

## Volatile emissions of six New Zealand fern species in response to physical damage and herbivory

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**Abstract:** Volatile compounds (scents) play an important role in mediating ecological interactions between plants and other organisms, including defence against biotic and abiotic stressors, such as herbivory or physical damage. Seed plants respond to wounding and herbivory by changing their volatile emissions and producing complex blends of compounds; this process is mediated by the phytohormone jasmonic acid (JA) that acts as a signalling molecule activating plant defence pathways. Exogenous application of JA can mimic the plant's responses to herbivory and is used in agriculture to enhance plant defences. Although this phenomenon has been well documented for seed plants, our knowledge on non-seed plants is scarce, leading to question whether the observed patterns are ubiquitous to all vascular plant species. This study aimed to characterise the volatile emissions of six native New Zealand fern species (*Cyathea dealbata*, *Cyathea medullaris*, *Dicksonia squarrosa*, *Asplenium bulbiferum*, *Asplenium oblongifolium*, and *Microsorium pustulatum*), and explore their changes in response to physical damage, exogenous application of JA, and herbivory. Physical damage caused a significant increase in volatile emissions, with odour blends dominated by green leaf volatiles (as observed in seed plants); whereas JA application only caused a moderate increase in volatile release and (unlike seed plants) did not produce a substantial increase in terpenoid emission. Compounds (*E*)-2-hexene and (*E*)-2-pentenol were unique to the headspace of herbivore-damaged plants, indicating that ferns may have specific responses to herbivory. This work suggests that changes in volatile emissions are common responses to biotic and abiotic stress in vascular plants, but prompts further research to elucidate the signalling and regulatory mechanisms in ferns.

**Keywords:** herbivore-induced plant volatiles; plant defences; plant-insect interactions; Pteridophytes; secondary metabolites

### Introduction

Secondary metabolites contribute to the specific odours, tastes, and colours observed in plants (Bennett & Wallsgrove 1994), and are key to plant survival. These chemicals have evolved to serve important ecological functions in plant reproduction, communication, and defence (Demain & Fang 2000). Many of these plant secondary metabolites are released into the environment in the form of volatile organic compounds (scents), which protect plants against abiotic stressors (Bartwal et al. 2013; Edreva et al. 2008), mediate plant-plant communication (Bais et al. 2004; Heil & Karban 2010), attract pollinators and seed-dispersers (Cipollini & Levey 1997; Pichersky & Gershenzon 2002), and control herbivores either directly, by deterring feeding stages and ovipositing females, or indirectly, by attracting their natural enemies towards herbivore-induced plant volatiles (Clavijo McCormick et al. 2012; De Moraes et al. 2001).

The majority of previous studies on herbivore-induced plant volatiles and their ecological roles come from angiosperm and gymnosperm species, particularly those of agricultural

importance. However, little is known about other vascular plants, including ferns and their allies, leading to question whether the patterns found in higher plants are universal to all plant species.

Contrary to the widespread belief that ferns have no attackers, research shows that ferns sustain similar levels of herbivore damage to seed-plants (Mehlreter et al. 2010). Furthermore, ferns and insects have a long-standing interaction, dating prior to the evolutionary appearance of flowering plants (Iannuzzi & Labandeira, 2014). Therefore, exploring fern volatiles and their responses to physical damage and herbivore attack can provide insight into the evolution of plant defences alongside valuable ecological information needed for their conservation. These kinds of studies can also lead to the discovery of compounds with insecticidal activity for use in biological control (Huang et al. 2020) or insecticide-producing proteins that could be expressed in crop plants (Shukla et al. 2016).

In seed plants, physical damage and chemical elicitors from insect oral secretions trigger changes in the emission of volatile compounds by initiating signalling cascades that will

activate the biosynthetic machinery of the plant (Arimura et al. 2005; Arimura et al. 2009). Jasmonic acid (JA) is the main signalling molecule involved in responses against chewing-herbivores, activating multiple defence pathways including the production and release of volatile organic compounds from damaged and undamaged tissues (Koorneef & Pieterse 2008; Walling 2000). In fact, exogenous JA application in combination with physical damage is sufficient to induce volatile emissions similar to those caused by herbivore damage (Clavijo McCormick et al. 2014; Hopke et al. 1994; Lou et al. 2005), and is used in agriculture to enhance plant defences (Horiuchi et al. 2001; Lou et al. 2005; Omer et al. 2000).

