent volatiles that promote *A. vittatum* emigration. In contrast, *C. p. pepo* has susceptible cotyledons and lacks leaf resistance traits.

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Chapter 2: *Cucurbita pepo* subspecies delineates striped cucumber beetle (*Acalymma vittatum*) preference[1]

2.1 Abstract

The striped cucumber beetle (*Acalymma vittatum* [F.]) is a destructive pest of cucurbit crops, and management could be improved by host plant resistance, especially in organic farming systems. However, despite the variation in striped cucumber beetle preference observed within the economically important species, *Cucurbita pepo* L., plant breeders and entomologists lacked a simple framework to classify and exploit these differences. This study used recent phylogenetic evidence and bioassays to organize striped cucumber beetle preference within *C. pepo*. Our results indicate preference contrasts between two agriculturally-relevant subspecies: *C. pepo* subsp. *texana* and *C. pepo* subsp. *pepo*. Plants of *C. pepo* subsp. *pepo* were more strongly preferred than *C. pepo* subsp. *texana* plants. This structure of beetle preference in *C. pepo* will allow plant breeders and entomologists to better focus research efforts on host plant non-preference to control striped cucumber beetles.

2.2 Introduction

The damage inflicted upon plants of the Cucurbitaceae family by the striped cucumber beetle, *Acalymma vittatum* (F.) (Coleoptera: Chrysomelidae), is a well-studied and

[1] *This is reprinted from* Brzozowski, L.*, Leckie, B.M.*, Gardner, J., Hoffmann, M.P., and Mazourek, M. 2016. *Cucurbita pepo* subspecies delineates striped cucumber beetle (*Acalymma vittatum*) preference. *Horticulture Research* 3 (2016): 16028 *under the Creative Commons License*

economically important phenomenon [1]–[8]. Striped cucumber beetles cause significant damage to cucurbit crops (squash, pumpkin, watermelon, cucumber and melon) via herbivory of foliage, flowers, fruit and roots [4], and by vectoring pathogens of major diseases like bacterial wilt (*Erwinia tracheiphila*)[9] and *Squash mosaic virus* [10]. All cucurbit crops are affected by these beetles.

The relative preference of striped cucumber beetles – and thus degree of economic damage – varies within the Cucurbitaceae family [5], [6], [11]. Because striped cucumber beetles can devastate a newly planted crop [4], some control strategies have been developed. For instance, growers using conventional methods have access to effective chemical controls, in particular, systemic neonicotinoid insecticides [12]. However, use of these pesticides has come under scrutiny because of their potential impact on pollinator health [13]–[15]. Cultural controls, such as row covers and trap cropping are also advised to growers, but they do not offer complete control and add expense, like labor, materials, or loss of production space to a trap crop [16]. One avenue for optimizing control of these beetles would be to elucidate mechanisms behind what drives striped cucumber beetle preference, and to exploit those variations in preference to develop effective plant-based control.

The underlying biology influencing herbivore preference within the Cucurbitaceae family has been extensively studied for a range of cucurbit species and a group of specialist diabroticite beetles (Chrysomelidae: Galerucinae: Luperini) to which striped cucumber beetles belong. It has been broadly established that cucurbitacins, bitter tetracyclic triterpenoids, are toxic to most generalist herbivores, but are feeding stimulants to this tribe of beetles [11], [17], [18]. However, *A. vittatum*

189 has been shown to be the least responsive to cucurbitacins of this group of beetles
190 [18], [19]. While *A. vittatum* adults do choose to feed, and larvae perform better, on
191 cucumber (*Cucumis sativus*) plants with functional production of cucurbitacin C [1],
192 [20], they do not require cucurbitacin C for key physiological processes, like
193 production of their aggregation pheromone [21], [22], and the feeding response
194 elicited by cucurbitacin C is highly dependent on their life history [23]. Other leaf
195 chemistry or nutrition could also influence preference [20], [24]. In addition,
196 interspecific differences in plant volatiles also play a role in attraction [3], [25]–[27].
197 There are systems where hypotheses about the role of these biochemical
198 factors driving preference can be tested. For instance, isogenic lines of cucurbitacin C
199 producing and non-producing lines of cucumber are available[28]. However, one
200 species where such resources have not existed is the economically important
201 *Cucurbita pepo* species. *C. pepo* houses two agriculturally important and genetically
202 diverse subspecies *C. pepo* subsp. *pepo*, and *C. pepo* subsp. *texana* [29], [30], that
203 likely arose from distinct domestication events [31]–[33]. These subspecies include a
204 variety of different market classes of squash: *C. pepo* subsp. *pepo* includes cocozelle,
205 pumpkin, vegetable marrow, and zucchini, while *C. pepo* subsp. *texana* includes
206 acorn, crookneck, scallop, and straightneck squash [34]. These market classes are
207 comprised of a phenotypically diverse array of crops [35], as some are eaten as
208 immature fruit (e.g. zucchini), while others are eaten mature (e. g. acorn squash).
209 Importantly, variation in striped cucumber beetle host preference has been
210 observed within this species [5], [6], [11], [36]. However, there does not yet appear to
211 be a singular metabolite predictor of striped cucumber beetle preference in *C. pepo*.

Instead, studies have indicated that there may be a myriad of factors that contribute to striped cucumber beetle preference within *C. pepo*, including beetle life history [23], aggregation pheromones [21], [22], nutrition [20], [24], and cucurbitacins levels [11].

Because of the abundance of complex relationships that drive preference, it would be valuable to develop a simple framework for organizing striped cucumber beetle preference within *C. pepo*. Having an established framework could unify studies on factors affecting beetle preference and allow for these to be more easily incorporated in plant breeding decisions.

Accordingly, the objective of this study was to structure *A. vittatum* preference in *C. pepo*. A wide variety of cultivated *C. pepo* varieties, including those of the different subspecies and market classes, were tested in greenhouse and field choice assays to evaluate host preference of striped cucumber beetles. Additionally, farm-scale field and greenhouse no-choice studies were performed to further elucidate host preference traits in *C. pepo* and inform deployment strategies of non-preference traits.

2.3 Materials and Methods

2.3.1 Plant material.

A panel of 29 cultivars from six *C. pepo* market classes cultivated for harvest of immature fruits (Table 1), hereafter "early-harvest panel", and a survey of 27 *C. pepo* cultivars from five market classes, including those harvested as immature and mature fruits, (Table 2), hereafter "mixed-harvest panel", were used in cultivar choice trials. In no-choice bioassays, one inbred cultivar from each subspecies that represented the phenotypic extremes of beetle preference, as determined by the panel surveys, was grown: Golden Zucchini (*C. pepo* subsp. *pepo*) as the highly

preferred cultivar, and Success PM straightneck summer squash (*C. pepo* subsp. *texana*) as the highly non-preferred cultivar. Plants for all bioassays were started from seed in Fort Light potting soil (Vermont Compost Company, Montpelier, VT) in the Cornell University Guterman Bioclimatic and Greenhouse Complex (Ithaca, NY), and bioassays were conducted when plants had two or more leaves, but had flowerjng.

2.3.2 Insects. All adult striped cucumber beetles (*Acalymma vittatum*) were obtained from the Organic Research Farm (Freeville, NY) managed by the Cornell University Agricultural Experiment Station. The field trials took advantage of naturally occurring beetle populations in fields which had been planted to representatives of every genus, species and subspecies of most commonly cultivated melons, watermelons, squash, and cucumbers. Two adult generations of striped cucumber beetles occur in this region[4]; all trials except the no-choice greenhouse trial used first generation adult beetles (beetles that emerged from overwintering in Spring 2014 or Spring 2015).

2.3.3 Field cultivar bioassays. Field bioassays of both cultivar panels were conducted at Freeville Organic Research Farm over two consecutive years (2014-2015). Seeds for the 2014 early-harvest panel field trial were sown in 50 cell trays in a greenhouse on 27 May, 2014, and transplanted into the field on 24 June, 2014. Seeds for the 2015 mixed-harvest panel field trial were sown in 72 cell trays in a greenhouse on 26 June, 2015, and transplanted into the field on 7 July, 2015. In both years, plants were hardened off in a cold frame and then transplanted into soil covered with black plastic mulch with 2.7m spacing between rows. Trials were arranged in a randomized

complete block design with each of five replicates containing three plants planted 0.3m apart, and cultivars separated by 0.9m, within rows. The soil was amended with compost to achieve recommended fertility levels for these crops. Trained scorers evaluated beetle damage by visual assessment once damage was evident by using a non-linear 1-5 scale for damage (1=0-10%, 2=11-30%, 3=31-60%. 4=61-90%. 5=91-100%). Backtransformation of this categorical data for analysis was then performed by using the mean percent defoliation of the range representing the categorical classification (for example 5% and 20.5% were used for scores 1 and 2, respectively).

2.3.4 Greenhouse cultivar bioassays. To complement the field bioassays, both cultivar panels were grown and evaluated in controlled conditions using enclosure cages at the Guterman Greenhouse. Seeds were sown into detachable nursery cell packs, and seeding occurred on 12 July, 2014 for the mixed-harvest panel greenhouse trial and on 26 June, 2015 for the early-harvest panel greenhouse trial. Three blocks were planted with four plants of each cultivar in a randomized complete block design, and placed within a 1 x 0.2 x 2 m spun-bound polyester cage (Agribon®, San Luis Potosí, México) in which they were collectively exposed to beetles on 22 July 2014, and on 7 July 2015. Field collected beetles were added to the cages until sufficient damage was achieved, as in Barber *et al*. (2012) [37]. Both trials were terminated once damage was visibly evident and deemed significant, about 72 hours after initial exposure to beetles. Individual leaves and cotyledons were destructively removed from every plant to be digitally imaged, and percent leaf defoliation was then calculated by measuring total leaf area and estimating missing leaf area in ImageJ [38].

2.3.5 Field no-choice bioassay. In addition to understanding the effect of subspecies on beetle preference given choice among a range of cultivars, the differences in herbivory elicited by subspecies when *A. vittatum* had no choice of food source were also explored. Previous greenhouse surveys with low numbers of plants (four to six plants) indicated that feeding behavior differed between 'Golden Zucchini' and 'Success PM' in a no-choice scenario (data not shown). To address the overarching goal of *A. vittatum* non-preferred cultivar development, a farm-scale no-choice bioassay was conducted. Accordingly, four large, 0.1ha square (30.5m x 30.5m) sites spaced at least 350m apart were chosen at the Pullyen-Tailby Farm managed by the Cornell University Agricultural Experiment Station in Varna, NY. Each site was prepared as 10 equally spaced rows 30m in length, covered with black plastic mulch, and managed organically. 'Golden Zucchini' and 'Success PM' seeds were sown into 50 cell flats in the Guterman Greenhouse between 1-2 June, 2015, hardened off, and then transplanted into two field sites each at 0.6m spacing (50 plants per row; 500 plants per site) on 17 June, 2015. All plants were also fertilized on 22 June, 2015with Perdue AgriRecycle microSTART60 Plus with feather meal in 7-1-1- Prill form to achieve recommended crop fertility. Leaf damage was then scored visually as percent defoliation using a linear 0-100 scale by units of five (0=0%, 5=1-5% defoliation, 10=6-10% defoliation, etc.) for all plants in all plots by a single trained observer beginning when *A. vittatum* was first observed in one plot, a 'Golden Zucchini' site, on 19 June, 2015. Plots were scored approximately three times weekly until 17 July, 2015, the point in the season when *A. vittatum* populations expectedly declined in our

304 region [4]. Since there were no feeding *A. vittatum* populations observed in the two
305 'Success PM' sites for over 15 days after beetles were first observed in the first
306 'Golden Zucchini' site, they were supplemented with approximately 500 beetles
307 collected from the Organic Research Farm (Freeville, NY) on 6 July, 2015. The
308 addition of beetles to the 'Success PM' sites was done in attempt to assess whether
309 damage would have occurred if striped cucumber beetles were present.
310
311 2.3.6 Greenhouse no-choice bioassay. To control for field variability, and the ability of
312 *A. vittatum* to leave the sites, large no-choice bioassays were conducted in the
313 Guterman Greenhouse. 'Golden Zucchini' and 'Success PM' seeds were sown in 72-
314 cell flats on 15 Sept., 2015. Two flats of each cultivar were then placed in individual
315 cages and exposed to field collected beetles per cage on 27 Sept., 2015, and this was
316 replicated twice (four total cages). Once damage was evident, on 9 Oct., 2015, leaves
317 were digitally imaged and scored for percent leaf damage.
318
319 2.3.7 Statistical Analysis. For all choice bioassays, percent defoliation was analyzed
320 by an ANOVA performed in JMP Pro 11 (JMP®, Version 11. SAS Institute Inc.,
321 Cary, NC, 1989-2007) using a generalized linear model. Subspecies, market class, and
322 cultivar were treated as fixed effects with cultivar nested within market class, and
323 market class nested within subspecies. The effects of blocking and replication were
324 treated as random effects. Least squared means were separated using a t-test for testing
325 between subspecies ($p<0.05$), and by Tukey's HSD test for between market classes
326 and cultivars ($p<0.05$.) To determine the distribution of beetle damage in the no-

choice assays, data was grouped into two categorical bins – minimal leaf damage (field: <10%, greenhouse: 0%), and significant leaf damage (field: ≥10%, greenhouse: >0%) – and Fisher's exact test was used to test the null hypothesis that there was no difference in distribution of beetle damage between subspecies. These data were blocked by plot in the field trials, and cage in greenhouse trials. In addition, in the greenhouse no-choice assay, an additional categorical grouping of extreme (>=80%) leaf damage was also examined by Fisher's Exact test.

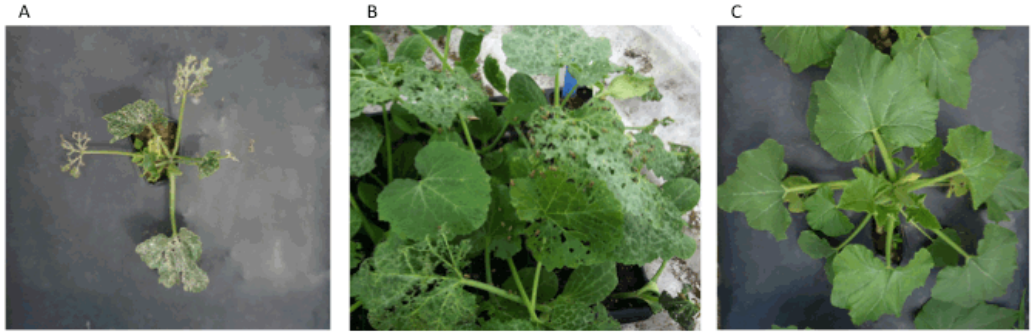
2.4 Results

2.4.1 Field cultivar bioassays. Early-harvest and mixed-harvest panels were evaluated at the Organic Research Farm to test *A. vittatum* preference in a real field setting, and it was found that transplanted seedlings of *C. pepo* subsp. *texana* suffered less herbivory damage by natural populations of striped cucumber beetles than plants of the other subspecies, *C. pepo* subsp. *pepo* (**Figure 2.1**).

In the early-harvest panel, the damage observed within the *C. pepo* subsp. *texana* cultivars ranged from 5% defoliation of several straightneck and crookneck cultivars, including Success PM, to 10% defoliation of the straightneck cultivar Lioness (**Table 2.1**). In contrast, every *C. pepo* subsp. *pepo* cultivar had higher mean percent defoliation than those in *C. pepo* subsp. *texana*, with a range from 20% in zucchini cultivar Partenon to 46% in vegetable marrow cultivar Romulus. There were significant differences in leaf defoliation between all market classes in the different subspecies, as well as between subspecies as a whole ($p<0.0001$), with the degree of

damage being more severe for *C. pepo* subsp. *pepo*. Likewise, in the mixed-harvest panel, expanded to include market classes of *C. pepo* harvested as mature fruit, there was again a significant difference in damage between subspecies ($p<0.0001$), which was also demonstrated within market class groupings (but not by individual cultivars) (**Table 2.2**). The extremes of leaf defoliation in each subspecies within the mixed-harvest panel were 1% to 9% in *C. pepo* subsp. *texana* ('Golden Bush Scallop', and 'Sweet REBA', respectively), and 9% to 54% in in *C. pepo* subsp. *pepo* ('Racer', and 'Golden Zucchini', respectively).

Figure 2.1. A comparison of adjacent young transplants in the field highlights the impact of striped cucumber beetle preference on plant health. *C. pepo* subsp. *pepo* cultivar 'Golden Zucchini' (A) is highly preferred and incurs substantial damage as the beetles aggregate and feed (B), while the non-preferred *C. pepo* subsp. *texana* cultivar 'Success PM' (C) is nearly free of herbivory damage.



360 **Table 2.1**. Striped cucumber beetle damage in field and greenhouse (GH) trials of early-harvest *C. pepo* cultivar panel

Subspecies	Damage[1,2]				Market Class	Damage[1,3]				Cultivar[4,5]	Seed source[6]	Damage[1,3]			
	Field		GH			Field		GH				Field		GH	
C. pepo subsp. *texana*	5.8	a	2.5	a	Crookneck	5.6	a	2.0	a	Dixie[α]	SM	5	ab	2	ab
										Gentry	JS	6	ab	2	a
					Scallop	5.7	a	2.9	a	Golden Bush Scallop[α]	SO	6	a-c	2	ab
										Flying Saucer	JS	6	ab	4	ab
					Straightneck	6.0	a	2.7	a	Cougar	HS	5	a	2	a
										Early Prolific Straightneck[α]	SO	5	a	1	a
										Success PM	CU	5	a	2	ab
										Superpik[β]	TS	5	a	2	ab
										Zephyr	JS	5	a	5	ab
										Slick Pik	JS	7	ab	5	ab
										Lioness	SC	10	a-c	1	a
C. pepo subsp. *pepo*	28.2	b	11.3	b	Cocozelle	22.8	b	13.6	b	Cocozelle	HM	22	a-d	12	a-c
										PMR Costata	CU	23	a-e	19	bc
										Costata Romanesco	HM	24	b-e	10	a-c
					Vegetable Marrow	30.1	bc	7.8	ab	Harukan[α]	SC	21	a-d	4	ab
										Magda[α]	CU	40	de	11	a-c
					Zucchini	31.5	c	12.6	b	Partenon	JS	20	a-d	10	ab
										Golden Arrow	FS	21	a-d	9	ab
										Cashflow	SY	25	a-e	13	a-c
										Dunja	JS	27	a-e	14	a-c
										Zucchini Elite[α]	HS	28	a-e	26	c
										Black Beauty[α]	TS	29	b-e	12	a-c
										Reward	OS	29	b-e	13	a-c
										Tigress	JS	31	c-e	8	ab
										Gold Rush	OS	35	de	9	ab

	Goldy	JS	37	de	13	a-c
	Midnight Lightning	HM	39	de	10	a-c
	Golden Zucchini	CU	42	de	12	a-c
	Romulus[αβ]	CU	46	e	14	a-c

[1]Damage is reported as the least mean square average percent defoliation.

[2]Means followed by different letters are significantly different at $p<0.001$ using Student's t-test.

[3]Means followed by different letters are significantly different at $p<0.05$ using a Tukey's HSD test.

[4]n=15 for field trials unless indicated by a [α] symbol: 'Dixie' n=5; 'Golden Bush Scallop' n=9; 'Early Prolific Straightneck' n=14; 'Harukan' n=12; 'Magda' n=12; 'Zucchini Elite' n=12; 'Black Beauty' n=14; 'Romulus' n=13.

[5]n=12 for greenhouse trials unless indicated by a [β] symbol: 'Superpik' n=14; 'Romulus' n=11.

[6]Letters represent source of seed: CU – Cornell University produced seed, Ithaca, NY; FS – Fedco Seeds, Waterville, ME; HS – Harris Seeds, Rochester, NY; HM – High Mowing Organic Seeds, Wolcott, VT; JS – Johnny's Selected Seeds, Winslow, ME; OS – Osborne Seed Company, LLC, Mount Vernon, WA; SC – Seeds of Change, Rancho Dominguez, CA; SM – Seminis, St. Louis, MO; SO – Southern Exposure Seed Exchange, Mineral, VA; SY – Syngenta Seeds, Inc., Minnetonka, MN; TS – Territorial Seed Company, Cottage Grove, OR

373 **Table 2.2**. Striped cucumber beetle damage in field and greenhouse (GH) trials of mixed-harvest *C. pepo* cultivar panel

Subspecies	Damage[1,2]		Market Class	Damage[1,3]		Cultivar[4,5]	Seed source[6]	Damage[1,3]	
	Field	GH		Field	GH			Field	GH
C. pepo subsp. *texana*	3.7 a	3 a	Scallop	3 a	4.8 ab	Golden Bush Scallop[β]	SO	1 a	3 ab
						Flying Saucer	JS	3 a	9 a-c
						Yellow Scallop[β]	SW	3 a	5 a-c
						Woods Prolific Bush Scallop[β]	SE	5 a	2 ab
			Straightneck	3.7 a	0.8 a	Cougar[α]	HS	3 a	1 a
						Multipik[β]	HS	3 ab	1 a
						Superpik	HS	3 a	1 a
						Early Prolific Straightneck[αβ]	SO	4 ab	0 a
						Success PM[α]	HM	5 ab	1 ab
			Acorn/Delicata	4.3 a	3.6 ab	Honey Bear	JS	2 ab	3 ab
						Jester	JS	2 ab	2 ab
						Sugar Loaf[αβ]	NG	3 ab	2 ab
						Royal Ace[αβ]	HS	4 ab	2 ab
						Bush Delicata	CU	4 a	1 ab
						Zeppelin	WG	5 ab	7 a-c
						Honeyboat	OS	6 ab	10 a-c
						Sweet REBA[β]	CU	9 a-c	2 ab
C. pepo subsp. *pepo*	23.3 b	10.9 b	Pumpkin	12.3 b	8.2 bc	Racer[β]	JS	9 a-c	9 a-c
						Triple Treat[β]	BR	10 a-c	9 a-c
						Magic Lantern	HS	11 a-c	5 a-c
						Aladdin	HS	14 a-d	9 a-c
						Howden	HS	20 b-e	9 a-c
			Zucchini	34.4 c	13.7 c	Dunja	JS	22 c-e	10 a-c
						Reward[β]	OS	32 de	13 a-c

	Black Beauty[αβ]	TS	32	de	4	a-c
	Zucchini Elite	HS	33	e	20	bc
	Golden Zucchini	SE	54	f	22	c

374

375 [1]Damage is reported as the least mean square average percent defoliation.
376 [2]Means followed by different letters are significantly different at $p<0.001$ using Student's t-test.
377 [3]Means followed by different letters are significantly different at $p<0.05$ using a Tukey's HSD test.
378 [4]n=15 for field trials unless indicated by a [α] symbol: 'Cougar' n=9; 'Early Prolific Straightneck' n=14; 'Success PM' n=14; 'Sugar Loaf' n=7; 'Royal Ace'
379 n=14; 'Black Beauty' n=14.
380 [5]n=12 for greenhouse trials unless indicated by a [β] symbol: 'Golden Bush Scallop' n=11; 'Yellow Scallop' n=9; 'Woods Prolific Bush Scallop' n=11;
381 'Multipik' n=11; 'Early Prolific Straightneck' n=10; 'Sugar Loaf' n=7; 'Royal Ace' n=11; 'Sweet REBA' n=10; 'Racer' n=10; 'Triple Treat' n=10; 'Reward'
382 n=6; 'Black Beauty' n=8.
383 [6]Letters represent source of seed: BR – Burpee, Warminster, PA; CU – Cornell University produced seed, Ithaca, NY; HS – Harris Seeds, Rochester, NY;
384 HM – High Mowing Organic Seeds, Wolcott, VT; JS – Johnny's Selected Seeds, Winslow, ME; NG – Nichols Garden Nursery, Albany, OR; OS – Osborne
385 Seed Company, LLC, Mount Vernon, WA; SE – Seed Savers Exchange, Decorah, IA; SO – Southern Exposure Seed Exchange, Mineral, VA; SW – Sow
386 True Seeds, Asheville, NC; TS – Territorial Seed Company, Cottage Grove, OR; WG – Wild Garden Seed, Philomath, OR
387

388 2.4.2 Greenhouse cultivar bioassays. To control for field variation, both panels were
389 also exposed to field-collected striped cucumber beetles in a restricted greenhouse
390 setting. The beetles caused significantly more beetle damage to the *C. pepo* subsp.
391 *pepo* cultivars, echoing the results of the field trial. In the early-harvest panel, the
392 difference in defoliation by subspecies was highly significant (p<0.0001) (**Table 2.1**).
393 The extremes of leaf defoliation in each subspecies were 1% to 5% in in *C. pepo*
394 subsp. *texana* ('Lioness', and 'Slick Pik', respectively), and 4% to 26% in *C. pepo*
395 subsp. *pepo* ('Harukan', and 'Zucchini Elite', respectively). The mixed-harvest panel
396 yielded similar results. Damage in *C. pepo* subsp. *texana* cultivars ranged from 0%
397 defoliation of straightneck cultivar Early Prolific Straightneck to 10% defoliation of
398 the acorn/delicata cultivar Honeyboat, while damage in *C. pepo* subsp. *pepo* ranged
399 from 5% defoliation of pumpkin cultivar Magic Lantern to 22% defoliation of the
400 zucchini cultivar Golden Zucchini (**Table 2.2**). Again, the difference in beetle damage
401 between subspecies was significant (p<0.0001). In neither cultivar panel did market
402 class or cultivar alone demonstrate as clear a significant divide between subspecies as
403 did evaluating all the cultivars together.
404
405 2.4.3 Field no-choice bioassay. No-choice bioassays with cultivars representing the
406 extremes of preference between subspecies were conducted to determine if *C. pepo*
407 subsp. *texana* (represented by straightneck cultivar 'Success PM') was not a suitable
408 host, or if *C. pepo* subsp. *pepo* (represented by zucchini cultivar 'Golden Zucchini')
409 was just so strongly preferred that *C. pepo* subsp. *texana* was never fed upon in a
410 mixed environment. These no-choice bioassays were also designed to understand the

agricultural relevance of the non-preference phenotype, and its applicability in plant breeding: for instance, if non-preference could be introgressed into all *C. pepo*, particularly *C. pepo* subsp. *pepo* cultivars, could non-preference be a stand-alone control measure at farm scale? Two farm-scale no-choice monocultures of 'Success PM' and two of 'Golden Zucchini' were observed. It was found that 'Success PM' plots had far more plants with less than 10% leaf defoliation than did the 'Golden Zucchini' plots (**Figure 2.2**) (Fishers exact two-tailed test, $p<0.0001$). Individual plant leaf defoliation ranged from 5% to 90% in 'Golden Zucchini' plots, and from 0% to 15% in 'Success PM' plots on the final day of observation. In addition, the 'Success PM' plots also attracted no natural feeding populations of striped cucumber beetles (LB, personal observation). Accordingly, *A. vittatum* from the Organic Research Farm were added to ensure evaluation of beetle damage was possible. However, even with the addition of beetles to the 'Success PM' plots, very few beetles remained in those plots, and those that did inflicted very little damage.

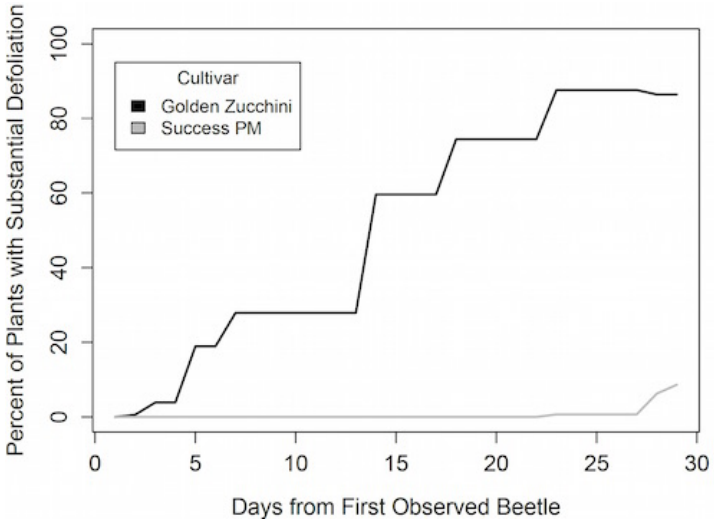


Figure 2.2. Leaf defoliation progress in field no-choice plots. Averaged number of plants with substantial (≥10% leaf defoliation) in no-choice field plots. Difference between cultivars in leaf damage was significant ($p<0.0001$) at day 2, and continued to be at the final day of observation, day 29 ($p<0.0001$).*A. vittatum* were added to 'Success PM' plots on day 18, about four days before reflected by an increase in defoliated 'Success PM'.

2.4.4 Greenhouse no-choice bioassay. Since one limitation of the field no-choice

experiment is that the beetles could not be contained, the field results were verified in a controlled greenhouse setting. There was no significant difference in the mean damage (total leaf defoliation) between cultivars in the greenhouse no-choice bioassay (**Table 2.3**), indicating that *C. pepo* subsp. *texana* is indeed a suitable host for the herbivore. However, the greenhouse no-choice bioassay revealed a substructure in the pattern and degree of beetle feeding (Table 3). Significantly more 'Success PM' plants had no leaf damage (0% leaf defoliation) than 'Golden Zucchini' plants (Fishers exact two-tailed test, $p<0.0001$). Intriguingly, significantly more 'Success PM' plants sustained what we termed "extreme" leaf damage, (≥80% leaf defoliation) than 'Golden Zucchini' plants (Fishers exact two-tailed test, $p=0.004$).

Table 2.3. Distribution of striped cucumber beetle damage to leaves in greenhouse no-choice bioassay

Cultivar	Subspecies	Mean damage[1,2]	Grouped by No damage[1,3,4]		Grouped by High damage[1,3,5]	
			0%	> 0%	< 80 %	≥ 80%
Success PM	*C. pepo* subsp. *texana*	29.8% (n.s.)	24.2%	75.8%	91.3%	8.7%
Golden Zucchini	*C. pepo* subsp. *pepo*	30.6% (n.s)	3.9%	96.1%	97.4%	2.6%

[1]Damage reported as mean percent leaf defoliation.
[2]No significant difference in mean leaf defoliation between cultivars was detected by a Student's t-Test.
[3]Numbers represent the percent of plants of that genotype with the indicated level of damage.
[4]No choice contingency table significant at $p<0.0001$ in two-sided table probability from Fisher's Exact Test.
[5]No choice contingency table significant at $p<0.01$ in two-sided table probability from Fisher's Exact Test.

2.5 Discussion

We have elucidated a significant factor affecting the structure of striped cucumber beetle (*A. vittatum*) preference within *C. pepo* by quantifying beetle damage on plants of two economically important subspecies, *C. pepo* subsp. *pepo*, and *C. pepo* subsp. *texana*. Our cultivar panels represented the range of the cultivated crop within each

452 subspecies [34], and are consistent with reports on the genomic differentiation of these
453 subspecies [29], [30]. Overall, in the variety of bioassays conducted, we found that *C.*
454 *pepo* subsp. *pepo* cultivars were more heavily damaged by the striped cucumber
455 beetles, and that *C. pepo* subsp. *texana* cultivars were less damaged when presented in
456 choice (**Figure 2.1**) or no-choice (**Figure 2.2**) experiments. In all, these results
457 implicate subspecies as a decisive driver of *A. vittatum* preference within *C. pepo*.
458 Previous work examined differential cucumber beetle preference within a
459 variety of cucurbit crops [1], [11], [36]. Researchers have employed various levels of
460 structure in which cucurbit plants are tested: some group by species [11], [39]–[41], or
461 market class [5], [6] or cultivar [24], [36] within a single species, like *C. pepo*.
462 However, applying this phylogenetic framework to previous studies demonstrates that
463 subspecies is predictive of beetle damage in a variety of experimental designs and
464 cultivar selections. Ferguson *et al.* (1983) conducted replicated field trials of many
465 *Cucurbita* crops, with a heavy representation of eighteen *C. pepo* cultivars. If these
466 cultivars are grouped into subspecies using information from the seed trade and online
467 databases (e.g. http://cuke.hort.ncsu.edu/cucurbit/wehner/vegcult/vgclintro.html), the
468 *C. pepo* subsp. *pepo* cultivars sustained significantly more damage. This is also true of
469 trial results for feeding damage published by McGrath (2002), where ten *C. pepo*
470 cultivars were grown among a variety of cultivated cucurbit crops. In addition, when
471 Hoffmann *et al.* (1996) focused on solely *C. pepo* cultivars, throughout the trial, the
472 number of beetles per plant, number of infested plants, and defoliation ratings, are
473 again substantially higher in *C. pepo* subsp. *pepo* cultivars. In all of these field trials,
474 authors relied on natural populations of beetles for damage, and reported the presence

of multiple beetle species of the tribe Luperini [6], [7], [11]. A smaller study[36], where four *C. pepo* cultivars – incidentally two from each subspecies – were grown in greenhouse trials with beetles separated by species, also upholds the validity of organizing *A. vittatum* preference by subspecies. Wiseman *et al.* (1961) reported significantly higher stem and cotyledon injury to *C. pepo* subsp. *pepo* cultivars by *A. vittatum* adults alone [36].

The plant metabolic processes driving beetle preference in *C. pepo*, however, are not well characterized. Broadly within the Cucurbitaceae family, cucurbitacins have been classically described as the key biomolecules dictating herbivore behavior. Generalist herbivores tend to perform poorly on cucurbitacin-rich plants; for instance, two-spotted mites (*Tetranuchys urticae* Koch.) suffered higher mortality [28], and had lower fecundity [42] when feeding on bitter cucumber. In contrast, over the course of a long co-evolutionary history, specialist herbivores of the Cucurbitaceae, like *A. vittatum* and its close relatives in the Luperini tribe (like the spotted cucumber beetle, *Diabrotica undecimpunctata howardi*), have adapted to tolerate cucurbitacins, and in some cases, find them to be feeding stimulants [17], [18], [43]. However, there is not strong evidence to support that differences in *A. vittatum* preference between subspecies in *C. pepo* can be attributed to the single qualitative factor of the differential presence of cucurbitacins.

Cucurbitacins B, D, and E have been detected in *C. pepo* [11], [19], [20], [24], [44], [45], but they are at extremely low concentrations compared to wild species, and cultivated species known to attract *A. vittatum*, such as several cultivars used for trap cropping [11], [19], [46]. Even though cucurbitacins have been detected in *C. pepo*,

most of the few precise measurements that exist were taken in root [20] and cotyledon tissue [11], [24], not leaf tissue. Cucurbitacins in leaves were found to reach a maximum of 5 – 10µg/g in fresh tissue in a preferred *C. pepo* subsp. *pepo* zucchini cultivar, Black [44], which is below the reported level of detection by diabroticite beetle feeding of 20 µg/g of cucurbitacins in fresh tissue [19]. Moreover, cucurbitacin content in the leaves of *C. pepo* plants is poorly correlated with cotyledon cucurbitacins, and thought to be controlled by a different genetic pathway [11]. The genetic basis of cucurbitacin production in *C. pepo*, in general, is also not well characterized. Unlike production of cucurbitacin C in *C. sativus*, cucurbitacin production in *C. pepo* is not controlled by a single gene [24], [47].

In addition, the foundation of most knowledge of the role of cucurbitacins in influencing the behavior of diabroticite beetles is known from Luperini beetle species other than *A. vittatum* [11], [17]–[19], most often *D. undecimpunctata howardi* [11], [17]–[19]. While these cucurbit specialist beetles are often discussed interchangeably [5], there are important differences. For instance, *A. vittatum* tends to excrete less and sequester more cucurbitacins [48] than its close relatives, and is far less sensitive to cucurbtaicins [18], [19]. Specifically, *A. vittatum* have been shown to react to 0.3µg pure cucurbitacin B, while *D. undecimpunctata howardi* is sensitive at a threshold of over two order of magnitudes lower, 0.001µg of pure cucurbitacin B [19]. Finally, behavioral studies also indicate that *A. vittatum* is not reliant on cucurbitacins. The preference of *A. vittatum* for cucurbitacin C present in *C. sativus*, is also inconsistent throughout its lifecycle [23], suggesting cucurbitacins may not play a critical role in preference. In addition, these cucurbitacins are neither a prerequisite, nor even

521 augment, *A. vittatum* aggregation pheromone production – an important behavior
522 implicated in increased pest pressure [21], [22]. Overall, from examining both *C. pepo*
523 and *A. vittatum*, cucurbitacins alone do not appear to be a likely candidate for the
524 biochemical basis of preference.

525 While cucurbitacins are certainly the most studied specialized defensive
526 metabolite from cucurbit crops, there is evidence that other classes of non-volatile and
527 volatile biomolecules may explain the disparities in herbivore preference between
528 subspecies. Differences in non-volatile nutritive compounds like cotyledon sugar
529 content [24], and root nitrogen [20], have been reported. Contrasts in both floral [25]–
530 [27] and non-floral [3] plant volatiles have also been shown to elicit differential beetle
531 preference. Other yet unknown factors may exist as well that drive the extreme
532 difference in preference considering that these two *C. pepo* subspecies are genetically
533 distinct [29], [30], and likely arose from different domestication events [31]–[33].
534 While future work should explore the broad range of biochemicals including nutrients,
535 cucurbitacin types and overall levels, and volatiles – and the interactions between
536 them – that may drive *A. vittatum* preference, it is important to recognize that
537 preference is likely controlled by a complex array of factors within a dynamic, and
538 environmentally-sensitive, biochemical system.

539 While efforts will be directed at achieving a more complete understanding of
540 the biochemical and genetic framework that underlie preference, this knowledge of *A.*
541 *vittatum* preference being structured by subspecies in *C. pepo* will be immediately
542 applicable in plant breeding and management decisions to benefit growers. Trap-
543 cropping strategies already take advantage of highly preferred cucurbits to pull beetles

away from the market crop [49]–[52]. A derivation of trap-cropping, a push-pull management system [53], has been suggested for *A. vittatum* control in *C. pepo* [27], where a non-preferred cultivar is paired with a highly preferred trap crop. Our results both identify *C. pepo* subsp. *texana* crops as a the non-preferred "push" planting and prompted the question of whether the "push" factor of the non-preferred cultivar can be strong enough alone to be an effective form of pest management. Specifically, how would *A. vittatum* respond if they were presented with no choice of food source on a farm-scale (e.g. 'Success PM' monoculture)? In the absence of a "preferred" food source, would the beetles still inflict minimal damage on the "non-preferred" plants?

Our results from both field and greenhouse trials indicate that, while non-preference does not equate to resistance *per se*, non-preference exploits a nuance in striped cucumber beetle feeding patterns, which leads to the non-preferred cultivar sustaining significantly less damage, even when a preferred cultivar is not locally available. In addition, in the greenhouse, concentrated damage on only a few plants of the non-preferred cultivar, were also observed, inviting the question of the role of host plant resistance in non-preference. This result, *A. vittatum* non-preference being manifested both in the presence and absence of a preferred genotype, is likewise corroborated by another no-choice trial of *A. vittatum* in cucumbers (*Cucumis sativus*) [1].

Although these no-choice tests only made use of two of the many available cultivars, by employing the structural framework for organizing *A. vittatum* preference by subspecies within *C. pepo*, countless other cultivars can be rigorously tested in both no-choice and push-pull settings. Overall, the no-choice component of this study has

provided new insights into striped cucumber beetle preference and the effectiveness in field deployment, and informs the breeding of *C. pepo* cultivars by providing evidence for the usefulness of introgression of non-preference across subspecies barriers.

This study was the first to consider subspecies as a driver of beetle preference within *C. pepo*, and provided a clear demarcation in preference between the two major commercial subspecies – *C. pepo* subsp. *pepo* and *C. pepo* subsp. *texana* – with *C. pepo* subsp. *texana* being more strongly non-preferred both alone, and among preferred *C. pepo* subsp. *pepo* cultivars. By basing this work in a biological context, this contrast provides an accessible structural framework for understanding variation in preference within *C. pepo*, and applying it in an agricultural setting has been discovered. This structure immediately allows plant breeders and entomologists to augment pest control options, and also promotes future work to understand the biochemical and genetic foundation of *A. vittatum* preference.

2.6 Bibliography

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Chapter 3: Mechanisms of Resistance to Insect Herbivores in Isolated Breeding Lineages of *Cucurbita pepo*[2]

3.1 Abstract

Although crop wild ancestors are often reservoirs of resistance traits lost during domestication, examining diverse cultivated germplasm may also reveal novel resistance traits due to distinct breeding histories. Using ten cultivars from two independent domestication events of *Cucurbita pepo* (ssp. *pepo* and *texana*), we identified divergences in constitutive and induced resistance measured by growth of generalist caterpillars and leaf traits. *C. p. texana* cultivars were consistently more resistant to *Trichoplusia ni* and *Spodoptera exigua*, and this was not due to expected mechanisms including cucurbitacins, nitrogen, sticky phloem sap, or toxicity. Although more susceptible on average, *C. p. pepo* cultivars showed stronger induced resistance, suggesting a trade-off between constitutive and induced resistance. To test the hypothesis that leaf volatiles accounted for differences in resistance to caterpillars, we devised a novel method to evaluate resistance on artificial diet while larvae are exposed to leaf volatiles. In both subspecies, cultivar-specific induced volatiles that reduced *T. ni* growth were present in highly inducible cultivars, but absent in those that showed no induction. These results have important agricultural implications as

[2] *Reprinted by permission from Springer Nature: Springer Journal of Chemical Ecology. Copyright license agreement number 4803650916823. Brzozowski, L. J., Mazourek, M., and Agrawal, A.A. Mechanisms of Resistance to Insect Herbivores in Isolated Breeding Lineages of Cucurbita pepo. 2019.* 4 5(3): 313-325

cultivar-specific resistance to caterpillars mirrored that of specialist beetles from field trials. Overall, the eponymous cucurbitacin defenses of the Cucurbitaceae are not the mechanistic basis of differences in constitutive or induced resistance between *C. pepo* subspecies or cultivars. Instead, deterrent cultivar-specific volatiles appear to provide general resistance to insect herbivores. Divergence during breeding history within and between subspecies revealed this pattern and novel resistance mechanism, defining new targets for plant breeding.

3.2 Introduction

Crop plants are often less resistant to herbivores than their wild ancestors [1], [2], yet variation for resistance persists within crop germplasm. During breeding history, traits impacting herbivore resistance were altered by direct (natural or human-directed) selection, indirect consequences of selection on other traits, and genetic drift [3]. The effect of breeding history on herbivore resistance is amplified when crop germplasm is isolated in distinct breeding pools, and comparisons between these pools provide opportunities to elucidate mechanisms of resistance. Indeed, tracking plant resistance through several genetic lineages revealed a decline in resistance from wild relatives to landraces to modern cultivars [4]–[6], and occasionally uncovered qualitative losses of major resistance traits between isolated breeding lineages [7]. Studying distinct lineages provides insight not only on losses, but other possible outcomes, such as novel resistance traits.

Elucidating resistance mechanisms through comparisons of cultivated plants

782 with distinct breeding histories is already situated within the context of crop lineages,
783 and thus may be advantageous for further agricultural applications. First, during
784 domestication, human consumers typically selected for palatability and against
785 toxicity [8], presumably eliminating sources of resistance that would be unacceptable
786 for consumption. Additionally, mechanisms of effective resistance in crop plants may
787 differ from resistance traits in their wild relatives. Indeed, some resistance traits are
788 important in wild plant populations, but neutral or detrimental under cultivation [9],
789 [10]. A separate suite of resistance traits may be more relevant in agricultural contexts
790 because the community and intensity of pests also differ [11]. For instance, potential
791 pest pressure from specialist insects at the onset of domestication of maize may have
792 disrupted balancing selective forces from generalist and specialist herbivores [10].
793 Evaluation of isolated breeding pools that arose post-domestication have also
794 provided insight into novel resistance mechanisms, including plant volatiles. Volatile
795 compounds serve as information to herbivores, for instance, a warning of competition
796 [12], or as feeding deterrents [13], [14]. A notable example is the discovery that
797 independent maize breeding programs in North America and Europe diverged in the
798 production of the volatile compound, (*E*)-β-caryophyllene [15]. This volatile is
799 induced in maize roots by the corn rootworm (*Diabrotica virgifera virgifera*) and
800 provides critical indirect resistance through recruitment of entomopathogenic
801 nematodes [7]. Across plant species, substantial variation in plant volatiles exists in
802 cultivated germplasm, and several domesticated plants have greater volatile induction
803 as compared to wild relatives [16]. Thus, use of multiple cultivars with independent
804 breeding histories provides a structure to identify novel resistance mechanisms, like

805 plant volatiles, some of which could become the target of breeding programs.

806 *Cucurbita pepo* provides an excellent system to test how divergent breeding
807 histories in independent lineages may have led to altered defensive traits. Within *C.*
808 *pepo*, there are two cultivated subspecies, *C. pepo* ssp. *pepo* (hereafter *C. p. pepo*) and
809 *C. pepo* ssp. *texana* (syn. *C. pepo* ssp. *ovifera*; hereafter *C. p. texana*), that have been
810 bred to include multiple market classes, including pumpkin and zucchini in *C. p. pepo*,
811 and acorn squash and summer squash in *C. p. texana* [17]. These subspecies arose
812 from two separate domestication events [18]–[20], and cross breeding is uncommon in
813 modern breeding programs [21]. *C. p. pepo* is thought to have been domesticated in
814 central Mexico from a yet unknown wild progenitor [20], but cultivar development
815 predominately occurred in Europe starting in the 16th century [17]. In contrast, *C. p.*
816 *texana* was domesticated and developed in the eastern United States and northern
817 Mexico [18], [20], [22]. Because *C. p. texana* has been developed in its center of
818 origin, it exclusively has been exposed to specialist beetle pests endemic to North
819 America [23] throughout its breeding history. North American specialist beetles in the
820 Diabroticina subtribe (Coleoptera: Chyrsomelidae), including *Acalymma vittatum* and
821 *Diabrotica* spp., strongly prefer *C. p. pepo* over *C. p. texana* cultivars [24]–[26].
822 Accordingly, these breeding lineages provide an opportunity to examine how
823 independent domestication events and breeding histories shaped the mechanistic basis
824 of plant resistance to these, and other, herbivores.

825 Cucurbitacins, intensely bitter and toxic triterpenoids, are an important form of
826 resistance across the Cucurbitaceae to generalist herbivores [23], [27], yet are tolerated
827 and sequestered as a defense against predators by some specialist beetles [28]–[31].

Fruits of wild *Cucurbita* spp. contain cucurbitacins, but today, *Cucurbita* spp. food crops lack cucurbitacins due to the identification of bitter-free mutants during the domestication process [19]. The implications of loss of fruit bitterness for leaf-chewing herbivores of *C. pepo* are unknown. While the full molecular pathway of cucurbitacin production is yet to be elucidated in *C. pepo*, loss of fruit bitterness does not preclude cucurbitacin production in leaves in other Cucurbitaceae, like cucumber (*Cucumis sativus*) [32], [33]. Indeed, cucurbitacins are present in cotyledons of some domesticated *C. pepo* [24], but have been reported to be nil or at low constitutive concentrations in true leaves [28], [34], [35]. Herbivory was previously shown to induce a substantial increase of a leaf cucurbitacin in one cultivar of *C. pepo* [36], but cucurbitacins have only been evaluated in a small number of cultivars, and their potential role in resistance and breeding requires additional investigation. Additional defenses of *C. pepo* have also been evaluated in some contexts: deterrent plant volatiles have been measured in one cultivar [37], and mucilaginous sap has been studied in other Cucurbitaceae [38], [39], but not *C. pepo*. Apart from defenses, leaf nutrient content is important for herbivore preference and performance [40], [41]. Nitrogen content was measured in a survey of Cucurbitaceae species but was not associated with specialist beetle (*A. vittatum*) abundance [35].

