



Effects of Host Plants on Development and Immunity of a Generalist Insect Herbivore

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Abstract

Secondary plant chemistry mediates a variety of communication signals among species, playing a fundamental role in the evolutionary diversification of communities and ecosystems. Herein, we explored diet-mediated host plant effects on development and immune response of a generalist insect herbivore. *Vanessa cardui* (Nymphalidae) caterpillars were reared on leaves of three host plants that vary in secondary metabolites, *Plantago lanceolata* (Plantaginaceae), *Taraxacum officinale* (Asteraceae) and *Tithonia diversifolia* (Asteraceae). Insect development was evaluated by larval and pupal viabilities, survivorship, and development rate. Immune response was measured as phenoloxidase (PO) activity. Additionally, chemical profiles of the host plants were obtained by liquid chromatograph-mass spectrometry (LC–MS) and the discriminant metabolites were determined using a metabolomic approach. Caterpillars reared on *P. lanceolata* exhibited the highest larval and pupal viabilities, as well as PO activity, and *P. lanceolata* leaves were chemically characterized by the presence of iridoid glycosides, phenylpropanoids and flavonoids. *Taraxacum officinale* leaves were characterized mainly by the presence of phenylpropanoids, flavones *O*-glycoside and germacranolide-type sesquiterpene lactones; caterpillars reared on this host plant fully developed to the adult stage, however they exhibited lower larval and pupal viabilities compared to individuals reared on *P. lanceolata*. Conversely, caterpillars reared on *T. diversifolia* leaves, which contain phenylpropanoids, flavones and diverse furanoheliangolide-type sesquiterpene lactones, were not able to complete larval development and exhibited the lowest PO activity. These findings suggested that *V. cardui* have adapted to tolerate potentially toxic metabolites occurring in *P. lanceolata* (iridoid glycosides), however caterpillars were not able to cope with potentially detrimental metabolites occurring in *T. diversifolia* (furanoheliangolides). Therefore, we suggest that furanoheliangolide-type sesquiterpene lactones were responsible for the poor development and immune response observed for caterpillars reared on *T. diversifolia*.

Keywords *Vanessa cardui* · *Plantago lanceolata* · *Taraxacum officinale* · *Tithonia diversifolia* · Phenoloxidase activity · LC–MS

Introduction

Interactions between plants and herbivores are greatly influenced by secondary metabolites, which enable chemically-mediated interactions among diverse organisms (Richards et al. 2015; Sedio 2017; Dyer et al. 2018). A long-standing

hypothesis in chemical ecology is that the evolution of diet breadth in insect herbivores is associated with host plant chemistry and natural enemies, highlighting the interactive roles that mediate tri-trophic interactions (Ehrlich and Raven 1964; Singer and Stireman 2003; Smilanich et al. 2009a; Price et al. 2011; Mooney et al. 2012; Muchoney et al. 2022).

Selection of host plants by insects depends on sequential behavioral responses to multiple sensory cues (e.g., visual, olfactory, gustatory and/or tactile stimuli) (Finch and Collier 2000; Renwick 2001). Insect herbivores (i.e., plant-feeding insects) acquire essential nutrients from plants that are required for their growth, development and reproduction (Behmer 2009). Therefore, host plant selection is pivotal for insect herbivores to achieve adequate

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dietary balance and avoid potentially toxic metabolites (Knolhoff and Heckel 2014; Simpson et al. 2015).

The quality and composition of the food consumed by insects may influence their physiological, behavioral and immunological responses (Lampert et al. 2014; Smilanich et al. 2018; Resnik and Smilanich 2020). Many plant metabolites play roles in plant defenses against herbivores by complex and interactive effects, and are described as toxic compounds (i.e., compounds that cause detrimental biological effects) (Duffey and Stout 1996). Over the years, a diversity of metabolite classes (e.g., alkaloids, monoterpenes, sesquiterpenes and phenolic compounds) has been described as being toxic to herbivores, depending upon synergistic effects with other compounds and the insect species consuming such toxic metabolites (Richards et al. 2010, 2012; Wink 2018).

Generalist insects are exposed to a wide variety of metabolites and usually possess general mechanisms and/or broad-substrate enzyme systems to cope with plant defensive metabolites (Ali and Agrawal 2012; Lampert 2012). Such interactions are reflected on insect immune responses and physiological processes (reviewed in Smilanich & Muchoney 2022). More specifically, strong immune responses of both generalist and specialist insects have been correlated with high-quality diets. In this study, we reared a generalist insect herbivore, *Vanessa cardui* (Linnaeus, 1758), on leaves of three host plant species that vary in secondary metabolite composition, *Plantago lanceolata* (Plantaginaceae) L., *Taraxacum officinale* (Asteraceae) L. and *Tithonia diversifolia* (Asteraceae) (Hemsl.) A. Gray to test whether the strength of the immune response, larval and pupal viability, and adult survivorship are host plant dependent.

Vanessa cardui (Nymphalidae), popularly known as painted lady or cosmopolitan, is a highly polyphagous insect herbivore that feeds on a variety of species across several plant families, but predominantly on Malvaceae, Asteraceae and Fabaceae species (Williams 1970; Stefanescu 1997). *Plantago lanceolata* is an invasive species originated from Eurasia that belongs to the Plantaginaceae family and is mainly characterized by the occurrence of iridoid glycosides, flavonoids, phenylpropanoids and coumarins (Rønsted et al. 2000; Beara et al. 2012; Budzianowska and Budzianowski 2021). *Taraxacum officinale* L. (a native species to Europe and Asia) and *Tithonia diversifolia* (Hemsl.) A. Gray (a native species to Mexico and Central America) are both members of the Asteraceae family and are chemically characterized by the presence of flavonoids, phenylpropanoids and sesquiterpenes lactones (Schuster et al. 1992; Williams et al. 1996; Gu et al. 2002; Schütz et al. 2005; Ambrósio et al. 2008). Whereas several metabolites are common to both species, different types of sesquiterpene lactones are associated with each one: *T. officinale* presents mostly germacranolide- and eudesmanolide-type sesquiterpene lactones (Kisiel and Barszcz 2000; Esatbeyoglu et al. 2017); while,

T. diversifolia exhibits mostly heliangolide- and furanoheliangolide-type sesquiterpene lactones (Gallon et al. 2019; Gallon and Gobbo-Neto 2021).

Diet quality is often associated with strengthened or weakened immune responses as well as with fitness traits (e.g., growth rates and larval and pupal masses). A general pattern points out that individuals experiencing favorable developmental conditions also exhibit strengthened immunity (Povey et al. 2013; Cotter et al. 2019; Yoon et al. 2019). To explore the effects of plant metabolites on insects, we investigated how larval development and immune system of *V. cardui* caterpillars are affected by host plant consumption. Additionally, we determined the varying metabolites in each plant species using a metabolomic approach and correlated the ingested plant metabolites with insect development and immunity. As both *P. lanceolata* and *T. officinale* are within the dietary repertoire of *V. cardui*, we expected caterpillars reared on these host plants to exhibit high immune response and developmental rate (Smilanich et al. 2018; Resnik and Smilanich 2020). In contrast, we anticipated caterpillars reared on the naïve host plant containing potentially toxic metabolites (*T. diversifolia*) to exhibit compromised development and immune response.

