# The effect of temperature on plant secondary metabolites and plant-insect interactions

Casey Hall BSc (Hons)

Environmental Futures Research Institute

School of Environment

Griffith University

Submitted in fulfilment of the requirements of the degree of

Doctor of Philosophy

December 2015

## Synopsis

Plant-herbivore-parasitoid systems include over half of all known species. These interactions are often chemically mediated and can be used to understand complex biotic communities. Increasing temperatures over the next century are predicted to disrupt plant-insect interactions. Existing studies have shown variable effects of increasing temperature on tri-trophic interactions, especially upon the higher trophic levels.

This thesis addresses the challenge of understanding how species interactions are affected by temperature by utilising a set of intimately interacting species: galling insects, their host plants and their parasitoid predators. I investigated a set of three co-occuring host plants *Solanum inaequilaterum*, *Rubus moorei* and *Rubus nebulosus*; their galling insects Cecidomyiidae sp. 1 and *Dasineura* sp. (Diptera); and their guild of nine species of parasitoids. I have quantified the interactions among these three trophic levels along an elevational gradient at five locations within eastern Australian subtropical rainforest. I use a combination of observational and experimental methods to determine the pathways through which temperature affects host plant chemistry, general herbivory, galling insects and their parasitoids.

Galling insects have the unique ability to actively manipulate plant secondary metabolites for their own benefit. We conducted a meta-analysis across 30 studies and found that the concentration of tannins and phenolics was significantly higher in galled compared to non-galled plant tissue. Host plant chemistry is expected to be important given the specificity of the interaction between the gall maker and host plant. As such, galling insects are ideal natural systems with which to study the relative effects of climate and plant defence chemicals in multitrophic interactions.

We found opposing trends in gall density with elevation between the two *Rubus* species, yet no clear trend in herbivory rates. However, we did find evidence for interguild competition between leaf chewers and galling insects, and also intraspecific competition among gallers leading to smaller adult body size. Galling insects may exhibit preferences for host plants with specific chemical profiles, leading to optimal plants with high densities of galling insects. We found differences in the chemistry between the two *Rubus* host species in response to galling and temperature. In particular, galled

*Rubus* leaves corresponded to triterpene signals in the $^{1}$H NMR spectra. While *Solanum* galled leaves corresponded to phenol amide signals.

One of the most notable results of our study was the increase in parasitoid species richness on more chemically diverse plants. We found that overall higher temperatures lead to increased phytochemical diversity. However, these results were observational in nature so to test this we experimentally warmed host plants using plastic enclosures along the elevational gradient. Experimentally warmed plants had reduced galling density, increased general herbivory, and increased phytochemical diversity. In contrast to our elevational gradient results, the experimentally induced increase in phytochemical diversity had a negative effect on parasitoids.

Through the combination of observational data and experimental manipulation we have shown that increased temperatures have significant bottom-up effects on food chains through changes in host plant chemistry. Such changes disproportionately affected parasitoids compared to their hosts, indicating that they are particularly vulnerable to changes in temperature. The results from this thesis have addressed a major gap in our understanding of the multiple and indirect effects of climate change on chemically-mediated mutlitrophic interactions.

## Statement of originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

_____

Casey Hall

## Table of Contents

## List of figures

## List of tables

## Journal articles arising from this thesis

Chapters 3, 4, 5, 6 and 7 in this thesis are co-authored manuscripts. My contribution to each co-authored paper is outlined at the front of the relevant chapter. Appropriate acknowledgements of those who contributed to the research but did not qualify as authors are included in each paper or in the *Acknowledgements* section of this thesis. Chapter 4 has been accepted for publication while Chapters 3, 5, 6 and 7 have been prepared for publication. The bibliographic details for these papers are:

Chapter 2:
Hall, C.R., Carroll, A.R. & Kitching, R.L. (prepared manuscript) The effect of insect galls on host plant chemical defence: a meta analysis.

Chapter 3:
Hall, C.R. & Kitching, R.L. (prepared manuscript) Interactions between galling insects and general herbivory along an elevational gradient in Australian subtropical rainforest.

Chapter 4:
Hall, C.R., Burwell, C.J. & Kitching, R.L. (2015) Changes in function and temporal variation in a guild of gall-parasitoids across a temperature gradient in Australian subtropical rainforest. *Austral Ecology*. doi:10.1111/aec.12283

Chapter 5:
Hall, C.R. & Carroll, A.R. (prepared manuscript) Gall induced changes in phytochemistry in three species of rainforest understory species across a temperature gradient.

Chapter 6:
Hall, C.R., Carroll, A.R. & Kitching, R.L. (prepared manuscript) Phytochemical diversity and gall-parasitoid interactions across a temperature gradient.

Chapter 7:
Hall, C.R., Carroll, A.R., Kitching, R.L. (prepared manuscript) The effect of experimental warming on phytochemistry and gall-parasitoid interactions in subtropical rainforest.

(Signed) _____ (Date) 12/12/2015

Casey Hall

(Countersigned) _____ (Date) 12/12/2015

Associate supervisor: Tony Carroll

(Countersigned) _____ (Date) 12/12/2015

Supervisor: Roger Kitching

## Acknowledgements

First and foremost, I thank my supervisors Professor Roger Kitching and Professor Tony Carroll for providing valuable advice, support and encouragement throughout my PhD. I am grateful to the Commonwealth Government of Australia for providing an Australian Postgraduate Award (APA) which funded this PhD program. I also thank the Griffith School of Environment, the Environmental Futures Research Institute and the Griffith University Higher Degrees by Research office for providing additional financial, administrative and academic development support. I also thank the Ecological Society of Australia for providing a grant to cover the experiment fieldwork costs.

I am grateful to the traditional custodians of the land on which this research was conducted: the Githabul people of Border Ranges national Park, NSW and the Mingunburri, Wangerriburri and Bundjalung groups of Lamington National Park, QLD. I thank the Queensland and New South Wales Governments for providing permits to work in Lamington and Border ranges National Parks.

This research could not have been achieved without the many volunteers that assisted me in the field. Particular thanks goes to Christina Kindermann, Emma Window, Sarah Maunsell, Timo Kellermann, Dennis Kimm, Luke Robertson, Kara Dalcin and Elliot Leech. I am also grateful to Bill McDonald for plant identifications, Chris Burwell and Peter Kolesik for insect identifications. I also thank Sarah Maunsell and Louise Ashton for establishing several of the Border Ranges sites and participants in the IBISCA Queensland project for establishing the Lamington sites, some of which were used in this study. I am grateful to the many people who have helped me with the chemistry component of this thesis, in particular Wendy Lou at Eskitis for helping me run NMR samples, Luke Robertson for teaching me how to run HPLC and solve NMR spectra and Leesa Habener and Joshua Hayton for helping me with lab equipment.

I am forever grateful to my parents for their unconditional support and encouragement throughout my higher education. I thank my cohort of PhD students, in particular Leesa Habener for her positivity and never-ending encouragement and Gary Palmer, who has been my housemate and good friend for the last three years. Finally, I thank Luke Robertson for his support and patience during the final writing up stage.

# Chapter 1

## Introduction and thesis outline

## 1.1 Introduction

*Effects of temperature on tri-trophic interactions*

Increasing temperatures over the next century are predicted to disrupt plant-insect interactions, potentially altering the structure and functioning of terrestrial ecosystems (Harrington et al. 1999; Tylianakis et al. 2008). Although there are an increasing number of studies on the effects of climate change on interacting species (Dyer et al. 2013; Gilman et al 2010), most climate change predictions are still focused on the distributions of individual species (Tylianakis et al. 2008). In order to make accurate predictions about ecosystem-level impacts, climate change research needs to incorporate trophic interactions. The overwhelming number and complexity of potential interactions makes this a challenging task (Harrington et al. 1999; Tylianakis et al. 2008; Woodward et al. 2010). One solution is to study community modules: small numbers of species (e.g. three to six) which are linked by a specific interaction structure, such as tri-trophic food chains (Holt 1997). These modules can be used, separately or in conjunction with other such modeules, to understand how species interactions will be affected by climate change (Gilman et al. 2010).

Existing studies have shown variable effects of increasing temperature on tri-trophic interactions, especially upon the higher trophic levels (Voigt et al. 2003). High temperatures have been shown to have direct negative effects on the survival, development and performance of parasitoids (Gillespie et al. 2012; Hance et al. 2007). In contrast, many studies predict increases in herbivore biomass and survival under warmer temperatures (Bale et al. 2002; Zvereva and Kozlov 2006). This potential mismatch between herbivore and parasitoid responses could result in herbivore outbreaks and consequent reduced plant biomass (de Sassi and Tylianakis 2012).

Increased temperature can also have significant effects on plant secondary metabolites (Bidart-Bouzat and Imeh-Nathaniel 2008). Changes in plant defence chemistry can have variable bottom-up effects on higher trophic levels (Kollberg et al. 2015; Dyer et al. 2013). Studies have shown both positive (Richards et al. 2015; Bukovinszky et al. 2008) and negative (Harvey et al. 2010; Ode 2006) effects of plant defence chemistry on parasitoids.

Despite the importance of phytochemistry in structuring plant-insect-parasitoid interactions, very few studies have included plant secondary metabolites when studying the effects of climate change on tri-trophic interactions.

*Effect of temperature on plant defence chemistry*

Plants have evolved an extensive array of secondary metabolites that are used in defence against herbivore and pathogen attack (Ehrlich and Raven 1964). These compounds are important mediators of plant-insect interactions. The effect of elevated temperature on the expression of plant secondary defence compounds seems to be specific to the type of compound and the plant species involved. The 'growth-differentiation balance' hypothesis states that plant defences are a result of a trade-off between growth and differentiation-related processes (Herms and Mattson 1992). Thus warming should decrease levels of carbon-based secondary compounds due to greater allocation to growth rather than defence, if resources are limited (Jamieson et al. 2012). A review by Bidart-Bouzat and Imeh-Nathaniel (2008) found carbon-based compounds including phenolics and condensed tannins, tend to decrease with increased temperatures. In contrast, hydrolyzable tannins, terpenes and volatile organic compounds have been shown to increase in concentration with warming (Hansen et al. 2006; Loreto et al. 2006; Sallas et al. 2003). Nitrogen containing compounds show variable responses to increased temperatures. Alkaloids may increase (Salminen et al. 2005), whereas glucosinolates may decrease or increase (Bidart-Bouzat and Imeh-Nathaniel 2008). The limited number of studies and variable responses among similar compounds, make it difficult predict how plant secondary defence compounds will respond to future climate warming.

Secondary metabolites are not only involved in defences against herbivores but also in generating tolerance to stressful abiotic conditions (Haugen et al. 2008). Siemens et al. (2009) suggest that evolution for tolerance of environmental stressors compromises the ability of a plant to produce chemical defences due to interference among plant hormone signalling pathways. This has important implications for how plants may adapt to climate change, as temperature-stressed plants may be more vulnerable to herbivores due to antagonistic interactions between stress hormone and chemical defence pathways (Siemens et al. 2012).

*Galling insects*

Plant galls involve active differentiation and growth of plant tissues in response to stimuli induced by the galling agent (Shorthouse and Rohfritsch 1992). The galling life-habit has arisen several times within the Insecta, including in the Thysanoptera, Hemiptera, Coleoptera, Diptera, Lepidoptera and Hymenoptera (Shorthouse and Rohfritsch 1992). Insect gallers are highly tissue and host-specific. Carneiro et al. (2009), for example, found 92% of recorded leaf-galling insect species in Brazil were monophagous, and only 2% induced galls on more than one genus of host plant.

Galling insects are ideal natural systems with which to study the relative effects of climate and plant defence chemicals in multitrophic interactions. Galling insects are especially vulnerable to environmental changes in temperature as their life cycles are intimately tied to the phenology of their host plant (Sumerford et al. 2000). Host plant chemistry is expected to be important given the specificity of the interaction between the gall maker and host plant (Abrahamson et al. 2003). Some galling insects can manipulate host plant chemistry, for example, high levels of tannins and other defensive chemicals are known to accumulate in the exterior of galls (discussed further in Chapter 2). In the past, oak galls were used for textile dyeing due to their high tannin content (Redfern 2011). Galled plants may be more susceptible to temperature stress due to trade-offs between secondary metabolites involved in stress tolerance and those induced by insect galls.

*Elevation and climate change research*

Elevation and other natural temperature gradients can be used as a space-for-time substitution for investigating how complex communities respond to changes in climate (Pickett 1989). Abiotic factors that change with elevation, however, are generally inter-correlated, making it hard to identify drivers of species distributions (Rahbek 2005). One solution is to use experimental manipulations, these, however, may be misleading as such short-term 'shocks' may not reflect the more gradual process of climate change (Barton 2011). Elevational gradients combined with experimental manipulation may overcome many of the limitations of using either method alone (Woodward et al. 2010, Dunne et al. 2004). In addition, using a

natural temperature gradient incorporates phenotypic plasticity and evolutionary responses of species to their local environment (Barton 2011).

## 1.2 Thesis outline

This thesis addresses the challenge of understanding how species interactions are affected by temperature by utilising a set of intimately interacting species: galling insects, their host plants and their parasitoid predators. I have quantified the interactions among these three trophic levels along an elevational gradient at five locations within eastern Australian subtropical rainforest. I use a combination of observational and experimental methods to determine the pathways through which temperature affects host plant chemistry, general herbivory, galling insects and their parasitoids (Figure 1.1, Table 1.1).

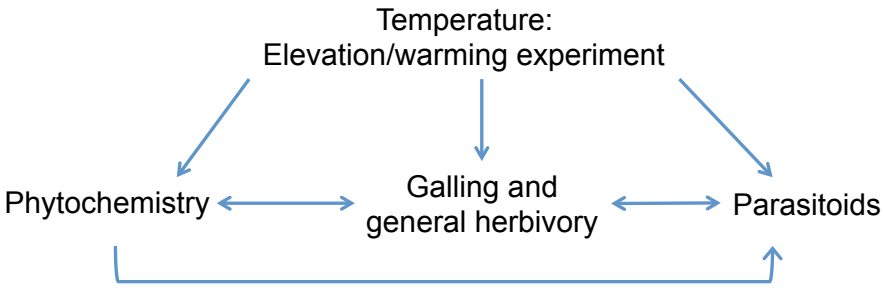



**Figure 1.1** A diagram of the pathways addressed in this thesis through which temperature can affect phytochemistry, herbivory and parasitoids.

Apart from Chapter 1 and Chapter 8, this thesis is organised as a series of published and unpublished manuscripts. Chapter 2 is a meta-analysis that summarises existing data examining the effects of galling by insects on concentrations of host plant defence chemistry. Chapter 3 focuses on how galling and general herbivory interact over a temperature gradient on two closely related host plant species. Chapter 4 examines how the species richness of gall parasitoids, parasitism rates and their relationships changes across a temperature gradient. Chapter 5 compares the gall-induced phytochemical changes in the three host plants across a temperature gradient. Chapter 6 uses path models to investigate how local temperature affects higher trophic levels through spatial differences in phytochemistry in two related host plant species. Chapter 7 describes a field-based warming experiment to assess the effect of

increased temperature on phytochemistry, herbivory and parasitoids on one host plant species along a natural temperature gradient. Chapter 8 concludes the thesis by providing a general discussion of the findings and directions for future research.

**Table 1.1** Data included in each results chapter of this thesis.

| | | Host plant species | Phyto-chemistry | Galling | Herbivory | Parasitoids |
|---|---|---|---|---|---|---|
| Chapter 2 | Meta analysis | 39 species | Level of defence | Presence/ absence | | |
| Chapter 3 | Elevation gradient | *Rubus* | | Density, body size | Rate (leaf area) | |
| Chapter 4 | | *Rubus, Solanum* | | | | Richness, parasitism |
| Chapter 5 | | *Rubus, Solanum* | $^{1}$H NMR, 2D NMR | Presence/ absence | | |
| Chapter 6 | | *Rubus* | $^{1}$H NMR, Diversity | Presence/ absence | Rate (leaf area) | Richness |
| Chapter 7 | Warming experiment | *Solanum* | $^{1}$H NMR, Diversity | Abundance | | Density |

The results chapters are in the form of manuscripts prepared for submission to peer reviewed journals in accordance with Griffith University policy on PhD theses as published and unpublished papers (Griffith University 2015). Relevant supplementary material is provided following each chapter. To avoid duplication a combined reference list is provided at the end of the thesis.

# Chapter 2

In this chapter we use meta-analytic techniques to summarise the effect that galling insects have on concentrations of host plant chemical defences across a range of plant species and habitat types.

**Statement of contribution to a co-authored paper**

Chapter 2 is a co-authored paper that has been prepared for publication. The citation is as follows:

Hall, C.R., Carroll, A.R. & Kitching, R.L. (prepared manuscript) The effect of insect galls on host plant chemical defence: a meta-analysis.

My contribution to the paper involved data collection, analysis and writing of the manuscript. My co-authors are my supervisors and were responsible for direction and editing of the manuscript. A. Carroll also collated and analysed host plant secondary metabolite information.

(Signed) _____ (Date) 12/12/2015
Casey Hall

(Countersigned) _____ (Date) 12/12/2015
Associate supervisor: Tony Carroll

(Countersigned) _____ (Date) 12/12/2015
Supervisor: Roger Kitching

# Chapter 2

## The effect of insect galls on host plant chemical defence: a meta-analysis

Casey R. Hall[1*], Anthony R. Carroll[1] and Roger L. Kitching[1]

[1]Environmental Futures Research Institute (EFRI), Griffith School of Environment, Griffith University, 170 Kessels Road 4111

*Corresponding author: casey.hall@griffithuni.edu.au

## 2.1 Abstract

The idea that galling insects actively manipulate host plant chemistry for their own benefit has been previously documented but has not been quantified across a range of galling and host plant taxa. We present the first meta-analysis that assesses quantitatively the effect of insect galling on levels of chemical defences in host plants across 39 gall-host plant species combinations. We use these data to investigate whether the ability to manipulate host plant chemistry is dependent on the galling insect taxa, the type of chemical and the climatic zone of the study location. Our results show that galling insects are able to induce increases in levels of phenolics and tannins in host plant tissue but appear to have little to no effect on volatiles. Hymenopteran galls increased host plant defence chemistry significantly, while Diptera and Hemiptera also increased levels but not significantly, whereas Lepidoptera galls had no effect. Galls from tropical forest, boreal forest and Mediterranean climatic zones significantly increased host-plant defence chemistry, whereas those from temperate forests showed no change. Galling insects exert considerable control over host plant defence chemistry for their own benefit by either increasing constitutive defences or minimising volatiles, although this is moderated by the type of chemical defence and insect taxa involved.

## 2.2 Introduction

Plants have evolved an impressive array of secondary metabolites which are used in defence against herbivore and pathogen attack (Ehrlich and Raven 1964, Feeny 1976, Fürstenberg-Hägg et al. 2013). These antifeedant compounds can directly, through toxic effects from ingestion, or indirectly, through attraction of natural enemies, affect herbivorous insects (War et al. 2012). Of all herbivores, insect gallers are one of the few guilds that are able to control both the physical and chemical aspects of their host plants; this is not surprising given the intimate nature of the relationship (Cornell 1983, Hartley 1998). Several studies have shown that gall tissue is often higher in phenolics and tannins compared to normal plant tissue (Taper and Case 1987, Abrahamson et al. 1991). It has been proposed that the accumulation of these compounds in gall tissue protects the galling insect against predators, other herbivores and pathogens (Cornell 1983; Hartley and Lawton 1992). In addition to direct chemical defences, galling insects can influence indirect plant defences, such as the emission of volatiles in response to herbivory (Turlings et al. 1990; Tooker and De Moraes 2007). For example, Tooker et al. (2008) found that galled *Solidago altissima* plants emitted significantly fewer volatiles compared to non-galled plants in response to subsequent herbivory. As parasitoids and other natural enemies use volatile cues to locate prey, the suppression of these chemicals is expected to benefit the gall maker (Turlings et al. 1990; Tooker et al. 2008).

Galling is clearly a successful strategy for providing organisms with both nutrition and protection as it has evolved multiple times in at least seven different insect orders (Roskam 1992) as well as in mites, nematodes, fungi and bacteria (Redfern 2011). With over 6000 species, the Cecidomyiidae (Diptera) is by far the most speciose group of galling insects, and occurs across a wide range of host plant taxa (Carneiro et al. 2009; Gagné and Jaschhof 2014). It is has been suggested that the galling habit in cecidomyiids resulted from a shift from precursor mycetophages that already possessed some prerequisites (such as extra-intestinal digestion) for the induction of galls (Roskam 1992). Once the galling habit was established, it radiated along with the host plants, with evidence of direct co-speciation in some cases (Roskam 1985). Galls produced by hymenopteran cynipids are structurally complex, and occur on very restricted groups of host plants. For example, approximately 87% of all North American cynipid galls occur exclusively on oak trees (Fagaceae: *Quercus sp.*) (Abrahamson et al. 2003). It is thought that they were able to radiate into enemy-free

space (in the sense of Atsatt 1981) after evolving mechanisms to cope with the high levels of tannins in oaks, which deter many other non-galling herbivores (Feeny 1970; Taper and Case 1987; Schultz 1988; Roskam 1992).

In general, the type and level of chemical defence differs greatly among plant species and in relation to abiotic factors (Endara and Coley 2011). Coley et al. (1985) argued that slower growing species would invest in more chemical defences than fast growing species; the so-called resource availability hypothesis (RAH). Thus in harsh climates and less fertile habitats, plants would be expected to be slow growing and produce longer-lived and more chemically defended leaves. Concurrently, Price et al. (1987) proposed the harsh environment hypothesis (HEH) to explain high galling diversity in Mediterranean-habitats, *cerrado* and rainforest canopies. These habitats share a common factor: longer-lived leaves under high amounts of hygrothermal stress (Julião et al. 2014). According to the RAH, plants in these habitats should be better defended chemically. The galling niche may have diversified in these habitats as a way to circumvent and subsequently utilise the plant's own chemical defences.

However, linking these two hypotheses leaves several key questions unanswered. Is the ability to manipulate host plant chemistry a universal trait among galling insects, or is it dependent on the insect taxa? In addition, is this ability restricted to certain classes of chemical compounds? Finally, is the influence of galling insects on host plant chemistry related to habitat type, i.e. greater influence in harsh and thus more chemically defended habitats? The idea that galling insects actively manipulate host plant defence chemistry has been previously documented but has not been quantified across a range of galling taxa and host plant habitats. To address these and related questions we have used meta-analytical techniques to assess the effect of galling on host plant chemistry in relation to: (1) the type of chemical (phenolics, tannins or volatiles); (2) galling insect taxa, (Hymenoptera, Diptera, Hemiptera or Lepidoptera); and (3) climatic zone (Mediterranean, tropical, temperate forest or boreal forest).

## 2.3 Methods

We carried out an extensive literature search in the ISI Web of Knowledge database from 1950 to 2015 using the following keywords: insect and gall* and (plant or parasit* or herbivor* or host or leaf) and (*chemi* or defen?e) and (resistance or metabolit* or secondary or induced or VOC* or volatile* or phenolic* or tannin* or terpen* or glucosin*)

in the topic field. We only considered further studies which quantified concentrations of plant chemical defences in relation to insect gall abundance, presence/absence or between galled and non-galled plant tissue.

When two different host plant species and their galling insects were analysed in the same paper, each galling and host plant species interaction was considered an independent record (cf Gurevitch and Hedges 1999; Aguilar et al. 2006). In addition, when more than one compound type was analysed, each was recorded separately. However, when an interaction was studied repeatedly over time, we used only the data for the first season and from mature leaves.

*Data analysis*

Mean and standard deviations of concentrations of defence chemicals in galled and ungalled plant tissue were extracted from tables and graphs of the selected studies. Some studies only provided Student's t, Fisher's F and correlation coefficients, these were used in further calculations. In some studies, mean and standard deviations were extracted from digitized graphs using WebPlotDigitizer v. 3.8 (Rohatgi 2015). To estimate effect sizes, we calculated Hedges' *d* (an unbiased standardized mean difference; Hedges and Olkin 1985; Gurevitch and Hedges 1999) from the original data using appropriate data transformations (see Supplemental S1 for the list of transformations used). The analyses were conducted using R statistical software (ver. 3.1.2; R Development Core Team 2014), using the *metafor* package (Viechtbauer 2010).

Mean effect sizes were calculated using random effects models, which assume that studies differ not only in sampling error (as fixed-effects models do) but also by a random component in effect sizes (Gurevitch and Hedges 1999). Random effects models are preferable in ecological data syntheses because their assumptions are more likely to be satisfied (Gurevitch and Hedges 1999). An effect was considered significant if the 95% confidence intervals (CI) of the effect size did not overlap zero. Positive effect sizes indicated that galling increased levels of defence chemicals in host plants. We analysed whether the following moderator (or predictor) variables influenced effect sizes: chemical type (phenolic, tannin or volatile), galling insect order (Hymenoptera, Hemiptera, Diptera or Lepidoptera) and climatic zone (Mediterranean, tropical, temperate forest and boreal forest). While tannins are a type of phenolic compound, we chose to examine them separately as the selected

studies generally measured tannins in terms of protein binding capacity and phenolics as total reducing capacity (by phenolic antioxidants) (Mole and Waterman 1987). While these methods are non-specific (i.e. any reducing agent is included in the phenolics analysis), different types of phenolic compounds are estimated based on the reference standard used (e.g. phenolic acids, anthocyanin, catechin, flavonoid glycosides) (Martin and Martin 1982). From an ecological perspective it makes sense to investigate tannins and phenolics separately as each has different effects, e.g. many tannins, especially larger molecular weight compounds, act as antifeedants by decreasing protein absorption efficiency while some phenolics exhibit direct toxicity against herbivores (War et al. 2012). Volatiles can show large variation in chemical classes, however the identified volatiles in our analysis were mainly terpenes.

To examine the heterogeneity of effect sizes, we used Q statistics, which are weighted sums of squares that follow an asymptotic chi-square distribution. We examined the *P*-values associated with $Q_{between}$ categories, which describe the variation in effect sizes that can be attributed to differences among the categories (Cooper et al. 2009).

*Publication bias*

An intrinsic problem when conducting quantitative reviews of published studies is the potential of publication bias; i.e. studies showing significant results having a greater possibility of publication than those showing non-significant results. We explored potential publication bias using both funnel plots, and by calculating a weighted fail-safe number (Rosenberg 2005). The funnel plot is a scatter plot of effect size vs. sample size, if no publication bias exists, the resulting plot is shaped like a funnel with the large 'opening' at the smallest sample sizes; i.e. the variation around the cumulative effect size should decrease as sample size increases (Palmer 1999). We used the fail-safe number calculator (Rosenberg 2005; http://www.rosenberglab.net/software.html#FailSafe) to estimate the number of non-significant, unpublished or missing studies that would need to be added to a meta-analysis to nullify its overall effect size. If the fail-safe number is larger than 5n+10, where n is the number of studies, then the results are robust regardless of any potential publication bias (Rosenberg 2005).

## 2.4 Results

We found 30 papers, published from 1982 to 2013, which evaluated changes in plant defence chemistry in relation to insect galling (S2). These studies yielded 60 data points from 39 unique gall-host plant interactions (Table S2.1). A summary of the number of gall species (and host plants) within each of the categories is summarised in Figure 2.1. Most gall species were related to either phenolics or tannins, whereas volatiles were measured in only 10 species (Figure 2.1a). The majority of galling species belonged to the Hymenoptera and Diptera, with both the Hemiptera and Lepidoptera accounting for less than 10 species together (Figure 2.1b). Tropical climatic zones contributed the most galling species, although numbers were fairly evenly spread among climate types (Figure 2.1c).

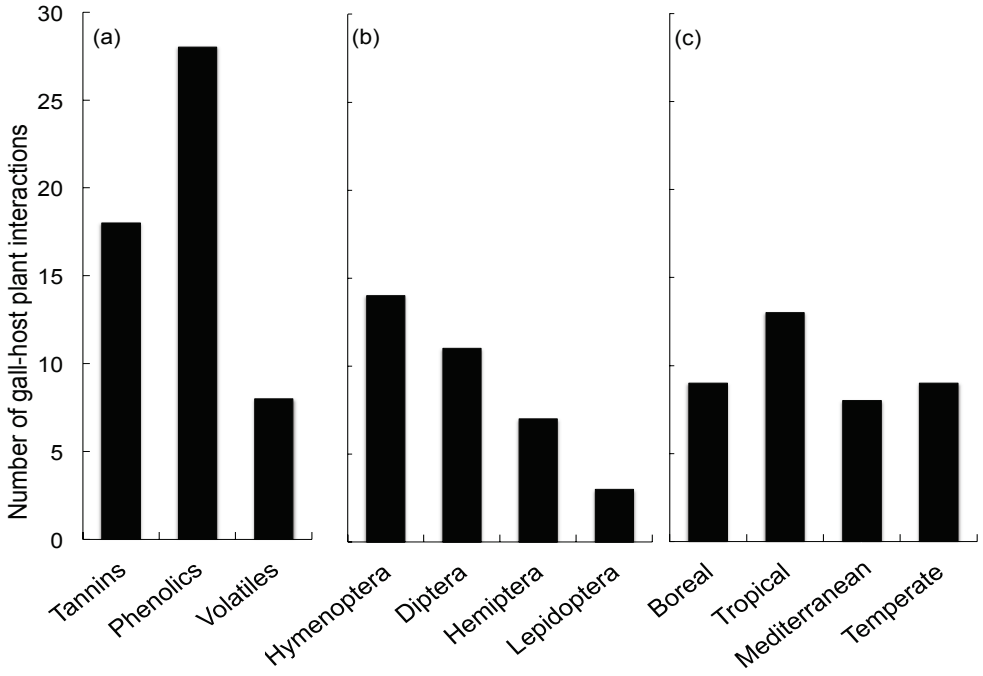



**Figure 2.1** Summary of the number of pair-wise galling-host plant species examined in the meta-analysis within each category: (a) the type of chemical defence; (b) the orders that each of the galling insects belong to and (c) the climatic zones examined.

However, the numbers of plant families galled by each order in each habitat show that tropical climates have the highest number of galled plant families, followed by temperate (Figure 2.2). This is driven largely by dipteran gall makers, which account for most of the tropical galls, but are absent from boreal climatic zones. The Hemiptera and Hymenoptera are more evenly distributed across climatic zones, although the latter are absent from tropical climates. In contrast to the high diversity of plant families, when looking at the main class of defence compound for each host plant species, most of the studied gall-plant interactions

occur on host plants dominated by flavonoid defences (Figure 2.3). This pattern is driven largely by the Hymenoptera, which gall plants from three chemical classes. In contrast, dipteran galls occur across a range of chemically different host plants (Figure 2.3).

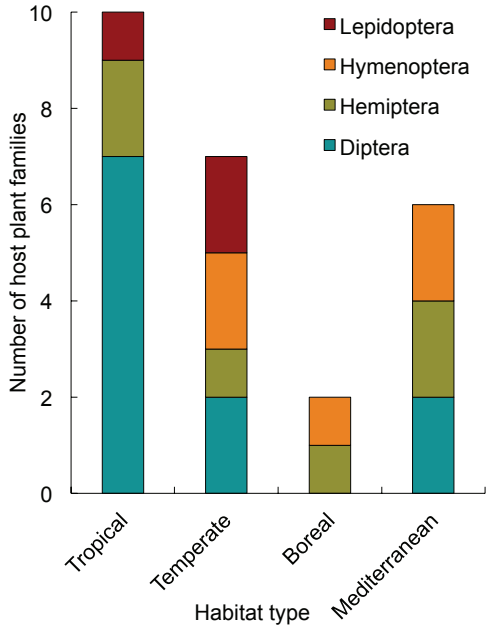



**Figure 2.2** The number of plant families galled in each habitat type (see Table S2.1 for plant families). Colours represent the four orders of galling insect included in the analysis.

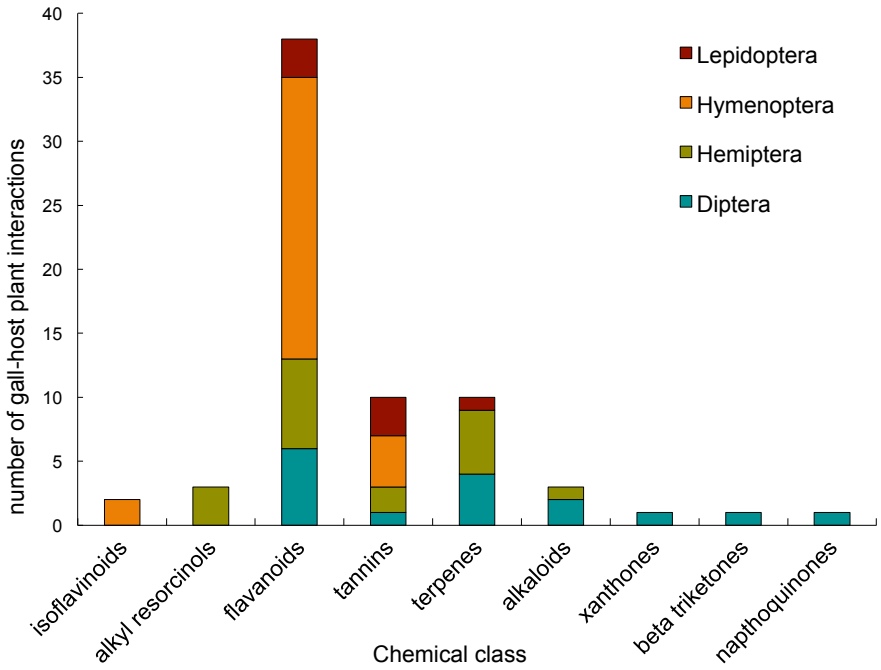



**Figure 2.3** The number of gall-plant interactions in each of the major secondary metabolite classes from each host plant species (see Table S2.1 for chemical classes).

The overall mean effect size of galling on concentrations of host plant secondary chemistry was positive (d = 0.97) and significantly different from zero (Figure 2.4a, Figure S2.1). The overall heterogeneity of effect sizes was large and significant ($Q_{total}$ = 541.26, n = 60, $P$ < 0.001), indicating that the positive effect of galling on plant secondary chemistry differed among the galling species and chemical classes. We then evaluated categorical variables in order to explain the observed heterogeneity.

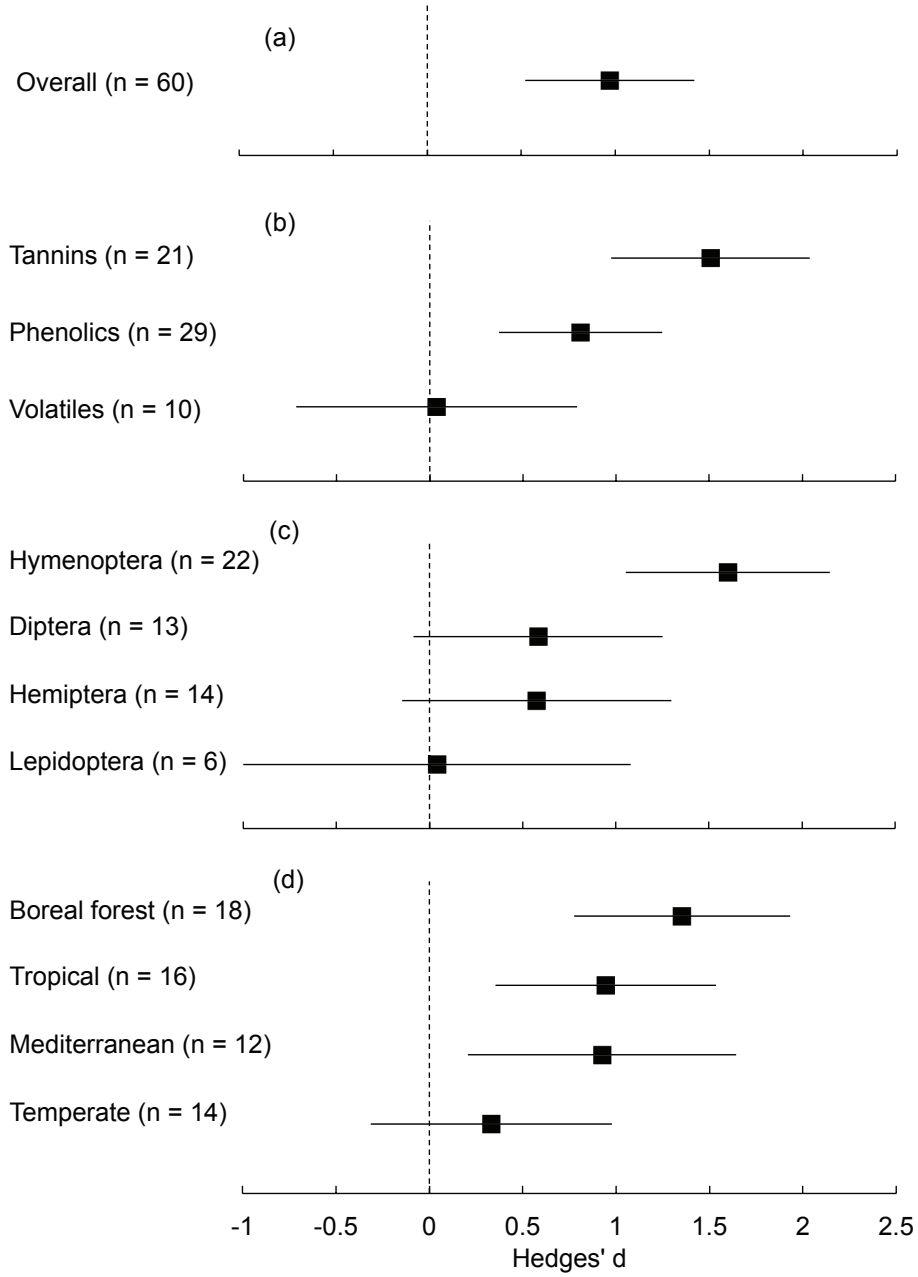



**Figure 2.4** Mean effect sizes (d) and 95% confidence intervals for the effect of insect galling on the concentration of secondary metabolites (a) overall (d = 0.97, $Q_{total}$ = 541.26, $P$ < 0.001). Categorical variables include: (b) tannins, phenolics and volatiles ($Q_{between}$ = 10.16, $P$ = 0.006); (c) Hymenoptera, Diptera, Hemiptera and Lepidoptera ($Q_{between}$ = 10.56, $P$ = 0.01); and (d) climatic zones: boreal forest, tropical, Mediterranean and temperate.

Among the categorical variables, the chemical class explained the highest proportion of variation among galling species ($Q_{between}$ = 10.16, $P$ = 0.006). Concentrations of both tannins and phenolics increased significantly in response to galling, whereas the effect on the concentration of volatile compounds was not significant (Figure 2.4b). When galling species were categorized by insect Order, there were also significant differences in their mean effect sizes ($Q_{between}$ = 10.56, $P$ = 0.01). Hymenoptera significantly increased levels of secondary chemistry in host plants: Diptera and Hemiptera also increased levels but the effect was not significant (Figure 2.4c). In contrast, lepidopteran galls had no effect on plant secondary chemistry (Figure 2.4c). The heterogeneity of effect sizes of galls from different climatic zones was not significant ($Q_{between}$ = 5.34, $P$ = 0.15). All habitat types showed positive mean effect sizes, although for temperate climates this positive effect was not significantly different from zero (Figure 2.4d).

Funnel plots of effect size vs. sample size and sample variance showed no evidence of significant publication bias (Figure 2.5). A Spearman rank correlation test between Hedges' *d* and effect sizes was non-significant (r = 0.018, df = 59, $P$ = 0.89) and sample variance (r = -0.19, df = 59, $P$ = 0.15). In addition, the calculated Rosenberg fail-safe number was much larger than the critical value (2377 > 310) supporting the robustness of our results.

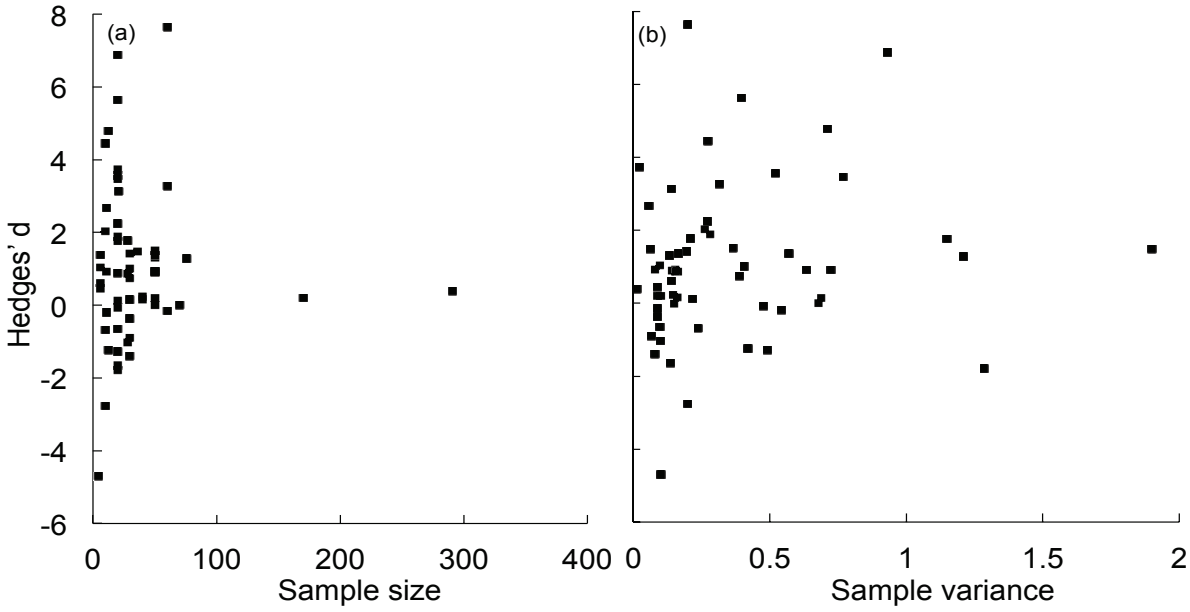



**Figure 2.5** Funnel plot of: (a) sample size vs. effect size (Hedges' d) values (r = 0.018, df = 59, $P$ = 0.89) and (b) sample variance vs. effect size (r = -0.19, df = 59, $P$ = 0.15), based on 60 data points from 39 gall-host plant interactions.

## 2.5 Discussion

This is the first study to assess quantitatively the effect of galling on plant defence chemistry based on empirical evidence across different chemical groups from a wide range of species and climatic zones. Our results suggest that galling insects increase concentrations of defence chemicals in their host plants. Effect sizes differed significantly depending on the chemical class and Order of the galling insects examined. In contrast, climatic zone did not explain significant differences in effect sizes.

We found that levels of both tannins and phenolics significantly increased in galled plants, whereas volatiles show no change. This may be due to the differing roles of these chemicals in plant defence. For example, tannins and phenolics are usually found in higher concentrations and act directly against chewing herbivores, whereas volatiles are usually an induced response to herbivore feeding which may be attractive to the herbivores' natural enemies (Turlings et al. 1990). Galling insects exert considerable control over their host plants and might be expected to minimise any defensive responses in the host-plants that render them more vulnerable to natural enemies (Tooker et al. 2008). By increasing levels of constitutive defence chemicals in some host plant species and supressing volatile emissions in others, galling insects may be able to not only reducing their risk of predation due to herbivory, but also reduce their risk of parasitism. While McKinnon et al. (1999) showed that galling aphids can manipulate both volatiles and phenolics simultaneously; it is unclear if this is widespread across galling taxa.

Across the examined insect Orders, Hymenoptera had the largest positive effect on concentrations of plant chemical defences. This result, however, should be interpreted with caution as just over half of the Hymenopteran data points come from a single study (Nyman and Julkunen-Tiitto 2000), with mostly positive effect sizes. With this in mind, Hymenopteran galls do have some characteristics that may explain the positive result. They are highly evolved with the ability to induce diverse and complex galls, but are generally restricted to just a few genera of host plants (Ronquist et al. 2015). In contrast, Cecidomyiidae (Diptera) and Hemiptera (principally psyllids and various Sternorryncha) induce galls across a wide range of unrelated plant taxa (Roskam 1992; Carneiro et al. 2009; Butterill and Novotny 2015). For example, in the results reviewed in this paper, 14 species of Hymenoptera induced galls across just four plant genera, while eleven Diptera and seven

Hemiptera species induced galls on eleven and seven plant genera, respectively. This may be why both Orders show more variation in the degree to which they increase host plant defence chemistry, as host plant genera vary widely with regard to their defence strategies and may use different compounds to the ones studied. Lepidopteran galls appear to produce no change in defence chemistry, although our sample size is small and shows large variation. This may reflect the rarity of the galling habit within the Lepidoptera compared with the major galling families found within the Diptera and Hymenoptera (Roskam 1992). In addition, lepidopteran herbivores commonly use host plant alkaloids, which were not included in this review due to a lack of data (Boppré 1990).