In this study, we determine how plant resistance mechanisms diverged between the two cultivated *C. pepo* subspecies, representing two independent domestication events where isolated breeding pools have been maintained. One subspecies, *C. p. texana*, has had continuous interaction with specialist beetle pests, while the other (*C. p. pepo*) was geographically separated. We thus hypothesized that these distinct

851 breeding histories would lead to disparate defense strategies, with different effects for
852 generalists and specialists. We used five cultivars each of *C. p. pepo* and *C. p. texana*
853 to test 1) for divergence in constitutive and induced resistance to the two leaf-feeding
854 generalist caterpillars, *Trichoplusia ni* and *Spodoptera exigua*, as measured by larval
855 performance, and 2) if differences in resistance were associated with secondary
856 metabolites or a suite of other foliar traits. Plant chemical traits including
857 cucurbitacins and nitrogen were measured and complemented by insect growth and
858 behavioral assays. To distinguish between deterrence and toxicity, we examined
859 growth efficiency of caterpillars as well as the impact of constitutive and induced
860 volatiles on caterpillar growth. Induction by caterpillar feeding was also compared to
861 elicitation by jasmonic acid, the plant hormone primarily responsible for orchestrating
862 induced resistance. And finally, given specialist beetle associations differentially
863 affected *C. pepo* breeding histories, 3) we sought to test how findings from generalist
864 herbivores related to specialist beetle preference. Specifically, we tested the generality
865 of resistance by relating resistance to caterpillars to field preference of a major
866 agricultural leaf-feeding specialist beetle pest (*A. vittatum*). In summary, we used the
867 independent domestication events of *C. pepo* as a means to identify mechanisms of
868 resistance to multiple herbivores that may be useful in plant breeding.
869

870 ## 3.3 Methods and Materials

871 3.3.1 Plant Material. Five *Cucurbita pepo* cultivars (**Table 3.1**) each were used from
872 two cultivated subspecies, *C. p. pepo* and *C. p. texana* [21], [42]. Plants were started
873 from untreated seed (source, **Table 3.1**) in the Cornell University Agricultural

Experiment Station greenhouses (Ithaca, NY, USA). In the greenhouses, a 14 hour photoperiod was maintained, and the day and night temperatures, were 27C and 21C, respectively. Plants were watered daily, treated with standard greenhouse fertilizing practices (150 ppm 21-5-20 NPK fertilizer, (Peters Company, Allentown PA, USA) five times a week), and non-chemical pest control (bio-control) was used as necessary. Lambert LM-111 potting mix (Rivière-Ouelle, Québec, Canada) was used in all assays except the volatile assay, in which McEnroe Organic Lite Growing mix (Millerton, NY, USA) was used to be in compliance with requirements of a certified organic greenhouse. Seeds were sown in individual 10 cm diameter pots for assays that required whole plants (whole plant feeding assays), and 72-cell flats for assays that used excised plant tissue (mass per unit area consumed of leaf discs, volatile assay).

Table 3.1. Cultivar list

Subspecies	Type	Cultivar Name	Abbreviation	Seed Source
C. p. pepo	pumpkin	Charisma PMR[a,f]	CH	Johnny's Selected Seeds
C. p. pepo	zucchini	Dunja[a,b,d]	DU	Johnny's Selected Seeds
C. p. pepo	pumpkin	Magic Lantern[a,c,e]	ML	Harris Seeds
C. p. pepo	zucchini	Costata[a,c,f]	CO	Cornell University
C. p. pepo	zucchini	Reward F1[a,c]	RE	Osborne Seed Company
C. p. texana	acorn	Honey Bear[a,c]	HB	Johnny's Selected Seeds
C. p. texana	delicata	PMR Bush Delicata[a]	BD	Cornell University
C. p. texana	straightneck	Success PM[a,b,c,e]	SP	Cornell University
C. p. texana	crookneck	Sunglo[a,f]	SU	Osborne Seed Company
C. p. texana	acorn	Sweet Reba[a,b,c,d]	SR	High Mowing Organic Seeds

[a]Included in induced resistance trial
[b]Included in jasmonic acid assays
[c]Included in leaf trenching assay
[d]Included in mass per unit leaf area assays, and volatile assays as most inducible cultivar
[e]Included in mass per unit leaf area assays, and volatile assays as least inducible cultivar
[f]Not included in beetle correlation estimates because defoliation data was not available

3.3.2 Insects. *Trichoplusia ni* is polyphagous on plants in at least 36 botanical families, including multiple Cucurbitaceae and *C. pepo* [43]. *Spodoptera exigua* is likewise a generalist herbivores feeding on more than 20 plant families, also including Cucurbitaceae [44]. Prior to conducting the experiments described here, we conducted a feeding trial (in February 2015) to assess degree of feeding on *C. pepo*, and we observed substantial feeding by both species (data not presented). *Spodoptera exigua* eggs were sourced from Benzon Research (Carlisle, PA, USA), and *Trichoplusia ni* eggs were supplied from colony at Cornell University (Dr. Ping Wang, Cornell AgriTech, Geneva, NY, USA). Due to similarity of results between caterpillar species we found at the subspecies level, and high *S. exigua* mortality in the induced resistance assay, *T. ni* was used in all subsequent assays. For assays where caterpillar mass was measured, two unfed neonate caterpillars were applied to each plant or diet. At the conclusion of each assay, all living caterpillars were placed in Eppendorf tubes and frozen at -20 C for individual weighing later (AT21 Comparator Microbalance, Mettler-Toledo, Columbus, OH, USA). In evaluating leaf trenching, *T. ni* were raised to second instar on high wheat germ diet [45] in a 26 C growth chamber before the assay. The same diet was also used in the volatile assay.

3.3.3 Induced Resistance and Plant Chemistry in the Two Subspecies – Induced Resistance Assay. All cultivars were grown to assess constitutive and induced leaf chemistry traits and plant resistance to *T. ni* and *S. exigua*. This experiment was conducted in two iterations in a randomized complete block design with three blocks per iteration. In each block, there were seven plants of each cultivar and each plant

was subjected to one of seven treatments ("t"): (t1) *T. ni* and (t2) *S. exigua* induction for chemical analyses, (t3) no herbivory control for chemical analyses, (t4) *T. ni* and (t5) *S. eixgua* induction to measure the effect on subsequent conspecific herbivory, and finally controls for induction by (t6) *T. ni* and (t7) *S. exigua*. Plants induction was achieved by five days (days 1-5) of herbivory by neonates immediately prior to chemical analyses or measuring effect on subsequent conspecific herbivory. The effect of induction was measured by caterpillar mass after five days (days 6-10) of feeding.

Seeds were sown in February 2015, approximately two weeks prior to treatment to allow for plants to reach the 1-2 leaf stage, and then plants were enclosed in mesh sleeves (30cm x 18cm). On the first day of the experiment (March 2015), plants were infested with *T. ni* (t1, t4), *S. exigua* (t2, t5), or left as is (t3, t6, t7). On day five, leaf tissue was collected from plants with treatments for chemical analyses (t1-t3; i.e. cv. Dunja with *T. ni* feeding, *S. exigua* feeding, and no herbivore control). The caterpillars were also weighed from those plants, and the plants were discarded. Also on day five, caterpillars were removed from the remaining *T. ni* and *S. exigua* feeding treatments (t4, t5), and weighed. Those plants (t4, t5) were then infested with new neonate conspecifics to test the effect of induction by conspecific prior herbivory. At the same time, the no herbivore control plants were infested with *T. ni* (t6), or *S. exigua* (t7) to compare to the effect of prior herbivory. The caterpillars applied on day five were allowed to feed until day 10 when they were removed and weighed. All caterpillars collected from plants on day five were analyzed to examine constitutive resistance in all cultivars (n = 12 per cultivar). Caterpillars collected from plants on day 10 were used to examine the degree of induced resistance (n = 6 per cultivar-

941 treatment combination).

942

943 3.3.4 Induced Resistance and Plant Chemistry in the Two Subspecies – Cucurbitacin
944 Analysis. Cucurbitacins were extracted in a method similar to Theis *et al* (2014).
945 Briefly, cucurbitacins were extracted from freeze-dried tissue with methanol, and were
946 then purified with solid phase extraction. Cucurbitacins were quantified in a triple-
947 quadrupole LC-MS/MS system (Accela-Quantum Access; Thermo Scientific)
948 equipped with a C18 reversed-phase column (Kinetex 2.6 µm EVO C18, 150 x 2.1
949 mm; Phenomenex).

950

951 3.3.5 Induced Resistance and Plant Chemistry in the Two Subspecies – Nitrogen
952 Analysis. Tissue for nitrogen analysis was sourced from the same freeze dried tissue
953 used for cucurbitacin analysis. Tissue was finely ground (120 s at 27 Hz, MM300,
954 Retsch, Haan, Germany) and submitted to the Cornell University Stable Isotope
955 Laboratory (Ithaca, NY, USA) for continuous flow analysis of percent nitrogen and
956 carbon with an elemental analyzer. Per cultivar, five to six samples of controls and
957 each treatment (*T. ni* and *S. exigua* prior herbivory) were measured, with the following
958 exceptions: n=4 for cv. Success PM – *S. exigua*; n=3 for cv. Sweet Reba – *S. exigua*,
959 *T. ni*.

960

961 3.3.6 Induced Resistance and Plant Chemistry in the Two Subspecies – Jasmonic Acid
962 Treatment. Three cultivars were used to test if jasmonic acid (JA) treatment had
963 similar induction effects of prior herbivory. We included two cultivars we had found

964 to be highly inducible, *C. p. pepo* (cv. Dunja) and *C. p. texana* cultivar (cv. Sweet
965 Reba), by *T. ni* in previous assays, and a less inducible *C. p. texana* cultivar (cv.
966 Success PM). Seed were sown in September 2015, and appropriate plants were
967 sprayed with jasmonic acid (0.5 mM JA, dissolved in ethanol) ten days after sowing.
968 In one test, JA treated plants were compared to plants sprayed with solvent (ethanol)
969 alone with two to six replicates per treatment-cultivar. A separate set of JA treated
970 plants were compared to plants that received two neonate *T. ni*, or nothing (control)
971 with 2 iterations of 6 blocks (where three blocks were complete, and three were nearly
972 so, typically missing a single treatment-cultivar combination) per cultivar and
973 treatment. For both tests, five days after the treatments commenced (for JA treatments,
974 there was one spray at the beginning of the five day period), initial *T. ni* were removed
975 from the appropriate treatments, and then all treatments and control were infested with
976 two neonate *T. ni*, which were allowed to feed for five days before weighing.
977
978 3.3.7 Feeding and Growth Bioassays – Qualitative Leaf Trenching. A subset of six
979 cultivars (three per subspecies, see Table 3.1) were sown on January 2017, and were
980 grown to the three leaf stage before a second instar *T. ni* were placed on the plants.
981 The plants were observed daily for eight days for evidence of trenching, and
982 qualitative notes and photographs were taken. Leaf trenching was observed in
983 *Epilachna borealis* in response to mucilaginous plant sap in *C. maxima*, *C. texana*,
984 and *C. okeechobeensis* [38], and also from *T. ni* in *C. sativus* and *C. moschata* [39].
985 Observation of *T. ni* trenching behavior between *C. pepo* subspecies was chosen to test
986 if *T. ni* exhibited a differential response that may be indicative of differences in plant

sap defenses.

3.3.8 Feeding and Growth Bioassays – Mass Gained per Unit Leaf Area. To address how larval growth was associated with leaf consumption, which can provide an indication of deterrence versus toxicity, we conducted a bioassay of *T. ni* on four cultivars. Using the most and least *T.ni* - inducible cultivars we identified in the full cultivar panel from each subspecies (**Table 3.1**), we presented *T. ni* with a single 10 cm^2 leaf disc on moistened filter paper in a plastic petri dish. Seeds were sown in March 2016, discs were removed from the newest fully expanded true leaf with a cork borer after two weeks of growth, and *T. ni* fed on the discs for five days. Cultivars cv. Dunja and cv. Success PM had 20 replicates, cv. Magic Lantern had 19 replicates, and cv. Sweet Reba had nine replicates. Leaf discs were imaged, and area damaged was measured in imageJ [46].

Later, the cultivars were grown to measure $10cm^2$ leaf disc fresh and dry weight. Seeds were sown in November 2017, and nine samples per cultivar from separate plants were removed and weighed two weeks after sowing (HR-120, A&D Company, Tokyo, Japan). The discs were lyophilized (FreeZone 2.5, Labconco, Kansas City, MO, USA) until dry and weighed immediately.

3.3.9 Feeding and Growth Bioassays – Volatile Deterrence. We tested if foliar volatiles influenced *T. ni* feeding on artificial diet using leaf tissue from cultivars we previously found to be the most and least inducible by *T. ni* from each subspecies (Table 1). This experiment was conducted in two iterations of a randomized block

design. Iteration 1 (August 2017) had three complete blocks, and iteration 2 (January 2018) had seven blocks (where three blocks were complete, and four were nearly so, typically missing a single treatment-cultivar combination). Each cultivar had an induction treatment (*T. ni* feeding on leaf tissue), or control treatment (leaf alone) per block. The seeds were sown approximately 14 days before the assay commenced. Photographs and a detailed description of the experimental arena are shown in **Fig. 3.1**, and described briefly here. In each arena, neonate *T. ni* were placed on an excess of diet in a small plastic cup covered by cheesecloth. Excised leaves were placed in water in 9.5mL floral tubes (Floral Supply, Fruit Heights, UT, USA) filled with water, and refilled as necessary. An organza mesh bag (SumDirect manufacturing, Dongguan, China) was secured around the leaf, and two neonate *T. ni* were added to leaves of the induction treatments. The diet cup and excised leaf were placed together in a 1L plastic container (Clear Lake Enterprises, Port Richey, FL, USA), and closed with a lid with small holes. The diet cup was on the bottom of the container, and the leaf was suspended 10cm above the diet cup, and was kept in place by the floral tube. As a check of the effect of the non-plant materials, a control treatment with no leaf but all accessories was also used. After three days, leaves with the *T. ni* induction treatment were scouted to confirm *T. ni* presence and feeding, and *T. ni* feeding on diet were recovered and weighed.

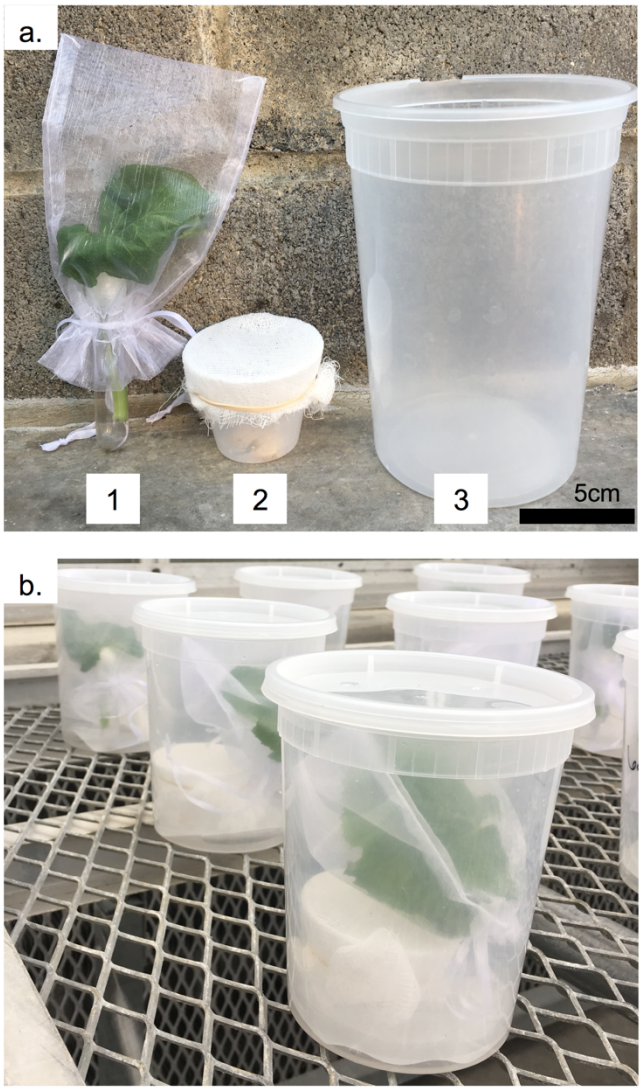

Fig. 3.1. Photographs of a (a) deconstructed and (b) set of fully assembled volatile assay arenas. The set up includes (a1) an excised leaf, (a2) artificial diet, and (a3) a 1L plastic container. The excised leaf (a1) is placed in a 9.5 mL plastic tube with water, and secured by gently wrapping the petiole in a cotton ball and then sealed with parafilm. The leaf is then enclosed in a drawstring mesh bag, and two nenonate *T. ni* are added to the leaf surface in the appropriate treatments. The drawstring is tightened around the plastic tube as to not damage the leaf petiole. An excess of artificial high wheat germ diet is placed in small cup, and the top is covered with cheesecloth and secured in place with a rubber band (a2). The enclosure (a3) is a 1L plastic container that will be supplied with saturated filter paper at the base (to keep humidity high in the arena) and a lid with a few small holes.

3.3.10 Resistance Comparison to *A. vittatum*. To address the generality of resistance mechanisms, the mass of *T. ni* caterpillars was compared to previously obtained

preference data of a specialist herbivore of cucurbit crops, *Acalymma vittatum* [26]. The experiment is detailed in [26], and the objective was to assess *A. vittatum* preference for cultivars in the two subspecies of *C. pepo* used in this experiment. Briefly, a field choice test with *n*=27 cultivars (*n*=17 *C. p. texana*, and *n*=10 *C. p. pepo*) ([26], Table 2) was conducted in 2015 under naturally occurring *A. vittatum* infestation in Freeville, NY. Cultivars were grown with five replicates in three-plant plots in a randomized complete block design, and *A. vittatum* preference was measured as estimated percent leaf defoliation of plants with one to three leaves (not flowering). Seven of the cultivars used in the field experiment were also used in experiments with *T. ni* (see **Table 3.1**). Importantly, in both experiments, plants were at the same growth stage (1-3 leaves, non-flowering). To test for *C. pepo* cross-resistance to these herbivores, we determined the correlation between *A. vittatum* preference and *T. ni* performance on these cultivars.

3.3.11 Statistical Analysis. Linear mixed models were used to model the response variables of caterpillar performance or chemical concentration as a function of plant cultivar and experimental parameters. For each caterpillar sample, the mass of the two caterpillars was averaged, and the average was used in further analysis. If only one caterpillar was recovered, that mass was used.

For the induced resistance and chemical assays, caterpillar mass or chemical concentration, respectively, were modeled with subspecies, treatment, subspecies by treatment interaction, and iteration as fixed effects, and cultivar nested within subspecies and block nested within iteration were included as random effects. For

assays with a subset of cultivars (jasmonic acid, mass per unit leaf area consumed, volatiles), caterpillar mass was modeled with cultivar, treatment, and cultivar by treatment interaction as fixed effects. In the jasmonic acid and volatile assay, iteration was treated as a fixed effect, and block nested within iteration was included as a random effect for the volatile assay.

All statistical analyses were performed in R [47]. Linear mixed models were calculated with the 'lmer' function in the 'lme4' R package [48], and linear models with the 'lm' function. In all cases, analysis of variance was used to test significance of fixed effects, except for in the mass per unit leaf area consumed assay where analysis of covariance was used. Tukey's honest significant difference test was used to separate effect levels with the 'TukeyHSD' function in 'agricolae' R package [49]. Finally, correlation between *A. vittatum* preference and *T. ni* mass was calculated using the 'cor.test' R function.

3.4 Results

3.4.1 Induced Resistance and Plant Chemistry in the Two Subspecies.

Both generalist caterpillars, *T. ni* and *S. exigua*, showed >40% lower mass after five days of feeding on *C. p. texana* cultivars compared to *C. p. pepo* cultivars (**Fig. 3.2a,c**; *T. ni:* $F_{1,93}$=20, P<0.001; *S. exigua*, $F_{1,89}$=10.08, P=0.002). Induced resistance following herbivory reduced growth of both species, although the effects were most pronounced in *C. p. pepo* with *T. ni*. Induced resistance reduced *T. ni* mass by 21% in *C. p. pepo*, and 9% in *C. p. texana* (**Table 3.2**; **Fig. 3.2b**). For *S. exigua*, induced resistance reduced mass by 7% in *C. p. pepo*, but had no effect in *C. p. texana* (**Table 3.2**; **Fig. 3.2d**).

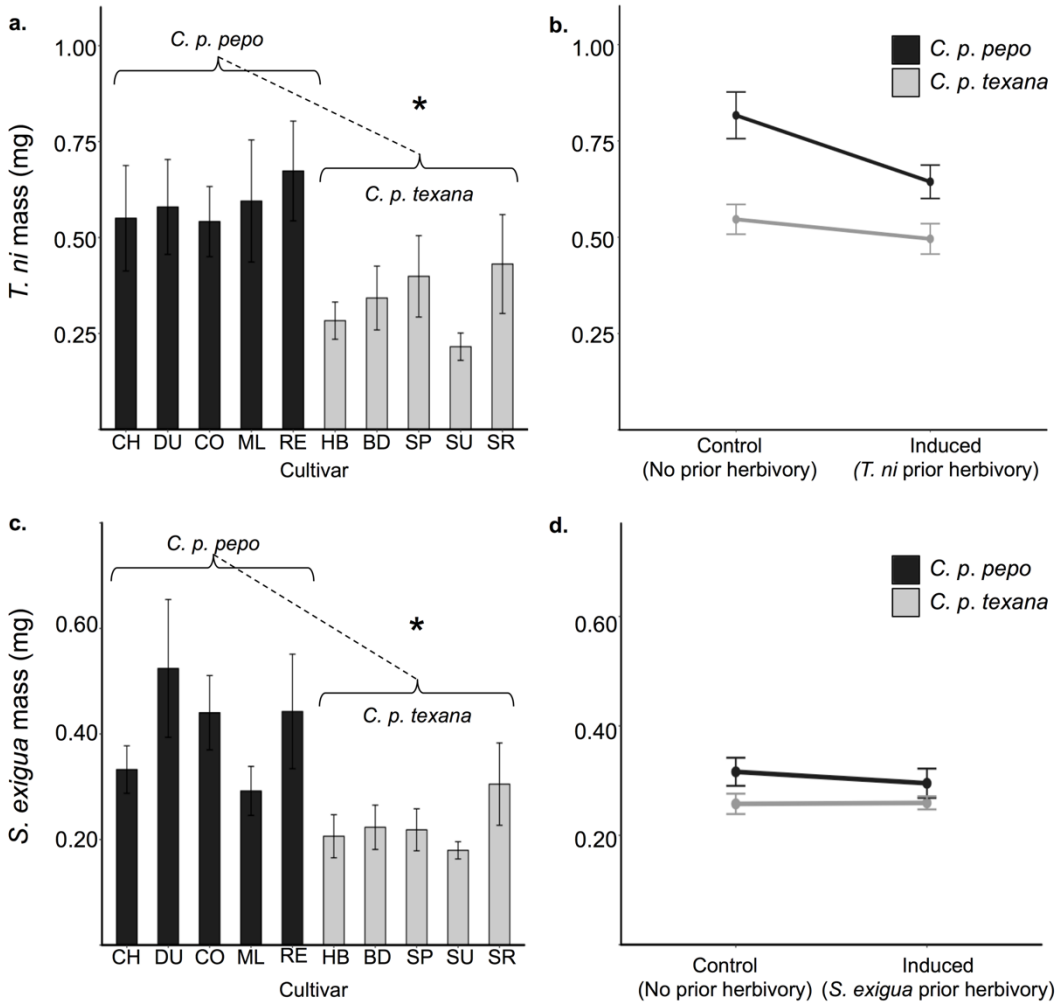

Fig. 3.2 Mass of generalist caterpillars after feeding on *C. pepo* subspecies with and without induction by prior conspecific herbivory**.** Differences in constitutive resistance by subspecies and cultivar are shown for (**a**) *T. ni* and (**c**) *S. exigua*. The effect of induction is summarized across varieties by subspecies (**b**, **d**). Shown are means ± 1 SE and asterisks indicate $P<0.05$ for the effect of subspecies in ANOVAs described in the text. Cultivar abbreviations are listed in Table 3.1

Table 3.2. ANOVA table from linear mixed effects model of herbivore mass in cultivar panel induced resistance assay

Insect	Effect[a,b]	DF	*F*-value	*P*-value
T. ni	Subspecies	1	8.241	**0.004**
	Treatment	1	6.133	**0.005**
	Subspecies X Treatment	1	2.344	0.130
	Iteration	1	4.834	**0.015**
	Residuals	97		
S. exigua	Subspecies	1	3.482	0.066
	Treatment	1	0.239	0.626
	Subspecies X Treatment	1	0.200	0.657
	Iteration	1	0.135	0.714
	Residuals	70		

[a]Treatment refers to induction by conspecific feeding.
[b]There were two nested random effects: randomized complete block nested within iterations of the experiment ("iteration") (*T. ni* model σ^2=0; *S. exigua* model σ^2=0.0002) and cultivar nested within subspecies (*T. ni* model σ^2=0.0104; *S. exigua* model σ^2=0.0009)

Based on these results, we selected the most and least inducible cultivars from each subspecies for further assays (**Table 3.1; Fig. 3.3**). Mean caterpillar mass reduction across both subspecies after induction by prior herbivory was greatest in cv. Dunja (*C. p. pepo*; 28% decrease; $F_{1,17}$=5.08, *P*=0.038), and cv. Sweet Reba (*C. p. texana*; 26% decrease; $F_{1,10}$=5.49, *P*=0.041). In contrast, cv. Magic Lantern (*C. p. pepo*) and cv. Success PM (*C. p. texana*) showed no trends of induction, and were chosen as the least inducible **(Fig. 3.3**).

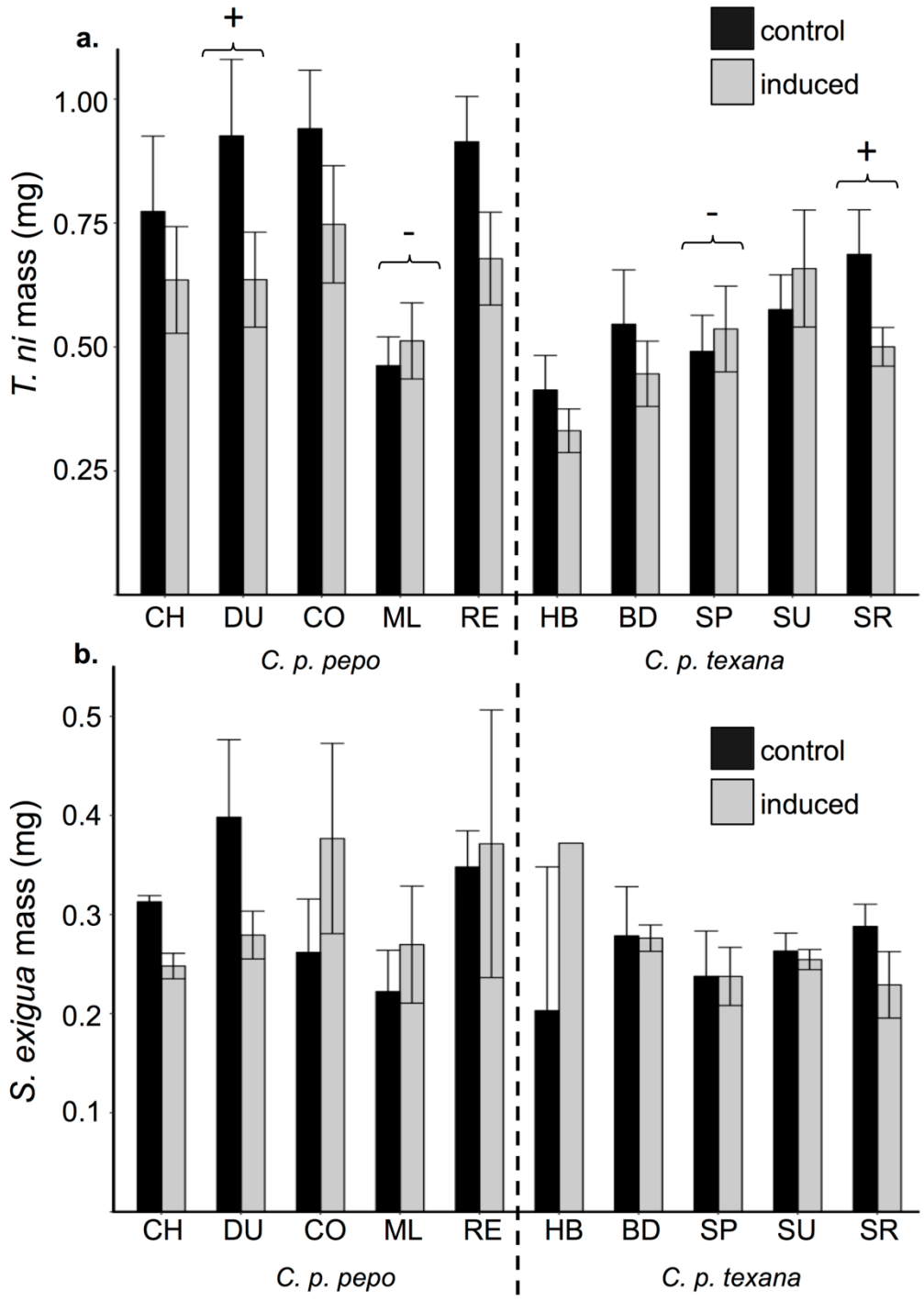

Fig. 3.3. Mass of (**a**) *T. ni* and (**b**) *S. exigua* caterpillars after feeding for five days on cultivars with prior conspecific herbivory ("induced") as compared to controls. Shown are means ± SE. Cultivar abbreviations are listed in Table 1. The "+" and "-" indicate the cultivar was selected as the most (*C. p. pepo* cv. Dunja, 'DU'; *C. p. texana* cv. Sweet Reba, 'SR') or least (*C. p. pepo* cv. Magic Lantern, 'ML'; *C. p. texana* cv. Success PM, 'SP'), inducible of each subspecies.

Cucurbitacins B, D, and E were detected in leaf tissue but only in trace concentrations; in the majority of samples, none of the cucurbitacins reached detectable levels (greater than 0.05 ng g^{-1} dry weight for cucurbitacins B and E, and above 1.0 ng g^{-1} dry weight for cucurbitacin D), and there was no pattern of cucurbitacins by subspecies or induction treatment (**Table 3.3**).

Table 3.3. Leaf cucurbitacin measurements

Cultivar	Subspecies	Cucurbitacin (ng g^{-1} dry tissue)[a,b] Control / *T. ni* Induced[c,d]		
		D	B	E
Charisma PMR	*C. p. pepo*	- / -	- / -	- / -
Dunja	*C. p. pepo*	- / -	- / -	- / -
Magic Lantern	*C. p. pepo*	- / -	- / -	- / -
PMR Costata	*C. p. pepo*	- / -	- / 0.06	0.47 / 1.13
Reward F1	*C. p. pepo*	- / -	- / -	- / -
Honey Bear	*C. p. texana*	- / -	- / -	- / -
PMR Bush Delicata	*C. p. texana*	- / -	0.07 / -	- / -
Success PM	*C. p. texana*	- / -	- / 0.10	- / 0.06
Sweet REBA	*C. p. texana*	5.30 / -	- / -	- / -

[a]"-" indicates that the particular cucurbitacin was not detected in the sample
[b]Cucurbitacin I was not detected in any samples, and not presented in this table
[c]"*T. ni* Induced" refers to five days of *T. ni* damage prior to sampling
[d]Induced cucurbitacins were additionally measured after *S. exigua* in cultivars Charisma PMR, Dunja, and Success PM, but none were detected.

In control plants, mean leaf nitrogen was nearly 9% higher in *C. p. texana* as compared to *C. p. pepo* cultivars ($F_{1,51}$=11.60, P=0.001). However, following induction by *T. ni* herbivory, nitrogen remained constant in *C. p. texana*, but dropped 5% in *C. p. pepo* as compared to controls (**Fig. 3.4a**; **Table 3.4**). Leaf nitrogen also slightly decreased in *C. p. pepo* with induction by *S. exigua*, and had a smaller change in *C. p. texana* as compared to controls (**Fig. 3.4b**; **Table 3.4**).

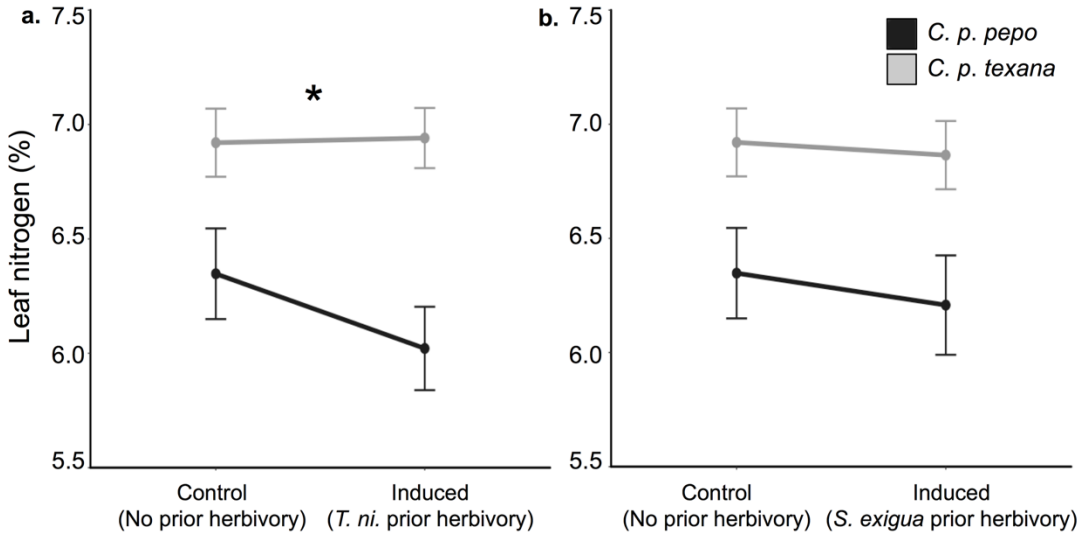

Fig. 3.4 Leaf nitrogen content in two *C. pepo* subspecies and induction by prior herbivory by (**a**) *T. ni* and (**b**) *S. exigua*. Shown are means ± 1 SE and the asterisk indicates $P<0.05$ for subspecies by induction treatment term in ANOVAs described in Table S2

Table 3.4. ANOVA table from linear mixed effects model of percent leaf nitrogen in induced resistance and plant chemistry assay

Insect[a]	Effect[b,c]	DF	*F*-value	*P*-value
T. ni	Subspecies	1	20.758	<0.001
	Treatment	1	2.472	0.119
	Subspecies X Treatment	1	6.594	0.012
	Iteration	1	3.316	0.071
	Residuals	104		
S. exigua	Subspecies	1	8.579	0.004
	Treatment	1	0.303	0.583
	Subspecies X Treatment	1	0.099	0.754
	Iteration	1	7.324	0.008
	Residuals	103		

[a]Effects of each herbivore (*T. ni*, *S. exigua*) were compared to the same set of control samples

[b]Treatment refers to induction by conspecific feeding

[c]There were two nested random effects: randomized complete block nested within iterations of the experiment ("iteration") (*T. ni* model $\sigma^2=0.373$; *S. exigua* model $\sigma^2=0.255$) and cultivar nested within subspecies (*T. ni* model $\sigma^2=0.039$; *S. exigua* model $\sigma^2=0.059$)

We tested whether the effects of induced resistance by prior herbivory could be reproduced by application of jasmonic acid (JA) using a subset of cultivars (one inducible *C. p. pepo*, one inducible *C. p. texana*, and one non-inducible *C. p. texana*; **Table 3.1**; **Fig. 3.3**). Effects of induction by JA were similar to induction via prior conspecific herbivory, and distinct from controls (**Fig. 3.5**; **Table 3.5**).

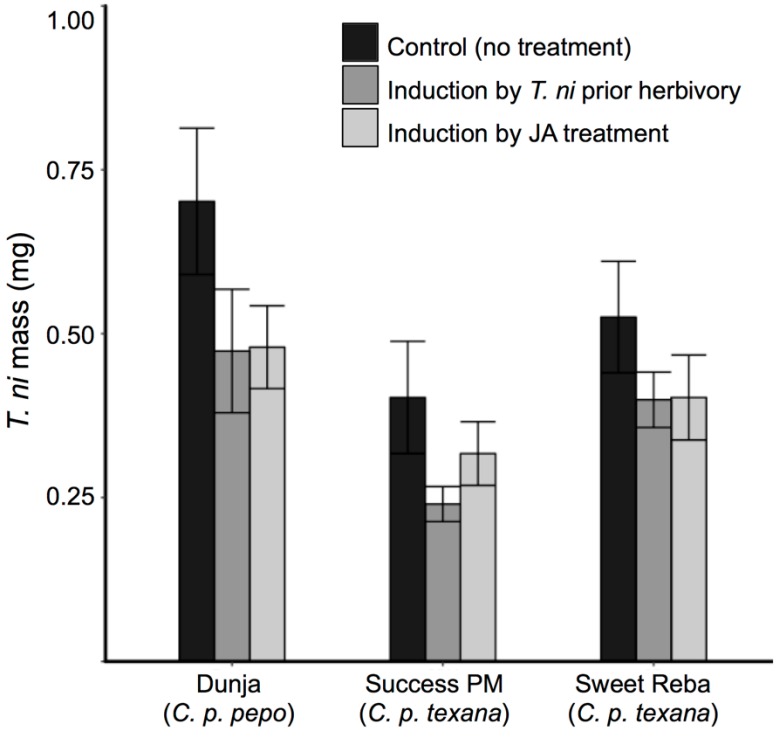

Fig. 3.5. Mass of *T. ni* feeding on cultivars after induction by *T. ni* prior herbivory, and jasmonic acid treatment (JA) as compared to control (no treatment). Shown are means ± SE, and the ANOVA is described in Table S4.

Table 3.5. ANOVA table to accompany jasmonic acid induction test

Effect[a]	DF	*F*-value	*P*-value
Cultivar	2	6.005	**0.004**
Treatment	2	3.530	**0.035**
Cultivar X Treatment	4	0.264	0.900
Iteration	1	2.323	0.132
Residuals	64		

[a]Treatment has three levels (control, induction by *T. ni* herbivory, and induction by JA treatment)

3.4.2 Feeding and Growth Bioassays. *T. ni* exhibited trenching (to avoid sticky phloem sap) on all *C. pepo* cultivars (**Fig. 3.6**), but appeared to avoid feeding on *C. p. texana* cultivars for a longer period of time before trenching (LB, personal observation).

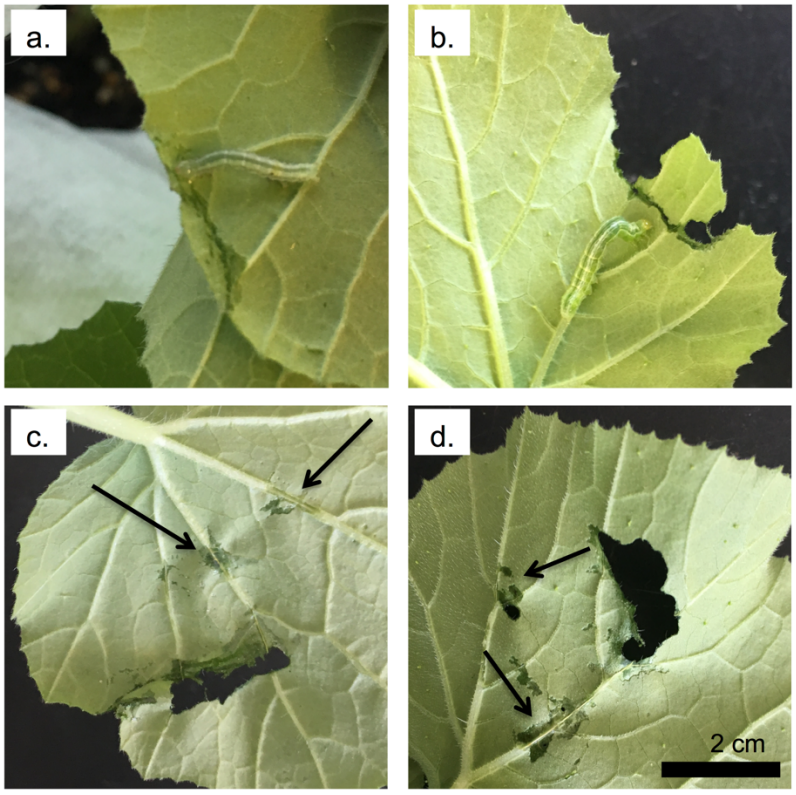

Fig. 3.6 Examples of semi-circular trenching by second instar *T. ni* on leaf edge when feeding on (**a**) Success PM (*C. p. texana*), (**b**) Reward (*C. p. pepo*), and examples of major vein cutting on (**c**) Honey Bear (*C. p. texana*), (**d**) Magic Lantern (*C. p. pepo*). All cultivars included in this assay are indicated in **Table 3.1**

To address the nutritional quality or potential toxicity of *C. pepo,* we conducted an assay to measure larval mass gained by *T. ni* per unit leaf area consumed using four cultivars (two of each subspecies, representing extremes of induction response **Table 3.1**; **Fig. 3.3**). *T. ni* mass on *C. p. pepo* cultivar leaf discs was 32% higher than those feeding on *C. p. texana* cultivars ($F_{3,64}$=6.77, P<0.001; Table S5). Similarly, *T. ni* caterpillars consumed 58% more leaf area of *C. p. pepo* discs

($F_{3,64}$=7.75, P<0.001). However, there was no difference between cultivars in *T. ni* mass attained per unit leaf area consumed (**Fig. 3.7**; **Table 3.6**). Separately, leaf disc fresh and dry mass was measured, and fresh mass was significantly different between cultivars ($F_{3,31}$=14.50, P<0.001); nonetheless dry mass per area was consistent ($F_{3,31}$=1.55, P=0.211). The results did not change when we converted area consumed to fresh mass consumed: *T. ni* caterpillars consumed 65% more fresh leaf mass of *C. p. pepo* discs ($F_{3,64}$=13.37, P<0.001), and *T. ni* mass gained per fresh mass consumed did not differ between cultivars ($F_{3,64}$=0.98, P=0.41). In summary, these feeding assays strongly indicate that caterpillar growth was proportional to feeding, and did not appear to be due to leaf toxicity.

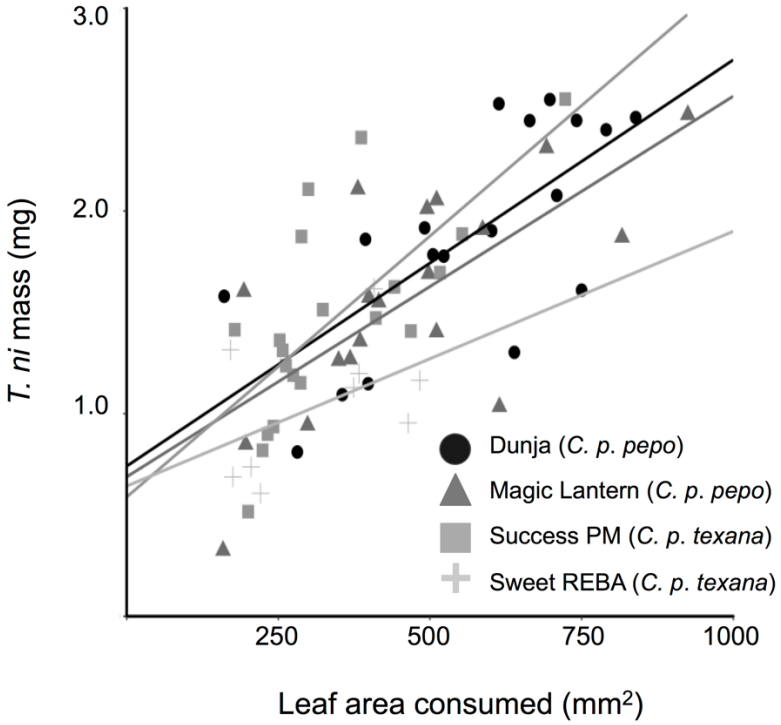

Fig. 3.7. The relationship between leaf area consumed and *T. ni* mass attained. Points represent individual caterpillars, and the lines best linear fit of *T. ni* mass by leaf area consumed.

Table 3.6. ANCOVA table for the leaf area consumed covariate for *T. ni* mass in the mass gained per unit leaf area bioassay.

Covariate	Effect	DF	*F*-value	*P*-value
Leaf area consumed	Cultivar	3	2.289	0.088
	Leaf area	1	37.563	**<0.001**
	Cultivar X Leaf area	3	0.692	0.561
	Residuals	60		

In our volatile bioassay, neonate *T. ni* were fed artificial diet with exposure to volatiles from an excised leaf of one of four cultivars, representing extremes of induction response in each subspecies (**Table 3.1**; **Fig. 3.3**; **Fig. 3.1**). Mass of *T. ni* feeding on diet was compared between those exposed to constitutive plant leaf volatiles and those exposed to plant volatiles induced by active *T. ni* feeding. The mass of *T. ni* feeding on artificial diet was significantly affected by cultivar of the excised leaf and induction treatment ($F_{3,57}$=3.82, P=0.015; **Table 3.7**; **Fig. 3.8**). Volatiles from leaves with active conspecific feeding significantly lowered mass of *T. ni* feeding on artificial diet in the two cultivars previously found to show the strongest induction response (*C. p. pepo* cv. Dunja: $F_{1,16}$=5.59, P=0.031; *C. p. texana* cv. Sweet Reba: $F_{1,13}$=7.44, P=0.017; **Fig. 3.8**), but not in the two cultivars with weak induction responses (*C. p. pepo* cv. Magic Lantern: $F_{1,17}$=0, P=0.99; *C. p. texana* cv. Success PM: $F_{1,8}$=0.80, P=0.40; **Fig. 3.8**).

Table 3.7. ANOVA table from linear mixed effects model of *T. ni* mass after feeding on artificial diet while exposed to plant volatiles

Effect[a,b]	DF	*F*-value	*P*-value
Cultivar	3	5.276	**0.003**
Treatment	1	6.939	**0.011**
Cultivar X Treatment	3	3.818	**0.015**
Iteration	1	2.136	0.149
Residuals	57		

[a]Treatment refers to conspecific leaf feeding

[b]Effect of block was treated as a nested random effect within experiment (σ^2=0.008)

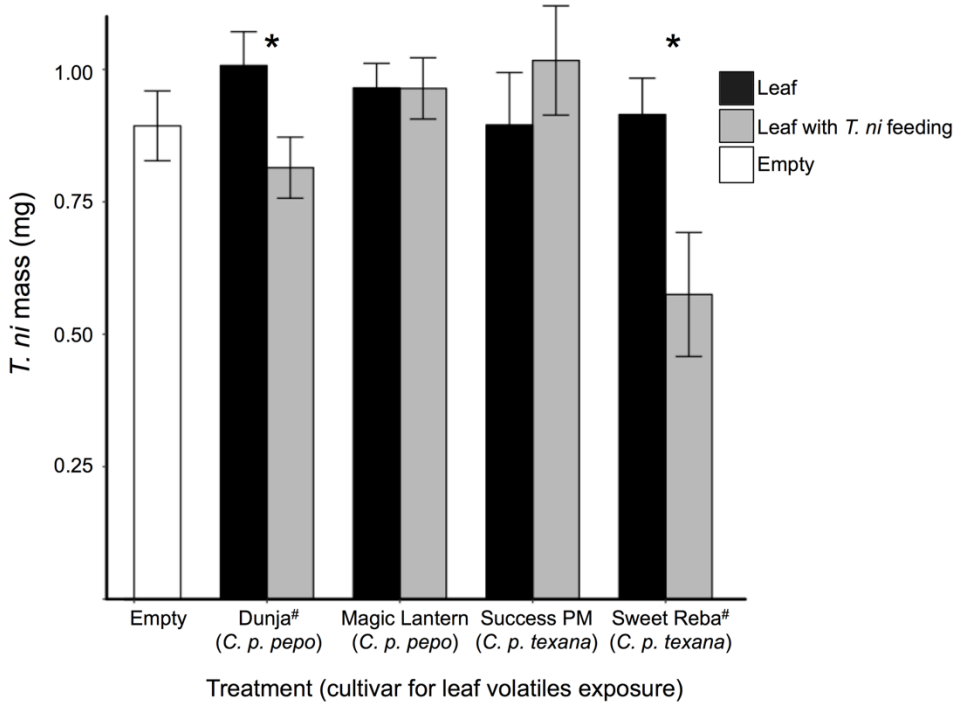

Fig. 3.8 Mass of *T. ni* feeding on artificial diet for three days when exposed to volatiles from leaves alone, leaves with *T. ni* feeding, or controls ("empty"). Shown are means ± 1 SE and asterisks indicates $P<0.05$ for effect of treatment (leaf alone, or with *T. ni* feeding) in varietal-specific ANOVAs described in the text. # indicates the two cultivars that demonstrated the strongest induction response in previous assays

3.4.3 Resistance Comparison to *A. vittatum.* Correlation between adult *A. vittatum* defoliation previously measured in the field [26] and *T. ni* mass gain on the same cultivars measured here (**Table 3.1**) was strongly positive (Pearson's $r=0.893$, $df = 5$, $P=0.007$; Spearman's rank correlation, $rho=0.964$, $P=0.003$) (**Fig. 3.9**).

