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Complementary effects of two plant defence traits on behaviour and performance of monarch butterfly caterpillars

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Abstract

1. Plants have evolved multiple defensive traits in response to herbivory; in turn, herbivore specialists evolved adaptive behaviours to avoid or tolerate such defences. Here, we employ milkweeds (*Asclepias* spp.) to test two defences, latex and trichomes, for their independent and interactive effects on behaviour and performance of monarch caterpillars (*Danaus plexippus*).
2. Latex exuded upon damage and the density of leaf trichomes positively correlate across milkweed species, suggesting they may have evolved together as synergistic defences. Nonetheless, the complementary roles of these two traits have been little-studied. We focus on two behaviours: shaving, or the removal of trichomes, and chewing, which encompasses both deactivation of latex and leaf consumption.
3. In an experiment with seven milkweed species, with and without manipulated latex flow, we found latex to be the primary determinant of reducing chewing, while both defences positively predicted shaving behaviour in the first instar. Next, we conducted a factorial experiment throughout the first three instars, manipulating latex and trichomes on a high-latex, high-trichome species, the woolly milkweed *Asclepias vestita*. On plants with latex and trichomes intact, caterpillars spent the most time shaving and least time chewing of all treatment groups, suggesting a possible synergism. These defence-driven behavioural effects decreased later in larval development.
4. Latex and trichomes both impacted monarch performance, additively increasing mortality and reducing growth of survivors. Thus, latex and trichomes represent two important plant defences with effects on specialist herbivore behaviour and implications for insect fitness.

KEYWORDS
Asclepias, latex, plant-insect interactions, synergism, trichomes

INTRODUCTION

Plants often display multiple defences, using a combination of physical, chemical, and metabolic traits to deter herbivory (Chen, 2008; Rasmann & Agrawal, 2009; Ryabushkina, 2005). Such defensive traits may interact (redundantly, synergistically, antagonistically or additively), modulating how they impact herbivore behaviour and performance. For example, tropical seaweeds produce calcium carbonate, a physical defence, as well as several secondary metabolites which act synergistically (i.e. their combined effects are greater than the sum of their individual effects) to reduce herbivore damage (Hay et al., 1994). In other plant systems, jasmonic and salicylic acid represent complementary chemical pathways which can act either antagonistically or synergistically to promote defence, depending on whether pathways are activated separately or simultaneously (Jiao et al., 2022). Such interactions play a role in the ecology of plant-herbivore interactions as well as the coevolution of plant defence and herbivore counter defence (Gatehouse, 2002).

Asclepias (milkweeds) is a well-studied genus of plants that occupy a wide range of habitats and show diverse phenotypic traits, including multiple defences (Agrawal, 2005). A host of caterpillars, beetles, and other insects specialise on milkweed as their main food source (Agrawal et al., 2024; Farrell, 2001). As a result, milkweeds and their insect specialists have coevolved to have unique, reciprocally adapted traits. While milkweed produces toxins, waxy coatings, and other physical defences against feeding herbivores, insects have evolved highly specialised behaviours to avoid and circumvent these defences (Agrawal & Fishbein, 2008; Dussourd, 1999; Robertson et al., 2015). Studying these behaviours, particularly as they relate to the deactivation of plant defence and insect survival, has been a topic of interest for several decades and raises the question of how adaptive behaviours, insect performance, and complementary plant defences interact (Dussourd & Denno, 1994; Dussourd & Eisner, 1987). Notably, milkweed species have a range of physical and chemical defences, and examples of both synergism and antagonism have been proposed in this system (Edwards et al., 2023).

In this study we focus on two defences, latex and trichomes, which function to reduce herbivore colonisation on milkweeds in the field (Agrawal, 2005). Latex is a thick, sticky liquid that exudes from damaged leaves and deters insects from feeding (Agrawal & Konno, 2009). Trichomes are hair-like structures which cover the exterior of leaves and make it difficult for herbivores to access the plant tissue (Levin, 1973). Both latex exudation and trichome density vary widely across milkweed species (Agrawal et al., 2008, 2009), within species, and among plants growing in different habitats (Agrawal et al., 2012). Further, these two traits show correlated evolution; species high in latex tend to have high-trichome densities and vice versa (Agrawal et al., 2009). Importantly, this correlation has made it difficult to determine how each of these factors individually affects insect behaviour and performance, and whether the traits act redundantly, additively or synergistically (Agrawal et al., 2009; Edwards et al., 2023; Rasmann & Agrawal, 2009). Although the nature of their interaction is not well-studied, the correlated evolution of latex and trichomes, as well as their shared function as defences, may indicate a potential synergism.

Milkweed specialists, including monarch caterpillars (*Danaus plexippus*), the focus of this study, exhibit adaptive behavioural patterns in response to each of these defences. The primary behaviour for reducing latex intake is termed *trenching*, in which an insect punctures the leaf vein and drains latex prior to feeding, especially in the early instars. This behaviour can be quite effective, reducing latex ingestion by up to 92% compared to intact leaves (Dussourd, 1999). Indeed, monarch caterpillars cut milkweed leaf veins in distinct ways depending on host milkweed species and developmental stage (Agrawal, 2017). Trichomes can also be removed by herbivores, using a *shaving* behaviour where the mandibles are used to cut trichomes from the leaf before feeding (Malcolm, 1994). Shaving has also been shown to significantly improve feeding and growth in monarchs (Robertson et al., 2015). While the general effects of latex and trichomes in the monarch-milkweed system have been studied, their independent and complementary roles in predicting specialised behaviours, as well as monarch performance across development, are not well-understood (Robertson et al., 2015; Zalucki et al., 2012). Furthermore, the causal role of these plant defences in predicting caterpillar behaviour and performance has been difficult to determine.