Methods and Materials

Experimental Overview

Vanessa cardui individuals were reared from eggs to adults in a climatic chamber (IN034, Darwin Chambers Company, St. Louis, MO, USA) with a 16 h photoperiod (day temperature of 25 °C and night temperature of 20 °C). Caterpillars were reared on the leaves of three different host plants: *P. lanceolata*, *T. officinale* or *T. diversifolia* ($n=25$ for each host plant). Pupal and larval survival and viabilities were measured across the insect life cycle, as well as development rate. The immune response was evaluated by means of phenoloxidase (PO) activity. Additionally, the chemical profile of the host plants was investigated using a liquid chromatograph-mass spectrometry (LC-MS)-based metabolomic approach.

Host Plants and Insect

Host plants were grown in climatic chambers (F36HO, Percival Scientific, Perry, IA, USA) at the University of Nevada, Reno using a 16 h photoperiod (day temperature of 25 °C and night temperature of 22 °C). *Plantago lanceolata* and *T. officinale* seeds were obtained commercially (Palm Beach Seed Company™, West Palm Beach, FL, USA and Seed Needs, New Baltimore, MI, USA) and *T. diversifolia* seeds were obtained from species growing in the field (Garden of

Medicinal Plants of the School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil). Plant seeds were sown in seed starters containers filled with soil (Miracle-Gro potting mix, The Scotts Company, LLC). After germination, individuals were transferred to 6-inch plastic nursery pots and allowed to grow for three months.

Vanessa cardui eggs were purchased from Carolina Biological (Carolina Biological Supply Company, Burlington, NC, USA). Approximately 30 to 40 insect eggs were jointly hatched on leaves of each host plant (i.e., three different treatments). After entering the 3rd instar, caterpillars ($n=25$) reared on each host plant were moved into individual 16 oz plastic cups and fed *ad libitum* on fresh leaves of each corresponding host plant, which were daily provisioned. To guarantee a consistent dietary intake in terms of secondary plant metabolites, we used young and mature host plant leaves (corresponding to different stages of leaf development).

Viability and Survival

Larval and pupal viabilities were measured as the percentage of larvae and pupae that were able to fully develop and complete the larval and pupal stages, respectively. Survival to adulthood was measured as the percentage of individuals that experienced full metamorphosis and eclosed as healthy adults. The probability of survival across the larval and pupal stages was analyzed using the GraphPad Prism software (version 9.3.1 for Windows, GraphPad Software, San Diego, CA, USA). Survival curves were created using the Kaplan–Meier method and analyzed whether time to death was dependent upon host plant consumption. For larval survival, we measured the elapsed time from hatch to caterpillar's death or pupae formation (censored observation). For pupal survival, we measured the elapsed time from pupation to pupa's death or eclosion as adults (censored observation). The generated curves for each larval and pupal survival data were compared using the overall Logrank test, where probability of survival is based on comparing entire survival curves.

Developmental Rate

Development time was measured for specific stages across the insect life cycle: from hatch to 3rd instar as well as the duration of 3rd instar, 4th instar, 5th instar, pupal stage and adult stage (i.e., adult longevity).

In addition to developmental data, larval weight during specific time points were collected. Larval weights were recorded at the beginning of the 3rd instar, at the beginning of the 4th instar, two days post-4th instar, five days post-4th instar (corresponding to the day of hemolymph extraction,

see below), seven days post-4th instar and nine days post-4th instar.

Statistical analyses were performed in the software GraphPad Prism (version 9.3.1 for Windows, GraphPad Software, San Diego, CA, USA). Development time was evaluated using one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test for the two first development time points (from hatch to 3rd instar and 3rd instar) and a t-test for further developmental stages (4th instar, 5th instar, pupal stage and adult stage). For larval weights, the interaction between consumed host plants and development time points was evaluated using mixed effects model followed by Tukey's multiple comparisons tests for comparing effects of host plants within each development time point.

Immune Response

Caterpillars reared on each host plant were evaluated for immune responses ($n=20$ for *P. lanceolata*; $n=14$ for *T. officinale* and $n=7$ for *T. diversifolia*) using a spectrophotometric assay of PO enzymatic activity. Hemolymph was extracted from caterpillars at the fifth day post entering the 4th instar (five days post-4th instar) by gently piercing the cuticle between the 5th and 6th abdominal segments with a fine needle. Hemolymph was collected using a 10 μ L micropipette. A total of 5 μ L of hemolymph was added to 250 μ L of ice-cold phosphate-buffered saline (PBS) (VWR Life Science, VWR International, Radnor, PA, USA) and vortexed. All supplies and solutions were kept on ice during the entire process to prevent enzymatic degradation. Once all PBS-bound hemolymph samples were prepared, 100 μ L of each sample was loaded in a 96-well microplate and 200 μ L of 4 mM dopamine (Alfa Aesar, Thermo Fisher Scientific, Haverhill, MA, USA) solution was added to each well. Colorimetric measurements were performed immediately using an iMark Microplate Absorbance Reader (Bio-Rad). The absorbance was measured at 490 nm every 30 s for 45 min and PO activity was calculated as the slope over the time the enzymatic reaction was linear (from 5 to 30 min). PO activity measurements with negative values ($n=6$ for *P. lanceolata*, $n=7$ for *T. officinale* and $n=2$ for *T. diversifolia*) were excluded from analyses. One-way ANOVA with Tukey's multiple comparisons test was performed to determine statistical differences in PO activity between the host plants using the software GraphPad Prism (version 9.3.1 for Windows, GraphPad Software, San Diego, CA, USA).

Trade-off in Resource Use

For caterpillars reared on *P. lanceolata* or *T. officinale* leaves, we compared the mass gained from the time of hemolymph extraction to the final larval instar and PO activities.

Mass gained was measured as the difference between the final recorded larval weight and the larval weight at the day of hemolymph extraction and calculated for all caterpillars that survived the larval stage and entered the pupal stage ($n=9$ and $n=4$, for caterpillars reared on *P. lanceolata* and *T. officinale* respectively). Mass gained and PO activities were compared using two-way ANOVA in the software GraphPad Prism (version 9.3.1 for Windows, GraphPad Software, San Diego, CA, USA).