According to the RAH, slow growing plants contain greater amounts of defence chemicals, perhaps explaining why we found the greatest gall-induced increase in chemical defence in data from boreal climates. In addition, a meta-analysis by Moles et al. (2011) found higher concentrations of plant resins and oils at higher latitudes. On the other hand, the same study found no latitudinal pattern in phenolics or tannins, although we note most of the studies were restricted to temperate latitudes. This may explain why we found that only galls from temperate climates showed no significant increase in defence chemicals. Galls from tropical forest and Mediterranean-like climatic zones also showed increases in defence chemistry. Both of these climatic zones also have long lived, and thus more chemically defended leaves (Julião et al. 2014). This supports the idea that the galling niche may have diversified in these climates as a way to both avoid and utilise the plant's own chemical defences.

*Conclusions*

Meta-analyses offer several advantages, namely that different studies are more readily comparable due to the use of standardised effect sizes, and we can investigate causes of heterogeneity among effect sizes (Palmer 1999). However, by their very nature meta-analyses are limited by publication bias and sample size. Although we found no evidence of publication bias, some categories had much smaller sample sizes than would have been ideal, thus limiting the statistical power in the analyses of their effects. Furthermore, the vast majority of the studies included in the meta-analysis are from the Americas and Europe. While this reflects a lack of studies in other continents, it is not clear whether the same patterns would have been found in Asia, Africa and Australia where the galling and plant species differ considerably from those in North America and Europe. Nevertheless, we show

strong effects from the available studies, which should provide stimulus for further work, especially in less studied regions of the world.

Galling insects, by either increasing constitutive defences or minimising volatiles, exert considerable control over host plant defence chemistry for their own benefit, although this is moderated by the type of chemical defence and insect taxa involved. However, only small subset of defensive chemicals have been thus far investigated, for example only one study looked at alkaloids, which constitutes some of the highest diversity of plant secondary compounds (Fürstenberg-Hägg et al. 2013). Future studies should include compounds from these neglected chemical classes in order to find out if our conclusions are more generally applicable across a range of secondary compounds. Also, any changes to host plant chemical defences would be expected to influence community-level dynamics associated with galled plants. Future studies should examine gall-induced chemical changes in a wider ecological context by incorporating other interacting species across a wider range of habitats. In addition, experimental manipulations that induce changes in plant chemistry (e.g. through manipulation of soil fertility or temperature) could be used to empirically investigate the role of defence chemistry in host plant selection by galling insects.

## S2 Supplementary material

**S1** Transformations used to calculate effect sizes for different statistics provided in the sampled studies:

Group means to Cohen's d:

$$d = \frac{\acute{X}_1 - \acute{X}_2}{SD_{within}}$$

where $X_1$ and $X_2$= group means

and $SD_{within}$ = the estimated common standard deviation of the two groups:

$$SD_{within} = \sqrt{\frac{(n_1 - 1)SD_1^2 + (n_2 - 1)SD_2^2}{n_1 + n_2 - 2}}$$

where $n_1$ and $n_2$= the sample sizes for groups $X_1$ and $X_2$ respectively and

$SD_1$ and $SD_2$= standard deviations for groups $X_1$ and $X_2$ respectively.

Student's t and Fisher's F to Cohen's d:

$$d = \frac{2t}{\sqrt{df_{error}}}$$

r to Cohen's d:

$$d = \frac{2r}{\sqrt{1 - r^2}}$$

The variance of d (converted from r) is:

$$V = \frac{4V}{(1 - r^2)^3}$$

Cohen's d to Hedge's g:

$$g = \frac{d}{\sqrt{\frac{n_1 + n_2}{df}}}$$

Hedge's g to Hedge's d:

$$d = g\left(1 - \frac{3}{4(n_1 + n_2 - 2) - 1}\right)$$

**S2** List of studies included in the meta-analysis:


[1] Abrahamson, W. G., McCrea, K. D., Whitwell, A. J., Vernieri, L. a. (1991) The role of phenolics in goldenrod ball gall resistance and formation. *Biochem. Syst. Ecol.* **19**, 615–622.

[2] Allison, S. D., Schultz, J. C. (2005) Biochemical responses of chestnut oak to a galling cynipid. *J. Chem. Ecol.* **31**, 151–166.

[3] Bailey, J. K., Wooley, S. C., Lindroth, R. L., Whitham, T. G. (2006) Importance of species interactions to community heritability: a genetic basis to trophic-level interactions. *Ecol. Lett.* **9**, 78–85.

[4] Cornelissen, T. G., Fernandes, G. W. (2001) Defence, growth and nutrient allocation in the tropical shrub *Bauhinia brevipes* (Leguminosae). *Austral Ecol.* **26**, 246–253.

[5] Cornelissen, T., Fernandes, G. W., Coelho, M. S. (2011) Induced responses in the neotropical shrub *Bauhinia brevipes* Vogel: does early season herbivory function as cue to plant resistance? *Arthropod Plant Interact.* **5**, 245–253.

[6] Coruh, S., Ercisli, S. (2010) Interactions between galling insects and plant total phenolic contents in *Rosa canina* L . genotypes. *Sci. Res. Essays*. **5**, 1935–1937.

[7] Damasceno, F. C., Nicolli, K. P., Caramão, E. B., Soares, G. L. G., Zini, C. A. (2010) Changes in the volatile organic profile of *Schinus polygamus* (Anacardiaceae) and *Baccharis spicata* (Asteraceae) induced by galling psyllids. *J. Brazillian Chem. Soc.* **21**, 556–563.

[8] Detoni, M. D. L., Vasconcelos, E. G., Rust, N. M., Isaias, R. M. D. S., Soares, G. L. G. (2011) Seasonal variation of phenolic content in galled and non-galled tissues of *Calliandra brevipes* Benth (Fabaceae: Mimosoidae). *Acta Bot. Brasilica*. **25**, 601–604.

[9] Formiga, A. T., Gonçalves, S. J. de M. R., Soares, G. L. G., Isaias, R. M. D. S. (2009) Relações entre o teor de fenóis totais e o ciclo das galhas de Cecidomyiidae em *Aspidosperma spruceanum* Müll. Arg. (Apocynaceae). *Acta Bot. Brasilica*. **23**, 93–99.

[10] Foss, L. K., Rieske, L. K. (2004) Stem galls affect oak foliage with potential consequences for herbivory. *Ecol. Entomol.* **29**, 273–280.

[11] Frost, C. J., Dean, J. M., Smyers, E. C., Mescher, M. C., Carlson, J. E., De Moraes, C. M., Tooker, J. F. (2012) A petiole-galling insect herbivore decelerates leaf lamina litter decomposition rates. *Funct. Ecol.* **26**, 628–636.

[12] Furlan, C. M., Salatino, A. and Domingos, M. (2004) Influence of air pollution on leaf chemistry, herbivore feeding and gall frequency on Tibouchina pulchra leaves in Cubatao (Brazil). *Biochem. Syst. Ecol*. **32**, 253-263.

[13] Gonçalves-Alvim, S. J., Collevatti, R. G., Fernandes, G. W. (2004) Effects of genetic variability and habitat of *Qualea parviflora* (Vochysiaceae) on herbivory by free-feeding and gall-forming insects. *Ann. Bot.* **94**, 259–68.

[14] Gopichandran, R., Peter, A. J. and Subramaniam, V. R. (1992) Age-correlated biochemical profiles of thrips galls in relation to population density of thrips. *J. Nat. Hist*. **26**, 609-619.

[15] Guimarães, A. L. A., Bizarri, C. H. B., Barbosa, L. S., Nakamura, M. J., Ramos, M. F. S., Vieira, A. C. M. (2013) Characterization of the effects of leaf galls of *Clusiamyia nitida* (Cecidomyiidae) on *Clusia lanceolata* Cambess. (Clusiaceae): anatomical aspects and chemical analysis of essential oil. *Flora.* **208**, 165–173.

[16] Hjältén, J., Niemi, L., Wennström, A., Ericson, L., Roininen, H., Julkunen-tiitto, R. (2007) Variable responses of natural enemies to *Salix triandra* phenotypes with different secondary chemistry. *Oikos*. **116**, 751–758.

[17] Kolehmainen, J., Roininen, H., Julkunen-tiitto, R., Tahvanainen, J. (1994) Importance of phenolic glucosides in host selection of shoot galling sawfly, *Euura amerinae*, on Salix pentandra. *J. Chem. Ecol.* **20**, 2455–2466.
[18] McKinnon, M. L., Quiring, D. T., Bauce, E. (1999) Influence of tree growth rate, shoot size and foliar chemistry on the abundance and performance of a galling adelgid. *Funct. Ecol.* **13**, 859–867.
[19] Motta, L. B., Kraus, J. E., Salatino, A., Salatino, M. L. F. (2005) Distribution of metabolites in galled and non-galled foliar tissues of *Tibouchina pulchra*. *Biochem. Syst. Ecol.* **33**, 971–981.
[20] Nyman, T., Julkunen-Tiitto, R. (2000) Manipulation of the phenolic chemistry of willows by gall-inducing sawflies. *Proc. Natl. Acad. Sci.* **97**, 13184–13187.
[21] Pascual-alvarado, E., Cuevas-reyes, P., Quesada, M., Oyama, K. (2008) Interactions between galling insects and leaf-feeding insects: the role of plant phenolic compounds and their possible interference with herbivores. *J. Trop. Ecol.* **24**, 329–336.
[22] Rehill, B. J., Schultz, J. C. (2012) *Hormaphis hamamelidis* fundatrices benefit by manipulating phenolic metabolism of their host. *J. Chem. Ecol.* **38**, 496–498.
[23] Rostás, M., Maag, D., Ikegami, M., Inbar, M. (2013) Gall volatiles defend aphids against a browsing mammal. *BMC Evol. Biol.* **13**, 193.
[24] Soetens, P., Rowell-Rahier, M., Pasteels, J. (1991) Influence of phenolglucosides and trichome density on the distribution of insect herbivores on willows. *Entomol. Exp. Appl.* **59**, 175–187.
[25] Sugiura, S., Yamazaki, K., Osono, T. (2006) Consequences of gall tissues as a food resource for a tortricid moth attacking cecidomyiid galls. *Can. Entomol.* **138**, 390–398.
[26] Taper, M., Case, T. (1987) Interactions between oak tannins and parasite community structure: unexpected benefits of tannins to cynipid gall-wasps. *Oecologia*. **71**, 254–261.
[27] Tooker, J. F., Rohr, J. R., Abrahamson, W. G. and De Moraes, C. M. (2008) Gall insects can avoid and alter indirect plant defences. *New Phytol*. **178,** 657-671.
[28] Torres-Gurrola, G., Delgado-Lamas, G. and Espinosa-Garcia, F. (2011) The foliar chemical profile of criollo avocado, *Persea Americana* var. *drymifolia* (Lauraceae), and its relationship with the incidence of a gall-forming insect, *Trioza anceps* (Triozidae). *Biochem. Sytem. Ecol.* **39**, 102-111.
[29] Valladares, G. R., Zapata, A., Zygaldo, J., Banchio, E. (2002) Phytochemical induction by herbivores could affect quality of essential oils from aromatic plants. *J. Agric. Food Chem.* **50**, 4059–4061.
[30] Zucker, W. V. (1982) How aphids choose leaves: the roles of phenolics in host selection by a galling aphid. *Ecology*. **63**, 972–981.

**Table S2.1** References, categories and effect sizes extracted from articles analysing effects of insect galling on host plant defence chemistry

| Study | Chemical Class | Gall species | Insect Order | Host plant species | Host Plant family | Main secondary chemistry class | Climatic zone | N | Effect size d |
|---|---|---|---|---|---|---|---|---|---|
| [1] | Phenolics | *Cecidomyiidae* | Diptera | *Ruprechtia fusca* | Polygoniaceae | flavanoids | Tropical forest | 50 | 0.9 |
| [2] | Phenolics | *Andricus petiolicolus* | Hymenoptera | *Quercus prinus* | Fagaceae | flavanoids, tannins | Temperate forest | 28 | 0.87 |
| [2] | Tannins | *Andricus petiolicolus* | Hymenoptera | *Quercus prinus* | Fagaceae | flavanoids, tannins | Temperate forest | 28 | 1.78 |
| [3] | Tannins | *Adelges abietis* | Hemiptera | *Picea glauca* | Pinaceae | terpenes | Boreal forest | 30 | 1.42 |
| [3] | Volatiles | *Adelges abietis* | Hemiptera | *Picea glauca* | Pinaceae | terpenes | Boreal forest | 30 | -0.9 |
| [4] | Phenolics | *Asteralobia soyogo* | Diptera | *Ilex pedunculosa* | Aquifoliaceae | | Temperate forest | 12 | -1.24 |
| [5] | Phenolics | *Eurosta solidaginis* | Diptera | *Solidago altissima* | Asteraceae | terpenes, flavanoids | Temperate forest | 20 | 1.89 |
| [6] | Phenolics | *Diplolepis sp.* | Hymenoptera | *Rosa canina* | Rosaceae | flavanoids, tannins | Mediterranean | 20 | -0.65 |
| [7] | Tannins | *Slavum wertheimae* | Hemiptera | *Pistacia atlantica* | Anacardiaceae | alkyl resorcinols | Mediterranean | 12 | 4.79 |
| [7] | Volatiles | *Psyllidae sp.* | Hemiptera | *Schinus polygamus* | Anacardiaceae | alkyl resorcinols | Mediterranean | 10 | 4.45 |
| [7] | Tannins | *Pemphigus betae* | Hemiptera | *Populus angustifolia* | Salicaceae | flavanoids | Temperate forest | 11 | 0.93 |
| [7] | Phenolics | *Pemphigus betae* | Hemiptera | *Populus angustifolia* | Salicaceae | flavanoids | Temperate forest | 4 | -4.69 |
| [8] | Phenolics | *Tanaostigmodes sp.* | Hymenoptera | *Calliandra brevipes* | Fabaceae | flavanoids and isoflavanoids | Mediterranean | 60 | 7.65 |
| [9] | Phenolics | *Cecidomyiidae sp.* | Diptera | *Aspidosperma spruceanum* | Apocynaceae | alkaloids, terpenes | Tropical forest | 60 | 3.27 |
| [10] | Tannins | *Callirhytis cornigera* | Hymenoptera | *Quercus palustris* | Fabaceae | flavanoids and isoflavanoids | Temperate forest | 60 | -0.15 |
| [11] | Volatiles | *Gnorimoschema gallesolidaginis* | Lepidoptera | *Solidago altissima* | Asteraceae | terpenes, flavanoids | Temperate forest | 40 | 0.24 |
| [11] | Phenolics | *Lepidoptera* | Lepidoptera | *Tibouchina pulchra* | Melastomaceae | tannins | Tropical forest | 6 | 1.04 |
| [12] | Tannins | *Lepidoptera* | Lepidoptera | *T. pulchra* | Melastomaceae | tannins | Tropical forest | 20 | 0.88 |
| [12] | Tannins | *Ectoedemia populella* | Lepidoptera | *Populus granidentata* | Salicaceae | flavanoids | Temperate forest | 30 | -0.36 |
| [13] | Phenolics | *Unknown* | Unknown | *Qualea parviflora* | Vochysiaceae | triterpenes, phenols | Mediterranean | 30 | 0.15 |
| [13] | Tannins | *Unknown* | Unknown | *Qualea parviflora* | Vochysiaceae | triterpenes, phenols | Mediterranean | 30 | 0.75 |
| [14] | Phenolics | *Austrothrips cochinchinensis* | Thysanoptera | *Calycopteris floribundus* | Combretaceae | tannins | Tropical forest | 50 | 1.51 |
| [14] | Phenolics | *Phorainothrips loranthi* | Thysanoptera | *Loranthus elasticus* | Loranthaceae | terpenes, xanthones | Tropical forest | 50 | 1.31 |
| [14] | Phenolics | *Liothrips viticola* | Thysanoptera | *Vitis lanceolaria* | Vitaceae | Flavonoids, tannins | Tropical forest | 50 | 0.92 |
| [15] | Volatiles | *Eurosta solidaginis* | Diptera | *Solidago altissima* | Asteraceae | Terpenes, flavanoids | Temperate forest | 40 | 0.16 |
| [16] | Phenolics | *Pontania proxima* | Hymenoptera | *Salix alba x fragilis* | Salicaceae | flavanoids | Temperate forest | 76 | 1.28 |
| [16] | Tannins | *P. triandrae* | Hymenoptera | *Salix triandra* | Salicaceae | flavanoids | Boreal forest | 36 | 1.48 |
| [17] | Phenolics | *Pontania arcticornis* | Hymenoptera | *Salix phylicifolia* | Salicaceae | flavanoids | Boreal forest | 20 | -1.64 |
| [18] | Phenolics | *Hormaphis hamamelidis* | Hemiptera | *Hamamelis virginiana* | Hamamelidaceae | Flavonoids, tannins | Tropical forest | 6 | 0.61 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| [18] | Tannins | *H. hamamelidis* | Hemiptera | *Hamamelis virginiana* | Hamamelidaceae | Flavonoids, tannins | Tropical forest | 6 | 1.37 |
| [18] | Volatiles | *Trioza anceps* | Hemiptera | *Persea americana* | Lauraceae | alkaloids | Tropical forest | 291 | 0.39 |
| [19] | Tannins | *Ectoedemia populella* | Lepidoptera | *Populus tremuloides* | Salicaceae | flavanoids | Temperate forest | 30 | -1.4 |
| [20] | Tannins | *Pontania aestiva* | Hymenoptera | *Salix borealis* | Salicaceae | flavanoids | Boreal forest | 20 | 5.64 |
| [20] | Phenolics | *Pontania nivalis* | Hymenoptera | *Salix glauca* | Salicaceae | flavanoids | Boreal forest | 20 | -1.79 |
| [20] | Tannins | *Pontania nivalis* | Hymenoptera | *Salix glauca* | Salicaceae | flavanoids | Boreal forest | 20 | 3.47 |
| [20] | Phenolics | *Pontania samolad* | Hymenoptera | *Salix lapponum* | Salicaceae | flavanoids | Boreal forest | 20 | -1.28 |
| [20] | Tannins | *Pontania samolad* | Hymenoptera | *Salix lapponum* | Salicaceae | flavanoids | Boreal forest | 20 | 1.77 |
| [20] | Phenolics | *Pontania myrsiniticola* | Hymenoptera | *Salix myrsinities* | Salicaceae | flavanoids | Boreal forest | 20 | 0.13 |
| [20] | Tannins | *Pontania myrsiniticola* | Hymenoptera | *Salix myrsinities* | Salicaceae | flavanoids | Boreal forest | 20 | 3.56 |
| [20] | Phenolics | *Euura amerinae* | Hymenoptera | *Salix pentandra* | Salicaceae | flavanoids | Boreal forest | 21 | 3.13 |
| [20] | Tannins | *Pontania arcticornis* | Hymenoptera | *Salix phylicifolia* | Salicaceae | flavanoids | Boreal forest | 20 | 6.88 |
| [20] | Phenolics | *Pontania reticulatae* | Hymenoptera | *Salix reticulata* | Salicaceae | flavanoids | Boreal forest | 20 | 2.25 |
| [20] | Tannins | *Pontania reticulatae* | Hymenoptera | *Salix reticulata* | Salicaceae | flavanoids | Boreal forest | 20 | 3.73 |
| [20] | Phenolics | *Pontania triandrae* | Hymenoptera | *Salix triandra* | Salicaceae | flavanoids | Boreal forest | 36 | 1.48 |
| [21] | Phenolics | *Cecidomyiidae sp.* | Diptera | *Achatocarpus gracilis* | Achatocarpacea | ? | Tropical forest | 50 | 0.93 |
| [21] | Phenolics | *Cecidomyiidae sp.* | Diptera | *Cordia alliodora* | Boraginaceae | terpenes and naphthoquinones | Tropical forest | 50 | 0.92 |
| [21] | Volatiles | *Clusiamyia nitida* | Diptera | *Clusia lanceolata* | Clusiaceae | beta triketones and xanthones | Tropical forest | 70 | 0 |
| [21] | Tannins | *Contarinia sp.* | Diptera | *Bauhinia brevipes* | Fabaceae | flavanoids and isoflavanoids | Mediterranean | 170 | 0.2 |
| [21] | Phenolics | *Cecidomyiidae sp.* | Diptera | *Guapira macrocarpa* | Nyctaginaceae | flavanoids | Tropical forest | 50 | 0 |
| [22] | Volatiles | *Psyllidae sp.* | Hemiptera | *Schinus polygamus* | Anacardiaceae | alkyl resorcinols | Mediterranean | 10 | -0.69 |
| [22] | Volatiles | *Psyllidae sp.* | Hemiptera | *Baccharis spicata* | Asteraceae | terpenes , flavanoids | Mediterranean | 10 | 2.04 |
| [23] | Phenolics | *Adelges abietis* | Hemiptera | *Picea glauca* | Pinaceae | terpenes | Boreal forest | 30 | 1.01 |
| [24] | Phenolics | *Pontania aestiva* | Hymenoptera | *Salix borealis* | Salicaceae | flavanoids | Boreal forest | 20 | -0.08 |
| [25] | Tannins | *Contarinia sp.* | Diptera | *Bauhinia brevipes* | Fabaceae | flavanoids and isoflavanoids | Mediterranean | 50 | 0.2 |
| [26] | Tannins | *Cynipidae sp.* | Hymenoptera | *Quercus sp.* | Fagaceae | flavanoids, tannins | Temperate forest | 11 | 2.67 |
| [27] | Phenolics | *Cecidomyiidae sp.* | Diptera | *Guettarda elliptica* | Rubiaceae | tannins, alkaloids | Tropical forest | 50 | 1.37 |
| [27] | Tannins | *Lepidoptera* | Lepidoptera | *Tibouchina pulchra* | Melastomaceae | tannins | Tropical forest | 6 | 0.45 |
| [28] | Volatiles | *Psyllidae sp.* | Hemiptera | *Baccharis spicata* | Asteraceae | terpenes, flavanoids | Mediterranean | 10 | -2.76 |
| [29] | Volatiles | *Cecydomyiidae sp.* | Diptera | *Mintosthachys mollis* | Lamiaceae | terpenes | Mediterranean | 28 | -1.02 |
| [30] | Phenolics | *Pemphigus betae* | Hemiptera | *Populus angustifolia* | Salicaceae | flavanoids | Temperate forest | 11 | -0.2 |

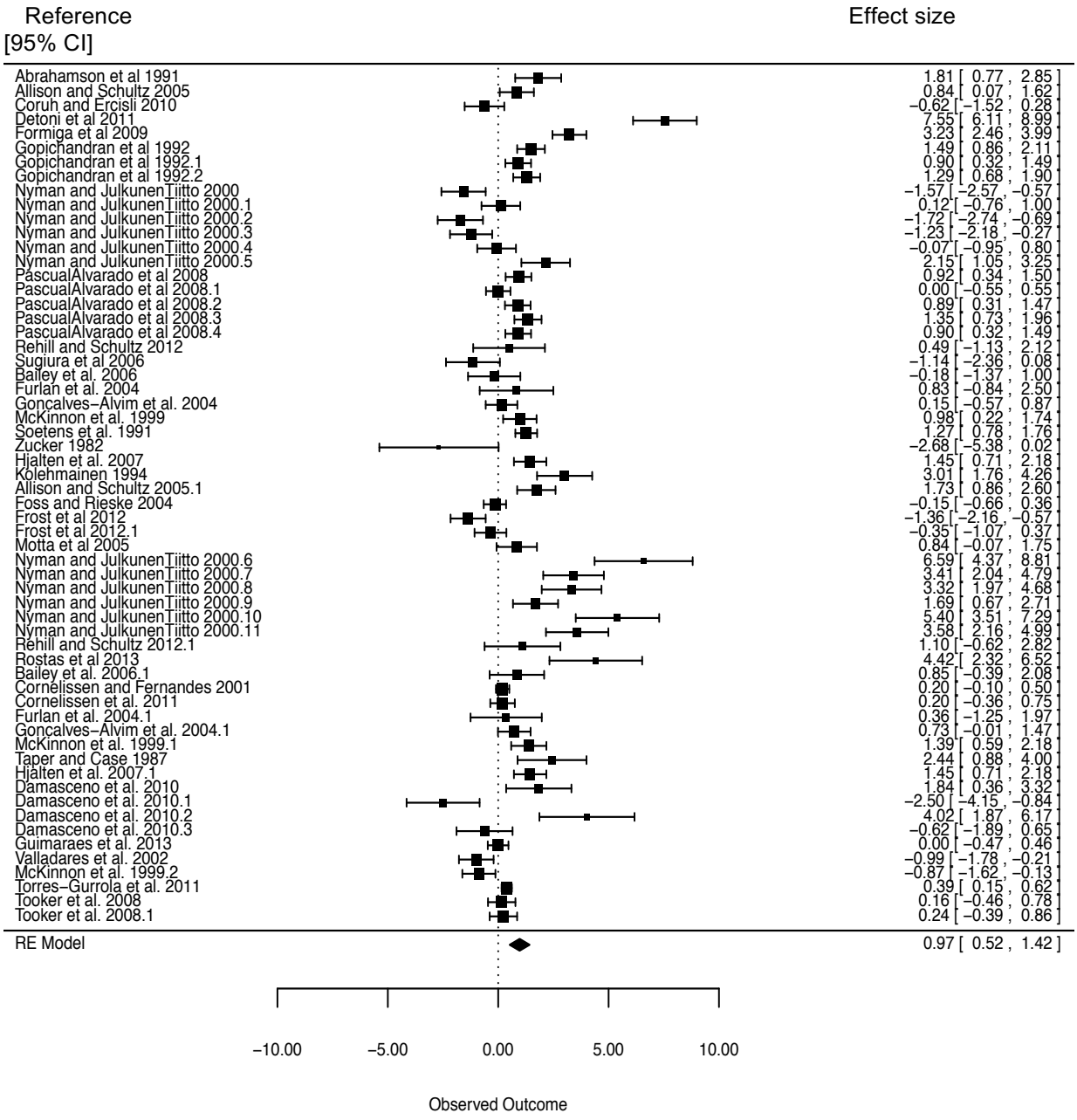



**Figure S2.1** Forest plot depicting the variation in effect sizes for each of the 60 data points (unique gall-host plant interactions for each chemical type) extracted from the studies included in the meta-analysis of the effect of insect galls on host plant defence chemistry.

# Chapter 3

In the previous chapter we found that many galling species increase concentrations of tannins and phenolics in galled host plant tissue. Such changes in phytochemistry could potentially affect other herbivores sharing the host plant. In this chapter we specifically examine the intra- and inter-specific interactions between galling insects and general herbivory on two closely related host plants.

**Statement of contribution to a co-authored paper**

Chapter 3 is a co-authored paper that has been prepared for publication. The citation is as follows:

Hall, C.R., Kitching, R.L. (prepared manuscript) Interactions between galling insects and general herbivory along an elevational gradient in Australian subtropical rainforest.

My contribution to the paper involved field work, data collection, analysis and writing of the manuscript. My co-author is my supervisor and was responsible for direction and guidance with regard to conception of ideas and sampling design and editing of the manuscript.

(Signed) _____ (Date) 12/12/2015
Casey Hall

(Countersigned) _____ (Date) 12/12/2015
Supervisor: Roger Kitching

# Chapter 3

## Interactions between galling insects and general herbivory along an elevational gradient in Australian subtropical rainforest

Casey R. Hall[1*] and Roger L. Kitching[1]

[1]Environmental Futures Research Institute (EFRI), Griffith School of Environment, Griffith University, 170 Kessels Road 4111

*Corresponding author: casey.hall@griffithuni.edu.au

## 3.1 Abstract

Plant species can potentially mediate the competition between guilds of herbivorous insects. Along with competitive interactions, abiotic factors can influence herbivorous insect functioning and development. In this study we use data from field-collected galls from two *Rubus* species to investigate the effect of elevation on leaf chewing herbivory, galling density and galler body size, and the interactions between gallers and leaf chewing herbivores. We found that gall density on *R. moorei* decreases with increasing elevation, while gall density on *R. nebulosus* peaks at a higher elevation. Reared galler body size was largest at mid elevations and decreased at both low and high elevations. There was a strong negative relationship between gall abundance and herbivory rate on *R. moorei*, yet a weak positive one for *R. nebulosus* and an overall negative relationship between gall abundance and galler body size. The relationship between leaf chewers and gallers appears to depend on gall abundance. The two guilds appear to compete on *R. moorei*, yet not on *R. nebulosus*, possibly due to the former having a higher overall abundance of galls. Both galling density and galler fitness are highest in the centre of host plant range, suggesting that several fitness related factors, such as optimal oviposition sites, are optimal within this range. Sites with high gall abundance also had reduced galler fitness, suggesting a strong effect of intraspecific competition among the galling insects.

## 3.2 Introduction

Competitive interactions between insect herbivores can play an important role in plant-insect interactions (Denno et al. 1995). Different guilds of herbivorous insects on a shared host plant may compete directly with each other for plant resources or indirectly through feeding-induced changes in host plant quality (Kaplan and Denno 2007). In addition, intra-specific competitive interactions may arise if optimal feeding sites are a limiting factor, as this leads to high population densities, potentially reducing plant quality and availability (Whitham 1978; Fisher et al. 2000). The gall-forming habit provides a useful ecological model for investigating such interactions as insect galls are sessile, often host specific, and can manipulate plant secondary compounds (Cornell 1983, Foss and Rieske 2004). This can indirectly affect other herbivorous guilds through the manipulation of shared host plant quality (Hartley and Lawton 1987). Leaf-chewers, in contrast, are generally mobile, cause actual loss of plant material, and are thus more likely to compete directly with other herbivores for resources (Hartley and Lawton 1987).

Galling insects, in general, have been shown to increase levels of plant secondary metabolites in galled plant material, which may confer some resistance against other herbivores on the same host plant (Taper and Case 1987, Abrahamsom et al. 1991). Pascual-Alvarado et al. (2008), for example, found that galled leaves not only had greater phenol concentrations but also significantly less folivore damage. In addition, Helms et al. (2013) found that exposure of host plants to the volatile emissions from adult galling insects was enough to deter subsequent leaf chewing herbivory. In contrast, several studies have found a positive effect of galling on leaf chewers (Cooper and Rieske 2009; Nakamura et al. 2003, Kunkler et al. 2013). The relationship, however, is a reciprocal one as the feeding of other herbivores may modify the quality of resources available to gallers and alter their fitness. For example, Crutsinger et al. (2013) found removal of other herbivores increased gall density and size.

Due to their sessile nature, galling insects may be more likely to encounter intraspecific competition, particularly if suitable feeding sites are limited. This can lead to localised patches of high gall density and ultimately decreases in leaf quality and availability of oviposition sites. For example, Wool and Manheim (1988) found that as gall density increased, gall size and success rate decreased. As galling insect body size, and

subsequently fitness, is positively related to gall size, this suggests that high gall density can lead to reduced fitness of the adult insects due to intra-specific competition (Stille 1984; McGinley 1989). Parasitoids are often responsible for high rates of mortality in galling insects (Price et al. 1987). They are important drivers of not just gall size (Price and Clancy 1986), but also host plant switching and speciation (Nyman et al. 2007). Host plant switches and changes in gall morphology (such as increased size) leads to fewer parasitoid species attacking a galling species, this "enemy-free space" could accelerate galling insect diversification (Nyman et al. 2007).

Along with competitive interactions, abiotic factors can influence herbivorous insect functioning and development. The variation in climate experienced along an elevation gradient exerts strong selection pressures on insect diversity and fitness (Hodkinson 2005). Insect herbivores can also be affected by elevation through the corresponding changes in host plant quality and productivity. In general, the intensity of herbivory has been shown to decline with elevation (Garibaldi et al. 2011; Rasman et al. 2013; Scheidel et al. 2003). Specialist herbivores, however, are less affected by climate than generalists (Scheidel et al. 2003). For example, Ribeiro et al. (2014) found increasing gall density with elevation, while Damken et al. (2012) found increased survivorship of a galling cecidomyiid with increasing elevation, due to increased habitat suitability. Insect gallers are more protected compared with free living herbivores and as such may not be responding directly to climate, but rather to host plant availability.

Both competition between herbivores and elevational changes in climate are important factors shaping insect herbivory. However, the combined effects of both elevation and competition on herbivory is largely unknown. In this study we investigated the interaction between galling by Cecidomyiidae sp.1 and leaf chewing herbivory on two closely related *Rubus* species along an elevational gradient in Australian subtropical rainforest. We use data from field-collected galls to investigate the following questions: (1) does leaf chewing herbivory, galling density and galling insect fitness (measured as body size) decrease with increasing elevation; (2) do chewing herbivores avoid leaves with galls; and, (3) is galling insect fitness correlated with gall abundance?

## 3.3 Methods

*Study site*

We sampled leaf galls at 15 sites within Lamington and Border Ranges National Parks in the Macpherson Ranges that span the Queensland/New South Wales border (Figure 3.1; see Chapter 4 for GPS coordinates). These parks contain a 36 519 ha expanse of continuous rainforest, ranging from approximately 250 m to 1200 m in elevation. The area is dominated by highly diverse and structurally complex subtropical rainforest and is described in further detail by Laidlaw et al. (2011). The moist subtropical climate is characterized by dominant summer rainfall, which peaks in January. The climate of the study area is described by McDonald (2010) and Strong et al. (2011).

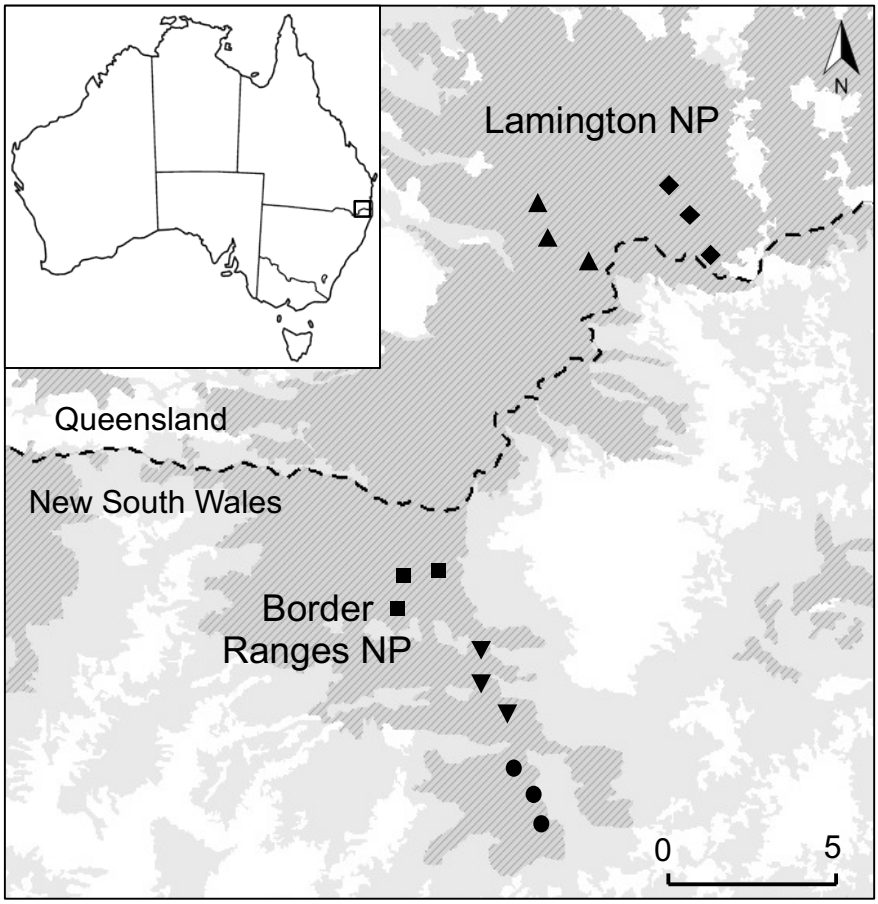



**Figure 3.1** Map of the sampling sites used in this study. Three sites, each separated by approximately 150 m in elevation were established at each of five locations: Binna Burra (diamonds), Lamington (triangles), Brindle creek (squares), Tweed road (inverse triangles) and Bar Mountain (circles). Grey shared areas indicate forest cover with rainforest cover shown in dark grey.

*Study system*

We studied the relationships between general leaf chewing herbivory and galling insect density and their effects on galling insect body size in two *Rubus* species (Rosaceae) along an elevational gradient. Galling insects use chemical stimuli to induce physical and metabolic changes in host plant tissue (Cornell 1983). They are usually monophagous and can often be assigned to morphospecies based on host plants and gall type. The galling species targeted is Cecidomyiidae sp. 1 (Diptera) and is currently being described by Peter Kolesik. Cecidomyiidae sp. 1 is a new genus and the first record of a cecidomyiid gall on native *Rubus* in Australia (personal communication). The vast majority of galls occurred on leaves, showing clumped distributions. Each species has multiple generations each year with peak gall abundance occurring in spring and autumn. Both gall types have overall similar morphological characteristics including soft tissue, a diameter of less than 1 cm, a spherical or ovoid shape with a small opening when mature, and a covering of trichomes (Figure S3.1). Each gall contains one cavity with a single fly larvae.

The two host plants sampled were *Rubus moorei* (F. Muell.) and *Rubus nebulosus* (A.R. Bean). Both plant species are native to Australia and have restricted distributions generally at higher altitudes (above 700 m asl) within subtropical rainforest, commonly in disturbed areas and light gaps. *Rubus nebulosus* is an evergreen climber found in moister rainforest along coastal areas north of Batemans Bay, NSW. *Rubus moorei* is similar to *R. nebulosus* but is restricted to subtropical rainforest in northern NSW and south-east Queensland (Harden et al. 2006). The two host plants were chosen as they often occur in close proximity to each other, are galled by the same species, the galls can be reared successfully and are linked through their shared parasitoids. They also gave us the opportunity to compare two closely related *Rubus* species with each other.

*Experimental design and sampling*

To generate a climatic gradient, we used an elevational gradient as a 'space for time substitution' (Pickett 1989; Hodkinson 2005). The use of frequently sampled plots distributed along a continuous elevation gradient is a commonly used approach to study insect variation along temperature gradients (see de Sassi *et al*. 2012; Reymond *et al*. 2013). We established five transects, with three sites each at approximately 150 m

intervals of elevation. Sites ranged from 762 m to 1145 m asl, a total elevation range of 383 m (see Chapter 4, Table 4.1). This provided a total median temperature difference of 3.5°C between the lowest and highest sites. All sites are located on north to north-west facing slopes to minimize temperature variation due to insolation.

At each site we sought three individuals of each plant species within a restricted 50 $m^2$ area in order to maintain a standard plot size and to decrease between-plant differences in abiotic conditions. *Rubus moorei* and *R. nebulosus* were rare at high and low elevations respectively, and as such not all sites contained both species. Accordingly, to standardize samples, gall density was calculated as number of galls/per gram of biomass sampled. Each site was sampled 6 times over a 1-year period with two months between each sampling occasion to allow adequate time for regrowth and for galls to re-establish. Each sampling occasion ranged from 1 to 2 weeks. Galls were sampled by closely inspecting each plant and removing all galled leaves. For consistency, all galls were collected, regardless of their development stage. Galled leaves were placed into sealed plastic boxes containing moistened tissue and checked every 3 days for two months for the emergence of adult insects. Reared adult cecidomyiids were preserved in ethanol. Adult cecidomyiid body length was measured from thorax to end of abdomen in ocular piece units and converted to mm.

Herbivory damage (missing leaf area) was calculated on both galled and non-galled leaves (collected for chemical analysis, see chapter 5) using the software package Image J (Rasband 2015). Leaves were arranged and photographed on a plain white background with a ruler. Each image was converted to grayscale and total existing leaf area ($cm^2$) measured in ImageJ and exported to Excel. Then for each image the missing leaf area due to herbivory was drawn in and calculated in image J. To calculate herbivory we divided missing leaf area by total leaf area (the sum of existing and missing leaf area).

To estimate plant biomass without destructive sampling, we estimated the total volume of each sampled plant. We measured height and crown diameter, and then calculated the cylindrical volume within which the above ground parts of the plant occurred. To convert plant volume to biomass, we measured the volume of 10 plants from each species following the same procedure as above. We then cut them at ground level and dried the leaf material in a drying cabinet for 48 h. We used linear regression to test how well volume approximated dry weight and found a significant relationship for both

plant species (*Rubus nebulosus* $F_{1,8} = 22.18$, $P = 0.002$, $R^2 = 0.73$; *Rubus moorei* $F_{1,8} = 5.91$, $P = 0.041$, $R^2 = 0.42$).

*Analysis*

All statistical analyses were carried out using R statistical software (R Development Core Team 2014). We used both linear and polynomial regression to assess the relationships between elevation, gall density, gall insect body length and herbivory rates. Gall density was used in analysis with elevation as it accounts for differences in host plant biomass. Gall abundance was used in herbivory comparisons as both were measured from the same sample size, thus controlling for plant biomass. Body length and gall abundance were ln(x+1) transformed prior to analysis to approximate a normal distribution. Gall abundance, herbivory rate and body length were averaged across the six sampling occasions for each site for the analysis of the relationship between gall abundance and herbivory rate as well as between gall abundance and body length.

## 3.4 Results

A total of 7068 galls, 4768 from *R. moorei* and 2300 from *R. nebulosus*, were collected across the 15 sampling sites and six occasions. Mean gall density was 3 and 1.7 galls/g, while herbivory rate was 0.05 and 0.04 for *R. moorei* and *R. nebulosus* respectively. *Rubus nebulosus* gall density peaked in the middle of its elevational range ($R^2 = 0.13$, $P = 0.02$), while *R. moorei* gall density decreased with elevation ($R^2 = 0.11$, $P = 0.02$) (Figure 3.2a). In contrast, herbivory rates showed no significant relationship with elevation for either *Rubus* species (Figure 3.2b, Table S3.1).

Rearing success rate of Cecidomyiidae sp 1. was low across all samples due to high rates of parasitism (see Chapter 4). Of the 113 reared Cecidomyiidae sp. 1 specimens that were able to be measured, only 18 were from *R. moorei*. Individuals from both *Rubus* species were not significantly different and were combined for all body length analyses. Both male and female Cecidomyiidae sp.1 show significant mid-elevation peaks in body size ($R^2 = 0.48$; $P < 0.001$ and $R^2 = 0.14$; $P = 0.01$ for males and females respectively) (Figure 3.3). Females, on average, were consistently larger than males across all elevations (Figure 3.3).

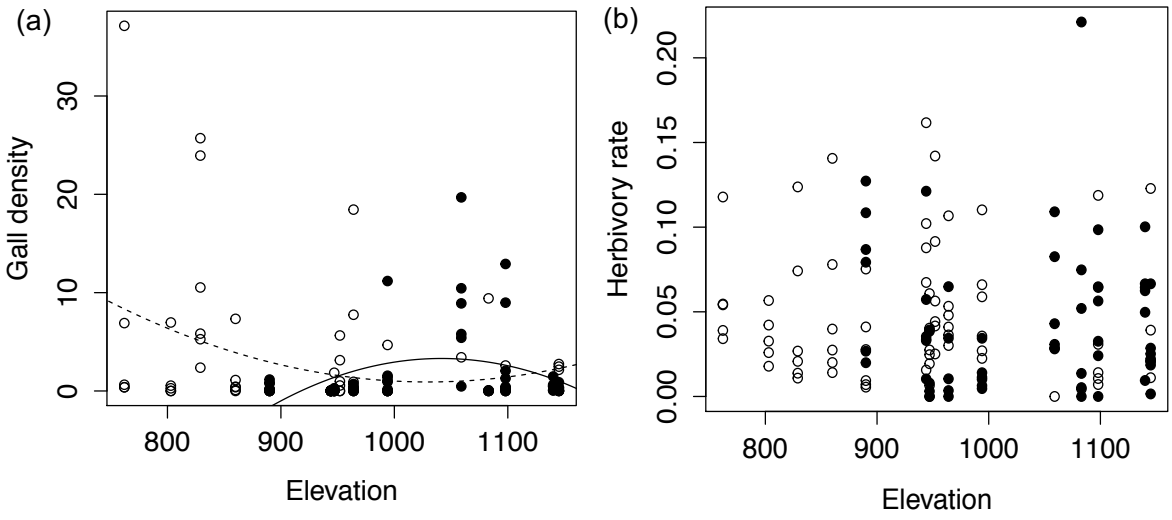



**Figure 3.2** Gall density (a) and herbivory rates (b) (mean per site and sampling occasion). *Rubus moorei* is represented by open circles and *R. nebulosus* by filled circles. Regression lines (dashed for *R. moorei* and solid for *R. nebulosus*) are shown when significant ($P$<0.05).

