



Experience may outweigh cue similarity in maintaining a persistent host-plant-based evolutionary trap

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Abstract. Rapid environmental change can decouple previously reliable cues from important resources, causing specialized recognition systems to result in maladaptive behaviors. For native herbivorous insects, such evolutionary traps are often imposed by attractive invasive plants that prove harmful to their offspring. Despite the costs of ovipositing on a poor-quality host, 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 or native resource. We evaluated the role of cue similarity in the persistence of maladaptive oviposition by a native butterfly on a lethal, invasive mustard. While the novel plant shares glucosinolate cues with at least one of the native hosts and the most abundant cue is a strong oviposition stimulant, we found that this cue was not a major driver of preference for either plant. Nor was preference for the two plants correlated, meaning decreased preference for the invasive mustard would not cause butterflies to miss potential oviposition opportunities on the superior native host. Instead, butterfly preference was modified by previous experience in a way that suggests that frequent encounters with native hosts in the wild may buffer butterflies against this evolutionary trap.

Key words: *behavioral plasticity; Brassicaceae; constraint; glucosinolates; herbivory; host plant recognition; maladaptation; Pieridae.*

INTRODUCTION

Many organisms use cue-response systems to process and react to information from their environment. These systems have been shaped through evolutionary time to link available, predictable cues with adaptive behavioral outcomes (Schlaepfer et al. 2005, Sih et al. 2011). Evolutionary traps occur when novel resources or habitats cause evolved cue-response systems to become unreliable, 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 (Schlaepfer et al. 2002, 2005, Robertson et al. 2013, Hale and Swearer 2016, Singer and Parmesan 2018).

Evolutionary traps are not permanent, but the conditions leading either to escape through rapid local adaptation or to long-term persistence of maladaptive interactions are not well understood. One potential escape trajectory is improved exploitation (Chew, 1975, Schlaepfer et al. 2002), in which selection favors individuals that are

better at using the novel resource. But, 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 novel resource is completely lethal and no variation in ability to exploit it exists in the population (Chew 1977, Davis 2015), improved exploitation is not an immediately available option.

Alternatively, populations can decrease their preference for poor-quality, novel resources. In addition to gene flow, selection strength, and underlying genetic variation, this escape trajectory depends on recognition system flexibility and similarities between available cue sets. Cue sets involved in mistakenly selecting low-quality novel resources often resemble those of historical resources, but the strength of that resemblance can vary (Sih et al. 2011, Robertson et al. 2013). Extensive cue set overlap, whether in composition or intensity, may necessitate the evolution of a narrower or more refined recognition system, increasing the chance individuals reject high-quality resources (Fox and Lalonde 1993, Macmillan and Creelman 2004). Therefore, cue similarity will constrain evolutionary escape when fitness costs of rejecting good resources balance or outweigh those of mistakenly using trap resources maintaining the maladaptive behavior in the population (Fox and Lalonde 1993, Robertson et al. 2013).

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Cue similarity is an especially relevant constraint in 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). Specialization depends on an efficient recognition system (Holmgren et al. 2007), often at the expense of behavioral flexibility or plasticity (Bernays 2001, Carrasco et al. 2015). Discrimination among host plants by specialist herbivores is shaped by the costs associated with both accepting poor-quality hosts and excluding high-quality hosts (Wood et al. 2018). Excluding high-quality hosts is expected to be especially costly if insects are time-limited, rather than reproductively limited, when searching for hosts (Rosenheim et al. 2000, Snell-Rood and Papaj 2009).

Most butterflies are host plant specialists (Futuyma 1976, Hamm and Fordyce 2015) and host plant recognition is often mediated by plant chemistry (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 stimulants or lack deterrents necessary for identifying 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* species have populations involved in invasive plant-based evolutionary traps (Chew 1977, Keeler and Chew 2008, Nakajima et al. 2013, Davis and Cipollini 2014). In some cases, the formation of butterfly–host-plant evolutionary traps has been followed by rapid shifts in female preference, larval performance or both (Keeler and Chew 2008, Knerl and Bowers 2013). In others, however, acceptance of novel host plants persists despite poor larval performance (Stefanescu et al. 2006, Casagrande and Dacey 2007, Nakajima et al. 2013), although whether these behaviors are ultimately maladaptive is uncertain (see Brown et al. 2017).

Here, we evaluate the role of cue similarity in maintaining long-term 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 the highest concentration (dominant) chemical cues 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.

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, Colorado, USA (38°57'33.0" N 106°59'23.0" W, 2,900 m above sea level). These butterflies oviposit on the invasive Eurasian species *Thlaspi arvense* L. (Chew 1977, Nakajima et al. 2013). 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). Co-occurrence with *T. arvense* 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). As in many other insect populations (Singer and Thomas 1988, Fox 1993, Nylin et al. 2005), variation in *P. macdunnoughii* oviposition preference is heritable (R. A. Steward, R. S. Epanchin-Niell, and C. L. Boggs, *unpublished manuscript*). Therefore, fitness costs associated with laying eggs on *T. arvense* are likely to represent strong selection for escape from this trap. However, no adaptive response has been detected in the half-century since this evolutionary trap was first recorded (Chew 1975, 1977) and up to a century-and-a-half since the plant first invaded the Rocky Mountains (Warwick et al. 2002).

