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THE PERSISTENT MALADAPTATION OF *PIERIS MACDUNNOUGHII*: CONSTRAINTS ON ADAPTATION TO AN EVOLUTIONARY TRAP

by

Rachel A. Steward

Bachelor of Science
Tufts University, 2011

Submitted in Partial Fulfillment of the Requirements

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University of South Carolina

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Accepted by:

Carol L. Boggs, Major Professor

Joseph M. Quattro, Committee Member

Ward B. Watt, Committee Member

David S. Wethey, Committee Member

Joel G. Kingsolver, Committee Member

Cheryl L. Addy, Vice Provost and Dean of the Graduate School

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DEDICATION

To Michael Carroll, with love.

To my mum, Becky Love, who taught me to be curious and confident. To my dad, Cleve Steward, who continues to be my best editor and supporter.

And to Francie Chew, who first showed me how to hold a butterfly and so started me on this journey, and without whose original work on *Pieris macdunnoughii* my dissertation would not have been possible. You have shaped my life in a profound way. I could not have done this without you.

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ABSTRACT

Evolutionary traps arise when organisms use novel, low-quality or even lethal resources based on previously reliable cues. Persistence of such maladaptive interactions depends not only on how individuals locate important resources, such as host plants, but also the mechanisms underlying poor performance. *Pieris macdunnoughii* (Remington) (Lepidoptera: Pieridae) lays eggs on a non-native mustard, *Thlaspi arvense* (L.) (Brassicaceae), which is lethal to its larvae. However, in the 150 years since *T. arvense* invaded this butterfly continues to recognize and oviposit on the invasive mustard.

I evaluated two possible constraints on the evolution of decreased preference within an invaded population. First, an evolutionary response to selection may be constrained by low heritable genetic variation for preference. Second, evolutionary traps are expected to persist when overlapping cue sets (cue similarity) link decreased preference for the novel, unsuitable plant with decreased preference for the historical, high-quality resources. I determined that while preference for the nonnative host over the native host is heritable, sex-linked, and varies considerably in the population, it is unlikely that this preference is correlated with preference for native hosts with similar defensive chemical profiles. Thus, neither a lack of heritable genetic variation nor an increased risk of excluding good host plants when avoiding *T. arvense* are likely to be constraining escape from this evolutionary trap. Instead, our results suggest behavioral

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plasticity may buffer populations from innate preference for the lethal host.

Finally, I tested the mechanisms underlying poor performance of neonate larvae on the novel host. Larvae were less likely to start eating *T. arvense* and starvation was a primary cause of mortality, indicating a pre-ingestive feeding deterrent. A primary oviposition stimulant, the glucosinolate sinigrin, increased this deterrent effect and mortality when added to *T. arvense* and native host plant leaves. Pre-ingestive deterrents, even those familiar to herbivorous insects, may significantly contribute to the persistence of evolutionary traps.

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CHAPTER I:

INTRODUCTION

1.1 Local (mal)adaptation

Local adaptation occurs when populations evolve in response to selection pressures that vary over small geographic scales (Hendry and Gonzalez 2008; Richardson et al. 2014; Tiffin and Ross-Ibarra 2014). The concept was first introduced by Turesson (1922) in his research on ‘ecotypes’, describing consistent heritable differences in form found among Swedish plant populations inhabiting irregular landscapes. The concept was quickly adopted (Gregor 1944; Turrill 1946) and it is now known that ecotypes and the local adaptation process are common (Hoeksema and Forde 2008; Hereford 2009). Locally adapted populations have higher fitness at their native site than members of other populations introduced to that site, and often have lower fitness when moved out of their native habitat (Savolainen et al. 2013). This antagonistic effect is not universal, but fitness outside of the native habitat is often negatively correlated with the degree of local adaptation (Hereford 2009), supporting the hypothesis that the process of specialization, even at large scales, represents a fitness trade-off (Hardy and Otto 2014; Vamosi et al. 2014).

Is local adaptation the norm? In his early work, Turesson surmised that all populations experiencing different selection pressures should be optimally adapted to their native sites (Turesson 1922). This is not the case. Maladaptation to the local

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environment persists in many natural populations, but the reasons why are poorly understood (Crespi 2000; Thompson et al. 2002; Hansen et al. 2006; Hendry and Gonzalez 2008; Hereford 2009; Anderson and Geber 2010; Fraser 2014). Populations are expected to fall short of optimal fitness peaks due to developmental variance and environmental stochasticity. However, if populations continue to express average fitness values well below the expected optima, it is likely that one or more of the conditions for local adaptation are not being met.

The potential for local (mal)adaptation depends on heterogeneous selection pressures across a landscape. Environmental variation does not inevitably lead to differences in selection, and traits or behaviors that appear costly to a fitness component may not always lead to a decline in overall fitness. Mosaics of selection interact with other evolutionary forces and genetic factors to produce differentiation, with the relative strength of selection affecting the importance of these interactions (Blanquart et al. 2013). Local maladaptation may therefore persist under conditions of low heritable genetic variation in traits under selection, balancing selection imposed by other fitness costs, gene flow between areas with different selection regimes, and temporal and spatial fluctuations in the strength of selection.

Populations may lack variation if the adaptive trait arises from a rare mutation and past drift, inbreeding or directional selection may have culled polymorphisms from the gene pool. The genetic basis for a trait and its mode of inheritance both control the rate at which beneficial alleles can increase in frequency in a population, or whether they can at all (Hoeksema and Forde 2008; Yeaman and Whitlock 2011; Savolainen et al. 2013). Pleiotropic genes that have effects on multiple traits may be under stabilizing

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selection from unknown forces. Linkage disequilibrium, dominance and whether a trait is autosomal or sex-linked also affect how quickly allele frequency changes can be observed in a population (Akerman and Buerger 2014; Tiffin and Ross-Ibarra 2014).

Unlike selection and drift, random gene flow is generally a homogenizing evolutionary force, decreasing genetic variation between populations. When both selection and migration occur in one of the populations, selection must be above a threshold set by the rate of gene flow in order to overcome this homogenizing effect (Wright 1931). Due to the importance of this equilibrium, many ecologists and population geneticists have focused on genetic isolation as a facilitator of local adaptation, using distance and time as proxies (Ehrlich and Raven 1969). Recent evidence suggests local adaptation can occur on much finer geographic and temporal scales in response to patchy selection pressures (Richardson et al. 2014; Tiffin and Ross-Ibarra 2014). For example, local adaptation of salamanders has occurred in ponds several hundred meters apart in response to intense selection by predation pressures that differ between ponds (Richardson and Urban 2013). Over the course of several decades, populations of Edith's Checkerspot (*Euphydryas editha*) evolved host plant preferences dependent on an invasive plant and human land use practices (which proved disastrous for the populations when human disturbance recently ended; Singer et al. 1993; Singer and Parmesan 2018). Even the hawkweeds Turesson (1922) was studying when developing the ecotype concept demonstrated local adaptation to immediately adjacent habitats. Although differentiation is dependent on some level of genetic isolation, it is possible that the geographic and temporal scales necessary to produce these differences are smaller and potentially shorter than once thought. The assumptions that too short a

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time has passed since the introduction of a novel selection pressure or too little distance exists between areas of different selection are not convincing explanations for persistent maladaptation.

1.2 Evolutionary traps

The scale of and constraints leading to maladaptation are especially important in the context of rapid environmental change. For species that are highly dependent on specialized recognition systems, anthropogenic changes to the environment (e.g. introduction of nonnative species, urbanization, modification of habitats, changes in land use patterns, climate change, etc.) can lead to preference/performance mismatches known as evolutionary traps (Schlaepfer et al. 2002, 2005; Robertson et al. 2013). Evolutionary traps occur when environmental change causes previously advantageous evolved cue-responses to become unreliable, and changes in the cue-response outcome (e.g. preferring low-quality resources, changing (or failing to change) the timing of life history events, misidentifying risks) have measurable fitness costs (Sih et al. 2011; Robertson et al. 2013).

Evolutionary traps are not ecological dead-ends, and the potential may exist for escape via rapid local adaptation to novel selection pressures (Carroll 2007a; Keeler and Chew 2008; Harvey et al. 2010). Interestingly, most of the theoretical and empirical literature focuses on the formation of (Schlaepfer et al. 2005; Sih et al. 2011; Sih 2013) or evolutionary escape from (Carroll et al. 2007; Keeler and Chew 2008; Hendry et al. 2011; Lankau et al. 2011) these traps, and not the processes involved in the maintenance of traps over long periods of time. However, some species encountering

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these preference/performance mismatches show no sign of escape and remain maladapted to changes in the local environment.

1.2.1 Types of traps

Most evolutionary traps can be categorized as resource-based. These involve maladaptive recognition and attempted exploitation of a novel resource (Robertson et al. 2013), whether that resource is food, mates, habitat, etc. Organisms can also be susceptible to risk-response (or disturbance-response) traps, resulting in excessively cautious (e.g. flight responses to harmless ecotourists; Beale 2007; Tablado and Jenni 2017) or hazardously naïve (e.g. failure to detect predator odor cues; Brown et al. 2018) responses to novel potential risks. The final major category is phenological traps. These evolutionary traps involve mismatches in the cue-response systems that mediate life-history decisions, such as entering or exiting diapause (wall brown butterfly; Dyck et al. 2015), onset of reproduction (great tits; Schaper et al. 2011), dispersal (Massot et al. 2008) or migration (Visser et al. 2010). The distinction among these trap types is important because the strength of the cue-response, the distribution of selection pressures (continuous or patchy across a landscape) and the potential escape trajectories are likely to differ considerably both within and between the three. Here and in the following chapters I focus on a resource-based trap, specifically egg-laying on a lethal invasive plant by a native arthropod, as a case study for persistent maladaptation.

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1.2.2 Resource-based traps: escape trajectories

Long before these mismatches were ever called traps, ecologists observed apparently maladaptive responses to environmental change and determined two main trajectories for escape, either by decreased preference for or improved exploitation of novel resources (Chew 1975, 1977; Wiklund 1975; Schlaepfer et al. 2002). Increased avoidance has evolved in response to invasive prey or hosts. For example, multiple endemic Australian species of snakes now tend to avoid invasive, toxic cane toads (*Rhinella marina*; Phillips and Shine 2006; Phillips et al. 2010). Decreased preference can be achieved by narrowing the range of positive cues involved in recognizing a resource, by expanding the range of negative cues, or a combination of the two. It is unclear which of these two cue-recognition shifts are more likely, and the relative importance of stimulants and deterrents, and their sensitivity to selection from maladaptive resource use have not been addressed in the evolutionary trap literature. Robertson and Chalfoun (2016) have pointed out that that it is also unclear whether evolutionary traps are more likely to arise as a result of the incapacity of existing sensory organs to detect novel cues, or inflexibility of processing algorithms once this information has reached the brain. Understanding of the cognitive basis of evolutionary traps will improve our ability to predict whether and when escape via decreased preference may be possible.

Increased exploitation is better documented, especially among herbivorous arthropods: the red-shouldered soapberry bug's (*Jadera haematoloma*) have adopted invasive golden train trees has suitable hosts after initial poor performance on the novel host (Carroll 2007b). Over the course of twenty years, a population of mustard white

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butterflies (*Pieris oleracea*) improved performance (development time and pupal weight) on invasive garlic mustard (*Alliaria petiolata*) to the extent that some families showed little difference in either development time or pupal mass on the native and nonnative diets (Courant et al. 1994; Keeler and Chew 2008; Steward, Acuna, Mei, Casagrande and Chew, unpubl.). Although not commonly characterized as evolutionary traps, among the best examples of escape via improved exploitation are rapid adaptation to pesticides. The application of pesticides and insecticides degrade the quality of a previously beneficial resource without changing the attractiveness of the cue-set, forming an evolutionary trap. There are countless examples of target insects evolving resistance to insecticidal compounds, both from standing genetic variation and *de novo* mutations (Hawkins et al. 2019)

For organisms using abiotic resources, the increased exploitation is usually not an option. Larval aquatic insects cannot develop on solar panels or asphalt roads no matter how attracted their mothers are to the polarized light reflected from these surfaces (Szaz et al. 2015; Robertson and Horváth 2019). Escape by this trajectory is not only limited by the potential of the resource, but also the current variance in fitness exhibited by organisms attempting to exploit that resource. If fitness is effectively zero, and there is no phenotypic variation, increased exploitation is an unlikely escape. This is especially true if the response is expected to depend largely on standing, rather than novel genetic variation.

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1.2.3 Resource-based traps: escape mechanisms

Whether the trajectory is decreased preference or increased exploitation, there are at least two mechanisms that can facilitate escape: phenotypic plasticity and rapid evolution in response to selection. Plasticity is commonly defined as the capacity for a single genotype to produce more than one phenotype, whether from a continuous or discontinuous suite of phenotypes, in response to environmental variation (West-Eberhard 2003 p. 33)and can be generalized to describe all environmentally-mediated phenotypic variation (West-Eberhard 2003 p. 35; DeWitt and Scheiner 2004 p. 2). Because within-generational phenotypic plasticity can operate at the individual level, it has the potential to provide the most rapid relief from maladaptive responses to rapid environmental change (Fox et al. 2019), and might even mean that effects of rapid environmental change are not detected at the population level (Berthon 2015). Inter- and transgenerational plasticity can also generate rapid responses to evolutionary traps, where parental or grandparental experiences result in nongenetic changes in preference or performance phenotypes (O'Dea et al. 2016). Plasticity contributes to escape by buffering populations against evolutionary traps, maintaining population size or genetic variation that could be eliminated under strong selection from the trap increasing the opportunity for an evolved response that may take longer to manifest (Strauss et al. 2006). For example, the European parasitoid wasp *Trissolcus cultratus* prefers invasive brown marmorated stinkbugs to native hosts but develops poorly in the eggs of these hosts. Recent evidence suggests trophic plasticity allows *T. cultratus* larvae to hyperparasitize nonnative parasitoid larvae developing in the same stinkbug egg,

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reducing the fitness costs associated with using this nonnative host insect (Abram et al. 2014; Konopka et al. 2016).

Without future evolutionary change, or decrease in trap prevalence, dependence on phenotypic plasticity is unlikely to be sufficient for organisms such as *T. cultratus* to cope with prolonged maladaptive resource use (Sih et al. 2011). Many organisms have demonstrated the capacity for rapid adaptive evolution in response to environmental change. This is especially evident in rapid shifts of both nonnative insects to use native plants as hosts, and of native insects expanding or switching their diets onto nonnative plants (Pearse and Hipp 2009; Pearse and Altermatt 2013). Although cultivated and feral alfalfa was originally identified as a poor host for the Melissa blue butterflies (*Lycaeides melissa*), recent evidence demonstrated that alfalfa-associated populations not only prefer the novel host (Forister et al. 2013), but that there are genomic signatures of differential host use in these populations compared to those that still use the native host (Chaturvedi et al. 2018). Even more compelling, it appears that these geographically distinct populations exhibited at least weak signs of parallel evolution of host-associated traits. This emphasizes the importance of considering organisms' evolutionary history and historical resource when considering whether escape via rapid adaptation is possible (Strauss et al. 2006; Sih et al. 2011). Both phenotypic plasticity and rapid adaptation operate against a background of potential constraints. These include existing capacity for plasticity, genetic variation within affected populations, the temporal and spatial strength of selection, gene flow from naïve populations, and conflicting costs associated with shifts in either preference or performance on the novel host.

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1.3 Persistent maladaptation: an evolutionary trap case study

Despite multiple trajectories and mechanisms for escape, maladaptive evolutionary traps appear to persist. In the following chapters I focus on the preference and performance of a native consumer stuck in an evolutionary trap with a nonnative resource. I evaluate the role of both genetic variation and heritability of preference, and the potential costs of a shift in preference as potential constraints on escape from this trap. Finally, I take a closer look at performance on the nonnative resource.

1.3.1 The Pieris macdunnoughii – Thlaspi arvense *trap*

The butterfly *Pieris macdunnoughii* is native to the southern Rocky Mountains. Like many insect herbivores, especially among the Lepidoptera, *P. macdunnoughii* has evolved a chemical cue- response system to identify suitable host plants on which to lay their eggs (Ehrlich and Raven 1964; Williams and Bowers 1987; Bernays and Graham 1988). Typical of the *Pieris napi* species complex and of many other butterflies in the Pierineae, *P. macdunnoughii* lays eggs preferentially on plants in the family Brassicaceae (Chew 1977; Edger et al. 2015). Discrimination between host plants is based on the presence of glucosinolate (GSL) stimulants, a group of sulfur-containing glycosides, and in some part cardenolide deterrents (Huang and Renwick 1993, 1994; Huang et al. 1994; Du et al. 1995; Städler et al. 1995; Appendix A).

This specialized cue-response system has recently been compromised in some populations of *P. macdunnoughii* by the invasion of Eurasian mustard *Thlaspi arvense*. *T. arvense* was first reported in the Gunnison basin of the southern Rockies in the 1880s

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(cf. Chew 1977). The plant forms dense populations in disturbed, well-drained habitats. Due to recreational, agricultural and developmental land use patterns, the abundance of *T. arvense* at a site can be stable, although the distribution over a small geographic scale might be highly variable.

In the early 1970s, (Chew 1975, 1977) observed that *P. macdunnoughii* females lay eggs on several invasive mustards, including *T. arvense*. At the time, Chew also demonstrated this plant was not suitable for larval development, leading to the death – usually in the early stages of development – of all offspring laid on the plant as a result of unknown defenses plant to which the butterflies are naïve. It seems likely that, under the right conditions, the costs of ovipositing on this plant might select for counteradaptation to the novel plant, either by increased avoidance during egg-laying or improved larval survival and growth (Chew 1977). However, in the forty years since the interaction was first described, at least one population of *P. macdunnoughii* exposed to *T. arvense* has shown no evidence of local adaptation, in either adult oviposition preference or larval performance (Nakajima et al. 2013). Based on the abundance and distribution of *T. arvense* at this site, measures of patch occupancy and egg distribution by *P. macdunnoughii* butterflies, and relative larval mortality estimates on the available invasive and native host plants, the fitness consequences of the acceptance behavior are estimated to be about 3% (Nakajima et al. 2013). This selection pressure should be strong enough to have an effect on trait frequencies, given other conditions for local adaptation are met (Tiffin and Ross-Ibarra 2014).

This system is well-suited for testing persistence of maladaptation. The general timeframe of the invasion is known, and we have access to historical data

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characterizing the interaction between the butterfly and the host plant in the 1970s (Chew 1977). Estimates of the potential fitness costs are significant, although expected to be specific to the abundance and distribution of *T. arvense* and host plant foraging strategies of the butterflies each generation (Nakajima et al. 2013). Finally, these butterflies currently only have one viable trajectory for evolutionary escape. Although some larvae may live longer than others on a diet of *T. arvense*, none survive to pupation or the adult reproductive stage, so any variation in larval growth that currently exists in the population is neither heritable (which is not to say it does not have a genetic basis) nor accessible to selection, as long as this variation is unlinked to performance on native hosts. Instead, all potential for an adaptive response lies with egg-laying behavior.

1.3.2 Constraints on escape

In chapter II, I evaluate the presence of heritable variation in *T. arvense* preference in invaded populations. In addition to selection pressure, an adaptive response to a novel herbivore-plant interaction requires heritable genetic variation (Hoffmann and Merilä 1999; Strauss et al. 2006). While butterflies from invaded populations show a wide range of preferences, from completely accepting to completely rejecting the novel host, the degree to which this represented a robust, heritable phenotype is unknown. In the presence of both selection and heritable variation, the rate of evolution will depend on the strength and consistency of selection, the degree of heritability and the genetic architecture of the selected trait. For example, the frequency of fully or partially recessive alleles of sex-linked genes evolves faster than similar

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alleles of genes located on autosomes (Charlesworth et al. 1987, 2018; Irwin 2018), because the effects of these recessive alleles are not masked in the heterogametic sex (Mank 2009; Irwin 2018). Additionally, ecological novelty in the form of nonnative, maladaptive host plants can affect the genetic basis for and variance of preference or performance traits (Kawecki 1995; Carroll et al. 2003). New cue sets may reveal previously neutral genetic variation (Hoffmann and Merilä 1999). We found that preference for whole plants and cut stems is heritable and sex-linked, but that these patterns are not upheld when butterflies are tested on plant chemical extracts.

Chapter III tests a second potential constraint on escape from the *T. arvense* trap: cue similarity. Cue similarity can constrain adaptive responses to evolutionary traps when overlapping cue sets link decreased preference for novel resources with decreased preference for historical or native resources. While cue sets involved in mistakenly selecting low-quality novel resources are expected to resemble those of historical resources – this is, after all, the basis of an evolutionary trap – they can vary in the strength of that resemblance (Sih et al. 2011; Robertson et al. 2013). A large overlap in the composition or intensity of cue sets may mean that narrowing or refining the recognition system causes individuals to reject high-quality resources, transitioning the maladaptive response from mistake to miss (Fox and Lalonde 1993; Macmillan and Creelman 2004). Fitness costs of rejecting good resources (misses) may outweigh those of using *T. arvense* (mistakes), thereby maintaining the maladaptive behavior in *P. macdunnoughii* populations (Fox and Lalonde 1993; Robertson et al. 2013). We found, however, that preference is uncorrelated between *T. arvense* and the high-quality host plant that shares several of the same GSL cues. Instead, experience with the native host

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significantly decreased preference for *T. arvense*, suggesting behavioral plasticity may mitigate selection pressures from this evolutionary trap.

1.3.3 Neonate larval mortality on a novel host

Finally, Chapter IV takes a closer look at the performance of neonate larvae on *T. arvense* to evaluate whether the negative consequences of feeding on the nonnative mustard are due primarily to pre-ingestive deterrents, or to a combination of factors that influence larvae once they have already started eating. *P. macdunnoughii* larvae demonstrate extreme neonate mortality on a *T. arvense* diet (Nakajima et al. 2013). However, later instars moved from native hosts on to the nonnative develop to pupation without a problem, and neonates rescued from *T. arvense* and moved to native hosts also develop successfully (Chew 1975). Whereas toxins are effective on naïve insects, deterrent responses require both the sensory anatomy and neural processing algorithms to identify and avoid a food source. It is unlikely that insects will demonstrate deterrent responses to entirely novel cues. Thus, the presence of a deterrent response suggests the responsible cue is one with which the insect has a shared evolutionary history. In host plant-based evolutionary traps escape through shifts in larval performance is possible but depends on the complexity of plant defenses. Unless susceptibility to active deterrents and toxins is genetically correlated, a combination of defenses that target both physiology and behavior could keep populations from evolving resistance to the evolutionary trap (Gould 1984; Bernays and Chapman 1987; Berenbaum and Zangerl 1992).

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CHAPTER II:

NOVEL PLANT UNMASKS HERITABLE VARIATION IN HOST PREFERENCE WITHIN AN INSECT POPULATION[1]

[1] Steward, RA, Epanchin-Niell, RS, Boggs, CL. To be submitted to *Evolution*.

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2.1 Introduction

For native herbivores, novel plant communities formed by the introduction of nonnative species represent both more complex and less reliable resource environments (Robertson et al. 2013). While native herbivores may fail to recognize non-native plants, or sometimes easily incorporate the nonnatives into their diets, in many cases native herbivores recognize a nonnative plant as a resource, despite not being able to successfully exploit it (Gripenberg et al. 2010; Pearse et al. 2013). Fitness costs associated with consistently using an unsuitable resource are expected to select against preference for the novel host or for improved physiological performance when feeding (Wiklund 1975; Schlaepfer et al. 2005; Strauss et al. 2006; Pearse et al. 2013).

In addition to selection pressure, an adaptive response to a novel herbivore-plant interaction also requires heritable genetic variation for either preference or performance of herbivores (Hoffmann and Merilä 1999; Strauss et al. 2006). In the presence of both selection and heritable variation, the rate of evolution will depend on the strength and consistency of selection, the degree of heritability and the genetic architecture of the selected trait. For example, the frequency of fully or partially recessive alleles of sex-linked genes evolves faster than similar alleles of genes located on autosomes (Charlesworth et al. 1987, 2018; Irwin 2018), because the effects of these recessive alleles are not masked in the heterogametic sex (Mank 2009; Irwin 2018). Additionally, ecological novelty in the form of nonnative, maladaptive host plants can affect the genetic basis for and variance of preference or performance traits (Kawecki 1995; Carroll et al. 2003). New cue sets may reveal previously neutral genetic variation (Hoffmann and Merilä 1999). It is not clear however, how heritability of preference for

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novel host plants may differ from heritability of preference for historical native hosts, and how this in turn may promote or constrain adaptive responses to novel hosts.

Lepidoptera, specifically butterflies, are particularly susceptible to maladaptive use of novel plants (Yoon and Read 2016; Singer and Parmesan 2018). Most butterfly species have very specialized diets, feeding on plants from no more than three families (Forister et al. 2015). Although individual variation in host plant preference is determined by many factors, the number (range) and preferred order (ranking) of plants used as hosts depend largely on evolved recognition systems to identify and evaluate host plant chemistry and quality (Thompson and Pellmyr 1991; Singer 2003). Rapid shifts in both host range and ranking have occurred in response to the introduction of novel plants (Keeler and Chew 2008; Singer and McBride 2010; Forister et al. 2013). Oviposition preference is an effective system in which to ask questions about how the structure of heritable genetic variation affects adaptive response to novel hosts.

A considerable body of work exists describing the heritability of oviposition preference between species of butterflies, within species between populations, and within populations that prefer different host plants. Oviposition preference traits tend to be sex-linked between species and geographically distant populations (Thompson 1988a; Scriber et al. 1991; Sperling 1994; Janz 1998, 2003; Nygren et al. 2006; Chaturvedi et al. 2018); but see Sheck and Gould 1995; Forister 2005; Hora et al. 2005). Females are the heterogametic sex, having both Z (= X) and W (= Y) chromosomes, while males are homogametic ZZ (Robinson 1971 in Sperling 1994). Host plant preference of interspecific or inter-populational hybrid females resembles that of their paternal grandmother. This pattern has been linked to the existence of

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stable host ranks in most populations, where the order in which females tend to prefer plants is based on intrinsic characteristics of the plant, such as secondary chemistry or nutritional quality (Janz 1998, 2003; Bossart and Scriber 1999).

Oviposition variation within populations has been attributed to individual differences in specificity, or the relative degree to which plants are preferred, while still maintaining the overall order of preferred plants (host rank). Specificity is expected to be inherent to the motivation of the female (Courtney et al. 1989), polygenic and particularly evolutionarily labile. Studies within butterfly and moth populations have found autosomal inheritance of oviposition preference (Tabashnik et al. 1981; Singer and Thomas 1988; Jaenike 1989; Bossart and Scriber 1995; Nylin et al. 2005). This previous evidence suggests that, within populations encountering a novel, maladaptive host plant, oviposition preference should be heritable, autosomal, and determined by many genes of small effect. However, to our knowledge, no one has yet examined whether this inheritance pattern persists within populations whose stable historical resource environment has been disturbed by maladaptive nonnative host plants.

In the southern Rocky Mountains of North America, the montane butterfly *Pieris macdunnoughii* (Remington 1952) recognizes and lays eggs on the invasive Eurasian mustard *Thlaspi arvense* (Brassicaceae), even though this novel host plant is completely lethal to the butterflies' larvae (Chew 1975; Nakajima et al. 2013; Steward et al. 2019). Spatially explicit models of butterfly-host plant interactions within a focal invaded population determined that this oviposition mistake results in a significant fitness cost in the modeled population (~3%, Nakajima et al. 2013) and should select for decreased preference for *T. arvense*. Whether evolution will, or even can, occur in

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populations where the invaded plant is abundant depends on whether there is heritable genetic variation in preference for the native host plant and *T. arvense*. However, in the 150 years since *T. arvense* was introduced to the region, and the 40 years since the maladaptive interaction was first recorded, the butterfly continues to recognize and oviposit on the invasive mustard.

Like many butterflies, *Pieris macdunnoughii* uses chemical cues to find its mustard host plants. Glucosinolates are alcohol soluble secondary metabolites that generally play a defensive role for plants in the Brassicaceae (Agerbirk and Olsen 2012). However, with the help of specialized detoxification mechanisms, butterfly larvae within the Pierinae can consume and develop on plant tissue containing glucosinolates (Wheat et al. 2007; Edger et al. 2015), and adult females use specific glucosinolate compounds as oviposition cues (Huang and Renwick 1993, 1994; Huang et al. 1994; Du et al. 1995). Maladaptive host plant recognition by *Pieris* butterflies in North America has largely been attributed to host plant chemistry, specifically glucosinolate composition and concentration (Keeler and Chew 2008; Nakajima et al. 2013; Davis and Cipollini 2014).

To understand preference variation and heritability in populations of *P. macdunnoughii* confronted by *T. arvense*, we tested the oviposition preference of wild and lab-reared generations of *P. macdunnoughii* females. Using simultaneous choice assays, we compared preference for *T. arvense* versus a preferred native host using whole plants, cut stems bearing leaves, and methanol-based leaf tissue extracts. We asked:

1. Within a population, is there variation in preference for *T. arvense*, and does this variation differ across whole plants, cut stems and methanol extracts?

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2. Do preferences for *T. arvense* vary temporally over the growing season or across a spatial gradient from invaded to uninvaded habitat from butterflies were collected?
3. Is preference for *T. arvense* heritable and is heritability of preference driven by and therefore higher on methanol soluble plant chemicals?

Based on the theory that within population variation is attributed to differences in individual butterfly motivation in relation to host plant chemistry (specificity; Singer 2003), we expected host preference to be similar when evaluated on whole plants and methanol-based leaf extracts. We expected spatial variation in preference would be similar between oviposition substrates, but that any temporal variation in preference detected on plants and stems would be in response to changes in the plants, and so would not occur when preference was tested on extracts. We expected to find that variation in preference is heritable, with an autosomal basis, resulting in daughters with similar preferences to their mothers. Because host plant chemistry is thought to be a primary mediator of the maladaptive host use, we expected heritability of preference to be stronger for extracts than for plants, since environmental variance would be reduced.

2.2 Methods

Part of a Holarctic expansion and speciation of the *Pieris napi* species complex, *Pieris macdunnoughii* (previously *Pieris napi macdunnoughii*) is a montane butterfly found in regions of Montana, Wyoming and Colorado (Geiger and Shapiro 1992; Chew and Watt 2006). Like most butterflies of the Pierinae, *P. macdunnoughii* specializes on Brassicaceae, including the native host plant *Cardamine cordifolia* (Gray) (Chew 1975, 1977). Previous published work on this species has focused on a population of

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butterflies near the Rocky Mountain Biological Laboratory (RMBL) in Gothic, CO, USA (38°57'33.0"N 106°59'23.0"W, 2900m a.s.l.; Chew 1975, 1977; Nakajima et al. 2013, 2014; Nakajima and Boggs 2015, Steward et al. 2019). Butterflies used in this study were collected over multiple summer field seasons (1997, 2006, 2015) from the Gothic population and several other populations in the upper East River Valley of the Gunnison Basin in Southern Colorado (Figure 2.1). Collection locations were mapped with *ggmap* (Kahle and Wickham 2013) using Google terrain maps (2018).

T. arvense is an early successional plant that rapidly colonizes exposed soil and is most consistently found in heavily disturbed areas (e.g., construction sites, roadways, recreational trailheads, and meadows open to cattle grazing). Already established in the Great Plains of North America in the early 1800s (reviewed in Warwick et al. 2002), it is likely *T. arvense* was introduced to the Elk Mountains and Gunnison Basin between 1850 and 1880 as disturbance increased with the influx of miners and ranchers. The plant is recorded as present in the Gunnison Basin from the beginning of RMBL herbarium records in 1929, and *T. arvense* has been abundant near Gothic, CO since at least the 1970s (Chew 1975).

All plants used in the preference assays were collected from sites near Gothic, CO for all three years of the study (Table B.1). Preference for *T. arvense* was tested in simultaneous choice assays against a preferred native host, *Cardamine cordifolia*, which is abundant throughout the East River Valley.

We conducted a total of three heritability studies over a span of eighteen years. During this time, *T. arvense* in the East River Valley remained abundant in areas of high recreation use and other frequent disturbance. Anecdotally, butterfly population sizes

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remained large in both invaded and uninvaded areas. Oviposition preference of all butterflies across 1997, 2006 and 2015 was assayed using simultaneous choice assays conducted in the same laboratory space and conditions. Butterflies were allowed to choose between *T. arvense* and native host *C. cordifolia* – in the form of either whole plants, cut stems bearing undamaged leaves, or filter paper treated with methanol-based leaf tissue extracts – and a negative control substrate.

2.2.1 Oviposition preference on whole plants

In 1997, adult gravid female *P. macdunnoughii* butterflies were collected over three days (June 25 – 27) from Parcel C, a tract of land on Mt. Crested Butte south of Gothic, CO, and adjacent to areas invaded by *T. arvense* (Figure 2.1). In the lab, the females were fed twice a day with a 25% honey-water solution. Females were kept in 0.23m x 0.23m x 0.23m screen cages, with one pot each of *T. arvense* and *C. cordifolia* and one pot of clover (*Trifolium pretense* F., Fabaceae), a non-host plant that does not stimulate oviposition. Empty space in the cage was filled with a neutral substrate, crumpled newspaper, on which the butterflies could land. In all years, larval host plants were matched by estimated foliage area. Whenever possible, plants were also matched phenologically (pre-flowering, flowering, seeding), although this was a lower priority. The cages were stored in an environmental chamber at 27-31ºC on a 16:8 L:D cycle. Eggs from each host plant were counted and collected every evening.

Larval offspring were reared in the environmental chambers under the same conditions as the ovipositing females. To reduce the level of maternally transmitted disease, eggs were first held in bleached coffee filters for eight minutes in 0.075%

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hypochlorite solution, followed by two five-minute rinses in water. After treatment, eggs hatched, and larvae developed on *C. cordifolia*, which supports rapid development (Chew 1975). Plants were replaced as needed during larval development. Pupae were collected after hardening of the cuticle, sex was determined, and pupae were grouped by sex and brood and left to emerge in screen cages in the environmental chamber.

Upon adult emergence, the F1 butterflies were numbered individually, and brood identity was recorded. Matings were obtained by placing up to 20 individuals from desired broods into 30cm x 45cm x 45cm net cages, which were placed outdoors in direct sunlight. We tried to mate offspring of mothers that laid at least 30 eggs. The ground surrounding the cages was kept moist in order to keep humidity high. Multiple mating cages were run at one time, allowing us to avoid sib-sib matings by placing males and females of the same brood in different cages. Cages were checked at least every 45 minutes, mating pairs removed, and mating combinations recorded. We aimed to mate each male with at least two females from different broods to produce pairs of half-sib families. Preference tests were repeated on the F1 generation. Their F2 offspring were reared, mated and also tested, creating a three-generation pedigree in which all grandmothers and both parents of the F2 generation were known.

Plants used in experiments were transplanted from the field (Table B.1) into 10 cm square pots filled with local soil, with one exception: *T. arvense* used in oviposition tests for the F2 generation was grown in potting soil from local seed.

