

Research article

Chemodiversity affects preference for *Tanacetum vulgare* chemotypes in two aphid species

Annika Neuhaus-Harr¹✉, Lina Ojeda-Prieto¹, Elisabeth Eilers^{2,3}, Caroline Müller², Wolfgang W. Weisser¹ and Robin Heinen¹

¹Department of Life Science Systems, Technical University of Munich, School of Life Sciences, Freising, Germany

²Department of Chemical Ecology, Bielefeld University, Bielefeld, Germany

³CTL GmbH Bielefeld, Bielefeld, Germany

Correspondence: Annika Neuhaus-Harr (annika.neuhaus@tum.de)

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Plants of the same species can strongly differ in their specialized metabolite profiles, which can affect insect presence and abundance in the field. However, how specialized chemistry shapes plant attractiveness to herbivorous insects is not fully understood. Here, we used common tansy *Tanacetum vulgare*, Asteraceae) – a perennial plant that is highly diverse in terpenoid composition and is known to have variable chemotypes – to test whether 1) plants with different chemotype profiles differ in attractiveness to two specialist aphid species, *Macrosiphoniella tanacetaria* and *Uroleucon tanacetii*, in pairwise choice assays; 2) the diversity of the terpenoid blend affects plant attractiveness to aphids; 3) how plant chemical traits relate to plant morphological traits and which traits best explain aphid preference. We found that *M. tanacetaria* preferred two out of five chemotypes, dominated by α -thujone/ β -thujone and β -trans-chrysanthenyl acetate, while avoiding a chemotype dominated by α -pinene/sabinene. *Uroleucon tanacetii* showed no clear preference towards chemotypes, but when given a choice between chemotypes dominated by α -thujone/ β -thujone and by α -pinene/sabinene, they preferred the former. Importantly, plant attractiveness to aphids was marginally negatively correlated with chemodiversity, i.e. the number of terpenoid compounds, in *M. tanacetaria*, but not in *U. tanacetii*. Interestingly, the relative concentration and number of terpenoids were generally higher in larger and bushier plants. Hence, we did not observe a tradeoff between plant growth and defence. We conclude that plant chemical composition affects plant attractiveness to aphids and hence may contribute to variation in natural aphid colonization patterns on plants of the same species.

Keywords: aphids, attractiveness, choice assays, genotypic variation, intraspecific phytochemical diversity, plant diversity, terpenoids

Introduction

Understanding relationships between plants and herbivores is an important goal in ecology. How variation in plant diversity shapes herbivory has been a subject of study



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for many decades (Scherber et al. 2010, Weisser et al. 2017). For a long time, between-species diversity was believed to be more important than within-species diversity as a driver of ecosystem processes (Des Roches et al. 2018). Intraspecific diversity, which includes the variation between individuals and the richness and abundance of genotypes and phenotypes within a population, recently gained more attention as an important driver of ecological processes (Raffard et al. 2019). It has been shown that intraspecific variation in plant genotype and phenotypes can structure plant–arthropod food webs (Crutsinger et al. 2006, Poelman et al. 2008, Bálint et al. 2016). As plants of the same species can differ strongly in various traits, understanding how this variation contributes to shaping interactions between plants and interaction partners is currently an important goal in plant ecology.

One important dimension of intraspecific variation in plants is chemodiversity, the variation in chemical composition between different plant individuals of the same species (Wetzel and Whitehead 2020). Primary metabolites are important regulators of basic physiological processes in plants, including growth, development and reproduction, and primary compounds are relatively similar across the plant kingdom (Weng et al. 2012). Specialized metabolites, on the other hand, play critical roles as mediators of interactions, and plants have evolved a much larger diversity in these specialized metabolites, both inter- and intraspecifically (Pichersky and Gang 2000, Iason et al. 2012). Intraspecific variation in specialized metabolite profiles is known to affect the structure of associated herbivore communities, including phloem-feeding insects such as aphids (Poelman et al. 2008, Bustos-Segura et al. 2017, Volf et al. 2019, Singh et al. 2021, Whitehead et al. 2021), but few studies have shown how chemodiversity structures herbivore abundance by affecting herbivore preference for plants (Ziaja and Müller 2023).

Chemodiversity may mediate the presence and abundance of insect herbivores on a plant growing in natural conditions. Natural patterns of insect herbivores are likely shaped by a combination of 1) direct attractiveness of the host plant to the insect herbivore, 2) direct effects via host plant resource quality (i.e. bottom–up effects) and 3) indirect effects, via the plant's attractiveness to natural enemies of the insect herbivore (i.e. top–down effects). Many specialized compounds likely evolved to deter and repel herbivores (Herms and Mattson 1992, Kessler and Baldwin 2001, Whitehead et al. 2021). However, some specialist herbivore species may also use these compounds to find their host (Nishida 2014, Wink 2018). As such, insects can be repelled or attracted by volatile organic compounds (VOCs) emitted into the headspace of a plant (Clancy et al. 2016, Jakobs and Müller 2019). Once an insect herbivore has arrived on the plant, stored and emitted compounds can then affect herbivore performance. For instance, stored compounds can act as feeding deterrents or influence the metabolism of the herbivores (Mithöfer and Boland 2012). But how these compounds act largely depends on the specific compound and the life history of the insect herbivore (Unsicker et al. 2009, McCormick et al. 2012, Jakobs et al. 2019).

Individual plants of the same species not only differ in their specialized metabolites, but often also differ pronouncedly in the expression of morphological traits. Various studies point out that traits related to growth or to structural defences may play an important role in driving interactions between plants and insects (Herms and Mattson 1992, Agrawal and Fischbein 2006). For instance, in a study on wheat plants, Batyrshina et al. (2020) found higher numbers of aphids on fast-maturing than on slow-maturing wheat plants. Indeed, tradeoffs between plant growth and defence are thought to be common in nature (Coley et al. 1985, Herms and Mattson 1992). Furthermore, plants with more pronounced mechanical defence traits, including stronger and tougher leaf tissues, tend to be better defended against insect herbivory (Caldwell et al. 2016). It is possible that at least some chemical traits are linked to morphological traits, together driving insect preference. For instance, Hayashi et al. (2005) found that trichome density and leaf mass per area differed between the two chemotypes of *Salix sachalinensis* (*Salicaceae*). Another striking example of differences in expression of chemical and morphological traits can be found in common tansy, *Tanacetum vulgare* (*Asteraceae*). In this species, plants with a higher storage of the terpenoid camphor were found to have taller shoots than those with lower camphor amounts, while plants containing davadone-D or artemisia ketone developed more flower heads, taller corymbs, and delayed flowering compared to plants with a lower content of these terpenoids (Keskitalo et al. 2001). Furthermore, *T. vulgare* from different origins (e.g. North America and Europe) have been found to differ in both morphological and chemical traits and exhibit negative correlations between reproductive biomass and terpenoid concentrations (Wolf et al. 2011). *Tanacetum vulgare* is a perennial, aromatic plant that has a large geographic distribution and is associated with a complex herbivore community including mono-, oligo- and polyphagous aphids (Schmitz 1998, Keskitalo et al. 2001, Kleine and Müller 2011). *Tanacetum vulgare* is rich in different mono- and sesquiterpenoids and plants can be divided into chemotypes based on the composition of leaf terpenoids (Keskitalo et al. 2001, Kleine and Müller 2011). Previous studies and breeding experiments showed that terpenoid composition has a genetic basis in *T. vulgare* (Keskitalo et al. 2001). Specialized aphids are thought to be adapted to the metabolites in *T. vulgare* and to use these for finding host plants (Schoonhoven et al. 2005, Jakobs and Müller 2019). Aphid colonization, growth rate and survival, and even the genotypic structure of aphid colonies were found to be affected by *T. vulgare* chemotypes under natural colonization in the field (Senft et al. 2017, 2019, Clancy et al. 2018, Zytynska et al. 2019). Specialized aphids show preferences towards specific chemotypes that differ between species (Jakobs and Müller 2018), but which characteristics of *T. vulgare* chemotypes (e.g. diversity, relative concentrations, individual compounds) drive these preferences needs further investigation.