FIGURE 1 *Asclepias vestita* with a monarch egg in Puma Canyon Ecological Preserve, California. Photo by Wendy Walker.

We conducted two experiments to determine the roles of latex exudation and trichome density on the behaviour and performance of first, second, and third instar *D. plexippus* larvae. We hypothesized that latex and trichomes would act synergistically to predict behaviour and performance, particularly in reducing chewing and inhibiting performance. In a comparative study of seven *Asclepias* species, with and without manipulated latex flow, we observed behaviour of first instar caterpillars on plant species with variable latex exudation and trichome density. We then conducted a factorial manipulation of latex and trichomes on a high-latex, high-trichome species, the woolly milkweed *Asclepias vestita* (Figure 1), and quantified behaviour and performance during the first three instars. Specifically, we addressed the following questions: (1) Are latex and trichomes predictive of monarch shaving and chewing behaviour and performance? (2) If so, do latex and trichomes act independently, additively or synergistically to predict behaviour and performance?

MATERIALS AND METHODS

Experiment 1: Early effects of latex and trichomes on caterpillar behaviour

To test caterpillar behaviour across a range of conditions, we grew seven species of North American milkweeds with known variability in latex exudation and trichome density: *Asclepias angustifolia*, *Asclepias incarnata*, *Asclepias exaltata*, *Asclepias syriaca*, *Asclepias speciosa*, *Asclepias californica*, and *A. vestita* (Agrawal & Fishbein, 2008). Seeds were collected by the authors or purchased from native plant nurseries. Seeds were stratified, germinated, and fertilised twice in the conditions described in Agrawal et al. (2021). We planted seedlings in 10-cm plastic pots for 6 weeks and watered them three times per week. Plants were raised in a growth chamber with approximately 350 μE of light on a LD 12:12 h cycle (28/26°C). At the end of the growth period, the five healthiest plants of each species (for a total of 35) were used for the experiment. Plants used were relatively young, with an average of around 8–12 leaves each.

Danaus plexippus eggs were obtained from Monarch Watch (Lawrence, Kansas) and stored at room temperature until hatching, at which point they were stored without food in an insulated cooler with an ice pack for less than 24 h. We used a total of 70 first instar larvae for all observations.

We conducted behavioural observations in growth chambers over 3 days, with a randomised block design of five temporal blocks of 14 treatments (7 species/2 latex treatments) with one replicate per block. In each round, 14 caterpillars were observed for 2 min at a time, revisiting each one five times approximately every 30 min (a total of 10 min of observation of each caterpillar, conducted over 3 h). The first observation started immediately after each caterpillar was placed on a leaf. Each caterpillar was placed on one of the seven plant species assigned to one of two treatments: a *cut* leaf treatment, in which it was placed in a petri dish on the adaxial surface of a cut leaf from its plant, or an *intact* treatment, in which it was placed on the terminal bud of its intact plant. This treatment manipulates latex flow, as latex no longer exudes from a severed leaf. Although some residual latex may remain after severing the leaf, we removed excess latex from the cut stem. Furthermore, no latex exudation occurs for caterpillars puncturing severed leaves, indicating that our treatment effectively removed latex. Thus, we will refer to cut leaves as a *no-latex* treatment, while an intact plant is a *latex* treatment. We recorded the amount of time spent performing each of five behaviours: chewing, shaving, walking, non-walking (typically head waving), and no movement. Durations of behaviours were recorded in seconds, out of a total of 600 s of observation (Table S1). We define chewing as all time spent severing latex canals and consuming leaves; at this point in development, feeding and trenching are indistinguishable so both are encompassed under 'chewing' behaviour. Shaving is defined as the removal of trichomes from the leaf surface. These two behaviours can be distinguished by whether only the trichomes or the plant tissue itself is damaged, which is visible under a magnifying glass for first instars.

To assess variability in physical defences across species, we measured the wet latex mass and trichome density of each of the 35 plants. These traits were measured after behavioural observations, so manipulations done to the plants in the process did not affect caterpillar behaviour. Latex mass was measured by cutting the tip of each leaf, absorbing latex exudation using a small disk of filter paper, and recording mass, following the procedure in Agrawal (2005). For trichome density, we took a hole punch (6 mm diameter) from the youngest fully developed leaf and took high resolution digital images of the abaxial surface of each punch. The camera system used was an Axiocam 506 colour camera with a Plan Apo S 1.0 3FWD 60 mm Macro Lens on a SteREO Discovery V20 microscope (Zeiss, Germany). A FIJI cell counter was used to count trichomes in a 2.3 mm × 2.3 mm square (not including the midrib) on each punch.

Experiment 2: Long-term effects of latex and trichomes on caterpillar behaviour and performance

We chose the milkweed species with the highest latex exudation and trichome density from experiment 1, *A. vestita*, for the next study. Monarch eggs were obtained and *A. vestita* plants were grown using the same procedures and conditions as experiment 1 ($n = 56$).