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2.2.2 Oviposition preference on cut stems with leaves

In 2006, butterflies used in the heritability tests were collected from five sites along the East River valley (QC, GC, EGS, and SEGS/SG; Figure 2.1). Females were kept in the lab in the same screen cages and cared for as described for 1997. They were provided with cuttings of *C. cordifolia* and *T. arvense* – again matched by size and, when possible, phenology – placed in separate 10cm deep florist's picks with water. A dandelion (*Taraxacum officinale* Weber Asteraceae) flower was placed in the cage in a florist's pick and spritzed with 25% honey-water twice a day to supplement its nectar. Butterflies were hand-fed on the flower twice a day. F1 and F2 generation butterflies were reared and mated as described for 1997 and tested on cut stems with leaves.

2.2.3 Oviposition preference on methanol leaf extracts

Butterflies were collected from many sites along a 5 km transect of the upper East River valley. Collection locations were recorded using GPS. Females were brought back to the lab, fed 25-30% honey-water solution and held at room temperature overnight. The following morning, females were placed into cylindrical clear plastic cages (0.18 m height x 0.15 m diam.) with 1 mm holes punched around the top to maintain airflow. The floor of the arena was lined with a damp paper towel. Four Pastilina modeling clay (Sargent Art) bases (1x1.5x1 cm lwh) were placed in a square formation ~ 3 cm apart. Filter paper disks (3 mm diam.; Grade 1, Whatman) were placed vertically in each clay base (Figure B.1). Filter paper disks were treated with 80 µL of either *T. arvense* or *C. cordifolia* methanol extract, as described below. The two other disks included a control (70% MeOH only) and a blank (untreated), to test

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whether the butterflies preferentially laid eggs on extract-treated disks. Eggs laid on each disk were counted and collected, and the disks were replaced with freshly treated disks daily for up to six days or until the butterfly died.

As in previous years, eggs were sterilized using a weak hypochlorite solution. Hatching larvae were transferred to rearing cages containing *C. cordifolia* leaves and kept in the environmental chamber (27-31ºC, 16:8 L:D). When *C. cordifolia* was unavailable, larvae were fed young radish leaves (*Raphanus sativus*) which support similar larval survival as native hosts (Chew 1975). Larvae were given constant access to fresh food plant until pupation, at which point they were removed from the larval rearing cages, grouped by sex and brood, and held at room temperature in screen cages. Eclosing butterflies were numbered individually and placed into mating cages as in 1997 and 2006. We primarily used offspring of females that laid at least 15 eggs. This lower cut-off was chosen because, although some butterflies laid many eggs on the filter paper, many laid fewer than our original 30 egg cutoff. This, combined with a viral infection and poor mating success, limited our sample size. Again, matings were arranged so no sib-sib matings occurred and to encourage re-mating of males with females from different broods. Mated females in the F1 and F2 generations were tested in the same way as the P generation.

Several butterflies in the F1 and F2 entered diapause, rather than developing directly. These pupae were held in an incubator (1-2ºC, 12:12 L:D cycle) for 5 months, at which point the temperature was raised and the light cycle adjusted (17:27ºC, 16:8 L:D) to bring the butterflies out of diapause. Butterflies were mated under artificial heat lamps in greenhouses in cages containing collard (*Brassica oleracea*) and radish

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seedlings. The F2 offspring of diapausing F1 individuals developed directly and were reared on juvenile radish plants grown from seed before mating and being tested. Preference did not differ between direct developing and diapausing females in the F1 generation (ANOVA, F = 1.23, df = 1,38, p = 0.275). In the F2 generation, diapausing butterflies laid 8.62% (+/- 8.37% CI) fewer of their eggs on *T. arvense* (ANOVA, F = 4.08, df = 1,44, p = 0.050). Only F1 and F2 had direct developing individuals, and the conditions for diapause experienced by wild P individuals differed drastically from pupae diapausing in the laboratory, so diapause was not included in the full model during statistical analysis.

2.2.3.1 Preparation of extracts

Fresh host plants were collected in the field. Leaves were removed from the stems of the freshly collected plants, weighed in small packets, and transferred to liquid nitrogen. To make the methanol extracts, we modified an extraction procedure from Agerbirk and Olsen (Agerbirk and Olsen 2011): once frozen, the leaves were lightly crushed, and boiled in 70% MeOH for several minutes before filtering. Excess MeOH was used to boil the leaves, so the filtrate was left to evaporate for 24 hours. We added a small amount of 70% MeOH to achieve equal concentrations (10g fresh weight/L) in the two extracts. Plant extracts were stored in a dark, cool fridge to prevent the light-sensitive glucosinolates from degrading. Throughout the experiment, we collected and froze 80 µL samples of the extracts to evaluate their glucosinolate content.

In addition to chemical oviposition stimulation, butterflies respond to visual stimuli (Traynier 1986; Snell-Rood et al. 2013). The colors of the two extracts differed slightly, so we added green food dye (McCormick Culinary Food Color: water,

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propylene glycol, FD&C Yellow 5, FD&C Blue 1, and propylparaben) to both extracts and the MeOH control (1 mL dye/15 mL extract or MeOH). In the rare cases when butterflies laid eggs on disks not treated with extracts, they were far more likely to lay on the green (1.086±0.406% of eggs) than the white disks (0. 113± 0. 106% of eggs, paired t-test, $t = 4.725$, $df = 181$, $P < 0.001$).

2.2.3.2 Glucosinolate desulfation and quantification

Glucosinolates in the methanol extracts were desulfated following Prasad et al. (2012) and Keith and Mitchell-Olds (2017). Briefly, Sephadex columns (DEAE 25) were prepared with 50 µL 1 mM Progoitrin [2(R)-Hydroxy-3-butenyl GSL] analytical reference standard (ChromaDex, Inc.). Samples were added to the columns and washed twice each with 70% MeOH and dH_2O. Excess liquid was drained from the column, and the samples were incubated with 30 µL sulfatase for at least 12 hours (2.5 mg/mL). Samples were eluted first with 75 µL MeOH followed by 75 µL HPLC-grade water. Eluants were transferred into 200 µL microinserts and left uncovered for 24 hours before storage at 4-5 ºC.

Desulfoglucosinolates were quantified in the University of South Carolina Mass Spectrometry Center using a Thermo Scientific Ultimate 3000 High Performance Liquid Chromatography system with a 3400RS binary pump. Chromatography was carried out using a Chromegabond WR C18 column (ES Industries; 150 x 2.1 mm, 3 µm particles, 120Å pore size). The mobile phase contained HPLC-grade water and acetonitrile, with a 0.2 mL/min flow rate and the following gradient: 0% acetonitrile (0-3 minutes), ramp to 20% (3-30 minutes), hold at 20% (30-37 minutes), ramp rapidly to

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85% (37-44 minutes), return to 0% acetonitrile (44-end). The injection volume of samples was 20 µL. Desulfoglucosinolates were detected and quantified with an Agilent 1100 G1315B diode array detector (DAD) monitoring absorbance at 229 nm and subsequently with a Thermo Scientific Corona Veo RS charged aerosol detector (CAD). Only desulfoglucosinolates appearing in both the DAD and CAD output were included. Glucosinolates were identified using positive ion electrospray ionization with a Waters QTof API US quadrupole time-of-flight mass spectrometer. Both mass spectra and comparative retention times from the literature (Tolrà et al. 2006; Kusznierewicz et al. 2013; Olsen et al. 2016; Humphrey et al. 2018) were used to identify desulfoglucosinolates (Table B.2).

At the time leaves were collected to make the extracts, we also collected fresh leaf samples to ensure the glucosinolate components of our methanol extracts captured the glucosinolate profile of fresh leaf tissue. The sixth leaf from the apical meristem of 15 plants of each species was collected directly into screw-cap microcentrifuge tubes containing 70% MeOH. Leaf samples were kept in a cool, dark location for 8 months, which allowed glucosinolates to leach into the surrounding MeOH. Glucosinolates in the leachate were desulfated and quantified as described for extracts.

2.2.4 Statistical analysis

Spatial, temporal and generational patterns of preference were analyzed using multiple linear regression (*stats* package; R Core Team 2018). While butterflies collected in 1997 and tested on whole plants all came from the same area, butterflies tested on cut stems and on extracts were collected over a 5 km transect in the upper

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East River valley. To account for any effect of source location on preference, we created a 'Paternal latitude' variable, which was equal to the collection latitude of the P generation, the maternal collection latitude of the F1 generation, and the paternal grandmother collection latitude of the F2 generation. The individual locations of butterflies were recorded by GPS in 2015, but not in 2006. We used the centroid of the collection site as the collection latitude of the 2006 parental generation. We also tested a 'Maternal latitude' variable, wherein the maternal grandmother collection latitude was used for the F2 generation, but this variable explained slightly less of the variation. The preference test start day was calculated as an ordinal day from the first test day within that generation. In the case of 2015, this numbering system restarted for diapausing individuals, so that the first direct-developing individual tested in the generation was assigned a start day of 1, and the first diapausing individual tested in the generation was also assigned a start day of 1. On whole plants and on cut stems, preference was considerably skewed toward *C. cordifolia*, so the proportion of eggs laid on *T. arvense* plants and cut stems was square-root transformed. To improve our confidence in this measure of preference, we only included butterflies that laid at least 30 total eggs, although this threshold was reduced to 15 in 2015 to improve the sample size in the F1 and F2 generations. This allowed us to retain 20 additional related pairs.

The full linear model for egg laying preference of butterflies tested on whole plants evaluated the two-way interaction of generation and start day on the square-root of the proportion of eggs laid on *T. arvense*. For butterflies tested on cut stems and those tested on extracts, the full model tested the interaction between generation, start day and paternal latitude. To check the effect of diapause in the context of the full

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extracts model, we nested diapause within generation. Models were hierarchically simplified using AICc (*MuMin* package; Barton 2018), and the fit of nested models were compared using likelihood ratio tests (Tables B.3-5). The significance of remaining model terms was compared using type II ANOVAs (car package; Fox and Weisburg 2011).

2.2.4.1 Heritability

We used dyadic mixed models (*dmm* package; Jackson 2016) to evaluate the contribution of sex-linked, autosomal and environmental variance to the proportion of eggs laid on *T. arvense* in our choice assays. The *dmm* package calculates variance estimates by first fitting a model using generalized least squares estimates of the fixed effects (bias-corrected maximum likelihood). The model residuals are then partitioned into environmental and genetic variance components using a dyadic model. Each dyad comprises paired residuals of related individuals, which is compared to the expected correlation based on the relatedness matrix. Standard errors and confidence intervals of variance components are estimated from the multiple linear regression of the dyads using GLS methods. We formulated a pedigree and calculated an autosomal and sex-linked relatedness matrix for each year. The *dmm* package relies on *nadiv* (Wolak 2012) to generate genetic relatedness matrices for autosomes and the shared sex chromosome (Z). The *dmm* package does not require all individuals following the parent generation to have known fathers and mothers. As gravid females were collected in the wild, we did not know the identities of the F1 fathers. We could not assume shared fathers because sperm precedence in this species is likely not complete (Wedell and Cook

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1998). We also reran the analyses with unique mates for all P females included in the pedigree, and while this slightly reduced the absolute values of the variance component estimates, the general contribution and significance of the genetic components were the same. The relatedness matrices were included as random effects in mixed models with proportion of eggs laid on *T. arvense* as the response variable. We ran two models for each year, one with no covariates to estimate heritability with spatial and temporal variability included within environmental variance. The second included the covariates from the best fit linear model. For each model, the program calculated the proportion of the phenotypic variance apportioned to autosomal additive genetic variance, sex-linked additive genetic variance, and environmental variance ($V_P = V_G + V_E$, where $V_G = V_A + V_Z$). Too few individuals were reared to test for maternal effects or dominance. The egg cut-off (30 for whole plants and cut stems, 15 for extracts) did not affect the overall phenotypic variance or the additive genetic variance. We ran the 2015 model with a 30-egg cut-off and the variance estimates were similar.

2.3 Results

2.3.1 Preference on whole plants and cut stems

In both 1997 when female oviposition preference was tested on whole plants and in 2006 when female oviposition was tested on cut stems, the proportion of eggs laid on *T. arvense* ranged from 0 to 1, but butterflies tended to prefer native *C. cordifolia* (skew toward 0, Figure 2.2). Preference variation for the two plants within the parental generation was similar (F-test of variances, $F_{(32,28)} = 0.703$, $p = 0.334$).

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Preference shifted toward *T. arvense* in subsequent generations, especially F2 (Figure 2.2), a main effect that was significant in 1997 (Table 1).

Oviposition preference was affected by the day females started choice trials (Figure 2.3). Rather than a constant decline in preference for *T. arvense* over the summer, the effect of start day differed among generations and was largely driven by declining preference for *T. arvense* by F2 individuals tested in late August and early September 1997 (Figure 2.3A, Table 2.1). On cut stems, the strongest effect of start day was again observed in declining preference for *T. arvense* in the F2 generation (Figure 2.3B, Table 2.1).

Butterflies tested on whole plants were all collected from a single location (Parcel C), but in 2006, butterflies were caught along a 5 km transect in the East River Valley before being tested on cut stems of *T. arvense* and *C. cordifolia* (QC, GC, EGS, SEGS/SG; Figure 2.1). This spatial variation was included in the best fit model, but there was no significant correlation between collection location (= likely paternal latitude) and proportion of eggs laid on *T. arvense* in any generation (Figure 2.4A, Table 2.1).

2.3.2 Preference on methanol leaf extracts

Unlike butterflies tested on plants, wild-caught (P) butterflies tested on extracts (2015) did not prefer *C. cordifolia* over *T. arvense*. The variance of preference was also significantly smaller on extracts than on plants (2015 v. 1997 F-test of variances, $F_{(93,28)} = 0.304$, $p < 0.001$; 2015 v. 2006 F-test of variances, $F_{(93,32)} = 0.432$, df = 93, 32, $p = 0.002$). None of the butterflies that laid enough eggs to be included in the data set oviposited exclusively on one plant extract (Figure 2.2, light gray). Testing butterflies

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on extracts effectively eliminated the temporal variation introduced by testing them on plants and stems, the characteristics of which may change with time. There was no effect of start day in any of the generations (Figure 2.3C, Table 2.1). However, preference for *T. arvense* significantly decreased with increasing paternal latitude (Figure 2.4B, Table 2.1).

2.3.3 Heritability

When taking only the pedigrees into account, additive genetic variance was attributed to sex-linked components. This evidence was strongest when butterflies were tested on whole plants (Figure 2.5A; $V_Z/V_P = 0.49 \pm 0.24$) and remained significant when covariates from the best fit linear model were included (generation x start day; $V_Z/V_P = 0.43 \pm 0.29$). Sex-linked genetic variance estimates were slightly lower, but still significant, for butterflies tested on cut stems (Figure 2.5B; $V_Z/V_P = 0.38 \pm 0.25$). While the model including covariates supported these results, the sex-linked additive genetic variance was not significant (generation x start day + paternal latitude; $V_Z/V_P = 0.10 \pm 0.32$). In both models, the phenotypic variance apportioned to autosomal additive genetic variance was negligible (Table 2.2). Rather, there was a general non-significant negative correlation between the proportion of eggs laid by mothers and daughters on the nonnative host (1997: slope = -0.063± 0.178, Figure B.2A; 2006 slope = -0.101±0.152, Figure B.2C). Estimates of other variance components did not change when V_A was removed from the model.

Variance components of preference for extracts did not reflect those for plants and stems (Table 2.2). The pedigree-only model attributed significant additive genetic variance to

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autosomal components (Figure 2.5C; $V_A/V_P = 0.35 \pm 0.31$), with negligible sex-linked components. However, inclusion of covariates from the best fit model (generation + start day + paternal latitude) shifted the variance attributions. The total estimated genetic variance was lower ($V_A/V_P + V_Z/V_P = 0.19$), but most of this was attributed to sex-linked rather than autosomal genetic variance ($V_Z/V_P = 0.14 \pm 0.43$).

2.3.4 Glucosinolate components

The majority of glucosinolates detected in leaf leachates (Figure 2.6A) were also recovered in methanol extracts (Figure 2.6B) for both *T. arvense* (2/3) and *C. cordifolia* (7/10). Sinigrin (2-propenyl) and glucocochlearin (1-methylpropyl) were the dominant glucosinolates in *T. arvense* and *C. cordifolia*, respectively. Concentrations applied to filter paper were considerably lower than those estimated for fresh leaves. Relative concentrations of different compounds were generally similar between leaves and extracts, with several exceptions. Glucoputranjivin (1-methylethyl, dGSL MW: 281) was higher, relative to other glucosinolates, in extracts than in leaf samples. For both plants, gluconapin (3-butenyl; dGSL MW 293) was found in leachates, but not in extracts, based on CAD peaks that were also detected by DAD.

2.4 Discussion

We found a sex-linked genetic basis of female preference for the novel, nonnative host plant *T. arvense*. Evidence was strongest when tested on whole plants but was replicated on cut stems and generally persisted when significant environmental covariates were considered in the heritability analyses. Z-linkage of *P. macdunnoughii* preference was unexpected. Within-population inheritance of lepidopteran oviposition

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preference, attributed more often to specificity and female motivation than differences in the overall attractiveness of available host plant species, has often been found to have an autosomal genetic basis (Tabashnik et al. 1981; Singer and Thomas 1988; Jaenike 1989; Bossart and Scriber 1995; Nylin et al. 2005). Z-linkage, on the other hand, is thought to be characteristic of fixed differences between populations and species (Janz 1998, 2003). Geographically distant populations of the Comma butterfly, *Polygonia c-album*, consistently demonstrated sex-linked differences in host-plant choice (Janz 1998; Nylin et al. 2005), whereas preference variation within populations was inherited autosomally (Nylin et al. 2005). Preference differences between *Papilio glaucus* and *P. canadensis* were also sex-linked (Scriber 1994), but sex-linkage disappeared within a late-flying hybrid population (Mercader and Scriber 2007).

Janz (1998, 2003) proposed that stable preference differences between populations and species are caused by accumulation and fixation of adaptive loci on the Z-chromosome resulting from extended association with different stable host plant—and selection—environments. Preference genes on the Z-chromosome under selection in the historical resource environment should be fixed (or have considerably lower variation) within populations, and the remaining detectable variation should have an autosomal genetic basis. Instead, within this *P. macdunnoughii* population, unfixed Z-linked variation is responsible for choice between *T. arvense* and *C. cordifolia* plants and stems, suggesting that the introduction of novel plants unmasks genetic variation for preference that may be analogous to that usually found between butterfly populations. If this is the case, we expect preference tests between pairs of native host plants to reveal autosomal, not Z-linked, inheritance patterns.

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Selection is expected to remove alleles with low fitness under "common" conditions, while deleterious mutations only expressed in stressful or novel environmental conditions are more likely to remain (Hoffmann and Merilä 1999). A similar hypothesis of mutation accumulation has been invoked to explain why herbivorous insects often have difficulty reverting to historical hosts after adoption of a new host (Grosman et al. 2015). Under selection for improved preference and performance on the novel host, selection is relaxed for use of the historical host, allowing deleterious mutations to accumulate. When insects once again have access to the historical host, fitness on the historical host is often lower than on the newly adopted host (Grosman et al. 2015). Fitness may also be lower than that of conspecifics that have continued to use the historical host (Buckley and Bridle 2014). Persistent genetic variation for oviposition on *T. arvense* and the inability of larvae to consume the plant may have resulted from a similar pattern of mutation accumulation. While the ancestral host ranges of *P. macdunnoughii* and its North American congeners are unknown, the European sister taxon to all North American *Pieris* species, *Pieris napi,* readily lays eggs and develops on *T. arvense* (Friberg et al. 2015). If we hypothesize the ability to eat *T. arvense* was lost in North American *Pieris* populations, *Pieris* butterflies on novel hosts (current native hosts) cannot revert to ancient host (*T. arvense*), because genes for accepting and eating this host that still exist in the population are accumulating deleterious mutations that are only revealed in the presence of *T. arvense.*

Although female butterflies rely on a variety of cues to identify host plants, host plant recognition and preference is overwhelmingly attributed to plant chemistry

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(Dethier 1954; Ehrlich and Raven 1964; Renwick 1989; Carrasco et al. 2015). However, plant chemistry can change rapidly as a result of contact, damage, oviposition or abiotic conditions (Louda and Rodman 1983; Cipollini et al. 2005; Mithöfer and Boland 2012). We expected methanol-soluble host plant chemistry captured in our extracts, specifically glucosinolates, to be the major driver behind variation in preference for *T. arvense*. As we predicted, butterflies were stimulated to lay eggs on methanol-based leaf extracts and extracts eliminated some of the preference variation that may have been caused by differences in plant quality or chemistry. Start day, which affected preference of both whole plants and cut stems, did not significantly affect preference of extracts, suggesting extracts successfully eliminated differences among cues presented to butterflies within and between generations.

Heritability analyses of extract preference did not match those for whole plants or cut stems. In fact, heritability patterns observed on extracts were primary autosomal, although with covariates there was no detectable genetic variance and again the phenotypic and genetic variance estimates were lower than on plants and stems. The combination of decreased phenotypic variance on extracts and autosomal inheritance suggest that responses to these cues may have been under selection for an extended period, and therefore part of the historical recognition system. While decreased phenotypic and genetic variance could be an effect of a more constant oviposition cue, this is also expected for loci that have experienced consistent, long-term purifying selection (Weis and Abrahamson 1986; Hoffmann and Merilä 1999; Huang et al. 2015). As an entire plant, *T. arvense* is a relatively novel resource. However, the cues available in the methanol extracts may have been less so. Considering only the glucosinolate

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components, both sinigrin and gluconapin are found in *Descurainia incana*, another of *P. macdunnoughii*'s native, high-quality host plants. Preference variation involving these specific cues may have preceded the introduction of *T. arvense.*

Furthermore, preference of wild-caught females for *T. arvense* consistently decreased to the north up the East River valley. When this spatial term was included in the dyadic mixed model, evidence for sex-linkage in tests of extracts was restored, though not significant. This may be evidence for spatial genetic structure within the population and may not have been detected in 2006 due to poor sample size from northernmost sample sites. Further investigation of the genetic structure of butterflies in the East River valley and the Gunnison basin will be necessary to evaluate whether this is true and whether heritability differs between putative populations.

Our conclusions about the relative importance of glucosinolates in the differences between plants and extracts are limited because the leaf extracts did not capture the full array of phytochemical cues available to ovipositing butterflies. The extracts captured most of the glucosinolate compounds found in *T. arvense* and *C. cordifolia* leaves, but the concentrations applied to filter paper were considerably lower than those of fresh leaves. Low glucosinolate concentrations likely contributed to the overall decreased preference variation on extracts. Acknowledging the simplified cue set provided in our preference assays, the loci responsible for responding to these particular cues are autosomal and unlikely to be playing a primary role when female butterflies are choosing between *T. arvense* and *C. cordifolia.*

It is possible the differences observed between the studies, conducted at nine-year intervals, were influenced by changes in preference for *T. arvense* within the

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population, rather than in response to the oviposition substrate. Although host plant preference can change within lepidopteran populations over a generation (Singer 2003), no major shifts in preference for *T. arvense* have been observed over the 45 years this population of *P. macdunnoughii* has been studied (Chew 1977, Nakajima et al. 2013, Steward, RA, Boggs, CL, unpubl.) It is also unlikely that sex-linked genetic variation was lost from the population in under two decades, when the population(s) have been exposed to the plant for closer to 15 decades.

We found that butterflies collected from the wild (P) generation consistently laid more eggs on *C. cordifolia* than *T arvense*, especially when tested on whole plants. This preference decreased in lab-reared generations. The trend for lab-reared individuals to prefer *T. arvense* warrants further examination, and may have resulted from learning, differences in mating experience or fecundity, life history, or variation in plant traits over time. Unlike the lab-reared F1 and F2 generation, wild-caught female butterflies mated in the field may have mated multiply and likely had prior oviposition experiences on hosts available in their habitat. Prior experience has been shown to affect *P. napi* preference for available host plants, especially when the historical relationship between the plant and host is old (Gamberale-Stille et al. 2018). Decisions by females are also influenced by fecundity which can modify the relative risk of poor host choice. *P. napi* butterflies with low fecundity were less likely to oviposit on lower ranked hosts (Schäpers et al. 2017). We expect that any effect of experience or fecundity in our experiment increased environmental variance, making the heritability estimates more conservative.

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Like the wild-caught P generation individuals, all of which overwintered as pupae, diapausing F1 and F2 individuals in our 2015 dataset tended to have lower preference for *T. arvense*, although diapause was not as significant predictor of preference. Although host plant specificity is linked to the number of generations a population goes through in a flight season (voltinism, Scriber 1994; Nylin et al. 2009), it is unclear whether diapause directly affects host plant preferences. This may be because it is difficult to separate the effect of diapause from the effect of seasonal and phenological changes in available host plants. For the most part, preference for *T. arvense* decreased with start day within generation when tests were conducted on more than three days (excluding F1 butterflies tested on cut stems). There is also evidence that preference for *T. arvense* tends to decrease over the season within this *P. macdunnoughii* population (RA Steward and CL Boggs unpubl.). This may be due to decreased plant nutritional quality or changes in available glucosinolate cues. Females prefer methanol-based extracts of budding and flowering *T. arvense* to extracts from plants that have already gone to seed (Steward, RA, Boggs, CL unpubl.).

One prediction for the evolution of host plant specialization is that the preference of mothers will be correlated with the performance of their offspring, and this is generally supported in long-term associations between insects that use a small subset of host plants (Gripenberg et al. 2010; Balagawi et al. 2013; Masselière et al. 2017), although preference-performance correlations range from good to poor (Thompson 1988b; Friberg et al. 2015). While reduced offspring performance is expected during initial stages of colonizing a novel host (Thompson 1988b; Garcia-Robledo and Horvitz 2012), preference-performance correlations have been implicated

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in rapid adaptation to novel hosts (Keeler and Chew 2008). Covariance of preference and performance traits at the genetic level are important to this process (Via 1986; Bossart 2003). Given that any oviposition on *T. arvense* by *P. macdunnoughii* is maladaptive, there is already strong evidence that preference and performance are decoupled in this system. The strong signal of sex-linked preference on whole plants and cut stems contrasts with evidence that performance has an autosomal basis. Short-term larval performance metrics correlated strongly within sibling groups (Steward et al. 2019), and the genomic basis for the ability of closely related *P. napi* to eat and survive on *T. arvense* has been localized to autosomes (Steward, RA, Wheat, CL, Wiklund, C, Boggs, CL, unpubl.). This decoupling, combined with the fact that preference precedes performance and in the absence of *de novo* mutation, means any adaptation by the butterfly to the plant will depend primarily on the evolution of preference.

2.5 Conclusions

Our study revealed an unexpected genetic basis for preference for a novel host plant. However, given heritable genetic preference variation and considerable selection against oviposition on *T. arvense*, it is even more puzzling that we have found no evidence for increased avoidance of the lethal host. There must be additional constraints on the evolution of host plant preference, such as gene flow between invaded and uninvaded areas. Cue similarity, the hypothesis that recognition of *T. arvense* depends on the same chemical, visual or tactile stimuli that are used to evaluate native host plants, is another possible constraint. Sex-linked preference for *T. arvense*

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may mean this cue response is dissimilar and not under the same selection pressures as normal host preferences. Cue similarity perhaps plays a role when butterflies assess certain chemical cues, but additional stimuli ultimately influence choice between available host plants. Further studies evaluating the genetic basis of preference for both native and nonnative hosts will be necessary to evaluate the mechanisms maintaining maladaptive preference in *P. macdunnoughii* populations.

2.6 Acknowledgments:

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2.7 Tables

Table 2.1. ANOVA hypothesis tests for significance of linear model terms. Models were hierarchically simplified using AICc values (Tables B.3-5).

Year	Response	Model Term	F	df	P-value	Sig.
1997	Sqrt. proportion of eggs laid on T. arvense plants	Generation	15.86	1, 115	< 0.001	*
		Start day	1.88	2, 115	0.172	
		Generation * Start day	3.79	2, 115	0.026	*
2006	Sqrt. proportion of eggs laid on T. arvense stems	Generation	1.98	2, 167	0.142	
		Start day	1.61	1, 167	0.206	
		Paternal latitude	2.39	1, 167	0.124	
		Generation * Start day	4.33	2, 167	0.015	*
2015	Proportion of eggs laid on T. arvense methanol extract	Generation	2.86	2, 172	0.060	•
		Start day	2.55	1, 172	0.112	
		Paternal latitude	4.45	1, 172	0.036	*

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Table 2.2. Dyadic mixed model estimates used to calculate the proportion of the phenotypic variance attributed to environmental (E), autosomal (A), and sex-linked (Z) variance components (Figure 2.5). Asterisks indicate estimates that are significant (95% confidence interval does not overlap zero).

Year	Model	Component	Var. Estimate	Lower 95% CI	Upper 95% CI	Sig
1997	Pedigree	V_E	0.073	0.049	0.097	*
		V_A	$1.00x10^{-9}$	-0.023	0.023	
		V_Z	0.073	0.027	0.118	*
		V_P	0.146	0.117	0.174	
	Pedigree + Covariates	V_E	0.046	0.031	0.062	*
		V_A	$3.00x10^{-4}$	-0.015	0.016	
		V_Z	0.035	0.005	0.066	*
		V_P	0.082	0.063	0.101	
2006	Pedigree	V_E	0.061	0.046	0.076	*
		V_A	$1.00x10^{-9}$	-0.013	0.013	
		V_Z	0.038	0.007	0.068	*
		V_P	0.099	0.080	0.117	
	Pedigree + Covariates	V_E	0.062	0.048	0.076	*
		V_A	$1.00 x10^{-9}$	-0.012	0.012	
		V_Z	0.007	-0.021	0.035	
		V_P	0.069	0.053	0.086	
2015	Pedigree	V_E	0.022	0.012	0.033	*
		V_A	0.012	0.004	0.020	*
		V_Z	$1.00 x10^{-9}$	-0.021	0.021	
		V_P	0.034	0.0226	0.0453	
	Pedigree + Covariates	V_E	0.025	0.015	0.034	*
		V_A	0.002	-0.006	0.009	
		V_Z	0.004	-0.015	0.024	
		V_P	0.031	0.020	0.041	

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2.8 Figures

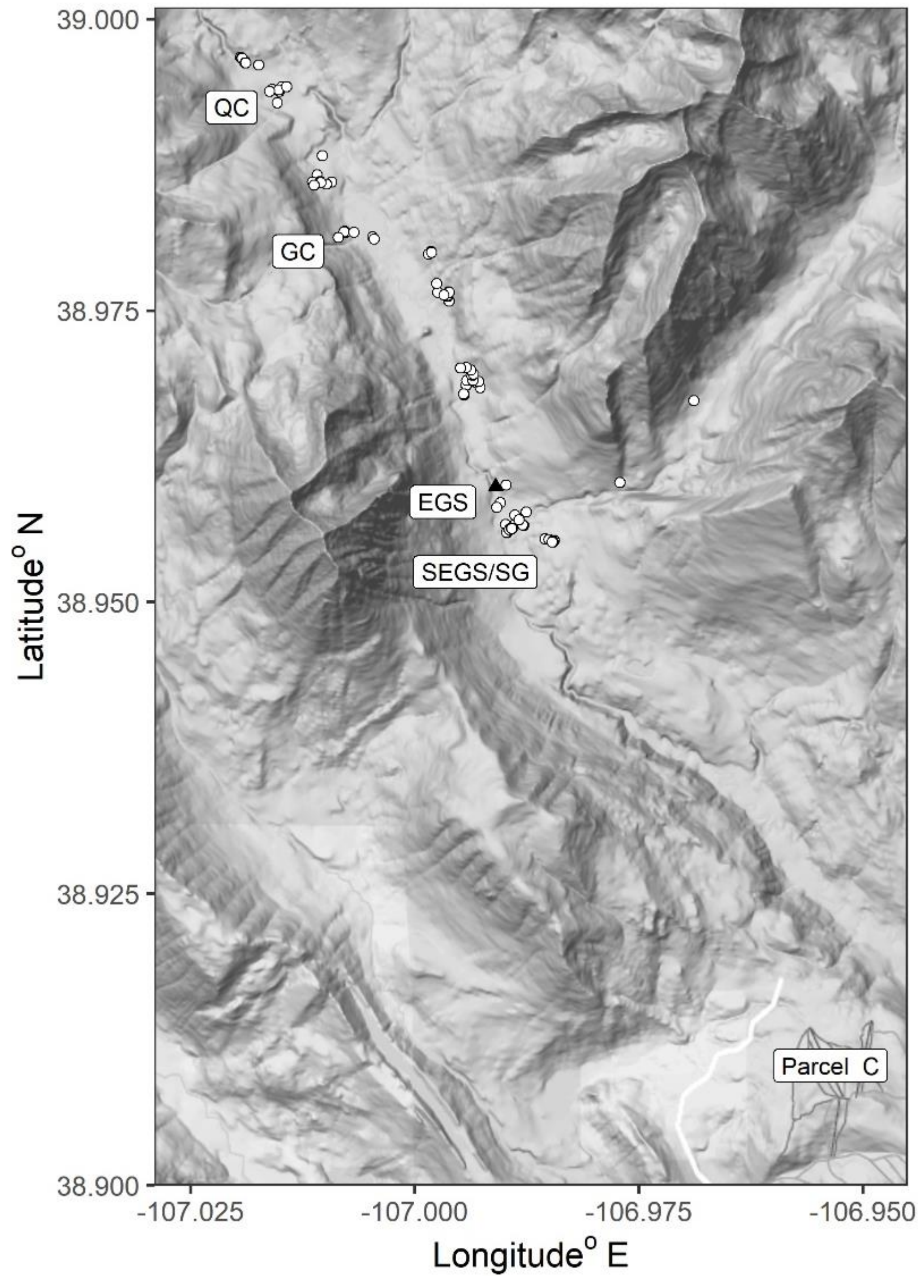

Figure 2.1. Collection sites for female butterflies caught in 1997 (Parcel C), 2006 (QC, GC, EGS, and SEGS/SG), and locations of individuals caught in 2015 (white circles) in the upper East River valley near Gothic, CO, USA. The triangle represents the northernmost extent of T. arvense within the valley as of 2015, which was likely similar to the extent in 1997 and 2006.