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 1977). Females accept a range of native and nonnative plants, including *T. arvense* (Chew 1977, Rodman and Chew 1980). Females in the East River Valley population generally lay more eggs on high-quality native hosts, but many butterflies lay a portion of their eggs on *T. arvense*. Oviposition by *Pieris* butterflies is stimulated by glucosinolates (GSLs; Huang and Renwick 1993, 1994, Huang et al. 1994, Du et al. 1995). These defensive chemicals are stored in plant tissues 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). The extent to which *Pieris* butterflies use these products when evaluating host plants is unclear (Wolfson 1980, Müller et al. 2015). Similar GSL structures occur in *T. arvense* and native host plants, but the extent of overlap and the relative concentrations of cue sets have not been quantified. The GSL profile of *T. arvense* appears to be very simple: only three unique GSL structures have 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). The primary native hosts of *P. macdunnoughii*, including *Cardamine cordifolia* (A. Gray) and *Descurainia incana* (Bernhardi ex Fischer & Meyer), produce GSLs, although only *D. incana* also produces sinigrin (Rodman and Chew 1980, Humphrey et al. 2018).

METHODS

Our study consists of three linked parts. First, we compared the GSL profiles of *T. arvense* and native hosts. Second, we tested the effect of sinigrin on female butterfly preference by comparing preference for sinigrin (1) against several other GSLs, (2) at different concentrations, and (3) in the presence of myrosinase. Third, we performed a multi-assay cross-over experiment to test whether decreased preference for *T. arvense* is correlated with decreased preference for *D. incana* and whether this is related to an overall decreased preference for sinigrin (Fig. 1).

Host plant chemistry

We collected fresh leaf samples from each of the focal host plants (*T. arvense*, *C. cordifolia* and *D. incana*) to confirm the GSL profiles. In 2015, samples were collected from three sites within the upper East River Valley, CO: *C. cordifolia* near the Judd Falls trailhead (38.967° N, 106.994° W), *D. incana* around the Gothic town site (38.959° N, 106.986° W), and *T. arvense* from the Snodgrass trailhead (38.920° N, 106.961° W). In 2017, we collected samples from a site within the Gothic town site (38.957° N, 106.989° W) where all three species co-occurred. We collected the fifth, sixth, or seventh leaf from the apical meristem of 5–15 plants of each species, placing leaves directly into screw-cap microcentrifuge

tubes containing 70% MeOH (Appendix S1: Table S1). Leaf samples were kept in a cool, dark location for 8–16 months, which allowed GSLs to leach into the MeOH.

Glucosinolates in MeOH leachates were desulfated following Prasad et al. (2012) and Keith and Mitchell-Olds (2017; Appendix S1). DesulfoGSLs were separated using high-performance liquid chromatography (HPLC). DesulfoGSLs structures were detected and quantified with a diode array detector (DAD) monitoring absorbance at 229 nm and subsequently with a charged aerosol detector (CAD). Only desulfoGSLs appearing in both the DAD and CAD output were included in analyses. Glucosinolates were identified using positive ion electrospray ionization with a mass spectrometer. Both mass spectra and comparative retention times from the literature (Tolrà et al. 2006, Clarke 2010, Kusznierevich et al. 2013, Olsen et al. 2016, Humphrey et al. 2018) were used to identify desulfoGSLs (Appendix S1: Table S2).

Glucosinolate preference assays

For all preference assays, gravid female butterflies were caught in the field, brought to the lab, weighed, and fed. We estimated 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°C) overnight before participating in an assay.

Assays were conducted in an environmental chamber (27–31°C: 17–19°C, 16 h: 8 h light: dark). Assay arenas consisted of cylindrical clear plastic cages (0.18 m height × 0.15 m diameter), in which the butterflies could fly and maneuver easily. Cage tops were perforated with 1-mm holes and the bottoms lined with damp paper towels to maintain airflow and humidity (Appendix S1: Fig. S1). Oviposition assays using GSL isolates were conducted using treated filter paper disks. Four Pastilina modeling clay (Sargent Art, Hazelton, PA, USA) bases (1 × 1.5 × 1 cm lwh) were placed in a square formation ~3 cm apart on the floor of the arena. Filter paper disks (3 cm diameter; 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 daily with freshly treated disks. While some pierid butterflies are deterred by the presence of conspecifics' eggs (Shapiro 1981, Schoonhoven 1990), the *P. macdunnoughii* used in these and other assays readily laid eggs on oviposition substrates (disks, leaves, etc.) already bearing their own eggs. We assumed for this study that the presence of eggs did not affect oviposition probability.

Glucosinolate choice.—Preference for sinigrin (SIN; GSL class, alkenyl) was tested against glucotropaeolin

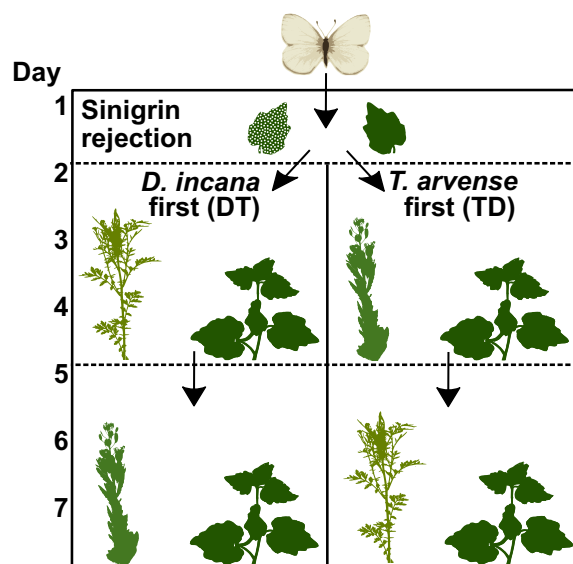


FIG. 1. Crossover design to test preference for sinigrin, *Thlaspi arvense*, and *Descurainia incana*. All butterflies were placed on a 1-d simultaneous choice assay between *Cardamine cordifolia* leaves painted with water or 25 mmol/L sinigrin solution (dotted leaf). Butterflies were then sequentially placed on choice assays between *C. cordifolia* and either *D. incana* (DT butterflies) or *T. arvense* (TD butterflies) for 3 d before being switched onto the second pair.

