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Novel host unmasks heritable variation in plant preference within an insect population

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Introductions of novel plant species can disturb the historical resource environment of herbivorous insects, resulting in strong selection to either adopt or exclude the novel host. However, an adaptive response depends on heritable genetic variation for preference or performance within the targeted herbivore population, and it is unclear how heritability of host-use preference may differ between novel and historical hosts. *Pieris macdunnoughii* butterflies in the Rocky Mountains lay eggs on the nonnative mustard *Thlaspi arvense*, which is lethal to their offspring. Heritability analyses revealed considerable sex-linked additive genetic variation in host preference within a population of this butterfly. This was contrary to general predictions about the genetic basis of preference variation, which are hypothesized to be sex linked between populations but autosomal within populations. Evidence of sex linkage disappeared when butterflies were tested on methanol-based chemical extracts, suggesting these chemicals in isolation may not be the primary driver of female choice among available host plants. Although unexpected, evidence for within-population sex-linked genetic variation in preference for *T. arvense* over native hosts indicates that persistent maladaptive oviposition on this lethal plant must be maintained by alternative evolutionary dynamics such as migration- or drift-selection balance or pleiotropic constraints.

KEY WORDS: **Glucosinolates, heritability, Lepidoptera, novel host plant, oviposition preference, sex linkage.**

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

In addition to selection pressure, an adaptive response of herbivores to a novel plant also requires heritable genetic variation for either preference or performance (Hoffmann and Merilä 1999; Strauss et al. 2006). In the presence of both selection and heritable variation, the rate of evolution will depend on the strength and consistency of selection, the degree of heritability, and the genetic architecture of the selected trait. For example, the frequency of beneficial fully or partially recessive alleles of sex-linked genes is likely to evolve faster than similar alleles of genes located on autosomes (Charlesworth et al. 1987, 2018; Irwin 2018), because the effects of these recessive alleles are not masked in the heterogametic sex (Mank 2009; Irwin 2018).

Additionally, ecological novelty in the form of nonnative, maladaptive host plants can affect the genetic basis for and variance of preference or performance traits (Kawecki 1995; Carroll et al. 2003), as new cue sets may reveal previously neutral genetic variation (Hoffmann and Merilä 1999). It is not clear, however, how heritability of preference for novel host plants may differ from heritability of preference for historical native hosts, and how this in turn may promote or constrain adaptive responses to novel hosts.

Lepidoptera, especially butterflies, are particularly susceptible to maladaptive use of novel plants (Yoon and Read 2016; Singer and Parmesan 2018). Most butterfly species have very specialized diets, feeding on plants from no more than three families (Forister et al. 2015). Although individual variation in host plant preference is determined by many factors, the number (range) and preferred order (ranking) of plants used as hosts depend largely on evolved recognition systems to identify and evaluate host plant chemistry and quality (Thompson and Pellmyr 1991). Rapid shifts in both host range and ranking have occurred in response to the introduction of novel plants (Singer et al. 1993; Keeler and Chew 2008; Forister et al. 2013).

In many cases, Lepidoptera and other insects first interact with a novel host through the egg-laying decisions of females, making oviposition preference an important phenotype for exploring how the structure of heritable genetic variation affects adaptive responses to novel hosts. Previous research detected a sex-linked genetic basis for oviposition preference differences between species of butterflies and between geographically distant populations (Thompson 1988; Scriber et al. 1991; Sperling 1994; Janz 1998, 2003; Nygren et al. 2006; Chaturvedi et al. 2018; but see Sheck and Gould 1995; Forister 2005; Hora et al. 2005). This pattern has been linked to the existence of stable host ranks in most populations, where the order in which females tend to prefer plants is based on intrinsic characteristics of the plant, such as secondary chemistry or nutritional quality (Janz 1998, 2003; Bossart and Scriber 1999). Given different and relatively stable plant communities, adaptive sex-linked genes for host preference may respond more quickly to selection, leading to fixed differences in preference among populations and species (Scriber 1994). Rapid accumulation of sex-linked differences in preference may subsequently act to reinforce population or species divergence, as has been observed for other adaptive sex-linked traits (Qvarnström and Bailey 2008). In contrast, as genetic variation decreases on the sex chromosomes, either by selection or by drift, the remaining variation affecting preference within populations is likely to be spread across the autosomes. Consistent with this prediction, studies within butterfly and moth populations have found that detectable variation in oviposition preference tends to exhibit autosomal inheritance (Tabashnik et al. 1981; Singer et al. 1988; Jaenike 1989; Bossart and Scriber 1995; Nylin et al. 2005; Singer and Parmesan 2021). However, to our knowledge, no one has examined whether this inheritance pattern persists within populations whose stable historical resource environment has been disturbed by novel host plants.

Part of a Holarctic expansion and speciation of the *Pieris napi* species complex, *Pieris macdunnoughii* (previously *Pieris napi macdunnoughii*) is a montane butterfly found in regions of Montana, Wyoming, and Colorado (Geiger and Shapiro 1992; Chew and Watt 2006). Like most butterflies of the Pierinae, *P. macdunnoughii* specializes on Brassicaceae, including the native host plant *Cardamine cordifolia* (Gray) (Chew 1975, 1977). In the southern Rocky Mountains of North America, *P. macdunnoughii* females also recognize and lay eggs on the invasive Eurasian mustard *Thlaspi arvense* (Brassicaceae), even though this novel host plant is completely lethal to the butterflies' larvae (Chew 1975; Nakajima et al. 2013; Steward et al. 2019). Spatially explicit models of butterfly-host plant interactions within a focal invaded population determined that this oviposition mistake results in a significant fitness cost in the modeled population (∼3%; Nakajima et al. 2013) and should select for decreased preference for *T. arvense*. However, in the 150 years since *T. arvense* was introduced to the region, and the 45 years since the maladaptive interaction was first recorded, the butterfly continues to recognize and oviposit on the invasive mustard. Whether evolution will, or even can, occur in populations where the invaded plant is abundant depends on whether there is heritable genetic variation in preference for native host plants over *T. arvense*.