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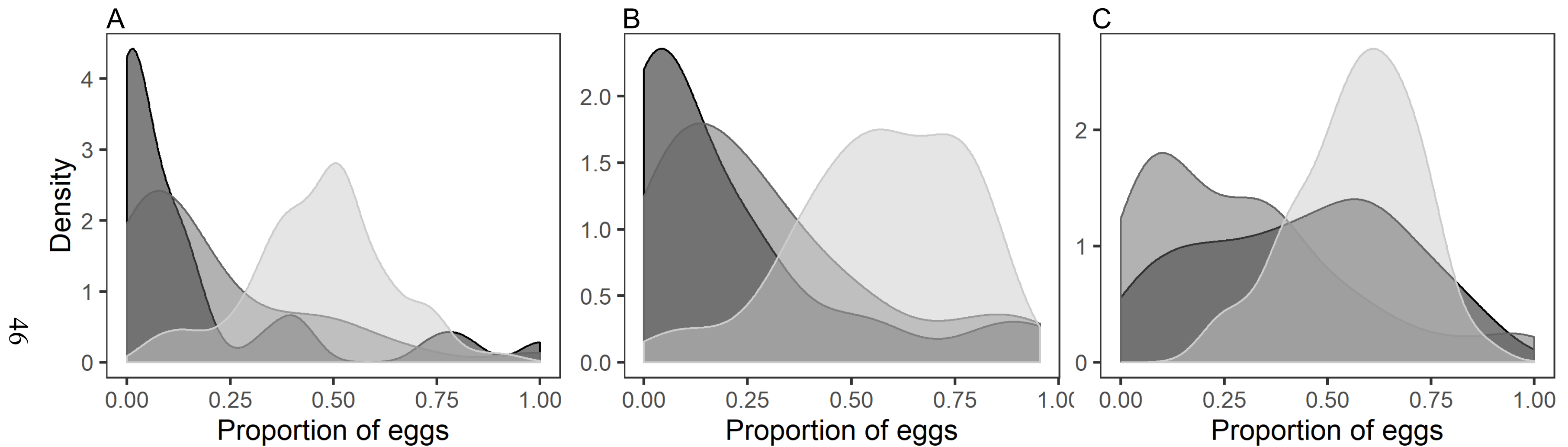

Figure 2.2. Density plots of untransformed proportion of eggs laid on T. arvense by (A) wild-caught P generation females and (B) lab-reared F1 and (C) F2 generations in simultaneous choice experiments conducted on whole plants (1997, dark gray), cut stems (2006, gray), and MeOH extracts (2015, light gray). Proportions of eggs were square-root transformed for all other 1997 and 2006 analyses.

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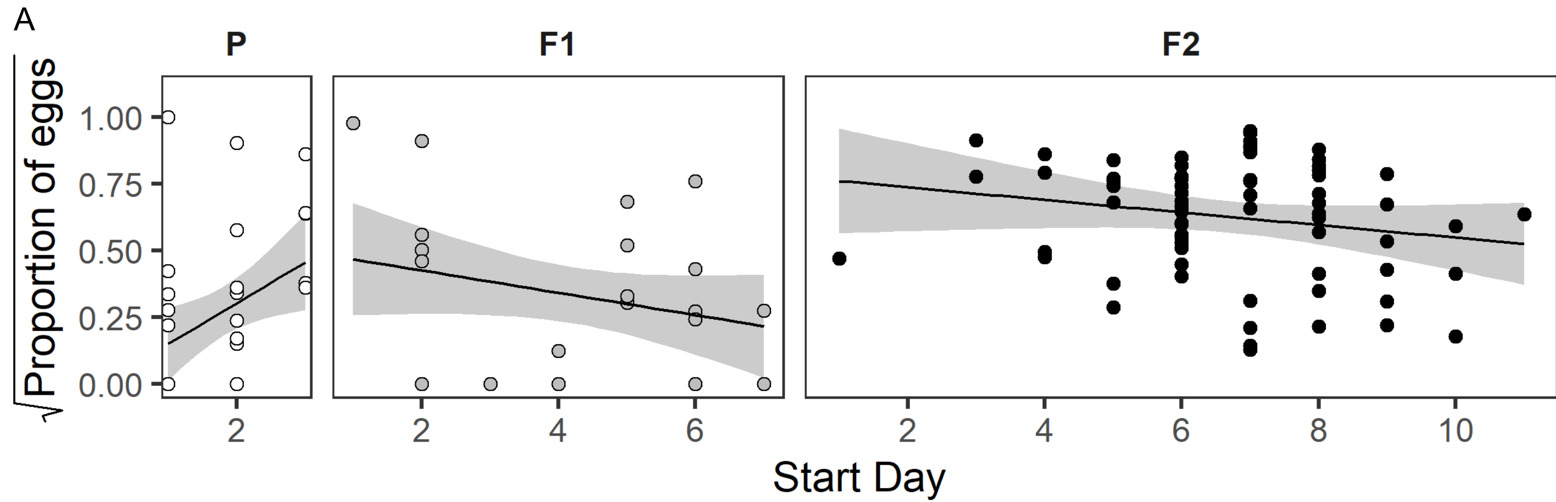

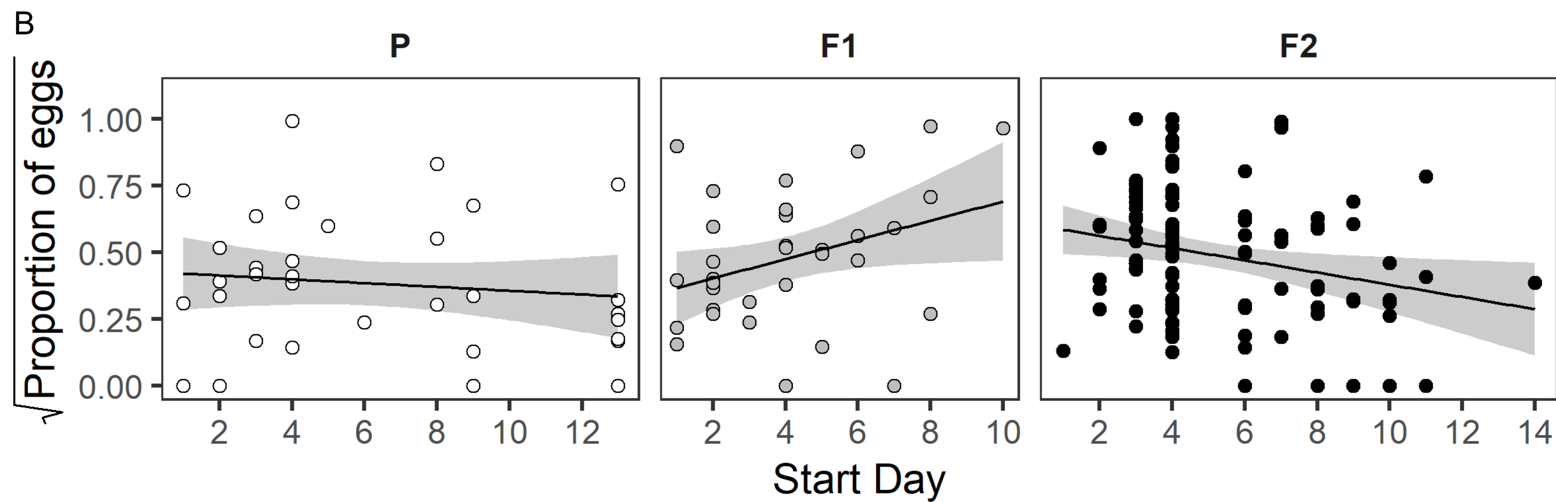

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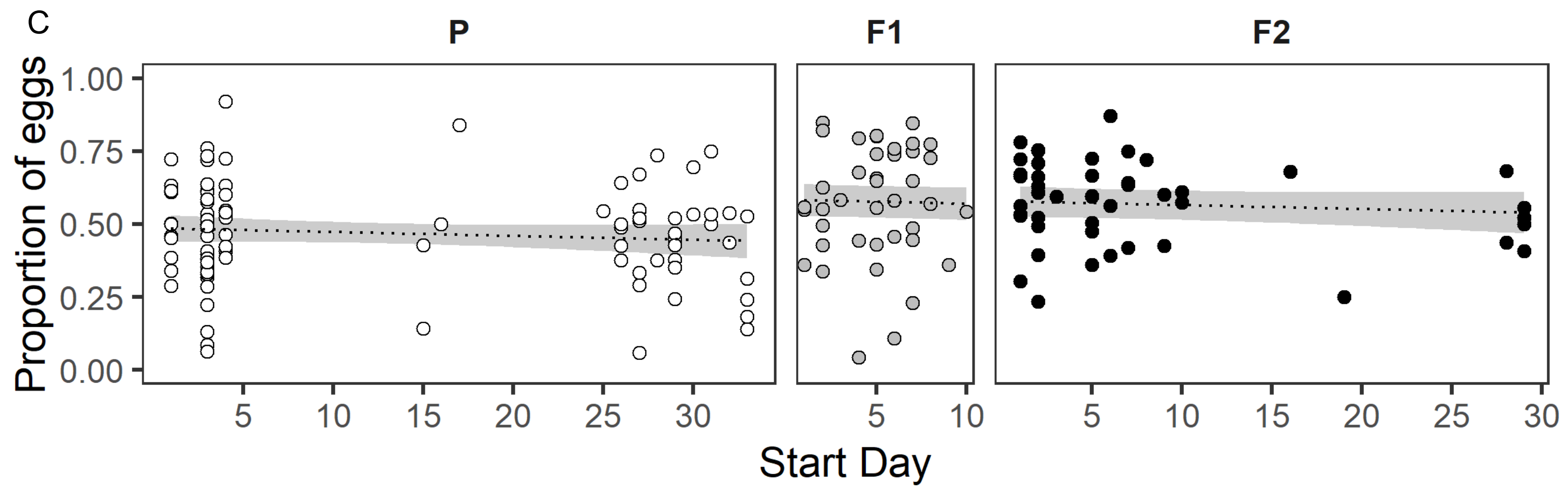

Figure 2.3. The effect of within-generation start day on the proportion of eggs (A, B: square-root transformed) laid on *T. arvense* plants by the parental (P), F1, and F2 generations in (A) 1997, (B) 2006 and (C) 2015. Lines and 95% confidence intervals (gray) are based on best fit linear models (Table 2.1, Tables B.3-5).

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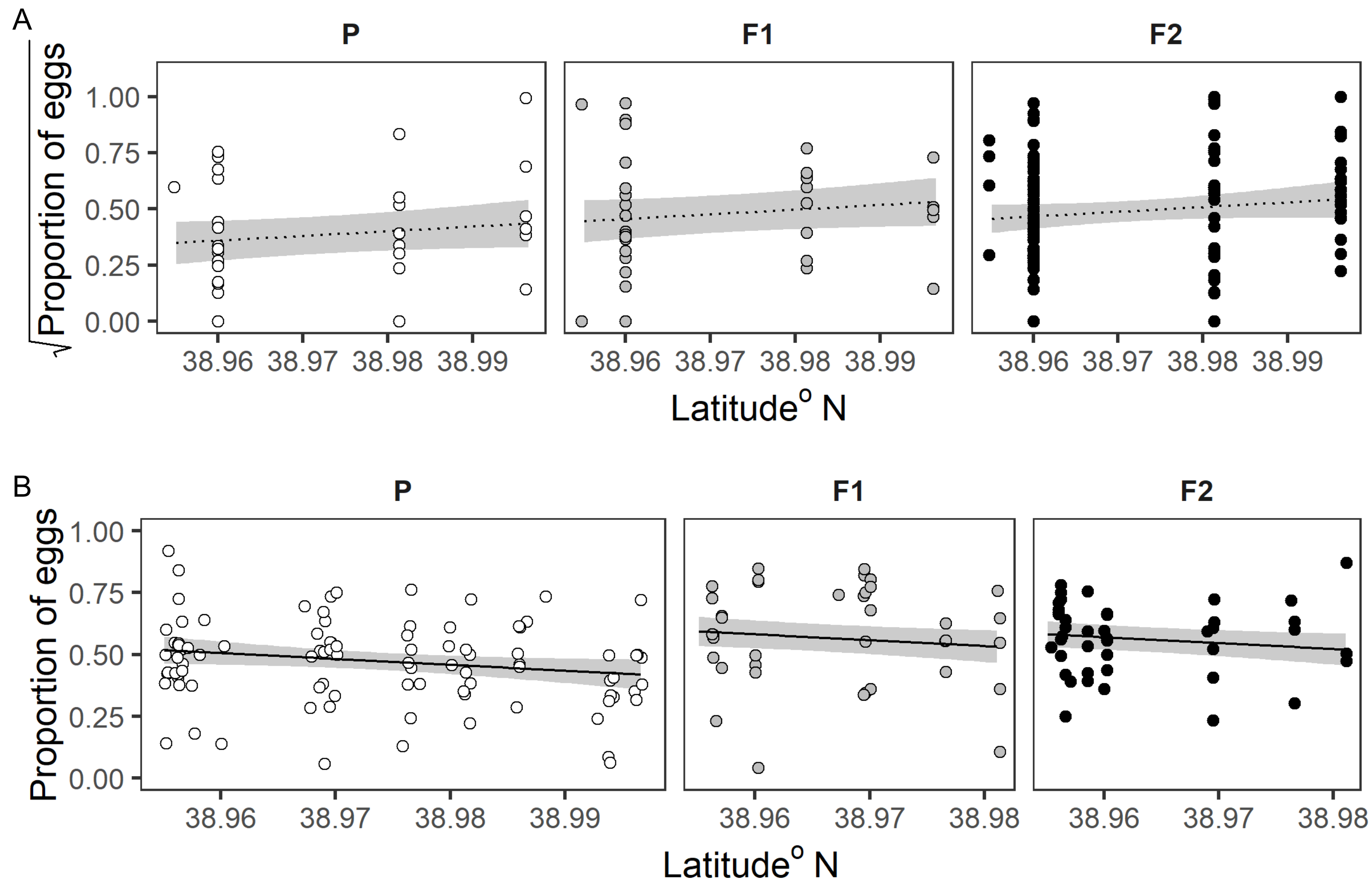

Figure 2.4. The effect of latitude on the proportion of eggs laid on *T. arvense* plants by the parental (P), F1, and F2 generations in (A) 2006 and (B) 2015. Lines and 95% confidence intervals (gray) are based on the best fit linear model (Table 2.1, Tables B.3-5). Dotted lines indicate model terms that were retained in the best fit model, but not significant.

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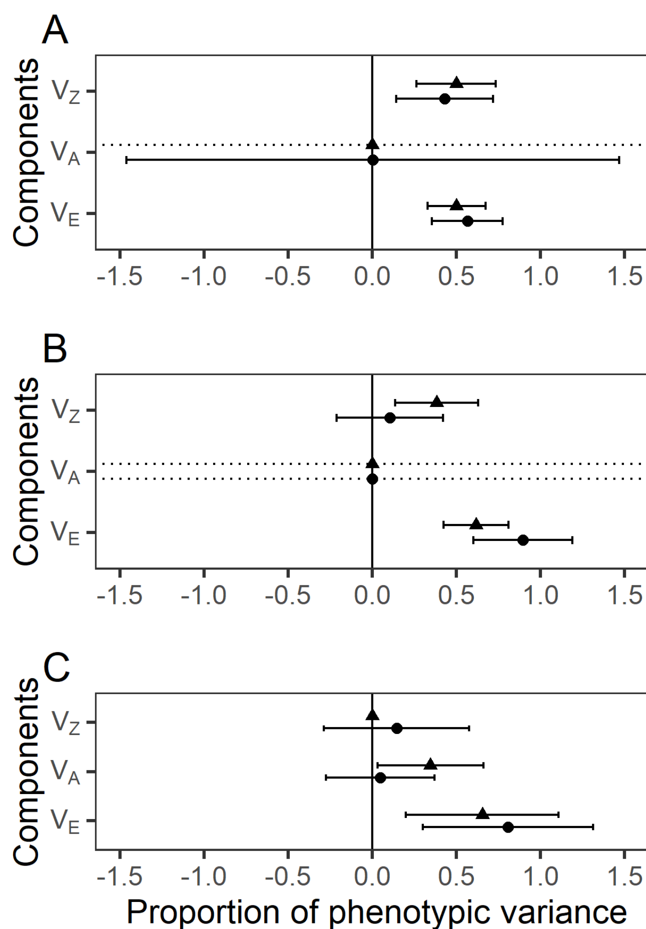

Figure 2.5. Proportion (+/- 95% CI) of the phenotypic variance (1.0) apportioned to additive sex-linked genetic variation (V_Z), additive autosomal genetic variation (V_A), and environmental variation (V_E) for preference tested on (A) whole plants, (B) cut stems and (C) methanol extracts. Variance components modeled without any covariates (triangles) were all significant, while those modeled using terms from the best fit models for each year (circles) often were not. Due to sparseness of the relatedness matrix, several of the confidence intervals were calculated as infinite (dotted lines).

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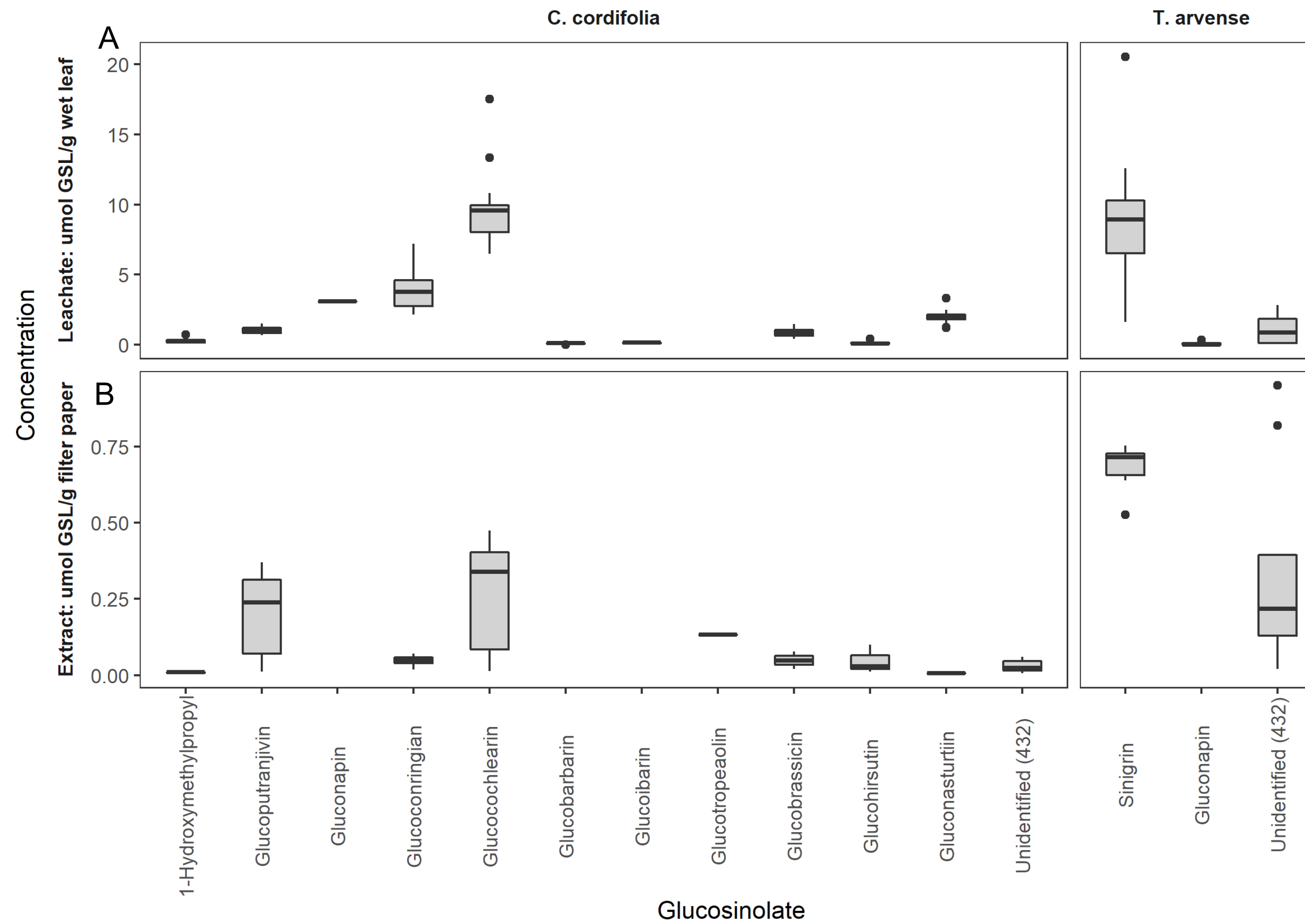

Figure 2.6. Glucosinolate concentrations in (A) leachates of leaves collected from the field and (B) methanol extracts made from leaves of the same plants and used in choice assays in 2015 (Table B.2).

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CHAPTER III:

THE ROLE OF CUE SIMILARITY IN MAINTAINING A PERSISTENT HOST-PLANT BASED EVOLUTIONARY TRAP[2]

[2] Steward, RA, Boggs, CL. *To be submitted to Ecological Monographs*.

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3.1 Introduction

As humans continue to rapidly modify natural environments, ecological and evolutionary traps are increasingly common (Schlaepfer et al. 2002; Robertson et al. 2013; Hale and Swearer 2016). Evolutionary traps occur when novel resources and habitats lead to preference-performance mismatches based on evolved recognition systems and are a subset of the maladaptive interactions that occur between organisms and their biotic and abiotic environments as a result of rapid anthropogenic environmental change (Magnan et al. 2016). As novel maladaptive interactions arise, it is important to consider how and when they are encountered (i.e. distribution and scale of maladaptive conditions), their potential fitness costs, and population level effects (e.g. local extinction, population sink; Hale and Swearer 2016). Whether maladaptation persists depends on the interaction of geographically heterogeneous selection pressures, underlying genetic variation, inflexible recognition systems and gene flow (Crespi 2000; Kawecki and Ebert 2004; Blanquart et al. 2013; Farkas et al. 2015). Yet, our understanding of these processes has come primarily from populations already in the process of locally adapting to recent environmental change. Cases of long-term maladaptation to specific, quantifiable selection pressures are less well-studied (Arnold 1992; Crespi 2000; Farkas et al. 2015).

Evolutionary traps are not inescapable and there is evidence of rapid local adaptation in response to such traps (Carroll 2007; Keeler and Chew 2008; Olivieri et al. 2016). Escape trajectories can be categorized as 'decreased preference' or 'improved exploitation' (Chew 1975; Schlaepfer et al. 2002; Carroll 2007), in which selection favors either individuals that avoid the novel resource or those that are better at using it.

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When abiotic features in the environment are mistaken for food or habitat (Gwynne and Rentz 1983; Szaz et al. 2015; Robertson et al. 2018), or the resource is completely lethal and no variation in ability to exploit it exists in the population (Chew 1977a; Davis 2015), increased exploitation is not an option.

One potential constraint on escape from such traps is cue similarity, whereby overlapping cue sets link decreased preference for the novel, unsuitable resource with decreased preference for the historical or native resource. In evolutionary traps, maladaptive interactions with novel resources or habitats are mediated by previously evolved cue-response systems that are no longer reliable (Sih et al. 2011). Cue sets involved in mistakenly selecting low-quality novel resources are expected to resemble those of historical resources but can vary in the strength of that resemblance. A large overlap in the composition or intensity of the cue sets may mean that narrowing or refining the recognition system causes individuals to reject high-quality resources, transitioning the maladaptive response from mistake to miss (Fox and Lalonde 1993; Macmillan and Creelman 2004). Fitness costs of rejecting good resources (misses) may outweigh those of using trap resources (mistakes), thereby maintaining the maladaptive behavior in a population (Fox and Lalonde 1993; Robertson et al. 2013).

Cue similarity may be especially important in maintaining host plant-based traps, in which native insects use attractive but harmful nonnative plants (Gripenberg et al. 2010; Pearse et al. 2013). The prevalence of host-plant based traps has been attributed to the high degree of specialization among herbivorous insects (Pearse and Altermatt 2013; Forister et al. 2015; Yoon and Read 2016). Host plant specialization depends on an efficient recognition system (Holmgren et al. 2007), often at the expense

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of behavioral flexibility or plasticity (Bernays 2001; Carrasco et al. 2015). Discrimination among host plants by specialist herbivores is shaped by the costs associated both with accepting poor quality hosts (mistakes) and with excluding high-quality hosts (misses) (Wood et al. 2018). The costs of misses are expected to be higher if insects are time-limited when searching for hosts rather than reproductively limited (Rosenheim et al. 2000; Snell-Rood and Papaj 2009).

Most butterflies are host plant specialists, with species feeding on few plant families (Ehrlich and Raven 1964; Futuyma 1976; Hamm and Fordyce 2015). Among butterflies, host plant recognition is often mediated by plant chemistry, which acts as a cue for feeding or egg-laying (Ehrlich and Raven 1964; Bernays and Graham 1988; Thompson and Pellmyr 1991; Renwick and Chew 1994; Futuyma and Agrawal 2009). Butterflies are particularly susceptible to evolutionary traps imposed by invasive plants, which may share the stimulants or lack the deterrents necessary for determining suitable hosts (Graves and Shapiro 2003; Schlaepfer et al. 2005; Yoon and Read 2016). For example, at least three of five recognized North American *Pieris* butterfly species have populations involved in invasive plant-based evolutionary traps (Chew 1977b; Chew and Courtney 1991; Keeler and Chew 2008; Nakajima et al. 2013; Davis and Cipollini 2014).

In many cases, the formation of butterfly-host plant evolutionary traps has been followed by rapid shifts in female preference, larval performance or both (Agosta 2006; Keeler and Chew 2008; Singer and McBride 2010). In others, however, apparently maladaptive recognition of novel host plants persists (Nakajima et al. 2013; Brown et al. 2017). Here, we evaluate the role of cue similarity in maintaining persistent

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maladaptive oviposition by a native North American butterfly species on a lethal, nonnative host plant. We predicted that there would be overlap in the composition and strength of cue-sets of native and nonnative host plants, that dominant components of the cue-set of the nonnative host would contribute to egg-laying decisions, and that preference for dominant cues and for the nonnative host would be correlated with preference for native hosts with similar cue sets.

3.2 Study System

Our study focused on a population of *Pieris macdunnoughii* (formerly *Pieris napi macdunnoughii* Remington) (Chew and Watt 2006) butterflies in the East River Valley near the Rocky Mountain Biological Laboratory in Gothic, CO, USA (38°57'33.0"N 106°59'23.0"W, 2900m a.s.l.). Like most pierine butterflies (Pieridae; Pierinae; Renwick 2002; Braby and Trueman 2006; Wheat et al. 2007), *P. macdunnoughii* recognizes and oviposits exclusively on mustards (Brassicaceae; Chew 1977b). Ovipositing females accept a range of native and nonnative plants, including the invasive Eurasian species *Thlaspi arvense* (Chew 1977a; Rodman and Chew 1980). Females in the East River Valley population generally lay more eggs on highly favorable native hosts, but some butterflies still lay many of their eggs on *T. arvense*. This variation in preference for plants is heritable and sex-linked within invaded populations (Steward, RA, Epanchin-Niell, RS, Boggs, CL, unpubl.). However, *T. arvense* is completely lethal to *P. macdunnoughii* larvae: no larvae raised on a diet of *T. arvense* in the lab or field survive past the pupal stage (Chew 1975; Nakajima et al. 2013; Steward et al. 2019; Boggs & Wiklund, unpubl.). Co-occurrence with *T. arvense*

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in one field setting has been estimated to reduce fitness of ovipositing females by around 3% based on larval survival, habitat occupancy by females, and the distribution and abundance of the lethal nonnative in relation to other hosts (Nakajima et al. 2013; Nakajima and Boggs 2015). This represents a significant selection pressure favoring escape from this trap. However, no adaptive response has occurred in the half century since this evolutionary trap was first recorded (Chew 1975, 1977a) and up to a century and a half since the plant first invaded the Rocky Mountains (Best and Mcintyre 1975).

Oviposition by *Pieris* butterflies is largely stimulated by the presence of glucosinolates (GSLs; Huang and Renwick 1993, 1994; Huang et al. 1994; Du et al. 1995). These defensive chemicals are stored in the leaves until damage brings them into contact with myrosinase enzymes, which catalyze degeneration into toxic products such as isothiocyanates, and in the presence of additional specifier proteins into thiocyanates, nitriles, and epithionitriles (Halkier and Gershenzon 2006). Similar GSL structures occur in *T. arvense* and native host plants, but the extent of overlap and the relative concentrations of cue sets are unclear. The GSL profile of *T. arvense* appears to be very simple: only three unique GSL structures have previously been identified in the leaf tissues and sinigrin (allyl- or 2-propenyl GSL) is by far the most dominant (Rodman and Chew 1980; Tolrà et al. 2006; Kuchernig et al. 2011). Each of the two primary native hosts of *P. macdunnoughii*, *Cardamine cordifolia* and *Descurainia incana*, produce GSLs, although only *D. incana* also produces sinigrin (Rodman and Chew 1980; Louda and Rodman 1983; Humphrey et al. 2018).

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3.3 Methods

Our study consists of several linked components. We confirmed the GSL profiles of *T. arvense* and native hosts. We tested the effect of sinigrin on female butterfly preference by comparing preference for sinigrin (a) against other commercially available GSLs, (b) at different concentrations and (c) in the presence of myrosinase. Finally, to test whether decreased preference for *T. arvense* is correlated with decreased preference for *D. incana* and that this is related to an overall decreased preference for the sinigrin cue, we performed a multi-assay cross-over experiment (Figure 3.1). We tested the preference for sinigrin of wild-caught butterflies, which were then transitioned onto assays testing preference for a sinigrin-containing plant (either *D. incana* or *T. arvense*) over *C. cordifolia*, a host that does not produced sinigrin. Butterflies were randomly assigned to the *D. incana* or *T. arvense* assay first, after which they were moved to the other plant assay.

3.3.1 Host plant chemistry

In 2015 and 2017, we collected fresh leaf samples from each of the focal host plants (*T. arvense*, *C. cordifolia* and *D. incana*) to confirm the glucosinolate profiles. Collection sites differed in 2015, but all samples in 2017 were collected from Gothic, CO (N 38.957, W106.989). The fifth, sixth or seventh leaf from the apical meristem of 5-15 plants of each species was collected directly into screw-cap microcentrifuge tubes containing 70% MeOH (Table C.1). Leaf samples were kept in a cool, dark location for 8-16 months, which allowed GSLs to leach into the surrounding MeOH.

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Glucosinolates in the MeOH extracts were desulfated following Prasad et al. (2012) and Keith and Mitchell-Olds (2017). Briefly, Sephadex columns (DEAE 25) were prepared with 50 µL 1 mM Progoitrin [2(R)-Hydroxy-3-butenyl GSL] analytical reference standard (ChromaDex, Inc., Irvine, CA, USA). Samples were added to the columns and washed twice each with 70% MeOH and dH2O. Excess liquid was drained from the column, and the samples were incubated with 30 µL sulfatase for at least 12 hours (2.5 mg/mL). Samples were eluted first with 75 µL MeOH followed by 75 µL dH_2O. Eluants were transferred into 200 µL microinserts and left uncovered for 24 hours before storage at 4-5 ºC.

DesulfoGSLs were quantified in the University of South Carolina Mass Spectrometry Center using a Thermo Scientific Ultimate 3000 High Performance Liquid Chromatography (HPLC) system with a 3400RS binary pump. Chromatography was carried out using a Chromegabond WR C18 column (ES Industries; 150 x 2.1 mm, 3 µm particles, 120Å pore size). The mobile phase contained HPLC-grade water and acetonitrile, with a 0.2 mL/min flow rate and the following gradient: 0% acetonitrile (0-3 minutes), ramp to 20% (3-30 minutes), hold at 20% (30-37 minutes), ramp rapidly to 85% (37-44 minutes), return to 0% acetonitrile (44-end). The injection volume of samples was 20 µL. DesulfoGSLs were detected and quantified with an Agilent 1100 G1315B diode array detector (DAD) monitoring absorbance at 229 nm and subsequently with a Thermo Scientific Corona Veo RS charged aerosol detector (CAD). Only desulfoGSLs appearing in both the DAD and CAD output were included. Glucosinolates were identified using positive ion electrospray ionization with a Waters QTof API US quadrupole time-of-flight mass spectrometer. Both mass spectra and

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comparative retention times from the literature (Tolrà et al. 2006; Kusznierewicz et al. 2013; Olsen et al. 2016; Humphrey et al. 2018) were used to identify desulfoGSLs (Table C.1).

We used multiple approaches to compare desulfoGSL quantities between plant species and collection year. First, we evaluated the presence or absence of desulfoGSLs in leaf samples from each plant using binomial generalized mixed models (lme4 package, Bates et al. 2015). Second, we compared log-transformed quantities (μmol dGSL g^{-1} of dry leaf mass) of GSLs detected in the leaf samples using linear mixed models. Third, we compared the subset of sinigrin concentrations in *D. incana* and *T. arvense* in 2015 and 2017. Finally, we used principal component analysis to identify axes of divergence among all the samples.

3.3.2 Oviposition preference assays: isolated glucosinolates

For all preference assays, gravid female butterflies were caught in the field and brought back to the lab where we weighed and fed them and estimated their age based on wing-wear. Wing-wear categories ranged from 0.5 (recently emerged, wings still flexible) to 3.5 (severely worn with portions of the wing margin missing), increasing by 0.5 increments. All butterflies in the lab were fed a 25-30% (v/v) honey-water solution ad-libitum twice daily. Freshly caught females were held at room temperature (17 – 25 ºC) overnight before participating in an assay.

Assays were conducted in an environmental chamber (27-31:17-19 ºC, 16:8 h L:D). Assay arenas consisted of cylindrical clear plastic cages (0.18 m height x 0.15 m diam.) with 1 mm holes punched around the top and damp paper towels lining the floor

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to maintain airflow and humidity (Figure C.1). Oviposition assays using GSL isolates (described below) were conducted using treated filter paper disks. Four Pastilina modeling clay (Sargent Art) bases (1x1.5x1 cm lwh) were placed in a square formation ~ 3 cm apart on the floor of the arena. Filter paper disks (3 cm diam.; Grade 1, Whatman) were treated with GSL or control solutions and placed vertically in each clay base. Eggs laid on each disk were counted, and disks were replaced with freshly treated disks daily. When testing leaves or cut plants, the petioles or stems were placed in flower picks, which were secured vertically to the side of the assay arena using floral wire. Water in the flower picks was refreshed daily, but except in cases of severe wilting, the same cut stems were used through the full three days of the host plant choice assays. Eggs were checked daily and counted at the end of the study.

3.3.2.1 Glucosinolate choice

In 2016, preference for sinigrin (SIN; GSL class = alkenyl) was tested against glucotropaeolin (benzyl GSL; TROP; aromatic) and glucoiberin (3-methylsulfinylpropyl GSL; GIB; aliphatic sulfur-containing side chain), which were the only GSLs previously detected in leaf samples from *T. arvense* in Colorado (Rodman and Chew 1980). Pure isolated glucosinolate potassium salts (Sinigrin hydrate potassium salt: Sigma Aldrich, Milwaukee, WI; Glucotropaeolin potassium salt: ChromaDex, Irvine, CA; Glucoiberin potassium salt: Cerilliant, Round Rock, TX) were used to make 1 mM solutions. Butterflies were provided with three filter paper disks treated with 100 µL of each of the GSL solutions or with a distilled water control, for an average of 1.63 µmol g^{-1} filter paper (based on an average filter paper mass of 61.2

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mg). In addition to chemical oviposition stimulation, butterflies respond to visual stimuli (Traynier 1986; Snell-Rood et al. 2013). We added green food dye (McCormick Culinary Food Color: water, propylene glycol, FD&C Yellow 5, FD&C Blue 1, and propylparaben) to all GSL solutions and the water control (1 mL dye/15 mL solution).