We used two experimental manipulations to isolate the effects of latex and trichomes. First, we repeated the intact versus cut leaf treatment from experiment 1 to manipulate latex exudation. Second, we manually removed the trichomes from the surface of half of the leaves by gently rubbing them off for 2 min using latex gloves. As a sham control, half of the plants were also handled for 2 min with approximately the same pressure applied to leaves, but without removing trichomes. Given these two manipulations, we had four treatment groups with and without latex and trichomes (L−/T−, L−/T+, L+/T−, L+/T+). Caterpillars on intact plants were confined to the six youngest leaves using a paper barrier fitted around the stem, and additional removal of trichomes or sham control handling was conducted as needed throughout the 7-day study period, when new leaves began to grow at the apex of the plant. For the no-latex treatment, caterpillars were given a cut leaf from one of the six youngest leaves on their plant, and damp cotton was used to keep the stem moist where cut. We replaced the cut leaves in each petri dish every 24 h. Thirteen caterpillars were assigned to each treatment group, and two additional caterpillars per treatment group were raised to be replacements in case of mortality.

Behavioural observations were conducted in a similar manner to experiment 1 (five rounds of 2-min observations), for both first instar and third instar caterpillars (Table S2). We used three temporal blocks of four treatments with four or five replicates per block (although the number of replicates decreased for third instar observations due to caterpillar mortality). As caterpillars matured, the same individuals were observed at the beginning of their third instar. For first instars, only chewing and shaving were recorded; feeding and trenching by third instar caterpillars are distinguishable, so these behaviours were recorded separately (in addition to shaving). We started observations on first instar caterpillars as soon as they were placed on a plant, and third instar caterpillars were transferred to a new leaf using a paintbrush upon starting observations.

We recorded performance data throughout the study period as proxies for fitness. Caterpillar mass was measured at the beginning of the first, second, third, and fourth instars, and mortality was noted at these times. Mortality data included the 52 original caterpillars, as well as eight replacements. The time from the start of the study period to the start of the second, third, and fourth instars was also recorded. Due to high mortality, four of the replacement caterpillars were added to the study (two L−/T+ and two L−/T−), although first instar behavioural data and initial and second instar mass were not collected for replacements.

Statistical analysis

For experiment 1, linear regressions were used to assess relationships between defence predictors and behaviours. All regressions from this experiment were calculated using species means ($n = 7$), representing an average value of five replicate plants from each species. We used regression *t* tests to assess if individual slopes were different from zero. We then used a second analysis of covariance approach with experiment 1, assessing the effect of a defence predictor, treatment, and their interaction on behaviours. This second analysis specifically addressed the hypothesis that the relationship between plant traits and insect behaviour changed when latex canals were severed. Most behavioural and performance data in experiment 2 was analysed using two-way analysis of variance with latex, trichomes, and their interaction as predictors. Due to high mortality, especially on intact plants with trichomes and latex, we could not include an interaction term for data collected after the first instar. All analyses were conducted in R version 2023.12.1+402, except for a *G*-test of first instar chewing behaviour and analysis of caterpillar mortality and third instar behaviour, which were assessed using logistic regression in JMP Pro (v16).

RESULTS

Effects of plant defence on caterpillar behaviour (Exp 1)

As we predicted based on past research, latex mass and trichome density were positively correlated across milkweed species (Figure 2; $n = 7$, slope *t* test $R^2 = 0.747$, $p = 0.012$). To test the roles of latex and trichomes in predicting chewing and shaving behaviour, we first fit linear regression models for eight relationships, testing separately latex and trichomes as predictors of shaving and chewing, with both latex and no-latex treatments (Table 1). Chewing and shaving durations are shown as time spent on each behaviour during 600 s across all observations. We found that trichome density was a strong predictor of shaving with and without latex. Latex was also predictive of shaving for both treatments, although this relationship was somewhat weaker. In contrast, latex was a strong predictor of chewing behaviour only for plants with intact latex. Lastly, trichome density was a poor predictor of chewing for both treatments, and this result was particularly notable on no-latex treatments.

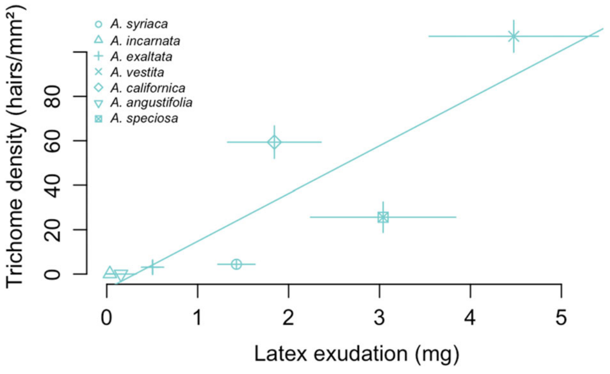

FIGURE 2 Species correlation between latex exudation (mg produced) and trichome density ($n = 5$ plants for each species). Shown are species means ± standard errors.

To aid in our interpretation of the effects of trichomes and latex on caterpillar behaviour, we next tested for interaction effects between our predictors (latex or trichomes) and treatment (latex or no-latex) on caterpillar behaviour. Here, an interaction term indicates that the effect of a predictor was modulated by the presence of latex (manipulated by the cut or intact treatments). Neither latex exudation nor trichome density showed interaction effects with treatment for shaving duration (Figure 3a; latex:treatment $F_{1,10} = 0.234$, $p = 0.639$; trichome:treatment $F_{1,10} = 1.068$, $p = 0.326$). Trichome density also did not interact with treatment to predict chewing duration (trichome:treatment $F_{1,10} = 2.479$, $p = 0.147$). We did, however, find an interaction between latex and treatment in predicting chewing duration (Figure 3b; latex:treatment $F_{1,10} = 11.504$, $p = 0.007$). Thus, a species' latex exudation predicted chewing duration only for the intact latex treatment. Lastly, chewing and shaving themselves were somewhat negatively correlated on plants with latex intact (Figure 4; latex slope *t* test $R^2 = 0.503$, $p = 0.074$; no-latex slope *t* test $R^2 = 0.098$, $p = 0.495$). In other words, with latex intact, caterpillar behaviour likely traded off between the two behaviours.