Chemical Profile

Host plants that were growing in the climatic chambers were sampled during insect development assays. Leaves of each host plant were collected at three different days with seven days between collections ($n=5$ for each day), immediately frozen and kept at $-10\text{ }^{\circ}\text{C}$. Leaves were freeze-dried for 48 h and pulverized with mortar and pestle. Extracts for the LC–MS analyses were prepared by adding 1 mL of a solution of $\text{MeOH:H}_2\text{O}$ (7:3, v:v) with hydrocortisone ($10\text{ }\mu\text{g mL}^{-1}$) to 20 mg of each plant sample. After vortex agitation (30 s, K-550-G, Scientific Industries, Bohemia, NY, USA), extractions were performed in an ultrasonic bath (10 min, 75 T Aquasonic, VWR Scientific Products, Radnor, PA, USA). Extracts were centrifuged (21130 g, 5 min, Centrifuge 5425, Eppendorf™, Fisher Scientific, Hampton, NH, USA) and the supernatant was filtered through a $0.22\text{ }\mu\text{m}$ PTFE syringe filter into glass vials. A quality control (QC) sample was prepared by combining aliquots ($20\text{ }\mu\text{L}$) of each extract.

LC–MS analyses were performed in a high-performance liquid chromatography (HPLC) system coupled to a time-of-fly (TOF) mass spectrometer (Agilent 6230, Agilent Technologies, Santa Clara, CA, USA), using a C18 column ($5\text{ }\mu\text{m}$, XB-C18 Kinetex, $100\text{ }\text{\AA}$, $150\times 3\text{ mm}$, Phenomenex, Torrance, CA, USA). Mobile phase was composed of water and acetonitrile (MeCN) both with 0.1% formic acid and the following gradient, at a flow rate of $0.75\text{ mL}\cdot\text{min}^{-1}$, was employed: 0–2 min, 5% MeCN; 2–30 min, 5–100% MeCN; 30–35 min, 100% MeCN; 35–38 min, 100–5% MeCN and 38–40 min, 5% MeCN. For each sample, an injection volume of $20\text{ }\mu\text{L}$ was used and samples were kept at $4\text{ }^{\circ}\text{C}$. Column oven was set at $45\text{ }^{\circ}\text{C}$. Chromatograms were acquired in both positive and negative ionization modes, and the following parameters were employed for the mass spectrometer: dry temperature, $325\text{ }^{\circ}\text{C}$; dry gas flow, $10\text{ L}\cdot\text{min}^{-1}$; nebulizer pressure, 20 psig; capillary voltage, 3500 V; fragmentor, 100 V; skimmer, 65 V; Oct 1RF Vpp, 750 V; acquisition range, m/z 100 to 1500; 2 spectra/sec; 500 ms/spectrum. Blank (i.e., extraction solution) and QC samples were injected at the beginning, middle and end of the LC–MS analysis, and used to certify the reliability and accuracy of

the data acquisition and processing (Supplementary Material, Fig. S1).

Raw LC–MS data from positive and negative ionization modes were converted to.mzXML format using the software ProteoWizard-MSconvert (version 3 for Windows, Proteowizard Software Foundation, Palo Alto, CA, USA) and separately processed with the software MzMine (version 2.53 for Windows, BMC Bioinformatics, United Kingdom). The following parameters were used for data processing: raw data methods—feature detection, mass detection, mass detector – centroid (noise level, 1.0×10^3); ADAP chromatogram builder, min group size in # of scans, 5; group intensity threshold, 1.0×10^3 ; min highest intensity, 1.0×10^4 ; m/z tolerance, 0.005 m/z ; feature list methods – feature detection, chromatogram deconvolution, algorithm—wavelets (ADAP) (S/N threshold, 10; S/N estimator, intensity window SN; min feature height, 1.0×10^3 ; coefficient/area threshold, 100; peak duration range, 0.02 – 2.0; RT wavelet range, 0.01–0.20), m/z center calculation—median; isotopes—isotopic peak grouper (m/z tolerance, 0.005 m/z ; retention time tolerance, 0.2 min (absolute); maximum charge, 2; representative isotope, most intense) and alignment—join aligner (m/z tolerance, 0.005 m/z ; weight for m/z , 50; retention time tolerance, 0.2 min (absolute) and weight for retention time, 50). Each set of data (from positive and negative ionization modes) were exported as.csv files, which were further combined into a single spreadsheet for multivariate statistical analysis.

Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA) were performed in the software SIMCA (version 13.0.3.0 for Windows, Umetrics, Umeå, Sweden), using the log transformed data from both ionization modes (combined spreadsheet). Each host plant was assigned as a class for the PLS-DA. Varying metabolites on each host plant were determined by examining the PLS-DA loading plot along with the variable importance on projection (VIP) plot (variables with VIP values greater than 1 were considered important for the discrimination between groups).

Discriminant metabolites were putatively identified using an in-house database composed of the metabolites previously reported in the host plants. The database was built by searching on the SciFinder website (<https://scifinder.cas.org>) for the metabolites reported in each host plant. We used host plant names (*P. lanceolata*, *T. officinale* and *T. diversifolia*) as keywords and examined the main references related to the isolation and identification of plant metabolites in aerial parts. A spreadsheet was created by gathering the following information for each reported metabolite: metabolite class, metabolite subclass, usual name, systematic name, molecular formula, CAS number, monoisotopic mass, m/z of the

protonated molecule (i.e., $[M+H]^+$), m/z of the deprotonated molecule (i.e., $[M-H]^-$) and reference (Supplementary Material 2, Database).

Results

Viability and Survival

Comparing larval and pupal viabilities throughout the insect developmental stages, we found that individuals reared on *P. lanceolata* leaves exhibited the highest larval and pupal viabilities (68% and 48%, respectively) (Table 1). When *T. officinale* leaves were provided as diet, larval and pupal viabilities were lower (36% and 32%, respectively). Interestingly, the survival to adulthood was 28% for both host plants, *P. lanceolata* and *T. officinale*. Individuals reared on *T. diversifolia* leaves were not able to complete the larval stage and exhibited mortality of 100% before entering the 5th larval instar.

Larval survival curves of *V. cardui* caterpillars reared on *P. lanceolata*, *T. officinale* or *T. diversifolia* leaves were significantly different from each other (Logrank test, $P=0.0095$) (Fig. 1). Caterpillars reared on *P. lanceolata* exhibited the highest probability of survival (68%), while caterpillars reared on *T. diversifolia* exhibited the lowest

probability of survival (i.e., no caterpillars survived to the pupal stage). When reared on *T. officinale* leaves, 36% of the caterpillars was able to survive throughout the larval stage and enter the pupal stage.

Pupal survival was evaluated for the individuals that were reared on *P. lanceolata* or *T. officinale*, since all caterpillars reared on *T. diversifolia* leaves died. The pupal survival curves showed no statistical differences between the host plants used as diet (Logrank test, $P=0.2761$) (Fig. 1). However, individuals reared on *T. officinale* exhibited higher probability of pupal survival (approximately 89%) than individuals reared on *P. lanceolata* (approximately 71%).

