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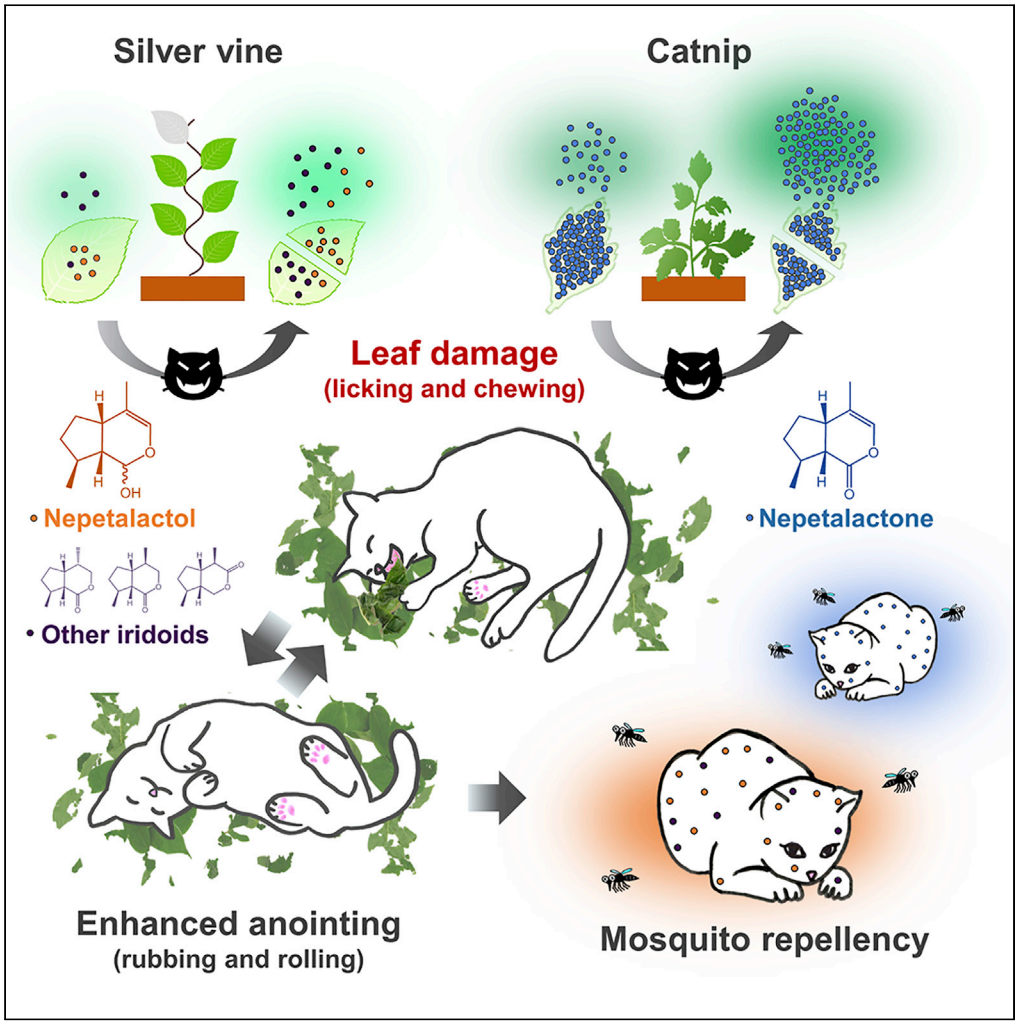

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Highlights

Feline damage of specific plants increases release of iridoids that repel mosquitoes

Damaged silver vine emits a relatively low amount of complex iridoids

Damaged catnip emits a high amount of the predominant iridoid nepetalactone

Cat responsiveness to these damaged plants is similar despite different iridoid emissions

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Domestic cat damage to plant leaves containing iridoids enhances chemical repellency to pests

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SUMMARY

Catnip (*Nepeta cataria*) and silver vine (*Actinidia polygama*) produce iridoids with arthropod-repellent effects. Cats rub and roll against these plants, transferring iridoids to their fur that repels mosquitoes. Cats also lick and chew plant leaves during this response, although the benefit of this additional behavior has remained unknown. Here, we show that feline leaf damage substantially increases iridoid emission from both plants while also diversifying iridoids in silver vine. Cats show an equivalent duration of response to the complex cocktail of iridoids in damaged silver vine and to the much higher level of a single iridoid produced by damaged catnip. The more complex iridoid cocktail produced when silver vine is licked and chewed by cats increases mosquito repellency at low concentration. In conclusion, feline leaf damage contributes by releasing more mosquito-repellent iridoids. Feline olfactory and behavioral sensitivity is fine-tuned to plant-specific iridoid production for maximizing the mosquito repellency gained.

INTRODUCTION

Plants produce compounds to defend themselves against herbivorous insects (War et al., 2018). Some herbivorous insects ingest and store the plant compounds in their body tissues or integuments, providing them with defense against predators and/or parasites (Opitz and Müller, 2009). Humans and other mammals exploit these compounds without needing to eat plants to gain chemical defense by using plant-emitted insect repellents in the local environment or a behavioral mechanism such as self-anointing their bodies with the plants. For example, a mosquito repellent produced from dalmatian pyrethrum (*Tanacetum cinerariifolium*) has been used for centuries in human history (Casida, 1980). Chimpanzees (*Pan troglodytes schweinfurthii*) make sleeping platforms using freshly cut *Cynometra* plants, which decreases their exposure to malarial vector mosquitoes (Samson et al., 2013). Tufted capuchin monkeys (*Cebus apella*) and white-nosed coatis (*Nasua narica*) anoint themselves with citrus-derived chemicals that repel ticks and yellow fever mosquitoes (*Aedes aegypti*) (Weldon et al., 2011). However, studies of the behavior toward plant metabolites related to chemical defense against parasites and pathogens by nonhuman animals are still very limited.

Domestic cats (*Felis silvestris catus*) show an unusual but characteristic response toward catnip (*Nepeta cataria*) and silver vine (*Actinidia polygama*) leaves that comprises licking and chewing the plants, face and head rubbing against the plants, and rolling over on the plants (Bol et al., 2017; Todd, 1962; Tucker and Tucker, 1988). Although this has often been interpreted by pet owners as a playful behavior among cats that appeared to be intoxicated by these specific plant species (Espin-Iturbe et al., 2017; Hatch, 1972), we demonstrated in a recent study that the rubbing and rolling behavior can protect cats from mosquito bites (Uenoyama et al., 2021). This characteristic feline behavioral response is induced by olfactory stimulation by iridoids that are emitted from the leaves of these plants and thought to play a role in plant chemical defense (Cronquist, 1977; Duplais et al., 2020). Iridoids have five-membered rings fused to six-membered rings with oxygen (Figure 1A). Although catnip produces the iridoid nepetalactone, silver vine produces a more complex mixture of nepetalactol, dihydronepetalactone, isodihydronepetalactone, iridomyrmecin, and isoiridomyrmecin (Meinwald, 1954; Sakan et al., 1959; Uenoyama et al., 2021). Some of these iridoids are known to be repellent to a broad range of insects, including non-herbivores such as *Aedes* and *Culex* mosquitoes and stable flies (*Stomoxys*) (Birkett et al., 2011; Feaster et al., 2009; Gkinis et al., 2014; Melo et al., 2021; Reichert et al., 2019; Uenoyama et al., 2021; Zhu et al., 2012). Our previous study showed that feline rubbing and rolling behavior against silver vine and catnip leaves transfers plant

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iridoids onto the face, head and body fur of cats, and this provides mosquito repellency that helps to protect cats from mosquito bites (Uenoyama et al., 2021).