(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, Wisconsin, USA; Glucotropaeolin potassium salt: ChromaDex, Irvine, California, USA; Glucoiberin potassium salt: Cerilliant, Round Rock, Texas, USA) were used to make 1 mmol/L solutions. Butterflies were provided with three filter paper disks treated with 100 μ L of each of the GSL solutions or with a distilled water control, for an average of 1.63 μ mol/g filter paper (based on an average filter paper mass of 61.2 mg). In addition to chemicals, butterflies respond to visual stimuli (Traynier 1986, Snell-Rood et al. 2013). We added green food dye (McCormick Culinary Food Color, Hunt Valley, MD, USA: 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).

Sinigrin concentration.—Glucosinolate concentrations vary dramatically between and within species. We tested female preference for increasing concentrations of sinigrin. Wild-caught butterflies (2016, $n = 19$) were provided with a control disk (water, 0 mmol/L sinigrin, increased to 1×10^{-6} mmol/L sinigrin in the statistical analysis) and three disks treated with 100 μ L of increasing concentrations of sinigrin: 1, 10, or 25 mmol/L (1.63, 16.3, or 40.9 μ mol sinigrin/g filter paper). Solutions were prepared and applied as already described. Butterflies were tested up to five consecutive days or until they died. We repeated the experiment with lab-reared females (2017) that mated in an unsupervised cage before being placed on preference tests. 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 mmol/L (92.2 μ mol sinigrin/g filter paper).

Myrosinase addition.—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 μ L 25 mmol/L = 40.9 μ mol sinigrin/g filter paper) with or without myrosinase (Sigma Aldrich, Milwaukee, Wisconsin, USA). The sinigrin solution was prepared as we have described, including the addition of green dye. Disks were treated with equal amounts of sinigrin. An aliquot of 20 μ L myrosinase solution (1.67 units/mL KH_2PO_4 buffer) or an equal quantity of buffer without myrosinase was added to the center of the disk. 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 directly measure isothiocyanate produced by the addition of myrosinase. However, we compared the amount of sinigrin recovered from disks treated with sinigrin and buffer and those treated with sinigrin and myrosinase. Treated disks were placed into screw-cap microcentrifuge tubes containing 70% MeOH. DesulfoGSLs were quantified as described in *Host plant chemistry*. We also compared sinigrin content of the GSL solutions of various concentrations.

Sinigrin rejection assay and linked host plant preference

Sinigrin rejection.—We tested the sinigrin preference of wild-caught butterflies using a simultaneous choice assay between *C. cordifolia* leaves painted with either water or a sinigrin solution (Fig. 1). We used leaves rather than paper disks to identify decreased preference for sinigrin given an attractive background GSL 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 μ L distilled water or 25 mmol/L sinigrin solution and allowed to dry. We placed sinigrin-treated and control leaves in flower picks secured to the sides of the choice arena. All 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 h for a maximum of three days at which point all butterflies that did not lay eggs were released back at their collection sites.

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* (Fig. 1). Butterflies were provided with flower picks holding cut stems bearing leaves and, in most cases, the apical meristem of each plant (Appendix S1: Fig. S1). Cut stems are appropriate stand-ins for whole plants when assessing relative oviposition preferences (Friberg and Wiklund 2016). Water in the flower picks was refreshed daily. Except in cases of severe wilting, the same cut stems were used through the full three days of the host plant choice assays. Female behavior appeared unaffected by the presence of previously laid eggs. 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 (Fig. 1). We recorded the total number of eggs laid on each plant over the 3-d period, and these counts were used as the response variable in statistical analysis.

Statistical analysis

All statistical analyses were performed using the R Statistical Analysis platform (version 3.5.3, R Core Team 2018). We used a hurdle model approach to compare desulfoGSL quantities between plant species and collection year (Data S1: CueSim_Fig2_PlantLeachates.R). First, we qualitatively compared each sample, evaluating the presence or absence of desulfoGSLs using binomial generalized mixed models (BGLMMs, lme4 package; Bates et al. 2015). Second, we compared log-transformed quantities ($\mu\text{mol dGSL/g}$ of dry leaf mass) of GSLs detected in the leaf samples using linear mixed models (LMMs). We compared sinigrin concentrations in *D. incana* and *T. arvense* in 2015 and 2017 with Tukey's multiple comparison tests. Finally, we used principal component analysis to identify axes of divergence in normalized GSL concentrations among all the samples (FactoMineR package; 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; Data S2: CueSim_Fig3AB_GSLcomp.R, CueSim_Fig3BC_SinigrinGradient.R, CueSim_Fig32B_SINvITC.R). The predictors included in full models differed between assays. Models were hierarchically simplified, and we selected the model with the lowest Akaike information criterion corrected for sample size (AIC_c ; 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).