Like many butterflies, *Pieris* species uses chemical cues to find their mustard host plants (Ikeura et al. 2010), and the primary chemicals linked to oviposition behavior by these butterflies are glucosinolates and their derivatives (Chew and Renwick 1995). Glucosinolates are alcohol-soluble secondary metabolites that generally play a defensive role for plants in the Brassicaceae (Agerbirk and Olsen 2012). However, with the help of specialized detoxification mechanisms, butterfly larvae within the Pierinae can consume and develop on plant tissue containing glucosinolates (Wittstock et al. 2004; Burow et al. 2006; Wheat et al. 2007; Edger et al. 2015), and adult females use specific glucosinolate compounds as oviposition cues (Huang and Renwick 1993, 1994; Huang et al. 1994; Du et al. 1995; Yang et al. 2021). Although gravid female butterflies use visual cues (e.g., color) and plant volatiles to identify potential hosts over larger spatial scales (Ikeura et al. 2010; Itoh et al. 2018; Lund et al. 2019), contact chemoreception of glucosinolates both on the leaf surface and within the leaf tissue, accessed by drumming with the foretarsae (Thiele et al. 2016), is critical for females ultimately ovipositing on a given host plant (van Loon et al. 1992; Städler et al. 1995; Yang et al. 2021). Thus, maladaptive host plant recognition by *Pieris* butterflies in North America has largely been attributed to host plant chemistry, specifically glucosinolate composition

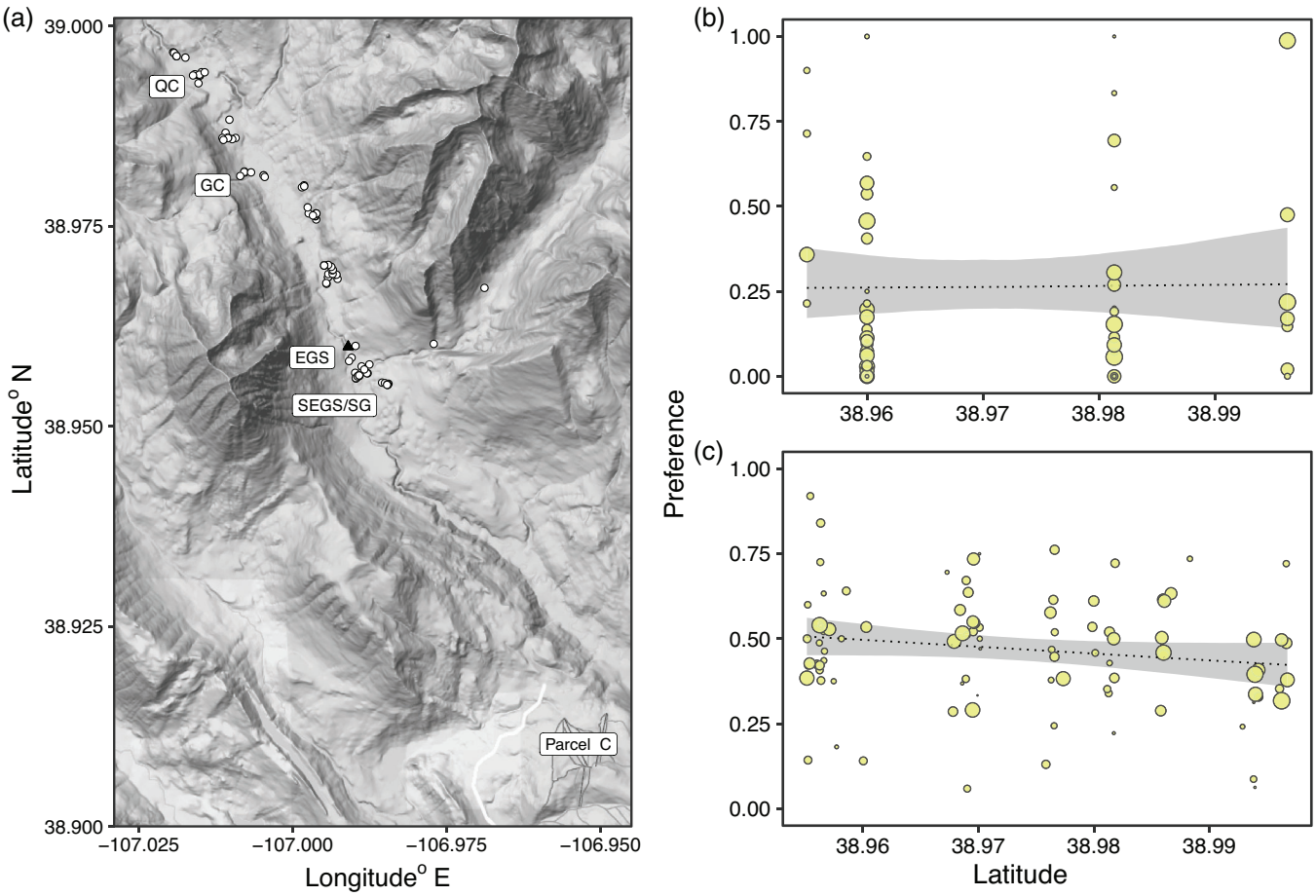

Figure 1. **Preference of wild-caught butterflies along a latitudinal gradient. (a) Collection sites for female butterflies tested on whole plants (Parcel C), cut stems (QC = Quigley Creek, GC = Gothic campsite, EGS = *Euphydryas gillettii* site, and SEGS/SG = South *E. gillettii* site and South Gothic), and locations of individuals tested on plant extracts (white circles) in the upper East River valley near Gothic, CO. The black triangle represents the northernmost extent of *Thlaspi arvense* within the valley as of 2015, which was similar to the extent in 1997 and 2006. Collection locations were mapped with *ggmap* (Kahle and Wickham 2013) using Google terrain maps (2018). Preference for *T. arvense* did not change with collection latitude when tested on (b) cut stems or (c) plant extracts. Dotted lines and 95% credible predicted intervals (gray) are based on Bayesian beta-binomial models (Tables S6 and S7). Point sizes are proportional to the total number of eggs laid (maximum on cut stems: 94, plant extracts: 274).**

and concentration (Keeler and Chew 2008; Nakajima et al. 2013; Davis and Cipollini 2014; Steward and Boggs 2020).

Here, we quantified heritability of preference for *T. arvense* within a population of *P. macdunnoughii* butterflies. Using simultaneous choice assays, we compared preference for *T. arvense* versus a common native host using whole plants, cut stems bearing leaves, and methanol-based leaf tissue extracts. We hypothesized that variation in preference within this population is heritable with an autosomal basis, resulting in daughters with similar preferences to their mothers. We further expected that evidence for autosomal inheritance would be strongest on plant extracts, as these directly assessed the butterflies' responses to host plant chemistry, thought to be a primary mediator of the maladaptive host use. We found that preference for *T. arvense* was weakly but significantly heritable and with a large sex-linked component, suggesting that lack of heritable genetic variation is not constraining the evolution of preference in this population.