Here, we used six *T. vulgare* chemotypes to investigate how intraspecific differences in chemical profiles shape

attractiveness to the specialized aphids *Macrosiphoniella tanacetaria* (Hemiptera, Aphididae) and *Uroleucon tanacetii* (Hemiptera, Aphididae). We tested the following hypotheses: (H1) two aphid species will show species-specific attraction to distinct *T. vulgare* chemotypes, as previously shown by [Jakobs and Müller \(2018, 2019\)](#); (H2) chemodiversity negatively correlates with attractiveness, under the assumption that most specialized metabolites repel antagonistic organisms; and (H3) growth-related traits will tradeoff with chemodiversity, under the assumption that maintaining chemodiversity is costly and limits available resources for growth. Specifically, we investigated whether chemical composition relates to morphological traits, whether chemical or morphological traits are more important in driving aphid preference towards specific plant chemotypes, and whether the quantity or quality of terpenoids contribute to plant attractiveness to aphids.

Material and methods

Chemotypic characterization of *T. vulgare* lines

In 2019, leaf and seed samples of 27 *T. vulgare* plants (hereafter: mothers) were collected in Jena, Germany (50°55'48"N, 11°34'48"E), and chemotyped based on their terpenoid profiles. Terpenoids were analyzed as in [Ziaja and Müller \(2023\)](#). The leaf material was freeze-dried, homogenized, weighed and extracted in heptane, adding one-bromodecane as internal standard. Extracts were centrifuged and the supernatants analyzed using gas chromatography coupled with mass spectrometry (GC-MS; GC 2010plus – MS QP2020, Shimadzu) on a semi-polar column (VF-5 MS, 30 m length, 0.2 mm ID, 10 m guard column, Varian) in electron impact ionization mode at 70 eV, with helium as carrier gas. Samples were injected at 240°C with a 1:10 split. A starting temperature of 50°C was kept for 5 min, ramping up to 250°C at 10°C min⁻¹, then increasing with 30°C min⁻¹ to a final temperature of 280°C, hold for 5 min. An alkane standard mix (C7–C40, Sigma Aldrich) was measured regularly between samples. For identifying the terpenoids, retention indices (RI) and mass spectra were compared with available synthetic reference compounds library entries of the National Institute of Standards and Technology (NIST) 2014, Pherobase ([El-Sayed 2014](#)) and mass spectra reported in [Adams \(2017\)](#). Terpenoids were semi-quantified using the peak areas of the total ion chromatograms, relative to the sample dry mass and the peak area of the internal standard.

Plant terpenoid profiles were clustered using unsupervised hierarchical k-means clustering with the *hclust()* function. A dissimilarity matrix was calculated based on the terpenoid concentrations using the *dist()* function. We used absolute values for this, as differentiation between chemical profiles (chemotypes) is likely to be the result of both the terpenoid composition and terpenoid concentration, and both aspects are considered to be ecologically meaningful in terms of mediating plant attractiveness to herbivores. The number

of clusters, *k*, was obtained using the elbow method. We selected a *k*=7 for mothers and used mothers from six clusters for further chemotype selection.

Two mothers per cluster were then randomly selected and the collected seeds were mass-sown in seedling trays in November 2020. Ten healthy seedlings were selected from each of 12 mother plants (6 clusters × 2 mothers), resulting in a total of 120 plants grown from seeds (hereafter: daughters). Seedlings were transplanted to 10 cm pots filled with standard potting substrate (Stender potting substrate C 700 coarse structure, 1 kg NPK minerals m⁻³, pH 5.5–6.0) and transferred to a greenhouse compartment at the Plant Technology Centre of the Technical University of Munich. Plants received supplemental lighting (standard Sylvania neon tube, 58W/125, universal white light) with 16:00:8:00 h L:D which was turned off when outdoor light was > 40 klx. After seven weeks, the ultimate 3–4 leaflets from the youngest fully expanded pinnate leaf of each plant were harvested. Samples were flash-frozen in liquid nitrogen, and subsequently freeze-dried for chemotyping. All daughters were transplanted to a common garden on-site in July 2021 and watered well until fully established.

As *T. vulgare* is outcrossing, daughter plants from the same mother can express different chemotypes in dependence of the pollen donors ([Lokki et al. 1973](#), [Holopainen et al. 1997](#), [Dussarrat et al. 2023](#)). Therefore, we conducted a second unsupervised hierarchical clustering on the daughter terpenoid composition as described above for mothers. For the daughter profile clustering, the elbow *k* was at 5–7 clusters, which is why *k*=6 clusters was used for daughter clustering. Clusters were based on the dominant compound(s) and their relative terpenoid concentration, for example 'Athu-Bthu' with α -thujone and β -thujone as dominant compounds.

Propagation of plant material for aphid choice assays

Based on the results of the daughter cluster analysis, we selected three daughters per mother plant for further experimentation. In August 2021, fresh plant material was taken from the 18 selected daughters (6 mothers × 3 daughters), and shoot cuttings were prepared by cutting stem parts 1–2 cm below and 4–5 cm above a leaf node. Leaves were clipped in half to reduce evapotranspiration and the risk of mold. The stem cuttings were pressed in seedling trays filled with the same substrate as used for the mother plants. Cuttings were immediately covered by a transparent plastic hood which was gradually opened after three weeks, as the plants established roots and shoots. After acclimatizing the cuttings for two weeks, they were repotted to 10 cm pots and later to 17 cm pots to avoid pot limitation before their use in choice assays in December 2021. Plants were bottom-watered automatically and fertilized with Universol Blue fertilizer (18% N – 11% P – 18% K; ICL Deutschland), to maintain a target electrical conductivity of 1.0. No chemical insecticides or fungicides were used during plant propagation. Due to low propagation success of the Bthu-low chemotype, only the

remaining five chemotypes (with the respective 15 daughters) were used in pairwise aphid choice assays, and between 6–10 clones of the daughters were used in the experiment. Note that clones from the same daughter were grown in different pots randomly spaced in the greenhouse, thus resulting in independent development of each of the plants.

Morphological traits

Morphological traits were measured non-destructively for each of the original 120 daughter plants in February 2021. The number of stems, leaves, and total number of nodes per plant were counted, height was recorded in cm, and internode length was derived by dividing height (in cm) by the number of nodes. From the youngest fully expanded leaf, the total number of leaflets from one leaf, leaf length, and leaflet density (number of leaflets/petiole length) were assessed, and chlorophyll content was measured using a chlorophyll meter (Konica Minolta SPAD-502Plus). The leaf was then harvested and leaf surface area was determined using ImageJ (Schneider et al. 2012). The leaves were then dried for 72 h at 60°C to determine dry weight, specific leaf area.

Aphid rearing

Adults and nymphs of *M. tanacetaria* and *U. tanacetii* were collected in August 2021 from *T. vulgare* plants in a field near Freising, Germany (48°24'17.7228"N, 11°41'23.50968"E) and kept in plastic cages at room temperature with supplemental light at long day regimes (16:00:8:00 h L:D). Plants for feeding the aphid colonies were unrelated to the used chemotypes for this study to avoid an induction of preferences in the aphid colonies. For each testing round, roughly 100 unwinged adult aphids of each species were placed on individual fresh plants. After 48 h, all adult aphids were removed, and all nymphs were left on the plants. Subsequently, the cohorts were kept in a Fitotron standard growth chamber 120 (21/16°C, 60% RH, Weiss Technik) for eight days. Before the start of the choice assays, the aphids were starved in a petri dish with a small piece of wet tissue for 24 h to ensure immediate feeding, as has been done in other insect species (Minoretto and Weisser 2000, Bustos-Segura and Foley 2018, Clancy et al. 2020).

Pairwise choice assays

For choice assays, we used clones from five out of the six *T. vulgare* maternal chemotypes with their respective three replicate daughters per chemotype. A priori, a full-factorial series of pairwise choice assays were designed, in which aphids could choose between cloned plants of two different chemotypes (Supporting information). Within each replicate series, all chemotypes were tested against each other using randomly picked clones from randomly picked daughters of the mother chemotypes, maximizing the number of unique pairwise choices between chemotype-daughter pairs. Choice assays were conducted in three rounds on three different days with different aphid cohorts that were standardized by age (9–10

days old), all executed within one week (9–16 December 2021). In total, we performed $n=23$ replicate rounds for *M. tanacetaria*, and $n=13$ for *U. tanacetii*, as their cohort numbers were substantially lower. The specific assignment of combinations of *T. vulgare* daughters for both aphid species can be found in the supplement (Supporting information).