In addition to NBGLMMs described above, we also compared the relative probability that a female laid eggs on each of the solutions in the GSL choice assay using a multinomial model (nnet package; Venables and Ripley 2002). The numbers of eggs laid on each substrate are not independent and make up a proportion of the total eggs laid in the assay. In the NBGLMMs, butterfly identity was included as a random effect linking egg counts. We repeated the analysis with the multinomial model to more appropriately account for the non-independence of eggs laid on each substrate. The response variable was the combined eggs laid on each of the solutions (e.g., c (sinigrin eggs, glucotropaeolin eggs, glucoiberin eggs) ~ model predictors). Similarly, relative preference for sinigrin and for sinigrin treated with myrosinase was analyzed using quasibinomial generalized linear models (QBGLM). These models were hierarchically simplified as previously described.

To understand 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 NBGLMMs with number of eggs laid on the sinigrin-treated leaf as the response variable (Data S3: CueSim_Fig4_S3_SinigrinRejection.R). 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 two 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 QBGLM with the proportion of eggs laid in the first assay as the explanatory variable and the proportion of eggs in the second assay as the response variable.

RESULTS

Host plant chemistry

Thlaspi arvense had the simplest GSL profile compared to *C. cordifolia* and *D. incana* (Fig. 2a; Appendix S1: Table S1). Despite this simplicity, *T. arvense* did not have lower GSL concentrations than either of the native hosts (Fig. 2b). Instead, *D. incana* had the lowest total GSL concentration (two-way ANOVA, $F_{2,51} = 5.70$, $P = 0.006$). Quantitative 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 (Fig. 2a; Appendix S1: Tables S3, S4). Comparing between the *T. arvense* and *D. incana* profiles, *T. arvense* had significantly more sinigrin than *D. incana* in both 2015 (Tukey's multiple comparisons, t ratio = -6.82 , $df = 33$, $P < 0.001$; Fig. 2a), and 2017 (Tukey's multiple comparisons, t ratio = -4.60 , $df = 33$, $P < 0.001$; Fig. 2a).

The *C. cordifolia* GSL profile diverged considerably from those of the other two plants. In a principal