Most volatile organic compounds belong to three chemical categories according to their biosynthetic origin: green leaf volatiles, terpenoids, or aromatics. Green leaf volatiles are produced by the lipoxygenase (LOX) pathway, terpenoids are derived from the mevalonate (MVA) and 2-C-methyl-D-erythritol 4-phosphate (MEP) pathways, and aromatic compounds are derived from amino acids through different routes starting with the shikimate pathway (Arimura et al. 2009; Maffei 2010). Terpenoid emissions, in particular, are expected to increase over time in response to JA treatment (Clavijo McCormick et al. 2014; Hopke et al. 1994; Lou et al. 2005).

In ferns, the few available studies suggest that induced volatiles are released upon physical damage and herbivory; and that the phytohormone JA may be acting as a signalling molecule. For instance, Imbiscuso et al. (2009) observed an “oxidative burst” in the fern species *Pteris vittata* and the subsequent emission of volatile terpenoids upon herbivore damage, which is a trend many higher plants express upon herbivory. Radhika et al. (2012) tested the changes in volatile emission in response to JA treatment, herbivory and physical damage on bracken fern *Pteridium aquilinum*, and reported that JA led to the emission of a blend of volatile organic compounds that was mainly comprised of terpenoids. Likewise, treatment with the JA precursors 12-oxo-phytodienoic acid (OPDA) and  $\alpha$ -linolenic acid also induced terpenoid emission, albeit at a lower intensity than the JA treatment (Radhika et al. 2012).

With the intention to expand our knowledge of the responses of ferns to physical damage and herbivory, and the role of JA in mediating these responses, we aimed to characterise the volatile emissions of six native New Zealand fern species. To do so, we first compared two volatile collection techniques: solvent extraction and headspace collection, using healthy plant material, to select the optimal method to test the remaining treatments. We hypothesized that undamaged ferns would vary in their volatile composition and that physical damage, phytohormone treatment (JA), and herbivory would induce changes in the ferns' volatile emission. However, we also expected some differences in the intensity of the responses, reflecting the different growth habits and phylogenetic relatedness of the tested species.

## Methods

### Biological material

The ferns used in this study were growing in two naturally vegetated areas within Massey University's Manawātū campus (Tennent Drive, Palmerston North, 4410 New Zealand) during the summer 2016–2017, or sourced from a tree nursery, Fronds NZ Ltd (Cambridge, New Zealand) for the herbivory experiment.

Six native fern species were used in this study: the silver

fern or ponga (*Cyathea dealbata*), the black tree fern or mamaku (*Cyathea medullaris*), the rough tree fern or whekī (*Dicksonia squarrosa*), the hen and chicken fern or mouku (*Asplenium bulbiferum*), the shining spleenwort or huruhuru whenua (*Asplenium oblongifolium*) and the hound's tongue or kōwaowao (*Microsorium pustulatum*) (Fig. 1). The first three species are tree ferns of the order Cyatheaales; the last three are epiphytic or terrestrial ferns of the order Polypodiales (Allan 1961). These species were selected due to their high abundance and ease of access at the study site. There is considerable indigenous knowledge about these species, often reporting medicinal or antimicrobial uses (Cambie and Ferguson, 2003; Burtenshaw et al. 2009; Landcare Research, 2009), suggesting that they produce bioactive compounds.

Adult Wellington tree wētā (*Hemideina crassidens*) were chosen as the herbivore for this experiment as Dewhurst (2012) made field observations of these insects feeding on the above fern species. Specimens were gathered from the Massey University Manawātū campus, six weeks prior to experiments and fed a non-fern diet consisting of leaves from the native tree māhoe (*Meliclytus ramiflorus*) and carrot (*Daucus carota* subsp. *sativus*) roots. The wētā were kept in a climate-controlled room (16°C) under a controlled light regime with a 12:12 hour Light:Darkness photoperiod (light between 07:00–19:00, with an irradiance of  $5 \pm 1 \mu\text{mol m}^{-2} \text{s}^{-1}$  to mimic low-light conditions in nature). Insects were starved for 24 hours prior to the experiments to increase food consumption, and released back into their collection sites at the conclusion of the experiment.

### Characterization of the volatile constituents of the six fern species

To identify the compounds that constitute the chemical profiles of the different species, we compared two collection methods that have been previously used to characterise fern scent in other species: solvent extraction (Fons et al. 2010; Froissard et al. 2011; Halarewicz & Szumny 2010), and headspace collection (Imbiscuso et al. 2009; Kessler et al. 2015; Radhika et al. 2012).