Development Rate

The development time of caterpillars was significantly different between host plants. Specifically, development time differed from hatch to 3rd instar ($F_{2,72}=174.80$; $df=2$; $P<0.0001$) and during 3rd instar ($F_{2,69}=22.39$; $df=2$; $P<0.0001$) for the caterpillars reared on all host plants, as well as during the 4th larval instar ($t=9.266$, $df=34$, $P<0.0001$) and adult stage ($t=2.704$, $df=12$, $P=0.0192$) for the caterpillars reared on *P. lanceolata* or *T. officinale* (Fig. 2). Caterpillars reared on *T. diversifolia* leaves took longer to enter the 3rd larval instar (mean=8.24 days, $SD=0.44$, $n=25$) than caterpillars reared on *P. lanceolata* or *T. officinale* (mean=6.08 days, $SD=0.28$, $n=25$ and mean=7.56 days, $SD=0.51$ and $n=25$, respectively). Similarly, the 3rd larval instar was longer for caterpillars reared on *T. diversifolia* (mean=4.83 days, $SD=1.53$, $n=23$) than when caterpillars were reared on *P. lanceolata* or *T. officinale* (mean=3.04 days, $SD=0.20$, $n=25$ and mean=3.88 days, $SD=0.54$, $n=24$, respectively). Caterpillars reared on *T. diversifolia* leaves presented longer development time during the 4th instar, yet they were unable to complete the instar (i.e., all caterpillars died before reaching the 5th instar). The 4th instar took approximately 4 days for

Table 1 Larval viability, pupal viability and survival to adulthood of *Vanessa cardui* caterpillars reared on *Plantago lanceolata*, *Taraxacum officinale* or *Tithonia diversifolia* leaves

Host plant	Larval viability (%)	Pupal viability (%)	Survival to adulthood (%)
<i>P. lanceolata</i>	68	48	28
<i>T. officinale</i>	36	32	28
<i>T. diversifolia</i>	0	–	–

Fig. 1 Probability of larval (A) and pupal (B) survivals for *Vanessa cardui* caterpillars reared on *Plantago lanceolata*, *Taraxacum officinale* or *Tithonia diversifolia* leaves. Dotted lines represent 95% confidence intervals

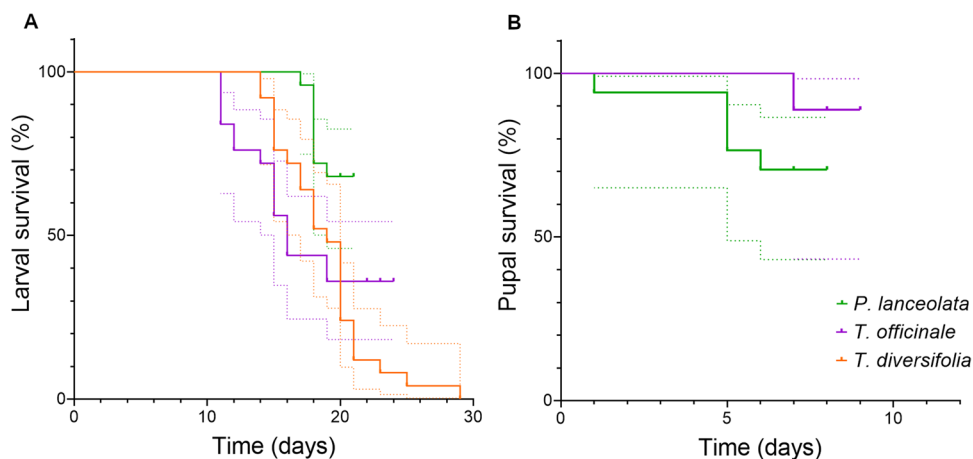
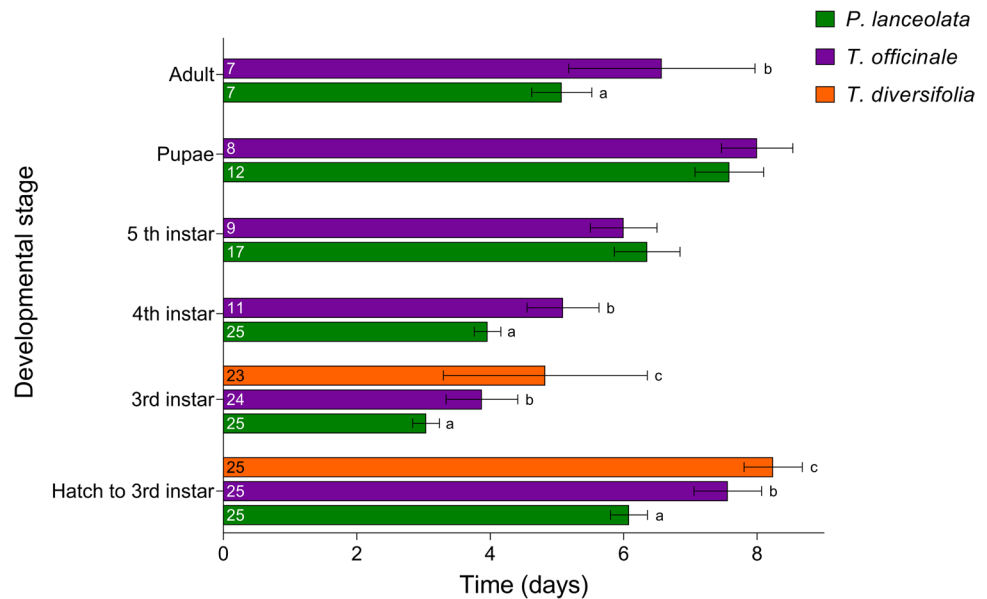


Fig. 2 Development time at selected stages of *Vanessa cardui* life cycle for caterpillars reared on *Plantago lanceolata*, *Taraxacum officinale* or *Tithonia diversifolia* leaves. Error bars represent standard deviations. Numbers within bars indicate sample sizes. Different letters indicate significant differences between the host plants within a developmental stage ($P < 0.01$, one-way ANOVA with Tukey's multiple comparisons test for hatch to 3rd instar and 3rd instar developmental stages; $P < 0.05$, t-test for 4th instar, 5th instar, pupal and adult developmental stages)



the caterpillars reared on *P. lanceolata* (mean = 3.96 days, SD = 0.20, $n = 25$) and approximately 5 days for the caterpillars reared on *T. officinale* (mean = 5.09 days, SD = 0.54, $n = 11$). Caterpillars reared on *P. lanceolata* or *T. officinale* leaves exhibited a similar development time during the 5th larval instar (mean = 6.35 days, SD = 0.49, $n = 17$ and mean = 6.00 days, SD = 0.50, $n = 9$, respectively), as well as during the pupal stage (mean = 7.58 days, SD = 0.51, $n = 12$ and mean = 8.00 days, SD = 0.53, $n = 8$, respectively). Adult longevity was higher when *T. officinale* leaves were provided as diet and the butterflies lived approximately 7 days (mean = 6.57 days, SD = 1.4, $n = 7$). Adults that were reared on *P. lanceolata* leaves lived approximately 5 days (mean = 5.07 days, SD = 0.45, $n = 7$).