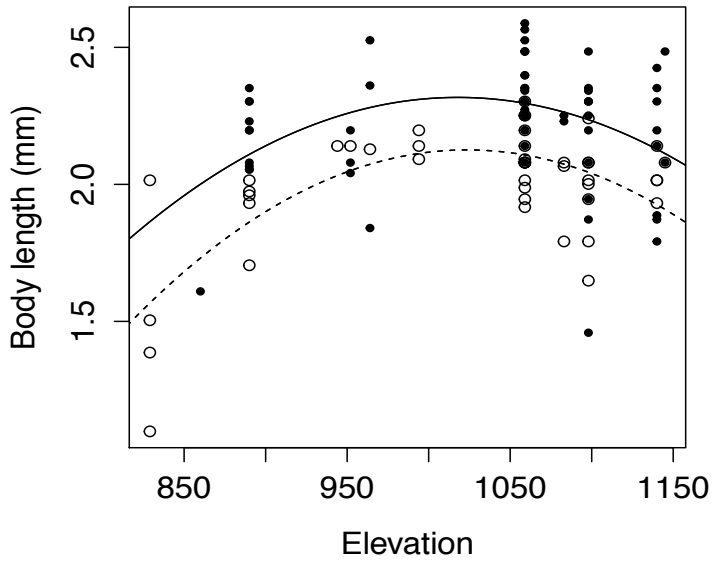



**Figure 3.3** Body length of Cecidomyiidae sp. 1 reared from both *Rubus* species at different elevations. Closed circles represent females with solid regression line, open circles males with dashed regression line.

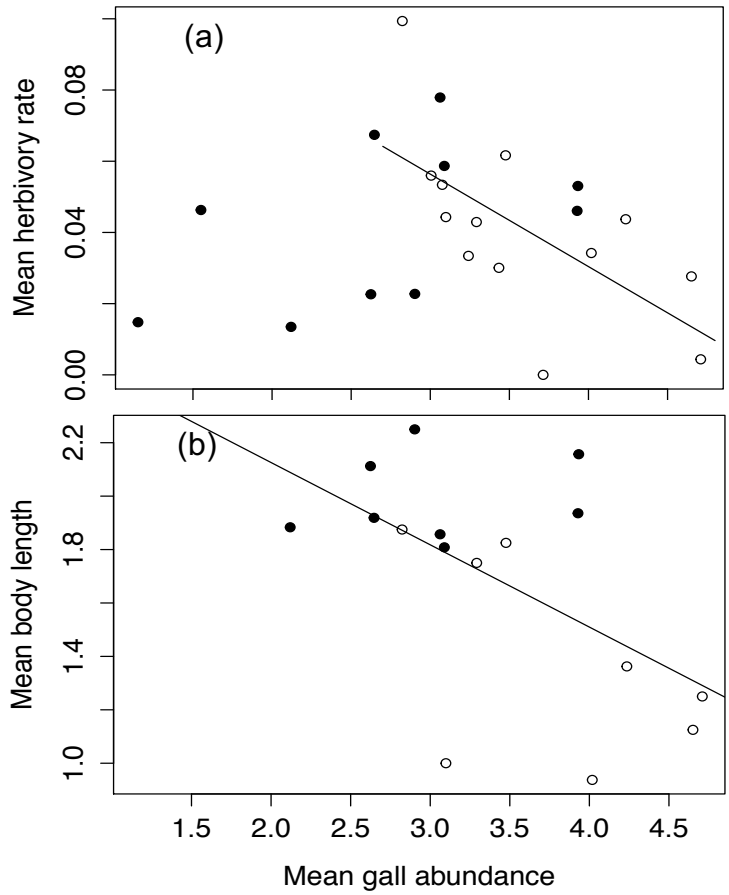



**Figure 3.4** The relationship between mean gall abundance and (a) mean herbivory rate for *R. moorei* (n=13) and *R. nebulosus* (n=10) and (b) mean body length per site (n=16). *Rubus moorei* open circles, *R. nebulosus* filled circles, significant regression lines ($P < 0.05$).

Herbivory rate decreased significantly with increasing gall abundance ($R^2 = 0.41$, $P = 0.02$) for *R. moorei*. *Rubus nebulosus*, however, showed the opposite trend, although this was not significant (Figure 3.4a). Mean body length significantly decreased with increasing gall abundance ($R^2 = 0.31$, $P = 0.02$) across Cecidomyiidae sp. 1 reared from both *Rubus* species (Figure 3.4b).

## 3.5 Discussion

The relationships among galling, general herbivory and elevation are complex and host plant dependent. We have demonstrated that two different guilds of insect herbivores respond differently to elevation, yet they also show evidence of interguild competition. The nature of the interaction, however, is contrasted on two closely related host plants.

In addition, we find evidence that intraspecific competition may significantly reduce adult fitness in galling insects.

Gall densities are to some extent partitioned between the two *Rubus* species along the elevational gradient. Gall density on *R. moorei* decreases with increasing elevation. In contrast, gall density on *R. nebulosus* peaks at a higher elevation. Previous studies have shown variable responses of galling to elevation and suggested that gallers are responding to factors other than elevation, such as host plant availability (Damken et al. 2012; Ribeiro et al. 2014). This seems to be the case in our system as the pattern of galling density was not only different between the two related host plants but also occurred where each host plant was more common.

In contrast to previous studies, we found no elevational pattern in herbivory rates, despite reduced gall density at higher elevations. Free living herbivores previously found on the host plants include a sawfly, *Philomastix glabra* from *R. nebulosus* (personal observation) and a beetle, *Aaaba fossicollis,* from *R. moorei* (Bruzzese 1980). Studies have found that lower temperatures at higher elevations reduces herbivore abundance through constraints on insect development (Rasman et al. 2013). Conversely, other studies have suggested that the effects of low temperature at high elevations may be obscured by positive effects due to escape from natural enemies (Koptur 1985; Hodkinson 2005). Maunsell et al. (2014), for example, found that leaf miners at high elevations escaped parasitism. In addition, it may be that in our study system, cooler temperatures at the highest elevations are not a limiting factor for free living insect herbivores due to the relatively mild subtropical climate. The vast majority of studies reporting declines in herbivory with increasing elevation are from temperate and alpine habitats which have extreme conditions at high elevations, whereas the continuously forested gradient used in this study may support a more constant abundance of herbivores. In addition, the small elevational range used in this study may not have been enough to detect climate driven patterns in herbivory.

Despite differences in elevational patterns of gall density between the two *Rubus* species, we found that overall, body size of reared gallers was largest at mid elevations and decreased at both low and high elevations. A similar mid-elevation peak was found for wing size of a Costa Rican butterfly (Hawkins and de Vries 1996). Often there is a general decrease in in the mean size of individual species within communities with

increasing elevation (Janzen et al. 1976). However, within species patterns show inconsistent and opposing trends even between closely related species (Hodkinson 2005). Gutierrez and Menendez (1997) found that body size was correlated with regional distribution for carabid beetles. These mid-elevation peaks may also be due to mid-range effects, where species are often best adapted to the central part of their range (Hochberg and Ives 1999). In our study system the percentage of forested area is greatest at mid to high elevations (Hall et al. 2015a), supporting the idea that body size may be related to the availability and suitability of habitat area. In addition, Cecidomyiidae sp. 1 hosts a guild of parasitoids across both *Rubus* species (Hall et al. 2015). Parasitoids have been found to be a strong selecting force on gall size as they are more successful at attacking small galls, thus selecting for larger gall size and thus larger adult galling insects (Price and Clancy 1986; Stille 1984; László et al. 2014). The shared parasitoids may select for larger gall size, and thus body size at mid elevations where both *Rubus* plant distibutions overlap due to a kind of intraspecific apparent competition between the two cecidomyiid populations from different *Rubus* host plants.

The relationship between gall abundance and herbivory rate showed opposing patterns on the two *Rubus* species, although this was only significant for *R. moorei*. Competitive interactions that are mediated via the host plant may be stronger on certain host plant species than on others - a phenomenon that has been termed interaction modification (*sensu* Wootton 1994). This may explain why there was a strong negative relationship between gall abundance and herbivory rate on *R. moorei*, yet a weak positive one for *R. nebulosus*. In addition, the lower gall abundance on *R. nebulosus* may mean that the gall's density is not high enough to cause significant changes in host plant quality. In contrast, the higher gall abundance on *R. moorei* may lead to more frequent competitive interactions with other feeding guilds. This may also be true for intraspecific interactions; most of the negative relationship between gall abundance and body size was driven by *R. moorei*. This species may have fewer optimal galling sites, leading to overcrowding and reduced fitness of the galling insects, whereas gall abundance on *R. nebulosus* was generally lower, leading to reduced intraspecific competition.

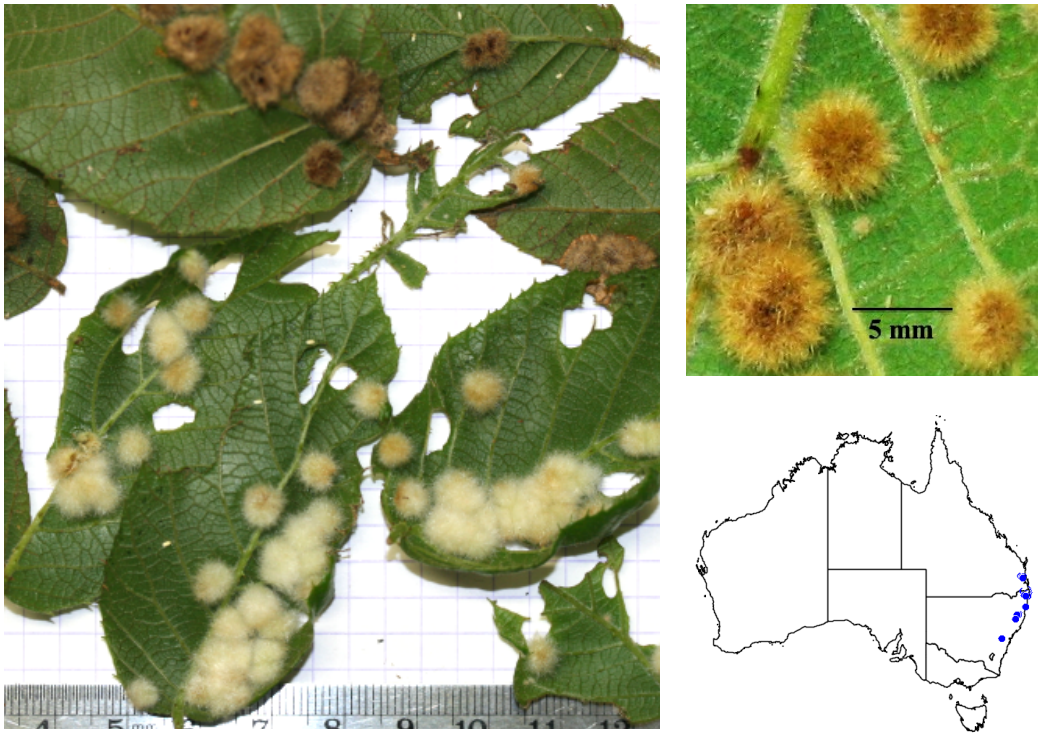
*Conclusions*

In this study, we found opposing trends in gall density with elevation between the two *Rubus* species, yet no clear trend in herbivory rates. However, we did find evidence for

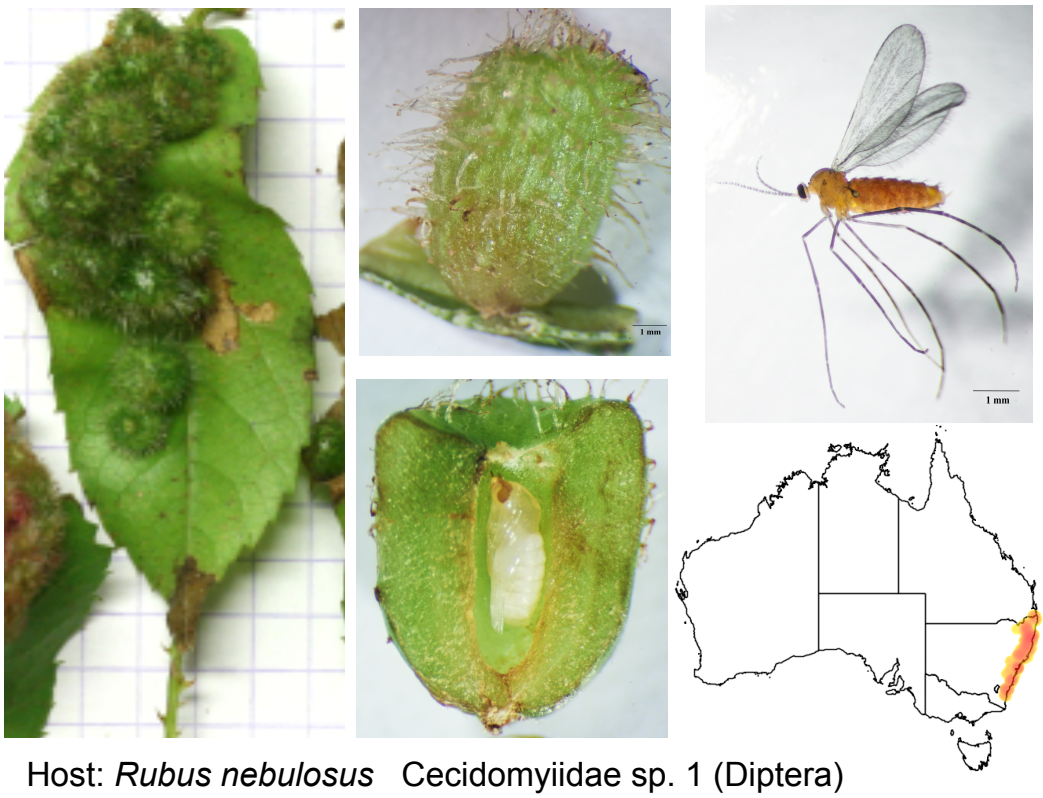
competition between leaf chewers and galling insects, and also intraspecific competition among gallers. Competitive interactions both between and within herbivorous insect species can be both direct, as with interference competition, or indirect, such as those mediated by a shared resource. The relationship between leaf chewers and gallers appears to depend on gall abundance. The two guilds only compete on *R. moorei*, not *R. nebulosus*, possibly due to the former having a higher overall abundance of galls. Both galling density and galler fitness are highest in the centre of host plant elevational range, suggesting that several fitness related factors are optimal in this range. Sites with high gall abundance also had reduced galler fitness, suggesting a strong effect of intraspecific competition among the galling insects.

This study provides evidence for the modification of competitive interactions (*sensu* Wootton 1994) between herbivores by their host plants, driven by differing abundances of galling insects between the two host plant species. It is evident that there is a great deal of variation in the mechanisms governing within and between guild competition. As such future studies need to focus on experimental manipulation to determine the mechanisms that modify competitive interactions along environmental gradients.

## S3 Supplementary Material




Host: *Rubus moorei* Cecidomyiidae sp. 1 (Diptera)




Host: *Rubus nebulosus* Cecidomyiidae sp. 1 (Diptera)

**Figure S3.1** Photos of R. moorei galls (top), R. nebulosus galls and Cecidomyiidae sp. 1 (bottom) and maps of host plant distributions. Photos by C. Hall and maps provided by Peter Kolesik.

**Table S3.1** Linear regressions between elevation, gall density, herbivory rate and body length calculated separately for R. moorei and R. nebulosus and male and female galling insects. Coefficients of determination ($R^2$) and F statistics with degrees of freedom are shown; significant relationships are in bold ($P<0.05$). Signs indicate the direction of the relationship.

| **Model** | **F (d.f)** | **$R^2$** | ***P*** |
|---|---|---|---|
| *R. moorei* gall density ~ elevation + elevation$^2$ | 4.16 (2,67) | **0.11** | **0.02** |
| *R. nebulosus* gall density ~ elevation + elevation$^2$ | 4.07 (2,56) | **-0.13** | **0.02** |
| *R. moorei* herbivory rate ~ elevation | 0.23 (1,68) | -0.003 | 0.63 |
| *R. nebulosus* herbivory rate ~ elevation | 0.04 (1,57) | 0.001 | 0.83 |
| Female ln(body length+1) ~ elevation + elevation$^2$ | 5.29 (2,64) | **-0.14** | **0.01** |
| Male ln(body length +1) ~ elevation + elevation$^2$ | 19.75 (2,42) | **-0.48** | **>0.01** |
| *R. moorei* herbivory rate ~ ln(gall abundance+1) | 7.71 (1,11) | **-0.41** | **0.02** |
| *R. nebulosus* herbivory rate ~ ln(gall abundance+1) | 2.31 (1,8) | 0.22 | 0.17 |
| ln(gall abundance +1) ~ body length | 6.36 (1,14) | **-0.31** | **0.02** |

# Chapter 4

The previous chapter provided evidence for strong inter- and intra-guild competition between galling insects and general herbivory. This galling species (Cecidomyiidae sp.1) and *Dasineura* sp. from *Solanum inaequilaterum* are both host to a guild of shared parasitoid species. This chapter focuses on this guild of gall parasitoids and examines the effect of temperature on the relationship between parasitoid richness and parasitism rates. Corrigendum: After publication of the following manuscript we discovered that Cecidomyiidae sp 2 and sp 3 reared from *R. nebulosus* and *R. moorei* are the same species (Chapter 3). This corrigendum will be included in the subsequent formal species description in collaboration with Peter Kolesik.

**Statement of contribution to a co-authored paper**

Chapter 4 is a co-authored paper that has been published in the journal Austral Ecology. Copyright permission to reproduce this article has been obtained from the publisher (John Wiley and Sons, licence number: 3753001430381). The citation is as follows:

Hall, C.R., Burwell, C.J., Kitching, R.L. (2015) Changes in function and temporal variation in a guild of gall-parasitoids across a temperature gradient in Australian subtropical rainforest. *Austral Ecology*. doi:10.1111/aec.12283

My contribution to the paper involved field work, data collection, analysis and writing of the manuscript. Roger Kitching was responsible for direction and guidance with regard to conception of ideas and sampling design and editing of the manuscript. Chris Burwell assisted with editing of the manuscript and morphospecies identification.

(Signed) _____ (Date) 12/12/2015
Casey Hall

(Countersigned) _____ (Date) 12/12/2015
Co-author: Chris Burwell

(Countersigned) _____ (Date) 12/12/2015
Supervisor: Roger Kitching

*Austral Ecology* (2015) ••, ••–••

# Changes in function and temporal variation in a guild of gall-parasitoids across a temperature gradient in Australian subtropical rainforest

CASEY R. HALL,[1,*] CHRIS J. BURWELL[1,2] AND ROGER L. KITCHING[1]
[1]*Environmental Futures Research Institute (EFRI), Griffith School of Environment, Griffith University, 170 Kessels Road, 4111 (Email: casey.hall@griffithuni.edu.au), and* [2]*Natural Environments Program, Queensland Museum, Brisbane, Queensland, Australia*

**Abstract** Parasitoids play an important role in ecosystem functioning through their influence on herbivorous insect populations. Theoretical and experimental evidence suggest that increased species richness can enhance and stabilize ecosystem function. It is important to understand how richness-driven functional relationships change across environmental gradients. We investigated how temperature affected the relationship between parasitoid richness and parasitism rate in a guild of gall-parasitoids along an elevational gradient. We collected galls at 15 sites along five elevational gradients (between 762 m and 1145 m asl) on six occasions over a year. A total of 1902 insects, including 1593 parasitoids, were reared from 12 402 galls. Parasitism rate increased significantly with temperature on all sampling occasions, except December and February. We found a significant, positive richness–parasitism relationship. This relationship, however, was weaker at higher elevations which may be linked to decreased functional efficiency of parasitoids at lower temperatures. Temporal variability in parasitism rate and parasitoid richness were significantly related, regardless of temperature. A stable functional guild of this kind may provide a more reliable ecosystem service under environmental changes.

**Key words:** Cecidomyiidae, elevation gradients, parasitism, rainforest, trophic interactions.

## INTRODUCTION

Environmental gradients, by selecting for certain functional traits, affect species in a non-random manner and can determine how species interact with each other (Hodkinson 2005). Elevational gradients, in particular, create pronounced patterns in species distributions due to the contrasting environmental conditions found over short geographical distances (Körner 2007). In general, decreasing temperatures with increasing elevation may constrain insect traits, such as thermal tolerance and phenology, leading to reduced species richness, turnover and functional diversity (Hodkinson 2005). Usually studies of elevational patterns in insect richness and assemblage turnover focus on a single taxon or trophic level (e.g. moths, Ashton *et al.* 2011; ants, Burwell & Nakamura 2011; dung beetles, Escobar *et al.* 2005), and elevational effects on species interactions are seldom taken into account (but see Maunsell *et al.* 2014).

Parasitoids provide an interesting case study as they play significant roles in ecosystem functioning (LaSalle & Gauld 1993), yet are constrained in their responses to environmental gradients by changes in host traits. Few studies have looked explicitly at how elevation affects host–parasitoid interactions. Those that have, have found that the decrease in parasitism rates with increasing elevation is disproportionately greater than that of their host populations (Virtanen & Neuvonen 1999; Maunsell *et al.* 2014). Such asymmetrical responses may explain why most studies report a decline in parasitism rates with increasing elevation (Hodkinson 2005; Péré *et al.* 2013). In some cases, parasitism rates reach zero before the upper elevational limits of the host species, thereby creating high elevation refuges from parasitism (Randall 1982; Maunsell *et al.* 2014). Even when present, the efficacy of parasitoids may be reduced at higher elevations. This is supported by laboratory experiments demonstrating decreased parasitoid functional efficiency at lower temperatures (Enkegaard 1994; Menon *et al.* 2002).

Other factors, however, may also be important determinants of parasitism rates. Complementary use of host resources by a guild of parasitoids may lead to increased rates of parasitism (Snyder *et al.* 2008). Different parasitoid species may be both temporally (e.g. egg *vs.* larval parasitoids) and spatially (e.g. ecto- *vs.* endoparasitoids) separated on the same host species (Bonsall *et al.* 2002; Hackett-Jones *et al.* 2009). In addition, the relative abundances of different parasitoids change seasonally (Sorribas *et al.* 2010). Such complementary host use may lead to a positive relationship between parasitoid richness and host

*Corresponding author.
Accepted for publication May 2015.

© 2015 Ecological Society of Australia
doi:10.1111/aec.12283

parasitism rates. The opposite may occur when parasitoid species overlap in host resource use, leading to intraguild competition and the occurrence of so-called 'redundant' species (Harvey *et al.* 2013). This may create a situation where an increase in richness does *not* lead to an increase in the associated ecological function (Straub *et al.* 2008). Such 'redundant' species, however, may become more important as environmental conditions change. In this way, diversity can act as a buffer, maintaining ecosystem function against changing conditions (Thébault & Loreau 2005). In addition, different responses among species to environmental fluctuations should lead to decreased temporal variability with increasing species richness (Lehman & Tilman 2000). Understanding how species richness maintains functional stability under environmental change is becoming increasingly important (Hooper *et al.* 2005). Field-based studies of stability in richness-parasitism relationships along environmental gradients are few, particularly in forests (Loreau *et al.* 2001).

In this paper we address this lack of field-based studies by examining a guild of interacting parasitoid species from three galling hosts across an elevational gradient in subtropical rainforest. We use data from field-collected galls and their reared parasitoids to investigate the following hypotheses:

1. Parasitism rate and parasitoid species richness will decline with decreasing temperature: The decrease in temperature associated with increasing elevation may reduce parasitoid development and functional efficiency (Menon *et al.* 2002), thus limiting their attack rate and species survival at cooler temperatures.
2. Parasitism rates will be positively related to parasitoid richness across the temperature gradient: Higher parasitoid richness may allow for greater complementarity in host use and saturation of niche space (Hooper *et al.* 2005), leading to increased rates of parasitism with increasing temperature.
3. Temporal variability in parasitism rate will be negatively related to parasitoid richness and positively related to temporal variability in richness: Related to the parasitism-richness hypothesis, increased parasitoid richness and decreased variability in richness may lead to greater temporal niche partitioning, leading to lower temporal variability in parasitism rates at higher temperatures (Lehman & Tilman 2000).

## METHODS

### Study area

We sampled leaf galls at 15 sites within Lamington and Border Ranges National Parks in the Macpherson Ranges that span the Queensland/New South Wales border (see Table 1 for GPS coordinates). These parks contain a 36 519 ha expanse of continuous rainforest, ranging from approximately 250 m to 1200 m in elevation. The area is dominated by highly diverse and structurally complex subtropical rainforest and is described in further detail by Laidlaw *et al.* (2011). The moist subtropical climate is characterized by dominant summer rainfall, which peaks in January. The climate of the study area is described in further detail by McDonald (2010).

### The study system

We studied differences in the richness, function and variability in a guild of parasitoids (Hymenoptera) reared from three

**Table 1.** Site information including latitude and longitude coordinates, elevation, median temperature and total number of reared insects (gallers + parasitoids)

| Transect | Latitude | Longitude | Elevation (m asl) | Median temperature (°C) | Total abundance |
|---|---|---|---|---|---|
| Binna Burra | −28.1994 | 153.1923 | 803 | 13.5 | 64 |
| | −28.2061 | 153.1911 | 952 | 12 | 117 |
| | −28.2559 | 153.2031 | 1083 | 10.5 | 173 |
| Lamington | −28.2284 | 153.1372 | 829 | 11.5 | 77 |
| | −28.2400 | 153.1490 | 944 | 12 | 100 |
| | −28.2620 | 153.1700 | 1140 | 10 | 169 |
| Brindle Creek | −28.3786 | 153.0669 | 762 | 11.5 | 54 |
| | −28.3883 | 153.0670 | 947 | 10.5 | 58 |
| | −28.3755 | 153.0960 | 1059 | 11 | 339 |
| Tweed Road | −28.4370 | 153.1420 | 860 | 11.5 | 38 |
| | −28.4333 | 153.1278 | 964 | 11 | 84 |
| | −28.4281 | 153.1256 | 1098 | 10.5 | 265 |
| Bar Mountain | −28.4818 | 153.1439 | 890 | 12 | 188 |
| | −28.4708 | 153.1410 | 994 | 11 | 46 |
| | −28.4592 | 153.1324 | 1145 | 10.5 | 130 |

doi:10.1111/aec.12283
© 2015 Ecological Society of Australia

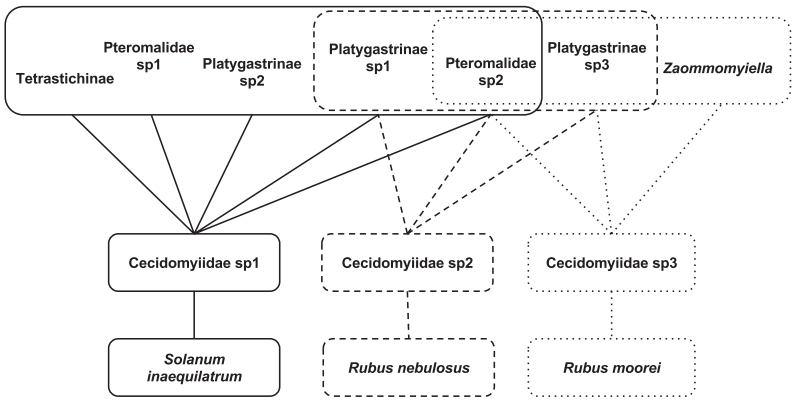



**Fig. 1.** Diagram of the study system, showing interactions among all species. Lines indicate a direct relationship between two species, line types represent host species.

species of galling insects (Cecidomyiidae) across a temperature gradient, generated by elevational differences in our study sites. Galling insects use chemical stimuli to induce physical and metabolic changes in host plant tissue (Cornell 1983). They are usually monophagous and can often be assigned to morphospecies based on host plants and gall type. The three galling species targeted belong to the dipteran family Cecidomyiidae (the gall midges). The vast majority of galls occurred on leaves, showing clumped distributions. Each species has multiple generations each year with peak gall abundance occurring in spring and autumn. All three gall types have overall similar morphological characteristics including soft tissue, less than 1 cm diameter, spherical or ovoid shape with a small opening when mature, and covered in trichomes (see Appendix S1 for photographs).

The three host plants sampled were *Rubus moorei* (F. Muell.), *Rubus nebulosus* (A. R. Bean) and *Solanum inaequilaterum* (Domin), each hosting a different morphospecies of galling Cecidomyiidae. All three plant species have restricted distributions and are found at higher altitudes (above 700 m asl) within subtropical rainforest, commonly in disturbed areas and light gaps. *Solanum inaequilaterum* is a perennial shrub, up to 2 m, that occurs in higher rainfall areas of southeast Queensland and northern New South Wales, in disturbed rainforest sites and margins (Bean 2004). *Rubus nebulosus* is an evergreen climber found in moister rainforest along coastal areas north of Batemans Bay, NSW. *Rubus moorei* is similar to *R. nebulosus* but is restricted to subtropical rainforest in northern NSW and south-east Queensland (Harden *et al.* 2006). The three host plants were chosen as they often occur in close proximity to each other, the galls can be reared successfully and are linked through their shared parasitoids. They also gave us the opportunity to compare two closely related *Rubus* species with each other and these, in turn, with a third more distantly related host species (*Solanum inaequilaterum*).

In total, nine species of parasitic Hymenoptera were reared from the three galling cecidomyiid species. Two species had total abundances less than 10 and were excluded from analysis. The seven most common species were identified to family and assigned to morphospecies. Three morphospecies were identified as Platygasteridae: Platygasterinae, two belonged to the Pteromalidae and two to the Eulophidae. The two eulophid species were identified as Tetrastichinae sp. and *Zaommomyiella* sp. by CJB. Platygastrinae sp. 2, Tetrastichinae and Pteromalidae sp. 1 were reared only from *Solanum* galls (Fig. 1). Pteromalidae sp. 2 was reared from all three hosts and Platygastrinae sp. 1 from both *R. nebulosus* and *Solanum*. Platygastrinae sp. 3 was reared from both species of *Rubus*, and *Zaommomyiella* sp. from *R. moorei* only (Fig. 1).

## Experimental design and sampling

To generate a climatic gradient, we used an elevational gradient as a 'space for time substitution' (Pickett 1989; Hodkinson 2005). The use of frequently sampled plots distributed along a continuous elevation gradient is a commonly used approach to study insect variation along temperature gradients (see de Sassi *et al.* 2012; Reymond *et al.* 2013). We established five transects, with three sites each at approximately 150 m intervals of elevation (Table 1).

Sites ranged from 762 m to 1145 m asl, a total elevation range of 383 m. This provided a total median temperature difference of 3.5°C between the lowest and highest sites. All sites are located on north to north-west facing slopes to minimize temperature variation due to insolation. Local topography, however, may still create significant microclimatic variation, in addition to the general temperature gradient (Weiss *et al.* 1988). To account for such natural variation, temperature was recorded at each site, every 3 h from June 2013 to September 2014, using temperature loggers (DS21 Thermochron iButton, Dallas Semiconductor/Maxim, CA, USA), suspended approximately 1.5 m above ground level. This allowed us to test the effects of temperature separately from the effects of other environmental variables that co-vary with elevation (such as humidity and radiation) (de Sassi *et al.* 2012). To overcome occasional data gaps, we have used median site temperature from March to September 2014 in all analyses, which included the peak galling seasons.

At each site we sought three individuals of each plant species within a restricted 50 $m^2$ area in order to maintain a standard plot size and to decrease between-plant differences in abiotic conditions. *Rubus moorei* and *R. nebulosus* were rare at high and low elevations, respectively as such not all sites contained all three species. Accordingly, to standardize

© 2015 Ecological Society of Australia
doi:10.1111/aec.12283

samples, gall density was calculated as number of galls/per gram of biomass sampled, and relative abundances of each species reared from galls were used in analyses. Each site was sampled 6 times over a 1-year period with 2 months between each sampling occasion to allow adequate time for regrowth and for galls to re-establish. Each sampling occasion ranged from 1 to 2 weeks; for clarity we refer to the month that sampling occurred in (i.e. March 2013, June 2013, October 2013, December 2013, February 2014 and May 2014). Galls were sampled by closely inspecting each plant and removing all galled leaves. For consistency, all galls were collected, regardless of their development stage. This may have led to an underestimate of parasitism (Stireman *et al.* 2005) but any bias should be consistent across all sites. Galled leaves were placed into sealed plastic boxes containing moistened tissue and checked every 3 days for two months for the emergence of adult insects. Reared adult insects were counted, preserved in ethanol and sorted to morphospecies by CH, with assistance from CJB for parasitoids. Insects were identified with varying degrees of taxonomic resolution, but morphospecies were used in all analyses. It was not possible to distinguish between parasitoids and hyper-parasitoids.

To estimate plant biomass without destructive sampling, we estimated the total volume of each sampled plant. We measured height and crown diameter, and then calculated the cylindrical volume within which the above-ground parts of the plant occurred. To convert plant volume to biomass, we measured the volume of 10 plants from each species following the same procedure as above. We then cut them at ground level and dried the leaf material in a drying cabinet for 48 h. We used linear regression to test how well volume approximated dry weight and found a significant relationship for each plant species (*Solanum* $F_{1,8} = 137.5$, $P < 0.001$, $R^2 = 0.95$; *Rubus nebulosus* $F_{1,8} = 22.18$, $P = 0.002$, $R^2 = 0.73$; *Rubus moorei* $F_{1,8} = 5.91$, $P = 0.041$, $R^2 = 0.42$).

### Analysis

All statistical analyses were carried out using R statistical software (R Development Core Team 2014), using the *LME4* package (Bates *et al.* 2013). All three host gall species were combined for analysis as we were interested in the parasitoid guild as a whole and some parasitoid species were shared across the three hosts (Fig. 1). In addition, we found no significant effect of plant host species on parasitoid richness ($\chi^2 = 5.03$, $P = 0.08$, d.f. = 2) or parasitism rates, corrected for overdispersion ($\chi^2 = 4.16$, $P = 0.12$, d.f. = 2) (see Appendix S2 for insect abundance from each host plant species). Parasitism rate (as a proportion) was calculated as the number of parasitoids divided by total reared insects (gallers + parasitoids) for each site by time combination. To test how parasitoid richness and parasitism rate varied across sampling occasions, and how this was related to median temperature, we used generalized linear mixed effects models (GLMMs) with Poisson (for count data) or binomial (for proportional parasitism rate data) error distributions. We included site as a random effect, to allow for non-independence of multiple samples per site, and sampling occasion as a categorical fixed predictor. To account for any effects of abundance on parasitoid richness and parasitism rate, we included the log of total reared insect abundance (gallers + parasitoids) as a covariate. We compared random intercept models to random intercept and slope models that also allowed for variation in the slope of the response, by calculating corrected Akaike (AICc) information statistics (Hurvich & Tsai 1989). In all cases, AICc values were lower for the random intercept model. Accordingly, this simpler model was used in further analyses. The fit of maximal models with either elevation or median temperature as fixed effects was also compared using AICc. In all cases, AICc values were lower for temperature, which was then used in all further analyses. Previous studies have shown that temperature is the main driver of insect assemblage patterns along elevational gradients (Hodkinson 2005; Hall *et al.* 2014). In this paper, we use 'temperature' hereafter as it is the elevation-related environmental variable which explained the greatest amount of variation. Model simplification was carried out to obtain the most parsimonious model for each dependent variable using likelihood ratio tests and calculated *P*-values from a chi-square distribution using the ANOVA function. We tested for overdispersion in GLMMs and for any obvious deviations from homoscedasticity and normality in all models. Models that were significantly overdispersed were fitted with an observation level random effect (OLRE) and retested.

To determine the effect of parasitoid richness and variability on variability in parasitism rate, we used two general linear models (GLMs) with backward stepwise elimination. The response variable was the coefficient of variation (CV) in parasitism rates (arcsine square root transformed to approximate assumptions of normality), with temperature as a fixed factor. The CV was calculated by dividing the standard deviation by the mean for each site. Interaction effects between temperature and covariables were included in the models. The first model used the mean species richness of parasitoids per elevation as a covariable. The second model used the variability (CV) in parasitoid species richness across months as a covariable, to determine whether stability in richness promotes stability in parasitism rate.

## RESULTS

### The effect of temperature on richness and parasitism

A total of 1902 insects, including 1593 parasitoids, were reared from 12 402 galls collected across the 15 sampling sites and six occasions. Average total rearing success rate across all sites was 14%, ranging between 6 and 28%, and was not correlated with elevation (Pearson's correlation coefficient = 0.18).

Mean parasitoid species richness (per sampling occasion) did not change significantly with median temperature (Table 2a). In contrast, parasitism rate showed significant variation with temperature, and this was not consistent across sampling occasions (i.e. there was a time × temperature interaction effect) (Appendix S3). Parasitism rate was positively related to temperature on four of the six sampling occasions (Fig. 2). The results for December showed the opposite pattern, and there was no change in parasitism rates during February (Fig. 2).

doi:10.1111/aec.12283
© 2015 Ecological Society of Australia

**Table 2.** Results for GLMMs for field observations on the relationships between median temperature, parasitoid richness and parasitism rate (a), and between richness and parasitism (b). *P*-values obtained from likelihood ratio tests comparing the minimum model to a null model. Sampling occasion is represented by the time variable in each model.

| Response variable | Minimum model | d.f. | $\chi^2$ | *P* |
|---|---|---|---|---|
| (a) Temperature × time | | | | |
| Richness | Temperature + log (insect abundance) + (1 \| site) | 1 | 0.077 | 0.781 |
| Parasitism rate† | Temperature × time + (1 \| site) + (1 \| OLRE) | 11 | 26.896 | **0.005** |
| (b) Parasitism–richness | | | | |
| Parasitism rate† | Temperature × richness + (1 \| site) + (1 \| OLRE) | 3 | 8.035 | **0.045** |

†Observation level random effect (OLRE) added to account for overdispersion. Bold terms indicate a significant effect of temperature ($P < 0.05$).

GLMM, generalized linear mixed effects model.

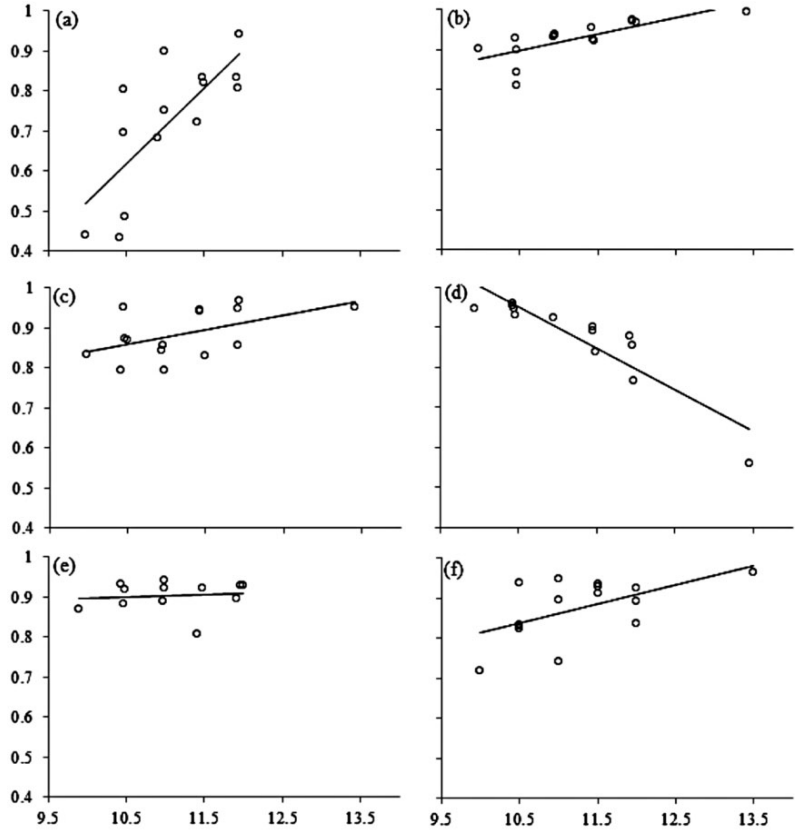



**Fig. 2.** Median temperature at each site *versus* parasitism rate for March 2013 (a), June 2013 (b), October 2013 (c), December 2013 (d), February 2014 (e) and May 2014 (f). Open circles represent fitted values from the GLMM of the parasitism–temperature relationship for each site. Lines represent the linear relationship between median temperature at each site and fitted values for parasitism rate for each sampling occasion. $n = 15$ for each sampling occasion except March 2013, December 2013 and February 2014, where $n = 14$, 13 and 12, respectively.

### Parasitism–richness relationship

There was a significant relationship between parasitism rate per month and mean parasitoid richness (Table 2b). However, the relationship had a significant interaction with temperature (Table 2b), such that the parasitism–richness relationship changed from being positive at higher temperatures, to negative at lower temperature sites (Fig. 3).

### Temporal variability in the parasitism–richness relationship

Variability in parasitism rate showed a negative relationship with mean parasitoid richness, such that sites with high mean parasitoid richness showed less variability (i.e. a lower CV) in parasitism rate (Fig. 4a). However, the final model, which included temperature, was not significant (Table 3a).

© 2015 Ecological Society of Australia
doi:10.1111/aec.12283

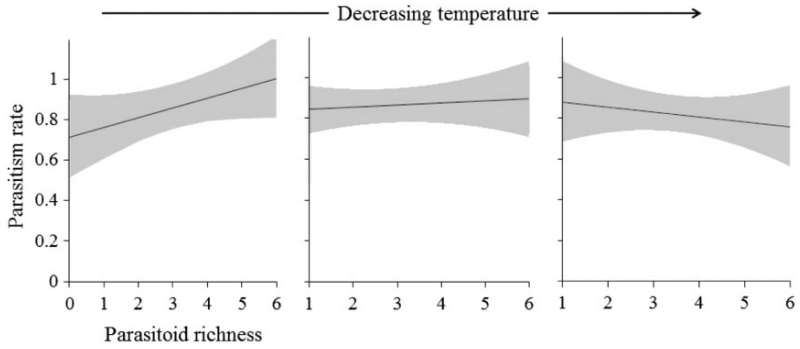



**Fig. 3.** The change in the relationship between parasitoid species richness and parasitism rate with median temperature per site and sampling occasion. Sites are separated into three temperature categories for visual clarity (high ≥12°C, $n = 22$; medium between 12°C and 10.5°C, $n = 33$; low ≤10.5°C, $n = 29$), ordered according to decreasing temperature from left to right. Analyses, however, treated temperature as a continuous variable. Black lines represent fitted values from linear models of the richness–parasitism relationship, grey areas show the upper and lower 95% confidence intervals.

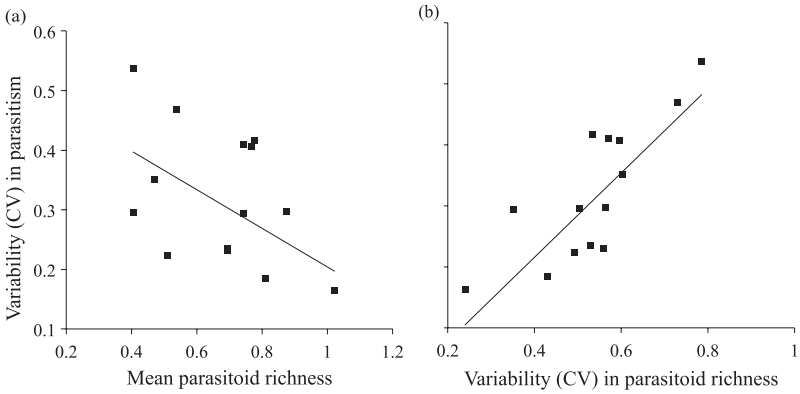



**Fig. 4.** The relationship between the mean number of parasitoid species per site and the coefficient of variation (CV) in parasitism rates (a) and the relationship between the CV in parasitoid richness and CV in parasitism rates per site (b).