For the solvent extraction, sections of fresh undamaged fronds were collected from five individuals of each species and cut into small pieces (approximately 1 cm<sup>2</sup>). A subsample of this tissue weighing exactly 3 g was used for the extraction. Using an adaptation of the method by Froissard et al. (2011), the samples were extracted using a 30 mL solution of high-grade hexane (CAS 110-5-3,  $\geq 99\%$ , Sigma Aldrich, St Louis, MO, USA) with 10 ng mL<sup>-1</sup> of nonyl acetate as an internal standard. Hexane is a good solvent for lipophilic/hydrophobic and non-polar substances making it ideal to recover green leaf volatiles and terpenoids (Lytchovchenko et al. 2009). The samples were incubated for 72 hours at 22°C with systematic shaking every 24 hours. Extracts were double-filtered, first through 70 mm glass microfibre filters and then using 0.2  $\mu\text{m}$  syringe filters, before Gas Chromatography-Mass Spectrometry (GC-MS) analysis. Three replicates per fern species were used.

For headspace collection, the fronds were bagged using heat-resistant polyethylene terephthalate bags (Easy cooking - Roasting sleeve, AWZ products Inc., China; See Appendix 1 in Supplementary Material). Collection of volatiles was conducted in the field using a portable volatile assay system (PVAS22, Volatile Assay Systems, Rensselaer, NY, USA) coupled with teflon and silicon tubing for dynamic (push-pull airflow) headspace collections. Air was pushed into the system at 0.9 L min<sup>-1</sup> and pulled out at 0.8 L min<sup>-1</sup>, creating a light





**Figure 1.** Representative fronds of the fern species used in this study; (a) rough tree fern or whekī (*Dicksonia squarrosa*), (b) hound's tongue or kōwaowao (*Microsorium pustulatum*), (c) hen and chicken fern or mouku (*Asplenium bulbiferum*), (d) shining spleenwort or huruhuru whenua (*Asplenium oblongifolium*), (e) silver fern or ponga (*Cyathea dealbata*), (f) black tree fern or mamaku (*Cyathea medullaris*).

over-pressure to avoid impurities entering the bag. Volatile organic compounds were collected using an adsorbent filter (HayeSep Q, Volatile Assay Systems, Rensselaer, NY, USA) placed at the 'pull' end of the system.

Volatiles were collected during a three-hour period. All collections were conducted in dry, sunny weather conditions (average temperature 18°C, 78% humidity). The fresh weight of sampled plant material was measured immediately after volatile collection (to estimate volatile emission per fresh weight), to have a comparable value to solvent extraction. Volatiles were extracted from the HayeSep Q filters by elution using 200  $\mu\text{L}$  of high-grade pentane (CAS, 109-66-0,  $\geq 99\%$ , Thermofisher Acros Organics, Geel, Belgium) containing 1 ng  $\text{mL}^{-1}$  nonyl acetate as an internal standard. The eluted samples were stored at  $-80^\circ\text{C}$  prior to analysis. Pentane is a non-polar solvent with similar properties to hexane but was preferred

over this for being less toxic due to the higher handling times of the samples (Takeuchi et al. 1980).

Analyses of frond extracts by both methods were performed using a Shimadzu benchtop gas chromatograph–mass spectrometer system (GC-MS) (QP2010, Shimadzu Corp., Kyoto, Japan) equipped with a TG-5MS capillary column (30 m  $\times$  0.25 mm  $\times$  0.25  $\mu\text{m}$ , Thermo Fisher Scientific, Waltham, MA, USA). The injection volume of each sample was 1  $\mu\text{L}$ . Helium (99.99%) was used as the carrier gas at a column flow-rate of 1  $\text{mL min}^{-1}$ . Carrier gas parameters include pressure control mode of 53.5 kPa, total flow (14.0  $\text{mL min}^{-1}$ ), linear velocity (36.3  $\text{cm sec}^{-1}$ ), purge flow (3.0  $\text{mL min}^{-1}$ ) and a split ratio of 10.0:1. The temperature programme was as follows: 50°C for 3 min, followed by an increase to 95°C at a rate of 5°C  $\text{min}^{-1}$ , an increase to 145°C at a rate of 15°C  $\text{min}^{-1}$ , an increase to 200°C at a rate of 10°C  $\text{min}^{-1}$  and maintenance



at 270°C for 3 min. The MS settings were as follows: ion source temperature (200°C), interface temperature (200°C), and solvent delay time (2 min), with a total program duration of 23.83 minutes.

Peaks in chromatograms of volatile and solvent profiles were manually integrated and analysed. The NIST05 MS library (National Institute of Standards and Technology, Gaithersburg, MD, USA) was used for mass spectral analyses and compound identification, according to reference compounds in the database. Only compounds with high similarity values ( $\geq 80$ ) are reported. Compound identity was confirmed using real standards, when available. Solvent-only samples, solvent plus internal standard, and volatile collections with clean air were used to identify contamination peaks, which were excluded from the analyses.