3.3.2.2 Sinigrin Concentration

We compared preference for increasing concentrations of sinigrin. In 2016, wild-caught butterflies (n = 19) were provided with a control disk (water, 0 mM sinigrin, increased to $1x10^{-6}$ in the statistical analysis) and three disks treated with 100 µL of increasing concentrations of sinigrin:1 mM, 10 mM, or 25 mM (1.63, 16.3, or 40.9 µmol sinigrin g^{-1} filter paper). Solutions were prepared and applied as described above. Butterflies were tested up to five consecutive days or until they died. \ We repeated the experiment in 2017 with lab-reared females that mated in an unsupervised cage. When females began to lay eggs, we moved them from the mating cage to individual assay arenas. Based on results from 2016, we excluded the control disk and instead added a fourth sinigrin treatment: 56.4 mM (92.2 µmol sinigrin g^{-1} filter paper).

3.3.2.3 Sinigrin + myrosinase

In the presence of myrosinase enzyme, sinigrin degrades into allyl-isothiocyanate. To test whether female preference is affected by allyl-isothiocyanate, we gave females a choice of sinigrin-treated disks (100 µl 25 mM = 40.9 µmol sinigrin g^{-1} filter paper) with or without myrosinase (Sigma Aldrich, Milwaukee, WI). The sinigrin solution was prepared as described above, including the addition of green dye.

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Disks were treated with equal amounts of sinigrin. Then, 20 μL myrosinase solution (1.667units mL^{-1} KH_2PO_4 buffer) or an equal quantity of buffer without myrosinase was added to the center of the disk and allowed to bleed to the edges. Butterflies were also provided with two controls: buffer with dye and an unaltered filter paper disk. No eggs were laid on the white disk, so this factor level was excluded from analyses.

We did not measure the amount of isothiocyanate produced by the addition of myrosinase. However, we did compare the amount of sinigrin recovered from disks treated with sinigrin and buffer and those treated with sinigrin and myrosinase by preparing the disks, allowing them to dry as we did for the preference assays and placing them into screw-cap microcentrifuge tubes containing 70% MeOH. DesulfoGSLs were quantified as described above (Host Plant Chemistry). We also compared sinigrin content of the GSL solutions of various concentrations.

3.3.3 Oviposition preference assays: Sinigrin rejection and linked host plant preference

We carried out a multi-assay crossover study to evaluate whether decreased preference for sinigrin was related to decreased preference for *T. arvense* and *D. incana*, *P. macdunnoughii*'s native host that produces sinigrin (Figure 3.1).

3.3.3.1 Sinigrin rejection

Butterfly preference for sinigrin was tested using a simultaneous choice assay between *C. cordifolia* leaves painted with either water or a sinigrin solution. We used leaves rather than filter paper disks to identify decreased preference for sinigrin given

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an attractive background glucosinolate profile. Fresh, undamaged (no signs of herbivory or previous oviposition) *C. cordifolia* leaves were taken from the same plant, matched by mass, painted with either 100 μL distilled water or 25 mM sinigrin solution and allowed to dry. We placed sinigrin-treated and control leaves in flower picks secured to the sides of the choice arena (Figure C.1). Butterflies were tested on the sinigrin-rejection assay for at least one day. If they laid eggs during that time period, they transitioned to the host plant choice assays. If they did not, they were held another 24 hours for a maximum of three days at which point all butterflies that did not lay eggs were released back at their collection sites.

3.3.3.2 Preference for sinigrin-containing host plants

Subsequently, butterflies were moved onto a choice assay between cut stems of *C. cordifolia* and a sinigrin-containing mustard: either the native host *D. incana* or the novel trap *T. arvense* (Figure 3.1). Butterflies were provided with flower picks holding cut stems bearing leaves and, in most cases, the apical meristem of each plant. Cut stems are appropriate stand-ins for whole plants when assessing relative oviposition preferences (Friberg and Wiklund 2016). When possible, we matched both species by phenology (e.g. budding, flowering, seeding) and size. Eggs laid on the cut stems were counted daily, but not removed from the plant to reduce the possibility of damaging the leaves. Butterflies were assigned to their first assay (*D. incana* or *T. arvense*) randomly, and after three days were switched onto the alternative assay (Figure 3.1). We recorded the total number of eggs laid on each plant over the three-day period, and these counts were used as the response variables in statistical analyses.

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3.3.4 Statistical analysis

We used a hurdle model approach to compare desulfoGSL quantities between plant species and collection year. First, we performed a qualitative analysis of the glucosinolates in each sample, evaluating the presence or absence of desulfoGSLs using binomial generalized mixed models (*lme4* package; Bates et al. 2015). Second, we compared log-transformed quantities (μmol dGSL g−1 of dry leaf mass) of GSLs detected in the leaf samples using linear mixed models. We also compared the subset of sinigrin concentrations in *D. incana* and *T. arvense* in 2015 and 2017 using linear models. Finally, we used principal component analysis to identify axes of divergence among all the samples (*FactoMineR* package; (Sébastien Lê et al. 2008).

Oviposition preference for GSL solutions was evaluated as the number of eggs laid on available substrates in each assay using negative binomial generalized linear mixed models with butterfly identity (ID) as a random effect (NBGLMM, *lme4* package). Predictors included in full models differed between assays (Table C.5). Models were hierarchically simplified, and we selected the model with the lowest AICc (*MuMin* package; Barton 2018) and BIC (*stats* package; (R Core Team 2018) that did not significantly differ from the next largest model (likelihood ratio test, χ^2 distribution). We performed additional analyses to compare the relative proportions of eggs laid in several of the assays. The probability of laying eggs on sinigrin, glucotropaeolin or glucoiberin solutions was assessed with multinomial models, while relative preference for sinigrin and sinigrin treated with myrosinase were analyzed

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using quasibinomial generalized linear models (QBGLM). These models were hierarchically simplified as described above.

To test the effects of butterfly age (i.e. wing wear) and mass, which are generally autocorrelated, we calculated a mass-wear residual from a polynomial linear regression of mass against log_2-transformed wing wear. Wing wear estimates are often comparable within but not between flight seasons (years), so mass-wear models were fit separately for every assay. The exception was the sinigrin concentration assay in 2017, which was conducted on butterflies reared in the lab. Butterflies mated either their first or second day after eclosion and were transitioned onto choice assays without being weighed. Thus, neither wing wear nor a mass-wear residual were included in the model.

We analyzed sinigrin rejection using NBGLMs with number of eggs laid on the sinigrin-treated leaf as the response variable. The full model included the three-way interaction between total eggs (log-transformed), mass-wear residual and age. We used NBGLMMs to analyze the number of eggs laid on the sinigrin-containing host in the host plant choice assays. The full model included the interaction between total eggs laid (log-transformed), assay (the plants being tested), order (DT or TD) and the proportion of eggs laid on the sinigrin-treated leaf in the sinigrin rejection assay. Because this last was included as a continuous explanatory variable, bounded by 0 and 1, we originally only included butterflies that laid at least 15 eggs in the sinigrin rejection assay. However, because this predictor was eventually dropped from the model, we reduced this threshold to 2 eggs, which did not change the form of the final best fit model. Finally, we tested the correlation in preference between host plants using a BGLM (quasibinomial distribution) with the proportion of eggs laid in the first assay as the

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explanatory variable and the proportion of eggs in the second assay as the response variable.

3.4 Results

3.4.1 Host plant chemistry

Thlaspi arvense had the simplest GSL profile of the three species. Only three known GSLs were detected in any of the samples (Table C.1; Figure 3.2A). Despite this simplicity, *T. arvense* did not have lower GSL concentrations than either of the native hosts. Instead, *D. incana* had the lowest total GSL concentration (two-way ANOVA, $F_{(2,51)} = 5.70$, $P = 5.81 \times 10^{-3}$). Total glucosinolate differences between species were driven by high concentrations of GSLs within dominant classes: alkenyl aliphatic GSLs (class D), such as sinigrin and gluconapin (3-butenyl GSL) in the profiles of both *D. incana* and *T. arvense*, and branched-chain aliphatic GSLs (class C) including glucoputranjivin (1-methylethyl GSL), glucoconringian (isobutyl / 2-methylpropyl GSL) and glucocochlearin (sec-butyl / 1-methylpropyl GSL) in *C. cordifolia* leaves (Table C.3, C.4; Figure 3.2A). Total GSL concentration differed significantly between 2015 and 2017, although while *C. cordifolia* GSLs decreased, both *D. incana* and *T. arvense* had more total GSLs in 2017 (two-way ANOVA, $F_{(2,51)} = 6.98$, $P = 2.09 \times 10^{-3}$; Figure 3.2B). The qualitative and quantitative GSL profile differed among species between years (Table 3.1, C.3). When analyzed by class, rather than GSL compound, the significant effect of species and year persisted (Table C.3, C.4). Comparing between the *T. arvense* and *D. incana* profiles, *T. arvense* had significantly more sinigrin than *D. incana* in both 2015 (Tukey's mult. comp., t-ratio = -6.82, df = 33, $P = 6.06 \times 10^{-7}$;

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Figure 3.2A), and 2017 (Tukey's mult. comp., t-ratio = -4.60, df = 33, P = 3.54×10^{-4}; Figure 3.2A,).

The *C. cordifolia* GSL profile diverged considerably from those of the other two plants. In a principle components analysis, *C cordifolia* samples diverged on a primary axis which accounted for 58.6% of the variance and was primarily driven by aliphatic branched-chain GSLs (Figure 3.2C). PCA axis 2 explained 15.8% of the variance with sinigrin, glucotropaeolin (benzyl GSL), gluconapin and glucobrassicanapin (4-pentenyl GSL) as major contributors.

3.4.2 Oviposition preference assays: Glucosinolate solutions

3.4.2.1 Glucosinolate choice

Butterflies were significantly more attracted to the sinigrin treated disk than to those treated with glucotropaeolin or glucoiberin (NBGLMM, $\chi^2 = 42.6$, d.f. = 2, p = 5.51×10^{-10}; Table 3.2, C.5; Figure3.2A), with butterflies laying an estimated average 72.0% of eggs on sinigrin, 25.2% on glucotropeaolin, and only 2.8% on glucoiberin at average mass-wear values (multinomial GLM, P-value < 0.001; Table 3.2, C.5; Figure 3.2B). While mass-wear residual was not a significant predictor of the number of eggs laid on each disk (Table C.5), it did affect the proportion of eggs laid on each of the GSL treated disks (multinomial model, $\chi^2 = 9.93$, d.f. = 2, P = 6.98×10^{-3}; Table 3.1; Figure 3.2B). Response rates were low: only 14 of the 35 butterflies responding to the GSL cues. Among responding butterflies, none oviposited on the untreated control filter paper disk.

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3.4.2.2 Sinigrin concentration

Preference increased with sinigrin concentration (Table 3.2, C.5; Figure 3.2C, D). Wild-caught butterflies (2016, n = 18) laid almost no eggs were laid on the control and relatively few on the 1mM sinigrin disks (NBGLMM, $\chi^2 = 55.5$, d.f. = 1, P = 9.261×10^{-14}; Figure 3.2C). Above 10mM, preference began to plateau, and females laid similar proportions of eggs on 10 mM and 25 mM sinigrin solutions. The same pattern was observed among (naïve) lab-reared females (2017, n = 11), although there was much more variation in the number of eggs laid by individual females, especially at lower sinigrin concentrations (NBGLMM, $\chi^2 = 4.84$, d.f. = 1, P = 0.0256; Table 3.2, C.5; Figure 3.2D).

3.4.2.3 Sinigrin + myrosinase

Butterflies (n = 18) laid more eggs on sinigrin treated disks than on the water control (NBGLMM, $\chi^2 = 33.5$, d.f. = 2, P = 5.18×10^{-8}; Table 3.2, C.5; Figure 3.2E). Relative to sinigrin disks treated with buffer, adding myrosinase did not affect the number of eggs laid by female butterflies. over the course of 3-5 days, butterflies laid an average of 14–18 eggs on both treatments). Nor was the proportion of eggs laid on each filter paper disk affected by butterfly wing-wear or mass-wear residual, or the presence of myrosinase (BGLM, Table C.6). Generally, butterflies laid an estimated 55.0% (95% CI: 47.5 - 62.3%) of their eggs on the myrosinase treated filter paper (BGLM, Table 3.2).

Compared to pure sinigrin solution or filter paper treated with sinigrin solution and buffer, myrosinase addition decreased the amount of desulfo-sinigrin recovered

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from sinigrin-treated filter paper, although this difference was not significant (LM, $F_{(2,3)}$ = 7.87, P = 0.064; Figure 3.2F). Each disk was treated with 100 µL 25 mM sinigrin solution. We recovered 25.9% (95% CI: 12.3 – 39.5%) less desulfo-GSL from disks with myrosinase (n=2) than disks with just buffer (n=2).

3.4.3 Sinigrin rejection

Among all butterflies (n = 83) there was no significant preference for sinigrin on a *C. cordifolia* leaf background and eggs laid on sinigrin-treated leaves increased proportionally to the total number of eggs laid in the assay (Table C.6; Figure C.2). However, individual butterflies' preferences ranged broadly: 10.8% of butterflies tested laid 10% or fewer of their eggs on the treated leaf, while 22.9% of butterflies laid at least 90% of their eggs on the sinigrin leaf.

3.4.4 Preference for sinigrin-containing host plants

Butterflies laid more eggs on the first choice test they experienced, regardless of whether they started on the *D. incana* (DT) or *T. arvense* (TD) assay (Table 3.3; Figure 3.4 A, B). Preference in the sinigrin-rejection assay did not affect the number of eggs laid on either sinigrin-containing host (NBGLM, likelihood ratio test, χ^2 = 0.014, df = 1, P = 0.907; Table C.6). However, while TD butterflies had equal preference for the sinigrin-containing host plant and the *C. cordifolia* control in both assays (NBGLM Tukey's multiple comparison, Z-ratio = -2.152, P = 0.137; Table C.7, Figure 3.4B), DT butterflies tended to prefer *D. incana*, but then laid significantly fewer eggs on *T.*

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arvense than on *C. cordifolia* in the second assay (Tukey's multiple comparison, Z-ratio = 4.178, $P < 0.001$; Table C.7; Figure 3.4A).

It is unlikely these differences were caused by differences in egg loads between DT and TD butterflies. Overall, DT and TD butterflies had laid the same number of eggs in in the sinigrin-rejection assay (LM, $F_{(1,77)} = 0.2.99$, $P = 0.088$) and laid similar numbers of eggs in the first assays (LM, $F_{(1,74)} = 0.0187$, $P = 0.892$) and the combined total eggs laid prior to the second assay were the same for both DT and TD butterflies (LM, $F_{(1,77)} = 1.408$, $P = 0.239$).

The proportion of eggs laid on the sinigrin-containing plant in the first assay was not correlated with the proportion of eggs laid on the sinigrin-containing plant in the second assay (QBGLM, analysis of deviance, deviance = 31.1, df = 2, P-value = 0.270; Table C.8; Figure 3.4C, D). There was a loose cluster of eight TD butterflies that laid fewer than 50% of their eggs on both *T. arvense* in the first assay and *D. incana* in the second assay. But, overall the proportion of eggs laid on the sinigrin-containing host plant in the second assay was only significantly affected by assay order (QBGLM, likelihood ratio test, $\chi^2 = 17.3$, df = 1, $P < 0.001$; Table 3.3)

3.5 Discussion

We found similarities between the glucosinolate cues of the lethal invasive mustard *T. arvense* and the native host plant *D. incana*, but these similarities did not constrain *P. macdunnoughii* preference for the two hosts. Both plants produced large quantities of sinigrin and other alkenyl GSLs. In isolation, sinigrin was a major oviposition stimulant. But, we found no evidence of correlated preferences for these

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two host plants, or for sinigrin and either of the host plants. Instead, we observed that butterflies' oviposition experiences modified their preferences. Our results suggest experience and behavioral plasticity play an important role in mediating the fitness consequences of maladaptive host plant preferences.

3.5.1 Cue similarity does not constrain host plant recognition

For the relative costs of host plant mistakes and misses to explain the persistence of the T. arvense trap, our minimum expectation was that low preference for *T. arvense* would be correlated with low preference for *D. incana* (Fox and Lalonde 1993; Mayhew 2001; Doak et al. 2006; Wood et al. 2018). But, individual *P. macdunnoughii* preferences for *T. arvense* and *D. incana* were not related. This result was surprising because contact chemoreception of GSL cues has generally been considered among the most important strategies used by pierine butterflies when evaluating available mustard hosts, especially at small spatial and temporal scales (Renwick and Chew 1994). Previously evidence that *Pieris* species respond differently to certain GSLs supported the assumption that these were primary cues for oviposition decisions. For example, *P. oleracea* females are more attracted to sinigrin and sinigrin-producing plants than *P. rapae* females (Huang et al. 1994; Du et al. 1995). Conspecific *Pieris* females also respond more strongly to certain GSLs than others, both *in plantae* and on abiotic substrates (Ma and Schoonhoven 1973; Traynier and Truscott 1991; Huang and Renwick 1994; Huang et al. 1994; Du et al. 1995; Newton et al. 2010; Rohr et al. 2012).

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Thlaspi arvense's foliar GSL profile is very simple. This is especially evident when compared with model Brassicaceae species, such as *Arabidopsis thaliana* (≥ 25 GSL structures; Fahey et al. 2001) or *Brassica rapa* (≥ 15, ISO 1992), but also compared to the native host plants *C. cordifolia* (we detected 10 structures) and *D. incana* (6). We consistently detected only three known GSL structures in *T. arvense* leaves: sinigrin, gluconapin (butenylGSL) and glucotropaeolin (benzylGSL) over two years of sampling. As in previous studies, (Rodman and Chew 1980; Tolrà et al. 2006; Kuchernig et al. 2011), sinigrin was by far the dominant GSL structure. Sinigrin and alkenyl GSL dominance is a defensive strategy found in other members of the Thlaspideae (e.g. *Alliaria petiolata* Cipollini 2002) and in the genus *Streptanthus* (Thelypodieae, Cacho et al. 2015), but the origin and effectiveness of this strategy against herbivores, especially naïve herbivores, is largely unknown (but see Cacho et al. 2015; Frisch et al. 2015).

Glucosinolate content was extremely different between the two years. This variation may have been caused by differences in abiotic conditions between years or collection locations. Constitutive and induced foliar GSL content is highly sensitive to environmental variation, including light intensity, soil moisture and ambient temperature (Louda and Rodman 1983; Siemens et al. 2012; Tong et al. 2014; Humphrey et al. 2018). The resulting spatial and temporal variation in the relative composition and strength of host plant cues could also limit the evolution of *P. macdunnoughii*'s host plant recognition system (Fox and Lalonde 1993).

When tested in isolation on a filter paper background, we found sinigrin was a stronger oviposition stimulant than aromatic or short sulfur-containing side chain GSLs.

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Stimulatory effects of isolated sinigrin are also well documented for native North American *Pieris* species *P. oleracea* (Huang and Renwick 1994; Du et al. 1995) and *P. virginiensis* (Davis et al. 2015). There is a direct mismatch between preference for sinigrin and performance of larvae. Sinigrin has a distinctly negative effect on feeding larvae, especially at high concentrations (Davis et al. 2015; Steward et al. 2019). For neonate *P. macdunnoughii,* sinigrin deters feeding and significantly increases mortality (Steward et al. 2019).

Yet, sinigrin in host plant leaves is not as important to preference as the filter paper assays implied. When tested on a *C. cordifolia* leaf background, sinigrin had no overall effect on preference, mirroring Davis et al (2015) who found sinigrin added to a native host plant did not affect the average preference of female *P. oleracea* butterflies. Like many herbivorous insects, *Pieris* butterflies may perceive and respond to cue blends differently than they do to each cue in isolation (Bruce and Pickett 2011; Cunningham 2012). We designed the assay with mixtures in mind. In the context of the cue similarity hypothesis, the primary phenotype of interest is rejection of an attractive host plant given the presence of a shared cue. And despite the overall lack of preference for treated or untreated leaves, there was considerable variation among individual preferences for sinigrin, with 15.7 % of responding butterflies laying less than 25% of their eggs on treated leaves. However, this individual variation did not have the preference consequences we expected. Preference for sinigrin was not an important predictor of preference for either sinigrin-producing host plant. So, while sinigrin is clearly contributing to egg-laying decisions, it is not the primary mediator for whether butterflies prefer *T. arvense* or *D. incana* to mustards to that do not produce sinigrin.

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These results, while unexpected in the context of the cue-similarity hypothesis, are supported by recent research showing that preference for *T. arvense* over *C. cordifolia* plants is heritable and sex-linked. When the same assays were repeated using only the methanol-soluble chemical cues extracted from the host plant leaves, the additive genetic variance decreased significantly and the remaining genetic variance was autosomal (Steward, RA, Espanchin-Niell, RS, Boggs, CL, unpubl.). Thus, GSL cues, considered among the most important part of post-alighting host plant assessment by pierine butterflies, may only play a supporting role to other leaf traits, such as nutritional quality (Hwang et al. 2008), gas exchange (but see Langan et al. 2001, 2004), leaf surface waxes (Eigenbrode and Espelie 1995), or water content (Wolfson 1980).

3.5.2 Experience modifies host plant preference

Rather than supporting innate correlated preferences between sinigrin-containing host plants, our results instead suggest that experience is a major determinant of oviposition preference. In the first set of assays, preference for the sinigrin-containing plant over *C. cordifolia* was similar. Generally, butterflies preferred *D. incana* more than they preferred *T. arvense*, but neither was significantly different from equal preference. However, while TD butterflies moved from the *T. arvense* assay onto the *D. incana* assay maintained this roughly equal preference, those that had already experienced the *D. incana* assay (DT) were far less likely to oviposit on *T. arvense*. These results demonstrate that not only do *P. macdunnoughii* females modify

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their preference based on previous experience, but the effect of experience only manifests on certain host plants.

Many lepidopteran species demonstrate behavioral plasticity and associative learning following experience with host plants, using visual and chemosensory cues (Traynier 1984, 1986; Traynier and Truscott 1991; Cunningham et al. 1998; Smallegange et al. 2006; Snell-Rood and Papaj 2009; Snell-Rood et al. 2009; Jones and Agrawal 2017, 2019; Gámez and León 2018). Butterflies also demonstrate biased behavioral plasticity in response to host plants and mates, demonstrating different responses as a result of experiences with different training cues (Westerman et al. 2012; Gamberale-Stille et al. 2019). Gamberale-Still et al. (2019) found *Polygonia c-album* and *Vanessa cardui* butterfly host-searching behavior was primed by prior experience alighting and ovipositing on host plants, but the strength of priming differed among host plants. Stronger responses, especially from *P. c-album* (the lesser generalist of the two), were induced by host plants with which the butterflies had a historically older association, much like *P. macdunnoughii* and native hosts *D. incana* and *C. cordifolia*. Whether behavioral plasticity observed in a lab setting will be adaptive in the wild is difficult to extrapolate (Morand-Ferron 2017; Nieberding et al. 2018). Testing biased behavioral plasticity in natural plant assemblages will be critical to understanding its effects on this evolutionary trap.

If *P. macdunnoughii* butterflies have biased behavioral plasticity in response to previous oviposition experiences, the fitness consequences of *T. arvense* invasion may be less extreme than those previously predicted (Nakajima et al. 2013; Nakajima and Boggs 2015). Nakajima et al. (2013) predicted increased fitness costs associated with

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the relative abundance of *T. arvense*. A second individual-based model determined that both the relative abundance and the proximity of *T. arvense* and native host plant patches would decrease population growth rates (Nakajima and Boggs 2015). In both models, butterflies were assigned fixed preference phenotypes. But, experience-based shifts in preferences are expected to streamline the foraging process and facilitate accurate host-finding based on the abundance of high-quality hosts (Cunningham and West 2008). Given our results, these models likely underestimate the degree to which the relative abundance and distribution of host plants affects fitness costs, because butterflies may be more likely to reject *T. arvense* after laying eggs on native hosts.

Plasticity in host plant preference may account for the persistence of maladaptive egg-laying on *T. arvense* in this population. While plasticity often buffers native species from strong negative selection pressure from invaders, it has the potential to decouple preference phenotypes from underlying genotypic frequencies, thereby dampening phenotypic evolution in response to environmental change (Mery and Kawecki 2004; Ghalambor et al. 2007; Berthon 2015; Murren et al. 2015). Plasticity is most likely to constrain phenotypic evolution when learning or a plastic behavior improves the performance of otherwise less-fit genotypes (Paenke et al. 2007). Furthermore, by decreasing the strength of selection acting directly on preference genotypes, plasticity may amplify the potential impacts of other evolutionary constraints, such as migration selection balance and temporal fluctuations in selection pressures.

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3.6 Acknowledgments

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3.7 Tables

Table 3.1. Analysis of deviance (Wald's χ^2 type II) comparisons of GSL differences between *C. cordifolia*, *D. incana*, and *T. arvense* in 2015 and 2017. The presence of glucosinolate structures was compared using a binomial generalized linear mixed model while log_2-transformed GSL quantities were compared using a linear mixed model.

Response	**Predictor**	χ^2	**d.f.**	**P-value**
Qualitative profile: Presence/absence of GSLs	Plant	34.6	5	<0.001
	GSL	48.0	14	<0.001
	Year	1.04	1	0.308
	Plant x GSL	33.9	24	0.086
	Plant x Year	2.26	2	0.322
	GSL x Year	22.2	12	0.036
Quantitative profile: GSL quantity (µmol g^{-1} dry leaf)	Plant	1.64	2	0.440
	GSL	947	12	<0.001
	Year	25.0	1	<0.001
	Plant x GSL	164	4	<0.001
	Plant x Year	0.048	2	0.976
	GSL x Year	55.8	12	<0.001
	Plant x GSL x Year	12.6	2	0.002

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Table 3.2. Effects of GSL compound (SIN = sinigrin, TROP = glucotropaeolin, GIR = glucoiberin), concentration and myrosinase (MYR) addition on oviposition preference. The negative binomial generalized linear mixed model (NBGLMM), multinomial model and quasibinomial generalized linear model (QBGLM) estimates and standard errors are given on the log, log and logit link scales, respectively.

Assay	**Model**	**Comparison**	**Estimate**	**SE**	**z-ratio**	**P-value**
GSL choice	NBGLMM – Tukey multiple comparison	SIN vs. TROP	1.605	0.392	4.10	<0.001
		SIN vs. GIR	3.509	0.584	6.01	<0.001
		TROP vs. GIR	1.904	0.608	3.13	0.005
	Multinomial model coefficients	SIN – TROP	-0.853	0.212	-5.02	<0.001
		SIN – GIR	-3.068	0.429	-7.15	<0.001
		TROP - GIR	-2.214	0.439	-5.05	<0.001
Sinigrin concentration	NBGLMM – fixed effect	Log_2 Concentration (2016)	0.362	0.071	5.12	<0.001
	NBGLMM – fixed effect	Log_2Concentration (2017)	0.162	0.073	2.23	0.026
Myrosinase	NBGLMM – Tukey multiple comparison	SIN vs. SIN + MYR	-0.224	0.213	-1.05	0.545
		SIN vs. Control	5.839	1.023	5.71	<0.001
		SIN+MYR vs. Control	6.062	1.023	5.92	<0.001
	QBGLM – Tukey multiple comparison	SIN vs. SIN + MYR	-0.202	0.153	-1.32	0.204

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Table 3.3. Coefficient estimates for best fit models evaluating the effects of model predictors on the number of eggs laid on the sinigrin containing host plant (*D. incana* or *T. arvense*) in the choice assays, and the correlation between the proportion of eggs laid on the sinigrin-containing host plant in the first assay and that in the second assay. Assay and order refer to the choice assay (*D. incana* or *T. arvense* tested against the *C. cordifolia* control) and the order in which the butterfly was tested (*D. incana* assay first or *T. arvense* assay first). The negative binomial generalized linear model (NBGLM) and quasibinomial generalized linear model (QBGLM) estimates and standard errors are given on the log and logit link scales, respectively.

Assay	**Model**	**Response**	**Fixed effects**	**Estimate**	**SE**	**z-ratio**	**P-value**
Sinigrin rejection	NBGLM (dispersion = 4.4242)	Eggs	Intercept	-0.239	0.449	-0.532	0.595
			Log_2(Total eggs)	0.902	0.130	6.92	<0.001
Sinigrin host plant preference	NBGLM (dispersion = 1.8367)	Eggs	Intercept	-0.660	0.504	-1.31	0.190
			Log_2(Total eggs)	1.05	0.118	8.92	<0.001
			Assay (T. arvense)	-1.22	0.300	-4.08	<0.001
			Order (TD)	-0.362	0.311	-1.16	0.246
			Assay x Order	1.05	0.458	2.30	0.021
	QBGLM (dispersion = 11.0410)	Prop. Eggs (second assay)	Intercept	-1.51	0.299	-5.07	<0.001
			Order (TD)	1.48	0.374	3.97	<0.001

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3.8 Figures

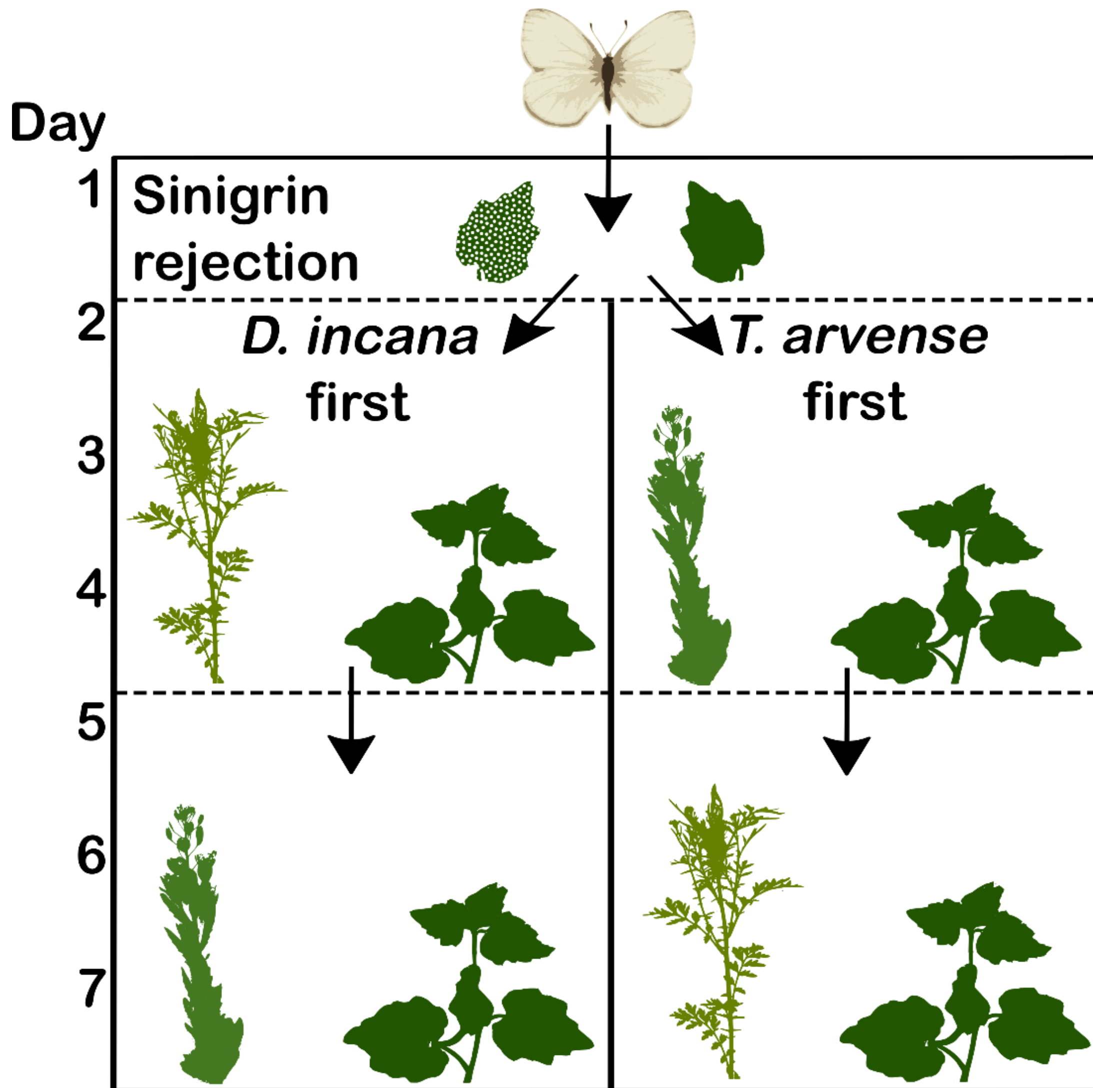

Figure 3.1. Crossover design to test preference for sinigrin, *T. arvense* and *D. incana*. All butterflies were first placed on a one-day simultaneous choice assay between *C. cordifolia* leaves painted with either water or 25 mM sinigrin solution (dotted leaf). Butterflies were then sequentially placed on choice assays between *C. cordifolia* and either *D. incana* or *T. arvense* for three days before being switched onto the second pair.