In addition to the rubbing and rolling behavior, cats also crumple and tear silver vine and catnip plants by extensive licking and chewing, which is a part of this very characteristic response (Figure 1B and Video S1). However, the adaptive function of this additional behavior has not been established. Although licking and chewing of plants support their intake and digestion in most animals, our preliminary studies showed that cats swallowed only a minimum, if any, of the plant material (Figure S1). A previous study reported that oral administration of nepetalactone in catnip induces no marked behavioral or physiological effects in cats (Waller et al., 1969). These findings suggest that the primary function of licking and chewing directed toward silver vine and catnip is to damage the leaves rather than to consume either plant material or iridoids. As cats exhibit both licking and chewing of plant leaves and rubbing and rolling throughout their characteristic response (Figure S2), it is likely that damage of the plants has an important adaptive function alongside rubbing and rolling that relates to insect repellency, but so far the contribution of plant-damaging behavior to gaining chemical pest defense has not been addressed in mammals.

This study aimed to uncover the functional significance of damaging silver vine and catnip leaves by licking and chewing in cats. As we observed that the silver vine leaves crumpled and torn by cats have a much stronger and characteristic aromatic odor to the human nose, we asked first whether the leaf damage promotes the emission of iridoids in these plants. Then, we manipulated the amount and types of iridoids in behavioral assays to test whether changes in iridoid composition and amount caused by the leaf damage enhance feline responsiveness to leaves and increase mosquito repellency. Our studies demonstrate that leaf damage of silver vine and catnip by cats manually increases the emission of iridoids from both damaged silver vine and catnip leaves and also increases the complexity of iridoids specifically in damaged silver vine. These changes in iridoids prolong the feline characteristic response to the leaves, which will increase the transfer of iridoids to the animal's fur. Although iridoid levels are much lower in silver vine compared to catnip leaves, we show that the complex iridoids induced by leaf damage in silver vine increases the mosquito repellency of silver vine iridoids at low concentration. Our findings highlight the benefit of the physical damage of insect repellent plants for chemical pest defense through external application in mammals, and provide valuable insight into how to obtain potent natural repellents against pest insects from plants.

RESULTS

Leaf damage changes the amount and composition of silver vine iridoids

To establish whether the physical damage of leaves caused by licking and chewing alters the emission of iridoids from silver vine leaves, we used GC/MS analysis to compare the amount and composition of iridoids in the headspace above intact leaves, leaves crumpled and torn by feline licking and chewing, and leaves manually crumpled and torn (Figures 1C and S3A; see STAR Methods for further details). Leaves damaged either by cats or manually showed a substantial increase in the airborne emission of total iridoids (Figure 1D). This increase was approximately 10-fold for leaves crumpled and torn by cats and 40-fold for those manually crumpled and torn compared to intact leaves. Five out of the six iridoids identified in silver vine leaf extracts (Sakan et al., 1959; Uenoyama et al., 2021) increased significantly in airborne emissions following either feline or manual damage. This was not the case for nepetalactone that is present only at very low levels in silver vine. Thus, physical damage of silver vine leaves by cats led to a substantially increased emission of airborne iridoids.

To establish whether the strong emission of these iridoids from damaged leaves reflects a greater level of these iridoids contained in the leaves themselves, we quantified the iridoids in solvent extracts of intact leaves and leaves damaged by cats or manually. In agreement with the results of the headspace analysis, damaged leaves showed a remarkable increase in the total iridoid level within damaged leaves ($F_{2,6}$ = 3018.6, $p < 0.0001$). The increase in iridoid levels within leaves was approximately 8-fold in response to feline damage (post hoc Bonferroni test, $p < 0.001$) and 6.5-fold in response to manual damage ($p < 0.001$, Figure 1E). The total iridoid level was also slightly greater in leaves damaged by cats compared to those damaged manually ($p < 0.001$). This contrasts with the greater release of total iridoids from manually damaged leaves (Figure 1D). Although airborne nepetalactol could not be detected in headspace analysis of intact leaves, nepetalactol accounted for 86.1 $\pm$ 1.2% of the total iridoids in solvent extracts from intact leaves. Damage treatments caused significant elevation of nepetalactol ($F_{2,6}$ = 1060.7, $p < 0.0001$),

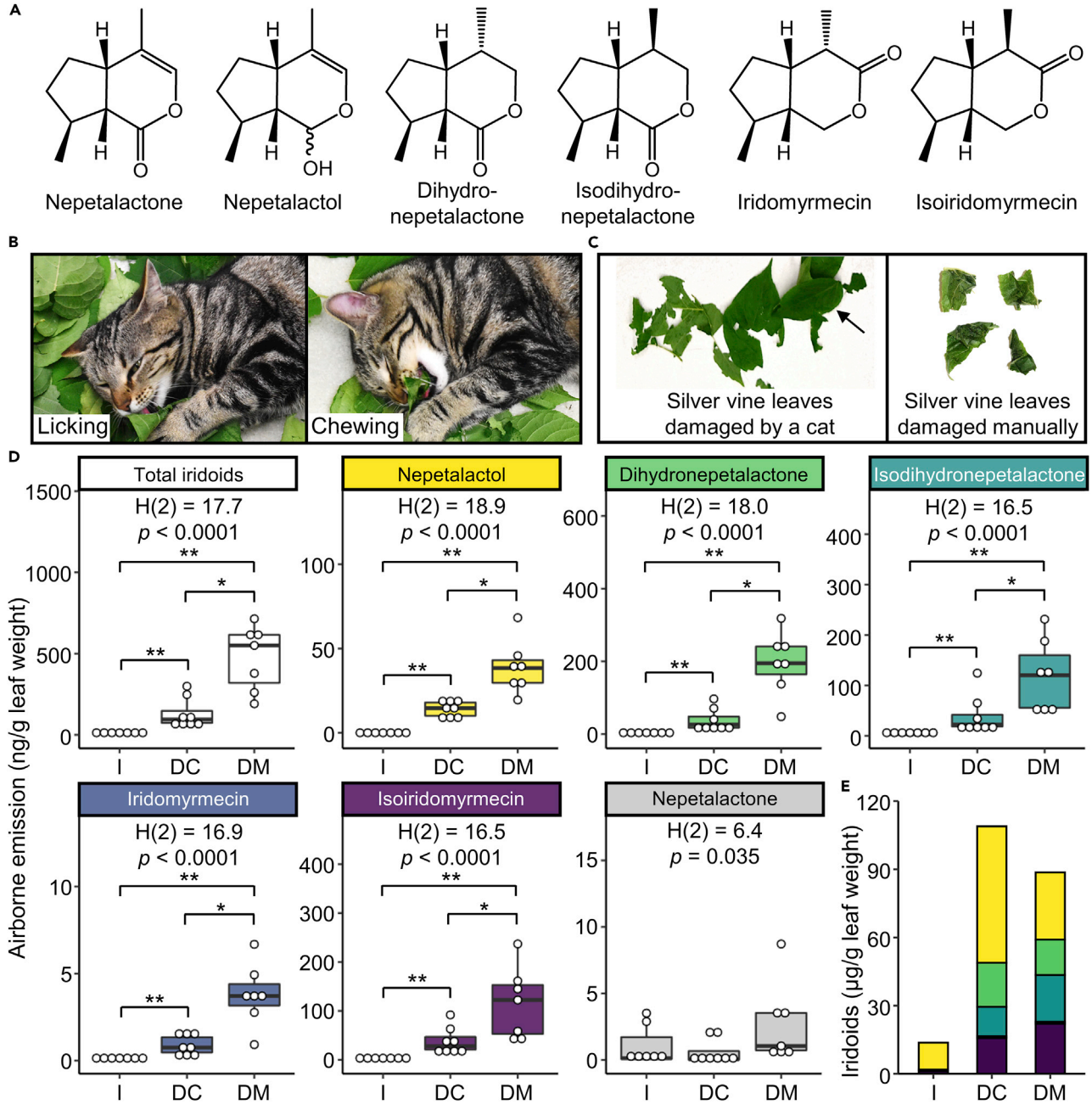

Figure 1. The amount and composition of iridoids in intact and damaged silver vine leaves

(A) Chemical structures of six plant iridoids detected in silver vine leaves.

(B) Images of a cat licking and chewing silver vine leaves. This behavior observed throughout the characteristic response (Figure S2) damages the leaves but is not for the consumption of the leaves (Figure S1).

(C) Images of silver vine leaves crumpled and torn by a cat or manually. An arrow indicates an intact leaf that was not licked and chewed by the cat.

