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Pre- and post-ingestive defenses affect larval feeding on a lethal invasive host plant

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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 the larvae. We first tested whether larval feeding behavior was affected before (pre-) ingestion or following (post-) ingestion of leaf material, indicating activity of feeding deterrents, toxins, or both in this evolutionary trap. Neonates were less likely to start feeding and eventually fed more slowly on *T. arvense* than on the native host plant *Cardamine cordifolia* (Gray) (Brassicaceae) in both laboratory and field. Starvation was a primary cause of mortality, indicating the role of a feeding deterrent. Feeding did not differ between larvae from invaded and uninvaded population. Second, *T. arvense* defensive chemistry is dominated by the glucosinolate sinigrin (allyl or 2-propenyl glucosinolate). Adding sinigrin to the leaves of *T. arvense* and native hosts *C. cordifolia* and *Descurainia incana* (Bernhardi ex Fischer & Meyer) (Brassicaceae) delayed the onset of feeding, caused larvae to feed more slowly, and decreased survival on the native hosts. This evolutionary trap may not be driven by a novel deterrent, but rather by a change in the concentration of a deterrent found in native hosts. Many insects have adapted to evolutionary traps posed by invasive plants, incorporating the new plant into their diets. *Thlaspi arvense* remains lethal to *P. macdunnoughii*, and pre-ingestive deterrents such as excess sinigrin may contribute to persistent maladaptation.

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

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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 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 naive. 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 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 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; Frisch et al., 2015; Gumz 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 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.

Materials and methods

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 non-native for approximately 45–160 years.

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.

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 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.

Larval feeding in the laboratory

We compared larval feeding on *T. arvense* with feeding on native host, *C. 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 1).

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 **9** 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 1).

Larval feeding with sinigrin addition

Larval feeding was assayed as described above on excised leaves painted with a synthetic sinigrin solution. We tested non-native *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 1mol 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 1mol 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 2).

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.

Statistical analysis

Larval feeding in the laboratory was analyzed using a multinomial generalized linear model (GLM) (nnet

Table 1 Initial sample sizes in the laboratory and field assays (N_{init}), the number of *Pieris macdunnoughii* larvae recovered after 4 h in the field ($N_{4\ h\text{-}rec}$), and the number of empty or fed larvae that were still alive at the end of the 6-h laboratory assay ($N_{6\ h\text{-}empty}$, $N_{6\ h\text{-}fed}$) and the 4-h field assay ($N_{4\ h\text{-}empty}$, $N_{4\ h\text{-}fed}$), summarized by host plant and population

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

Table 2 Sinigrin assay initial sample sizes (N_{init}), and the number of living *Pieris macdunnoughii* larvae empty or fed after 6 h ($N_{6\ h\text{-}empty}$, $N_{6\ h\text{-}fed}$), summarized by host plant and sinigrin-addition treatment

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

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 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 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 with 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 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 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 (1 000 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, 2018) evaluating the effect of host plant and sinigrin addition over 6 days, with a random effect of family. Multiple comparisons (Tukey method) were conducted to compare survival differences among treatment levels.

Results

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 1A and B, Tables 3 and S1). 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 1A and B). Once feeding had begun, larvae on *T. arvense* leaves also fed significantly more slowly than those on *C. cordifolia* (Figure 1E, Tables 1, S3, and S4). 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 S4). 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 S1).

Differences in larval feeding onset and gut fullness between host plants were also observed in field conditions (Figure 1C, D and F, Tables 3, S5, and S6). The host plant effect was smaller in the field. But larval gut fullness was