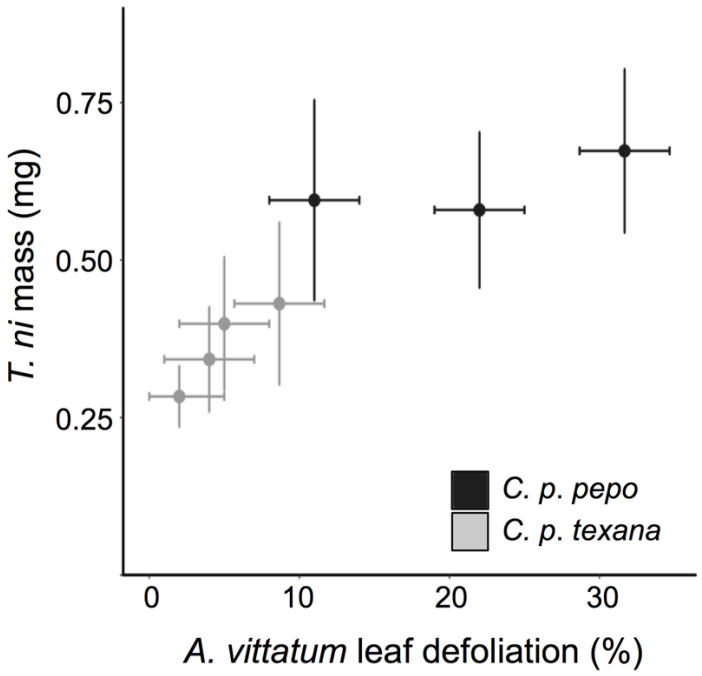

Fig. 3.9 Correlation of mass of *T. ni* after feeding for five days on control (non-induced) plants compared to percent defoliation by *A. vittatum* in field plots (Brzozowski et al. 2016) of the same cultivars (**Table 1**). Each point represents the mean of an individual cultivar ± 1 SE

3.5 Discussion

Evaluating defensive traits in multiple cultivars from two parallel domestication events revealed contrasting levels of resistance and a novel mechanism of plant resistance to insects in *C. pepo*. While *C. p. pepo* had lower constitutive resistance, it was more strongly inducible when assayed by measuring growth of two generalist, leaf-chewing herbivores (*T. ni* and *S. exigua*). Lower resistance to these generalists in *C. pepo* mirrored greater preference of an important specialist beetle pest, *A. vittatum*. Specific analyses of multiple metabolites, including leaf cucurbitacins, did not explain these differences, and growth assay demonstrated that caterpillars gained equal mass per unit leaf consumed in cultivars from both subspecies (consistent with the lack of a toxic principle between cultivars). These

1257 results echo Theis *et al* (2014), where they likewise found that neither leaf nitrogen
1258 nor cucurbitacins (nor other measured leaf traits) predicted leaf damage by *A. vittatum*
1259 on 20 diverse Cucurbitaceae species.

1260 An important result of our work is that volatiles induced by active *T. ni*
1261 damage in some varieties had a deterrent effect on *T. ni* growth when feeding on a
1262 standard diet. As discussed below, the impact of plant volatiles on larval feeding has
1263 been little studied, and is an area in need of further investigation. The volatile
1264 deterrent effect we found was not specific to either *C. pepo* subspecies, but instead
1265 was detected in cultivars that we found to have the strongest inducible resistance.
1266 While floral volatile composition in *Cucurbita* spp. and the implications for specific
1267 herbivores and pollinators are well-characterized [35], [50]–[52], knowledge of leaf
1268 volatile components is limited. Volatiles from leaf trichomes of one *C. p. texana*
1269 cultivar not used in this study were shown to have attractant and repellent compounds
1270 to the pickleworm moth (*Diaphania nitidalis*) [37]. Volatiles from induced by
1271 bacterial wilt infection (*Erwinia tracheiphila*) in wild *C. pepo* are also known to
1272 attract more *A. vittatum* than healthy plants [53]. Finally, in other Cucurbitaceae,
1273 induced volatiles have been implicated in the attraction of natural enemies of
1274 herbivores [54]–[56].

1275 Plant domestication and breeding trajectory has a range of effects on
1276 herbivore-induced plant volatiles. Overall, recent meta-analyses across systems
1277 showed that domesticated plants have greater induced volatile production than wild
1278 plants [16], although parasitoid and predator attraction are not consistently greater in
1279 cultivated plants [57]. In maize, there was considerable genetic variability in induced

1280 volatile production in a diverse group of cultivars and wild accessions, and this was
1281 not predicted by domestication status or subsequent breeding [58]. Nonetheless, by
1282 examining finer-scaled contrasts in maize germplasm, it was revealed that plant
1283 breeding history shaped herbivore-induced root volatiles critical for indirect defense:
1284 isolated North American and European breeding programs diverge in production of
1285 (E)-ß-caryophyllene [7], and modern hybrids lack oviposition induced volatiles
1286 present in landraces [59]. In a more recently domesticated crop with a known
1287 pedigree, cranberry (*Vaccinium macrocarpon*), induced volatiles measured following
1288 treatment with exogenous jasmonic acid were consistent across pedigree and state of
1289 cultivar improvement [5]. These results indicate that while varietal-specific induced
1290 volatiles are common, we may be best able to elucidate changes to herbivore-induced
1291 plant volatile production by evaluating clearly defined contrasts of plant breeding
1292 lineages.

1293 Mechanistically, volatile repellents can affect herbivores through signaling
1294 status as a non-host with criterion as subtle as one component of a volatile blend, or
1295 warning herbivores of plant defenses, potential competition, or natural enemies [12].
1296 The impact of plant volatiles on larval feeding behavior has thus far not received
1297 substantial attention, although it may have potential as a resistance mechanism. In
1298 maize, indole is an herbivore induced plant volatile and was recently shown to reduce
1299 feeding and increase mortality of *S. littoralis* [14]. Deterrent volatiles produced during
1300 the day also drive the nocturnal feeding behavior of *Mythimna separate* on maize [13].
1301 Overall, deterrent plant volatiles can impact caterpillar feeding behavior, but more

work is needed to understand the mechanistic basis and ecological relevance of such signals.

While we found cultivar-specific induced volatile deterrence in both subspecies, there are remaining questions about what mechanisms support the large and repeatable differences in overall resistance between *C. pepo* subspecies. It is possible that induced volatiles are more ubiquitous in one subspecies, and that could be addressed by testing induced volatile deterrence in a broader *C. pepo* panel. We observed no evidence of leaf toxicity, but instead less consumption of *C. p. texana* cultivars in no-choice assays, which indicates that there may be differences in constitutive deterrent volatiles between subspecies that our volatile assay did not detect. Moving beyond secondary metabolites, there are morphological differences in plant traits between subspecies, including leaf color and shape, but their relation to herbivore resistance is unknown [60]. Perhaps examination of these traits, or other more multi-functional traits, like leaf water content [35], or leaf turgor pressure [38], would provide insight into the differences in plant resistance in *C. pepo*.

Additional work on mechanisms of preference in *C. pepo* should also examine causes of divergence in constitutive and induced resistance between the independent domestication events. Induction is overall stronger in susceptible *C. p. pepo*, but there is greater constitutive resistance in the resistant *C. p. texana*. Induced resistance may be favored when herbivore pressure is intermittent and cost of defense is high [61], although tradeoffs between constitutive and induced resistance strategies are apparently less common in domesticated than wild plants [62]. An exploration of costs of endemic herbivore damage in the region of domestication and subsequent breeding

may provide insight into whether natural or human-mediated selection may have favored one type of resistance over another in each subspecies.

Because *C. p. pepo* appears to be most palatable for both generalists assayed and specialist beetles, whatever mechanism increases *C. p. pepo* susceptibility to the two generalist herbivores assayed here could be the same as that which increases preference of specialist beetles (**Fig. 3.9**) (Chrysomelidae: Galerucinae; Ferguson et al. 1983; Hoffmann et al. 1996; Brzozowski et al. 2016). Literature on the preference of these beetles has been overwhelmingly associated with cucurbitacin content, where these specialists compulsively feed on and sequester cucurbitacins [28], and cucurbitacins increase larval performance [63], [64]. However, loss of fruit bitterness is a hallmark of all six domestications of the cultivated *Cucurbita* spp. (i.e. *C. pepo*, *C. moschata*, *C. maxima*, etc) [18]–[20], and we demonstrate here that leaf cucurbitacin content is minimal in domesticated *C. pepo*. These results are inconsistent with cucurbitacins being the principal determinant of generalist or specialist herbivore behavior, yet these diverse herbivores still find common ground in increased susceptibility of *C. p. pepo*.

Our results have interesting implications for how specialist and generalist herbivores respond to plant resistance traits in an agricultural context, and whether there is a dichotomy between them. For instance, while many specialists have evolved to overcome specific chemical defenses that are effective against generalist herbivores through sequestration or avoidance [65], such “resistance” traits may often be lost in the domestication process [1], [2]. In the current study, we found a similar outcome of the breeding process for well-adapted specialists and non-adapted generalists of *C.*

pepo, indicating substantial cross resistance.

Overall, this work highlights the benefits of studying the chemical ecology of diverse pools of cultivated germplasm with distinct breeding histories for discovering new mechanisms of resistance to insect herbivores. Dogma in plant breeding is that we must look to the genetic diversity of wild species for traits to introgress into elite germplasm to steel it against our most pressing biotic and abiotic challenges [66]–[70]. With this approach, more than 2000 biotic stress resistance traits have been identified in crop wild relatives; however, the vast majority of traits identified are for disease resistance, and less than one quarter of these target insect pests [70]. Thus, strategies for breeding for resistance to insect pests must also be inclusive of secondary centers of diversity, and contemporary breeding pools. The context in which these breeding pools were developed may also better reflect the context of agricultural plant-herbivore interactions than wild systems, where major secondary metabolites (like cucurbitacins) may have different effects. In the diverse pools of cultivated germplasm with distinct breeding histories, plant breeders may discover alternative, perhaps quantitative, and likely smaller-impact resistance traits. Screening material for the most promising, but less obvious traits will benefit by being informed by chemical ecology, and incorporating these traits into agroecosystems will require tools from associated disciplines [71].

In conclusion, by using two independent domestication events to evaluate divergence in plant resistance traits in *C. pepo*, we identified differences in resistance that spanned diverse leaf-chewing herbivores. We found that these differences did not align to major known resistance traits, like cucurbitacins, or nitrogen, but instead

found varietal-specific induced volatile deterrents in both subspecies. This work contributes to the growing evidence that plant defenses in the context of cultivated systems may be distinct from those in the wild [11], and has implications for plant breeders. By exploring defensive novelty through contrasting domestications and breeding histories in *C. pepo*, we found susceptibility in one lineage that persisted through the behavior of diverse herbivores, and discovered a previously unknown defense trait in *C. pepo* that may provide a new target for plant breeders.

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Chapter 4: Attack and aggregation of a major squash pest: parsing the role of plant chemistry and beetle pheromones

4.1 Abstract

1. Successful management of insect crop pests requires an understanding of the cues and spatial scales at which they function to affect rates of attack of preferred and non-preferred host plants. A long-standing conceptual framework in insect-plant ecology posits that there is hierarchical structure spanning host location, acceptance, and attack that could be exploited for integrated pest management.

2. We investigated how plant- and insect-derived chemical cues affect successive decisions of host choice in aggregating insects, and tested predictions in the *Cucurbita pepo* - *Acalymma vittatum* system. *Acalymma vittatum* is an aggregating specialist beetle pest that strongly prefers zucchini (*C. p. pepo*) to summer squash (*C. p. ovifera*), two independent domesticates of *C. pepo*. We hypothesized that subspecies-specific plant traits, especially volatile cues, interact with the male-produced aggregation pheromone to amplify beetle preference for *C. p. pepo*.

3. Differential beetle attack of *C. pepo* subspecies in the field is not determined by plant traits that affect host finding or differential aggregation due to pheromones: across two years, beetles had strong density-dependent attraction to both subspecies when male beetles were feeding, and no interactions between plant volatiles and the male-produced pheromone were detected. In absence of male pheromone emission, beetles were equally unattracted to plants with or without beetle feeding.

4. In contrast, plant traits that mediate insect acceptance appear to underlie differences in preference. At a local scale, beetles did not accept and emigrated from

C. p. ovifera compared to *C. p. pepo*. Distinct volatile emissions were observed between subspecies, but further work is needed to identify if these volatiles promote emigration.

5. *Synthesis and applications*: By dissecting pest preference during successive host choice decisions, we isolated a trait with implications for pest management. Beetles on cucurbits can be managed by employing cultivars with differential susceptibility (e.g. trap cropping), and the mechanistic knowledge presented here informs best practices and limitations for on-farm applications. More broadly, pest management in diversified cropping systems can be enhanced through understanding how plant preference gradients affect herbivore movement and behavior, and plant breeders can target traits to reduce herbivory in such systems.

4.2 Introduction

Herbivory by insect pests follows from successive decisions (host finding, local examination of plant cues and acceptance, attack) that can be differentially impacted by plant and insect factors [1], [2]. The contributions of plant and insect cues intersect when aggregating insects rely upon plant chemistry to initiate or enhance aggregations [3], [4], making it challenging to isolate a single factor in aggregation and preference. Nonetheless, the successive steps of host choice provide a framework to test if and how plant chemistry contributes to scenarios leading to proliferation of and herbivory by aggregating insects on plants.

For beetle pests, once an individual arrives on a host, plant chemistry can be incorporated into [5], or promote release of [6], [7], aggregation pheromones.

Responding conspecific beetles may then be attracted to induced plant volatiles from beetle feeding [8], [9], pheromone alone or amplification of pheromone attractiveness by plant volatiles [10], [11]. Finally, upon arriving, beetles may use local cues to reject non-preferred or accept preferred hosts [12], [13]. Dissecting the role of plant chemistry throughout the process of host choice would enhance our understanding of mechanisms of preference in aggregating beetles and inform ecological management strategies.

Given the extensive natural variation in plant chemistry, aggregation outcomes may be distinct on preferred and non-preferred plants. Indeed, aggregating beetles have shown differences in pheromone emission [14] and attraction [15] on different host plant species. This may be caused by non-preferred plants lacking chemistry to facilitate pheromone release, having less attractive plant volatiles or other traits that promote emigration. Plant variation in precursors and synergists are known in pine-feeding bark beetle systems [16], and interspecific chemical differences quantitatively alter pheromone output [17]. However, there is limited knowledge of how intraspecific plant trait variation affects aggregation [18]–[20], especially in agricultural systems. This information could allow growers to choose cultivars that modify aggregation behavior as a means of pest management on diversified farms. Thus, resolving the role of intraspecific plant chemical variation in preference and aggregation may lead to improved strategies for management.

Cucurbitaceae and the major specialist beetle pest, *Acalymma vittatum* (Coleoptera: Chrysomelidae), provide an excellent system to dissect the relationship between plant cues and herbivore aggregation in preference. Cucurbitaceae specialist

beetles have been extensively studied for their association with cucurbitacins, non-volatile and bitter triterpenoids of the Cucurbitaceae that cause compulsive feeding behavior [21]. However, release and perception of the *A. vittatum* male-produced aggregation pheromone, vittatalactone, is not affected by cucurbitacin content [22], [23]. This suggests that other aspects of plant chemistry, including plant volatiles, may affect *A. vittatum* host plant preferences resulting in beetle aggregation.

Acalymma vittatum preferentially consumes leaves of cultivars of *Cucurbita pepo* ssp. *pepo* over *C. pepo* ssp. *ovifera* (syn *texana*, [24], [25]) although the chemical mechanistic bases remain elusive [26], [27]. We thus tested if subspecies-specific factors affect beetle behavior through which observed differences in preference may be explained. Specifically, does plant subspecies alter attraction of conspecifics, aggregation pheromone release, or promote beetle behaviors like emigration? And which has a greater contribution to ultimate differences in attack?

In this study, using representative cultivars from each subspecies, we dissected the successive steps in *A. vittatum* host choice that result in *C. p. pepo* sustaining more damage than *C. p. ovifera*. We tested the relative contributions of *C. pepo* volatiles, beetle male-produced aggregation pheromone release, and potential for synergistic action between those in preferred and non-preferred *C. pepo*. We predicted that *C. p. pepo* would be more attractive to *A. vittatum* during host location and more conducive to density-dependent attraction than *C. p. ovifera*, while *C. p. ovifera* may be deterrent (or promote emigration). Over three years of field experiments, we tested the effect of subspecies on (1) density-dependent beetle aggregation, (2) relative contributions of plant and beetle chemical cues to aggregation during field colonization, (3) beetle host

acceptance and (4) attack, and (5) foliar headspace volatiles. We use these findings to recommend management practices for *C. pepo* crops in particular, and generally for diversified farming systems.

4.3 Materials and Methods

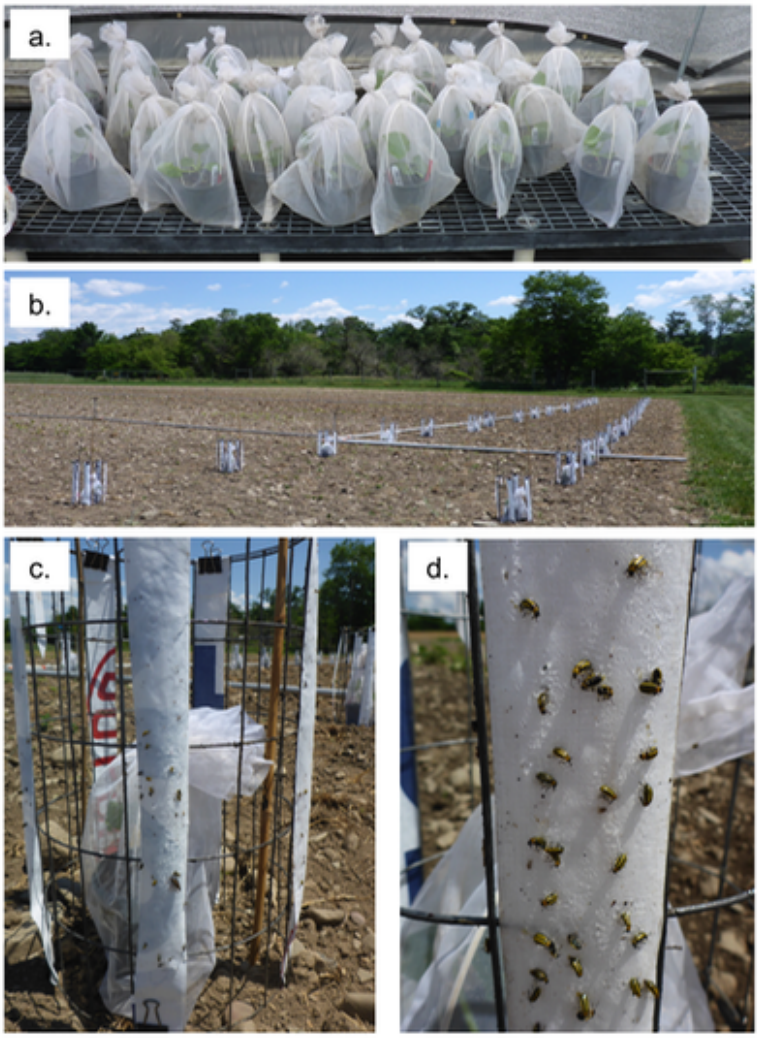
4.3.1 Plants. Two cultivars, *C. p. ovifera* cv. Success PM (yellow summer squash) and *C. p. pepo* cv. Golden Zucchini were previously established to be, respectively, non-preferred and preferred by *Acalymma vittatum* [25]. Untreated seeds were acquired from commercial sources in 2016 (High Mowing Organic Seeds, Wolcott, VT, USA; Seed Savers Exchange, Decorah, IA, USA), and Cornell University seed stocks were used in all other experiments. All seeds were started in 72-cell flats with McEnroe Organic Lite Growing mix (Millerton, NY, USA), and then transplanted into 1.74 L pots 10 days after sowing at the Cornell University Agricultural Experiment Station (CUAES) greenhouses (Ithaca, NY, USA). The greenhouses were maintained with a 14 h light, 10 h dark photoperiod with supplemental metal halide lighting, with 27 C day and 21 C night temperatures. Plants were watered daily, and *Amblyseius cucumeris* (BioBest, Westerlo, Belgium) was applied to prevent establishment of thrips. In all experiments, plants had 1-3 fully expanded true leaves and were not flowering. Plant damage was recorded as percent leaf defoliation was visually estimated with a 0-100% scale with 5% increments at the end experiments by a single observer (LB).

4.3.2 Insects. Adult *Acalymma vittatum* were collected from mixed *Cucurbita* spp. crops at the CUAES Homer C. Thompson Organic Vegetable Farm (Freeville, NY, USA, 42°31'05.7"N 76°20'07.1"W). When appropriate, *A. vittatum* were separated by sex by visually examining abdominal apex morphology [28]. Assays were conducted when *A. vittatum* are abundant in the region (mid June – July).

4.3.3 Beetle host location. Traps were used to test the effects of subspecies, beetle infestation type (male or female beetles, as only males produce vittatalactone), and density (number of beetles) on *A. vittatum* host colonization. Traps were similar to those used by [22], [23], are pictured in **Fig. 4.1**, and detailed below.

Plants were enclosed in opaque mesh bags (25 cm by 30 cm), and beetles were added to the appropriate treatments and allowed to establish for 24h (**Fig 4.1a**). Plants were then brought to Thompson Farm and spaced 3 m apart in an empty field (**Fig. 4.1b**). Wire cages (30.5 cm x 51 cm) were placed around the pots and secured with bamboo poles (**Fig 4.1c,d**). Then, five 12 cm wide strips of white spunbonded polyethylene (Tyvek HomeWrap, Dupont, Wilmington, DE, USA) were attached to the outside of the cages with binder clips at equal intervals, and coated with Tangle-trap sticky coating (Tanglefoot, Marysville, OH, USA) (**Fig 4.1c,d**). Overall, this design obscured visual cues for colonizing beetles. In both years (2016, 2017), each week from mid June through mid July, a new set of plants and insects were assembled and assayed as a temporal experimental block with 30 total traps per block. The design is detailed as follows.

Figure 4.1. Photographs of beetle field trap set up used for discrete and quantitative colonization experiments. (a) Plants were infested with beetles at varying densities 24 hours prior to setting traps up in field. These traps were then placed 3m apart in the field (b), and enclosed by wire cages with Tyvek strips covered with Tangle-trap (c). The traps were secured in place with bamboo stakes. These traps were effective in catching *A. vittatum*, and *A. vittatum* was the predominant species trapped (d).



In the first assay year (2016), beetles were caught on traps with one of four density treatments per subspecies ("discrete treatments" hereafter): four males, four females, eight males, and controls (plants without beetles). Four female beetles were chosen because we expected females to consume more tissue than males, and thus produce leaf damage intermediate to the male treatments. There were five temporal

experimental blocks with four replicate traps of each of the beetle treatments, and three replicate control traps per subspecies. One block was dropped from analysis after an organic pesticide application on an adjacent squash field lead to insect dispersal overwhelming nearby traps. Over the course of three days, beetles trapped were counted daily but not removed.

The following year (2017), the treatments were adjusted so that the quantitative relationship between infestation density by male and female beetles and number of beetles trapped could be assessed ("quantitative treatments" hereafter). Over three temporal blocks, there were two replicates of controls (plants without beetles), one trap each of nine male treatments (1, 2, 3, 4, 6, 7, 8, 10, 12) and one trap each of four female treatments (2, 4, 8, 10) per subspecies. After three days, trapped beetles were recorded. Then, a subset of treatments were retained and modified ("separated treatments" hereafter) to test the attractiveness of plants with prior beetle feeding (beetles removed), and of beetles that had previously fed (and placed on a pot of fresh soil). The treatments with 6, 8, 10 and 12 male, and 8 female beetles feeding on plants were thus separated to form ten new traps per subspecies. In addition, the 3, 4, and 7 male, and 4 female beetle treatments, and one control (plant only) per subspecies were retained as positive controls. The number of trapped beetles were counted after two days. Data from one of the three blocks of the separated treatments was not analyzed due to the low number of beetles caught (10 total).

4.3.4 Beetle host acceptance. Beetle emigration from (rejection of) each subspecies was measured at a CUEAS site isolated from known squash production to reduce

confounding beetle immigration (Varna, NY, USA, 42°27’52.9”N 76°26’41.7”W). The experiment was conducted in three temporally separated blocks in 2018 with 10 replicates of each subspecies per block. Plants were enclosed in a mesh bag and infested with eight beetles. After allowing the beetles to establish for at least 8 h, the plants were brought to the field, and the bags were opened after sunset, when the beetles were less active. The following day, beetles were counted four times (08:00, 10:00, 14:00, 16:00).

4.3.5 Beetle attack. Beetle preference in attack between subspecies, measured as defoliation, was evaluated at a local scale in the greenhouse. Since cucurbitacins are present in some *C. pepo* cotyledons [29], cotyledons were removed from some plants 24 h prior to infestation to test leaf chemistry alone. One plant of each subspecies was placed 25 cm apart in mesh bags (25 by 30 cm, two plants per bag, both with or both without cotyledons). Five beetles were released and fed for 48 h. Beetle preference for leaf tissue alone was tested in 2017 in two temporally separated blocks with 22 total replicates, and for intact plants in three blocks with 43 total replicates in 2018.

4.3.6 Chemical analyses. In 2017, for each subspecies, headspace volatiles were collected from plants with no beetles, plants with a range of males (two replicates of 2, 3, 4, 8, and one instance of 1, 6, 10), plants with a range of females (one instance of 2, 4, 10), and two replicates of ‘soil controls’ that consisted solely of the pot with soil and bag. The samples were prepared in the same fashion as the traps used in the quantitative treatment field assays, and the appropriate treatments were infested with

beetles 24 h prior to volatile collection. Oven bags (Reynolds, Lake Forest, IL, USA) were placed over each sample, and a small hole was punctured in the bags for an ORBO^{TM} 32S activated charcoal 100/50 mg ampoule (Supelco, Bellefonte, PA, USA). Volatiles were collected from 11:00 to 16:00 with two collection manifolds connected to separate 12 V vacuum pumps (Gast™ Manufacturing Inc., Benton Harbor, MI, USA at a flow rate of 350 mL / min). Ampoules were stored at -20C.

Ampoules were spiked with 4µL of 90 ng/µL tetraline (internal standard) and eluted twice with 200 uL dichloromethane into glass vials. The samples were then run on a GC-MS (Varian Saturn 2200 GC/MS/MS, Agilent Technologies, Santa Clara, CA, USA), equipped with a DB-WAX FAME column (Agilent J&W GC Column, 30 m × 0.25 mm ID, df = 0.25). Peak area was quantified in Varian MS Workstation Version 6.9.1 (Agilent Technologies, Santa Clara, CA, USA), and normalized by tetraline peak area. Unfortunately, multiple samples were lost during preparation (remaining samples are shown in Table 1, Results).

4.3.7 Statistics. Data from field experiments with beetle traps were analyzed as linear mixed models in the 'lme4' package [30] in R [31].

For tests of beetle colonization by subspecies, the number of beetles caught or plant defoliation were the response variables, block was a random effect, and ANOVA were conducted on the fixed effects. For the discrete treatment experiment, the categorical variables of subspecies, density treatment (n=4), and their interaction were fixed effects. In the quantitative treatment experiment with all intact traps, the quantitative variable of beetle density, and the categorical variables of beetle sex and

subspecies with all pairwise interactions, were fixed effects. In the separated treatments, the fixed effects of treatment (male beetles only, plants previously fed upon by male beetles, or positive controls; traps with female beetles were excluded from the analysis), plant subspecies and their interaction were tested. The effect of plant damage on number of beetles caught was also tested in the discrete treatment experiment, where linear mixed models with fixed effects of subspecies, defoliation, and their interaction were used for each beetle treatment separately.

The emigration assay was analyzed by categorizing the plants as either with or without complete emigration (zero beetles), summing each category across all three blocks, and comparing between subspecies with a William's corrected G-test for a 2x2 table. The local-scale greenhouse choice assay was analyzed by categorizing all of the plants as either with or without damage, summing the number in each category across all blocks, and comparing between subspecies with a Fisher's exact test for a 2x2 contingency table.

Differences in subspecies headspace volatiles were analyzed (n= 8 samples per subspecies; n= 6 volatiles above limit of quantification in at least two samples). VOCs present in soil controls (n=2) were removed from analysis. Difference in total plant VOC emission was tested with an ANOVA and fixed effects of subspecies, beetle number, their interaction, and manifold, and subspecies differences in specific compounds were tested with a one-tailed t-test. Compositional differences in beetle induced volatiles between subspecies were assessed with permutational multivariate analysis of variance using Bray-Curtis dissimilarities with 100 permutations in R/vegan [32] where all induced treatments were pooled. Random forest analysis was

conducted in R/randomForest [33], and variable selection on out-of-box error rate was conducted in R/varSelRF [34], with 5000 trees.

4.4 Results

4.4.1 Host location. Field insect traps were first used to test the effects of volatile cues from visually masked plants and discrete levels of beetle density on *A. vittatum* host location. In total, 3824 beetles were trapped. Traps of both *C. pepo* subspecies infested with female beetles and controls (no beetles) were equivalent in beetle catch, and caught 56% fewer beetles than traps infested with four or eight pheromone-producing male beetles (**Fig. 4.2a**, **Table 4.1**). There was a 71% increase in beetle capture from traps containing four males to those containing eight males for less preferred *C. p. ovifera*. In contrast, similar numbers of beetles were caught in four and eight male traps for preferred *C. p. pepo* (8% more in traps with eight males; no subspecies-by-treatment interaction, **Table 4.1**). Analyses of counts from earlier time points (1, 2 days) were consistent with results from 3 days (**Table 4.2**).

Table 4.1. A linear mixed effects analysis used to model beetles caught on each trap after three days during the discrete treatment assay. An ANOVA was conducted on fixed effects, and there was a random effect of block (σ^2=186.4; 48.9% of variance explained). Treatments indicates variable beetle density, and include four male, four female, eight male beetles, or control (no beetles), and the two plants are *C. p. pepo* cv. 'Golden Zucchini' and *C. p. ovifera* cv. 'Success PM'.

Effect	DF	*F*-value	*P*-value
Plant subspecies	1	2.188	0.142
Treatment	3	34.908	**<0.001**
Plant subspecies X Treatment	3	2.458	0.067
Residuals	111		

Table 4.2. Linear mixed effects models were used to model beetles caught on each trap after one and two days during the discrete treatment assay. An ANOVA was conducted on fixed effects, and there was a random effect of block. Treatments indicates variable beetle density, and include four male, four female, eight male beetles, or control (no beetles), and the two plants are *C. p. pepo* cv. 'Golden Zucchini' and *C. p. ovifera* cv. 'Success PM'.

Effect	1 day[1]		2 day[2]	
Plant subspecies	$F_{1,111}$=1.818	*P*=0.180	$F_{1,111}$=2.038	*P*=0.156
Treatment	$F_{3,111}$=23.339	***P*<0.001**	$F_{3,111}$=21.992	***P*<0.001**
Plant subspecies X Treatment	$F_{3,111}$=1.793	*P*=0.153	$F_{3,111}$=1.990	*P*=0.120

[1] Random effect of block (σ^2=123.8; 47.4% of variance explained)
[2] Random effect of block (σ^2=52.0; 49.5% of variance explained)

Percent leaf defoliation within traps was similar for both of the four beetle density treatments (male and female) and increased for the eight beetle treatment (**Fig. 4.2b**). Caged beetle density was the best predictor of beetle damage ($F_{1,87}$=81.33, P<0.001), but there were also significant effects of subspecies ($F_{1,87}$=4.75, P=0.032), and their interaction ($F_{1,87}$=9.14, P=0.003); this interaction was driven by unexpectedly higher damage on *C. p. ovifera* plants infested with eight male beetles (**Fig. 4.2b**). However, there was no effect of damage, or interaction between damage and subspecies, on number of beetles caught (**Table 4.3**).

The following year, traps were set up with an expanded range of caged beetles of each sex to quantitatively test for interactions between beetle infestation density, beetle sex, foliar damage and subspecies. Fewer total beetles were trapped (592), but traps infested with female beetles at any density and plant controls were again equivalently unattractive, and increasing the number of male beetles in cages linearly increased the number of beetles trapped (**Fig. 4.3a**). There were no significant interactions between subspecies, damage, or beetle density in the trap (**Table 4.4**). The interaction between beetle density and sex was significant, where exclusively more

males increased trap catch (**Table 4.4**). Plant defoliation was similar between subspecies, increased with beetle density, and female beetles caused more damage than males (**Fig. 4.3b**, **Table 4.4**).

Table 4.3. Linear mixed effects models were used to model beetles caught on each trap with different treatments after three days during the discrete treatment assay. An ANOVA was conducted on fixed effects of damage and plant subspecies, and there was a random effect of block. The two plants are *C. p. pepo* cv. 'Golden Zucchini' and *C. p. ovifera* cv. 'Success PM'.

Effect	Four females[1]		Four males[2]		Eight males[3]	
Plant subspecies	$F_{1,25}$=0.921	*P*=0.346	$F_{1,25}$=6.715	***P*=0.016**	$F_{1,27}$=1.477	*P*=0.235
Damage	$F_{1,25}$=0.084	*P*=0.774	$F_{1,25}$=0.826	*P*=0.372	$F_{1,27}$=1.159	*P*=0.291
Plant subspecies x Damage	$F_{1,25}$=0.209	*P*=0.652	$F_{1,25}$=0.365	*P*=0.551	$F_{1,27}$=3.492	*P*=0.073

[1] Random effect of block (σ^2=131.5; 56.1% of variance explained)
[2] Random effect of block (σ^2=196.1; 46.1% of variance explained)
[3] Random effect of block (σ^2=580.6; 74.8% of variance explained)

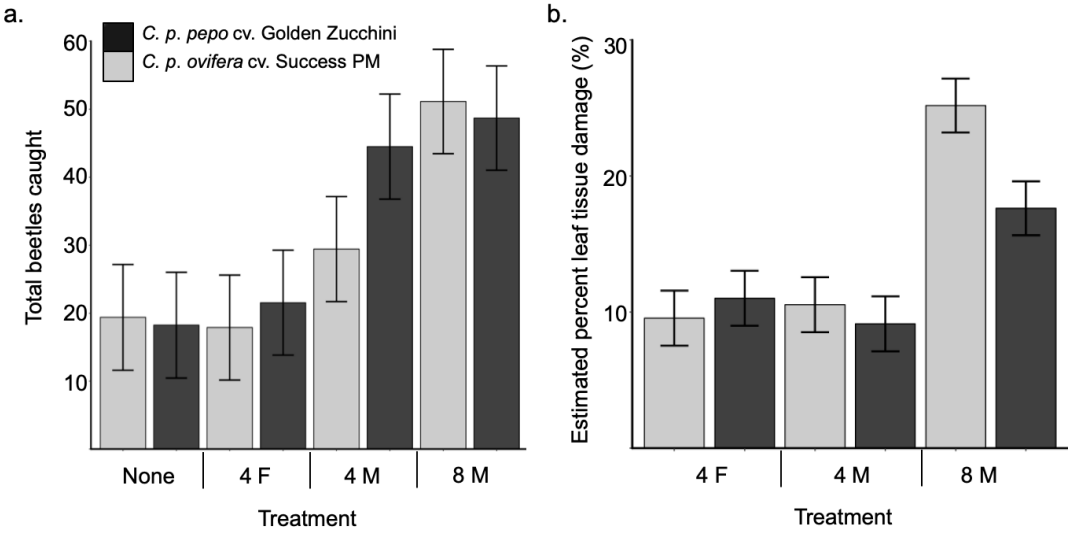


Figure 4.2. Beetle attraction to volatiles emitted from discrete plant-beetle treatment combinations in visually masked field cages: (a) number of beetles caught in 2016, and (b) leaf damage inside the traps, grouped by plant cultivar and treatment. Treatment refers to level of infestation: four female ("4F"), four male ("4M"), or eight male beetles ("8M"), or control (no beetles; "none") on both plant cultivars differentiated by color). The height of bars is the least-squared mean per block, and error bars represent one standard error.

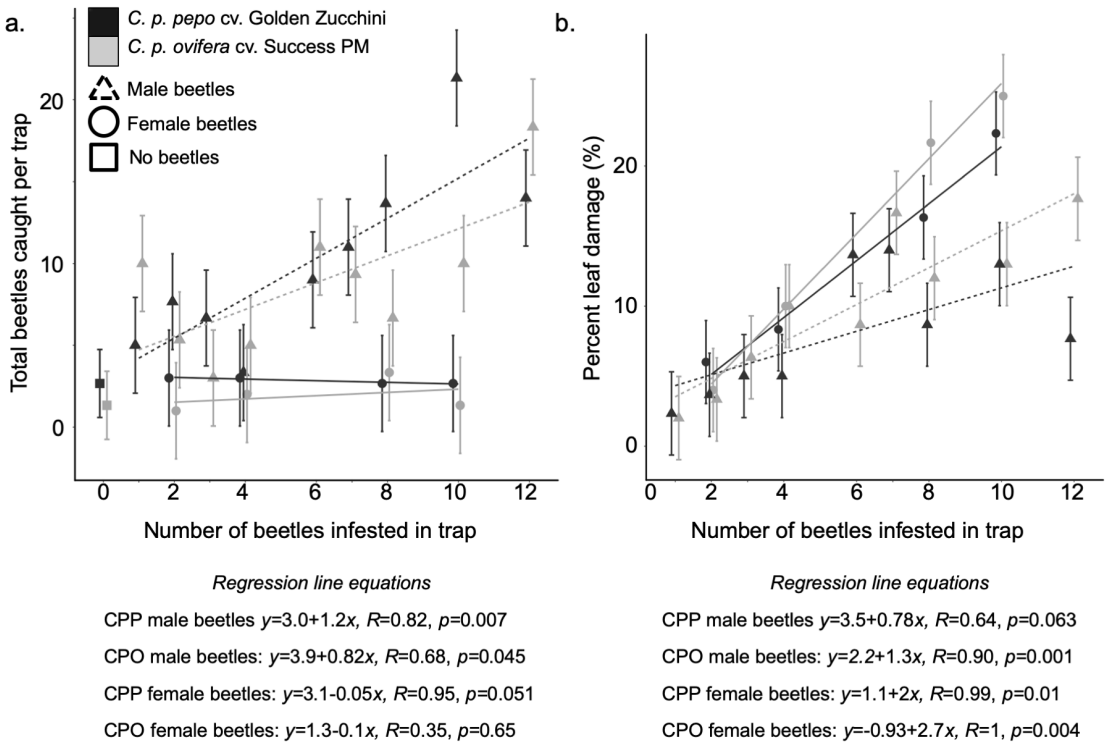


Figure 4.3. Beetle attraction to volatiles emitted from continuous plant-beetle treatment combinations in visually masked field cages: (a) number of beetles caught in 2017, and (b) the leaf damage inside the traps, grouped by cultivar and beetle sex. Plant cultivars are differentiated by color, and sex of beetles in trap is indicated by shape. Points of all shapes represent the least-squared mean, and bars represent one standard error. The lines are linear regressions for each plant cultivar and beetle sex combination.

Table 4.4. We modeled the number of beetles trapped over three days during as well as leaf damage during the quantitative treatment assay with a linear mixed effects model. An ANOVA was conducted on fixed effects. There was a random effect of block (σ^2=0.02, 0.1% and σ^2=5.18; 19% of variance explained for number of beetles trapped, and leaf damage, respectively). Beetle density ('D') ranged from 1-12 for males, and 4-10 for females, and plant subspecies ('P') included *C. p. pepo* cv. 'Golden Zucchini' and *C. p. ovifera* cv. 'Success PM'.

		Total beetles caught		Leaf damage	
Effect	DF	*F*-value	*P*-value	*F*-value	*P*-value
Beetle density (D)	1	17.463	**<0.001**	79.682	**<0.001**
Beetle sex (S)	1	28.062	**<0.001**	20.087	**<0.001**
Plant subspecies (P)	1	1.078	0.303	3.089	0.083
D x S	1	5.890	**0.018**	13.692	**<0.001**
D x P	1	0.494	0.485	3.338	0.072
S x P	1	0.039	0.844	0.001	0.975
D x S x P	1	0.450	0.505	0.023	0.880
Residuals	69				

At the end of the quantitative treatment assay, a subset of the cages were separated into traps with male beetles alone, plants with previous male feeding alone, and male beetles and plants together (positive control); beetles were trapped for two days.

While fewer beetles were caught (150), there was an effect of treatment ($F_{2,41}$=12.18, P<0.001) where traps with male beetles alone or plants previously fed by male beetles caught 72% and 79% fewer beetles than positive controls (male beetles feeding on plants), respectively (**Fig. 4.4**). Higher numbers of male beetles only increased trap catch in positive controls ($F_{1,7}$=8.48, P=0.023), not the other treatments (male beetles alone, $F_{1,11}$=0.20, p=0.665; plants alone, $F_{2,41}$=4.00, P=0.071) (**Fig. 4.4**).

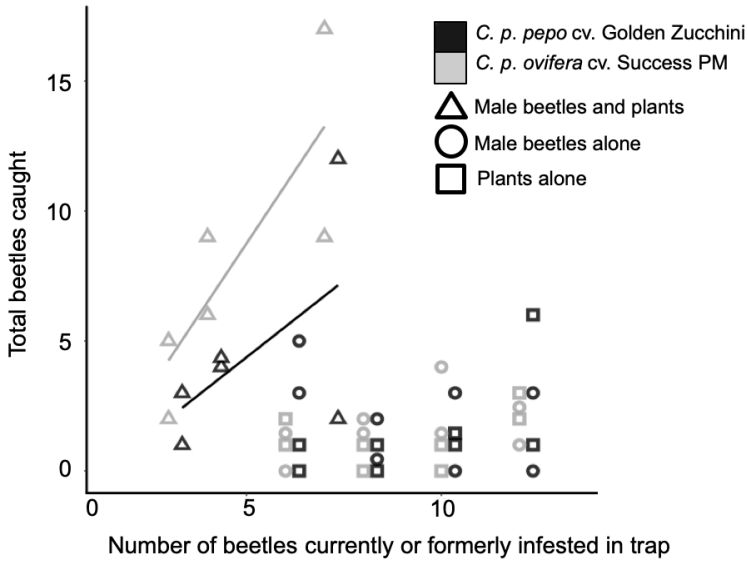


Figure 4.4. Beetle attraction to volatiles emitted from separated plant-beetle combinations (traps containing male beetles, plants alone, or positive controls of male plants and beetles together) in visually masked field cages in 2017. The points (circles, triangles, and squares) are raw values, and lines are linear regressions for each plant cultivar and beetle sex combination where the there was a significant effect of number beetles (current, or previously feeding; see Results).

4.4.2 Host acceptance. When beetles were given the opportunity to emigrate, fewer *C. p. pepo* plants were deserted (rejected) by beetles compared to *C. p. ovifera* over one day (William's corrected G-test 08:00h G=5.56, P=0.018; 10:00h G=3.25 P=0.072; 14:00h G=9.74 P=0.002; 16:00h G=13.03 P=<0.001). Less than 50% of leaf tissue was removed from any plant, and percent defoliation was consistent between subspecies (*C. p. pepo* 10.7%; *C. p. ovifera* 11.7%).

4.4.3 Beetle attack. Leaf damage was assessed in neighboring *C. p. ovifera* and *C. p. pepo* plant-beetle-choice assays. Significantly more *C. p. ovifera* had no damage, both when cotyledons were intact (plants with no damage: CPT 13/22, CPP 0/22, Fisher's exact P<0.001) or removed (plants with no damage: CPT 38/43, CPP 2/44, Fisher's exact P<0.001).

4.4.4 Beetle aggregation pheromone and plant volatiles. Headspace volatiles were collected to determine if aggregation pheromone (vittatalactone) release from beetles or plant volatile emissions differed between subspecies. We detected vittatalactone only in samples with at least one male beetle (**Table 4.5**). Six additional volatiles were detected in the headspace of plants under field conditions (**Table 4.6**). . *p. ovifera* samples had 2.5-fold greater volatile emission than *C. p. pepo* ($F_{1,11}$=20.43, P<0.001), and more beetles enhanced volatile emissions in both subspecies ($F_{1,11}$=7.86, P=0.017). Neither the interaction between subspecies and beetle number ($F_{1,11}$=0.07, P=0.802) nor effect of manifold ($F_{1,11}$=0.05, P=0.827) were significant. Headspace composition differed between subspecies (PERMANOVA subspecies pseudo-

$F_{1,13}$=7.69, P=0.001; manifold pseudo-$F_{1,13}$=0.89, p=0.535). Random forest classification had an out-of-bag error rate of 12.5%, and found linalool and an unidentified compound ("RT11.8") were sufficient to parse subspecies through variable selection (**Table 4.6**). Most *C. p. pepo* samples lacked RT11.8 and linalool concentration was 4-fold higher in *C. p. ovifera* than *C. p. pepo* (**Table 4.5**, **Fig. 4.5**).

Table 4.5. Normalized signal intensity (*k*-counts) of selected plant volatiles (linalool, RT11.8) and male aggregation pheromone (vittatalactone) detection and quantification by GC-MS.

	C. p. pepo			*C. p. ovifera*		
Treatment	Linalool	RT11.8	Vittatalactone	Linalool	RT11.8	Vittatalactone
Field cages						
1M	0.7	N.D.	<LOQ	6.2	1.8	N.D.
2M	1.5	N.D.	<LOQ	4.5	1.0	<LOQ
3M	1.2	N.D.	<LOQ	6.0	0.8	<LOQ
4M	-	-	-	4.9	0.9	<LOQ
6M	0.8	N.D.	<LOQ	-	-	-
8M	5.0	0.4	1.07	13.0	0.8	<LOQ
10M	-	-	-	9.0	0.7	0.660
2F	1.3	N.D.	N.D.	-	-	-
10F	1.8	0.5	N.D	8.5	0.8	N.D.

Notes: The treatments are male beetles "M" and female beetles "F", and the abbreviations are defined as follows: "N.D", not detected; "<LOQ", less than limit of quantification; "–", no sample. All values are a single instance, except the values reported for two males of *C. p. pepo*, and three males of *C. p. ovifera* are the mean of two samples.