(D) Amount of total iridoids, nepetalactol, dihydronepetalactone, isodihydronepetalactone, iridomyrmecin, and isoiridomyrmecin and nepetalactone in headspace samples emitted over 4 h from intact silver vine leaves (I), leaves damaged by cats (DC), and leaves damaged manually (DM) (aliquot 5 g) (n = 7–8 per treatment). Intact leaves emitted (median values) 11.4 ng dihydronepetalactone, 18.7 ng isodihydronepetalactone, 0.7 ng iridomyrmecin, 17.2 ng isoiridomyrmecin, and 0.6 ng nepetalactone. Nepetalactol was below the limit of detection. H values, degrees of freedom, and p values from Kruskal-Wallis tests. *p < 0.05, **p < 0.01 with the Steel–Dwass post hoc test. Box and whisker plots show the median, interquartile range, first data points within 1.5 interquartile ranges, and individual values. GC/MS total ion chromatograms of volatile compounds other than iridoids in headspace of I, DC, and DM are shown in Figure S3.

(E) Mean content of nepetalactol, dihydronepetalactone, isodihydronepetalactone, iridomyrmecin, and isoiridomyrmecin in extracts of silver vine leaves (aliquot 5 g) after headspace sampling (n = 3). See text for analyses. Colors for each compound correspond to the panel in (D).

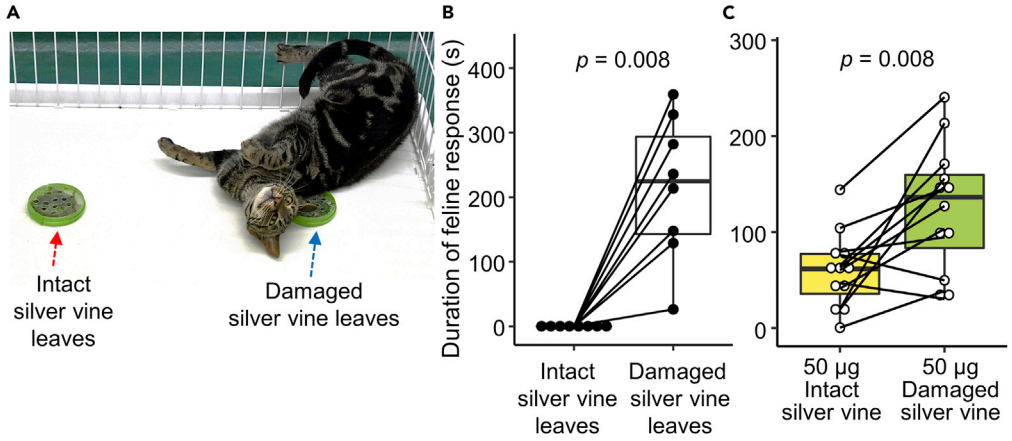

Figure 2. The responsiveness of cats to iridoids contained in intact and damaged silver vine leaves

(A) An image of the behavioral assay using cats to compare the total duration of the feline characteristic response (licking, chewing, rubbing, and rolling) to stimulant 1 (red arrow) and stimulant 2 (blue arrow) that were presented simultaneously. In this assay, stimulants 1 and 2 were intact silver vine leaves and manually damaged silver vine leaves, respectively.

(B) Duration of the feline response to the dishes containing intact and manually damaged silver vine leaves (n = 8 cats).

(C) Duration of the characteristic response of cats to 50 µg of synthetic iridoid cocktails corresponding to the ratio of the iridoids in leaf extracts of intact silver vine (intact silver vine-cocktail) and manually crumpled and torn leaves (damaged silver vine-cocktail) (n = 12). Table 1 gives the iridoid cocktail formulations. (B and C) Box and whisker plots show the median, inter-quartile range, first data point within 1.5 interquartile ranges, and individual values. Points connected by lines indicate paired dishes in the same trial. p values from Wilcoxon matched-pair test, two-tailed.

dihydronepetalactone ($F_{2,6}$ = 2015.6, p < 0.0001), isodihydronepetalactone ($F_{2,6}$ = 9437.5, p < 0.0001), and isoiridomyrmecin ($F_{2,6}$ = 2980.4, p < 0.0001) in silver vine leaf extracts (Figure 1E). Levels of each of these iridoids differed significantly between intact and damaged treatments and between feline and manually damaged silver vine leaves (post hoc Bonferroni tests, all p < 0.001). Feline damage led to the highest levels of nepetalactol and dihydronepetalactone, whereas manual damage led to the highest levels of isodihydronepetalactone and isoiridomyrmecin within leaves (Figure 1E). The increases in additional iridoids in response to leaf damage meant that nepetalactol accounted for only 55.1 ± 0.4% of total iridoids in leaves damaged by cats or 33.4 ± 0.4% in leaves damaged manually. In summary, the physical damage of silver vine leaves caused by feline licking and chewing resulted in an increased amount and complexity of the iridoids in the leaves and a substantial increase in the emission of these iridoids from the leaves.

Iridoid composition in damaged silver vine leaves is more effective than that in the intact leaves in inducing the feline response

To examine whether changes in silver vine leaf iridoids induced by leaf damage influence the behavioral response of cats, we compared the duration of their characteristic response toward silver vine leaves without and with manually crumpling and tearing in a two-choice test. The intact and damaged leaves were placed in separate dishes covered with perforated plastic lids to protect them from physical contact with cats and then presented to cats simultaneously (Figure 2A). The duration of the characteristic response (licking and chewing of the lid and rubbing and rolling against the lid or surrounding floor) was more prolonged toward manually damaged compared to intact leaves (Figure 2B; exact p = 0.008).

Given the much greater emission of iridoids from leaves damaged by cats or manually, it was unsurprising that the cats showed a more prolonged response to the damaged than to intact leaves. This was consistent with the dose-dependent response of the cats to iridoids reported previously (Sakurai et al., 1988). However, any changes in iridoid composition in response to physical damage might also have contributed to the increased feline characteristic response. To test this possibility, we compared the duration of feline response (n = 12) to a synthetic iridoid cocktail that corresponded to the iridoid ratio in intact silver vine leaf extract (intact silver vine-cocktail, dominated by nepetalactol) versus the more complex iridoid cocktail in manually crumpled and torn leaves (damaged silver vine-cocktail) while controlling the total amount of iridoids presented (50 µg of each iridoid cocktail; formulations shown in Table 1). The damaged-cocktail induced a more prolonged feline response than the same amount of intact-cocktail (Figure 2C; p = 0.008). The behavioral responses to the iridoid chemicals alone were consistent

Table 1. Formulations for plant iridoid cocktails used in Figures 2, 3, and 4

Percentage of compounds (%)	Intact silver vine-cocktail	Damaged silver vine-cocktail	Damaged catnip-cocktail	Modified damaged silver vine-cocktail
Nepetalactone	0	0	99.8	46.3
Nepetalactol	94.7	46.3	0	0
Dihydronepetalactone	3.4	29.7	0	29.7
Isodihydronepetalactone	0.1	15.7	0.2	15.7
Iridomyrmecin	0	0.2	0	0.2
Isoiridomyrmecin	1.7	8.2	0	8.2
Total	100.0	100.0	100.0	100.0

with a more prolonged response to damaged silver vine than intact silver vine, regardless of changes in other volatile compounds in response to leaf damage (Figure S3). Thus, the altered iridoid mixture in damaged silver vine leaves contributed to promoting a prolonged behavioral response from cats.

Leaf damage changes the emission — but not the composition — of catnip iridoids

We next examined whether the physical damage caused by crumpling and tearing of leaves has similar effects on the amount and composition of iridoids in catnip leaves to those shown in silver vine. In contrast to silver vine, manual crumpling and tearing of catnip led to little if any change in the total amount of iridoids in the leaf extract (Figure 3A). Notably though, iridoid levels were substantially higher in catnip leaves compared to silver vine leaves regardless of treatment [catnip intact (365.4 $\pm$ 6.4 μg/100 mg leaf wet weight) versus silver vine intact (2.9 $\pm$ 0.2 μg/100 mg), $p < 0.0001$; catnip manually damaged (343.5 $\pm$ 5.3 μg/100 mg) versus silver vine manually damaged (8.9 $\pm$ 0.5 μg/100 mg), $p < 0.0001$].