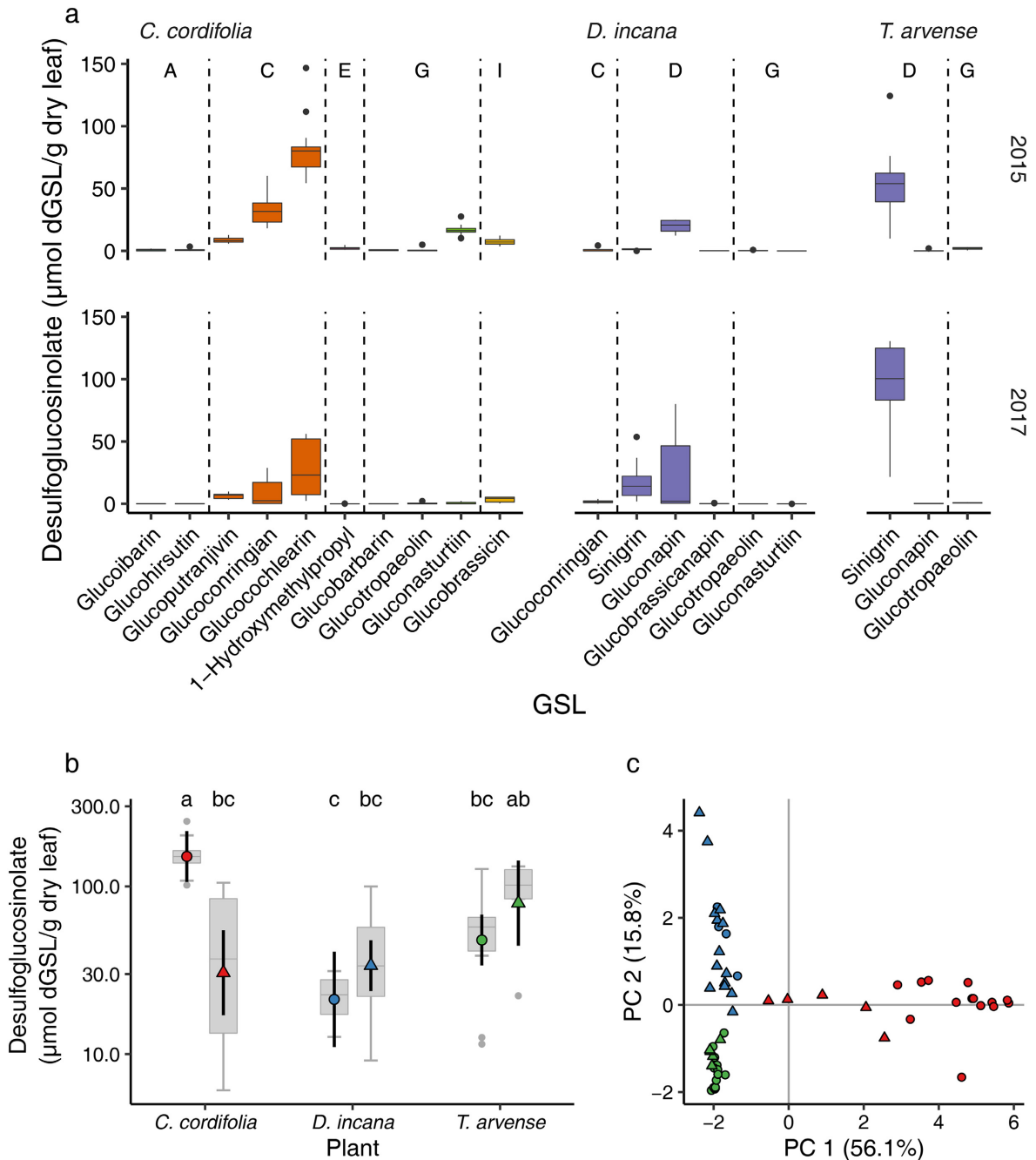


FIG. 2. (a) Desulfoglucosinolate quantities ($\mu\text{mol dGSL/g dry leaf}$) detected by CAD in *Cardamine cordifolia*, *Descurainia incana*, and *Thlaspi arvense* leaves. Dashed lines delineate GSL classes: A, sulfur-containing side chain; C, aliphatic branched chain; D, olefin/alkenyl; E, aliphatic straight and branched chain alcohols; G, aromatic; I, Indole. (b) Total dGSL quantities ($\mu\text{mol/g dry leaf}$) 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 box plots show distribution of the data. Box plot components are mid line, median; box edges, interquartile ranges; and whiskers, largest or smallest observation falling within 1.5 IQRs of the upper or lower quantiles. Letters indicate groups that are not significantly different (Tukey's multiple comparisons). (c) Principal component analysis of dGSL profiles. Points represent leaf samples of the three host plants. Shapes and colors are the same as Fig. 2b. Together, PC1 and PC2 explained 71.9% of the variance in dGSL profiles.

component analysis, *C. cordifolia* samples diverged on a primary axis that accounted for 58.6% of the variance and was primarily driven by aliphatic branched-chain GSLs (Class C, Fig. 2c). PCA axis 2 explained 15.8% of the variance with sinigrin, glucotropaeolin (benzyl GSL), gluconapin, and glucobrassicinapin (4-pentenyl GSL) as major contributors.

Glucosinolate preference assays

Glucosinolate choice.—Butterflies significantly preferred sinigrin-treated disks to those treated with glucotropaeolin or glucoiberin (NBGLMM, $\chi^2 = 42.6$, $df = 2$, $P < 0.001$; Table 2; Appendix S1: Table S5; Fig. 3a), with butterflies laying an estimated average 72.0% of eggs on sinigrin, 25.2% on glucotropaeolin, and only 2.8% on glucoiberin at average mass-wear values (multinomial GLM, $P < 0.001$; Table 2, Fig. 3b; Appendix S1: Table S5). While mass-wear residual was not a significant predictor of the number of eggs laid on each disk (Appendix S1: Table S5), larger butterflies laid a greater proportion of their eggs on sinigrin disks (multinomial model, $\chi^2 = 9.93$, $df = 2$, $P = 0.007$; Table 1; Fig. 3b). Response rates were low: only 14 of the 35 butterflies responded to the GSL cues. Among responding butterflies, none oviposited on the untreated control filter paper disk.

Sinigrin concentration and myrosinase addition.—For both wild-caught ($n = 18$) and lab-reared ($n = 11$) butterflies, preference for sinigrin plateaued at concentrations above 10 mmol/L (NBGLMM, Table 2, Fig. 3c, d; Appendix S1: Table S5). Myrosinase addition decreased the amount of desulfo-sinigrin recovered from 25 mmol/L sinigrin disks by 25.9% (95% CI 12.3–39.5%; Appendix S1: Fig. S2a). Despite the presence of allyl-isothiocyanate, butterfly ($n = 18$) preference did not differ between myrosinase-treated and buffer-treated disks (Table 2; Appendix S1: Table S5, Fig. S2b). In both assays, untreated controls were largely ignored, and butterfly preference was not affected by mass-wear residual (NBGLMM and QBGLM, Appendix S1: Table S5). Details can be found in Appendix S1.

Sinigrin rejection assay and linked host plant preference

Sinigrin rejection.—Among all butterflies ($n = 83$) there was no significant preference for sinigrin on a *C. cordifolia* leaf background (Appendix S1: Table S6, Fig. S3). 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.

Preference for sinigrin-containing host plants.—Butterflies laid more eggs on the first choice test they experienced, regardless of whether they started on *D. incana* and were then tested on *T. arvensis* (DT butterflies) or experienced the plants in the reverse

order (TD butterflies; Table 3, Fig. 4a, b). Preference in the sinigrin-rejection assay did not affect the number of eggs laid on either sinigrin-containing host (NBGLM, likelihood ratio test, $\chi^2 = 0.014$, $df = 1$, $P = 0.907$; Appendix S1: Table S6). However, while TD butterflies had equal preference for the sinigrin-containing host plant (*T. arvensis* or *D. incana*) and the *C. cordifolia* control in both assays (NBGLM Tukey's multiple comparison, Z ratio = -2.152 , $P = 0.137$; Fig. 4b; Appendix S1: Table S7), DT butterflies tended to prefer *D. incana* over *C. cordifolia*, but then laid significantly fewer eggs on *T. arvensis* than on *C. cordifolia* in the second assay (Tukey's multiple comparison, Z ratio = 4.178 , $P < 0.001$; Fig. 4a; Appendix S1: Table S7).

It is unlikely these differences were caused by differences in egg loads between DT and TD butterflies. Overall, DT and TD butterflies laid the same number of eggs in the sinigrin-rejection assay (LM, $F_{1,77} = 0.299$, $P = 0.088$) and laid similar numbers of eggs in the first host plant assay (LM, $F_{1,74} = 0.0187$, $P = 0.892$). 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, $df = 2$, $P = 0.270$; Fig. 4c, d; Appendix S1: Table S8). There was a loose cluster of eight TD butterflies that laid fewer than 50% of their eggs on both *T. arvensis* 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 prior experience (order; QBGLM, likelihood ratio test, $\chi^2 = 17.3$, $df = 1$, $P < 0.001$; Table 3).