### Fern responses to physical damage and jasmonic acid treatment

To test ferns' responses to physical damage and to jasmonic acid (JA) treatment, the headspace collection technique as described above was used. This method was selected since it yielded a higher compound abundance and was non-destructive. Five individuals of each species were subjected to the following treatments: (1) control (no damage), (2) physical damage, (3) physical damage plus JA. Each treatment had a total volatile collection time of 3 hours during the same time of day (10:30–13:30). Physical wounding of the plant material was conducted via standardised linear tissue scarring (six 5 cm cuts) with a razor blade on the adaxial surface of the fronds. One mL of a 10 mg L<sup>-1</sup> JA solution was applied evenly across the artificially damaged portions of the fronds using a spray bottle prior to headspace collection.

### Fern responses to herbivory

Herbivory trials using the Wellington tree wētā (*H. crassidens*) were held under laboratory conditions using potted ferns of the four species that were commercially available: *C. dealbata*, *D. squarrosa*, *A. bulbiferum*, and *A. oblongifolium*.

During the course of this experiment, plants were transferred to a climate-controlled room three days before the volatile collection. Room conditions were 17°C and a 12:12 hour Light:Darkness photoperiod, with light between 08:00 and 20:00 at an irradiance of  $5 \pm 1 \mu\text{mol m}^{-2} \text{s}^{-1}$  to resemble shaded conditions in nature. Treatments were control (without herbivores) and herbivory, consisting of a single tree wētā that was allowed to feed for two hours between 17:00–20:00 (their normal feeding times). Volatiles were measured during the feeding period and four replicates were used per treatment. Afterwards, volatiles from insects only were collected following the same procedures and subtracted from the measurements to separate the smell of the insect from that of the plant it was feeding on.

### Statistical analyses

A one-way ANOVA on ranks (Kruskal-Wallis test) was used to establish differences between species and within species for each treatment (control, JA, and physical damage) within each fern species. Mann-Whitney pairwise comparisons were used to identify differing treatments when Kruskal-Wallis tests were significant. All statistical analyses were conducted using the SPSS statistical computing software (IBM, Version 25). Due to the high variability in the amount of damage and volatile emission from the herbivory treatment, only presence and absence of the compounds in each treatment are reported.

## Results

### Characterization of the volatile constituents of the six fern species

Eight volatile organic compounds were detected using the solvent extraction method (Table 1), most of which are green leaf volatiles produced by the LOX pathway. Nine volatile organic compounds were detected using the headspace collection method, including one green leaf volatile, various terpenoids (derived from the MEP and MEV pathways) and C5 and C8 compounds from unknown biosynthetic origin (i.e. not products of the main volatile biosynthetic pathways known in seed plants). Some compounds were identified in one replicate only (Tables 1, 2), but we report them here as indicators of the metabolic ability of the fern species to produce them. The headspace collection method yielded higher amounts of total volatile organic compounds for all species, while the solvent extraction method identified more compounds for each individual species. We selected the headspace collection method for further comparisons due to its higher yield and the fact that it did not involve sample destruction. We only report compounds having clearly identifiable peaks in the chromatogram; however, other compounds may also be present in minor amounts below our detection level.

### Fern responses to physical damage and jasmonic acid treatment

According to GC-MS analysis of headspace samples, nine volatile organic compounds were detected within the control treatment, ten in the phytohormone treatment, and eleven in the physical damage treatment (see Appendices 2 and 3 in Supplementary Materials). The C8 compounds (*E*)-2-octenol, 3-octanol, and 3-octanone were consistently detected in most fern species across all treatments, while green leaf volatiles (*Z*)-3-hexenol, (*Z*)-2-hexenal, and 1-hexanol were present only in the physical damage and jasmonic acid (JA) treatments. The compound (*Z*)-3-hexenal was only detected in the physical damage treatment, where it was found to be one of the most abundant volatiles for all species.

When comparing treatments (control, JA, and physical damage) within species (Fig. 2), the Kruskal-Wallis tests revealed significant differences for four of the studied species: *C. medullaris* ( $H = 11.18$ ,  $N = 15$ ,  $\text{d.f.} = 2$ ,  $P = 0.004$ ), *C. dealbata* ( $H = 11.8$ ,  $N = 15$ ,  $\text{d.f.} = 2$ ,  $P = 0.004$ ), *D. squarrosa* ( $H = 11.06$ ,  $N = 15$ ,  $\text{d.f.} = 2$ ,  $P = 0.004$ ), and *A. bulbiferum* ( $H = 6.86$ ,  $N = 15$ ,  $\text{d.f.} = 2$ ,  $P = 0.032$ ). For three of these species (*D. squarrosa*, *C. medullaris* and *C. dealbata*), all treatments (control, JA, and mechanical damage) differed from one another, whereas for *A. bulbiferum* no differences were observed between the JA and physical damage treatments.