Although physical leaf damage diversified the iridoid composition of silver vine from mostly nepetalactol to a combination of five iridoids, catnip iridoids consisted almost solely of nepetalactone (over 99%) in both intact and damaged leaf extracts (Figure 3B). Headspace analysis revealed that the crumpling and tearing of catnip leaves led to a more than 20-fold increase in the total amount of iridoid emitted, similar to the effect on emission of total iridoids from silver vine (Figures 3C and S3B; median of total iridoids in headspace of intact catnip: 0.5 μg/g leaf wet weight/10 min, manually damaged catnip: 11.3 μg/g/10 min).

Equivalent response of cats to silver vine and catnip despite differences in plant iridoid profiles

To determine if differences in the total amount of iridoids and the iridoid profile complexity between catnip and silver vine resulted in a different duration of response to these two plants among cats, we compared their responses to damaged catnip and silver vine leaves that were manually crumpled and torn. Even though damaged catnip leaves emit a substantially higher concentration of iridoids than damaged silver vine leaves (median of total iridoids in headspace of manually damaged silver vine: 22.9 ng/g leaf wet weight/10min, Figure 1D, versus manually damaged catnip: 11.3 μg/g/10 min, Figure 3C; $z = -3.00$, $p = 0.001$), cats showed a similar duration of response toward extracts from the same weight of manually crumpled and torn leaves (100 mg wet weight) from the two plants presented simultaneously (Figure 3D; $p = 0.101$). To exclude any inhibitory or enhancing effects of compounds other than iridoids in the damaged leaf extracts, we compared the duration of the feline response toward synthetic iridoid cocktails prepared to match the amount and the ratio of the different iridoids in extracts of 100 mg of manually damaged catnip (damaged catnip-cocktail; total iridoids: 400 μg) and equivalently damaged silver vine (damaged silver vine-cocktail; total iridoids: 10 μg) (Figures 3A and Table 1). In agreement with Figure 3D, there was no significant difference in the duration of the feline response between 400 μg of damaged catnip-cocktail and 10 μg of damaged silver vine-cocktail (Figure 3E; $p = 0.638$); both types of cocktails stimulated a similarly prolonged response despite the substantial difference in the total amount of iridoid presented. To confirm that cats are more sensitive to the complex cocktail of iridoids in damaged silver vine leaves compared to the nepetalactone-dominated iridoids in damaged catnip, we compared equivalent amounts of iridoids corresponding to the damaged catnip and damaged silver vine (50 μg of each). As expected, cats showed a more prolonged response to the damaged silver vine-cocktail than to the damaged catnip-cocktail (Figure 3F; $p = 0.002$). This was not the case when the damaged silver vine-cocktail was modified by replacing nepetalactol in the mixture with nepetalactone (Figure 3G; $p = 0.133$; the formulation is shown in Table 1).

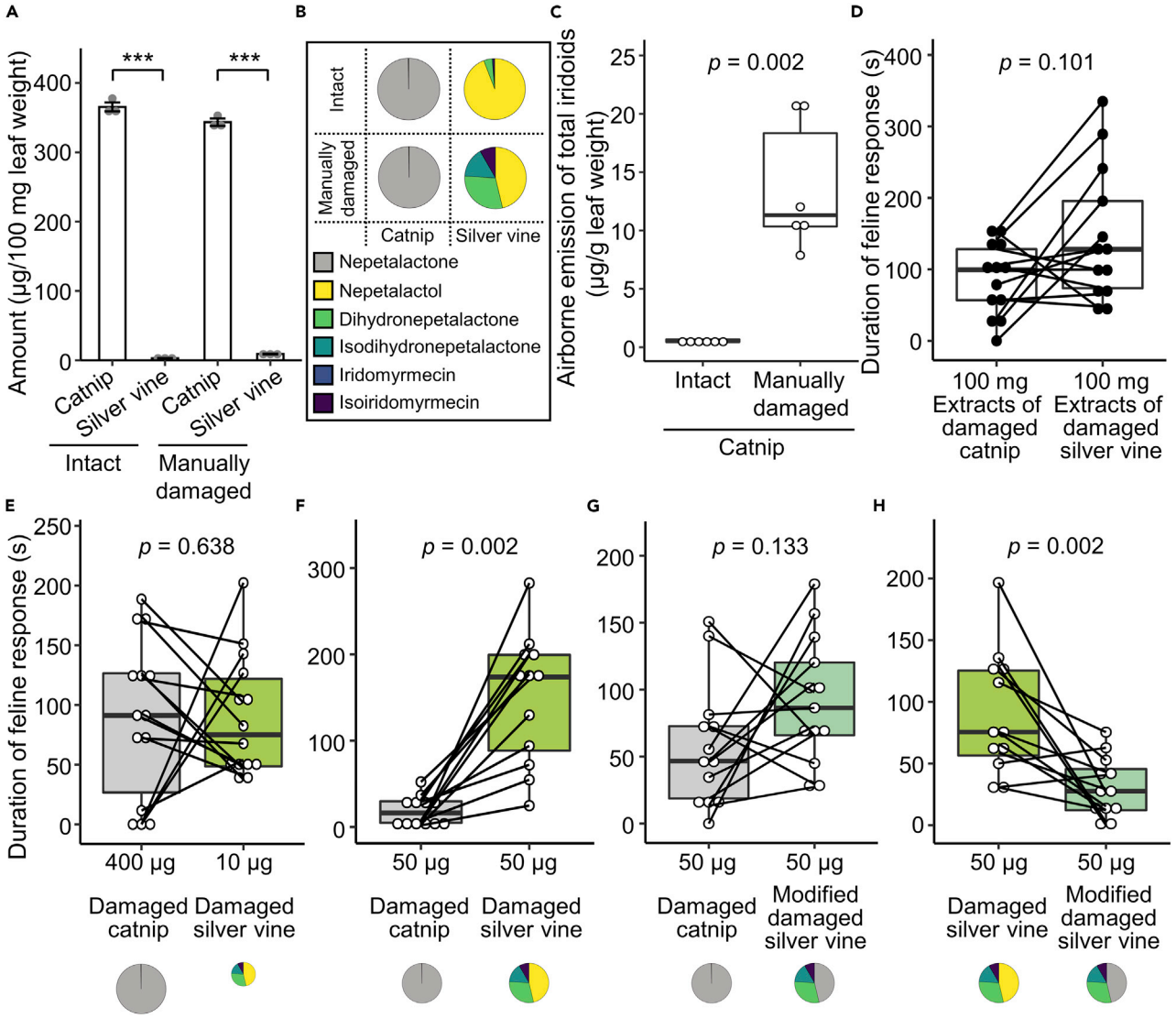

Figure 3. The differences in iridoid profiles between catnip and silver vine and the responsiveness of cats to these plants

(A and B) Total amount (A; mean ± *SE*; n = 3) and composition (B) of iridoids in the extracts of catnip and silver vine leaves that were intact or damaged manually 10 min before extraction. Points in (A) are individual values.

(C) Headspace amount of total iridoids emitted from catnip intact leaves and leaves manually damaged over a 10 min period (n = 6).

(D) Duration of the feline response to manually damaged leaf extracts of silver vine and catnip (corresponding to 100 mg leaf wet weight, n = 13 cats).

(E) Duration of the feline response to synthetic iridoid cocktails prepared to match the ratio and the amount of iridoids in the extracts of 100 mg of equivalently damaged catnip (damaged catnip-cocktail; total iridoids: 400 µg) and silver vine (damaged silver vine-cocktail; total iridoids: 10 µg) (n = 14 cats).

(F) Duration of the feline response to 50 µg damaged catnip-cocktail and 50 µg damaged silver vine-cocktail (n = 12 cats).