Methods

Pieris macdunnoughii butterflies used in this study were collected over multiple summer field seasons (1997, 2006, 2015) from the sites near Gothic, CO in the upper East River Valley of the Gunnison Basin in central Colorado (Fig. 1a). Depending on snowmelt, overwintering pupae eclose in late May or early June and adult butterflies can be seen into early August. The adult population peaks in late June (Nakajima et al. 2014). A second smaller peak has been observed in mid-July; however, larval development times under natural conditions (40–51 days, depending on host plant; Chew 1975) are too long for the offspring of

this second peak to reach diapause before winter, meaning the population is functionally univoltine. However, direct development remains plastic in the population, and multiple generations can be reared under lab conditions by manipulating larval exposure to light (similar to *P. napi*; Pruisscher et al. 2017). *Thlaspi arvense* is an early successional plant that rapidly colonizes exposed soil and is most consistently found in heavily disturbed areas (e.g., construction sites, roadways, recreational trailheads, and meadows open to cattle grazing). Already established in the Great Plains of North America in the early 1800s (reviewed by Warwick et al. 2002), it is likely *T. arvense* was introduced to the Elk Mountains and Gunnison Basin between 1850 and 1880 as disturbance increased with the influx of miners and ranchers. *Thlaspi arvense* is recorded as present in the Gunnison Basin from the beginning of Rocky Mountain Biological Laboratory (RMBL) herbarium records in 1929, and it has been abundant near Gothic since at least the 1970s (Chew 1975).

All plants used in the preference assays were collected from sites near Gothic for all 3 years of the study (Table S1). Preference for *T. arvense* was tested in simultaneous choice assays against a preferred native host, *C. cordifolia*, which is abundant throughout the East River Valley. Although sourcing plants from the field by transplanting whole plants or cutting stems has the potential to introduce variation in plant chemistry, either from natural environmental variation or by damage incurred when bringing the plants into the lab, there is mixed evidence whether this damage produces a directional bias in host preference (Friberg and Wiklund 2016; but see Bruinsma et al. 2007).

We conducted a total of three heritability studies over a span of 18 years. During this time, *T. arvense* in the East River Valley remained abundant in areas of high recreation use and other frequent disturbance. Anecdotally, butterfly population sizes remained large in both invaded and uninvaded areas. Oviposition preferences of all butterflies across 1997, 2006, and 2015 were tested using simultaneous choice assays conducted in the same laboratory space and conditions. Butterflies were allowed to choose between *T. arvense* and a native host (*C. cordifolia)*, in the form of either whole plants, cut stems bearing undamaged leaves, or filter paper treated with methanol-based leaf tissue extracts and a negative control substrate, and in all cases, preference was measured as the number of eggs laid on *T. arvense* out of the total eggs laid.

OVIPOSITION PREFERENCE ON WHOLE PLANTS

Adult gravid female *P. macdunnoughii* butterflies were collected over 3 days (June 25–27, 1997) from Parcel C (now known as "Prospect"), a tract of land on the north side of Mt. Crested Butte south of Gothic, CO, and adjacent to areas invaded by *T. arvense* (Fig. 1). In the lab, the females were fed twice a day with a 25% honey-water solution. Females were kept in $0.23 \times 0.23 \times 0.23$ m screen cages, with one pot each of *T. arvense* and *C. cordifolia* and one pot of clover (*Trifolium pretense* F., Fabaceae), a nonhost plant that does not stimulate oviposition. Empty space in the cage was filled with a neutral substrate, crumpled newspaper, on which the butterflies could land. The size of the cages was small enough to ensure that butterflies would encounter both plants easily, minimizing the role of visual cues (e.g., plant height) and plant volatiles in mediating first contact with a host plant. In all generations, larval host plants were matched by estimated leaf area and whenever possible, plants were also matched phenologically (preflowering, flowering, seeding). Due to natural phenological differences between the host plants, this was not always possible and was a lower priority than leaf area. The cages were stored in an environmental chamber at 27–31°C during the day and 20–22°C at night on a 16:8 L:D cycle and were rotated arbitrarily each day. Eggs from each host plant were counted and collected every evening.

Larval offspring were reared in the environmental chambers under the same conditions as the ovipositing females. To reduce the level of maternally transmitted disease, eggs were briefly submerged in 0.075% hypochlorite solution, then rinsed with water. After treatment, eggs hatched, and larvae developed on *C. cordifolia*, which supports rapid development (Chew 1975). Plants were replaced as needed during larval development. Pupae were collected after hardening of the cuticle, sex was determined, and pupae were grouped by sex and brood and left to emerge in screen cages in the environmental chamber.

Upon adult emergence, the F1 butterflies were numbered individually on the hindwing with permanent fine-tip pen, and maternal identity was recorded. Matings were obtained by placing up to 20 individuals from desired broods into $30 \times 45 \times 45$ cm net cages, which were placed outdoors in direct sunlight. We tried to mate offspring of mothers that laid at least 30 eggs in preference trials. The ground surrounding the cages was kept moist to maintain high humidity. Multiple mating cages were run at one time, and by placing males and females of the same brood in different cages, we avoided sib-sib matings. Cages were checked at least every 45 min, mating pairs removed, and mating combinations recorded. We aimed to mate each male with at least two females from different broods to produce pairs of half-sib families. Preference tests were repeated on the F1 generation. Their F2 offspring were reared, mated, and also tested, creating a three-generation pedigree in which all grandmothers and both parents of the F2 generation were known. Although over 1000 butterflies were reared in the lab, the final dataset included 37 P, 34 F1, and 138 F2 females that laid eggs in the preferences tests.

Plants used in preference tests were transplanted from the field (Table S1) into 10-cm-square pots filled with local soil, with one exception: *T. arvense* used in oviposition tests for the F2 generation was grown in potting soil from local seed.

OVIPOSITION PREFERENCE ON CUT STEMS WITH LEAVES

Butterflies used in heritability tests were collected from five sites along the East River valley (QC, GC, EGS, and SEGS/SG; Fig. 1) over 1 week (June 23–30, 2006). Females were kept in the lab in the same screen cages and cared for as described for 1997. They were provided with cuttings of *C. cordifolia* and *T. arvense*—again matched by size and, when possible, phenology—placed in separate 10-cm-deep florist picks with water. Previous work on congener *Pieris rapae* found that preference hierarchy does not differ between whole plants and cut stems (Friberg and Wiklund 2016). A dandelion (*Taraxacum officinale* Weber Asteraceae) flower was placed in the cage in a florist pick and spritzed with 25% honey-water twice a day to supplement its nectar. Butterflies were hand-fed on the flower twice a day. F1 and F2 generation butterflies were reared and mated as described for whole plants and tested on cut stems with leaves. A total of 65 P, 44 F1, and 121 F2 females laid eggs in the 2006 preference tests and were included in the final models. However, only 36 P females had F1 offspring that also mated and laid eggs.