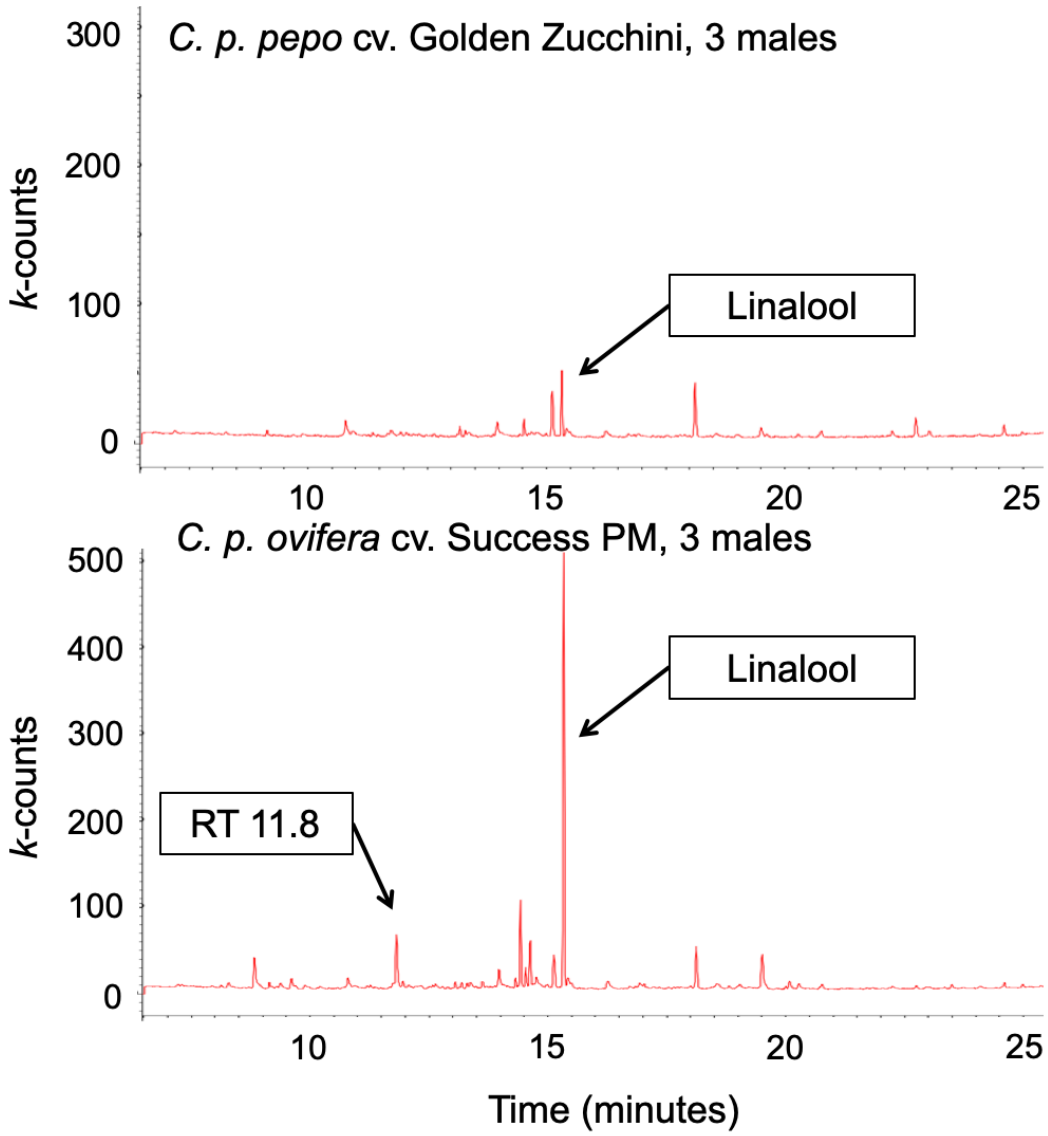


Figure 4.5. Example GC-MS chromatogram of plant volatile organic compounds of each plant subspecies with three male beetles feeding.

Table 4.6. Summary statistics of plant headspace volatile organic compounds

Retention time (min)	Kovats Index	Major *m/z* peaks of unknowns	Compound	*C. p pepo* mean ng/h (% comp.)	*C. p, ovifera* mean ng/h (% comp.)	t-test results	MDA
8.8	1158	57	Unknown	43 +/- 14 (18.9%)	58 +/- 14 (7.7%)	$t_{1,15}$=-0.67, P=0.51	-3.9
11.8	1302	69, 79	Unknown	7 +/- 7 (1.3%)	65 +/- 14 (8.7%)	$t_{1,15}$=-4.82, ***P*<0.001**	44.8
14.4	1473		Cyclohexane carboxylic acid, hexyl ester	86 +/- 29 (35%)	108 +/- 22 (14.9%)	$t_{1,15}$=-0.72, P=0.49	-3.8
14.6	1487		2,4-dimethyl-2-pentene	50 +/- 14 (20.6%)	50 +/- 14 (6.8%)	$t_{1,15}$=-0.18, P=0.86	-0.5
15.3	1536		Linalool	122 +/- 36 (41.7%)	526 +/- 79 (63.9%)	$t_{1,15}$=-4.43, ***P*=0.001**	38.5
18.1	1717		2-cyclopentyl cyclopentanone	21 +/- 14 (10.2%)	29 +/- 14 (4.4%)	$t_{1,15}$=-0.39, P=0.70	0.2
Total			NA	331 +/- 72 (100%)	842 +/- 108 (100%)	NA	NA

Notes: Mean values and standard errors are reported in ng/h, and the value in parentheses is the mean percent composition of total. For each subspecies, there were eight samples, 'MDA' is mean decrease in accuracy from random forest analysis

4.5 Discussion

Disentangling the roles of plant chemistry and pheromone production or perception on the hierarchy of host choice can provide insight as to why aggregating insects demonstrate preference for particular plants and inform management strategies. Here, we sought to determine how plant chemistry affects successive steps in *Acalymma vittatum* aggregation, and if this differed between preferred *Cucurbita pepo* ssp. *pepo* and non-preferred *Cucurbita pepo* ssp. *ovifera*. Unexpectedly, we found no differences in conspecific attraction to plant volatiles from *C. p. pepo* or *C. p. ovifera* during field colonization under any treatment tested – controls (constitutive plant volatiles), female-infested plants (induced plant volatiles, lacking pheromone), plants previously infested with males (induced plant volatiles, lacking pheromone), and male-infested plants (induced plant volatiles, with pheromone). Instead, the density of feeding male beetles was the strongest predictor of beetle attraction, and plants lacking active male beetle feeding were unattractive. Thus, we tentatively conclude that active male feeding is a sufficient stimulus to release aggregation pheromone and resulted in density-dependent field-scale attraction and colonization on both plant subspecies. However, *A. vittatum* rejected *C. p. ovifera* through emigration, likely due to deterrent plant volatiles.

4.5.1 Beetle attraction to plant chemistry, pheromones in host location.

Our work compared *A. vittatum* aggregation behavior between two cultivated *C. pepo* subspecies. Given the established dichotomy in *A. vittatum* preference between subspecies across multiple scales [24], [25], we hypothesized that vittatalactone

release, plant volatiles, or the interactions thereof, influenced *A. vittatum* preference during field colonization. These functions are non-exclusive as, for instance, *Leptinotarsa decemlineata* is attracted to plant volatiles alone, and volatiles synergize with the aggregation pheromone [11].

While we lacked sufficient sensitivity to quantitatively characterize vittatalactone concentrations, we confirmed that vittatalactone was released by male beetles feeding upon both *C. pepo* subspecies, as is true for both cucurbitacin-rich and -poor cucumber plants [22], [23]. Food sources commonly increase aggregation responses across diverse beetles (reviewed in [35]), and further work is needed to understand how ingesting food facilitates release in this species. Nonetheless, our results indicate that differences in pheromone production while *A. vittatum* feeds upon *C. pepo* is not the cause of observed preference differences between subspecies.

There was also no evidence that plant volatiles themselves or differential synergism with aggregation pheromone affect host plant attraction. While attraction to volatiles (especially from damaged tissue) is widespread in aggregating beetles [8], [9], [18], [19], our result is consistent with previous work that constitutive and induced cucumber volatiles were unattractive to *A. vittatum* [23]. Further, the consistent density-dependent attraction of conspecifics to both *C. pepo* subspecies when infested with male beetles indicates that volatiles from both subspecies were either equally positive or neutral. In the discrete treatment assay, there was greater attraction to the preferred *C. p. pepo* infested with fewer male beetles than non-preferred *C. p. ovifera*. However, when we assayed a quantitative range of male beetles the following year, conspecific attraction was proportional to male beetle density.

We thus conclude that beetles do not demonstrate preference for *C. p. pepo* during host location due to long distance plant volatile cues or vittatalactone pheromones, indicating that observed differences in attack are due to other behaviors.

4.5.2 Local cues, and host acceptance. Local cues that lead to individual movement decisions are an important component of insect host acceptance [12], [13], and appear to affect *A. vittatum* preference. There was greater *A. vittatum* emigration from the non-preferred *C. p. ovifera* even when undamaged leaf tissue remained. Similarly, *A. vittatum* was equally attracted to cucumber monocultures (preferred) and cucumbers in a multi-species polyculture (corn, broccoli; less preferred) [36], [37]. However, *A. vittatum* remained longer in monocultures, and more frequently moved from polycultures to monocultures [36], [37]. Thus, emigration after host plant quality assessment appears to be a key component of *A. vittatum* preference. However, mechanistically, previous work found no associations between common metrics of leaf quality like nitrogen, trichomes, or water content and *A. vittatum* damage across diverse Cucurbitaceae [26].

In local choice assays, *A. vittatum* almost exclusively damaged the preferred *C. p. pepo*. Most *C. p. ovifera* had no indications that the beetles sampled the plant before choosing to consume *C. p. pepo*, potentially due to locally acting deterrents. While specific compounds were not measured, induced deterrent volatiles to generalist caterpillars were previously reported in other *C. pepo* cultivars [27].

4.5.3 Scale dependent cues? We found that two volatiles, linalool and an

unidentified compound, had differential emission between subspecies. Such differences are often sufficient cues for insects about host plant identity [38] and may indicate plant resistance status (but see [39]). Although we cannot establish causality, the greater emission of linalool in *C. p. ovifera* may be responsible for short distance deterrence and host rejection. Plant-produced linalool has been reported to deter insect herbivory [40] and oviposition [41], and have contrasting scale-dependent effects [42] in other systems. Increased replication and assays with isolated linalool would provide stronger evidence for deterrence of *C. p. ovifera.* However, short-range repellency is still consistent with our host location findings, as insects typically rely on scale-dependent cues [12], [13].

4.5.4 Implications for pest management on diversified farms. Our work advances efforts to reduce beetle damage on *C. pepo* crops via influencing *A. vittatum* movement by plant germplasm deployment. First, if exclusively non-preferred *C. p. ovifera* crops are grown, there may be aggregation hotspots: beetles confined on *C. p. ovifera* and *C. p. pepo* generate similar amounts of overall damage, but it is concentrated on fewer *C. p. ovifera* plants [25], indicating that beetles will eventually aggregate on some *C. p. ovifera* plants after emigrating. Thus, managers could use trap cropping (discussed below) to provide a destination for emigrating beetles. Plant breeding for enhanced *C. p. ovifera* deterrence could further promote beetle movement towards trap crops. On farms growing both *C. p. pepo* and *C. p. ovifera*, beetle rejection of *C. p. ovifera* could exacerbate damage on *C. p. pepo* (not necessarily due to attractiveness of *C. p. pepo*). Growers could reduce *C. p. pepo* damage by locating

plantings where selective management efforts are feasible (e.g. floating row cover to physically shield crops). Finally, *Cucurbita maxima* cultivars have floral volatile attractants to beetles [43], and are used as trap crops [44]. For *C. pepo*, we show that trap cropping to minimize damage on *C. p. pepo* likely relies on the attractiveness of *C. maxima* alone, whereas the trap effectiveness may be amplified by beetles emigrating from *C. p. ovifera*. While our work further confirms that vittatalactone is sufficient for beetle host location on diverse *C. pepo*, data on the effectiveness of synthetic lures is mixed and requires additional development (JG and MH, unpublished data, 2012; [45])

This study provides mechanistic information on how *A. vittatum* behavior is affected by *Cucurbita* spp. germplasm, and broadly highlights the value of crop diversification and suggests strategies by which it improves pest management. Approaches like push-pull, where non-preferred and preferred varieties are used in spatial arrangements to 'push' pests away from the main crop and 'pull' towards the trap crop [46] already target insect movement behavior as a means of pest management. For farms without an explicit push-pull design and management, our work emphasizes that pest movement along a preference gradient could have substantial impacts on pest densities and ultimately damage. Thus, by considering interactions between diverse crop varieties in the system, management efforts may be more successful. Importantly, and complementary to management efforts, plant breeders should seek to augment this range of traits from attraction to deterrence to increase performance of the system as a whole, not only for individual crops.

4.6 Conclusions

We found that *A. vittatum* demonstrated no preference in field colonization for *C. p. pepo* over *C. p. ovifera* plants and released pheromone if confined on either plant. Instead, beetles more readily rejected and emigrated from the non-preferred *C. p. ovifera* and attacked preferred *C. p. pepo* at local scales, potentially due local perception of plant volatiles. These results add to our understanding of why *C. p. pepo* cultivars are more consistently damaged by *A. vittatum*: it is not because of aggregation behaviors, but likely due to deterrent cues from *C. p. ovifera* that beetles respond to when close to plants. Broadly, variation in factors that affect host preference may be commonly limited to cues that mediate insect behavior at local scales and host acceptance, not field colonization. These preference differentials should be applied in diversified farming systems to modulate pest movement and reduce crop damage.

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Chapter 5: Divergence of defensive cucurbitacins in independent *Cucurbita pepo* domestication events leads to differences in specialist herbivore preference

5.1 Abstract

Crop domestication and improvement often affect plant resistance to pests and production of secondary metabolites, creating challenges for isolating the role of specific metabolites and the genetic targets of past selection. Cucurbitacins are bitter triterpenoids with extreme phenotypic differences between Cucurbitaceae lineages, yet we lack integrated models of herbivore preference, cucurbitacin accumulation, and genetic mechanisms. In *Cucurbita pepo*, we dissected the effect of cotyledon cucurbitacins on preference of a specialist insect pest (*Acalymma vittatum*) for multiple tissues, assessed genetic loci underlying cucurbitacin accumulation in diverse germplasm and a biparental F_2 population (from a cross between two independent domesticates), and characterized quantitative associations between gene expression and metabolites during seedling development. *Acalymma vittatum* affinity for cotyledons is mediated by cucurbitacins, but other traits contribute to whole-plant resistance. Cotyledon cucurbitacin accumulation was associated with population structure, and our genetic mapping identified a single locus, *Bi-4*, containing genes relevant to transport and regulation – not biosynthesis – that diverged between lineages. These genes were expressed during seedling development, most prominently a transporter of secondary metabolites. Thus, past selection and future breeding for plant resistance to insects involves targeting genes for regulation and transport of defensive metabolites, in addition to core biosynthesis genes.

5.2 Introduction

Plants produce a diverse array of secondary metabolites, yet many are restricted to certain plant populations or lineages, complicating efforts to characterize their specific roles in ecological interactions and the genetic basis of production and accumulation [1]. In agricultural plants, lineage-specificity is amplified by the genetic bottlenecks associated with domestication and subsequent improvement [2]. This process has often been associated with a decrease in resistance to insect herbivores, but is not always correlated with plant-wide loss of defensive metabolites [3]. Thus, a challenge for modern plant breeding is understanding the degree to which past selective pressures shaped the genetic and mechanistic bases of intraspecific variation in defensive metabolites, and how this can be leveraged to augment resistance in future breeding efforts.

Intraspecific variation in defensive chemistry in crop plants can have major effects on interactions with herbivores both when there is a complete loss of biosynthesis (e.g. [4]) or a reduction of metabolites [5]. Connecting the ecological phenotype of resistance in the field to the genetic mechanisms can be straightforward in the case of complete pathway knockouts. In the context of plant breeding, naturally occurring mutants are identified, selected, and widely introgressed through intensive plant breeding (e.g., loss of fruit pungency in *Capsicum annuum* [6]), or specifically developed by genetic engineering (e.g., nicotine in *Nicotiana attenuata* [7]). However, variation can also stem from spatially and temporally dynamic process like induction by insect feeding or other stressors [8], transport [9], or changes associated with ontogeny [10], [11]. Thus, investigations into the genetic basis of intraspecific

variation in defensive metabolites should involve study of how biosynthesis interacts with spatiotemporally-sensitive processes like regulation, storage and transport [12], [13].

Cucurbitacins, bitter triterpenoids predominately found in the Cucurbitaceae, are an ideal class of compounds for investigating the relationship between herbivory and the dynamic accumulation of defensive metabolites. Eighteen cucurbitacins (named by letters A-T) have been described [14], and abundance varies within and between species, tissues [15]–[17] and developmental stages [18]. Disruption of cucurbitacin accumulation has strong effects on herbivore preference: mutants that have complete pathway disruption of cucurbitacins have been invaluable in demonstrating that absence of species-specific cucurbitacin C from cucumber (*Cucumis sativus*) confers susceptibility to generalist herbivores, and resistance to specialist Diabroticite beetles (Coleoptera: Chrysomelidae) that prefer and sequester cucurbitacins [19]–[21]. However, this finding is restricted to a single lineage (*Cucumis sativus*), and despite discovery of the mutant phenotype over half a century ago [22], the genetic mechanism remains unconfirmed.

Studies of genetic mechanisms of cucurbitacin accumulation and herbivore preference have been largely independent. The genetic pathway of cucurbitacin accumulation in aboveground tissues has been facilitated by the characterization of cucurbitacin C biosynthesis in cucumber [23]. In cucumber, melon (*Cucumis melo*), and watermelon (*Citrullus lanatus*), biosynthesis occurs locally in tissues through activation of the first committed step of a oxidosqualene cyclase (followed by numerous oxidations and acetylation) by leaf-, root- or fruit-specific transcription

2314 factors [23], [24]. Mutations in fruit-specific regulation [23], [25], [26] were fixed in
2315 selective sweeps during domestication of multiple species, but selection for loss of
2316 oxidosqualene cyclase function was also implicated in loss of fruit bitterness in
2317 *Cucumis melo* ssp. *melo* [26]. In contrast, there is a paucity of research on genetic
2318 mechanisms differentiating lineages (especially intraspecific lineages, but see [26])
2319 and in tissues relevant for herbivores.
2320 Building upon this work in cultivated Cucurbitaceae, *Cucurbita pepo*, provides an
2321 excellent opportunity to connect the genetic mechanisms of cucurbitacin accumulation
2322 and herbivory because of the evolutionary association between *C. pepo* and
2323 cucurbitacin-sequestering specialized Diabroticite beetles [27], [28]. Both the
2324 Diabroticite beetles [21] and *Cucurbita* spp. [29] are native to the Americas, where the
2325 two cultivated *C. pepo* subspecies, *C. pepo* ssp. *pepo* ("CPP", e.g. zucchini) and *C.*
2326 *pepo* ssp. *ovifera* ("CPO", e.g. summer squash, syn. *C. pepo* ssp. *texana*), were
2327 independently domesticated [29], [30]. After domestication, the subspecies further
2328 diverged as CPP cultivars were largely developed in Europe, free of specialist
2329 herbivores, while CPO cultivars were bred in the Americas (Paris, 2000).
2330 Cucurbitacins are absent in true leaves and fruits of cultivated *C. pepo* [15]. At the
2331 seedling stage, however, at least a few cultivars of CPP are rich in cucurbitacins in
2332 cotyledons and roots, while CPO lack cucurbitacins plant-wide [18], [32]–[34]. While
2333 this pattern suggested pathway disruption in CPO, likely by a single gene [35], the
2334 genetic mechanisms differentiating the subspecies are unknown. Preference of the
2335 specialized herbivore *Acalymma vittatum* is furthermore associated with subspecies:
2336 both the cotyledons (high cucurbitacins) and leaves (no cucurbitacins) of CPP are

preferred over CPO [34], [36], [37]. Here we generated a new mapping population by crossing CPP and CPO to help disentangle the association between cucurbitacins and selection history, allowing us to identify causal genetic loci in biosynthesis or accumulation, and to understand if plant-wide herbivore preference is indeed predicated on cucurbitacins.

Overall, we sought to characterize the role of cotyledon cucurbitacins in herbivore preference between *C. pepo* subspecies and to elucidate the genetic and mechanistic bases of cotyledon cucurbitacin production. We hypothesized that cotyledon cucurbitacins affect *A. vittatum* preference independently of other factors associated with the divergence between *C. pepo* subspecies. To address this, we selected for phenotypic extremes for multiple generations in a mapping population, and measured *A. vittatum* preference in the field over two seasons. We also tested whether a lack of cotyledon cucurbitacins in CPO relative to CPP is due to loss of function of a major pathway gene affecting all tissues. In addition to conducting biparental genetic mapping and screening diverse *C. pepo* germplasm, we also measured gene expression and phenotypes over seedling development to identify transporters or regulatory factors contributing to spatiotemporal variation in cucurbitacin accumulation. These approaches allowed us to assess the role of different genetic mechanisms contributing to cucurbitacin variation, as well as insights into past selection processes and the potential for future breeding strategies to manage a major agricultural pest.

5.3 Materials and methods

5.3.1 Plants. Seeds for expression, mapping and selection studies were Cornell University stock seed: parent lines *Cucurbita pepo* ssp. *pepo* cv. Black Beauty (hereafter "CPP"), and *C. p*. ssp *ovifera* cv. Success PM (hereafter "CPO"), and derived F_1 plant and F_2 mapping population. Seeds of the *C. pepo* diversity panel were from the selfed progeny of USDA PI lines. Plants were started from untreated seeds in organic potting soil, and no additional fertilizer or pest control was used. Plants used in insect bioassays were started in Cornell University Agricultural Experiment Station Guterman greenhouses (Ithaca, NY, USA), and all others were grown in an indoor growing room (Ithaca, NY, USA). Both environments were certified organic, and had a 16 h day, 8 h night photoperiod with supplemental lighting with 27 °C day and 21 °C night temperatures.

5.3.2 Cucurbitacin extractions. Cucurbitacins were extracted from root, cotyledon, and leaf tissue at different developmental stages using protocols similar to [38]. Briefly, fresh tissue was homogenized in methanol and purified by solid phase extraction. Samples were then suspended in acetonitrile before quantification on UHPLC-MS system equipped with a C18 column (150 mm×2.1 mm, 2.6 μm particle size).

5.3.3 Cucurbitacin gene homolog identification. We identified potential homologs of known cucurbitacin biosynthetic genes in *Cucurbita pepo* to test for allelic variation, and to examine gene expression patterns. Gene lists from cucumber (*Cucumis sativus*),

melon (*Cucumis melo*) and watermelon (*Citrullus lanatus*) were compiled from Shang *et al.* (2014) and Zhou *et al.* (2016), and BLAST [39] was used to identify homologs in *Cucurbita pepo*. Gene function and location are shown in **Table 5.1**, and *C. pepo* homologs follow the naming convention of Zhou *et al.* (2016).

5.3.4 Isolating effects of cotyledon cucurbitacins on herbivore preference – Biparental F2 population evaluation. Cucurbitacins B, D, E and I were measured in the F_2 mapping population derived from high cotyledon cucurbitacin CPP and low cotyledon cucurbitacin CPO. Cucurbitacins were extracted from 188 F_2 individuals in four blocks of 55-58 plants, each with three replicates of both parents and the F_1 as checks in an augmented incomplete block design. The extraction blocks were staggered over four weeks in January 2018, but all cucurbitacins were quantified together. Summary statistics and Pearson's correlations were calculated for all checks and the F_2 population for concentration of all individual and total cucurbitacins, and the B:D, E:I and BD:EI ratios. Best linear unbiased predictors (BLUPs) were then calculated for all phenotypes to be used in genetic mapping.

Total cucurbitacin content of checks (CPO, CPP, F1) were individually examined with linear mixed models to determine the variation due to block, and to detect outlying values. For this and future analysis, outliers were defined as individuals with a Studentized residual with an absolute value greater than three. One outlying CPO individual with was removed.

Table 5.1. Identification of cucurbitacin biosynthetic and regulatory gene homologs from other Cucurbitaceae species. Genes lists from cucumber ("C", *Cucumis sativus*), melon ("M", *Cucumis melo*) and watermelon ("W", *Citrullus lanatus*) were compiled from Shang *et al.* (2014) and Zhou *et al.* (2016), and BLAST was used to identify homologs in *Cucurbita pepo*. The name given to the homolog in *C. pepo* follows the naming convention of Zhou *et al.* (2016).

Gene function	*C. pepo* name	Species	Homolog name	*C. pepo* gene	perID (%)	length (bp)	E-value
Cucurbitadienol synthase (oxidosqualene cyclase, OSC)	CpBi	C	CsBi	Cp4.1LG12g10070	84.62	429	4E-119
		M	CmBi	Cp4.1LG12g10070	85.28	428	2E-123
		W	ClBi	Cp4.1LG12g10070	88.12	320	5E-104
Oxidation of cucurbitadienol (C11 carboxylase and C20 hydroxylase)	Cp890	C	Cs890	Cp4.1LG09g10760	88.07	327	1E-107
		M	Cm890	Cp4.1LG09g10760	88.51	235	2E-76
		W	Cl890	Cp4.1LG09g10760	85.61	264	5E-74
Acetyltransferase	CpACT	C	CsACT	-			
		M	CmACT	-			
		W	ClACT	Cp4.1LG17g09410	78.18	1297	0
Tissue specific transcription factors	CpBx	C	CsBl, CsBt	-			
		M	CmBr, CmBt	Cp4.1LG13g02020	77.55	441	1E-69
		W	ClBt, ClBr	-			
Potential oxidizer (C25 hydoxylase)	Cp160	C	Cs160	Cp4.1LG17g09580	82.93	615	6E-155
		M	Cm160	Cp4.1LG17g09580	84.42	616	7E-170
		W	Cl160	Cp4.1LG17g09580	87.68	617	0
Potential oxidizer	Cp170	C	Cs170	Cp4.1LG12g10100	78.26	1490	0
		M	Cm170	Cp4.1LG12g10100	77.9	1489	0
		W	Cl170	Cp4.1LG12g10100	80.09	1060	0
Potential oxidizer (C2 hydoxylase)	Cp180	C	Cs180	-			
		M	Cm180	-			
		W	Cl180	Cp4.1LG17g09580	97.62	42	1E-12
Potential oxidizer	Cp710	C	Cs710	Cp4.1LG17g09580	88.52	122	2E-035
		M	Cm710	Cp4.1LG09g10760	88.51	235	2E-76

		W	Cl710	Cp4.1LG17g09580	82.13	414	2E-95
Potential oxidizer	Cp490	C	Cs490	Cp4.1LG02g15440	86.54	661	0
		M	Cm490	Cp4.1LG02g15440	87.08	658	0
		W	Cl490	Cp4.1LG02g15440	86.48	1154	0
Potential oxidizer	Cp510	C	Cs510	Cp4.1LG05g02130	88.17	507	4E-171
		C	Cs510	Cp4.1LG15g03520	88.38	499	1E-170
		M	Cm510	Cp4.1LG05g02130	83.82	785	0
		M	Cm510	Cp4.1LG15g03520	88.18	499	5E-169
		W	Cl510	Cp4.1LG05g02130	90.14	497	0
		W	Cl510	Cp4.1LG15g03520	88.68	521	2E-180
Potential oxidizer	Cp560	C	Cs560	Cp4.1LG15g03520	80.12	488	1E-97
		M	Cm560	Cp4.1LG15g03520	92	125	3E-44
		W	Cl560B	Cp4.1LG15g03520	85.66	272	4E-75
Potential oxidizer	Cp530	W	Cs530	Cp4.1LG15g03520	83.7	135	7E-29
		W	Cs550	Cp4.1LG15g03520	77.8	473	2E-075

2407

Then, prior to BLUP calculation, samples with cucurbitacin D, E, or I values below the limit of detection were replaced with values drawn from a random uniform distribution between zero and the limit of detection (D: 0.05, E: 0.002, I: 0.005 ng/g) to allow for data transformation. Ratio values, however, were calculated from raw data, and were set to NA when the denominator value was below the limit of detection. Due to the non-normal distributions of phenotypes, all data was log transformed, except for the B:D ratio where a Box-Cox transformation with lambda=-2 was most appropriate and was conducted in the 'MASS' R package.

Best linear unbiased predictors (BLUPs) were calculated with the following model: y ~ check + (1:block) + (1|geno:new). After outlier removal, the number of F2 individuals were reduced in to the following for some phenotypes phenotypes: log(D)=187, log(E)=186, log(I)=185, box-cox(-2)BtoD=136. BLUPs were re-calculated with outliers removed, and then deregressed by dividing by 1- (BLUP error variance / genetic variance) (Garrick *et al.*, 2009). Broad-sense heritability on a plot basis was then calculated by dividing the genetic variance by total variance.

5.3.5 Isolating effects of cotyledon cucurbitacins on herbivore preference – Selection experiment. We tested the effect of selection for cotyledon cucurbitacins on *A. vittatum* preference for cotyledon, leaf, and floral tissues in June-July 2018 and 2019 in the CPP and CPO parents, as well as intersubspecific families derived from their cross. This allowed us to test the effect of cotyledon cucurbitacins on *A. vittatum* preference phenotypes independent of *C. pepo* population structure.

To generate the intersubspecific families, F_2 individuals from the previously

described F_2 population with the 10% highest and lowest extremes of total cucurbitacins (n = 14 of each extreme) were selected and self-pollinated to create $F_{2:3}$ families. Then, individuals from those $F_{2:3}$ families (n = 6, 7 individuals from different low and high $F_{2:3}$ families, respectively) were again selfed to create F_4 families. This process is summarized in **Fig. 5.1**. The $F_{2:3}$ families were evaluated in 2018 and the F_4 families were evaluated in 2019. It was necessary to evaluate succeeding generation each year due to limited seed quantity in the $F_{2:3}$ generation.

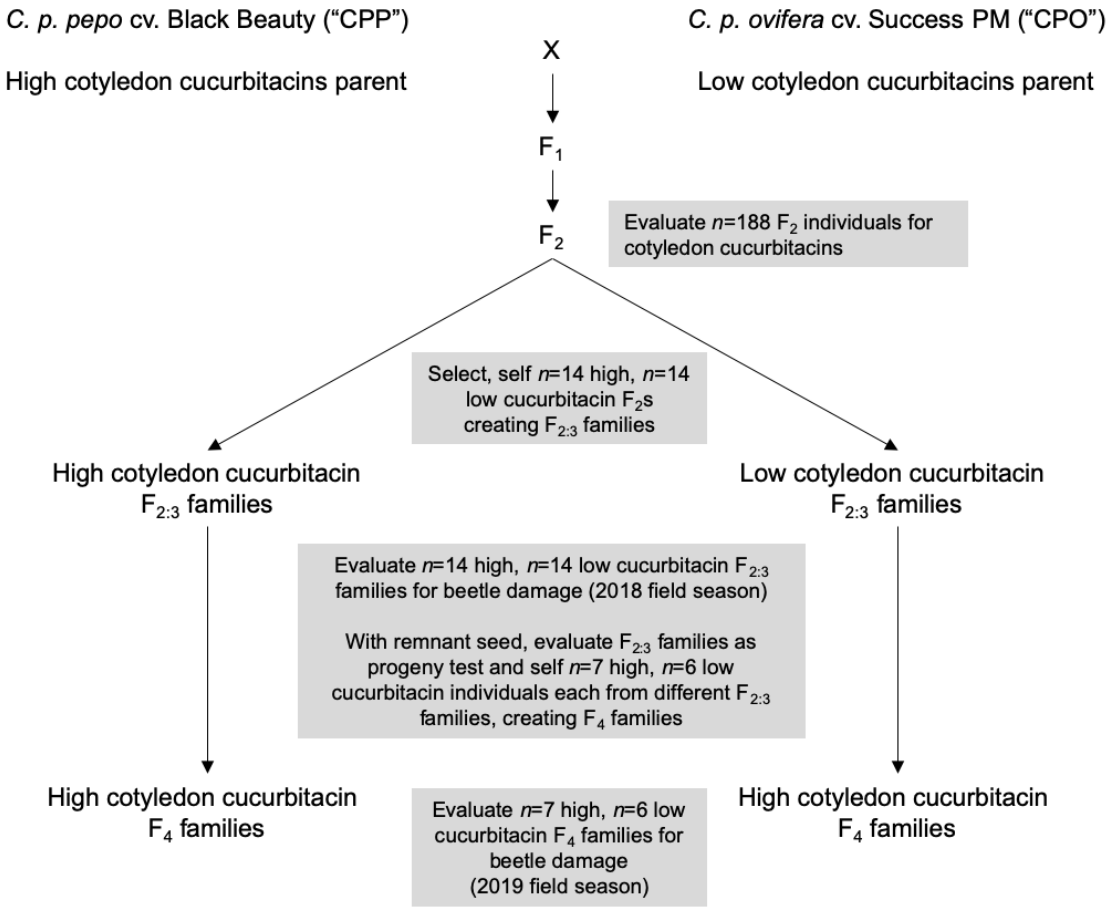

Figure 5.1. Pedigree and selection scheme of biparental population used in selection experiment for high and low cotyledon cucurbitacins.

Trials were conducted on the Homer C. Thompson Organic Vegetable Research Farm (Freeville, NY, USA), where there was naturally abundant *A. vittatum* infestation. Families and parents were evaluated by being: (1) sown into 72-cell peat pots and transplanted immediately after germination to measure damage to newly emerged cotyledons ("cotyledon plots"), and (2) sown into plastic 72-cell trays and transplanted with two fully expanded leaves to independently evaluate leaf damage ("leaf plots"). Both were transplanted into raised beds with black plastic mulch and drip irrigation in staggered double rows as plots of 12 plants each. Cotyledon plots had 15 cm spacing between plants within plots and 30 cm spacing between plots, and leaf plots had 45 cm between plants and 60 cm spacing between plots. There were four replicates of cotyledon plots, and three replicates of leaf plots.

Damage was visually estimated as percent defoliation of individual plants one week after transplanting for cotyledons in cotyledon plots, and two weeks after transplanting for leaves in both leaf and cotyledon plots. Beetles were counted at the plot level on foliage in cotyledon plots three weeks after transplanting (2018), and in floral tissue four weeks after transplanting in cotyledon (2018, 2019) and leaf (2019) plots.

Statistical analyses were conducted separately by year, and plot means (of a maximum of n=12 plants) were used for analysis of leaf and cotyledon damage. Plots with fewer than two flowers were excluded from analysis of floral count data, and $F_{2:3}$ plots (2018) with less than five (of 12) individuals were excluded from all analyses. Phenotypic differences between the CPP and CPO parent genotypes were evaluated with linear mixed models with a random effect of replicate and fixed effect of

genotype (CPO or CPP). To evaluate phenotypic differences due to cotyledon cucurbitacins in the intersubspecific families, there was a fixed effect of selection direction (high or low cotyledon cucurbitacins), random effect of family nested within selection direction, and a random effect of replicate. The single phenotype of floral counts from 2019 was evaluated with a different model for both parents and families: since floral counts were taken in both leaf and cotyledon plots, there was an additional fixed effect of plot type (cotyledon or leaf), along with its interaction with genotype (for parents) or selection direction (for families), and the random effect of replicate was nested within plot type. In all models, an ANOVA was conducted on the fixed effect.

5.3.6 Isolating effects of cotyledon cucurbitacins on herbivore preference – Induction experiment. In the selection experiment, we measured the correlation between *A. vittatum* damage to cotyledons and leaves, so we thus also sought to test if *A. vittatum* feeding induced cucurbitacin accumulation in leaves as a mechanistic connection between preference for the two tissues. Using the two parents (CPP and CPO), we tested induction by five *A. vittatum* feeding on cotyledons, or on leaves, and had a control of no beetles and measured cucurbitacins in both tissues at 24 h and 72 h after the start of beetle feeding.

Seeds were sown in June 2018 and transplanted into 0.52 L pots 12 days after sowing (1-2 expanded true leaves). Leaves and cotyledons were separated by mesh bags. Leaves were enclosed in small drawstring organza mesh bags (8 cm by 11 cm, SumDirect manufacturing, Dongguan, China) with an unraveled cotton ball wrapped

around the petioles to prevent beetle movement between leaf and cotyledon tissues. In the leaf induction treatments, five beetles were added to these bags. All plants were then enclosed in a larger mesh bag (25 cm by 30 cm; not shown).

At that point, five beetles *A. vittatum* were released on the appropriate tissue. After 24 h, beetles were removed from all plants. Cucurbitacins were immediately extracted from half of the plants (24 h time point) and extracted 48 h later from the rest (72 h time point). For each genotype and time, samples were taken from cotyledons (n=6 for beetle treatments, n=4 for controls) and leaves (n=4, n=3) of unique plants. Fewer leaf samples were taken because constitutive and induced cucurbitacins were expected to be low.

5.3.7 Genetic basis of cotyledon cucurbitacins – Biparental F2:3 family progeny evaluations. Cucurbitacins were measured in fully expanded cotyledons of individuals from eight low, and nine high cucurbitacin $F_{2:3}$ families derived from the intersubspecific cross between CPO and CPP (**Fig. 5.1**) to determine the inheritance of the trait. Seed availability limited sample sizes to ten or fewer individuals.

5.3.8 Genetic basis of cotyledon cucurbitacins– OSC marker development and testing in biparental population. Prior to genetic mapping in the biparental F_2 population, we tested if polymorphisms in the homolog of the oxidosqualene cyclase ("OSC") controlling the first committed step of cucurbitacin biosynthesis in cucumber [23] co-segregated with the F_2 phenotypes. We identified the homolog, which appears to be a single copy gene in *C. pepo*, developed four PCR primers and protocols, and

sequenced the products of the CPO and CPP parent lines with Sanger sequencing.

We developed four PCR primer pairs anchored in exons of the oxidosqualene cyclase ("OSC") gene homolog (**Table 5.1**) to amplify the gene and ordered from Integrated DNA Technologies (Coralville, Iowa, USA). Products were prepared by mixing 10 µL of 2 ng/µL DNA, 6.25 µL of sterile distilled water, 2 µL of 10x PCR buffer, 1 µL of 2.5 mM dNTPs, and 0.25 µL of each 10 µM forward primer, reverse primer, and Taq polymerase, and then amplifying with the thermocycler program of initial denaturation at 94˚C for 3 m, 35 cycles of 94˚C for 30 s, 60˚C for 30 s, and 72˚C for 90 s, and a final extension at 72˚C for 15 m. Products were visualized on 1% agarose gel stained with ethidium bromide. Products were prepared for Sanger sequencing with 5 µL of 10 ng/µL PCR product, 1 µL of 10 µM forward primer, and 12 µL of sterile distilled water. Sanger sequencing was performed at the Cornell University Institute of Biotechnology Genomics Facility (Ithaca, NY, USA) using Big Dye Terminator chemistry and AmpliTaq-FS DNA Polymerase on an Applied Biosystems Automated 3730xl DNA Analyzer (Thermo Fisher Scientific, Waltham, MA, USA) to assess sequence similarity.

Potential intronic regions were identified by aligning sequences to annotated sequences from the *C. pepo* genome annotated for introns in the web based Clustal Omega (EMBL-EBI). With the single primer pair with a 200 bp difference in product length between parent lines ("OSC5"), we screened nine each of high and low cucurbitacin individuals to determine if the marker co-segregated with the phenotype of interest.

5.3.9 Genetic basis of cotyledon cucurbitacins – Biparental F2 genetic mapping. DNA was extracted from 184 F_2 individuals, and two replicates of each parent (CPO and CPP) and their F_1 with a DNeasy 96 Plant Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A 192-plex genotyping-by-sequencing library was prepared at the University of Wisconsin-Madison Biotechnology Center (Madison, WI, USA), and sequenced at Cornell University Biotechnology Resource Center (Ithaca, NY, USA) on a NextSeq 500 (Illumina, San Diego, CA, USA) with single-end 75bp reads.

Reads were aligned to the *Cucurbita pepo* genome (v4.1) [40] with the 'bwa' aligner in the GBSv2 pipeline in TASSEL 5 [41]. In VCFtools [42], SNPs that were not biallelic, had extreme mean read depths (<5, or >75), low minor allele frequency (<0.05), or were missing in >20% of samples were removed. Then, only SNPs polymorphic between parents were retained. Using LB-Impute and a window size of five [43], SNPs were error-corrected and imputed with an accuracy of 92%. Finally, segregation ratios were evaluated in the 'qtl' R package with the 'geno.table' function [44] and those with $p<0.001$ from the expected 1:2:1 ratio were removed. In all, there were 183 remaining F_2 individuals, and 8962 remaining SNPs. This set of SNPs was retained for future multi-locus mixed model GWAS and TWAS analysis and was further pruned to be computationally manageable for linkage map construction and analysis.

A linkage map was constructed in R/qtl using the est.rf() and est.map() functions with a Kosambi mapping function. In this package, redundant SNPs were removed with the 'findDupMarkers' function, and unlinked markers and those

substantially increasing map length were removed (using the 'dropone' function), and individuals with more crossovers than expected were also removed. Chromosome 3 was split into two linkage groups presumably because of lack of recombination in the pericentromeric region between chromosome arms. In all, the 2927 SNPs were used in the linkage map, and total map length was 2407.7 cM (**Table 5.2**). QTL mapping was conducted in R/qtl using 'scanone' and 'scantwo' functions with Haley-Knott regression, and significant LOD thresholds were determined by 1000 permutations.

Table 5.2. Summary of linkage map developed for QTL mapping. N_{mar} is the number of markers in used in each linkage group.

Chromosome	N_{mar}	Length (cM)	Avg. spacing (cM)	Max. spacing (cM)
CP4.1LG01	272	191.2	0.7	4.7
CP4.1LG02	169	136.3	0.8	9.8
CP4.1LG03.1	96	68.5	0.7	8.5
CP4.1LG03.2	23	14.3	0.7	3.4
CP4.1LG04	212	156.1	0.7	11.1
CP4.1LG05	158	128	0.8	5.4
CP4.1LG06	105	104.1	1	20.5
CP4.1LG07	125	116.5	0.9	6.3
CP4.1LG08	145	130.5	0.9	8.6
CP4.1LG09	124	117.7	1	10.1
CP4.1LG10	171	120.3	0.7	5.5
CP4.1LG11	152	128.9	0.9	8.9
CP4.1LG12	151	135.1	0.9	5.8
CP4.1LG13	159	131.1	0.8	6.1
CP4.1LG14	120	108.2	0.9	6.8
CP4.1LG15	102	93.1	0.9	4.9
CP4.1LG16	122	115.2	1	8.2
CP4.1LG17	123	107	0.9	9.6
CP4.1LG18	114	97.5	0.9	6.1
CP4.1LG19	149	100.7	0.7	3.6
CP4.1LG20	135	107.6	0.8	5.6
Total	***2924***	***2407.9***		

A multi-locus mixed-model genome wide association study (GWAS) approach [45] was used to test for associations independently of a linkage map. It was implemented in R/mlmm.gwas [46], with the filtered (8962) and linkage map filtered (2927) SNP sets. An additive model was used, and set at a maximum of ten forward steps, and optimal models were selected based on lowest extended BIC [45].

5.3.10 Genetic basis of cotyledon cucurbitacins - Population structure in diverse germplasm. In addition to the biparental population, we extracted cucurbitacins from 117 diverse individuals from USDA *Cucurbita pepo* PI collection. Cucurbitacin B only was measured from 2-4 biological replicates of each genetic accession, where each replicate was pooled from two individuals to smooth heterogeneity within accessions. Extractions were conducted in an augmented incomplete block design of six blocks each with three replicates of CPO and CPP as checks. Then, BLUPs were calculated with the following model: log(cucB concentration) ~ check + (1|block) + (1|genotype:new), where 'check' was the identity of the checks and 'new' refers to whether the genotype was a check or not.

Publicly available GBS SNP data from the accessions [47] was filtered with a minor allele frequency of 0.05 and 25% missingness in sample calls, leaving 24,236 SNPs. Principal component analyses (PCA) was conducted on the genome-wide SNP marker matrix, and the marker matrix of SNPs within the interval identified by QTL mapping (n=16 SNPs) in TASSEL [48]. The first three principal components accounted for the following percent of variance (genome-wide: PC1: 85.2%, PC2: 9.2%, PC3: 5.6%; QTL mapping interval only: PC1: 61.0%, PC2: 30.8%, PC3: 8.2%),

and were used in analysis.

Using the linear model y ~ PC1 + PC2 + PC3, we calculated the coefficient of determination to determine the percent of variation in cotyledon cucurbitacin phenotype due to population structure. Then, a linear model was used to test the effect of region the accession was collected from and an ANOVA was conducted on the fixed effect. Both models were assessed with and without wild accessions (*n*=3 wild accessions) included.

5.3.11 Genetic basis of cotyledon cucurbitacins – Gene expression in the biparental F2 population. Cotyledon tissue was flash frozen on liquid nitrogen from all F_2 plants immediately prior to removing tissue for phenotyping cucurbitacins in the mapping population. Later, 21 high (range: 0.37 – 31.91 µg/g, mean: 11.72 µg/g) and 14 low (range: 0.006 – 0.022 µg/g, mean: 0.012 µg/g) cucurbitacin individuals were selected for gene expression analysis. RNA was extracted from the F_2 individuals and four samples of each parent and their F_1 using a modified hot-borate protocol. A 3′ RNA-seq library was then prepared and sequenced on an Illumina NextSeq 500 with single-end 75bp reads at the Cornell University Biotechnology Resource Center. The sequencing reads were processed by removing the first 12 base pairs and reads less than 25 base pairs in length with Trimmomatic [49], adapter sequence contaminants and polyA stretches greater than 12 in length stretches with cutadapt [50], and base calls with phred<5 with fastq_quality_trimmer [51]. Then the reads were aligned to the *C. pepo* v4.1 genome and counted with STAR [52]. The counts were normalized by library size by median ratio method in R/DEseq [53].

A transcriptome-wide association study (TWAS) was conducted by a linear regression of normalized count of each expressed gene (genes expressed in less than five individuals or with less than one count per million were dropped from the analysis, from 27868 to 22088) using a custom R script. Differential expression was assessed between F_2 individuals with cucurbitacins present and absent, and separately between parent lines in R/DEseq [53].

5.3.12 Mechanistic basis of cotyledon cucurbitacins - Temporal and spatial variation of cucurbitacins and gene expression during seedling development. To connect the genetic mapping results to biochemical mechanisms, cucurbitacins were measured in root, cotyledon, and leaf tissue over the course of seedling development from CPP and CPO at the stages of: (1) dry seed, (2) seeds imbibed for 24 h, (3) seedlings with radical emerging, (4) seedlings with cotyledons emerged but not green, (5) seedlings with cotyledons emerged and green, and (6) seedlings with cotyledons fully expanded and a leaf emerging. There were three biological replicates of each sample, where each sample was pooled from two to six individuals, and most had a mass of 0.5 g (but some were lower because of tissue limitations). Cucurbitacins were extracted from developing tissues in two independent experiments conducted in April and May 2019. In both, seeds sown daily for two weeks in saturated paper towels in a growth chamber held at 27 °C, and visually sorted into the categories for concurrent extractions. In April 2019, seedlings planted in potting soil (like those used in mapping populations) were also included with three to four replications each of cotyledon and root tissue.

Data were analyzed with linear models, where there was a fixed effect of

experiment (April or May 2019) in every analysis. First, within a genotype and tissue, the effect of developmental stage was tested. Then, for root cucurbitacin concentration alone, the interactive effects of genotype and developmental stage were tested. Finally, for CPP only, the interactive effects of tissue and developmental stage were tested. In all cases, effect significance was determined with a one-way ANOVA, and differences between effect levels were determined by Tukey honest significant differences at $p < 0.05$.

Because of size limitations, cotyledon, root, and leaf tissue was collected for RNA from an independent set of plants in October 2019. Two time points were sampled - radical emergence, and fully expanded cotyledons - and there were three replicates of leaf tissue, and five to six replicates of all other tissues. Tissue was ground in liquid nitrogen with a mortar and pestle, and RNA was extracted using a Plant RNeasy kit (Qiagen, Hilden, Germany) according to manufacturer protocols. Library preparation, sequencing, read processing, alignment and generation of normalized counts were conducted as previously described. Genome-wide differential expression was analyzed in R/DEseq between genotypes within tissue and developmental stage, and between times within genotype and tissue. Genome wide differential expression statistics are reported for expression of key biosynthetic genes (**Table 5.1**). Genome-wide differential expression results are also reported for genes in the interval identified in QTL mapping of total cucurbitacin concentration in cotyledons at the radical emerging stage.

5.3.13 Statistics. Statistical computations were done in R [54]. Basic summary statistics and linear models were evaluated in base R with the 'lm' function. Linear mixed models were evaluated with the 'lmer' function R/lme4 [55]. Pearson's correlations were calculated with the 'rcorr' function in R/Hmisc.