**Table 3.** Results from general linear models testing effects of (a) mean parasitoid richness per site and (b) temporal variability (CV) in parasitoid richness on the temporal variability (CV) in parasitism rate. NS indicates non-significant interactions removed from the models during backward stepwise elimination. Final models are shown with F and adjusted $R^2$ values. Bold terms indicate a significant effect of the predictor variable ($P < 0.05$).

| Predictor variable | d.f. | t | *P* | $R^2$ |
|---|---|---|---|---|
| (a) CV parasitism ~ Mean richness | | | | |
| Temperature | 1 | −1.397 | 0.188 | |
| Mean richness | 1 | −2.196 | **0.048** | |
| Temperature × richness | | | NS | |
| Final model: Temperature + mean richness | 12 | F = 3.01 | 0.087 | 0.22 |
| (b) CV parasitism ~ CV richness | | | | |
| Temperature | 1 | −1.319 | 0.211 | |
| CV Richness | 1 | 3.816 | **0.002** | |
| Temperature × CV richness | | | NS | |
| Final model: Temperature + CV richness | 12 | F = 8.23 | **0.006** | 0.51 |

In contrast, variability in parasitism rate was positively correlated with variability (CV) in parasitoid richness (Table 3b, Fig. 4b), such that sites with greater variability in parasitoid richness also had a more variable parasitism rate. In both models temperature and its interaction with the main effect were not significant; however, temperature was still included in final models.

doi:10.1111/aec.12283
© 2015 Ecological Society of Australia

## DISCUSSION

We found either partial or full support for our three hypotheses. Elevation driven differences in temperature had a significant effect on parasitism rate, but this changed through time. Our results provide rare field-based evidence that temperature affects richness/functional relationships significantly. However, the positive relationship between temporal variability in parasitism rate and variability in parasitoid richness remained unaffected by temperature. Despite collecting a large number of galls, we observed a low rearing success rate. Similarly, Ribeiro and Basset (2007) found that only 5.4 and 15.6% of galls contained live larvae in consecutive years.

### The effect of temperature on parasitoid richness and parasitism rate

We found a significant decrease in parasitism with decreasing temperatures at high elevations, but not parasitoid richness. In contrast to our results, a recent meta-analysis found a significant decline in both parasitoid species richness and parasitism rates with increasing elevation (Péré *et al.* 2013). Linear decreases in parasitism rate with increasing elevation have also been found in the same study area in both leaf-miners (Maunsell *et al.* 2014) and cavity nesting Hymenoptera (Morris *et al.* 2014). These authors found that parasitism rates reached zero above 1000 m in elevation. In contrast, parasitism rate remained relatively high in our study, possibly due to the number of endoparasitoids in our system. Endoparasitoids develop within the host's body and, as such, may be better protected from cooler temperatures compared with ectoparasitoids (Quicke 1997). Among the parasitoids encountered, the three species of Platygasterinae belong to a known endoparasitic group (Austin *et al.* 2005; CSIRO Australia 2005). In addition, the *Zaommomiella* species, which had previously unknown biology, is also suspected to be endoparasitic (Bouček 1988). Péré *et al.* (2013) found that among a variety of parasitoid lifestyles, only endoparasitoids on endophagous hosts did not show a decline in species richness with elevation. In addition, it is possible that ectoparasitoids, which are typically more generalist compared to endoparasitoids, are more affected by possible absence of alternative host species at higher elevations (Gaston & Williams 1996).

We found significant temporal variation in parasitism rates, with December showing the opposite pattern compared with the other sampling occasions. Short-term seasonal differences in parasitoid assemblage turnover have also been recorded from the same study area (Hall *et al.* 2014). Tylianakis *et al.* (2006) found significant temporal variation in parasitism rates of cavity nesting Hymenoptera across a habitat gradient. This highlights the importance of examining host–parasitoid relationships across temporal scales, as pooling data neglect the importance of temporal turnover (Tylianakis *et al.* 2006).

### Parasitism ~ richness relationship

We found support for our second hypothesis of a significant relationship between parasitoid richness and parasitism rate (an ecosystem function). Such a relationship has also been found in assemblages of cavity-nesting Hymenoptera across habitat types (Tylianakis *et al.* 2006) and in coffee agro-forests (Veddeler *et al.* 2010). Positive relationships between species richness and measures of ecosystem function are thought to be a result of the effects of multiple species increasing functional diversity (Tylianakis *et al.* 2006). In this way a more species-rich and functionally diverse guild of parasitoids may lead to increased parasitism rates through increased and complementary use of host niche space (Tylianakis *et al.* 2006). In contrast, both Macfadyen *et al.* (2011) and Rodríguez and Hawkins (2000) found no relationship between species richness and parasitism rates. However, the latter two studies pooled long-term data, neglecting the importance of temporal turnover in parasitoid species (Tylianakis *et al.* 2006).

Perhaps of more importance, we found that the richness–parasitism relationship was dependent on temperature, such that, on average, the relationship shifted from being positive at warmer temperatures to increasingly negative at cooler temperatures. Theoretical modelling has shown that there may be no general diversity–functional relationship as the ecosystem services provided by each species depends on the environmental context (Cardinale *et al.* 2000). These authors found that spatial heterogeneity strengthened the richness–ecosystem functioning relationship due to greater complementary habitat use by different species. Habitat complexity generally decreases with elevation (Diamond 1988; Vázquez-Garcia & Givnish 1998), which may explain our observed decrease in the slope of the richness–parasitism relationship at cooler, high elevation sites.

### Temporal variability in the parasitism–richness relationship

We found only partial support for the third hypothesis of relationship between temporal variability in species richness and temporal variability in parasitism. Temporal variability of parasitism rate decreased with increasing species richness but not significantly. We did, however, find a positive relationship between temporal variability in parasitoid species richness and tem-

© 2015 Ecological Society of Australia
doi:10.1111/aec.12283

poral variability in parasitism rates as did Tylianakis *et al*. (2006) and Veddeler *et al*. (2010). A more temporally consistent functional guild is more likely to provide a constant ecosystem service (Veddeler *et al*. 2010). In addition, a more functionally diverse guild may act as biological insurance for ecosystem services through an increased ability to compensate for individual species fluctuations (Thébault & Loreau 2005).

Surprisingly, and in contrast to the richness–parasitism relationship, the relationship between temporal variability of both species richness and parasitism rate was constant across the temperature gradient. In an analogous result, Tylianakis *et al*. (2006) found that habitat type had no effect on the relationship between temporal stability in richness and parasitism. In contrast, Macfadyen *et al*. (2011) found significantly less temporal variation in parasitism rate on organic compared with conventional farms.

## CONCLUSIONS

We have demonstrated that the positive richness–function relationship in a multi-trophic system is significantly affected by changes in temperature along an elevation gradient. The temporal stability of the relationship, however, is less affected. It is an important observation that stability in species richness is able to maintain ecosystem stability despite changes in temperature. Parasitoid richness and the ecosystem functions it supports may vary across temperature gradients and through time. Understanding of the processes affecting these relationships is essential for conservation and management (Loreau *et al*. 2003). Future changes in climatic conditions may significantly change the richness–parasitism relationship. The temporal stability in the ecosystem services provided by parasitoids, however, may be more resilient to change. The extent to which our results can be generalized to fit other diversity/functional relationships remains unclear as parasitoids are very host-specific compared with other guilds of predators. Only further field-based experimental studies under different climatic conditions will throw light on such relationships.

## ACKNOWLEDGEMENTS

This study was funded by the Griffith University School of Environment PhD program. We thank T. Kellermann, C. Kindermann, E. Window, K. Dalcin and S. Maunsell for their assistance in the field. B. McDonald identified the plant species, for which we are most grateful. We also thank S. Maunsell and L. Sam for their support and feedback on analysis during our R Geeks discussion groups. This study was carried out under National Parks and Wildlife Scientific Licence SL101083 and Scientific Purposes Permit WITK12222212.

## REFERENCES

Ashton L. A., Kitching R. L. & Maunsell S. C. (2011) Macrolepidopteran assemblages along an altitudinal gradient in subtropical rainforest–exploring indicators of climate change. *Mem. Queensl. Mus.* **55,** 375–90.

Austin A. D., Johnson N. F. & Dowton M. (2005) Systematics, evolution, and biology of scelionid and platygastrid wasps. *Annu. Rev. Entomol.* **50,** 553–82.

Bates D., Maechler M. & Bolker B. (2013) lme4: Linear mixed-effects models using S4 classes. R package, version 0.999999-0. [online]. [Cited 13 December 2014]. Available from URL: http://CRAN.R-project.org/package=lme4

Bean A. R. (2004) The taxonomy and ecology of *Solanum* subg. *Leptostemonum* (Dunal) Bitter (Solanaceae) in Queensland and far north-eastern New South Wales, Australia. *Austrobaileya* **6,** 639–816.

Bonsall M. B., Hassell M. P. & Asefa G. (2002) Ecological trade-offs, resource partitioning, and coexistence in a host-parasitoid assemblage. *Ecology* **83,** 925–34.

Bouček Z. (1988) *Australasian Chalcidoidea (Hymenoptera). A Biosystematic Revision of Genera of Fourteen Families, with a Reclassification of Species*. CAB International, Wallingford.

Burwell C. J. & Nakamura A. (2011) Distribution of ant species along an altitudinal transect in continuous rainforest in subtropical Queensland, Australia. *Mem. Queensl. Mus.* **55,** 391–412.

Cardinale B. J., Nelson K. & Palmer M. A. (2000) Linking species diversity to the functioning of ecosystems: on the importance of environmental context. *Oikos* **91,** 175–83.

Cornell H. V. (1983) The secondary chemistry and complex morphology of galls formed by the Cynipinae (Hymenoptera): why and how? *Am. Midl. Nat.* **110,** 225–34.

CSIRO Australia (2005) Entedoninae. *Liriomyza Parasitoids in Southeast Asi*a. [online]. [Cited 11 January 2015]. Available from URL: http://www.ento.csiro.au/science/Liriomyza_ver3/key/Liriomyza_Parasitoids_Key/Media/Html/entedoninae.html

de Sassi C., Lewis O.T. & Tylianakis J. M. (2012) Plant-mediated and nonadditive effects of two global change drivers on an insect herbivore community. *Ecology* **93,** 1892–901.

Diamond J. (1988) Factors controlling species diversity: overview and synthesis. *Ann. Mo. Bot. Gard.* **75,** 117–29.

Enkegaard A. (1994) Temperature dependent functional response of *Encarsia formosa* parasitizing the Poinsettia-strain of the cotton whitefly, *Bemisia tabaci*, on Poinsettia. *Entomol. Exp. Appl.* **73,** 19–29.

Escobar F., Lobo J. M. & Halffter G. (2005) Altitudinal variation of dung beetle (Scarabaeidae: Scarabaeinae) assemblages in the Colombian Andes. *Glob. Ecol. Biogeogr.* **14,** 327–37.

Gaston K. J. & Williams P. H. (1996) Spatial patterns in taxonomic diversity. In: *Biodiversity: A Biology of Numbers and Difference* (ed. K. J. Gaston) pp. 202–29. Blackwell Science Oxford, Oxford.

Hackett-Jones E., Cobbold C. & White A. (2009) Coexistence of multiple parasitoids on a single host due to differences in parasitoid phenology. *Theor. Ecol.* **2,** 19–31.

Hall C. R., Burwell C. J., Nakamura A. & Kitching R. L. (2014) Altitudinal variation of parasitic Hymenoptera assemblages in Australian subtropical rainforest. *Aust. Entomol.*. doi: 10.1111/aen.12114

doi:10.1111/aec.12283

© 2015 Ecological Society of Australia

Harden G. J., McDonald B., McDonald W. J. F. & Williams J. B. (2006) *Rainforest Trees and Shrubs: A Field Guide to Their Identification in Victoria, New South Wales and Subtropical Queensland Using Vegetative Features*. Gwen Harden Publishing, Nambucca Heads.

Harvey J. A., Poelman E. H. & Tanaka T. (2013) Intrinsic inter- and intraspecific competition in parasitoid wasps. *Annu. Rev. Entomol.* **58,** 333–51.

Hodkinson I. D. (2005) Terrestrial insects along elevation gradients: species and community responses to altitude. *Biol. Rev. Camb. Philos. Soc*. **80,** 489–513.

Hooper D. U., Chapin F. S. III, Ewel J. J. *et al*. (2005) Effects of biodiversity on ecosystem functioning: a consensus of current knowledge. *Ecol. Monogr.* **75,** 3–35.

Hurvich C. M. & Tsai C. L. (1989) Regression and time series model selection in small samples. *Biometrika* **76,** 297–307.

Körner C. (2007) The use of 'altitude' in ecological research. *Trends Ecol. Evol.* **22,** 569–74.

Laidlaw M. J., McDonald W., Hunter R. J. & Kitching R. L. (2011) Subtropical rainforest turnover along an altitudinal gradient. *Mem. Queensl. Mus.* **55,** 271–90.

LaSalle J. & Gauld I. D. (1993) Hymenoptera: their biodiversity, and their impact on the diversity of other organisms. In: *Hymenoptera and Biodiversity* (eds J. LaSalle & I. D. Gauld) pp. 1–26. CAB International, Wallingford.

Lehman C. L. & Tilman D. (2000) Biodiversity, stability, and productivity in competitive communities. *Am. Nat.* **156,** 534–52.

Loreau M., Mouquet N. & Gonzalez A. (2003) Biodiversity as spatial insurance in heterogeneous landscapes. *Proc. Natl. Acad. Sci. U.S.A.* **100,** 12765–70.

Loreau M., Naeem S., Inchausti P. *et al*. (2001) Biodiversity and ecosystem functioning: current knowledge and future challenges. *Science* **294,** 804–8.

McDonald B. (2010) The Tweed caldera group. In: *Remnants of Gondwana: A Natural and Social History of the Gondwana Rainforests of Australia* (eds R. L. Kitching, R. Braithwaite & J. Cavanaugh) pp. 39–50. Surrey Beatty and Sons, Sydney.

Macfadyen S., Craze P. G., Polaszek A., van Achterberg K. & Memmott J. (2011) Parasitoid diversity reduces the variability in pest control services across time on farms. *Proc. Biol. Sci.* **278,** 3387–94.

Maunsell S. C., Kitching R. L., Burwell C. J. & Morris R. J. (2014) Changes in host-parasitoid food web structure with elevation. *J. Anim. Ecol.* **84,** 353–63. doi: 10.1111/1365-2656.12285

Menon A., Flinn P. W. & Dover B. A. (2002) Influence of temperature on the functional response of *Anisopteromalus calandrae* (Hymenoptera: Pteromalidae), a parasitoid of *Rhyzopertha dominica* (Coleoptera: Bostrichidae). *J. Stored Prod. Res.* **38,** 463–9.

Morris R. J., Sinclair F. H. & Burwell C. J. (2014) Food web structure changes with elevation but not rainforest stratum. *Ecography*. doi: 10.1111/ecog.01078

Péré C., Jactel H. & Kenis M. (2013) Response of insect parasitism to elevation depends on host and parasitoid life-history strategies. *Biol. Lett.* **9,** 20130028. doi:10.1098/rsbl.2013.0028

Pickett S. (1989) Space-for-time substitution as an alternative to long-term studies. In: *Long Term Studies in ecology* (ed. G. E. Likens) pp. 110–35. Springer-Verlag, Berlin.

Quicke D. L. J. (1997) *Parasitic Wasps*. Chapman & Hall Ltd, London.

R Development Core Team (2014) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna.

Randall M. G. M. (1982) The ectoparasitization of *Coleophora alticolella* (Lepidoptera) in relation to its altitudinal distribution. *Ecol. Entomol.* **7,** 177–85.

Reymond A., Purcell J., Cherix D., Guisan A. & Pellissier L. (2013) Functional diversity decreases with temperature in high elevation ant fauna. *Ecol. Entomol.* **4,** 364–73.

Ribeiro S. P. & Basset Y. (2007) Gall-forming and free-feeding herbivory along vertical gradients in a lowland tropical rainforest: the importance of leaf sclerophylly. *Ecography* **30,** 663–72.

Rodríguez M. Á. & Hawkins B. A. (2000) Diversity, function and stability in parasitoid communities. *Ecol. Lett.* **3,** 35–40.

Snyder G. B., Finke D. L. & Snyder W. E. (2008) Predator biodiversity strengthens aphid suppression across single- and multiple-species prey communities. *Biol. Control* **44,** 52–60.

Sorribas J., Rodríguez R. & Garcia-Mari F. (2010) Parasitoid competitive displacement and coexistence in citrus agroecosystems: linking species distribution with climate. *Ecol. Appl.* **20,** 1101–13.

Stireman J. O. 3rd, Dyer L. A., Janzen D. H. *et al*. (2005) Climatic unpredictability and parasitism of caterpillars: implications of global warming. *Proc. Natl. Acad. Sci. U.S.A.* **102,** 17384–7.

Straub C. S., Finke D. L. & Snyder W. E. (2008) Are the conservation of natural enemy biodiversity and biological control compatible goals? *Biol. Control* **45,** 225–37.

Thébault E. & Loreau M. (2005) Trophic interactions and the relationship between species diversity and ecosystem stability. *Am. Nat.* **166,** E95–114.

Tylianakis J. M., Tscharntke T. & Klein A.-M. (2006) Diversity, ecosystem function, and stability of parasitoid-host interactions across a tropical habitat gradient. *Ecology* **87,** 3047–57.

Vázquez-Garcia J. A. & Givnish T. J. (1998) Altitudinal gradients in tropical forest composition, structure, and diversity in the Sierra de Manantlán. *J. Ecol.* **86,** 999–1020.

Veddeler D., Tylianakis J., Tscharntke T. & Klein A.-M. (2010) Natural enemy diversity reduces temporal variability in wasp but not bee parasitism. *Oecologia* **162,** 755–62.

Virtanen T. & Neuvonen S. (1999) Performance of moth larvae on birch in relation to altitude, climate, host quality and parasitoids. *Oecologia* **120,** 92–101.

Weiss S. B., Murphy D. D. & White R. R. (1988) Sun, slope, and butterflies: topographic determinants of habitat quality for *Euphydryas editha*. *Ecology* **69,** 1486–96.

## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Appendix S1.** Photos of dissected cecidomyiid pupae from two plant species.

**Appendix S2.** Total gall, parasitoid and cecidomyiid abundance for each host plant species at each site.

**Appendix S3.** Full coefficient table for model of changes in parasitism rate through time at different temperatures with binomial error distribution.

© 2015 Ecological Society of Australia

doi:10.1111/aec.12283

## S4 Supplementary material


**Figure S4.1** (a) dissected *Rubus nebulosus* containing a cecidomyiid pupa, and (b) dissected *Solanum inaequilaterum* gall with cecidomyiidae pupa extracted from pupal chamber. Photos by C Hall.

**Table S4.1** Full coefficient table for model of changes in parasitism rate through time at different temperatures with binomial error distribution. A colon between two variables indicates an interaction effect. Asterisks indicate significant effects ($P$<0.05).

| | **Estimate** | **Std. error** | **z value** | **P(>\|z\|)** |
|---|---|---|---|---|
| (Intercept) | 11.58 | 5.438 | 2.129 | 0.03323 * |
| temp | -9.403 | 5.359 | -1.755 | 0.07932 |
| Feb-14 | -10.105 | 8.222 | -1.229 | 0.21906 |
| Jun-13 | -18.599 | 7.645 | -2.433 | 0.01498 * |
| Mar-13 | -21.504 | 6.953 | -3.093 | 0.00198 * |
| May-14 | -14.727 | 7.439 | -1.98 | 0.04773 * |
| Oct-13 | -14.257 | 6.548 | -2.177 | 0.02945 * |
| temp:Feb-14 | 10.266 | 8.321 | 1.234 | 0.21728 |
| temp:Jun-13 | 19.286 | 7.687 | 2.509 | 0.01212 * |
| temp:Mar-13 | 20.684 | 6.952 | 2.975 | 0.00293 * |
| temp:May-14 | 14.719 | 7.48 | 1.968 | 0.04909 * |
| temp:Oct-13 | 14.336 | 6.523 | 2.198 | 0.02797 * |

**Table S4.2** Total gall, parasitoid and cecidomyiid abundance for each host plant species at each site. Location refers to transect name: Brindle Creek (BC), Bar Mountain (BM), Lamington (LM), Tweed Road (TR) and Binna Burra (BB).

| Location | Elevation (m asl) | Plant host species | Gall abundance | Parasitoid abundance | Cecidomyiid abundance |
|---|---|---|---|---|---|
| BC | 947 | *R. nebulosus* | 13 | 1 | 0 |
| BC | 1059 | *R. nebulosus* | 751 | 197 | 59 |
| BM | 890 | *R. nebulosus* | 326 | 121 | 20 |
| BM | 994 | *R. nebulosus* | 44 | 7 | 3 |
| BM | 1145 | *R. nebulosus* | 155 | 49 | 2 |
| LM | 944 | *R. nebulosus* | 26 | 5 | 0 |
| LM | 1140 | *R. nebulosus* | 440 | 63 | 20 |
| TR | 964 | *R. nebulosus* | 128 | 25 | 9 |
| TR | 1098 | *R. nebulosus* | 299 | 85 | 22 |
| BB | 1083 | *R. nebulosus* | 118 | 5 | 0 |
| BC | 762 | *R. moorei* | 681 | 12 | 2 |
| BC | 947 | *R. moorei* | 330 | 7 | 0 |
| BC | 1059 | *R. moorei* | 40 | 3 | 0 |
| BM | 890 | *R. moorei* | 622 | 22 | 1 |
| BM | 994 | *R. moorei* | 248 | 25 | 0 |
| BM | 1145 | *R. moorei* | 259 | 0 | 4 |
| LM | 829 | *R. moorei* | 546 | 53 | 4 |
| LM | 944 | *R. moorei* | 95 | 19 | 1 |
| TR | 860 | *R. moorei* | 254 | 2 | 1 |
| TR | 964 | *R. moorei* | 192 | 5 | 1 |
| TR | 1098 | *R. moorei* | 749 | 67 | 5 |
| BB | 803 | *R. moorei* | 172 | 14 | 0 |
| BB | 952 | *R. moorei* | 470 | 30 | 5 |
| BB | 1083 | *R. moorei* | 110 | 5 | 6 |
| BC | 762 | *Solanum* | 194 | 31 | 9 |
| BC | 947 | *Solanum* | 261 | 41 | 9 |
| BC | 1059 | *Solanum* | 434 | 75 | 5 |
| BM | 890 | *Solanum* | 225 | 24 | 0 |
| BM | 994 | *Solanum* | 135 | 11 | 0 |
| BM | 1145 | *Solanum* | 422 | 62 | 14 |
| LM | 829 | *Solanum* | 229 | 16 | 4 |
| LM | 944 | *Solanum* | 613 | 65 | 10 |
| LM | 1140 | *Solanum* | 595 | 70 | 16 |
| TR | 860 | *Solanum* | 172 | 30 | 5 |
| TR | 964 | *Solanum* | 204 | 33 | 11 |
| TR | 1098 | *Solanum* | 389 | 55 | 31 |
| BB | 803 | *Solanum* | 231 | 47 | 3 |
| BB | 952 | *Solanum* | 429 | 76 | 6 |
| BB | 1083 | *Solanum* | 801 | 135 | 22 |

# Chapter 5

In the previous chapter we found that the positive relationship between parasitoid richness and parasitism rate was dependent on temperature. There is increasing evidence to suggest that temperature also has strong bottom-up effects on herbivores and their parasitoids through changes in host plant chemistry. In this chapter we examine the spatial differences in phytochemistry in galled and non-galled leaves along a temperature gradient. We also isolate and identify several defence compounds from the host plant species.

**Statement of contribution to a co-authored paper**

Chapter 5 is a co-authored paper that has been prepared for publication. The citation is as follows:

Hall, C.R., Carroll, A.R. (prepared manuscript) Gall induced changes in phytochemistry in three species of rainforest understory species across a temperature gradient.

My contribution to the paper involved data collection, analysis and writing of the manuscript. Tony Carroll was responsible for direction and guidance with regard to chemical separation and identification of compounds and editing of the manuscript.

(Signed) _____ (Date) 12/12/2015
Casey Hall

(Countersigned) _____ (Date) 12/12/2015
Associate supervisor: Tony Carroll

# Chapter 5

## Gall induced changes in the phytochemical profile in three rainforest understory species across a temperature gradient

Casey R. Hall[1*] and Anthony R. Carroll[1]

[1]Environmental Futures Research Institute, School of Environment, Griffith University, Nathan, QLD 4111

[*]Corresponding author: casey.hall@griffithuni.edu.au

## 5.1 Abstract

Plant populations exhibit phenotypic variation in secondary metabolites over their geographical ranges. The amount and spatial distribution of this variation in secondary metabolites can influence interactions with herbivores. In this study we investigate galling induced differences in phytochemistry of three rainforest understory species; *Solanum inaquilaterum*, *Rubus moorei* and *Rubus nebulosus,* over a temperature gradient. We used $^{1}$H NMR to characterize plant chemical profiles and HPLC and 2D NMR to separate and identify compounds of interest. Galled leaves of both *Rubus nebulosus* and *Solanum inaequilaterum* showed distinct variation in their chemical profile based on site temperature. We isolated four known triterpenes (maslinic acid, 2-oxopolomic acid and cis- and trans-*p*-coumaroyltormentic acid) from *R. nebulosus*, and two known compounds ($N^{1}$,$N^{8}$-bis(dihydrocaffeoyl) spermidine and N-methyl nicotinic acid) from *S. inaequilaterum*. $^{1}$H NMR signals from these compounds were found to be indicative of the observed differences in the phytochemistry of galled leaves. In this study, we have shown that gall induced changes in the phytochemical profile of three rainforest plants is mediated by site temperature. Our results have led us to identify several secondary metabolites that may be responding to both abiotic and biotic pressures in each plant species. This may lead to new insights into the roles of these compounds in plant defence and environmental adaptation.

## 5.2 Introduction

Plants produce a wide array of secondary metabolites and this diversity is thought to be the result of their coevolution with natural enemies such as pathogens and herbivores (Ehrlich and Raven 1964). Plants express both inter and intra-specific differences in mixtures of the secondary metabolites they produce, and also show differences in total and relative concentrations of individual compounds (Moore et al. 2014). As a result, individuals within plant populations exhibit phenotypic variation in secondary metabolites over their geographical ranges (Brenes-Arguedas and Coley 2005). The amount and spatial distribution of this variation in secondary metabolites can influence interactions with herbivores (Richards et al. 2015; Lankau and Strauss 2008), and accordingly can have an ecologically significant effect on community composition (Dyer et al. 2014). In this study we investigate the spatial differences in secondary metabolites of three co-occurring rainforest understory species, *Solanum inaquilaterum, Rubus moorei* (F. Muell.) and *Rubus nebulosus* (A.R. Bean).

*Rubus* is a large genus within the Rosaceae, containing around 700 species (Alice and Campbell 1999). The genus has a long history of traditional uses, not only as a food but also as medicines (Patel et al. 2004), and several species are grown commercially. As such the phytochemistry of the genus is relatively well documented (see Patel et al. 2004 for a review). Most species investigated contain a diverse mix of secondary metabolites. *Rubus* leaves have been found to be especially rich in tannins (ellagic acid), flavanoid derivatives of kaempferol and quercetin and phenolic acids. They are also rich in terpenoid compounds, including monoterpenes (α-pinene, β-myrcene and limonene), several diterpene glycosides (rubusoside and labdane- and kauren-type) and ursane type triterpenes (Patel et al. 2004). *Solanum* is a large genus in the Solanaceae with approximately 1700 species (Weese and Bohs 2007). Several species in this genus have a long history of traditional medicinal use while others are important food species (Amir and Kumar 2004). Various chemical constituents have been reported from this genus such as alkaloids (solasidine), phenolics, flavonoids (scopoletin), sterols, saponins and their glycosides (see Amir and Kumar 2004 for a review).

Many of these compounds potentially play a role in plant defence. Many terpenoids, for example, are specifically induced in response to herbivore damage (Cheng et al. 2007).

Other compound classes also show potent anti herbivore effects, although these compounds can also be used as host plant cues by specialist herbivores. For example, the unique glycoalkaloid profile found in some *Solanum* species is used as cues by specialist herbivores which have evolved adaptations to these otherwise toxic compounds (Altesor et al. 2014). Galling insects are a unique guild of specialist herbivores that are known to actively manipulate plant secondary compounds (Chapter 2). This varies, however, depending on the species involved and the type of chemical defence. Galling insects for example, generally increase concentrations of phenolics and tannins while having no effect on volatile compounds (Chapter 2).

The specific aims in this study were to characterise galling-induced differences in phytochemistry of three plant species over a temperature gradient. The three plant species are galled by two species of Cecidomyiidae (Diptera). They co-occur in the understory of subtropical rainforest in south-east Queensland and northern New South Wales. Investigation of two closely related and one different plant species allowed us to look at similarities and differences both among and between genera. Leaf chemistry is used here as an indicator of possible changes to plant-insect interactions over a temperature gradient.

The ecological study of secondary metabolites has traditionally been limited to either broad classes of compounds, such as phenolics, or the identification of single well known compounds (Moore et al. 2013). However, it is likely that more than one compound is involved in any specific plant-insect interaction. Nuclear magnetic resonance spectroscopy (NMR) was the analytical method of choice to study the chemical profile of the plants because of its discriminating power to differentiate functional groups within different molecules, identify the structures of specific compounds and the proportion of different molecules present within a mixture. $^{1}$H NMR measures the specific electron environment surrounding each proton in a compound while the intensity of individual proton signals relative to each other in a spectrum allows one to identify and quantify mixtures of compounds in an extract (Figure S5.1). As such $^{1}$H NMR spectra can be compared across a range of samples and this can be useful to detect small changes across the entire plant metabolome (Krishnan et al. 2004). NMR enables quantification of metabolites through integrations of their corresponding $^{1}$H NMR signals. This method can determine the composition of plant

chemical constituents in a crude extract without the need for separation techniques or the use of standards for each compound. This method is also useful to highlight regions of the spectra correlating to potential compounds of interest. Further 2D NMR techniques can be used to identify these compounds.

## 5. 3 Methods

*Study system*

Galled and non-galled leaves from the three target plant species were sampled during April and October 2013 at 15 sites within Lamington and Border Ranges National Parks in the Macpherson Ranges. These national parks span the Queensland/New South Wales border (see Chapter 3 for a map of study sites). The three focal plant species; *Solanum inaequilaterum*, *Rubus moorei* and *Rubus nebulosus* co occur in the understory of subtropical rainforest. Both *Rubus* species are galled by Cecidomyiidae sp. 1 and *S. inaequilaterum* is galled by *Dasineura* sp. These and the host plants are described in further detail in Chapters 3 and 4.

*Chemical analysis of crude extract*

For chemical analysis both galled and non-galled leaves were collected from each plant across two sampling occasions (March and October 2013). The samples were dried with silica and transported to Griffith University, Gold Coast campus. Leaf samples were ground to a fine powder. Approximately 0.1 g was transferred to a test tube and combined with 10 mL MeOH. The samples were sonicated for 15 minutes and the supernatant was transferred into a separate test tube. This step was repeated twice more and the supernatants combined and evaporated. The dried extract from each leaf sample was then dissolved in 600 uL of deuterated dimethyl sulfoxide (DMSO) and transferred to an NMR tube. The $^{1}$H NMR spectra were obtained at 25°C on a 600 MHz Varian NMR spectrometer. The parameters used were 16 scans and a spectral width of 15 ppm (from $\delta_H$ 0-15 ppm).

*Statistical analysis of crude extract*

Data from both sampling occasions were combined for all further analyses. The NMR spectroscopic data were processed using MestReNova software (version 10.0.2-15465,

Mestrelab Research S.L. 2015). NMR spectra were processed using common metabolomics methods (Kim et al. 2010). The chemical shift and integration values of the peaks observed in the spectra were used to create a multivariate data set that was subsequently analysed using principle component analysis (PCA). This method has two functions; it groups the most similar samples and identifies regions of the spectra that are contributing most to differences among the groups (Wishart 2008). Signals in different regions of the $^{1}H$ NMR spectra can be related to different types of compounds (see Figure S5.1). This data can then be used as a guide to find compounds within the mixture that are responsible for the similarities and difference within groups. These compounds can then be isolated using separation techniques, such as HPLC and their molecular structures identified using 2D NMR techniques. Spectra were aligned using the solvent peak (DMSO), base-line corrected, phase corrected, normalized to a total area of 100 and integrated into 0.04 ppm bins corresponding to the region of $\delta_H$ 0.0-12.5 for the two *Rubus* species and $\delta_H$ 0.0-10.0 for the *Solanum* species. The regions between $\delta_H$ 3.0-4.0 and $\delta_H$ 2.48-2.52 were excluded from the analysis because of the residual signal of water and DMSO. Binning reduces the number of variables from potentially 30 000 to approximately 200 and reduces differences in signal fluctuations among samples (Kim et al. 2010). The data for each sample were exported and combined into one dataset for analysis. Principle component analysis (PCA) was performed in R with Pareto scaling (R development team 2015).

*HPLC separation and compound identification*

To separate individual compounds, HPLC separations were performed on crude leaf extracts. Bulk dry leaf material; 39.25 g of *R. nebulosus*, 38.3 g of *R. moorei* and 31.77 g of *S. inaequilaterum* was extracted by repeated sonication in MeOH (4 x 200mL). The MeOH extracts for each plant were combined, evaporated and 1 g of the extract obtained from each species was adsorbed onto $C_{18}$ silica gel. The extract impregnated gel was placed in a HPLC pre-column cartridge (10 mm x 20 mm), connected in series to a $C_{18}$–bonded silica HPLC column (21 mm x 150 mm) and eluted with a gradient from 100% $H_2O$/0.1% TFA (trifluoroacetic acid) to 100% MeOH/0.1% TFA over 60 minutes at a flow rate of 9mL/min. Seventy fractions were collected, evaporated and every second fraction was further analysed by $^{1}H$ NMR spectroscopy. Data was acquired using a Bruker 500MHz NMR with 16 scans at 25°C. Fraction 24 from *S.*

*inaequilaterum* was semi-pure and analysed further using 2D NMR spectroscopy ($^1$H, COSY, HSQC, HMBC). Both *Rubus* species had similar compounds that were not separated fully using reverse phase HPLC. To further separate these compounds normal phase HPLC was undertaken on two fractions. These were obtained by combining fractions 28 to 38 and 40 to 62 from the $C_{18}$ separation of the extract from *R. nebulosus* as the compounds of interest were more concentrated in this species. This second HPLC separation involved a gradient from 100% Hexane to DCM (dichloromethane) to 20% MeOH in diol bonded silica gel (21mm x 150 mm). $^1$H NMR analysis of fractions obtained from this separation indicated that fraction 36 was pure and that fraction 48 contained a mixture of two isomers of two different compounds. Both of these fractions were analysed by 2D NMR ($^1$H, COSY, HSQC, HMBC).

## 5.4 Results

*Phytochemical profile*

A total of 173 samples, 76 from March and 97 from October, were analysed using $^1$H NMR. Overall 58 were from galled and 115 from non-galled leaves. Analysis using PCA was used to identify any groupings or trends in the dataset. Results from the PCA scores plot for non-galled leaves from *R. nebulosus* show no clear groupings in chemical profile based on site temperature (Figure 5.1a). The regions of the spectra correlating with the PC axes are shown as a loadings graph (Figure 5.1a). For galled leaves the PCA shows some clustering based on site temperature (Figure 5.1b). The loadings correlating with PC2 show the greatest difference between galled and non-galled leaves, particularly in the regions $\delta_H$ 4-5 and 6-7ppm (Figure 5.1a,b).

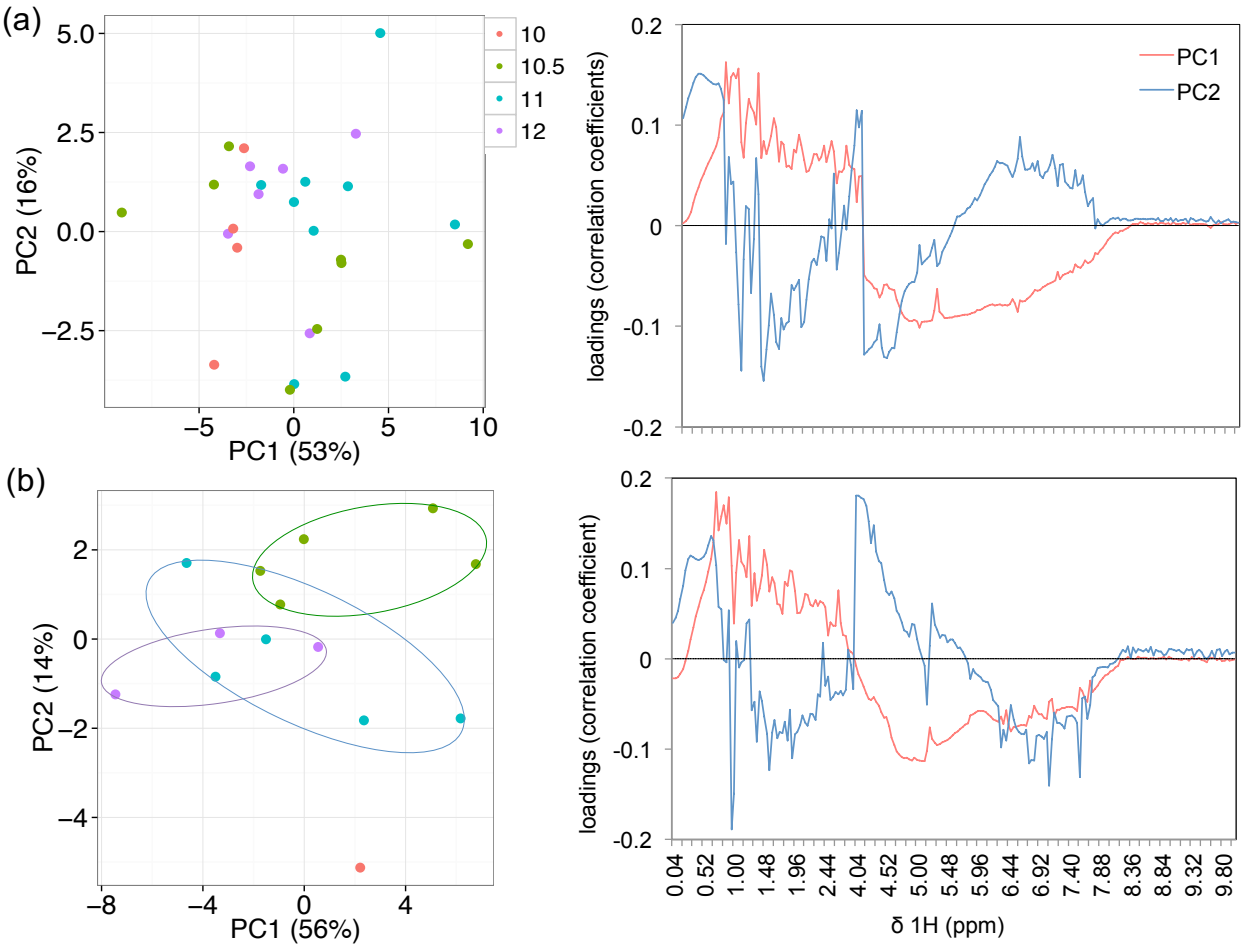



**Figure 5.1** PCA and loading plots for *Rubus nebulosus* (a) non galled and (b) galled leaves. Colours on PCA plots represent mean site temperatures (pink = 10°C, green = 10.5°C, blue = 11°C and purple = 12°C), loading correlations are represented by pink lines for PC1 and blue for PC2.

Results from the PCA scores plots for both galled and non-galled leaves from *R. moorei* show no clear groupings in chemical profile based on site temperature (Figure 5.2a,b). There are, however, changes in the loadings correlated with both PC1 and PC2 showing fewer correlations in the $\delta_H$ 0-1.5ppm region, and more in the $\delta_H$ 2-3ppm region of the sprectra (Figure 5.2b). The downfield signals, in the $\delta_H$ 6-8ppm region, correlating with PC1 in galled leaves are mostly associated with the cinnamate ester region of the *p*-coumaroyltormentic acid compounds we identified (Figure 5.4).

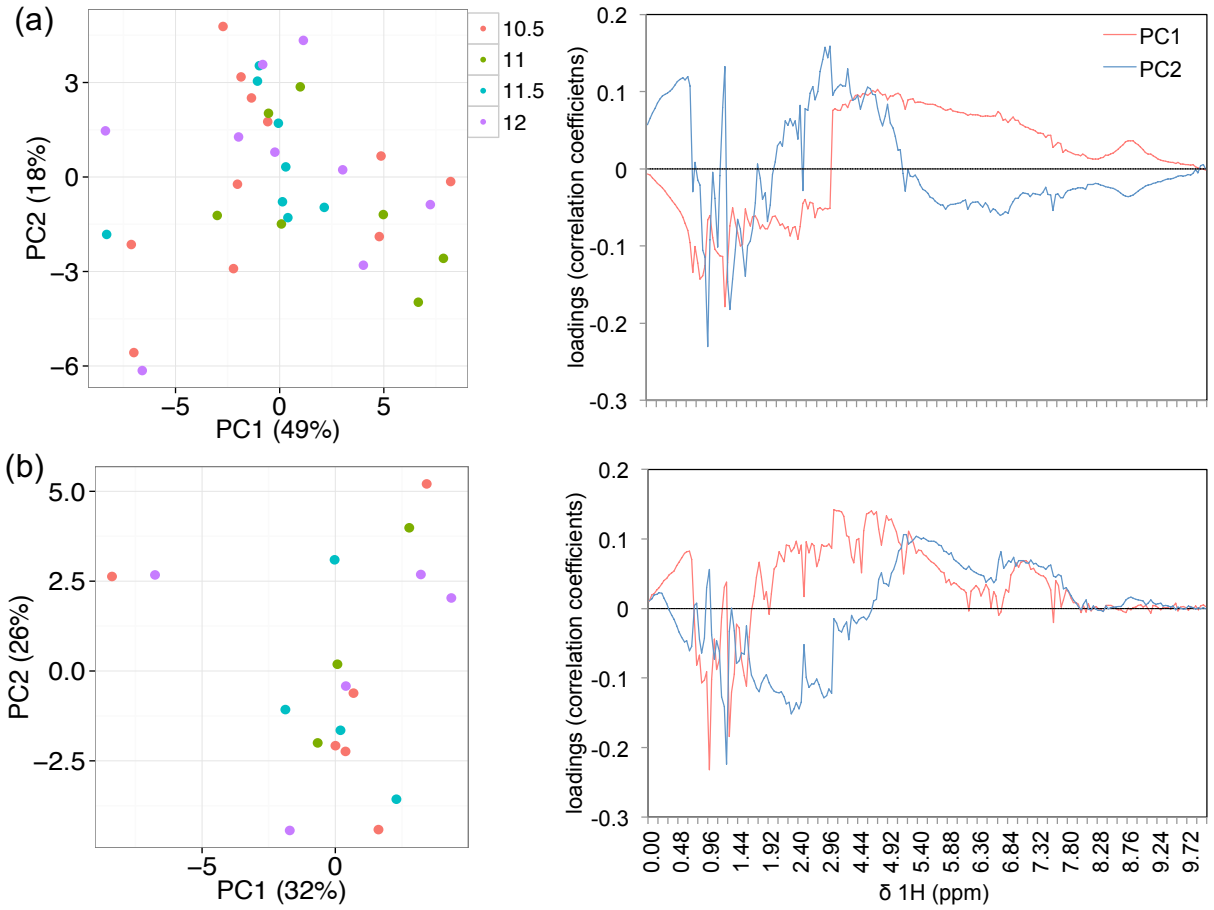



**Figure 5.2** PCA and loading plots for *Rubus moorei* (a) non galled and (b) galled leaves. Colours on PCA plots represent mean site temperatures (pink = 10°C, green = 10.5°C, blue = 11°C and purple = 12°C), loading correlations are respresented by pink lines for PC1 and blue for PC2.

Results from the plot os PCA scores for non-galled leaf phytochemistry for *S. inaequilaterum* show no clear groupings in chemical profile based on site temperature (Figure 5.3a). For galled leaves, however, the PCA shows some clustering based on site temperature (Figure 5.3b). The loadings graph shows increased correlations with PC1 in the $\delta_H$ 7-8 ppm region and PC2 in the $\delta_H$ 0.5-2 ppm region of the spectra for galled leaves compared to non galled (Figure 5.3a,b). The correlations with PC2 in both galled and non galled leaves at $\delta_H$ 6.5 ppm are directly related to the caffeoyl spermidine compound identified (Figure 5.5).