Larval Weight

Overall, mean weights of caterpillars reared on *T. diversifolia* leaves were lower than the mean weights of caterpillars reared on *P. lanceolata* or *T. officinale* leaves (Fig. 3) and a significant interaction was found between the consumed host plants and developmental time points ($F_{10,224} = 90.38$, $df = 10$, $P < 0.0001$). For caterpillars reared on *T. diversifolia*, the mean larval weights were significantly different from the mean larval weights of caterpillars reared on *P. lanceolata* starting at the 3rd instar ($P < 0.0001$ for the 3rd instar time point and further development time points). Compared to caterpillars reared on *T. officinale*, caterpillars reared on *T. diversifolia* exhibited significantly different mean larval weights starting at the 4th instar ($P = 0.0006$ for the 4th instar time point; $P = 0.0013$ for three days post-4th instar time point and $P < 0.0001$ for further time points). Additionally, mean weights of caterpillars reared on *P. lanceolata* or *T.*

officinale leaves were significantly different at the beginning of the 3rd instar and at nine days post-4th instar ($P < 0.0001$ for both time points) (i.e., mean larval weights were similar during most of the larval development).

Immune Response

Caterpillars reared on *P. lanceolata* leaves exhibited significantly higher PO activity than caterpillars reared on *T. officinale* or *T. diversifolia* leaves ($F_{2,22} = 197.5$, $df = 2$, $P < 0.0001$) (Fig. 4). For the caterpillars reared on *T. diversifolia*, PO activity was close to zero and did not differ significantly from the PO activity of caterpillars reared on *T. officinale* ($P = 0.1356$). At the time of the hemolymph extraction, all caterpillars reared on *P. lanceolata* and most of the caterpillars reared on *T. officinale* were in the 5th larval instar. However, all caterpillars reared on *T. diversifolia* were still in the 4th larval instar.

Trade-off in Resource Use

Caterpillars reared on *T. officinale* exhibited increased mass gain during the last larval instar and lower PO activity than caterpillars reared on *P. lanceolata* (Fig. 5). Additionally, a significant interaction was found between mass gained, PO activity and host plant consumption ($F_{1,22} = 49.69$, $df = 1$, $P < 0.0001$), which suggested a potential trade-off in resource use.

Chemical Profile

The varying chemical content in terms of secondary metabolites among the three host plants was confirmed in the PCA

Fig. 3 Mean larval weights of *Vanessa cardui* caterpillars reared on *Plantago lanceolata*, *Taraxacum officinale* or *Tithonia diversifolia* leaves. Error bars represent standard deviations. Lv indicates the larval viability for caterpillars reared on each host plant. Different letters indicate significant differences between host plants within a development time point ($P < 0.001$, mixed effects model with Tukey's multiple comparisons test). Mean larval weights, standard deviations and sample sizes of caterpillars reared on each host plants are presented in Table S1 (Supplementary Material)

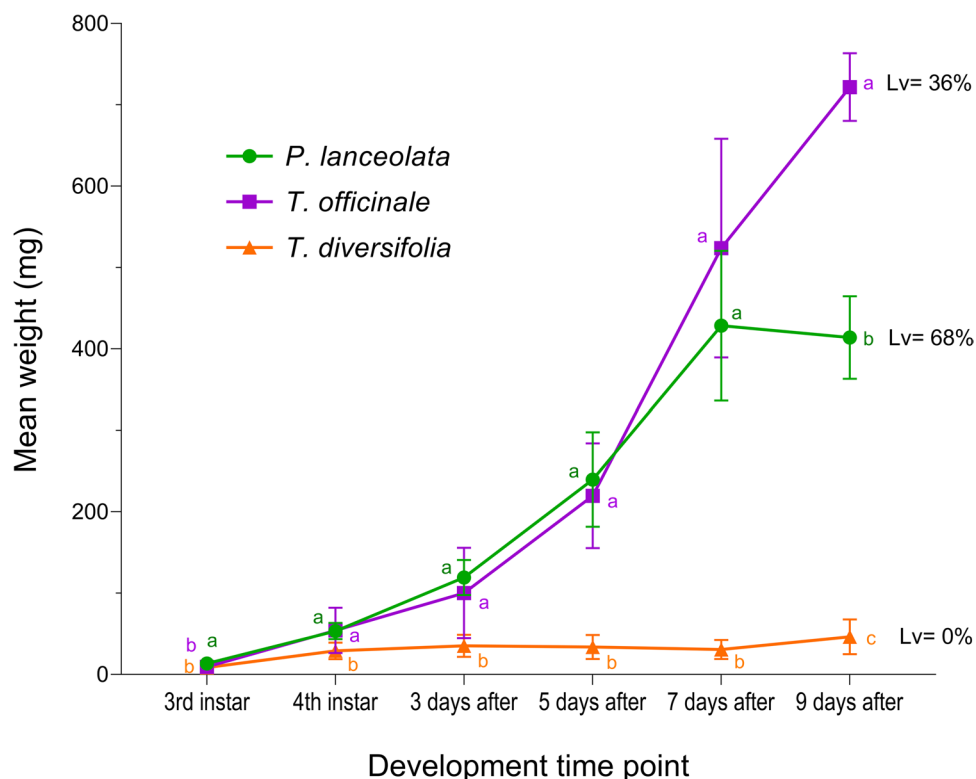
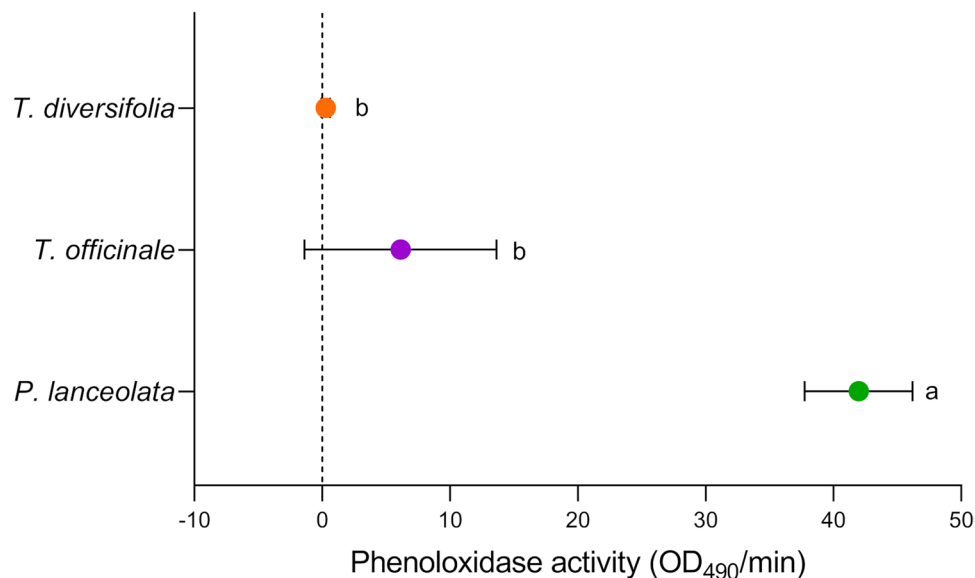


Fig. 4 Mean phenoloxidase activity for *Vanessa cardui* caterpillars reared on *Plantago lanceolata*, *Taraxacum officinale* or *Tithonia diversifolia* leaves. Error bars represent standard deviations. Different letters indicate significantly differences between the host plants ($P < 0.0001$, one-way ANOVA with Tukey's multiple comparisons test)



plot ($PC=4$; $R^2X=0.708$; $Q^2=0.646$), which showed a sample cluster tendency according to the plant species (Fig. 6).