5.4 Results

5.4.1 Isolating effects of cotyledon cucurbitacins on herbivore preference. We developed a biparental F_2 population between a high cotyledon cucurbitacin *C. pepo* ssp. *pepo* (CPP, 8.04 +/- 1.14 µg/g) and low cotyledon cucurbitacin *C. pepo* ssp. *ovifera* (CPO, 0.02 +/- 0.01 µg/g) and selected progeny with phenotypic extremes of cotyledon cucurbitacins (**Fig. 5.1**). This allowed us to assess the effect of cotyledon cucurbitacins on specialist herbivore, *Acalymma vittatum*, preference of multiple tissues independent of the confounding population structure of the two subspecies.

Over two years of field trials, CPP sustained greater cotyledon (2018: 149% more, $F_{1,5}$=9.17 p=0.03; 2019: 61% more, $F_{1,5}$=37.07 p=0.002) and leaf (2018: 213% more, $F_{1,3}$=19856 p<0.001; 2019: 531% more, $F_{1,3}$=38.10 p=0.008) damage than CPO, despite neither having appreciable leaf cucurbitacin concentration (**Fig. 5.2**). In contrast, while fully expanded cotyledons of the families derived from selecting for high cotyledon cucurbitacins sustained more cotyledon damage than families selected for low cotyledon cucurbitacins (2018: 69% more, $F_{1,95}$=23.02 p<0.001; 2019: 19.2% more, $F_{1,48}$=30.73 p<0.001), leaf damage when three true leaves were present was equivalent between selection directions (2018: $F_{1,56}$=0.63, p=0.43; 2019: $F_{1,35}$=0.70, p=0.41) (**Fig. 5.2**).

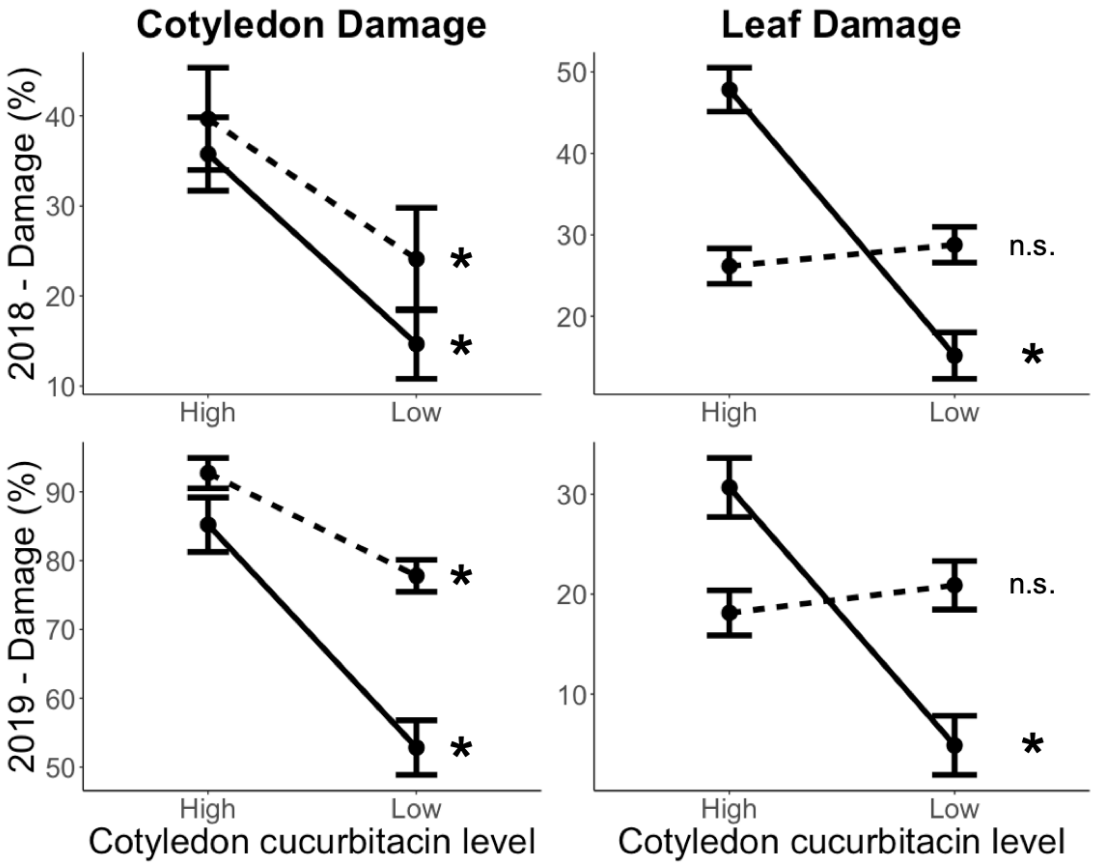

Figure 5.2. Damage to cotyledons and leaves of parent lines and selected families in field trials from 2018 and 2019. The parents (solid lines) and families (dashed lines) are classified as 'low' (*C. p. ovifera* and derived from low cucurbitacin F_2 individuals, respectively) or 'high' (*C. p. pepo* and derived from high cucurbitacin F_2 individuals, respectively). The position of the dots is the estimated marginal mean and error bars are +/- 1 SE and the statistics are given in text.

In addition, there was no induction of cucurbitacins in leaf or cotyledon tissue by *A. vittatum* feeding on either tissue of CPP or CPO, further indicating that leaf preference is mechanistically independent of cotyledon cucurbitacins (**Table 5.3**).

Table 5.3. Cucurbitacin concentration in leaf and cotyledon tissue ("Tissue measured") as a result of *Acalymma vittatum* feeding for 24 h (and then removed) measured at that point (24 h from start of feeding) or two days after removing beetles (72 h from start of feeding). Beetles were either allowed to feed on cotyledon or leaf tissue, and there were control plants (no beetle feeding). Values are means +/- 1 SE in ng/g FW. ANOVA results are given from the model of cucurbitacins as a function of treatment for each genotype and time.

Tissue measured	Time after start (h)	Beetle feeding treatment	*C. p. ovifera* cv Success PM		*C. p. pepo* cv Black Beauty	
			ng/g (FW)	ANOVA	ng/g (FW)	ANOVA
Cotyledons	24h	None	4+/-0.2	$F_{2,12}$=0.12	873+/-264	$F_{2,13}$=0.12
		Cotyledon	4+/-1	*p*=0.89	717+/-152	*p*=0.82
		Leaf	4+/-1		797+/-118	
	72h	None	2+/-0.4	$F_{2,13}$=0.45	863+/-220	$F_{2,13}$=0.70
		Cotyledon	2+/-0.2	*p*=0.65	623+/-82	*p*=0.51
		Leaf	1+/-0.2		680+/-135	
Leaves	24h	None	0.2+/-0.1	$F_{2,8}$=0.78	0.5+/-0.2	$F_{2,8}$=0.12
		Cotyledon	16.9+/-16.5	*p*=0.49	0.4+/-0.2	*p*=0.90
		Leaf	1.4+/-1.2		0.4+/-0.0	
	72h	None	0.2+/-0.1	$F_{2,8}$=0.34	0.3+/-0.0	$F_{2,8}$=0.84
		Cotyledon	0.4+/-0.2	p=0.72	0.2+/-0.0	*p*=0.47
		Leaf	0.4+/-0.0		1.8+/-1.6	

At a later developmental stage, foliar beetle density was equivalent on parental lines ($F_{1,5}$=0.01 *p*=0.95), but there were 34% fewer on low cotyledon cucurbitacin families ($F_{1,92}$=4.32 *p*=0.04). Beetle density in flowers of reproductively mature plants varied between parents in only one of the two years (2018: equivalent, $F_{1,5}$=0.50 *p*=0.51; 2019: 64% fewer beetles in CPO, $F_{1,6}$=6.78 *p*=0.04), but did not differ between high and low families (2018: $F_{1,87}$<0.001 *p*=0.99; 2019: $F_{1,81}$=0.73 *p*=0.40).

Due to the importance of cotyledon cucurbitacins on herbivore preference of cotyledon tissue, our continued efforts focused on characterizing the genetic and mechanistic bases of cotyledon cucurbitacins.

5.4.2 Genetic basis of cotyledon cucurbitacins – Biparental population mapping. We assessed cotyledon cucurbitacins in fully expanded cotyledons of the parents, F_1 and F_2 biparental population. The cucurbitacin phenotype of the F_1 resembled CPP, and the F_2 progeny cucurbitacin concertation ranged from 0.003-36.3 µg/g, where cucurbitacin B was the most abundant compound (**Table 5.4**) and all cucurbitacins were positively correlated (**Table 5.5**). The broad-sense heritability on a plot basis was high for total cotyledon cucurbitacins (0.97), as well as individual compounds (**Table 5.4**). When we categorized F_2 individuals with total cucurbitacin concentration at or below the CPO mean as "low" cucurbitacins, there was a 3:1 ratio of high to low cucurbitacin individuals (Observed: 131:57, Expected: 141:47, X^2=1.08, df=1, p=0.30). Together, this result is consistent with a single dominant Mendelian gene model for cotyledon cucurbitacin accumulation, as previously demonstrated by Sharma and Hall (1971).

Table 5.4. Summary statistics of cotyledon cucurbitacin concentrations (ug/g fresh weight) from low (*C. p. ovifera*) and high (*C. p. pepo*) cotyledon cucurbitacin parent lines, their F_1, and the F_2 population as well as broad sense heritability (H^2) calculated from F_2 phenotypes. The following numbers of individuals were used: high parent, *n*=12; low parent, F_1, *n*=11; F_2 individual and total cucurbitacins, *n*=188; F_2 BD:EI ratio, *n*=154; F_2 B:D ratio, *n*=138; F_2 E:I ratio, *n*=47.

Phenotype	Genotype	Mean	SE	Min	Max	H^2
Cucurbitacin B	High parent	7.01	1.02	2.15	12.53	0.965
	Low parent	0.01	0.00	0.01	0.02	
	F_1	8.15	2.06	3.62	27.26	
	F_2	5.22	0.46	0.00	33.77	
Cucurbitacin D	High parent	0.90	0.12	0.23	1.55	0.771
	Low parent	0.01	0.01	0.00	0.06	
	F_1	0.74	0.13	0.20	1.79	
	F_2	0.63	0.06	0.00	3.95	
Cucurbitacin E	High parent	0.11	0.02	0.01	0.23	0.464
	Low parent	0.00	0.00	0.00	0.00	
	F_1	0.05	0.02	0.01	0.20	
	F_2	0.02	0.00	0.00	0.25	
Cucurbitacin I	High parent	0.03	0.00	0.00	0.05	0.336
	Low parent	0.00	0.00	0.00	0.00	
	F_1	0.01	0.00	0.00	0.02	
	F_2	0.00	0.00	0.00	0.04	
Total cucurbitacins	High parent	8.04	1.14	2.66	14.24	0.968
	Low parent	0.02	0.01	0.01	0.08	
	F_1	8.94	2.21	3.90	29.27	
	F_2	5.88	0.51	0.00	36.32	
B:D ratio	High parent	8.4	0.9	4.6	14.3	0.998
	Low parent	0.4	NA	0.4	0.4	
	F_1	11.0	1.0	7.2	18.1	
	F_2	8.5	0.4	0.4	23.1	
E:I ratio	High parent	4.0	0.5	1.9	6.9	0.706
	Low parent	NA	NA	NA	NA	
	F_1	6.5	0.7	4.8	9.6	
	F_2	4.7	0.2	1.5	13.7	
BD:EI ratio	High parent	122.4	49.6	33.9	647.2	0.797
	Low parent	4.3	1.0	1.9	6.6	
	F_1	322.7	84.1	115.0	1041.8	
	F_2	478.5	37.4	1.2	2318.7	

Table 5.5. Pearson correlation coefficients between cotyledon concentrations of cucurbitacins from F_2 population, where numbers in bold indicate $p<0.05$.

Genotype	Cucurbitacin	D	E	I
High parent	B	**0.81**	**0.90**	**0.61**
	D	1	**0.81**	**0.82**
	E		1	**0.81**
Low parent	B	0.52	-0.05	NA
	D	1	-0.15	NA
	E		1	NA
F_1	B	**0.95**	**0.98**	**0.89**
	D	1	**0.93**	**0.90**
	E		1	**0.94**
F_2	B	**0.87**	**0.68**	**0.56**
	D	1	**0.47**	**0.42**
	E		1	**0.87**

We tested if different alleles for the first committed biosynthetic step (oxidosqualene cyclase, 'OSC', **Table 5.1**) caused the observed F2 phenotypes. We sequenced OSC in the parents, identified only intronic indels, and developed a codominant size-polymorphic PCR marker. Marker state in the F_2s did not co-segregate with the phenotype, but rather there was a 1:2:1 segregation ratio expected of an unlinked gene within phenotypic class (high: observed, 3:5:1; Fisher's exact test p=1; low: observed, 2:6:1; Fisher's exact test p=1).

We then conducted QTL mapping with BLUPs calculated from the quantitative phenotype and found a single locus for total cotyledon cucurbitacins on chromosome 5 (LOD 77, $p<0.001$), explaining 87% of phenotypic variation (**Fig. 5.3**, **Table 5.6**), hereafter referred to as "*Bi-4*" (Paris & Padley, 2014). The *Bi-4* interval (recombination breakpoint markers, SCP4.1LG05_1542768, SCP4.1LG05_1802502) is a 259.7 kB, 3.5 cM region containing 47 genes, none of which are cucurbitacin biosynthetic candidate gene homologs (**Table 5.1**). This locus was significant for

cucurbitacins B, D, E and the BD:EI ratio, but other marginally significant loci were detected for the B:D and E:I ratios, and none were detected for cucurbitacin I (**Table 5.6**).

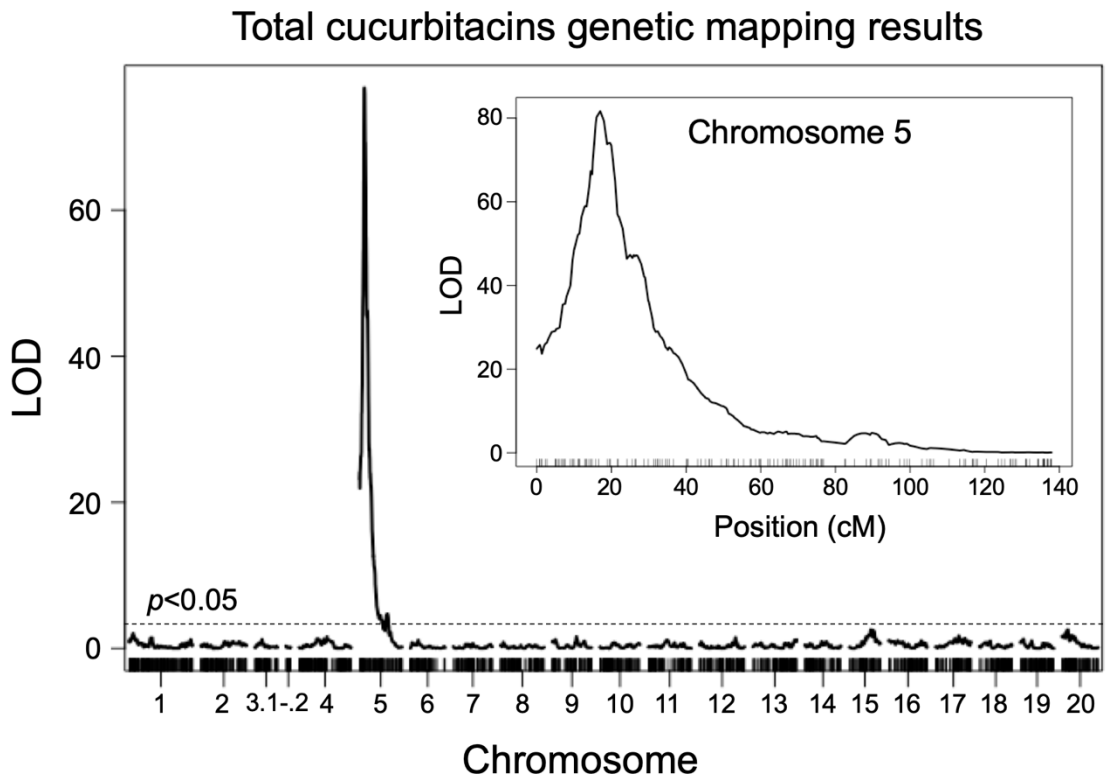

Figure 5.3. Single QTL mapping result for total cucurbitacins. The main plot is genome-wide logarithm of odds (LOD) score, and the inset is chromosome 5 alone. The dashed line indicates significance at $p<0.05$ as determined by 1000 permutations.

Table 5.6. Single QTL mapping model summary for all cotyledon cucurbitacin phenotypes. Results are given by the chromosome (Chr), position in linkage map, logarithm of odds (LOD) score, associated *p*-value. PVE is the phenotypic variance explained (%) by the locus, and *n* is the number of individuals used in mapping.

Phenotype	Chr	Position (cM)	LOD	*p*	PVE (%)	*n*
Total cucurbitacins	5	15	76.7	**<0.001**	87.0	173
Cucurbitacin B	5	15	69.2	**<0.001**	84.2	173
Cucurbitacin D	5	15	48.2	**<0.001**	72.5	172
Cucurbitacin E	5	18	26.1	**<0.001**	50.5	171
Cucurbitacins BD:EI ratio	5	14.9	34.8	**<0.001**	67.9	141
Cucurbitacins B:D ratio	12	107	4.1	0.068	13.9	125
Cucurbitacins E:I ratio	2	122	4.8	**0.024**	40.3	42

Three additional loci, two additive and one epistatic, were identified using a two QTL model (**Table 5.7**). The same genomic regions were associated with cucurbitacins using a multi-locus mixed-model GWAS approach (**Table 5.8**), verifying our results independent of a genetic linkage map. In addition, evaluations of $F_{2:3}$ progeny where the haplotype of the F_2 progenitor in the *Bi-4* interval was known demonstrated phenotypes are predicted by the genotype at this single Mendelian locus (**Fig. 5.4**).

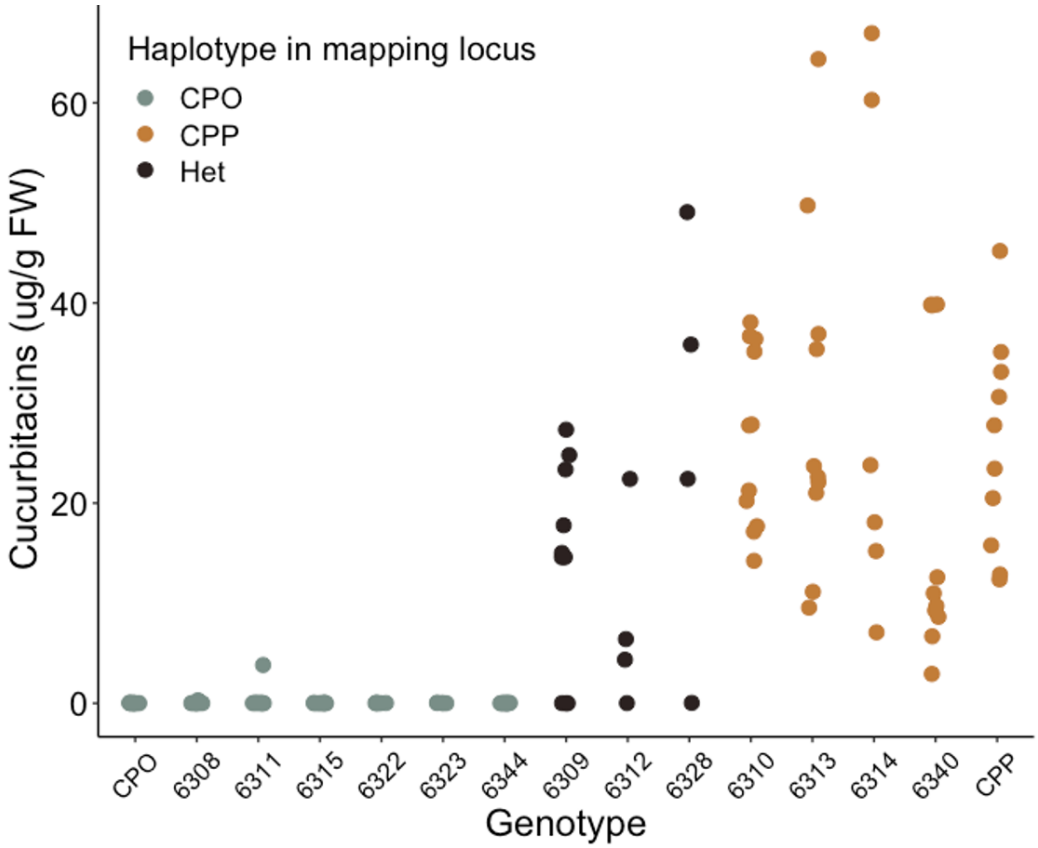

Figure 5.4. Results of $F_{2:3}$ progeny evaluations where total cucurbitacins (ug/g FW) were measured from F_2 individuals with known haplotypes in the *Bi-4* interval. The points represent phenotype of a single individual from a given $F_{2:3}$ family ("63NN"), or parent checks *C. p. ovifera* ("CPO") and *C. p. pepo* ("CPP"). The color is the haplotype of the F_2 progenitor at the locus identified by genetic mapping.

Table 5.7. Multiple QTL mapping model summary for all cotyledon cucurbitacin phenotypes where there were significant additive or interactive effects of multiple QTL. Results are given for the full model, and individual interaction and additive components.

		QTL-1		QTL-2		Full model			Interaction		Additive	
Phenotype	*n*	Chr	Pos(cM)	Chr	Pos(cM)	LOD	*p*	PVE (%)	LOD	*p*	LOD	*p*
Total cucurb.	173	5	15	2	120	95.0	**<0.001**	92.0	7.8	**0.009**	87.2	**<0.001**
Cucurbitacin B	173	5	15	2	120	85.1	**<0.001**	89.6	7.1	**0.033**	78.0	**<0.001**
Cucurbitacin D	172	5	15	2	120	65.2	**<0.001**	82.4	7.1	0.076	58.1	**<0.001**
Cucurbitacin E	171	5	16	2	121	42.4	**<0.001**	67.7	4.7	0.962	37.8	**<0.001**
Total cucurb.	173	5	15	15	68	84.2	**<0.001**	89.4	2.8	1	81.4	**<0.001**
Cucurbitacin B	173	5	16	15	68	77.1	**<0.001**	87.2	3.5	1	73.6	**<0.001**
Cucurbitacin E	171	5	18	20	81	37.6	**<0.001**	63.2	6.5	0.098	31.2	**<0.001**
BD:EI ratio	141	5	15	20	79	44.6	**<0.001**	69.5	1.8	1	42.8	**<0.001**

Table 5.8. Multi-locus mixed-model genome wide association study results. Results are presented from analysis with filtered SNPs (8962) and the subset used in linkage map construction (2927). The model cofactors are indicated with a (*), and significant SNPs are reported with false discovery rate adjusted *p* values. There were no significant hits for cucurbitacin I or the B:D ratio.

	QTL mapping SNP set			*Filtered SNP set*		
Phenotype	SNP	p_{adj}	cofactor	SNP	p_{adj}	cofactor
Total	SCP4.1LG05_1935823	9.43E-32	*	SCP4.1LG05_1948397	4.00E-35	*
B	SCP4.1LG05_1935823	1.17E-30	*	SCP4.1LG05_1948397	4.47E-34	*
D	SCP4.1LG05_1935823	4.87E-21	*	SCP4.1LG05_1948397	1.73E-22	*
	SCP4.1LG02_12370592	1.67E-02		SCP4.1LG02_12756285	4.69E-02	
	SCP4.1LG02_12495410	2.24E-02		SCP4.1LG02_12778505	4.21E-02	
	SCP4.1LG02_12507558	3.17E-02		SCP4.1LG02_12778597	4.21E-02	
	SCP4.1LG02_12596140	3.26E-02		SCP4.1LG02_12789916	4.21E-02	
	SCP4.1LG02_12639192	1.67E-02		SCP4.1LG02_12790047	4.21E-02	
	SCP4.1LG02_12696386	2.60E-02		SCP4.1LG02_12855045	4.21E-02	
	SCP4.1LG02_12739902	1.67E-02		SCP4.1LG02_12855055	4.21E-02	
	SCP4.1LG02_12778505	1.67E-02		SCP4.1LG02_12893842	4.21E-02	
	SCP4.1LG02_12855045	1.67E-02		SCP4.1LG02_12893932	4.21E-02	
	SCP4.1LG02_12893842	1.67E-02		SCP4.1LG02_12893935	4.21E-02	
	SCP4.1LG02_12932024	1.67E-02		SCP4.1LG02_12932024	4.21E-02	
				SCP4.1LG02_12932155	4.21E-02	
E	SCP4.1LG05_1639082	1.10E-17	*	SCP4.1LG05_1948397	2.59E-20	*
	SCP4.1LG02_12739902	2.64E-07	*	SCP4.1LG02_12756285	4.81E-06	*
BD:EI ratio	SCP4.1LG05_1935823	2.46E-07	*	SCP4.1LG05_1948397	1.03E-08	*

5.4.3 Genetic basis of cotyledon cucurbitacins – Diverse germplasm evaluation. We measured cotyledon cucurbitacins in 117 diverse, globally-sourced USDA PI *C. pepo* accessions and inferred population structure from genetic data beyond that previously characterized in US germplasm [34] to evaluate our QTL mapping results more widely. First, accessions collected from the Americas had lower cotyledon cucurbitacin concentration than those collected from Europe, Africa and Asia (including wild accessions: $F_{3,113}$=6.27, p<0.001; excluding wild accessions: $F_{3,110}$=7.56, p<0.001, **Fig. 5.5**). While the USDA collection is not indexed by subspecies, CPP germplasm is widely distributed globally, but CPO germplasm is uncommon outside of the Americas (Paris, 2000). In addition, we found that population structure accounts for a substantial amount of phenotypic variance in cotyledon cucurbitacins both when population structure is inferred based on genome-wide markers those in the *Bi-4* interval (**Table 5.9**).

Table 5.9. Percent variation of cotyledon cucurbitacin B concentration explained by population structure of diverse USDA *C. pepo* plant accessions.

R^2_{adj}	Genome-wide	*Bi-4* interval
Including wild accessions	0.276	0.345
Excluding wild accessions	0.353	0.436

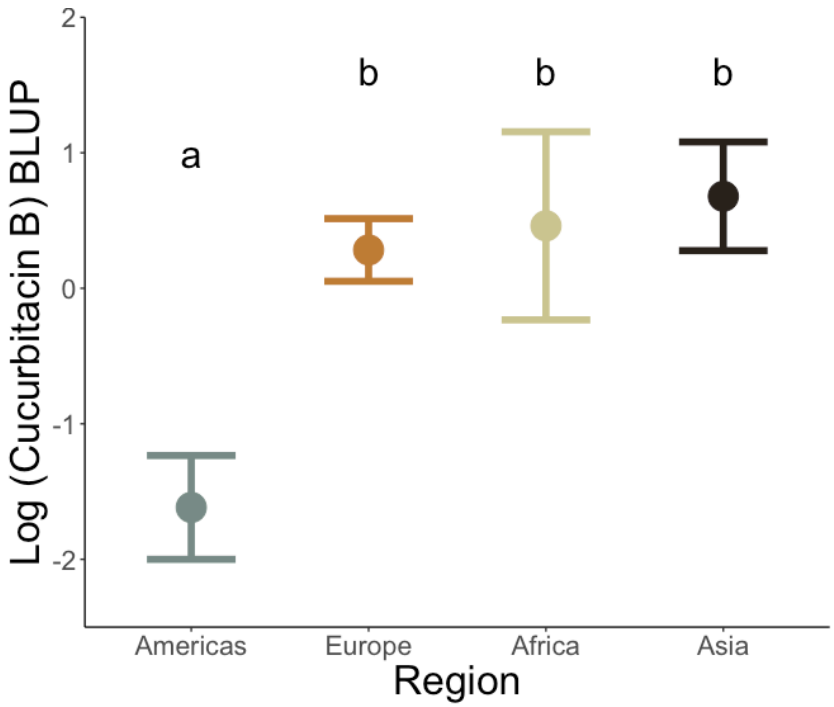

Figure 5.5. Cucurbitacin B in diverse *C. pepo* accessions from the USDA PI collection by geographical region from which the accession was collected. The point is the estimated marginal mean of all BLUP values with error bars +/- 1 SE, and letters are Tukey HSD $p<0.05$.

5.4.4 Genetic basis of cotyledon cucurbitacins – Biparental population gene expression. We next measured gene expression in 35 select F_2 individuals in tissue collected concurrently with cucurbitacin extraction (fully expanded cotyledons). In a TWAS to quantitatively associate gene expression levels and cucurbitacins, six genes met a modest genome-wide significance threshold ($p_{adj}<0.20$; **Table 5.10**). Two genes were in the *Bi-4* interval: Cp4.1LG05g02530, a multidrug and toxic compound extrusion (MATE) transporter and Cp4.1LG05g03720, an unknown protein. When we classified F_2 phenotypes qualitatively as present or absent for cucurbitacins, we found 41 differentially expressed (DE) genes between F_2 individuals. The MATE transporter was the most significant DE gene genome-wide in F_2 individuals (log2foldChange = 7.06, $p_{adj}<0.001$) and was also DE between parents (log2foldChange = 44.06,

p_{adj}<0.001). Biosynthetic genes were not expressed in cotyledons of F_2 individuals or parents at the developmental stage sampled (fully expanded cotyledons).

Table 5.10. Results from transcriptome wide association analysis (TWAS) of F_2 individuals. Genes and FDR-adjusted *p*-values are presented for the most significant results.

Gene	p_{adj}	In *Bi-4* interval	Gene description
Cp4.1LG05g02690	0.006	No, upstream	Ribosomal protein L15
Cp4.1LG05g02850	0.110	No, downstream	SNF1-related protein kinase regulatory subunit beta-2
Cp4.1LG05g03720	0.110	yes	Unknown
Cp4.1LG05g02530	0.119	yes	MATE transporter
Cp4.1LG04g06320	0.135	No, different chromosome	Unknown
Cp4.1LG05g02570	0.151	No, upstream	Oxidoreductase family protein

5.4.5 Mechanistic basis of cotyledon cucurbitacins. Finally, we sought to connect the genetic mapping results and observed cotyledon cucurbitacin phenotypes by profiling cucurbitacin accumulation and gene expression in multiple tissues over the course of seedling development. This allowed us to evaluate evidence for cross-tissue transport (as suggested by the MATE transporter), and for potential regulators in the *Bi-4* interval that may be expressed prior to full cotyledon expansion.

Cotyledon cucurbitacins in CPP steadily rose beginning at radical emergence and diminished once cotyledons were fully expanded (developmental stage effect: $F_{5,36}$=33.92, p<0.001; **Fig. 5.6a**), while cotyledon cucurbitacins in CPO were minimal and did not vary over development ($F_{5,16}$=0.92, p=0.49; **Fig. 5.6c**). Unexpectedly, there were cucurbitacins in roots of CPO and CPP, but CPP had 80% greater concentration ($F_{1,38}$=29.04, p<0.001). Root cucurbitacin concentrations did not vary

by developmental stage within or between genotypes (within CPP, $F_{3,26}$=2.49, p=0.08 **Fig. 5.6b**; within CPO, $F_{3,11}$=0.98, p=0.44 **Fig. 5.6d**; between, $F_{3,38}$=2.52, p=0.07). Cucurbitacin composition varied between tissues: cucurbitacin B was most abundant in cotyledons, but E was highly abundant in root tissue of both CPP and CPO (**Fig. 5.6b, d**). In a parallel analysis of soil-grown plants at the fully expanded time point, cucurbitacin concentration was 9-fold lower, due to the larger size, but we observed the same magnitude of contrasts (data not presented). Overall, these results indicate that both CPP and CPO can synthesize cucurbitacins, but in CPO cucurbitacins exclusively accumulate in roots.

We evaluated expression of cucurbitacin biosynthetic gene homologs at two time points of seeding development (radical emergence, fully expanded cotyledons). We found that biosynthetic genes were expressed in all tissues where cucurbitacins accumulated (CPP cotyledons, **Fig**. **5.6a**; CPP roots, **Fig**. **5.6b**; CPO roots, **Fig**. **5.6d**), and not in tissues lacking accumulation (CPO cots **Fig**. **5.6c**; leaves of both, **Fig**. **5.6e**). There was also a temporal shift in biosynthetic gene expression: expression was greatest when the radical was emerging but attenuated by full cotyledon expansion. While this suggests a regulatory component of cucurbitacin biosynthesis, the homolog of the known tissue-specific regulatory bHLH transcription factor homolog from other Cucurbitaceae was not expressed (**Table 5.11**). Count data and differential expression statistical results for all tissues are presented in **Table 5.11**.

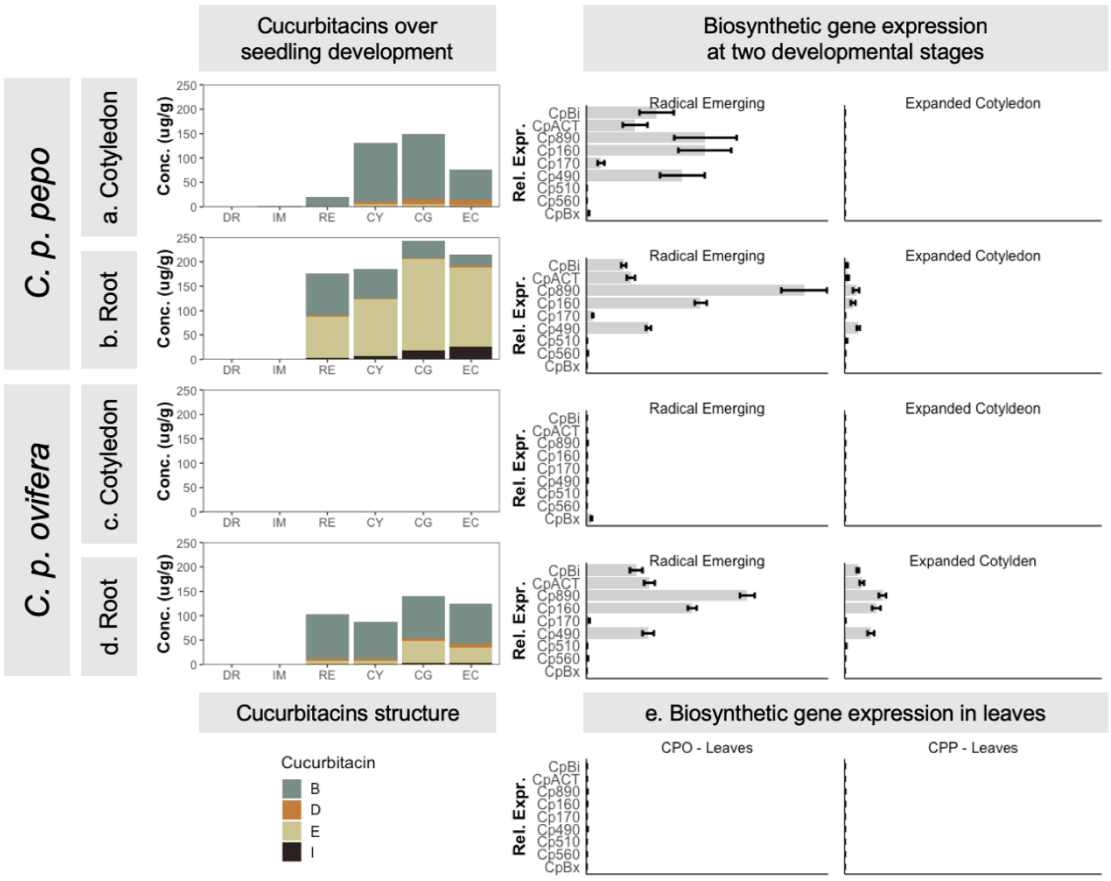

Figure 5.6. Cucurbitacin accumulation and gene expression over seedling development. Each row represents a specific genotype and tissue: (a) high cotyledon cucurbitacin *C. p. pepo*, "CPP" cotyledons, "cot.", (b) CPP roots, (c) low cotyledon cucurbitacin *C. p. ovifera*, "CPO" cotyledons, (d) CPO roots, (e) leaves of CPO and CPP. The leftmost column is cucurbitacin concentration (ug/g FW) over seedling development where the x-axis is developmental stage (DR: dry seed, IM: imbibed seed, RE: radical emerging, CY: cotyledons emerged, not yet green, CG cotyledons emerged and green, EC: full expanded cotyledons, and leaf emerging). The middle and right columns are relative gene counts (relative to highest count) on identical scales for biosynthetic homologs in the cucurbitacin pathways, ordered by importance, for the RE and EC time points. Complete gene homolog descriptions are provided in Table S2 (CpBi: oxidosqualene cyclase, CpACT: acetyltransferase, Cp890: cucurbitadienol oxidizer, CpNNN: other potential oxidizers, CpBx: putative tissue-specific transcription factor). Statistical results of differential gene expression are given in Table S4.

Table 5.11. Expression of major biosynthetic gene homologs at two points in seedling development, radical emergence ('RE') and expanded cotyledons ('EC'), in root, cotyledon and leaf tissue, to accompany Figure 5. The counts given are library-size normalized mean counts +/- 1 standard error. The *p*-values are genome wide differential expression between genotypes (*C. p. pepo*, 'CPP'; *C. p. ovifera*, 'CPO'), within time points and tissues horizontally, and then between time points, within genotypes and tissues vertically. Bold values indicated p-values <0.05.

		ROOTS			**COTYLEDONS**			**LEAVES**		
	Time	CPP	CPO	*p*	CPP	CPO	*p*	CPP	CPO	*p*
Oxidosqualene cyclase Cp4.1LG12g10070 (CpBi)	RE	564 +/- 39	745 +/- 95	0.23	1051 +/255	2 +/- 0.8	**8E-46**	NA	NA	
	EC	36 +/- 9	184 +/- 17	**3E-09**	2+/- 0.7	2 +/- 0.9	0.95	2 +/- 2	4 +/- 2	0.76
	p	**2E-38**	**7E-18**		**1.3E-37**	0.65				
Cucurbitadienol oxidation Cp4.1LG09g10760 (Cp890)	RE	3278+/- 45	2401 +/- 11	**0.02**	1776 +/-465	14 +/- 6	**3E-18**	NA	NA	
	EC	159 +/- 45	529 +/- 52	**2E-05**	4 +/- 1.7	6.6 +/- 2.4	0.76	6 +/- 4	11 +/- 6	0.75
	p	**3E-37**	**6E-52**		**1E-31**	0.51				
Acetylation Cp4.1LG17g09410 (CpACT)	RE	673 +/- 61	944 +/- 82	**0.01**	729 +/- 184	5 +/- 2	**1E-21**	NA	NA	
	EC	43 +/- 14	240 +/- 30	**3E-08**	0.4 +/- 0.2	4 +/- 1	0.06	1 +/- 1	4 +/- 2	0.5
	p	**3E-31**	**3E-26**		**1E-22**	0.87				
Potential oxidizer Cp4.1LG17g09580 (Cp160)	RE	1724 +/- 90	1584 +/- 65	0.29	1767 +/- 396	3 +/- 0.2	**2E-64**	NA	NA	
	EC	118 +/- 32	443+/-60	**5E-05**	4+/-1	6+/- 1	0.74	8 +/- 2	10 +/- 3	0.81
	p	**2E-29**	**5E-26**		**3E-53**	**0.02**				
Potential oxidizer Cp4.1LG12g10100 (Cp170)	RE	98 +/-9	43 +/- 7	**3E-05**	219 +/- 48	2 +/- 0.4	**9E-28**	NA	NA	
	EC	6 +/- 2	10 +/- 2	0.35	0.6 +/- 0.2	1+/- 0.7	0.76	0	0.3 +/- 0.3	NA
	p	**6E-26**	**3E-09**		**2E-16**	0.773				
Potential oxidizer Cp4.1LG02g15440	RE	937 +/- 38	925 +/- 85	0.70	1425+/-334	13 +/- 1.9	**1E-58**	NA	NA	
	EC	191 +/- 22	366 +/- 48	**7E-03**	1.8+/- 0.6	3.6 +/-1.3	0.553	4 +/- 1.5	14 +/- 3	0.25

(Cp490)	*p*	**2E-37**	**5E-11**		**3E-43**	**0.05**				
Potential oxidizer Cp4.1LG05g02130 (Cp510)	RE	5 +/- 2	8 +/- 3	0.54	0	0.2 +/- 0.2	NA	NA	NA	
	EC	27 +/- 10	21+/- 3	0.52	0	0.6 +/- 0.4	NA	0.7 +/- 0.7	1 +/- 1	NA
	p	**1E-04**	**0.01**		NA	0.54				
Potential oxidizer Cp4.1LG15g03520 (Cp560)	RE	21 +/- 3	20+/- 5	0.88	0	0.4 +/- 0.2	NA	NA	NA	
	EC	8 +/- 3	6 +/-1	0.75	0.6 +/- 0.4	0.2 +/- 0.2	NA	3+/- 2.5	3.3 +/- 0.7	0.97
	p	**9.8E-03**	**2E-03**		NA	NA				
Tissue TF Cp4.1LG13g02020 (CpBx)	Rad	0	0.2 +/- 0.2	NA	40 +/- 4	76 +/- 11	**1E-03**	NA	NA	
	Full	0.2 +/- 0.0	0	NA	2 +/- 0.4	1.4 +/- 1	0.86	0	0	NA
	p	NA	NA		**2E-10**	**2E-16**				

We also examined expression of the 47 genes in the *Bi-4* interval in CPP and CPO during seedling development. Thirty-four of these genes were expressed in cotyledons at the time of radical emergence, 10 of which were DE, including five that have been implicated in other species as relevant for regulation or transmembrane transport (**Table 5.12**). Of particular interest are two genes with greater expression in CPP: the MATE transporter, and Cp4.1LG05g03830, an ACT containing regulatory protein induced by abscisic acid (ABA). The ACT protein is also DE between parents and F_2 individuals at the fully expanded cotyledon stage.

Table 5.12. Candidate genes from the *Bi-4* interval based on differential expression in cotyledons of parent lines at the radical emerging time point. A positive Log2FoldChange value indicates greater expression in CPP, and negative corresponds to greater expression in CPO. The hypothesized role of genes in regulation ("R") or transport ("T") is given in the column 'Role'.

Gene name	Gene function	Role	Base mean	Log2FoldChange	p_{adj}
Cp4.1LG05g03830	ACT containing protein, ACR8	R	34.0	1.25	<0.01
Cp4.1LG05g02530	MATE transporter	T	4.4	5.72	<0.01
Cp4.1LG05g03700	40S Ribosomal protein S13		3125.4	0.69	0.04
Cp4.1LG05g03660	Amine oxidase		108.0	0.74	0.03
Cp4.1LG05g03670	Rhomboid-like protein	T	272.6	-1.30	<0.01
Cp4.1LG05g03910	Protein IQ-Domain 31		120.5	-1.17	<0.01
Cp4.1LG05g03520	Putative myosin heavy-chain protein		50.4	-2.04	<0.01
Cp4.1LG05g03620	Molybdenum cofactor sulferase		30.1	-1.84	0.002
Cp4.1LG05g03560	Putative LRR receptor kinase	R	18.2	-1.40	0.002
Cp4.1LG05g03690	Stay Green protein	R	10.5	-1.85	0.042

5.1 Discussion

Plant secondary metabolite accumulation relies on functional biosynthetic genes, regulation – often specific to tissues, developmental stage, or attack by pests – and transport and storage. Selection on any of these components leads to spatial and temporal variation in metabolite accumulation, and has implications for plant-herbivore interactions [1]. We examined the ecological effects and genetic basis of cucurbitacin accumulation in two independently domesticated lineages of *Cucurbita pepo* across developmental stages. Overall, we found that herbivore preference is driven both by cotyledon cucurbitacins and cucurbitacin-independent chemistry in the true leaves. Divergence in cotyledon cucurbitacins was attributed to a single Mendelian locus that affects accumulation early in seedling development, not through disruption of known biosynthetic gene function, but instead through regulation or transport.

5.5.1 Isolating effects of cotyledon cucurbitacins on herbivore preference.

The specialist beetle and agricultural pest, *Acalymma vittatum*, preferentially consumes leaves and cotyledons of *C. pepo* ssp. *pepo* (CPP) over *C. pepo* ssp. *ovifera* (CPO) [34], [36], [37], despite neither domesticate having cucurbitacins in leaf tissue [15], [21], [38]. We sought to isolate the effect of cotyledon cucurbitacins on herbivore preference from other factors associated with subspecies, like defense inducibility [38] and morphological traits [57]. Both physical [58] and chemical [4] traits implicated in herbivore resistance can be confounded by plant population structure; accordingly,

2935 divergent selection for phenotypic extremes in an intermated population is an
2936 important method to establish or reject causality. By selecting for high and low
2937 cotyledon cucurbitacins in our biparental population, we found that cotyledon
2938 cucurbitacins were indeed predictive of *A. vittatum* preference for cotyledons, but not
2939 other tissues in two years of field experiments. Thus, cotyledon cucurbitacins are
2940 causal for cotyledon preference independent of other (unknown) differences in
2941 cotyledons associated with subspecies, but leaf and cotyledon preference are
2942 correlated at the subspecies level because of other traits associated with population
2943 structure, not pleiotropic effects of cotyledon cucurbitacins. Feeding by *A. vittatum*
2944 also failed to induce cucurbitacin accumulation in leaves or cotyledons, further
2945 indicating a lack of a mechanistic connection between those tissues. *Acalymma*
2946 *vittatum* likewise did not induce cucurbitacins in cucumber (*Cucumis sativus*) by root
2947 feeding [59], but induction has been reported at a different developmental stage (prior
2948 to leaf expansion) by a generalist mite [19].
2949
2950 5.5.2 Genetic basis of cotyledon cucurbitacins – Genetic mapping. Cotyledon
2951 cucurbitacin accumulation in *C. pepo* has single Mendelian gene inheritance [35], but
2952 may interact with other loci [56], and has not been mechanistically characterized. With
2953 a biparental mapping population, we indeed identified a single Mendelian locus, *Bi-4*,
2954 restricted to a genomic interval containing 47 genes. Based on the most recent *C. pepo*
2955 genome sequence (v4.1 [40]), none of these genes are homologs of described
2956 cucurbitacin biosynthesis genes, indicating that selection against cotyledon
2957 cucurbitacins in CPO did not result in entire pathway disruption.

In a more diverse set of *C. pepo* germplasm, variation in cotyledon cucurbitacins was predominately explained by population structure, and accessions collected from the Americas had the lowest cucurbitacin concentration. This divergence could be due to the biogeographical split in cultivar development [31]: CPO cultivars were exclusively bred within the range of cucurbitacin-sequestering specialist beetles, the Americas, and thus potentially subject to selective pressure against high cucurbitacin content. Recent work showed divergent selective sweeps for fruit cucurbitacins between subspecies of *C. melo* [26] and evaluations of high resolution genomic data of independent domestication events in *Cucurbita* spp. would likely also identify targets of selection. Across systems, divergent past selection processes affected plant wide secondary metabolite concentration and often herbivory; parallels can be drawn to glucosinolates and herbivory in the cultivated Brassicaceae, where there was selection for high and low concentrations in multiple tissues [60].

5.5.3 Genetic basis of cotyledon cucurbitacins – Gene expression. We also measured gene expression in fully expanded cotyledons of the biparental population. A key result was that expression of Cp4.1LG05g02530, a multidrug and toxic compound extrusion (MATE) transporter in the *Bi-4* interval is quantitatively and positively correlated with the phenotype. MATE transporters facilitate movement of diverse secondary metabolites like flavonoids, alkaloids, and cyanogenic glycosides in many species [61]. Notably, MATE transporters are necessary for vacuolar transport of nicotine within and between biosynthetically active and inactive tissues in *Nicotiana* [62]–[64].

The cellular localization of cucurbitacins, as well as movement within or between tissues remains uncharacterized, making it difficult to generate hypotheses about how the MATE transporter affects cucurbitacin phenotypes. Nonetheless, cucurbitacins are found in roots of multiple *Cucurbita* spp. [16], and recent work demonstrated that grafting non-bitter *Cucumis melo* onto a *Cucurbita maxima* rootstock rendered the fruit bitter [65], suggesting cross-tissue transport.