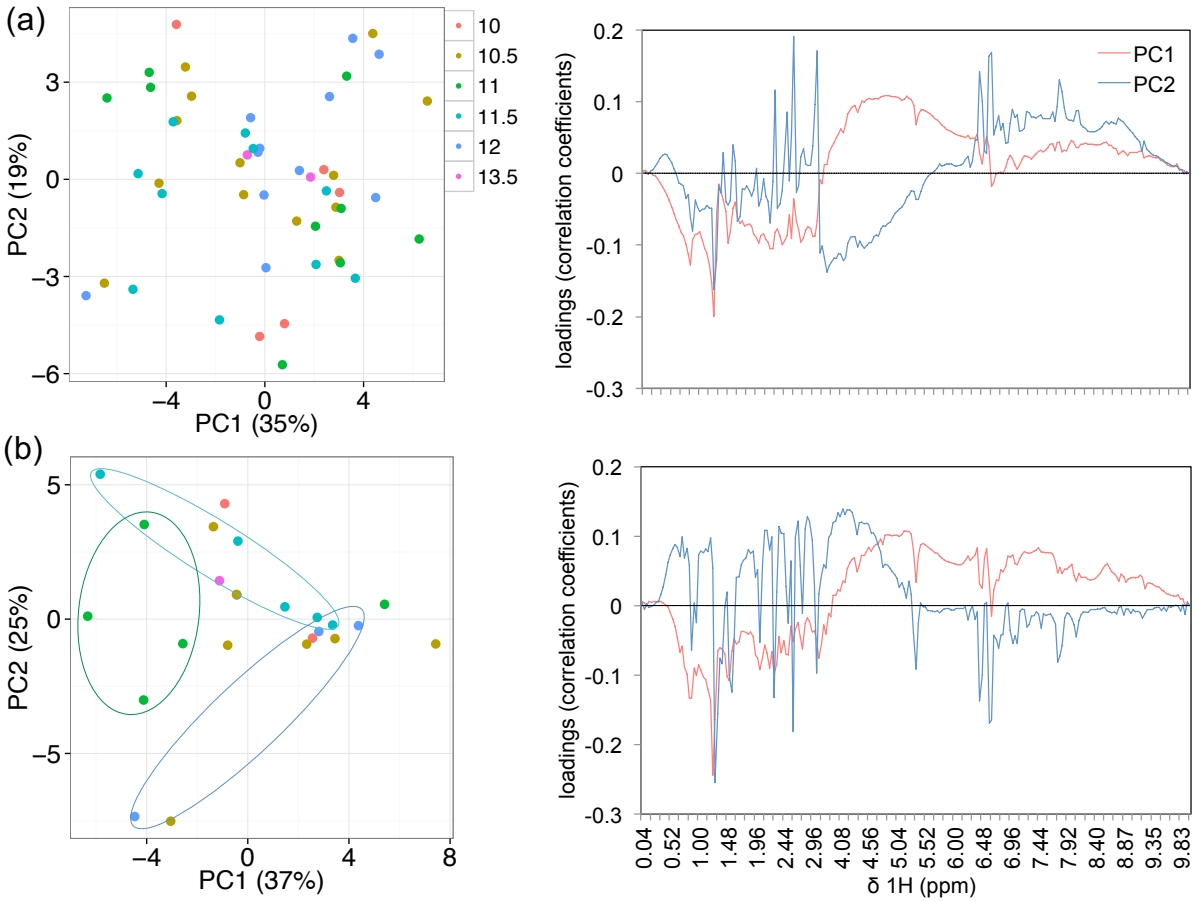



**Figure 5.3** PCA and loading plots for *Solanum inaequilaterum* (a) non galled and (b) galled leaves. Colours on PCA plots represent mean site temperatures (pink = 10°C, green = 10.5°C, blue = 11°C and purple = 12°C), loading correlations are represented by pink lines for PC1 and blue for PC2.

*Compound identification*

The PCA analyses visually showed some differences in phytochemical profiles based on galling and site temperature. With this in mind the main secondary metabolites from both *Rubus* species and *S. inaequilaterum* were purified and structurally identified in order to assess their contribution to the observed differences. The $^{1}$H NMR spectrum for compound 1 exhibited signals of a double bond proton ($\delta_H$ 5.17), a carboxylic acid group ($\delta_H$ 12, C28a), six methyl singlets and a methyl doublet in the upfield region and these signals were characteristic of an ursolic acid-type triterpenoid (Table 5.1). Protons at $\delta_H$ 4.60 and 3.79 and a downfield carbon at $\delta_C$ 210 (C2) are characteristic of two hydroxyl groups and a ketone respectively (Table 5.1, Figure 5.4). HMBC showed correlations from the methyl groups at C23 and C24 ($\delta_H$ 0.61, 1.09) into each other and into carbons at C3, 4 and 10. This indicated that both methyl groups were attached to

the same quaternary carbon at $\delta_C$ 44.9 (C4). The methyl doublet at $\delta_H$ 0.85 ($\delta_C$ 16.1, C29, $J$ = 6.4Hz) showed HMBC correlations into carbons at $\delta_C$ 25.9, 41.2 and 71.4. While the methyl singlet at $\delta_H$ 1.07 ($\delta_C$ 26.0, C30) showed HMBC correlations into carbons at $\delta_C$ 41.2, 71.4 and 53.0. The downfield quaternary carbon at $\delta_C$ 71.4 indicated the presence of an attached hydroxyl group, along with the methyl at δ 1.07. This was also supported by the adjacent carbon at $\delta_C$ 53.0 with a downfield proton at δ 2.38. The compound was identified as 3,19-dihydroxy-2-oxo-urs-12-en-28-oic acid (2-oxopomolic acid) based on 2D NMR (Figure S5.1) and comparisons with published data (Kim et al. 2012; Jia et al. 1993).

Compounds 2, 3 and 4 were obtained as a mixture. The $^1$H NMR spectrum indicated the presence of cis- and trans-coumaroyl moieties as revealed by the respective signals (Table 5.2). The $^1$H NMR data indicated the presence of a phenol with proton doublets at $\delta_H$ 7.55 and 6.8 with a hydroxyl group associated with the carbon signal at $\delta_C$ 159.5 (C4') (Table 5.2, Figure 5.4). Downfield carbons at $\delta_C$ 144 and 115.3 with HMBC correlations into $\delta_H$ 7.55 indicated the presence of a double bond. The attached proton doublets at $\delta_H$ 7.53 and 6.39 had a coupling constant of 16Hz indicating a trans double bond. Similar signals shifted upfield with half the intensity and a coupling constant of 12Hz indicated the presence of a cis isomer at half the concentration of the trans isomer. Besides these signals, proton signals of the triterpene residue, similar to compound 1 were observed. The only difference between structure 1 and the triterpene residue of structure 2 was the replacement of the ketone at carbon 2 with a hydroxyl group, indicated by the presence of a downfield proton at $\delta_H$ 3.7 attached to a carbon at $\delta_C$ 64.6 (C2). The two isomers were identified as 3-*O*-*trans*-*p*-coumaroyltormentic acid and 3-*O*-*cis*-*p*-coumaroyltormentic acid based on 2D NMR analysis and subsequent comparison to published data (Figure S5.2) (Taniguchi et al. 2002; Numata et al. 1989).

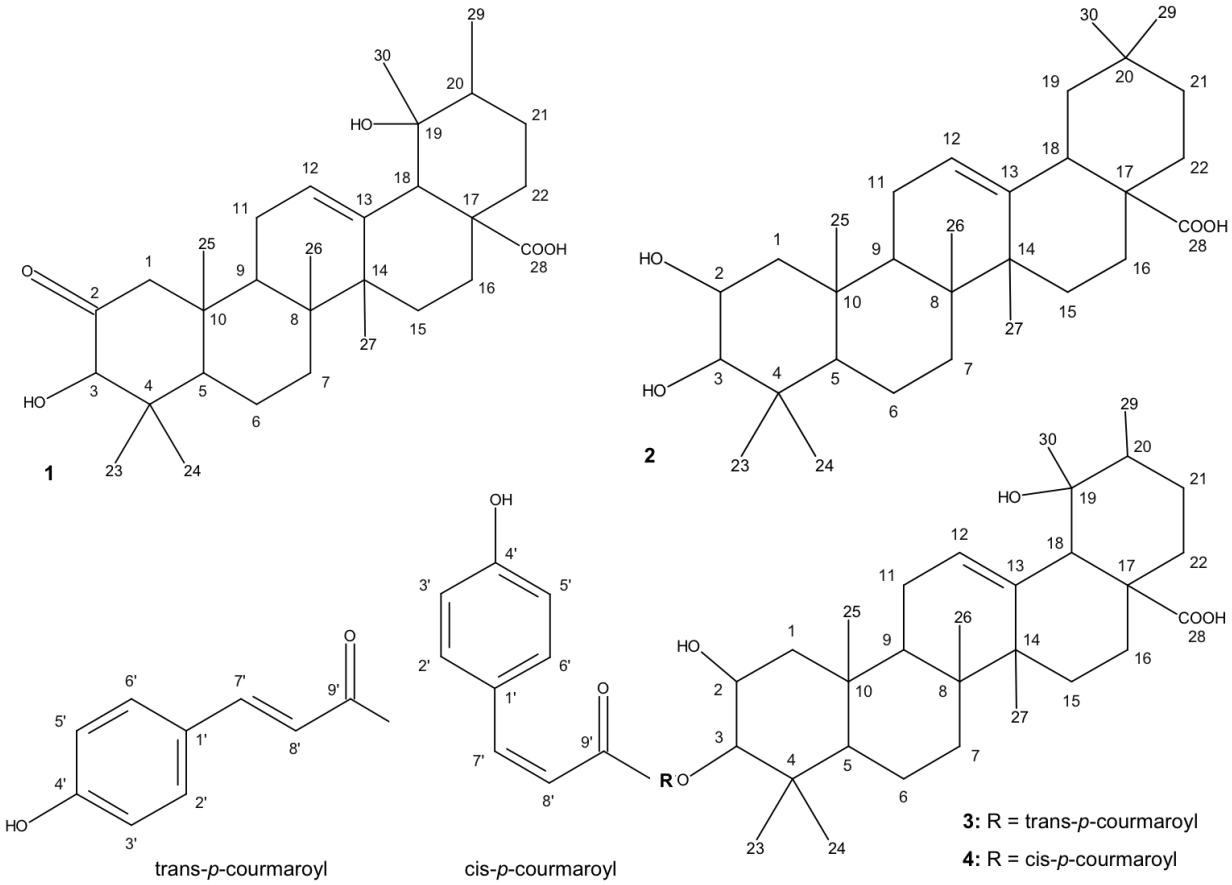



**Figure 5.4** Compounds isolated from leaves of *Rubus nebulosus*.

The $^{1}$H NMR spectrum also showed a H-3 signal at $\delta_H$ 2.75, which was shifted upfield from that of the corresponding signals in 3 and 4, indicating the presence of another triterpene without the coumaroyl group. In addition, H-12 was $\delta_H$ 5.15, also shifted upfield from that of compounds 3 and 4, indicating the presence of compound 2 (Table 5.1). The distinguishing features of structure 2 is the presence of two hydroxyl groups at carbons 2 and 3, indicated by proton and carbon chemical shifts of $\delta_H$ 3.42, 2.75 and $\delta_C$ 67.2 and 82.5 respectively. In addition the methyl groups at $\delta_H$ 0.91 and 0.93 both showed HMBC correlations into $\delta_C$ 45.1 and 32.6 and into each others carbons, indicating that both were attached to the same quaternary carbon at $\delta_C$ 30.0 (C20). This compound was identified as 2,3-dihydroxyolean-12-en-28-oic acid (maslinic acid) based on 2D NMR analysis and subsequent comparison to published data (Figure S5.2) (Ikuta et al. 1995).

**Table 5.1** NMR spectral data of compounds 1 and 2.

| | | compound 1 | | compound 2 | |
|---|---|---|---|---|---|
| Position | δC | δH (mult, *J*Hz) | HMBC (C no.) | δC | δH |
| 1 | 52.7 | 2.15, 2.27 (d, *J* = 12Hz) | 3, 5, 10 | 46.7 | 0.78, 1.79 |
| 2 | 210 | | | 67.2 | 3.42 |
| 3 | 81.9 | 3.92 (m) | 23, 24 | 82.5 | 2.75 |
| 3a | | 4.60 (d, *J* = 4.4 Hz) | | | |
| 4 | 44.9 | | | 39.1 | |
| 5 | 53.6 | 1.53 (m) | | 54.8 | 0.75 |
| 6 | 18.3 | 1.55, 1.42 (m) | 5, 8, 10 | 18 | 1.29, 1.52 |
| 7 | 32.1 | 1.58, 1.30 (m) | 5, 8 | 33.4 | 1.24, 1.48 |
| 8 | 41.0 | | | 39.3 | |
| 9 | 45.8 | 1.96 (m) | 8, 10, 11, 25, 26 | 46.8 | 1.5 |
| 10 | 43.9 | | | 37.5 | |
| 11 | 22.8 | 1.85, 1.95 (m) | 9, 12, 13 | 23.4 | 1.93, 1.96 |
| 12 | 126.1 | 5.17 (m) | 9, 14, 18 | 124 | 5.15 |
| 13 | 138.7 | | | 138 | |
| 14 | 40.5 | | | 41.6 | |
| 15 | 28.0 | 0.94, 1.70 (m) | 8, 13, 14, 17, 27 | 27.1 | 1.0, 1.8 |
| 16 | 25.0 | 1.39, 2.50 (m) | 15, 17 | 23.4 | 1.55, 1.93 |
| 17 | 47.0 | | | 46.5 | |
| 18 | 53.0 | 2.38 (s) | 12, 13, 14, 17 | 52.5 | 2.11 |
| 19 | 71.4 | | | 45.1 | 1.06, 1.64 |
| 19a | | 3.79 (s) | | | |
| 20 | 41.2 | 1.26 (m) | 19, 21 | 30 | |
| 21 | 25.9 | 1.15, 1.63 (m) | 20, 22 | 32.6 | 1.13, 1.62 |
| 22 | 36.9 | 1.50, 1.59 (m) | 17, 20, 21 | 37 | 1.5, 1.59 |
| 23 | 16.7 | 0.61 (s) 3H | 3, 4, 5, 24 | 16.8 | 0.71 |
| 24 | 28.7 | 1.09 (s) 3H | 3, 4, 5, 23 | 28.9 | 0.92 |
| 25 | 15.7 | 0.79 (s) 3H | 1, 2, 5, 9, 10 | 16.2 | 0.97 |
| 26 | 16.0 | 0.69 (s) 3H | 7, 8, 9, 14 | 16.2 | 0.9 |
| 27 | 23.7 | 1.35 (s) 3H | 8, 13, 14, 15 | 23.2 | 1.07 |
| 28 | 179 | | | 178 | |
| 28a | | 12.0 (s) | | | |
| 29 | 16.1 | 0.85 (d, *J* = 6.4 Hz) 3H | 18, 19, 20 | 21.1 | 0.91 |
| 30 | 26.0 | 1.07 (s) 3H | 19, 20, 21 | 28.9 | 0.93 |

**Table 5.2** NMR spectral data of compounds 3 and 4.

| | compound 3 (trans) | | compound 4 (cis) | |
|---|---|---|---|---|
| Position | δC | δH (mult, *J*Hz) | δC | δH (mult, *J*Hz) |
| 1 | 47.7 | 0.95, 1.88 | 47.7 | 0.95, 1.88 |
| 2 | 64.6 | 3.7 | 64.6 | 3.7 |
| 3 | 83.7 | 4.5 | 83.7 | 4.47 |
| 3a | | | | |
| 4 | 38.8 | | 38.8 | |
| 5 | 54.6 | 0.92 | 54.6 | 0.92 |
| 6 | 18 | 1.3, 1.49 | 18 | 1.3, 1.49 |
| 7 | 32.8 | 1.25, 1.47 | 32.8 | 1.25, 1.47 |
| 8 | 39.5 | | 39.5 | |
| 9 | 46.9 | 1.55 | 46.9 | 1.55 |
| 10 | 43 | | 43 | |
| 11 | 23.09 | 1.88, 1.89 | 23.09 | 1.88, 1.89 |
| 12 | 126.9 | 5.17 | 126.9 | 5.17 |
| 13 | 138 | | 138 | |
| 14 | 41.8 | | 41.8 | |
| 15 | 28 | 0.88, 1.68 | 28 | 0.88, 1.68 |
| 16 | 25 | 1.38, 2.5 | 25 | 1.38, 2.5 |
| 17 | 47 | | 47 | |
| 18 | 53.25 | 2.37 | 53.25 | 2.37 |
| 19 | 71.6 | | 71.6 | |
| 19a | | | | |
| 20 | 38.3 | 1.3 | 38.3 | 1.3 |
| 21 | 25.9 | 1.14, 1.6 | 25.9 | 1.14, 1.6 |
| 22 | 36.7 | 1.52, 1.58 | 36.7 | 1.52, 1.58 |
| 23 | 17.8 | 0.85 | 17.8 | 0.85 |
| 24 | 28.5 | 0.8 | 28.5 | 0.8 |
| 25 | 17 | 0.75 | 17 | 0.75 |
| 26 | 17 | 0.7 | 17 | 0.7 |
| 27 | 23.9 | 1.29 | 23.9 | 1.29 |
| 28 | 179 | | 179 | |
| 28a | | | | |
| 29 | 16.8 | 0.84 | 16.8 | 0.84 |
| 30 | 26.3 | 1.08 | 26.3 | 1.08 |
| 1' | 125.3 | | 125.5 | |
| 2' | 130 | 7.55 (d, *J* = 8Hz) | 132.7 | 7.68 (d, *J* = 8Hz) |
| 3' | 116 | 6.8 (d, *J* = 8Hz) | 114.5 | 6.73 (d, *J* = 8Hz) |
| 4' | 159.5 | | 158.5 | |
| 4'a | | 10 | | 9.85 |
| 5' | 116 | 6.8 (d, *J* = 8Hz) | 114.5 | 6.73 (d, *J* = 8Hz) |
| 6' | 130 | 7.55 (d, *J* = 8Hz) | 132.7 | 7.68 (d, *J* = 8Hz) |
| 7' | 144 | 7.53 (d, *J* = 16Hz) | 142 | 6.85 (d, *J* = 12Hz) |
| 8' | 115.3 | 6.39 (d, *J* = 16Hz) | 116.5 | 5.82 (d, *J* = 12Hz) |
| 9' | 166.7 | | 166.2 | |

Fraction 24 from the $C_{18}$ separation of *S. inaequilaterum* was deduced from analysis of 2D NMR data to be the spermine alkaloid 1(Figure 5.5). The $^{1}H$ NMR data indicated the presence of a tri-substituted benzene ring with proton signals at $\delta_H$ 6.43, 6.56 and $\delta_H$ 6.6 with two hydroxyl groups associated with proton signals at $\delta_H$ 8.72 (C5, $\delta_C$ 145) and $\delta_H$ 8.64 (C6, $\delta_C$ 145) (Table 5.3, Figure 5.5). A downfield carbon at $\delta_C$ 172 and HMBC correlations into an upfield proton at $\delta_H$ 2.25 (C2' δ 37.5) indicated the presence of a propanamide group (Table 5.3, Figure S5.3). These signals indicate that structure 1 contained a 3,4-dihydroxyphenyl-N-propanamide. The remaining signals were observed at δ 3.01 (2 x CH2), 1.50, 1.45, 1.28 and 3.18 (2x $CH_2$). COSY correlations from $\delta_H$ 1.50 to 3.01 and 3.18 and between $\delta_H$ 3.01 and 1.45, 1.45 and 1.28 and from $\delta_H$ 1.28 to 3.18, indicated that the molecule contained butyl and propyl groups. The 13C chemical shift of the carbons attached directly to the protons resonances at $\delta_H$ 3.01 and 3.18 ($\delta_C$ 38.7, 37.4, 45.8, 44.7) suggested that nitrogen atoms substituted these four carbons. The intensity of the aromatic signals relative to the upfield signal suggested that the molecule contained two 3,4-dihydroxyphenyl propanamide groups and these partial structures could be most logically joined to form structure 1. Structure 1 was identified as $N^1$,$N^8$-bis(dihydrocaffeoyl) spermidine and has been isolated previously from *Solanum* (Gancel et al. 2008). The $^{1}H$ NMR spectrum also possessed signals associated with a long chain fatty acid. This partial structure (2) was characterised by the presence of methylene protons, which showed COSY correlations to each other and HMBC correlations into a carboxylic acid carbonyl carbon suggesting a carbon chain of unknown length (Figure 5.5). This fatty acid is likely to be a counter-ion to the spermine alkaloid. Structure 3 is N-methyl nicotinic acid which eluted in many of the early fractions and is associated with the downfield signals in the PCA, along with structure 1 (Figure S5.5).

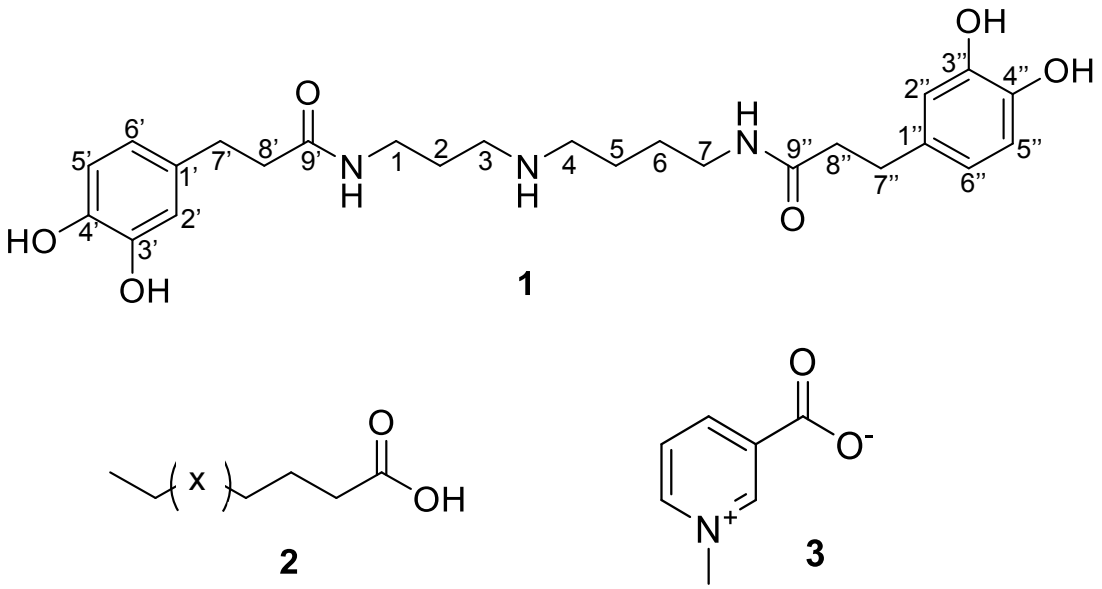



**Figure 5.5** Structures isolated from leaves of *Solanum inaequilaterum*. X denotes an unknown number of methylene groups.

**Table 5.3** NMR spectroscopic data from structure 1 from *Solanum inaequilaterum*.

| Position | δC | δH | HMBC |
|---|---|---|---|
| 1 | 38.3 | 3.01 | |
| 2 | 25.2 | 1.54 | |
| 3 | 45.8 | 3.18 | |
| 4 | 44.7 | 3.17 | |
| 5 | 26.1 | 1.45 | |
| 6 | 27.9 | 1.29 | |
| 7 | 37.4 | 3.01 | |
| 1' | 132 | | 2', 6', 7' |
| 2' | 115 | 6.56 | 1', 6' |
| 3' | 145 | 8.64 | 5' |
| 4' | 143 | 8.72 | 2', 6' |
| 5' | 115 | 6.6 (dd) | 6' |
| 6' | 118 | 6.43 (dd) | 1', 2', 5' |
| 7' | 31.1 | 2.62 | 1', 2', 6' |
| 8' | 38.2 | 2.25 | 1' |
| 9' | 172 | | 7' |
| 1" | 132 | | 2", 6", 7" |
| 2" | 115 | 6.56 | 1", 6" |
| 3" | 145 | 8.64 | 5" |
| 4" | 143 | 8.72 | 2", 6" |
| 5" | 115 | 6.6 (dd) | 6" |
| 6" | 118 | 6.43 (dd) | 1", 2", 5" |
| 7" | 31.1 | 2.62 | 1", 2", 6" |
| 8" | 38.2 | 2.25 | 1" |
| 9" | 172 | | 7" |

## 5.5 Discussion

To date most studies of changes in phytochemistry related to insect herbivory have focused on a limited number of compounds and compound classes. Knowledge of the effects of herbivory on the whole secondary metabolite profile can provide insight into the complex chemical changes that occur from specific ecological interactions. Here we have applied NMR techniques to characterise how the phytochemical profile of three species of rainforest understory plants responds to galling herbivory and local temperature differences. Our results have led us to identify several secondary metabolites that may be responding to both abiotic and biotic pressures in each plant species. This may lead to new insights into the roles of these compounds in plant defence and environmental adaptation.

Galling in general has been shown to induce chemical changes in host plants (Chapter 2), the results in this chapter, however, highlight the role of specific compounds in galler-plant interactions. Both *Rubus* species contained several different triterpenoid compounds, similar to those previously found in this genus (Patel et al. 2004). This is, however, the first chemical investigation of *R. nebulosus* and *R. moorei*. The PCA of galled *R. nebulosus* leaves showed regions in the $^{1}$H NMR spectra corresponding to peaks from the triterpene coumaroyltormentic acid compounds that we isolated. This suggests that galling may be changing the concentrations of these compounds. Galled *R. moorei* leaves showed chemical differences mainly in the upfield region, indicating changes in the expression of the triterpenes, maslinic and oxopomolic acid. Gall-induced changes in triterpene compounds may protect the galling insect from predation and other forms of herbivory. For example, the increased triterpenes produced from aphid galls on pistachio trees deters mammalian herbivory (Rostás et al. 2013). Both *Rubus* species are galled by a single species, Cecidomyiidae sp.1, so differences in the phytochemical responses of the two *Rubus* species may have different consequences for galler fitness and their parasitiods. In Chapter 3 we found that competition between general herbivory and galling was only apparent on *R. moorei*. This chapter provides evidence that the modification of competitive interactions between herbivores by their host plants may be chemically mediated (Wootton 1994).

The increase in downfield signals correlating with PC1 in galled *S. inaequilaterum* leaves could be indicative of increased concentrations of spermine alkaloids. Phenolamides such as spermine and its derivatives play an important role in plant development and defence against natural enemies (Bassard et al. 2010). Insect herbivory has been shown to induce increased levels of phenolamides in wild tobacco (*Nicotiana attenuate*), resulting from changes in several metabolic pathways (Gaquerel et al. 2014). Similar compounds have been found to be deterrent against leaf miners (Tebayashi et al. 2007) and reduce the performance of specialist and generalist caterpillars (Kaur et al. 2010). Amides from *Piper* plants have been found to be deterrent to both specialist and generalist herbivores and work synergistically in defence, causing total mortality of caterpillars when multiple amides were present (Dyer et al. 2003).

Along with biotic interactions, abiotic factors can influence phytochemical profiles. For example, terpene chemotypes of aphid galls on pistachio trees vary considerably across their geographic range (Sifi et al. 2015). This indicates that these compounds induced by the gall maker also respond to environmental factors, although at this scale it is likely that genetic differences may also play a role. Polyamides also play a role in plant adaptation to abiotic stressors such as UV, salinity and temperature (Bassard et al. 2010). Polyamides have been shown to accumulate in bean plant tissue in response to heat shock (Edreva et al. 1998). The dual roles of many plant secondary metabolites in defence against both abiotic stress and natural enemies may constrain a plants ability to adapt to particular stessors (Siemens et al. 2012). For example, Siemens et al. (2009) found that *Boechera stricta* plants with higher glucosinolate defence compounds were less tolerant to abiotic stress. This may be due to anatagonistic crosstalk between signalling pathways. In Solananceae, for example, production of the defence compound caffeoylputrescine is controlled by the jasmonate signalling pathway (Tebayashi et al. 2007). However, jasmonate is anatagonistic to the abscisic acid signalling pathway, which plays an important role in plant response to abiotic stress (Fujita et al. 2006).

The strength of NMR over other approaches lies in its ability to identify unknown compounds in a complex mixture. The metabolomics approach is limited due to overlapping peaks in crude $^{1}$H NMR spectra, thus requiring separation and further 2D NMR to isolate specific compounds. One potential solution is to use 2D NMR techniques such as HMBC on crude extracts, which would generate peaks with both

carbon and proton chemical shifts, thus significantly reducing peak overlap (Mahrous and Farag 2015). Farag et al. (2013) used this technique to distinguish hop cultivar metabolite fingerprints while also identifying major secondary metabolites. Nevertheless, we found distinct changes in $^{1}$H NMR phytochemical profiles within species related to galling herbivory and site temperature.

The combination of NMR-based metabolic profiling and multivariate data analysis enables more insight into chemically mediated interactions than would be gained by targeting only specific metabolites or broad chemical classes such as condensed tannins. Sutter and Müller (2011), for example, found that different types of herbivory and damage lead to significant changes in plant metabolomes that were not detected when looking at single metabolites. The metabolomics approach is preferable for determining the response of plants to multiple challenges in a complex environment. In this study, we have shown that gall induced changes in the phytochemical profile of three rainforest plants is mediated by site temperature. This provides insight into how plants may respond to simultaneous abiotic and biotic stressors. The combined effects of increased herbivory and extreme weather events predicted under climate change may affect plant secondary metabolites in ways not predicted by looking at either stress in isolation.

## S5 Supplementary material

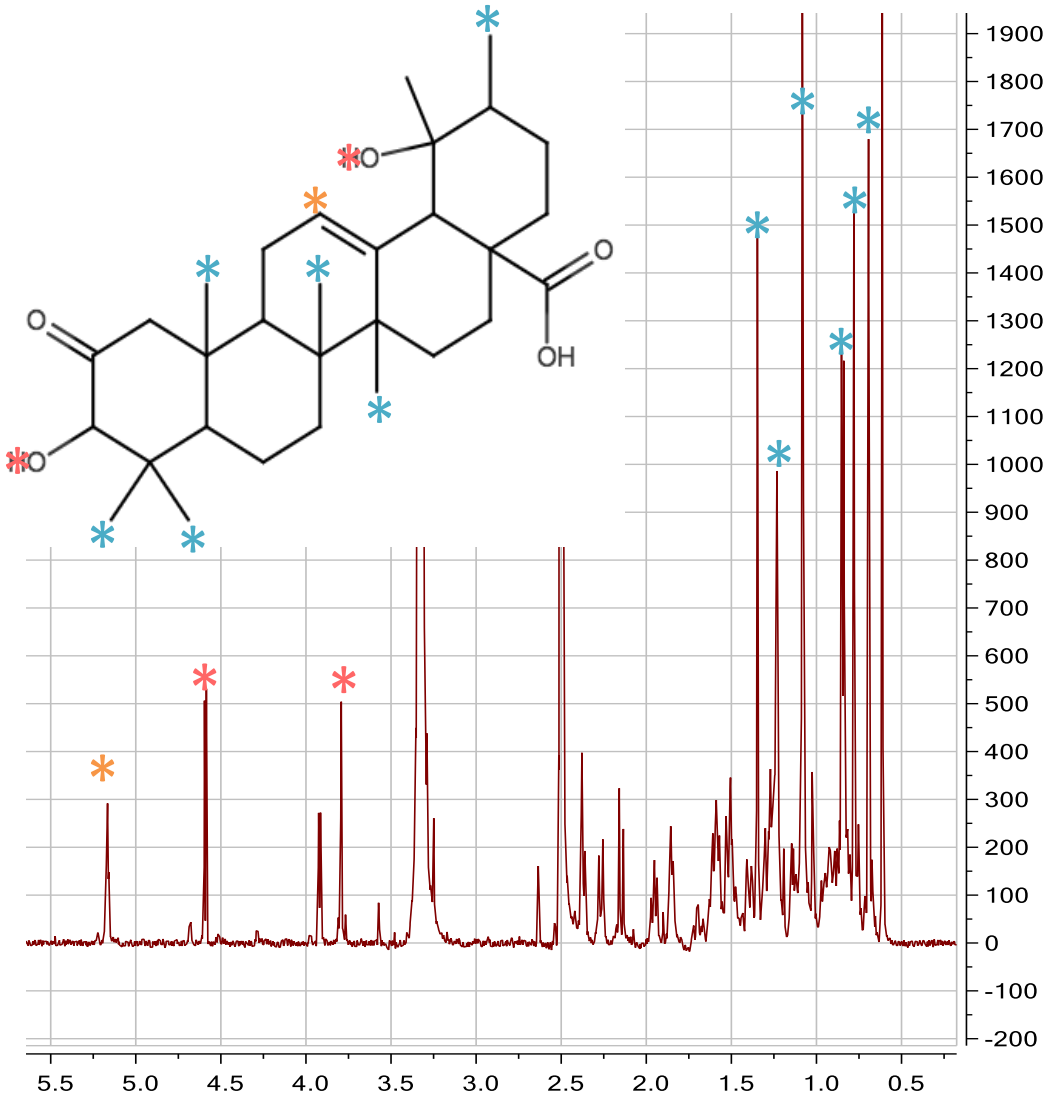



**Figure S5.1** Example of a $^{1}$H NMR spectrum with different protons marked. Protons associated with methyl groups are indicated by blue asterisks, hydroxyl by red asterisks and the double bond proton by an orange asterisk.


Figure S5.2 *(Continued on next page).*

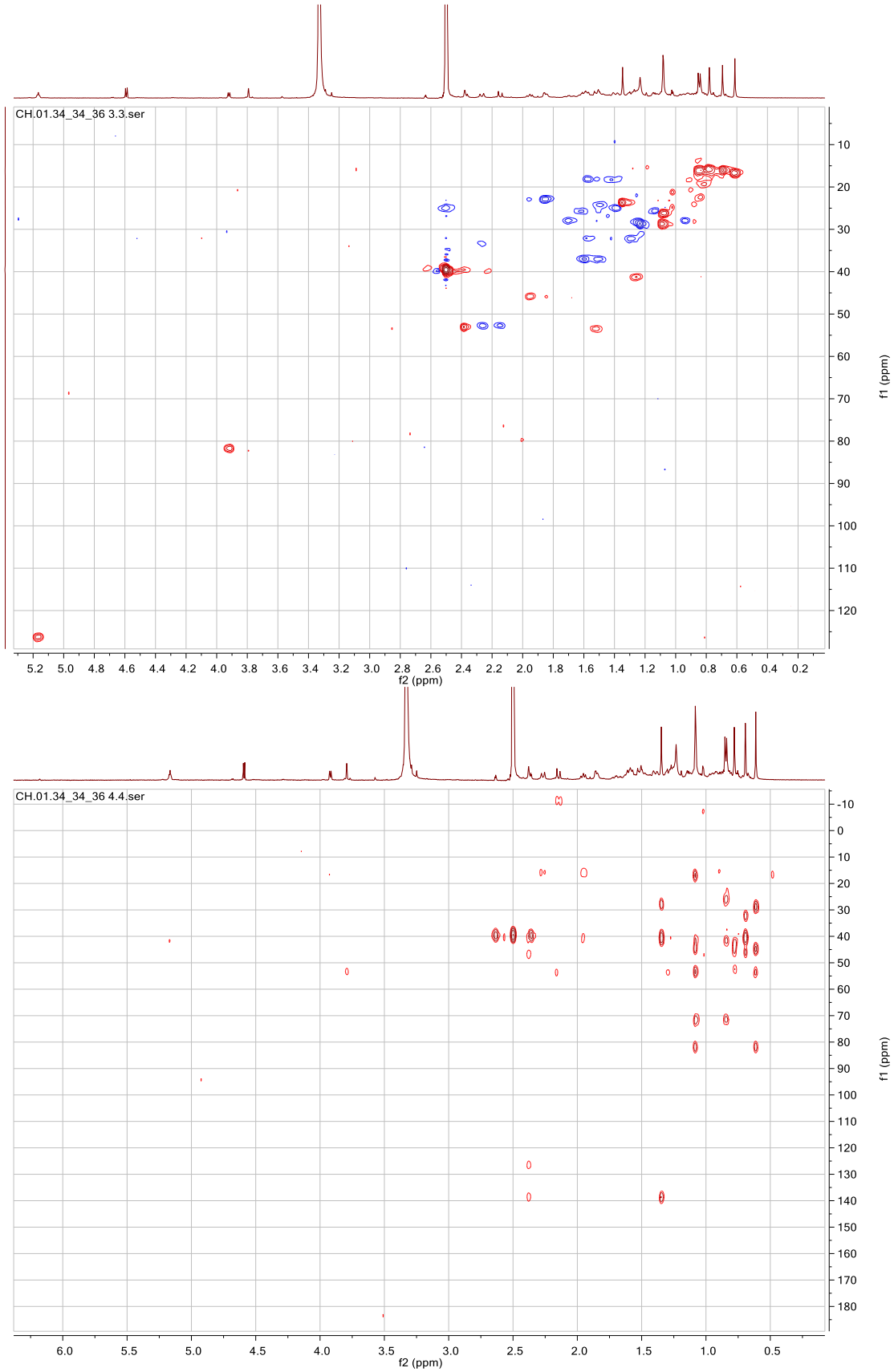



**Figure S5.2** $^{1}$H NMR, COSY, HSQC and HMBC spectrum (500MHz) of oxopomolic acid in DMSO-$d_6$.

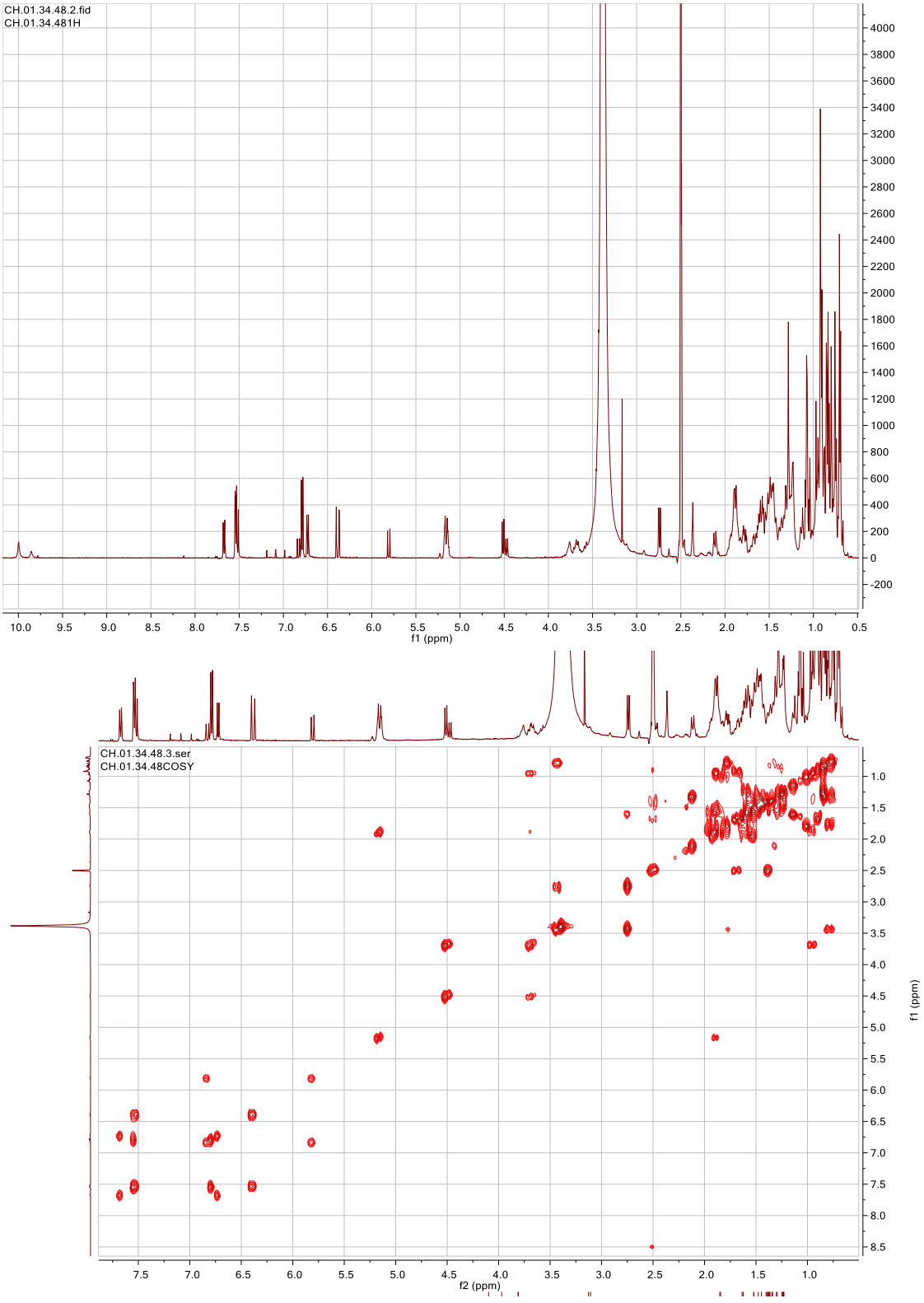



Figure S5.3 *(Continued on next page).*

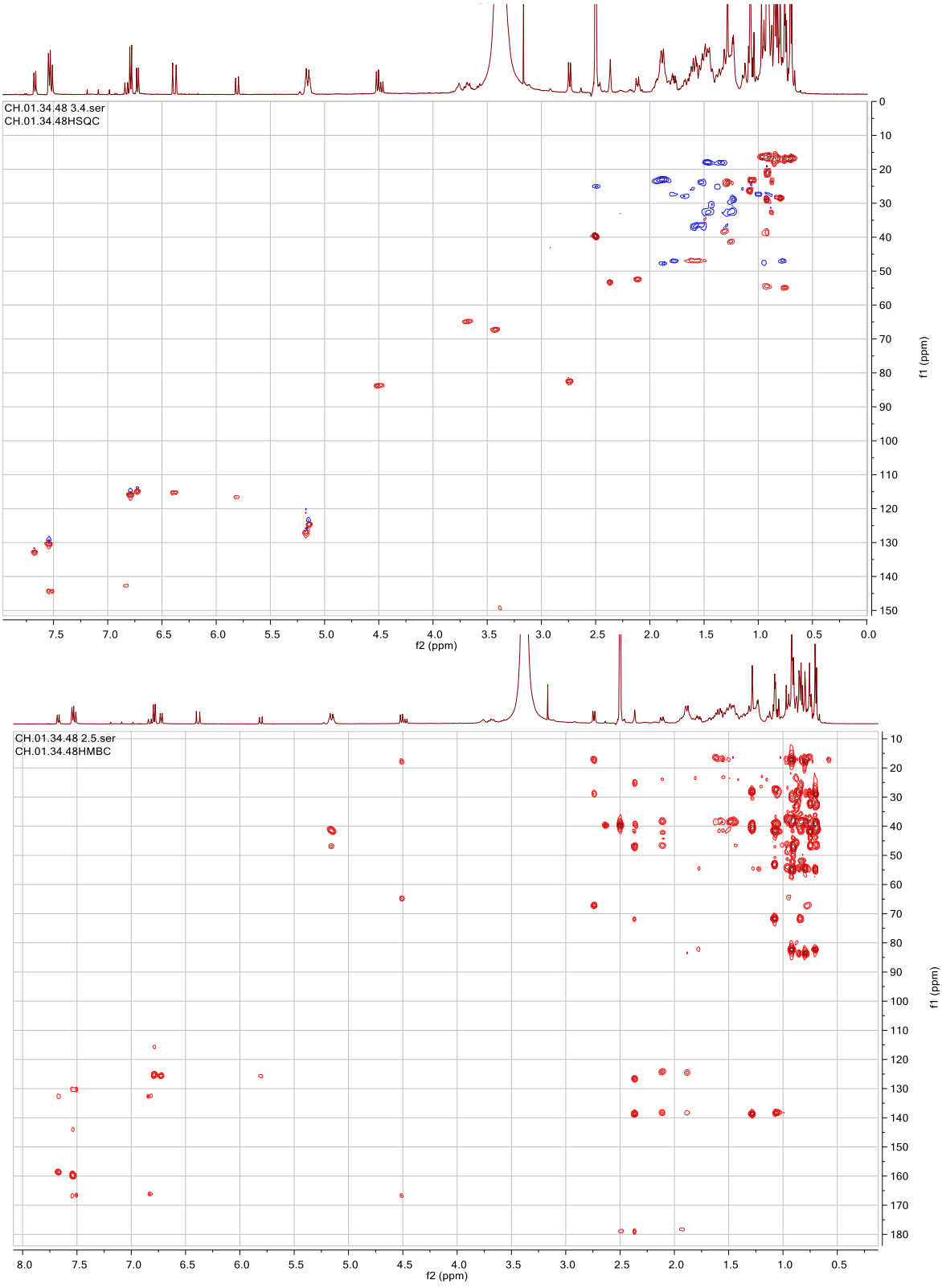



**Figure S5.3** $^{1}$H NMR, COSY, HSQC and HMBC (500MHz) spectrum of maslinic acid, 3-*O*-*trans*-*p*-coumaroyltormentic acid and 3-*O*-*cis*-*p*-coumaroyltormentic acid in DMSO-$d_6$.