PLS-DA along with the VIP plot allowed the determination of the discriminant metabolites between groups (i.e., each host plant) (Fig. 7). Using our in-house database along with LC-MS data (retention times, mass spectra and fragmentation patterns) (Supplementary Material, Table S2) and VIP value (Supplementary Material, Table S3), 40 discriminant metabolites were putatively

identified (Table 2). Iridoid glycosides, phenylethanoid glycosides and flavonoids (one flavone and one flavone *O*-glycoside) were the main discriminant metabolites putatively identified in *P. lanceolata* leaves. *Taraxacum officinale* leaves were characterized by the presence of phenylpropanoids, sesquiterpene lactones (germacranolides, eudesmanolides and guaianolide), two flavones *O*-glycoside and one phenylacetyl glycoside. For *T. diversifolia*, phenylpropanoids, sesquiterpene lactones

Fig. 5 Aligned dot plot with line at mean for mass gained (A) and phenoloxidase activity (B) of *Vanessa cardui* caterpillars reared on *Plantago lanceolata* or *Taraxacum officinale* leaves, showing a potential trade-off in resource use

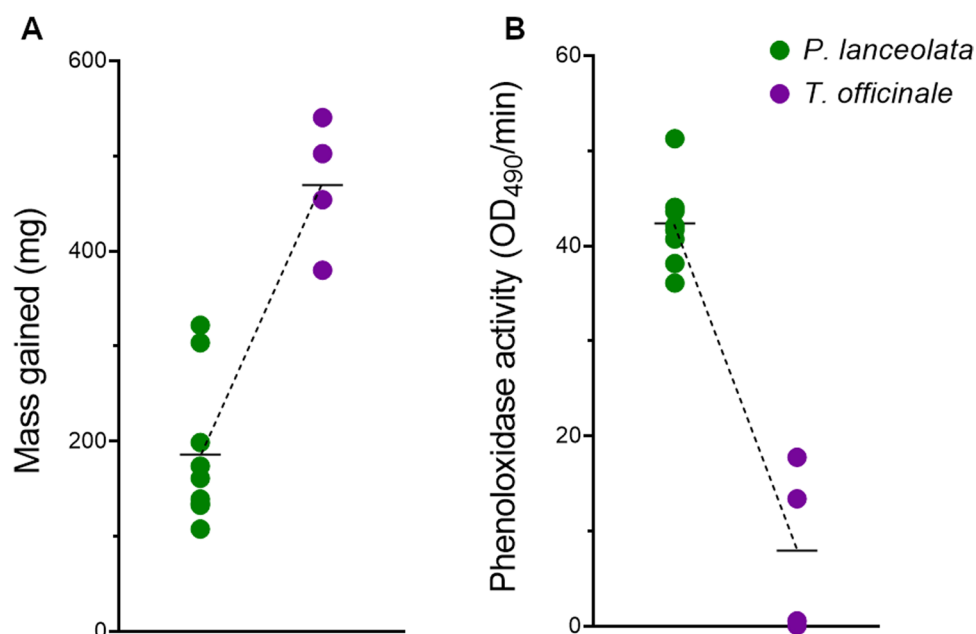
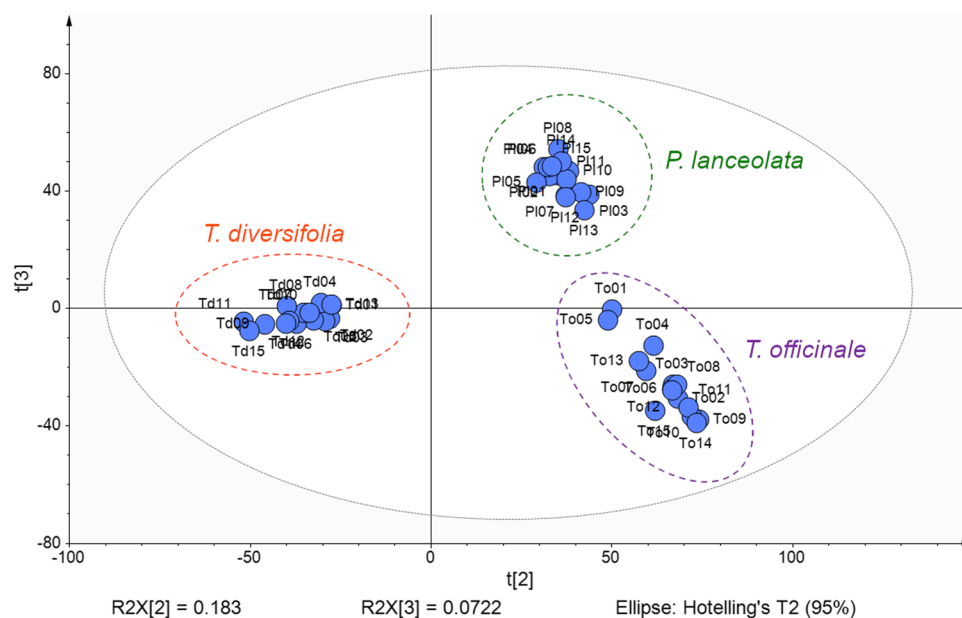


Fig. 6 PCA score plot for the LC-MS data of *Plantago lanceolata*, *Taraxacum officinale* and *Tithonia diversifolia* leaves



(furanoheliangolides, heliangolide and eudesmanolide) and flavones were appointed as discriminant metabolites.