5.5.4 Mechanistic basis of cotyledon cucurbitacins. Because our expression analysis of F_2 individuals was conducted in fully expanded cotyledons, it is possible that regulation or transport were not actively occurring. To address this, we also tracked cucurbitacin accumulation and expression of biosynthetic gene homologs and genes in the *Bi-4* interval to assess evidence for cucurbitacin transport or regulation during seedling development of CPO and CPP.

Previous work showed that there is a pulse of cucurbitacin accumulation in seedlings early in development in many Cucurbitaceae species [18], [66], and we found that cotyledon cucurbitacins increase only in CPP starting at radical emergence, but remain steady in roots of both lineages. Tissue-specific accumulation was associated with local conserved biosynthetic gene expression at the stage of radical emergence. Thus, while transport of cucurbitacins may be important in some capacity (e.g. vacuolar storage), cross-tissue transport does not appear to be required for cotyledon cucurbitacin accumulation. Instead, the coordination of biosynthesis with seedling development indicates potentially novel regulatory mechanisms of cucurbitacin accumulation. In other Cucurbitaceae (*Cucumis sativus*, *Cucumis melo*,

3004 *Citrullus lanatus*), biosynthesis occurs locally through activation by leaf-, root- or
3005 fruit-specific bHLH transcription factors [23], [24]. However, biosynthetic gene
3006 expression during radical emergence in CPP does not coincide with expression of
3007 known bHLH transcription factor homologs, suggesting novel regulatory mechanisms
3008 in *Cucurbita pepo*, or regulation at different ontogenetic stages. Local regulation,
3009 synthesis, and transport are likewise jointly important in secondary metabolite
3010 accumulation in other systems such as glucosinolates in the Brassicaceae [12].
3011 To address if there were candidate genes related to regulation of cucurbitacin
3012 biosynthesis, we evaluated differentially expressed genes in the *Bi-4* interval in
3013 cotyledons at the radical stage between CPP and CPO. We propose one additional
3014 candidate gene; Cp4.1LG05g03830 is an ACT-domain containing protein similar to
3015 ACR8 in Arabidopsis [67], where it acts as a regulator and expression increases in
3016 response to abscisic acid (ABA) [68]. There is higher expression in CPP, and the
3017 connection to regulation via ABA is intriguing: ABA is important in seed germination
3018 [69], and ABA increases cucurbitacin biosynthetic gene expression in cucumber [23].
3019 If validated, this candidate gene represents a link between regulation of seed
3020 germination and cucurbitacin biosynthesis. This candidate, and the MATE transporter,
3021 should be further explored in immortal populations where phenotyping gene
3022 expression and cucurbitacin accumulation at multiple time points is feasible.
3023 More broadly, examining ontogenetic trajectories of defensive metabolites early in
3024 seedling development may provide important insights into growth-defense tradeoffs.
3025 Cotyledons are a particularly vulnerable life stage [70], and we observe that
3026 cotyledons of *Cucurbita pepo* are heavily defended by cucurbitacins, yet have

contrasting ontogenetic trajectories and cucurbitacin structural diversity than root tissue, which is higher in cucurbitacin E and does not vary with development. This raises questions about the selective pressures or developmental constraints that shaped this phenotype [11]. While our study is limited to two lineages, broad studies within the family could lead to insights about growth-defense tradeoffs across multiple species and domestication events, as we expect that tradeoffs are scale dependent [71].

5.6 Conclusions

Our study demonstrates that cucurbitacins are certainly relevant for herbivore preference, even when isolated from biogeographically diverged lineages. Through genetic mapping and gene expression analysis, we showed that a potential target of selection for cotyledon cucurbitacins was regulators or transporters that contribute to dynamic variation of cucurbitacins throughout development and between tissues. The complementary role that such genes play alongside core biosynthetic genes is critical, yet perhaps understudied in genetics of secondary metabolite expression. By characterizing both the ecological effects of cucurbitacins and the genetic factors contributing to intraspecific variation, this work informs present plant breeding efforts to improve insect resistance in *C. pepo*.

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Chapter 6: Evaluation of selection methods for resistance in *Cucurbita pepo* to a specialist pest

6.1 Abstract

Plant varieties resistant to insect pests are a critical component of integrated pest management, but challenges associated with plant breeding for insect resistance, such as long breeding cycle duration and low trait heritability, slow progress in the field. In this study, we tested two novel selection schemes to improve gain from selection for resistance to the major pest, the striped cucumber beetle (*Acalymma vittatum*), in squash (*Cucurbita pepo*, e.g. zucchini). First, we tested an indirect selection scheme using a proxy insect with correlated resistance phenotypes, *Trichoplusia ni*, in place of the seasonally available *A. vittatum*. We found that while *T. ni* performance was heritable, there was no reciprocal benefit for *A. vittatum* resistance. Second, we tested genomic selection, a method that allows for selection without phenotyping every generation. Although there was moderate genomic predictive ability, we did not observe realized gains from selection in the field. Overall, strategies that minimize investment in phenotyping for insect resistance and shorten breeding cycle duration are needed to develop insect resistant varieties, and this work provides examples and empirical data of two such strategies deployed in an applied context.

6.2 Introduction

Development and cultivation of varieties resistant to insect damage is a

cornerstone of integrated pest management [1], yet the use of resistant fruit and vegetable crops is limited [2]. Successful breeding for resistance to insect pests has been largely restricted to cases where the genetic architecture is oligogenic and heritability is high, as in gene-for-gene interactions in some Hemipteran and Dipteran pests [3], [4]. For many other pests, such as beetles (Coleoptera), plant breeders are challenged by factors including quantitative inheritance, low heritability, and limited knowledge of resistance mechanisms [5]. Thus, improved plant breeding strategies are needed to increase genetic gain in an economically viable manner.

The amount of genetic gain plant breeders can attain by selection is governed by trait heritability, phenotypic variance and selection intensity [6]. Broadly, heritability can be improved through enhanced phenotyping and experimental design [7]; however, if phenotyping insect resistance relies on natural infestation, the degree to which heritability can be improved may be limited due to stochastic fluctuations in pest density or by confounded pest behaviors (e.g. aggregation). As a consequence, plant breeders may instead evaluate and select upon correlated traits, such as defensive chemistry [8], [9] or physical defenses [10], [11]. When traits have genetic correlation, comparable or higher heritability, or more individuals can be phenotyped in a high-throughput manner, indirect selection on those traits can translate to greater genetic gain [12]. A barrier to this method is that correlated traits may not be identified, and mechanism discovery may be beyond the scope of some breeding programs.

A final component of the breeders' equation is to measure genetic gain per unit time, and is a more relevant metric for assessing success in applied programs [13]. Decreasing breeding cycle duration may also be more accessible than other strategies

(e.g. increasing selection intensity may require larger populations and field space, [14]). One readily apparent solution for breeding for insect resistance would be incorporating, or exclusively using, indirect selection on traits that allow for more generations of selection per year. Another method increasingly used in breeding programs is genomic selection, a method with the goal to increase genetic gain in a shorter time span in part by allowing for population advancement without phenotyping [15]. Thus, phenotypes that are difficult or expensive to measure, such as insect resistance, are likely to benefit from genomic selection [16], [17]. However, genomic selection for insect resistance has received little attention (we are aware of a single study of resistance to the pine weevil (*Pissodes strobi*, Coleoptera: Curculionidae) in Norway spruce (*Picea abies*), [18]) compared to more widespread application for traits like yield and disease resistance [19]. Overall, methods that decrease breeding cycle duration, and shift (indirect selection) or reduce (genomic selection) phenotyping should be explored to improve breeding for resistance to insect pests.

We tested such selection methods in an applied breeding program for *Cucurbita pepo* (zucchini) resistance to a major agricultural pest, the striped cucumber beetle, *Acalymma vittatum* (Coleoptera: Chrysomelidae). *Acalymma vittatum* causes damage through herbivory of all plant tissues in both larval and adult stages [20], and vectors bacterial wilt disease (pathogen, *Erwinia tracheiphila*; [21]) as well as seed-transmissible *Squash mosaic virus* [22]. There is variation for foliar damage in *C. pepo* cultivars [23], [24] at economically meaningful levels [25]. However, there are numerous challenges to breeding for resistance. First, *A. vittatum* adults group in pheromone-mediated aggregations [26], amplifying spatial variation in damage and

thus decreasing heritability. In addition, maintaining *A. vittatum* colonies large enough to screen breeding populations is prohibitive, so phenotypic selection typically occurs only once per year during natural *A. vittatum* field infestation and carries risks of failure due to disease occurrence. Finally, accurately measuring damage is time-intensive and requires trained observers, making phenotyping a significant investment. Overall, there is a clear need for innovative methods to increase genetic gain for resistance that is sensitive to low heritability, mitigates risks of beetle-vectored disease and decreases cycle length in a manner that is attainable for breeding programs.

One approach would be to indirectly select for resistance to leaf damage by *A. vittatum* based on a correlated trait. Unfortunately, mechanisms of leaf resistance to *A. vittatum* have yet to be characterized in cultivated *C. pepo*. Cucurbitacins, bitter triterpenoids of the Cucurbitaceae that *A. vittatum* seeks and sequesters for its own defense [27], are not found in leaves of cultivated *C. pepo* [28], [29], nor do other traits frequently associated with resistance like nutrient content and leaf thickness [29], [30] affect leaf preference. As a result, there is not a chemical or physical leaf trait can be targeted for indirect selection. However, we previously observed that larval performance of *Trichoplusia ni* (Lepidoptera: Noctuidae), an occasional herbivore of squash [31], was highly correlated with *A. vittatum* preference at the cultivar level (r_P=0.893), where lower larval performance is associated with reduced beetle preference [29]. In addition, *T. ni* performance on *C. pepo* can be evaluated on small plants in controlled environments year-round as *T. ni* are available through commercial sources, making it a promising 'proxy' chewing insect to use in a rapid-cycling indirect selection scheme.

We also evaluated the gain from genomic selection for *A. vittatum* resistance. The benefits of genomic selection are magnified in this system in both allowing for additional generations of selection per year and also for alleviating issues with seasonal *A. vittatum* availability and disease transmission. In the only other genomic selection study we are aware of for insect resistance, the authors demonstrated a prediction accuracy of 0.83 for resistance to pine weevils in Norway spruce with a multi-trait prediction model incorporating traits correlated to resistance (e.g. tree height), although realized gains were not measured [18]. More specifically to this system of study, genomic selection has been shown to be a viable method for improving other quantitative traits in *Cucurbita* spp. [32].

To test selection methods for selection to reduce breeding cycle time and increase genetic gain for resistance to *A. vittatum* in *C. pepo*, we developed an intermated *C. pepo* population between a highly preferred cultivar with good fruit quality and a non-preferred cultivar with lower fruit quality, selected for two generations, and measured realized gains. In testing indirect selection through use of *T. ni* as a proxy, we hypothesized that there would be cross-resistance between *T. ni* performance and *A. vittatum* preference, but that *T. ni* performance would have greater heritability than *A. vittatum* preference due to the inability of *T. ni* larvae to migrate and choose different host plants. We also predicted that genomic selection would lead to population improvement. Overall, these methods test different strategies to develop resistant varieties through reducing breeding cycle length.

6.3 Methods

6.3.1 Plants. All germplasm was sourced from the Cornell University breeding program. This includes population founders, *Cucurbita pepo* ssp. *pepo* cv. Black Beauty (zucchini), and *C. pepo* ssp. *ovifera* (syn *texana*) cv. Success PM (summer squash), the intermated F_2 population as the base population, and all derived populations. The selection scheme shown in **Figure 6.1**. All plants were started from untreated seeds in the Cornell University Agricultural Experiment Station greenhouses (Ithaca, NY) and grown with a 16 h day, 8 h night photoperiod and temperatures of 27 °C and 21 °C, respectively. Plants used in founder phenotype evaluations, *T. ni* indirect selection and common garden experiments, and genomic selection were sown individually into 10 cm pots and kept in the greenhouse. Plants used in *A. vittatum* realized gain field trials were sown into 72-cell trays in the greenhouse and subsequently transplanted in certified organic fields of the Homer C. Thompson Vegetable Research Farm (Freeville, NY). Plants had 1-3 fully expanded true leaves at the beginning of assays.

6.3.2 Acalymma vittatum assays. All *Acalymma vittatum* used in field evaluations were naturally occurring populations at the Homer C. Thompson Vegetable Research Farm (Freeville, NY). For greenhouse assays, *A. vittatum* were collected up to 24 h before trials commenced. *Acalymma vittatum* preference was measured as leaf damage to plants. Leaf damage was both visually estimated as percent defoliation, and measured from photographs using ImageJ [33].

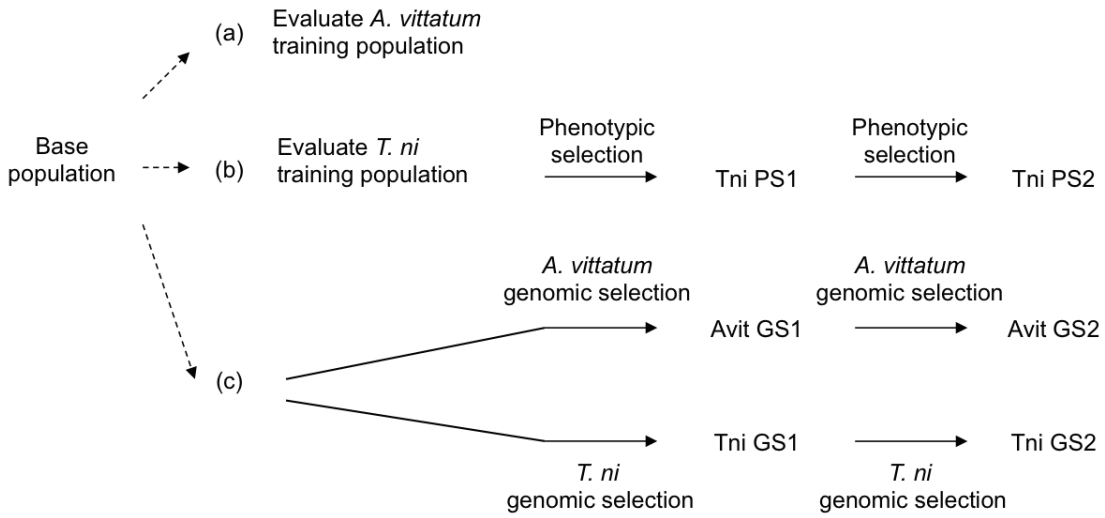

Figure 6.1. Diagram of selection schemes. The (a) *A. vittatum* training population, (b) *T. ni* training population and starting material for the indirect selection scheme, and (c) starting material for the genomic selection schemes were sourced from the Base Population. The dashed arrows represent source of germplasm, and the solid arrows represent a generation of selection. The type of selection is described above the solid arrows.

6.3.3 Trichoplusia ni assays. Eggs were supplied from a colony maintained at Cornell University (Dr. Ping Wang, Cornell AgriTech, Geneva, NY) and held in a 30 °C growth chamber. Immediately upon hatching, 2-3 neonates were applied to the newest fully expanded true leaf of plants in the greenhouse. Plants were then individually enclosed in a mesh sleeve (30 cm x 18 cm) and watered daily. After five days, larvae were removed from plants, placed in Eppendorf tubes, frozen and weighed (AT21 Comparator Microbalance, Mettler-Toledo, Columbus, OH, USA). Larval mass was used as the metric of *T. ni* performance, as in [29]. Most larvae were recovered at the end of the five day period.

6.3.4 Founder phenotype evaluation. While previous work had established that *C. p.*

3403 *pepo* cultivars are more preferred by *A. vittatum* [23], [24] and sustain greater *T. ni*
3404 performance [29] than *C. p. ovifera*, we tested differences between the two founders
3405 for both metrics. We evaluated *A. vittatum* preference using paired choice tests. In
3406 June 2018, one plant of each founder was placed 25 cm apart in a 25 cm x by 30 cm
3407 mesh bag, five beetles were added, and leaf damage was visually estimated after 48 h.
3408 Thirty-one pairs were evaluated over four temporally separated blocks each with six to
3409 nine replicates each. Differences in estimated leaf defoliation between founders was
3410 assessed with a linear mixed effects model with founder genotype and block as fixed
3411 effects, individual pairing nested within block as a random effect. To test for
3412 differences in *T. ni* performance, we measured *T. ni* mass on a total of 25 *C. pepo* cv.
3413 Black Beauty and 20 *C. pepo* cv. Success PM plants in two blocks in January-
3414 February 2018. Average mass of recovered larvae was analyzed as a linear model with
3415 fixed effects of genotype and block.
3416
3417 6.3.5 Trichoplusia ni indirect selection. In March 2018, 131 plants from the base
3418 population were evaluated for *T. ni* performance (**Fig. 6.1b**) in an augmented
3419 incomplete block design of seven blocks of 18-20 base population plants each with
3420 both founders as checks. Mass of *T. ni* larvae was measured from each plant (most had
3421 2-3 individuals). Best linear unbiased predictors (BLUPs) were calculated with the
3422 following model: *T. ni* mass ~ (1|genotype) + (1|block) + check, and then deregressed
3423 [34].
3424 The 15% of individuals (*n*=20) with lowest BLUPs (reduced *T. ni*
3425 performance) were randomly intermated with one pollination per plant to complete the

first generation of phenotypic selection and create “TniPS1” (**Fig. 6.1b**). Seeds from TniPS1 were then mixed in equal numbers per maternal genotype, and a second generation of phenotypic selection was conducted in July 2018. An augmented incomplete block design was used with 14 blocks of 14-15 TniPS1 plants (*n*=209 total), and with a check of each founder per block. Using BLUPs calculated by the same method, the best 29 plants (14%) were randomly intermated to create “TniPS2” (**Fig. 6.1b**). Seeds of TniPS2 were then mixed in equal numbers per maternal genotype for subsequent evaluation.

6.3.6 Training population evaluation – *T. ni*. The base population used in the first generation of *T. ni* phenotypic selection was used as the *T. ni* genomic selection training population (**Fig. 6.1b**). The BLUPs described above were used for training genomic selection models, and tissue was collected and lyophilized for DNA extraction from the 131 individuals.

6.3.7 Training population evaluation – *A. vittatum*. Individuals from the base population were evaluated in the field for *A. vittatum* damage to provide phenotypes for training the *A. vittatum* genomic selection model (**Fig. 6.1a**). The field was prepared by creating four raised bed rows running East to West with black plastic mulch and drip irrigation spaced 2.1 m apart. The *A. vittatum* training population was then transplanted into the field in mid-June 2018 with 0.45 m between individual plants in an augmented incomplete block design. Each of the four rows was split into two blocks (North, South), and founder checks were included in each block. Although

only 190 plants could be genotyped in the training population, 240 plants were transplanted (60 per row) to create a buffer for plant mortality associated with transplanting or causes unrelated to beetle damage, or sample loss. Plant damage was evaluated once founder checks had substantial differences in defoliation, early July 2018, and tissue was collected for DNA extraction.

Best linear unbiased predictors were calculated from the 209 phenotyped individuals using the linear mixed effect model $y \sim (1|genotype) + (1|row) + (1|row:block) + check$. The following response variables were used: estimated percent leaf defoliation (%), actual measured percent leaf defoliation (%), log(actual measured percent leaf defoliation), actual measured leaf defoliation (cm^2). Broad sense heritability (H^2) and phenotypic correlations were also calculated, and deregressed BLUPs were used to train the *A. vittatum* genomic selection model.

6.3.8 Genotyping and SNP calling – training populations and genomic selection. DNA was extracted from all populations with a Qiagen DNeasy 96 Plant kit from lyophilized tissue according to the manufacturer's instructions (Qiagen, Hilden, Germany). 192-plex GBS library preparation was done at University of Wisconsin-Madison Biotechnology Center (Madison, WI, USA), and the libraries were sequenced at Cornell University Biotechnology Resource Center (Ithaca, NY, USA) on an Illumina NextSeq 500 (Illumina, San Diego, CA, USA) with single-end 75bp reads.

Reads were aligned to the most recent *Cucurbita pepo* genome (v4.1) [35] with the 'bwa' aligner in the GBSv2 pipeline in TASSEL 5 [36], and 129,953 SNPs were called. SNPs were filtered to be biallelic, minor allele frequency of 0.05, and missing

in less than 50% of samples with VCFtools [37], leaving 22,213 SNPs. Then, only the 16,052 SNPs polymorphic between the population parents were retained. Finally, missing genotypes were imputed with an accuracy of 87% with LB-Impute with a window size of five, imputation software developed for biallelic populations [38].

6.3.9 Genomic selection – model training. The training populations for both *A. vittatum* and *T. ni* were used to train genomic selection models in Summer 2018. For both, previously described BLUPs were used as phenotype and there were 190, 130 individuals with both phenotypes and genotypes for *A. vittatum* and *T. ni*, respectively. Predictive ability of different genomic selection models were evaluated in the R package PopVar [39] with five-fold cross-validation and 100 replications. Ultimately, genomic BLUP (GBLUP) was used to train the genomic selection model, and was implemented in TASSEL 5 [36]. Narrow sense heritability (h^2) was also calculated in TASSEL 5 using the mixed linear model (MLM) function with kinship derived from all genomic SNPs.

6.3.10 Genomic selection – generations of selection. In Fall 2018, 190 seeds from the base population were sown and 23 (12%), 27 (14%)best individuals were selected from the population based on the genomic estimated breeding values (GEBVs) calculated from the *A. vittatum* and *T. ni* GBLUP models, respectively. Within each population, individuals were randomly intermated. Seeds from pollinations (AvitGS1, TniGS1) were then mixed within selection models in equal numbers per maternal genotype for the subsequent generation. In Spring 2019, another cycle of selection was

conducted on both populations using the same model. For the *A. vittatum* selection scheme, 190 individuals were genotyped, and 31 (16%) were selected, and for the *T. ni* selection scheme, 174 individuals were genotyped, and 19 (11%) were selected. Again, within each population, individuals were randomly intermated and seeds from pollinations (AvitGS2, TniGS2) were then mixed within selection models in equal numbers per maternal genotype for subsequent evaluation. After completing all cycles of selection, population differentiation was visualized using principal component analysis of genome-wide SNPs in TASSEL, and quantified by mean weighted F_{ST} values calculated in VCFtools [37].

6.3.11 Realized gains – *A. vittatum* preference. Populations were evaluated for realized gain for reduced *A. vittatum* preference from different selection methods in the field during Summer 2019. The field was prepared with raised beds with drip irrigation and black plastic mulch with each row spaced 3 m apart. There were sentinel plots of six individuals of each founder line at the end of each row to assess damage progression. Populations were evaluated in a randomized complete block design of 20 plant plots with 0.45 m between plants in four blocks (rows).

Populations were evaluated over two periods due to seed availability. First, the base population, both *T. ni* phenotypically selected populations (TniPS1, TniPS2), and both first generations of genomic selection (TniGS1, AvitGS1) were transplanted in mid-June and evaluated in early July. The second set evaluated included the base population and all genomic selection populations (TniGS1, TniGS2, AvitGS1, AvitGS2), and was transplanted mid-August and evaluated late August. In both trials,

the populations were phenotyped as soon as sentinel founder plots had substantial differences in defoliation. The following linear mixed model was used for each trial *A. vittatum* preference (measured percent damage) ~ genotype + (1|block).

6.3.12 Realized gains – *T. ni* performance. We assessed the effect of selection on *T. ni* performance in two common gardens. First, both generations of *T. ni* phenotypic selection (TniPS1, TniPS2) were compared to the base population in Summer 2019. Then, in Fall 2019, both first generations of genomic selection (TniGS1, AvitGS1) were compared to the base population. Two independent experiment iterations were conducted for each common garden. In each iteration, we used a randomized block design to account for spatial variation in the greenhouse, and there were four blocks with approximately 12 plants per block. In sum, $n_{BasePop}$=87, n_{TniPS1}=94, n_{TniPS2}=95 plants were evaluated in the first common garden and $n_{BasePop}$=82, $n_{AvitPS1}$=92, n_{TniGS1}=90 in the second. All *T. ni* recovered from each plant were weighed together to calculated average mass of the two to three larvae. Differences between generations or selection type was evaluated with the linear mixed effect model *T. ni* performance ~ genotype + experiment:(1|block), and an ANOVA was conducted on the fixed effects.

6.3.13 Statistics. All statistical analyses were conducted in R [40]. Linear models were assessed with the 'lm' function, and linear mixed effect models were evaluated with the 'lmer' function R/lme4 [41]. Significance of fixed effects was determined with ANOVA.

6.4 Results

6.4.1 Phenotypic extremes in parent lines. We confirmed that founder genotypes had substantial phenotypic differences in *Acalymma vittatum* preference and *Trichoplusia ni* performance. *Acalymma vittatum* caused four times greater percent leaf damage to the susceptible than resistant founder ($F_{1,56}$=47.529, p<0.001), and *T. ni* larvae attained 61.1% greater mass feeding upon the susceptible founder than the resistant founder ($F_{1,42}$=34.563, p<0.001).

6.4.2 Indirect selection approach. We tested an indirect selection approach for *A. vittatum* resistance with a proxy insect (*T. ni*) that could decrease breeding cycle duration, and may have higher heritability than *A. vittatum* damage. By phenotypically selecting the top 15% of individuals (lowest *T. ni* performance) for two generations, we observed moderate heritability (**Table 6.1**). Later, the base population and both generations of phenotypic selection were evaluated in a common garden and there was a significant effect of selection on *T. ni* performance ($F_{2,269}$=5.637, p=0.004). Mean *T. ni* mass in base population mean was close to the mid-parent values (3.52% less), dropped after one generation of selection (TniPS1, 15.44% less), and was maintained after another generation of selection (TniPS2, 15.46% less).

Table 6.1. *Trichoplusia ni* mass in each generation of indirect selection where lower mass indicates reduced performance. μ_0 refers to the mean of the evaluated population, $\mu_{selected}$ refers to the mean of selected individuals, and H^2 is the broad sense heritability on a plot basis.

Generation	As % less than mid-parent		As % less than high parent		H^2
	μ_0	$\mu_{selected}$	μ_0	$\mu_{selected}$	
Generation 1 (Base -> TniPS1)	1.04 %	27.60%	23.69%	44.18%	0.228
Generation 2 (TniPS1 -> TniPS2)	18.81%	59.40%	46.25%	73.12%	0.289

6.4.3 Genomic selection. We first phenotypically evaluated individuals from the base population to train genomic selection models (**Fig. 6.1a-b**). For the *A. vittatum* training population, of the three foliar damage metrics quantified, actual measured percent defoliation had the highest broad-sense heritability on a plot basis (**Table 6.2**), even though estimated and actual percent damage were correlated (Pearson's r=0.838, p<0.001). The *T. ni* training population phenotype, mean larvae mass, had lower broad-sense heritability than most *A. vittatum* phenotypes (**Table 6.2**). Although distinct individuals were used for the base population and both training populations, the training populations are representative samples of the base population (**Fig. 6.2a**).

Table 6.2. Training population phenotypes, where H^2 refers to broad sense heritability on a plot basis from phenotypes alone (genomic data not incorporated).

Training population	Phenotype	H^2
Acalymma vittatum	Estimated percent defoliation (%)	0.066
	Measured percent defoliation (%)	0.369
	Log(Measured percent defoliation)	0.314
	Measured total damage (cm^2)	0
Trichoplusia ni	Larvae mass	0.228

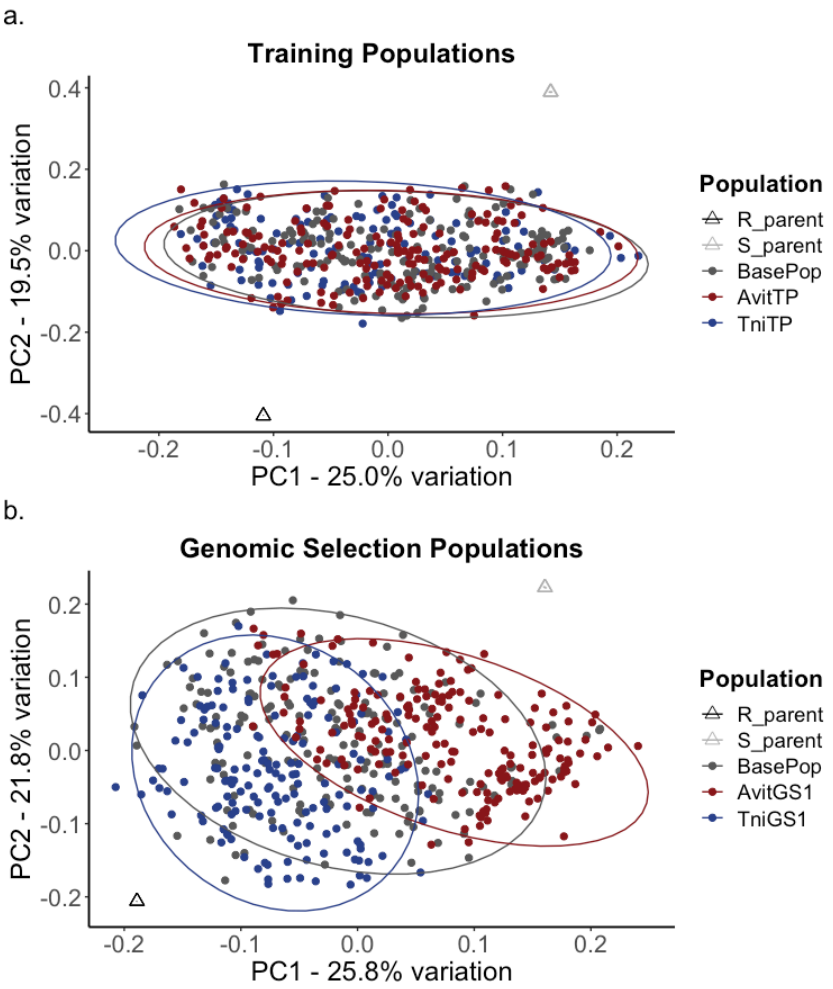

Figure 6.2. Principal component analyses (PCA) plots from genome-wide SNP marker matrix for genomic selection (a) training populations and (b) after one generation of selection. The percent variation explained by the first two principal components are given on the x- and y-axis respectively. The points representing the resistant ("R_parent") and susceptible parent ("S_parent") are triangle shaped and the points representing the base population ("BasePop"), *A. vittatum* and *T. ni* training and genomic selection populations are circles. Ellipses represent normal confidence intervals for each group.

Genomic selection models had similar cross-fold validation accuracies (**Fig. 6.3**), and thus GBLUP (equivalent to rrBLUP when the genomic relationship matrix is used, [42]) was chosen for future selection. Predictive ability was similar between models, but *T. ni* performance had slightly higher narrow sense heritability (**Table 6.3**).

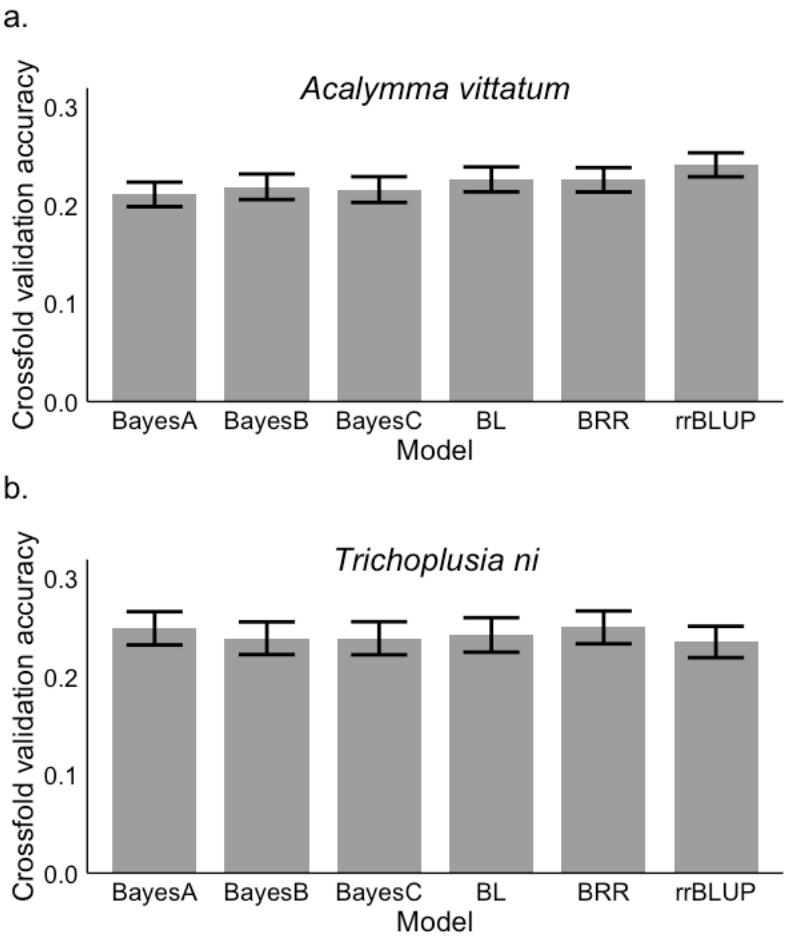

Figure 6.3. Comparison of different genomic selection models for training population cross-fold validation accuracy for (a) *Acalymma vittatum* and (b) *Trichoplusia ni*. The model abbreviations are as follows: "BL" is Bayesian Lasso, "BRR" is Bayesian Ridge Regression, and rrBLUP is ridge-regression BLUP, which is equivalent to GBLUP. The error bars represent one standard error.

Table 6.3. Genomic selection model predictive ability +/- one standard deviation of mean and narrow sense heritability. For *A. vittatum* the training phenotype used was log(Measured percent defoliation).

Model	Predictive ability	h^2
Acalymma vittatum	0.248 +/- 0.010	0.192
Trichoplusia ni	0.230 +/- 0.018	0.290

There was differentiation between populations from one generation of

selection (Base to TniGS1 F_{ST} =0.020; Base to AvitGS1 F_{ST} =0.031, **Fig. 6.2b**; AvitGS2 and TniGS2 were not genotyped). While we predicted there would be some shared genetic basis between resistance to *T. ni* and *A. vittatum*, a completely different set of individuals were selected from the base population, and there was population divergence (F_{ST} = 0.059).

6.4.4 Realized gains for *A. vittatum* resistance. The response to selection for increased resistance to *A. vittatum* was mixed. In general, there was no effect of either indirect or genomic selection, but in the second field experiment, TniGS1 and AvitGS1 sustained substantially less damage than the other populations evaluated (**Table 6.4**). TniGS1 and AvitGS1 were also evaluated in greenhouse for realized gain from selection for *T. ni* mass and there was no differentiation between either and the starting population ($F_{2,259}$=0.330, p=0.719).

Table 6.4. Realized response of *A. vittatum* measured percent leaf defoliation to selected populations in field.

Experiment	Population	Damage (%)		SE		Statistics
June 2019	Base	12.05	+/-	2.90	a	$F_{4,386}$=1.008 p=0.403
	TniPS1	14.71	+/-	2.90	a	
	TniPS2	14.79	+/-	2.90	a	
	TniGS1	11.85	+/-	2.90	a	
	AvitGS1	14.35	+/-	2.90	a	
August 2019	Base	30.94	+/-	5.07	b	$F_{4,388}$=9.496 **p<0.001**
	TniGS1	17.32	+/-	5.07	a	
	TniGS2	32.60	+/-	5.06	b	
	AvitGS1	14.98	+/-	5.06	a	
	AvitGS2	22.98	+/-	5.06	ab	

6.5 Discussion

Breeding for quantitative resistance to insect pests is a major obstacle for plant

breeders, and testing methods to increase genetic gain in time and cost-effective manner addresses this challenge. In this study, we evaluated new selection methods for resistance to the specialist insect pest, the striped cucumber beetle, *Acalymma vittatum*, in *Cucurbita pepo* (zucchini). Using a biparental population, we conducted two generations of an indirect selection scheme using a proxy insect, and genomic selection. Overall, we had moderate predictive ability from genomic selection and mixed results of realized gain from both selection schemes. This study highlights opportunities to incorporate new selection methods for insect resistance into applied breeding programs, and specific insights into future directions for breeding for resistance to *A. vittatum*.

6.5.1 Indirect selection approaches. We tested a novel indirect selection scheme by selecting *C. pepo* based on performance of *Trichoplusia ni*, as *T. ni* performance is highly phenotypically correlated (r_P=0.893) with *A. vittatum* preference at the cultivar level [29]. *Trichoplusia ni* was chosen also because we predicted *T. ni* performance would have higher heritability than *A. vittatum* preference, and up to three cycles of selection per year could occur (instead of one by phenotyping *A. vittatum* in the field). Thus, we expected to observe gain from selection on the measured trait of *T. ni* performance and target trait of resistance to *A. vittatum*. We found that there was heritable variation for resistance to *T. ni* and resistance increased by selection. However, contrary to our predictions, heritability of *T. ni* resistance was on par with *A. vittatum* preference, and we did not observe cross-resistance to *A. vittatum* or strong evidence of genetic correlation. These results indicate that resistance to *T. ni* and *A.*

vittatum likely have distinct mechanistic bases.

While this study was predicated on the phenotypic correlation between traits, future work would be more compelling if the genetic correlation was first concretely established. For instance, estimates of heritability and trait covariance from phenotyping both traits in structured populations (e.g. parent-offspring regression) [12], or from investigating the underlying shared genetic basis using genotypes and phenotypes with structural equation modeling [43]. Then, knowing those components, the likelihood of success in selection upon the correlated trait would be known. From the heritability estimates derived from our genotyping and phenotyping associated with genomic selection, we can now estimate that the genetic correlation would need to exceed r_A>0.8 to have the same response for *A. vittatum* resistance for correlated and direct selection. So, while *T ni* performance did have slightly greater heritability than *A. vittatum* damage, it was not high enough to be productive lacking a strong genetic correlation between traits.

More broadly, indirect selection is a promising selection method for the emergent trait of insect resistance, but a major drawback to this approach is that the measured trait may only account for a portion of the variation in resistance to a herbivore (e.g. maysin, a defensive flavone in silk tissue of *Zea mays*, to corn earworm, [44], [45]). Our study attempts to integrate multiple potential resistance traits by selecting with a proxy insect, and is the first of its kind to our knowledge. A critique may be to instead use insects more closely related to each other than those used in our work; however, while more closely related organisms would have greater genetic correlation, they likely also share similar barriers to genetic gain (e.g. breeding

3672 cycle duration, ease of phenotyping).

3673

3674 6.5.2 Genomic selection approaches. We also applied genomic selection, a method
3675 that can be especially important for phenotypes that are difficult or expensive to
3676 measure [16], [17], but has only been evaluated in one other instance for insect
3677 resistance [18]. Our genomic predictive abilities were moderate and realized gains
3678 were mixed, and several factors would have likely led to more successful outcomes.
3679 For instance, using replicated inbred lines instead of a segregating population as a
3680 model training population would have likely improved prediction accuracies. More
3681 broadly, while simulation studies indicate that traits with low heritability ($h^2 = 0.2$)
3682 will still realize gains by genomic selection, [45], there are few studies that report
3683 realized gains [46]. In studies that reported realized gains for quantitative traits
3684 (nutritional quality [47]; disease resistance [48]) trait heritability has been higher ($h^2 >$
3685 0.4).

3686

3687 6.5.3 Insights into mechanisms of resistance to *A. vittatum* and future breeding
3688 strategies. While the motivation of this study was to evaluate different selection
3689 methods, our observations will influence future efforts to phenotype *A. vittatum*
3690 preference. In the *A. vittatum* training population, we measured beetle damage by
3691 estimated percent damage rating, measured percent damage, and amount of tissue
3692 consumed (in cm^2). The results demonstrated that measuring exact percent damage
3693 dramatically increased heritability, even though measured percent damage is highly
3694 correlated with estimates and estimates reliably distinguish differences in beetle

damage at the cultivar level [23], [24]. As percent leaf area consumed is also the metric associated with economic loss [25], it may be worthwhile for plant breeders to invest in precise phenotyping methods.

This work has also provided insight into mechanisms of *A. vittatum* host choice and resistance. Leaf volume consumed and feeding efficiency are often important indicators of food quality [49], but we found that total leaf tissue area consumed by *A. vittatum* was not a heritable trait. Intriguingly, we also previously found that *T. ni* did not differ in feeding efficiency across *C. pepo* cultivars [29]. Instead, given that percent damage was heritable indicates that visual cues (e.g. green leaf area, or contrast) may be important for host choice in *A. vittatum*, as it is for other Chrysomelid beetles [50]. Additional support for the importance of visual cues in host choice is that *A. vittatum* does not demonstrate preference in host choice when plants are visually masked [26], [51]. Continued work to identify mechanisms of resistance to *A. vittatum* in *C. pepo* leaves is critical to inform phenotyping efforts as candidates for indirect selection or to integrate into a multi-trait genomic selection scheme, as has been shown to increase genomic prediction accuracies for insect resistance [18].

6.6 Conclusions

In summary, we tested two selection strategies to accelerate genetic gain in improving resistance to *A. vittatum* in *C. pepo*. Both an indirect selection scheme and genomic selection scheme decreased breeding cycle length and had moderate predictive abilities, but mixed realized gains from selection. While genomic selection model training could be improved through more precise phenotyping (e.g. of inbred lines)

and realized gains may be evident in inbred lines derived from segregating populations instead of the populations themselves, this study nonetheless provides of applying new selection techniques and evaluating empirical results in an applied program. Overall, improving resistance to insect pests is a persistent challenge for plant breeding, and implementing selection methods that reduce breeding cycle duration may be a productive strategy for improving genetic gain.

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Chapter 7: Understanding genetic drivers of plant defenses in squash to herbivory by a beetle pest

7.1 Abstract

Plants respond dynamically to herbivory, and the qualities and temporal trajectories of defense expression can vary dramatically even within species. Identifying which of these diverse traits are effective in reducing herbivory provides not only a better understanding of ecological and evolutionary processes, but also informs applied agricultural plant breeding work. We sought to investigate these relationships in cultivated *Cucurbita pepo* (Cucurbitaceae) and the specialist pest, *Acalymma vittatum* (Coleoptera: Chrysomelidae) where there is distinct intraspecific variation in defense: *Acalymma vittatum* exhibits a strong preference for *C. pepo* ssp. *pepo* (e.g. zucchini, "CPP"), and non-preference for *C. pepo* ssp. *ovifera* (e.g. summer squash, "CPO"). However, the mechanistic bases of preference and defense induction are not yet understood. Accordingly, we assessed these mechanisms by measuring gene expression in *C. pepo* over the course of five days of herbivory by *A. vittatum*. Using 3' RNAseq, we characterized the type and temporal trajectory of *C. pepo* plant defense cascades, both between subspecies and over time, to ultimately deduce how *A. vittatum* may cope with these defenses. We found that susceptible CPP has a greater induced response to herbivory, including potential induced susceptibility, but exhibits more tolerance to herbivory than resistant CPO. In contrast, CPO may be rendered resistant through secondary metabolite production. Thus, an important insight is that

plant breeders could seek to combine beneficial traits from both CPP and CPO (tolerance, resistance, respectively) to develop improved varieties. Overall, this approach both illuminates how plants respond to herbivory in systems where mechanisms have not been tractable, and also sets targets for applied agricultural research.

7.2 Introduction

Plants must be defended against multiple antagonistic organisms to complete their life cycle, and such organisms may possess counteradaptations to overcome these defenses. Plants produce a diverse array of defensive traits that vary even between closely related plants [1], [2], and yet not all defenses are effective in mitigating damage against the spectrum of herbivores encountered [3]. For both plants and their antagonists, the parameters of this interaction can also temporally shift through induction of plant metabolites and responsive changes to the herbivore's behavior or performance [4], [5]. Given the dynamic array of defenses plant produce, distilling in which context defenses mediate specific biotic interactions will improve our understanding of ecological and evolutionary processes, and inform applied plant breeding and pest management work.

Across clades and in response to diverse herbivores, targeted observations of key metabolites have been integral in shaping our knowledge of how plants respond to herbivores in space and time [6]–[8]. With the increase in accessibility of gene expression profiling, transcriptional changes underlying hormone responses, defensive metabolites, growth-related processes, and putative indirect defense (induced plant

volatiles) have been observed in multiple species over time during herbivory [9]–[13]. Importantly, these responses demonstrate intraspecific variation; for instance, genes associated with defensive hormone signaling, like jasmonic acid, are differentially expressed between genotypes in response to herbivory in cultivated crop varieties [14] and wild populations [15]. Contrasts between resistant and susceptible plant genotypes have also been integral in highlighting differential investment in constitutive versus induced defensive strategies [16], [17]. However, comparative transcriptomic profiling between resistant and susceptible plants still leaves open questions about which of these changes in defense (or susceptibility) are perceived and elicit a response in insect herbivores.

A system with ecological and agricultural applications where plant defense variation and its implications for herbivores can be explored is *Cucurbita pepo* (Cucurbitaceae) and the specialist pest, *Acalymma vittatum* (Coleoptera: Chrysomelidae). *Acalymma vittatum* exhibits a strong preference for *C. pepo* ssp. *pepo* (e.g. zucchini, "CPP"), and non-preference for *C. pepo* ssp. *ovifera* (syn *texana*; e.g. summer squash, "CPO") [18], [19], subspecies that arose from two separate domestication events in North America [20]–[22]. Post-domestication, most CPO breeding and development occurred in North America, the endemic range of *A. vittatum*, while CPP cultivars were largely bred in Europe [23] and thus removed from *A. vittatum* and other specialist beetle pressure. While the divergence in biogeography and preference are well-known, the dynamic mechanistic bases driving this interaction remain uncharacterized [24], [25]. Thus, use of non-targeted transcriptomics can provide insight into co-evolved adaptations, and how defenses and responses shift

3937 throughout the course of herbivory.

3938 Both *C. pepo* subspecies have an induced response to herbivory as measured
3939 by performance of a generalist caterpillar five days after induction [25]. However, the
3940 magnitude of induction is substantially greater in CPP, and CPO has greater
3941 constitutive defense and remains more effectively defended at all times [25]. More
3942 broadly in the Cucurbitaceae family, defense induction and changes to plant
3943 palatability have been demonstrated in numerous species in response to herbivores
3944 from multiple feeding guilds [26]–[29], but has not been characterized at the
3945 transcriptome-wide level. We thus predict that *C. pepo* temporally alters defenses in
3946 response to herbivory by *A. vittatum*, and that we can predict the counter response of
3947 *A. vittatum* through investigating changes in gene expression during herbivory.

3948 In this study, we investigated the genetic drivers of changes in plant defense
3949 during herbivory between cultivars representing two subspecies of *C. pepo* (CPP and
3950 CPO) and the specialist beetle pest, *A. vittatum*. Specifically, we sought to characterize
3951 the type and temporal trajectory of *C. pepo* plant defense cascades, both between
3952 subspecies and over time, and to ultimately identify how *A. vittatum* copes with these
3953 defenses. We thus evaluated gene expression with 3' RNAseq in *C. pepo* leaf tissue
3954 four time points over the course of five days of herbivory. Through conducting
3955 differential gene expression analysis and gene ontology enrichment analysis, we then
3956 characterized the responses of *C. pepo* to feeding by *A. vittatum*. Overall, distilling
3957 which molecular mechanisms have the potential to affect the degree of plant damage
3958 or success of the herbivores broadens our ecological understanding of plant-herbivore
3959 interactions and can identify genetic variation that could be the target of plant breeding

efforts in agricultural systems.

7.3 Methods

7.3.1 Bioassay. Using cultivated *Cucurbita pepo* and the specialist pest, *Acalymma vittatum*, we asked how the organisms respond on a transcriptomic level during herbivory. We used two inbred lines to capture the extremes of *A. vittatum* preference in *C. pepo*: 'Golden Zucchini' (preferred; *C. pepo* ssp. *pepo*, "CPP"), and 'Success PM' (non-preferred; *C. pepo* ssp. *ovifera*, "CPO") [18]. Untreated seeds were sourced from the Cornell University breeding program. Seeds were sown in late June 2017 in McEnroe Organic Lite Growing Mix soil (McEnroe Organic Farm, Millerton, NY) in the Cornell University Agricultural Experiment Station Guterman greenhouse (Ithaca, NY), and greenhouse conditions with 16 h day, 8 h night photoperiod and respective temperatures of 27 °C and 21 °C. At the time of feeding assays, plants had two to three true leaves and were not flowering.