DISCUSSION

We found similarities between the glucosinolate cues of the lethal invasive mustard *T. arvensis* 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 or other alkenyl GSLs. In isolation, sinigrin was a major oviposition stimulant. But we found no evidence of correlated preferences for these 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.

Shared sinigrin cues do not constrain host plant recognition

For the relative costs of host plant mistakes and misses to explain the persistence of the *T. arvensis*

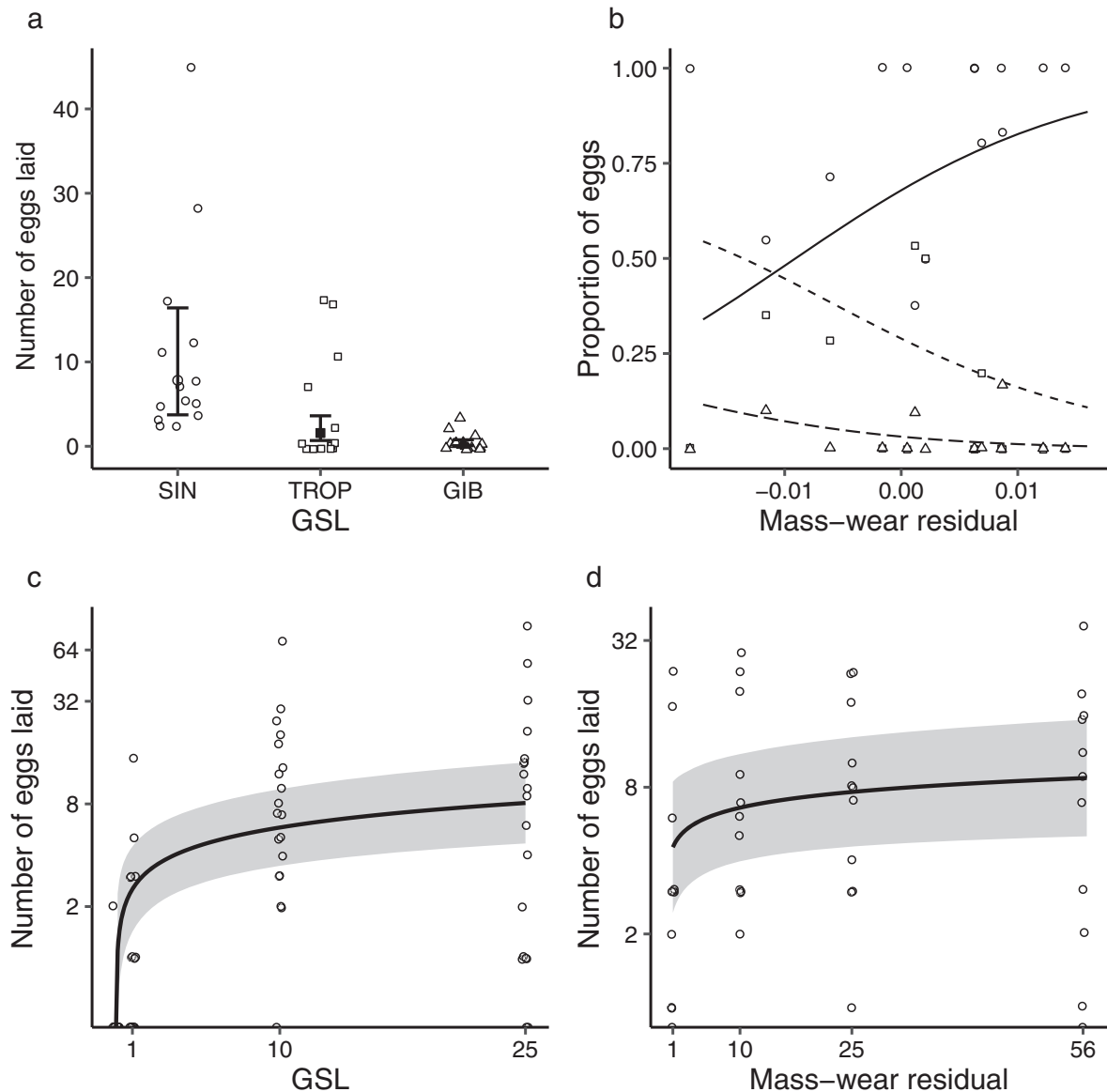


FIG. 3. (a) Total eggs laid and (b) proportion of eggs laid as a function of age-corrected mass (mass-wear residual) on disks treated with sinigrin (SIN, circles, solid line), glucotropaeolin (TROP, squares, short-dashed line), and glucoiberin (GIB, triangles, long-dashed line) in the GSL choice assay. Black points (a) represent negative binomial mixed model (NBGLMM) estimates (and 95% CI). 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 and (d) lab-reared females. Lines show NBGLMM model estimates (and 95% CI).

evolutionary 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 (Chew and Renwick 1995). Previous

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 planta* and on abiotic substrates (Ma and Schoonhoven 1973, Traynier and Truscott 1991, Huang et al. 1994, Huang and Renwick 1994, Du et al. 1995, Newton et al. 2010, Rohr et al. 2012).

TABLE 1. Analysis of deviance (Wald's χ^2 type II) comparisons of glucosinolate (GSL) differences between *Cardamine cordifolia*, *Descurainia incana*, and *Thlaspi arvense* in 2015 and 2017.