After comparing the emission patterns between different species, we found significant differences in the constitutive (control:  $H = 13.88$ ,  $N = 30$ ,  $\text{d.f.} = 5$ ,  $P = 0.016$ ) and induced emissions (JA:  $H = 19.37$ ,  $N = 30$ ,  $\text{d.f.} = 5$ ,  $P = 0.001$ ; physical damage:  $H = 13.6$ ,  $N = 30$ ,  $\text{d.f.} = 5$ ,  $P = 0.018$ ). The  $P$ -values for the Mann-Whitney post-hoc tests (Table 3), suggest that the treatments differ from one another. The main observable trend is that tree ferns (Cyatheales) had lower amounts of constitutive (control) emissions than terrestrial or epiphytic ferns of the order Polypodiales, but the intensity of the responses to physical damage and JA was quite variable across species.

**Table 1.** Average concentration of volatile organic compounds from six fern species using solvent extraction. Values indicated with an asterisk ‘\*’ represent data from a single replicate. Values are presented in ng g<sup>-1</sup> of fresh weight per hour. Note: n.d. = not detected.

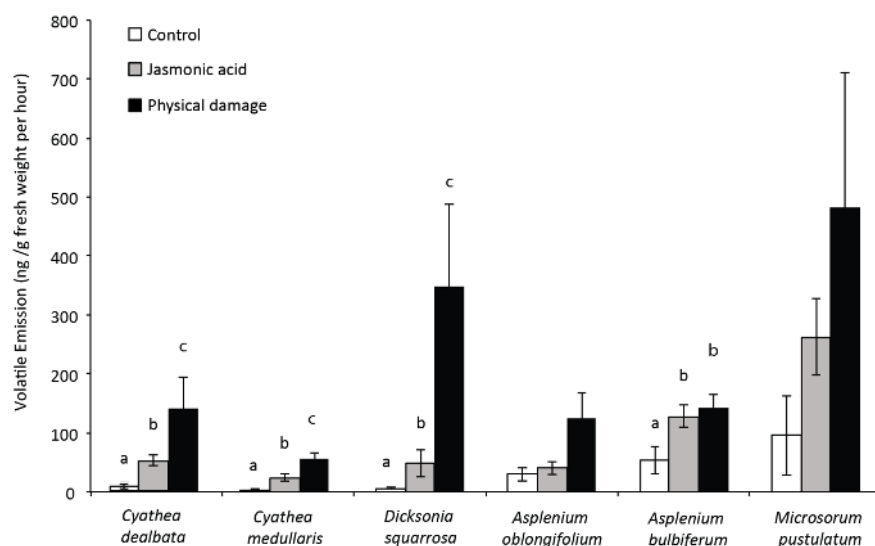
Compound	Tree ferns (Cyatheales)			Terrestrial or epiphytic ferns (Polypodiales)		
	<i>C. dealbata</i>	<i>C. medullaris</i>	<i>D. squarrosa</i>	<i>A. oblongifolium</i>	<i>A. bulbiferum</i>	<i>M. pustulatum</i>
<b>Green leaf volatiles</b>						
Hexenal	0.47 ± 0.02	0.27 ± 0.03	2.03 ± 0.06	0.13 ± 0.02	0.50 ± 0.05	0.07*
(Z)-2-Hexenal	2.00 ± 0.16	0.59 ± 0.12	6.83 ± 0.12	0.26 ± 0.03	0.69 ± 0.03	n.d.
(Z)-3-Hexenol	0.80 ± 0.01	0.48 ± 0.06	0.54 ± 0.07	0.47 ± 0.01	n.d.	n.d.
(Z)-2-Hexenol	n.d.	n.d.	1.06 ± 0.12	0.65 ± 0.06	0.58 ± 0.06	n.d.
1-Hexanol	n.d.	n.d.	1.01 ± 0.23	0.82 ± 0.03	1.37 ± 0.08	n.d.
<b>Terpenoids</b>						
Linalool	n.d.	n.d.	n.d.	n.d.	n.d.	0.18 ± 0.03
<b>Other</b>						
(E)-2-Octenol	0.06 ± 0.01	0.57*	0.15 ± 0.03	n.d.	n.d.	0.14 ± 0.03
2-Nonenol	0.30 ± 0.02	n.d.	n.d.	0.13 ± 0.03	0.14 ± 0.01	n.d.
Mean Volatile Emission <sup>1</sup>	0.36 ± 0.22	0.36 ± 0.21	0.72 ± 0.63	2.46 ± 0.18	3.28 ± 0.23	0.32 ± 0.06

<sup>1</sup> Excluding compounds present in one sample only

**Table 2.** Average concentration of volatile organic compounds from six fern species using a headspace collection method. Values are presented in ng g<sup>-1</sup> of fresh weight per hour. Note: n.d. = not detected.