For the choice assays, the second and third youngest fully expanded leaf of a plant were selected, the first three leaflets discarded, and the following leaflets used in assays. Two different leaflets were placed in new 14.5 cm petri dishes at equal distances from the centre, and 8 cm apart from each other. The sides for each replicate alternated between replicate rounds to account for external bias. One starved aphid was placed in the centre of the petri dish, and the petri dishes were sealed with parafilm, to prevent the leaves from drying quickly. Petri dishes were kept in a climate chamber at Technical University of Munich (TUM Model EcoSystem Analyzer TUM_{mesa}). The LED lighting system in these chambers generates homogenous light conditions, aimed at reducing spatial effects. Petri dishes were left for 24 h (16:00:8:00 h L:D photoperiod, 21°C, 60% RH). Aphid preference was recorded after two, five and 24 h. However, as leaf quality visibly decreased after 24 h, but as aphids rely on leaf turgor for feeding, most were not on the leaves, or had died. Therefore, we excluded the 24 h time point from all following analyses. Each aphid and leaf were only used once. Dead aphids were excluded from further analyzes.

Statistical analysis

All statistical analyzes were performed in R (www.r-project.org). All used R code can be found in the Supporting information.

To address H1, we conducted binomial tests to test if aphids preferred one chemotype over another for each pairwise combination. We tested whether the number of choices made for one chemotype in a specific combination was significantly different from what would be expected in a random choice. Binomial tests were also used to check whether spatial (left/right side of the petri dish) effects on aphid preference occurred, using all observations per species ($n=360$ for *M. tanacetaria*, $n=130$ for *U. tanacetii*) as well as observer effects, using all observations ($n=690$). Four separate logit models were used, to determine the attractiveness of specific *T. vulgare* chemotypes for each aphid species, and the attractiveness of specific *T. vulgare* daughters for each aphid species, across all combined pairwise tests, using the R 'survival' package (Therneau 2021). Z-values were obtained from the models for each *T. vulgare* chemotype and for each *T. vulgare* daughter as a proxy for attractiveness, with plants with more positive z-values being more and negative z-values being less attractive to aphids.

To test H2, we calculated plant chemodiversity metrics for each daughter plant (Shannon diversity, terpenoid evenness, terpenoid richness and relative total terpenoid concentration) from the absolute terpenoid profiles, using the 'vegan' package (Oksanen et al. 2020). In four separate models for

each aphid species, we tested for relationships between plant attractiveness (z-value, response variable) and 1) Shannon diversity; 2) terpenoid evenness; 3) terpenoid richness; and 4) relative total terpenoid concentration (1–4 explanatory variables). These models were replicated at the *T. vulgare* daughter level (n=15). We further tested for correlations between the relative concentrations of individual compounds in a plant and the attractiveness of the plant separately for each of the two aphid species. We used unadjusted correlation plots and Holm-adjusted plots for multiple correlations, using the 'RcmdrMisc' package (Fox 2022) and present both for visualization purposes. For verification purposes, we ran a multiple regression model testing the effect of all compounds on attractiveness. As there was a substantial number of compounds (46), our models were limited by available degrees of freedom (n=15). Therefore, we removed all compounds that were measured in less than two-thirds of all samples. Using step() in combination with variance inflation factors to address multicollinearity, we reduced the model to the minimum adequate version, which only included individual compounds, which did not have significant effects on plant attractiveness to aphids.

To address H3, we tested for differences among chemotypes in plant height, number of stems, number of leaves, leaflets density, specific leaf area and chlorophyll, Shannon diversity, terpenoid evenness, terpenoid richness and relative total terpenoid concentration, by using one-way ANOVA with chemotype as a fixed factor (n=18 replicates in total for chemotypes).

Results

Chemical profile of chemotypes

Hierarchical k-means clustering of terpenoid profiles resulted in six large daughter clusters, without any small outlier clusters driven by strongly deviating individual profiles (Fig. 1). Daughter lineages did not typically resemble their mother lineages in their terpenoid profiles or their clustering and, therefore, the observed daughter clusters typically included daughters from multiple mother lineages (Fig. 1). From each cluster, we selected three daughters from the same mother lineage. When more than three daughters were available from a mother in a cluster, we selected the three daughters clustering closest together. Our chemotype selection resulted in six maternal chemotype lines with three genetically different daughter replicates for each cluster (Fig. 1) that were used for preparing plants for the preference experiment.

Selected chemotypes showed a high diversity of compounds (Fig. 2a), and significantly differed in their Shannon diversity, terpenoid evenness and relative total terpenoid concentration, but not in their terpenoid richness (Fig. 2b–e). Specifically, the chemotype 'Athu-Bthu' had both α - and β -thujone as prevalent compounds. The chemotypes 'Bthu-low' and 'Bthu-high' were both dominated by β -thujone but characterized by a low or high relative total terpenoid concentration, respectively. The

chemotype 'Chrys-acet' was strongly dominated by β -trans-chrysantenyl acetate. In the chemotype 'Mixed-low' several terpenoids contributed more evenly to the total profile, and had overall a low relative total terpenoid concentration. In the chemotype 'Mixed-high', terpenoids had overall a higher relative total terpenoid concentration. The chemotypes Mixed-high and Mixed-low showed a significantly higher terpenoid Shannon diversity and terpenoid evenness than the other four chemotypes (Fig. 2b–c). The six selected chemotypes did not significantly differ in terpenoid richness (Fig. 2d), but did differ in their relative total terpenoid concentrations, with the chemotypes Mixed-low and Bthu-low expressing significantly lower concentrations compared to the other chemotypes (Fig. 2e). Every chemotype consisted of three daughters (biological replicates) which were number coded e.g. Mixed_high_21, Mixed_high_23 and Mixed_high_30.

Aphid preference for chemotypes (H1)

After two hours, seven *M. tanacetaria* and five *U. tanacetii* individuals were found dead and were excluded from the analyzes. In total, 178 *M. tanacetaria* and 69 *U. tanacetii* had chosen the leaflet of one chemotype. A binomial test showed that *M. tanacetaria* aphids tended to marginally prefer leaflets from Athu-Bthu over leaflets from the chemotypes Mixed-low and Bthu-high (Table 1, Fig. 3a). After five hours of observation, *M. tanacetaria* showed a significant preference for chemotype Athu-Bthu over chemotype Mixed-low (p=0.013) and tended to prefer Chrys-acet over the chemotypes Mixed-low and Mixed-high (Table 1, Fig. 3b). *Uroleucon tanacetii* aphids significantly preferred the Athu-Bthu over the Mixed-low chemotype after two hours (p=0.039; Table 1, Fig. 3c), but after five hours no more significant preferences were observed for this species (Table 1, Fig. 3d).

Considering all aphid choices made across all pairwise comparisons in a clogit model, *M. tanacetaria* aphids showed a significant attraction to Chrys-acet (z=2.331, p=0.020) and a marginally significant attraction to the Athu-Bthu chemotype (Table 2, Fig. 4a), while *U. tanacetii* aphids did not show a clear preference for any chemotype (Table 2, Fig. 4b). However, we found that aphids also exhibited preferences at the individual plant daughter level, with *M. tanacetaria* showing significant preferences for the daughters Chrys-acet_95, Chrys-acet_100, Athu-Bthu_55 and Athu-Bthu_56, whereas *U. tanacetii* did not show significant preferences to any *T. vulgare* daughters (Table 3).

Aphid preference to plant chemodiversity (H2)

For *M. tanacetaria*, we observed a non-significant trend of decreasing attraction to plants with higher terpenoid Shannon diversity (Fig. 4c) and higher terpenoid evenness (Fig. 4d), when testing independent variables separately in linear regression models, but a non-significant trend of increased attraction to plants with a higher relative total terpenoid concentration (Fig. 4e). For *U. tanacetii*, we observed no significant relationships between attractiveness and chemodiversity indices.