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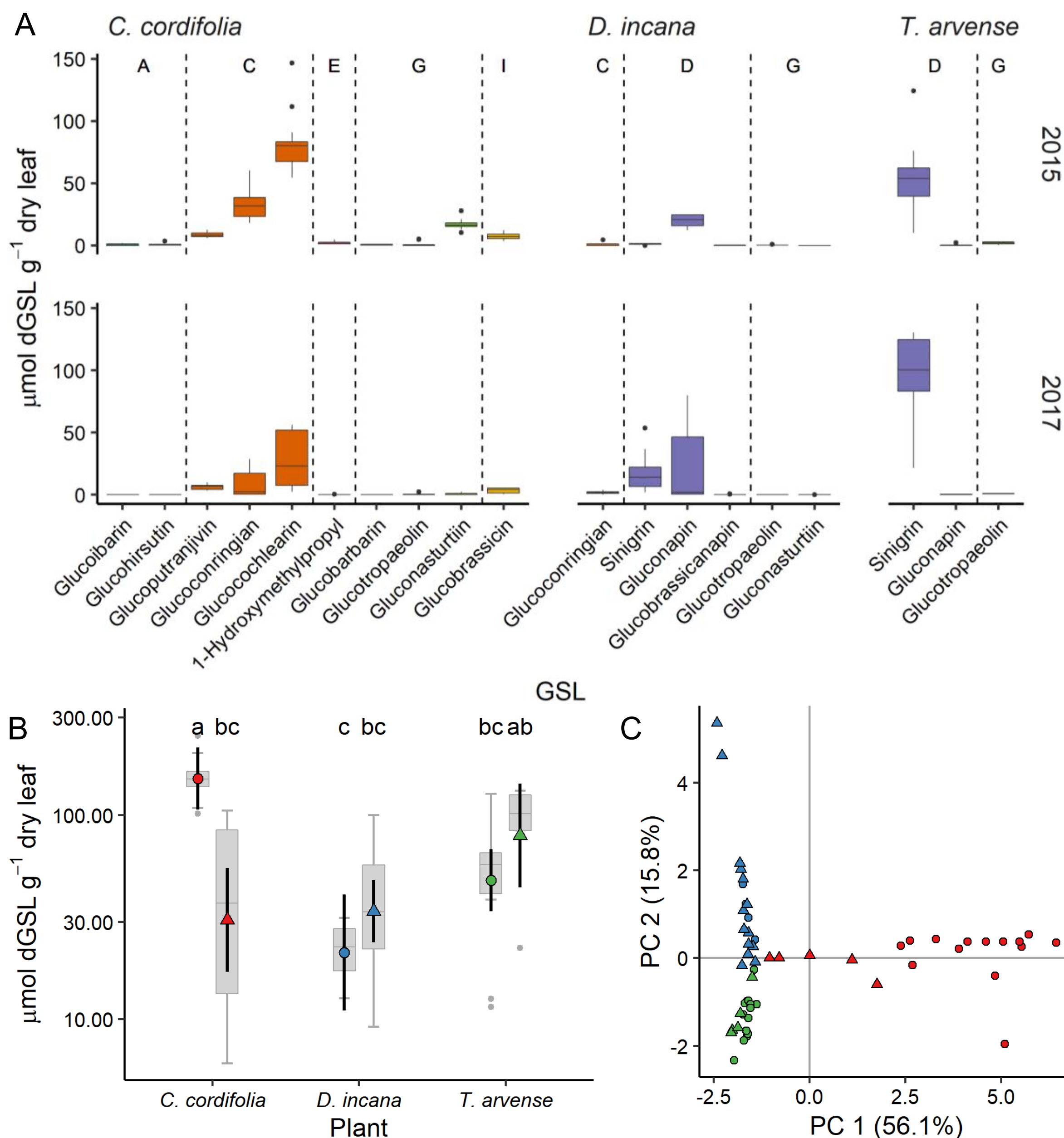

Figure 3.2. (A) Desulfoglucosinolate quantities (µmol dGSL g^{-1} dry leaf) detected by CAD in *C. cordifolia*, *D. incana* and *T. arvense* leaves in 2015 and 2017. Dashed lines delineate different GSL classes: A = sulfur containing side-chain, C (orange) = aliphatic branched chain, D (purple) = olefin/alkenyl, E = aliphatic straight and branched chain alcohols, G = aromatic, I = Indole. (B) Total dGSL quantities (µmol g^{-1} dry leaf) in *C. cordifolia* (red), *D. incana* (blue), and *T. arvense* (green) in 2015 (circles) and 2017 (triangles). Points and error bars represent linear model estimates, while boxplots show distribution of the data. Letters indicate groups that are not significantly different (Tukey's multiple comparisons). (C) Principle component analysis of dGSL profiles. Points represent leaf samples of the three host plants collected in 2015 and 2017. Together, PC1 and PC2 explained 71.9% of the variance in dGSL profiles.

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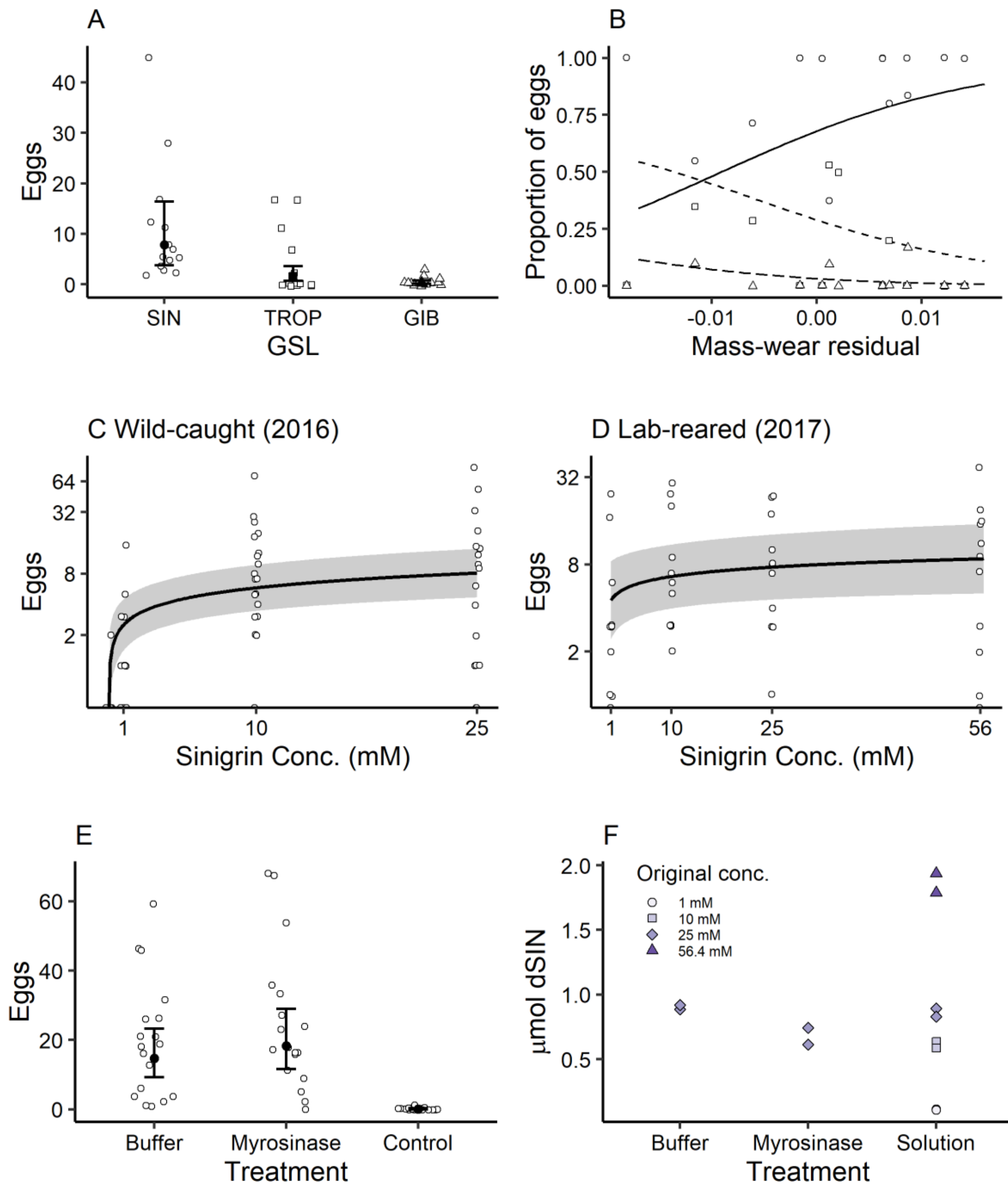

Figure 3.3. Butterfly preference for isolated glucosinolates. (A) Total eggs laid and (B) proportion of eggs laid as a function of age-corrected mass (mass-wear residual) on disks treated with sinigrin (SIN, circles, solid), glucotropaeolin (TROP, squares, short-dash) and glucoiberin (GIB, triangles, long-dash) in the GSL choice assay. Black points (A) represent negative binomial mixed model (NBGLMM) estimates (+/- 95% confidence intervals). Solid and dashed lines (B) represent multinomial model estimates. (C) Total eggs laid on disks treated with increasing concentrations of sinigrin by wild-caught females in 2016, and (D) lab-reared females in 2017. Lines show NBGLMM model estimates (+/- 95% confidence intervals). (E) Total eggs (and

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NBGLMM estimates +/- 95% confidence intervals) laid on filter paper disks treated with 25 mM sinigrin solution and 20µL either KH_2PO_4 buffer or myrosinase (1.67 units/mL), or an untreated control. (F) Desulfo-sinigrin (dSIN, µmol) detected by CAD from solutions differing in sinigrin concentration, or filter paper disks treated with 25 mM sinigrin solution and 20µL either KH_2PO_4 buffer or myrosinase (1.67 units/mL). Original sinigrin concentration affected the amount of desulfo-sinigrin recovered from solutions ($F_{(1,6)} = 511.8$, $P = 4.88 \times 10^{-7}$). There was a marginally nonsignificant difference between 25mM sinigrin solution, buffer and myrosinase ($F_{(2,3)} = 7.87$, $P = 0.064$). When non-myrosinase samples were pooled, however, desulfo-sinigrin in myrosinase treated samples was significantly lower ($F_{(1,4)} = 17.6$, $P = 0.0138$).

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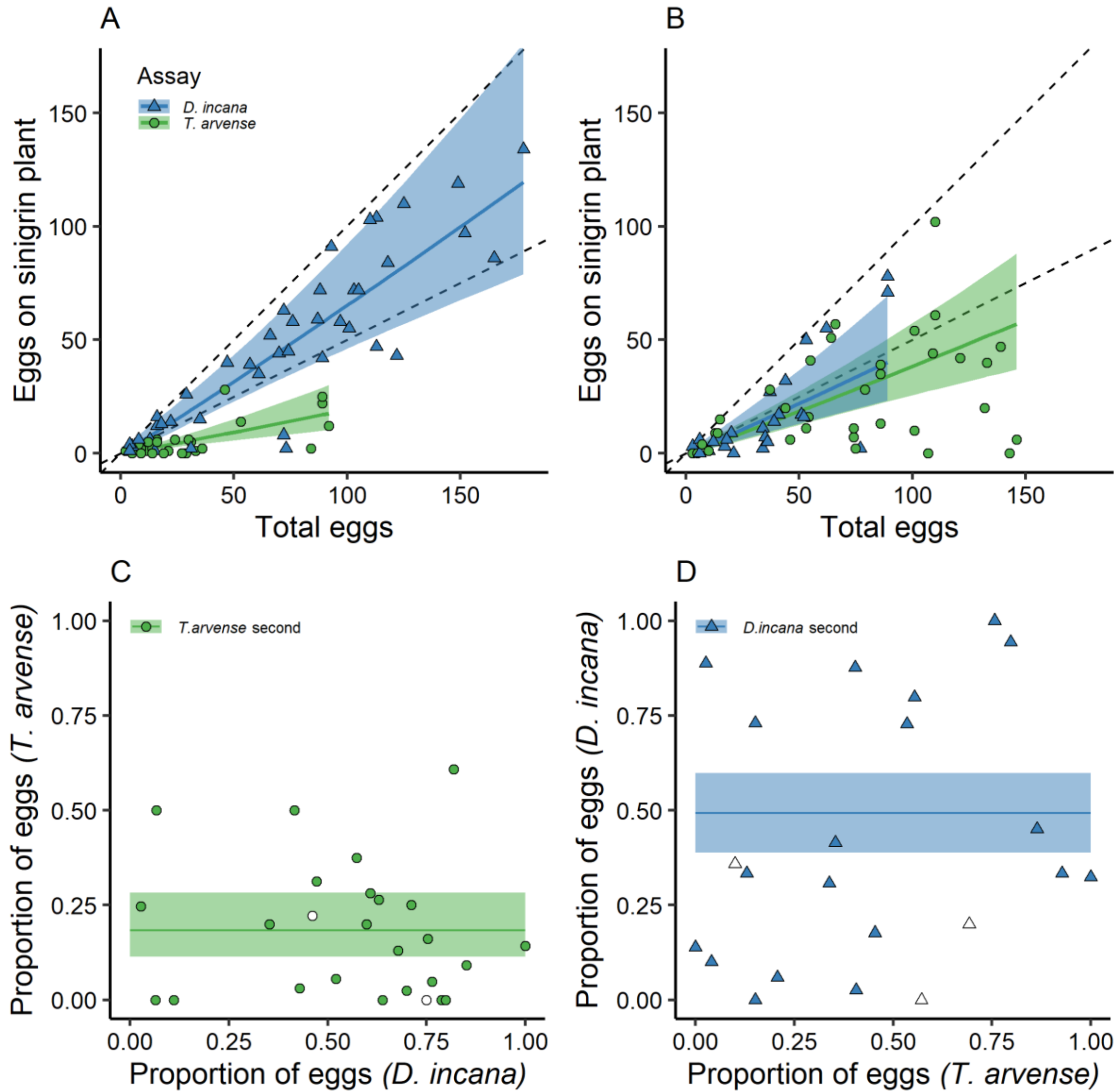

Figure 3.4. Eggs laid on the sinigrin containing host plant (*D. incana* = blue triangles; *T. arvense* = green circles) in simultaneous choice assays by (A) butterflies starting on the *D. incana* assay and (B) those starting on the *T arvense* assay. Dashed lines (slope = 0.5) indicate equal preference for the sinigrin-containing host and the control (C. cordifolia). Solid lines represent negative binomial GLM estimates (± 95% confidence intervals, Table 3.3, C.6). Correlation between the proportion of eggs laid on sinigrin containing host plants by butterflies experiencing (C) *D. incana* first and *T. arvense* second or (D) *T. arvense* first and *D. incana* second. White symbols indicate individuals laying fewer than 15 eggs in the first assay. Solid lines represent quasibinomial GLM model estimates (± 95% confidence intervals, table 3.3, C.8).

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CHAPTER IV:

PRE- AND POST-INGESTIVE DEFENSES AFFECT LARVAL FEEDING ON A LETHAL INVASIVE HOST PLANT[3]

[3] Steward, RA, Fisher, LM, Boggs, CL. 2019. *Entomologia Experimentalis Applicata. 167:292-305.*

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4.1 Introduction

The opportunity for insects to interact with novel non-native plants has increased with shifts in species distribution, whether by range expansion or human-mediated introductions (Morriën et al., 2010; Rasmann et al., 2014). For specialized insects, the consequences of these novel interactions depend heavily on plant chemistry and its role in both host recognition and feeding (Wiklund, 1975; Pearse et al., 2013; Sunny et al., 2015). Host plant-based evolutionary traps arise when non-native plants present cues for host plant recognition while also exhibiting defenses – especially novel chemical defenses – to which the native specialists are vulnerable (Schlaepfer et al., 2002; Casagrande & Dacey, 2007; Verhoeven et al., 2009; Robertson et al., 2013; Yoon & Read, 2016). This vulnerability is often attributed to chemical novelty: invasive plants tend to be well defended, especially against generalists, by chemical defenses not found in the native plant community (Cappuccino & Arnason, 2006; Macel et al., 2014; but see Lind & Parker, 2010).

Whether chemical novelty underlies poor performance in host plant-based evolutionary traps is unclear and may be associated with how the invasive host affects larval feeding patterns. To respond pre-ingestively to a novel food plant, an insect must have the physical and neural anatomy necessary to perceive deterrent cues, and an evolved aversive response to those cues, all of which might be costly to evolve and maintain (Schoonhoven, 1987). Although this does not require deterrents to be currently linked to toxicity in a plant (Bernays & Chapman, 1987; Bernays & Graham, 1988), it does suggest that defensive chemicals acting as pre-ingestive deterrents in introduced plants are unlikely to be evolutionarily novel, or significantly different from those

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encountered in the native plant community (Berenbaum, 1986). Toxicity, affecting larval performance post-ingestively, is more likely to result from chemicals to which native specialist insects are evolutionarily naïve. Given the context of chemical familiarity but poor performance, insect feeding in evolutionary traps might be affected by either deterrents, or toxins, or both pre- and post-ingestive defenses together.

Butterflies are particularly susceptible to evolutionary traps set by invasive plants (Graves & Shapiro, 2003; Schlaepfer et al., 2005; Yoon & Read, 2016). Many butterfly species, especially within populations, use a very narrow range of host plants. Adult females often identify suitable host plants using chemical cues that may be unique to host plants in the historical host plant community, but are shared by related, invasive species (Renwick & Chew, 1994). Furthermore, neonate larvae are largely immobile and especially dependent on the egg-laying choices, or mistakes, of their mothers (Zalucki et al., 2002). In many cases, traps have selected for rapid shifts in female preference, larval performance, or both (Agosta, 2006; Keeler & Chew, 2008; Singer & McBride, 2010). No such shift has occurred for the native North American butterfly, *Pieris macdunnoughii* (Remington) (Lepidoptera: Pieridae) [formerly *Pieris napi macdunnoughii* (Chew & Watt, 2006)]. Females of this species recognize and lay eggs on the invasive Eurasian mustard *Thlaspi arvense* (L.) (Brassicaceae) where they co-occur in the Rocky Mountains of Colorado, USA (Chew, 1975, 1977; Nakajima et al., 2013). *Thlaspi arvense* is completely lethal to *P. macdunnoughii* larvae (Chew, 1975; Nakajima et al., 2013): no larvae reared on a diet of *T. arvense* in the lab or field survived past the pupal stage (Nakajima et al., 2013).

The basis for larval mortality on *T. arvense* is unknown. It has been

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characterized as poorly defended both physically and nutritionally, with low trichome density, high specific leaf area, and a low C:N ratio, all of which are associated with increased palatability to herbivorous insects (Okamura et al., 2016). However, as a mustard, it is well defended chemically.

Like most pierine butterflies, *P. macdunnoughii* oviposits exclusively on mustards (Brassicaceae), and oviposition is largely stimulated by the presence of glucosinolates, defensive secondary metabolites (Huang & Renwick, 1993; Renwick, 2002). Considerable variation in glucosinolate profiles (including the diversity and abundance of different glucosinolate forms) has been observed among individuals, populations, and species of mustards (Fahey et al., 2001; Agerbirk & Olsen, 2012). Pierine larvae have evolved resistance to the toxic products of glucosinolates by rerouting the hydrolysis pathway that typically forms isothiocyanates – or, in the presence of plant specifier proteins, alternative hydrolysis products such as thiocyanates and epithionitriles – in the larval gut to instead form less toxic nitriles that can be excreted (Wittstock et al., 2003; Wheat et al., 2007; Edger et al., 2015). Although pierine butterflies are broadly resistant to glucosinolates, certain glucosinolates have deterrent or toxic properties for particular species (Renwick, 2002).

One of the most remarkable chemical differences between *T. arvense* and *P. macdunnoughii*'s native hosts is the simplicity of the *T. arvense* glucosinolate profile, which is dominated by the aliphatic glucosinolate sinigrin (allyl- or 2-propenyl-glucosinolate) (Rodman & Chew, 1980; RA Steward, unpubl.). Sinigrin is a highly attractive oviposition stimulant to several native North American *Pieris* species (Huang & Renwick, 1994; Du et al., 1995). However, the effect of sinigrin on *Pieris* larval

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performance, and specifically on feeding patterns at various stages of larval development, is unclear (David & Gardiner, 1966; Blau et al., 1978; Olsson & Jonasson, 1994; Renwick & Lopez, 1999; Smallegange et al., 2007; Müller et al., 2010; Santolamazza-Carbone et al., 2014; Davis et al., 2015). Early larval feeding studies using the congener *P. rapae* attributed poor larval performance to alternative sinigrin-derived products including cyanic compounds (Slansky & Feeny, 1977). Biochemical pathways necessary for producing toxic sinigrin derivatives have been identified in *T. arvense* and several other mustards (Kuchernig et al., 2012; Gumz et al., 2015; Frisch et al., 2015), but have not been tested in *P. macdunnoughii*'s native hosts.

Here, we first explored the action of *T. arvense* defenses against neonate *P. macdunnoughii* larvae with the goal of determining whether defenses were inhibiting the onset of feeding (pre-ingestive deterrent properties) or slowing feeding once it had begun (post-ingestive deterrent or toxic properties), compared to larvae on a normal host, *Cardamine cordifolia* (Gray) (Brassicaceae). Second, we compared the probability of dying before and after the onset of feeding in order to understand whether starvation or ingestion of *T. arvense* leaf tissue had greater consequences for neonate mortality. Third, we tested butterfly populations from an invaded and an uninvaded habitat, to see whether there was evidence for population-level differences. Finally, anticipating differences in both abiotic and biotic conditions, we compared feeding on whole plants in the field with laboratory assays on excised leaves to evaluate whether laboratory results effectively captured patterns that might occur in the wild.

Due to its dominance in the *T. arvense* glucosinolate profile, we also hypothesized that sinigrin negatively affects larval feeding on *T. arvense*. Larger

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negative effects of sinigrin addition to *T. arvense* would be preliminary evidence that *P. macdunnoughii* larvae are affected by alternative sinigrin-derived defenses not found in its normal hosts. We first compared the neonate feeding patterns when sinigrin was added to non-native *T. arvense* leaves and those of two native host plants, *C. cordifolia*, which does not naturally produce sinigrin (Rodman & Chew, 1980, Humphrey et al., 2018), and *Descurainia incana* (Bernhardi ex Fischer & Meyer) (Brassicaceae), which naturally produces sinigrin in small quantities (Rodman & Chew, 1980). Second, we monitored survival on treated and untreated leaves over the first 6 days of larval growth.

4.2 Methods

4.2.1 Study system

Thlaspi arvense was likely introduced to the Elk Mountains and Gunnison Basin in Colorado, USA, between the 1850s and 1880s with an influx of miners and ranchers. The plant was already established in the Great Plains of North America, with herbarium records dating back to the early 1800s (reviewed in Warwick et al., 2002). An early successional plant, *T. arvense* rapidly colonizes exposed soil, and is most consistently found in heavily disturbed areas (e.g., construction sites, roadways, recreational trailheads, and meadows open to cattle grazing). It was abundant at Gothic (Gunnison County, CO, USA; 38°57'33.0"N, 106°59'23.0"W; 2 900 m above sea level) in the 1970s, when the *P. macdunnoughii* / *T. arvense* evolutionary trap was first described (Chew, 1975), so populations of *P. macdunnoughii* in the Gunnison basin have been patchily exposed to this lethal nonnative for approximately 45-160 years.

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4.2.2. Butterfly collection and care

In June 2016, we collected adult butterflies from Gothic, where native hosts *C. cordifolia* and *D. incana* and invasive *T. arvense* were sympatric and abundant, and from an uninvaded site 4.5 km to the north, Quigley Creek (38°59'46.9"N, 107°01'05.3"W). In the laboratory, females were stored individually in 15 × 18 cm clear PVC cylinders in a growth chamber, at L16(27-32 °C):D8(16-22 °C) photo-thermoperiod. They were fed twice daily with 25% (vol/vol) honey-water. Females were provided with freshly cut stems of *C. cordifolia* and *T. arvense.* We removed egg-bearing stems from the enclosures and refrigerated (4-7 °C) them for 1-3 days to delay hatching. We sterilized eggs in a weak (<5%) bleach solution and water rinse before transferring them onto strips of parafilm, which were stored in sterile dishes with dampened paper towel. Sterilized eggs were kept in the growth chamber and checked daily for first-instar larvae. In the first two experiments, we tested the offspring of butterflies from both sites, splitting sibling larvae evenly among treatments. In the final experiment, in which sinigrin was added to host plant leaves, we only used offspring from butterflies collected near Gothic.

4.2.3 First-instar feeding metrics

In all laboratory trials, we used three metrics for feeding behavior: larval feeding onset, relative gut fullness, and leaf area consumed. Upon hatching, first-instar *P. macdunnoughii* are translucent. Ingested leaf material is visible as it passes along the fore- and mid-gut, becoming more diffuse upon entering the hind gut and passing to the

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rectum for excretion. To assess larval feeding onset, larvae were categorized based on the absence of leaf material in the gut (empty), presence of leaf material (fed), or they were found dead. For all living larvae that had begun feeding, a relative measure of gut fullness was calculated by dividing the length of the gut contents by the full body length. Gut contents were measured using ImageJ (Schneider et al., 2012; Rasband, 2016) as the length along the dorsal midline from the base of the head to the end of the visible leaf material. Because the gut contents are less distinct upon entering the hind gut, relative gut fullness levels off between 60-70%. This novel approach was used because larval mass is highly variable among newly hatched larvae and may fluctuate independently of feeding (Zalucki et al., 2012). Previous studies have weighed larvae in groups or over longer periods of time (Bowers et al., 1992), but we were able to quantify feeding for individual larvae over several hours.

We calculated change in leaf area for all assays on excised leaves. First instars eat very little, so leaf area is less accurate when detecting feeding differences over short time periods but served as a useful comparison. Leaves were photographed under a Plexiglas window before and after the 6-h laboratory trials. Leaf area was calculated using Easy Leaf Area (Easlon & Bloom, 2014). A solid 1 × 1 cm red square was included in each photo as the calibration area. Photographs were analyzed using the default algorithm, visually inspected for accuracy, and run again using batch-specific settings to account for different light conditions across photographs.

4.2.4 Larval feeding in the laboratory

We compared larval feeding on *T. arvense* with feeding on native host, *C.*

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cordifolia. Plants were transplanted from populations in the Gothic Valley and kept in pots in the laboratory. Newly hatched, unfed larvae were placed individually on entire excised leaves in 45-mm-diameter Petri dishes lined with moist filter paper. We used leaves with no visible signs of previous abiotic or biotic damage. Before and after the trial, each leaf was photographed from a fixed distance alongside the red calibration square (1 cm^2). We photographed larvae using a Leica S6D Greenough stereomicroscope at 2, 4, and 6 h. When not being photographed, larvae were kept in the growth chamber. Larval feeding onset and relative gut fullness were measured as described above. We tested a total of 237 larvae (Table 4.1).

4.2.5 Larval feeding in the field

To evaluate whether our results on excised leaves in the laboratory reflected larval feeding in the field, we placed recently hatched unfed first instars from the laboratory on whole plants of both *T. arvense* and *C. cordifolia* growing interspersed within the same 4 × 4 m patch in Gothic. Larvae were from the same families used in the laboratory assay. Four, five, or six larvae were placed onto each plant, always on the top 6-8 leaves, and the plants were covered with organza bags secured tightly with thread. The entire plant stems were brought into the laboratory after 4 h. Recovered larvae were photographed with the stereomicroscope. We recovered 90.1% of the larvae, and the final sample size was 254 (Table 4.1).

4.2.6 Larval feeding with sinigrin addition

Larval feeding was assayed as described above on excised leaves painted with a

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synthetic sinigrin solution. We tested nonnative *T. arvense* and natives *C. cordifolia* and *D. incana*. Unlike *C. cordifolia*, *D. incana* produces small amounts of sinigrin (~1% of glucosinolates in the leaves; Rodman & Chew, 1980). Entire and undamaged excised leaves were photographed, weighed, and painted either with 0.0564 M sinigrin solution (sinigrin hydrate, >99% purity; Sigma Aldrich, St. Louis, MO, USA) to add 50 μmol g^{-1} of dry leaf mass, as estimated by a standard curve for *T. arvense*, *C. cordifolia*, and *D. incana* (adjusted $R^2 = 0.954$), or with a distilled-water control. We previously determined the concentration of sinigrin in *T. arvense* leaves from populations in the Gothic valley as [mean ± 95% confidence interval (95% CI) =] 53.87 ± 14.82 μmol g^{-1} dry leaf (RA Steward & CL Boggs, unpubl.). Mustard species differ in their distribution of glucosinolates within the leaf and on the leaf surface (Badenes-Pérez et al., 2011), and although our estimates of glucosinolate concentration are based on whole leaves, previous work has shown that sinigrin is found on the leaf surface of *T. arvense* (Griffiths et al., 2001). Painting the leaves with sinigrin solution is unlikely to have replicated *T. arvense* leaf surface encountered by feeding larvae but achieved our goal of exposing larvae to increased amounts of sinigrin.

After the leaf surfaces dried, larvae from within families were evenly assigned to treatments. Larvae were placed individually onto treated or control leaves of one of the three species, stored in dishes in the growth chamber, and photographed with the stereomicroscope at 2, 4, and 6 h. Larval feeding onset and relative gut content was measured as described above. A total of 199 larvae were tested (Table 4.2).

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4.2.7 Larval survival with sinigrin addition

We continued to observe the larvae over the 6 days following the sinigrin addition assay, replacing leaves (treated as described above) every other day. Larvae remained in individual Petri dishes and were kept in the growth chambers under the same conditions as for adults above. Larval survival was assessed every 24 h up to 144 h.

4.2.8 Statistical analysis

Larval feeding in the laboratory was analyzed using a multinomial generalized linear model (GLM) (nnet package; Venables & Ripley, 2002) to identify significant predictors for the probability of larvae being empty, fed, or dead. The full model included a three-way interaction of time (ordered factor), host plant, and population. This model was hierarchically simplified, and nested models were compared using Akaike's information criterion (AIC) and Wald's χ^2 tests (stats package; R Core Team, 2016). The significance of remaining predictors was analyzed with type II ANOVA (car package; Fox & Weisburg, 2011).

Transition probabilities from empty to fed, empty to dead, and dead to fed were compared using multi-state models (msm package; Jackson, 2011). The Q-matrix was constrained to allow the above transitions, or remaining in the empty or fed states, whereas dead was an absorbing state. Time was included as a continuous variable in the model, with the specification that observation times did not represent exact transition times. Significant predictors from the multinomial GLM were included as covariates in the multi-state model. There were few cases of fed larvae dying on *T.* arvense, but none

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on *C. cordifolia.* As this negatively affected confidence interval estimates for *C. cordifolia*, we reran the analysis excluding *T. arvense*, resulting in similar transition estimates as the original model but more confined confidence intervals.

Not all larvae initially distributed onto plants in the field assay were recovered after 4 h. Rates of recovery ranged from 77.2 to 90.2%. We again used a multinomial GLM to compare the proportions of empty, fed, and dead larvae at the 4-h timepoint in the laboratory and field assays. We excluded all larvae tested on the 2nd day of the field assay, when the ambient temperature was much lower in the field due to inclement weather, resulting in delayed feeding onset and reduced relative gut content compared to the other three assay days. The full multinomial model tested the effects of population, host plant, and assay (field or laboratory), and was hierarchically simplified as described for the laboratory assay analysis.

Relative gut fullness was analyzed using a linear mixed model (LMM) (lme4 package; Bates et al., 2015) for all living larvae that that had started to eat (gut fullness >0) from families represented across treatment combinations. The full model included a three-way interaction of time, host plant, and population. To isolate post-ingestive feeding differences, timepoints were adjusted to reflect the time since a larva was last observed empty rather than the time since the beginning of the assay. For example, if a larva first had visible leaf tissue in its gut at 4 h, this was adjusted to 2 h in the model. Family and larva identity were included as random effects. Models were simplified and analyzed as described for the multinomial GLM. Least-squares means (LSM) tests (multivariate method) were used to compare differences among treatment levels. As the data are proportions, we also tested a logit transformation of the relative gut fullness

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variable, but it did not improve the fit. We compared relative gut fullness at the 4-h timepoint (unadjusted) of larvae in the laboratory assay with fed larvae recovered from the field assay using an LMM, with family identity as a random effect. The model was hierarchically simplified and analyzed as above.

For the subset of larvae that began feeding, we also analyzed change in leaf area over the entire laboratory assay. Leaf area was transformed with rank normalization (GenABEL package; Aulchenko et al., 2007) and analyzed with type II ANOVA. These were verified using an in-house script for parametric bootstrapping (1000 repetitions). LSM tests (multivariate method) were used to compare differences among treatment levels.

For the sinigrin addition assay, models were fit and analyzed following the methods described above for larval feeding and relative gut content in the laboratory. The effect of population was excluded because all butterflies were collected from locations near Gothic, and we had previously found no difference between larvae from the populations tested in the laboratory assay. Only a single individual that started feeding died during the first 6 h of the assay, which reduced the ability of the multi-state model to estimate transition probabilities and confidence intervals, so this individual was excluded from that analysis.

Again, we analyzed the leaf area consumed over the entire sinigrin assay. Leaf area was transformed, analyzed with type II ANOVA, and compared between treatments as described above. Larval survival was analyzed using cox mixed effects proportional hazards models (coxme package; Therneau, 2015), evaluating the effect of host plant and sinigrin addition over 6 days, with a random effect of family. Multiple

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comparisons (Tukey method) were conducted to compare survival differences among treatment levels.

4.3 Results

4.3.1 Larval feeding in the laboratory and field

Larvae were 82% as likely to have started eating *T. arvense* as *C. cordifolia* after 2 h, a deficit that did not improve by the end of the assay (Figure 4.1A,B, Tables 4.3 and D.1). After 6 h, only 70.8% of larvae had started eating *T. arvense* compared to 90.6% on the native host. Most larvae that died had not started eating, although two larvae that began feeding on *T. arvense* died by the end of the assay (Figure 4.1A,B). Once feeding had begun, larvae on *T. arvense* leaves also fed significantly more slowly than those on *C. cordifolia* (Figure 4.1E, Tables 4.1, D.3, and D.4). Most larvae eating *C. cordifolia* were able to fill their guts entirely by the 4th hour of feeding: gut fullness did not change significantly between 4 and 6 h (LSM multiple comparison: t-ratio = -1.330, P = 0.75; Table D.4). Relative gut fullness of larvae on *T. arvense*, on the other hand, increased slightly from the 4-h to the 6-h timepoint.

Feeding differences between treatments after 6 h were not detectable using change in leaf area, although leaf consumption was generally higher on the native normal host (rank normalization transformed ANOVA: $F_{1,152} = 3.015$, P = 0.082; Figure D.1).

Differences in larval feeding onset and gut fullness between host plants were also observed in field conditions (Figure 4.1C, D, F, Tables 4.3, D.5, and D.6). The host plant effect was smaller in the field. But larval gut fullness was still greater on *C.*

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cordifolia than on *T. arvense* (LSM multiple comparison: t-ratio = 5.782, P<0.001; Figure 4.1F, Table D.7).

Generally, there were no differences between the Gothic and Quigley Creek populations, apart from onset of larval feeding in the field (Figure 4.1C, D). There was a significant interaction between assay and population: Gothic larvae were less likely to start eating in the field, regardless of host plant (Table 4.3).

4.3.2 Larval feeding with sinigrin addition

Larvae were less likely to start feeding on all leaves treated with sinigrin, whether native or non-native. This effect was only significant for *T. arvense* and *C. cordifolia* on which the addition of sinigrin decreased the onset of feeding by 25-45% at all time points (Figure 4.2A,B, Table 4.4). The effect of sinigrin on *C. cordifolia* was so great that the probability of transitioning from empty to fed was not significantly different between treated leaves of these two host plants (Table D.9). On *D. incana*, over 90% of living larvae had started eating after 2 h, whether leaves were treated with sinigrin or not (Hazard ratio treated: control = 0.886, 95% CI = 0.454-1.727), and by the end of 6 h all living larvae had started feeding (Figure 4.2C).

Mortality among unfed larvae ranged from 0 to 26.5% and was generally higher on sinigrin-treated plants of all species (Figure 4.2A-C). However, larvae were not statistically more likely to die on sinigrin-treated than on control leaves during the first 6 h of the study (Table D.9). These estimates were likely influenced by the lack of any larval death in the control *D. incana* treatment.

Sinigrin addition decreased larval gut fullness on both native host plants but had

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no effect on larval gut fullness on *T. arvense* (Figure 4.2D-F, Tables 4.4 and D.11). For both the sinigrin and control treatments using *T. arvense*, relative gut fullness reached 30% by the 2nd h, and did not change significantly over the next 4 h. On *C. cordifolia*, the addition of sinigrin decreased larval feeding to *T. arvense* levels, and after 2 h larval relative gut fullness on control *T. arvense* leaves and treated *C. cordifolia* leaves was not different (LSM multiple comparison: t-ratio = -1.677, P = 0.79; Table D.11).