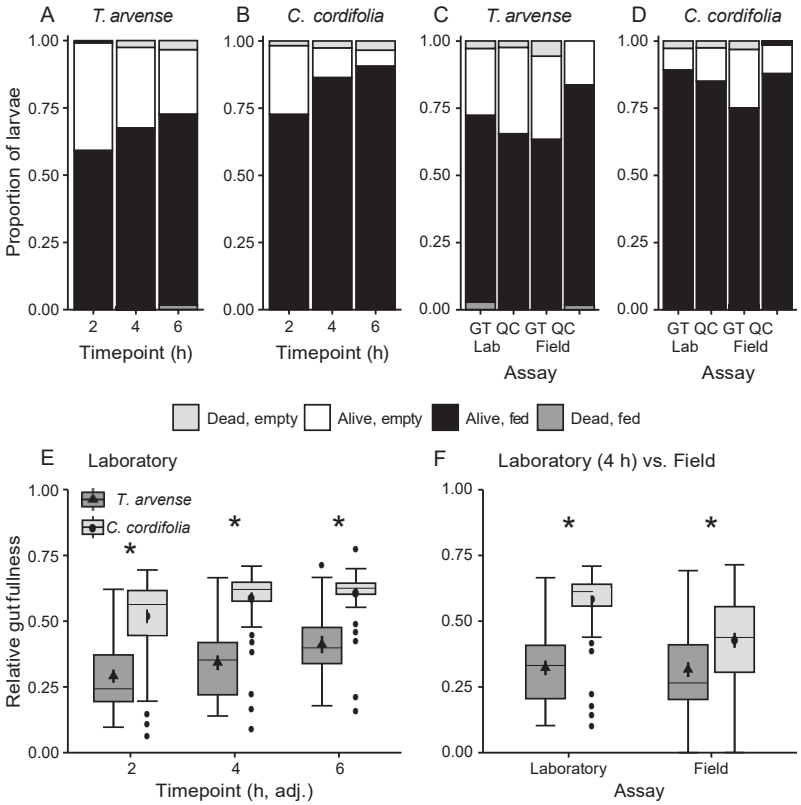

Figure 1 Proportion of *Pieris macdunnoughii* larvae feeding on *Thlaspi arvense* and *Cardamine cordifolia* (A, B) after 2, 4, and 6 h in the laboratory and (C, D) in the field. Differences in larval feeding between Gothic (GT) and Quigley Creek (QC) larvae were only found in the field. Relative gut fullness (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. Error bars around the mean relative gut fullness of larvae eating *T. arvense* (triangles) or *C. cordifolia* (circles) represent 95% confidence intervals. 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 dots. Asterisks indicate significant differences between *T. arvense* and *C. cordifolia* diets within a timepoint or assay type (LSM multiple comparison: P<0.05). Timepoints in the laboratory assay were adjusted for the onset of feeding.

Table 3 ANOVA (Wald's v^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	v^2	d.f.	P
Larval feeding				
Laboratory, multinomial GLM (Figure 1A and B, Table S1)	Host plant	31.926	2	<0.001
	Timepoint	25.287	4	<0.001
Laboratory vs. field, multinomial GLM (Figure 1C and D, Table S5)	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 1E, Table S3)	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 1F, Table S6)	Host plant	165.027	1	<0.001
	Assay	42.049	1	<0.001
	Host plant*assay	29.117	1	<0.001

still greater on *C. cordifolia* than on *T. arvense* (LSM multiple comparison: t-ratio = 5.782, P<0.001; Figure 1F, Table S7).

Generally, there were no differences between the Gothic and Quigley Creek populations, apart from the onset of larval feeding in the field (Figure 1C and D). There was a significant interaction between the assay and population: Gothic larvae were less likely to start eating in the field, regardless of host plant (Table 3).

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 2A and B, Table 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 S9). 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 2C).

Mortality among unfed larvae ranged from 0 to 26.5% and was generally higher on sinigrin-treated plants of all species (Figure 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 S9). 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 no effect on larval gut fullness on *T. arvense* (Figure 2D–F, Tables 4 and S11). For both the sinigrin and control treatments using *T. arvense*, relative gut fullness reached 30% by the 2nd hour, 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 S11).

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 S2).

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 3A, Table S13). Between the two treatments, only a single larva on *T. arvense* survived to the 6th day of observations.

Larvae that fed on sinigrin-treated *D. incana* leaves survived at a higher rate than those fed on sinigrin-treated *T. arvense* leaves (z = 3.564, P = 0.005; Figure 3B, Table S13). In contrast, the survival of larvae fed on

sinigrin-treated *C. cordifolia* and *T. arvense* leaves was not different (z = 4.468, P<0.001; Figure 3C, Table S13). There was significant variation among families, which was included in the model as a random effect (Table S12).