Compound	Tree ferns (Cyatheales)			Terrestrial or epiphytic ferns (Polypodiales)		
	<i>C. dealbata</i>	<i>C. medullaris</i>	<i>D. squarrosa</i>	<i>A. oblongifolium</i>	<i>A. bulbiferum</i>	<i>M. pustulatum</i>
<b>Green leaf volatiles</b>						
(Z)-3-Hexenyl acetate	n.d.	n.d.	6.39*	n.d.	n.d.	n.d.
<b>Terpenoids</b>						
(Z,Z)- $\alpha$ -Farnesene	n.d.	n.d.	n.d.	n.d.	n.d.	24.23 ± 20.45
1S- $\alpha$ -Pinene	n.d.	8.57*	1.26*	n.d.	n.d.	24.58*
Copaene	3.34*	n.d.	n.d.	5.65 ± 2.44	6.40 ± 1.27	5.23*
Linalool	n.d.	1.82*	n.d.	n.d.	n.d.	n.d.
<b>Other</b>						
4-Hydroxy-4-methyl-2-pentanone	n.d.	n.d.	6.39*	3.64 *	n.d.	n.d.
(E)-2-Octenol	7.79 ± 3.49	4.49 ± 1.12	7.62 ± 0.39	19.49 ± 6.23	31.64 ± 13.39	102.35 ± 66.26
3-Octanol	n.d.	0.97 *	1.09*	6.63 ± 4.83	15.27 ± 6.02	n.d.
3-Octanone	1.97*	2.99*	n.d.	7.06 ± 1.88	7.59 ± 2.58	16.61*
Mean volatile Emission <sup>1</sup>	7.79 ± 3.49	4.49 ± 1.12	7.62 ± 0.39	38.83 ± 15.38	60.9 ± 23.26	102.35 ± 66.26

<sup>1</sup> Excluding compounds present in one sample only



**Figure 2.** Comparison of total volatile emission rates for three treatments: control, jasmonic acid, and physical damage, according to fern species. Letters above the bars indicate significant differences between treatments for each species as revealed by a Kruskal-Wallis test, followed by Mann-Whitney pairwise comparisons.

**Table 3.** *P*-values of the Mann-Whitney pairwise comparisons between the total volatile emissions of six fern species for three treatments: control, jasmonic acid and physical damage. Numbers in bold indicate significant differences ( $P < 0.05$ ).

Control	<i>C. dealbata</i>	<i>C. medullaris</i>	<i>D. squarrosa</i>	<i>A. oblongifolium</i>	<i>A. bulbiferum</i>
<i>Cyathea medullaris</i>	0.14				
<i>Dicksonia squarrosa</i>	0.50	<b>0.04</b>			
<i>Asplenium oblongifolium</i>	0.07	<b>0.02</b>	<b>0.04</b>		
<i>Asplenium bulbiferum</i>	<b>0.03</b>	0.20	<b>0.04</b>	0.26	
<i>Microsorium pustulatum</i>	<b>0.02</b>	<b>0.01</b>	<b>0.03</b>	0.34	0.32
Jasmonic acid	<i>C. dealbata</i>	<i>C. medullaris</i>	<i>D. squarrosa</i>	<i>A. oblongifolium</i>	<i>A. bulbiferum</i>
<i>Cyathea medullaris</i>	<b>0.03</b>				
<i>Dicksonia squarrosa</i>	0.33	5.00			
<i>Asplenium oblongifolium</i>	0.26	0.20	5.00		
<i>Asplenium bulbiferum</i>	<b>0.01</b>	<b>0.01</b>	<b>0.03</b>	<b>0.01</b>	
<i>Microsorium pustulatum</i>	<b>0.01</b>	<b>0.01</b>	<b>0.01</b>	<b>0.01</b>	0.72
Physical damage	<i>C. dealbata</i>	<i>C. medullaris</i>	<i>D. squarrosa</i>	<i>A. oblongifolium</i>	<i>A. bulbiferum</i>
<i>Cyathea medullaris</i>	<b>0.03</b>				
<i>Dicksonia squarrosa</i>	0.15	<b>0.02</b>			
<i>Asplenium oblongifolium</i>	5.00	0.14	0.14		
<i>Asplenium bulbiferum</i>	0.20	<b>0.01</b>	0.20	0.26	
<i>Microsorium pustulatum</i>	<b>0.03</b>	<b>0.01</b>	0.33	<b>0.03</b>	<b>0.02</b>

**Table 4.** Compounds detected in the herbivory experiments and corresponding treatments in which they were observed. Control (CT), wētā (*H. crassidens*; W), n.d. = not detected.