(G) Duration of the feline response to 50 µg damaged catnip-cocktail and 50 µg modified damaged silver vine-cocktail in which nepetalactol was replaced by nepetalactone (n = 13 cats). (H) Duration of the feline response to damaged silver vine-cocktail and modified damaged silver vine-cocktail (n = 12 cats). Table 1 gives the iridoid cocktail formulations. (C to H) Box and whisker plots show the median, interquartile range, first data point within 1.5 interquartile ranges, and individual values. Points connected by lines indicate paired dishes in the same trial. p values from Mann-Whitney *U* test (C) and Wilcoxon matched-pair test, two-tailed (D to H). ***p < 0.0001 from Bonferroni post hoc tests.

The importance of the combination of nepetalactol with additional iridoids was further evidenced by a more prolonged feline response to the damaged silver vine-cocktail than to the modified damaged silver vine-cocktail (Figure 3H; p = 0.002). Thus, the more prolonged response of cats to the complex iridoids emitted from damaged silver vine leaves depended on the mixture of nepetalactol with other iridoids such as isodihydronepetalactone; it was not simply a response to high levels of iridoids other than nepetalactol that are emitted when leaves are licked and chewed.

Iridoid composition of damaged silver vine repels mosquitoes faster than that of the intact leaves

Previous studies have reported mosquito-repelling activity of individual iridoids such as nepetalactone, nepetalactol, and dihydronepetalactone (Birkett et al., 2011; Feaster et al., 2009; Gkinis et al., 2014; Melo et al., 2021; Reichert et al., 2019; Uenoyama et al., 2021; Zhu et al., 2012) but have not examined responses to the iridoid cocktails reported in the current study. To examine whether mosquitoes are more sensitive to the complex cocktail of iridoids in damaged silver vine leaves compared to the nepetalactol-dominated iridoids in intact silver vine, we compared the repellency of the synthetic iridoid cocktails that correspond to extracts from intact and manually crumpled and torn silver vine leaves (Table 1) against *Aedes albopictus*. This is a common mosquito in Japan and China (Medley, 2010) that is a vector of *Dirofilaria immitis* which infects the heart and pulmonary arteries of dogs and cats (Traversa and Di Cesare, 2014). We tested each cocktail at two concentrations (400 μg and 800 μg total iridoids) against groups of 18–26 mosquitoes in each replicate; these concentrations correspond to the iridoid content of approximately 4 g and 8 g damaged silver vine leaves, respectively. We measured the percentage of mosquitoes in each replicate trial that had moved into a shelter, placed 15 cm from the test stimulus, 10 min and 20 min after one of the test solutions was introduced into an enclosed acrylic box and compared this to a solvent control treatment to assess repellency (Figure S4; see STAR Methods for further details). Ten minutes after introduction, the proportion of mosquitoes in the shelter differed significantly between treatment groups ($H(4) = 24.2$, $p < 0.0001$). *A. albopictus* avoided the lower concentration (400 μg) of damaged silver vine-cocktail compared to the solvent control, but not the intact-cocktail at the same low concentration (Figure 4A). However, *A. albopictus* avoided both the intact and damaged iridoid cocktails presented at higher concentration (800 μg) compared to the solvent control. By 20 min after introduction, *A. albopictus* avoided both intact and damaged cocktails at both low and high concentrations (Figure 4B; overall difference between groups: $H(4) = 24.0$, $p < 0.0001$).

We also examined mosquito repellency of nepetalactone-dominated iridoids in damaged catnip. In contrast to silver vine-cocktails, damaged catnip-cocktail did not repel *A. albopictus* at 400 μg or 800 μg at either time point after introduction, but there was significant avoidance only toward 1600 μg of damaged catnip-cocktail 20 min after introduction (10 min: Figure 4C, overall difference between groups: $H(3) = 7.1$, $p = 0.069$; 20 min: Figure 4D, overall difference between groups: $H(3) = 9.4$, $p = 0.024$).

These results indicate that the iridoid compositions of intact and damaged silver vine leaves both induced mosquito repellency, but the chemical constituent profile of iridoids from the damaged leaves had a faster repellent effect on *A. albopictus* than the simpler iridoid profile of the intact leaves at low concentration. A greater amount of damaged catnip-cocktail was required to repel *A. albopictus* compared to both intact and damaged silver vine-cocktails.

DISCUSSION

The present study provides evidence that the physical damage of silver vine and catnip leaves by feline licking and chewing makes an important contribution to their chemical pest defense in combination with rubbing and rolling when cats are exposed to these plants. Our data show clearly that physical damage of silver vine and catnip promotes the immediate emission of plant iridoids. Besides, such damage also changes the composition of plant iridoids in silver vine, though not in catnip. These changes in both the amount of plant iridoid emission (both plant species) and composition (silver vine only) induced significantly extended response to these plants, promoting increased self-anointing (rubbing and rolling) behavior that transfers plant iridoids to the feline fur. Moreover, the diversification of iridoids in damaged silver vine leaves provides a stimulus that is more repellent to mosquitoes at low concentration, inducing a faster response than nepetalactol-dominated or nepetalactone-dominated iridoids in plants. Thus, leaf damage by licking and chewing acts in combination with rubbing and rolling as an adaptive response that allows cats to gain effective mosquito repellency from iridoid-producing plants, helping to reduce the health risks and irritation associated with mosquitoes and possibly other arthropod pests that are sensitive to plant iridoids.

We have shown previously that cats respond to each of the iridoids presented individually (Uenoyama et al., 2021). In addition to this, our current study demonstrates that the combination of iridoids produced by silver vine in response to physical damage is even more effective in inducing a prolonged feline response than intact silver vine. Comparison of the feline response duration toward damaged silver vine-cocktails and

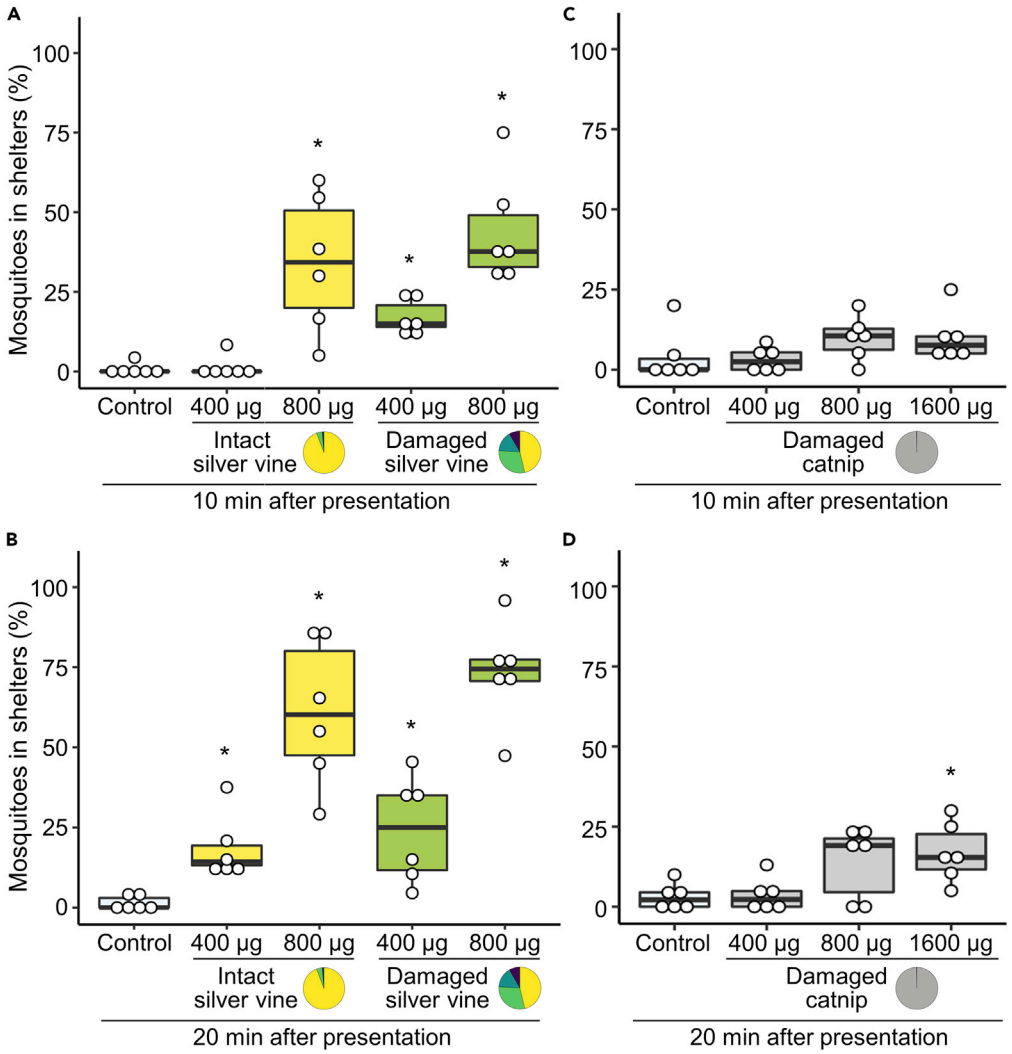

Figure 4. Mosquito repellency of synthetic iridoid cocktails corresponding to extracts from intact or manually damaged silver vine leaves