Discussion

We demonstrate 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 that the 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.

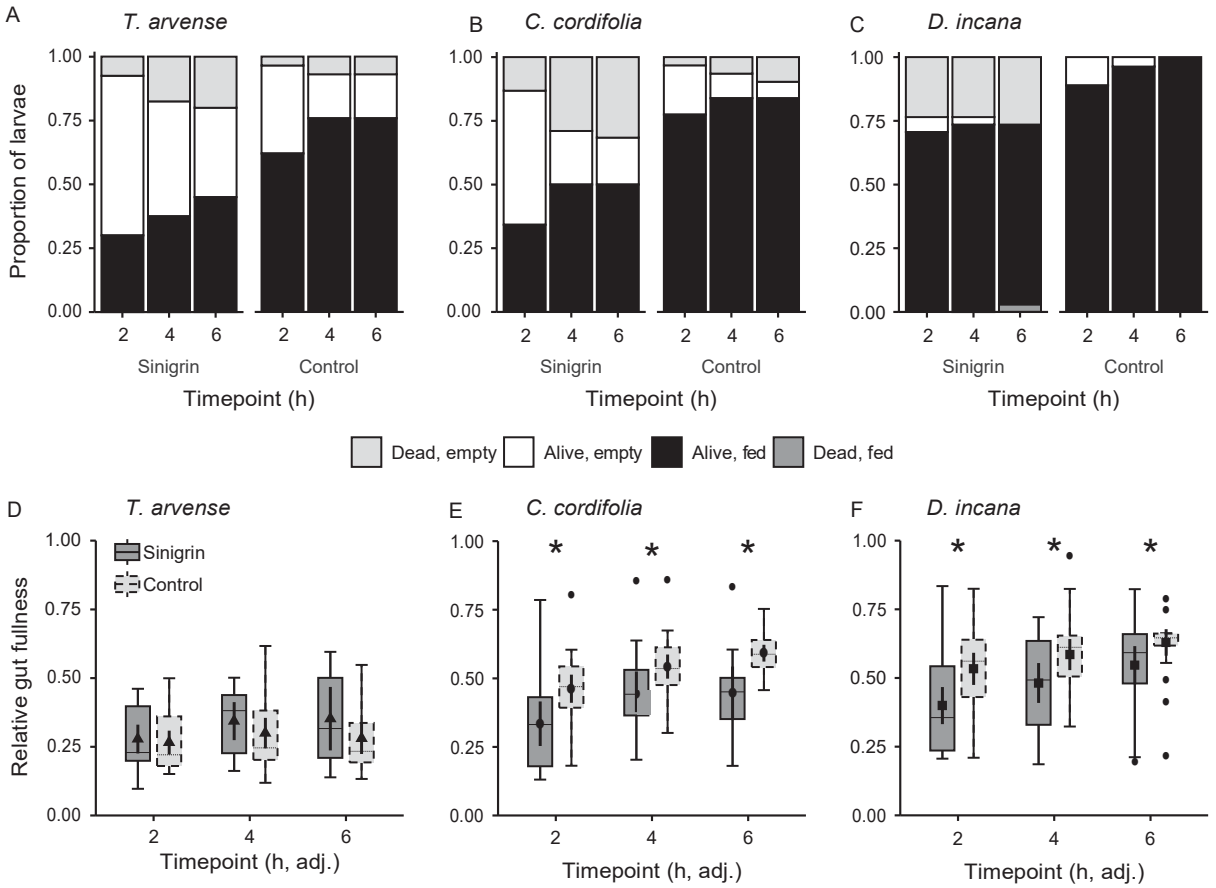

Figure 2 Proportion of *Pieris macdunnoughii* larvae after 2, 4, and 6 h (A–C) feeding and (D–F) relative larval gut fullness on leaves of *Thlaspi arvense* (triangles) and native host plants *Cardamine cordifolia* (circles) and *Descurainia incana* (squares) treated with water (dashed line) or sinigrin (50 1mol g^{-1} dry weight; solid line). Error bars around the mean relative gut fullness of larvae eating *T. arvense* or *C. cordifolia* represent 95% confidence intervals. 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 dots. Asterisks indicate significant differences between sinigrin and control treatments at each timepoint (LSM multiple comparison: P<0.05). Timepoints in the laboratory assay were adjusted for the onset of feeding.