OVIPOSITION PREFERENCE ON METHANOL LEAF EXTRACTS

Butterflies were collected from many locations along a 5-km transect of the upper East River valley (Fig. 1; June 18 to July 21, 2015). Females were brought back to the lab, fed 25%–30% honey-water solution, and held at room temperature overnight. The following morning, females were placed into cylindrical clear plastic cages (0.18 m height × 0.15 m diameter) with 1-mm holes punched around the top to maintain airflow. The floor of the arena was lined with a damp paper towel. Four Pastilina modeling clay (Sargent Art) bases (0.5 × 1 × 0.5 cm) were placed in a square formation ∼3 cm apart. Filter paper disks (3 mm diameter; Grade 1, Whatman) were placed vertically in each clay base (Fig. S1). Filter paper disks were treated with 80 μL of either *T. arvense* or *C. cordifolia* methanol extract, as described below. The two other disks included a control (70% MeOH only) and a blank (untreated), to test whether the butterflies preferentially laid eggs on extract-treated disks. This design allowed us to test preference for the major glucosinolates and other alcohol-soluble secondary metabolites in *T. arvense* and *C. cordifolia* leaves. Eggs laid on each disk were counted and collected, and the disks were replaced with freshly treated disks daily for up to 6 days or until the butterfly died.

Eggs were sterilized, then hatching larvae were transferred to rearing cages containing *C. cordifolia* leaves and kept in the environmental chamber (27–31:20–22°C, 16:8 L:D). When *C. cordifolia* was unavailable, larvae were fed young radish leaves (*Raphanus sativus*), which support similar larval survival as native hosts (Chew 1975). Larvae were given constant access to fresh food plant until pupation, at which point they were removed from the larval rearing cages, grouped by sex and brood, and held at room temperature in screen cages. Eclosing butterflies were numbered individually and placed into mating cages as in 1997 and 2006. We primarily used offspring of females that laid at least 15 eggs. This lower cutoff was chosen because, although some butterflies laid many eggs on the filter paper, many laid fewer than our original 30 egg cutoff. This, combined with a viral infection and poor mating success, limited our sample size. Again, matings were arranged so no sib-sib matings occurred and to encourage re-mating of males with females from different broods. Mated females in the F1 and F2 generations were tested in the same way as the P generation. Over 1000 butterflies were raised in the lab, but the final dataset comprises 104 P, 41 F1, and 48 F2 females.

Several butterflies in the F1 and F2 entered diapause, rather than developing directly. These pupae were held in an incubator (1–2°C, 12:12 L:D cycle) for 5 months, at which point the temperature was raised and the light cycle adjusted (27:17°C, 16:8 L:D) to bring the butterflies out of diapause. Butterflies were mated under artificial heat lamps in greenhouses. The F2 offspring of diapausing F1 individuals developed directly and were reared on juvenile radish plants grown from seed before mating and being tested. Although diapausing butterflies in the F2 generation laid 8.62% (±8.37% credible interval) fewer of their eggs on *T. arvense* extracts (ANOVA, $F = 4.08$, df = 1,44, $P = 0.050$), preference did not differ between direct developing and diapausing females in the F1 generation (ANOVA, $F = 1.23$, df = 1,38, $P = 0.275$). We concluded there was not enough support to include diapause as a covariate in the models and both diapausing and direct-developing individuals were included in the final analyses.

PREPARATION OF EXTRACTS

Fresh host plants were collected in the field. Leaves were removed from the stems of the freshly collected plants, weighed in small packets, and transferred to liquid nitrogen. To make the methanol extracts, we modified an extraction procedure from Agerbirk and Olsen (2011): once frozen, the leaves were lightly crushed, and boiled in 70% MeOH for several minutes before filtering. Excess MeOH was used to boil the leaves, so the filtrate was left to evaporate for 24 h. We added a small amount of 70% MeOH to achieve equal concentrations (10 g fresh weight/L) in the two extracts. Plant extracts were stored in a dark, cool fridge to prevent the light-sensitive glucosinolates from degrading. Throughout the experiment, we collected and froze 80 μL samples of the extracts (24 of *C. cordifolia*, 19 of *T. arvense*) to evaluate their glucosinolate content.

In addition to chemical oviposition stimulation, butterflies respond to visual stimuli (Traynier 1986; Snell-Rood 2013). The

colors of the two extracts differed slightly, so we added green food dye (McCormick Culinary Food Color: water, propylene glycol, FD&C Yellow 5, FD&C Blue 1, and propylparaben) to both extracts and the MeOH control (1 mL dye/15 mL extract or MeOH). In the rare cases when butterflies laid eggs on disks not treated with extracts, they were far more likely to lay on the green (1.086% ± 0.406% of eggs) than the white disks (0.113% ± 0.106% of eggs, paired *t*-test on number of eggs, $t = 4.725$, df = 181, $P < 0.001$).

GLUCOSINOLATE DESULFATION AND QUANTIFICATION

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

Desulfoglucosinolates were quantified in the University of South Carolina Mass Spectrometry Center using a Thermo Scientific Ultimate 3000 High Performance Liquid Chromatography system with a 3400RS binary pump. Chromatography was carried out using a Chromegabond WR C18 column (ES Industries; 150 × 2.1 mm, 3 μm particles, 120 Å pore size). The mobile phase contained HPLC-grade water and acetonitrile, with a 0.2 mL/min flow rate and the following gradient: 0% acetonitrile (0–3 min), ramp to 20% (3–30 min), hold at 20% (30–37 min), ramp rapidly to 85% (37–44 min), return to 0% acetonitrile (44-end). The injection volume of samples was 20 μL. Desulfoglucosinolates were detected and quantified with an Agilent 1100 G1315B diode array detector (DAD) monitoring absorbance at 229 nm and subsequently with a Thermo Scientific Corona Veo RS charged aerosol detector (CAD). Only desulfoglucosinolates appearing in both the DAD and CAD output were included. Glucosinolates were identified using positive ion electrospray ionization with a Waters QTof API US quadrupole time-of-flight mass spectrometer. Both mass spectra and comparative retention times from the literature (Tolrà et al. 2006; Kusznierewicz et al. 2013; Olsen et al. 2016; Humphrey et al. 2018) were used to identify desulfoglucosinolates (Table S2).

At the time leaves were collected to make the extracts, we also collected fresh leaf samples to ensure the glucosinolate components of our methanol extracts captured the glucosinolate profile of fresh leaf tissue. The sixth leaf from the apical meristem of 15 plants of each species was collected directly into screw-cap microcentrifuge tubes containing 70% MeOH. Leaf samples were kept in a cool, dark location for 8 months, which allowed glucosinolates to leach into the surrounding MeOH. Glucosinolates in the leachate were desulfated and quantified as described for extracts. One *C. cordifolia* sample was discarded due to contamination. Our glucosinolate identification and quantification was sufficient for comparing host plant leaves and extracts but should not be used for comparative analyses or reviews of plant chemical defenses.