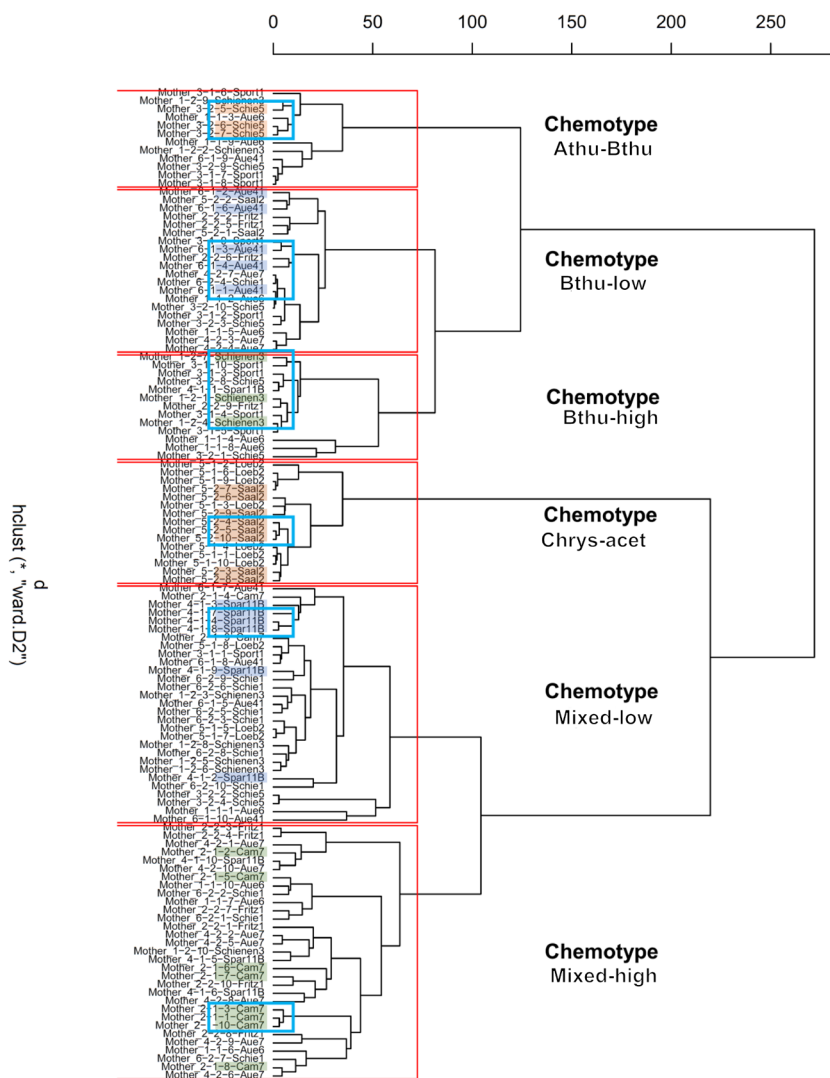


Figure 1. Hierarchical clustering tree for terpenoid profiles of all 120 daughter plants from *Tanacetum vulgare*. Clusters ($k=6$) are indicated in red boxes. For the selection, in each cluster a different mother lineage was chosen, and three daughters were manually selected and highlighted. The final selection of daughters of each cluster is indicated in blue boxes.

Unadjusted correlation plots of individual terpenoids revealed that various relationships existed between individual terpenoids and the attractiveness to aphid species, but when Holm-adjusted for multiple correlations were applied, this reduced the relationships between attractiveness and individual terpenoids to only a single weak negative relationship between 1-terpinen-4-yl acetate and plant attractiveness to *M. tanacetaria* (Fig. 4f). This was further verified with simplified multiple regression models corrected for collinearity, which reduced the models to single terpenoid compounds as factors, which did not significantly affect plant attractiveness.

Relationships between chemical diversity and morphological plant traits (H3)

Significant positive relationships were observed between the number of stems per *T. vulgare* daughter and terpenoid Shannon diversity ($n=120$ daughter plants, Table 4, Fig. 5a),

T. vulgare daughter height and terpenoid Shannon diversity (Fig. 5b), and *T. vulgare* daughter height and relative terpenoid concentration (Fig. 5c).

Discussion

In this study, we found that the investigated *T. vulgare* plants clustered into six distinct chemotypes that differed in their terpenoid Shannon diversity, terpenoid evenness and relative total terpenoid concentration. We used five of these chemotype lines to test how leaf chemical profiles affected aphid preference in pairwise choice assays and found that two specialist aphid species showed species-specific preferences to specific chemotypes. Across all pairwise combinations *M. tanacetaria* showed stronger preferences and patterns of attraction than *U. tanacetii*. In line with our expectations, we observed a trend of higher attractiveness of plants with a higher relative total