Compound	<i>C. dealbata</i>	<i>D. squarrosa</i>	<i>A. oblongifolium</i>	<i>A. bulbiferum</i>
<b>Green leaf volatiles</b>				
( <i>E</i> )-2-Hexene	W	W	W	W
<b>Terpenoids</b>				
( <i>Z,Z</i> )- $\alpha$ -Farnesene	n.d.	n.d.	n.d.	CT
Copaene	n.d.	n.d.	n.d.	CT
<b>Other</b>				
( <i>E</i> )-2-Pentenol	W	W	W	W
( <i>E</i> )-2-Octenol	CT/W	CT/W	CT/W	CT/W
3-Octanol	n.d.	n.d.	n.d.	CT
3-Octanone	W	W	CT/W	CT/W

### Fern responses to Wellington tree wētā herbivory

We found high variability in the feeding amounts by *H. crassidens* on different fern species and even on individual ferns. However, seven volatile organic compounds were detected in the control and herbivory treatments. *Asplenium bulbiferum* was found to have the highest number of volatile compounds emitted (seven) in contrast to the other fern species, which emitted four each (Table 4). Compounds (*Z*)-2-hexene and (*E*)-2-pentenol were characteristic of the headspace of tree wētā-damaged plants.

### Discussion

This work characterises for the first time the chemical profiles of six ecologically different New Zealand fern species, showing that ferns can respond to physical wounding, exogenous phytohormone application, and herbivory by changing their volatile profiles. Our study is also the first to test fern responses under natural conditions, and to use an insect species that naturally feeds on ferns. The effects of physical damage, phytohormone treatment and direct herbivory on fern-volatile

emissions had previously only been investigated in greenhouse-propagated plants (Imbiscuso et al. 2009; Radhika et al. 2012), which does not reflect natural conditions where other stressors may occur alongside herbivory. Furthermore, these studies used a herbivore that does not naturally feed on ferns *Spodoptera littoralis* – the Egyptian cotton leafworm.

After comparing two extraction methods: solvent extraction and headspace collection, we conclude that the latter is more suitable for ecological studies involving fern compounds as it yielded higher amounts of volatiles collected (Tables 1, 2). In addition, this method is non-destructive and more biologically relevant, as it collects only those compounds naturally released into the atmosphere, which are available for insects to detect, rather than other compounds that can only be extracted after long periods of soaking in solvent. Previous studies have used solvent extraction to analyse secondary metabolites in other fern species (Fons et al. 2010; Froissard et al. 2011; Halarewicz & Szumny, 2010). These studies report a high abundance and diversity of compounds, but involve different extraction procedures to those used here, including maceration and longer extraction times. It is possible that due to the nature of fern frond structure, longer extraction periods

and the crushing of plant cells may be necessary to recover a higher number of secondary metabolites. Although crushing damages cell walls, releasing other compounds, which are not naturally found in the fern scent. Studies investigating fern volatiles using headspace sampling techniques (Imbiscuso et al. 2009; Kessler et al. 2015; Radhika et al. 2012) typically yield fewer compounds, but are less invasive and do not require the destruction of the sample (unless it is part of the treatment) and are therefore more similar to natural emissions (Imbiscuso et al. 2009; Kessler et al. 2015; Radhika et al. 2012).

The headspace collection of undamaged ferns yielded a total of nine compounds including one green leaf volatile, terpenoids, and other C5 and C8 compounds of unknown biosynthetic origin. Research by Fall et al. (2001) reported that C5 compounds can be produced during leaf drying, senescence, and following freezing damage, and their appearance is oxygen-dependent, consistent with the involvement of the enzyme lipoxygenase. Therefore, their emission is likely associated with environmental stress and physiological changes of the plant under natural conditions. C8 compounds are known derivatives from fungal metabolism (Combet et al. 2006) and yeasts are well known to colonise leaf surfaces of plants (Sláviková et al. 2007), suggesting that the fern scent under field conditions is a combination of plant and microbial metabolism. Further studies are needed to elucidate the origin of these compounds and their biological activity.