STATISTICAL ANALYSIS

We first tested for spatial differences in preference of wild-caught females in the parental generation using Bayesian beta-binomial models (Data S1; *brms* package; Buerkner 2017, 2018, 2020). Although butterflies tested on whole plants all came from the same area (Fig. 1a, Parcel C), butterflies tested on cut stems and plant extracts were collected over a 5-km transect in the Upper East River Valley. For the parental generation of butterflies tested on cut stems, we used the centroid of the collection site as the collection latitude. For butterflies tested on plant extracts, individual collection locations were recorded by GPS. Models were run with uninformative priors for 40,000 iterations with a warmup of 10,000 and thin of 5. The effect of collection location was assessed using Leave One Out information criteria (LOOIC) and Bayes Factors calculated with BayesTestR (Makowski et al. 2019) to determine whether the sampling area comprised multiple source populations that could affect heritability estimates. We were unable to directly test this in the heritability models described below as the F1 matings did not retain this collection location spatial structure.

We then used Bayesian beta-binomial multilevel models to evaluate the contribution of Z-linked (V_z), autosomal (V_a), and environmental variance (V_e) to the proportion of eggs laid on *T. arvense* in our choice assays (phenotypic variance, V_p) (Data S1). Heritability (h^2) was calculated as the proportion of V_p attributable to genetic variance (e.g., ${h_a}^2 = V_a/V_p$, where $V_p = V_z + V_a + V_e$). The environmental variance (i.e., V_e) comprised the product of the overdispersion parameter (ω) and the fixed variance of the binomial distribution that is proportional to $\pi^2/3$ (Nakagawa and Schielzeth 2010; Reid et al. 2011; see the Supporting Information for details).

Models followed the form: *response* ~ *predictors* + *(1|individual, cov = A)* + *(1|individual, cov = Z)*, where the response was the number of eggs laid on *T. arvense* out of the total eggs laid. Predictors included generation and trial start day. *A* and *Z* specified the covariance of the random effects based on the autosomal and Z-linked relatedness matrices, respectively. Relatedness matrices were generated from pedigrees using *nadiv* (Wolak 2012), taking into account that among Lepidoptera females are the heterogametic sex, having both Z (=X) and W (=Y)

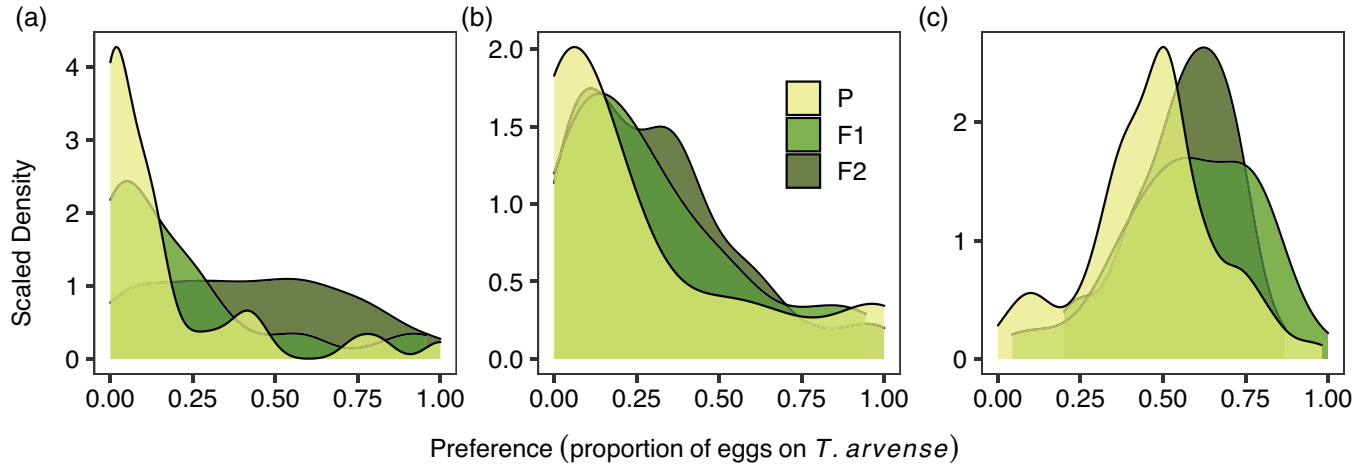

Figure 2. **Preference variation among generations. Preference (proportion of eggs laid on *Thlaspi arvense* in simultaneous choice experiments) was measured for wild-caught parental, lab-reared F1, and lab-reared F2 generations tested on (a) whole plants, (b) cut stems, and (c) plant extracts. Preference for *T. arvense* whole plants was also affected by within-generation start day (Table S4).**

chromosomes, whereas males are homogametic ZZ (Robinson 1971; Sperling 1994). The preference test start day was calculated as an ordinal day from first test day within each generation. All models were run using the same set of partially informative priors for 40,000 iterations with a 10,000-iteration warmup and a thin of 5. Full models tested the interaction of generation and start day and were compared to reduced models using Leave One Out information criteria (LOOIC) and Bayes Factors calculated with BayesTestR. We consistently chose the model with the highest relative evidence based on the Bayes factor comparisons (Table S3), and in most cases these concorded with the lowest LOOIC estimate (Table S3). The contributions of fixed predictors were evaluated by comparing the 95% credible interval of posterior distributions to zero.

Autosomal and Z-linked variances were calculated from the standard deviations of the random intercepts of A and Z in the model output. We used the "hypothesis" method in *brms* to evaluate whether the 95% credible intervals for heritability estimates provided support for heritable variation in oviposition preference. In many cases, it is appropriate to account for the variance in the fixed effects in the total phenotypic variance when calculating heritability (de Villemereuil et al. 2018). This can be difficult for non-Gaussian distributions, so in addition to calculating heritability based on best-fit models, we also calculated heritability based on the intercept-only models to characterize the possible influence of the fixed effects.

Results

SPATIAL VARIATION

Butterflies tested on whole plants were all collected from a single location (Parcel C), but butterflies tested on cut stems and extracts were collected along a 5-km transect in the East River Valley before being tested (Fig. 1a). This spatial variation had no effect on preference for cut stems (Fig. 1b; Bayesian beta-binomial model; Table S3) among wild-caught females. Butterflies from areas were *T. arvense* is absent (higher collection latitudes) were slightly less likely to oviposit on *T. arvense* (Bayesian beta-binomial model, coefficient = –8.2, 95% credible interval = –18.0 to 1.6); however, this trend was not statistically robust (Table S3). Overall, there was no evidence that the spatial structure of our sampling locations influenced egg laying preference and subsequent heritability estimates.