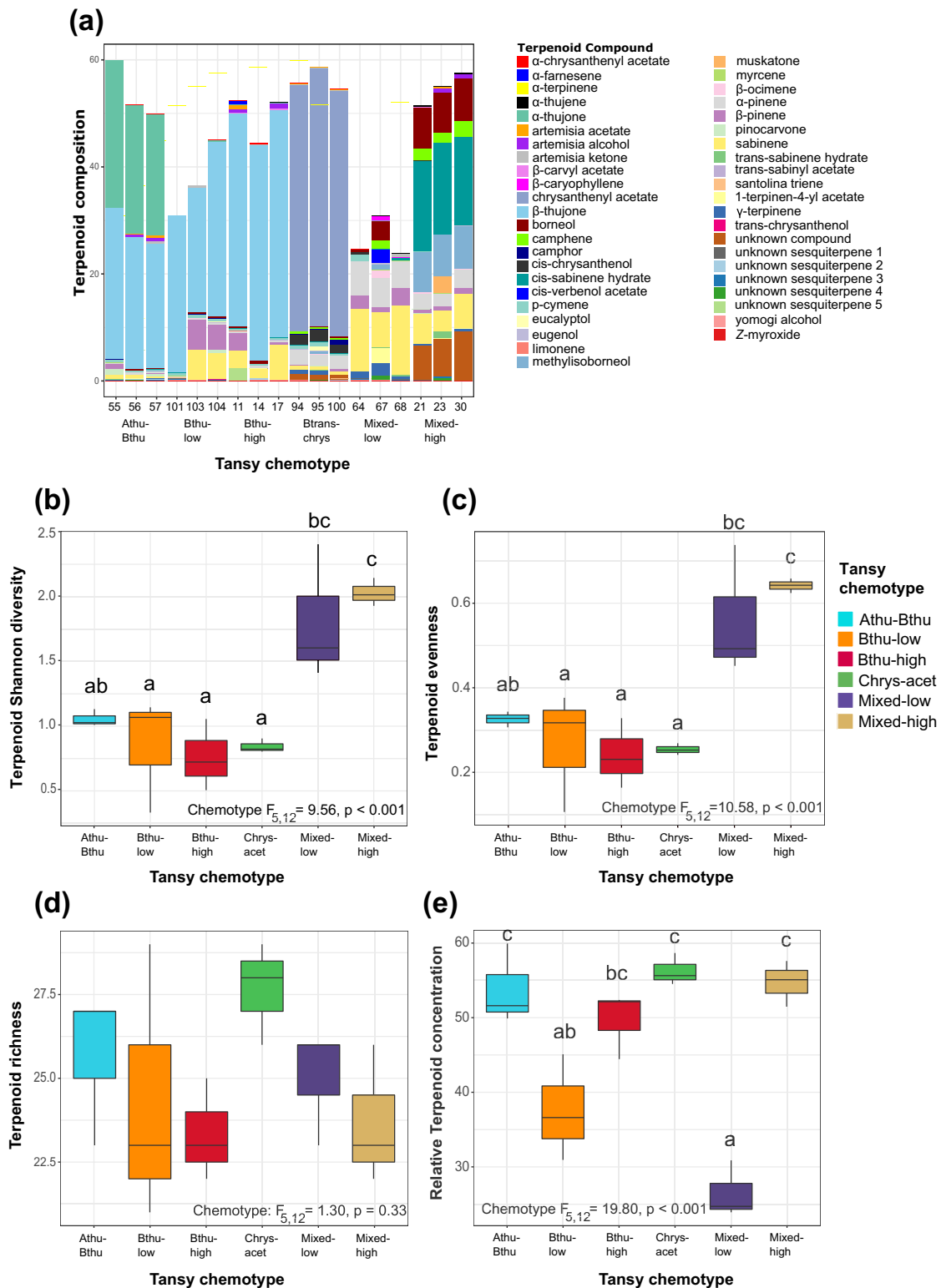


Figure 2. Differences in chemical composition of leaves of different *T. vulgare* chemotypes. (a) Stacked bar chart showing the composition of the chemical profiles of each of the three daughters of the selected six maternal chemotypes. Box plots (b–e) show differences among chemotypes as interquartile ranges of (b) terpenoid Shannon diversity, (c) terpenoid evenness, (d) terpenoid richness, and (e) relative total terpenoid concentration for each chemotype. The lower hinge corresponds to the first quartile (25th percentile) and the upper hinge depicts the third quartile (75th percentile). Whiskers extend to the 5 and 95% percentiles; solid lines represent the medians. Chemotype effect is indicated in panels, letters above bars indicate significant differences ($p < 0.05$) between chemotypes based on post hoc Tukey tests. Boxplots represent $n = 3$ daughters per chemotype.

Table 1. Effects of five *Tanacetum vulgare* chemotypes on their attractiveness to *Macrosiphoniella tanacetaria* and *Uroleucon tanacetii*, when offered in pairwise choices. A binomial test was used to obtain credible intervals and p-values. Significant values are highlighted in bold, marginally significant values in italics.

Time (h)	Chemotype 1	Chemotype 2	<i>Macrosiphoniella tanacetaria</i>			<i>Uroleucon tanacetii</i>		
			n	95% CI	p-value	N	95% CI	p-value
2	Athu-Bthu	Mixed-low	18	<i>0.465, 0.903</i>	<i>0.096</i>	9	0.518, 0.997	0.039
2	Athu-Bthu	Bthu-high	19	<i>0.488, 0.909</i>	<i>0.064</i>	9	0.212, 0.863	1
2	Athu-Bthu	Mixed-high	20	0.231, 0.685	0.824	7	0.184, 0.901	1
2	Athu-Bthu	Chrys-acet	17	0.230, 0.722	1	6	0.118, 0.882	1
2	Mixed-low	Chrys-acet	16	0.110, 0.587	0.210	7	0.184, 0.901	1
2	Mixed-high	Chrys-acet	15	0.078, 0.51	0.119	6	0.043, 0.778	0.688
2	Bthu-high	Chrys-acet	18	0.133, 0.590	0.238	6	0.043, 0.778	0.688
2	Bthu-high	Mixed-low	17	0.330, 0.816	0.629	8	0.085, 0.755	0.727
2	Bthu-high	Mixed-high	19	0.435, 0.874	0.167	6	0.043, 0.778	0.688
2	Mixed-low	Mixed-high	18	0.215, 0.692	0.815	5	0.284, 0.995	0.375
5	Athu-Bthu	Mixed-low	17	0.566, 0.962	0.013	5	0.147, 0.947	1
5	Athu-Bthu	Bthu-high	18	0.410, 0.867	0.238	8	0.157, 0.843	1
5	Athu-Bthu	Mixed-high	19	0.335, 0.798	0.648	4	0.194, 0.994	0.625
5	Athu-Bthu	Chrys-acet	16	0.247, 0.754	1	7	0.099, 0.816	1
5	Mixed-low	Chrys-acet	18	<i>0.097, 0.535</i>	<i>0.096</i>	6	0.043, 0.778	0.688
5	Mixed-high	Chrys-acet	19	<i>0.092, 0.512</i>	<i>0.064</i>	5	0.147, 0.947	1
5	Bthu-high	Chrys-acet	16	0.247, 0.754	1	6	0.118, 0.882	1
5	Bthu-high	Mixed-low	19	0.289, 0.756	1	6	0.118, 0.882	1
5	Bthu-high	Mixed-high	21	0.430, 0.854	0.189	8	0.085, 0.755	0.727
5	Mixed-low	Mixed-high	18	0.215, 0.692	0.815	5	0.147, 0.947	1

terpenoid concentration and lower terpenoid Shannon diversity and evenness, despite our low power to detect these relationships, and only for *M. tanacetaria*. Remarkably, although characteristics of the chemical blend correlated with plant attractiveness to aphids, it could not be clearly linked to individual terpenoids, suggesting that the interaction between compounds is responsible for preferences.

In line with our first hypothesis, we found that aphids preferred different chemotypes in pairwise choice assays. *Macrosiphoniella tanacetaria* preferred leaves from plants of Athu-Bthu and Chrys-acet chemotypes, with preference patterns becoming more pronounced at later time points of the assay. Previous studies found higher numbers of *M. tanacetaria* present on plants dominated by β -thujone compared to those dominated by trans-caryyl acetate in a climate chamber experiment (Jakobs and Müller 2018) but a higher abundance of *M. tanacetaria* on plants with camphor compared

to those with β -thujone as the dominant terpenoid in a field study (Benedek et al. 2019a). We also observed that *U. tanacetii* was significantly attracted towards plants of the Athu-Bthu chemotype, with the preference becoming less pronounced over time. This is interesting, as previously negative effects of β -thujone on *U. tanacetii* numbers have been found in the field (Benedek et al. 2019b), though *U. tanacetii* were attracted to plants with high relative levels of α -thujone and β -thujone growing in homogenous plant patches in

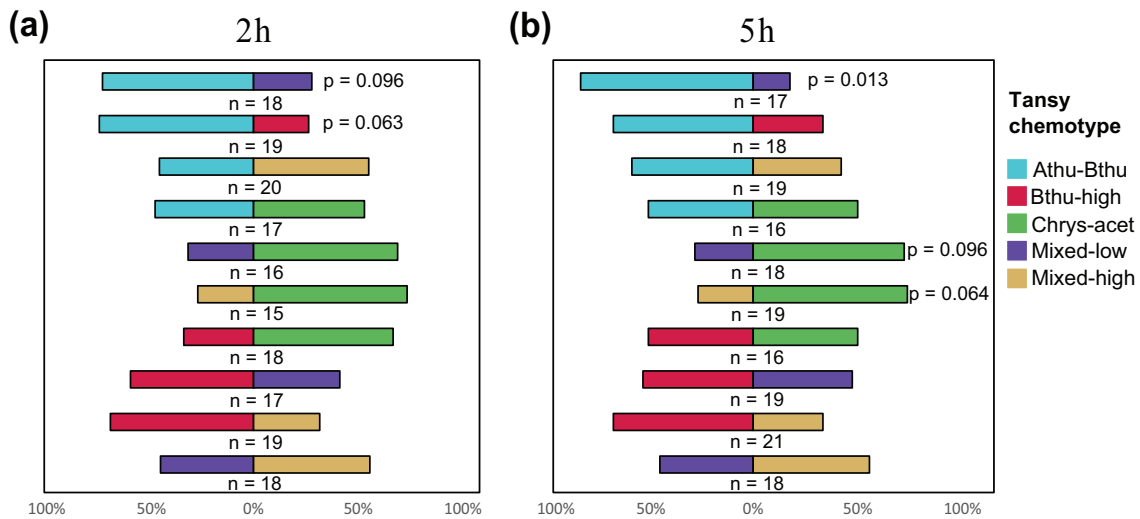
Table 3. Effects of *T. vulgare* daughters on their attractiveness to *M. tanacetaria* and *U. tanacetii* based on all pairwise combinations in the choice assays after two hours of observation. A c-logit model, including effects of daughter (five chemotypes \times three daughters) on aphid choices, was used to obtain z- and p-values. Significant values are highlighted in bold, marginally significant values in italics.

Table 2. Effects of five *T. vulgare* chemotypes on their attractiveness to *M. tanacetaria* and *Uroleucon tanacetii* based on all pairwise combinations of the choice assays after two hours of observation. A c-logit model was used to obtain z- and p-values (for visualization see Fig. 4). Significant values are highlighted in bold, marginally significant values in italics.