Effects of plant defence on caterpillar behaviour (Exp 2)

In experiment 2 on *A. vestita*, we assessed behaviour of first instar caterpillars. Shaving duration was 29-fold higher on plants with

TABLE 1 Linear regressions with R^2 and *p* values for defence predictors on monarch caterpillar behaviours in experiment 1.

Predictor	Behaviour	Treatment	R^2	*p* value
Trichomes	Shaving	Latex	0.942	**<0.001**
Trichomes	Shaving	No latex	0.964	**<0.001**
Trichomes	Chewing	Latex	0.428	0.111
Trichomes	Chewing	No latex	0.085	0.526
Latex	Shaving	Latex	0.711	**0.017**
Latex	Shaving	No latex	0.642	**0.030**
Latex	Chewing	Latex	0.822	**0.005**
Latex	Chewing	No latex	0.33	0.177

Note: All regressions were based on $n = 7$ (using species means). Bold lettering indicates that the result was statistically significant.

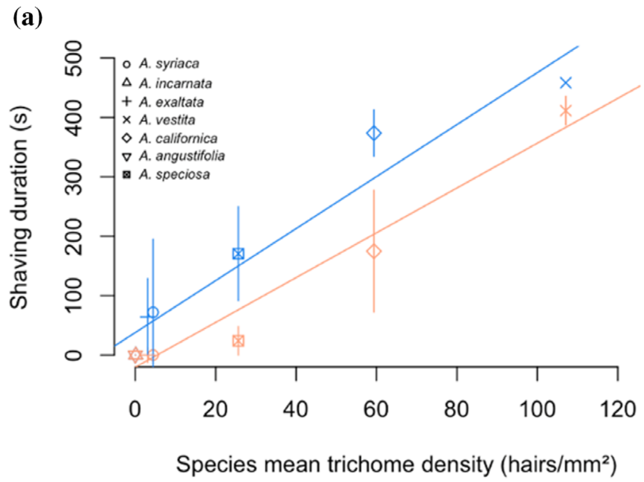

FIGURE 3 Species correlations between two milkweed defences (a) trichome density; (b) latex exudation and monarch behaviour. Separate regressions are shown for intact latex (blue) and no-latex treatments (orange). Shown are species means ± standard errors.

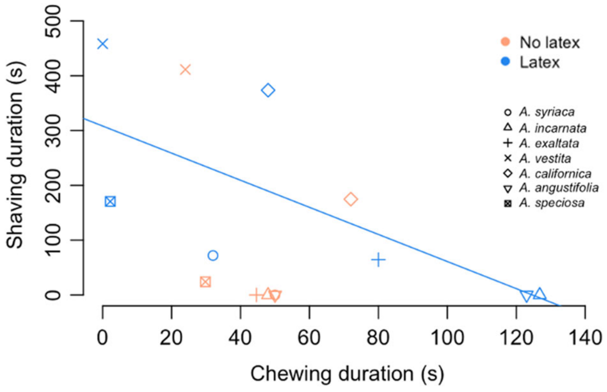

FIGURE 4 Species correlation between duration of shaving and chewing for latex (blue) and no-latex treatments (orange). Each dot represents a species mean.

trichomes, and 1.6-fold higher on plants with latex intact (Figure 5b; Table 2; $n = 52$, trichomes $F_{1,48} = 88.903$, $p < 0.001$; latex $F_{1,48} = 5.658$, $p = 0.021$). Importantly, we found an interaction between latex and trichomes for shaving duration (trichome:latex $F_{1,48} = 4.344$, $p = 0.043$). In the absence of trichomes, there was little shaving in either latex treatment; however, on intact trichome plants, caterpillars spent 58% more time shaving on latex treatments compared to no latex (Figure 5b). Trichomes also had a strong effect on chewing behaviour: 12 of 26 first instar caterpillars on plants without trichomes spent time chewing, while none chewed on leaves with trichomes (Figure 5a; G-test with Williams correction for 2 × 2 table, adjusted $G = 19.426$, $p < 0.001$). There was also 107% more chewing among caterpillars raised without latex, but no significant interaction between the effects of latex and trichomes ($n = 52$, latex $F_{1,48} = 1.43$, $p = 0.238$; trichome:latex $F_{1,48} = 1.43$, $p = 0.238$).

Among caterpillars surviving until the third instar (37% of the original 60), we found little effect of either latex or trichomes on feeding duration (logistic regression, $n = 22$, trichomes L-R $\chi^2 = 0.010$, $p = 0.920$; latex L-R $\chi^2 = 2.885$, $p = 0.089$). Nonetheless, like the first instar caterpillars, third instars shaved much more on plants with trichomes, but at this time point, latex made little difference (logistic regression, $n = 22$, trichomes L-R $\chi^2 = 15.428$, $p < 0.001$; latex L-R $\chi^2 = 3.256$, $p = 0.071$).