VARIATION AMONG GENERATIONS

When female oviposition preference was tested on whole plants, butterflies tended to prefer native *C. cordifolia* (the proportion of eggs laid on *T. arvense* ranged from 0 to 1, with a skew toward 0; Fig. 2a). This was also true of butterflies tested on cut stems (Fig. 2b; Table 1). In both years, preference shifted toward *T. arvense* in subsequent F1 and F2 generations, especially F2 females (Table S4). Start day, as a proxy for plant age and quality, was only retained as a covariate in the final model explaining preference for whole plants (Table S3). Rather than a constant decline in preference for *T. arvense* over the summer, the effect of start day differed among generations and was largely driven by declining preference for *T. arvense* by F2 individuals tested in late August and early September (Table S5). Unlike butterflies tested on plants, wild-caught butterflies tested on extracts did not prefer *C. cordifolia* over *T. arvense* (Fig. 2c; Table 1). Very few butterflies, and none from the later generations, oviposited exclusively on one plant extract. Testing butterflies on extracts effectively eliminated the temporal variation introduced by testing them on plants

Table 1. Sample sizes and estimated preferences, defined as the proportion of eggs laid on *Thlaspi arvense* out of the total eggs laid in a simultaneous choice assay, of the parental (P), first lab-reared (F1), and second lab-reared (F2) generations. Preference was estimated using beta-binomial models and the median total eggs laid in each generation.

					Effect of generation	
Year	Substrate	Generation	Number of females	Median total eggs	Preference	95% credible interval
1997	Whole plants	P	37	54	0.139	0.079–0.225
		F1	34	60	0.167	0.097–0.263
		F2	140	49	0.430	0.323–0.544
2006	Cut stems	P	36	34	0.219	0.152–0.298
		F1	44	59	0.299	0.198–0.423
		F2	121	55	0.312	0.233–0.399
2015	Methanol extracts	P	104	64	0.468	0.424–0.512
		F1	41	68	0.579	0.504–0.651
		F2	48	85	0.560	0.485–0.631

and stems, the characteristics of which may change with time (Tables S5 and S6).

HERITABILITY

On both whole plants and cut stems, the majority of additive genetic variance was attributed to Z-linked components (Fig. 3a), with narrow-sense heritability estimated at 0.089 (95% credible interval: 0.0.039–0.015) using whole plants and 0.095 (0.044–0.153) using cut stems (Fig. 3b; Table S7). In both experiments, the phenotypic variance apportioned to autosomal additive genetic variance (V_a) was consistently, although not statistically, lower than Z-linked components. These results are loosely supported by marginally nonsignificant correlations between the preferences of F2 butterflies and their paternal grandmothers (Fig. S2) on both whole plants ($R = 0.19$, $P = 0.056$) and cut stems ($R = 0.13$, $P = 0.15$).

Heritability estimates for preference tested on extracts were negligible (Fig. 3b; Table S7). Interestingly, although the variance attributed to the relatedness matrices was lower than those estimated for whole plants and cut stems, the relative variance attributed to autosomal and Z-linked components (Fig. 3c) was similar to the other studies (Fig. 3a,b). There was no correlation between paternal grandmother and granddaughter preferences (Fig. S2f).

Although we did not explicitly account for the variance of the fixed effects in our models (see de Villemereuil et al. 2018), comparing all models for each oviposition substrate (Fig. 3d, gray points and credible intervals), we found that the fixed effects trivially impacted the heritability estimates, marginally decreasing estimates for both V_z and V_a.

GLUCOSINOLATE COMPONENTS

The majority of glucosinolates detected in leaf leachates (Fig. S3a) were also recovered in methanol extracts (Fig. S3b) for both *T. arvense* and *C. cordifolia*. Olefins and branched chain aliphatic glucosinolates were the dominant glucosinolates in *T. arvense* and *C. cordifolia*, respectively. Absolute concentrations applied to filter paper were considerably lower than those estimated for fresh leaves. Relative concentrations of different compounds were generally similar between leaves and extracts, with several exceptions. For example, aromatic glucosinolates were found in *T. arvense* leaf samples, and have previously been found in the *T. arvense* glucosinolate profile (Rodman and Chew 1980), but were not detected in methanol extracts (Fig. S3a).

Discussion

SEX-LINKED HOST PREFERENCE WITHIN A POPULATION

Our analysis of within-population hostplant preference using over 600 adult females revealed significant, heritable additive genetic variation in host plant preference. Contrary to our expectations, estimates of sex-linked heritability were almost twice those of autosomal heritability when preference was measured with whole plants and cut leaves (average $h_Z = 0.092$, $h_A = 0.050$). Within-population inheritance of lepidopteran oviposition preference, attributed more often to specificity and female motivation than differences in the overall attractiveness of available host plant species, has previously been ascribed an autosomal genetic basis (Tabashnik et al. 1981; Singer et al. 1988; Jaenike 1989; Bossart and Scriber 1995; Nylin et al. 2005). Sex linkage, on the other hand, is more common for fixed differences between populations and species (Janz 1998, 2003). For example, geographically distant populations of the Comma butterfly, *Polygonia c-album*, consistently demonstrated sex-linked differences in host-plant choice (Janz 1998; Nygren et al. 2006), whereas preference variation within populations was inherited autosomally (Nylin et al. 2005). Preference differences between *Papilio*