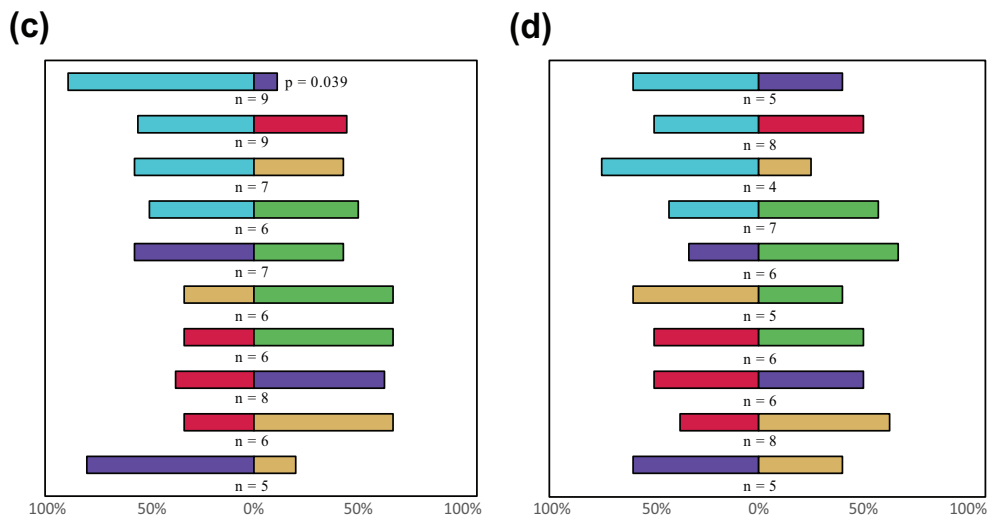
Chemotype	<i>Macrosiphoniella tanacetaria</i>		<i>Uroleucon tanacetii</i>	
	z-value	p-value	z-value	p-value
Mixed-high	-0.669	0.503	-1.226	0.220
Chrys-acet	2.331	0.020	0.783	0.434
Athu-Bthu	1.730	<i>0.084</i>	1.206	0.228
Bthu-high	0.340	0.734	-0.448	0.654
Mixed-low	-0.752	0.452	0.404	0.686

Daughter	<i>Macrosiphoniella tanacetaria</i>		<i>Uroleucon tanacetii</i>	
	z-value	p-value	z-value	p-value
Mixed-high_21	-0.792	0.429	-1.203	0.229
Mixed-high_23	1.064	0.288	-1.447	0.148
Mixed-high_30	1.602	0.110	0.506	0.613
Chrys-acet_100	2.171	0.030	0.302	0.762
Chrys-acet_94	1.589	0.112	-0.659	0.510
Chrys-acet_95	2.439	0.015	0.442	0.658
Athu-Bthu_55	2.183	0.029	-0.207	0.836
Athu-Bthu_56	1.983	0.047	0.100	0.921
Athu-Bthu_57	1.119	0.263	0.467	0.640
Bthu-high_11	1.058	0.290	-1.597	0.110
Bthu-high_14	1.451	0.147	-1.103	0.270
Bthu-high_17	0.858	0.391	0.434	0.664
Mixed-low_64	-0.246	0.806	-0.292	0.770
Mixed-low_67	0.666	0.505	-0.446	0.656
Mixed-low_68	1.075	0.283	-0.297	0.766

M. tanacetaria



U. tanacetii



Percentage of choices for chemotype per pairwise combination

Figure 3. Chemotype effects on pair wise choices by aphids. Depicted are mean percentage of choices by *Macrosiphoniella tanacetaria* after two (a) and five hours (b), and *Uroleucon tanacetii* after two (c) and five (d) hours, for all possible pairwise combinations of chemotypes. Effective sample size is indicated for each pairwise comparison, and no-choice replicates were excluded. Note that chemotype Bthu-low was not included in pairwise comparisons due to propagation difficulties. p-values next to bars indicate (marginally) significant preferences (for test statistics see Table 1). No-choice replicates were removed from the analyzes. Realized replication levels for the choice assays ranged between n = 15–21 per chemotype pair for *M. tanacetaria*, and between 4–9 for *U. tanacetii*.

another study (Ziaja and Müller 2023). However, in another common garden study no clear preference was observed towards chemotypes for this aphid species (Kleine and Müller 2011). Under field conditions, different factors (including insect preference, bottom–up and top–down processes) affect observed aphid numbers, which may explain some discrepancies between field observations and choice assays. In ‘snapshot’ field observations, these independent factors can be hard to disentangle. In addition, concentrating only on

the dominant compounds may be misleading, as minor compounds or the blend itself can result in the biological effect (Unsicker et al. 2009). Therefore, studies under laboratory conditions are necessary to understand the influence of these individual factors on aphid behavior and performance. Our results suggest that aphid preference for plants might not always reflect how they perform on plants.

In our experiment, 70–91% of *M. tanacetaria* individuals made a choice after five hours for one chemotype in each