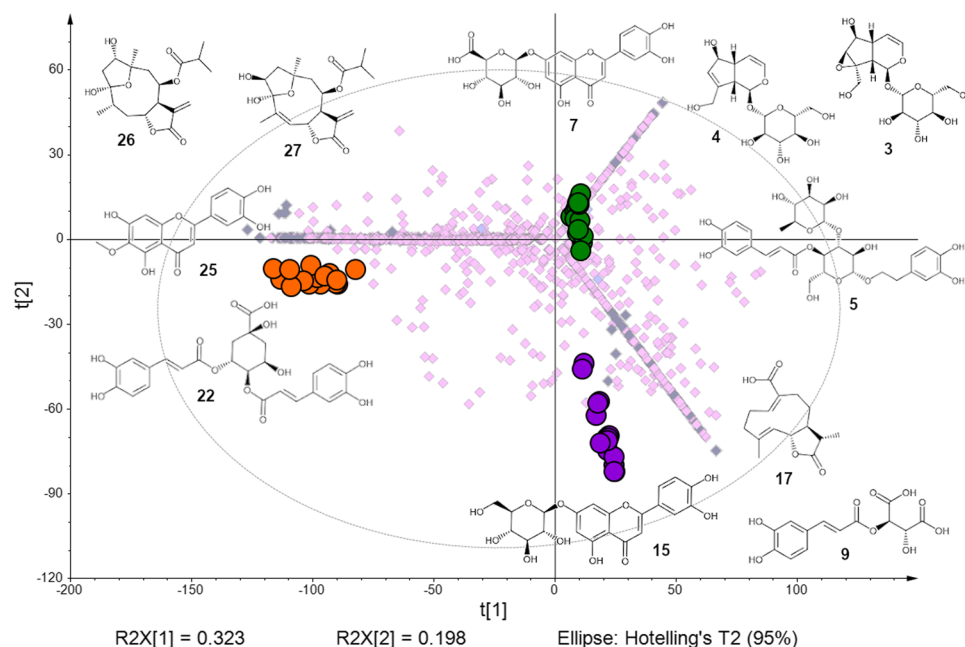
Discussion

Vanessa cardui caterpillars thrived and fully developed on *P. lanceolata* leaves containing iridoid glycosides and phenylethanoid glycosides. However, they were not able to properly develop and complete metamorphosis when reared on *T. diversifolia* leaves, which contains a variety of furanoheliangolide-type sesquiterpene lactones. Concurrently,

caterpillars reared on *T. officinale* leaves, which do not have iridoid glycosides, phenylethanoid glycoside or furanoheliangolide-type sesquiterpene lactones, were able to develop throughout the larval and adult stages, yet they presented lower larval and pupal viabilities compared to caterpillars reared on *P. lanceolata* leaves. These findings suggest that iridoid and phenylethanoid glycosides are not detrimental for *V. cardui* development compared with furanoheliangolide-type sesquiterpene lactones that showed possible detrimental effects on *V. cardui* caterpillars.

Iridoid glycosides are the typical defense metabolites of Plantaginaceae family and have been related to toxic

Fig. 7 PLS-DA score plot superimposed to the loading plot and structures of metabolites corresponding to the main chemical classes appointed as discriminants between the host plants (phenylpropanoids, furanoheliangolide- and germacranolide-type sesquiterpene lactones, flavonoids, phenylethanoids and iridoids glycosides). Colored circles represent host plant samples (green, *Plantago lanceolata*; purple, *Taraxacum officinale*; orange, *Tithonia diversifolia*). Light pink diamonds represent the variables (molecules and fragments) detected by LC-MS, with the putatively identified discriminant metabolites highlighted in dark blue



effects on non-adapted insect herbivores (e.g., decreased growth rate, increased mortality and feeding deterrence) (Bowers 1991; Dobler et al. 2011). Among the diverse iridoid glycosides, aucubin and catapol play important roles in plant–insect interactions. For insects that specialize on plants containing iridoid glycosides, a negative correlation has been demonstrated between sequestration of defensive metabolites and immune response (i.e., high iridoid glycoside sequestration was associated with reduced immunity) (Smilanich et al. 2009a; Lampert and Bowers 2015; Muchoney et al. 2022). Even though sequestration of plant secondary metabolites by insects may compromise their immune response, some sequestered metabolites may be effective as direct defense against natural enemies and pathogens (Muchoney et al. 2022). *Vanessa cardui* are capable of sequestering iridoid glycosides, but at lower concentrations compared to adapted specialists like the common buckeye (*Junonia coenia*) and the Baltimore checkerspot (*Euphydryas phaeton*) (Bowers and Puttick 1986; Lampert et al. 2014), thus any detrimental effects on the immune response due to sequestered iridoid glycosides may not be fully realized with *V. cardui*.

Similarly, sesquiterpene lactones are characteristic metabolites of the Asteraceae family and are widely associated with protective roles in plants (e.g., anti-herbivore and antimicrobial activities) (Padilla-Gonzalez et al. 2016). Germacranolides, eudesmanolides and guaianolides are the most representative structural types of sesquiterpene lactones. The occurrence of specific types of sesquiterpene lactones within Asteraceae taxa have been used to infer taxonomic relationships (Da Costa et al. 2005). In the tribe Heliantheae (in which *T. diversifolia* is located), heliangolides

and furanoheliangolides are the main types of sesquiterpene lactones described (Pereira et al. 1997; Ambrósio et al. 2008; Gallon et al. 2019). Our results suggest that *V. cardui* have adapted to tolerate the iridoid glycosides detected in *P. lanceolata* leaves, as well as some types of sesquiterpene lactones presented in *T. officinale* leaves (germacranolide-, eudesmanolides- and guaianolide-type), however they were susceptible to the toxic effects of furanoheliangolide-type sesquiterpene lactones and/or other metabolites occurring in *T. diversifolia*.

We found that, upon hatching, neonate caterpillars started feeding on *T. diversifolia* leaves, however, across larval development they exhibited reduced feeding, which was reflected in low weights and failed to reach the 5th larval instar. Indeed, most of the caterpillars reared on *T. diversifolia* died during the 3rd larval instar; few caterpillars were able to enter the 4th instar and none survived to the 5th instar. Additionally, from visual inspections we observed that caterpillars reared on *T. diversifolia* consumed less plant material (i.e., smaller consumed leaf area) than caterpillars reared on *P. lanceolata* or *T. officinale*. Recently, tagitinin C, a heliangolide-type sesquiterpene lactone obtained from *T. diversifolia* leaves, was indicated as a feeding deterrent compound against *Helicoverpa armigera* (Inácio et al. 2020). Considering that furanoheliangolide-type sesquiterpene lactones were the main discriminant metabolites detected for *T. diversifolia*, our data suggest that these types of sesquiterpene lactones along with tagitinin C may act as feeding deterrents against *V. cardui*, especially since they are naïve to this plant. However, other mechanisms besides chemistry may be involved in the detrimental effects observed in caterpillars reared on *T. diversifolia*, and further experiments

Table 2 Discriminant metabolites putatively identified in *Plantago lanceolata*, *Taraxacum officinale* and *Tithonia diversifolia* leaves