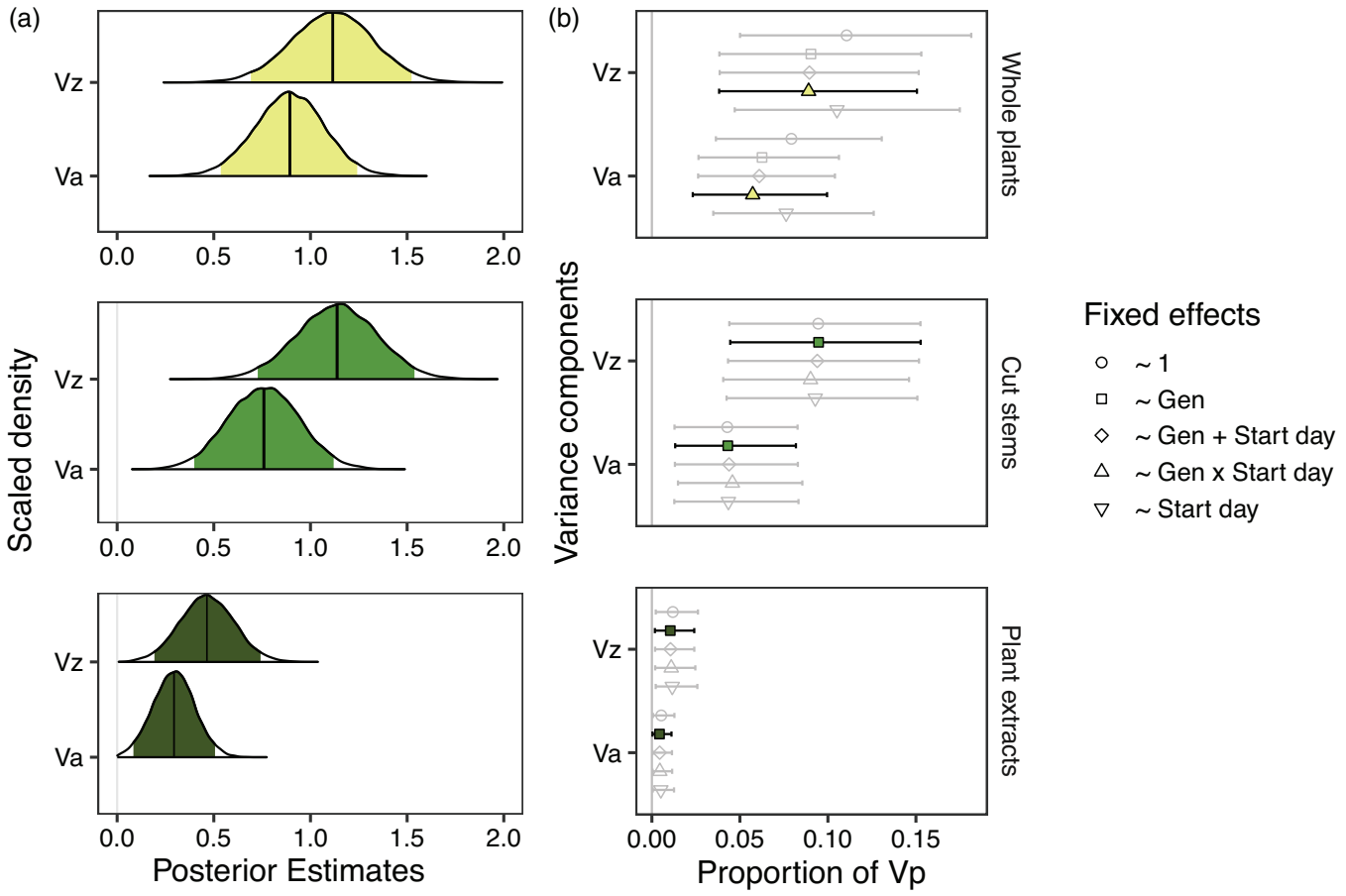

Figure 3. Heritability of preference. (a) Posterior density estimates for the random effects of autosomal (V_a) and Z-linked (V_z) relatedness matrices for butterflies tested on whole plants, cut stems, and extracts. Distributions very close to zero have weaker support than those with higher estimates and normal distributions. (b) Proportion of the phenotypic variance (V_p) attributed to additive autosomal genetic variance (V_a) and additive Z-linked genetic variance (V_z; Table S5). Credibility intervals are constrained by the hypothesis-testing approach to not overlap zero. Values further from zero indicate greater contributions of the variance component to V_p and higher heritability. Changing the fixed effects in the models trivially affected estimates of V_z and V_a, and these model results are shown using gray points and credible intervals.

glaucus and *Papilio canadensis* were also sex linked (Scriber 1994), but sex linkage disappeared within a late-flying hybrid population (Mercader and Scriber 2007).

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

LACK OF HERITABILITY OF PREFERENCE FOR PLANT EXTRACTS

Although female butterflies rely on a variety of cues to identify host plants, host plant recognition and preference is overwhelmingly attributed to plant chemistry (Dethier 1954; Ehrlich and Raven 1964; Renwick 1989; Carrasco et al. 2015). However, plant chemistry can change rapidly because of contact, damage, oviposition, or abiotic conditions (Louda and Rodman 1983; Cipollini et al. 2005; Mithöfer and Boland 2012). We expected methanol-soluble host plant chemistry captured in our extracts, specifically glucosinolates, to be the major driver behind

variation in preference for *T. arvense*. As we predicted, butterflies were stimulated to lay eggs on methanol-based leaf extracts and extracts eliminated some of the preference variation that may have been caused by differences in plant quality or chemistry. Start day, which affected preference of whole plants, did not significantly affect preference of extracts, suggesting extracts successfully eliminated differences among cues presented to butterflies within and between generations.

Although butterflies were able to discriminate between extracts from the two plants, heritability of this preference was very low. In fact, heritability estimates were only weakly supported by the model, with slightly more support for sex-linked over autosomal inheritance of preference based on the distribution of posterior estimates. The stark differences in both heritability estimates and overall phenotypic variance among the studies suggest that the methanol extracts were missing cues crucial to oviposition preference variation in this population.

The leaf extracts used in our study did not capture the full array of phytochemical cues available to ovipositing butterflies (Fig. S3), limiting our conclusions about the relative importance of glucosinolates in the differences we observed between plants and extracts. The extracts captured most of the glucosinolate compounds found in *T. arvense* and *C. cordifolia* leaves, but the concentrations applied to filter paper were lower than those of fresh leaves and these low concentrations likely contributed to the overall decreased preference variation on extracts. Furthermore, although glucosinolates are expressed on the leaf surface of other Brassicaceae (e.g., *Arabidopsis thaliana*; Shroff et al. 2015) and females likely have access to interior leaf glucosinolates through drumming, the exact glucosinolate profiles available to females when alighting on *C. cordifolia* or *T. arvense* are unknown. However, taste sensilla on the tarsal forelegs of *P. rapae* are sensitive to low concentrations of glucosinolates (Yang et al. 2021) and our methanol extracts were strong enough to elicit oviposition (Fig. S1). Rather, the large differences between heritability estimates on extracts compared to whole plants and cut stems suggest that the loci responsible for responding to the particular cues captured in our extracts are unlikely to be playing a primary role when female butterflies are choosing between *T. arvense* and *C. cordifolia*.

It is possible the differences observed between the studies, conducted at 9-year intervals, were influenced by changes in preference for *T. arvense* within the population, rather than in response to the oviposition substrate. Although host plant preference can change within lepidopteran populations over a generation (Singer 2003), no major shifts in preference for *T. arvense* have been observed over the 45 years this population of *P. macdunnoughii* has been studied (Chew 1975; Nakajima et al. 2013; Steward and Boggs 2020). It is also unlikely that sex-linked genetic variation was lost from the population in under two decades, when the population(s) have been exposed to the plant for closer to 15 decades. Although beyond the scope of this paper, these heritability estimates combined with knowledge of both the role of experience in older wild females (Steward and Boggs 2020) and the impact of the relative spatial and temporal distributions of potential host plants (Nakajima et al. 2013), provide a solid foundation for modeling the complex dynamics of preference evolution in *P. macdunnoughii* in response to *T. arvense*.