Headspace analysis also indicates that tree ferns (Cyatheales) have lower amounts of constitutive (control) emissions than terrestrial or epiphytic ferns of the order Polypodiales. This may reflect their phylogenetic affinities, similar to the appearance of mustard oils in the Brassicaceae (Rask et al. 2000), salicinoids in the Salicaceae (Boeckler et al. 2011), or alkaloids in the Solanaceae (Chowański et al. 2016). However, this result may also reflect trade-offs between growth and defence, or between different defence strategies (i.e. chemical and structural defences; Levin, 1976), where tree ferns invest more in growth or structural defences than their epiphytic and terrestrial counterparts. These hypotheses require further exploration.

Our results also show that ferns can respond to wounding, jasmonic acid, and herbivore damage by changing their volatile emissions in a similar fashion to gymnosperms and angiosperms. This aligns with the findings of previous studies (Imbiscuso et al. 2009; Radhika et al. 2012), and suggests that the use of volatiles as defence mechanisms is common to all vascular plants. Physical damage is known to affect plant volatile emissions in gymnosperms and angiosperms by augmenting the release of green leaf volatiles due to the rupture of cell membranes, which releases lipids that are used as substrates for the lipoxygenase (LOX) pathway (Arimura et al. 2009). Lipid peroxidation (oxidative degradation of lipids) is common to all biological systems (Feussner & Wasternack, 2002), so green leaf volatile emissions are expected to occur in all plants in response to physical injury irrespective of their taxonomy.

Jasmonic acid (JA) is a naturally occurring organic compound that regulates plant responses to biotic and abiotic stresses, and it is also involved in plant growth and development (Delker et al. 2006). In a similar study on bracken fern (*Pteridium aquilinum*), Radhika et al. (2012) reported that JA and its precursors (12-oxo-phytodienoic acid (OPDA) and  $\alpha$ -linolenic acid), caused a significant increase in the emission of volatile organic compounds. Exogenous application of JA is known to induce the emission of volatile organic compounds

(mainly terpenoids) in seed-plants (Clavijo McCormick et al. 2014; Hopke et al. 1994; Lou et al. 2005). However, the fern species investigated here had variable responses to JA induction and none them responded by significantly increasing terpenoid emissions during the collection period. There are two possible explanations for this outcome: (1) JA is not involved in anti-herbivore responses in all ferns, (2) all ferns use JA but differ in their thresholds or the time needed to trigger anti-herbivore-responses. Recent studies indicate that the water fern *Azolla filiculoides* has genes for the production and sensing of jasmonic and salicylic acids, indicating that JA signalling can be a highly conservative mechanism (de Vries et al. 2018). More studies are needed to establish if JA is a universal molecule mediating anti-herbivore responses in plants, and if so, the threshold levels and response times required by different fern species. Future studies should test different concentrations of phytohormones and involve volatile collections at different time-frames, including several hours after phytohormone induction.

The high variability in the feeding amounts (and corresponding volatile emissions) in our herbivory treatments may be due to the Wellington tree wētā having different degrees of preference between fern species (which is currently under investigation). In spite of the high variability, it was possible to identify two compounds that were characteristic of tree wētā herbivory in all treatments; the green leaf volatile (*E*)-2-hexene and (*E*)-2-pentenol (a C5 compound of unknown biosynthetic origin), indicating that ferns might have unique responses to herbivory differing from those to physical damage. More studies are needed to explore other fern herbivores, understand their feeding modes, patterns, and preferences, and how these affect plant volatile emissions.

This work supports the notion that changes in volatile emissions are common defence responses in vascular plants (not only in seed plants) to physical damage and herbivory, and suggest that ferns might have unique responses to herbivore damage differing from those to physical damage alone. However, the role of phytohormone jasmonic acid in mediating these responses remains unclear, prompting further research to elucidate the signalling and regulation mechanisms behind responses to damage and herbivory in non-seed plants. Furthermore, the results suggest that some compounds of unknown biosynthetic origin can be essential components of fern-volatile emissions, so additional studies are needed to establish their biosynthetic origin and biological activity.

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## Supplementary material

Additional supporting information may be found in the supplementary material file for this article:

**Appendix S1.** Experimental set-up used for volatile collections.

**Appendix S2.** Average emissions ( $\pm$  SE) of volatile organic compounds from fronds of six fern species treated with the phytohormone jasmonic acid.

**Appendix S3.** Average emissions ( $\pm$  SE) of volatile organic compounds from fronds of six fern species exposed to physical damage.

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