Response and predictor	χ^2	df	P
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 $\times$ GSL	33.9	24	0.086
Plant $\times$ Year	2.26	2	0.322
GSL $\times$ Year	22.2	12	0.036
Quantitative profile:			
GSL quantity ($\mu\text{mol/g}$ dry leaf)			
Plant	1.64	2	0.440
GSL	947	12	<0.001
Year	25.0	1	<0.001
Plant $\times$ GSL	164	4	<0.001
Plant $\times$ Year	0.048	2	0.976
GSL $\times$ Year	55.8	12	<0.001
Plant $\times$ GSL $\times$ Year	12.6	2	0.002

Note: The presence of GSL structures was compared using a binomial generalized linear mixed model while $\log_2$ -transformed GSL quantities were compared using a linear mixed model.

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 (butenyl GSL) and glucotropaeolin (benzyl GSL) over 2 yr 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. Our results suggest that variable sinigrin concentrations above a threshold are likely equally attractive to ovipositing females, and we did not detect any decrease in preference at very high concentrations. Sinigrin and alkenyl GSL dominance is a defensive strategy found in other members of the Thlaspidaceae (e.g., *Alliaria petiolata*; Cipollini 2002) and in the genus *Streptanthus* (Thelypodaceae; 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

TABLE 2. Effects of glucosinolate (GSL) compound (SIN, sinigrin; TROP, glucotropaeolin; GIR, glucoiberin), concentration and myrosinase (MYR) addition on oviposition preference.

Assay, model, and comparison	Estimate	SE	z ratio	P
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 vs. TROP	-0.853	0.212	-5.02	<0.001
SIN vs. GIR	-3.068	0.429	-7.15	<0.001
TROP vs. GIR	-2.214	0.439	-5.05	<0.001
Sinigrin concentration				
NBGLMM, fixed effect				
$\log_2$ (2016 concentration)	0.362	0.071	5.12	<0.001
NBGLMM, fixed effect				
$\log_2$ (2017 concentration)	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

Note: The negative binomial generalized linear mixed model (NBGLMM), multinomial model and quasibinomial generalized linear model (BGLM) estimates and standard errors are given on the log, log and logit link scales, respectively.

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, we found that sinigrin was a stronger oviposition stimulant than aromatic or short sulfur-containing side-chain GSLs, and this preference is likely to be equally strong when hydrolysis products (ITCs) are also present. Stimulatory effects of isolated sinigrin are also well documented for North American *Pieris* species *P. oleracea* (Huang and Renwick 1994, Du et al. 1995) and *P. virginensis* (Davis et al. 2015). There is a mismatch between preference for sinigrin and larval performance. Sinigrin has a 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

TABLE 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 (*Descurainia incana* or *Thlaspi 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, model, response variable, and fixed effects	Estimate	SE	z ratio	P
Sinigrin rejection				
NBGLM (dispersion = 4.42), response variable number of 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 = 1.84), response variable eggs				
Intercept	-0.660	0.504	-1.31	0.190
log ₂ (total eggs)	1.05	0.118	8.92	<0.001
Assay (<i>T. arvense</i>)	-1.22	0.300	-4.08	<0.001
Order (TD)	-0.362	0.311	-1.16	0.246
Assay × Order	1.05	0.458	2.30	0.021
QBGLM (dispersion = 11.0), response proportion of eggs (second assay)				
Intercept	-1.51	0.299	-5.07	<0.001
Order (TD)	1.48	0.374	3.97	<0.001

Notes: Assay and order refer to the choice assay (*D. incana* or *T. arvense* tested against the *Cardamine 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.

did not affect the average preference of female *P. virginiensis* 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. 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.

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 methanol-soluble chemical cues extracted from host plant leaves, sex-linked genetic variance decreased significantly and the remaining genetic variance was autosomal (R. A. Steward, R. S. Epanchin-Niell, and C. L. Boggs, *unpublished manuscript*). 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).

Experience modifies host plant preference

Rather than supporting innate correlated preferences between sinigrin-containing host plants, our results instead suggest experience is a major determinant of oviposition preference. Butterflies responded similarly to the sinigrin-containing plants in the first set of assays. Generally, butterflies preferred *D. incana* over *C. cordifolia* more than they preferred *T. arvense* over *C. cordifolia*, but neither trend was significantly different from equal preference. However, while butterflies moved from the *T. arvense* assay onto the *D. incana* assay maintained this roughly equal preference, those that had experienced the *D. incana* assay first were far less likely to oviposit on *T. arvense*. These results reveal that not only do *P. macdunnoughii* females modify their preference based on previous experience, but the effect of experience only manifests on certain host plants. This is an intriguing alternative hypothesis for the persistence of maladaptive oviposition preference that merits further direct testing.

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 et al. 2009, Snell-Rood and Papaj 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-Stille 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 infer (Morand-Ferron 2017, Nieberding et al. 2018). Testing biased behavioral plasticity in natural plant assemblages will be