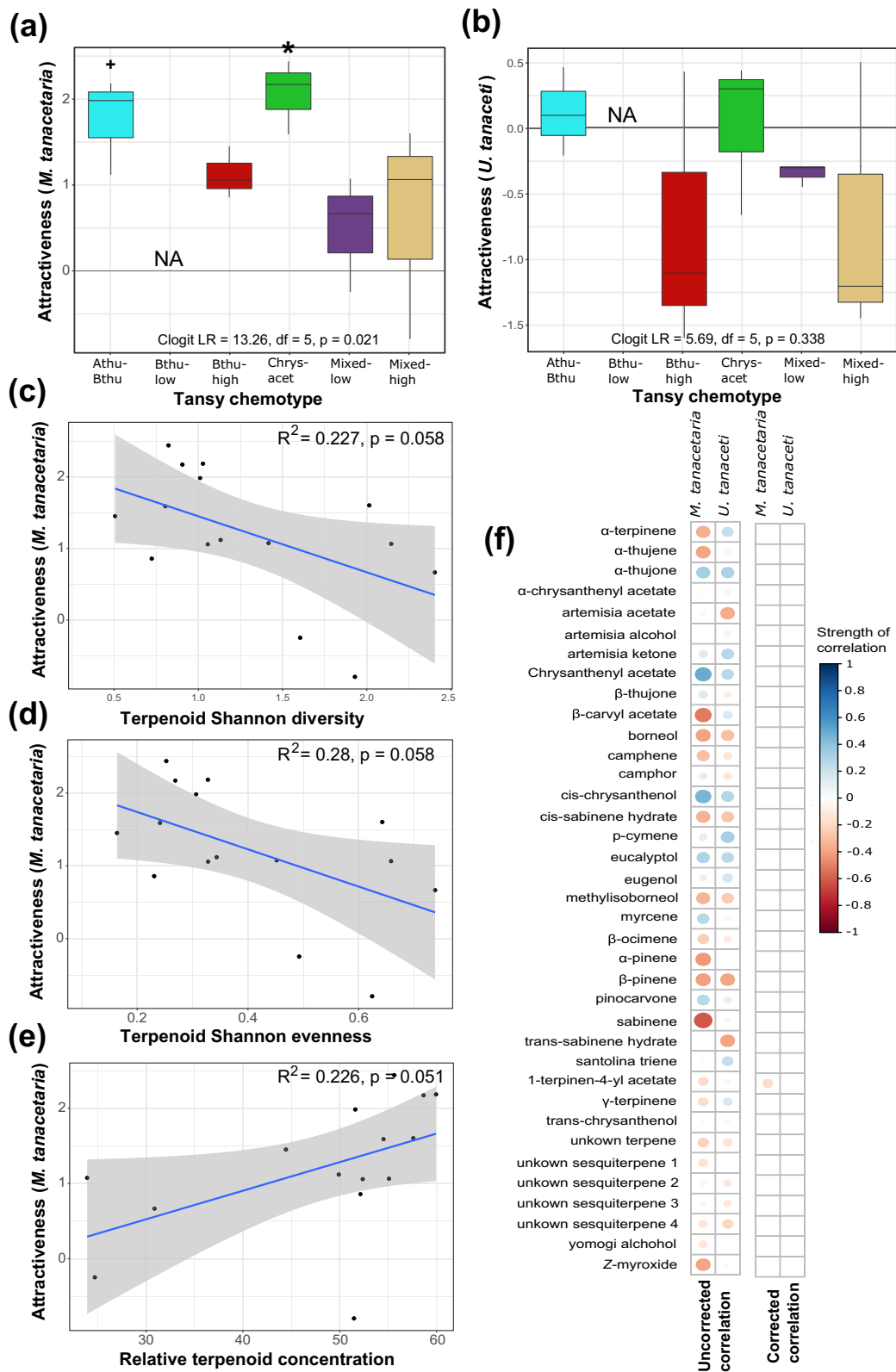


Figure 4. Relationships between *T. vulgare* attractiveness and chemodiversity for both aphid species. Attractiveness (reflected by the z-value) was computed by summarizing all decisions made towards a specific chemotype over all combinations after two hours and using a clogit model to test whether a certain chemotype was chosen more often than expected compared to a random choice. (a) Mean attractiveness per chemotype for *M. tanacetaria* after two hours. (b) Mean attractiveness per chemotype for *U. tanacetii* after two hours. Symbols indicate

Figure 4. (Continued)

significance levels (* $p < 0.05$; + $0.05 < p < 0.10$). For detailed model output for (a) and (b) Table 3. (c) Relationship between *T. vulgare* terpenoid Shannon diversity and attractiveness to *M. tanacetaria*. (d) Relationship between *T. vulgare* terpenoid evenness and attractiveness to *M. tanacetaria*. (e) Relationship between *T. vulgare* terpenoid abundance and attractiveness to *M. tanacetaria*; in (c–e) dots represent the mean z-value (attractiveness) for each of the 15 daughters (5 chemotypes \times 3 daughters). (f) Correlation plots showing relationship between individual terpenoid compounds and attractiveness to both aphid species. The size of the dot depicts the strength of the correlation. Blue colors indicate positive and red colors indicate negative correlations. The left correlation plot shows all unadjusted correlations with a significant p-value. The right correlation plot shows all correlations with a significant p-value after using Holm-adjusted for multiple correlations.

combination, compared to only 30–61% of *U. tanacetii* individuals. These species-specific differences between the aphids may be explained by differences in their life histories and preferred niches (Jakobs et al. 2019). In various field and greenhouse experiments, we observed that these aphid species showed different behaviors on and off the plant. For instance, *M. tanacetaria* tends to be more mobile than *U. tanacetii*, and readily searches for new host plants when its current host deteriorates. *Uroleucon tanacetii* typically remains on leaves until yellowing, and then move to the next upper non-infested leaf. We found more deaths and fewer choices made at later time points (i.e. after 24 h), which could be because individual leaflets do dry out after some time. However, as individual choices were already apparent and similar after two and five hours, we believe that deteriorating leaf quality did not affect choices made after two hours.

Across all experimental choice combinations, we found significant effects of chemotype on the attractiveness of those chemotypes for *M. tanacetaria*, but not for *U. tanacetii*. This is in line with previous research from Kleine and Müller (2011), who found that *M. tanacetaria*, but not *U. tanacetii* exhibited distinct preferences towards specific chemotypes. To understand what may drive the attractiveness of a plant to *M. tanacetaria*, we investigated relationships between *T. vulgare* individual chemical properties and their level of attractiveness to aphids. We observed that terpenoid Shannon diversity negatively correlated with attractiveness to *M. tanacetaria* aphids. This is consistent with the hypothesis on the evolution of chemodiversity in plants, and particularly the evolution of a breadth of specialized compounds to repel antagonists (Wetzel and Whitehead 2020). Furthermore, we found that terpenoid evenness marginally negatively correlated with attractiveness to *M. tanacetaria*, indicating that blends that were more evenly distributed in terpenoid composition were

less attractive to aphids than those dominated by one or several compounds. A plausible explanation might be that having some highly dominant compounds could serve as strong cues for plant recognition (Kleine and Müller 2011). However, once an aphid has arrived on a plant, subsequent aphid performance on *T. vulgare* is likely affected more pronouncedly by the phloem sap composition (Jakobs and Müller 2019). We also found a positive effect of relative total terpenoid concentration on attractiveness to *M. tanacetaria*. This might be an indication that although Shannon diversity can have a deterrent effect (Whitehead et al. 2021), the relative total terpenoid concentration (as a proxy for potential emission) can also be an important cue for host finding. A role of specialized metabolite concentrations in herbivore attraction and repellence has been found in numerous studies (reviewed by Macel 2011). For instance, contrasting effects have been observed in the specialized aphid *Aphis jacobaea* (Hemiptera, Aphididae) on *Jacobaea vulgaris* (Asteraceae), where plants high in pyrrolizidine alkaloids hosted fewer aphids than plants with low concentrations, although this could not be related to aphid preference behavior alone (Vrieling et al. 1991). Our results align with those from a recent study that shows that chemodiversity is an important driver of dietary specialization in insects (Leong et al. 2022). The relationships between *M. tanacetaria* and terpenoid Shannon diversity, evenness and relative total terpenoid concentration were only marginally significant, which may have been a result of the limiting number of chemotype lines ($n = 15$) included in our current study. Our study was limited by the poor propagation success of one of the chemotype lines, and the low number of daughters selected for choice assays. Future studies on a range of terpenoid diversity profiles should be performed to strengthen the empirical evidence for chemodiversity as driver of herbivore preference.

Table 4. Model output of multiple linear regression models testing the effects of plant morphological characteristics on plant attractiveness for *M. tanacetaria* and *U. tanacetii*, and on plant chemodiversity characteristics. Models were checked for collinearity by variance inflation factors and simplified via stepwise model selection. Dashes indicate variables were removed from the model.

Explanatory variable	Attractiveness <i>Macrosiphoniella tanacetaria</i>	Attractiveness <i>Uroleucon tanacetii</i>	Terpenoid richness	Terpenoid Shannon diversity	Terpenoid Shannon evenness	Terpenoid concentration (relative)
	F (p-value)	F (p-value)	F (p-value)	F (p-value)	F (p-value)	F (p-value)
Height	–	–	0.20 (0.665)	6.03 (0.028)	6.44 (0.024)	7.05 (0.017)
No. of stems	10.20 (0.010)	–	0.11 (0.741)	8.33 (0.012)	9.12 (0.009)	–
No. of leaves	5.02 (0.049)	–	0.07 (0.801)	–	–	–
Leaflets density	3.14 (0.107)	1.85 (0.199)	–	–	–	–
SLA	–	–	–	–	–	–
Chlorophyll	6.94 (0.0250)	2.42 (0.146)	–	2.55 (0.133)	2.54 (0.133)	–