Larvae ate the greatest leaf area on *D. incana*, and the smallest on *T. arvense*. Less leaf area was consumed for leaves treated with sinigrin, regardless of host plant. However, neither the effect of host plant nor the effect of sinigrin addition was significant for leaf area consumed (Figure D.2).

4.3.3 Larval survival with sinigrin addition

The addition of sinigrin to leaves of the two native hosts, *C. cordifolia* and *D. incana*, resulted in lower larval survival in comparison to control leaves. The difference in survival between treated and control groups, however, was only significant for *C. cordifolia* (Cox proportional hazards, Tukey multiple comparisons: z = 3.403, P = 0.009). On *T. arvense*, poor survival did not differ between leaves treated with sinigrin and those treated with water (Cox proportional hazards, Tukey multiple comparisons, z = 0.974, P = 0.93; Figure 4.3A, Table D.13). Between the two treatments, only a single larva on *T. arvense* survived to the 6th day of observations.

Larvae fed sinigrin-treated *D. incana* leaves survived at a higher rate than those fed sinigrin-treated *T. arvense* leaves (z = 3.564, P = 0.005; Figure 4.3B, Table D.13). In contrast, the survival of larvae fed sinigrin-treated *C. cordifolia* and *T. arvense* leaves

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was not different (z = 4.468, P<0.001; Figure 4.3C, Table D.13). There was significant variation among families, which was included in the model as a random effect (Table D.12).

4.4 Discussion

We demonstrated that feeding deterrents play a major role in poor performance on this host plant-based evolutionary trap. The distinction between pre- and post-ingestive consequences for *P. macdunnoughii* larvae feeding on *T. arvense* emphasizes a role for both larval chemosensation and gut physiology in evolutionary trap formation, maintenance, or escape. After the first 6 h of exposure to host plants, we found larvae were only 80% as likely to have started feeding on *T. arvense* as on native host *C. cordifolia*. Among larvae that began feeding, those eating *T. arvense* ate more slowly. We also observed that the risk of dying was much higher among unfed than among fed neonate larvae, in both the laboratory and the field. Although toxic post-ingestive effects may be present but masked in our data, our results suggest pre-ingestive deterrence may contribute significantly to poor neonate survival over the first several hours of feeding.

Sinigrin in *T. arvense*'s glucosinolate profile may contribute to pre-ingestive deterrence. Topical addition of sinigrin solution decreased the odds of feeding on all three host plants, at the same time increasing the proportion of dead, unfed larvae. Sinigrin addition also slowed feeding and significantly decreased survival on treated leaves of both native host plants.

The *Pieris* genus is well-known for resistance to sinigrin, and many European

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species are either unaffected by or attracted to high concentrations of sinigrin in the leaves and flowers of their food plants (Blau et al., 1978; Renwick & Lopez, 1999; Smallegange et al., 2007; Santolamazza-Carbone et al., 2016). Nonetheless, *P. macdunnoughii*'s sensitivity to sinigrin also occurs in at least one North American congener. Using a similar experimental design, Davis et al. (2015) tested *Pieris virginiensis* Edwards survival on the leaves of native host *Cardamine diphylla* (Michx.) Alph. Wood (little or no sinigrin) and non-native *Brassica juncea* (L.) Czern. (high sinigrin), treated with sinigrin solution or water. Over the entire larval stage, there was lower survival when feeding on sinigrin-treated leaves of sinigrin-containing non-native *B. juncea.* There was no difference in survival between treated and untreated leaves of the native host. However, this was primarily the result of late-instar mortality on the control (water) treatment. As in our study, there was considerably more neonate mortality on the sinigrin-treated leaves in both treatments. Native *Pieris* larvae may be most sensitive to sinigrin-based defenses in the earliest stages of development.

Davis et al. (2015) suggested their results supported the hypothesis that the non-native, but not the native, plants generate alternative sinigrin-derived toxic metabolites, including hydrogen cyanide, that negatively affected larval feeding and survival (Kuchernig et al., 2012; Frisch et al., 2015; Gumz et al., 2015; van Ohlen et al., 2016). If this pattern is generalizable to sinigrin-dominant Eurasian mustards, we expected to see an increase in post-ingestive effects and mortality when sinigrin was added to *T. arvense*, with little effect on the native plants. Alternative hydrolysis products are likely in *T. arvense* due to the presence of thiocyanate forming protein (TaTFP; Kuchernig et al., 2012). However, although feeding patterns differed across the three host plants in

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our study, these patterns did not suggest the negative consequences of sinigrin were unique to *T. arvense*.

The different consequences for larvae on the three host plant backgrounds emphasize the synergistic roles played by secondary plant chemistry in mediating larval feeding (Gershenzon et al., 2012; Robin et al., 2017). For example, larval feeding on *D. incana* – generally considered to be the preferred and best-quality native host (Chew, 1975; Nakajima et al., 2013) – was least affected by the addition of sinigrin. In both the control and sinigrin-addition groups, all living larvae started feeding by the end of the 6-h assay, suggesting the presence of a feeding stimulant that can overcome any deterrent effects of small quantities of sinigrin in the leaves (Rodman & Chew, 1980). Further experiments manipulating both the leaf surface glucosinolates and those within the leaf tissues would be beneficial to confirm deterrent or stimulant effects.

Our post-ingestive feeding metric of relative gut fullness may be capturing ingestion differences caused by an unwillingness to start feeding. Pre-ingestive deterrents have consequences for both how rapidly larvae begin feeding and the rate at which feeding continues. Differences in gut fullness were not consistently associated with differences in willingness to start eating. Despite minimal pre-ingestive deterrence on sinigrin-treated *D. incana* leaves, there was still a significant difference in gut content between the two treatments after 6 h. These results show that gut fullness reflects feeding consequences for the larvae beyond pre-ingestive deterrence. However, post-ingestive consequences might include activation of sensitivity to additional deterrents. Glendinning (1996) determined that only after ingestion of leaf material did *Manduca sexta* (L.) neonates reject high-nicotine diets. This response happened rapidly,

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within 30 s of feeding onset (Glendinning, 1996). More frequent observation of larvae over a shorter feeding timeline may help in distinguishing the nature of the post-ingestive feeding consequences of both *T. arvense* and sinigrin on its own.

Vulnerability to evolutionary traps is determined by the responses of neonate insects, which can change as juveniles age. Thus, deterrent or toxic effects on neonate insects should not be inferred from feeding tests conducted on older stages. For example, late-stage *P. macdunnoughii* were not vulnerable to *T. arvense* chemical defenses (Chew, 1975). The cardiac glycoside alliarinoside in invasive garlic mustard, *Alliaria petiolata* (M. Bieb.) Cavara & Grande, reduces consumption by *P. oleracea* neonates but has little effect on feeding in the fourth instar. Fourth-instar caterpillars, on the other hand, are susceptible to a flavonoid deterrent, but only on certain diet backgrounds (Haribal & Renwick, 1998; Renwick et al., 2001). Besides direct effects on larval feeding and performance, neonate experiences can shape preference and the ability to shift between host plants. *Pieris rapae* larvae can consume non-host cowpea foliage when transferred as neonates, but after experience feeding on mustard host plants they lose this diet flexibility (Renwick & Lopez, 1999). Such facultative monophagy is common among specialist insects and may be a function of differences in gut gene expression (Celorio-Mancera et al., 2012). Preferential use of late instars may skew our understanding of larval performance on novel plants and limit the opportunity to identify mechanisms underlying performance.

Under certain conditions, insects can rapidly adapt to and escape from evolutionary traps posed by invasive plants, by decreasing preference for or improving performance on the novel resource. For example, after colonizing introduced species

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that supported lower larval survival than native hosts, several populations of *Euphydryas editha* (Boisduval) reverted to their historical host plant associations. Among native insects that have rapidly increased fitness on novel hosts, *E. phaeton* larvae from invaded populations were better able to grow and survive on invasive *Plantago lanceolata* L. than were those from uninvaded populations (Bowers et al., 1992). Congener of *P. macdunnoughii*, *P. oleracea* populations have improved development time and survival on invasive *A. petiolata* in under 20 years (Keeler & Chew, 2008; RA Steward, W Acuna, M Mei, RA Casagrande, FS Chew, unpubl.).

Rapid adaptation by way of improved larval performance does not appear to be an evolutionary option currently available to *P. macdunnoughii* on *T. arvense.* Our results confirmed those of previous studies (Chew, 1975; Nakajima et al., 2013). It is probable we have not captured all variation in the population, but over 45 years of research, no larvae from Gothic townsite or surrounding populations have survived to pupation when fed solely on *T. arvense*. Complete mortality on *T. arvense* prior to adulthood suggests there is little to no fitness variation in *P. macdunnoughii* populations on which selection pressures quantified by Nakajima et al. (2013) can act. Furthermore, we found no evidence for differences between the invaded Gothic and uninvaded Quigley Creek populations. The exception was a significant main effect of population in the field assay, where larvae from Quigley Creek were more likely to have fed than Gothic larvae, regardless of host plant. These population-level differences may have emerged due to the increased environmental variation in the field compared to the lab. Temperatures in the field tended to be lower and more variable. Anecdotally, larvae and adult butterflies from the Gothic population tend to be less hardy than those from other

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populations, and may have been more sensitive to field conditions, explaining why larvae from this population took longer to start feeding on both host plants. Although a comparison of only two populations cannot effectively identify patterns resulting from natural selection, lack of evidence for either faster onset of feeding or increased consumption of *T. arvense* in the Gothic population is consistent with expectations that this population is not improving larval performance on the novel host.

In the face of rapid anthropogenic environmental change, the importance of predicting the eco-evolutionary outcomes of novel insect-plant interactions has been widely acknowledged (Reznick & Ghalambor, 2001; Pearse et al., 2013). Similarly, recent efforts have been made to explain conditions for susceptibility to ecological and evolutionary traps (Sih et al., 2011; Fletcher et al., 2012; Robertson et al., 2013, 2018). The overwhelming focus has been on preference over performance, perhaps because several well-studied evolutionary traps involve novel resources on which fitness cannot improve, such as ovipositing aquatic insects mistaking terrestrial surfaces that reflect polarized light for water (Robertson et al., 2018). In host plant-based evolutionary traps, however, escape through shifts in larval performance is possible, but depends in part on the complexity of plant defenses. Unless susceptibility to active deterrents and toxins is genetically correlated, a combination of defenses that target both physiology and behavior would constrain selection for resistance (Gould, 1984; Bernays & Chapman, 1987; Berenbaum & Zangerl, 1992). Simple two-locus models predict that evolution of insect resistance will take much longer when toxicity is accompanied by feeding deterrents, compared to toxicity alone (Gould, 1984, 1988). Comparisons of larval feeding behaviors – and the plant defenses that mediated them – in persistent and

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escaped traps may reveal patterns of defensive complexity that could be incorporated into a predictive framework for escaping host plant-based evolutionary traps by improving larval performance.

4.5 Acknowledgments

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4.6 Tables

Table 4.1 Initial samples sizes in the laboratory and field assays (N_{init}), the number of *Pieris macdunnoughii* larvae recovered after 4 h in the field ($N_{4h\text{-}rec}$), and the number of empty or fed larvae that were still alive at the end of the 6-h laboratory assay ($N_{6h\text{-}empty}$, $N_{6h\text{-}fed}$) and the 4-h field assay ($N_{4h\text{-}empty}$, $N_{4h\text{-}fed}$), summarized by host plant and population

	Gothic				Quigley Creek			
Host plant	**N_{init}**		**$N_{6h\text{-}empty}$**	**$N_{6h\text{-}fed}$**	**N_{init}**		**$N_{6h\text{-}empty}$**	**$N_{6h\text{-}fed}$**
Thlaspi arvense (lab)	36		7	26	84		22	59
Cardamine cordifolia (lab)	37		2	33	80		10	68
	N_{init}	**$N_{4h\text{-}rec}$**	**$N_{4h\text{-}empty}$**	**$N_{4h\text{-}fed}$**	**N_{init}**	**$N_{4h\text{-}rec}$**	**$N_{4h\text{-}empty}$**	**$N_{4h\text{-}fed}$**
T. arvense (field)	83	71	22	45	79	61	10	51
C. cordifolia (field)	76	64	14	48	82	74	8	65

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Table 4.2 Sinigrin assay initial sample sizes (N_{init}), and the number of living *Pieris macdunnoughii* larvae empty or fed after 6 h ($N_{6h\text{-}empty}$, $N_{6h\text{-}fed}$), summarized by host plant and sinigrin-addition treatment.

	Sinigrin			**Control**		
Host plant	N_{init}	$N_{6h\text{-}empty}$	$N_{6h\text{-}fed}$	N_{init}	$N_{6h\text{-}empty}$	$N_{6h\text{-}fed}$
Thlaspi arvense	40	14	18	29	5	22
Cardamine cordifolia	38	6	19	31	2	26
Descurainia incana	34	0	25	27	0	27

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Table 4.3 ANOVA (Wald's χ^2) of model predictors for multinomial generalized linear models (GLMs) comparing the proportions of empty, dead, and fed larvae (larval feeding) and linear mixed models (LMMs) of relative gut fullness of larvae in the laboratory and field assays.

Assay		Predictor	χ^2	d.f.	P
Larval feeding	Laboratory, multinomial GLM (Figure 4.1A,B, Table D.1)	Host plant	31.926	2	<0.001
		Timepoint	25.287	4	<0.001
	Laboratory vs. field, multinomial GLM (Figure 4.1C,D, Table D.5)	Host plant	13.667	2	0.0011
		Assay	0.164	2	0.92
		Population	5.01	2	0.082
		Assay*population	7.113	2	0.029
Relative gut fullness	Laboratory, LMM (Figure 4.1E, Table D.3)	Host plant	206.274	1	<0.001
		Timepoint (adjusted)	136.237	2	<0.001
		Timepoint (adjusted)*host plant	9.917	2	0.0070
	Laboratory vs. field, LMM (Figure 4.1F, Table D.6)	Host plant	165.027	1	<0.001
		Assay	42.049	1	<0.001
		Host plant*assay	29.117	1	<0.001

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Table 4.4 ANOVA (Wald's χ^2) of final model predictors for multinomial generalized linear models (GLMs) comparing the proportions of empty, dead, and fed larvae (larval feeding) and linear mixed models (LMMs) of relative gut fullness of larvae in the sinigrin addition assay.

Assay		Predictor	χ^2	df	P
Larval feeding	Sinigrin addition, multinomial GLM (Figure 4.2A-C, Table D.8)	Timepoint	31.324	4	<0.001
		Host plant	81.429	4	<0.001
		Sinigrin addition	75.668	2	<0.001
		Host plant*sinigrin addition	12.060	4	0.020
Relative gut fullness	Sinigrin addition, LMM (Figure 4.2D-F, Table D.10)	Timepoint (adjusted)	46.397	2	<0.001
		Host plant	100.548	2	<0.001
		Sinigrin addition	16.800	1	<0.001
		Host plant*sinigrin addition	11.551	2	0.0031

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4.7 Figures

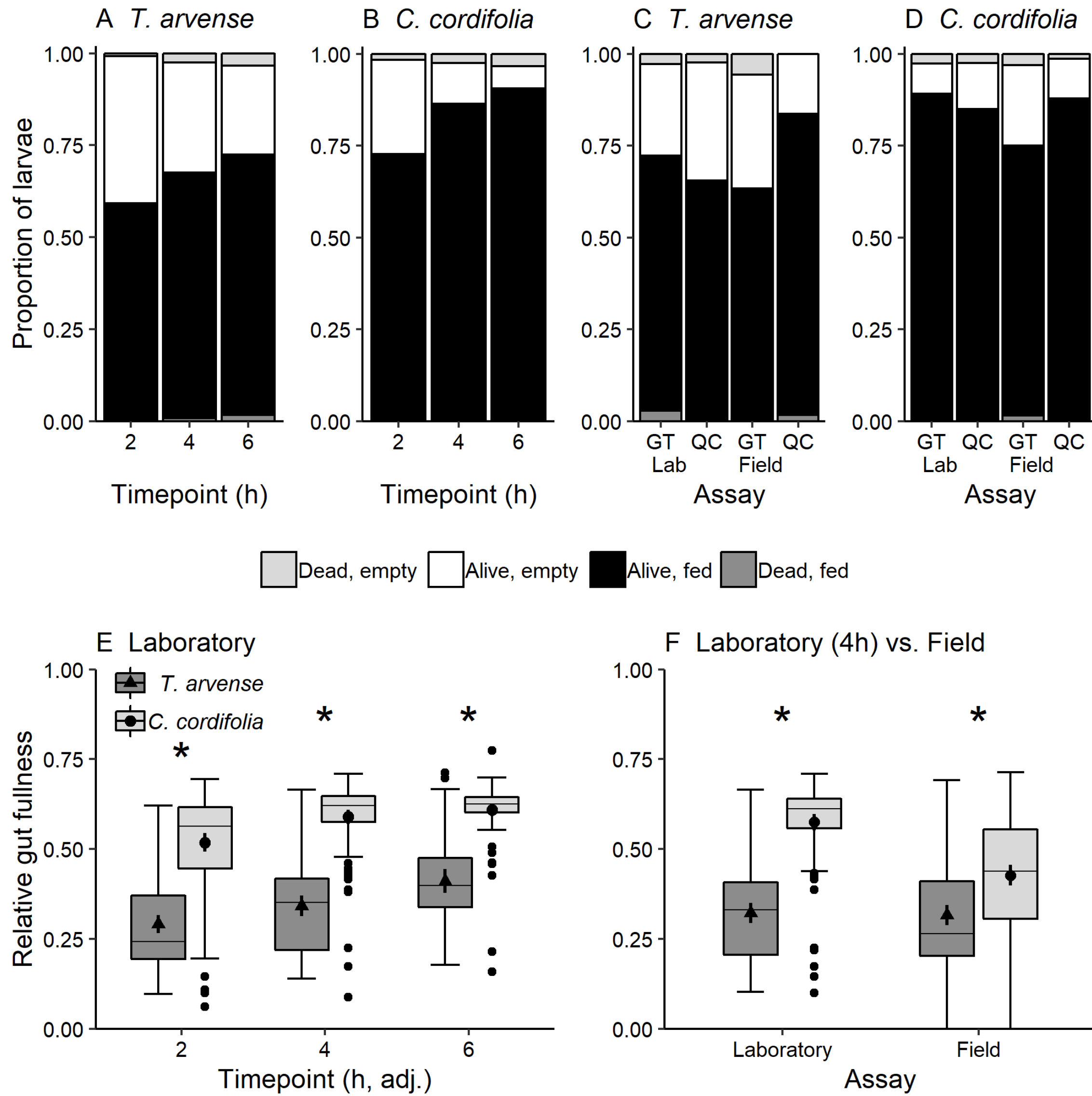

Figure 4.1 Proportion of *Pieris macdunnoughii* larvae feeding on *Thlaspi arvense* and *Cardamine cordifolia* (A,B) after 2, 4, and 6 h in the laboratory and (C,D) in the field. Differences in larval feeding between Gothic (GT) and Quigley Creek (QC) larvae were only found in the field. Relative gut fullness (mean ± 95% confidence intervals; E) after 2, 4, and 6 h in the laboratory and (F) after 4 h in the laboratory vs. field was averaged for larvae that had started eating. Boxes represent the interquartile range (IQR) with a horizontal line at the median and whiskers extending to the largest or smallest observation falling within 1.5 IQRs of the upper or lower quantiles. Asterisks indicate significant differences between *T. arvense* and *C. cordifolia* diets within a timepoint or assay type LSM multiple comparison: $P<0.05$). Timepoints for relative gut fullness measurements in the laboratory assay were adjusted for the onset of feeding.

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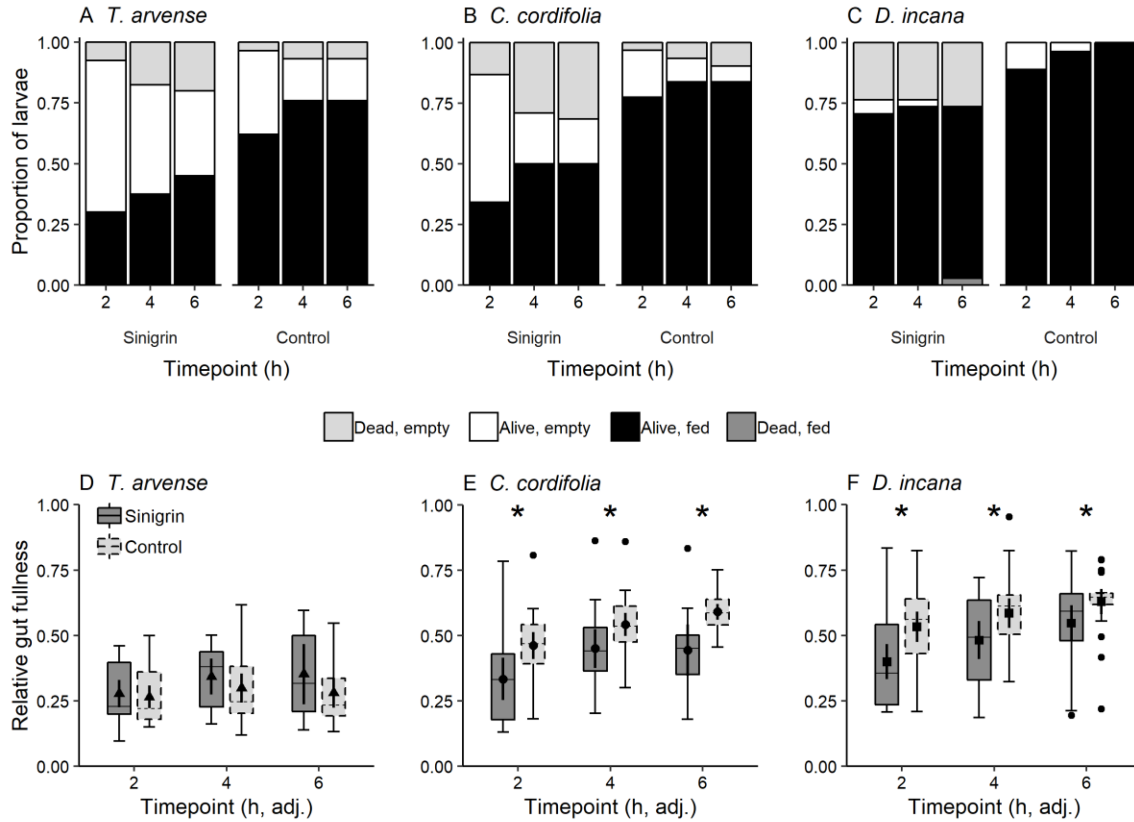

Figure 4.2 Proportion of *Pieris macdunnoughii* larvae after 2, 4, and 6 h (A-C) feeding and (D-F) relative larval gut fullness on leaves of *Thlaspi arvense* (triangles) and native host plants *Cardamine cordifolia* (circles) and *Descurainia incana* (squares) treated with water (dashed line) or sinigrin (50 µmol g^{-1} dry weight; solid line). Error bars represent 95% confidence intervals around the mean relative gut fullness of larvae eating *T. arvense* (triangles) or *C. cordifolia* (circles). Boxes represent the interquartile range (IQR) with a horizontal line at the median and whiskers extending to the largest or smallest observation falling within 1.5 IQRs of the upper or lower quantiles. Outliers appear as black points. Asterisks indicate significant differences between sinigrin and control treatments at each timepoint (LSM multiple comparison: P<0.05). Timepoints for relative gut fullness measurements were adjusted for the onset of feeding.

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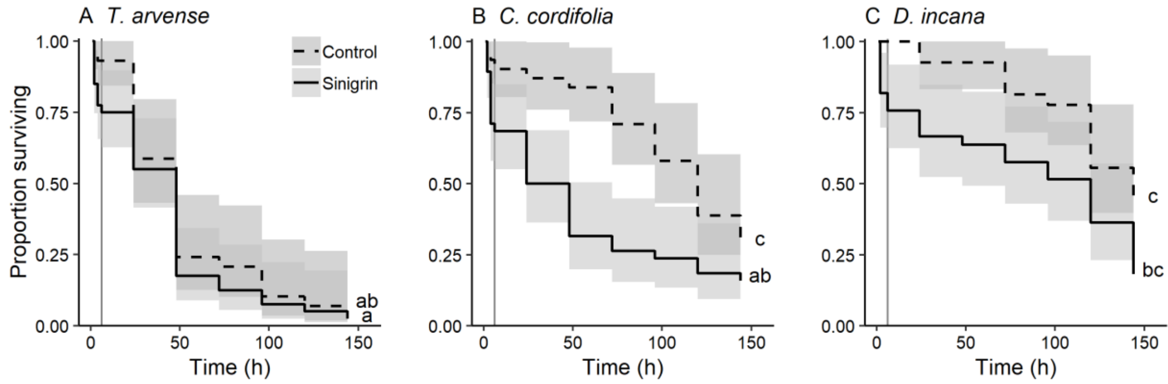

Figure 4.3 Survivorship curves of *Pieris macdunnoughii* larvae reared on cut (A) *Thlaspi arvense*, (B) *Cardamine cordifolia*, and (C) *Descurainia incana* leaves treated with sinigrin (50 µl g^{-1} dry leaf weight; solid line) and distilled water (control; dashed line). Vertical gray lines indicate the end of the 6-h assay and letters indicate significant differences among treatments (Cox proportional hazards, Tukey multiple comparisons: $P<0.05$).

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CHAPTER V:

CONCLUSION

As described in Chapter I, both rapid adaptation and phenotypic plasticity can facilitate escape from resource-based traps via decreased preference or improved exploitation. Yet, previous research on oviposition by *Pieris macdunnoughii* females on the lethal invasive mustard *Thlaspi arvense* has not identified any response – adaptive or plastic – to the presence of the invasive plant. I set out to evaluate potential constraints leading to the persistence of this maladaptive behavior, demonstrating that while preference for the nonnative host over the native host is heritable and varies considerably in the population (Chapter II), it is unlikely that this preference is correlated with preference for native hosts with a similar defense chemical profile (Chapter III). Thus, neither a lack of heritable genetic variation nor an increased risk of excluding good host plants when avoiding *T. arvense* are likely to be constraining escape from this evolutionary trap.

Both Chapter II and III suggest considerable environmental variance associated with oviposition preference, from the lack of any significant additive genetic variance when choosing between plant methanol extracts to modifications in preference after experiencing specific host plants. These results provoke the question, how is behavioral plasticity mediating interactions with the invasive plant in the wild? In chapter III, experience with a set of native hosts clearly decreased subsequent preference for *T.*

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arvense. In the wild, experienced-based preference plasticity will have to arise through trial-and-error sampling of the available resources. Rather than a direct reward as with nectar foraging, 'good' host plants are reinforced by the presence of additional contact cues that strengthen innate preferences. Although chapter III demonstrates modifications to preference are possible, the temporal and spatial scale over which they might occur in the wild are still unclear. It is also uncertain whether this behavioral plasticity might arise due to cue processing or cue sensitivity, whether in restricted sensitivity to the gustatory cues of *T. arvense* or increased sensitivity to other characteristics of the plant.

Consistent with the hypothesis that oviposition experience in the field modifies preference, results from Chapter II and additional preference assays suggest that field-collected butterflies from invaded areas are more likely to lay eggs on *T. arvense*, in contrast to the expectation that selection imposed by the lethal plant would increase female avoidance in these populations. This pattern may be the result of butterflies modifying innate preferences based on plants encountered frequently in the first several days after eclosion. We have also seen that lab-reared butterflies often have much more even preference for *T. arvense* and *C. cordifolia* than do wild-caught butterflies.

Even though phenotypic plasticity can operate as a partial, temporary escape from this evolutionary trap, it can also slow rapid evolution in response to selection. Furthermore, it can interact with other evolutionary constraints to maintain maladaptive genotypes in the population. Chew (1977) was the first to point out that migration of *P. macdunnoughii* from naïve populations into invaded populations may be enough to swamp out the selection pressures introduced by *T. arvense.* The impacts of this

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migration-selection balance would be amplified by preference plasticity. As Nakajima et al (2013, 2015) showed, the fitness consequences of *T. arvense* invasion arise from combination of its abundance, its proximity to native hosts, and the probability that a female butterfly encounters it during early oviposition sampling events. Experience-modified preference would decrease the selection differential within invaded populations and would increase the migration load, the number of maladaptive genotypes introduced by migration (Bolnick & Nosil 2007). Butterflies dispersing from naive areas will be more likely to prefer native plants because all prior oviposition experience has been on native plants. This modified preference would mimic the beneficial phenotype (rejection of *T. arvense*) in the invaded population. However, offspring of these butterflies would be more likely to have the costly phenotype (accepts *T. arvense*). Under equilibrium forces (constant migration, constant selection), the effect of migration with preference plasticity should be equal to migration without this plasticity, but in a system with fluctuating migration and selection, this plasticity could significantly reduce the frequency of the locally beneficial rejection phenotype.

It would be tidy to conclude that sinigrin is the pivotal player in this evolutionary trap. It is at the center of its very own preference-performance mismatch, clearly acting as an oviposition stimulant when in isolation (Chapter III) and compromising larval feeding and survival (Chapter IV). But this is misleading. While sufficient for egg-laying, it is not necessary and does not override other beneficial cues. Nor does preference for sinigrin appear to mediate preference for sinigrin-containing plants. Future research should evaluate the role of non-glucosinolate plant components on both adult preference and larval feeding, and how they work in concert with

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glucosinolate mixtures to mediate interactions with *T. arvense* and other mustard food plants.

In addition to neonate deterrence, the key to larval performance on *T. arvense* may lie in its ability to produce diverse hydrolysis products from its deceptively simple glucosinolate profile. Following hydrolysis by myrosinase, glucosinolates spontaneously rearrange to form isothiocyanates. If the rearrangement is catalyzed by a specifier protein, however, the reaction may alternatively form nitriles (promoted by nitrile specifier protein, NSP), epithionitriles (epithiospecifier protein, ESP, but only for alkenyl glucosinolates, otherwise ESPs just produce nitriles) or thiocyanates (thiocyanate specifier protein, TFP; Lambrix et al. 2001; Wittstock and Burow 2007). While the origin of both NSP and ESP appear to predate the radiation of the core Brassicaceae, TFPs have only been identified in a few species, including *T. arvense*. Of the other two species, both can also be described as specializing on a single glucosinolate. *Lepidium sativum* produces mainly glucotropaeolin (benzylglucosinolate), *Alliaria petiolata*, a member of the Thlaspidae tribe, is dominated by sinigrin (allylglucosinolate). The TFPs in these plants are highly structure specific (Burow et al. 2006; Kuchernig et al. 2011; Eisenschmidt-Bönn et al. 2019), and the thiocyanates they produce can be degraded into additional toxic compounds (Frisch et al. 2015).

The radiation and success of Brassicaceae species has often been attributed to the proliferation of different glucosinolate structures (Edger et al. 2015, 2018). These mustard species present an alternative strategy for success, investing heavily in a single glucosinolate and relying on the alternative hydrolysis products for defense. Further

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exploration of these alternative products will be important to understanding the physiological consequences of consuming *T. arvense* for *P. macdunnoughii* larvae and may also shed light on how defensive novelty can exploit existing deterrent responses and present new physiological hurdles to insect herbivores.

Whatever the basis for poor *P. macdunnoughii* performance, *T. arvense* does not pose problems for all *Pieris* larvae. The northern European congeners *Pieris rapae* and *P. napi* survive perfectly well on *T. arvense* (Forsberg 1987; Friberg et al. 2015). This difference provokes the question, was the ability to eat *T. arvense* gained in the northern European species after the Holarctic expansion of this species complex, or was it lost in the North American species as they began to specialize on suites of North American mustard? Placing maladaptive plant-insect interactions within a phylogenetic framework will help to elucidate how shared evolutionary history with putatively novel plants explain susceptibility to host plant-based evolutionary traps. A first step to answering this question – currently underway – will be to determine the genetic basis for *P. macdunnoughii'*s inability to eat *T. arvense*. With this information, it will be possible to compare allele frequencies, expression levels and associated feeding phenotypes within and between Northern European, Siberian and North American species in the *Pieris napi* species complex. Patterns of conservation or loss of major effect loci may reveal whether the ability to eat *T. arvense* is ancestral and could identify populations that are safeguarded against *T. arvense* invasion by relict polymorphisms for successful development on the invasive plant (Bowden 1979).

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APPENDIX A:

AN INTRODUCTION TO PIERINE SPECIALIZATION ON GLUCOSINOLATES

Glucosinolates are secondary defensive metabolites produced by plants in the order Brassicales and convergently in the distant genus Drypetes (Putranjivaceae, Malpighiales; Rodman et al. 1998; Edger et al. 2015, 2018). Glucosinolates are degraded by myrosinase enzymes – also synthesized and stored by the plants – into 'mustard oils': primarily isothiocyanates, but also thiocyanates and nitriles as mediated by specifier proteins (Agerbirk and Olsen 2011; Kuchernig et al. 2011, 2012; Frisch et al. 2015). Glucosinolate compounds, of which there are more than 130 believed to occur naturally, have a common central thioglucoside structure and a side chain that differs based on its amino acid precursor and various chain elongation steps in the biosynthetic pathway (Agerbirk and Olsen 2011). Side chain differences affect the volatility and toxicity of isothiocyanates. Insect consume glucosinolates in immense quantities when feeding on species in the Brassicales (although the compounds and toxicities vary widely). The putative insecticidal activity of isothiocyanates is disruption of proteins by conjugation to nucleophilic residues (Winde and Wittstock 2011). Nevertheless, there are many specialist and generalist herbivorous insects that feed glucosinolate-producing plants.

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In intact tissues, myrosinases are stored in small pockets. Although glucosinolates are expressed constitutively, mechanical damage to the plant is necessary for glucosinolates to be degraded into toxic isothiocyanates, making it an especially effective defense against chewing insects (Hopkins et al. 2009; Agerbirk and Olsen 2011; Winde and Wittstock 2011). Key innovations for herbivore resistance to the mustard oil bomb have not only allowed the exploitation of a well-defended clade of plants, but in some cases have facilitated adaptive radiations (Wheat et al. 2007; Edger et al. 2015).

The key mechanism that allowed pierine butterflies to transition from their ancestral host plants in the order Fabales onto glucosinolate-producing Brassicales targeted the myrosinase-dependent hydrolysis of glucosinolates (Wittstock et al. 2004; Wheat et al. 2007; Edger et al. 2015). Following hydrolysis by myrosinase, aglycones are rerouted to form nitriles by a protein expressed in the larval midgut: nitrile specifier protein (NSP; Wittstock et al. 2003). Subsequent studies have found additional downstream steps that help larvae convert and excrete glucosinolate hydrolysis products and that the efficacy of NSP resistance varies across individuals and species of host plants (Burow et al. 2006; Agerbirk et al. 2007; Stauber et al. 2012; van Ohlen et al. 2016).