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

Table 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	d.f.	P
Larval feeding				
Sinigrin addition, multinomial GLM (Figure 2A–C, Table S8)	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 2D–F, Table S10)	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

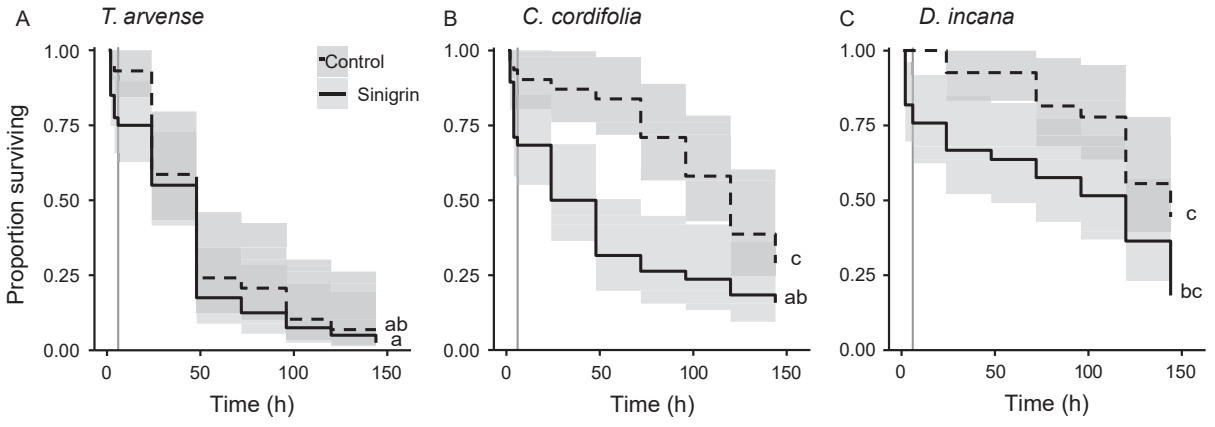

Figure 3 Survivorship curves of *Pieris macdunnoughii* larvae reared on cut (A) *Thlaspi arvense*, (B) *Cardamine cordifolia*, and (C) *Descurainia incana* leaves treated with sinigrin (50 1mol g^{-1} dry weight; solid line) and distilled water (control; dashed line). Vertical gray lines indicate the end of the 6-h assay and letters indicate significant differences among treatments (Cox proportional hazards, Tukey multiple comparisons: P<0.05).

presence of thiocyanate forming protein (TaTFP; Kuchernig et al., 2012). However, although feeding patterns differed across the three host plants in 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, 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 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 that we have not captured all variation in the population, but over 45 years of research, no larvae from Gothic townsite or the 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 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 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.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Leaf area consumed (cm^2) over the 6 h of the laboratory feeding assay.

Figure S2 Leaf area consumed (cm^2) over the first 6 h of the sinigrin feeding assay.

Table S1 Hierarchical simplification of multinomial generalized linear model (GLM) of larval feeding in the laboratory assay

Table S2 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*

Table S3 Hierarchical simplification for linear mixed model (LMM) of relative gut fullness in the laboratory assay

Table S4 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)

Table S5 Hierarchical simplification of multinomial generalized linear model (GLM) of larval feeding in the laboratory and field assays

Table S6 Hierarchical simplification for binomial mixed models of relative gut fullness between the 4-h timepoint in the field and laboratory

Table S7 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*)

Table S8 Hierarchical simplification of multinomial generalized linear model (GLM) of larval feeding in the sinigrin assay

Table S9 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)

Table S10 Hierarchical simplification for linear models of relative gut fullness in the sinigrin assay

Table S11 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*)

Table S12 Hierarchical simplification for cox mixed effects proportional hazards models of larval survival

Table S13 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*)