Effects of plant defence on caterpillar performance (Exp 2)

By the end of the first instar, 62% of caterpillars had died, and both latex and trichomes (but not their interaction) impacted mortality (Figure 5c,d; logistic regression, $n = 60$, trichomes L-R $\chi^2 = 7.833$, $p = 0.005$; latex L-R $\chi^2 = 7.833$, $p = 0.005$; trichome:latex L-R $\chi^2 = 0.031$, $p = 0.860$). Five additional caterpillars died by the end of the second instar (logistic regression, $n = 60$, trichomes L-R $\chi^2 = 3.792$, $p = 0.052$; latex L-R $\chi^2 = 8.589$, $p = 0.003$; trichome:latex L-R $\chi^2 = 1.140$, $p = 0.286$), but there was no further mortality after this point. At the beginning of the second instar, surviving caterpillars raised without trichomes weighed 27% more compared to with trichomes, while those raised without latex weighed 10% more (but not significantly more) than those raised with latex (Figure 5e,f; $n = 23$, trichome $F_{1,20} = 6.67$, $p = 0.018$; latex $F_{1,20} = 3.587$, $p = 0.073$). At the beginning of the third instar, caterpillars on no-trichome plants grew 50% more, and caterpillars in the no-latex treatment grew 16% more than controls, although, again, these differences were not significant ($n = 22$, trichome $F_{1,19} = 3.511$, $p = 0.076$; latex $F_{1,19} = 2.4$, $p = 0.138$). Lastly, at the beginning of the fourth instar, there was little difference in caterpillar weights on plants with or without trichomes, but 57% higher weights for caterpillars in the no-latex treatment compared to controls ($n = 22$, trichome main effect $F_{1,19} = 0.073$, $p = 0.790$; latex main effect $F_{1,19} = 6.6$, $p = 0.019$).

In terms of development, all caterpillars had progressed from the first to the second instar within 24 h, but there was some variability in duration of the second and third instars. There was no difference between treatment groups in the time to completion of the second (Table 2; $n = 22$, trichome $F_{1,19} = 0.035$, $p = 0.853$; latex $F_{1,19} = 1.939$,