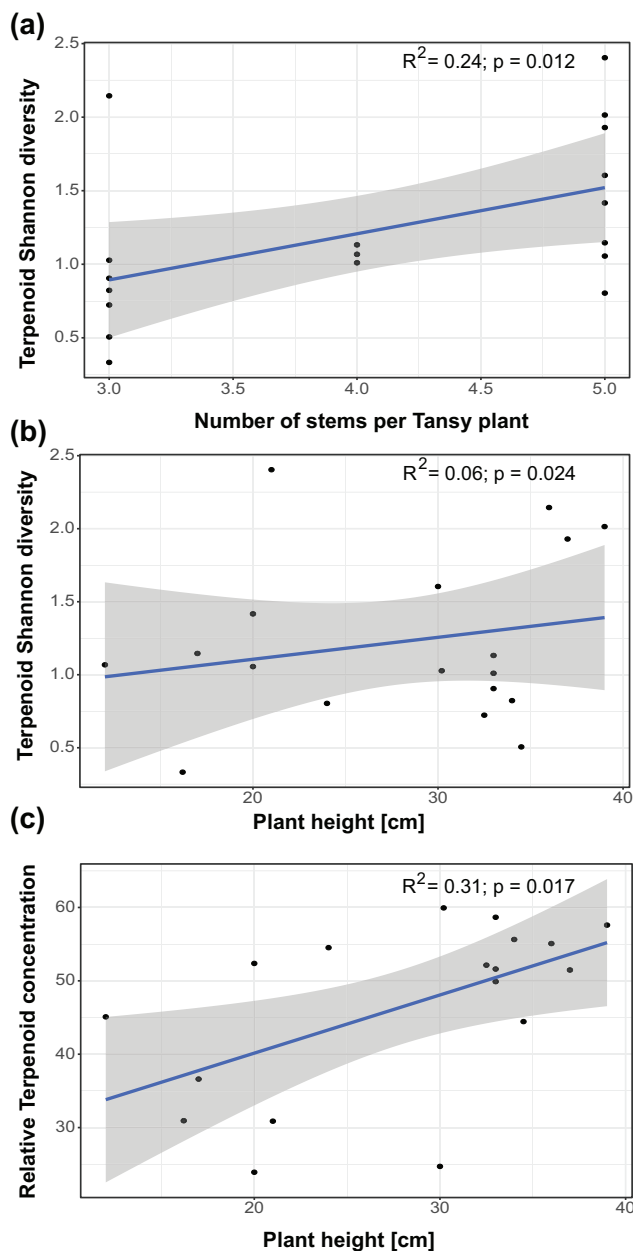


Figure 5. Relationships between chemical and morphological parameters. Each dot represents one of the 18 daughters (6 chemotypes \times 3 daughters). (a) Relationship between the number of stems per *T. vulgare* plant and terpenoid Shannon diversity. (b) Relationship between *T. vulgare* height and terpenoid Shannon diversity. (c) Relationship between *T. vulgare* height and relative total terpenoid concentration. Values represent single measurements on individual daughters for each chemotype, which were used in the correlations (six chemotypes, three daughters, $n = 18$ total).

Our analyses of the role of individual compounds in influencing attractiveness indicated that the effect of individual terpenoids of *T. vulgare* attractiveness to aphids is minimal. After corrections for multiple correlations, we observed a weak negative effect of 1-terpinen-4-yl acetate on *T. vulgare* attractiveness to *M. tanacetaria*. Several studies have shown

relationships between chemotypes, or dominant compounds and aphid abundance in the field. For instance, β -thujone has been related to decreased colony distribution and colony numbers in *U. tanacetii* and abundances in *M. tanacetaria* in field studies (Bálint et al. 2016, Benedek et al. 2019b). Other studies have found an increased density of *Metopeurum fuscoviride* (Hemiptera, Aphididae) on chemotypes with high concentrations of borneol or camphor (Bálint et al. 2016, Senft et al. 2019), while plants with high amounts of α -thujone, (*E*)-dihydrocarvone, α -copaene and β -cubebene were colonized earlier (Clancy et al. 2016). Although we found only limited evidence that individual compounds impact aphid attractiveness, it cannot be ruled out that these compounds play a role in feeding deterrence once aphids have settled on the plant or in the attraction of natural enemies of herbivores. Importantly, our study included terpenoids only, but other compounds, such as, for example flavonoids that were not measured here, may also affect the preference behavior of the aphids. This calls for future studies disentangling the role of broader metabolome-wide chemodiversity in driving aphid and natural enemy dynamics on the plant.

It is important to disentangle the relation between chemical and growth traits, as both traits have been shown to influence plant–insect interactions, including preference behavior. While growth–defence tradeoffs are a fundamental principle in plant ecology (Herms and Mattson 1992, Karasov et al. 2017), we did not observe such a tradeoff in *T. vulgare*. In a previous study only inflorescence biomass but not other growth factors (e.g. plant height, total biomass, no. of stems) were significantly negatively correlated to terpenoid concentration (Wolf et al. 2011). However, instead we found a synergistic effect between variables associated with plant growth and terpenoid Shannon diversity and relative total terpenoid concentration in our plants. It is commonly assumed that plants have to partition their resources between growth and defence traits, leading to either smaller and better defended plants, or vice versa (Coley et al. 1985, Herms and Mattson 1992, He et al. 2022). Terpenoids are more expensive to produce than many other metabolites, as they require a wide array of different enzymes, posing substantial production and storage costs (Gershenson 1994). However, contrary to these hypotheses, we found that larger and bushier plants had a higher relative total terpenoid concentration and terpenoid Shannon diversity. Similarly, positive relationships between growth and defence have been found in two *Plantago* species (Barton 2007, Plantaginaceae). One plausible explanation for our findings may be that larger plants photosynthesize more and have a larger energy budget, which can be used for elevated and diversified local terpenoid synthesis. Furthermore, it is vital for plants to grow and defend to optimize their fitness within a dynamic environment (Huot et al. 2014), and tradeoff patterns might also change with different plant ontogeny stages (Boege and Marquis 2005). Recent work has shown strong effects of maternal ‘chemo-genotypes’ on leaf metabolic composition in *T. vulgare* (Dussarrat et al. 2023). Given that our chemotypes were confounded by their maternal origin (chemo-genotypes), this may also affect broader

metabolic profiles, and future work should investigate to what extent the maternal fingerprint on chemo-genotypes also influences aphid attraction and performance.

In this study, we used unwinged (apterae) aphids. Although in early colonization in the field, host plant selection is more likely to occur by winged (alate) aphids (Mehrpavar et al. 2014), the dispersal to other plants is also commonly observed by non-winged (apterae) aphids, both in experimental colonies and the field (the authors pers. obs.). Although performing choice assays with winged early colonizers may be an important next step to understand seasonal colonization dynamics, obtaining such early-season alates from eggs is experimentally challenging. However, choice assays with asexual alates would also be interesting to investigate aphid colonization dynamics later in the season, as these often display clear preferences. For instance, in a choice assay study, Mehrparvar et al. (2014) found that alate dispersal morphs of *M. tanacetaria* displayed preferences for plants with specific herbivore infestation history, while unwinged aphids did not show any such host plant preferences. Our results indicate that preferences and choices in unwinged morphs do occur at the chemotype level, which likely represents a stronger chemical contrast for aphids than the herbivory history studied in previous work.

Conclusion

We found that terpenoid chemodiversity characteristics are one potential driver for aphid host plant preference in *T. vulgare*, which contributes to our mechanistic understanding of the link between chemodiversity in plants and insect communities that interact with them. We found that different chemotypes have distinct attraction patterns, which can be partly explained by diversity metrics of the terpenoid blend and the relative total terpenoid concentration. Insects use chemical cues, including host plant metabolites, to inform their decisions. It is eminent that we develop a better understanding of how intraspecific plant chemodiversity shapes the various aspects of the (herbivorous) insect life cycle, including development, survival, defence and overall fitness.

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Author contributions

Annika Neuhaus-Harr and **Lina Ojeda-Prieto** share first authorship and contributed equally to this publication. **Annika Neuhaus-Harr**: Conceptualization (supporting);

Data curation (equal); Formal analysis (lead); Investigation (lead); Visualization (equal); Writing – original draft (lead). **Lina Ojeda-Prieto**: Conceptualization (equal); Data curation (equal); Formal analysis (supporting); Visualization (equal); Writing – original draft (equal); Writing – review and editing (supporting). **Elisabeth Eilers**: Conceptualization (equal); Formal analysis (supporting); Writing – review and editing (supporting). **Caroline Müller**: Formal analysis (supporting); Investigation (supporting); Methodology (supporting); Writing – review and editing (supporting). **Wolfgang W. Weisser**: Conceptualization (supporting); Formal analysis (supporting); Funding acquisition (lead); Investigation (supporting); Methodology (supporting); Project administration (equal); Supervision (supporting); Validation (supporting); Visualization (supporting); Writing – review and editing (supporting). **Robin Heinen**: Conceptualization (equal); Data curation (supporting); Formal analysis (supporting); Investigation (supporting); Methodology (supporting); Project administration (supporting); Supervision (lead); Validation (supporting); Visualization (equal); Writing – original draft (supporting); Writing – review and editing (supporting).

Data availability statement

Data are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.vhhmqp1k> (Neuhaus-Harr et al. 2023).

Supporting information

The Supporting information associated with this article is available with the online version.

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