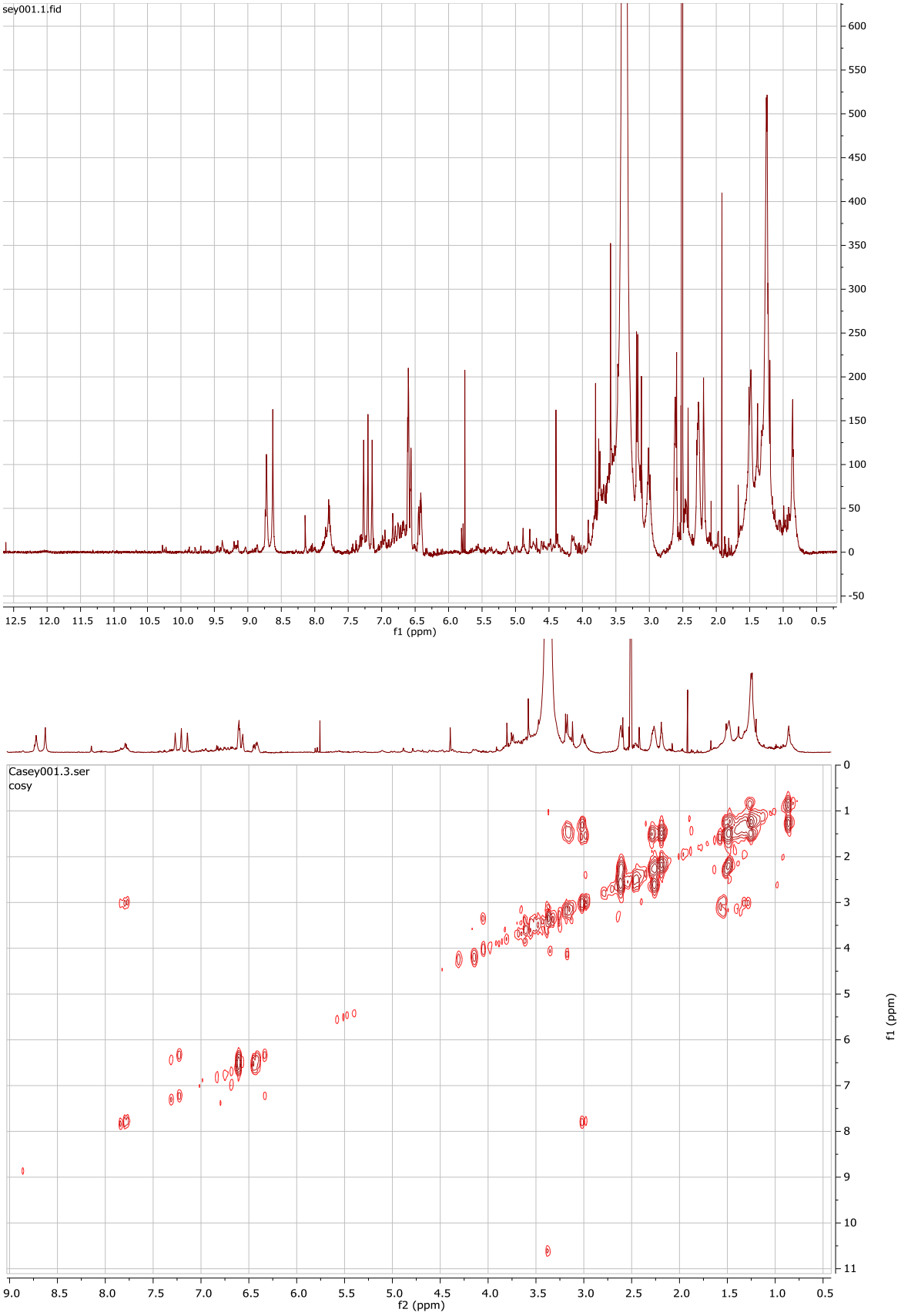



Figure S5.4 *(Continued on next page).*

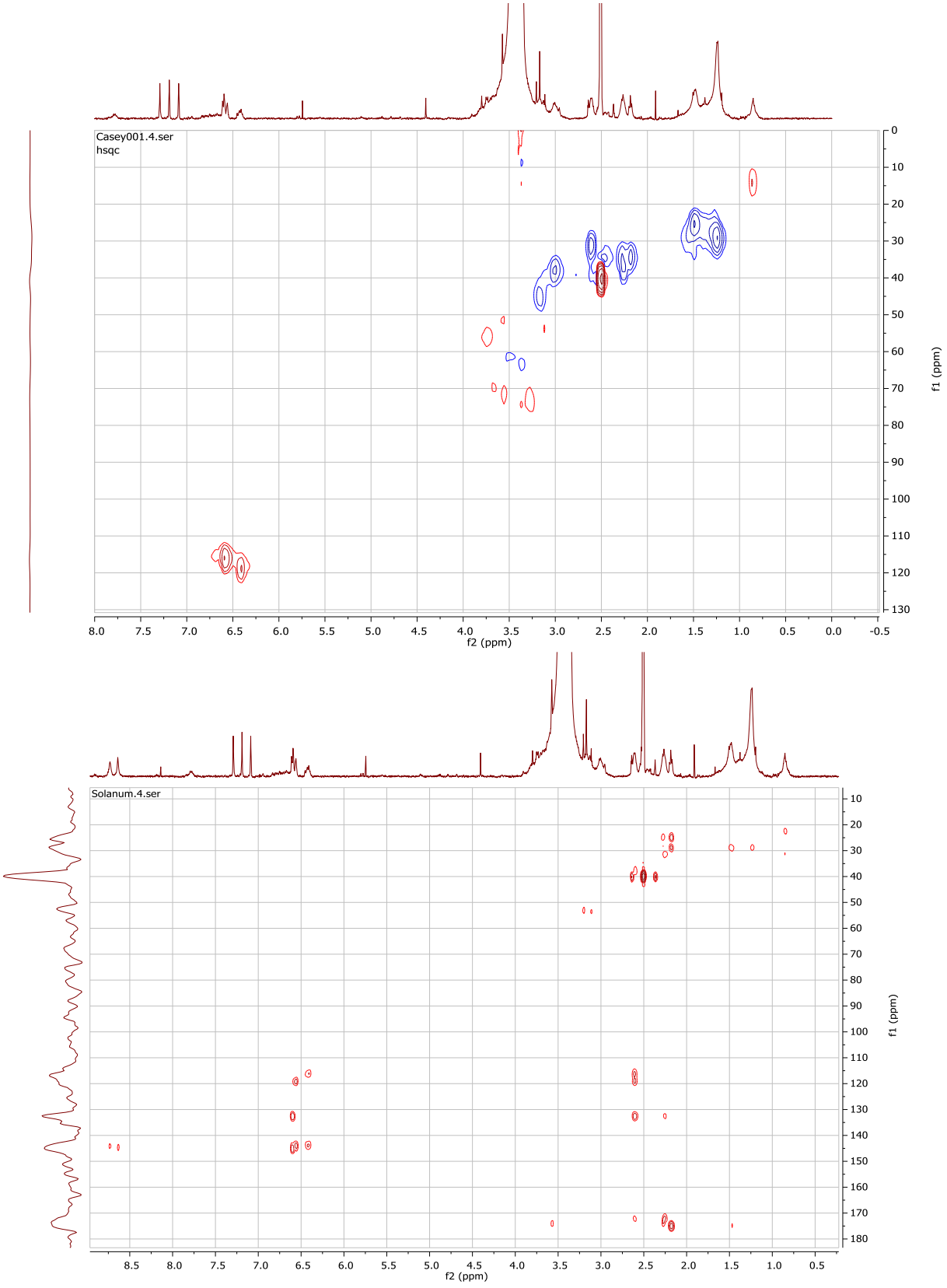



**Figure S5.4** $^{1}$H NMR, COSY, HSQC (800MHz) and HMBC spectrum (500MHz) of $N^{1}$,$N^{8}$-bis(dihydrocaffeoyl) spermidine and fatty acid chain partial structures in DMSO-$d_6$.

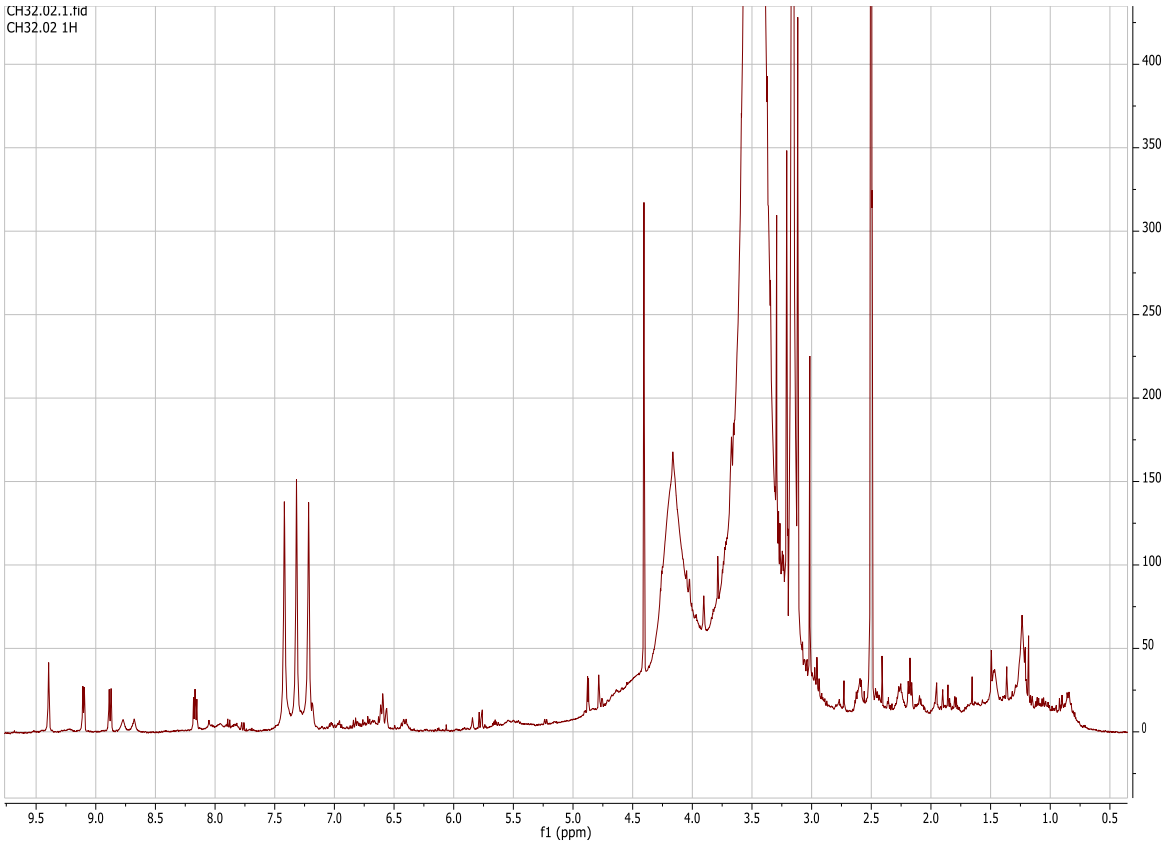



**Figure S5.5** $^{1}$H NMR spectrum (600MHz) of N-methyl nicotinic acid.

# Chapter 6

In the previous chapters we have found that temperature has a significant effect on parasitoids and also on host plant chemistry of galled leaves. In this chapter we use path models to assess the hypothesis that these temperature related differences in phytochemistry have significant indirect effects on higher trophic levels.

**Statement of contribution to a co-authored paper**

Chapter 6 is a co-authored paper that has been prepared for publication. The citation is as follows:

Hall, C.R., Carroll, A.R., Kitching, R.L. (prepared manuscript) Phytochemical diversity and gall-parasitoid interactions across a temperature gradient.

My contribution to the paper involved field work, data collection, analysis and writing of the manuscript. Roger Kitching provided direction and guidance with the project and editing of the manuscript. Tony Carroll guided the chemical analysis and also edited the manuscript.

(Signed) _____ (Date) 12/12/2015
Casey Hall

(Countersigned) _____ (Date) 12/12/2015
Associate supervisor: Tony Carroll

(Countersigned) _____ (Date) 12/12/2015
Supervisor: Roger Kitching

# Chapter 6

## Phytochemical structural diversity and gall-parasitoid interactions across a temperature gradient

Casey R. Hall[1*], Anthony R. Carroll[1] and Roger L. Kitching[1]

[1]Environmental Futures Research Institute, School of Environment, Griffith University, Nathan, QLD 4111

[*]Corresponding author: casey.hall@griffithuni.edu.au

## 6.1 Abstract

It is generally thought that plants maintain diverse chemical mixtures to defend against a variety of natural enemies. Environmental variables, such as temperature, drive intraspecific variation in phytochemistry over relatively small geographic scales. Recent studies have shown that changes in plant chemistry can have strong bottom-up effects on parasitoid functioning and development. Temperature and plant secondary metabolites can affect tritrophic interactions through a number of direct and indirect pathways. We use field collected data from a tritrophic system consisting of *Rubus* host plants, their galling insect and parasitoids over an elevational gradient to investigate the relative strengths of the bottom up phytochemically-mediated effect of temperature on parasitoids and the direct top down effect of temperature of parasitoids. We found a significant indirect effect of temperature on parasitoids. Changes in the phytochemical profile of galled leaves at higher temperatures resulted in a strong positive effect on parasitoid species richness. Our results demonstrate that increased chemical structural diversity may attract a higher number of parasitoid species, potentially benefiting the plants due to reduced herbivore damage. The results are also consistent with the safe haven hypothesis, i.e. chemically defended herbivores deter other general predators, but are safe hosts for parasitoids.

## 6.2 Introduction

Plants have evolved a wide array of secondary metabolites used in defence against natural enemies (Feeny 1970, Fürstenberg-Hägg et al. 2013). Natural plant populations can exhibit considerable variation in overall concentration of secondary metabolites and also in their phytochemical diversity, defined as the richness and abundance of individual compounds (Hilker 2014; Moore et al. 2013). In general, phytochemical diversity is thought to benefit plants due to the increased chance that one or a combination of compounds are active against specific enemies (Berenbaum and Zangerl 1996). Phytochemical defence, particularly against specialist herbivores, is usually linked to specific compounds, which make up only a small fraction of all available compounds in a plant (Adler et al. 1995).

Secondary metabolites can reduce insect herbivory directly, through toxic and deterrent properties, and/or indirectly through the attraction of natural enemies of the herbivore (War et al. 2012). Terpenes, for example, have a direct toxic effect on herbivores but also act as volatile cues to attract parasitoids (Fürstenberg-Hägg et al. 2013). Several herbivore-induced volatile plant compounds are known to be used by parasitoids during host location (Kessler and Bladwin 2001; Arimura et al. 2009). Insect host derived chemicals are used generally for host identification by parasitoids at close range (Quicke 1997). Endophytic hosts such as galling insects, however, present greater challenges to searching parasitoids as they do not have direct access to the herbivore. Parasitoids use leaf chemistry as a proxy for insect host selection and are very sensitive to changes in leaf chemistry. They can accurately assess plant chemistry, as this is a good predictor of the insect host performance, which is directly linked to parasitoid performance (Harvey 2005). As a result parasitoids are able to distinguish even slight differences in plant quality based on chemical cues (Gols et al. 2009).

Galling insects use chemical stimuli to induce physical and metabolic changes in host plant tissue (Cornell 1983). They can actively manipulate host plant chemical defences for their own benefit. Studies have shown increased concentrations of tannins and phenolics (Chapter 2; Hartley 1998) and suppressed herbivore-induced plant volatile emissions in galled plant tissues (Tooker et al. 2008). Recent studies have shown that changes in plant chemistry can have strong bottom-up effects on parasitoid functioning

and development (Bukovinszky et al. 2008; Harvey et al. 2010; Richards et al. 2015). Phytochemical defences can have variable effects on parasitoid performance. For example, herbivores feeding on more chemically defended plants may have decreased fitness leading to lowered immune defences against parasitoids (Smilanich et al. 2009). Conversely, some studies have shown the opposite, where herbivores that sequester plant compounds have reduced parasitoid performance (Müller et al. 2001). These studies not only illustrate the importance of phytochemical diversity in plant-insect interactions, but also the potential cascading effect of phytochemical diversity on higher trophic levels.

In natural populations there is often considerable variation in phytochemistry across different local and geographic scales due to a variety of selection pressures (Harvey et al. 2010; Moles et al. 2011). Climatic conditions such as water availability and temperature can have particularly strong effects on phytochemistry (Gutbrodt et al. 2011; Hallam and Read 2006). Elevational gradients, such as those described in this work, provide a 'natural experiment' where localised changes in temperature drive intraspecific variation in phytochemistry over relatively small geographic scales (Salmore and Hunter 2001; Pellissier et al. 2014; Zidorn 2010).

In addition to phytochemically mediated effects, temperature also has direct effects on parasitoid functioning and efficiency, potentially causing top-down changes in tritrophic interactions (Hall et al. 2015; Romo and Tylianakis 2013). It is clear that temperature and phytochemical diversity can affect tritrophic interactions through a number of direct and indirect pathways, but little is known about how the interaction between galling and temperature affect phytochemical diversity. Does temperature have stronger bottom-up effects on higher trophic levels through changes in phytochemical diversity? Does temperature have a greater direct top down effects on parasitoids? Or is the effect of temperature on parasitoids due to host density mediated effects rather than phytochemical diversity?

In this study we used field collected insect data from a tritrophic plant-gall-parasitoid system. We construct a path model based on existing literature (Table 6.1, Figure 6.1) to address the questions about how different temperatures and insect galling interact to affect phytochemical diversity and gall-parasitoids. Path analysis is an extension of multiple regression where causal modelling among variables allows for the analysis of

both direct and indirect relationships (Streiner 2005). Traditional chemical methods have restricted our understanding of phytochemistry to either broad classes of compounds, such as phenolics, or to a few well known compounds (Moore et al. 2013). In this study we used $^{1}$H NMR spectroscopy to quantify phytochemical diversity (see Richards et al. 2015). The chemical structure information that can be derived from analysis of $^{1}$H NMR spectra is such that it provides a snapshot of the plant metabolome and can be used to provide a measure of intra- and intermolecular complexity, both have been shown to be important components of chemical diversity and have an important effect on biological function (Richards et al. 2015).

**Table 6.1** Hypothesised effects of temperature on phytochemistry mediated tritrophic interactions based on previous studies (paths from Figure 6.1).

| Path | Hypothesis |
|---|---|
| **A** | Temperature has a direct effect on phytochemistry, higher temperatures leading to increases in phytochemical diversity (Dyer et al. 2013; Pellisier et al. 2014). |
| **B** | Parasitoids are positively affected by phytochemical diversity of host plants (Bukovinsky et al. 2008; Richards et al. 2015). |
| **C** | Phytochemical difference between galled and non-galled leaves is positively correlated with phytochemical diversity (Richards et al. 2015). |
| **D** | Similar to phytochemical diversity, phytochemical difference between galled and non-galled leaves affects parasitoids. |
| **E** | Higher temperatures can have direct effects on parasitoid function and survival (Hall et al. 2015; Romo and Tylianakis 2013). |

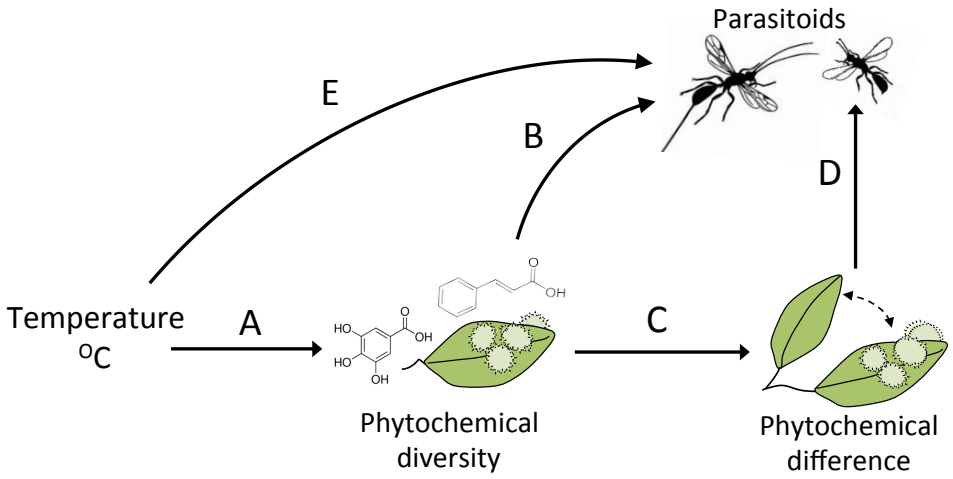



**Figure 6.1** Path diagram summarising direct and indirect effects of increased temperature on phytochemical diversity and gall parasitoids. The letters included as path coefficients correspond to direct effects (Table 6.1).

## 6.3 Methods

*Study System*

We studied the relationships between host plant phytochemical diversity, galling insects and their parasitoids in two naturally occurring *Rubus* species along an elevational gradient. The two host plants sampled were *Rubus moorei* (F. Muell.) and *Rubus nebulosus* (A.R. Bean), both hosting Cecidomyiidae sp. 1 (see Chapter 3). Both plant species have restricted distributions and are only found at higher altitudes (above 700 m asl) within subtropical rainforest. The host plants and galling cecidomyiidae are described in further detail in Chapter 3. From these two galls we reared four species of parasitoid: these were assigned to the following morphospecies; Platygasteridae sp 1, Platygasteridae sp 3, Pteromalidae sp 2 and *Zaommomyiella* sp. and checked by Chris Burwell from the Queensland Museum. The two host plants were chosen as they often occur in close proximity to each other. The galls can be reared successfully and are linked through their shared parasitoids. They also gave us the opportunity to compare two closely related *Rubus* species with each other.

*Study Site*

We sampled leaf galls at 15 sites within Lamington and Border Ranges National Parks in the Macpherson Ranges that span the Queensland/New South Wales border (see Chapters 3 and 4 for a description of the study sites). The area is dominated by highly diverse and structurally complex subtropical rainforest and is described in detail by (Laidlaw et al. 2011). The climate of the study area is described by McDonald (2010).

*Experimental design and sampling*

To generate a climatic gradient, we used an elevational gradient as a 'space for time substitution' (Pickett 1989, Hodkinson 2005). At each site we sought three individuals of each plant species within a restricted 50 $m^2$ area in order to maintain a standard plot size and to decrease between-plant differences due to abiotic conditions. Each site was sampled 6 times over a 1-year period with 2 months between each sampling occasion to allow adequate time for regrowth and for galls to re-establish. The experimental design and sampling protocol is described in Chapter 4.

*Chemical data*

Galled and non-galled leaf material from each sampled plant were collected. The chemical analysis of crude plant extract is described in Chapter 5.

*Analysis*

The NMR spectral data were processed using MestReNova software (version 10.0.2-15465, Mestrelab Research S.L. 2015). NMR spectra were processed using common metabolomics methods (Kim et al. 2010). Spectra were aligned using the solvent peak (DMSO), base-line corrected, phase corrected, normalized to a total area of 100 and integrated into 0.04 ppm bins along the width of the spectra. Binning reduces the number of variables from potentially 30 000 to approximately 200 and reduces differences in signal fluctuations among samples (Kim et al. 2010). The data for each sample were exported and combined into one dataset for analysis.

We used $^{1}$H NMR peak diversity (richness and abundance) as a measure of chemical structural composition (as per Richrads et al. 2015). The benefits of $^{1}$H NMR have been described in Chapter 5. There are, however, some limitations to this method. Namely that there are multiple peaks per compound in an $^{1}$H NMR spectrum (see Chapter 5 Supplementary material) and the number of compounds in a crude extract mean that many signals will overlap, reducing the spectral resolution. As such, the diversity of $^{1}$H NMR signals may not necessarily correlate with chemical diversity, but could be indicative of an increase in only one or two structurally different compounds. Nevertheless, NMR has the potential to rapidly an accurately quantify chemical diversity across many samples. Here we use $^{1}$H NMR peak diversity as an approximation of inter and intra-molecular complexity and structural diversity. Throughout this study we refer to this measure as phytochemical diversity.

We calculated the diversity index (1-D; where $D = \sum \frac{n}{N^2}$ , n is the integral at a specific binned frequency range, and N is the total number of binned frequency ranges) for each sample (Richards et al. 2015). The raw diversity values (D) were multiplied by one hundred before analysis. We calculated phytochemical diversity from $\delta_H$ 0-12.5 ppm, excluding the solvent peak at $\delta_H$ 2.5 ppm and the region from $\delta_H$ 3-4 ppm which contained a broad water signal and overlapping peaks.

All statistical analyses were carried out using R statistical software (R core development team 2015). Phytochemical diversity data was calculated on samples collected across two sampling occasions. This resulted in 32 galled and 61 non galled chemical diversity data points. One extreme outlier was removed from phytochemical diversity data to improve the normality of the distribution. Welch's t-tests, which accounts for un-equal variance, were used to test for significant differences in phytochemical diversity between galled and non-galled leaves for both *Rubus* species. Gall and parasitoid density were square root transformed to meet assumptions of normality. Linear regression was used to assess the relationship between elevation and temperature and phytochemical diversity of galled and non-galled leaves separately. Phytochemical diversity was not significantly related to elevation ($R^2 = 0.037$, $F_{1,31} = 1.20$, $P = 0.28$), so temperature was used for all further analysis. The difference in phytochemical diversity between galled and non-galled leaves was calculated by subtracting mean phytochemical diversity of non-galled leaves from galled leaves for each site, for convenience we will refer to this measure as 'phytochemical difference'. The phytochemical difference gives an estimate of the magnitude of the chemical response of plants to galling at each site.

We examined direct and indirect effects of temperature on phytochemical diversity and parasitoids with path analysis using the Lavaan package in R (Rosseel 2012). Gall and parasitoid data from the six sampling occasions were averaged for each site, this resulted in 12 and 10 data points for *R. moorei* and *R. nebulosus* respectively. Methods of calculating gall density are described in Chapter 3. Mean site temperature, gall density, parasitoid species richness, phytochemical diversity and phytochemical difference for each *Rubus* species at each site were used. All variables were standardised before path analysis. We proposed a model based on causal directional relationships between variables from our hypotheses and compared the model fit with a null model consisting of unresolved causal relationships among variables. Models were assessed using *P* values from chi-square tests, where the *P* values close to 1 indicate a good fit. The chi square tests the difference between the expected and observed covariance matrices, thus a chi-square closer to zero (and thus a *P* value closer to 1) indicates that the model fits the data. We used an alpha level of 0.05 for all statistical tests.

## 6.4 Results

A total of 7068 galls, 4768 from *R. moorei* and 2300 from *R. nebulosus*, were collected across the 15 sampling sites and six occasions. We reared a total of 29 Cecidomyiidae individuals and 264 parasitoid individuals of four species from the collected *R. moorei* galls (Table S6.1). From *R. nebulosus* galls we reared 135 Cecidomyiidae and 558 parasitoids of three species (Table S6.1). Phytochemical diversity was not significantly different between galled and non-galled leaves for either *Rubus moorei* ($t = 0.87$, $df = 43$, $P = 0.39$) or *R. nebulosus* ($t = -1.58$, $df = 20$, $P = 0.13$) (Figure 6.2). There was no significant difference in non galled phytochemical diversity between the two *Rubus* species ($t = 0.12$, $df = 60$, $P = 0.91$). *Rubus nebulosus,* however, had significantly lower galled leaf phytochemical diversity compared with *Rubus moorei* ($t = 2.21$, $df = 22$, $P = 0.038$) (Figure 6.2).

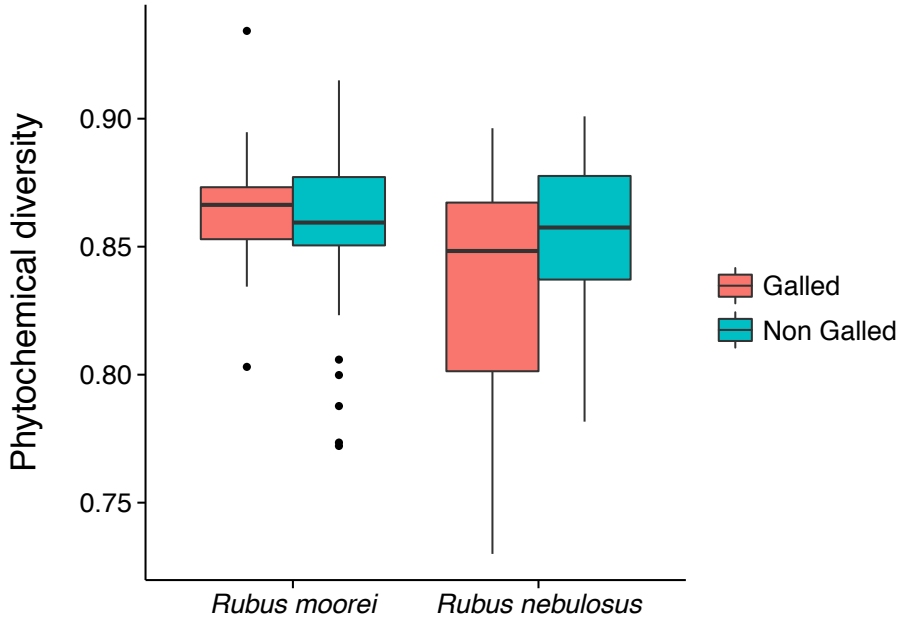



**Figure 6.2** Boxplots of phytochemical diversity of galled (pink) and non galled (blue) leaves for Rubus moorei and Rubus nebulosus.

Phytochemical diversity of galled leaves was significantly related to site temperature ($R^2 = 0.18$, $F_{1,31} = 6.81$, $P = 0.014$). Phytochemical diversity of non galled leaves, however, was not significant ($R^2 = 0.00$, $F_{1,60} = 0.05$, $P = 0.82$). Parasitoid species richness was significantly positively correlated with phytochemical diversity of galled leaves ($R^2 = 0.30$, $F_{1,20} = 8.39$, $P = 0.009$) (Figure 6.4).

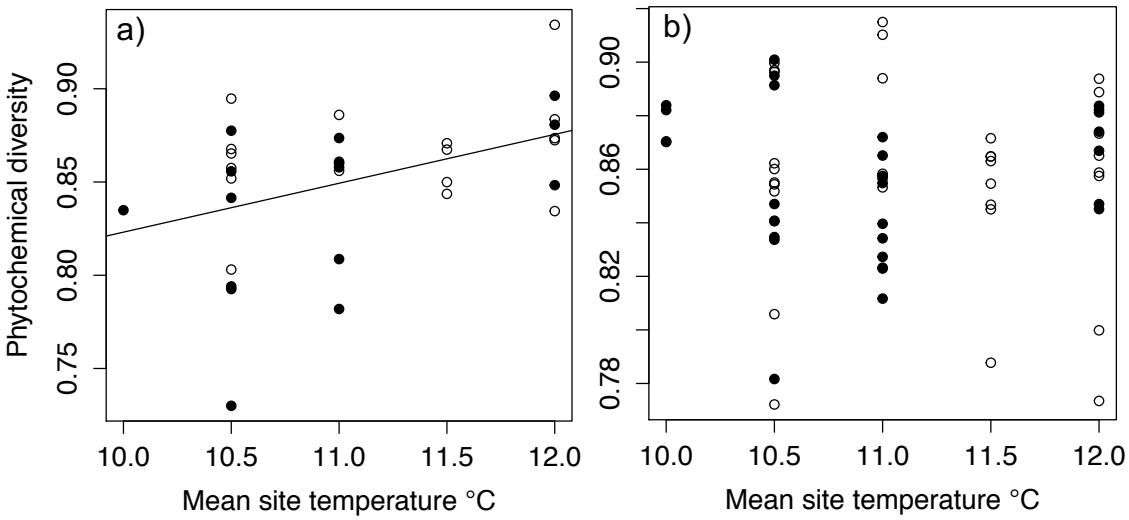



**Figure 6.3** Phytochemical diversity of a) galled leaves and b) non galled leaves. *Rubus nebulosus* is represented by filled circles and *R. moorei* by open circles, significant linear regression is represented by the solid line.

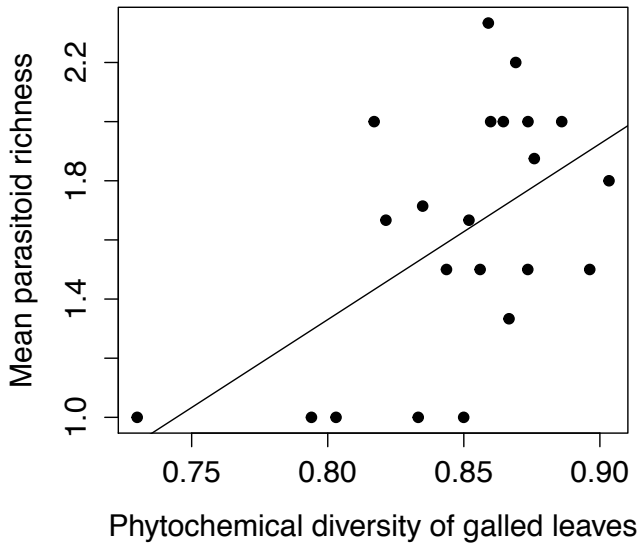



**Figure 6.4** The relationship between mean site parasitoid species richness and phytochemical diversity of galled leaves (*R. moorei* n = 12, *R. nebulosus* n = 10). Significant linear regression shown by solid line.

Our initial path model, which included the top-down effect of temperature on parasitoids, fit the data ($P = 0.621$, df = 1, $\chi^2 = 0.245$; null $P = 0.032$) (Figure 6.5a). Average site temperature was positively associated with host phytochemical diversity, which subsequently predicted parasitoid species richness. Temperature had a weak direct negative effect on parasitoid species richness, however, this path was not significant (Figure 6.5a).

A refined model was tested, removing the non significant top down pathway of temperature on parasitoid richness. This refined model showed an improved fit ($P$ = 0.874, df=2, $\chi^2$ = 0.268; null $P$ = 0.08) (Figure 6.5b). Again, temperature was positively associated with phytochemical diversity and this positively predicted parasitoid richness. To test if the observed relationships were related to general plant chemistry rather than gall induced plant chemistry we ran the model with phytochemical diversity of non-galled leaves. This model did not fit the data ($P$ = 0.02).

In line with previous studies (Bukovinszky 2008), it could be that the effect of temperature on higher trophic levels is mediated by changes in resource density. To test this alternative hypothesis, we specified a model with gall density and parasitoid density predicting parasitoid species richness in place of both phytochemical diversity variables (Figure 6.5c). This alternative model was a poorer fit and was not significantly different from the null model ($P$ = 0.58, df = 2, $\chi^2$ = 1.09; null $P$ = 0.64) (Figure 6.5c).

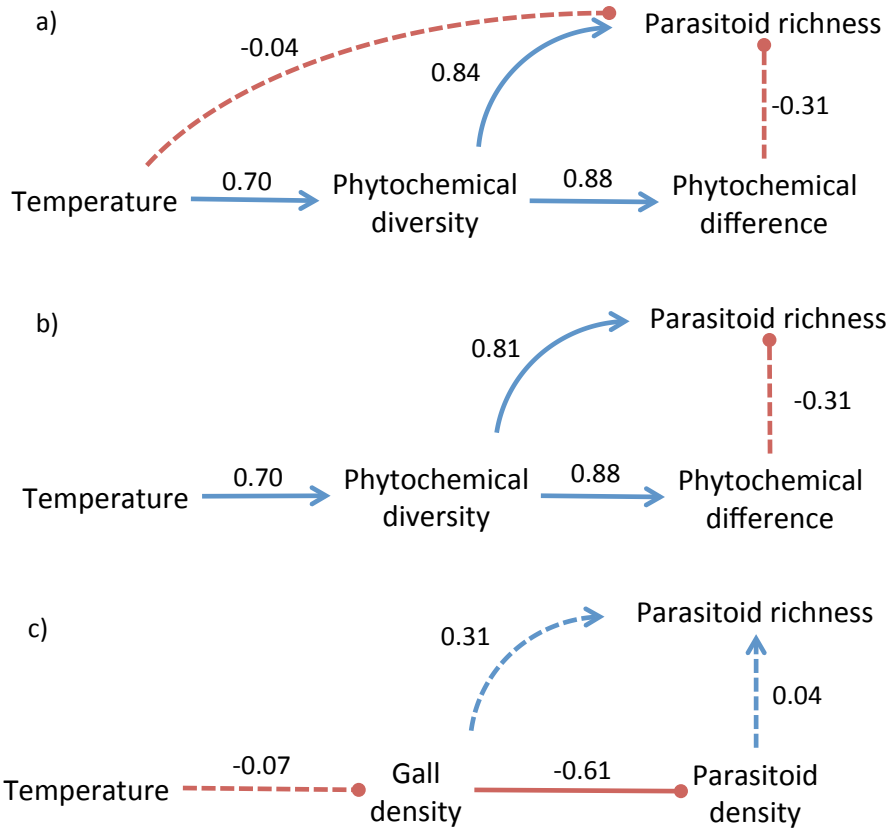



**Figure 6.5** Path models tested a) initial model includes direct effect of temperature on parasitoids; b) refined model; and c) alternate model of density mediated effects. Standardised path coefficients are included next to each path. Significant paths are shown by solid lines and non significant by broken lines. Blue arrows indicate positive effects and red circles negative effects.

## 6. 5 Discussion

The most notable result of the study was the indirect effect of temperature, which caused increased phytochemical diversity of galled leaves, resulting in a strong positive effect on parasitoid species richness. Despite there being no significant difference in phytochemical diversity between galled and non-galled leaves, only galled leaves showed a relationship with temperature. This indicates an interaction among galling, temperature and phytochemical diversity, possibly due to trade-offs in plant secondary metabolites reflecting the relative impacts of biotic and abiotic factors (Siemens et al. 2009).

Galling insects have been shown to manipulate host plant chemistry (Chapter 2). In this study only galled leaves show a positive relationship between phytochemical diversity and site temperature. This indicates that the compounds present in galled leaves are not only different to non-galled leaves but these compounds are either sensitive to, or are used by, the plant to cope with increased temperature. Most of the identified compounds from both *Rubus* species are triterpenoids (see Chapter 5). These are common surface-active plant compounds that play an important role in plant ecology (Harborne 1997). The quantity and quality of triterpenoids is influenced by biotic stress, including herbivory (Dutton et al. 2002), and also abiotic stress factors such as humidity and temperature (Liu et al. 2015; Moses et al. 2014). Both *Rubus* species are restricted to higher elevations, which may be due to trade-offs between chemical defence and stress tolerance. For example, Siemens et al. (2009) have shown that the evolution of tolerance to abiotic stress across a range boundary is constrained by allocation to chemical defence. In our study, galling and high temperatures may have an additive effect on plant defence chemistry, ultimately leading to a reduction in available plant resources, potentially restricting their distribution to cooler, higher elevations.

It is generally thought that plants maintain diverse chemical mixtures to defend against a variety of natural enemies (Kursar et al. 2009). Our results demonstrate that more chemically diverse galled leaves have a higher number of parasitoid species, potentially benefiting the plants due to reduced herbivore damage. The results are also consistent with the safe haven hypothesis, i.e. chemically defended herbivores deter other general predators, but are safe hosts for parasitoids (Gentry and Dyer 2002). The hypothesis

predicts that plant chemical defence will indirectly benefit parasitoids by providing enemy-free space in the form of chemically defended hosts. Similar positive effects of host plant chemistry on parasitoids have been found in a number of studies (Bukovinszky 2008; Richards et al. 2015; Gentry and Dyer 2002), although see Ode (2006) for a review of negative effects of phytochemistry on parasitoids.

Parasitoids are able to distinguish even slight differences in host plant quality based on chemical cues (Gols et al. 2009). For parasitoids, it is essential to asses host plant chemistry, as this can be a predictor of host performance which is linked to parasitoid performance (Harvey 2005). Triterpenoids, in particular, are often found in leaf cuticle and are thus part of the first chemical contact between searching parasitoids and the host plant. Dutton et al. (2002), for example, show that parasitoids of apple leaf miners respond to squalene, a triterpenoid present only in mined leaves. Parasitoids have been shown to respond to altered chemical composition of leaf cuticle waxes (Rostás et al. 2008). Although stressed plants may have qualitatively different chemistry, parasitoids are not impaired in their ability to locate host plants (Mumm and Dicke 2010).

Plant-herbivore-parasitoid interactions are affected by a combination of abiotic factors, natural enemies, and plant quality. Temperature has direct effects on host plant quality via changes in phytochemistry but as shown in our observational study, indirect effects can significantly modify herbivore-parasitoid interactions. Looking at only direct effects does not uncover important indirect mechanisms, such as trade-offs in plant chemical defence between herbivory and abiotic stress. Studies on simple tritrophic systems that include host plant chemical response to abiotic factors allows insight into how changes in temperature will affect parasitism, herbivores, and ecosystem services. This is particularly important as warmer temperatures are predicted to disrupt herbivore-parasitoid interactions, potentially leading to herbivore outbreaks.

Our results are to be interpreted with caution as the use of $^{1}$H NMR peak diversity as a measure of chemical diversity has not been thoroughly verified (although see Richards et al. 2015 for justification). It may be that rather than measuring changes in chemical diversity, we are just measuring changes in the concentration of one or two metabolites. The limitations and potential solutions using 2D NMR have been discussed in Chapter 5. Nevertheless, in this study we have found that changes across the whole phytochemical profile may be important for higher trophic levels. The use of NMR

based metabolite profiling provides greater insight into chemically mediated ecological interactions when compared to targeting only a few specific metabolites.

Our results are limited to observational data only. We did not manipulate temperature but used elevation as a proxy. However, tritrophic studies on field-based natural communities are rare, and thus our study provides relevant insight into chemically mediated trophic interactions in a realistic ecological setting. The effects arising from multiple compounds, while rarely studied, suggest that phytochemical diversity may be a key functional trait in its own right (Richards et al. 2010, Raguso et al. 2015). Understanding the role of phytochemical diversity in driving biodiversity and ecosystem processes is particularly important in complex and threatened habitats such as rainforest.

## S6 Supplementary Material

**Table S6.1** Total reared insect abundances from both *Rubus* species at each site.

| Transect | Elevation | Cecidomyiid | Platy sp1 | Platy sp3 | Pter sp2 | *Zaom* | **Total** |
|---|---|---|---|---|---|---|---|
| ***Rubus moorei*** | | | | | | | |
| Binna Burra | 803 | | | 3 | 11 | | **14** |
| | 952 | 5 | | 16 | 10 | 4 | **35** |
| | 1083 | 6 | | | 5 | | **11** |
| Brindle Creek | 762 | 2 | | 3 | 2 | 7 | **14** |
| | 947 | | | 3 | 2 | 2 | **7** |
| | 1059 | | | 2 | 1 | | **3** |
| Bar Mountain | 890 | 1 | 2 | 10 | 5 | 5 | **23** |
| | 994 | | 1 | 8 | 9 | 7 | **25** |
| | 1145 | 3 | | | | | **3** |
| Lamington | 829 | 4 | 2 | 23 | 24 | 4 | **57** |
| | 944 | 1 | | | 16 | 3 | **20** |
| Tweed Road | 860 | 1 | | | 1 | 1 | **3** |
| | 964 | 1 | | 2 | 3 | | **6** |
| | 1098 | 5 | 6 | 8 | 34 | 19 | **72** |
| | Sub total | **29** | **11** | **78** | **123** | **52** | **293** |
| ***Rubus nebulosus*** | | | | | | | |
| Binna Burra | 1083 | | 5 | | | | **5** |
| Brindle Creek | 947 | | 1 | | | | **1** |
| | 1059 | 59 | 39 | 70 | 88 | | **256** |
| Bar Mountain | 890 | 20 | 10 | 66 | 45 | | **141** |
| | 994 | 3 | | 6 | 1 | | **10** |
| | 1145 | 2 | | 2 | 47 | | **51** |
| Lamington | 944 | | 2 | 1 | 2 | | **5** |
| | 1140 | 20 | 8 | 20 | 35 | | **83** |
| Tweed Road | 964 | 9 | | 1 | 24 | | **34** |
| | 1098 | 22 | 4 | 32 | 49 | | **107** |
| | Sub total | **135** | **69** | **198** | **291** | | **693** |
| **Grand Total** | | **164** | **80** | **276** | **414** | **52** | **986** |

# Chapter 7

In the previous chapters we found that temperature had a significant indirect effect on parasitoids through changes in phytochemical diversity. Previous chapters used observations from an elevational gradient. It can be difficult, however, to tease apart the effects of individual environmental variables as they are often highly inter-correlated with elevation. In this chapter we use in-field warming enclosures to experimentally increase temperatures around the focal plant species *Solanum inaequilaterum*. We use these experiments to investigate the effect of increased temperature on phytochemical diversity, general herbivory, galling herbivores and their parasitoids.