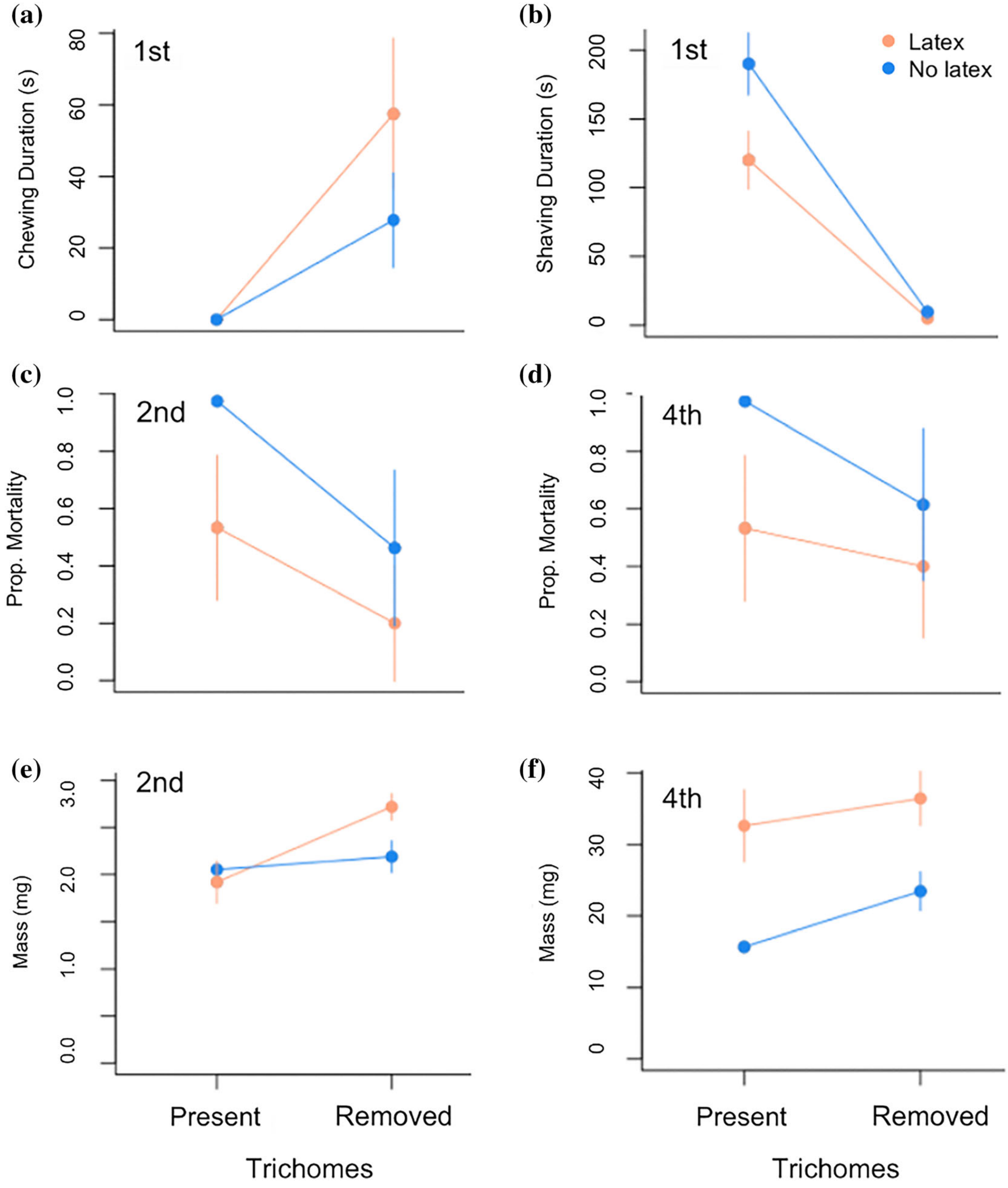

FIGURE 5 Behaviour and performance of (a) first instar chewing, (b) first instar shaving, (c) second instar mortality, (d) fourth instar mortality, (e) second instar surviving caterpillar mass, and (f) fourth instar surviving caterpillar mass for factorial manipulations of latex and trichomes on *Asclepias vestita*. Instar (first, second, or fourth) is indicated in the upper left of each panel. Orange points represent the no-latex treatment while blue points represent the intact latex treatment. Shown are means ± standard errors (a, b, e, f) or proportions and 95% confidence intervals (c, d).

$p = 0.18$) or the third instar ($n = 22$, trichome $F_{1,19} = 0.306$, $p = 0.586$; latex $F_{1,19} = 0.175$, $p = 0.681$).

DISCUSSION

Effects of plant defence on caterpillar behaviour

Here, we report behavioural differences in monarch caterpillars across seven species of milkweed, modulated by the presence and magnitude of latex exudation and trichome density. In our first experiment, we found that, as expected, trichome density was a strong positive predictor of time spent shaving (Figure 3a). Shaving removes trichomes from leaves, where they may either be discarded or ingested. This nearly always preceded chewing, particularly on high-latex species, so this behaviour likely represents a costly investment in disarming plant defence (Malcolm, 1994; Zalucki et al., 2002). Furthermore, shaving requires active movement of caterpillars, potentially making them vulnerable to predation and acting as an additional barrier to feeding. Interestingly, we noted that caterpillars often moved to the abaxial surface of the leaf, where trichomes densities tend to be higher (Zalucki et al., 2012). This behaviour may be an adaptation to avoid

TABLE 2 *p* Values for behavioural and performance-related response variables from experiment 2.

Response variable (instar)	Trichome *p* value	Latex *p* value	Trichome:latex *p* value	*N*
Shaving (1)	**<0.001**	**0.021**	**0.043**	52
Chewing (1)	***0.001***	*0.238*	0.238	52
Shaving (3)	**0.007**	0.517	N/A	22
Feeding (3)	*0.459*	*0.153*	N/A	22
Mortality (2)	**0.005**	**0.005**	0.860	60
Mortality (4)	0.052	**0.003**	0.286	60
Mass (2)	***0.018***	*0.073*	N/A	23
Mass (3)	*0.076*	*0.138*	N/A	22
Mass (4)	*0.790*	***0.019***	N/A	22
Instar duration (2)	0.853	0.18	N/A	22
Instar duration (3)	0.586	0.681	N/A	22

Note: Italics text denotes mean response variables that were lower with latex or trichomes intact, and bold lettering indicates statistical significance. Data was collected at the beginning of each instar (given parenthetically). N/A indicates the test was not conducted.

predation or desiccation and could represent a trade-off larvae face between prioritising feeding, disarming of plant defences, and avoidance of other risks. We also found that our manipulation of latex (cutting leaves) had an effect on shaving duration, with more time spent shaving on intact latex treatments. Young monarchs have been documented to make circular or semi-circular trenches on leaves, cutting off latex flow to the encircled part of the leaf (Agrawal, 2017; Zalucki & Brower, 1992; Zalucki, Brower, et al., 2001; Zalucki, Malcolm, et al., 2001). This enables safer feeding and may require more extensive shaving on an intact plant with active latex flow. Accordingly, our finding suggests either more cautious disarming of defences on plants with trichomes and latex, or the need to shave trichomes over a larger area when also disarming latex.

In addition to shaving, we found that chewing behaviour could be predicted by plant defence. For latex treatments, there was a strong negative correlation between latex exudation and chewing duration, with essentially no effect without latex (Figure 3b). The interactive effect between latex amount and treatment (cutting leaves) for this relationship indicates that the presence of latex reduces chewing. Furthermore, trichome density was not a strong predictor of chewing duration on either latex treatment (Table 1). As such, we conclude that latex exudation, but not trichome density, was the primary predictor of chewing behaviour across milkweed species during the first instar. Notably, chewing in our experiment encompasses both trenching and feeding, as it was not distinguishable for first instar caterpillars. We expect two effects of removing latex: caterpillars should spend less time trenching and more time feeding, and these changes should be particularly evident on high-latex species. Although we could not distinguish the two, we note that removing latex increased chewing duration particularly on higher latex species (Figure 3b). Thus, we expect that the increase in feeding, particularly among caterpillars raised on high-latex species, may be the main contributor to our finding. Lastly, shaving and chewing duration themselves were negatively associated when latex was intact (Figure 4). This result may reflect that caterpillars shaving for long durations were reared on plants with high latex, which was a deterrent of chewing. It may also be a product of time constraints, as each caterpillar was observed for a fixed amount of time, and thus any individual spending a majority of their time on one behaviour would necessarily show lower durations of the other.