ID	Metabolite name	Metabolite class (subclass)
<i>Plantago lanceolata</i>		
1	asperuloside	Iridoid glycoside
2	plantamajoside	Phenylethanoid glycoside
3	catapol	Iridoid glycoside
4	aucubin	Iridoid glycoside
5	acteoside	Phenylethanoid glycoside
6	isoacteoside	Phenylethanoid glycoside
7	luteolin 7- <i>O</i> -glucuronide	Flavone <i>O</i> -glycoside
8	apigenin	Flavone
<i>Taraxacum officinale</i>		
9	caftaric acid	Phenylpropanoid (hydroxycinnamic acid derivative)
10	coutaric acid	Phenylpropanoid (hydroxycinnamic acid derivative)
11	taraxacolide 1-glucopyranoside	Sesquiterpene lactone (eudesmanolide)
12	ixerin D	Sesquiterpene lactone (guaianolide)
13	luteolin 7- <i>O</i> -rutinose	Flavone <i>O</i> -glycoside
14	11,13-dihydrotaraxinic acid glucopyranosyl ester	Sesquiterpene lactone (germacranolide)
15	luteolin 7- <i>O</i> -glucoside	Flavone <i>O</i> -glycoside
16	chicoric acid	Phenylpropanoid (hydroxycinnamic acid derivative)
17	11,13-dihydrotaraxinic acid	Sesquiterpene lactone (germacranolide)
18	taraxiroside B	Phenylacetyl glycoside (butyrolactone)
<i>Tithonia diversifolia</i>		
19	5- <i>O</i> -caffeoylquinic acid	Phenylpropanoid (hydroxycinnamic acid derivative)
20	4- <i>O</i> -caffeoylquinic acid	Phenylpropanoid (hydroxycinnamic acid derivative)
21	orizabin	Sesquiterpene lactone (furanoheliangolide)
22	3,4-di- <i>O</i> -caffeoylquinic acid	Phenylpropanoid (hydroxycinnamic acid derivative)
23	4,5-di- <i>O</i> -caffeoylquinic acid	Phenylpropanoid (hydroxycinnamic acid derivative)
24	1-hydroxy-3- <i>O</i> methyltirotundin	Sesquiterpene lactone (furanoheliangolide)
25	nepetin	Flavone
26	tagitinin A	Sesquiterpene lactone (furanoheliangolide)
27	tagitinin B	Sesquiterpene lactone (furanoheliangolide)
28	2-hydroxitirotudin	Sesquiterpene lactone (furanoheliangolide)
29	hispidulin	Flavone
30	tagitinin C	Sesquiterpene lactone (heliangolide)
31	diversifolin	Sesquiterpene lactone (furanoheliangolide)
32	tagitinin F	Sesquiterpene lactone (furanoheliangolide)
33	2- <i>O</i> -methyltagitinin B	Sesquiterpene lactone (furanoheliangolide)
34	1-methoxydiversifolin	Sesquiterpene lactone (furanoheliangolide)
35	tirotundin	Sesquiterpene lactone (furanoheliangolide)
36	1-hydroxy-3- <i>O</i> -methyldiversifolin	Sesquiterpene lactone (furanoheliangolide)
37	3-acetoxy-8-isobutyroyloxyreynosin	Sesquiterpene lactone (eudesmanolide)
38	3- <i>O</i> -methyltirotundin	Sesquiterpene lactone (furanoheliangolide)
39	1-methoxy-3- <i>O</i> -methyldiversifolin	Sesquiterpene lactone (furanoheliangolide)
40	tagitinin I	Sesquiterpene lactone (furanoheliangolide)

are required to confirm the proposed detrimental effects of sesquiterpene lactones on *V. cardui* development.

Besides the potential toxic effects of furanoheliangolide-type sesquiterpene lactones occurring in *T. diversifolia*, the poor development of *V. cardui* caterpillars reared

on this plant species may be explained by the absence of some essential nutrients (e.g., protein, carbohydrates, vitamins and minerals). The diet composition and quality have been associated with direct and indirect effects on insect physiology, performance, and immunological functions

(Singer et al. 2014; Cotter et al. 2019; Yoon et al. 2019). Therefore, *T. diversifolia* may lack essential nutrients that would allow *V. cardui* caterpillars to fully develop.

Regarding the immune response, *V. cardui* caterpillars reared on *P. lanceolata* exhibited the highest PO activity. Whereas caterpillars reared on *T. diversifolia* or *T. officinale* presented lower and similar PO activity. In insects, PO is an enzyme responsible for activating melanogenesis (i.e., production of the pigment melanin), which in turn is an important component of the immune response (González-Santoyo and Córdoba-Aguilar 2012). Upon attack by foreign invaders or mechanical injury, melanin is deposited around the foreign object or damaged tissues to initiate encapsulation processes and immune responses. Therefore, PO is a key indicator of the immune status in insects, since it is involved in the defense response against parasitoids, pathogens and virus (Smilanich et al. 2009b, 2018; Hansen et al. 2017; Resnik and Smilanich 2020). Several studies have demonstrated that PO activity is affected by the ingested diet composition, especially in terms of protein and carbohydrate ratios (Lee et al. 2006; Povey et al. 2009; Cotter et al. 2019). The assumption that PO production and maintenance is diet-dependent predicts that healthy individuals ought to exhibit high levels of PO (González-Santoyo and Córdoba-Aguilar 2012). Our results corroborate this assumption, since caterpillars reared on *P. lanceolata* presented higher larval and pupal viabilities, as well as higher PO activity, compared to caterpillars reared on *T. officinale* or *T. diversifolia* leaves.

Interestingly, caterpillars reared on *P. lanceolata* and *T. officinale* exhibited similar larval weights at equivalent developmental points during most of the larval stage; however, their weights were significantly different at the final stage of larval development. Caterpillars reared on *T. officinale* reached higher weights at the end of the 5th instar and consequently increased mass gain from the time of hemolymph extraction to final larval instar (mean larval weight = 721.76 mg, SD = 41.52, $n = 5$; mean mass gain = 469.23 mg, SD = 69.24, $n = 4$) compared to caterpillars reared on *P. lanceolata* (mean larval weight = 413.88 mg, SD = 50.65, $n = 20$; mean mass gain = 185.96 mg, SD = 76.73, $n = 9$). Since extracting hemolymph involves puncturing the hemocoel, it causes an injury to the body and elicits a wound response by the caterpillar. Thus, the weight gained post-hemolymph extraction represents a period when the immune response was activated. In insects, immune investment is often costly, and hence trade-offs between immunity and other fitness traits (e.g., fecundity, longevity, survival and development) are expected (Schwarzenbach and Ward 2006; Schwenke et al. 2016; Hanson et al. 2019). Considering that caterpillars reared on *P. lanceolata* leaves exhibited higher PO activity than caterpillars reared on *T. officinale*, the difference found for larval weights and mass gained, as well as adult longevity, may

reflect a trade-off in resource use between investing on larval development versus mounting an effective immune response. Alternatively, *T. officinale* leaves may present high lipidic and/or carbohydrate content, which would increase mass gained in the final stage of larval development.

Taken together, our findings suggest that furanohelian-golide-type sesquiterpene lactones may deploy detrimental effects on *V. cardui* larval development and immune response, whereas, iridoid glycosides and phenylethanoid glycosides from *P. lanceolata* were not associated with toxic effects on *V. cardui* development or immune system. As initially predicted, caterpillars reared on *P. lanceolata* and *T. officinale* (common host plants used by *V. cardui* in the wild) exhibited higher immune responses and developmental rates than caterpillars reared on *T. diversifolia* (a naïve host plant). Additionally, we provided evidence for a trade-off between fitness traits and immunity in this generalist insect herbivore. Moving forward, evaluating the influence of essential nutrients along with secondary plant metabolites may contribute to clarify and unravel the diet-mediated roles in plant–insect interactions.

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Data Availability The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing Interests The authors have no relevant financial or non-financial interests to disclose.

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