**Statement of contribution to a co-authored paper**

Chapter 7 is a co-authored paper that has been prepared for publication. The citation is as follows:

Hall, C.R., Carroll, A.R., Kitching, R.L. (prepared manuscript) The effect of experimental warming on phytochemistry and gall-parasitoid interactions in subtropical rainforest.

My contribution to the paper involved field work, data collection, analysis and writing of the manuscript. Roger Kitching provided direction and guidance with the project and editing of the manuscript. Tony Carroll guided the chemical analysis and also edited the manuscript.

(Signed) _____ (Date) 12/12/2015
Casey Hall

(Countersigned) _____ (Date) 12/12/2015
Associate supervisor: Tony Carroll

(Countersigned) _____ (Date) 12/12/2015
Supervisor: Roger Kitching

# Chapter 7

## The effect of experimental warming on phytochemistry and gall-parasitoid interactions in subtropical rainforest

Casey R. Hall[1*], Anthony R. Carroll[1] and Roger L. Kitching[1]

[1]Environmental Futures Research Institute, School of Environment, Griffith University, Nathan, QLD 4111

[*]Corresponding author: casey.hall@griffithuni.edu.au

## 7.1 Abstract

Simulating climate change by using field-based warming experiments allows for greatly improved understanding of highly complex systems and offers the opportunity to test theories developed using observational and laboratory studies. We ran warming experiments along an elevational gradient in subtropical rainforest for 14 consecutive months. We set up passive warming enclosures around our focal plant species, *Solanum inaequilaterum* at six sites along an elevational gradient in subtropical rainforest. Using this tritrophic system we investigated the direct and indirect effects of increased temperature on phytochemical diversity, general herbivory, galling herbivores and their parasitoids. We effectively increased average daytime temperatures by 0.9°C around individual plants, although the effect of the warming varied from -0.1°C to 2.2°C. We found that warming above 0.8°C led to warmed plants having higher phytochemical diversity compared with controls. Experimental warming significantly decreased gall abundance and increased general herbivory. Neither galling nor general herbivory, however, were affected by plant chemistry. In contrast we found significant negative effects of increased phytochemical diversity on gall parasitoids. These results provide the first field-based experimental evidence for the phytochemically mediated effects of increased temperature on tritrophic interactions in a rainforest system. The effects of increased temperatures acting through different direct or indirect pathways could cancel each other out or lead to changes not predicted in mesocosm experiments or observational studies.

## 7.2 Introduction

The ecological effects of climate change are gradual, requiring long-term observations. These types of studies are rarely possible. Instead, ecologists use natural environmental gradients, such as elevation, as a powerful tool for investigating ecological responses to climate (Körner 2007). The change in climatic variables along elevational gradients can be used as a 'space for time' substitution for making predictions about the impacts of future climate warming (Pickett 1989). It can be difficult, however, to tease apart single environmental variables, as they are often highly inter-correlated with elevation. Ecologists also use laboratory experiments on simplified systems to test specific factors while controlling for other variables (Lawton 1995). Laboratory experiments, however, are unable to capture the complexity of real world species interactions and even at best are a highly simplified version of 'real' ecological communities (Carpenter 1996).

One solution is to use *in-situ* manipulative experiments. Field-based experiments allow for greatly improved representations of highly complex systems and offer the opportunity to test theories developed using observational and laboratory studies. Field-based warming experiments rely on either passive or active warming. Actively warmed experiments use buried heating coils (e.g. de Sassi et al. 2012) or above ground radiators (Barton and Schmitz 2009), while passively warmed experiments use plastic enclosures, simulating a miniature 'greenhouse effect' by trapping warm air (Kharouba et al. 2015; Scherber et al. 2013). The majority of *in-situ* warming experiments are from grassland and temperate forest ecosystems: to date only two warming experiments have been set up in rainforest (Cavaleri et al. 2014; Slot et al. 2014). This lack of studies reflects the logistic (e.g. remote locations) and environmental challenges (e.g. high biodiversity and complexity) inherent when working in rainforest ecosystems (Cavaleri et al. 2014).

Manipulative experiments have also been criticised as they often inflict only short-term, high stress conditions that do not reflect the gradual long-term effects of climate change. Short term, spatially confined experiments, for example, have been found to chronically overestimate net ecosystem responses to perturbation (Leuzinger et al. 2011). In addition, generalising the effects of gradual climate change from short-term 'shock' experiments may be misleading as they do not take into account local

adaptation and evolutionary responses (Barton 2011). This can lead to seemingly contradictory results from observational studies compared to manipulation experiments. Most observational studies, for example, report declines in parasitism with increasing elevation and decreasing temperatures (Hall et al. 2015; Maunsell et al. 2014; Péré et al. 2013), suggesting that parasitoids benefit from warmer temperatures. In contrast, warming experiments show that parasitoids are significantly negatively affected by increased temperatures (de Sassi et al. 2012; Gillespie et al. 2011; Dyer et al. 2013). A solution is to combine environmental gradients with experimental manipulations, thus incorporating local adaptation into an ecological relevant study system.

In previous studies we investigated gall-parasitoid interactions along an elevational gradient in subtropical rainforest (Chapters 4 and 6). We found that parasitism rate increased at elevations with higher temperatures leading to stronger relationships between parasitism and parasitoid species richness (Hall et al. 2015). This implies that warmer temperatures improve parasitoid functioning. Furthermore, we found that parasitoids respond strongly to the chemical diversity of their host plants (Chapter 6), and that changes in phytochemistry are influenced by local temperatures (Chapter 5). The strong response of parastoids to both temperature and phytochemistry implies that increased temperatures may benefit parasitoids not only through increased functioning but also through enhanced host location and/or protection through changes in host plant chemistry.

Using these results we asked the following questions: (1) Does increased temperature lead to increased phytochemical diversity? (2) Does specialist (galling) and generalist herbivory respond differently to increased temperatures and changes in phytochemistry? (3) Are parasitoids positively affected by temperature related changes in host plant phytochemical diversity? To investigate these questions we ran warming experiments along an elevational gradient in subtropical rainforest for 14 consecutive months, incorporating both spatial and seasonal variation. By combining both an elevational (i.e. climatic) gradient and experimental manipulation, we can start to tease apart the effects of temperature from other variables while accounting for local adaptation within a natural ecosystem.

## 7.3 Methods

*Study sites*

We set up the experiment at six sites in subtropical rainforest in Border Ranges National Park, Northern New South Wales. These sites had originally been established for our observational study of leaf gallers and their parasitoids (Chapter 4). The six sites were from the Brindle Creek and Bar Mountain transects, ranging from 762m asl to 1145m asl. Detailed site descriptions are provided elsewhere (Chapters 3, 4, 5 and 6).

*Warming experiments*

Based on our observational surveys, we selected *Solanum inaequilaterum* as the focal species for the experiments as it is evenly distributed along the gradient, sufficiently abundant to enable experimental manipulations and grows upright so that making it possible to build enclosures around the plants.

At each site we sought ten individuals of *S. inaequilaterum* within a restricted 50 $m^2$ area in order to maintain a standard plot size and to decrease variation in abiotic conditions and genetic differences among control and experiment plants. Plants were selected with height varying between 1m and 1.5m, so controlling for plant age and size. Five of these plants were designated controls and five had warming enclosures built around them. Warming enclosures consisted of three plastic coated steel rods hammered into the soil at equal intervals around the plant. A double layer of clear polythene plastic (100μm thick) was wrapped around the steel rods and secured with duct tape and cable ties (Figure 7.1). Temperature was recorded within warming enclosures and at a control plant at each site using temperature data loggers (DS21 Thermochron, iButton, Dallas Semiconductor/Maxim, CA, USA). These recorded the temperature every three hours from July 2014 to September 2015.

Enclosures were constructed so most of the top part of the plant was exposed in order to prevent the plastic acting as a barrier to insect access to the plants. In addition, three open mesh control plants (Figure 7.1c) were added at each site in January 2015 to control for the plastic enclosures potentially acting as a barrier. Due to fewer sampling occasions and several tree falls destroying many of the mesh controls, there was not

enough data to use for comparisons. Consequently, all analyses in this paper are on the original control plants (i.e. no mesh enclosure).


**Figure 7.1** a) Plastic warming enclosure, b) early trial plastic enclosure (ibutton visible) built using bamboo and c) control plant with fine nylon mesh as a barrier control with experiment plant in the background.

Three humidity ibuttons were set up to test whether the warming enclosures also had an effect on humidity. These sensors did not work properly in the rainforest and consistently recorded 100% humidity due to saturation of the sensors. Conditions above 60% relative humidity can offset the humidity readings for a prolonged period of time (Cavlier 2012). Kharouba et al. (2015) used plastic bags as warming enclosures on tree branches and found no significant difference in humidity between control and experiments. In addition, Godfree et al. (2011) found only minimal differences in relative humidity using open top warming chambers.

Galled and non-galled leaves from control and experiment plants were sampled on four separate occasions; September 2014, January 2015, May 2015 and August 2015. Each sampling occasion was separated by three months to allow for regrowth. Galls were

sampled by closely inspecting each plant and removing all galled leaves. For consistency, all galls were collected, regardless of their stage of development. Approximately 15 non-galled leaves were randomly sampled from each plant for herbivory and chemical analyses. Galled leaves were placed into sealed plastic boxes containing moistened tissue and checked every 3 days for two months for the emergence of adult insects. Reared adult insects were preserved in ethanol. Both galled and non-galled leaves were assessed for herbivory damage using the methods detailed in Chapter 3. Each gall contains one cavity with a single fly larvae, but often multiple parasitoids.

*Chemical analysis*

The non-galled leaves from control and experiment plants 1, 2 and 3 from each site were collected for a total of six chemical samples for each site. These were stored in silica gel and dried in a drying cabinet in preparation for chemical analysis. Dried leaves were ground and 0.1g (dry weight) was extracted in methanol and evaporated for $^{1}$H NMR analysis. NMR methods are detailed in Chapters 5 and 6.

*Data analysis*

Temperature data were averaged across the 14 months for each plant. As warming was only effective during the day, diurnal temperature variation was calculated for each plant as the difference between average 12AM and 12PM temperatures to account for microclimatic differences among the plants within each site. The effect of the warming experiments at each site was calculated as the difference in diurnal variation between treatment and control plants.

The NMR spectroscopic data were processed in MestReNova software (version 10.0.2-15465, Mestrelab Research S.L. 2015) using common metabolics methods detailed in Chapters 5 and 6 (Kim et al. 2010). Phytochemical diversity was calculated using methods detailed in Chapter 6. The limitations of using $^{1}$H NMR peak diversity as a measure of chemical diversity are discussed in Chapter 6. All statistical analyses were carried out using R statistical software (R core development team 2015). A two way ANOVA was used to test the effect of experimental warming and elevation on phytochemical diversity. Post-hoc analysis was used to test for significant differences among elevations. Walch t tests were used to test for differences between control and

experiments for herbivory, gall abundance and parasitoid density. A Principle Component Analysis (PCA) was conducted using binned NMR data to visualise any groupings of samples and to identify significant signals in the NMR spectra. Linear models were used to test for significant relationships between: the effect size of the warming experiments and the difference between experiment and control plant phytochemical diversity; gall abundance and herbivory; and parasitoid density and phytochemical diversity of control and experiment plants.

## 7.4 Results

*Warming experiments*

The warming experiments increased daytime temperatures by 0.9°C on average, although this varied from 2.2°C to -0.1°C (Table 7.1). Maximum temperature was on average 3.0°C higher for warmed plants, again this varied across the sites, ranging from 9.8°C to -0.8°C. Overall there was no significant difference in phytochemical diversity between experiment and control plants (df = 1, F = 1.09, $P = 0.31$). There was a significant effect of elevation (df = 5, F = 4.22, $P = 0.006$), however, post-hoc tests show that this was due to the 947 m site having significantly lower phytochemical diversity compared to all other sites except the site at 890 m asl (Table S7.1).

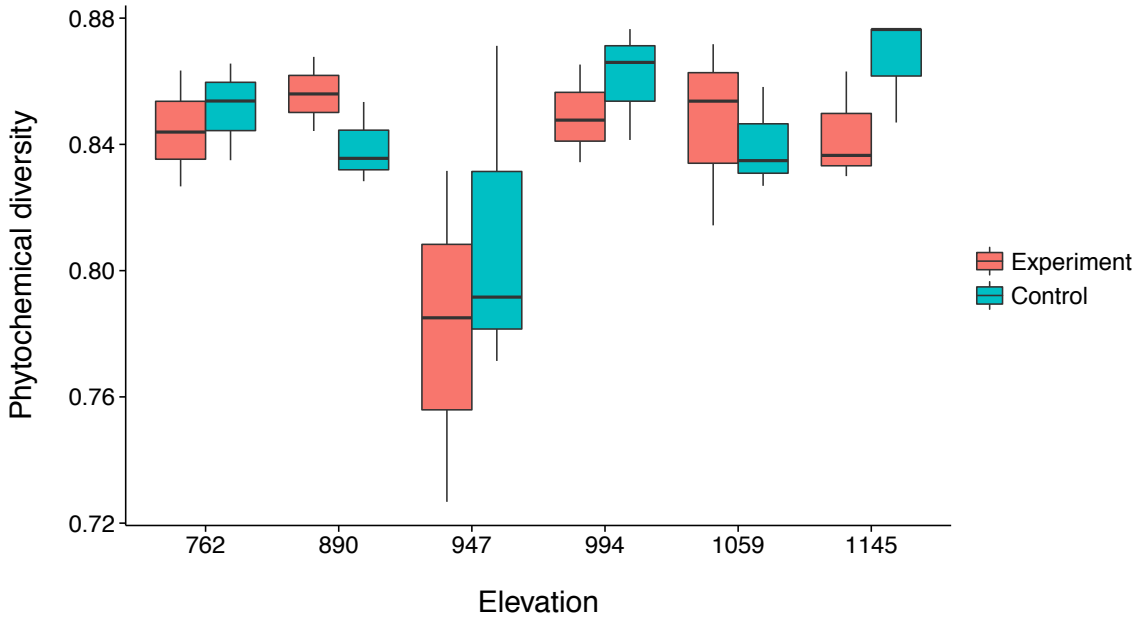



**Figure 7.2** Boxplots showing average phytochemical diversity of warmed and control plants at each elevation (m asl). Experiments are shown in red and controls in blue.

**Table 7.1** The difference between day (12 PM) and night (12 AM) average and maximum temperatures (°C) for control and treatment plants at each site. The effect of treatments at each site was calculated as the difference in diurnal variation between treatment and control plants.

| | | **Average temperature (°C)** | | | **Maximum temperature (°C)** | | |
|---|---|---|---|---|---|---|---|
| Elevation | Time | Experiment | Control | Difference | Experiment | Control | Difference |
| **Brindle Creek** | | | | | | | |
| 762 | 12 PM | 15 | 14.3 | | 34 | 33 | |
| | 12 AM | 12.25 | 12.2 | | 21.5 | 22 | |
| | **Diurnal variation** | **2.75** | **2.1** | **0.65** | **12.5** | **11** | **1.5** |
| 947 | 12 PM | 15.5 | 13.6 | | 31.3 | 24 | |
| | 12 AM | 12.1 | 12.4 | | 21 | 23.5 | |
| | **Diurnal variation** | **3.4** | **1.2** | **2.2** | **10.3** | **0.5** | **9.8** |
| 1059 | 12 PM | 15.4 | 14.7 | | 31.2 | 27.5 | |
| | 12 AM | 11.6 | 12 | | 23.5 | 23.5 | |
| | **Diurnal variation** | **3.8** | **2.7** | **1.1** | **7.7** | **4** | **3.7** |
| **Bar Mountain** | | | | | | | |
| 890 | 12 PM | 14.7 | 13.1 | | 27.5 | 26 | |
| | 12 AM | 12.4 | 12.3 | | 21.2 | 22 | |
| | **Diurnal variation** | **2.3** | **0.8** | **1.2** | **6.3** | **4** | **2.3** |
| 994 | 12 PM | 15.3 | 14.8 | | 29.8 | 28 | |
| | 12 AM | 12.1 | 11.9 | | 22.4 | 22.5 | |
| | **Diurnal variation** | **3.2** | **2.9** | **0.3** | **7.4** | **5.5** | **1.9** |
| 1145 | 12 PM | 14.1 | 14.6 | | 27.2 | 28 | |
| | 12 AM | 11.1 | 11.5 | | 22 | 22 | |
| | **Diurnal variation** | **3** | **3.1** | **-0.1** | **5.2** | **6** | **-0.8** |

*Phytochemical diversity*

There was a significant relationship between the effect size of the warming experiments and the difference in phytochemical diversity between warmed and control plants ($R^2$ = 0.42, $F_{1,14}$ = 10.2, $P$ = 0.006) (Figure 7.2). Figure 7.2 shows that most plants warmed by greater than 0.8°C had higher phytochemical diversity compared with control plants, and this was reversed for those plants were the effect of warming was less than 0.8°C.

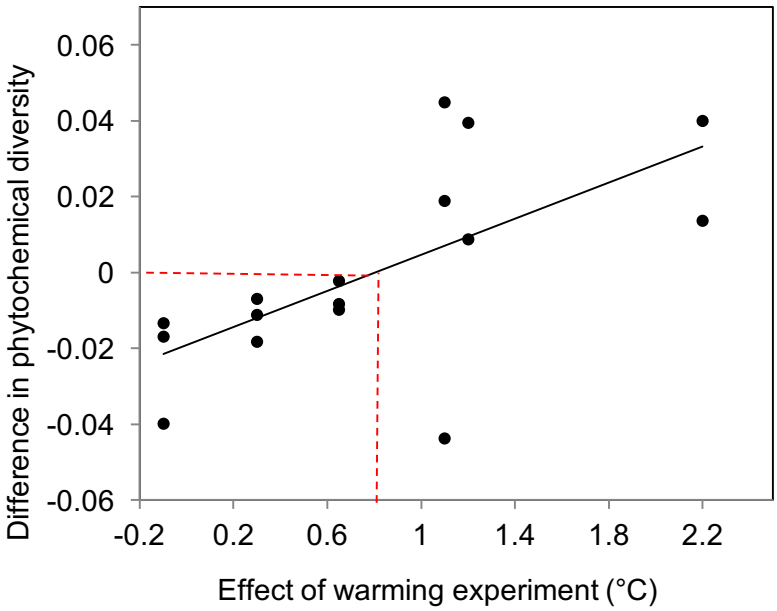



**Figure 7.3** Relationship between the effect size of the warming experiment and the difference between experiment and control phytochemical diversity ($R^2 = 0.42$, $F_{1,14} = 10.2$, $P = 0.006$). Three control and experiment pairs were sampled at each site, n=16 due to two missing values. The solid line indicates a significant correlation. The broken lines show the intersection of 0.8°C warming and the change from negative to positive phytochemical difference.

The PCA shows two clear groupings (Figure 7.4), reflecting the 0.8°C warming threshold seen in Figure 7.3. The plants from more effectively warmed sites had a greater spread and distance between warmed and control plants, while plants from the less warmed sites show a clumped distribution with both warmed and control plants together. Figure 7.5 shows that several areas in the upfield ($\delta_H$ 0.8-1,2 ppm) region and around $\delta_H$ 6.5 ppm of the NMR spectra correlate with both PC1 and PC3. While the region $\delta_H$ 7.0 to 7.5 ppm shows negative correlations with PC3. Protons in this region are usually associated with aromatic rings, such as the phenol amide identified from *S. inaequilaterum* in Chapter 5. This region ($\delta_H$ 7-7.5ppm) is strongly associated with the separation of sites based on the 0.8°C warming threshold (Figure 7.4).

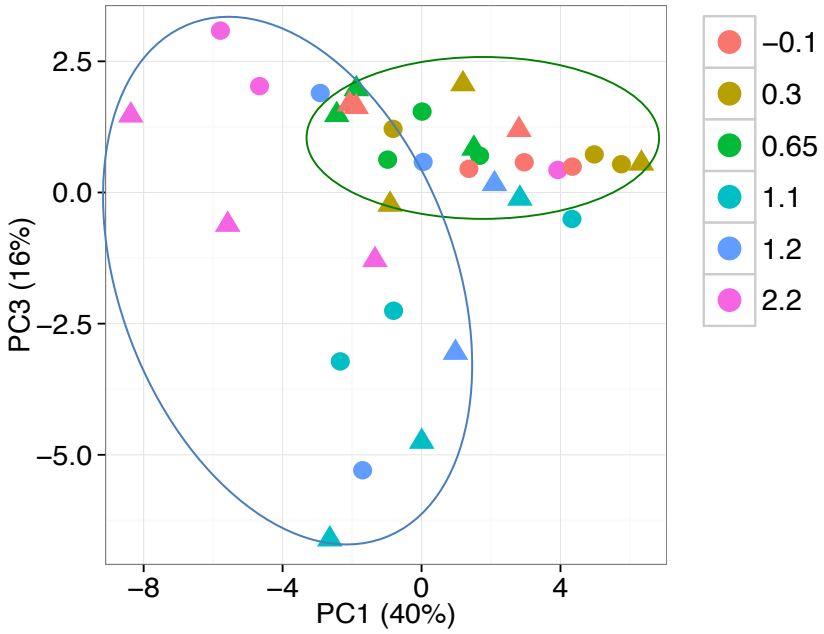



**Figure 7.4** PC1 vs PC3 of NMR from experiment and control plants at each site. The colours represent different sites and are based on the effect size of the warming. Filled circles represent control plants and triangles treatment plants. The ellipses show groupings of sites warmed above (blue) and below (green) 0.8°C.

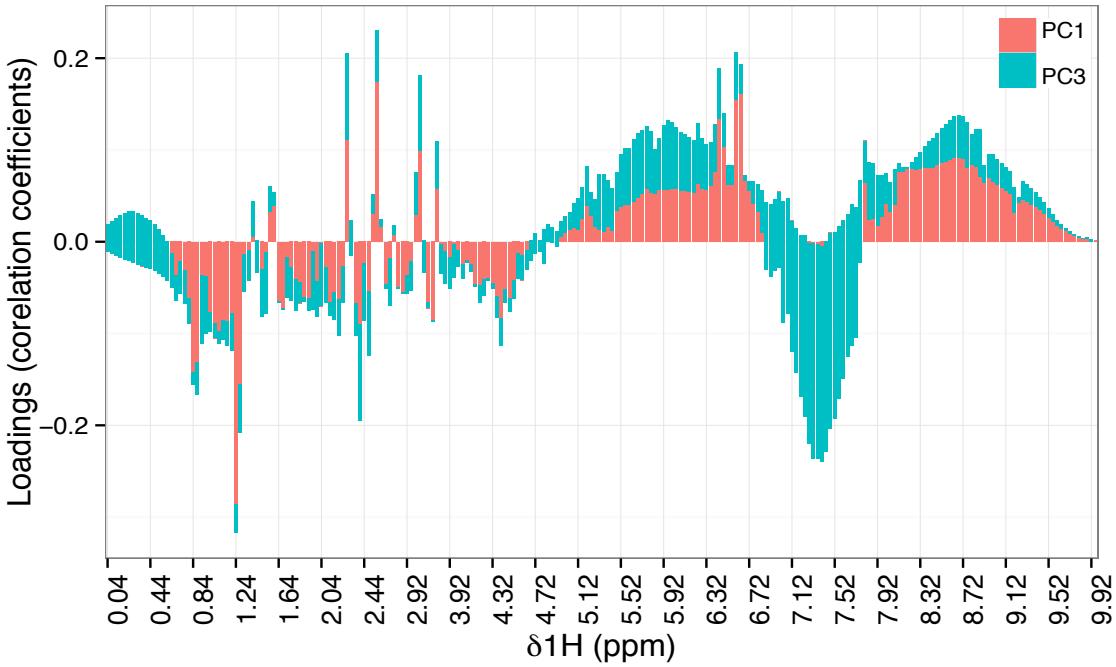



**Figure 7.5** Bar plot showing correlations from PC1 (red) and PC3 (blue) against binned NMR chemical shifts (loadings).

*Herbivory*

A total of 844 galls were collected across the six sites and four sampling occasions. From these we reared a total 43 *Dasineura* sp individuals. Mean herbivory, measured as percentage of leaf area missing, was significantly higher on warmed plants (t = -5.1, df = 10, $P = 0.001$), particularly at low elevations (Figure 7.5a). Mean gall abundance showed the opposite response to herbivory and was significantly lower on warmed plants (t = 2.7, df = 6, $P = 0.04$), and this was strongest at high elevations (Figure 7.5b). Gall abundance significantly decreased with increasing herbivory ($R^2 = 0.67$, $F_{1,10} = 19.9$, P = 0.001) (Figure 7.5c). Both gall abundance and herbivory were not significantly related to warming effect size or phytochemical diversity.

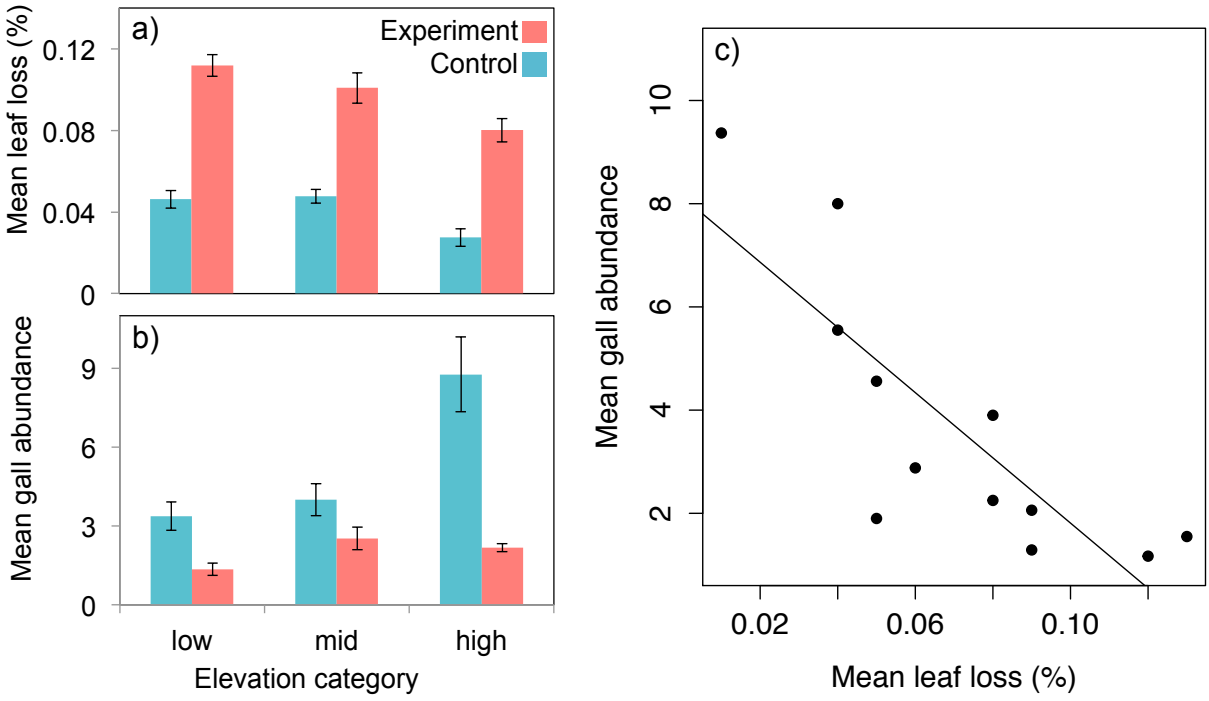



**Figure 7.6** a) Mean herbivory (leaf loss) and b) mean gall abundance for experiment and control plants from low (762 and 890), mid (947 and 994) and high (1059 and 1145) elevation sites; c) relationship between mean gall abundance and mean herbivory, significant regression shown by a solid line.

*Parasitism*

A total of 124 parasitoids were reared belonging to two morphospecies, 34 Tetrastichinae sp. and 90 Platygasteridae sp. 1. Only 19 parasitoids were reared from experimental plants compared with 105 from controls. Parasitoid density, which takes into account the significantly lower gall abundance on experimental plants, was not

significantly different between control and experiment plants (t = 1.58, df = 38, *P* = 0.12). Mean parasitoid density decreased significantly with increasing phytochemical diversity for experimental plants ($R^2 = 0.32$, $F_{1,11} = 5.06$, *P* = 0.046) (Figure 7.7). A similar trend was seen in control plants, although this was not significant ($R^2 = 0.03$, $F_{1,14} = 0.4$, *P* = 0.5) (Figure 7.7).

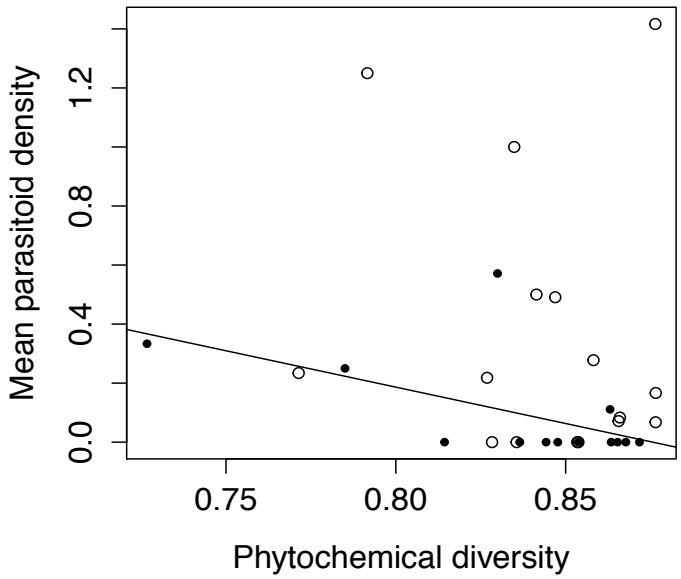



**Figure 7.7** Mean parasitoid density and phytochemical diversity. Filled circles represent experiments and open circles control plants. Significant regression indicated by solid line ($R^2 = 0.32$, $F_{1,11} = 5.06$, *P* = 0.046).

## 7.5 Discussion

Field based manipulation experiments are a valuable tool which can be used to detect potential effects of climate change that may not be apparent using observational studies alone (Cavaleri et al. 2015; Luo et al. 2011). In rainforests, however, field-based manipulation experiments remain rare. Using passive warming enclosures we effectively increased average daytime temperatures by 0.9°C on average around individual plants along an elevational gradient in subtropical rainforest. We found overall no difference in photochemical diversity between control and warmed plants. We did find, however, that warming above 0.8°C led to warmed plants having higher phytochemical diversity compared with controls. We found that experimental warming caused contrasting responses in specialist (galling) compared to generalist (chewing) herbivory. Neither galling nor general herbivory was affected by plant chemistry. In contrast we found significant negative effects of phytochemical diversity on gall

parasitoids. These results provide the first field-based experimental evidence for the phytochemical mediated effects of increased temperature on tritrophic interactions in a rainforest system.

We found no difference in phytochemical diversity between control and warmed plants due to overall low overall variation in diversity and large within-site variation. One site (947) had lower chemical diversity compared to all other sites. This result may be due to low genetic diversity at this particular site. We did find what appeared to be a threshold of warming, where experimental plants warmed above 0.8°C on average had higher phytochemical diversity compared with control plants. This is supported by our previous study (Chapter 6) where we found increased phytochemical diversity at higher temperatures along an elevational gradient in *Rubus* species. This pattern, however, was only significant in galled leaves, indicating that phytochemical diversity is likely driven by complex interactions between temperature and galling (Chapter 6). Both observation gradient studies and manipulation experiments have found positive relationships between temperature and chemical defence. For example, phenolics and iridoid glycosides along elevational gradients (Pellisier et al. 2014; Zidorn 2010), phenolics and tannins along a latitudinal gradient (Hallam and Read 2006) and saponins in mesocosm experiments (Dyer et al. 2013) have all shown increased concentration or production due to higher temperatures. Increased production of secondary metabolites with increasing temperatures may function as protection against heat stress induced oxidative damage, UV damage and help prevent water loss (Bita and Gerats 2013).

The temperature induced production of secondary metabolites may have unpredictable effects on insect herbivores. Surprisingly we did not find any relationship between host plant chemical diversity and herbivory rates. This is in contrast to previous studies, which found that the negative effects of plant defence compounds on herbivores are exacerbated by increased temperatures (Rodriguez-Casteneda 2013; Kollberg et al. 2015). In line with our previous results (Chapter 3), we found that increased leaf herbivory significantly decreased gall abundance, suggesting that strong inter-guild competition may be masking the responses of herbivory to plant chemistry. Increased herbivory by general leaf chewing herbivores can potentially have negative affects on sessile herbivores such as gallers due to direct resource competition (Crutsinger et al. 2013). The relationship, however, is a reciprocal one, as galling insects have been

shown to increase concentrations of chemical defences, deterring subsequent leaf chewing herbivory. Pascual-Alvarado *et al.* (2008), for example, found that galled leaves not only had greater phenol concentrations but also significantly less folivore damage.

Interestingly, we found contrasting responses to experimental warming between galling and general herbivores. Galling significantly decreased and herbivory increased significantly on experimental plants. Increased temperatures are predicted to benefit insect herbivores due to faster development times and increased fitness (Hillebrand et al. 2009). While the response of herbivores to increased temperatures is highly variable (Lemoine et al. 2014), studies generally show positive effects (de Sassi and Tylianakis 2012; Dyer et al. 2013; see Bale et al. 2002 for a review). In this study, however, we cannot determine if the reduced galling is due to direct effects of increased temperature or resource competition due to increased herbivory or both. Nevertheless, our results suggest that generalist herbivores may perform better than specialists under future climate warming.

We found no significant difference in parasitoid density on warmed compared to control plants. This suggests that the warming experiments significantly negatively affected the galling fly but not its parasitoids. Parasitoids were, however, significantly negatively related to phytochemical diversity of warmed plants. Our results, however, should be interpreted with caution due to the low numbers of insects reared from experiment plants. A number of previous studies have found that the negative effects of plant defence chemistry on parasitoids is mediated through changes in the host insect (Dyer et al. 2013; Gols et al 2008; Harvey 2005; Ode 2006). One caveat is that these studies (and ours) all used simplified tritrophic systems with only one or two host-parasitoid relationships. These results contrast to our previous results (Chapter 6) and several previous studies (Bukovinszky 2008; Richards et al. 2015; Gentry and Dyer 2002), which found that parasitoids benefited from increased host plant chemical defence. The main differences are that these studies looked at community level responses rather than single tritrophic food chains. This suggests that results found using simplified systems may not accurately reflect community level responses, highlighting the need for manipulative experiments at the community level (de Sassi and Tylianakis 2012).

Our results are limited as the experiments raised only daytime temperatures. The asymmetric increases in night temperatures predicted under future climate change may negatively affect insects (Zhao et al. 2014). For passive experiments like ours in remote areas, night temperature could be increased with the addition of thermal mass (Godfree et al. 2011). We chose not to include this due to the large amount of thermal mass needed to have a significant effect in our study design. Nevertheless, our experiments successfully increased average day temperatures along with average maximum temperatures, both of which are predicted to increase under future climate change scenarios (IPCC 2007). In addition we found large variation in the effectiveness of the treatments across the sites and a negative effect on phytochemical diversity below warming of 0.8°C. This was possibly due to varying amounts of sunlight, wind and other climatic variables across the gradient altering the treatment effect size. This allowed us, however, to look at the effects of the intensity of warming, rather than just a single temperature increase. Some of our results could have been influenced by treatment effects, that is, the artificial conditions created inside the warming enclosures. However, the size of the enclosures and amount of plant material left exposed would have had minimal impacts on insect movement. We set up mesh controls to account for this potential artefact, however we did not get enough data for comparison (see methods). Despite these drawbacks, our passive warming experiments, the first to be used in rainforest, successfully raised ambient daytime air temperatures across an elevational gradient.

The responses of biotic interactions to climate change are complex. The effects of increased temperatures acting through different direct or indirect pathways could cancel each other out or lead to changes not predicted in mesocosm experiments or observational studies. Predicted increases in plant biomass due to temperature may be counteracted by changes in herbivory, parasitism and increases in secondary metabolites, such that the overall effect of temperature is negative. It is clear that complex interactions must be examined in order to produce realistic predictions for future impacts of climate change at the community level.

**Acknowledgments**

This study was funded by Griffith University School of Environment and a grant from the Ecological Society of Australia.

## S7 Supplementary Material

**Table S7.1** Tukey post-hoc comparisons of means, 95% family-wise confidence level for phytochemical diversity at each elevation. Significant differences are in bold ($P<0.05$).

| Elevation | Difference | Lower | Upper | P |
|---|---|---|---|---|
| 890-762 | -0.0031 | -0.052 | 0.046 | 0.99 |
| 947-762 | -0.052 | -0.098 | -0.005 | **0.02** |
| 994-762 | 0.0072 | -0.0395 | 0.054 | 0.99 |
| 1059-762 | -0.0048 | -0.0514 | 0.04192 | 0.99 |
| 1145-762 | 0.0068 | -0.0399 | 0.05353 | 0.99 |
| 947-890 | -0.0486 | -0.0976 | 0.00032 | 0.05 |
| 994-890 | 0.0103 | -0.0387 | 0.059 | 0.98 |
| 1059-890 | -0.0016 | -0.051 | 0.047 | 0.99 |
| 1145-890 | 0.00996 | -0.039 | 0.0589 | 0.98 |
| 994-947 | 0.0589 | 0.012 | 0.1056 | **0.01** |
| 1059-947 | 0.047 | 0.0003 | 0.0937 | **0.04** |
| 1145-947 | 0.059 | 0.012 | 0.105 | **0.01** |
| 1059-994 | -0.012 | -0.059 | 0.0348 | 0.97 |
| 1145-994 | -0.00031 | -0.047 | 0.046 | 1.0 |
| 1145-1059 | 0.012 | -0.035 | 0.058 | 0.97 |

# Chapter 8

## General discussion and conclusions

## 8.1 Discussion

Plant-herbivore-parasitoid systems involve most known insect species (La Salle 1993). These complex and diverse interactions are often chemically mediated (Dyer et al. 2014). Increasing temperatures over the next century are predicted to disrupt these interactions, altering terrestrial ecosystems (Tylianakis et al. 2008). This has prompted the call for research that incorporates complex interactions into climate change research (Harrington et al. 1999; Woodward et al. 2010). The aim of this thesis has been to determine the pathways through which temperature mediates the interactions among host plant chemistry, galling herbivores and their parasitoids.

In this thesis we have shown that:

- galling insects lead to increased levels of defence chemicals in host plants, although this varies depending on the type of compound and the galling insect taxa (Chapter 2);
- high gall density leads to both inter- and intra-specific competition among leaf gallers and general herbivory and that these competitive interactions are stronger than the effect of elevation on herbivory (Chapter 3);
- there are strong effects of site temperature on parasitism rates and on the relationship between parasitoid richness and parasitism rates (Chapter 4);
- site temperatures have a greater effect on the phytochemistry of galled leaves compared with non-galled leaves (Chapter 5);
- higher site temperatures have an indirect, positive effect on parasitoid species richness through increases in phytochemical diversity (Chapter 6); and
- experimental warming decreased galling, increased general herbivory and increased phytochemical diversity, negatively affecting parasitoid density (Chapter 7).

We found that galling insects are able to induce increased levels of defence chemicals in host plants (Chapter 2). There is, however, variation in this response depending on the

type of compound and the galling insect taxon. Galled plants in boreal and Mediterranean habitats showed increased chemical defences, supporting the idea that the galling niche is successful in these habitats due to the plant community as a whole being more chemically defended, as predicted by the harsh environment hypothesis (Price et al. 1987). Galling insects, however, are also under significant selection pressure from parasitoids (Csóka et al. 2005; Stone and Schönrogge 2003). The radiation of galling insects, particularly the ability to increase secondary metabolities on certain host plant taxa, may be a response to both environmental factors and their natural enemies. Finding evidence for a unified theory on galling insect distributions and plant chemical defence is likely to be difficult. More studies are needed linking plant chemistry, galling insects, and their parasitoids at the community level.

In an ecological landscape where concentrations of plant secondary metabolites differ both in amount and in composition, there may be a preference by galling insects for plants with specific chemical profiles. This may lead to optimal plants (from the galling insect's point-of-view) with high densities of galling insects. In Chapter 3 we found that high densities of galling insects on *R. moorei* lead to decreased adult gall fly body size. We also found that high gall density was correlated with decreased general herbivory. The subsequent discovery in Chapter 3 that only one morphospecies galls two different host plants has interesting implications in terms of host plant chemistry and differences in the parasitoid guild. Further molecular analysis could determine if this morphospecies is undergoing speciation driven by host plant switching, and whether this has resulted in 'enemy-free space' for the galling fly (Nyman et al. 2007). Gall-induced increases in defence compounds have been shown to negatively affect herbivores sharing the galled host plant (Helms et al. 2013; Pascual-Alvarado et al. 2008). We found differences in the chemistry between the two *Rubus* host species in response to galling and temperature (Chapter 5). In particular, galled *R. moorei* leaves corresponded to triterpene signals in the $^{1}$H NMR spectra. Triterpenes have many ecological roles in plants and can be influenced by both temperature and herbivory (Moses et al. 2014). Aphid galls on pistachio trees, for example, increase production of terpenes, resulting in a defensive role against mammalian herbivory (Rostás et al. 2013). It is possible that the galling-induced changes in host plant secondary metabolites act as a deterrent against general herbivory. Confirming these effects, however, will require further experimentation using herbivore exclusion.

Triterpenes can also affect higher trophic levels. Parasitoids, for example, have been shown to respond to specific triterpenes (Dutton et al. 2002). Parasitoids are tied to their hosts' development which in turn can be affected by plant chemistry. At the community level, parasitoids have been shown to be attracted to plants with increased chemical diversity (Richards et al. 2015). One of the most notable results of our study was the increase in parasitoid species richness on more chemically diverse plants (Chapter 6). We found that overall higher temperatures lead to increased phytochemical diversity (as defined in Chapter 6) in both the elevational gradient and the experimental study (Chapters 6 and 7). We found, however, that this had opposing effects on parasitoids between the two studies. Existing studies have shown variable effects of increased temperature on higher trophic levels, highlighting the uncertainty surrounding the predicted effects of climate change on natural enemies (Voigt et al. 2003). Gillespie et al. (2012), for example, found opposing effects of warming experiments on two species of parasitoids on a shared aphid host. While warming experiments have shown negative effects of temperature on parasitoids (Dyer et al. 2013; de Sassi and Tylianakis 2012), observational studies show that parasitoids benefit from warmer temperatures (Maunsell et al. 2014; Péré et al. 2013). In chapter 4, for example, we found that parasitism rates increased with increasing temperature and that higher temperatures led to a stronger richness-parasitism relationship. A more functionally diverse guild may act as biological insurance through an increased ability to compensate for individual species fluctuations. While experiments show negative effects of temperature on a single parasitoid species, the overall impact at the community level is less clear. It is difficult to scale up the effects of temperature at the individual level to effects on even a simple community, let alone a complex naturally occurring one (Gillespie et al. 2012).

As is the case with temperature effects, studies linking phytochemistry and parasitoid performance, show varying results. Most, again, have examined only simplified crop systems and single compounds. Many previous studies have taken what may be considered an oversimplified approach to plant chemistry by looking at single classes of compounds. These studies tend to find negative effects of chemical defence on parasitoids mediated through negative changes upon host insects (Gols et al. 2007; Ode 2006). In the same way that community responses cannot be determined by looking at single species alone, the synergistic effects of high phytochemical diversity cannot be extrapolated from looking at single compound types. We used $^{1}$H NMR to examine

phytochemical diversity and, while it is an extremely powerful tool, it does, however, have some limitations. $^{1}$H NMR spectra from crude plant extracts often have multiple overlapping peaks and many peaks may correspond to a single compound. As such, $^{1}$H NMR based diversity is more a measure of peak diversity, which may not necessarily correlate with compound diversity. One solution for future consideration may be to use 2D NMR, such as heteronuclear single quantum correlation (HSQC), as a phytochemical fingerprint. HSQC gives data on both the $^{1}$H and C chemical shifts of protons within the sample, providing higher resolution (Pierens et al. 2009).

As I have shown in Chapter 7, manipulative experiments produce insights not clearly available from pattern analyses. In trying to overcome the limitations of using either elevation or experiments alone, we combined the two methods for looking at the effects of increased temperature on tritrophic interactions. The experiments I used, however, have limitations which need to be addressed in future work. We relied on passive warming only in our experiments. Further, the experiments were limited to single plants and daytime warming only, producing inconsistent warming along the gradient. A viable solution will be to use large scale active warming. Published results of such experiments so far only exist for grasslands and temperate forests. One is currently underway in tropical rainforest in Costa Rica (Cavaleri et al. 2015) and it will be valuable to replicate this in other rainforest types and locations.