ENVIRONMENTAL VARIATION IN OVIPOSITION PREFERENCE

We found that butterflies collected from the wild consistently laid more eggs on *C. cordifolia* than *T. arvense*, especially when tested on whole plants. In lab-reared individuals, preference shifted toward *T. arvense*, a trend that may have resulted from learning, differences in mating experience or fecundity, life history, or variation in plant traits over time. Unlike the F1 and F2 generation, wild-caught female butterflies likely had prior oviposition experiences on hosts available in their habitat. Experience ovipositing on native host plants decreases preference for *T. arvense* (Steward and Boggs 2020). Additionally, prior experience has been shown to affect *P. napi* preference for available host plants, especially when the historical relationship between the butterfly and host plant is old (Gamberale-Stille et al. 2019). Wild-caught butterflies also had the opportunity to mate multiple times. Decisions by females are influenced by fecundity, which can modify the relative risk of poor host choice. *Pieris napi* butterflies with low fecundity were less likely to oviposit on lower ranked hosts (Schäpers et al. 2017). We expect that any effect of experience or fecundity in our experiment increased environmental variance, making the heritability estimates more conservative. Additionally, female host use in the wild and host preference as measured in the lab are sensitive to many environmental factors, including habitat characteristics and plant apparency, phenology, and quality (Chew and Renwick 1995). The importance of different cues from these environmental factors varies over spatial scales (Chew 1977; Nakajima et al. 2013; Lund et al. 2019). Here, we have focused on testing preference in small cages ($<$0.23 m between substrates) where females were highly likely to alight on both plant or extract alternatives, with the goal of limiting the role of visual cues (e.g., plant apparency or height) and plant volatiles affecting butterfly search behavior at larger distances (Itoh et al. 2018). Importantly, this design is comparable with previous studies of the heritability of host preference (Scriber et al. 1991; Janz 1998; Nylin et al. 2005; Nygren et al. 2006). However, given the importance of these cues, and the different sensory systems necessary for evaluating them, it would be highly interesting to quantify heritability of preference at larger spatial scales.

Conclusions

Our study revealed an unexpected genetic basis for preference for a novel host plant. However, given heritable genetic preference variation and considerable selection against oviposition on *T. arvense*, it is even more puzzling that we have found no evidence for increased avoidance of the lethal host. Evolutionary stasis in this population of *Pieris macdunnoughii* is likely due to other constraints, such as genetic drift, pleiotropy, temporal and spatial variation in the strength of selection, gene flow from naïve populations, or phenotypic plasticity that constrain or mitigate selection pressures from this evolutionary trap. For example, recent evidence suggests that experience-based plasticity in preference by individual butterflies indeed supersedes preference for specific glucosinolates (Steward and Boggs 2020). Combined with the results of our extract assays, it appears that glucosinolates remain an important cue for host plant preference, but additional stimuli may ultimately influence choice between available host plants. Such stimuli could be chemical (e.g., nitrogen or water content), environmental (e.g., local humidity, temperature, light), or structural (e.g., plant architecture, leaf hairiness). Studies evaluating the genomic basis of preference for both native and nonnative hosts will be necessary to evaluate the heritable mechanisms maintaining maladaptive preference in *P. macdunnoughii* populations.

AUTHOR CONTRIBUTIONS

RAS, CLB, and RSEN designed the experiments. RSEN and CLB collected data in 1997, CLB collected data in 2006, and RAS collected data in 2015. RAS analyzed the data. RAS wrote the first draft of the manuscript. RAS, CLB, and RSEN contributed to subsequent revisions.

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DATA ARCHIVING

All data and scripts used to generate results and create figures are available in the Supporting Information. In addition, all data and scripts have been archived on DRYAD, and can be found at https://doi.org/10.5061/dryad.5tb2rbp6w.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Collection sites for Thlaspi arvense and Cardamine cordifolia plants used in choice assays.

Table S2. Glucosinolates detected both by diode array detection (DAD) and charged anion detection (CAD). Glucosinolates were identified by mass spectra and comparative retention times. Glucosinolate identification and quantification was sufficient for comparing the chemistry of host plant leaves and extracts but should not be used for comparative analyses or reviews of plant chemical defenses.

Table S3. Estimates and 95% credible intervals of models testing the effect of collection location on wild-caught butterfly preference for cut stems or methanol extracts for all three generations. Models were compared to the null model (∼1) using bayes factors and the expected log predictive density (ELPD). Coefficient estimates and credible intervals (CI) are reported on the logit scale. Credible intervals from two-sided hypothesis tests (Intercept = Collection location; brms::hypothesis()) that overlap 0 are good indicators that the effect of collection location on preference for T. arvense is not statistically meaningful.

Table S4. Relative evidence (Bayes factors, larger numbers indicate greater evidence) for models listed across the top compared to the model on the left. Model predictors: Gen = generation, Date = adjusted start date, (1|A) = random intercept of the autosomal covariance matrix, (1|Z) = random intercept of the Z-linked covariance matrix.

Table S5. Expected log predictive density (ELPD) and Leave one out information criteria (LOOIC) estimated for Bayesian beta-binomial models of host plant preference. Final models indicated in bold. Model predictors: Gen = generation, Date = adjusted start date, (1|A) = random intercept of the autosomal covariance matrix, (1|Z) = random intercept of the Z-linked covariance matrix.

Table S6. Estimates and 95% credible intervals of fixed effects in final models for butterfly preference of whole plants, cut stems or methanol extracts for all three generations. Estimates are reported on the logit scale. The percentage of the full posterior distribution inside the region of practical equivalence (ROPE) and the probability of direction (PD) indicate the significance of each fixed effect.

Table S7. Variance component and heritability estimates and associated 95% credible intervals from best fit models for T. arvense preference for whole plants, cut stems and plant extracts.

Figure S1. Wild-caught P. macdunnoughii female with eggs on filter paper treated with MeOH leaf extract in 2015 (photo credit: C. Cerrilla).

Figure S2. Mother-daughter and paternal grandmother-granddaughter correlations of proportions of eggs laid on T. arvense approximate the autosomal and sex-linked inheritance patterns, respectively, for butterflies tested on (a, b) whole plants, (c, d) cut stems and (e, f) plant extracts. In the mother-daughter correlations, points are colored according to the daughter generation (F1 = light green, F2 = olive green). Only females laying more than 25 eggs were included in the correlation, and point size is scaled by the average total eggs laid both butterflies to visually represent confidence in each point estimate.

Figure S3. Glucosinolate concentrations in (a) methanol extracts made from leaves of the same plants and used in choice assays (Table S2) and (b) from leachates of C. cordifolia and T. arvense leaves collected in the field. Concentrations were calculated using high performance liquid chromatography coupled with charged anion detection (HPLC-CAD). Glucosinolate numbers correspond to those from Fahey et al., (2001; Table S2) and are ordered and colored by structural class (A = sulfur-containing side-chains, C = Aliphatic, branched chains, D = Olefins, NA = Unidentified, G = Aromatic, I = Indolic). Our glucosinolate identification and quantification was sufficient for comparing host plant leaves and extracts but should not be used for comparative analyses or reviews of plant chemical defenses.

DataS1

DataS2