Percentage of mosquitoes (*Aedes albopictus*) that had moved into shelters, placed 15 cm from (A and B) intact silver vine-cocktail, damaged silver vine-cocktail (400 µg and 800 µg), (C and D) damaged catnip-cocktail (400 µg, 800 µg, and 1600 µg), or a solvent control (n = 6 replicates per treatment) assessed 10 min (A and C) and 20 min (B and D) after presenting the stimuli (Figure S4). Table 1 gives the cocktail formulations. Box and whisker plots show the median, interquartile range, first data point within 1.5 interquartile ranges, and individual values. *$p < 0.05$ from the Steel post hoc tests, with planned comparison of each test stimulus with the control.

catnip-cocktails indicates that the olfactory and behavioral sensitivity of cats to plant iridoids has been fine-tuned to show a prolonged characteristic response toward either a low level of complex iridoids in damaged silver vine or a much higher level of nepetalactone in damaged catnip. Notably though, nepetalactol remains an important component for inducing the prolonged response to a low level of damaged silver vine-cocktail as replacing this with nepetalactone in the mixture significantly shortened the feline response.

Manual crumpling and tearing of silver vine leaves changed their iridoid composition in a very similar way to the leaves crumpled and torn by cats during the characteristic response which usually lasts for 5 to 15 min (Hart and Leedy, 1985). The even greater emission of this iridoid complex following the manual damage treatment is likely to be because cats were less thorough in tearing the leaves as shown in Figure 1C. However, the increase in total iridoids in the leaves themselves was greater in response to feline damage than

manual damage, perhaps because of a smaller amount of iridoid emission from leaves torn less thoroughly by cats. Our findings suggest that changes in the amount and composition of iridoids in silver vine leaves in response to feline licking and chewing are because of the physical damage inflicted on the leaves. In addition to the crumpling and tearing of catnip and silver vine leaves by chewing, feline tongues have sharp spine-shaped papillae on their surfaces that can also scratch the leaves and cause physical damage during licking (Noel and Hu, 2018). This study did not examine the molecular mechanisms underlying the changes in iridoids in response to silver vine leaf damage. However, as nepetalactol is an intermediate product of other iridoids such as nepetalactone in many plant species (Alagna et al., 2016; Geu-Flores et al., 2012; Kries et al., 2017; Lichman et al., 2019; Miettinen et al., 2014; Munkert et al., 2015), it seems likely that dihydronepetalactone, iridomyrmecin, and their isomers may have been produced from nepetalactol through the action of plant enzymes that respond to leaf damage. No study so far has elucidated the mechanism of biosynthesis of the five types of iridoids in silver vine. Our findings may lead to new clues to identify key plant enzymes for the biosynthesis of plant iridoids that may be usefully utilized as repellents against a broad range of pests including mosquitoes. For example, it may be helpful to explore the enzymes with upregulated expression and/or activity that occur within 10 min of leaf damage (the typical duration of a feline response).

In contrast to silver vine, leaf damage only increased the emission of nepetalactone from catnip leaves. Both the amount of nepetalactone and the composition of iridoids were similar between the intact and damaged leaf extract of catnip. Thus, the increased emission of nepetalactone from damaged catnip might be a release of nepetalactone already contained in the plant cells rather than any *de novo* biosynthesis in response to licking and chewing.

In conclusion, the physical damage of the plant leaves enhances the release of iridoids repellent to arthropods such as mosquitoes and, in combination with enhanced self-anointing to transfer iridoids to the fur, acts to provide stronger chemical pest defense. It is already known that some mammals, including cats and birds, rub their bodies against plants and invertebrates, resulting in the transfer of anti-pest compounds to their bodies (Uenoyama et al., 2021; Valderrama et al., 2000; Weldon et al., 2011). The present study provides important additional evidence that plant-damaging behavior increases the emission of plant iridoids that have anti-mosquito activity and stimulates cats to anoint themselves with the iridoids. Notably, other mammals such as spider monkeys (*Ateles geoffroyi*) exhibit similar behavioral patterns that include biting of some plants that contain anti-pest or antibacterial compounds and body rubbing against the plants (Laska et al., 2007). Thus, plant-damaging behavior, which occurs alongside self-anointing, might play an important role in gaining pest-repellency in other mammals as well as cats.

Limitations of the study

The present study could not explain the molecular mechanism underlying the synergistic effects of nepetalactol on cats and mosquitoes when this is combined with other iridoids. A recent study found that transient receptor potential channel A1 (TRPA1) is the major mediator of repellency of nepetalactone to mosquitoes, but this is not involved in the perception of nepetalactone among mammals (Melo et al., 2021). Further study including the chemoreception mechanism of these compounds in both cats and mosquitoes will be necessary to understand the increased bioactivity of iridoid mixtures.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104455.

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AUTHOR CONTRIBUTIONS

R.U. and M.M. conceived the project and designed the experiments. R.U. and M.M. conducted all of the experiments with additional contribution by T.M. to behavioral experiments. R.U., J.L.H., and M.M. analyzed the data. M.A. and T.N. synthesized iridoids. R.U. and M.M. wrote the draft manuscript, which was edited by J.L.H. M.M. supervised the project.

DECLARATION OF INTERESTS

M.M., R.U., and T.N. have filed a patent application covering the use of nepetalactol as an insect repellent (patent application 2020–140755, PCT/JP2021/030942). The authors declare that they have no other competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Nepetalactone	Adachi et al. (2016); Schreiber et al. (1986); Uenoyama et al. (2021)	CAS: 21651-62-7
Nepetalactol	Adachi et al. (2016); Schreiber et al. (1986)	CAS: 109215-55-6
Dihydronepetalactone	Adachi et al. (2016); Schreiber et al. (1986); Uenoyama et al. (2021)	CAS: 21950-33-4
Isodihydronepetalactone	Adachi et al. (2016); Schreiber et al. (1986); Uenoyama et al. (2021)	CAS: 24190-27-0
Iridomyrmecin	Adachi et al. (2016); Schreiber et al. (1986)	CAS: 485-43-8
Isoiridomyrmecin	Adachi et al. (2016); Schreiber et al. (1986)	CAS: 107538-14-7
Chloroform	FUJIFILM Wako Pure Chemical	034-02608
Methanol	FUJIFILM Wako Pure Chemical	139-01827
n-Hexane	FUJIFILM Wako Pure Chemical	080-03423
Ethanol	Sigma–Aldrich	09-0770-4
Deposited data		
Raw and analyzed data	This paper	Supplemental All data set
Experimental models: Organisms/strains		
Domestic cat (*Felis silvestris catus*)	Kitayama Labes Co.,Ltd.	N/A
Asian tiger mosquito (*Aedes albopictus*)	Sumika Technoservice Corporation	N/A
Silver vine (*Actinidia polygama*)	Wild populations in Takizawa, Iwate Prefecture, Japan (coordinate: 39°80′ north latitude, 141°10′ east longitude)	N/A
Catnip (*Nepeta cataria*)	A garden in Morioka, Iwate Prefecture, Japan	N/A
Software and algorithms		
SPSS version 28	IBM	https://www.ibm.com/analytics/spss-statisticssoftware
JMP version 10.0.2	SAS Institute Inc.	https://www.jmp.com/en_us/software/data-analysis-software.html
Behavioral Observation Research Interactive Software (BORIS) version 7.10.7	Olivier Friard and Marco Gamba	http://www.boris.unito.it/pages/download.html
GCMSsolution version 4.53	Shimadzu Co.	https://www.an.shimadzu.co.jp/gcms/support/download/gcms_s/gcms453sp1dl.htm
Other		
Digital video camera	Sony	Handycam HDR-CX680
GC/MS	Shimadzu Co.	QP-2010 Ultra
Thermal desorption system	Shimadzu Co.	TD-20
Autosampler	Shimadzu Co.	AOC-20i/s
DB-WAX column	Agilent Technologies, Inc.	122-7062UI
Tenax-TA absorbent	Shimadzu Co.	223-57102-91
Septum for headspace sampling	GERSTEL	093640-057-00