We repeated our behavioural observations for first instar caterpillars on *A. vestita* plants in experiment 2, here manipulating both latex and trichomes. Concordant with our first experiment, we found that trichomes and latex both strongly influenced shaving duration, with greater shaving on plants with trichomes and latex intact (Figure 5b). Surprisingly, the presence of trichomes, but not latex, had a negative effect on chewing duration (Figure 5a). Although this appears to contradict our previous finding that latex was the primary predictor of chewing, we note that *A. vestita* is a species with particularly high-trichome density and perhaps time constraints and tradeoffs resulted in higher shaving at the expense of chewing in this treatment. Of the 36 caterpillars across both experiments that were raised on *A. vestita* with trichomes intact (26 from L+/T+ and L−/T+ treatments in experiment 2, 10 from experiment 1), we only observed chewing in one caterpillar during the first 3 h. From our observations, it appeared that with trichomes intact, most caterpillars were unable to shave enough to reach the leaf tissue within that time. As such, we concluded that this finding was likely a product of time constraints rather than a deviation from the general trend that latex primarily predicts chewing behaviour. Furthermore, the role of latex as a strong deterrent of leaf damage by young caterpillars is consistent with earlier studies documenting poor performance of caterpillars raised on high-latex species (Zalucki et al., 2012; Zalucki & Brower, 1992; Zalucki & Kitching, 1982; Zalucki & Malcolm, 1999; Zalucki, Brower, et al., 2001; Zalucki, Malcolm, et al., 2001). A potential unmeasured factor from our first experiment was the influence of cardenolides on behaviour. In addition to latex and trichomes, cardenolides are plant chemical defences which vary across our seven study species. In particular, cardenolides may have played a role in mediating chewing behaviour, as they are present in both leaf tissue and latex. Regardless, given that we found similar behavioural patterns on a single species in experiment 2, we expect that the confounding effects of cardenolides on behaviour in our first experiment were minimal.

Lastly, we observed feeding, trenching, and shaving behaviour in the surviving third instar caterpillars reared on *A. vestita* (mortality was 63% to this stage). At this point, trichome density alone had strong effects on shaving, while neither predictor influenced feeding duration (Table 2). While the role of latex in performance and trenching behaviour has been well-studied for first instar caterpillars (Zalucki et al., 2012; Zalucki & Brower, 1992; Zalucki & Kitching, 1982), there is much less known about its influence in later instars. Nonetheless, first and third instar caterpillars are known to behave very distinctly, with older larvae spending more time feeding and less time moving or avoiding plant defences (Johnson &

Zalucki, 2007). Additionally, monarchs have been shown to display different techniques to avoid latex at different stages of development (Agrawal, 2017). Finally, a recent study suggested that instead of trenching to disarm plant defence, later instar monarchs actively ingest latex to increase sequestration of cardenolides and improve their own defence against predators (Betz et al., 2024). While we did not directly observe any trenching, damage to leaf veins, or latex ingestion during our observations, it follows that latex would no longer be a deterrent to feeding or an instigator of shaving at this point in development. A potential limitation of this part of our experiment is the possibility for changes in the expression of defences on cut leaf treatments. Several studies have documented the presence of inducible defences in milkweeds, in which plants increase expression of defence traits following plant damage (Bingham & Agrawal, 2010; Rasmann et al., 2009; Tan et al., 2018). Given that we cut leaves off of plants to feed caterpillars throughout the week in the no-latex treatment, it is possible that this may have impacted the development of existing leaves. We tried to reduce this effect by using separate plants for each caterpillar. Additionally, most research on inducible defences has documented changes in expression of latex and secondary metabolites; given that our cut leaf treatments removed latex, and plant defences overall showed a reduced impact at this point, we hope that these effects were minimal. Thus, we showed that caterpillar behaviour is dynamic and distinct at different developmental stages. While trichome density is an important predictor of shaving behaviour throughout development, latex primarily affects shaving and chewing behaviours during early instars.

Effects of plant defence on caterpillar performance

Consistent with existing research, we found that the majority of caterpillar mortality occurred during the first instar (Figure 5c,d; Zalucki et al., 2012; Zalucki & Brower, 1992; Zalucki & Kitching, 1982). Furthermore, at the beginning of the second and fourth instars, both latex and trichomes had significant effects on mortality. This supports our general finding that latex was a strong deterrent of chewing for first instars, as well as the specific result that trichomes reduced early chewing on *A. vestita*. Caterpillar growth results supported the roles of both latex and trichomes on caterpillar performance, with lower mass of survivors raised on plants with latex or trichomes intact (Figure 5e,f). These results are consistent with previous studies on the early roles of trichomes and latex on insect performance. Though generally low, survival rates of first instar caterpillars are variable, and are negatively correlated with plant defences, including latex, trichomes, and cardenolides (Edwards et al., 2023; Zalucki, Brower, et al., 2001; Zalucki, Malcolm, et al., 2001). This effect is particularly pronounced in experiments which control for latex flow. On no-latex plants, caterpillar growth rates are improved for larvae compared to those raised on intact plants. Although the impact of trichomes is less well-studied, *Battus philenor* caterpillars raised on high-trichome plants performed better on, and showed a preference for, plant regions with trichomes removed (Fordyce & Agrawal, 2001). Another study showed that, although they showed no aversion, ingestion of trichomes by *Manduca sexta* caterpillars reduced growth and increased time to pupation compared to controls (Kariyat et al., 2019). Lastly, a study on *Trichoplusia ni* caterpillars noted similar results, with lower mass gain and plant damage on high-trichome plants (Kaur & Kariyat, 2023). In all, both in our experiments and previous work, outcomes for young caterpillars were improved on species with lower latex exudation and trichome densities (Zalucki, Brower, et al., 2001; Zalucki, Malcolm, et al., 2001).