Despite the NSP innovation, pierine butterflies like P. macdunnoughii struggle to feed on certain mustards (Keeler and Chew 2008; Davis and Cipollini 2014). The basis for larval success might depend on deterrence, toxicity or both (Frisch et al. 2015; Müller et al. 2015; Steward et al. 2019). Both glucosinolates and their hydrolysis products have been found to act as feeding deterrents for different Pieris species

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(Müller et al. 2015; Steward et al. 2019). Unlike adult butterflies, larvae primarily taste with their mouths, using sensilla on the maxillae and epipharynx (Schoonhoven and van Loon 2002). Neurons enervating the medial and lateral sensilla styloconicum – a highly conserved structure on the maxilla – are involved in feeding responses to glucosinolates and their hydrolysis products (Du et al. 1995; van Loon and Schoonhoven 1999; Schoonhoven and van Loon 2002; Müller et al. 2015). Deterrent responses to novel host plant are likely to depend on preadapted receptors, neural processing and cue-response behaviors (Dethier 1980).

Different mustard species may also present novel toxic defenses. For example, toxicity of garlic mustard (A. petiolata) to North American Pieris species is partly due to the presence of glucosinolate-derived cyanogenic glucosides (ex. alliarinoside) (Frisch et al. 2014; Davis et al. 2015). Garlic mustard is also known to produce hydrogen cyanide from allyl-glucosinolate (sinigrin, 2-propenyl glucosinolate), guided by a thiocyanate forming protein (TFP) (Frisch et al. 2015). A similar process occurs in T. arvense (Kuchernig et al. 2011). An efficient detoxification strategy for glucosinolate-derived hydrogen cyanide has been identified in the generalist (relative to other butterflies in the genus) P. rapae (van Ohlen et al. 2016). It is unclear whether the β-cyanoalanine synthase involved in this process are shared with P. napi, or by any of the North American Pieris.

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APPENDIX B:

CHAPTER II SUPPLEMENTARY INFORMATION

B.1 Tables

Table B.1. Collection sites for Thlaspi arvense and Cardamine cordifolia plants used in choice assays.

Year	Plant	Dates	Location	Latitude °N	Longitude °E
1997	C. cordifolia	6/23/1997 6/24/1997 7/19/1997	South Gothic (SG)	38.955442	-106.985915
		8/17/1997	Schofield Pass	39.016578	-107.047588
	T. arvense	6/23/1997 6/25/1997	Snodgrass Trailhead	38.919119	-106.960338
		7/19/1997	Kettle Ponds	38.942863	-106.975173
		NA	Sprouted from seeds collected at Gothic, CO	NA	NA
2006	C. cordifolia	Multiple	South Gothic (SG)	38.955442	-106.985915
	T. arvense	Multiple	Snodgrass Trailhead	38.919119	-106.960338
2015	C. cordifolia	6/14/2015	N. Judd Falls Trailhead	38.968579	-106.994234
	T. arvense	6/14/2015	Snodgrass Trailhead	38.919119	-106.960338

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Table B.2. Glucosinolates detected both by diode array detection (DAD) and charged anion detection (CAD). Glucosinolates were identified by mass spectra and comparative retention times.

Num.[a]	Common Name[b]	Chemical name	Class[c]	dMW[d]	RT(minutes) DAD (CAD)
24a	Progoitrin (STD)	2(R)-2-Hydroxy-3-butenyl	D	309	5.2 (5.3)
30	1-Hydroxymethylpropyl	1-(Hydroxymethyl)propyl	E	311	6.2 (6.3)
107	Sinigrin	2-Propenyl	D	279	7.1 (7.2)
56	Glucoputranjivin	1-Methylethyl (isopropyl)	C	281	12.2 (12.3)
12	Gluconapin	3-Butenyl	D	293	13.9 (14.0)
62	Glucoconringian (Isobutyl)	2-Methylpropyl	C	295	15.7 (15.8)
61	Glucocochlearin (sec-Butyl)	1-Methylpropyl	C	295	16.8 (16.9)
40	Glucobarbarin	2(R)-Hydroxy-2-phenylethyl	G	359	17.8 (17.9)
66	Glucoibarin	7-(Methylsulfinyl)heptyl	A	399	19.2 (19.3)
11	Glucotropeaolin	Benzyl	G	329	19.9 (20.0)
43	Glucobrassicin	Indol-3-ylmethyl	I	368	22.4 (22.5)
10	Glucohirsutin	8-(Methylsulfinyl)octyl	A	413	23.7 (23.8)
11	Gluconasturtiin	2-Phenylethyl	G	343	25.9 (26.0)
NA	Unidentified	possible: Cysteine disulfanyl propyl	(A)	432	29.8 (29.9)

a Structure-based number (Fahey et al. 2001)
b (Fahey et al. 2001; Clarke 2010)
c Chemical class: A – Sulfur-containing side-chains; C – Aliphatic, branched chain; D – Olefins; E – Aliphatic alcohols (straight and branched chain); G – Aromatic; I – Indole (Fahey et al. 2001; Clarke 2010)
d desulfoglucosinolate molecular weight

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Table B.3. Hierarchical simplification of environmental and generational variation in 1997. The proportion of eggs laid on T. arvense was square-root transformed. Nested models were compared using corrected AIC and likelihood ratio tests.

Model	AICc	F	df	P-value
Generation * Start day	**3078.4**	**NA**	**NA**	**NA**
Generation + Start day	3165.0	1.981	2	0.143

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Table B.4. Hierarchical simplification of environmental and generational variation in 2006. The proportion of eggs laid on T. arvense was square-root transformed. Nested models were compared using corrected AIC and likelihood ratio tests.

Model	AICc	χ^2	df	P-value
Generation * Start day * Paternal latitude	12.94			
Generation * Start day + Generation * Paternal latitude + Start day * Paternal latitude	17.10	8.80	2	0.012
Generation * Start day + Start day * Paternal latitude	12.89	0.31	2	0.855
Generation * Start day + Paternal latitude	**11.54**	**0.89**	**1**	**0.346**
Generation * Start day	11.81	2.47	1	0.116
Generation + Start day	17.14	9.64	2	0.008

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Table B.5. Hierarchical simplification of environmental and generational variation in 2015. The response variable was proportion of eggs laid on T. arvense treated disks, untransformed. Nested models were compared using corrected AIC and likelihood ratio tests.

Model	AICc	χ^2	df	p-value
Generation/Diapause * Start day * Paternal latitude	-94.27			
Generation/Diapause * Start day + Generation/Diapause * Paternal latitude + Generation * Start day * Paternal latitude	-97.49	1.90	2	0.387
Generation/Diapause * Paternal latitude + Generation * Start day * Paternal latitude	-102.06	0.43	2	0.807
Generation/Diapause + Generation * Start day * Paternal latitude	-106.09	0.83	2	0.661
(Generation/Diapause) + Generation * Start day + Generation * Paternal latitude + Start day * Paternal latitude	-110.02	0.82	2	0.665
Generation/Diapause + Generation * Paternal latitude + Start day * Paternal latitude	-114.16	0.49	2	0.781
Generation/Diapause + Start day * Paternal latitude	-117.98	0.70	2	0.704
Generation/Diapause + Start day + Paternal latitude	-119.66	0.53	1	0.459
Generation + Start day + Paternal latitude	**-120.61**	**3.41**	**2**	**0.182**
Generation + Paternal latitude	-120.14	2.61	1	0.106
Start day + Paternal latitude	-119.08	5.79	2	0.055
Generation + Start day	-118.24	4.52	1	0.034

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B.2 Figures

Figure B.1. Wild-caught P. macdunnoughii female with eggs on filter paper treated with MeOH leaf extract in 2015 (credit: C. Cerrilla).

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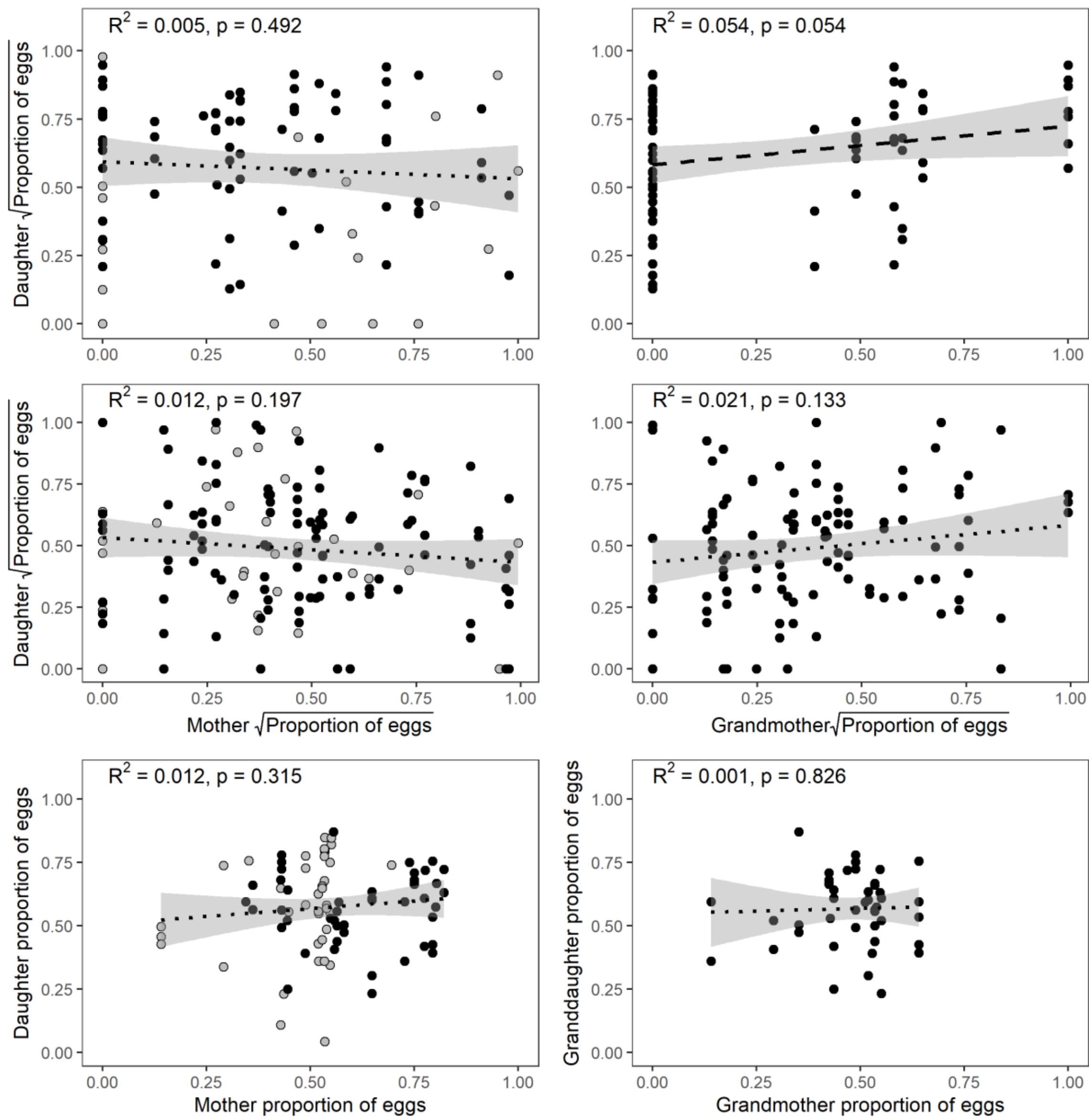

Figure B.2. Correlations between the proportion of eggs laid by daughters and their mothers (A, C, E; Gray circles = F1 daughters, black circles = F2 daughters), and by granddaughters and their paternal grandmothers (B, D, F). Dashed lines represent marginally nonsignificant correlations ($p<0.1$) while dotted lines are nonsignificant. Neither relatedness nor spatial temporal variation found to significantly affect preference were accounted for, so results do not reflect dmm heritability estimates.

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APPENDIX C:

CHAPTER III SUPPLEMENTARY INFORMATION

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C.1 Tables

Table C.1. Diode array detection (DAD) and charged anion detection (CAD) retention times (RT) of glucosinolates (GSLs) found in *Cardamine cordifolia*, *Descurainia incana* and *Thlaspi arvense* leaf samples.

Num[a]	GSL common name[b]	GSL structural name	Class[c]	desulfoMW[c]	RT(min) DAD (CAD) 2015	RT (min) DAD(CAD) 2017
24a	Progoitrin (STD)	2(R)-2-Hydroxy-3-butenyl	D	309	5.22 (5.33)	3.70 (3.82)
24b	Epiprogroitrin (STD)	2(S)-2-Hydroxy-3-butenyl	D	309	NA	4.27 (4.38)
30	1-Hydroxymethylpropyl	1-(Hydroxymethyl)propyl	E	311	6.18 (6.30)	NA
107	Sinigrin	2-Propenyl	D	279	7.14 (7.24)	4.97 (5.11)
31	Glucoconringiin	2-Hydroxy-2-methylpropyl	E	311	- (10.8)	NA
56	Glucoputranjivin	1-Methylethyl	C	281	12.2 (12.3)	8.79 (8.89)
12	Gluconapin	3-Butenyl	D	293	13.9 (14.0)	11.2(11.4)
62	Glucoconringian (Isobutyl)	2-Methylpropyl	C	295	15.7 (15.8)	13.2 (13.3)
61	Glucocochlearin (sec-Butyl)	1-Methylpropyl	C	295	16.8 (16.9)	14.3 (14.4)
40	Glucobarbarin	2(R)-Hydroxy-2-phenylethyl	G	359	17.8 (17.9)	15.2 (15.3)
66	Glucoibarin	7-(Methylsul®nyl)heptyl	A	399	19.2 (19.3)	16.8 (16.9)
11	Glucotropeaolin	Benzyl	G	329	19.9 (20.0)	17.2 (17.3)
101	Glucobrassicanapin	4-pentenyl	D	307	NA	17.3 (17.4)
43	Glucobrassicin	Indol-3-ylmethyl	I	368	22.4 (22.5)	19.6 (19.7)
10	Glucohirsutin	8-(Methylsulfinyl)octyl	A	413	23.7 (23.3)	21.3 (21.4)
11	Gluconasturtiin	2-Phenylethyl	G	343	25.9 (26.0)	23.3 (23.4)

a Structure-based number (Fahey et al. 2001)
b (Fahey et al. 2001; Clarke 2010)
c Chemical class: A – Sulfur-containing side-chains; C – Aliphatic, branched chain; D – Olefins; E – Aliphatic alcohols (straight and branched chain); G – Aromatic; I – Indole (Fahey et al. 2001; Clarke 2010)
d desulfoglucosinolate molecular weight
NA : none/ not applicable

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Table C.2. Detected glucosinolates and total leaves sampled for chemical analysis in 2015 and 2017.

Plant	GSL	Class	Detected (Total samples)	
			2015	**2017**
C. cordifolia	Glucoibarin	A	9 (15)	3 (5)
C. cordifolia	Glucohirsutin	A	11 (15)	4 (5)
C. cordifolia	Glucoputranjivin	C	14 (15)	5 (5)
C. cordifolia	Glucoconringian	C	14 (15)	4 (5)
C. cordifolia	Glucocochlearin	C	14 (15)	5 (5)
C. cordifolia	1-Hydroxymethylpropyl	E	15 (15)	1 (5)
C. cordifolia	Glucobarbarin	G	11 (15)	2 (5)
C. cordifolia	Glucotropaeolin	G	10 (15)	2 (5)
C. cordifolia	Gluconasturtiin	G	14 (15)	3 (5)
C. cordifolia	Glucobrassicin	I	14 (15)	5 (5)
D. incana	Glucoconringian	C	1 (4)	14 (14)
D. incana	Glucobrassicanapin	D	3 (4)	13 (14)
D. incana	Sinigrin	D	3 (4)	14 (14)
D. incana	Gluconapin	D	4 (4)	14 (14)
D. incana	Glucotropaeolin	G	3 (4)	0 (14)
D. incana	Gluconasturtiin	G	0 (4)	1 (14)
T. arvense	Sinigrin	D	14 (14)	5 (5)
T. arvense	Gluconapin	D	6 (14)	5 (5)
T. arvense	Glucotropaeolin	G	14 (14)	5 (5)

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Table C.3. Hierarchical simplification of binomial generalized linear mixed models (BGLMMs) and linear mixed models (LMMs) evaluating differences in the presence and total quantities of GSL structures and classes in *C. cordifolia*, *D. incana*, and *T. arvense* in 2015 and 2017. Models were compared using AICc, BIC and likelihood ratio tests between nested models.

Model	Response	Fixed effects	Random effects	AICca	BICb	χ^2	d.f.	P-value
BGLMM	Presence/absence of GSL structure	Plant * GSL * Year	Sample ID	367.6	710.8	NA	NA	NA
		Plant * GSL + Plant * Year + GSL * Year	Sample ID	327.0	570.3	17.7	24	0.815
		Plant * GSL + GSL * Year	**Sample ID**	**329.2**	**564.0**	**6.8**	**1**	**0.033**
		Plant * GSL + Plant * Year + GSL * Year	NA	350.1	589.2	25.4	1	<0.001
LMM	Log2[GSL quantity (µmol g-1 dry leaf)]	Plant * GSL * Year	Sample ID	785.8	911.2	NA	NA	NA
		Plant * GSL + Plant * Year + GSL * Year	Sample ID	797.0	916.6	14.0	2	<0.001
		Plant * GSL * Year	**NA**	**782.5**	**905.0**	**7.22**	**1**	**0.007**
BGLMM	Presence/absence of GSL class	Plant * Class * Year	Sample ID	129.4	261.2	NA	NA	NA
		Plant * Class + Plant * Year + Class * Year	Sample ID	104.9	203.0	<0.001	10	<0.999
		Plant * Class + Class * Year	**Sample ID**	**98.4**	**189.6**	**<0.001**	**10**	**<0.999**
		Plant * Class + Year	Sample ID	134.9	208.6	48.0	5	<0.001
		Plant * Class + Class * Year	NA	121.5	209.3	25.5	1	<0.001
LMM	Log2[GSL class quantity (µmol g-1 dry leaf)]	Plant * Class * Year	Sample ID	414.2	481.2	NA	NA	NA
		Plant * Class + Plant * Year + Class * Year	Sample ID	412.2	474.3	0.372	2	0.833
		Plant * Class + Plant * Year	Sample ID	437.6	486.9	40.4	5	<0.001
		Plant * Class + Plant * Year + Class * Year	**NA**	**406.1**	**465.6**	**14.1**	**1**	**<0.001**

a Corrected Akaike's Information Criterion
b Bayesian Information Criterion
NA: none/ not applicable

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Table C.4. Analysis of deviance (Wald's χ^2 type II) and analysis of variance (F-test) comparisons of best fit models (Table C.3) of the presence of different GSL classes and total quantities of GSL classes in *C. cordifolia*, *D. incana*, and *T. arvense* in 2015 and 2017. The presence of glucosinolate compounds was compared using a binomial generalized linear mixed model (GLMMs) while log-transformed GSL quantities were compared using a linear mixed model.

Model	Response	Predictor	χ^2	d.f.	P-value
BGLMM	Presence/absence of GSL class	Plant	0.449	2	0.799
		Class	0.441	6	0.993
		Year	0.038	1	0.844
		Plant * Class	0.0439	10	>0.999
		Class * Year	0.404	5	0.995
			F	**d.f.**	**P-value**
LMM	GSL class quantity (µmol g^{-1} dry leaf)	Plant	183	2,150	<0.001
		Class	190	5,150	<0.001
		Year	32.6	1,150	<0.001
		Plant * Class	11.1	4,150	<0.001
		Plant * Year	18.0	2,150	<0.001
		Class * Year	5.29	5,150	<0.001

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Table C.5. Hierarchical simplification of negative binomial generalized mixed models (NBGLMMs), multinomial models, and quasibinomial generalized linear models (QBGLM) comparing preferences of butterflies for filter paper disks treated with different GSL compounds (1mM solution), increasing sinigrin concentrations in 2016 and 2017, and 25mM sinigrin with or without myrosinase added. Mass-wear residuals and wing wear were included as covariates. Only butterflies laying at least two eggs were included in the analyses. We selected the model that minimized AICc and BIC and was not significantly different from the next largest model.

Assay	Model	Response	Fixed effects	Random effects	AICc	BIC	χ^2	d.f.	P-value
GSL compounds	NBGLMM	Eggs	GSL * mass-wear residual * wing wear	Butterfly ID	213.0	221.8	NA	NA	NA
			GSL * mass-wear residual + GSL * wing wear + mass-wear residual * wing wear	Butterfly ID	205.2	215.3	0.949	2	0.622
			GSL * mass-wear residual + GSL * wing wear	Butterfly ID	202.6	212.9	1.41	1	0.235
			GSL * mass-wear residual + wing wear	Butterfly ID	199.2	209.2	3.75	2	0.154
			GSL * mass-wear residual	Butterfly ID	196.1	205.6	0.152	1	0.697
			GSL + mass-wear residual	**Butterfly ID**	**193.7**	**201.7**	**3.56**	**2**	**0.169**
			GSL	Butterfly ID	190.9	198.0	0.001	1	0.970
			1	Butterfly ID	209.2	213.8	23.3	2	<0.001
			GSL	NA	194.7	200.5	6.32	1	0.012
	Multinomial	Eggs	Mass-wear residual * Wing wear	NA	289.3	315.5	NA	NA	NA
			Mass-wear residual + Wing wear	NA	287.5	307.3	2.53	2	0.282
			Mass-wear residual	**NA**	**287.9**	**301.2**	**4.56**	**2**	**0.100**
			1	NA	297.0	303.7	13.2	2	0.001
Sinigrin concentration (conc.)	NBGLMM	Eggs (2016)	Log$_2$(conc.) * mass-wear residual + Log$_2$(conc.) * wing wear	Butterfly ID	350.5	366.4	NA	NA	NA
			Log$_2$(conc.) * mass-wear residual + wing wear	Butterfly ID	348.9	363.1	0.965	1	0.326
			Log$_2$(conc.) + mass-wear residual + wing wear	Butterfly ID	346.5	358.8	0.039	1	0.843
			Log$_2$(conc.) + wing wear	Butterfly ID	344.2	354.7	0.125	1	0.724
			Log$_2$(conc.)	**Butterfly ID**	**343.7**	**352.2**	**1.77**	**1**	**0.184**
			1	Butterfly ID	394.1	400.6	52.7	1	<0.001
			Log$_2$(conc.)	NA	355.0	361.5	13.61	1	<0.001

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		Eggs (2017)	**Log$_2$(concentration)**	**Butterfly ID**	**281.4**	**287.5**	**NA**	**NA**	**NA**
			1	Butterfly ID	283.1	287.8	4.15	1	0.042
			Log$_2$(concentration)	NA	291.2	296.0	12.3	1	<0.001
Sinigrin + Myrosinase	NBGLMM	Eggs	Treatment * mass-wear residual * wing wear	Butterfly ID	329.0	346.0	NA	NA	NA
			Treatment * mass-wear residual + treatment * wing wear + mass-wear residual * wing wear	Butterfly ID	322.4	338.7	0.638	2	0.727
			Treatment * wing wear + mass-wear residual	Butterfly ID	317.4	331.2	0.483	3	0.214
			Treatment + mass-wear residual + wing wear	Butterfly ID	315.8	327.3	4.03	2	0.134
			Treatment + wing wear	Butterfly ID	313.1	323.3	0.012	1	0.914
			Treatment	**Butterfly ID**	**310.9**	**319.6**	**0.321**	**1**	**0.571**
			1	Butterfly ID	985.1	990.6	679	2	<0.001
			Treatment	NA	312.6	319.7	4.11	1	0.043
	QBGLM	Eggs	Mass-wear residual * wing wear	NA	NA	NA	NA	NA	NA
			Mass-wear residual + wing wear	NA	NA	NA	3.79	1	0.400
			Mass-wear residual	NA	NA	NA	0.029	1	0.941
			1	**NA**	**NA**	**NA**	**0.475**	**1**	**0.921**

NA: none/ not applicable

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Table C.6. Hierarchical simplification of negative binomial generalized linear (mixed) models (NBGLM, NBGLMM) for the number of eggs laid on *C. cordifolia* leaves treated with sinigrin (sinigrin rejection assay) and the number of eggs laid on sinigrin-containing host plants (Assay: *D. incana* or *T. arvense*). We selected the model that minimized AICc and BIC and was not significantly different from the next largest model.

Assay	Model	Response	Fixed effects	Random Effects	AICc	BIC	χ2 (F)	d.f.	P-value
Sinigrin rejection	NBGLM	Eggs	Log2(total eggs) * mass-wear residual * age	NA	552.5	571.8	NA	NA	NA
			Log2(total eggs) * mass-wear residual + log2(total eggs) * age + mass-wear residual * age	NA	554.1	571.5	4.15	1	0.042
			Log2(total eggs) * age + mass-wear residual * age	NA	551.7	567.1	0.022	1	0.883
			Log2(total eggs) + mass-wear residual * age	NA	549.3	562.7	0.027	1	0.869
			Log2(total eggs) + mass-wear residual + age	NA	548.4	559.7	1.348	1	0.246
			Log2(total eggs) + age	NA	546.1	555.2	0.002	1	0.968
			Log2(total eggs)	**NA**	**544.2**	**551.2**	**0.331**	**1**	**0.565**
			1	NA	647.9	652.6	105.9	1	<0.001
Sinigrin host plant preference	NBGLMM	Eggs	Log2(total eggs) * assay * order * prop. eggs (sin rej.)	Order/ butterfly ID	889.5	933.6	NA	NA	NA
			Log2(total eggs) * assay * order * prop. eggs (sin rej.)	Order	886.6	928.9	<0.001	1	>0.999
			Log2(total eggs) * assay * order * prop. eggs (sin rej.)	NA	883.8	924.2	<0.001	1	>0.999
			Log2(total eggs) * assay * order + log2(total eggs) * assay * prop. eggs (sin rej.) + log2(total eggs) * order * prop. eggs (sin rej.) + assay * order * prop. eggs (sin rej.)	NA	882.3	920.7	1.285	1	0.257
			Log2(total eggs) * assay * order + log2(total eggs) * assay * prop. eggs (sin rej.) + assay * order * prop. eggs (sin rej.)	NA	880.0	916.3	0.383	1	0.536
			Log2(total eggs) * assay * order + log2(total eggs) * assay * prop. eggs (sin rej.) + order * prop. eggs (sin rej.)	NA	878.0	912.2	0.606	1	0.436
			Log2(total eggs) * assay * order + log2(total eggs) * assay * prop. eggs (sin rej.)	NA	875.8	907.9	0.423	1	0.515

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Log2(total eggs) * assay * order + log2(total eggs) * prop. eggs (sin rej.) + assay * prop. eggs (sin rej.)	NA	875.4	905.3	2.134	1	0.144
Log2(total eggs) * assay * order + log2(total eggs) * prop. eggs (sin rej.)	NA	873.3	901.0	0.470	1	0.493
Log2(total eggs) * assay * order + prop. eggs (sin rej.)	NA	872.5	897.8	1.600	1	0.206
Log2(total eggs) * assay * order	NA	870.1	893.1	0.014	1	0.907
Log2(total eggs) *assay + log2(total eggs) *order + assay * order	NA	871.9	892.5	4.105	1	0.043
Log2(total eggs) *assay + assay * order	NA	870.8	889.0	1.26	1	0.261
Log2(total eggs) + assay * order	**NA**	**869.8**	**885.5**	**1.29**	**1**	**0.255**
Log2(total eggs) + assay + order	NA	877.1	890.3	9.48	1	0.002

NA: none/ not applicable

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Table C.7. Tukey's multiple comparison of model estimates (Table C.6) of number of eggs laid on sinigrin-containing host plants (*D. incana* or *T. arvense*) in simultaneous choice assays, where butterflies were either tested on *D. incana* first (DT) or *T. arvense* first (TD) before being tested on the second assay. Ratio and standard error (SE) estimates were calculated on the log-scale for the mean number of total eggs laid by butterflies in all assays (58.8 eggs), accounting for dispersion (1.8367). P-values were adjusted using Tukey's method for a family of four estimates.

Contrast	Ratio	SE	z-ratio	P-value	Sig.
D. incana (DT) – *T. arvense* (DT)	3.688	1.152	4.178	<0.001	***
D. incana (DT) – *D. incana* (TD)	1.409	0.442	1.093	0.694	
D. incana (DT) – *T. arvense* (TD)	1.721	0.437	2.140	0.141	
D. incana (TD) – *T. arvense* (DT)	0.382	0.145	-2.543	0.054	•
D. incana (TD) – *T. arvense* (TD)	0.467	0.154	-2.303	0.097	•
T. arvense (DT) – *T. arvense* (TD)	1.221	0.405	0.603	0.931	

NA: none/ not applicable

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Table C.8. Hierarchical simplification of quasibinomial generalized linear (QBGLMs) for the relative proportion of eggs laid on sinigrin containing host plants in the second assay as a function of the proportion laid in the first assay. We selected the simplest model that was not significantly different from the next largest model.

Assay	Model	Response	Fixed effects	Deviance	d.f.	P-value
Sinigrin host plant preference	QBGLMM	Prop. eggs on sinigrin-containing host (second assay)	Order * prop. eggs (first assay) * mass-wear residual	NA	NA	NA
			Order * prop. eggs (first assay) + order * mass-wear residual + prop. eggs (first assay) * mass-wear residual	11.4	2	0.620
			Order * mass-wear residual + prop. eggs (first assay) * mass-wear residual	14.7	2	0.539
			Order * mass-wear residual + prop. eggs (first assay	31.1	2	0.270
			Order * mass-wear residual	18.5	2	0.460
			Order + mass-wear residual	23.2	1	0.162
			Order	**26.7**	**1**	**0.134**
			1	191.4	1	<0.001

NA: none/ not applicable

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C.2 Figures

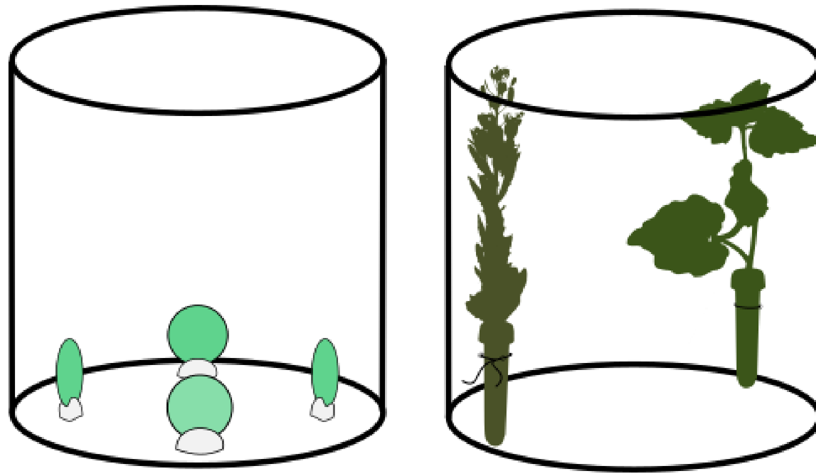

Figure C.1. Set-up of clear plastic oviposition arenas using filter paper disks (right) or whole plants in flower picks.

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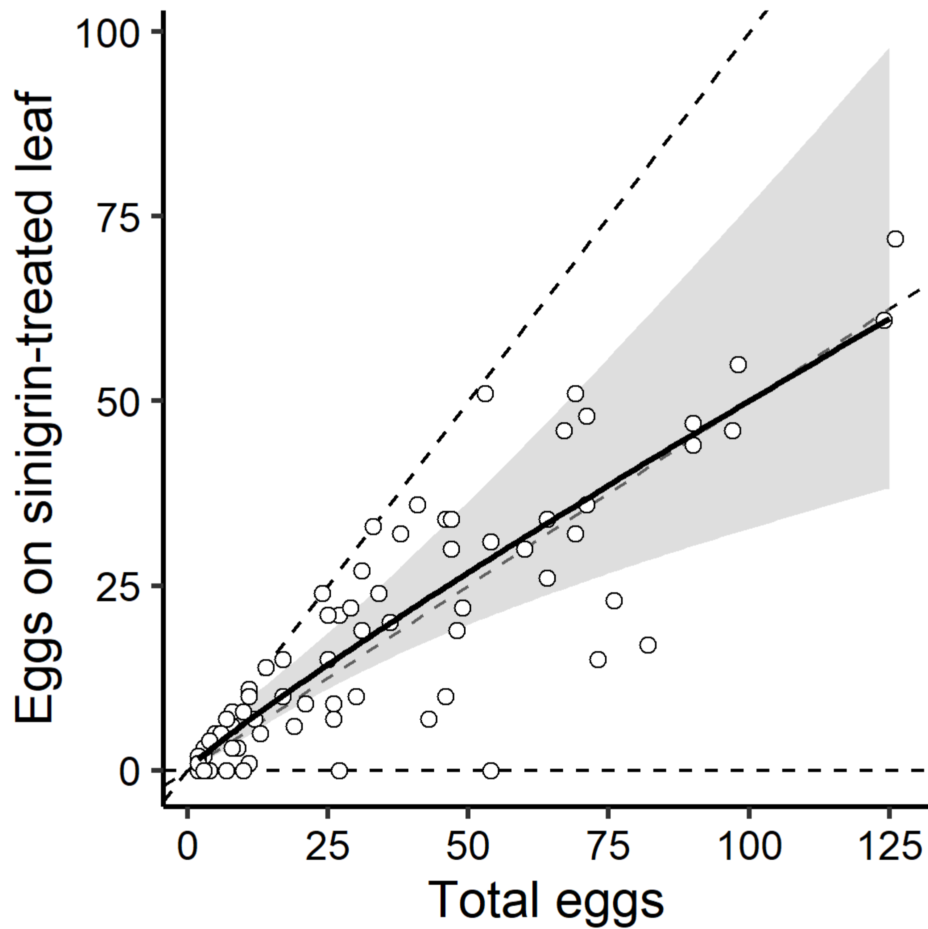

Figure C.2. Butterflies laid equal numbers of eggs on untreated *C. cordifolia* leaves and leaves treated with 100uL of 25mM sinigrin. The number of eggs laid on the sinigrin-treated leaf was unaffected by either age or mass-wear residual of individual butterflies (Table C.6). Dashed lines have a slope of 1 and 0.5, indicating 100% and 50% preference for sinigrin-treated leaves, respectively.

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APPENDIX D:

CHAPTER IV SUPPORTING INFORMATION

D.1 Tables

Table D.1 Hierarchical simplification of multinomial generalized linear model (GLM) of larval feeding in the laboratory assay. Wald's χ^2 values indicate comparisons with the next largest model. The final model is indicated in bold.

Fixed effects	AIC	χ^2	d.f.	P-value
Timepoint*host plant*population	918.84	NA	NA	NA
Timepoint*host plant + time*population + host plant*population	912.19	1.352	4	0.85
Timepoint*host plant + host plant*population	904.73	0.543	4	0.97
Timepoint*host plant + population	900.80	0.061	2	0.97
Timepoint + host plant + population	897.51	4.717	4	0.32
Time + host plant	**896.93**	**3.420**	**2**	**0.18**

AIC, Akaike's information criterion; NA, no comparison

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Table D.2 Hazard ratios and 95% confidence intervals (95% CI) for transitioning from empty to dead, empty to fed, and fed to dead for larvae on *Thlaspi arvense* compared to those on *Cardamine cordifolia*. Hazard ratios were calculated over the entire assay period. Confidence intervals were calculated by randomly sampling (1000 samples) the assumed multivariate normal distribution of the likelihood estimates and covariance matrix. No fed larvae died on *C. cordifolia*, so a 95% CI could not accurately be estimated

Transition	Hazard ratio	95% CI
Empty to dead	0.268	0.013-5.207
Empty to fed	0.512	0.381-0.689
Fed to dead	7139.859	NA

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Table D.3 Hierarchical simplification for linear mixed model (LMM) of relative gut fullness in the laboratory assay. Model fit was compared using Akaike's information criterion (AIC) and Wald's χ^2 tests. χ^2 test values indicate comparisons with the next largest model, usually directly above. Indented models were both compared back to the next largest model.