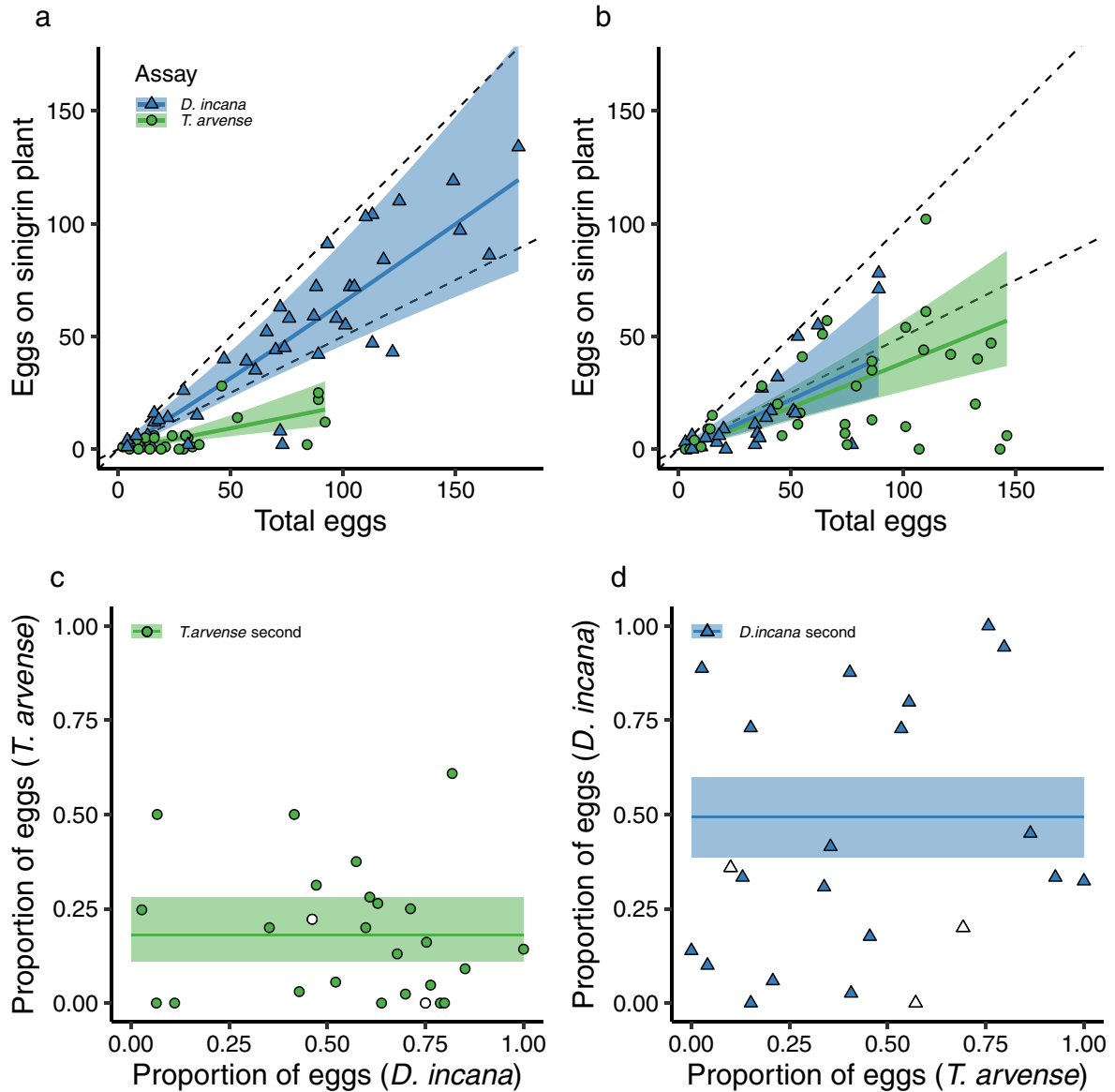


FIG. 4. Eggs laid on the sinigrin-containing host plant (*Descurainia incana*, blue triangles; *Thlaspi arvense*, green circles) in simultaneous choice assays by (a) butterflies starting on the *D. incana* assay and (b) those starting on the *T. arvense* assay. Dashed lines (slope = 0.5 and 1.0) indicate equal preference for the sinigrin-containing host and the control (*Cardamine cordifolia*) or complete preference for the sinigrin-containing host. Solid lines represent negative binomial GLM estimates (and 95% CI, Table 3; Appendix S1: Table S6). 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 ($\pm 95\%$ CIs, Table 3; Appendix S1: Table S8).

critical to understanding its effects on this evolutionary trap.

As our results support, lepidopteran oviposition preferences are not static, changing both in response to the environment and as a result of experience. Here, we made the simplifying assumption that eggs laid on available host plants were independent events, and that presence of eggs per se or plant induced changes to eggs do

not affect subsequent oviposition probabilities. *Pieris macdunnoughii* generally lay eggs singly in the wild but, in our host plant choice assays, butterflies were only provided with a single stem of each host plant, which is a common approach for testing *Pieris* preference (e.g., Friberg et al. 2015). Attraction to eggs could falsely exaggerate host plant preferences, whereas avoidance of eggs may obscure preference differences between host plants.

Raitanen et al. (2014) found that conspecific eggs were attractive to ovipositing *Pieris napi*, although *P. rapae* and *P. brassicae* avoid conspecific larvae and the chemical cues of conspecific eggs (Schoonhoven et al. 1990, Blaakmeer et al. 1994, Sato et al. 1999). In addition, Brassicaceae are well known for their induced defensive responses to egg-laying (Hilker and Meiners 2006, Geiselhardt et al. 2013, Firtzlaff et al. 2016), which, in some cases, deter subsequent oviposition (Blaakmeer et al. 1994). The precise effects of the presence of eggs on oviposition behavior of *P. macdunnoughii* are unknown.

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 the relative abundance of *T. arvense*. A second 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 species from strong negative selection pressure from environmental change, it has the potential to decouple preference phenotypes from underlying genotypic frequencies, thereby dampening phenotypic evolution in response to environmental change (Ghalambor et al. 2007, Berthon 2015, Murren et al. 2015, Gibert et al. 2019). For example, learning slowed the evolution of innate diet preferences in artificially selected *Drosophila melanogaster* lines, although this effect was diet specific (Mery and Kawecki 2004). 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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