Trichomes had the greatest effect on mass of surviving second instar caterpillars, with notably decreasing effects in later instars, again supporting the important early role of trichomes in deterring feeding. The effect of latex increased over the study period, although this increase was marginal. We also found some variability in the duration of the second and third instars, but effects from either plant defence were insignificant (Table 2). A limitation of our study was that we only recorded components of performance every several hours. While mass and duration were observed at the beginning of each instar, there was likely some degree of inconsistency in the exact point of development in which these measures were recorded. As such, some variability in the effects of latex and trichomes on mass are expected. Furthermore, instar duration was only recorded every 24 h. Although average instar durations were higher with both latex and trichomes intact, these differences were not statistically significant. Given the difference we found in mass across treatment groups, it follows that there may also be corresponding differences in the timing of development. We expect that the lack of a difference we found may reflect a limitation of our study design, and we acknowledge that finer scale differences in instar duration may not have been captured. Nonetheless, we document clearly that latex and trichomes each contribute to mortality and differential mass of survivors, with particular effects early in development.

Defensive synergism of latex and trichomes

As predicted, we found a strong positive relationship between latex exudation and trichome density across the seven species (Figure 2). We were thus interested in separating the two and testing for synergism by manipulating both predictors in a separate experiment. For first instar caterpillars, we found that both latex and trichomes, as well as their interaction, had effects on shaving duration (Table 2). Given that both defences independently led to increased shaving, this finding may suggest a potential synergism of the two in terms of behaviour. We also calculated interaction terms for first instar chewing and mortality, although none of these were significant. Due to high mortality (particularly of the L+/T+ treatment, with 93% mortality in the first instar), we did not have sufficient sample sizes to calculate an interaction term for any later response variables recorded in experiment 2. In a previous observational study of monarch survival on *A. syriaca*, Edwards et al. (2023) reported evidence of two defence synergies in milkweed, latex by trichomes and latex by secondary metabolites (cardenolides), measured by insect performance. Shaving of trichomes typically precedes deactivation of latex, both of which precede consumption of leaf material. This, combined with the fact that latex and trichomes show correlated evolution, suggests the synergism may indeed be possible in *A. vestita*, even if not universal.

Examples of plant defence interactions are well-documented in other plant systems. Most plants utilise multiple defences against herbivore specialists, and these traits usually interact in an additive or synergistic manner (Chen, 2008). In non-milkweed systems, some plants accumulate multiple types of toxic minerals, which are theorised to have joint effects. Experimentation in these systems has documented synergistic and additive effects between different metal ions and combinations of metals and organic compounds (Boyd, 2012). Similarly, plants tend to produce a small but unique combination of secondary metabolites, which may all act together to additively or synergistically defend the plant (Chen, 2008). We predict that under conditions of environmental stress (high temperatures, herbivore exposure, etc.) plant tissue would be more vulnerable and thus more important to defend (Coley et al., 1985). Defence synergism, or at least multiple additive defences, may allow plants in such conditions to defend against external stressors. *A. vestita* and other high-latex, high-trichome milkweed species tend to live in hot, dry environments (Agrawal et al., 2009). While trichomes play a role in defence, they have also been shown to be useful in gas exchange and preventing desiccation (Li et al., 2022). We expect that *A. vestita* and other high-latex, high-trichome milkweed species experience such stressful conditions, thus leading to the evolution of latex and trichomes as complementary defence mechanisms. In turn, these environmental stressors may also exert pressure for insects to evade defences and access sufficient plant tissue, resulting in the close coevolution of plant defence and insect counter defence as seen in the milkweed-monarch system.

CONCLUSION

We combined comparative and experimental approaches to test the independent and synergistic roles of latex and trichomes in mediating monarch caterpillar behaviour and performance on milkweeds. Previous research has documented the importance of these defences in adaptive behaviours and early caterpillar fitness, but little work had been conducted to disentangle the two throughout caterpillar development. We found that latex and trichomes interacted to impact behavioural strategies, especially under time constraints during the first instar. Latex exudation was the primary predictor of chewing for first instars across the seven species, although trichome density was also important on a very high-trichome species, *A. vestita*. The impact of these predictors on behaviour diminished over development, and by the third instar, neither had an effect on feeding. We also report evidence of latex- and trichome-mediated performance effects on mortality and mass of survivors, which were particularly notable during early stages of development. In all, 80% of caterpillars died on intact *A. vestita* despite our experiment being conducted in a controlled growth chamber environment. Although milkweeds and monarchs represent a well-studied system in ecology, most plants exhibit multiple defensive traits which may similarly interact. Our work holds implications for the interactions of plant defences on herbivore specialists, and highlights the complex, complementary roles of two defences in predicting herbivore behaviour and performance.

AUTHOR CONTRIBUTIONS

Emma J. Craig: Conceptualization; investigation; writing – original draft; formal analysis; visualization. **Anurag A. Agrawal:** Conceptualization; funding acquisition; writing – review and editing; formal analysis.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data from this study will be accessible after publication from Dryad Digital Repository at: https://doi.org/10.5061/dryad.63xsj3vb6.

ETHICS STATEMENT

The authors declare all ethical guidelines have been followed.

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SUPPORTING INFORMATION

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

Table S1. Proportions of time caterpillars spent on each of five recorded behaviors during Experiment 1.

Table S2. Proportions of time caterpillars spent on each of three recorded behaviors during Experiment 2 for caterpillars raised on *A. vestita*. L+/L− indicates latex intact/removed, and T+/T− indicates trichomes intact/removed, respectively.

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