## 8.2 Conclusions and future directions

In natural communities there is a geographic mosaic of chemically mediated plant-herbivore-parasitoid interactions (Dyer et al. 2014). In addition, the responses of these interactions to increased temperatures are complex. The effects can act on multiple different pathways such that the responses of pairs of interacting species may be countered or damped by the large number and complexity of interactions within a community. As such it will be difficult to predict how communities will respond to increasing temperatures. More studies are needed that include multitrophic interactions with coincident measures of phytochemical diversity. Our understanding of the effects of secondary metabolites on multiple trophic levels is still at a basic level, however this area is particularly important if we are to understand the multiple effects of warmer temperatures on communities. This thesis adds significantly to our understanding of this

area, for example, we found that the effect of temperature on parasitoids is mediated by changes in phytochemical diversity. The results from this thesis provide many avenues for future studies. In addition to the experimental approaches discussed above, one obvious next step is to expand the study to include more plant and insect species. In so doing we could asses the effects of climate warming on plants with varying levels of phytochemical diversity and how this will affect different guilds of herbivores and their parasitoids at the community level. Increase in our understanding of these areas, particularly in the unexplored area of how chemical diversity affects interaction and thus species diversity, is needed to accurately assess the relationship between climate change, diversity and ecosystem function.

## References


Abrahamson, W.G., McCrea, K.D., Whitwell, A.J. & Vernieri, L. (1991) The role of phenolics in goldenrod ball gall resistance and formation. *Biochemical Systematics and Ecology,* **19**, 615–622.

Abrahamson, W.G., Hunter, M.D., Melika, G. & Price, P.W. (2003) Cynipid gall-wasp communities correlate with oak chemistry. *Journal of Chemical Ecology,* **29**, 209-223.

Adler, L., Schmitt, J. & Bowers, M. (1995) Genetic variation in defensive chemistry in *Plantago lanceolata* (Plantaginaceae) and its effect on the specialist herbivore *Junionia coenia* (Nymphalidae). *Oecologia,* **101**, 75–85.

Aguilar, R., Ashworth, A., Galetto, L. & Aizen, M.A. (2006) Plant reproductive susceptibility to habitat fragmentation: review and synthesis through a meta-analysis. *Ecology Letters*, **9**, 968-980.

Alice, L.A. & Campbell, C.S. (1999) Phylogeny of *Rubus* (Rosaceae) based on nuclear ribosomal DNA internal transcribed spacer region sequences. *American Journal of Botany,* **86**, 81-97.

Altesor, P., García, Á., Font, E., Rodríguez-Haralambides, A., Vilaró, F., Oesterheld, M., Soler, R. & González, A. (2014) Glycoalkaloids of wild and cultivated *Solanum*: effects on specialist and generalist insect herbivores. *Journal of Chemical Ecology*, **40**, 599-608.

Amir, M. & Kumar, S. (2004) Possible industrial applications of genus *Solanum* in twentyfirst century – A review. *Journal of Scientific & Industrial Research*, **63**, 116-124.

Arimura, G., Matsui, K. & Takabayashi, J. (2009) Chemical and molecular ecology of herbivore-induced plant volatiles: Proximate factors and their ultimate functions. *Plant and Cell Physiology*, **50**, 911–923.

Atsatt, P.R. (1981) Lycaenid butterflies and ants. Selection for enemy free space. *American Naturalist*, **118**, 638-654.

Bale, J.S., Masters, G.J., Hodkinson, I.D., Awmack, C., Bezemer, T.M., Brown, V.K., Butterfield, J., Buse, A., Coulson, J., Farrar, J., Good, J., Harrington, R., Hartley, S., Jones, T., Lindroth, R.L., Press, M., Symrnioudis, I., Watt, A. & Whittaker, J.B. (2002) Herbivory in global climate change research: direct effects of rising temperature on insect herbivores. *Global Change Biology*, **8**, 1-16.

Barton, B.T. (2011) Local adaptation to temperature conserves top-down control in a grassland food web. *Proceedings of the Royal Society B: Biological Sciences,* **278**, 3102-3107.

Barton, B.T. & Schmitz, O.J. (2009) Experimental warming transforms multiple predator effects in a grassland food web. *Ecology Letters*, **12**, 1317–1325.

Bassard, J.E., Ullmann, P., Bernier, F. & Werck-Reichhart, D. (2010) Phenolamides: bridging polyamides to the phenolic metabolism. *Phytochemistry,* **71**,1808-1824.

Berenbaum, M. & Zangerl, A. (1996) Phytochemical diversity and redundancy in ecological interactions. In: *Recent advances in phytochemistry* (eds J. Romeo, J. Saunders, & P. Barbosa) pp. 1–24 Springer US.

Bidart-Bouzat, M.G. & Imeh-Nathaniel, A. (2008) Global change effects on plant chemical defenses against insect herbivores. *Journal of Integrative Plant Biology,* **50**, 1339-1354.

Bita, C.E. & Gerats, T. (2013) Plant tolerance to high temperature in a changing environment: Scientific fundamentals and production of heat stress-tolerant crops. *Frontiers in Plant Science*, **4**, Article 273.

Boppré, M. (1990) Lepidoptera and pyrrolizidine alkaloids: exemplification of complexity in chemical ecology. *Journal of Chemical Ecology*, **16**, 165-185.

Brenes-Arguedas, T. & Coley, P.D. (2005) Phenotypic variation and spatial structure of secondary chemistry in a natural population of a tropical tree species. *Oikos*, **108**, 410-420.

Bruzzese, E. (1980) The phytophagous insect fauna of *Rubus* spp. (Rosaceae) in Victoria, a study on the biological control of blackberry (*Rubus fruticosus* L. agg.). *Journal of the Australian Entomological Society,* **19**, 1-6.

Bukovinszky, T., van Veen, F.J.F., Jongema, Y. & Dicke, M. (2008) Direct and indirect effects of resource quality on food web structure. *Science,* **319**, 804-807.

Butterill, P.T. & Novotny, V. (2015) Gall-forming insects in a lowland tropical rainforest: low species diversity in an extremely specialised guild. *Ecological Entomology,* **40,** 409-419.

Carneiro, M.A., Branco, C.A., Braga, C.D., Almada, E.D., Costa, M.B., Maia, V.C. & Fernandes, G.W. (2009) Are gall midge species (Diptera, Cecidomyiidae) host-plant specialists? *Revista Brasileira de Entomologia,* **53**, 365-378.

Carpenter, S.R. (1996) Microcosm experiments have limited relevance for community and ecosystem ecology. *Ecology*, **77**, 677-680.

Cavaleri, M.A., Reed, S., Smith, W.K. & Wood, T.E. (2015) Urgent need for warming experiments in tropical forests. *Global Change Biology,* **21**, 2111–2121.

Cavlier, S. (2012) *White paper: Relative humidity sensor behavior and care*. Delmhorst Instrument Co. Available at: http://www.delmhorst.com/Documents/PDFs/ Product -Support/White-Paper---Relative-Humidity-Sensor-Behavior-an.pdf

Cheng, A., Lou, Y., Mao, Y., Lu, S., Wang, L. & Chen, X. (2007) Plant terpenoids: biosynthesis and ecological functions. *Journal of Integrative Plant Biology*, **29**, 179-186.

Coley, P.D., Bryant, J.P. & Chapin, F.S. (1985) Resource availability and plant anti-herbivore defence. *Science,* **230**, 895-899.

Cooper, H.M., Hegdes, L.V. & Valentine, J.C. (2009) *The handbook of research synthesis and meta-analysis.* Russel Sage Foundation Publications, NY, USA.

Cooper, W.R. & Rieske, L.K. (2009) Woody stem galls interact with foliage to affect community associations. *Environmental Entomology*, **38,** 417–424.

Cornell, H. (1983) The secondary chemistry and complex morphology of galls formed by the Cynipinae (Hymenoptera): why and how? *American Midland Naturalist,* **110,** 225–234,

Crutsinger, G.M., Gonzalez, A.L., Crawford, K.M. & Sanders, N.J. (2013) Local and latitudinal variation in abundance: the mechanisms shaping the distribution of an ecosystem engineer. *PeerJ,* **1**, e100, https://dx.doi.org/10.7717/peerj.100

Csóka, G., Stone, G.N. & Melika, G. (2005) The biology, ecology and evolution of gall wasps. In *Biology, ecology and evolution of gall-inducing arthropods* (eds. A. Raman, C.W. Schaeffer & T.M. Withers) pp. 573–642. Enfield, New Hampshire: Science Publishers Inc.

Damken, C., Perry, G.L. & Beggs, J.R. (2012) Complex habitat changes along elevational gradients interact with resource requirements of insect specialist herbivores. *Ecosphere*, **3**, Article 123.

de Sassi, C., Lewis, O.T. & Tylianakis, J.M. (2012) Plant-mediated and nonadditive effects of two global change drivers on an insect herbivore community. *Ecology,* **93**, 1892-1901.

de Sassi, C., & Tylianakis, J.M. (2012) Climate change disproportionately increases herbivore over plant or parasitoid biomass. *PloS one,* **7**, e40557.

Denno, R.F., McClure, M.S. & Ott, J.R. (1995) Interspecific interactions in phytophagous insects: competition re-examined and resurrected. *Annual Review of Entomology*, **40**, 297-331.

Dunne, J., Saleska, S., Fischer, M. & Harte, J. (2004) Integrating experimental and gradient methods in ecological climate change research. *Ecology,* **85**, 904-916.

Dutton, A., Mattiacci, L., Amado, R. & Dorn, S. (2002) A novel function of the triterpene squalene in a tritrophic system. *Journal of Chemical Ecology*, **28**, 103–116.

Dyer, L.A., Dodson, C.D., Stireman, J.O., Tobler, M.A., Smilanich, A.M., Fincher, R.M. & Letourneau, D.K. (2003) Synergistic effects of three *Piper* amides on generalist and specialist herbivores. *Journal of Chemical Ecology*, **29**, 2499-2514.

Dyer, L.A., Parchman, T.L., Jeffrey, C.S., Richards, L.A. (2014) New dimensions of tropical diversity: an inordinate fondness for insect molecules, taxa, and trophic interactions. *Current Opinion in Insect Science,* **2**, 1-6.

Dyer, L.A., Richards, L.A., Short, S.A. & Dodson, C.D. (2013) Effects of CO2 and temperature on tritrophic interactions. *PLoS ONE*, **8**, e62528.

Edreva, A., Yordanov, I., Kardjieva, R. & Gesheva, E. (1998) Heat shock responses of bean plants: involvement of free radicals, antioxidants and free radical/active oxygen scavenging systems. *Biologia Plantarum*, **41**, 185‑191.

Ehrlich, P.R. & Raven, P.H. (1964) Butterflies and plants: a study in coevolution. *Evolution,* **18**, 586-608.

Endara, M.J. & Coley, P.D. (2011) The resource availability hypothesis revisted: a meta-analysis. *Functional Ecology*, **25**, 389-398.

Farag, M.A., Mahrous, E.A., Lübken, T., Porzel, A. & Wessjohann, L. (2013) Classification of commercial cultivars of *Humulus lupulus* L. (hop) by chemometric pixel analysis of two dimensional nuclear magnetic resonance spectra. *Metabolomics*, **10**, 21-32.

Feeny, P. (1970) Seasonal changes in oak leaf tannins and nutrients as a cause of spring feeding by winter moth caterpillars. *Ecology,* **51**, 565-581.

Feeny, P. (1976) Plant apparency and chemical defence. In *Biochemical Interaction between Plants and Insects* (eds. J. Wallace & R. Mansell). Plenum Press, New York, pp 1-40.

Fink, U. & Völkl, W. (1995) The effect of abiotic factors on foraging and oviposition success of the aphid parasitoid, *Aphidius rosae*. *Oecologia,* **103**, 371-378.

Fisher, A.E., Hartley, S.E. & Young, M. (2000) Direct and indirect competitive effects of foliage feeding guilds on the performance of the birch leaf-miner *Eriocrania*. *Journal of Animal Ecology,* **69**, 165-176.

Foss, L.K. & Rieske, L.K. (2004) Stem galls affect oak foliage with potential consequences for herbivory. *Ecological Entomology*, **29**, 273–280.

Fujita, M., Fujita, Y., Noutoshi, Y., Takahashi, F., Narusaka, Y., Yamaguchi-Shinozaki, K. & Shinozaki, K. (2006) Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. *Current Opinion in Plant Biology*, **9**, 436–442.

Fürstenberg-Hägg, J., Zagrobelny, M. & Bak, S. (2013) Plant defence against insect herbivores. *International Journal of Molecular Sciences*, **14**, 10242-10297.

Gagné, R.J. & Jaschhof, M. (2014) *A catalogue of the Cecidomyiidae (Diptera) of the World. 3rd Edition*. Digital version 2, Available at: http://www.ars.usda.gov/SP2UserFiles/Place/12454900/Gagne_2014_World_Cecidomyiidae_Catalog_3rd_Edition.pdf

Gancel, A.L., Alter, P., Dhuique-Mayer, C., Ruales, J. & Vaillant, F. (2008) Identifying carotenoids and phenolic compounds in Naranjilla (*Solanum quitoense* lam. var. puyo hybrid), an Andean fruit. *Journal of Agricultural and Food Chemistry*, **56**, 11890-11899

Gaquerel, E., Gulati, J. & Baldwin I.T. (2014) Revealing insect herbviory-induced phenolamide metabolism: from single genes to metabolic network plasticity analysis. *The Plant Journal*, **79**, 679-692.

Garibaldi, L., Kitzberger, T. & Chaneton, E. (2011) Environmental and genetic control of insect abundance and herbivory along a forest elevational gradient. *Oecologia,* **167**, 117–129.

Gentry, G.L. & Dyer, L.A. (2002) On the conditional nature of neotropical caterpillar defenses against their natural enemies. *Ecology*, **83**, 3108–3119.

Gillespie, D.R., Nasreen, A., Moffat, C.E., Clarke, P. & Roitberg, B.D. (2012) Effects of simulated heat waves on an experimental community of pepper plants, green peach aphids and two parasitoid species. *Oikos*, **121**, 149-159.

Gilman, S.E., Urban, M.C., Tewksbury, J., Gilchrist, G.W. & Holt, R.D. (2010) A framework for community interactions under climate change. *Trends in Ecology and Evolution,* **25**, 325-331.

Godfree, R., Robertson, B., Bolger, T., Carnegie, M. & Young, A. (2011) An improved hexagon open-top chamber system for stable diurnal and nocturnal warming and atmospheric carbon dioxide enrichment. *Global Change Biology*, **17**, 439–451.

Gols, R., Raaijmakers, C.E., van Dam, N.M., Dicke, M., Bukovinszky, T. & Harvey, J.A. (2007) Temporal changes affect plant chemistry and tritrophic interactions. *Basic and Applied Ecology,* **8**, 421-433.

Gols, R., van Dam, N.M., Raaijmakers, C.E., Dicke, M., Harvey, J.A. (2009) Are population differences in plant quality reflected in the preference and performance of two endoparasitoid wasps? *Oikos*, **118**, 733–742.

Gols, R., Wagenaar, R., Bukovinszky, T., van Dam, N.M., Dicke, M., Bullock, J.M., Harvey, J.A. (2008) Genetic variation in defence chemistry in wild cabbages affects herbivores and their endoparasitoids. *Ecology*, **89**, 1616–1626.

Griffith University (2015) *Thesis as a series of published and unpublished papers*. Available at https://www.griffith.edu.au/higher-degrees-research/current-research-students/thesis/preparation/thesis-as-a-series-of-published-unpublished-papers

Gurevitch, J. & Hedges L.V. (1999) Statistical issues in ecological meta-analyses. *Ecology*, **80**, 1142-1149.

Gutbrodt, B., Mody, K. & Dorn, S. (2011) Drought changes plant chemistry and causes contrasting responses in lepidopteran herbivores. *Oikos*, **120**, 1732–1740.

Gutierrez, D. & Menendez, R. (1997). Patterns in the distribution, abundance and body size of carabid beetles (Coleoptera: Caraboidea) in relation to dispersal ability. *Journal of Biogeography,* **24**, 903–914.

Hall, C.R., Burwell, C.J. & Kitching, R.L. (2015) Changes in function and temporal variation in a guild of gall-parasitoids across a temperature gradient in Australian subtropical rainforest. *Austral Ecology*, doi: 10.1111/aec.12283

Hall, C.R., Burwell, C.J., Nakamura, A. & Kitching R.L. (2015a) Altitudinal variation of parasitic Hymenoptera assemblages in Australian subtropical rainforest. *Austral Entomology*, **54**, 246-258.

Hallam, A. & Read, J. (2006) Do tropical species invest more in anti-herbivore defence than temperate species? A test in *Eucryphia* (Cunoniaceae) in eastern Australia. *Journal of Tropical Ecology*, **22**, 41-51.

Hance, T., van Baaren, J., Vernon, P. & Boivin, G. (2007) Impact of extreme temperatures on parasitoids in a climate change perspective. *Annual Review of Entomology*, **52**, 107-126.

Hansen, A., Jonasson, S., Michelsen, A. & Julkunen-Tiitto, R. (2006) Long-term experimental warming, shading and nutrient addition affect the concentration of phenolic compounds in arctic-alpine deciduous and evergreen dwarf shrubs. *Oecologia,* **147**, 1-11.

Harborne, J.B. (1997) Plant secondary metabolism. In *Plant ecology* (ed M.J. Crawley) pp. 132–155. Blackwell Science Ltd.

Harden, G.J., McDonald, B. & Williams, J.B. (2006) *Rainforest climbing plants: A field guide to their identification*. Gwen Harden Publishing.

Harrington, R., Woiwod, I. & Sparks, T. (1999) Climate change and trophic interactions. *Trends in Ecology & Evolution,* **14**, 146-150.

Hartley, S.E. (1998) The chemical composition of plant galls: are levels of nutrients and secondary compounds controlled by the gall-former? *Oecologia,* **113**, 492–501.

Hartley, S.E. & Lawton, J.H. (1987) Effects of different damage types on the chemistry of birch foliage, and the responses of birch feeding insects. *Oecologia*, **74**, 432-437.

Hartley, S.E. & Lawton, J.H. (1992) Host-plant manipulation by gall-insects: a test of the nutrition hypothesis. *Journal of Animal Ecology,* **61**, 113-119.

Harvey, J.A. (2005) Factors affecting the evolution of development strategies in parasitoid wasps: The importance of functional constraints and incorporating complexity. *Entomologia Experimentalis et Applicata*, **117**, 1–13.

Harvey, J.A., van Dam, N.M., Raaijmakers, C.E., Bullock, J.M. & Gols, R. (2010) Tri-trophic effects of inter- and intra-population variation in defence chemistry of wild cabbage (*Brassica oleracea*). *Oecologia*, **166**, 421–431.

Haugen, R., Steffes, L., Wolf, J., Brown, P., Matzner, S. & Siemens, D.H. (2008) Evolution of drought tolerance and defense: dependence of tradeoffs on mechanism, environment and defense switching. *Oikos,* **117**, 231-244.

Hawkins, B.A. & de Vries, P.J. (1996). Altitudinal gradients in the body sizes of Costa Rican butterflies. *Acta Oecologica,* **17**, 185–194.

Hedges, L.V. & Olkin, I. (1985) *Statistical methods for meta-analysis*. Academic Press, Orlando, USA.

Helms, A.M., De Moraes, C., Tooker, J.F. & Mescher, M.C. (2013) Exposure of *Solidago altissima* plants to volatile emissions of an insect antagonist (*Eurosta solidaginis*) deters subsequent herbivory. *Proceedings of the National Academy of Science*, **110**, 199-204.

Herms, D.A. & Mattson, W.J. (1992) The dilemma of plants: to grow or defend. *The Quarterly Review of Biology,* **67**, 283-335.

Hilker, M. (2014) New synthesis: Parallels between biodiversity and chemodiversity. *Journal of Chemical Ecology*, **40**, 225–226.

Hillebrand, H., Borer, E.T., Bracken, M.S., Cardinale, B.J., Cebrian, J., Cleland, E.E., Elser, J.J., Gruner, D.S., Harpole, W.S., Ngai, J.T., Sandin, S., Seabloom, E.W., Shurin, J.B., Smith, J.E. & Smith, M.D. (2009) Herbivore metabolism and stoichiometry each constrain herbivory at different organizational scales across ecosystems. *Ecology Letters*, **12**, 516–527.

Hochberg, E. & Ives, A.R. (1999) Can natural enemies enforce geographical range limits? *Ecography*, **22**, 268-276.

Hodkinson, I.D. (2005) Terrestrial insects along elevation gradients: species and community responses to altitude. *Biological Reviews*, **80**, 489–513.

Holt, R.D. (1997) Community modules. In *Multitrophic interactions in terrestrial systems* (eds. A.C. Gange & V.K. Brown) pp. 333-350. London: Blackwell.

Ikuta, A., Kamiya, K., Satake, T. & Saiki, Y. (1995) Triterpenoids from callus tissue cultures of *Paeonia species*. *Phytochemistry*, **38**, 1203-1207.

IPCC (2007) *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the fourth assessment report of the Intergovernmental Panel on Climate Change.* Cambridge University Press, New York, NY.

Jamieson, M.A., Trowbridge, A.M., Raffa, K.F. & Lindroth, R.L. (2012) Consequences of climate warming and altered precipitation patterns for plant-insect and multitrophic interactions. *Plant Physiology,* **160**, 1719-1727.

Janzen, D.H., Ataroff, M., Farinas, M., Reyes, S., Rincon, N., Soler, A., Soriano, P. & Vera, M. (1976). Changes in the arthropod community along an elevational transect in the Venezuelan Andes. *Biotropica, 8*, 193–203.

Jia, Z.J., Liu, X.Q. & Liu, Z.M. (1993) Triterpenoids from *Sanguisorba alpina*. *Phytochemistry*, **32**, 155–159.

Julião, G.R., Venticinque, E.M., Fernandes, G.W. & Price, P.W. (2014) Unexpected high diversity of galling insects in the upper canopy: The savannah out there. *PLoS ONE,* **9**, e114986.

Kaplan, I. & R.F. Denno. (2007) Interspecific interactions in phytophagous insects revisited: a quantitative assessment of competition theory. *Ecology Letters,* **10**, 977–994.

Kaur, H., Heinzel, N., Schottner, M., Baldwin, I.T. & Galis, I. (2010) R2R3-NaMYB8 regulates the accumulation of phenylpropanoid-polyamine conjugates which are essential for local and systemic defense against insect herbivores in *Nicotiana attenuata, Plant Physiology*, **152**, 1731‑1747.

Kessler, A. & Baldwin, I. (2001) Defensive function of herbivore-induced plant volatile emissions in nature. *Science*, **291**, 2141–2144.

Kharouba, H.M., Vellend, M., Sarfraz, R.M. & Myers, J.H. (2015) The effects of experimental warming on the timing of a plant-insect herbivore interaction. *Journal of Animal Ecology*, **84**, 785–796.

Kim, K.H, Choi, Y.H. & Verpoorte, R. (2010) NMR-based metabolomics analysis of plants. *Nature Protocols,* **5,** 536-549.

Kim, K.H., Choi, S.U. & Lee, K.R. (2012) Cytotoxic triterpenoids from *Berberis koreana*. *Planta Medica*, **78**, 86–89.

Kollberg, I., Bylund, H., Jonsson, T., Schmidt, A., Gershenzon, J. & Björkman, C. (2015) Temperature affects insect outbreak risk through tritrophic interactions mediated by plant secondary compounds. *Ecosphere*, **6**, Article 102.

Koptur, S. (1985) Alternative defenses against herbivores in *Inga* (Fabaceae: Mimosoideae) over an elevational gradient. *Ecology,* **66**, 1639-1650.

Körner, C. (2007) The use of 'altitude' in ecological research. *Trends in Ecology & Evolution*, **22**, 569–574.

Krishnan, P., Kruger, N.J. & Ratcliffe, R.G. (2004) Metabolite fingerprinting and profiling in plants using NMR. *Journal of Experimental Botany*, **56**, 255-265.

Künkler, N., Brandl, R. & Brändle, M. (2013) Changes in clonal poplar leaf chemistry caused by stem galls alter herbivory and leaf littler decomposition. *PloS One,* **8,** e79994. doi:10.1371/journal.pone.0079994

Kursar, T.A., Dexter, K.G., Lokvam, J., Pennington, R.T., Richardson, J.E., Weber, M.G., Murakami, E.T., Drake, C., McGregor, R. & Coley, P.D. (2009) The evolution of antiherbivore defenses and their contribution to species coexistence

in the tropical tree genus *Inga*. *Proceedings of the National Academy of Sciences*, **106**, 18073–18078.
La Salle, J. (1993) Parasitic hymenoptera, biological control and biodiversity. In *Hymenoptera and Biodiversity* (eds. J. La Salle & I.D. Gauld) pp. 197-211. Wallingford, UK: C.A.B. International.
Laidlaw, M.J., McDonald, B., Hunter, R.J. & Kitching, R.L. (2011) Subtropical rainforest turnover along an altitudinal gradient. *Memoirs of the Queensland Museum*, **55**, 271–290.
Lankau, R. & Strauss, S.Y. (2008) Community complexity drives patterns of natural selection on a chemical defense of *Brassica nigra*. *The American Naturalist*, **171**, 150-161.
László, Z., Sólyom, K., Prázsmári, H., Barta, Z. & Tóthmérész, B. (2014) Predation on rose galls: parasitoids and predators determine gall size through directional selection. *PLoS ONE,* **9**, e99806. doi:10.1371/journal.pone.0099806
Lawton, J.H. (1995) Ecological experiments with model systems. *Science*, **269**, 328–331.
Lemoine, N.P., Burkepile, D.E. & Parker, J.D. (2014) Variable effects of temperature on insect herbivory. *PeerJ,* **2**, e376 https://doi: 10.7717/peerj.376
Leuzinger, S., Luo, Y., Beier, C., Dieleman, W., Vicca, S. & Körner, C. (2011) Do global change experiments overestimate impacts on terrestrial ecosystems? *Trends in Ecology & Evolution*, **26**, 236–241.
Liu, B., Lei, C., Shu, T., Zhang, Y., Jin, J., Li, S. & Liu, W. (2015) Effects of low-temperature stress on secondary metabolism in mosses exposed to simulated n deposition. *Plant Ecology & Diversity*, **8**, 415–426.
Loreto, F., Barta, C., Brilli, F. & Nogues, I. (2006) On the induction of volatile organic compound emissions by plants as consequence of wounding or fluctuations of light and temperature. *Plant, Cell & Environment,* **29**, 1820-1828.
Luo, Y., Melillo, J., Niu, S., Beier, C., Clark, J.S., Classen, A.T., Davidson, E., Dukes, J.S., Evans, R.D., Field, C.B., Czimczik, C.I., Keller, M., Kimball, B.A., Kueppers, L.M., Norby, R.J., Pelini, S.L., Pendall, E., Rastetter, E., Six, J., Smith, M., Tjoelker, M.G. & Torn, M.S. (2011) Coordinated approaches to quantify long-term ecosystem dynamics in response to global change. *Global Change Biology*, **17**, 843–854.
Mahrous, E.A. & Farag, M.A. (2015) Two dimensional NMR spectroscopic approaches for exploring plant metabolome: a review. *Journal of Advanced Research*, **6**, 3-15.
Martin, J.S. & Martin, M.M. (1982) Tannin assays in ecological studies: lack of correlation between phenolics, proanthocyanidins and protein-precipitating constituents in mature foliage of six oak species. *Oecologia,* **54**, 205-211.
Maunsell, S.C., Kitching, R.L., Burwell, C.J. & Morris R.J. (2014) Changes in host-parasitoid food web structure with elevation. *Journal of Animal Ecology,* **84,** 353–363.
McDonald, B. (2010) The Tweed caldera group. In *Remnants of Gondwana: a natural and social history of the Gondwana rainforests of Australia* (eds. R.L. Kitching, R. Braithwaite & J. Cavanaugh) pp. 39–50. Surrey Beatty and Sons.
McGinley, M. (1989) The influence of a positive correlation between clutch size and offspring fitness on the optimal offspring size. *Evolutionary Ecology,* **3**, 150–156.

McKinnon, M.L., Quiring, D.T. & Bauce, E. (1999) Influence of tree growth rate, shoot size and foliar chemistry on the abundance and performance of a galling adelgid. *Functional Ecology*, **13**, 859–867.

Mole, S. & Waterman, P.G. (1987) A critical analysis of techniques for measuring tannins in ecological studies. I. Techniques for chemically defining tannins. *Oecologia,* **72**, 137-147.

Moles, A.T., Bonser, S.P., Poore, A.B., Wallis, I.R. & Foley, W.J. (2011) Assessing the evidence for latitudinal gradients in plant defence and herbivory. *Functional Ecology*, **25**, 380-388.

Moore, B.D., Andrew, R.L., Külheim, C. & Foley, W.J. (2014) Explaining intraspecific diversity in plant secondary metabolites in an ecological context. *New Phytologist*, **201**, 733–750.

Moses, T., Papadopoulou, K.K. & Osbourn, A. (2014) Metabolic and functional diversity of saponins, biosynthetic intermediates and semi-synthetic derivatives. *Critical Reviews in Biochemistry and Molecular Biology*, **49**, 439–462.

Müller, C., Agerbirk, N., Olsen, C.E., Boeve, J.L., Schaffner, U. & Brakefield, P.M (2001) Sequestration of host plant glucosinolates in the defensive hemolymph of the sawfly *Athalia rosae*. *Journal of Chemical Ecology,* **27**, 2505–2516.

Mumm, R. & Dicke, M. (2010) Variation in natural plant products and the attraction of bodyguards involved in indirect plant defense. *Canadian Journal of Zoology,* **88**, 628–667.

Nakamura, M., Miyamoto, Y. & Ohgushi, T. (2003) Gall initiation enhances the availability of food resources for herbivorous insects. *Functional Ecology*, **17**, 851–857.

Numata, A., Yang, P., Takahashi, C., Fujiki, R., Nabae, M. & Fujita, E. (1989) Cytotoxic triterpenes from a chinese medicine, Goreishi. *Chemical and Pharmaceutical Bulletin*, **37**, 648-651.

Ode, P.J. (2006) Plant chemistry and natural enemy fitness: effects on herbivore and natural enemy interactions. *Annual Review of Entomology*, **51**, 163–185.

Palmer, R. (1999) Detecting publication bias in meta-analyses: A case study of fluctuating asymmetry and sexual selection. *American Naturalist*, **154**, 220-233.

Pascual-Alvarado, E., Cuevas-Reyes, P., Quesada, M. & Oyama, K. (2008) Interactions between galling insects and leaf-feeding insects: the role of plant phenolic compounds and their possible interference with herbivores. *Journal of Tropical Ecology,* **24**, 329-336.

Patel, A.V., Rojas-Vera, J. & Dacke, C.G. (2004) Therapeutic constituents and actions of *Rubus* species. *Current Medicinal Chemistry*, **11**, 1501-1512.

Pellissier, L., Roger, A., Bilat, J. & Rasmann, S. (2014) High elevation *Plantago lanceolata* plants are less resistant to herbivory than their low elevation conspecifics: Is it just temperature? *Ecography*, **37**, 950–959.

Péré, C., Jactel, H. & Kenis, M. (2013) Response of insect parasitism to elevation depends on host and parasitoid life-history strategies. *Biology Letters*, **9**, Article 20130028.

Pickett, S.T. (1989) Space-for-time substitution as an alternative to long-term studies. In *Long-term studies in ecology: approaches and alternatives* (ed G.E. Likes) pp. 110-135. New York, U.S.A: Springer-Verlag.

Pierens, G.K., Mobli, M. & Vegh, V. (2009) Effective protocol for database similarity searching of heteronuclear single quantum coherence spectra. *Analytical Chemistry*, **81**, 9329-9335.

Price, P.W. & Clancy, K.M. (1986) Interactions among three trophic levels: gall size and parasitoid attack. *Ecology,* **67**, 1593–1600.
Price, P.W., Fernandes, G.W. & Waring, G.L. (1987) Adaptive nature of insect galls. *Environmental Entomology*, **16**, 15-24.
Quicke, D.J. (1997) *Parasitic Wasps*. Chapman & Hall, London.
R Development Core Team (2014) R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria. Available at: http://www.R-project.org/
Raguso, R.A., Agrawal, A.A., Douglas, A.E., Jander, G., Kessler, A., Poveda, K. & Thaler, J.S. (2015) The raison dêtre of chemical ecology. *Ecology*, **96**, 617–630.
Rahbek, C. (2005) The role of spatial scale and the perception of large-scale species-richness patterns. *Ecology Letters,* **8**, 224-239.
Rasband, W.S. (2015) ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, Available at: http://imagej.nih.gov/ij/
Rasman, S., Pellissier, L., Defossez, E., Jactel, H. & Kunstler, G. (2013) Climate-driven change in plant-insect interactions along elevation gradients. *Functional Ecology*, **28**, 46-54.
Redfern, M. (2011) *Plant Galls*. HarperCollins Publishers, London.
Reymond, A., Purcell, J., Cherix, D., Guisan, A. & Pellissier, L. (2013) Functional diversity decreases with temperature in high elevation ant fauna. *Ecological Entomology*, **4**, 364–373.
Ribeiro, S.P., Basset, Y. & Kitching, R.L. (2014) Density of insect galls in the forest understory and canopy: neotropical, Gondwana or global patterns? In *Neotropical Insect Galls* (eds. G.W. Fernandes & J.C. Santos) pp. 129-141. Springer, London.
Richards, L.A., Dyer, L.A., Forister, M.L., Smilanich, A.M., Dodson, C.D., Leonard, M.D. & Jeffrey, C.S. (2015) Phytochemical diversity drives plant insect community diversity. *Proceedings of the National Academy of Sciences*, **112**, 10973–10978.
Richards, L.A., Dyer, L.A., Smilanich, A.M. & Dodson, C.D. (2010) Synergistic effects of amides from two *Piper* species on generalist and specialist herbivores. *Journal of Chemical Ecology,* **36**, 1105–1113.
Rodriguez-Castañeda, G. (2012) The world and its shades of green: a meta-analysis on trophic cascades across temperature and precipitation gradients. *Global Ecology and Biogeography*, **22**, 118–130.
Rohatgi, A. (2015) *WebPlotDigitizer*, Available at http://arohatgi.info/WebPlot Digitizer/
Romo, C.M. & Tylianakis, J.M. (2013) Elevated temperature and drought interact to reduce parasitoid effectiveness in suppressing hosts. *PLoS ONE*, **8**, e58136.
Ronquist, F., Nieves-Aldrey, J-L., Buffington, M.L., Liu, Z., Liljeblad, J., Nylander, J.A. (2015) Phylogeny, evolution and classification of gall wasps: the plot thickens. *PLoS ONE,* **10**, e0123301.
Rosenberg, M.S. (2005) The file-drawer problem revisited: a general weighted method for calculating fail-safe numbers in meta-analysis. *Evolution,* **59**, 464-468.
Roskam, J.C. (1985) Evolutionary patterns in gall midge-host plant associations (Diptera, Cecidomyiidae). *Tijschrift voor Entomologie*, **128**, 193-213
Roskam, J.C. (1992) Evolution of the gall-inducing guild. In *Biology of insect-induced galls* (eds. J.D. Shorthouse & O. Rohfritsch) pp. 34-48. Oxford University Press, New York.

Rosseel, Y. (2012) Lavaan: an R package for structural equation modeling. *Journal of Statistical Software*, **48**, 1-36, doi:10.18637/jss.v048.i02

Rostás, M., Maag, D., Ikegami, M. & Inbar, M. (2013) Gall volatiles defend aphids against a browsing mammal. *BMC Evolutionary Biology*, **13**, Article 193.

Rostás, M., Ruf, D., Zabka, V. & Hildebrandt, U. (2008) Plant surface wax affects parasitoid's response to host footprints. *Naturwissenschaften*, **95**, 997–1002.

Sallas, L., Luomala, E.-M., Utriainen, J., Kainulainen, P. & Holopainen, J.K. (2003) Contrasting effects of elevated carbon dioxide concentration and temperature on Rubisco activity, chlorophyll fluorescence, needle ultrastructure and secondary metabolites in conifer seedlings. *Tree Physiology,* **23**, 97-108.

Salminen, S.O., Richmond, D.S., Grewal, S.K. & Grewal, P.S. (2005) Influence of temperature on alkaloid levels and fall armyworm performance in endophytic tall fescue and perennial ryegrass. *Entomologia Experimentalis et Applicata,* **115**, 417-426.

Salmore, A. & Hunter, M. (2001) Elevational trends in defense chemistry, vegetation, and reproduction in *Sanguinaria canadensis*. *Journal of Chemical Ecology*, **27**, 1713–1727.

Scheidel, U., Rohl, S. & Bruelheide, H. (2003) Altitudinal gradients of generalist and specialist herbivory on three montane Asteraceae. *Acta Oecologica*, **24**, 275–283.

Scherber, C., Gladbach, D.J., Stevnbak, K., Karsten, R.J., Schmidt, I.K., Michelsen, A., Albert, K.R., Larsen, K.S., Mikkelsen, T.N., Beier, C. & Christensen, S. (2013) Multi-factor climate change effects on insect herbivore performance. *Ecology and Evolution*, **3**, 1449–1460.

Schultz, J.C. (1988) Many factors influence the evolution of herbivore diets, but plant chemistry is central. *Ecology,* **69**, 896-897.

Scifinder (2015) Chemical Abstracts Service. Columbus, OH

Shorthouse, J.D. & Rohfritsch, O. (1992) *Biology of Insect-Induced Galls*. New York, Oxford University Press.

Siemens, D.H., Duvall-Jisha, J., Jacobs, J., Manthey, J., Haugen, R. & Matzner, S. (2012) Water deficiency induces evolutionary tradeoff between stress tolerance and chemical defense allocation that may help explain range limits in plants. *Oikos,* **121**, 790-800.

Siemens, D.H., Haugen, R., Matzner, S. & Vanasma, N. (2009) Plant chemical defence allocation constrains evolution of local range. *Molecular Ecology,* **18**, 4974-4983.

Sifi, I., Gourine, N., Gaydou, E.M. & Yousfi, M. (2015) Chemotypes of essential oil of unripe galls of *Pistacia atlantica* Desf. from Algeria. *Natural Products Research*, **29**, 1945-1949.

Slot, M., Rey-Sánchez, C., Gerber, S., Lichstein, J.W., Winter, K. & Kitajima, K. (2014) Thermal acclimation of leaf respiration of tropical trees and lianas: Response to experimental canopy warming, and consequences for tropical forest carbon balance. *Global Change Biology*, **20**, 2915–2926.

Smilanich, A.M., Dyer, L.A., Chambers, J.Q. & Bowers, M.D. (2009) Immunological cost of chemical defence and the evolution of herbivore diet breadth. *Ecology Letters*, **12**, 612–621.

Stille, B. (1984) The effect of hostplant and parasitoids on the reproductive success of the parthenogenetic gall wasp *Diplolepis rosae*. *Oecologia*, **63**, 364–369.

Stone, G.N. & Schönrogge, K. (2003) The adaptive significance of insect gall morphology. *Trends in Ecology & Evolution*, **18**, 512–522.

Streiner, D.L. (2005) Finding our way: an introduction to path analysis. *Canadian Journal of Psychiatry*, **50**, 115-122.

Strong, C.L., Boulter, S.L., Laidlaw, M.J., Maunsell, S.C., Putland, D.A. & Kitching, R.L. (2011) The physical environment of an altitudinal gradient in the rainfreost of Lamington National Park, southeast Queensland. *Memiors of the Queensland Museum,* **55,** 251- 270.

Sumerford, D.V., Abrahamson, W.G. & Weis, A.E. (2000) The effects of drought on the *Solidago altissima-Eurosta solidaginis*-natural enemy complex: population dynamics, local extirpations, and measures of selection intensity on gall size. *Oecologia,* **122**, 240-248.

Sutter, R. & Müller, C. (2011) Mining for treatment-specific and general changes in target compounds and metabolic fingerprints in response to herbivory and phytohormones in *Plantago lanceolata*. *New Phytologist*, **191**, 1069-1082.

Taniguchi, S., Imayoshi, Y., Kobayashi, E., Takamatsu, Y., Ito, H., Hatano, T., Sakagami, H., Tokuda, H., Nishino, H., Sugita, D., Shimura, S. & Yoshida, T. (2002) Production of bioactive triterpenes by *Eriobotrya japonica* calli. *Phytochemistry*, **59**, 315-323.

Taper, M. & Case, T. (1987) Interactions between oak tannins and parasite community structure: unexpected benefits of tannins to cynipid gall-wasps. *Oecologia,* **71**, 254–261.

Tebayashi, S., Horibata, Y., Mikagi, E., Kashiwagi, T., Mekuria, D.B., Dekebo, A., Ishihara, A. & Kim, C.S. (2007) Induction of resistance against the leafminer, *Liriomyza trifolii*, by jasmonic acid in sweet pepper. *Bioscience, Biotechnology & Biochemistry*, **71**, 1521–1526.

Tooker, J.F. & De Moraes, C.M. (2007) Feeding by the Hessian fly [*Mayetiola destructor* (Say)] larvae does not induce plant indirect defences. *Ecological Entomology*, 32, 473-477.

Tooker, J.F., Rohr, J.R., Abrahamson, W.G. & De Moraes, C.M. (2008) Gall insects can avoid and alter indirect plant defenses. *New Phytologist*, **178**, 657-671.

Turlings, T.J., Tumlinson, J.H. & Lewis, W.J. (1990) Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science*, **250**, 1251–1253.

Tylianakis, J.M., Didham, R.K., Bascompte, J. & Wardle, D.A. (2008) Global change and species interactions in terrestrial ecosystems. *Ecology Letters,* **11**, 1351-1363.

Viechtbauer, W. (2010) Conducting meta-analyses in R with the metafor package. *Journal of Statistical Software*, **36**, 1-48.

Voigt, W., Perner, J., Davis, A.J., Eggers, T., Schumacher, J., Bährmann, R., Fabian, B., Heinrich, W., Köhler, G., Lichter, D., Marstaller, R. & Sander, F.W. (2003) Trophic levels are differentially sensitive to climate. *Ecology*, **84**, 2444-2453.

War, A.R., Paulraj, M.G., Ahmad, T., Buhroo, A.A., Hussain, B., Ignacimuthu, S., Sharma, H.C. (2012) Mechanisms of plant defense against insect herbivores. *Plant Signalling & Behaviour*, **7**, 1306–1320.

Weese, T.L. & Bohs, L. (2007) A three-gene phylogeny of the genus *Solanum* (Solanaceae). *Systematic Botany*, **32**, 445-463.

Wishart, D.S. (2008). Quantitative metabolomics using NMR. *Trends in Analytical Chemistry*, **27**, 228-237.

Whitham, T.G. (1978) Habitat selection by *Pemphigus* aphids in response to response limitation and competition. *Ecology,* **59**, 1164–1176.

Woodward, G., Benstead, J.P., Beveridge, O.S., Blanchard, J., Brey, T., Brown, L.E., Cross, W.F., Friberg, N., Ings, T.C., Jacob, U., Jennings, S., Ledger, M.E.,

Milner, A.M., Montoya, J.M., O'Gorman, E., Olesen, J.M., Petchey, O.L., Pichler, D.E., Reuman, D.C., Thompson, M.A., Van Veen, F.J.F. & Yvon-Durocher, G. (2010) Ecological networks in a changing climate. *Advances in ecological research,* **42**, 71-138.

Wool, D. & Manheim, O. (1988) The effects of host-plant properties on gall density, gall weight and clone size in the aphid *Aploneura lentisci* (Pass.) (Aphididae: Fordinae) in Israel. *Researches on Population Ecology*, **30**, 227-234.

Wootton, J.T. (1994) The nature and consequences of indirect effects in ecological communities. *Annual Review of Ecology and Systematics*, **25**, 443-446.

Zhao, F., Zhang, W., Hoffmann, A. & Ma, C.S. (2014) Night warming on hot days produces novel impacts on development, survival and reproduction in a small arthropod. *Journal of Animal Ecology*, **83**, 769-778.

Zidorn, C. (2009) Altitudinal variation of secondary metabolites in flowering heads of the Asteraceae: Trends and causes. *Phytochemical Reviews,* **9**, 197–203.

Zvereva, E.L. & Kozlov, M.V. (2006) Consequences of simultaneous elevation of carbon dioxide and temperature for plant-herbivore interactions: a meta-analysis. *Global Change Biology*, **12**: 27–41.