Adult *Acalymma vittatum* were collected from *C. pepo* flowers on the Homer C. Thompson Vegetable Research Farm (Freeville, NY) immediately prior to starting the experiment. Captured beetles were sexed according to abdomen morphology [30] and only male beetles were used to eliminate (unknown) confounding factors associated with sex.

We conducted a bioassay to allow us to measure transcriptomic responses at 6, 24, 48 and 120 hours to capture a spectrum of constitutive and induced plant defenses. Six hours was the initial sampling point because *A. vittatum* often will take a few hours to begin feeding when enclosed in mesh bags (LB, personal observation). For

each time point, a separate set of genetically identical plants was used to ensure that destructive tissue sampling did not impact the results. The bioassays consisted of four treatments: 1) single CPP plant with beetles (six replicates per time point [p.t.p.]), 2) single CPO plant with beetles (six replicates p.t.p.), 3) plants alone (four replicates per genotype p.t.p), and 4) beetles alone (four replicates p.t.p). In early July 2017, plants were individually enclosed in mesh bags (25 cm x 30 cm), in a randomized complete block design with six blocks to capture spatial variation in the greenhouse. More plants were used in the bioassay than were ultimately sequenced. Five adult male beetles were added to all appropriate entries.

At the end of each time point, estimated percent leaf damage in five percent increments (0%, 5%, 10% …) was also recorded at the end of the bioassay. The effect of genotype, time point, treatment and block were assessed by ANOVA. Then, bulked leaf tissue was collected in triplicate, flash frozen in liquid nitrogen, and stored at -80C.

7.3.2 RNA extraction, sequencing and read counts. We conducted 3' RNA sequencing of 48 samples plant leaf tissue comprising were three replicates of each plant genotype, treatment and time combination. 3' RNAseq is ideal for comparing expression levels in plant and animal tissues with only one read per transcript, and is more cost effective than RNA-sequencing [31]. Plant RNA was extracted with Qiagen Plant RNeasy kit (Qiagen, Hilden, Germany) according the manufacturer's instructions. RNA quality was established on an agarose gel and plate reader. The Cornell University Biotechnology Resource Center (BRC) Genomics Facility (Ithaca,

NY, USA) performed 96-plex 3'RNAseq library construction and sequencing with the Illumina NextSeq 500 (Illumina, San Diego, CA, USA) with single-end 75bp reads.

For quality control of the 3' RNAseq reads, we removed the first 12 base pairs and short reads (< 25 bp) with Trimmomatic [32], adapter sequences and polyA stretches > 12 bp with cutadapt [33], and low quality (phred < 5) base calls with fastq_quality_trimmer [34]. Reads were then aligned to the most recent version of the *C. pepo* genome (v4.1 [35]) using STAR [36] to generate read counts. On average, there were 3580611 reads aligned per sample (83% aligned), and no significant differences in number of reads aligned between samples due to genotype, treatment, time, or experimental block.

7.3.3 Differential gene expression analysis. We normalized read counts by library size using the median ratio method and conducted differential gene expression analysis with the DESeq2 [37] package in R [38]. We first examined differentially expressed genes (DEGs) between treatments (beetle-infested, controls) within time point and genotype (e.g. susceptible genotype at 6 h). We also evaluated DEGs between genotypes (susceptible, resistant) and within treatment and time points (e.g. controls at 6 h). We then identified DEGs to exclusive to beetle-infestation treatments as the genes not differentially expressed in control plants ($p_{adj\text{-}FDR} > 0.1$).

7.3.4 Gene ontology expression analysis. We conducted gene ontology (GO) enrichment analysis [39] using the Bioconductor topGO package [40] in R. We

4029 evaluated enriched GO terms in significant ($p_{adj\text{-}FDR} > 0.1$) upregulated
4030 (log2foldchange > 0) and downregulated (log2foldchange < 0) DEGs between
4031 treatments within time point and genotype and then between genotypes of plants
4032 infested with beetles at all time points. We extracted biological processes (BP) GO
4033 terms from the Cucurbit Genomics database [41], and there were 1898 BP GO terms.
4034 For enrichment analysis in topGO, we used a node size of 10, and then adjusted *p*-
4035 values using the false discovery rate correction.
4036

4037 ## 7.4 Results

4038 7.4.1 Bioassay. We infested a resistant (*C. overifa* ssp. *pepo*, "CPO") and susceptible
4039 (*C. pepo* ssp. *pepo*, "CPP") cultivar with the specialist beetle pest, *Acalymma vittatum*,
4040 and allowed *A. vittatum* to feed for up to five days. While CPO is less preferred in
4041 choice scenarios, both experience a similar degree damage in no-choice scenarios [18],
4042 as we observed in this experiment (**Fig. 7.1**). Damage increased over time
4043 ($F_{3,38}$=24.97, p<0.001), but there was no difference between genotypes ($F_{1,38}$=1.30,
4044 p=0.26), or interaction between genotype and time ($F_{3,38}$=0.37, p=0.77), allowing us to
4045 conclude that observed differences in gene expression trajectories between cultivars
4046 are not confounded with amount of tissue removed.
4047
4048

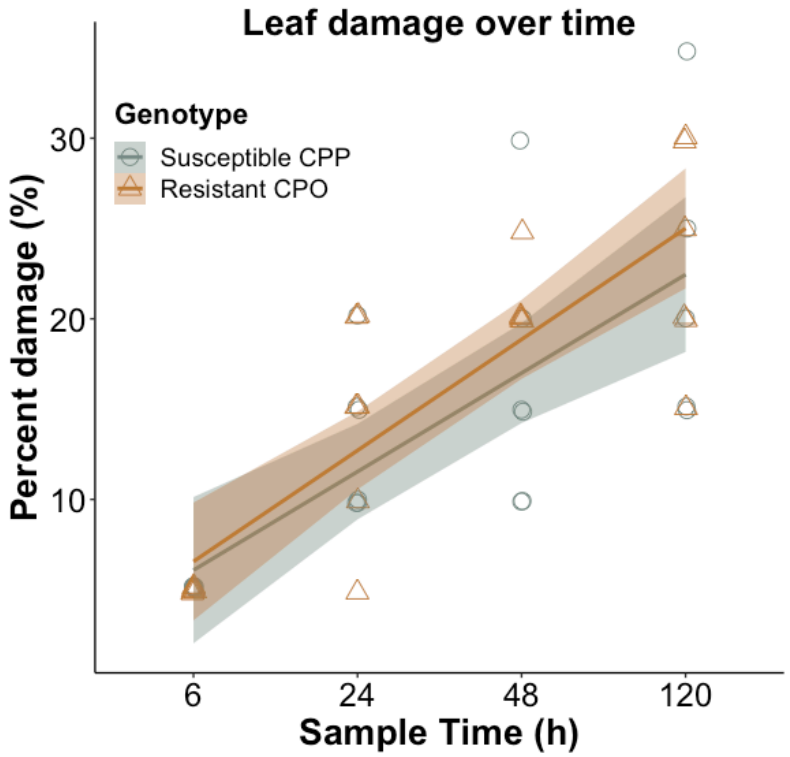

Figure 7.1. Leaf damage over time. Each point represents and individual sample, and the line is a linear model of damage over time by genotype with shading of one standard error.

7.4.2 Differential gene expression – *Cucurbita pepo*. We evaluated differentially expressed genes (DEGs) between treatments (control, beetle-infested) within cultivars at each time point, and found that the susceptible CPP has a faster and higher magnitude of transcriptomic response than the resistant CPO (**Fig. 7.2**). CPP has an immediate response of 2540 DEGs at 6 h, and then attenuates at 24 h and 48 h before building again at 120 h. In CPO, we observe a similar pattern, but delayed in starting until the 24 h time point and many fewer DEGs (220 at 24h). For both, there are more unique DEGs than shared across time points. Of the DEGs at all time points (CPP: 3404 CPO: 383), 174 upregulated and 10 downregulated DEGs are shared between cultivars during at least one time point. In comparing DEGs exclusive to beetle-

infested treatments between cultivars at all time points, there were more upregulated DEGs in the susceptible CPP (1009 total) than the resistant CPO (622 total) (**Fig 7.3**).

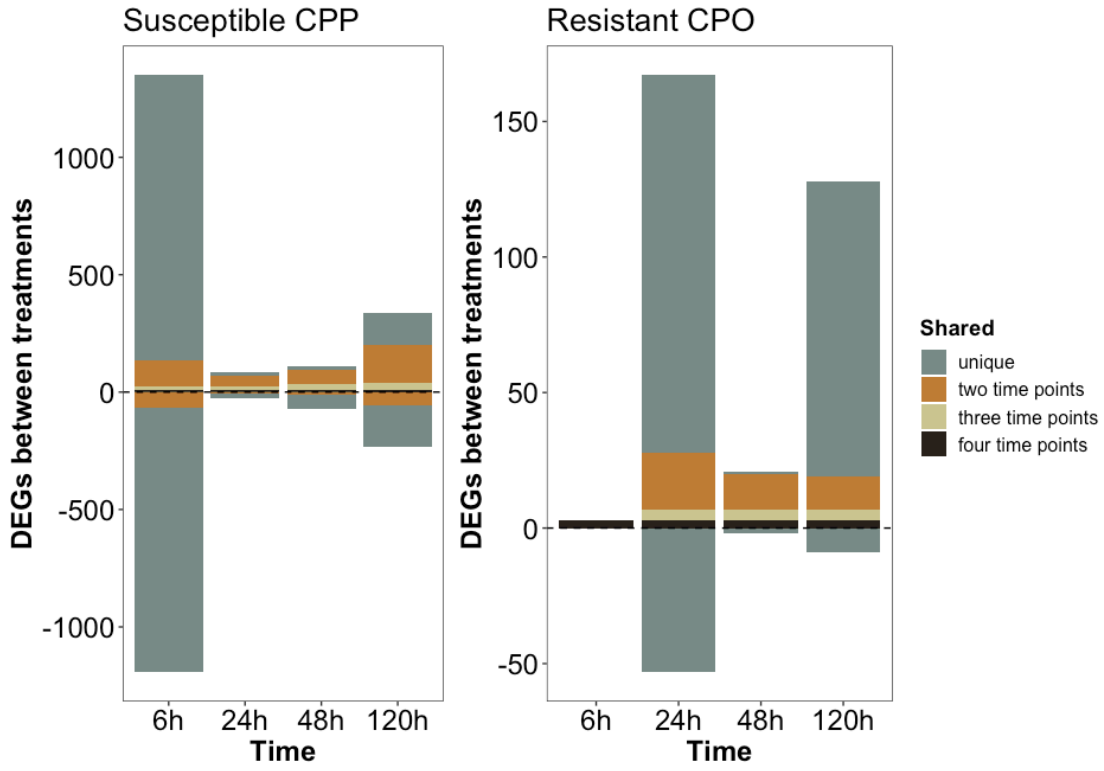

Figure 7.2. Differentially expressed genes (DEGs) between treatments (control, beetle-infested) within genotype at each time point. Positive values indicate DEGs upregulated in response to infestation, and negative values were down regulated. The color corresponds to if the DEG was shared between two or more time points, or unique to the time point.

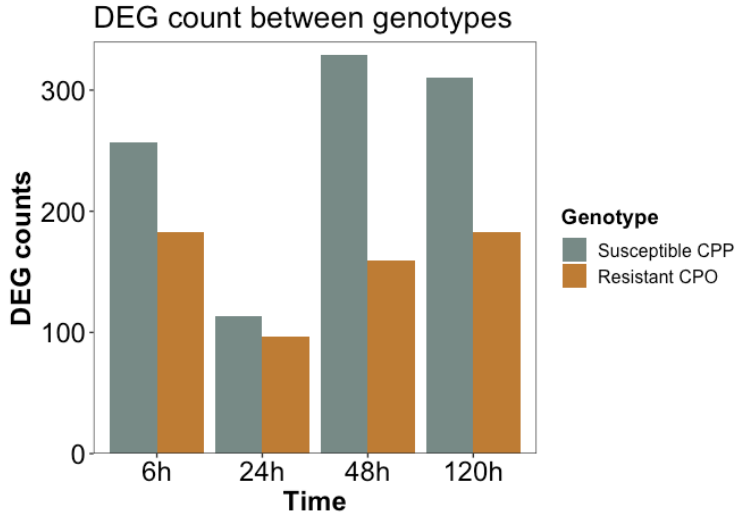

Figure 7.3. Differentially expressed genes (DEGs) between genotypes at all time points exclusive to beetle-infested plants (excluding DEGs between controls). The color corresponds to the genotype that had greater expression of the DEG.

7.4.3 GO term enrichment analysis – *Cucurbita pepo*. We assessed the biological relevance of DEGs by examining enriched gene ontology (GO) terms of upregulated and downregulated genes in the resistant and susceptible cultivars. In the resistant CPO (**Table 1**), DEGs upregulated after 24 h of beetle feeding had significant enrichment of wound response, and then there was a transition to terms for terpene biosynthesis (48 h), and then to other secondary metabolic processes and response to chitin (a component of the insect exoskeleton). In parallel, there was a progressive enrichment of growth-related GO terms in down-regulated genes beginning at 24 h (photosynthesis, cell walls, and tissue development). In the susceptible CPP (**Table 2**) at the earliest time point (6 h), there was significant enrichment of GO terms in upregulated genes related to both growth and light perception processes (photosynthesis; carbon utilization; response to far red light), as well as hormone-mediated defense signaling processes (jasmonic acid and salicylic acid signaling). Later, the top enriched GO terms from upregulated genes (24 h, 48 h) were related to secondary metabolism and wound response and then there was a significant enrichment of terms related to jasmonic acid by 120 h. While growth processes are elevated at 6 h in CPP, there is a significant enrichment of those terms in downregulated genes by 120 h.

Table 7.1. Enriched gene ontology (GO) terms between treatments (beetle-infested and controls) for resistant CPO at all time points. GO terms are ranked by the most significant within section (top three are listed) and those with $p_{adj\text{-}FDR} < 0.20$ are in bold.

Time	GO.ID	Term	Rank	$p_{adj\text{-}FDR}$
		Upregulated genes		
6 h	GO:0006508	proteolysis	1	>0.5
	GO:0019538	protein metabolic process	2	>0.5
	GO:1901564	organonitrogen compound metabolic process	3	>0.5
24 h	GO:0009611	response to wounding	1	**9.49E-03**
	GO:0010951	negative regulation of endopeptidase activity	2	**0.03**
	GO:0055114	oxidation-reduction process	3	**0.08**
48 h	GO:0042214	terpene metabolic process	1	>0.5
	GO:0008152	metabolic process	2	>0.5
	GO:0043067	regulation of programmed cell death	3	>0.5
120 h	GO:0006835	dicarboxylic acid transport	1	>0.5
	GO:0010200	response to chitin	2	>0.5
	GO:0006535	cysteine biosynthetic process from serine	3	>0.5
		Downregulated genes		
6 h	GO:0000003	reproduction	1	>0.5
	GO:0000023	maltose metabolic process	2	>0.5
	GO:0000038	very long-chain fatty acid metabolic process	3	>0.5
24 h	GO:0019344	cysteine biosynthetic process	1	**3.42E-08**
	GO:0018298	protein-chromophore linkage	2	**1.42E-05**
	GO:0015979	photosynthesis	3	**1.42E-05**
	GO:0009765	photosynthesis, light harvesting	4	**1.42E-05**
	GO:0000103	sulfate assimilation	5	**0.15**
48 h	GO:0010411	xyloglucan metabolic process	1	>0.5
	GO:0042546	cell wall biogenesis	2	>0.5
	GO:0071555	cell wall organization	3	>0.5
120 h	GO:2000280	regulation of root development	1	>0.5
	GO:0010229	inflorescence development	2	>0.5
	GO:0010305	leaf vascular tissue pattern formation	3	>0.5

Table 7.2. Enriched gene ontology (GO) terms between treatments (beetle-infested and controls) for susceptible CPP at all time points. GO terms are ranked by the most significant within section (top three are listed) and those with $p_{adj\text{-}FDR} < 0.20$ are in bold

Time	GO.ID	Term	Rank	$p_{adj\text{-}FDR}$
		Upregulated genes		
6 h	GO:0006098	pentose-phosphate shunt	1	**2.47E-09**
	GO:0010027	thylakoid membrane organization	2	**2.28E-07**
	GO:0019252	starch biosynthetic process	3	**2.28E-07**
	GO:0010207	photosystem II assembly	4	**3.89E-07**
	GO:0015979	photosynthesis	5	**4.43E-07**
	GO:0019288	isopentenyl diphosphate biosynthetic process, MEP	6	**4.43E-07**
	GO:0009637	response to blue light	7	**1.41E-06**
	GO:0009902	chloroplast relocation	8	**2.61E-06**
	GO:0006364	rRNA processing	9	**9.49E-06**
	GO:0000023	maltose metabolic process	10	**9.49E-06**
	GO:0015995	chlorophyll biosynthetic process	11	**1.09E-05**
	GO:0019344	cysteine biosynthetic process	12	**9.81E-05**
	GO:0009773	photosynthetic electron transport in photosystem I	13	**2.34E-04**
	GO:0010114	response to red light	14	**3.39E-04**
	GO:0035304	regulation of protein dephosphorylation	15	**6.83E-04**
	GO:0009735	response to cytokinin	16	**7.71E-04**
	GO:0010218	response to far red light	17	**6.54E-03**
	GO:0019761	glucosinolate biosynthetic process	18	**6.54E-03**
	GO:0006563	L-serine metabolic process	19	**7.29E-03**
	GO:0016117	carotenoid biosynthetic process	20	**9.49E-03**
	GO:0006544	glycine metabolic process	21	**0.02**
	GO:0009595	detection of biotic stimulus	22	**0.02**
	GO:1901068	guanosine-containing compound metabolic process	23	**0.03**
	GO:0006412	translation	24	**0.03**
	GO:0009765	photosynthesis, light harvesting	25	**0.04**
	GO:0010155	regulation of proton transport	26	**0.04**
	GO:0009862	systemic acquired resistance, salicylic acid mediated	27	**0.04**
	GO:0009867	jasmonic acid mediated signaling pathway	28	**0.05**
	GO:0042742	defense response to bacterium	29	**0.05**
	GO:0010310	regulation of hydrogen peroxide metabolic process	30	**0.05**
	GO:0010200	response to chitin	31	**0.06**
	GO:0031348	negative regulation of defense response	32	**0.06**
	GO:0019684	photosynthesis, light reaction	33	**0.09**
	GO:0042793	plastid transcription	34	**0.09**
	GO:0018298	protein-chromophore linkage	35	**0.10**
	GO:0015976	carbon utilization	36	**0.10**
	GO:0016226	iron-sulfur cluster assembly	37	**0.10**
	GO:0006566	threonine metabolic process	38	**0.12**
	GO:0006767	water-soluble vitamin metabolic process	39	**0.17**
	GO:0042823	pyridoxal phosphate biosynthetic process	40	**0.17**
24 h	GO:0055114	oxidation-reduction process	1	0.25
	GO:0046417	chorismate metabolic process	2	> 0.5
	GO:0051260	protein homooligomerization	3	> 0.5
48 h	GO:0010951	negative regulation of endopeptidase activity	1	0.38
	GO:0009414	response to water deprivation	2	0.44
	GO:0009611	response to wounding	3	> 0.5
120 h	GO:0009611	response to wounding	1	**0.16**

	GO:0009753	response to jasmonic acid	2	**0.16**
	GO:0009694	jasmonic acid metabolic process	3	**0.16**
	GO:0010951	negative regulation of endopeptidase activity	4	**0.18**
		Downregulated genes		
6 h	GO:0006412	translation	1	**4.37E-19**
	GO:0042254	ribosome biogenesis	2	**4.37E-19**
	GO:0006457	protein folding	3	**0.07**
	GO:0006096	glycolytic process	4	**0.09**
	GO:0007093	mitotic cell cycle checkpoint	5	**0.11**
	GO:0009908	flower development	6	**0.11**
	GO:0006334	nucleosome assembly	7	**0.11**
	GO:0043248	proteasome assembly	8	**0.18**
	GO:0006342	chromatin silencing	9	**0.18**
	GO:0051788	response to misfolded protein	10	**0.18**
	GO:1901991	negative regulation of mitotic cell cycle phase	11	**0.18**
	GO:0051646	mitochondrion localization	12	**0.18**
24 h	GO:0016573	histone acetylation	1	> 0.5
	GO:0032880	regulation of protein localization	2	> 0.5
	GO:0009741	response to brassinosteroid	3	> 0.5
48 h	GO:0006662	glycerol ether metabolic process	1	> 0.5
	GO:0006550	isoleucine catabolic process	2	> 0.5
	GO:0006574	valine catabolic process	3	> 0.5
120 h	GO:0019288	isopentenyl diphosphate biosynthetic process, MEP	1	**9.49E-11**
	GO:0045036	protein targeting to chloroplast	2	**2.18E-08**
	GO:0006412	translation	3	**1.58E-05**
	GO:0015995	chlorophyll biosynthetic process	4	**7.12E-05**
	GO:0006457	protein folding	5	**1.63E-04**
	GO:0042254	ribosome biogenesis	6	**1.27E-03**
	GO:0009658	chloroplast organization	7	**2.03E-03**
	GO:0009697	salicylic acid biosynthetic process	8	**2.37E-03**
	GO:0019344	cysteine biosynthetic process	9	**2.74E-03**
	GO:0006364	rRNA processing	10	**4.93E-03**
	GO:0042026	protein refolding	11	**0.03**
	GO:0045893	positive regulation of transcription, DNA-templated	12	**0.04**
	GO:0009089	lysine biosynthetic process via diaminopimelate	13	**0.05**
	GO:0019685	photosynthesis, dark reaction	14	**0.05**
	GO:0010103	stomatal complex morphogenesis	15	**0.09**
	GO:0019684	photosynthesis, light reaction	16	**0.10**
	GO:0010075	regulation of meristem growth	17	**0.11**

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7.5 Discussion

Using two independently domesticated lineages of squash (*Cucurbita pepo*), and the endemic specialist herbivore, *Acalymma vittatum*, we investigated how *C. pepo* is dynamically defended and predict the implications for the response of *A. vittatum*. We found that the *C. pepo* domesticates had divergent transcriptomic responses to herbivory, where the susceptible *C. p. pepo* ("CPP") was more inducible and the resistant *C. p. ovifera* ("CPO") appeared to have greater constitutive defense. While CPO demonstrated a more canonical response to herbivory, CPP appeared to retain greater resource acquisition during herbivory and thus greater tolerance. Overall, these results provide insight into induced defenses, and identify traits of importance for plant breeding efforts.

7.5.1 *Cucurbita pepo* response to herbivory. We demonstrated that two *C. pepo* domesticates representing extremes of *A. vittatum* preference differ in their transcriptomic response to herbivory in magnitude, strategy and temporal trajectory. Our previous work had shown that CPP is more inducible than CPO in bioassays with generalist herbivores [25], and we observe the same trend here: CPP has more differentially expressed genes than CPO at all times even with similar levels of tissue damage. Intraspecific comparisons of crop plants has sometimes shown that resistant or tolerant cultivars demonstrate greater transcriptional changes in response to herbivory [17], [42], whereas it is posited that fewer transcriptional changes in resistant lines indicate greater investment in constitutive resistance [16]. During agricultural history, most CPO cultivars were bred in the endemic range of specialist

beetles, while many CPP cultivars were not [23]; our finding of higher constitutive versus induced responses in *C. pepo* from locales with higher pest incidence is consistent with predictions from optimal defense theory [43].

Multiple species demonstrate intraspecific variation in temporal patterns of induced responses to herbivory [15], [16], [42], as do the two domesticates of *C. pepo*. The resistant CPO first has gene expression signatures of wound response, and then transitions to expression related to metabolites and transport. In parallel, there is progressive downregulation of photosynthesis, and then growth processes. Plant primary metabolism can respond in dichotomous ways to herbivory; in some cases, photosynthesis may increase to accommodate metabolic demand for defense response, but in other cases, plants may sequester and store carbohydrates [44]. While CPO appears to sequester resources, in contrast, CPP initially upregulates both growth (photosynthesis) and defense (hormones jasmonic acid and salicylic acid) responses. Increased resource acquisition early during herbivory by CPP may be the causative factor in the previously observed greater induced resistance in CPP [25]. In addition, changes in light perception responses in CPP should also be considered in connection with jasmonic acid signaling [45]. Later during herbivory, growth related genes (photosynthesis) are down regulated in CPP, but seemingly not to the same extent as CPO. Overall, these *C. pepo* domesticates have different defense trajectories, where the susceptible CPP has an early burst of resource acquisition, while CPO sequesters resources and has more obvious investment in secondary metabolites, which may have implications for strategies of response by *A. vittatum*.

7.5.2 Implications for *Acalymma vittatum*. The mechanistic knowledge generated in this study has three major implications for host suitability for *A. vittatum*. First, the initial herbivory-induced upregulation of growth-related genes in preferred CPP suggests changes in food quality that may render the preferred CPP more susceptible to *A. vittatum*. Broadly, changes to growth-related gene expression has been shown to affect susceptibility to diverse herbivores [9], [42]. In addition, the early activation of both jasmonic acid and salicylic acid defensive hormone signaling pathways, coupled with changes in light perception, by herbivory in CPP is intriguing for future research. As these pathways are often antagonistic, some herbivores can elicit one pathway to their benefit (e.g. oviposition fluid increases salicylic acid responses, mitigating the jasmonic acid response [46]). Together, these results suggest that herbivory by *A. vittatum* affects growth-related and defensive hormone gene expression in a manner that may render CPP more susceptible to attack, potentially via light perception signaling [45]. Finally, we posit that CPO is non-preferred primarily due to induction of terpenoid secondary metabolism based on our gene expression results and previous work. In an independent bioassay, we found greater linalool emission from foliar tissue of CPO after wounding by *A. vittatum* than from CPP (Brzozowski *et al.*, *in revision*). Thus, more extensive evaluation of the effect of plant terpenoid volatiles on both *A. vittatum* behaviors as well as indirect defense, like recruitment of parasitoids is warranted. Parasitism of *A. vittatum* is widespread [47], and gene expression evaluations in other systems also indicate investment in indirect defense following herbivory [16]. Overall, in comparing intraspecific variation for gene expression in response to herbivory, we identified (potentially independent) factors that contribute to

susceptibility in CPP and resistance in CPO.

7.5.3 Directions for plant breeding. A core goal of this project was to identify targets for plant breeding for enhanced resistance to *A. vittatum* in *C. pepo*, and there are two key outcomes. First, this work provides additional support for secondary metabolism being important for (in)direct defense in CPO. By further verifying mechanisms and scales at which *A. vittatum* responds to secondary metabolites, this trait can be incorporated into breeding programs. Intriguingly, this work also indicates that the susceptible CPP may have increased tolerance to herbivory by maintaining resource acquisition for longer. Tolerance to herbivory is often less well studied than other types of resistance, but can be an important tool in pest management [47], [48]; thus combining resistance traits of CPO and tolerance traits of CPP could be a long term goal of breeding programs. An important caveat of this work is that *A. vittatum* was confined on both CPO and CPP, a situation that may not be realistic for agricultural settings. This work demonstrates that traits relevant for pest management may be present in germplasm that we identify as resistant (CPO) and susceptible (CPP).

7.6 Conclusions

By evaluating gene expression in two domesticates of *C. pepo*, resistant CPO and susceptible CPP, we identified patterns of gene expression indicating divergent strategies for response to herbivory. The resistant CPO has fewer changes to gene expression immediately following herbivory, suggesting investment in constitutive defense, but later increases secondary metabolism while restricting growth. In

contrast, the susceptible CPP is highly inducible and continues resource acquisition for longer periods of time following herbivory, indicating some degree of plant tolerance. Overall, this work provides insight as to how *A. vittatum* interacts with *C. pepo*: CPP may be a better food source, while CPO may rely more heavily on indirect defense. The approach of using non-targeted transcriptomics has provided insight into genetic drivers of resistance mechanisms and thus structured applied plant breeding goals.

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Chapter 8: Synthesis

8.1 Summary

The motivation of this work was to breed for reduced foliar damage in squash (*Cucurbita pepo*) from the major agricultural pest, the striped cucumber beetle (*Acalymma vittatum*). In this work, we built a mechanistic model of factors that contribute to foliar damage and evaluated variation in these factors between the two cultivated subspecies of squash, *C. p. pepo* (e.g. zucchini) and *C. p. ovifera* (e.g. summer squash). All evaluations were conducted on plants with up to four fully expanded true leaves and pre-flowering.

In summary, we confirmed that cucurbitacins, ubiquitous terpenoids of the Cucurbitaceae plant family, confer susceptibility to beetle attack in cotyledons. However, cotyledon susceptibility does not render leaves susceptible. Instead, after dissecting components that could contribute to leaf resistance, we now define leaf resistance as factors in leaves that reduce beetle retention on a given plant. These results are diagramed in **Figure 8.1** and detailed in **Table 8.1**.

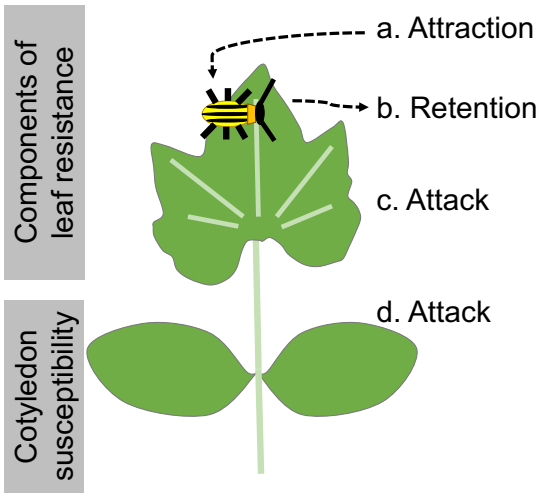

Figure 8.1. Schematic representing our current understanding of factors contributing to leaf resistance and cotyledon susceptibility in *Cucurbita pepo* where each point is elaborated upon in Table 8.1.

Table 8.1. Summary of factors that affect foliar damage by *Acalymma vittatum* on the two subspecies of *Cucurbita pepo*, *C. p. ovifera* ("CPO") and *C. p. pepo* ("CPP").

Factor	Evidence	Support	Future directions
Leaves			
a. Attraction			
There was not beetle attraction to foliar volatiles, and there is no variation in pheromone-mediated density dependent attraction	Using opaque cages to trap field-colonizing beetles, cages that were not infested with pheromone-producing males were not attractive to incoming beetles (Chapter 4) With the same cages, there is equal pheromone-mediated density dependent attraction to both CPO and CPP. This indicates lack of differential pheromone release or synergism with plant volatiles. (Chapter 4)	***Strong***. This was consistently observed in multiple field seasons and demonstrates the same pattern as was observed in another cucurbit (cucumber) [1], [2].	Further work could elucidate how beetles locate squash plantings in general (e.g. random sampling, visual cues), but may not have direct breeding applications
b. Retention			
There was less beetle retention on CPO and damage was concentrated on fewer CPO plants relative to CPP.	In an experiment where beetles were initially confined on plants and then allowed to leave, fewer beetles remained on CPO. (Chapter 4) In no-choice greenhouse and field experiments, damage was fairly uniform across CPP, but was concentrated on fewer CPO plants, consistent with pheromone-mediated settling after movement from CPO. (Chapter 2) To generate hypotheses about plant chemistry that could promote this behavior, foliar volatiles were measured in a representative susceptible CPP and resistant CPO, and differential volatile emissions were identified. (Chapter 4)	***Moderate*** for behavior. Independent field experiments indicate the importance of beetle movement and retention. ***Preliminary*** for role of volatiles.	This is a promising area for future work to understand mechanisms that affect retention (e.g. testing specific plant chemistry) and facets of beetle movement (e.g. how far?)

c. Attack			
No chemical factors that affect leaf attack (area consumed) in no-choice tests were identified	In a suite of experiments, no known factors (e.g. cucurbitacins, nitrogen) were implicated in affecting amount or rate of leaf consumption. However, differences in induction of leaf defenses between CPP and CPO were observed, although their role in beetle feeding remains undetermined. (Chapters 3, 5, 7)	***Strong***. Multiple independent experiments with consistent results for both factors	Non-targeted analysis of leaf tissue (metabolomics or transcriptomics) could lead to breeding applications if traits that affected beetle metabolism were identified
In choice tests, leaf attack was greater in CPP than CPO	In field and greenhouse cultivar panels, beetles consistently preferentially attacked CPP cultivars over CPO cultivars (Chapters 2, 6)		
Cotyledons			
d. Attack			
Beetles consume more cotyledon tissue of plants with high cotyledon cucurbitacin concentration	It was previously established that cotyledons of CPP are rich in cucurbitacins and sustain greater damage than the cotyledons of CPO, which are low in cucurbitacins. To decouple cotyledon cucurbitacin concentration from other factors associated with subspecies, divergent selection was conducted in an intermated population, and foliar damage was evaluated. Cotyledons of plants with high cotyledon cucurbitacins were more susceptible to beetles than those with low cotyledon cucurbitacins; leaf damage was unaffected by cotyledon cucurbitacins. (Chapter 5)	***Strong***. Multiple years of field data, consistent with broader literature on cucurbitacins and specialist beetles	Ready for molecular marker development and application in breeding programs

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8.2 Key limitations and considerations

8.2.1 Leaf resistance is movement. Our working model is that leaf resistance is due to beetle emigration – not factors that confer resistance (or susceptibility) via attraction or attack. As we also demonstrate, beetles will feed upon, and male beetles will release aggregation pheromone from, plants classified as 'resistant' and 'susceptible', if confined. This could be extended to conclude that the manifestation of leaf resistance is because beetles fail to initiate density dependent aggregation due to dispersal. This result further encourages use of trap crops or other beetle attractants such that dispersing beetles have a destination removed from the market crop, where the resistant plants could amplify effectiveness of the trap as a 'push' in a push-pull system [3]–[5].

8.2.2 Limited germplasm screened. A key caveat of this work is that all experiments were done with a few varieties: *C. p. ovifera* cv. 'Success PM' summer squash, *C. p. pepo* cv 'Golden Zucchini', and *C. p. pepo* cv 'Black Beauty' zucchini. This was done to allow for necessary replication in these intensive and often noisy field experiments. While this work and others demonstrate consistencies in degree of resistance among varieties within subspecies [6]–[8], further work should expand the breadth of germplasm screened.

8.2.3 Flowering affects beetle behavior. This work was done on young squash with the rationale being that tissue loss shortly after gemination or transplanting can majorly affect plant growth and thus yield. At this early-season stage, it can be unlikely that

4396 flowering Cucurbitaceae are available. However, once flowers are available, it is
4397 widely established that flowers (color, pollen food source and volatiles) attract beetles
4398 [9]–[11] and this can be applied in trap cropping efforts [12].
4399

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Appendix A: DMR-NY401[3]

A.1 Introduction

The Cornell University vegetable breeding program has developed cucumbers (*Cucumis sativus* L.) resistant to a spectrum of diseases, including powdery mildew [1], [2] and viruses [3]. The program has also released a number of cultivars with multiple disease resistances, like the 'Marketmore' series [4]. The most recent release from this breeding program was a green slicing cucumber inbred line, DMR-NY264, that is resistant to cucurbit downy mildew [5]. Here, we report the development of a new cucumber cultivar, DMR-NY401, with downy mildew resistance similar to 'DMR-NY264', but characterized by earlier maturation and higher yields.

Development of these newest cultivars was initiated in response to the rapid rise of cucurbit downy mildew as one of the greatest worldwide contemporary disease threats to cucumber production. Cucurbit downy mildew is characterized by angular chlorotic foliar lesions that quickly turn necrotic, and often lead to rapid plant death [6]. Diagnosis is aided by the presence of purplish-black sporangia of the causal oomycete pathogen, *Pseudoperonospora cubensis* (Berk. & Curt.) Rostov., which are often visible on the abaxial leaf surface. In the United States, sporangia are widely disseminated from overwintering sites in southern Florida to the eastern United States

[3] This work has been published: Brzozowski, L.*, Holdsworth, W.L.*, and Mazourek, M. 2016. 'DMR-NY401': A New Downy Mildew-Resistant Slicing Cucumber. *HortScience* 51 (10): 1294-1296. doi: 10.21273/HORTSCI10857-16

via wind currents [7]–[9]. In recent years, it has been proposed that inoculum could be originating from new sources, like greenhouses in colder locales [10], and new evidence suggests that the pathogen can be seed transmitted through infected plants [11].

Managing this disease on cucumber in the United States became a challenge after the appearance of a new strain of the pathogen in 2004 in the southern U.S. [10]. The pathogen overcame host plant resistance that had lasted for decades, causing devastating yield losses [12], [13]. The ability of the pathogen to evolve rapidly has also reduced the efficacy of many fungicides, and resistance to a range of fungicides has been reported [14]–[16]. Achieving durable control with fungicides is challenged by the recent spread of a new mating type (A2) of the pathogen to four continents within five years of its initial appearance [17].

In response to the lack of host plant resistance available in commercially suitable germplasm after 2004 [18], Cornell University developed and released 'DMR-NY264', which built on earlier downy mildew resistance breeding work in the 'Marketmore' and 'Poinsett' series [5]. While the genetic basis of downy mildew resistance is unknown [17], 'DMR-NY264' likely derives its resistance from the additive genetic effects of its moderately downy mildew-resistant ("DMR") parents [5]. While 'DMR-NY264' exhibits exceptional cucurbit downy mildew resistance, it is late to produce fruit, making it most useful in regions where growers are planting in anticipation of severe downy mildew pressure or have sufficient growing degree days to offset this lag. The next step in the breeding process was developing an earlier and more prolific cucumber that retained the resistance of 'DMR-NY264' while

continuing to improve on fruit type.

To develop this cucumber, 'DMR-NY264' was crossed to 'Dasher II', an early, green slicing cucumber (**Fig. A.1**). Large F_2 populations of progeny from this cross were evaluated in the field under natural cucurbit downy mildew inoculum, harvested regularly, and the top-performing progeny were selected. Cuttings were taken from these selections, and were then intermated in the greenhouse. By opting to not pollinate in the field, many more plants at earlier generations could be observed without bias from fruit load. These intermated progeny were subsequently selfed, and the families were evaluated in, and selected from, the field. After that, selected progeny were selfed for two more generations to increase uniformity. From this process, an earlier and more prolific downy mildew resistant line, 'DMR-NY401', was developed.

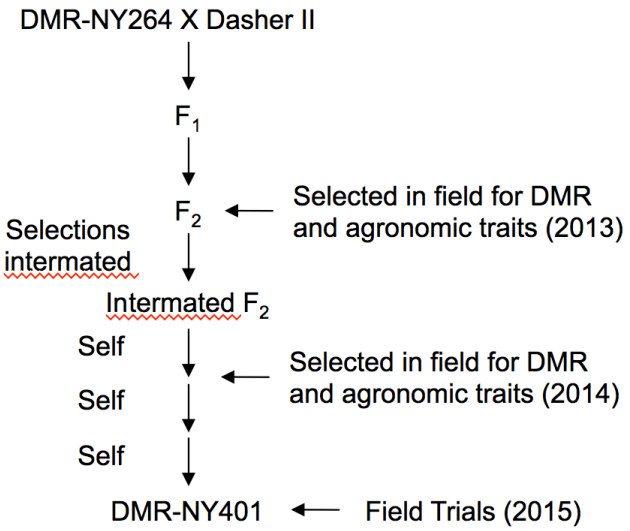


Figure A.1 Pedigree of Cornell downy mildew-resistant breeding line DMR-NY401. Between each field season, two generations were advanced in a winter greenhouse.

A.2 Description and Performance

''DMR-NY401' is a slicing cucumber, medium-long in length (8-10"), with

uniform green color and white spines (**Fig. A.2**). The average marketable fruit weight was 0.2 +/- 0.05 kg in conventional and 0.19 +/- 0.02 kg in organic trials. Importantly, 'DMR-NY401' retained the disease resistance of 'DMR-NY264' while increasing fruit length, yield, and earliness of initial harvest.

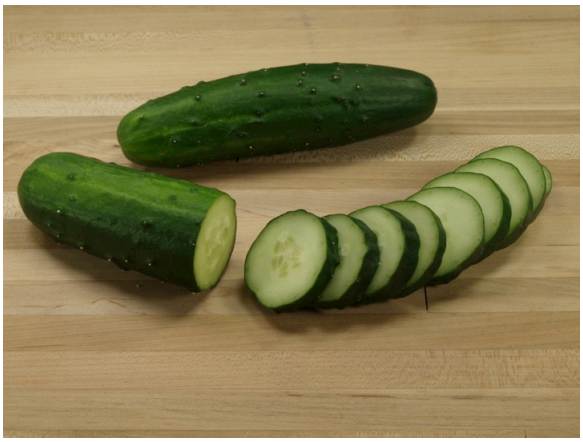


Figure A.2. Fruit of Cornell downy mildew-resistant inbred line, DMR-NY401.

Disease resistance and yield were evaluated in conventional and organic trials for 'DMR-NY401' alongside Cornell University top early DMR breeding lines (15-402 to 15-408), commercial green slicing cultivars with advertised resistance to the post-2004 strain of the downy mildew pathogen (see **Table A.1**), and susceptible and resistant check cultivars, Straight 8 and DMR-NY264, respectively.

Seeds for the organic and conventional trials were sown on 16 July 2015 in Guterman Greenhouse (Ithaca, NY), and transplanted on 31 July 2015 at Freeville Organic Research Farm (Freeville, NY), and 3 Aug. 2015 at Homer C. Thompson Vegetable Research Farm (Freeville, NY), respectively, late in the season after the pathogen was reported in the region. Both trials were planted into rows covered in black plastic mulch, with 2.7m spacing between rows, and arranged in a randomized complete block design with three replications of 10 plant plots. Plants were separated

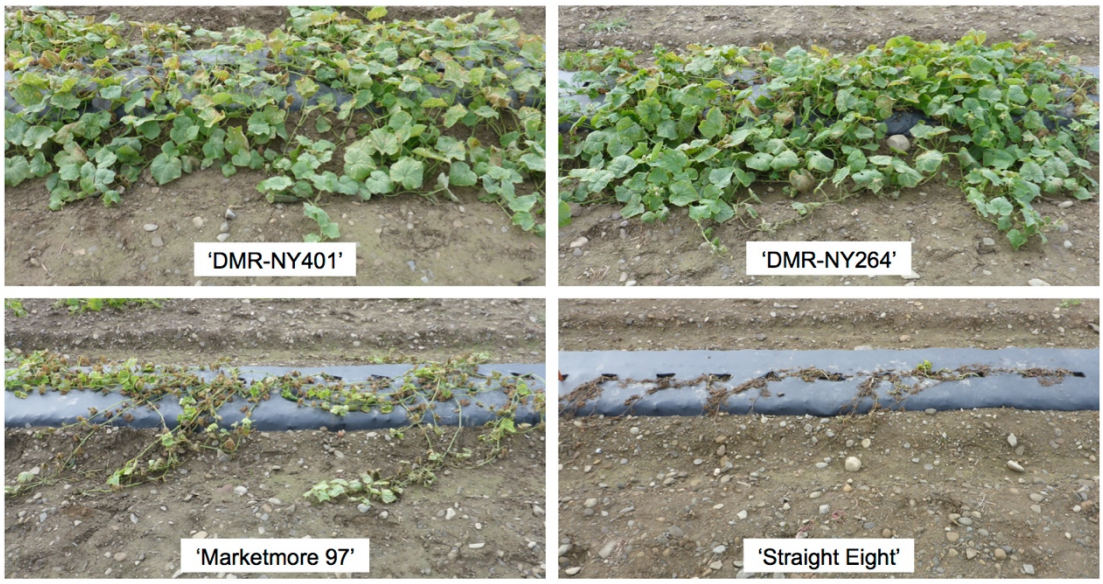
by 0.6m within the plot, and by 1.8m between plots. In addition, transplants for the conventional trial were treated with imidacloprid (Marathon®, Bayer Environmental Science, Research Triangle Park, NC) to control insect pests, and azoxystrobin (Heritage®, Syngenta Crop Protection, Greensboro, NC) to control fungal diseases, like powdery mildew, at labeled rates on 27 July 2015.

Downy mildew symptoms were first recorded in both trials on 14 Aug. 2015 (see Table 1) and percent foliar disease was then recorded weekly. Other minor foliar diseases, including angular leaf spot (*Pseudomonas syringae* pv. *lachrymans*), Alternaria leaf blight (*Alternaria cucumerina*) and powdery mildew (*Podosphaera xanthii*), were present in the organic trial, but their severity was extremely limited compared to downy mildew, and efforts were made to ensure symptoms due to these diseases were not recorded as percent foliar disease due to downy mildew. Other multistate trials in the Eastern United States that included 'DMR-NY401' and its progenitors have also not reported significant disease due to downy mildew, and have observed field resistance to powdery mildew (Mazourek, M., unpublished data). Marketable fruits were harvested, counted and weighed three times weekly beginning 4 Sept. 2015.

Trial data was assessed with a one-way ANOVA, and the differences between individual trial entries were evaluated with the Tukey-Kramer HSD test in JMP Pro 11 (JMP®, Version 11. SAS Institute Inc., Cary, NC, 1989-2007).

The downy mildew resistance of 'DMR-NY401', measured by AUDPC, was comparable to that of 'DMR-NY264' (**Table A.1**), and these plants continued to grow up until frost (**Fig. A.3**). This is consistent with AUDPC measured in breeding plots of

4540 the progenitor of ‘DMR-NY401’ compared to key representative commercial cultivars
4541 in the year prior (2014) that were grown under the conventional management regime
4542 previously described (**Table A.2**). These data demonstrate consistency of the
4543 resistance of DMR-NY401 in separate downy mildew epidemics. In addition, both
4544 days to harvest and yield were improved in two very different open field production
4545 systems. The date of first harvest for ‘DMR-NY401’ was significantly shortened by
4546 approximately nine days compared to ‘DMR-NY264’ under both management
4547 regimes, and not statistically distinguishable from any of the commercial cultivars
4548 trialed (**Table A.3**). In addition, ‘DMR-NY401’ had the highest fruit production of
4549 both trials – it outperformed commercial counterparts and ‘DMR-NY264’ (**Table**
4550 **A.3**). Overall, ‘DMR-NY401’ has a timely harvest window and good yield while
4551 maintaining strong disease resistance.
4552



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4554 **Figure A.3.** Images of plots from conventional trial on 8 Oct. 2015

Table A.1. AUDPC measurements for all trial entries under both organic and conventional management

Trial Entry[z,y]	Organic Trial AUDPC[x]	Conventional Trial AUDPC[x]
DMR-NY264	306.8 a	528.3 a
DMR-NY401	473.5 a b	608.5 a
15-402	576.8 a b	550.8 a
15-407	708.2 a b c	944.2 a
15-403	826.0 a b c	687.0 a
15-404	879.7 b c	697.3 a
15-408	1131.0 c	618.0 a
15-405	1754.2 d	1456.2 b
Marketmore 97	2449.3 e	1876.3 b c
SV4719CS (Seminis)	2558.5 e	2470.2 d
SV4220CS (Seminis)[w]	n.d.	2622.3 d e
Darlington (Seminis)	2595.2 e	2930.7 d e f
15-406	2671.7 e f	1928.8 c
Dasher II (Seminis)	3144.3 f g	3025.2 e f
Centella (Harris)	3223.8 g	3140.2 f
Straight Eight (Ferry-Morse)	4196.8 h	3678.7 g

[z]Data for all entries are reported as the mean of three replications.
[y]Trial entry was highly significant in a one-way ANOVA for both trials ($P < 0.0001$), and block was significant in the organic trial ($P = 0.0027$).
[x]Means in the same column followed by different letters are significantly different as determined by Tukey-Kramer honestly significant difference ($P < 0.05$) test.
[w]'SV4220CS' was not evaluated in the organic trial.