Model	Random effects	AIC	χ^2	d.f.	P-value
Time (adj.)*host plant*population	(1|family_ID) + (1|larva_ID)	-872.83	NA	NA	NA
Time (adj.)*host plant + time (adj.)*population + host plant*population	(1|family_ID) + (1|larva_ID)	-875.88	0.950	2	0.62
Time (adj.)*host plant+ host plant*population	(1|family_ID) + (1|larva_ID)	-877.67	2.208	2	0.33
Time (adj.)*host plant + population	(1|family_ID) + (1|larva_ID)	-877.60	2.063	1	0.15
Time (adj.)*host plant	(1|family_ID) + (1|larva_ID)	-878.46	1.144	1	0.29
Time (adj.)*host plant	**(1|larva_ID)**	**-878.63**	**1.831**	**1**	**0.18**
Time (adj.)*host plant	NA	-733.12	147.51	1	<0.001

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Table D.4 Multiple comparison results of final linear mixed model (LMM) for relative gut fullness in the laboratory assay, comparing the effects of time (2, 4, and 6 h), host plant (Cc, *Cardamine cordifolia*; Ta, *Thlaspi arvense*) and population (GT, Gothic; QC, Quigley Creek). P-values were adjusted using the multivariate method for 15 tests, significance is indicated with asterisks

Contrast	Log odds ratio	SE	d.f.	t-ratio	P-value	
2-Cc vs. 4-Cc	-0.067	0.109	322.350	-6.107	<0.001	***
2-Cc vs. 6-Cc	-0.082	0.012	328.630	-7.080	<0.001	***
2-Cc vs. 2-Ta	0.228	0.018	302.190	12.801	<0.001	***
2-Cc vs. 4-Ta	0.175	0.018	313.560	9.655	<0.001	***
2-Cc vs. 6-Ta	0.106	0.019	333.410	5.745	<0.001	***
4-Cc vs. 6-Cc	-0.015	0.012	324.180	-1.330	0.75	
4-Cc vs. 2-Ta	0.295	0.018	307.390	16.419	<0.001	***
4-Cc vs. 4-Ta	0.241	0.018	318.580	13.241	<0.001	***
4-Cc vs. 6-Ta	0.173	0.019	338.140	9.279	<0.001	***
6-Cc vs. 2-Ta	0.310	0.018	327.900	16.880	<0.001	***
6-Cc vs. 4-Ta	0.257	0.019	338.440	13.772	<0.001	***
6-Cc vs. 6-Ta	0.188	0.019	356.930	9.891	<0.001	***
2-Ta vs. 4-Ta	-0.053	0.012	326.000	-4.348	<0.001	***
2-Ta vs. 6-Ta	-0.122	0.013	330.690	-9.449	<0.001	***
4-Ta vs. 6-Ta	-0.068	0.013	322.760	-5.266	<0.001	***

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Table D.5 Hierarchical simplification of multinomial generalized linear model (GLM) of larval feeding in the laboratory and field assays. Wald's χ^2 values indicate comparisons with the next largest model. The final model is indicated in bold

Fixed effects	AIC	χ^2	d.f.	P-value
Assay*host plant*population	648.74	NA	NA	NA
Assay*host plant + assay*population + host plant*population	644.85	0.108	2	0.95
Assay*host plant + assay*population	641.00	0.149	2	0.93
Assay*host plant + population	**639.58**	**2.587**	**2**	**0.27**

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Table D.6 Hierarchical simplification for binomial mixed models of relative gut fullness between the 4-h timepoint in the field and laboratory. χ^2 test values indicate comparisons with the next largest model, usually directly above. Indented models were both compared back to the next largest model.

Fixed effects	Random effects	AIC	χ^2	d.f.	P
Assay*host plant*population	(1|family_ID)	-400.94	NA	NA	NA
Assay*host plant + assay*population + host plant*population	(1|family_ID)	-405.95	0.951	1	0.33
Assay*host plant + assay*population	(1|family_ID)	-412.95	0.340	1	0.56
Assay*host plant + population	(1|family_ID)	-419.76	0.401	1	0.53
Assay*host plant	**(1|family_ID)**	**-426.73**	**0.723**	**1**	**0.40**
Assay*host plant	NA	-439.17	10.432	2	0.001

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Table D.7 Multiple comparison results of final linear mixed model (LMM) for relative gut fullness between the laboratory and field after 4 h, comparing the effects of population (GT, Gothic; QC, Quigley Creek) and host plant (Cc, *Cardamine cordifolia*; Ta, *Thlaspi arvense*). P-values were adjusted using the multivariate method for six tests, significance is indicated with asterisks

Contrast	Estimate	SE	d.f.	t-ratio	P	
Lab-Cc vs. Field-Cc	0.157	0.019	385.578	8.436	<0.001	***
Lab-Cc vs. Lab-Ta	0.250	0.020	376.679	12.686	<0.001	***
Lab-Cc vs. Field-Ta	0.262	0.019	385.989	13.595	<0.001	***
Field-Cc vs. Lab-Ta	0.093	0.020	381.588	4.645	<0.001	***
Field-Cc vs. Field-Ta	0.105	0.018	373.964	5.782	<0.001	***
Lab-Ta vs. Field-Ta	0.012	0.021	383.773	0.590	0.94	

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Table D.8 Hierarchical simplification of multinomial generalized linear model (GLM) of larval feeding in the sinigrin assay. Wald's χ^2 values indicate comparisons with the next largest model.

Fixed effects	AIC	χ^2	d.f.	P
Timepoint*host plant*sinigrin addition	930.89	NA	NA	NA
Timepoint*host plant + time*sinigrin addition + host plant*sinigrin addition	915.78	0.882	8	0.99
Timepoint*host plant + host plant*sinigrin addition	908.01	0.237	4	0.99
Timepoint + host plant*sinigrin addition	**896.60**	**4.591**	**8**	**0.80**

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Table D.9 Hazard ratios and 95% confidence intervals (95% CI) for transitioning from empty to fed and empty to dead for sinigrin- and water-treated leaves of *Thlaspi arvense* (Ta), *Cardamine cordifolia* (Cc) and *Descurainia incana* (Di). Hazard ratios were calculated over the entire assay period from a dataset excluding the single larva that died after feeding.

Transition	Treatment	With respect to	Hazard ratio	95% CI
Empty to dead	Ta + sinigrin	Ta + water	1.859	0.393-8.797
	Cc + water	Ta + water	2.034	0.336-12.299
	Cc + sinigrin	Ta + water	1.050	0.140-7.844
	Di + water	Ta + water	1.10E-04	5.33E-90-2.27E+81
	Di + sinigrin	Ta + water	4.67E+04	2.25E-81-9.66E+89
	Cc + water	Ta + sinigrin	0.952	0.127-7.108
	Cc + sinigrin	Ta + sinigrin	2.135	0.869-5.242
	Di + water	Ta + sinigrin	2.22E-05	3.14E-89-1.56E+79
	Di + sinigrin	Ta + sinigrin	5.135	1.905-13.845
	Cc + sinigrin	Cc + water	1.951	0.544-6.991
	Di + water	Cc + water	4.48E-05	8.23E-99-2.44E+89
	Di + sinigrin	Cc + water	5.37 E+04	9.84E-90-2.93E+98
	Di + water	Cc + sinigrin	1.24E-05	1.4E-120-1.1E+110
	Di + sinigrin	Cc + sinigrin	2.406	0.969-5.971
	Di + sinigrin	Di + water	2.90E+04	1.41E-45-5.94E+53
Empty to Fed	Ta + sinigrin	Ta + water	0.379	0.393-8.797
	Cc + water	Ta + water	1.599	0.336-12.299
	Cc + sinigrin	Ta + water	0.989	0.140-7.844
	Di + water	Ta + water	2.607	5.33E-90 –2.27E+81
	Di + sinigrin	Ta + water	2.334	2.25E-81-9.66E+89
	Cc + water	Ta + sinigrin	1.012	0.421-2.430
	Cc + sinigrin	Ta + sinigrin	1.581	0.832-3.005
	Di + water	Ta + sinigrin	0.428	0.171-1.075
	Di + sinigrin	Ta + sinigrin	6.086	3.111-11.904
	Cc + sinigrin	Cc + water	0.375	0.204-0.688
	Di + water	Cc + water	1.630	0.878-3.026
	Di + sinigrin	Cc + water	2.361	0.958-5.822
	Di + water	Cc + sinigrin	0.424	0.172-1.044
	Di + sinigrin	Cc + sinigrin	3.849	1.995-7.425
	Di + sinigrin	Di + water	0.886	0.454-1.727

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Table D.10 Hierarchical simplification for linear models of relative gut fullness in the sinigrin assay. χ^2 test values indicate comparisons with the next largest model, usually directly above. Indented models were both compared back to the next largest model. The final model is indicated in bold.

Model	Random effects	AIC	χ^2	d.f.	P
Time*host plant*sinigrin addition	(1|larva_ID) + (1|family_ID)	-304.8	NA	NA	NA
Time*host plant + time*sinigrin addition + host plant*sinigrin addition	(1|larva_ID) + (1|family_ID)	-365.4	1.734	4	0.79
Time*host plant + host plant*sinigrin addition	(1|larva_ID) + (1|family_ID)	-36	1.701	2	0.43
Time + host plant*sinigrin addition	**(1|larva_ID) + (1|family_ID)**	**-365.4**	**6.514**	**4**	**0.16**
Time + host plant*sinigrin addition	(1|larva_ID)	362.2	5.200	1	0.023
Time + host plant*sinigrin addition	(1|family_ID)	-342.3	23.392	1	<0.001

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Table D.11 Multiple comparison results of final linear mixed model (LMM) for relative gut fullness in the sinigrin assay, comparing the effects of sinigrin addition (sin; control: con, water) and host plant (Cc, *Cardamine cordifolia*; Ta, *Thlaspi arvense*; Di, *Descurainia incana*). Results are given on the log odds ratio and P-values were adjusted using the multivariate method for 153 tests

Contrast	Log odds ratio	SE	df	t-ratio	P	
2-Sin-Ta vs. 4-Sin-Ta	-0.068	0.014	230.250	-4.769	<0.001	***
2-Sin-Ta vs. 6-Sin-Ta	-0.097	0.015	236.029	-6.515	<0.001	***
2-Sin-Ta vs. 2-Con-Ta	0.030	0.037	135.754	0.804	0.995	
2-Sin-Ta vs. 4-Con-Ta	-0.038	0.039	167.134	-0.972	0.986	
2-Sin-Ta vs. 6-Con-Ta	-0.067	0.039	169.413	-1.722	0.748	
2-Sin-Ta vs. 2-Sin-Cc	-0.082	0.038	131.210	-2.174	0.463	
2-Sin-Ta vs. 4-Sin-Cc	-0.150	0.040	161.165	-3.765	0.010	*
2-Sin-Ta vs. 6-Sin-Cc	-0.180	0.040	164.584	-4.468	0.001	**
2-Sin-Ta vs. 2-Con-Cc	-0.207	0.036	134.255	-5.822	<0.001	***
2-Sin-Ta vs. 4-Con-Cc	-0.274	0.037	167.531	-7.337	<0.001	***
2-Sin-Ta vs. 6-Con-Cc	-0.304	0.038	168.029	-8.107	<0.001	***
2-Sin-Ta vs. 2-Sin-Di	-0.150	0.036	136.927	-4.148	0.003	**
2-Sin-Ta vs. 4-Sin-Di	-0.217	0.038	169.604	-5.729	<0.001	***
2-Sin-Ta vs. 6-Sin-Di	-0.247	0.038	170.411	-6.487	<0.001	***
2-Sin-Ta vs. 2-Con-Di	-0.255	0.035	134.480	-7.248	<0.001	***
2-Sin-Ta vs. 4-Con-Di	-0.323	0.037	169.154	-8.676	<0.001	***
2-Sin-Ta vs. 6-Con-Di	-0.353	0.037	169.018	-9.458	<0.001	***
4-Sin-Ta vs. 6-Sin-Ta	-0.030	0.015	229.442	-1.958	0.602	
4-Sin-Ta vs. 2-Con-Ta	0.097	0.040	182.207	2.415	0.319	
4-Sin-Ta vs. 4-Con-Ta	0.030	0.037	135.754	0.804	0.995	
4-Sin-Ta vs. 6-Con-Ta	<0.001	0.040	178.782	0.004	1.000	
4-Sin-Ta vs. 2-Sin-Cc	-0.015	0.041	173.463	-0.360	1.000	
4-Sin-Ta vs. 4-Sin-Cc	-0.082	0.038	131.210	-2.174	0.463	
4-Sin-Ta vs. 6-Sin-Cc	-0.112	0.041	172.354	-2.739	0.171	
4-Sin-Ta vs. 2-Con-Cc	-0.139	0.039	184.934	-3.564	0.020	*
4-Sin-Ta vs. 4-Con-Cc	-0.207	0.036	134.255	-5.822	<0.001	***
4-Sin-Ta vs. 6-Con-Cc	-0.236	0.038	178.802	-6.154	<0.001	***
4-Sin-Ta vs. 2-Sin-Di	-0.082	0.040	186.947	-2.072	0.528	
4-Sin-Ta vs. 4-Sin-Di	-0.150	0.036	136.927	-4.148	0.003	**
4-Sin-Ta vs. 6-Sin-Di	-0.179	0.039	181.117	-4.600	0.001	**
4-Sin-Ta vs. 2-Con-Di	-0.188	0.039	185.056	-4.850	<0.001	***
4-Sin-Ta vs. 4-Con-Di	-0.255	0.035	134.480	-7.248	<0.001	***
4-Sin-Ta vs. 6-Con-Di	-0.285	0.038	179.050	-7.478	<0.001	***
6-Sin-Ta vs. 2-Con-Ta	0.127	0.041	186.922	3.125	0.069	•
6-Sin-Ta vs. 4-Con-Ta	0.059	0.040	181.381	1.483	0.866	
6-Sin-Ta vs. 6-Con-Ta	0.030	0.037	135.754	0.804	0.995	
6-Sin-Ta vs. 2-Sin-Cc	0.015	0.041	176.662	0.359	1.000	
6-Sin-Ta vs. 4-Sin-Cc	-0.053	0.041	172.274	-1.296	0.930	
6-Sin-Ta vs. 6-Sin-Cc	-0.082	0.038	131.210	-2.174	0.463	
6-Sin-Ta vs. 2-Con-Cc	-0.110	0.040	191.863	-2.774	0.158	
6-Sin-Ta vs. 4-Con-Cc	-0.177	0.039	185.475	-4.569	0.001	**
6-Sin-Ta vs. 6-Con-Cc	-0.207	0.036	134.255	-5.822	<0.001	***
6-Sin-Ta vs. 2-Sin-Di	-0.052	0.040	193.461	-1.310	0.927	
6-Sin-Ta vs. 4-Sin-Di	-0.120	0.039	187.064	-3.057	0.081	•
6-Sin-Ta vs. 6-Sin-Di	-0.150	0.036	136.927	-4.148	0.003	**
6-Sin-Ta vs. 2-Con-Di	-0.158	0.039	192.681	-4.033	0.004	**
6-Sin-Ta vs. 4-Con-Di	-0.226	0.039	187.033	-5.854	<0.001	***
6-Sin-Ta vs. 6-Con-Di	-0.255	0.035	134.480	-7.248	<0.001	***

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2-Con-Ta vs. 4-Con-Ta	-0.068	0.014	230.250	-4.769	<0.001	***
2-Con-Ta vs. 6-Con-Ta	-0.097	0.015	236.029	-6.515	<0.001	***
2-Con-Ta vs. 2-Sin-Cc	-0.112	0.035	111.294	-3.246	0.052	•
2-Con-Ta vs. 4-Sin-Cc	-0.180	0.037	151.614	-4.806	<0.001	***
2-Con-Ta vs. 6-Sin-Cc	-0.209	0.038	156.769	-5.536	<0.001	***
2-Con-Ta vs. 2-Con-Cc	-0.237	0.031	99.422	-7.654	<0.001	***
2-Con-Ta vs. 4-Con-Cc	-0.304	0.034	143.794	-8.982	<0.001	***
2-Con-Ta vs. 6-Con-Cc	-0.334	0.034	145.885	-9.813	<0.001	***
2-Con-Ta vs. 2-Sin-Di	-0.179	0.031	100.238	-5.726	<0.001	***
2-Con-Ta vs. 4-Sin-Di	-0.247	0.034	143.516	-7.213	<0.001	***
2-Con-Ta vs. 6-Sin-Di	-0.276	0.034	145.732	-8.034	<0.001	***
2-Con-Ta vs. 2-Con-Di	-0.285	0.031	101.480	-9.234	<0.001	***
2-Con-Ta vs. 4-Con-Di	-0.353	0.034	147.593	-10.392	<0.001	***
2-Con-Ta vs. 6-Con-Di	-0.382	0.034	149.067	-11.230	<0.001	***
4-Con-Ta vs. 6-Con-Ta	-0.030	0.015	229.442	-1.958	0.602	
4-Con-Ta vs. 2-Sin-Cc	-0.045	0.037	149.236	-1.194	0.955	
4-Con-Ta vs. 4-Sin-Cc	-0.112	0.035	111.294	-3.246	0.053	•
4-Con-Ta vs. 6-Sin-Cc	-0.142	0.038	157.347	-3.746	0.011	*
4-Con-Ta vs. 2-Con-Cc	-0.169	0.034	144.951	-4.949	<0.001	***
4-Con-Ta vs. 4-Con-Cc	-0.237	0.031	99.422	-7.654	<0.001	***
4-Con-Ta vs. 6-Con-Cc	-0.266	0.034	148.481	-7.774	<0.001	***
4-Con-Ta vs. 2-Sin-Di	-0.112	0.035	145.116	-3.236	0.052	•
4-Con-Ta vs. 4-Sin-Di	-0.179	0.031	100.238	-5.726	<0.001	***
4-Con-Ta vs. 6-Sin-Di	-0.209	0.035	148.503	-6.030	<0.001	***
4-Con-Ta vs. 2-Con-Di	-0.217	0.034	146.874	-6.396	<0.001	***
4-Con-Ta vs. 4-Con-Di	-0.285	0.031	101.480	-9.234	<0.001	***
4-Con-Ta vs. 6-Con-Di	-0.315	0.034	150.764	-9.213	<0.001	***
6-Con-Ta vs. 2-Sin-Cc	-0.015	0.037	151.402	-0.398	1.000	
6-Con-Ta vs. 4-Sin-Cc	-0.083	0.038	154.376	-2.196	0.448	
6-Con-Ta vs. 6-Sin-Cc	-0.112	0.035	111.294	-3.246	0.053	•
6-Con-Ta vs. 2-Con-Cc	-0.139	0.035	151.342	-4.026	0.004	**
6-Con-Ta vs. 4-Con-Cc	-0.207	0.035	152.846	-5.987	<0.001	***
6-Con-Ta vs. 6-Con-Cc	-0.237	0.031	99.422	-7.654	<0.001	***
6-Con-Ta vs. 2-Sin-Di	-0.082	0.035	151.237	-2.349	0.357	
6-Con-Ta vs. 4-Sin-Di	-0.150	0.035	152.474	-4.290	0.002	**
6-Con-Ta vs. 6-Sin-Di	-0.179	0.031	100.238	-5.726	<0.001	***
6-Con-Ta vs. 2-Con-Di	-0.188	0.035	153.997	-5.441	<0.001	***
6-Con-Ta vs. 4-Con-Di	-0.255	0.035	156.446	-7.387	<0.001	***
6-Con-Ta vs. 6-Con-Di	-0.285	0.031	101.480	-9.234	<0.001	***
2-Sin-Cc vs. 4-Sin-Cc	-0.068	0.014	230.250	-4.769	<0.001	***
2-Sin-Cc vs. 6-Sin-Cc	-0.097	0.015	236.029	-6.515	<0.001	***
2-Sin-Cc vs. 2-Con-Cc	-0.125	0.033	106.963	-3.797	0.011	*
2-Sin-Cc vs. 4-Con-Cc	-0.192	0.036	147.309	-5.407	<0.001	***
2-Sin-Cc vs. 6-Con-Cc	-0.222	0.036	147.621	-6.238	<0.001	***
2-Sin-Cc vs. 2-Sin-Di	-0.067	0.033	108.539	-2.022	0.562	
2-Sin-Cc vs. 4-Sin-Di	-0.135	0.036	148.100	-3.751	0.011	*
2-Sin-Cc vs. 6-Sin-Di	-0.164	0.036	148.585	-4.569	0.001	**
2-Sin-Cc vs. 2-Con-Di	-0.173	0.033	107.607	-5.311	<0.001	***
2-Sin-Cc vs. 4-Con-Di	-0.241	0.035	149.514	-6.791	<0.001	***
2-Sin-Cc vs. 6-Con-Di	-0.270	0.035	149.250	-7.634	<0.001	***
4-Sin-Cc vs. 6-Sin-Cc	-0.030	0.015	229.442	-1.958	0.602	
4-Sin-Cc vs. 2-Con-Cc	-0.057	0.036	151.096	-1.584	0.820	
4-Sin-Cc vs. 4-Con-Cc	-0.125	0.033	106.963	-3.797	0.011	*
4-Sin-Cc vs. 6-Con-Cc	-0.154	0.036	151.441	-4.303	0.002	**
4-Sin-Cc vs. 2-Sin-Di	<0.001	0.036	152.180	0.011	1.000	
4-Sin-Cc vs. 4-Sin-Di	-0.067	0.033	108.539	-2.022	0.562	

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4-Sin-Cc vs. 6-Sin-Di	-0.097	0.036	152.520	-2.669	0.199	
4-Sin-Cc vs. 2-Con-Di	-0.105	0.036	151.602	-2.959	0.105	
4-Sin-Cc vs. 4-Con-Di	-0.173	0.033	107.607	-5.311	<0.001	***
4-Sin-Cc vs. 6-Con-Di	-0.203	0.036	152.244	-5.696	<0.001	***
6-Sin-Cc vs. 2-Con-Cc	-0.027	0.037	158.647	-0.748	0.997	
6-Sin-Cc vs. 4-Con-Cc	-0.095	0.036	158.712	-2.608	0.225	
6-Sin-Cc vs. 6-Con-Cc	-0.125	0.033	106.963	-3.797	0.011	*
6-Sin-Cc vs. 2-Sin-Di	0.030	0.037	159.454	0.813	0.995	
6-Sin-Cc vs. 4-Sin-Di	-0.038	0.037	159.347	-1.023	0.981	
6-Sin-Cc vs. 6-Sin-Di	-0.067	0.033	108.539	-2.022	0.562	
6-Sin-Cc vs. 2-Con-Di	-0.076	0.036	159.865	-2.091	0.516	
6-Sin-Cc vs. 4-Con-Di	-0.143	0.036	160.775	-3.957	0.005	**
6-Sin-Cc vs. 6-Con-Di	-0.173	0.033	107.607	-5.311	<0.001	***
2-Con-Cc vs. 4-Con-Cc	-0.068	0.014	230.250	-4.769	<0.001	***
2-Con-Cc vs. 6-Con-Cc	-0.097	0.015	236.029	-6.515	<0.001	***
2-Con-Cc vs. 2-Sin-Di	0.057	0.030	96.329	1.940	0.614	
2-Con-Cc vs. 4-Sin-Di	-0.010	0.033	144.538	-0.314	1.000	
2-Con-Cc vs. 6-Sin-Di	-0.040	0.033	149.320	-1.204	0.952	
2-Con-Cc vs. 2-Con-Di	-0.048	0.029	97.819	-1.666	0.778	
2-Con-Cc vs. 4-Con-Di	-0.116	0.032	149.468	-3.575	0.019	*
2-Con-Cc vs. 6-Con-Di	-0.146	0.033	153.539	-4.447	0.001	**
4-Con-Cc vs. 6-Con-Cc	-0.030	0.015	229.442	-1.958	0.602	
4-Con-Cc vs. 2-Sin-Di	0.125	0.033	145.032	3.812	0.009	**
4-Con-Cc vs. 4-Sin-Di	0.057	0.030	96.329	1.940	0.614	
4-Con-Cc vs. 6-Sin-Di	0.028	0.033	151.763	0.834	0.994	
4-Con-Cc vs. 2-Con-Di	0.019	0.032	147.388	0.593	0.999	
4-Con-Cc vs. 4-Con-Di	-0.048	0.029	97.819	-1.666	0.778	
4-Con-Cc vs. 6-Con-Di	-0.078	0.033	154.822	-2.385	0.335	
6-Con-Cc vs. 2-Sin-Di	0.154	0.033	149.464	4.674	<0.001	***
6-Con-Cc vs. 4-Sin-Di	0.087	0.033	151.420	2.623	0.219	
6-Con-Cc vs. 6-Sin-Di	0.057	0.030	96.329	1.940	0.615	
6-Con-Cc vs. 2-Con-Di	0.049	0.033	152.902	1.493	0.862	
6-Con-Cc vs. 4-Con-Di	-0.019	0.033	156.226	-0.575	0.999	
6-Con-Cc vs. 6-Con-Di	-0.048	0.029	97.819	-1.666	0.778	
2-Sin-Di vs. 4-Sin-Di	-0.068	0.014	230.250	-4.769	<0.001	***
2-Sin-Di vs. 6-Sin-Di	-0.097	0.015	236.029	-6.515	<0.001	***
2-Sin-Di vs. 2-Con-Di	-0.106	0.029	96.739	-3.599	0.020	*
2-Sin-Di vs. 4-Con-Di	-0.173	0.033	146.973	-5.294	<0.001	***
2-Sin-Di vs. 6-Con-Di	-0.203	0.033	150.759	-6.152	<0.001	***
4-Sin-Di vs. 6-Sin-Di	-0.030	0.015	229.442	-1.958	0.602	
4-Sin-Di vs. 2-Con-Di	-0.038	0.033	144.452	-1.174	0.958	
4-Sin-Di vs. 4-Con-Di	-0.106	0.029	96.739	-3.599	0.020	*
4-Sin-Di vs. 6-Con-Di	-0.135	0.033	151.733	-4.107	0.003	**
6-Sin-Di vs. 2-Con-Di	-0.009	0.033	149.971	-0.261	1.000	
6-Sin-Di vs. 4-Con-Di	-0.076	0.033	153.423	-2.299	0.386	
6-Sin-Di vs. 6-Con-Di	-0.106	0.029	96.739	-3.599	0.021	*
2-Con-Di vs. 4-Con-Di	-0.068	0.014	230.250	-4.769	<0.001	***
2-Con-Di vs. 6-Con-Di	-0.097	0.015	236.029	-6.515	<0.001	***
4-Con-Di vs. 6-Con-Di	-0.030	0.015	229.442	-1.958	0.602	

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Table D.12 Hierarchical simplification for cox mixed effects proportional hazards models of larval survival. χ^2 test values indicate comparisons with the next largest model- usually directly above. Indented models were both compared back to the next largest model. The final model is indicated in bold.

Model	Random effects	AIC	χ^2	df	P
Host plant*sinigrin addition	**(1|family_ID)**	**1455.8**	**NA**	**NA**	**NA**
Host plant + sinigrin addition	(1|family_ID)	1454.3	2.522	2	0.28
Host plant*sinigrin addition	NA	1475.0	11.107	1	<0.001

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Table D.13 Multiple comparison (Tukey contrasts) of final cox mixed effects proportional hazards models of larval survival, comparing the effects of sinigrin addition (sin; control: con, water) and host plant (Cc, *Cardamine cordifolia*; Ta, *Thlaspi arvense*; Di, *Descurainia incana*). P-values were adjusted for 15 tests.

Contrast	Estimate	SE	z	P	
Con-Di vs. Con-Cc	-0.424	0.339	-1.251	0.809	
Con-Ta vs. Con-Cc	1.081	0.300	3.602	0.004	**
Sin-Cc vs. Con-Cc	0.977	0.287	3.403	0.008	**
Sin-Di vs. Con-Cc	0.409	0.295	1.386	0.733	
Sin-Ta vs. Con-Cc	1.478	0.289	5.119	<0.001	***
Con-Ta vs. Con-Di	1.505	0.335	4.492	<0.001	***
Sin-Cc vs. Con-Di	1.402	0.322	4.355	<0.001	***
Sin-Di vs. Con-Di	0.833	0.328	2.539	0.111	
Sin-Ta vs. Con-Di	1.903	0.325	5.850	<0.001	***
Sin-Cc vs. Con-Ta	-0.104	0.274	-0.378	0.999	
Sin-Di vs. Con-Ta	-0.672	0.286	-2.351	0.172	
Sin-Ta vs. Con-Ta	0.397	0.263	1.509	0.655	
Sin-Di vs. Sin-Cc	-0.569	0.272	-2.088	0.291	
Sin-Ta vs. Sin-Cc	0.501	0.252	1.984	0.348	
Sin-Ta vs. Sin-Di	1.070	0.272	3.932	0.001	**

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D.2 Figures

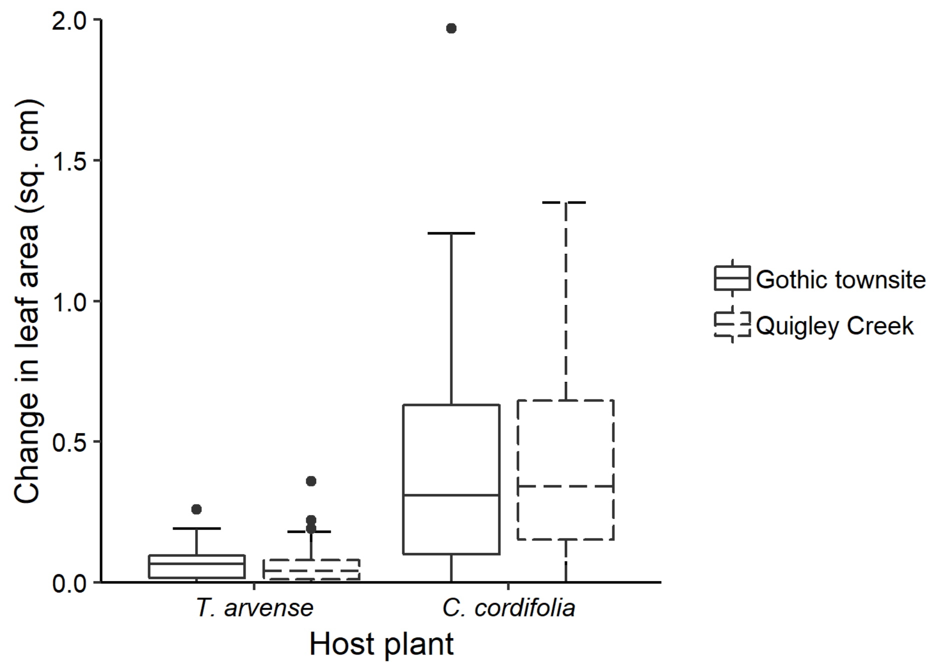

Figure D.1 Leaf area consumed (cm^2) over the 6 h of the laboratory feeding assay. Boxes represent the interquartile range (IQR) with a horizontal line at the median and whiskers extending to the largest or smallest observation falling within 1.5 IQRs of the upper or lower quantiles, respectively. Outliers appear as black points. There was no main effect of population (rank normalization transformed ANOVA: $F_{1,152} = 0.032$, $P = 0.86$). The difference in leaf area change between the two host plants was nearly significant ($F_{1,152} = 3.015$, $P = 0.082$).

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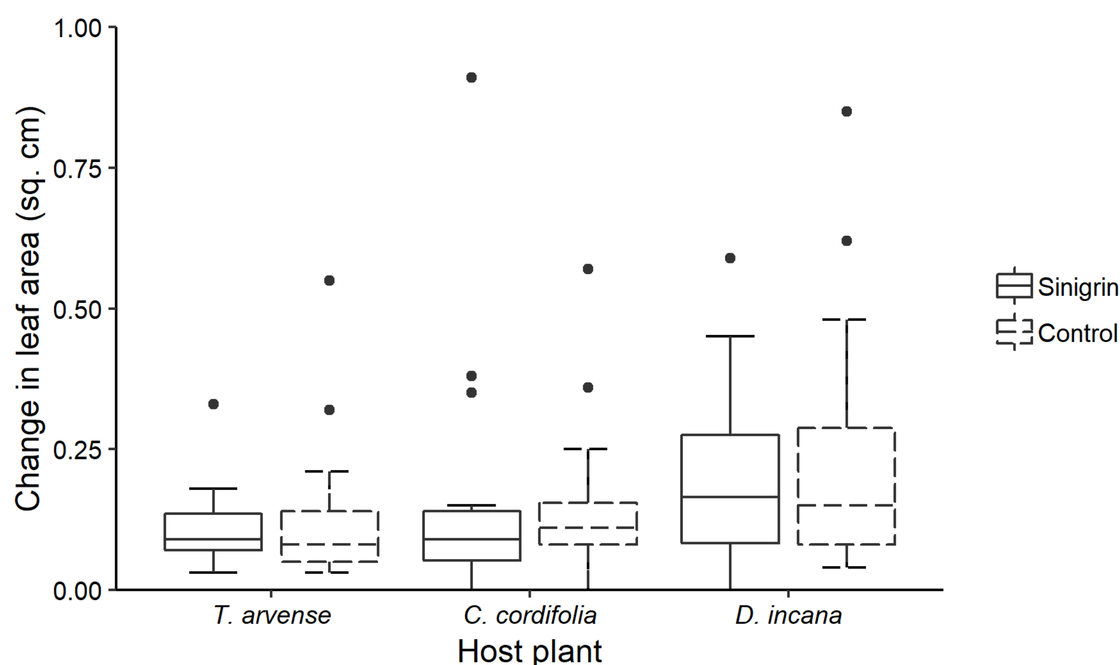

Figure D.2 Leaf area consumed (cm^2) over the first 6 h of the sinigrin feeding assay. Boxes represent the interquartile range (IQR) with a horizontal line at the median and whiskers extending to the largest or smallest observation falling within 1.5 IQR-lengths of the upper or lower quantiles, respectively. Outliers appear as black points. The interaction between host plant and sinigrin addition was not significant (rank normalization transformed ANOVA, $F_{2,103} = 0.671$, $P = 0.51$). There was neither a significant main effect of host plant ($F_{2,103} = 1.120$, $P = 0.33$), nor of sinigrin addition ($F_{1,103} = 0.061$, $P = 0.81$).

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CHAPTER IV REPRINT LICENSE TERMS AND CONDITIONS

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