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Cat cage	DCM Co., Ltd.	456063
Glove	Kimberly-Clark	52816
Petri dish (9 cm diameter)	Sansyo Co., Ltd.	36-3407
Petri dish (6 cm diameter)	AGC Techno Glass Co., Ltd.	3010-060
Acrylic chamber	Crew's Co.	AB-200
Plastic bag	Jointex Co.	365-245
Filter paper	Toyo Roshi Kaisha Ltd.	Advantec qualitative no. 1, 70 mm

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Masao Miyazaki (mmasao@iwate-u.ac.jp).

Materials availability

All data supporting the synthesized iridoids can be received from the lead contact upon request. The synthesized iridoids used in this study will be made available on request by the lead contact with a completed Materials Transfer Agreement.

Data and code availability

- All source data to generate all the figures are included in Table S1 (Supplemental all data set).
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

Sixteen healthy mixed-breed laboratory cats (aged 10–139 months; five intact males, nine intact females, and two spayed females) participated in this study. The cats were housed in pairs or individually in three-storied cages (93 cm × 63 cm × 178 cm; DCM Co., Ltd., Tokyo, Japan) that were kept at 24 °C by an air conditioner under a 12-/12-h light/dark photoperiod (lights on at 7:00 am). They received dry food (Royal Canin, Aimargues, France) twice daily and had continuous visual, auditory, and olfactory contact with other cats in the room. All subject cats were confirmed to exhibit the characteristic licking, chewing, rubbing, and rolling response to nepetalactone, nepetalactol, dihydronepetalactone, isodihydronepetalactone, iridomyrmecin, and isoiridomyrmecin, which were synthesized in our previous studies (Uenoyama et al., 2021). The cats that participated in each experiment are described in Table S1. All procedures were approved by the animal research committee of Iwate University (Approval numbers A202027, A202038, and A202124).

Female *Aedes albopictus* mosquitoes (3–8 days old) were purchased from Sumika Technoservice Corporation (Hyogo, Japan) and maintained at 24 °C by an air conditioner until mosquito repellence assays (Figure 4).

Plants

Fresh silver vine was sampled from wild populations in Takizawa, Iwate Prefecture, Japan, in June and July 2021 and was used for behavioral assays within 1 hour of sampling. Catnip seeds were purchased from a flower shop and grown in a garden in Morioka, Iwate Prefecture. Catnip was harvested immediately before the experiments in June through August 2021.

METHOD DETAILS

Preparation of leaf extracts of plants

Leaf extracts were prepared using a modification of our previous method (Uenoyama et al., 2021). In brief, silver vine and catnip leaves were ground with a mortar and pestle in the presence of liquid nitrogen, and

the leaf powder was suspended overnight in a 20-fold (w/v) organic solvent [2:1 (v/v) cocktail of chloroform (reagent grade, > 99.0% purity; FUJIFILM Wako Pure Chemical, Osaka, Japan) and methanol (reagent grade, > 99.8% purity; FUJIFILM Wako Pure Chemical)]. After removing the leaf residue by centrifugation at 3,000 rpm for 5 min, the leaf extract solvent was removed by rotary evaporation and the extract was dissolved in *n*-hexane (HPLC grade, ≥ 96.0% purity; FUJIFILM Wako Pure Chemical) to a concentration of 1 g leaves per milliliter. The iridoid contents of each extract were analyzed by GC/MS, as described below.

Fresh silver vine leaves were subjected to untreated (intact) and manual crumpling and tearing treatment (n = 3 per treatment). Each leaf in the artificial-damage treatment was crumpled by crushing in the experimenter's hand wearing gloves (LAVENDER NITRILE Powder-Free Exam Gloves, Kimberly-Clark, Roswell, GA, USA) and torn into quarters to simulate feline licking and chewing. Ten minutes after the treatments, extracts were prepared as described above.

Behavioral assays using cats

In all experiments, the cats were placed in individual test cages (93 cm × 63 cm × 59 cm; DCM Co., Ltd., Tokyo, Japan) for a few minutes before each assay for habituation. The behavioral response of each cat to silver vine leaves or toward two Petri dishes (9 cm diameter; Sansyo Co., Ltd., Tokyo, Japan) containing test stimuli was recorded using a digital video camera (Handycam HDR-CX680; Sony, Tokyo, Japan) placed in front of the cage, until the cat showed a lack of interest in the plants or dishes for at least 10 min. The location of each dish on the right or left of the cage floor was randomized in each assay. The duration of the feline response (licking, chewing, rubbing, and rolling) toward the dish was assessed blinded to dish identity using Behavioral Observation Research Interactive Software (BORIS) ver. 7.10.7 (Friard and Gamba, 2016).

Experiment 1 examined the responsiveness of eight cats to the intact and manually crumpled and torn silver vine leaves. Subjects were presented simultaneously with two Petri dishes fixed to the cage floor with gummed cloth tape: one dish contained 2 g of intact silver vine leaves and the other contained 2 g of manually crumpled and torn silver vine leaves, both covered by perforated lids.

Experiment 2 examined the responsiveness of 12 cats to two types of synthetic iridoid cocktails that corresponded to the ratio of iridoids in the leaf extracts of intact silver vine leaves (intact silver vine-cocktail) or to leaves crumpled and torn manually 10 min before extraction (damaged silver vine-cocktail). The two iridoid cocktails were prepared at 5 mg/ml using the formulations shown in Table 1, with ethanol (reagent grade, > 99.5 % purity, Sigma–Aldrich, St. Louis, MO, USA) as a solvent. Fifty micrograms of each cocktail were applied to the base of separate dishes. After the solvent evaporated, the subjects were tested with a choice of open dishes containing intact silver vine-cocktail versus a damaged silver vine-cocktail, as described above.

Experiment 3 examined the responsiveness of 13 cats to catnip and silver vine leaf extracts. Subjects were presented with a choice of open dishes containing extracts of catnip versus silver vine leaves (100 μl aliquot corresponding to 100 mg leaf wet weight) that had been crumpled and torn manually 10 min before extraction. Our previous study showed that this dose of intact silver vine leaf extract was enough to induce the full characteristic response shown toward silver vine plants by positive responder cats (Uenoyama et al., 2021).

Experiment 4 examined the responsiveness of 14 cats to synthetic iridoid cocktails prepared to match the ratio and amounts of iridoids in the extracts of 100 mg of damaged catnip (damaged catnip-cocktail; total iridoids: 400 μg) and damaged silver vine leaves (damaged silver vine-cocktail; total iridoids: 10 μg) that were equivalently crumpled and torn manually 10 min before extraction. Damaged catnip-cocktail and damaged silver vine-cocktail were prepared at 5 mg/ml and 125 μg/ml, respectively, using the formulations shown in Table 1, with eighty microliters of each cocktail applied to separate open dishes.

Experiment 5 examined the responsiveness of 12 cats to 50 μg damaged catnip-cocktail versus 50 μg damaged silver vine-cocktail.

Experiment 6 examined the responsiveness of 13 cats to 50 μg damaged catnip-cocktail versus 50 μg modified damaged silver vine-cocktail in which nepetalactol was replaced by nepetalactone (the formulation is shown in Table 1).