Table A.2. AUDPC measurements for trial entries grown in 2014 under conventional management

Trial Entry[z,y]	2014 AUDPC[x]
DMR-NY401 progenitor[w]	156.3 a
DMR-NY264	253.8 a
SV4719CS (Seminis)	730.8 b
Dasher II (Seminis)	835.3 b c
Straight Eight (Stokes)	991.8 c

[z]Data for all entries are reported as the mean of two replications.
[y]Trial entry was highly significant in a one-way ANOVA ($P < 0.0001$)
[x]Means in the same column followed by different letters are significantly different as determined by Tukey-Kramer honestly significant difference ($P < 0.05$) test.
[w]DMR-NY401 is a selection of the second selfed generation from the DMR-NY401 progenitor
.

Table A.3. Date of first marketable fruit harvest, and cumulative marketable fruit harvest and yield for all trial entries under both organic and conventional management

Trial Entry[z,y]	Organic Trial			Conventional Trial		
	Fruit harvested per plot[x]	Fruit yield per plot (kg)[x]	Date of first harvest (days after sowing)[x]	Fruit harvested per plot[x]	Fruit yield per plot (kg)[x]	Date of first harvest (days after sowing)[x]
DMR-NY401	45.7 a	8.5 a	57 a b	47.7 a	9.6 a	57 a b
15-402	37 a b	6.8 a b	57 a b	30.7 a b c d	6.2 a b c d	55 a b
15-405	30.7 a b c	5.5 a b c	54 a	39 a b c	6.3 a b c d	57 a b
15-403	29.3 a b c	5.5 a b c	55 a b	43 a b	9 a b	58 a b
15-407	24 a b c d	4.9 a b c d	56 a b	23.7 b c d	4.7 b c d e	61 b c
15-408	22.3 b c d e	4.2 a b c d e	56 a b	34 a b c d	7.5 a b c	61 b c
DMR-NY264	17 b c d e	3.1 b c d e	65 c	13.7 d e	2.6 d e	66 c
SV4719CS (Seminis)	11.3 c d e	2.9 b c d e	53 a	29 a b c d	3.9 c d e	51 a
SV4220CS (Seminis)[v]	n.d.	n.d.	n.d.	28 a b c d	3.8 c d e	51 a
Marketmore 97	9.7 c d e	1.9 c d e	54 a	17 d e	2.4 d e	55 a b
15-404	9.3 c d e	1.8 c d e	62 b c	17 d e	3.1 c d e	61 b c
15-406	5 d e	0.7 d e	54 a	15 d e	2.1 d e	56 a b
Centella (Harris)	4.7 d e	1 d e	54 a	17.7 d e	2.3 d e	51 a
Dasher II (Seminis)[w]	4.3 d e	0.7 d e	55 a b	20.7 c d e	2.5 d e	51 a
Darlington (Stokes)[w]	3.3 d e	0.4 e	61 a b c	16 d e	1.6 d e	52 a
Straight Eight (Stokes)	0 e	0 e	N/A	0 e	0 e	N/A

[z]Data for all entries are reported as the mean of three replications.

[y]Trial entry was highly significant in a one-way ANOVA for both trials ($P < 0.0001$) for all reported results (number of fruit harvested per plot, fruit yield per plot, and date of first harvest), and block was significant in the organic trial for both cumulative fruit harvest ($P = 0.0021$) and yield ($P = 0.0021$).

[x]Means in the same column followed by different letters are significantly different as determined by Tukey-Kramer honestly significant difference ($P < 0.05$) test.

[w]Averages of "Date of first harvest" for entry is the mean of two plots rather than three because one plot produced no marketable fruit prior to plant death.

[v]'SV4220CS' was not evaluated in the organic trial.

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Appendix B: Pickling cucumber breeding

B.1 Selection within the breeding program

After releasing downy mildew resistant (DMR) slicing cucumbers (DMR-NY264 and DMR-NY401), a goal of the breeding program was to develop DMR pickling cucumbers. The process involved crossing DMR slicer germplasm to pickling cucumbers ('Vlaspik', 'Eureka') to introgress resistance from slicers while retaining acceptable pickling fruit quality traits. The selection scheme is outlined in **Fig. B.1**. Throughout the selection process, there was a stringent threshold for DMR, and increasingly strict criteria for earliness, fruit number, shape and quality characteristics.

Beginning in 2015, single plants were selected from F2 families, and then four different selection schemes were used. First, some of these selections were selfed and selected in each generation through creating F4 families (**Fig. B.1a**). Then, some F2 selections were intermated within family (**Fig. B.1b**), and then subsequently selfed for two generations, or formed the basis of a recurrent selection population that was selected for another generation. Finally, some F2 selections were crossed to 'Eureka', a market standard with slightly better yield and disease resistance than 'Vlaspik', although poorer fruit quality (**Fig. B.1c**). These were then subsequently selfed for four generations.

In the penultimate generation described **Fig. B.1**, extensive notes were taken on fruit quality characteristics, plant architecture, prolificacy, earliness and disease resistance. In addition, grower feedback was solicited on some fruit samples in 2017 at field days

in Michigan and New York. Then, F4 and (F2 intermated)S2 families were evaluated in replicated trials in 2018. The (F2 intermated)S2 families were the most heavily represented in trials, as they were identified as generally more DMR and having better fruit quality. Additional generations of recurrent selection also occurred in 2018-2019 to continue population improvement.

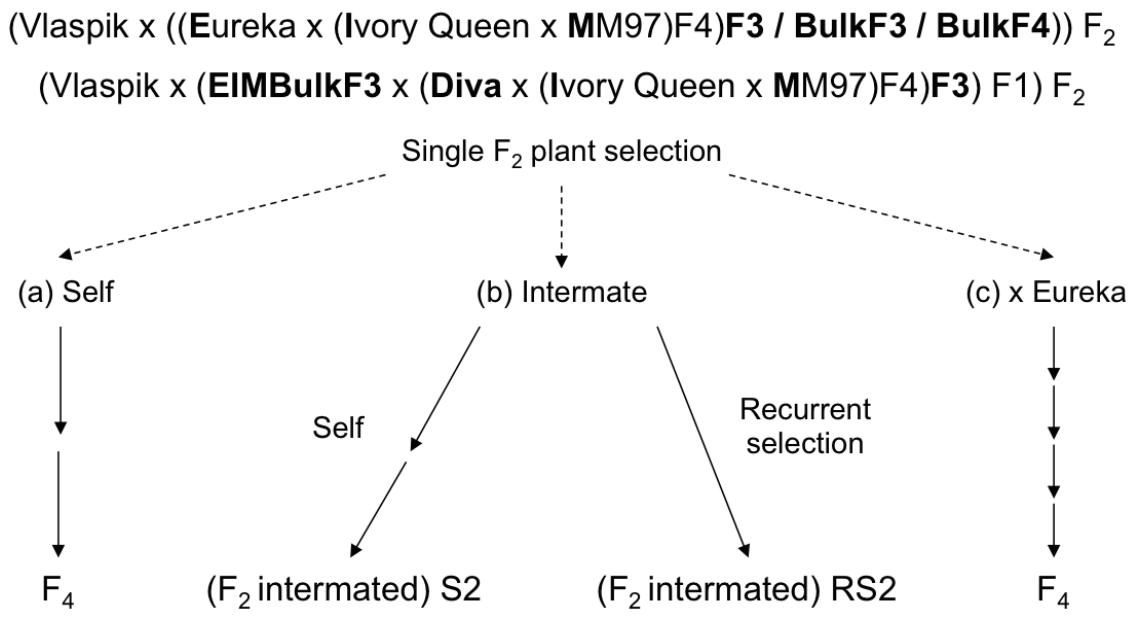


Fig. B.1 Pedigree selection process for developing downy mildew resistant pickling cucumbers

B.2 Participatory selection

Downy mildew resistant pickling cucumber germplasm was improved through participatory breeding involving growers and processors to ensure that the germplasm met grower needs and market requirements, and could lead to more rapid adoption of new varieties. This collaboration involved researchers and growers at three sites: Florida (University of Florida), Michigan (Michigan State University), and New York

(Cornell University).

First, replicated trials of parent lines were conducted to test if sources of resistance ('DMR-NY401', 'DMR-NY264') were effective in all locations compared to susceptible market standard pickling cucumbers ('Vlaspik', 'Eureka'). Trials occurred in New York and Florida in 2016 and 2017 with four spatial blocks of 15 plant plots. Across all four site-years, resistant cucumbers from the New York-based breeding program had lower disease severity (**Fig. B.2**), indicating that this germplasm is appropriate for developing DMR pickling cucumbers for multiple locations. In general, the susceptible market standards had greater yield early in the season, but the resistant parent lines met or exceeded those yields by the end of the season (**Fig. B.3**). This difference in early yield was more pronounced in New York than Florida. In summary, the resistant parents lines appear to be suitable as resistance donors for pickling germplasm because the resistance is effective in multiple locations and the yield penalty is not insurmountable in any location.

With confirmation that the starting germplasm was appropriate, participatory selection was conducted over the following two years (**Fig. B.4**). The starting population was an intermated and selfed F2 population that was selected in the breeding program for resistance and fruit quality traits. Three hundred seed from that population was provided to all participatory sites (FL, MI, NY) in 2017. Then, participatory evaluation and individual plant selection was conducted with growers, extension agents and university researchers at each site, with MI and FL sites being on a university research station, and the NY site on a grower's farm. From each site, up to 12 individuals were selected, selfed and intermated within site to maintain a

population for recurrent selection (thus maintaining three separate recurrent selection populations).

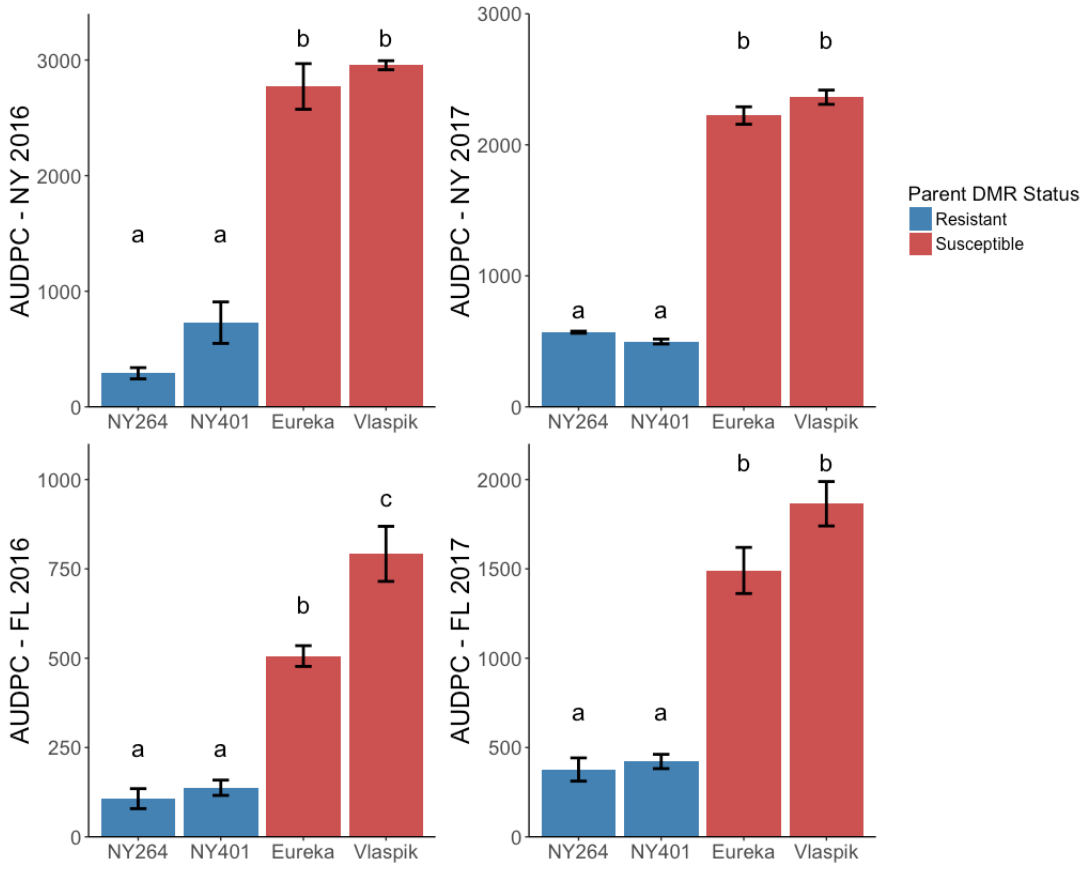
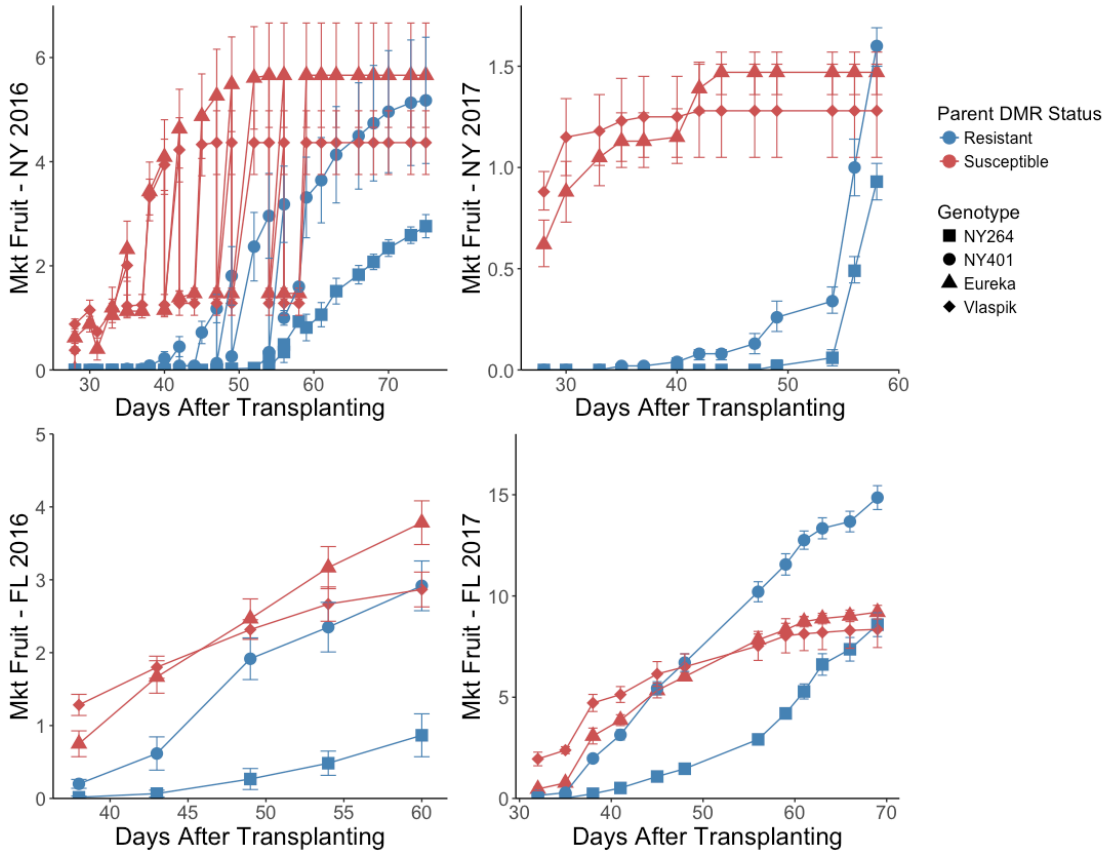


Figure B.2. Area under the disease progress curve (AUDPC) for all locations and years where height of the bar is the mean, and error bars are +/- 1 standard error, and letters are $p<0.05$ with Tukey HSD test. Each site was analyzed with a linear model with response variable of AUDPC and fixed effect of genotype and block. Genotype was significant in all instances (NY16: $F_{3,9}=112.8$, $p<0.001$; NY17: $F_{3,9}=614.0$, $p<0.001$; FL16: $F_{3,9}=67.38$, $p<0.001$; FL17: $F_{3,9}=49.94$, $p<0.001$). Block was not significant in any model.



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4712 **Figure B.3**. Cumulative marketable fruit count per plant for all locations and years
4713 where point height is the mean, and error bars are +/- 1 standard error. Each point
4714 represents harvest by day after transplanting.
4715

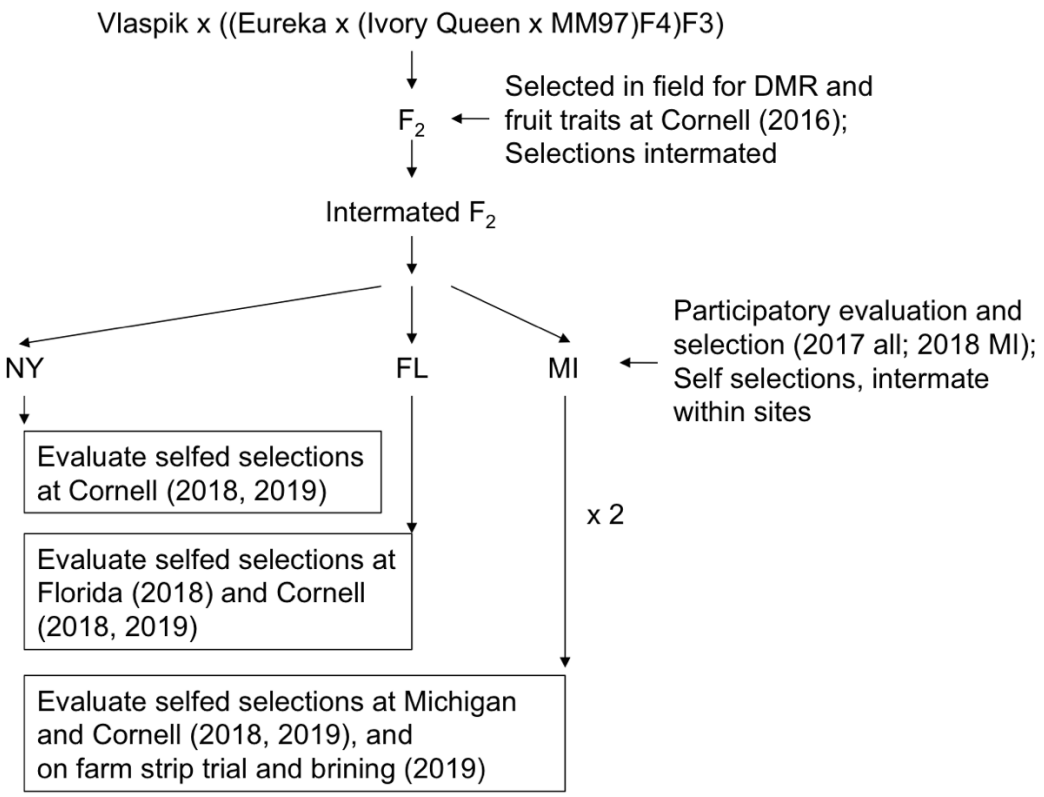

Figure B.4. Diagram of participatory selection scheme, where arrows indicate a generation.

The initial intention was to create a single variety. However, upon talking with growers and researchers at field days, it was evident that grower needs dramatically differed between sites based on harvest method (**Table B.1**). For instance, growers in Michigan harvest pickling cucumbers by machine in a single harvest, necessitating small plants with concentrated fruit set, while growers that sold to wholesale markets (NY, FL) preferred a longer harvest window.

Table B.1. Summary of feedback from each regional site.

Site	Characteristics
Wimauma, Florida	- *Primary market*: mostly fresh market and wholesale, some processing - *Important characteristics*: 3:1 length:width ratio, small seed cavity, concentrated fruit set, darker color with no yellow belly
Lansing, Michigan	- *Primary market*: processing - *Important characteristics*: small seed cavity, blocky ends, 3:1 length:width ratio, small plant size and concentrated fruit set for machine harvest
Ransomville, New York	- *Primary market*: wholesale and fresh market - *Important characteristics*: small seed cavity, blocky ends, no speckles or spines, ability to harvest 4 times weekly for 4 weeks

After one cycle of selfing and intermating, the selfed progeny and populations were evaluated in FL and MI in 2018 in a similar manner. In Michigan, there was a second cycle of selection and generation advancement, and those progeny were evaluated on the research station, on-farm and in a brining trial the following year (2019). Selfed progeny from all sites were also evaluated at the research station at Cornell University. Overall, based on grower feedback, fruit quality metrics increased, and resistance was maintained. On farm trials in 2019 were hampered by low disease pressure as market standard pickling cucumbers tend to outperform our breeding lines in the absence of disease.

Appendix C: Striped Pepper Breeding

A goal of the breeding program was to develop non-pungent, snacking-sized, variegated peppers that had bold stripes on the fruit, good fruit eating quality, and adequate productivity. The target audience for this pepper was home gardeners and small market growers that could market this pepper as a high value product. There were two breeding projects: (1) pedigree selection within the breeding program, and (2) a nation-wide participatory breeding project through the Northern Organic Vegetable Improvement Collaborative (NOVIC).

For pedigree selection (**Fig. C.1**), selections of existing BC1F2 families derived from a cross between 'Lipstick', a sweet red pepper, and F3 individuals from either crosses between 'Fish' pepper, a variegated hot pepper, and 'Veggie Sweet', a sweet pepper, or 'Fish' and 'Czech Black', a hot purple pepper were made in the field. In early generations, it was critical to select for non-pungent peppers with the desired fruit variegation, and in later generations, quantitative traits like yield and flavor were prioritized. From these initial selections, three generations of selection and selfing were conducted to create stable varieties, and there was a replicated trial of F5 families (**Fig. C.1a**). Concurrently, superior non-pungent F3 families were crossed to cultivars 'Oranos' and 'Xanthii' with the goal of introgressing resistance to *Tobacco Mosaic Virus* (TMV) and improving quality (**Fig. C.1b**). In addition, there was an unexpected diversity of unique flavors in the progeny. Thus, these peppers were primarily selected for quality and flavor, but prolificacy and vigor were also considered.

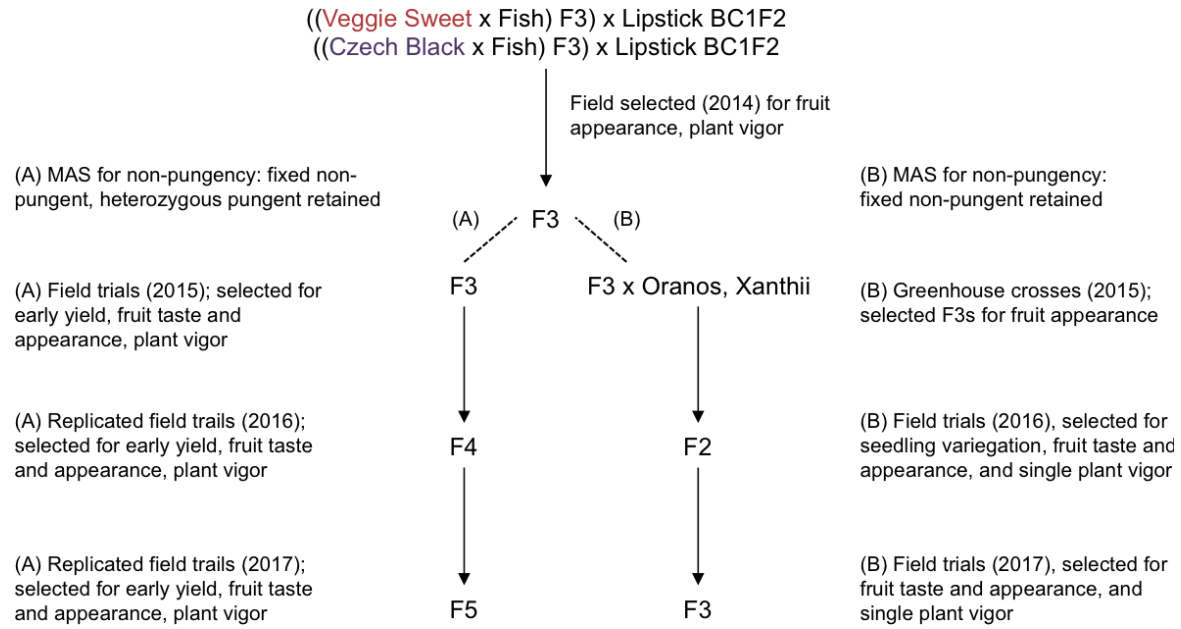

Figure C.1. Pedigree selection scheme within program

For NOVIC participatory breeding (**Fig. C.2**), seed mixed from purple and non-purple non-pungent F3 families from the pedigree selection scheme were provided to collaborators (research hubs, research stations, growers). Collaborators selected their favorite three plants, sent back ripe fruit, and provided written qualitative descriptions of their selection criteria. Selections made within this population at Cornell were intermated to create a 'Local' population (**Fig. C.2a**), and all selections nationwide were intermated to create a 'National' population (**Fig. C.2b**). After two generations of selection, the single site selection ('Local') population was overall less vigorous and later than the 'National' population. Selection criteria also varied across sites. Overall, yield and fruit appearance were consistently highly important, and taste was moderately important. In contrast, selection for some traits

like foliage appearance, earliness, pest resistance only explicitly occurred at a few sites.

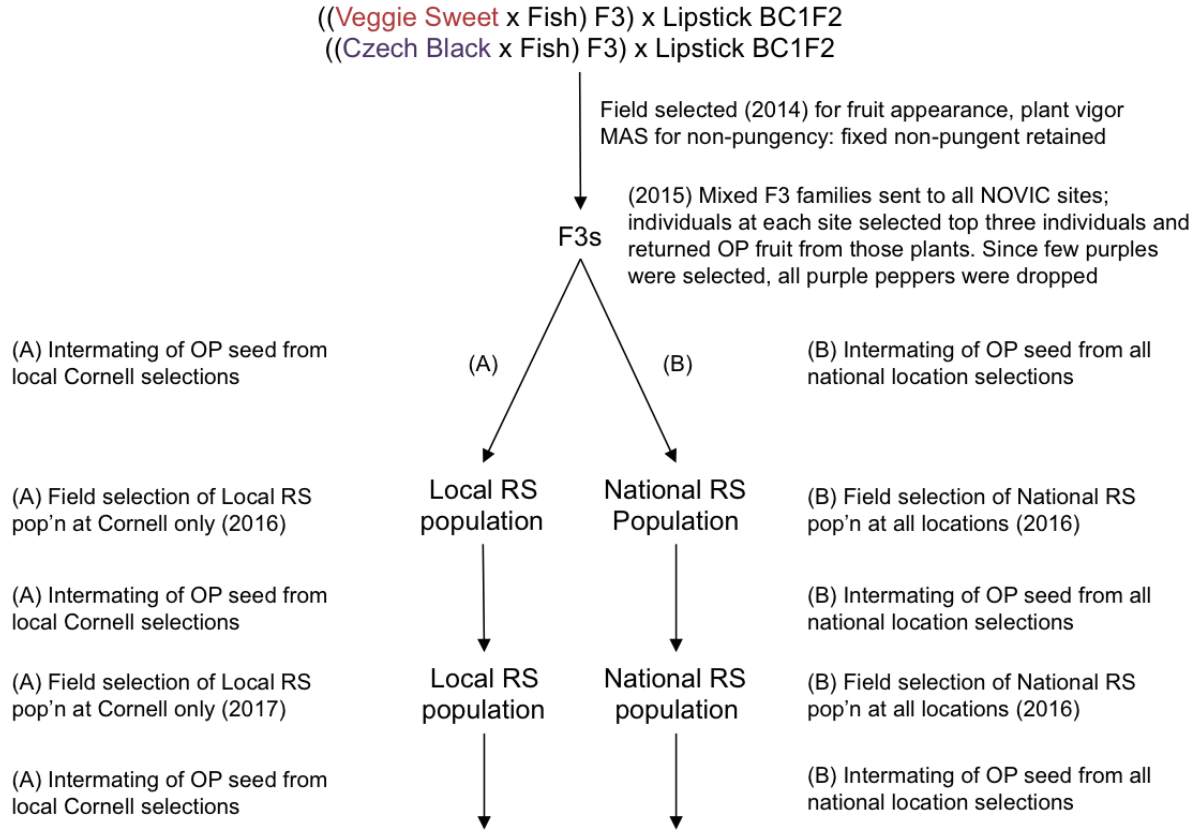

Figure C.2. NOVIC recurrent selection scheme

Appendix D: Tradeoffs and synergies in management of two co-occurring specialist squash pests

D.1 Abstract

Squash bugs (*Anasa tristis*, Hemiptera: Coreidae) are specialist pests of Cucurbitaceae crops, especially *Cucurbita pepo* (summer squash, zucchini). However, intraspecific contrasts in preference are inconsistent, making it challenging to recommend varieties to growers, and necessitating additional management strategies. Using cultivars that typify the two cultivated *Cucurbita pepo* subspecies, *C. p. pepo* and *C. p. ovifera*, we dissected squash bug oviposition preference and performance, and the interactions and tradeoffs with the aggregation and feeding behavior of another specialist cucurbit pest, the striped cucumber beetle (*Acalymma vittatum*, Coleoptera: Chrysomelidae). We demonstrated in independent assays that squash bugs prefer to oviposit on *C. p. ovifera*, representing a tradeoff with beetle preference. However, there was no advantage to squash bug nymph survival or developmental timing on *C. p. ovifera*. We also observed increased squash bug oviposition on plants with greater beetle damage. Mechanistically, over two years of field studies, we found that squash bugs appear to eavesdrop on the male-produced beetle aggregation pheromone. These observations suggest that squash bugs use the cue of a co-occurring endemic Cucurbitaceae specialist beetle for host choice, and has implications for management of both: while there are tradeoffs in varietal selection, synthetic aggregation pheromone could improve trapping of both squash bug and striped cucumber beetles.

D.2 Background

D.2.1 Squash bugs as agricultural pests. Squash bugs (*Anasa tristis*, Hemiptera: Corideae) are specialist pests of the Cucurbitaceae family, most severely of *Cucurbita* spp. (squash and pumpkins), causing significant agricultural damage through direct herbivory of plant tissues, and vectoring a bacterial disease, cucurbit yellow vine decline (CYVD) [1]. In the United States, squash bugs are a significant pest in the southeast and southern plains states but squash bugs and CYVD have been advancing northward [2]. The current damage caused by this pest and range expansion heightens the importance of refining and developing new management strategies.

D.2.2 Current management strategies and considerations. Squash bugs are difficult to control with insecticides [3], thus motivating research for other management techniques such as cultural controls like cover crops [4], [5] and trap crops [6], and recruitment of biological control [7], [8]. While host plant resistance is a cornerstone of integrated pest management, use of resistant varieties in this system is impeded in part by lack of systematic preference trials within a species in the Cucurbitaceae. Multiple efforts have demonstrated interspecific variation in preference (e.g. [9]–[11]), but there is less information on intraspecific preference that can be used to inform grower decisions at the field scale [1], [12].

Growers may also have to balance management decisions for squash bugs with other co-occurring insect pest populations. Squash bugs are part of a suite of Cucurbitaceae specialist herbivores endemic to the Americas, that also include

Diabroticite beetles (Coleoptera: Chrysomelidae), such as the striped cucumber beetle (*Acalymma vittatum*) [13]. Thus, tradeoffs or synergies in management of these co-occurring species have applied agricultural significance and also provide insight into the long-standing ecological interactions between these native herbivores and plants.

D.2.3 Motivation. In our work, we sought to better understand host plant preference and performance of the squash bug within *Cucurbita pepo*, its ecological interactions with the striped cucumber beetle, and the implications for management. We thus leveraged existing knowledge of striped cucumber beetle preferences in *C. pepo* germplasm to conduct field surveys and manipulative experiments over two years of field trails. First, we examined squash bug oviposition preference and nymph performance in *C. pepo*. Then, we interrogated the connection between beetle damage and squash bug oviposition preference and tested the role of beetle male-produced aggregation pheromone in mediating these phenotypes.

D.3. Materials and Methods

D.3.1 Insects. *Anasa tristis* (Hemiptera: Coreidae) and *Acalymma vittatum* (Coleoptera: Chrysomelidae) were all collected or observed in certified organic fields on the Cornell University Agricultural Experiment Station Homer C. Thompson Vegetable Research Farm (Freeville, NY). Male *A. vittatum* were identified and selected from mating pairs based on abdomen morphology [14]. Exclusively male *A. vittatum* produce aggregation pheromone, vittatalactone [15].

D.3.2 Plants. Two cultivars representing established differences in extremes of *A. vittatum* preference between *Cucurbita pepo* subspecies, *C. p. pepo* cv 'Golden Zucchini' and *C. p. ovifera* cv 'Success PM' (Brzozowski *et al.*, 2016) were sourced from Cornell University seed stocks and used in all experiments except the field survey involving intersubspecific breeding lines (detailed below).

Pants were started from untreated seed in 72-cell trays at the Cornell University Agricultural Experiment Station greenhouses (Ithaca, NY) in custom organic potting mix. For field surveys, seedlings were transplanted into 0.6 m wide raised beds covered with black plastic mulch equipped with drip irrigation. For controlled oviposition choice tests, nymph development assays, and trapping experiments, seedlings were instead transplanted into 1.74 L (15 cm diameter) pots. No pest control or additional fertilizer was applied, and plants were irrigated as needed.

D.3.3 Squash bug oviposition preference between C. pepo cultivars. We conducted a field survey of large plantings of the two *C. pepo* cultivars to assess squash bug oviposition preference and relationship to beetle activity. The field was designed such that there were two 50 m rows each containing plants of each cultivar. Individual plants were censused weekly for three weeks in July 2019 for counts of beetles, squash bugs, and squash bug egg clutches. Beetle damage was recorded once during the survey on a 0-5 scale of defoliation (0 = 0% defoliation, 1 = 1-20% defoliation, 2 = 21-40% defoliation, etc)

Cumulative presence or absence of squash bug adults and egg clutches on plants in total was modeled with a logistic regression model with fixed effects of cultivar, row, their interaction and plant position (numeric, 1-50 to account for north to south gradient in the row). Cumulative beetle count was modeled in the same fashion but fit with a negative binomial model. In both cases, variable significance was determined with a likelihood ratio test. Finally, beetle damage was assessed with a linear model with the effects described above, and effect significance was tested by ANOVA.

D.3.4 Squash bug nymph development on *C. pepo* cultivars. Using the *C. pepo* cultivars, we assessed differences in squash bug nymph development and survival between *C. p. pepo* and *C. p. ovifera*. Ten newly hatched nymphs (first instar) were released on to single bagged plants (n = 10, 7 for *C. p. pepo* and *C. p. ovifera*, respectively). Start date was staggered over a four day period in August 2019 due to nymph availability. Nymph counts and instar classification were assessed nine times over 30 days. Instars were classified as 1st instar, 2nd instar, 3-5th instar, or mature nymphs; the 3-5th instar nymphs were classified together because of visual similarity.

To assess the effect of cultivar on nymph survival and development, we used two separate generalized linear mixed effect models with Poisson distributions. For survival, there were main effects of cultivar and days post infestation, and for development, there was an additional main effect of instar category. In both there were random effects to match the experimental design (start date, position in field, and individual plant). Using a likelihood ratio test, we compared models with all potential

fixed effect interactions to a reduced model with genotype and all genotype interactions removed to assess the effect of genotype on survival and developmental timing.

D.3.5 Relationship between squash bug oviposition and beetle damage. To remove the effect of cultivar on squash bug oviposition preference and provide greater mechanistic insight into the interaction between beetles and squash bugs, we measured beetle damage and squash bug egg clutch frequency in intersubspecific *Cucurbita pepo* F_4 generation breeding lines. Thirteen breeding lines were planted in 12 plant plots, and were replicated three times (as described in Brzozowski et al, *submitted*). Plants were evaluated at the 3-5 leaf stage (not flowering) between late June and early July 2019. Beetle damage was visually estimated as leaf defoliation between 0-100% defoliation (in increments of 5%) and squash bug egg clutch number was recorded on the plot level. Clutch number was modeled with a generalized linear mixed model with a Poisson distribution with random effects of genotype and replicate and fixed effect of beetle damage, and variable significance was determined with a likelihood ratio test.

D.3.6 Relationship between squash bug oviposition and beetle aggregation pheromone - Squash bug oviposition choice tests on *C. pepo* cultivars. Within *C. pepo* cultivar, we tested squash bug oviposition preference between plants with and without male beetle infestation (with and without aggregation pheromone). Five male beetles were enclosed on a single leaf with a small mesh bag on a plant with 3-5 leaves for half of

the plants, and an empty mesh bag was placed on the other half as controls. We then paired plants within cultivar and placed both treatments (male beetles, control) in a 1 m^3 field cage and released one mating pair of squash bugs (one male, one female). Eggs were counted every at least every three days, and assays were terminated within six days.

Assays were conducted over five independent experiments in July 2019. In each experiment, there were at least two pairs of each genotype, and a total of 30 *C. p. ovifera* and 20 *C. p. pepo* pairs evaluated. However, there was no oviposition in the fifth iteration of the experiment, and that was dropped from the analysis, leaving 25 *C. p. ovifera* and 17 *C. p. pepo* pairs evaluated over four times. Differences in oviposition frequency between cultivars (eggs on either treatment in cage) was assessed with a Fisher's exact two-tailed test. Differences between treatments within cultivar was tested using a Wilcoxon signed-rank test using number of eggs on a plant as a response variable and only cages where there was oviposition on either or both plants were included.

D.3.7 Relationship between squash bug oviposition and beetle aggregation pheromone - Squash bug attraction to beetle pheromone. We used visually masked traps to test the effect of plant cultivar, *A. vittatum* infestation density, and beetle sex on relative attractiveness to *A. vittatum* and other squash pests in June-July of 2016 as described in detail in Brzozowski *et al* (*in revision*). With the same *C. pepo* cultivars, there were 62 traps that contained male beetles (able to produce aggregation pheromone), 30 traps with female beetles (no aggregation pheromone), and 28 traps without beetles. The

number of squash bugs trapped over a three day period were recorded. Traps were classified by presence or absence of squash bugs. A William's corrected G-test was used to test if the distribution of squash bugs was different between traps with male beetles and all others, male and female beetles, male beetles and no beetles, and female beetles and no beetles.

D.3.8 Statistics. All statistical analyses were conducted in R [17]. Generalized linear models were evaluated with the 'glm' function with the family set to "poisson" and "binomial" for Poisson and logistic regressions, respectively. Generalized linear mixed effect models were evaluated with the 'glmer' function from the R package 'lme4' [18]. The linear model was fitted with the 'lm' function.

D.4 Results

D.4.1 Squash bug oviposition preference between *C. pepo* cultivars. Using representative cultivars of *C. p. ovifera* and *C. p. pepo*, squash bug adults and egg clutches were more frequently found on *C. p. ovifera* whereas *C. p. pepo* had greater beetle densities throughout the season (**Fig. D.1**). In sum, we observed 41 adult *A. tristis*, and 154 egg clutches on *C. p. ovifera* compared to 7 adults and 104 egg clutches on *C. p. pepo*. The presence versus absence of adults and egg clutches was predicted by subspecies (**Table D.1**). In contrast, almost three times as many beetles were found on *C. p. pepo* (4029 total) than *C. p. ovifera* (1432 total) (**Table D.1**), and damage was also greater on *C. p. pepo* ($F_{1.219}$=113.1, p<0.001).

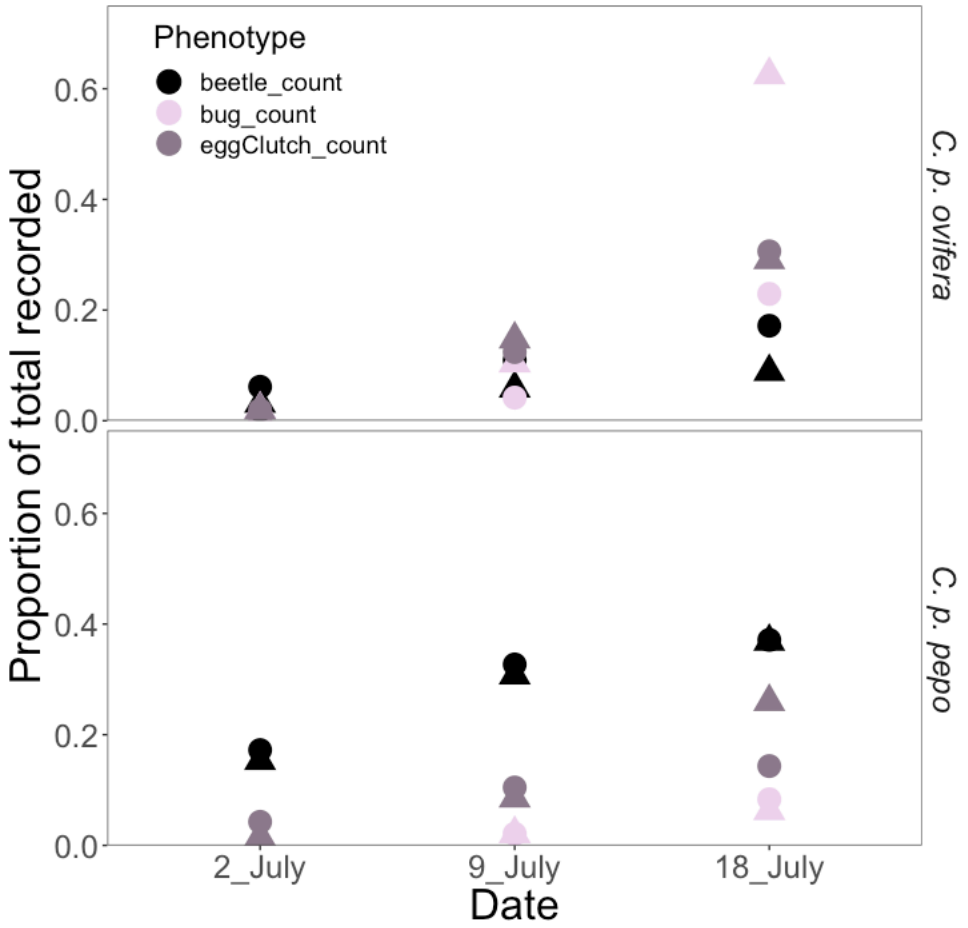

Fig. D.1. Number of squash bug adults, egg clutches and beetles in field survey represented relative to total recorded over the course of the experiment. The color indicates the phenotype and the shape represents each of the two rows per cultivar.

Table D.1. Analysis of deviance table for squash bug adults, egg clutches and beetles in field survey. The *P*-value recorded is from a likelihood ratio test.

		Squash bug adults		Squash bug egg clutches		Beetle adults	
Effect	DF	Deviance	*P*	Deviance	*P*	Deviance	*P*
Genotype	1	17.7	**<0.001**	4.0	**0.04**	239.5	**<0.001**
Row (E-W)	1	3.1	0.08	6.6	**0.01**	18.6	**<0.001**
Plant position (N-S)	1	0.3	0.60	0.001	0.97	13.3	**<0.001**
Genotype*Row	1	3.7	0.06	1.4	0.24	25.8	**<0.001**

Squash bug adults: Model deviance(df) – Null: 187.28(223), Residual 162.53(219)
Eggs: Model deviance(df) – Null: 310.51(223), Residual 298.48(219)
Beetle counts: Model deviance(df) – Null: 531.89(222), Residual 234.61(218)

In cages where squash bugs were presented with two plants of the same cultivar (no choice between cultivars), squash bugs also had more frequent oviposition on *C. p. ovifera* (16 with, 9 without eggs) than *C. p. pepo* (3 with, 14 without eggs) (Fisher's exact two-tailed p=0.0045).

D.4.2 Squash bug nymph development on *C. pepo* cultivars. Overall, there was no difference between *C. p. pepo* and *C. p. ovifera* in squash bug nymph survival (X^2 = 2.391, df=2, p=0.302) or development timing (X^2 = 9.7613, df=8, p=0.282) (**Fig. D.2**).

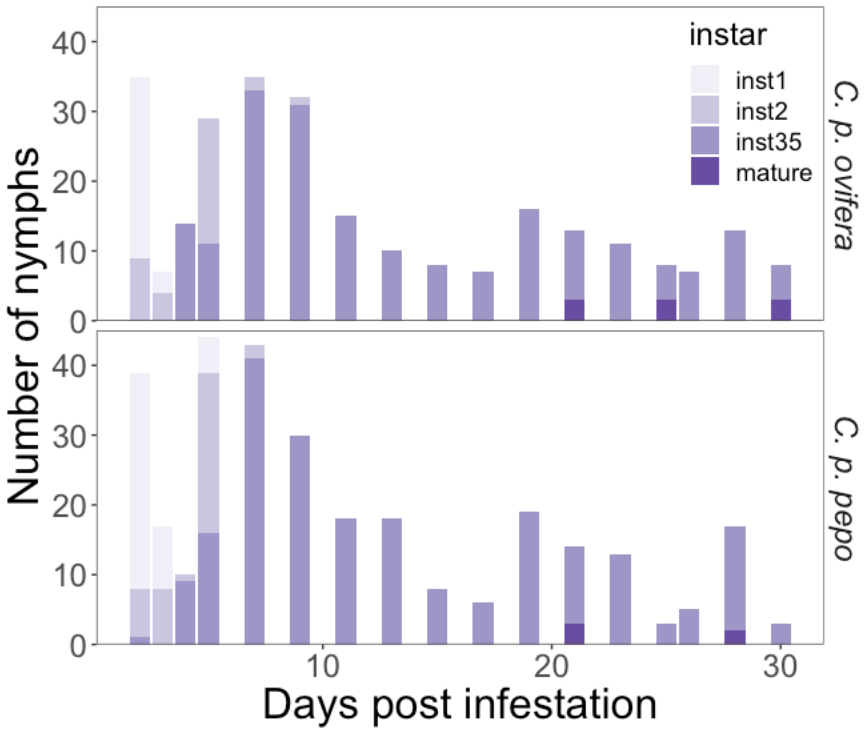

Fig. D.2. Number of nymphs colored by each developmental stage on *C. pepo* cultivars over time.

D.4.3 Relationship between squash bug oviposition and beetle damage. Using plots of intersubspecific *C. pepo* breeding lines to remove strong biases in preference of both squash bugs and striped cucumber beetles, squash bugs oviposited in at least one of three plots of each of the 13 genotypes, and 22 of 39 total plots had eggs. Plots had up to five egg clutches, and beetle damage to plots increased the number of egg clutches in plots ($F_{1,23}$=3.921, p=0.056).

D.4.4 Relationship between squash bug oviposition on *C. pepo* cultivars and beetle aggregation pheromone. Using paired choice tests, we assessed whether squash bugs preferentially oviposited on plants with male beetle feeding (and emitting aggregation pheromone) compared to those lacking male beetles. Across cultivars, oviposition declined over the course of the season, from oviposition in 72% of tests in the earliest trial, but only in 25% by the last trial. Only three egg clutches were found on *C. p. pepo* across all trials (two on plants with male beetles, and one on a plant lacking male beetles). For *C. p. ovifera*, over the course of the season, there was no overall difference in oviposition on control plants versus those with male beetles. However, there was a strong interaction with trial time: squash bugs preferred *C. p. ovifera* plants with male beetles in early trials when there was more frequent oviposition, but did not demonstrate preference in later trials when there was less frequent overall oviposition (**Table D.2**).

Table D.2. Wilcoxon signed rank test results for total number of squash bug eggs between treatments on *C. p. ovifera* from all cages with eggs

		Treatment difference		
Trial	*N* tests with oviposition	By trial	By early, late season	Result - overall
1	7	***P* = 0.0389**	***P* = 0.013**	*P* = 0.488
2	5	*P* = 0.233		
3	1	NA	*P* = 0.089	
4	3	*P* = 0.149		

In a separate experiment, we tested squash bug attraction to visually masked traps of *C. pepo* cultivars with different beetle infestation treatments. Traps containing aggregation pheromone producing male beetles more frequently caught squash bugs than all other types of traps (traps with females, no beetles). Squash bugs were trapped on 14 of traps with male beetles, as compared to five all other traps (William's corrected G=4.43, P=0.035). Comparisons of traps with males compared to traps with no beetles, or females alone were marginally significant (no beetles: squash bugs caught on 2 traps, William's corrected G=3.459, P=0.063; female beetles: squash bugs caught on 3 traps William's corrected G=2.235, P=0.135). However, there was no difference in frequency of squash bugs trapped between traps with female beetles versus no beetles (William's corrected G=0.137, P=0.711), suggesting that pheromone, not plant volatiles induced by beetle feeding were responsible for squash bug attraction.

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