Experiment 7 examined the responsiveness of 12 cats to 50 μg damaged silver vine-cocktail versus 50 μg modified damaged silver vine-cocktail.

Headspace sampling of plant leaves

Five grams of fresh silver vine leaves were subjected to different treatments: intact (n = 7), feline response (n = 8), and manual crumpling and tearing (n = 7). In the feline response, the leaves placed in individual test cages were damaged by the typical response including licking and chewing for approximately 10 min. Immediately after damage by cats or manually, the leaves were carefully transferred into a 5-cm-diameter, 10-cm-high cylindrical glass bottle to avoid further damaging the leaves. The bottle was closed with a stainless-steel screw-cap with a 9-mm-diameter hole in the center that was covered by a septum [silicone (blue)/PTFE (white), 45° Shore A, 1.3 mm; GERSTEL, Mülheim an der Ruhr, Germany]. The headspace above the leaves was concentrated into an adsorption glass tube containing 300 mg of Tenax-TA adsorbent (Shimadzu Co., Kyoto, Japan) by purging with pure nitrogen gas at a rate of 65 ml/min, at room temperature for 4 hours. Fresh catnip (1 g aliquot) leaves were subjected to intact and manual crumpling and tearing treatments (n = 6 per treatment), and the leaf headspace was then concentrated into Tenax-TA adsorbent for 10 min as described above. Immediately after sampling the headspace, organic solvent extracts of the leaves were prepared as described above.

Gas chromatography / mass spectrometry

For the analysis of organic solvent extracts of silver vine and catnip, one microliter of each sample was injected into GC/MS (QP-2010 Ultra device, Shimadzu Co.) at 250°C injector temperature using an AOC-20i/s autosampler (Shimadzu Co.). Within calibration curves, leaf extracts corresponding to 1 mg and/or 0.1 mg leaf wet weight were injected into the GC/MS. For the headspace analysis, volatile compounds trapped in Tenax-TA were desorbed at 250°C by purging helium gas at 60 ml/min for 10 min using a thermal desorption device (TD-20; Shimadzu Co.) and were introduced directly into the GC/MS. All samples were introduced in splitless mode.

The GC equipped with DB-WAX column (60 m × 0.25 mm internal diameter, 0.25 μm film thickness; Agilent Technologies, Inc., Santa Clara, CA, USA) was operated with helium carrier gas, 1.5 ml/min column flow. The GC oven temperature was maintained at 40°C for 2 min, increased to 250°C at a rate of 4°C/min, and held at 250°C for 10 min. Mass spectrometry was operated in electron impact mode (70 eV) at 200°C of ion-source temperature. Mass spectra were obtained in full-scan mode from *m/z* values of 35 to 500. GCMSsolution software (ver. 4.53; Shimadzu Co.) was used to process the raw data, peak identification from total ion chromatograms, peak–peak signal–noise (S/N) ratio calculation, and peak area measurement. The amounts of nepetalactone, nepetalactol, dihydronepetalactone, isodihydronepetalactone, iridomyrmecin, and isoiridomyrmecin were quantified based on the areas of the *m/z* 81, *m/z* 135, *m/z* 81, *m/z* 81, *m/z* 95, and *m/z* 67 peaks, respectively, extracted from the full-scan data. The limit of detection (LOD) and the lower limit of quantification (LLOQ) were assessed in each analytical run at an S/N ratio greater than 3 and 10, respectively. Calibration curves for nepetalactol and other iridoids were generated using 4.27, 8.53, 17.06, 34.1, 68.2, 136.5, 273.0, 546.0, 1092.0, 2184.0, and 4368.0 μg/ml, and using 0.71, 1.42, 2.84, 5.69, 11.4, 22.8, 45.5, 91.0, 182.0, 364.0, and 728.0 μg/ml, respectively.

Mosquito repellent assays

Mosquito repellent properties of intact silver vine-cocktail, damaged silver vine-cocktail, and damaged catnip-cocktail were assessed following our previous study (Uenoyama et al., 2021). Eighteen to twenty-six female *Aedes albopictus* mosquitoes (3–8 days old) were transferred to an acrylic chamber (20 cm × 20 cm × 20 cm; Crew's Co., Osaka, Japan) that had seven air vents (3 mm diameter) covered with a filter paper (Advantec qualitative no. 1, 70 mm; Toyo Roshi Kaisha Ltd., Tokyo, Japan) and was connected to a plastic bag as a shelter (Figure S4; 24 cm width, 26 cm depth, 0.04 mm thickness; Jointex Co., Tokyo, Japan). An open Petri dish (6 cm diameter; AGC Techno Glass Co., Ltd., Shizuoka, Japan, rubbed with 40-grit sandpaper) treated with an iridoid cocktail (400 μg or 800 μg of aliquot) or an appropriate ethanol solvent control was placed on the floor of separate cages after solvent evaporation, on the opposite side of the shelter (n = 6 replicates per treatment). As our previous study showed that 5 g silver vine leaves were sufficient to repel around 30 % of *Aedes albopictus* mosquitoes, while cats that had rubbed against 50 g silver vine leaves were significantly repellent to this mosquito species (Uenoyama et al., 2021), here we used 400 μg and 800 μg doses that corresponded to the iridoid contents of contained in approximately 4 g and 8 g damaged silver vine leaves, respectively. We also tested repellence of

1600 μg of the damaged catnip-cocktail because 400 μg and 800 μg of the cocktails failed to induce significant repellence of *Aedes albopictus* compared to the control stimulus. Ten and twenty minutes after the presentation of stimuli, the number of mosquitoes present in the shelter was counted and expressed as a percentage of the total group tested. Mosquito repellency was assessed by comparing the percentage of mosquitoes in the shelter in each treatment replicate with control replicates.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed in SPSS (version 28, IBM) or JMP (version 10.0.2, SAS institute inc.)

The amount of total iridoids in the headspace above silver vine leaves was compared between intact, cat-exposed, and manually damaged treatments using a Kruskal-Wallis test (exact p value), with Steel-Dwass post-hoc comparisons between each pair of treatments. Similar tests assessed differences between treatments in each individual iridoid in headspace samples. A Mann-Whitney *U* test (exact p value) compared the amount of total iridoids in the headspace above in catnip leaves between intact and manual-damage treatments. Univariate ANOVAs compared the amount of total iridoids in solvent extracts from intact, cat-exposed, and manually damaged silver vine leaves, as well as the amounts of each separate iridoid (nepetalactol, dihydronepetalactone, isodihydronepetalactone, and isoiridomyrmecin) after checking that residuals from each model approximated normality. Post hoc tests with Bonferroni correction for multiple comparisons assessed differences between each pair of treatments.

Wilcoxon matched-pair signed ranks test (two-tailed, exact p value calculated where $n < 10$) compared the total duration of the characteristic response (licking, chewing, rubbing, and rolling) to intact versus manually damaged silver vine leaves that were presented simultaneously. Similar tests compared the duration of response to 50 μg intact silver vine-cocktail versus 50 μg damaged silver vine-cocktail; extracts from manually damaged leaves of catnip versus silver vine (corresponding to 100 mg leaf weight); 400 μg damaged catnip-cocktail versus 10 μg damaged silver vine-cocktail; 50 μg damaged catnip-cocktail versus 50 μg damaged silver vine-cocktail; 50 μg damaged catnip-cocktail versus 50 μg modified damaged silver vine-cocktail; and 50 μg damaged silver vine-cocktail versus 50 μg modified damaged silver vine-cocktail.

The percentage of mosquitoes in each replicate group found in the shelter was compared between intact silver vine-cocktail, damaged silver vine-cocktail (400 μg and 800 μg), or solvent control treatments using a non-parametric Kruskal-Wallis test (exact p value) after 10 min and 20 min exposure, with Steel post-hoc tests comparing each test stimulus to the control, as data did not meet the assumptions required for parametric analysis. Similar tests compared mosquito response to different amounts of damaged catnip-cocktail (400 μg, 800 μg, and 1600 μg) or solvent control treatments.